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REVIEW

Microbiome-liver crosstalk: A multihit therapeutic target for liver disease

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Abstract

Liver disease has become a leading cause of death, particularly in the West, where it is attributed to more than two million deaths annually. The correlation between gut microbiota and liver disease is still not fully understood. However, it is well known that gut dysbiosis accompanied by a leaky gut causes an increase in lipopolysaccharides in circulation, which in turn evoke massive hepatic inflammation promoting liver cirrhosis. Microbial dysbiosis also leads to poor bile acid metabolism and low short-chain fatty acids, all of which exacerbate the inflammatory response of liver cells. Gut microbial homeostasis is maintained through intricate processes that ensure that commensal microbes adapt to the low oxygen potential of the gut and that they rapidly occupy all the intestinal niches, thus outcompeting any potential pathogens for available nutrients. The crosstalk between the gut microbiota and its metabolites also guarantee an intact gut barrier. These processes that protect against destabilization of gut microbes by potential entry of pathogenic bacteria are collectively called colonization resistance and are equally essential for liver health. In this review, we shall investigate how the mechanisms of colonization resistance influence the liver in health and disease and the microbial-liver crosstalk potential as therapeutic target areas.

Key Words: Microbiome; Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Liver disease; Microbiome-host crosstalk; Gut homeostasis; Microbial metabolites

Core Tip: The influence of the gut microbiome on various body systems has important implications for health and disease, such as liver disease. While the exact mechanisms of how the microbiome contributes to liver disease are unknown, there is strong evidence that the translocation of various metabolites across the mucosal barrier plays a strong role, which is precipitated by dysbiotic gut microbiota. Considering the importance of the microbiome in liver disease, powerful therapeutic options that can manipulate the gut microbiome are being explored. These approaches could have the potential for effective treatments for various stages of liver disease. This review will explore how the mechanisms of colonization resistance influence the liver in health and disease and finally examine potential therapeutic targets in the gut-liver axis.

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INTRODUCTION

A healthy gut microbiota plays a significant role in maintaining a homeostatic gut environment. One such role is colonization resistance, which is defined as the microbial capacity to resist invasion of exogenous microorganisms (for example, pathogens) and/or prevent uncontrolled overgrowth of endogenous microbes (for example, pathobionts). For gut homeostasis to be achieved, microbial alpha diversity must remain high, gut mucosal integrity must be maintained, and tolerance to the billions of microbial immunogens present in the gut must be established. This is all achieved through intricate microbe-to-microbe and microbe-to-host interactions mediated by microbial metabolites, such as short-chain fatty acids, or microbial cell wall components, such as lipopolysaccharides, lipoteichoic acid, peptidoglycans and flagellin. Homeostasis is also achieved through the production of antimicrobial peptides, resource and oxygen competition, host immunomodulation, and conjugation of bile acids. The mechanisms by which these inter/intramicrobial interactions mediate colonization resistance or how their perturbation leads to disease have not yet been fully elucidated. However, it is known that an imbalance in microbial composition, otherwise known as dysbiosis, which may arise from dietary changes, ingestion of exogenous toxins such as antibiotics or xenobiotics, or through infections that suppress the immune system, has serious and sometimes long-term clinical implications. Diseases such as diabetes, obesity, atherosclerosis, and liver disease are associated with dysbiosis and the translocation of gut microbial products into circulation. As the liver is the first organ to be exposed to the gut bacterial products and digested food delivered through the portal vein, any leakage of microbial products into circulation will lead to hepatocellular immune activation, thereby promoting systemic and hepatic inflammation, which may lead to liver disease[1]. An understanding of the mechanisms involved in colonization resistance and its influencing factors is therefore crucial to establish their link to the etiology of liver disease as well as to identify possible hit points along the gut-liver axis that can be utilized as therapeutic targets for liver disease[2]. This review explores some of the mechanisms of colonization resistance and their importance to the etiology of the different stages of nonalcoholic fatty liver disease (NAFLD) from simple steatosis to liver inflammation, as well as alcohol-associated liver disease (ALD), and highlights potential entry points that may be used as therapeutic targets for liver disease. A summary of the interplay between the microbiome, liver, immune system, and metabolome is presented in Figure 1.

GUT MICROBIAL EUBIOSIS

The gut microbiome starts taking shape at birth, where it is initially influenced by the mode of delivery. Vaginally born babies will have a gut microbial composition very close to the maternal vaginal microbiota, while the caesarian born will adopt mainly the skin microbiota[3]. Mammals have five phyla that predominate the gut: Firmicutes (*e.g.*, *Lactobacillus, Clostridium, Ruminococcus, Eubacterium, Fecalibacterium and Roseburia*), Actinobacteria (with *Bifidobacterium* as one of its most important members), Bacteroidetes (*e.g.*, *Bacteroides, Prevotella, and Xylanibacter*), Proteobacteria (*e.g.*, *Escherichia* and *Desulfovibrio*) and Verrucomicrobia (*e.g.*, *Akkermansia*)[4]. The earliest colonizers are mainly facultative aerobes of the phyla Firmicutes and Actinobacteria, which play a significant role in lowering the gut's oxygen level to allow for the colonization of obligate anaerobes. These aerotolerant microbes reside in the upper gut, where they continue to reduce the amount of oxygen in the gut for life. *Escherichia coli* and *Enterococcus faecalis* are the most abundant in the oxygen-high neonatal gut, and they rapidly expand in the early phase, leading to a gradual depression of oxygen levels and allowing growth of the facultative

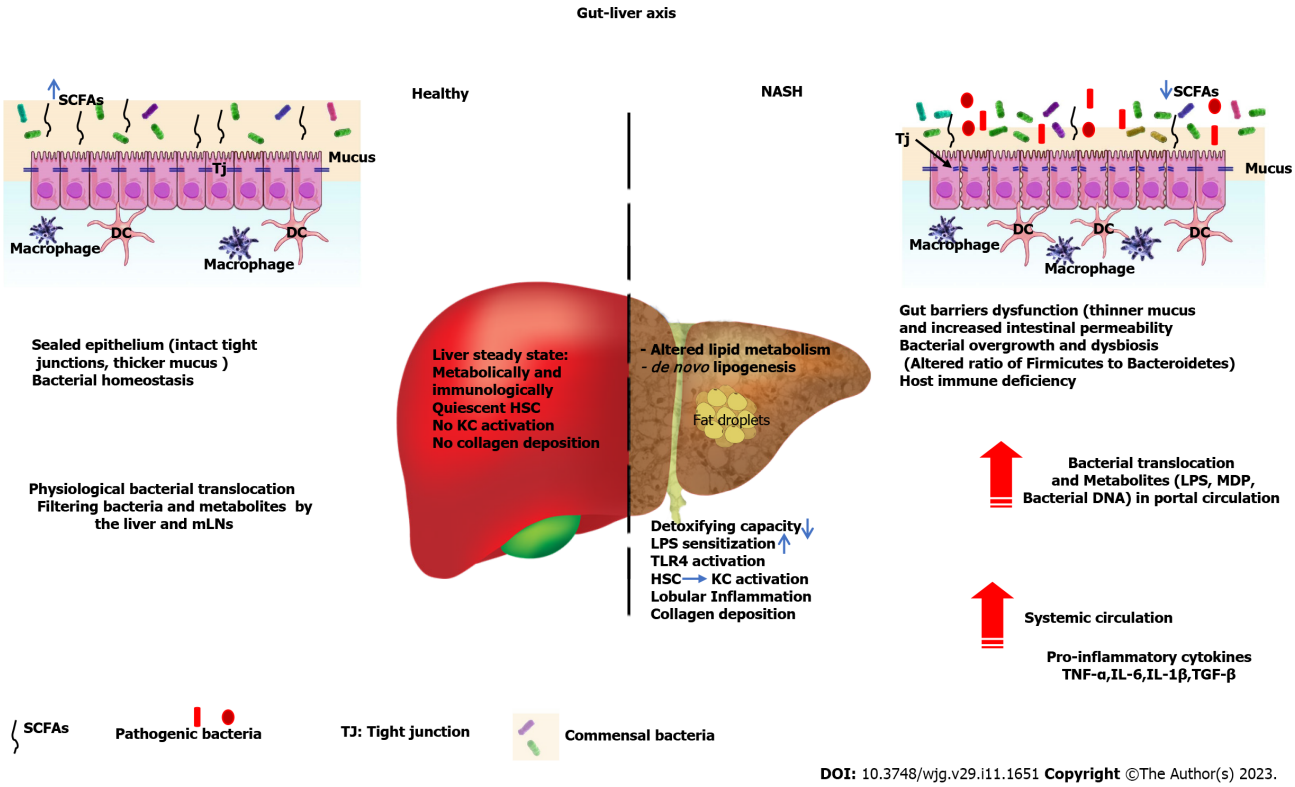

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Figure 1 During development of nonalcoholic steatohepatitis, several immunological and metabolic pathways intersect, thus promoting progression of liver injury and nonalcoholic steatohepatitis. In healthy conditions, gut-liver axis homeostasis is guaranteed by intact intestinal epithelium barriers and proper liver-host immune functions that limit the translocation of bacteria and their metabolites. In nonalcoholic steatohepatitis (NASH), on the one hand, the intestinal barriers are disrupted (thin mucus layer, decreased expression of tight junction proteins, altered ratio of Firmicutes to Bacteroidetes, dysbiosis, decreased short-chain fatty acids that result in increased leakage of bacteria and their metabolites (Lipopolysaccharide, MDP, flagellin, bacterial DNA) into the portal vein and systemic circulation, consequently stimulating the production of inflammatory cytokines in the systemic circulation. On the other hand, liver function is compromised because of the accumulation of fat, altered lipid metabolism, and increased microbial burden, which in turn elicits hepatic inflammation, hepatic stellate cell activation and collagen deposition, Kupffer cell activation, and triggering of the toll-like receptor 4 signaling pathway, which altogether contribute to the development of NASH. Tj: Tight junction; SCFAs: Short-chain fatty acids; KC: Kupffer cell; HSC: Hepatic stellate cell; LPS: Lipopolysaccharide; mLN: Mesenteric lymph nodes; TLR4: Toll-like receptor 4; TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; TGF-β: Transforming growth factor-β; IL-1β: Interleukin-1β.

anaerobes *Bifidobacterium, Bacteroides* and *Clostridium,* which colonize most of the lower gut[4,5]. The neonatal microbiota is also influenced by the mode of feeding, where breast-fed babies show a more stable microbiota that has a higher copy number of *Bacteroides* and *Bifidobacterium* but a lower abundance of *Enterococcus* and *Streptococcus* species, while formula-fed babies have a higher abundance of *Clostridium, Streptococcus* and *Enterococcus*[6]. The early life microbiota only begins to take a semblance of adult microbiota when solid food is introduced and will remain relatively unstable until 3-5 years after birth[7]. The rapid expansion of early life microbiota and the adaptation to oxygen levels signify the earliest mechanisms for initiating gut microbial homeostasis[8].

The colon has the highest density of microbes in the gastrointestinal tract, harboring approximately 70% of all gut microbes, which are mostly members of the Firmicutes and Bacteroidetes phyla[9]. The Firmicutes to Bacteroidetes axis is important in maintaining gut homeostasis, as members of each phylum have specialized metabolic roles (*i.e.*, metabolism of sugar vs. indigestible fibers) that impact the microbiome and the host. It is believed that the role in homeostasis is optimized when the relative abundance is 80% Firmicutes and 15% Bacteroidetes[8,10,11]. However, the significance of this value and the actual impact it has on the host have been questioned by some researchers[12], emphasizing the importance of more research on the role of Firmicutes and Bacteroidetes in gut microbial homeostasis, health and disease. Nutrients, metabolic byproducts and the competition between exogenous microbes and commensals help prevent colonization of pathogens and maintain homeostasis. Different animal studies have shown that nutrient competition occurs between metabolically related microbiota members. For example, germ-free mice colonized with three human commensal strains of *Escherichia coli* (*E. coli* HS, *E. coli* Nissle 1917, *E. coli* MG1655) successfully prevented colonization of the cecum by the pathogen enterohaemorrhagic *Escherichia coli* (EHEC) EDL933, an *E. coli* 0157:H7 biotype, due to the three precolonized commensal biotypes outcompeting *E. coli* EDL933 for nutrients[13]. This colonization resistance was further shown to occur using multiple sugars as metabolic substrates for probiotic *E. coli* Nissle 1917 and commensal subtype *E. coli* HS, whose rapid growth effectively limited the colonization of EHEC *E. coli* EDL933 in a mouse model[14]. Competition for a shared nutritional niche of proline was

similarly demonstrated in a gnotobiotic mouse model colonized with early life microbiota where early-life *E. coli* 1 was shown to outcompete *E. coli* 0157:H7[15]. This colonization resistance was also thought to be attributed to the production of lactate and acetate by bifidobacteria and enterococci, which can suppress the motility of *E. coli* 0157:H7 under cecal anaerobic conditions[15]. Colonization resistance is also aided by the production of toxic antimicrobial peptides by commensals. For example, many members of the phylum Bacteroidetes produce toxic antimicrobial peptides through their type 6 secretion systems (T6SS)[16], *E. coli* produces narrow-spectrum antibiotics called microcins that effectively kill competitors within their niche[17,18], and the probiotic *Bifidobacterium* secretes broad-spectrum bacteriocins[19].

Overall, any extrinsic or intrinsic factors that upset the stable microbial communities will in essence destabilize the colonization resistance mechanisms and lead to disease by allowing colonization of pathogenic microbes and/or leakage of microbes and microbial toxins into circulation.

COLONIZATION RESISTANCE THROUGH MICROBIAL ENHANCEMENT OF GUT BARRIER FUNCTION

The gut is lined with a thick mucus layer made of a highly glycosylated mucin 2 protein, which is densely packed and insoluble in the layer closest to the epithelium but loosely packed and soluble on the outer layer[20,21]. This mucus layer prevents direct contact of bacteria with the gut epithelium, thereby reducing the potential for pathogen colonization[20,21]. The development of the mucus layer is enhanced by the gut microbiota and depends on the intestinal microbial composition. It has been shown that germ-free rodents have a much thinner mucus layer than their conventionally colonized counterparts[22]. Petersson and colleagues have shown that a thin colon mucosal layer in a colitis germ-free mouse model can be restored by administering lipopolysaccharides or peptidoglycans to germ-free mice[22]. Bacteria enhance the mucus layer in numerous ways, such as through the production of secondary metabolites. Short chain fatty acids (SCFAs), such as acetate produced by *Bifidobacterium* or butyrate produced by gram-positive Firmicutes such as *Faecalibacterium prausnitzii, Roseburia sp, and Butyricicoccus pullicaecorum*[23,24], are known to strengthen gut barrier function, normalize permeability, improve intestinal epithelium defense, protect against pathogenic infections, and reduce inflammation[25-28].

Intestinal epithelial cells are held together by a set of tight junction proteins that are molecules situated at the tight junctions of epithelial cells. The integrity of these tight junctions can be influenced by commensal bacteria and their effects on tight junction proteins. For example, *Lactobacillus rhamnosus* GG induces claudin-3 expression, *L. acidophilus* and *L. plantarum* stimulate the expression of occludin, and *Bifidobacterium infantis* preserves claudin-4 and occludin deposition at tight junctions[29,30]. In a mouse necrotizing enterocolitis model, *Bifidobacterium* was found to preserve claudin 4 and occludin localization in tight junctions, thereby preventing gut permeability[31]. In mouse models, probiotics have been shown to improve the integrity of the intestinal barrier, which has also been observed in Crohn's and colitis patients[28]. *In vitro* treatment of Caco-2 cells with the probiotic *E. coli* Nissle 1917 increases the expression and peripheral migration of ZO-2[32]. Treatment of Caco2 cells with the probiotic *Lactobacillus plantarum* MB452 increased occludin and cingulin gene expression[33]. These results indicate that *in vitro*, certain probiotics can improve gut barrier function. Maintaining the integrity of the intestinal barrier is essential due to the high levels of microbial lipopolysaccharide (LPS) present within the lumen of the gut, as LPS is a potent immunological signal that can induce an inflammatory cascade if detected systemically, which a healthy intestinal barrier effectively prevents[34]. Leakage of LPS and other microbial polypeptides into circulation due to dysbiosis can lead to inflammation in the liver (among other organs), which can lead to the development of liver disease[34].

COLONIZATION RESISTANCE AND BILE ACID METABOLISM

Pericentral hepatocytes primarily produce bile acids from cholesterol[35]. In humans, these acids are then transported to the gut, where they are dehydroxylated, epimerized, or dehydrogenated into different secondary bile acids, such as deoxycholic acid (DCA), ursodeoxycholic acid (UDCA), ursocholic acid, or lithocholic acid (LCA)[35]. In mice, murideoxycholic acid and hyodeoxycholic acid are also produced[35]. Secondary bile acids are known to bind to the intestinal farnesoid X receptor (FXR) and G-protein coupled receptor 5 (TGR5)[36]. Some bile acid metabolites have also been shown to have a contradictory effect on gut barrier tight junctions[36]. UDCA and LCA, for example, have opposing effects on the barrier of human colonic T84 cells[36]. Treatment of these cells with primary bile acid-chenodeoxycholic acid (CDCA) combined with LCA leads to an increase in barrier permeability and the inflammatory cytokine IL-8[37]. Using a Caco-2 cell model, it was demonstrated that DCA led to an increase in the phosphorylation of epithelial growth factor receptor, which induced barrier dysfunction[38]. Prematurely weaned piglets treated with CDCA showed an improvement in the gut

barrier with higher ZO-1 expression and increased expression of the proinflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-6 and the anti-inflammatory cytokine IL-10[39]. The authors speculated that the anti-inflammatory effects of both IL-10 and ZO-1 counteracted the inflammatory effects of IL-6 and TNF-α, thus precipitating a net improvement in the intestinal barrier[39]. These examples demonstrate that bile acid metabolism is a significant key player in gut health, and it can be utilized as a therapeutic target for liver disease and other metabolic disorders, as will be discussed later.

MICROBIAL ASSOCIATION WITH LIVER DISEASE

Liver disease has been shown through preclinical and clinical trials to be accompanied by gut dysbiosis [40-44]. It has been shown that liver cirrhosis is also correlated with bacteremia, increased gut permeability, and increased circulatory LPS[43]. Dysbiosis has been noted in many mouse models of liver disease, such as secondary biliary fibrosis (common) induced by bile duct ligation, alcoholic liver disease induced by alcohol uptake in drinking water and hepatotoxicity-induced liver cirrhosis using carbon tetrachloride (CCL_4) treatment[42,43]. In humans, several gram-positive bacteria, including members of the genera *Clostridium* XI, *Anaerobacter*, *Streptococcus*, and *Lactobacillus,* were found to be more abundant in the gut in NAFLD patient biopsies than in healthy volunteers[45]. In contrast, *Oscillibacter* and *Flavonifractor* of the family *Ruminococcaceae* were abundant in healthy volunteers relative to NAFLD patients[45]. In severe fibrosis forms of NAFLD, the bacteria *Bacteroidetes vulgatus* and *Escherichia coli* were identified as the most abundant[46]. Although there has not yet been a general consensus on what microbial ratios of different strains exist in NAFLD patients, many research findings indicate that a lower Firmicutes to Bacteroidetes ratio is associated with liver disease[11,47]. Dysbiosis may be caused by a reduction in bile acids (which are bacteriostatic) of a cirrhotic liver, which precipitates inflammation and immunosuppression, factors that can positively feedback on cirrhosis[42]. Dysbiosis may also arise from increased saprophytic fungal growth in the alimentary canal. Cirrhotic liver patients who routinely receive antimicrobial treatment have an overgrowth of fungi, especially *Candida,* leading to fungal-bacterial balance in the gut and worsening dysbiosis[42]. Although cirrhosis is a systemic disease, it is believed to be worsened by dysbiosis both in the gut liver axis and outside this axis, such as in saliva and serum[42,48].

While there is a knowledge gap on the use of microbial interventions for NAFLD therapy, there are data showing that nonalcoholic steatohepatitis (NASH) patients improve following treatment with the antibiotic rifaximin, which is used for the treatment of traveler's diarrhea caused by *Escherichia coli*[49]. In a study examining the gut microbiota of stage 4 hepatitis C virus (HCV) patients, *Prevotella* and *Faecalibacterium* were found to be more abundant in HCV patients than in healthy controls, while *Ruminococcus* and some *Clostridium* species were more abundant in healthy controls than in HCV patients. *Bifidobacterium* was found only in healthy individuals[50]. Germ-free mice were shown to develop NAFLD following fecal microbial transplantation from donor hyperglycemic mice with systemic inflammation when fed a high-fat diet[51]. On the other hand, germ-free recipients that received fecal transplantation from normal donors (*i.e.*, normoglycemic with negligible systemic inflammation) did not develop NAFLD and were normoglycemic when fed a high-fat diet[51]. Rabot *et al*[52] also showed that germ-free mice fed a high-fat diet were more resistant to hepatic steatosis than colonized controls. In an experimental mouse model of cholestasis-induced liver fibrosis induced either through bile duct ligation or by CCl_4 treatments, colonization with complex microbiota (specific pathogen-free mice) was protective against severe fibrosis when compared to limited colonization (Altered Schaedler Flora)[53]. How the gut microbiota induces a leaky gut, bacteriaemia and an inflammatory flare leading to liver disease has been the subject of intense research. Brown and colleagues fed mice a high carbohydrate diet to induce a leaky gut[54]. This high carbohydrate diet caused a sloughing of the intestinal villi and reduced tight junction integrity, which allowed bacteria to translocate into the circulatory system[54]. In cirrhotic patients, it has been shown that microbial components leaking through the intestinal barrier, such as LPS, lipoteichoic acid, lipopolypeptides, and peptidoglycans, activate Toll-like receptors (TLRs) in hepatic stellate cells, Kupffer cells, and hepatocytes (all of which are differentially populated with TLRs 1-9), inducing severe inflammatory responses and fibrosis in the liver[43,55] Microbial activation of TLR2 in monocytes has especially been identified as significant in liver fibrosis through the production of TNF alpha, which initiates a cascade of reactions leading to increased gut permeability[43].

MICROBIAL METABOLITES IN LIVER DISEASE

Gut microbiota-host crosstalk in liver disease remains widely unclear. However, in recent years, many studies have established a correlation between different microbial metabolites and liver disease[56]. LPS are gut microbiota-derived endotoxins that form the major component of the gram-negative bacterial outer cell wall. High plasma levels of LPS have been identified in NAFLD patients and are associated with gram-negative intestinal bacterial overgrowth and compromised gut lining epithelial tight

junctions[57,58]. LPS induces an inflammatory response by activating hepatic Kupffer cells through TLR4. Apart from inducing proinflammatory cytokines and chemokines from hepatic Kupffer cells, LPS also activates hepatic stellate cells (HSCs) to differentiate into myofibroblast-like cells by producing extracellular matrix proteins, thus promoting liver fibrosis[49,59-61]. Other important metabolites are SCFAs from the fermentation of indigestible dietary fiber, which are mostly found in the colon, where most of them are produced and absorbed[62]. The major microbial fermentation products following microbial degradation of fiber are the SCFAs butyrate, propionate, and acetate. The body utilizes approximately 10% of the energy supply from microbially derived SCFAs, meaning that 90% is stored in white adipose tissue[63]. Several studies have revealed that gut microbial dysbiosis is associated with chronic liver diseases such as NAFLD or ALD[45,64]. In a metabolomic study in children with NASH, serum levels of 2-butanone and 4-methyl-2-pentanone were found to be elevated compared to those in healthy individuals[65]. Adults with NAFLD were found to have higher levels of fecal propionate and isobutyric acid, which are part of the fecal SCFA family[66]. Obese patients with NAFLD were also found to have high levels of propanoic acid and butanoic acid[67]. SCFAs such as acetate and butyrate modulate the host immune response by dampening the LPS-induced hepatocellular inflammatory response and restoring mucosal and systemic immunologic homeostasis, thus minimizing liver injury [68,69]. SCFAs can act as hormonal molecules by binding to G-protein-coupled receptors (GPCRs), which leads to activation of the GPCR pathway, slowing gut motility and increasing energy harvest[70-72]. Upon activation, glucagon-like peptide-1 is secreted from epithelial L-cells, enters circulation, and induces insulin release from the pancreas[70]. GPCR pathway activation also limits insulin-mediated hepatic and muscular fat accumulation and stimulates energy expenditure[71]. In adipocytes, SCFAs activate G protein-coupled receptor (GPR) 41 and GPR43 to inhibit lipolysis and activate adipocyte differentiation[70]. SCFAs also regulate immune cell functions through GPR43, which is widely expressed in most immune cells[73-75]. SCFAs have also been shown to inhibit histone deacetylases, which downregulate gene expression and reduce the production of inflammatory cytokines, particularly in macrophages and blood mononuclear cells during acute inflammatory hepatitis[69]. Therefore, it can be argued that dysbiosis that reduces microbial SCFA generation will result in a dysregulated inflammatory response and thus contribute to the progression of liver disease"

Indole and its derivatives are microbial metabolites of tryptophan breakdown. Indole upregulates tight junction proteins in the gut and downregulates colonic epithelium inflammatory genes through the aryl hydrocarbon receptor[76]. Indole-3-propionate activates pregnane X receptor to downregulate proinflammatory cytokine production and has been associated with protection against injury through oxidative stress signaling[76,77]. Indole-3-acetate has been shown to modulate hepatocyte lipogenesis, thus playing a protective role against NAFLD[78]. Microbial metabolism of dietary choline and L-carnitine produces trimethylamine (TMA), which is oxidized to trimethylamine N-oxide (TMAO) during hepatic detoxification of the blood through catalysis of the liver enzyme hepatic flavin monooxygenases[79]. TMAO is excreted in urine, and recent findings in animal NAFLD models fed a high-fat diet have shown increased urine levels of TMAO[80]. In a Chinese cohort study, the severity of NAFLD was closely associated with circulatory TMAO[81]. Bacteria are essential for the conversion of dietary choline to TMA, which is oxidized in the liver through the catalysis of hepatic flavin monooxygenase to generate trimethylamine-N-oxide, whose accumulation has been associated with both cardiac and renal disease[82,83]. Phosphatidylcholine is also metabolized by gut microbes to generate TMA, whose oxidation in the liver yields TMAO and, as previously described, may lead to kidney and cardiac disease[84,85]. It is now thought that accumulation of TMAO in the liver causes NASH through the inhibition of FXR and alteration of bile acid homeostasis[86]. SCFAs are significant microbial metabolites in the etiology of liver disease. More studies are required to target SCFAs as diagnostic or therapeutic tools for predicting or treating liver disease.

DIET AND XENOBIOTICS IN LIVER DISEASE

Liver disease is highly influenced by exposure to different environmental factors, which has recently been referred to as the exposome. It is now known that liver disease is impacted by an interaction between the genetic makeup of the host, exposome, and gut microbiome[87,88]. Certain types of gut microbiota have been associated with endogenous alcohol generation, which may in turn be hepatotoxic, leading to NASH[89]. The gut microbiota is important for the metabolism of bile acids, and in the absence or deficiency of bacteria that can convert primary bile acids to secondary bile acids, there is an accumulation of circulatory bile acids, which in turn activate TGR5, leading to monocyte dysfunction, which may exacerbate the hepatic inflammatory response and lead to liver disease[90]. High circulatory bile acids reflect a dysfunctional FXR, the nuclear receptor responsible for bile acid homeostasis, whose function is to facilitate enterohepatic bile acid circulation[91]. Dysbiosis affecting 7α-dehydroxylation-rich Firmicutes, which convert primary bile acids to FXR-low-binding secondary bile acids, will inevitably affect the function of FXR, leading to liver disease[92]. The liver is a crucial filter for toxins that find their way into the body either accidentally or deliberately. Alcohol is by far the most significant xenobiotic causing liver disease in humans, and it has been identified as the cause of ALD

[93]. It can be argued that alcohol consumption causes both destruction of microbial communities and rupture of the barrier wall integrity in the gut and leads to induction of inflammation during detoxification in the liver. A compromised gut barrier leads to leakage of LPS and other microbial ligands into circulation, triggering inflammation of liver cells.

A high-fat diet and environmental pollutants are further risk factors for liver disease, and their effects are exacerbated by microbial metabolites[94,95]. It is likely that most xenobiotics, in addition to being directly toxic to hepatic cells, will cause dysbiosis that favors changes in microbial composition that generate toxic liver disease-causing metabolites. The changes in these microbial metabolites may therefore be used as noninvasive diagnostic biomarkers for liver disease[96] but may also become significant therapeutic targets for the treatment of this disease[96-98]. A high carbohydrate diet has been demonstrated in environmental enteropathy animal models to lead to intestinal wall epithelial brush-border shortening and loosening of tight junctions[54]. Furthermore, small intestinal gram-negative bacterial overgrowth and high plasma LPS levels can lead to liver disease[56]. In the absence of dietary fibers, the gut microbiota cannot produce sufficient SCFAs, which may lead to a dysregulated inflammatory response and liver disease[99,100]. Most liver metabolism occurs through the catalysis of cytochrome P-450 (CYP-450), and it is known that many dietary biproducts can influence the activity of CYP-450[101]. Dietary retinoids, for example, are metabolized by hepatic cells, including hepatic stellate cells. An alteration in the uptake and metabolism of retinoids may influence retinoic acid signaling, which may activate hepatic stellate cells, resulting in loss of retinoid stores, aberrant extracellular matrix generation and the onset of fibrosis, which inevitably precipitate liver disease[102]. Additionally, alcohol consumption affects hepatic retinoid metabolism through inhibition of retinoid oxidation, induction of CYP2E1 enzymes to increase retinoic acid metabolism, or increased peripheral tissue damping of retinoic acid, all of which leads to activation of hepatic stellate cells and development of liver disease[103]. Retinoic acid is a gut microbial metabolite of vitamin A whose intestinal concentration is modulated by suppression of retinol dehydrogenase 7 expression by commensal *Clostridia* microbes[104]. Retinoic acid not only regulates bile acid homeostasis but also shares with it the receptors retinoid X receptor and FXR and therefore shares the functions of lipid metabolism and insulin sensitivity[105]. In a rat model, a high-fat diet in combination with high glucocorticoid treatment resulted in a fourfold hepatic lipid deposition and an almost threefold increase in circulatory alanine aminotransferase indicative of liver injury[106]. A high-fat diet also caused severe liver damage with high levels of circulatory alanine transaminases (ALT) and aspartate aminotransferases (AST) in a mouse model[107]. Mice fed a high-fat diet developed high intestinal gram-negative microbial growth and an increase in ethanol-producing bacteria when compared to mice fed normal chow[107]. This result is consistent with findings from clinical studies where it has been documented that microbial diversity rapidly changes with a change in diet[108]. Therefore, it can be concluded that diet, food additives, and xenobiotics affect liver disease by influencing gut microbial composition, gut permeability, and microbial metabolites. The liver plays a major role in metabolism and blood detoxification and is thus prone to damage from microbial endotoxins, environmental toxins, and microbial dietary metabolites, all of which work together in cascaded inflammatory responses to cause liver injury. Understanding the individualized microbial signatures and their influence on gut permeability, immunologic inflammatory responses, and the hepatic response to insult will expose multientry avenues to precision liver disease therapy.

MICROBIOME-HOST INTERACTION IN LIVER DISEASE

The intestine is heavily colonized with microbiota, yet the surrounding tissues remain sterile. This barrier is maintained by intricate crosstalk between gut microbes, the gut wall epithelium, and the innate immune system[109,110]. The expression of intercellular tight junction proteins between the intestinal epithelium is regulated by cytokines such as interferon gamma and TNF and other regulatory cytokines that interact with immunoglobulin A (IgA)-coated gut microbiota to maintain gut and immune homeostasis[111]. A change in diet or intake of xenobiotics such as alcohol, prescription/over-the-counter drugs, or other environmental chemicals may lead to destabilization of the intestinal homeostatic environment either through selective overgrowth or reduction of specific microbial strains or injury to the mucosal lining. A destabilization of the homeostatic environment will give way to a shift in the immunological signaling molecules protective to the gut tight junctions and a sloughing of intestinal villi. This breach in the barrier allows leakage of microbial endotoxins into circulation and microbial translocation into the liver, thus triggering an immunological inflammatory response once the microbial products are detected by the liver's pathogen recognition receptors, mainly the TLR and nucleotide oligomerization domain-like receptors[109,112]. HSCs are endowed with TLR 2, 4, and 9, which are associated with promoting TLR4 fibrosis[43,60]. Kupffer cells are lined with TLR 2, 3, 4, and 9 and are hepatic macrophages that form the main targets of microbial ligands within the liver[43]. Furthermore, hepatocytes express TLR 1-9 and are the most abundant cells in the liver, playing a critical role in the acute phase of the immunologic response through cytokine-like IL-6[113]. The inflammatory response of the liver to leaked gut-microbial endotoxins is not yet fully understood. However, it is

known that upon activation, Kupffer cells release proinflammatory and profibrogenic cytokines, such as TNF-α, Transforming Growth Factor (TGF)-β and IL-1β, and a few more members of the inflammasome whose effect is to induce inflammation and accumulation of lipids in the liver, and if this is not resolved, it leads to fibrosis NAFLD[114]. Therapeutic target efforts are geared toward minimizing the hepatic inflammation seen after proinflammatory cytokine release. Chemokine receptor antagonists such as C-C motif chemochine receptor (CCR) 2 and CCR5 [Cenicrivinoc (CVC)] have been used with some success to decrease leukocyte infiltration, and when used in a diet-induced NASH mouse model and a thioacetamide-induced fibrosis rat model, liver fibrosis was effectively reduced[115,116]. This outcome has since been replicated in phase 2 clinical trials with a remarkable reduction in fibrosis[117]. Several other proinflammatory cytokines, including IL-17, IL-11, and IL-1, are still under investigation. A clinical trial therapy utilizing an IL-1 pathway anti-inflammatory drug, diacerelin, achieved a remarkable reduction in fibrosis in NAFLD patients with diabetes[118].

THE GUT MICROBIOME AS A DIAGNOSTIC BIOMARKER FOR LIVER DISEASE

The dynamics of the gut microbiome could be used as a noninvasive diagnostic tool for liver cirrhosis and hepatocellular carcinoma (HCC)[119]. In a cross-regional prospective validation study in China, human fecal samples analyzed for microbial diversity revealed a significant rise in diversity as the liver condition advanced from cirrhosis to HCC with cirrhosis[119]. There was also a high level of butyrate-producing bacteria in healthy controls relative to early cirrhosis patients and a notable rise in LPS-producing bacteria in HCC patients[119]. In a different experiment, gut microbiota known to originate from the oral cavity were found to be enriched in liver cirrhosis patients relative to healthy volunteers [120]. In an Asian NAFLD cohort, *Ruminococcaceae* and *Veillonellaceae* species were found to be more predominant in NAFLD patients relative to healthy individuals[121]. These microbiome changes could not be associated with genetic predispositions known to influence NAFLD and were thought to be environmentally driven[121]. *Bacteroides* and *Escherichia* spp. have, on the other hand, been associated with liver fibrosis in NAFLD patients[122]. Overall, these multiregional studies indicate that there is great potential for the gut microbiota as a noninvasive diagnostic biomarker for liver disease with distinct indications of the staging of fibrosis and inflammation[121,123]. There is also great potential for the gut microbiota and associated metabolites to be utilized as therapeutic biomarkers[119-121]. It must, however, be appreciated that as of yet, a single microbial signature indicative of liver disease does not exist mainly because disease outcome is influenced by multiple factors such as diet, genetic background, age, and lifestyle (such as alcohol consumption), all of which must be considered while interpreting data on the predictive value of fecal microbiota on liver disease[124].

THERAPEUTIC APPROACHES

As we have discussed above, dysbiosis and a dysfunctional gut barrier promote the leakage of microbial endotoxins and components, as well as bile acid metabolites, into circulation, which can eventually lead to liver injury. Various therapeutic approaches (which are at various stages of testing) could be used to address these different factors for the treatment or prevention of liver disease, which will be highlighted below. Although SCFA supplements could be an attractive therapeutic approach in liver disease, their taste is normally not well tolerated. However, methods such as microencapsulation[125], either as soft gels or liquid capsules, are available that mask the taste of bitter medications and could be used for oral delivery of SCFA, which has the added benefit of being slow release and helps prevent evaporation of some volatile SCFAs, such as butyrate. Butyrate enemas have been used in a rat model, with the treatment group showing improved mucosal repair and reduced colonic damage compared to the untreated control groups[126]. However, butyrate enemas did not show any improvement in clinical studies with ulcerative colitis patients[127]. There is potential for the use of SCFA as a therapeutic approach, but more research is required to develop an optimal approach. Prebiotics such as inulin represent a substitute approach for the supply of SCFAs[98]. Multiple agonists of FXR are under investigation, including GS-9674 and LJN452, in phase 2 trials for NASH[98]. Some fibroblast growth factors (FGFs), such as FGF19 and FGF21, have shown encouraging results for NAFLD therapy[128,129].

Probiotic interventions

Treating dysbiosis and restoring homeostasis is complicated due to the wide range of associated factors that lead to a loss of important microbial populations or diversity in the first place. In most cases, treating dysbiosis with a single approach usually gives discouraging outcomes. However, studies involving probiotics have shown encouraging results in terms of safety, tolerance, and efficacy[130]. In a Phase 1 clinical trial, *Lactobacillus rhamnosus* GG administered to cirrhotic patients resulted in reduced *Enterobacteraceae* and increased relative abundance of *Clostridiales incertae* Sedis XIV and *Lachnospiriceae* with reduced endotoxemia and decreased pathogenic bacterial growth indicative of improved health

[131]. In another study using multiple probiotic strains, a reduction in inflammatory cytokine flares in cirrhotic patients was observed[132]. In obese, sonographically identified NAFLD children, treatment with a probiotic combination of *Bifidobacteria (B. bifidum and B. lactis)* and two *Lactobacilli* (*L. rhamnosus* DSMZ 21690 and *L. acidoph*ilus) strains significantly lowered intrahepatic fat content and ALT levels as well as AST relative to the placebo treatment[133]. This reduction in hepatic steatosis was replicated in NAFLD patients treated with a multistrain probiotic[134]. In another study, a twelve-week treatment of 30 NAFLD volunteers with six strains of bacteria containing *Bifidobacterium breve* and *B. lactis*, *Lactobacillus rhamnosus, L. acidophilus* and *L. paracasei pacasei* and *Pediococcus pentosaceus* in a randomized, double-blind, placebo-controlled study led to an improvement in proinflammatory cytokines, a reduction in cholesterol and a decrease in body weight[135]. When probiotics are mixed with compatible prebiotics, better outcomes have been achieved in clinical trials, but more studies are needed to determine the most effective combinations[136,137]. Hepatic steatosis has, for example, been reported to decrease in patients with NASH following symbiotic and prebiotic treatment. Serum alkaline phosphatase was decreased following treatment with probiotics, prebiotics and synbiotics[136] However, it is noteworthy that the outcomes are dependent on the composition of probiotics, the exposure time, and the dosage[136]. Studies in animal models have shown similar outcomes as in human studies. In rats fed a high-fat diet, treatment with *Bifidobacteria longum* or *Lactobacillus acidophilus* significantly reduced hepatic fat accumulation[138]. There was also a strong negative correlation between fat liver content and probiotic concentration in the stool[138]. In addition, hepatic steatosis was markedly reduced after 12 wk of treatment with *B. longum*, but this was not the case with *L. acidophilus* treatment[138]. In a diabetic rat model, treatment with *Akkermansia muciniphila* led to a decreased inflammatory response and improved liver function[139]. In hepatic encephalopathy, a mixture of *Lactobacillus plantarum*, *L. casei, L. delbrueckii* subsp. Bulgaricus, *Bifidobacterium infantis, B. longum, B, breve, and Streptococcus salivarius* subsp. Thermophilius has been associated with both primary and secondary prophylaxis[140,141]. Yogurts containing *L. bulgaricus, S. thermophilus, L. acidopilus* La5 and *B. lactis* Bb12 as well as a prebiotic mixture of fruco-oligosaccharides and *L. casei, L. rhamnosus, S. thermophilus, B. breve, L. acidophilus, B. longum,* and *L. bulgaricus* have been shown to improve aminotransferase in NAFLD patients[142-144]. In NASH patients, probiotics containing *L. bulgaricus* and *S. thermophilus* have also shown improvement in aminotransferase[145]. A combination of *B. longum* W11 and fructooligosaccharides, on the other hand, has shown improvement in aminotransferase and the histological score activity of NASH patients[146]

Fecal microbiota transplantation

Fecal microbiota transplantation (FMT) is the administration of a solution containing fecal material from a "healthy" donor into the intestinal tract of a recipient to modify that recipient's gut microbial composition for targeted health benefits[147]. To date, FMT has been successfully used in the treatment of recurrent *Clostridium difficile* infection, and there is growing evidence that FMT can be used to treat noninfectious diseases such as inflammatory bowel disease, obesity, and other metabolic disorders [147]. FMT has also been tried as a therapeutic option for liver disease. In a diet-induced steatohepatitis mouse model, FMT-treated mice showed increased SCFAs, improved expression of tight junction proteins, reduced proinflammatory cytokines and less intrahepatic lipid deposition compared to controls (*i.e.*, no FMT)[148]. There have also been several human clinical trials but with mixed outcomes, with some achieving a significant reduction in proinflammatory cytokines and improved gut barrier function and others not responding to therapy[149,150]. Future experiments should address the question of who qualifies as a healthy donor, how should we deal with the variation in gut microbial diversity among the recipients, and how best to package the product for better acceptability.

Bile acid metabolism

A recent study in mice indicated that during antibiotic-induced dysbiosis, the homeostasis of bile acids was equally destabilized[151,152]. Treatment of these mice with flavanones and total phenolic extracts of citrus aurantium L. (TPE-CA) restored bile acid homeostasis and gut barrier integrity[152]. TPE-CA also regulates the enterohepatic circulation entry of bile acids through the farnesoid X receptor-fibroblast-growth factor 15 pathway[152]. The effects of dysbiosis and increased intestinal unconjugated bile acid that are observed in ALD were reversed through improved FXR activity and gut barrier function following treatment with fexaramine, which is an intestine restricted FXR agonist. These results indicate that modulation of cyp7a1 and lipid metabolism can be achieved in a mouse model and thereby minimize ethanol-derived liver damage by targeting the bile acid-FXR-fibroblast-growth factor 15 signaling pathway[153]. Future experiments to verify these findings in higher mammals and translate the results to therapeutic interventions for human liver disease are warranted.

Precision microbial engineering

The mechanisms by which the intestinal microbiota influences the development and/or progression of liver disease are only beginning to unfold, but to fully elucidate the microbiome role in liver disease, a more comprehensive picture of the dynamics of the gut ecosystem is needed. Unfortunately, most of our knowledge about the intestinal microbiota arises from fecal or biopsy sample analysis, which is not

representative of the entire gut microbiome. However, novel technologies are being developed to address this knowledge gap. One such innovation is a capsule sampler and drug delivery system that is swallowed and utilizes mechanical gut peristaltic movements to guide the capsule down the entire length of the gut as the capsule collects samples[154]. Recently, a capsule robot was designed from a shape memory alloy spring with a chamber of a storage capacity of 500 μL, which showed enhanced sample preservation[155]. Another approach consists of an inexpensive 3D-printed sampler containing a hydrogel whose swelling ability seal and protects the liquid gut samples[156]. Such strategies that analyze small samples from various sites will provide information on microbiota distribution and will make microbial engineering and microbial targeting more feasible.

One such microbial engineering approach being developed is the use of Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR) Cas-based instructions to precisely cut off targeted genetic sequences of the microbial genome and thus change their function *in vivo*[157]. A conjugative plasmid, TP114, was recently used as a delivery vehicle for CRISPR-Cas9, targeted at drug-resistant *Escherichia coli* and *Citrobacter rodentium*, which led to full clearance of these organisms in a mouse model four days after administration[157]. More recent delivery systems for CRISPR-Cas9 have been designed to utilize probiotics as a genetically engineered conjugative vehicle that are more efficient and practical to use than bacteriophage-based systems[158,157]. The use of CRISPR-Cas9 as antimicrobial therapy is still in its early stages but has the potential to be an effective therapy for targeting specific, undesired microbes in the dysbiotic gut of liver disease. Other approaches to manipulate the gut microbiome are mucosal vaccines. IgA is the predominant antibody in the gut that binds to pathogens and commensals, preventing their translocation across the mucosal barrier. Using a probiotic-based mucosal vaccine with *Lactobacillus acidophilus*, Fox *et al*[159] showed that a potent, diverse IgA response could be elicited which could help with colonization resistance. In another study, Slack and colleagues designed an oral vaccine using genetically modified *Salmonella enterica* capable of setting evolutionary traps for prophylaxis treatment in a mouse model[160,161]. While this technology was advanced into a pig model and is currently being tested on human neonates to treat neonatal sepsis and necrotizing enterocolitis, it has hallmarks to be equally beneficial as therapeutic approaches for liver disease.

Diet and lifestyle changes as therapeutic targets

There are many therapeutic options for NAFLD that are being explored, some of which are in advanced levels of clinical trials; however, no treatment is yet available[124]. Diet and lifestyle changes remain the most effective methods of managing liver disease[162]. Low caloric diets, low carbohydrate intake and low protein diets have all been shown to be effective in the management of liver disease[163,162]. It should, however, be noted that dietary changes alone cannot achieve the intended long-term weight loss goals to reduce liver inflammation. It is rather a combination of correct diet and exercise that is most effective against NAFLD[162]. The response to dietary changes and exercise on both gut microbiota that are negatively associated with liver disease and the amount of fat in the liver is different between individuals and between races[164] The amount of *Bacteroides*, for example, is lower in Chinese NAFLD individuals after diet and exercise compared to people from the West, and this is correlated with lower hepatic fat[164]. It has also been noted that Bacteroides increases in obese volunteers but decreases in lean volunteers following exercise and diet intervention[165]. This is suggestive of personalized intervention approaches of diet and lifestyle changes[164].

CONCLUSION

The influence of the gut microbiome on various body systems has important implications for health and disease, such as liver disease. While the exact mechanisms by which the microbiome contributes to liver disease are unknown, there is strong evidence that translocation of various metabolites across the mucosal barrier plays a strong role, which is precipitated by a dysbiotic gut microbiota. Considering the importance of the microbiome in liver disease, powerful therapeutic options that can manipulate the gut microbiome are being explored. These approaches could have the potential for effective treatments for various stages of liver disease. More research needs to be done to understand the crosstalk between the microbiome and host as it relates to liver disease so that more effective and targeted preventative and therapeutic options can be developed.

FOOTNOTES

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REVIEW

Radiological findings in non-surgical recurrent hepatocellular carcinoma: From locoregional treatments to immunotherapy

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Abstract

Since hepatocellular carcinoma (HCC) represents an important cause of mortality and morbidity all over the world. Currently, it is fundamental not only to achieve a curative treatment but also to manage in the best way any possible recurrence. Even if the latest update of the Barcelona Clinic Liver Cancer guidelines for HCC treatment has introduced new locoregional techniques and confirmed others as well-established clinical practices, there is still no consensus about the treatment of recurrent HCC (RHCC). Locoregional treatments and medical therapy represent two of the most widely accepted approaches for disease control, especially in the advanced stage of liver disease. Different medical treatments are now approved, and others are under investigation. On this basis, radiology plays a central role in the diagnosis of RHCC and the assessment of response to locoregional treatments and medical therapy for RHCC. This review summarized the actual clinical practice by underlining the importance of the radiological approach both in the diagnosis and treatment of RHCC.

Key Words: Carcinoma; Hepatocellular; Liver; Ablation; Catheter; Radio frequency ablation; Ablation techniques; Medication therapy management; RECIST

Core Tip: During the follow-up of patients affected by hepatocellular carcinoma (HCC), radiology is considered the key to the diagnosis of recurrence, by taking advantage of cross-sectional imaging with a special focus on computed tomography and magnetic resonance imaging. As in the case of active surveillance in a patient with mild to moderate risk for developing HCC, cross-section imaging can help in the quick identification of signs of recurrence. Moreover, radiology plays a key role in the evaluation of treatment response during medical therapy for HCC, recently approved in the revised version of the Barcelona Clinic Liver Cancer staging.

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INTRODUCTION

Hepatocellular carcinoma (HCC) represents the sixth-leading cause of cancer-related deaths worldwide, and it is the most frequent primary liver tumor, accounting for about 85% of primary liver malignancies. Cirrhosis is the histological substrate on which 80% of HCCs arise[1]. According to the European Association for the Study of the Liver and the American Association for the Study of Liver Diseases, all patients with a high risk of developing HCC should undergo a surveillance program[2,3]. Treatment options with curative intent are liver resection (LR), locoregional treatments (LRT), or orthotopic liver transplantation (OLT), and the choice of treatment is influenced by intrinsic features of the lesion, aspects related to the patient, and medical and economic resources available in each center[4,5].

Many HCCs are detected at an intermediate or advanced stage, which are not eligible, at least in the first instance, for curative treatment. In such cases, several treatment options are available, which can also be used in a combined or sequential manner including local termoablation [radiofrequency ablation (RFA), microwave ablation (MWA)], traditional transarterial embolization with traditional chemotherapy or microparticles [transcatheter arterial embolization, transarterial chemoembolization (TACE), TACE with drug-eluting beads (DEB-TACE)], transarterial radioembolization (TARE), and stereotactic ablative radiotherapy[6]. Finally, in cases of metastatic disease, the most common and widely used and approved approach remains systemic therapy with sorafenib, a tyrosine kinase inhibitor drug implicated in several pathogenetic mechanisms[7].

However, even if the primary goal is to have a curative intent, recurrence rate after transplantation is between 8% and 21% despite the use of new predictive models[8]. By contrast to OLT, both LRT and LR suffer from a high recurrence rate (60%-80%). When occurring, tumor recurrence may be considered non-transplantable if it exceeds the transplantation criteria such as those defined by the alpha-fetoprotein or Milan/up-to-seven criteria. Non-transplantable recurrence is a major cause of precluding salvage OLT, which showed comparable overall survival (OS) to primary OLT in patients with HCC with compensated cirrhosis[9].

Even if the latest update of the Barcelona Clinic Liver Cancer (BCLC) guidelines[10] for HCC treatment has introduced new locoregional techniques and confirmed others as well-established clinical practices, there is still no consensus about the treatment of recurrent HCC (RHCC)[11]. For these reasons, the multidisciplinary approach should be considered to define the best option for each RHCC patient[12]. On this basis, this review summarized the actual clinical practice by underlining the importance of the radiological approach both in the diagnosis and treatment of RHCC.

LRT

To date, the available options for RHCC were similar to naïve-HCC options and include LR, OLT, and LRT for patients with liver-only recurrence, TACE, TARE, and stereotactic ablative radiotherapy for patients with unresectable disease, and systemic therapies or enrollment in clinical trials for patients with extrahepatic disease recurrence[13-15].

Ablative treatments

Since only 15%-30% of patients with RHCC are suitable for an LR due to progressive liver dysfunction,

presence of multiple nodules, tumor location, or donor shortage for LT, the ablative treatments play a crucial role in early-stage RHCC[16]. RFA for RHCC is a safe and feasible technique, offering no significant difference in OS compared to RFA for primary HCC[17]. As both RFA and LR are indicated in RHCC tumors with similar features, many studies have compared the two treatments.

Three interesting and recent meta-analyses[13,18] established that LR provided better outcomes than RFA, especially in long-term survival outcomes. RFA is associated with a decreased risk of major complications and requires shorter hospitalization time, a more cost-effective approach in comparison with LR. Moreover, in well-selected patients, RFA may be an optimal choice for RHCC with similar outcomes of LR, notably for a single lesion < 3 cm or in patients with three or fewer nodules, following the guidelines for primary HCC[10]. Also, other studies, including one randomized controlled trial[19], confirmed the same results[20-22].

RFA performances are found to be worse than LR in disease free-survival (DFS), because the LR may ensure removal of the tumor-bearing portal territory where micrometastases and microscopic vascular invasion are present and usually impossible to detect through external ultrasonography[13].

To overcome the shortcomings of RFA, MWA has been assessed in the treatment of HCC, as it produces significantly larger areas of necrosis, faster ablation times, higher intratumor temperature, less tumor seeding risk, and less susceptibility to heat-sink effect over RFA[15,23] (Figure 1). However, there are few studies about percutaneous MWA performance in RHCC. Only one has compared surgical MWA and LR for RHCC showing the safety and feasibility of surgical MWA for RHCC within 3 cm in size and no more than three nodules[24]. Nevertheless, MWA was proven to be superior to RFA[25] and competing with LR when the tumor is > 3 cm and < 5 cm and close to the large vessels[26]. During treatment of very early and early HCC, RFA, MWA, and cryoablation have substantially similar outcomes[23].

A multicentric randomized controlled trial comparing RFA with cryoablation in HCC < 4 cm reported no differences in terms of OS and DFS but found differences regarding local tumor control in favor of cryoablation (7.7% *vs* 18.2%, $P = 0.04$)[27]. While another study conducted on 3239 patients showed a significant advantage in liver cancer-specific survival for RFA[28]. Therefore, the results regarding cryoablation are still unclear[29]. However, data are currently lacking concerning outcomes following the use of cryoablation in RHCC, and future studies should be focused on these aspects.

TACE

TACE is the most common treatment modality used for RHCC following initial resection[16,17]. However, as with LR, appropriate candidates for TACE should be carefully chosen based on their hepatic reserve[16,30] (Figure 2). However, there may exist a significant risk of worsened liver dysfunction following TACE among patients who have undergone prior hepatectomy[15,16,30]. Scores such as up-to-seven criteria or biomarkers such as Mac-2 binding protein glycosylation isomer to assess liver fibrosis can be used to identify patients who tolerate TACE less[16,30].

Regarding TACE in RHCC, Zu *et al*[31] demonstrated that the 1-, 2-, and 3-year OS rates after TACE were 73%, 52%, and 32%, respectively, while the number of resected HCC nodules (≥ 2), size (> 5 cm) of the RHCCs, and the number of TACE sessions (≤ 3) are independent risk factors for poor outcomes after TACE for recurrent HCC. Comparing TACE in naïve-HCC and RHCC, Liu *et al*[32] showed that RHCC treated with TACE accomplished acceptable results. After the propensity score matching analysis, there were no statistically significant differences between the naïve-HCC group and RHCC group in objective tumor regression and disease control rate. On the other side, the RHCC group had a shorter median OS (24 mo *vs* 33 mo) and PFS (10 mo *vs* 12 mo) in comparison with the naïve-HCC group.

Since it is a non-curative treatment, a recent meta-analysis demonstrated that TACE had worse outcomes (OS and DFS) than liver transplantation, LR, and RFA in RHCC patients[33]. Even comparing the two LRTs, Gou *et al*[34] showed that RFA had better short-term and long-term OS than TACE. Conversely, TACE may improve survival in patients with inoperable tumors, with large lesions or multifocal RHCC (beyond the Milan Criteria), and early (< 1 year) recurrence[35,36]. Interestingly, TACE proved to be a more effective option than LR/RFA in RHCC of BCLC stage 0 or A with microvascular invasion, especially in those that recur early after curative resection[37].

Among transarterial procedures, DEB-TACE, which uses doxorubicin, and TARE, using yttrium-90-labeled spheres, have been developed[12]. Even if it has been demonstrated that DEB-TACE facilitates higher concentrations of drugs within the target tumor and lower systemic concentrations with fewer adverse events than conventional-TACE in the management of HCC, especially on RHCC, there is no strong evidence showing the superiority of DEB-TACE over conventional TACE[38,39]. There are a lack of studies considering DEB-TACE as monotherapy for RHCC.

TARE may be an option for intermediate or advanced-stage HCC. It could also be used as an alternative to TACE especially for patients with portal vein thrombosis or for patients with earlier stages who are not eligible for curative procedures[16]. It is a safe and effective procedure for RHCC following LR, with satisfactory outcomes (median time-to-progression and OS were 11.3 mo and 22.1 mo, respectively)[40].

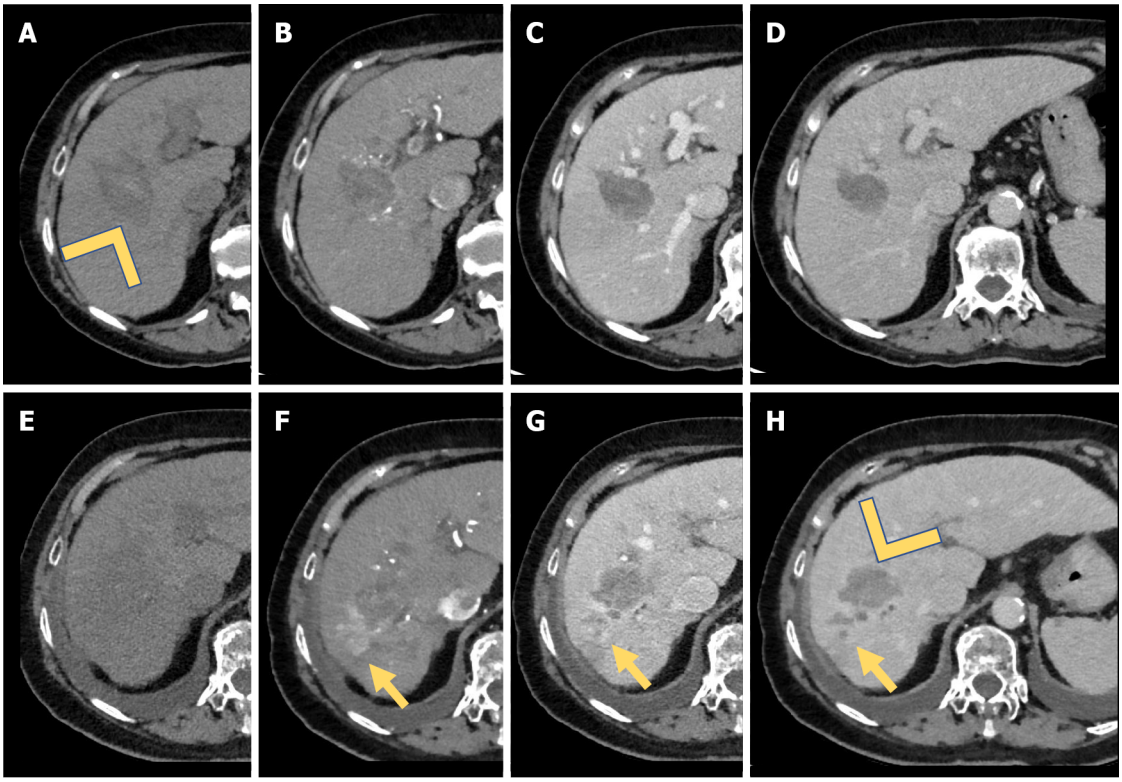

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Figure 1 Computed tomography study for assessment of treatment response (after microwave ablation). A: A 65-year-old male underwent microwave ablation of a hepatocellular carcinoma located in the V-VIII hepatic segment. Computed tomography scans were acquired after 2 wk of treatment. A large hypoattenuating area in the unenhanced (arrowhead) phase located in the V-VIII hepatic segment represented the treatment zone; B-D: During the dynamic study, no enhancement during the arterial phase (B) was seen, underlying the complete treatment response. Also, during the portal venous phase (C) and delayed phase (D) no wash-out was seen; E-H: After 1 year, the area of treatment was less hypoattenuating in the unenhanced phase (E), with a pseudonodular peripheral area of hypervascularization during the arterial phase (F, yellow arrow), with a wash-out during the portal venous and delayed phases (G and H, yellow arrow). On the other hand, the area of treatment did not show any arterial phase hyperenhancement or wash-out (H, arrowhead). The final diagnosis was hepatocellular carcinoma recurrence after microwave ablation (yellow arrows).

Combined therapies

Since RHCC frequently requires aggressive treatment to reach good therapeutic outcomes, the combined approaches have been evaluated by several studies for RHCC[16]. It has been proven that TACE alone is unable to cause complete tumor necrosis[41] and that RFA cannot detect satellite lesions [13]. Therefore, combined therapies may have a synergistic effect and be beneficial for patients with RHCC. TACE-RFA combined treatment can cause tumor necrosis up to 7 cm in diameter in one session [42].

The combination of TACE and RFA leads to theoretical advantages over either monotherapy. TACE can reduce the heat sink effect of the RFA, thereby increasing the ablation range. On the other hand, satellite lesions can be detected through TACE[41]. Furthermore, TACE with the intralesional accumulation of radio-opaque iodized oil used or drug-eluting beads increases the echogenicity and conspicuity of small HCC, otherwise hardly visible on ultrasound (US) guidance during RFA[43].

Song *et al*[44] showed that TACE-RFA had better DFS in comparison with TACE alone in patients with RHCC ≤ 5 cm. However, there were no significant differences between the two groups in OS and adverse events. Ascites is a frequent complication in the TACE-RFA group (Figure 3). Moreover, TACE-RFA provides comparable local efficacy and long-term survival results for patients with RHCC after hepatectomy, both for tumor size < 5 cm and > 5 cm. Furthermore, the TACE-RFA group has fewer complications[41,45] and lower hospitalization time in comparison with the LR group[45].

Zhang *et al*[46] demonstrated that DEB-TACE combined with RFA can increase the survival of patients with RHCC. Notably, OS rates were similar to primary HCC, while DFS rates were lower. A recent study[47] comparing MWA-TACE with TACE alone for small RHCC showed that the 5-year PFS of the combined therapy (37.5%) was higher than that of patients receiving TACE alone (18.7%), while the cumulative OS rates at 5 years were 61.1% for TACE-MWA and 50.3% for TACE alone, with no significant differences. Song *et al*[44] and Ji *et al*[47] demonstrated that combined therapies improve tumor control but not long-term survival outcomes.

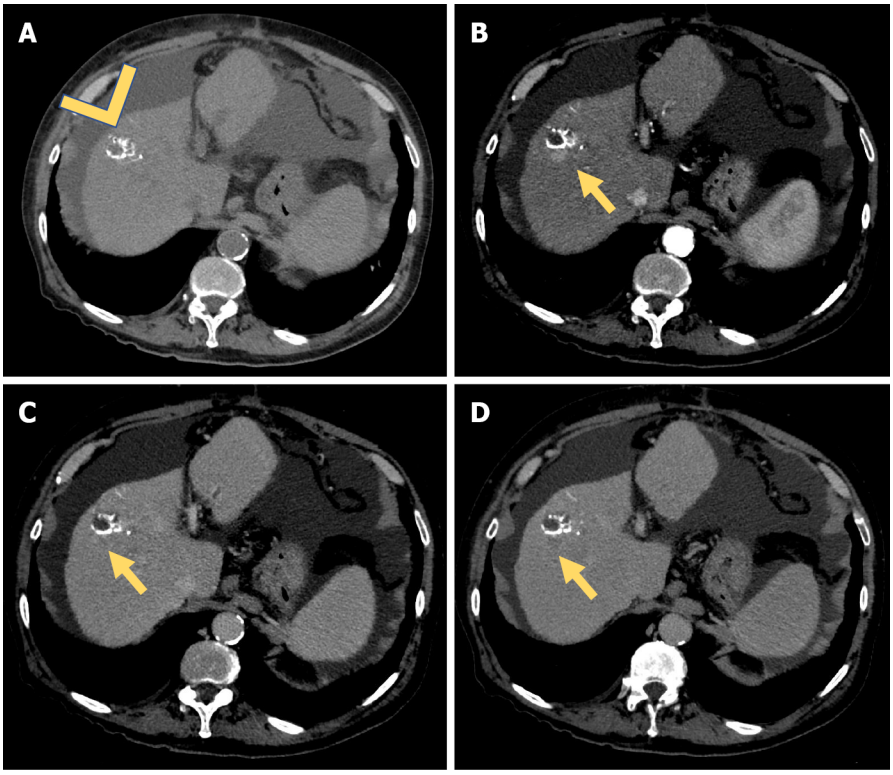

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Figure 2 Computed tomography study for assessment of treatment response (after transarterial chemoembolization). A: A 55-year-old male underwent conventional transarterial chemoembolization of a hepatocellular carcinoma lesion located in the VIII hepatic segment. Two years after treatment, a computed tomography scan showed areas of hyperattenuating components in the unenhanced phase (A), representing the ethiodized oil (arrowhead); B-D: During the arterial (B) phase, a pseudonodular area of hypervascularization during the arterial phase (B, yellow arrow) was seen, with a slight hypoattenuating appearance during the portal venous phase (C) and a clear washout during the delayed phase (D). This represents an example of recurrent hepatocellular carcinoma after transarterial chemoembolization.

MEDICAL THERAPY

Since 2007, sorafenib represented the standard medical treatment of advanced HCC[48] (Figure 4). Sorafenib was the first multityrosine-kinase inhibitor, blocking different receptors, including Raf, the vascular endothelial growth factor, and platelet-derived growth factor, expressed by signaling pathways in HCC. Considering its large approval worldwide, sorafenib was employed not only for patients in an advanced stage of the disease but also as a bridging therapy to downstage the disease and include patients in the transplantation list[49].

Currently, the clinical landscape for patients with advanced liver cancer has changed quickly. Different agents were approved for clinical use, including lavatinib, cabozantinib, regorafenib, and ramucirumab, all addressed to the aforementioned pathways[50]. Moreover, different signs of progress have been made in immunotherapy, in particular with the advent of immune check-point blockers. Nivolumab (anti-PD-1 antibody), pembrolizumab (anti-PD-1 antibody), tremelimumab (anti-CTLA-4 antibody), and atezolizumab (anti-PD-L1 antibody) were tested for advanced HCC[51].

In 2022, Reig *et al*[10] refreshed the BCLC strategy for prognosis prediction and treatment recommendations. It has been established that the first line treatment of advanced HCC should be based on a combined approach. Atezolizumab with bevacizumab (anti-vascular endothelial growth factor antibody) is currently the first-choice first-line treatment. Finn *et al*[52], in a global, open-label, phase 3 trial, demonstrated the best OS and PFS of the combined therapy in comparison with sorafenib alone. Conversely, the atezolizumab-bevacizumab treatment can be used in patients with compensated Child-Pugh A cirrhosis and risk of upper gastrointestinal bleeding.

The second-line treatment is not well established yet. If patients underwent sorafenib treatment, then it is possible to evaluate the benefit from regorafenib[53], cabozatinib[54], or ramucirumab[55]. If the second-line treatment cannot add a clinical benefit or is not feasible due to patient contraindications, then the third-line treatment with cabozatinib can be considered to increase OS[56]. Finally, if all previously mentioned cases are not manageable, patients should be enrolled in clinical trials. Clinical and laboratory data used to choose the preferred medical treatment are out of the scope of the present review.

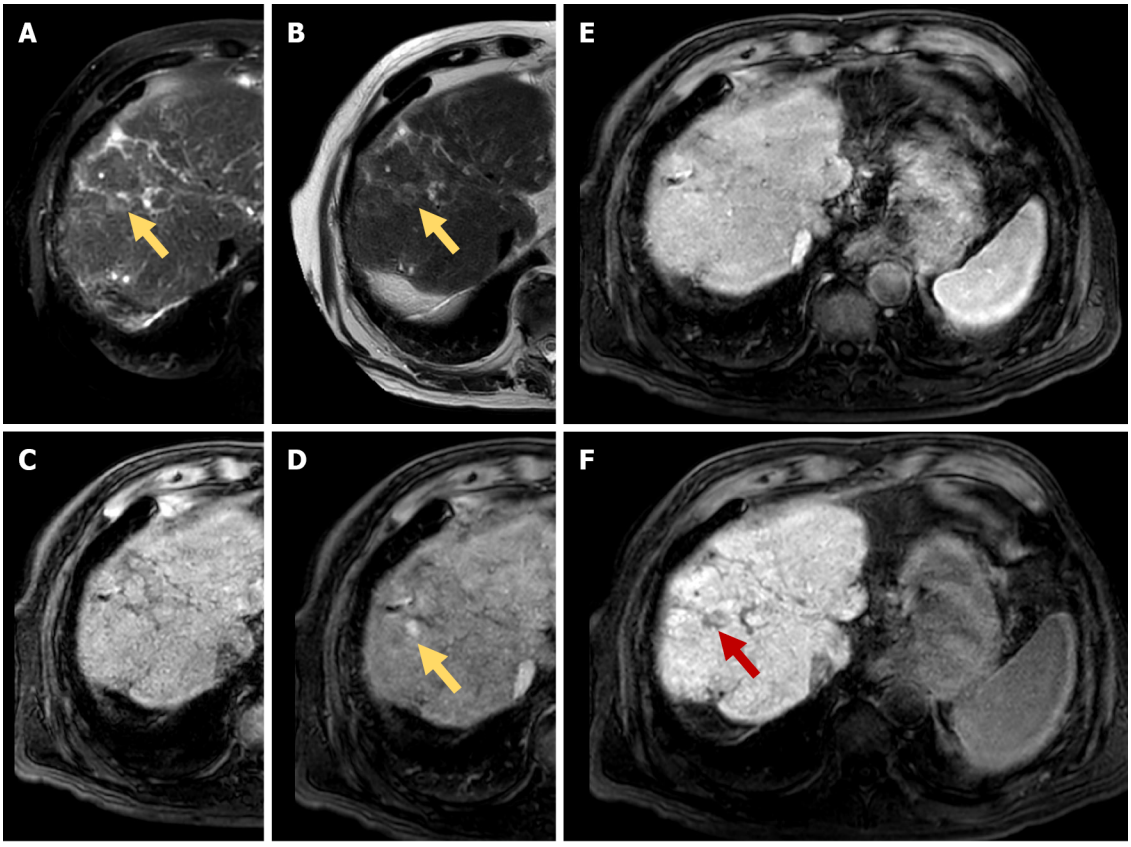

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Figure 3 Magnetic resonance imaging follow-up study with GD-EOB-DTPA for assessment of treatment response (after transarterial chemoembolization and radiofrequency ablation). A and B: A 70-year-old female underwent conventional transarterial chemoembolization-radiofrequency ablation of a hepatocellular carcinoma lesion located in the VIII hepatic segment. Eighteen months after treatment, confluent areas of hyperintense signal on T2 weighted imaging, with and without fat saturation, represented fibrosis. In this context a small slightly hyperintense nodular lesion was seen on T2 weighted imaging (T2 and T2 fs, yellow arrows); C-F: This lesion was isointense to the liver parenchyma in the unenhanced phase (C), with a non-peripheral wash-in appearance during the arterial phase (D), isointense during the portal venous phase (E), and hypointense during the hepatobiliary phase acquired after 20 min of Gd-EOB-DTPA administration (F). The final diagnosis was recurrent hepatocellular carcinoma after transarterial chemoembolization-radiofrequency ablation.

In this setting, patients who underwent LRTs should be followed up due to the risk of recurrence. In patients who underwent medical approaches it is important to monitor tumor response. All the above-mentioned medical strategies can determine apoptosis or necrosis of tumoral cells. One of the most important common findings to evaluate during follow-up is the change in tumor size. A significant increase in tumor volume or maximum axial diameter should be considered as a progression, according to the World Health Organization (WHO) criteria[56]. However, over time, different clinical studies were focused on the main issues related to the WHO classification. Consequently, RECIST 1.1 was introduced in clinical practice. However, RECIST 1.1 has some limitations, including the increase or decrease in size and necrosis, not being taken into account[57]. This last aspect is extremely important during medical treatments since the majority of drugs employed for HCC induce a reduction in tumor vascularization. For these reasons it is important to acquire images with complete protocols, to detect typical radiological findings of the primitive tumor, and to collect every significant change. First, increased dimensions of hypervascular areas or nodules should be considered as a main finding of tumor recurrence or progression[58,59]. To evaluate these, it is of utmost importance to acquire a correct arterial phase both on computed tomography (CT) and magnetic resonance imaging (MRI).

In 2014, Salvaggio *et al*[60] aimed to collect HCC enhancement changes after sorafenib treatment. The authors demonstrated that after medical treatment both arterial and portal venous enhancement was significantly reduced. In particular, the authors demonstrated that patients with partial response can manifest a greater decrease in arterial phase enhancement. However, they did not demonstrate the opposite. Patients with progressive disease did not show any statistically significant difference in arterial phase enhancement before and after treatment. To better understand the medical response, the international literature moved to the usefulness of MRI. Choi *et al*[61] reviewed the most common imaging findings of HCC during medical treatment by using MRI. The authors reported the importance of the hypervascular appearance during the arterial phase, as reported for CT. Moreover, MRI can help to detect early responders from non-responders by using diffusion-weighted imaging (DWI) and apparent diffusion coefficient maps, showing in the first group of patients an increase of DWI signal

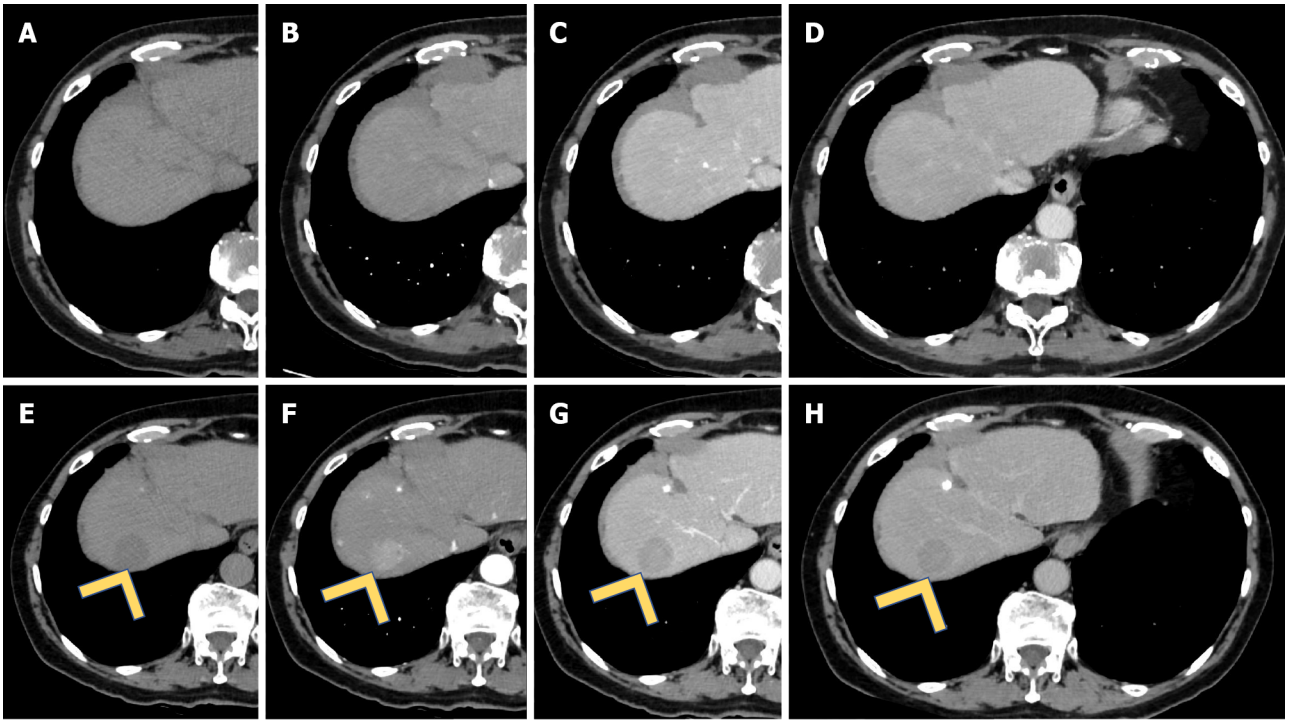

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Figure 4 Multiphasic computed tomography study for assessment of treatment response (after sorafenib). A-D: A 66-year-old female underwent conventional medical therapy (sorafenib) for an advanced hepatocellular carcinoma (HCC) lesion. Computed tomography images represented the complete response to the medical therapy, with no areas or nodular lesions suspected for HCC; E-H: During the follow-up, 2 years after completion of therapy, a nodular hypoattenuating lesion in the unenhanced phase (E) appeared in the VII hepatic segment. This lesion had similar features of primary HCC, with non-rim hyperenhancement during the arterial phase (F), wash-out during the portal venous phase (G) and delayed phase (H). This is an example of HCC recurrence after sorafenib.

with correspondence on apparent diffusion coefficient map due to necrosis and reduced tumor cellularity. Finally, MR can benefit from the usefulness of hepatobiliary contrast agents, as demonstrated in the SORAMIC trial[62]. However, by searching PubMed and EMBASE no important studies have been published yet about this promising added value, and future studies should be focused on these aspects.

The advent of all the above-mentioned strategies, alone or combined, introduced a new class of response[52]. While about 8% can show a hyperprogression, a new atypical response is included in the iRECIST criteria[63]. However, no predictive biomarkers can help clinicians to determine the risk of atypical response during immunotherapy, and only the radiological approach, both with CT and MRI, can help follow patients during the treatment. Even if in the past medical treatment was considered the last useful medical treatment in advanced HCC, different ongoing studies are testing a combination of only medical drugs and in combination with LRTs, such as TACE, as reported by Pinter *et al*[64].

Combined strategies may be useful in advanced RHCC. Peng *et al*[65] showed that sorafenib combined with TACE-RFA was superior to therapy with sorafenib alone concerning time to progression and OS in patients with RHCC with one intrahepatic tumor size ≤ 7 cm or ≤ 5 cm intrahepatic nodules, with each tumor ≤ 3 cm.

RADIOLOGICAL APPROACH TO RHCC

Radiology plays a central role in the assessment of patient response LRT for RHCC. The identification of viable tumor treatment guides for further management, and it potentially affects transplantation eligibility. In these instances, it is often helpful to engage in a multidisciplinary discussion to determine how to best manage each patient. The Liver Imaging Reporting and Data System (LI-RADS) was developed in 2011 to relay the likelihood of HCC on CT or MRI in a standardized manner, in patients at risk for HCC. In 2017, the LI-RADS treatment response algorithm (LI-RADS TRA) was introduced for the assessment of lesions that have been previously treated with LRT[66]. Unlike the prior response criteria RECIST and WHO that focus on disease progression on a systemic level, LI-RADS TRA is based on enhancement features to predict viability on a lesion level[67]. Although modified RECIST (mRECIST) has historically been used for the evaluation of HCC after locoregional therapy, differences from LI-RADS TRA include a lack of equivocal category and a lack of additional features for diagnosing tumor viability[68]. mRECIST uses the presence of arterial enhancing components alone to diagnose

viability while LI-RADS TRA includes additional imaging features such as washout during the portal venous or delayed phases and enhancement similar to pre-treatment to define viable tumors and encompass the equivocal category in addition to the binary evaluation[69].

RECOMMENDATIONS FOR HCC RECURRENCE DETECTION

Non-invasive imaging is superior to any other method for the surveillance of patients at risk of developing RHCC, either after OLT or other curative treatments. However, robust data lacks the optimal follow-up schedule of HCC-treated patients. Notably, international guidelines slightly differ in the recommended follow-up intervals, ranging from 3 mo to 6 mo, and duration of cross-sectional imaging after curative treatments. The National Comprehensive Cancer Network panel recommends ongoing total-body surveillance with multiphasic cross-sectional imaging (*i.e.* CT or MRI) every 3 mo to 6 mo for 2 years, then every 6 mo to 12 mo after curative therapies[70]. The 2018 Practice Guidance by the American Association for the Study of Liver Diseases suggests surveillance for HCC recurrence in posttransplant patients with abdominal and chest CT scan, though timing and duration as well as the impact of surveillance are not univocally defined[71]. After ablative therapies, the American Association for the Study of Liver Diseases recommends surveillance with contrast-enhanced CT or MRI every 3-6 mo[71].

The 2018 European Society for Medical Oncology Clinical Practice Guidelines were endorsed by the pan-Asian consensus conference, which included experts from several Asian societies. However, the Asian-adapted version slightly changed the follow-up timing after curative treatment, limiting the 3-mo interval by dynamic CT or MRI studies to the 1st year instead of 2[72,73]. Also, the European Association for the Study of the Liver recommends a follow-up after resection with curative intent with 3-4 mo intervals limited to the 1st year after treatment, with a return to regular surveillance thereafter[4].

Interestingly, Kim *et al*[74] found that HCC patients who undergo curative treatments with complete response and who present with increasing alpha-fetoprotein levels have a high probability of impending tumor recurrence even in the presence of a negative MRI. The follow-up schedule proposed within the European Society for Medical Oncology guidelines for patients treated with TACE or systemic therapies includes contrast-enhanced CT or MRI every 3 mo[74].

All the above-mentioned guidelines converge on the equivalent role of CT and MRI in clinical practice, given that the most important aspect for the diagnosis of HCC is the definition of criteria with the highest achievable accuracy, regardless of the imaging technique. Erkan *et al*[75] reviewed 3491 pathologically examined liver lesions, either studied by CT or MRI, comparing the diagnostic performance of different non-invasive diagnostic criteria of HCC. They found no statistically significant differences among criteria in diagnostic accuracy, with LI-RADS performing the best in terms of sensitivity and accuracy. Nevertheless, though CT and MRI have comparable performance in clinical practice, they present specific features to be considered.

CT

CT has the advantage of being the most practical and widely available tool to perform surveillance in HCC-treated patients. Its main limitations consist of ionizing radiation exposure and iodinated contrast agents-related nephrotoxicity. The detection and characterization of liver nodules with conventional contrast-enhanced CT is substantially limited to the size, morphology, and enhancement pattern of the lesions, which are sufficient elements to reach a confident diagnosis according to LI-RADS. RHCC imaging findings are analogous to the primary lesion. In particular, the typical hallmarks in the imaging diagnosis of RHCC are the combination of hyperenhancement in the arterial phase and washout on the portal venous or delayed phases[4]. Several studies and meta-analyses have compared the performance of CT with other imaging techniques. In a multicenter prospective trial including 544 nodules in 381 patients, the sensitivity and specificity for the diagnosis of 10-20 mm HCC nodules were 67.9% and 76.8%, respectively, while for the 20-30 mm HCC nodules, the sensitivity and specificity were higher (71.6% and 93.6%, respectively)[76]. In a meta-analysis, CT had an overall sensitivity of 72% with a subgroup analysis revealing a sensitivity of 31% *vs* 82% for sub-centimetric lesions compared to ≥ 1 cm ones[77]. Of note, this data did not consider the prevalence of HCC diagnosis in HCC-naïve patients compared to previously treated patients, for whom the pre-test probability of disease is expected to be increased. A multicenter prospective study that enrolled patients scheduled for liver imaging before surgery showed a sensitivity of 70%[78].

MRI

The accuracy of MRI in detecting HCC, especially small nodules, is superior to that of CT as shown by several studies and meta-analyses, one of which reported a sensitivity of 82% compared with 66% of CT and a comparable specificity[4,79]. However, MRI is yet to be definitively recommended over CT, given that the quality of the available evidence is considered low[79]. Moreover, a distinction between extracellular contrast agents (ECA) and hepatobiliary contrast agents (HBCA) should be considered. Analogous to those used in CT, ECA detects and characterizes lesions through the enhancement pattern.

Conversely, HBCA provides information on the hepatocellular function and bile excretion. Typical nodule hypointensity against a strongly enhanced background parenchyma in the hepatobiliary phase increases RHCC conspicuity and delineation, facilitating detection and consequently the diagnosis[80]. Despite this advantage, it must be pointed out that, if considered alone, hepatobiliary phase imaging is non-specific. Therefore, it always requires interpretation together with the dynamic study[81]. Martino *et al*[82] reported significantly higher diagnostic accuracy, sensitivity, and negative predictive value when dynamic and hepatobiliary phase MRI were combined compared to CT and dynamic phase MRI alone; a particular diagnostic benefit was obtained for lesions between 1 cm and 2 cm.

Nevertheless, although most HCC lesions are typically hypointense during the hepatobiliary phase, about 5%-12% HCC lesions can be hyperintense, owing to the overexpression of OATP[81]; conversely, some benign nodules may show no contrast uptake[83]. The knowledge of the pathological features of the originally treated nodules may predict the behavior of recurrent disease on hepatobiliary phase imaging, improving diagnostic confidence.

Different HBCA molecules have specific pharmacokinetic profiles. Gadoxetate disodium presents a 50% hepatic excretion, which contributes to an early liver parenchyma enhancement. Conversely, gadobenate-dimeglumine has a 3%-5% hepatic excretion that delays the hepatobiliary phase imaging onset. As a consequence, gadoxetate disodium does not provide a conventional delayed vascular phase but instead shows a transitional phase that lasts for several minutes, representing a transition from extracellular-dominant to intracellular-dominant enhancement[81]. Interestingly, Yim *et al*[84] recently observed that, in a retrospective cohort of patients who underwent both ECA and HBCA, RHCC was diagnosticated with higher accuracy using ECA.

DWI has been shown to improve the accuracy of RHCC detection, especially when combined with gadoxetic acid-enhanced imaging[85,86]. Finally, a recent meta-analysis confirmed that DWI may improve the ability to detect residual HCC or RHCC after TACE[87].

MRI likely has the highest accuracy compared to other imaging techniques in the detection of small recurrence after curative treatments[43]. However, results interpretation according to the standard LI-RADS may suffer from reduced sensitivity and specificity for disease recurrence detection. Wang *et al*[88] found that non-rim arterial phase hyperenhancement and three ancillary features (hepatobiliary phase hypointensity, mild-moderate T2 hyperintensity, and restriction of diffusion) were significantly related to RHCCs < 20 mm and concluded that the characterization of < 10 mm recurrence may show improved specificity compared with the LI-RADS 4 category combining at least two ancillary features. However, in patients treated with systemic therapies, according to the mRECIST criteria, new HCC lesions must measure at least 1 cm to define disease progression[58]. Despite the high sensitivity of MRI to detect recurrence after curative treatments, it has been shown that small viable RHCC may hide behind false-negative studies. This warrants regular short-term imaging surveillance[89].

However, in the absence of evidence to recommend a particular method or contrast agent over the other, practitioners are encouraged to base the choice on their judgment on an individual basis, considering the local availability of resources, personal experience, and imaging features of the previously-treated HCC[71].

Contrast-enhanced US

The use of contrast-enhanced US (CEUS) is encouraged as it has been demonstrated that its specificity can be even superior compared to CT/MRI[76]. Although CEUS is inferior to both CT and MRI in terms of objectivity and panoramic view, it provides advantages in cases of renal dysfunction and iodine allergy. The current indications for CEUS are multiple, the most important of which are equivocal or inconclusive findings on CT or MRI studies and assessment of treatment response after TACE or ablation[90]. Bansal *et al*[91] proposed an algorithm with alternating MRI and CEUS for secondary surveillance following potentially curative therapy of HCC. In their prospective studies, the authors found similar diagnostic performance of the two techniques; of note, CEUS was able to confirm or disprove equivocal findings on MRI. The comparable diagnostic performance of CEUS, CT, and MRI was previously reported[92].

It has been reported that RHCC may differ from the initial tumor at imaging, and this may help to distinguish recurrence form residual diseases, which may have a prognostic relevance. Wu *et al*[93] recently described different CEUS patterns of RHCC compared to initial tumors: Among the others, more homogeneous enhancement, poorly defined borders, and marked washout were found to be typical features of recurrent disease.

The application of artificial intelligence and radiomics to preoperative CEUS has recently gained large interest, and it has been demonstrated to potentially predict the prognosis in terms of HCC recurrence and overall survival[94-97]. CEUS, added to other conventional US-based techniques, has also shown the ability to improve the prediction of microvascular invasion, which is probably the most important factor associated with a worse prognosis[98]. Finally, CEUS can be useful as guidance for ablative therapies, especially to target recurrence of previously treated lesions[99,100].

Perfusion CT/MRI

Perfusion imaging does not have a definite role in clinical practice, and it is mainly performed for investigative purposes. Although several authors have independently demonstrated that perfusion CT-

derived parameters can discriminate between normal liver parenchyma, HCC, and hypervascular pseudolesions[101-103], they are yet to be included in clinical practice guidelines due to the absence of standardization among different centers[104]. However, perfusion imaging that provides quantitative parameters that could potentially be more reliable than qualitative/subjective parameters seems promising in the assessment of tumor response both to locoregional and systemic therapies[105-114].

Compared to CT, perfusion MRI has been investigated more regarding the possibility of predicting microvascular invasion of HCC before treatment. The microvascular invasion has been demonstrated to be correlated with poor outcomes of curative therapies due to higher rates of disease recurrence[115]. Perfusion MRI can be performed either with dynamic contrast-enhanced studies or with the intravoxel incoherent motion diffusion-weighted technique[116-119].

Nuclear medicine

The role of nuclear medicine in the diagnosis and staging of HCC is debated. If on the one hand there is insufficient evidence to recommend the use of fluorodeoxyglucose positron emission tomography preoperatively, it has been demonstrated that nuclear medicine studies are able to predict tumor aggressiveness and may aid in identifying those patients at risk for HCC recurrence after liver transplantation, resection, or ablation for better treatment allocation[120,121]. Fluorodeoxyglucose positron emission tomography, with or without CT, has also been shown to present low sensitivity but high specificity for diagnosing extrahepatic metastases or local residual/recurrent HCC after treatment [121].

CONCLUSION

On the one hand, the assessment of the response to LRTs has been widely described[121-123]. On the other hand, histologic modifications induced by molecular therapies may explain different imaging findings of recurrent disease. Differentiation between treatment-induced tumor necrosis and viable tumor with reduced arterial perfusion may be challenging. After treatment with systemic targeted therapy, the tumor may show areas of necrosis without any contrast enhancement that must be distinguished from areas of reduced but still unequivocal arterial uptake consistent with viable tumor[44]. Even RHCC under systemic treatments may present with atypical enhancing patterns, especially lacking arterial hyperenhancement, which makes radiological assessment more difficult. All these aspects should be considered, and multimodal imaging evaluation combined with multidisciplinary framework can improve image interpretation. Conventional non-invasive imaging techniques provide robust criteria for HCC residual/recurrence detection, with high accuracy, representing the current standard of practice. Advanced imaging tools, either hardware- or software-based, have a double potential role: to predict HCC treatment response or the risk of recurrence, to increase sensitivity, specificity, and thus operator confidence in early RHCC detection.

FOOTNOTES

Author contributions: Ippolito D and Maino C designed the research; Maino C, Gatti M, Marra P, and Cortese F performed the research; Maino C, Gatti M, Marra P, and Cortese F analyzed the data; All authors wrote the paper.

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REVIEW

Factors affecting the quality of bowel preparation for colonoscopy in hard-to-prepare patients: Evidence from the literature

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Abstract

Adequate bowel cleansing is critical for a high-quality colonoscopy because it affects diagnostic accuracy and adenoma detection. Nevertheless, almost a quarter of procedures are still carried out with suboptimal preparation, resulting in longer procedure times, higher risk of complications, and higher likelihood of missing lesions. Current guidelines recommend high-volume or low-volume polyethylene glycol (PEG)/non-PEG-based split-dose regimens. In patients who have had insufficient bowel cleansing, the colonoscopy should be repeated the same day or the next day with additional bowel cleansing as a salvage option. A strategy that includes a prolonged low-fiber diet, a split preparation regimen, and a colonoscopy within 5 h of the end of preparation may increase cleansing success rates in the elderly. Furthermore, even though no specific product is specifically recommended in the other cases for difficult-to-prepare patients, clinical evidence suggests that 1-L PEG plus ascorbic acid preparation are associated with higher cleansing success in hospitalized and inflammatory bowel disease patients. Patients with severe renal insufficiency (creatinine clearance < 30 mL/min) should be prepared with isotonic high volume PEG solutions. Few data on

cirrhotic patients are currently available, and no trials have been conducted in this population. An accurate characterization of procedural and patient variables may lead to a more personalized approach to bowel preparation, especially in patients undergoing resection of left colon lesions, where intestinal preparation has a poor outcome. The purpose of this review was to summarize the evidence on the risk factors influencing the quality of bowel cleansing in difficult-to-prepare patients, as well as strategies to improve colonoscopy preparation in these patients.

Key Words: Colorectal cancer; Colonoscopy; Adenoma detection rate; Bowel preparation; Polyethylene glycol

Core Tip: Almost a quarter of procedures are still performed with inadequate preparation. A strategy that includes a low-fiber diet for an extended period of time, a split preparation regimen, and a colonoscopy within 5 h of the end of preparation may improve cleansing success rates in the elderly. In addition, while no specific product is recommended for difficult-to-prepare patients, clinical evidence suggests that 1-L polyethylene glycol (PEG) plus ascorbic acid preparation is associated with higher cleansing success in hospitalized and inflammatory bowel disease patients. Isotonic high volume PEG solutions should be given to patients with severe renal failure.

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INTRODUCTION

One of the most commonly used techniques for diagnosing colorectal diseases is colonoscopy. Furthermore, it is important in colorectal cancer (CRC) screening because early detection is linked to a long-term reduction in malignancy incidence and mortality[1,2].

As is well known, the quality of a colonoscopy is entirely dependent on adequate bowel cleansing, which can affect diagnostic accuracy and the rate of adenoma detection (ADR)[3]. Inadequate bowel preparation, on the other hand, leads to decreased sensitivity to colonoscopy, increased procedural time, a higher risk of adverse events, and a greater likelihood of having to repeat the exam at a higher cost[4-7].

This is a critical topic because data from the literature show that a quarter of procedures still have suboptimal preparation[8]. Similarly, a large Italian study discovered that poor preparation occurs in approximately 17% of colonoscopies[9], and results from the United Kingdom screening program confirmed that inadequate preparation accounts for more than 20% of incomplete procedures[10]. Finally, data from a recent survey of sixty-four Italian screening centers revealed that only 29% of centers meet the minimum standard of at least 90% colonoscopies with adequate cleansing[11].

The type of bowel preparation, split-dose regimen, low-fiber diet, comorbidities, concomitant medications, inpatient status, and elderly age have all been found to affect the quality of bowel cleansing (Figure 1)[12]. Some of these variables, such as the type of solution, preparation regimen, and diet, are modifiable risk factors. In this regard, recent scientific advances have resulted in the introduction of new effective bowel cleansing solutions and reinforced the importance of specific preparation regimens.

On the other side, factors such as age or comorbidities are not modifiable and not susceptible to intervention. This makes it more difficult to intervene to increase the quality of bowel cleansing in specific conditions, also considering that guidelines do not provide specific recommendations for patients with multiple risk factors[13].

The purpose of this review is to summarize the evidence on the risk factors that influence the quality of cleansing in difficult-to-prepare patients, as well as strategies to improve colonoscopy preparation in these patients.

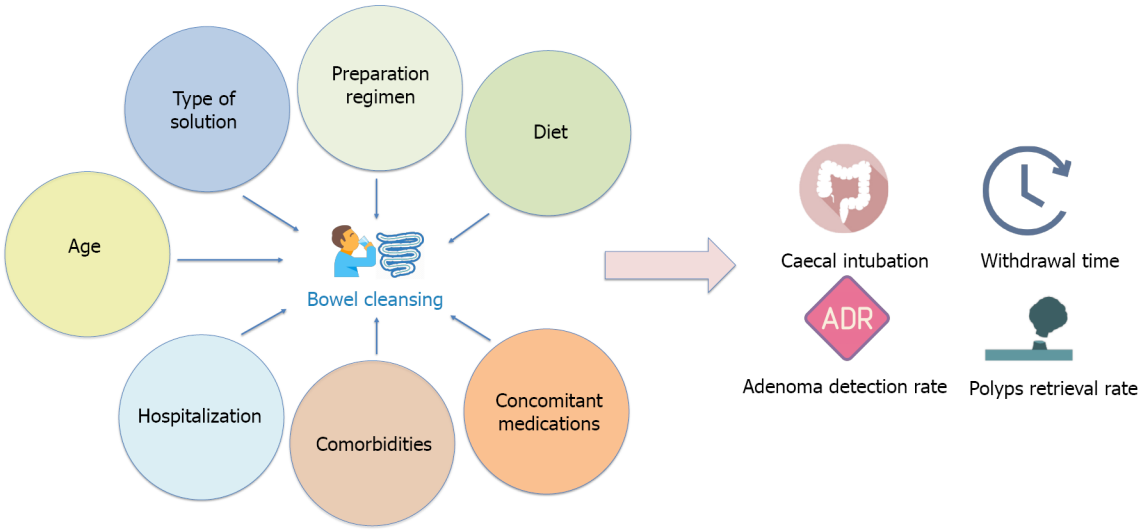

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Figure 1 Factors affecting bowel preparation and impact on colonoscopy outcomes.

FACTORS INFLUENCING THE QUALITY OF BOWEL PREPARATION

Type of solution and tolerability

Several laxatives are currently available and have been studied, with varying efficacy and tolerability.

Among these solutions are polyethylene glycol plus ascorbate (PEG-ASC) (4-L, 2-L, and 1-L), 2-L PEG plus citrate, 2-L PEG plus Bisacodyl, magnesium citrate plus picosulphate (MCSP), and trisulfate (magnesium sulfate, sodium sulfate, and potassium sulfate), also known as oral sulfate solution (OSS).

A meta-analysis of PEG solutions discovered that split-dose high-volume PEG was more effective than other options, including low-volume regimens [odds ratio (OR) = 3.46, 95% confidence interval (95%CI) 2.45-4.89][14]. Another meta-analysis comparing split-dose high-volume PEG to split-dose low-volume PEG found that high-volume regimens were superior (OR = 1.89, 95%CI 1.01-3.46)[15]. Nonetheless, because high-volume solutions may reduce patient compliance, resulting in suboptimal preparation, new low-volume laxatives have been introduced in the last decade.

Following that, a 2-L PEG plus citrate and simethicone solution was added. Randomized controlled trials (RCTs) comparing this formulation 2-L PEG plus citrate *vs* 4-L PEG found similar cleansing efficacy (73.6% *vs* 72.3%, 95%CI difference -7.5 to 10.1), but with greater tolerability (25.4% *vs* 37.0%, $P <$ 0.01) and acceptability (93.9% *vs* 82.2%, $P < 0.001$)[16].

Moreover, 2-L PEG plus citrate showed similar efficacy (78.3% *vs* 74.3%, $P = 0.37$) and acceptability (81.4% *vs* 80.8%, $P = 0.74$) also when compared to 2-L PEG-ASC[17].

Recently, a 1-L PEG-ASC solution with a very low volume has been introduced to improve patients' experience during colonoscopy by reducing the total oral intake of liquids to be consumed. The development of this very low-volume solution was made possible by increasing the ascorbate content, which improves the laxative effect and allows the solution to be delivered in a smaller volume.

Three phase-3 RCTs comparing the effectiveness of 1-L PEG-ASC (Plenvu, Norgine, Harefield, United Kingdom) *vs* trisulfate[18], sodium picosulfate plus magnesium citrate[19], and 2-L PEG[20] revealed that 1-L PEG-ASC was non-inferior to the comparator.

Moreover, a multicenter prospective study was performed to assess the effectiveness of 1-L PEG-ASC compared to 2-L and 4-L PEG preparation in a real-life setting on a cohort of 1289 patients ($n = 490$ performing a 4-L PEG preparation, $n = 566$ a 2-L PEG and $n = 233$ a 1-L PEG)[21].

In this study, cleansing success was achieved in 72.4%, 74.1%, and 90.1% ($P < 0.001$), respectively, while high-quality cleansing of the right colon was achieved in 15.9%, 12.0%, and 41.4% ($P < 0.001$) for the 4-L, 2-L, and 1-L-PEG preparation groups, respectively. The 1-L PEG-ASC preparation was an independent predictor of overall cleansing success and high-quality cleansing of the right colon in multiple regression analysis. In that study, 44.8% of patients were over the age of 65, confirming the validity of the safety of 1-L PEG-ASC in the elderly.

A real-life study performed on a cohort of hospitalized subjects confirmed that, among all variables, the 1-L PEG-ASC solution (OR = 0.39, 95%CI 0.23-0.65) and a > 75% intake of bowel preparation (OR = 0.09, 95%CI 0.05-0.15) significantly reduced the risk of inadequate colon cleansing[22].

This information is undoubtedly valuable and should be considered even in the care of hospitalized patients.

Nonetheless, the same studies mentioned above found that 1-L PEG-ASC is associated with a higher incidence of adverse events, such as nausea and vomiting, even if none of these were serious enough to

impair bowel cleansing quality[18-21].

Based on current data, guidelines recommend using high-volume or low-volume PEG-based regimens, and non-PEG-based validated products, in a split-dose fashion[13].

Even though no specific product is recommended for difficult-to-prepare patients, 1-L PEG-ASC appears to be promisingly superior in hospitalized patients and could be a reasonable choice in this setting; however, these data were obtained after the guidelines were issued.

Preparation regimen

The preparation regimen has a significant impact on the quality of bowel cleansing. A day before preparation was traditionally performed before a colonoscopy. Nonetheless, the most recent evidence suggests that dividing the preparation between the day before the exam and the day of the exam leads to better intestinal cleansing and a shorter time between the end of the preparation and the same colonoscopy.

A meta-analysis of 47 trials involving 13487 patients revealed that a split-dose regimen is associated with significantly better cleansing than day-before preparations (OR = 2.51, 95%CI 1.86-3.39), regardless of solution type and dose[15].

Furthermore, subgroup analysis by solution type confirmed that split was more effective than the day-before regimen for PEG, sodium phosphate, and picosulfate (OR = 2.60, 95%CI 1.46-4.63; OR = 9.34, 95%CI 2.12-41.11; OR = 3.54, 95%CI 1.95-6.45, respectively). Likewise, a higher proportion of patients (OR = 1.90, 95%CI 1.05-3.46) were willing to repeat preparation with split-dose *vs* day-before cleansing [15].

Subsequent RCTs evaluating the effectiveness of high-volume PEG[23,24], low-volume PEG[25,26], and sodium picosulfate[27-29] confirmed the split regimen's superiority over the day-before regimen.

In terms of the effectiveness of a split regimen on the detection of neoplastic lesions, an RCT comparing 2-L PEG-ASC in a split *vs* day-before fashion found that a split regimen resulted in a higher detection rate of adenomas (53.0% *vs* 40.9%; RR = 1.22, 95%CI 1.03-1.46) and advanced adenomas (26.4% *vs* 20.0%; RR = 1.35, 95%CI 1.06-1.73)[26].

Another RCT comparing 2-L PEG-ASC in split dose *vs* split-dose sodium picosulfate/magnesium citrate (SPMC) in a day-before dose showed a non-significant higher polyp detection rate, and a significantly higher detection rate of right-sided polyps and adenomas (51.5% *vs* 44.0%, $P = 0.14$; 28.0% *vs* 16.6%, $P = 0.007$; 21.0% *vs* 11.9%, $P = 0.015$, respectively) in favor of split regimen[30].

Also, two meta-analyses (including 11 and 14 RCTs) compared split-dose bowel preparation with same-day bowel preparation and found similar results in terms of bowel preparation quality, patient willingness to repeat it, and overall tolerability[31,32].

In addition to the split preparation, patients who need to have a colonoscopy in the afternoon should consider same-day preparation. In this regard, data from two meta-analyses revealed that when split and same-day regimens were used, the quality of bowel preparation, tolerability, and willingness to repeat were similar.

As a result, for patients undergoing afternoon colonoscopy, current guidelines recommend split-dose bowel preparation and same-day bowel preparation[13].

Diet before the procedure

Before a colonoscopy, a low-residue diet or clear liquids are usually advised. Patients should avoid foods high in fiber, such as fruits, vegetables, and whole grains, to achieve a low-residue diet. Colored liquids should be avoided because they can obscure proper mucosal visualization. Water, broth, coffee or tea, ice, gelatin, and fruit juices are the best clear liquid choices (apple, lemonade, and grapefruit).

On the day before the colonoscopy, two meta-analyses compared a low-residue diet to a clear liquid diet, with both arms using the same laxative. When compared to a clear liquid diet, Nguyen *et al*[33] discovered that a low residue diet was associated with better tolerability (OR = 1.92, 95%CI 1.36-2.70, $P < 0.01$) and higher willingness to repeat bowel preparation (OR = 1.86, 95%CI 1.34-2.59, $P < 0.01$). However, no differences in adequate bowel cleansing (OR = 1.21, 95%CI 0.64-2.28, $P = 0.58$) or adverse event incidence (OR = 0.88, 95%CI 0.58-1.35, $P = 0.57$) were found[33]. Another meta-analysis, this time by Avalos *et al*[34], compared a low-residue diet or a regular diet to a clear liquid diet. There were no differences in adequate cleansing (RR = 1.00, 95%CI 0.97-1.04). The low residue-regular diet was linked to a higher likelihood of repeating the procedure, better tolerability, and more frequent consumption of the solution (RR = 1.08, 95%CI 1.01-1.16; RR = 1.04, 95%CI 1.01-1.08; RR = 1.04, 95%CI 1.01-1.08, respectively). Except for more hungriness in the clear liquid diet group, there were no differences in ADR or adverse events between groups (RR = 1.93, 95%CI 1.13-3.30)[34]. In a third meta-analysis by Song *et al*[35] including 7 RCTs using different laxatives, a low residue diet showed higher tolerability (RR = 1.06, 95 %CI 1.02-1.11) and a higher likelihood of repeating the exam (RR = 1.17, 95%CI 1.09-1.26) compared with a clear liquid diet.

Concerning the duration of the diet, current guidelines recommend a low-residue diet or clear liquids for at least one day before the examination[13]. In this regard, two recent RCTs compared the 1-d *vs* 3-d diet, finding no significant difference after diet prolongation. The first RCT compared a 3-d *vs* 1-d low-fiber diet with a 4-L PEG split-dose preparation and found a similar rate of adequate bowel cleansing (91.7% for 3-d diet *vs* 94.7% for 1-d diet, $P = 0.24$)[36]. Similarly, the second RCT compared a 1-d *vs* 3-d

low-residue diet with a 2-L PEG + ASC split-dose preparation (82.7% *vs* 85.6%), with no differences in adherence, satisfaction, or polyp or ADR[37].

However, it should be noted that fiber consumption varies greatly between people and regions of the world. As a result, in countries where fiber consumption is naturally low, a single day's diet is more likely to suffice. On the contrary, in people or countries where fiber consumption is abundant, the diet should be tailored accordingly, particularly in hard-to-prepare patients.

Timing of colonoscopy after bowel preparation

The timing of colon preparation administration has a significant impact on both its tolerability and efficacy. Starting the preparation, the day before the colonoscopy improves the quality of colonic cleansing and the likelihood of finding flat lesions.

According to recent research, there is a negative correlation between the start of the colonoscopy, the interval since the last dose of bowel preparation, and the cleanliness of the mucosa. It is recommended to perform the colonoscopy sooner after the bowel preparation is complete to improve the quality of the examination[38,39].

Patients with intervals of 7 h or less between the start of PEG intake and the start of the colonoscopy had better bowel preparation than patients with intervals of more than 7 h (P = 0.03)[40].

Furthermore, bowel cleansing with the fractional regimen was superior within three hours of the last dose intake, declined gradually after 4-5 h, and became statistically insignificant at five hours, according to Bucci *et al*'s meta-analysis in 2014 including 29 RCTs comparing split *vs* day-before regimens[41].

Additionally, a subsequent multicenter, randomized, endoscopist-blinded study found that the best window of time for bowel preparation is the same for all preparations. Regardless of the preparation (PEG, PEG-ASC, ≤ 11.8, sodium picosulphate/magnesium citrate, ≤ 13.3 h), the ideal time before the colonoscopy was ≤ 11.8 h. The timing of the preparation did not affect the tolerability[42].

Anyway, the overall findings support the recommendation in the European Society of Gastrointestinal Endoscopy (ESGE) guidelines to begin the final dose of bowel preparation no later than 5 h before the colonoscopy and to finish it no later than 2 h before the procedure[13].

Of note, compared to other days of the week, Monday had the highest rate of inadequate preparation [Boston bowel preparation scale (BBPS) 6] of 16.5% in a 2022 retrospective review of 4279 colonoscopies. Notably, poor bowel preparation was not linked to post-holiday procedures[43].

However, in clinical practice, patients do not always adhere to the recommended timing due to a need related to the potential long distance to travel before having to perform the endoscopic exam, or as a result of the stress that they will have an urgent necessity for the public bathroom during the trip.

As a result of more accurate characterization of procedural and patient variables, bowel preparation may become more personalized.

SPECIFIC CLINICAL SETTINGS

Hospitalized patients

Hospitalization is associated with a nearly twofold increase in the risk of unsuccessful bowel preparation before colonoscopy when compared to the ambulatory setting[44,45].

Furthermore, the percentage of inpatients with an adequately prepared colon does not exceed 50% because they are typically elderly, frail, and suffering from comorbidities that either prevent successful bowel prep ingestion or affect patients' comprehension and compliance with the regimen's instructions [44,45].

Inadequate bowel preparation increases the risk of missed adenomatous polyps or CRC, as well as a patient inconvenience; it also harms healthcare systems by causing procedures to be delayed or repeated, as well as prolonged hospital stays[45-47].

Several studies have evaluated the efficacy of various interventions, such as different laxatives, changes in preparation administration timing, and promotion of educational programs for physicians, nurses, and patients, to overcome these challenges. Lower socioeconomic class, opiate/tricyclic antidepressant use, afternoon colonoscopies, American Society of Anaesthesiologists (ASA) physical status classification system class 3, and pre-preparation nausea/vomiting were identified as the main potential predictors of inadequate inpatient preparation[46].

Furthermore, Gkolfakis *et al*[45] recently published a systematic review with meta-analysis to evaluate the efficacy of various interventions to improve the quality of colon preparation in inpatients.

In this study, which included 17 studies and 2733 inpatients, the authors concluded that, despite several interventions, only nearly two-thirds of inpatients achieve adequate colon preparation before colonoscopy, and that educational interventions significantly improve inpatients' bowel preparation quality[45].

In the absence of standardized guidelines or recommendations, the ideal bowel preparation regimen for inpatients has yet to be determined[45].

In any preparation strategy, PEG-based regimens should be considered first because they are more likely to achieve adequate bowel cleansing while maintaining an optimal patient safety profile. Furthermore, case-by-case application of multiple and combined strategies (*e.g.*, written educational material, nurse facilitation of the process, *etc.*) may have the potential to influence the outcome[45,48].

Elderly

With the extension of life expectancy, the opportunities for medical care for elderly patients increase [49]. The rise in cancer patients, in particular, is a global challenge; the International Agency for Research on Cancer estimates that the number of cancer patients will rise dramatically[49]. Regardless of the procedure's risks, bowel preparation can be problematic in terms of purgative effect and risk[50].

Patients must also go through a period of dietary restriction, fasting, and later fluid restriction, which can be difficult for people who have diabetes or chronic kidney disease (CKD)[50]. Furthermore, an elderly patient whose only access to the toilet is *via* stair-lift may be unable to prepare at home, necessitating in-patient admission for bowel preparation or alternative investigation to colonoscopy[50].

Despite the fact that little research has been conducted on bowel preparation in the elderly and very elderly population, a 2016 study discovered that diabetes, difficulty walking, or performing activities of daily living were associated with poor bowel preparation in patients over the age of 65[50,51]. Furthermore, poor bowel preparation is the leading cause of colonoscopy failure in patients aged 90 and up[50,52].

In five years, a British Patient Safety Agency alert reported one death and 218 patient safety incidents [53]. Although the vast majority of these incidents (93%) resulted in no or minor harm, 6% resulted in moderate harm, and one patient died[53].

Omitted medication (29%), incorrect drug (23%), and incorrect or unclear dose, strength, or frequency (11%) were all considered medicine errors. Side effects of bowel preparation include hypovolaemia, renal failure, and electrolyte disturbances[54]. An accurate bowel preparation prescription, as well as consideration of potential interactions and side effects, is especially important in older adults, who are more likely to have multiple comorbidities and polypharmacy, both of which may exacerbate these side effects. They may also have less physical reserve to deal with any unforeseen complications. In patients over the age of 65, serum albumin concentration before bowel preparation, for example, may predict hypovolaemia[55].

In elderly patients with pre-existing chronic renal failure, all oral laxatives should be used with caution, and patients undergoing dialysis or with advanced CKD should consult with the renal team. Sodium phosphate preparations should be avoided entirely in patients with renal failure[54]. Furthermore, any medications that, when combined with bowel preparation, may worsen renal failure, such as diuretics, angiotensin-converting enzyme inhibitors, and angiotensin II receptor antagonists, should be avoided[54].

Cleaning success was achieved in 70.3% of patients aged > 65 years with comorbidities undergoing colonoscopy after a 1-L PEG-ASC, 2-L PEG/PEG-ASC, or 4-L-PEG-based preparation, according to a recent retrospective analysis of a prospective cohort. Notably, elderly patients had a higher rate of inadequate colonoscopy cleansing than non-elderly patients (7.0% *vs* 3.8%, $P = 0.012$)[56]. Split regimen, adequate cleansing at last colonoscopy, tolerability score, a low-fiber diet for at least 3 days, and colonoscopy within 5 h after the end of preparation (OR = 2.43, $P = 0.003$; OR = 2.29, $P = 0.02$; OR = 1.29, $P < 0.001$; OR = 2.45, $P = 0.001$; OR = 2.67, $P = 0.008$, respectively) were predictors of bowel cleansing in the elderly[56]. Also, 1-L PEG had a higher tolerability score than 2-L and 4-L PEG in elderly (7.7 *vs* 7.2 and 7.2, $P = 0.099$). Interestingly, when compared to the other preparations, the 1-L PEG-ASC preparation was associated with higher quality cleansing of the right colon (39.6% *vs* 17.0% *vs* 9.4%, respectively, $P < 0.001$) and may thus be the preferred option for the elderly.

Methods for achieving safe and adequate bowel preparations in the elderly should include clear instructions, reminder calls, and case management for potential confounding patient-related factors[57].

Comorbidities

Endoscopic techniques have become increasingly important in recent years, particularly in the treatment of colorectal flat lesions[58,59].

At the same time, the significance of accurate detection and assessment of such lesions in predicting malignancy has become clear. Indeed, proper bowel preparation is critical for colonoscopy because it allows for visualization of the entire colonic mucosa and improves the safety of therapeutic maneuvers [6,60].

In contrast, poor preparation lengthens the procedure, increases the risk of complications, and increases the likelihood of missing lesions[61].

The percentage of colonoscopies performed with inadequate bowel cleansing ranges between 5% and 35%[12,44,62-66].

Because a proper bowel cleansing regimen increases the likelihood of success, identifying risk factors for inadequate bowel cleansing is critical. Patient-related predictors of colonoscopy preparation failure include prior inadequate bowel cleansing, a history of constipation, increasing age (> 65 years), male gender, low health literacy (*e.g.*, cognitive skills), inpatient status, obesity, diabetes mellitus (DM), inflammatory bowel disease (IBD), unexplained chronic diarrhea, megacolon, cirrhosis, stroke,

dementia or Parkinson's disease, patients at increased risk for electrolyte abnormalities (*e.g.*, patients on diuretics), uncontrolled hypertension, severe congestive heart failure (New York Heart Association class III or IV), severe CKD (creatinine clearance < 30 mL/min/1.73 m), previous colorectal surgery, use of constipation-related medications (narcotics and tricyclic antidepressants), severe colonic stricture or obstructing tumor or perforation, dysphagia, gastroparesis, or gastric outlet obstruction, pregnancy or lactation[12,64,65,67-69].

Administration of the entire preparation the night before the colonoscopy, rather than split-dosing, and a later start time for the colonoscopy are procedure-related risk factors for inadequate bowel preparations[44]. The presence of one or more of these risk factors can influence bowel cleansing regimens and choices[70].

ESGE recommends the use of high-volume or low-volume PEG-based regimens, as well as non-PEG-based agents that have been clinically validated for routine bowel preparation. For elective colonoscopy, split-dose bowel preparation (with or without additional measures) should be used, as it has been linked to improved preparation quality[13].

In 2015, Dik *et al*[71] conducted a Dutch study that included only patients who who received a split-dose regimen. In total, 1331 colonoscopies were included in the study, with 12.9% having insufficient bowel preparation. Diabetes, chronic constipation, a history of abdominal or pelvic surgery, and recent hospitalization are all risk factors for poor bowel cleansing quality.

Gandhi *et al*[65] conducted a meta-analysis of independent risk factors in over 75,000 people receiving a split-dose bowel preparation. Constipation, diabetes, and medication use were identified as predictors of colonoscopy preparation failure despite the studies' heterogeneity. In a 2018 meta-analysis by Mahmood *et al*[12], age, male sex, inpatient status, DM, hypertension, cirrhosis, narcotic use, constipation, stroke and tricyclic antidepressants were associated with inadequate bowel cleansing (OR = -1.20, OR = 0.85, OR = 0.57, OR = 0.58, OR = 0.58, OR = 0.49, OR = 0.59, OR = 0.61, OR = 0.51, respectively). Furthermore, in Western countries, diabetes, cirrhosis, male sex, stroke history, and tricyclic antidepressant use were found to be stronger risk factors for inadequate bowel preparation than in Asian countries.

In a 2022 United States retrospective study of 1029 patients, Agrawal *et al*[66] discovered the following factors to be associated with colonoscopy cancellations: Graduate school education, Hispanic ethnicity, a hemoglobin level of 10 g/dL, and if the colonoscopy was done for other indications (OR = 1.93, $P = 0.04$; OR = 0.47, $P = 0.01$; OR = 1.41, $P = 0.05$; OR = 0.53, $P = 0.04$, respectively). Dementia (OR = 2.44, $P = 0.02$) and gastroparesis (OR = 3.97, $P = 0.01$) were factors associated with poor bowel preparation in a multivariate analysis.

Ultimately, in a 2016 United States study of 2401 colonoscopies, African Americans were 70% more likely to have suboptimal preparation (95%CI 1.2-2.4); DM, tricyclic antidepressant use, narcotic use, and Miralax-Gatorade prep *vs* 4-L PEG 3350 were all associated with suboptimal preparation quality in a multivariable analysis (OR = 2.3, 95%CI 1.6-3.2; OR = 2.5, 95%CI 1.3-4.9; OR = 1.7, 95%CI 1.2-2.5; OR = 0.6, 95%CI 0.4-0.9, respectively)[72].

Obesity: Obesity, when combined with other risk factors, is an independent predictor of poor bowel preparation during a colonoscopy in practice.

In a 2013 retrospective study of 2163 consecutive patients, mostly men, who had colonoscopies in Indiana, one of the independent risk factors for inadequate preparation was a body mass index (BMI) of ≥ 30 Kg/m^2 (OR = 1.46, 95%CI 1.21-1.75, $P < 0.0001$)[73].

Sharara *et al*[74] discovered that BMI was an independent risk factor for inadequate preparation in a 2016 Arabic study involving 541 patients. Obesity was associated with an OR of 5.3 (95%CI 1.4-19.8, $P = 0.01$) when compared to normal BMI. In a prospective study of 195 patients, obese patients had comparable rates of inadequate preparation to normal-weight individuals (OR = 0.7, 95%CI 1.10-3.96, $P = 0.68$). Patients who were underweight performed significantly worse than those with normal BMI (OR = 8.0, 95%CI 1.1-58.0, $P = 0.04$).

A high BMI had a significant difference in the effect of bowel cleansing between studies with mostly female patients (OR = 1.05) and studies with mostly male patients (OR = 1.30) ($P = 0.013$ for the difference), according to a 2018 systematic review and meta-analysis[65]. Inadequate bowel preparation was linked to diabetes (OR = 1.79) and hypertension (OR = 1.25), among other risk factors.

According to a recent study by Passi *et al*[75] in the United States, 49.4% of 27696 colonoscopies had insufficient bowel preparation, which was most common in the class III obesity group. When compared to the normal body mass index (BMI) group, a BMI of 30 kg/m^2 and 40 kg/m^2 was associated with an increased risk of an incomplete colonoscopy ($P = 0.001$ for overweight, $P = 0.0004$ for class I/II obesity), a longer procedure ($P < 0.05$ for all), and poorer tolerance ($P < 0.0001$ for class I/II obesity, $P = 0.016$ for class III obesity).

According to some studies, distinct bowel preparations are beneficial and safe for obese patients. In a 2012 prospective Australian study of 104 patients showing a similar bowel preparation quality after using sodium picosulphate, 90% of non-obese and 89% of obese patients had good bowel preparation ($P > 0.99$)[76].

Patients were randomized to receive split-dosing of either NER1006, 2-L PEG-ASC, or OSS in a recent (2021) two phases III Spanish trials[77]. Split-dose NER1006 (1-L-PEG-ASC) was associated with high

levels of cleansing, ranging from 87% to 94% in a total of 551 patients, including those who were obese or diabetic. Obese males aged above 60 had significantly higher overall and high-quality bowel-cleansing success rates with 1-L-PEG-ASC, at 100.0% and 72.7%, respectively, compared to 86.7% and 50.0% in the control group (P = 0.015 and P = 0.033, respectively).

Diabetes mellitus: Due to the high prevalence of gastrointestinal symptoms and the increased risk of CRC, diabetic patients have a higher demand for colonoscopies than the general population[78-80].

As a result, adults with diabetes should be properly screened, and a longer bowel preparation may be necessary to ensure an adequate endoscopic examination[81].

Due to dietary/medication regimen changes, narcotic use, and diabetes-related complications/comorbidities such as hypoglycemia, electrolyte imbalance, acute renal failure, and ketoacidosis, diabetic patients are at risk of poor bowel preparation[82,83].

DM has been identified as an independent risk factor influencing bowel preparation quality by decreasing colonic motility[71,84-86].

The rate of insufficient bowel preparation in diabetic patients ranges from 9% to 30%[84,87,88], which should be significantly reduced by implementing a multifactorial strategy. Surprisingly, even though DM patients are notoriously difficult to prepare, few studies have looked into the best bowel preparation management strategy in this setting. In diabetic patients, taking 10 ounces of magnesium citrate two days before colonoscopies, in addition to a single 4-L PEG dose, improved colon cleansing (from 54% to 70%)[89].

Another single-blind prospective trial on DM patients discovered that adding lubiprostone, a highly selective locally-acting activator of chloride channels used in functional constipation, to a single 4-L PEG the day before the procedure improved colon cleansing; however, the improvement was statistically non-significant due to the small sample size[90]. A small trial in DM outpatients examined additional bowel cleansing strategies with 6-L PEG, but the results were not encouraging[84].

Current United States guidelines do not endorse any of these recommendations, instead recommending a split-dose bowel cleansing regimen for DM patients with no adjustments[8]. A subsequent European randomized, single-blind, superiority trial compared a conventional bowel preparation protocol with a diabetic-specific preparation protocol, which included a low-fiber diet for three days, a clear liquid diet for one day, and a 4-L split-dose PEG regimen[88].

The latter group was given a special education program that included diet, laxative intake, and blood glucose-lowering agent adjustment instructions. In the conventional protocol, inadequate bowel cleansing was statistically more common than in the diabetic-specific protocol (20% *vs* 7%; RR = 3.1, 95%CI 1.2-8.0, P = 0.014).

Chronic constipation: The most prevalent type of constipation, functional chronic constipation, frequently affects women and the elderly who undergo colonoscopies often and ranges in prevalence from 2% to 27% in Western countries[91-93]. Constipation has been identified as a risk factor for inadequate bowel preparation[13,94]. Currently, ESGE does not recommend any specific bowel preparation in patients suffering from constipation chronically[13].

In elderly patients, slow transit constipation, defined by decreased bowel movements, may result in insufficient laxative wash-out and bowel preparation. This hypothesis was confirmed in a 2015 Korean study[95], which discovered that colonic transit time of more than 30 h was associated with inadequate bowel preparation. Furthermore, slow-transit constipation, as determined by radiopaque marker colonic transit testing, was linked to a more than 2-fold increased risk of poor bowel preparation in a 2022 study of 274 American patients with chronic constipation (OR = 2.2, 95%CI 1.1-4.4)[96].

In patients with a history of constipation, additional bowel purgatives should be considered[8]. Numerous studies in recent years have suggested different bowel preparation regimens in patients with chronic constipation, with good results using a variety of laxatives.

In a double-blind 2008 United States trial, 200 CRC screening patients were randomly assigned to receive a 24 g dose of lubiprostone or placebo before a split-dose PEG with electrolytes bowel preparation in the absence of dietary restriction[97].

Split-dose PEG, electrolytes, and lubiprostone pretreatment was found to be more effective (P = 0.001) and tolerable (P = 0.003) than placebo, most likely due to a reduction in abdominal bloating (P = 0.049)[97].

In a 2015 Italian randomized, single-blind study, 400 constipated patients were enrolled and randomly assigned to one of two arms: Split 2-L PEG-citrate-simethicone plus 2-day bisacodyl or split 4-L PEG[98]. In a 2016 Chinese RCT[99], the addition of lactulose one day before colonoscopy in combination with 4-L split-dose PEG was shown to be significantly superior (P < 0.05) to the conventional preparation with oral PEG and electrolytes for colonoscopy bowel preparation.

In terms of ease of administration (P < 0.001), willingness to repeat (P < 0.001), and compliance (P = 0.002), the 2-L PEG-citrate-simethicone/bisacodyl solution was found to be significantly more acceptable[98]. According to a 2019 RCT[100], the optimal dose of crystalline lactulose for Japanese constipated patients is 26 g/day. A short therapy cycle of PEG plus electrolytes was effective and safe in improving bowel preparation in chronic constipation patients in a 2020 and 2021 Japanese study[101,102]. In 2016, a larger Asian population was studied in a randomized, double-blind, placebo-controlled trial[103]. Surprisingly, when lower doses of PEG were combined with lubiprostone, no significant

difference in preparation quality was observed.

In a 2021 systematic review and meta-analysis of three RCTs, Dang *et al*[104] enrolled 225 chronically constipated patients, with 47.6% receiving sodium phosphate and 52.4% receiving PEG. Despite the low quality of evidence, patients who received sodium phosphate before their colonoscopy had cleaner colons than those who received PEG (OR = 1.87, 95%CI 1.06-3.32, P = 0.003).

IBD: Inadequate bowel preparation has also been linked to comorbidities such as IBD[105]. This was demonstrated in an Italian multicenter, randomized, single-blind study of 211 adult outpatients with ulcerative colitis (UC) undergoing colonoscopy and receiving either 2-L PEG plus bisacodyl or 4-L PEG [106]. Low-volume PEG was not inferior to 4-L PEG for bowel cleansing in UC (P = NS), but it was better tolerated (P < 0.0001) and accepted (P < 0.0001). The split dosage was associated with better cleansing regardless of preparation. A period of more than 6 h between the end of preparation and the colonoscopy predicted poor cleansing.

In a 2021 retrospective analysis of a prospective cohort, Maida *et al*[107] demonstrated the efficacy and safety of 1-L PEG-ASC in 45% of 411 patients.

IBD patients had higher cleansing success (92.9% *vs* 85.4%, P = 0.02) than controls, with a similar number of patients experiencing adverse events (22.2% *vs* 21.2%, P = 0.821) and treatment-emergent adverse events (51 *vs* 62%, P = 0.821). Furthermore, the presence of IBD (OR = 2.51, P = 0.019), lower age (OR = 0.98, P = 0.014), a split regimen (OR = 2.43, P = 0.033), the absence of diabetes (OR = 2.85, P = 0.015), and chronic constipation (OR = 3.35, P = 0.005) were all independently associated with cleansing success[107].

Endoscopic disease activity has recently been discovered to predict suboptimal bowel preparation, and biological therapy has been shown to protect IBD patients from it.

In a 2022 United States study by Kumar *et al*[108], the moderate-to-severe endoscopic disease was associated with higher odds of suboptimal bowel preparation *vs* mild or inactive disease [adjusted OR (aOR) 2.7; (95%CI 1.52-4.94)], whereas baseline biologic use was associated with a lower odds of suboptimal bowel preparation [aOR, 0.24 (0.09-0.65)] among the overall IBD cohort. Furthermore, age > 65 years and single-dose *vs* split-dose bowel preparation were independent predictors of suboptimal bowel preparation [aOR, 2.99 (1.19-7.54); aOR, 2.37 (1.43-3.95), respectively].

Liver cirrhosis: Liver cirrhosis predicts poor bowel preparation at screening colonoscopy[64,109].

This finding is most likely due to multiple factors impairing intestinal motility in cirrhotic patients [110,111]. The role of chronic liver disease in predisposing to inadequate bowel preparation in the absence of cirrhosis is unknown. In a 2016 United States study, Anam *et al*[112] compared 120 cirrhotics to 220 non-cirrhotics with chronic liver disease, and the first group performed significantly worse on bowel preparation. Cirrhotics had lower bowel preparation scores than non-cirrhotics (P = 0.0027), with cirrhotics having the lowest (48%) and non-cirrhotics having the highest (30%), with no effect of the MELD score.

The rate of failure to complete the bowel preparation and the incidence of side effects were comparable in 53 cirrhotics compared to 52 healthy subjects undergoing screening colonoscopy, according to an Italian 2015 study by Salso *et al*[113]. Despite this, nearly half of the cirrhotics (49% *vs* 5% control; P < 0.001) had poor bowel cleansing.

In a 2017 Chinese retrospective study, Lee *et al*[114] compared the safety of two bowel-cleansing agents in patients with liver cirrhosis (2-L PEG-ASC *vs* 4-L PEG). Patients preferred the 2-L PEG-ASC over the 4-L PEG group for acceptability and compliance. Finally, because both groups were successfully cleansed, the authors concluded that using 2-L PEG-ASC for colonoscopy in cirrhotics was a safe option.

Decompensated cirrhosis patients are more prone to frailty, cognitive abnormalities, and decreased ambulation. Clayton *et al*[115] discovered that patient educational video did not improve bowel preparations (split-prep) in the pre/post-intervention period in 121 patients with decompensated cirrhosis undergoing colonoscopy during the initial liver transplantation evaluation (29.8% *vs* 31.9%, respectively).

Furthermore, patients with moderate to severe ascites had a significantly higher rate of inadequate colonoscopy bowel preparation than non-ascites patients[115].

CKD: The use of cleansing agents in patients with CKD should be carefully evaluated due to the risk of electrolyte imbalance or worsening renal function[116]. No significant changes in vital or biochemical parameters have been linked to high volume osmotically balanced solutions containing PEG and electrolytes capable of maintaining bowel lumen isosmosis[13].

According to previous research[117,118], PEG is generally safe in CKD patients; however, adequate hydration and renal function monitoring should be ensured before and after colonoscopy in some cases to avoid acute kidney failure[119]. Individualized laxative choice is strongly advised for patients at risk of hydroelectrolyte disturbances (moderate quality evidence)[13].

Because of hyperosmolarity and the risk of magnesium toxicity, as well as acute phosphate nephropathy, magnesium-based preparations and sodium phosphate should be avoided in CKD patients[13,120,121].

Furthermore, due to the poor tolerability of high-volume PEG-based regimens, low-volume PEG (2-L) solutions with ascorbic acid (PEG-ASC) solutions have been proposed to reduce the patient's excessive fluid intake. Ascorbic acid can act as an osmotic agent and enhance the laxative effect of PEG due to its hexose structure[122], and its pleasant taste makes it easier for patients to swallow. Ascorbic acid, on the other hand, has been linked to the formation of renal stones and acidosis, with contradictory results[123, 124]. As a result, low-volume preparations continue to be a challenge for many CKD patients.

Notably, ESGE guidelines do not recommend aspartame and ascorbate-containing solutions (such as 2-L and 1-L PEG-ASC solutions) for patients with renal insufficiency and creatinine clearance less than 30 mL/min. A high rate of hypernatremia has been observed following the administration of 1-L PEG-ASC, owing primarily to the product's sodium content[13]. If low volume PEG solutions combined with citrate and simethicone are administered to patients with creatinine clearance less than 30 mL/min, caution is advised[13].

In a 2016 retrospective study, a same-day 1-L low-volume PEG regimen with a previous-day low-residue diet and laxative was tested to improve tolerability[125]. The study included 5,427 patients who were instructed to consume a low-residue fiber diet with 10 mL sodium picosulfate one day before the colonoscopy, followed by 1-L low-volume PEG and 0.5-L water four hours before the exam. In 86 CKD patients (creatinine 1.1 mg/dL), the BBPS 6 success rate was 94.1%, and there were no serious complications[125]. Lee *et al*[123] found that the 2-L PEG-ASC was a safe choice for bowel preparation before colonoscopy in patients with impaired renal function in a 2016 study.

In one retrospective cohort, patients with a GFR of 60 mL/min were given either 4-L PEG or 2-L PEG-ASC solutions. Patients in the 2-L PEG-ASC group (n = 61) rated their tolerance and acceptability higher than those in the 4-L PEG group (n = 80)[123]. After either preparation, there was no statistically significant change in electrolytes, blood urea nitrogen, or creatinine. When the regimens were compared, 7.5% of 4-L PEG patients and 11.5% of 2-L PEG-ASC patients had a transient > 30% increase in creatinine levels, though the differences were not statistically significant[123]. Ohmiya *et al*[126] discovered that same-day conventional bowel preparation with PEG electrolyte lavage solution plus Ascorbate (PEG-ELS-ASC) was safe and effective in 56 CKD patients in the Japanese 2021 study.

Only retrospective cohorts have found PEG to be safer than other formulations in patients with impaired renal function[127].

The most severe kidney injury case reported reversible post-colonoscopy acute renal failure within a few weeks of oral sodium phosphate (OSP) intake, necessitating renal replacement therapy in 19% of patients[128]. Furthermore, during the 2006-2007 time period, the Food and Drug Administration received reports of 171 cases of renal failure caused by the use of OSP and 10 cases caused by the use of PEG[128]. A 2005 retrospective population-based Iceland study found that the risk of biopsy-proven acute phosphate nephropathy is about one in every 1000 OSP doses sold[128].

Three RCTs comparing OSS preparation to 4-L PEG found that split OSS was noninferior to split high-volume PEG in terms of efficacy, safety, and tolerability. Although real-world data on OSS in the setting of renal insufficiency are limited, and despite no significant differences in the frequency of acute renal failure reported with this preparation, European guidelines[13] recommend that it be avoided in patients with severe renal insufficiency (glomerular filtration rate 30 mL/min).

According to ESGE guidelines and current evidence, patients with severe renal insufficiency should be prepared with isotonic high volume PEG solutions rather than low volume PEG or non-PEG regimens.

Heart disease: Previously, it was thought that bowel preparation (particularly after administration of PEG-ELS solution) could worsen heart failure[129], as a result, except in urgent or emergency cases, exposing such patients to a colonoscopy was risky. Also, coronary heart disease has been identified as a risk factor for severe desaturation and relevant electrocardiographic changes during endoscopic sedation[130]. Furthermore, several studies have found that these solutions may be harmful to patients with heart disease due to the potential increase in plasma volume and their effects on electrolyte disturbances[131].

Thiazide diuretics and SSRIs, which have the potential to cause fluid and electrolyte imbalances, should be avoided in at-risk patients while undergoing bowel preparation[132].

Heart disease and CRC were the only predictors strongly associated with poor bowel cleansing in a 2019 Spanish single-center, endoscopist-blinded RCT of 136 patients (OR = 3.37, 95%CI 1.34-8.46, P = 0.010; OR = 3.82, 95%CI 1.26-11.61, P = 0.018, respectively)[133]. In a 2020 study, Poola *et al*[134] discovered that 44% of 315 inpatients' bowel preparation was fair/poor. Poor bowel preparation was associated with elderly people who had a history of congestive heart failure.

Concomitant medications

Most medications can be taken with a small sip of water until the day of the colonoscopy. Because of the decreased oral intake prior to the procedure, some medications, such as diabetes medications or anticoagulants, may need to be adjusted. Because it causes residual feces, oral iron should be stopped at least five days before the colonoscopy[135]. Additionally, the procedure's urgency and the availability of alternative tests must be taken into account.

In a 2017 Chinese systematic review and meta-analysis of RCTs (overall 3217 patients with chronic pain), Huang *et al*[136] discovered that treatment with a fixed-ratio combination of prolonged-release oxycodone/naloxone reduces the incidence of opioid-induced constipation and provides clinically significant intermediate-term bowel function improvement while maintaining pain relief. According to a 2019 Spanish study by Velázquez Rivera *et al*[137], tapentadol and oxycodone had better bowel function profiles with no differences in a cross-sectional observational study of 180 Spanish patients with opioid-induced constipation during long-term treatment.

Previous history of colorectal surgery

Inadequate bowel cleansing is detrimental to the examination, resulting in lower ADR, longer procedural time, lower rates of cecal intubation, shorter intervals between examinations, and a 12%-22% increase in overall colonoscopy cost[4]. Previous colorectal surgery is a risk factor for patients who are difficult to prepare[64,138,139].

Previous colorectal surgery (along with diabetes, Parkinson's disease, and liver cirrhosis) is a condition that significantly predicts inadequate colon preparation, according to a prospective study enrolling 2811 outpatients in 2012[64].

Another study published in 2015 discovered that diabetes, chronic constipation, a history of abdominal and/or pelvic surgery, and current hospitalization were all significant predictors of poor bowel cleansing in patients using a split-dose preparation scheme[71]. Improved bowel preparation strategies may be difficult to achieve in this patient population. The long-term effects of colonic surgery and anastomoses on colonic motor function are currently unknown[140]. Patients who have had colonic surgery are usually excluded from trials on the efficacy of bowel preparations due to this lack of knowledge. As a result, no evidence-based guidelines for preparing this subset of patients are now available, and the various bowel cleansing schemes rely on single-center experience or expert opinions recommending high-volume regimens[141]. Following surgery, some mechanisms affecting the enteric nervous system and autonomic innervation are known to be altered.

Indeed, Vather *et al*[142] discovered that distal colonic motor patterns traversed healed anastomosis sites regularly, indicating possible cellular regeneration. Some of the causes could be attributed to colorectal anastomoses' effect on the enteric nervous system and autonomic innervation, which can result in changes in colonic retrograde and antegrade motor patterns[142-144]. Remarkably, patients who have had a previous colectomy appear to be the most difficult to prepare. While right colectomy reduces absorption and determines rapid transit, left colectomy may worsen peristalsis in moving luminal content outside the body[145].

Mussetto *et al*[139] found that a low-volume mixed preparation (15 mg bisacodyl plus 2-L PEG) was not inferior to 4-L PEG for adequate bowel cleansing during surveillance colonoscopy in a 2015 study of 120 patients who had prior colorectal resection for cancer (85.0% *vs* 81.7%, $P = 0.624$). Notably, the mixed low-volume regimen had a higher success rate and tolerability in patients who had previously undergone left colectomy *vs* right colectomy ($P = 0.025$ and $P < 0.001$, respectively). The only predictor of unsuccessful cleansing using logistic regression was previous left colectomy ($P = 0.012$). Similarly, Yoo *et al*[146] conducted a case-control study in Turkey in 2018, enrolling 200 patients who received either a low-volume or high-volume bowel preparation regimen. In terms of adequate cleansing (modified BBPS of 6-9), there was no statistical difference between the resection and control groups (88% *vs* 88%). Patients with a left colon resection had an OR of 0.27 ($P = 0.003$) for successful cleansing, according to the logistic regression analysis of the resection group, and low-volume preparation (OR = 3.09, $P = 0.023$) was the best predictor of an effective cleansing procedure.

According to Kim *et al*[147], 12.1% of the 12,881 participants in the National Cancer Center's health screening cohort who underwent screening or surveillance colonoscopy had a history of abdominopelvic surgery. Poor bowel preparation was linked to gastric or minor intestinal surgery in a multivariate analysis (OR = 1.76, 95%CI 1.23-2.53, $P = 0.002$). Other types of surgery, on the other hand, did not affect bowel preparation quality.

Chung *et al*[148] looked at 247 patients who had previously had a colorectal resection and had a surveillance colonoscopy in a 2017 study. The right colon preservation group had a significant association with bowel cleansing quality in both univariate (22.3% *vs* 7.5%, $P = 0.028$) and multivariate (OR = 3.6, 95%CI 1.0-12.3, $P = 0.038$) analysis.

In contrast, Gandhi *et al*[65] discovered that a history of abdominal surgery (OR = 0.99) did not correlate with inadequate bowel preparation in a 2018 systematic review and meta-analysis of 67 studies involving 75818 patients.

A study (NCT02761317) is currently being conducted on patients who have had colorectal resection to compare the efficacy of bowel cleansing between the standard preparation (2-L PEG solution, 2-L PEG-ELS), low-volume preparation (10 mg bisacodyl plus 2-L PEG-ELS), and high-volume preparation (10 mg bisacodyl plus 2-L PEG-ELS) (4-L PEG-ELS).

History of poor bowel preparation

The presence of one or more risk factors for inadequate preparation will influence preparation selection and regimen. Due to the high risk of missing clinically relevant lesions, patients with insufficient cleansing must have the colonoscopy repeated after a more thorough bowel cleansing attempt[13].

Patients who did not prepare adequately because they misinterpreted the instructions can be counseled and then directed to repeat the same bowel regimen. For patients who did not tolerate or respond adequately to the original preparation[149] alternative preparations should be tried.

Despite the lack of strong evidence from RCTs, the ESGE guidelines and some studies recommend repeating colonoscopy on the same or the next day with additional bowel cleansing (*e.g.*, 500 mL PEG-ASC) or using enema as a salvage option in patients with insufficient bowel cleansing[72,149-152]. The bowel preparation regimen outlined below should be tailored to the potential causes of failure[13].

Some patients continue to have inadequate preparation despite following the regimen, switching regimens, and using a split-dose lavage. In such cases, two days of clear liquids are usually prescribed, followed by a morning procedure. If the patient's preparation was extremely poor, options include adding a second laxative if contraindications exist (*e.g.*, using 4-L PEG-ELS solution followed by a 1-L solution of magnesium citrate) or repeating the preparation administration over two days (except for sodium phosphate).

Within one year, 90% of patients who did not do adequate bowel cleansing require a repeat colonoscopy after one or more attempts at bowel cleansing[13,70]. Similarly, experts advise patients who did not tolerate or respond well to the first bowel cleansing to try another. Split-dosing should also be used for improved bowel cleansing[70]. Despite adherence to the regimen, switching to different regimens, and using a split-dose lavage, some patients continue to have insufficient bowel cleansing for the next exam, particularly those with severe comorbidities (*e.g.*, stroke, dementia, DM, obesity), the elderly, men, and people taking psychotropic drugs, all of which are commonly associated with a chronic constipation condition[6,64,153]. The efficacy of a next-day or same-day colonoscopy after additional bowel preparation *vs* a later colonoscopy is limited and contradictory. In a 2009 Israelian single-center study of 235 patients with inadequate preparation, next-day colonoscopy was associated with a lower risk of secondary failure (OR = 0.31, 95%CI 0.1-0.92)[63]. In a 2013 retrospective study of 3047 procedures with inadequate cleansing, patients advised to have a next-day colonoscopy were more likely to follow the recommendation for a repeat colonoscopy[154]. In a larger 2016 United States single-center series of 397 patients with inadequate procedures, recurrent failure was observed in 30.0% of the next-day group and 23.5% of the non-next-day group (P = 0.48)[155].

In a single-center Korean prospective nonrandomized study conducted in 2014, 87 patients with insufficient preparation after an initial 4-L PEG were given either an additional 2-L PEG on the same day or a 4-L PEG plus bisacodyl one week later after 3 days of a low residue diet, with no difference found between the two regimens[149]. A 2017 Spanish randomized trial demonstrated the superiority of a high-volume PEG-based regimen over a low-volume PEG-based regimen when combined with an intensive preparation regimen[150]. In a 2018 observational study, 60 Turkish patients who had received inadequate preparation underwent a same-day repeat colonoscopy after receiving an additional laxative of 250 mL senna alkaloids with 1.5-L water, and 83% of patients had the repeat colonoscopy reach the cecum[151].

In a 2012 United States study, Sohn *et al*[152] found that direct administration of laxative enemas through the colonoscope into the right colon *via* the biopsy channel was effective in 21 patients with inadequate preparation after low-volume PEG and bisacodyl preparation. In a 2012 Japanese study, Horiuchi *et al*[156] published a study in which 26 patients with inadequate preparation after low-volume PEG were given 500 mL of PEG *via* the colonoscope biopsy channel, with 96.1% (25/26) of cases successfully prepared.

Yang *et al*[157] on the other hand, conducted a randomized trial in 125 patients with inadequate preparation, comparing administration of a 1-L PEG enema *via* colonoscope to additional oral ingestion of 2-L PEG, with 35 of 66 (53%) of the enema group and 53 of 67 (81%) of the oral group obtaining suitable preparation. 42 patients with inadequate preparation were randomly assigned to either pump irrigation or syringe irrigation in a 2012 Belgian monocenter study. In terms of pre-procedure preparation, pump irrigation outperformed hand irrigation, with a significant difference in the right colon[158].

Predictive models of inadequate bowel preparation in outpatients

Identifying risk factors for poor bowel cleansing can aid in determining which patients require more intensive bowel preparation. Six predictive models of inadequate bowel preparation have been developed to date based on patient potential risk factors[64,71,159-162].

Hassan *et al*[64] identified several factors that can significantly influence preparation quality in a 2012 Italian multicenter prospective study of 2811 consecutive outpatients, which were then used to develop an accurate predictive model. Overweight, male sex, having a high BMI, older age, previous colorectal surgery, cirrhosis, Parkinson's disease, diabetes, and positive fecal occult test results were all associated with inadequate bowel preparation. With 60% sensitivity, 59% specificity, 41% positive predictive value, and 76% negative predictive value, these factors predicted which patients would have inadequate cleansing.

In the validation cohort, the scale's discriminative ability was strong, with the area under the curve (AUC) of 0.77. An ASA score of 3, use of tricyclic antidepressants or narcotics, diabetes, constipation, previous abdominal and/or pelvic surgery, history of inadequate bowel preparation, and hospitalization were identified as independent predictors of inadequate bowel preparation in a 2015 Dutch

study by Dik *et al*[71]. The scale's discriminative ability was strong in the validation cohort, with an AUC of 0.77. Finally, in the multivariate analysis of 667 consecutive Spanish outpatients in 2017, antidepressants (OR = 4.25, 95%CI 1.91-9.47), co-morbidity (OR = 3.35, 95%CI 2.16-5.18), constipation (OR = 2.09, 95%CI 1.29-3.40), and abdominal/pelvic surgery (OR = 1.60, 95%CI 1.03-2.47) were independent predictors of inadequate cleansing. According to these findings, the model with all of these variables had an AUC of 0.72 in the development cohort and 0.70 in the validation cohort of 409 patients[150].

Berger *et al*[160] developed a predictive score called "Prepa-Co" in a recent French single-center study, allowing the identification of patients at high risk of inadequate bowel preparation. In total, 561 patients were included, with 25% having inadequate bowel preparation. In the prediction model of inadequate bowel preparation, the risk score includes seven variables: Diabetes or obesity, irregular physical activity, cirrhosis, use of antidepressants or neuroleptics, use of opiate medication, surgery history, and history of inadequate bowel preparation. With an AUC of 0.62, Prepa-Co correctly predicted bowel cleanliness in 68.3% of cases, with a specificity of 75.8% and a negative predictive value of 80.8%.

Sadeghi *et al*[161] conducted a population-based study on 2476 Iranian adults in 2022, and age, gender, ethnicity, BMI, abdominal circumference, fruit consumption, smoking, NSAIDs, SSRIs, education, constipation, physical activity, and diabetes were all factored into the predictive model, with the AUC reaching 0.70 in the final step.

Kurlander *et al*[162] developed prediction models for bowel preparation inadequacy in a retrospective cohort of 6885 United States veterans who underwent colonoscopy. The AUC for the validation cohort was 0.66 (95%CI 0.62-0.69), whereas the AUC for the validation cohort using random forest machine learning was 0.61 (95%CI 0.58-0.65).

So far, none of these predictive models have been tested outside of their validation cohorts, and no study has attempted to use a different regimen on patients who have risk factors for poor colon cleanliness. Furthermore, the ESGE concluded in 2019 that there was insufficient data to recommend the use of specific predictive models for inadequate bowel preparation in clinical practice[13].

CONCLUSION

To date, numerous efforts have been applied to increase the sensitivity of colonoscopy in the detection of polyps and advanced adenomas, reducing the risk of I-CRC[163,164]. These include the application of key-performance quality measures for colonoscopy[165], the use of distal attachment devices[166,167], and new intestinal preparations improving the quality of bowel cleansing[168].

Despite these efforts, adequate bowel cleansing remains a basic prerequisite for a quality colonoscopy, since it can affect other quality measures including ADR and cecal intubation rate.

Prior inadequate bowel cleansing, chronic constipation, elderly, male gender, low health literacy, obesity, diabetes, IBD, cirrhosis, neurologic disease, risk of electrolyte abnormalities, severe heart and renal failure, previous colorectal surgery, use of constipation-related medications, gastroparesis, and severe colonic stricture are the major patient-related predictors of colonoscopy preparation failure.

In the elderly, a strategy including a prolonged low-fiber diet, split preparation regimen, and colonoscopy within 5 h of the end of preparation, may increase the cleansing success rates. Furthermore, even though no specific product is specifically recommended in the other cases for difficult-to-prepare patients, recent evidence suggests that 1-L PEG-ASC preparation may be preferred in hospitalized and IBD patients. Figure 2 depicts a schematic view of the main bowel preparation tips to achieve a successful bowel cleansing.

According to current guidelines, patients with severe renal insufficiency (creatinine clearance less than 30mL/min) should be prepared with isotonic high volume PEG solutions, whereas low volume PEG plus adjuvants (*e.g.*, 1-L/2-L-PEG-ASC, and 1-L PEG plus citrate) or non-PEG regimens (*e.g.*, MCSP or OSS) are not advised.

To determine the most effective bowel preparation for difficult-to-prepare elderly, constipated, and cirrhotic patients, more high-quality research, including prospective studies with randomized designs, is required. Larger, multicenter, prospective studies are needed to determine the best bowel preparation for patients with prior abdominal-pelvic surgery, most importantly to improve ADR, especially for flat lesions in the right colon in patients who had a left colon resection.

Moreover, regardless of type of bowel solution, in very elderly patients with comorbidities, a careful assistance from family members or those who care for the elderly is necessary to guarantee the compliance to diet and preparation modalities.

In conclusion, an accurate characterization of procedural and patient variables may lead to a more personalized approach to bowel preparation (Table 1), reducing the risk of missed lesions and of I-CRC.

Table 1 Recommendation for bowel preparation in specific clinical settings

Clinical scenario	Remarks
Hospitalization	PEG-based regimens should be considered first in any preparation strategy because they are more likely to achieve adequate bowel cleansing while maintaining an optimal patient safety profile[13,45,48]
	Furthermore, multiple, combined strategies (*e.g.*, written educational material, nurse facilitation of the process, *etc.*) based on a case-by-case decision could influence the outcome[45,48]
	Although no specific product is strongly recommended for difficult-to-prepare patients, clinical evidence suggests that a 1-L PEG-ASC preparation may be preferred in hospitalized patients[22]
Elderly	A strategy that includes a low-fiber diet for an extended period of time, a split preparation regimen, and a colonoscopy within 5 h of the end of preparation may improve cleansing success rates in the elderly[57]
	A 1-L PEG preparation may be preferred for the elderly due to higher cleansing quality and higher compliance due to lower volume[56,57,77]
Obesity	ESGE recommends the use of high volume or low volume PEG-based regimens, as well as non-PEG-based agents that have been clinically validated for routine bowel preparation[13]
	For elective colonoscopy, split-dose bowel preparation (with or without the additional measures) should be used, as it has been linked to improved preparation quality[13]
Diabetes mellitus	Current US guidelines do not support assumption of lubiprostone or magnesium citrate, instead recommending a split-dose bowel cleansing regimen with no adjustments for DM patients[8]
Chronic constipation	ESGE does not recommend any specific bowel preparation in constipation patients[13]
Inflammatory bowel disease	Split dosage was associated with better cleansing regardless of preparation in some studies[106,108]
	1-L PEG-ASC is associate to higher cleansing success and good safety and should be preferred[107]
Liver cirrhosis	The use of 2-L PEG-ASC for colonoscopy in liver cirrhosis to be a safe option[114]
Chronic kidney disease	All oral laxatives should be used with caution in patients with pre-existing chronic renal failure and liaison with the renal team is advised in patients undergoing dialysis or with advanced chronic kidney disease[54,106]
	Individualized laxative selection is strongly recommended for patients at risk for hydroelectrolyte disturbances (moderate quality evidence)[13]
	Because of the risk of magnesium toxicity and acute phosphate nephropathy, magnesium-based preparations and sodium phosphate should be avoided in chronic kidney disease patients[54,120,121]
	Also, because high-volume PEG-based regimens are poorly tolerated, low-volume PEG (2-L) solutions with ascorbic acid (PEG-ASC) have been proposed to reduce the patient's excessive fluid intake[122]
	According to ESGE guidelines, patients with severe renal insufficiency (creatinine clearance less than 30 mL/min) should be prepared with isotonic high volume PEG solutions, whereas low volume PEG plus adjuvants (*e.g.*, 1L-, 2L-PEG-ASC, 1 L PEG plus citrate) or non-PEG regimens (*e.g.*, MCSP or oral sulfate solution) are not advised[13]
Heart disease	Bowel preparation (particularly after administration of PEG-ELS solution) could worsen heart failure[129]
Polypharmacy	Most medications can be taken up until the day of the colonoscopy and are taken with a small sip of water[135]
	Some medications, such as diabetes medications or anticoagulants, may need to be adjusted due to decreased oral intake prior to the procedure[13]
	Oral iron should also be discontinued at least five days before the colonoscopy because it causes residual feces[135]
History of colorectal surgery	In a study of 120 patients with prior colorectal resection for colorectal cancer, a low-volume mixed preparation (15 mg bisacodyl plus 2-L PEG) was not inferior to a high-volume regimen (4-L PEG) for adequate bowel cleansing during surveillance colonoscopy [139]
	In patients who had previously undergone left colectomy vs right colectomy, the mixed low-volume regimen had a higher success rate and tolerability[139,146]
History of poor bowel preparation	Despite a lack of strong evidence from randomized controlled trials, the ESGE guidelines and some studies recommend repeating colonoscopy using same-day or the next day with additional bowel cleansing (*e.g.*, 500 mL PEG plus ascorbate) or using enema as a salvage option in patients with inadequate bowel cleansing[73,149-152,156]
	The next bowel preparation regimen should be tailored to the potential causes of failure[13]

PEG-ASC: Polyethylene glycol plus ascorbate; MCSP: Magnesium citrate plus picosulphate; DM: Diabetes mellitus; ESGE: European Society of Gastrointestinal Endoscopy.

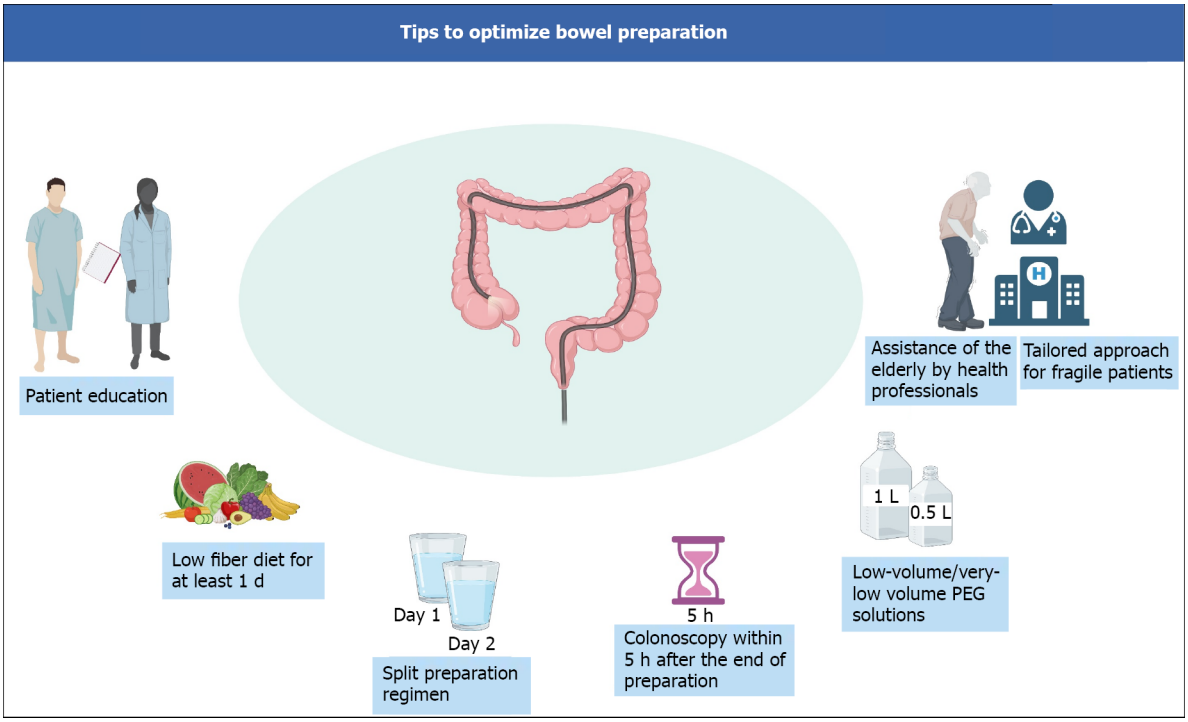

Figure 2 A schematic view of the main bowel preparation tips to achieve a successful bowel cleansing. PEG: Polyethylene glycol. PEG: Polyethylene glycol. Citation: The authors has been granted a license to use the BioRender content, including icons, templates and other original artwork, appearing in the attached completed graphic pursuant to BioRender's Academic License Terms, created with BioRender.com (Supplementary material).

FOOTNOTES

Author contributions: Shahini E and Maida M are the guarantors of the integrity of the entire study, and contributed to the manuscript drafting and revision for important intellectual content; all authors contributed to the manuscript editing and had full control over the preparation of the manuscript.

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MINIREVIEWS

Gut microbiome therapeutic modulation to alleviate drug-induced hepatic damage in COVID-19 patients

Khansa Ahsan, Munir Ahmad Anwar, Nayla Munawar

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Abstract

Coronavirus disease 2019 (COVID-19) infection caused by the severe acute respiratory syndrome coronavirus 2 virus, its symptoms, treatment, and post-COVID-19 effects have been a major focus of research since 2020. In addition to respiratory symptoms, different clinical variants of the virus have been associated with dynamic symptoms and multiorgan diseases, including liver abnormalities. The release of cytokines by the activation of innate immune cells during viral infection and the high doses of drugs used for COVID-19 treatment are considered major drivers of liver injury in COVID-19 patients. The degree of hepatic inflammation in patients suffering from chronic liver disease and having COVID-19 could be severe and can be estimated through different liver chemistry abnormality markers. Gut microbiota influences liver chemistry through its metabolites. Gut dysbiosis during COVID-19 treatment can promote liver inflammation. Here, we highlighted the bidirectional association of liver physiology and gut microbiota (gut-liver axis) and its potential to manipulate drug-induced chemical abnormalities in the livers of COVID-19 patients.

Key Words: COVID-19; Gut-liver axis; Probiotics; Prebiotics; Cytokines; Gut microbiome

Core Tip: There are several reviews in the literature focused on the pathophysiology of liver damage during severe acute respiratory syndrome coronavirus 2 infection. However, we highlighted the potential role of gut microbiota in managing drug-induced liver damage during and after coronavirus disease 2019. We shed light on various metabolites produced by gut microorganisms that have a significant role in reducing liver damage in coronavirus disease 2019 with the use of different probiotics and prebiotics.

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) is an infectious viral disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that spreads easily from person to person *via* respiratory droplets in the form of aerosols. COVID-19 has a global overall mortality rate of 2%-3%[1]. After the first case was reported in Wuhan, China in November 2019[2], SARS-CoV-2 rapidly spread throughout the world. Because of the consequential health crisis worldwide, the World Health Organization declared COVID-19 a global pandemic disease in March 2020[3]. To date, more than 6 million deaths caused by the virus have been reported around the globe.

Similar to all other RNA viruses, when SARS-CoV-2 enters and adapts to a new human host, its nucleic acids mutate, which results in new viral progeny. Several variants have gained concern during the course of the pandemic due to their impact on human health, such as alpha (B.1.1.7), beta (B.1.351), gamma (P.1), delta (B.1.617.2), and omicron (B.1.1.529)[4]. SARS-CoV-2 binds to angiotensin-converting enzyme 2 (ACE2) receptors of the host prior to entry into the cells. ACE2 is a protein receptor found on the epithelial lining of many cells and tissues such as the nose, mouth, and lungs. It is also present on blood vessels, the heart, kidneys, the liver, and the gastrointestinal (GI) tract[5,6].

Patients during the first outbreak of COVID-19 exhibited only respiratory symptoms. Approximately 2.6% suffered from diarrhea, and 2% suffered from chronic liver illness[7]. With the progression of the disease, several patients started to report gastric issues, including diarrhea, nausea, anorexia, vomiting, abdominal pain, anxiety, and intestinal bleeding[8]. These gastric symptoms lead to gut dysbiosis, which is associated with bacterial translocation into the blood during the course of COVID-19 infection, resulting in lethal secondary infections[9]. Similarly, COVID-19 damages the liver to various degrees. Numerous factors that could contribute to liver damage during infection include direct viral cytopathic effects, immune-mediated injury (systemic inflammatory response syndrome), passive hepatic congestion due to right-sided heart failure, liver hypoxia, and drug-induced liver injury[10]. Hence, COVID-19 is not limited to the respiratory tract but is also a multiorgan disease with dynamic symptoms.

To date, there is no effective therapy or antivirals for SARS-CoV-2 due to rapid genomic changes in the virus; however, symptoms are treated with various drugs. Clinical trials are being conducted to evaluate these drugs, although some of them have adverse effects on human health including liver damage or abnormal liver function[10]. It is well known that the use of drugs can cause dysregulation of gut microbiota (gut dysbiosis) as well[11]. Gut dysbiosis can result in hepatic inflammation through the biliary tract, portal vein, and systemic circulation. The translocation of endotoxins and bacteria due to increased intestinal permeability and reduction in the production of commensal gut microbial metabolites such as butyric acid, bile acids, phenolic compounds, indole and bile acid derivatives, and carotenoids promote liver inflammation. The intestine and the liver communication with each other through the gut microbiota and their metabolites have been highlighted in several studies[12]. In addition, the molding of gut community structure with drugs used for the treatment of COVID-19 infection is also well-documented[13,14]. It ultimately disturbs the gut-liver link and participates in the severity of COVID-19 consequently.

This review was conducted with the aim of evaluating and explaining the bidirectional association of the gut microbiota and liver and resolving drug-induced liver damage by investigating the role of the microbiota in restoring liver chemistry. We understand that the incorporation of microbiome-targeted therapeutics may potentially create a new way of alleviating or preventing drug-induced hepatic damage in COVID-19 patients.

SARS-COV-2 PATHOGENESIS

SARS-CoV-2 invades host cells by binding its spike protein to ACE2 host cell receptors, which is a transmembrane protein. More specifically, spike S1 of SARS-CoV-2 binds to the enzymatic domain of mACE2 (membrane-bound ACE2) of epithelial cells, resulting in the invasion of the virus in the form of endosomes. Since these membrane receptors are known to be expressed on the epithelial lining of the nose, lungs, GI tract, heart, liver, and blood vessels, they are prone to viral attachment[6]. Viral entry occurs *via* host proteases such as transmembrane serine protease types 2 and 4 followed by viral replication[15].

The SARS-CoV-2 virus also disrupts the normal intestinal microbiome, leading to digestive issues such as diarrhea. Meta-analysis of 60 studies comprising 4243 patients from China, Singapore, South Korea, the United Kingdom, and the United States showed a frequency of GI symptoms (nausea, vomiting, diarrhea, anorexia) of 17.6%. Among these, anorexia and diarrhea were most common at 26.8% and 12.5%, respectively. These symptoms usually appear after 1-2 d of respiratory symptoms (dry cough, sore throat, shortness of breath, pneumonia, and lung infection)[16,17]. Furthermore, there is an increase in the number of lung-derived C-C chemokine receptor 9 and cluster of differentiation 4 (CD4+) T cells in the small intestine by elevated levels of chemokine ligand 25, causing intestinal inflammation [18].

Several studies have shown the association of the GI tract in the pathogenesis of COVID-19, signifying the link between gut microbiota and the clinical outcome of the disease. Consequently, gut microbial dysbiosis has been found to be related to the development and severity of the disease[19,20]. When the gut microbial composition of patients with COVID-19 was compared with healthy individuals, numerous gut commensals with well-known immunomodulatory potential including *Faecalibacterium praunitzii, Eubacterium rectale,* and bifidobacteria were observed to be depleted in COVID-19 individuals and persisted to be low in stool and blood samples collected up to 1 mo after recovery from the disease. This unbalanced microbial composition indicated stratification according to disease severity corresponding to higher levels of inflammatory cytokines and blood markers including C-reactive protein, lactate dehydrogenase, γ-glutamyl transferase, and aspartate aminotransferase. The study not only specified the association of gut microbiome with disease severity but also indicated that altered gut microbiota may contribute to persistence of symptoms[20].

Similarly, Zuo *et al*[21] observed that the gut microbiome profiles of COVID-19 patients had prominent alterations with an increase in opportunistic pathogens such as *Coprobacillus*, *Clostridium ramosum*, and *Clostridium hathewayi* and a decrease in *Faecalibacterium prausnitzii* (butyrate producing anti-inflammatory bacterium), leading to severity of the disease[21]. Correspondingly, patients had higher proportions of opportunistic fungal pathogens in their fecal samples including *Candida albicans, Candida auris*, and *Aspergillus flavus* when compared to controls. Prolonged gut dysbiosis was observed during the hospitalization of these patients even after nasopharyngeal clearance of SARS-CoV-2, which indicates the long-term influence of the disease on the microbiota composition[21].

A cross-sectional study involving 30 patients suffering from COVID-19, 24 influenza A (H1N1) patients and 30 healthy individuals was conducted by Gu *et al*[22]. 16S rRNA analysis of V_3-V_4 regions revealed a decrease in gut microbial diversity of COVID-19 patients and a relative increase in opportunistic pathogens including *Streptococcus, Rothia, Actinomyces, Vellionella,* and *Erysipelatoclostridium* compared to healthy controls. Total number of *Streptococcus* and *Escherichia/Shigella* significantly increased in COVID-19 and H1N1 patients, respectively[22]. Disturbance in the normal gut microbiome and abundance of opportunistic pathogens leads to intestinal inflammation. Hence, the GI epithelium may become susceptible to SARS-CoV2 infection under certain circumstances such as viral load, coexisting disease, age, medication, gut dysbiosis, and inflammation[23]. Correspondingly, levels of interleukin (IL-17A) rise, triggering neutrophils to migrate. Hence, the lungs become prone to cytokines and bacterial invasions *via* the bloodstream, resulting in inflamed lungs[24].

The bidirectional axis of the intestine, microbiome, and liver *via* the portal vein is also affected. The portal vein transports host and microbial metabolites such as ammonia to the liver, which has an impact on liver functioning. Gut microflora that are involved in the fermentation of amino acids, constantly produce ammonia as metabolic waste, which is transported to the liver and converted into urea to be excreted in the urine. Opportunistic pathogens such as *Clostridium* and *Peptostreptococcus* are known to produce high levels of ammonia causing disruption in body nitrogen homeostasis. This eventually leads to hepatocellular metabolic dysfunction and liver injury[25] that might increase by gut dysbiosis during antiviral drug treatment of COVID-19 infection.

PATHOPHYSIOLOGY OF THE LIVER IN COVID-19 INFECTION

Despite the fact that SARS-CoV-2 is a respiratory infection, it is also primarily associated with the liver. As mentioned above, the presence of ACE2 receptors makes the host's liver prone to injury. The expression of the ACE2 receptor is significantly higher in cholangiocytes, *i.e.* 57.7% (bile duct epithelial cells) *vs* 2.6% in hepatocytes[26]. Multiple factors are involved in liver damage during COVID-19, such

as direct cytopathic attack by SARS-CoV-2, inflammation, intrahepatic immunity, multidrug-induced liver damage, drug toxicity, hypoxia, and gut dysbiosis (Figure 1)[27-30]. A direct viral attack can also lyse or induce hepatic apoptosis. Virus-specific protein 7a induces a caspase-dependent apoptosis pathway that is usually present in the lungs, kidneys, and liver[31]. Viral replication has also been observed in hepatic cells with viral spikes present in the host cytoplasm[32]. These observations suggest the cytopathic effect of SARS-CoV-2 on hepatocytes. Wang *et al*[33] further observed dilatation of the endoplasmic reticulum, reduced glycogen granules, mitochondrial swelling, and membrane disruption in hepatocytes followed by hepatic apoptosis and binuclear hepatocytes.

Another significant cause of liver damage is immune-mediated liver injury. It typically occurs due to a cytokine storm with elevated levels of IL-1, IL-6, viral-induced cytotoxic T cells (CD8), and tumor necrosis factor produced by host cells against viral infection[32,34-36]. Liver tissues of 40 patients who died due to COVID-19 were subjected to PCR for viral RNA in a study by Lagana *et al*[37]. Mitochondrial enlargement, dilation of the endoplasmic reticulum, and membrane dysfunction were observed in autopsies. Additionally, hepatic enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were found to be elevated to 68 U/L (normal 46 U/L) and 102 U/L (normal 37 U/L), respectively. Seventy-five percent of patients had macrovesicular steatosis, 50% had lobular necroinflammation (acute hepatitis), and 15% had sinusoidal microthrombi, indicating that COVID-19-infected individuals had biochemical evidence of liver damage.

Neutrophils, Kupffer cells, and plasmocytes were observed in hepatic lobules and sinusoidal and portal regions in autopsies of 48 COVID-infected patients[38]. Liver damage related to COVID-19 infection results in a change in liver enzyme serum levels, with an increase ranging from 16% to 62% for aminotransferases and 5%-20% for bilirubin[39]. Another study by Guan *et al*[40] indicated increased aminotransferase levels in serum by 22% in 757 hospitalized patients, increased AST in 18.2% of patients with mild symptoms, 39% of patients with severe symptoms, and 50% of patients admitted to the intensive care unit (ICU). Serum bilirubin levels were also above the upper limit of normal in 13.3% of patients with mild symptoms and 20.8% of patients with severe symptoms[40].

DRUG-INDUCED LIVER DAMAGE (HEPATOTOXICITY)

The use of drugs for underlying chronic diseases, including antibiotics, antivirals, anti-inflammatory drugs, and anticoagulants, during COVID-19 infection is a major cause of liver damage (Figure 1)[41]. Cai *et al*[42] revealed that over 10% of the patients had elevated liver enzymes when they were admitted to the hospital, which may have been caused by prescribed medications[42]. A systematic review/meta-analysis consisting of 20874 SARS-CoV-2 patients summarized from 107 articles showed that 25.4% of patients had drug-induced liver toxicity. Among 208 patients who received remdesivir treatment, 15.2% had a drug-induced liver injury. Lopinavir/ritonavir had a higher incidence rate of 37.2% in 775 patients[43]. Furthermore, antiviral drugs (such as favipiravir, remdesivir, lopinavir/ritonavir, chloroquine, oseltamivir, and ribavirin) and antipyretics (acetaminophen) can lead to hepatotoxicity during the course of COVID-19 infection[34]. One of the most common causes of liver damage was underlying liver disease (chronic liver disease, hepatitis, cirrhosis, and non-alcoholic steatohepatitis), which was found in a meta-analysis of 13 studies including 3046 COVID-19 patients. Of these, 25% of individuals had a hepatic injury, 21% had elevated ALT, and 24% had elevated AST. These injuries lead to the severity of COVID-19 symptoms[44]. Therefore, it was suggested that patients with underlying liver diseases must not be prescribed hepatotoxic drugs since most of the drugs are metabolized in the liver, including oseltamivir, lopinavir/ritonavir, and chloroquines. Moreover, gamma-glutamyl transferase (GGT) and bilirubin levels mainly increase with the use of antiviral drugs in COVID-19 patients. The GGT enzyme is primarily found in liver cells. In the case of liver damage, the enzyme may leak out into the bloodstream, resulting in high levels of GGT in the blood and causing liver damage.

Drugs such as lopinavir (ritonavir) may cause a transient and slight increase in liver enzymes. Patients with advanced liver disease had elevated lopinavir plasma levels. Approximately 57.8% of patients taking lopinavir developed liver damage[45]. Lopinavir is a protease inhibitor. It is usually given in combination with ritonavir to increase the plasma half-life for the treatment of human immunodeficiency virus (HIV). Since it has low efficacy toward SARS-CoV-1, it must be prescribed as early as possible after the initial diagnosis of COVID-19[46]. Severe hepatotoxicity could be caused by a high dose of ritonavir (*i.e.* 1200 mg/day). However, in lower doses (200-400 mg), it could boost other drugs (such as lopinavir and indinavir)[47].

Lopinavir/ritonavir showed 63% adverse drug reactions in 217 COVID-19 patients. However, other drugs (umifenovir, chloroquine, and antibacterial) contributed to 47% of adverse drug reactions in total [48]. Fan *et al*[49] further indicated that out of 148 COVID-19 patients, 45 individuals had a normal baseline liver function test, among which 48% developed liver abnormalities after hospital admission. When compared to patients with normal liver function (31.3%), a significantly higher percentage of patients with abnormal liver function (57.8%) had received lopinavir/ritonavir after admission[49]. Similarly, in a study involving 417 COVID-19 patients, Cai *et al*[42] indicated that liver dysfunction was considerably more prevalent in the lopinavir/ritonavir-treated groups. Within 2 wk of admission, the

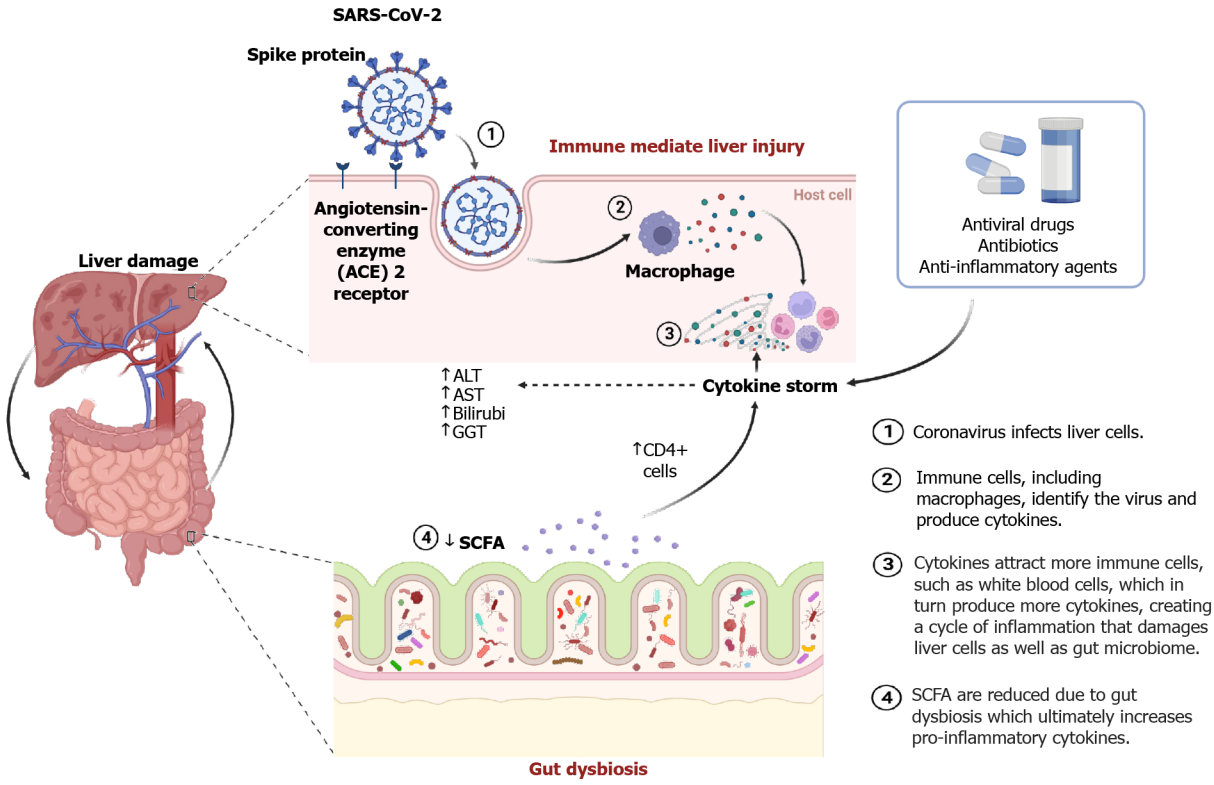

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Figure 1 Potential causes of liver injury during coronavirus disease 2019. Following severe acute respiratory syndrome coronavirus 2 infection, liver injury may arise due to direct viral entry [*via* angiotensin-converting enzyme 2 (ACE2) receptors on hepatocytes] or gut microbial dysbiosis leading to cytokine storm in the liver. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CD4+: Cluster of differentiation; GGT: Gamma-glutamyl transpeptidase; SCFA: Short-chain fatty acids.

presence of abnormal liver tests became more pronounced, with levels of ALT, AST, total bilirubin, and GGT exceeding the upper limit of normal in 49 (23.4%), 31 (14.8%), 24 (11.5%), and 51 (24.4%) patients, respectively. However, the definition of drug-induced liver injury by clinical guidelines from the European Association for the Study of the Liver was not applicable to this study due to the lack of evidence demonstrating the role of drugs in observed liver injury[42].

Simultaneous use of lopinavir/ritonavir and arbidol in COVID-19 patients with mild symptoms increased the odds of liver function, up to 3.58 times greater than in those who did not receive the medications. Human liver microsomes were used to examine the metabolic interactions between the two drugs in an effort to determine the cause of this unexpected increase. The following chain of evidence revealed that the use of tocilizumab was observed to improve both lung and liver functions within 3 wk in a case series of 7 patients who had significant abnormal liver tests in addition to worsening respiratory system function 5-7 d after receiving treatment with lopinavir/ritonavir, hydroxychloroquine, and azithromycin[50].

Since the direct effect of lopinavir/ritonavir on gut microbiota in SARS-CoV2 patients is still unknown, changes in bacterial diversity of HIV-1 patients have been observed when lopinavir/ritonavir was administered in antiretroviral therapy. Predominantly affected phylum include *Firmicutes*, *Proteobacteria*, *Bacteriodetes*, and *Actinobacteria*. Moreover, levels of gut microbial genera such as *Lachnospira, Butyricicoccus, Oscillospir,* and *Prevotella* were reduced. The *Provetella* population has been previously linked to HIV-induced inflammatory response in the host[51]. Also, a decrease in *Lachnospira, Butyricicoccus,* and *Oscillospir* has adverse effects on the host immune system since these are beneficial microbiota of the human gut[52]. Short-chain fatty acids (SCFA) such as butyrate produced by gut microbiota suppress colon inflammation. Consequently, it protects against liver damage and regulates insulin signaling in adipose tissues[53,54]. Hence, the change in gut microbiota related to lopinavir/ritonavir treatment could pose a risk of liver damage during COVID-19 infection.

Another drug used for COVID-19 patients is remdesivir, which is a nucleotide prodrug of an adenosine analog. It terminates viral replication by binding to viral RNA-dependent RNA polymerase enzyme without interfering with host RNA or DNA polymerases[55]. Remdesivir has been used for the treatment of the Filoviridae viral family, including the Ebola and Marburg viruses[56]. Its efficacy extends to Lassa fever virus and pathogenic CoV (including Middle East respiratory syndrome and SARS CoVs)[2]. Grein *et al*[57] reported the first study in a cohort of 53 COVID-19 patients. Drug effects were observed from 5-10 d of administration. Elevated levels of hepatic enzymes with a 23% incidence

rate were the most frequent adverse effect. Additionally, elevated liver aminotransferase was the reason why 1 of the 4 patients stopped receiving treatment[57].

A similar pattern was observed in the study by Kalil *et al*[58] that included 402 patients to determine the best time course for intravenous remdesivir. The most frequent adverse effects on the liver in that grade were reported to be ALT and AST elevations of 1-2 (*i.e.* 7% and 6%, respectively). Moreover, Wang *et al*[33] demonstrated a placebo-controlled double-blind clinical trial on a group of 255 patients. Grade 1-2 elevated AST was identified as an adverse liver effect (12% in the placebo group, 7% in the drug-treated group), and grade 1-2 elevated ALT resulted in discontinuation of the drug (1%). Nevertheless, grade 1–2 hypoalbuminemia (15% in the placebo group, 13% in the drug-treated group) and grade 1–2 increased bilirubin (9% in the placebo group, 10% in the drug-treated group) were the most frequent liver adverse effects[33].

Interestingly, the harmful effects of remdesivir were studied in COVID-19 patients from intensive care units (ICUs) and infectious disease wards (IDWs). The approximately same level of increase in aminotransferase was observed in both groups (ICU: 44.4%; IDW: 41.2%); however, the level of bilirubin increased more in the ICU group than in IDW individuals, indicating that the difference in enzyme levels may be related to the difference in symptomatic severity in patients[59]. In another case, an acute rise in ALT was noted after the start of remdesivir for 2 d and was immediately reversed after stopping it. The patient developed hepatotoxicity, which was proposed to be due to a drug-drug interaction of remdesivir and P-glycoprotein inhibitors. Hence, it was suggested to use the drug with caution[60]. Remdesivir-associated liver failure improved in a study reported by Carothers *et al*[61], suggesting that the use of acetylcysteine could be beneficial. However, there is a limitation in data regarding the management of acetaminophen-associated liver failure with acetylcysteine.

Similarly, favipiravir (avigan) is also considered for the treatment of SARS-CoV-2 infection. It is a broad-spectrum antiviral medicine that was initially administered for the treatment of influenza in Japan. Favipiravir is a prodrug that is taken up by viral RNA polymerase as a purine nucleotide after being intracellularly phosphorylated to form the active metabolite (favipiravir ibofuranosyl-5′-triphosphate), effectively inhibiting RNA-dependent RNA polymerase. It has been effective against RNA viruses such as West Nile virus, yellow fever virus, foot-and-mouth disease, Ebola, and Lassa virus[62]. In a case study, favipiravir was found to cause cholestatic liver injury. The authors suggested that liver injury developed due to the use of antibacterials followed by a high dose of favipiravir (6000 mg on the 1st d and 2400 mg for 14 d), which worsened liver function with elevated transaminase and total bilirubin levels[63].

A recent study comparing patients in the favipiravir group to those in the control group receiving lopinavir/ritonavir 400 mg/100 mg twice daily for 14 d plus aerosolized interferon-α by inhalation (5 million U twice daily) showed that the favipiravir group significantly reduced the amount of time needed for viral clearance (median 4 d *vs* 11 d) while also experiencing fewer side effects[64]. Additionally, teratogenicity (abnormal fetal development), hyperuricemia[50], diarrhea, and neutropenia are the known side effects of this drug[65]. Although the dose regimen for clinical trials or experimental drugs used for COVID-19 patients is aided by information from the treatment of influenza, more clinical testing is necessary to determine the exact effectiveness of favipiravir[65].

Guaraldi *et al*[66] in a retrospective cohort study found that tocilizumab (an IL-6 receptor antagonist) does not have any harmful effects on the liver when administered to 1351 COVID-19 patients. However, serum transaminase levels were elevated up to 40 fold[67]. Similarly, a significant correlation was later found between the administration of lopinavir/ritonavir, hydroxychloroquine, remdesivir, and tocilizumab, leading to liver damage in 1827 patients[68]. Other drugs, including hydroxychloroquine and azithromycin, are known to be used for SARS-CoV-2 infections leading to liver damage[69].

Most of these drugs also inhibit liver transporters including ABCB11/BSEP, ABCC2/MRP2, SLC47A1/ MATE1, ABCC3/MRP3, ABCC4/MRP4, SLC22A1/OCT1, SLCO1B1/OATP1B1, SLCO1B3/OATP1B3, and SLC10A1/NTCP. Since these transporters play a significant role in the clearance of endobiotics and other toxic compounds, their inhibition may affect liver functioning and cause alterations in the GI tract and kidney damage[1].

Since inflammatory cytokines are produced in response to COVID-19 infection, excessive cytokine (cytokine storm) causes septic shock, tissue damage, and organ failure. The microbial imbalance found in the blood analysis of COVID-19 patients was also linked to elevated levels of inflammatory cytokines and blood markers, such as C-reactive protein and specific enzymes, as a result of tissue damage[20]. The gut dysbiosis-associated hepatic inflammation led to severe symptoms of COVID-19 and increased mortality. Therefore, incorporation of the drug-induced gut dysbiosis and its connection to hepatic injury could better elucidate the gut-liver-bidirectional-axis association with COVID-19 severity. A precise understanding of interaction between gut microbiota and liver physiology will facilitate the development of targeted therapeutics to improve the condition of COVID-19 patients.

GI MANIFESTATION AND GUT DYSBIOSIS (METABOLIC DYSFUNCTION)

Studies have suggested that altered gut microbiota (dysbiosis) can play a significant role in immune-

mediated inflammatory diseases[70]. Similarly, dysbiosis of the gut microbiota might determine the clinical outcome of patients with underlying comorbid illnesses such as type 2 diabetes, hypertension, and obesity in COVID-19[71]. A potential role of gut microbiota in overall pathogenesis and outcomes is implied by the fact that gut microbial diversity generally decreases with age and that COVID-19 severity and fatality increase in older individuals. The impact of this disease can be minimized by improving the gut microbiota profile *via* personalized nutrition and supplements that improve immunity in older patients and immunocompromised individuals[72].

It has further been suggested that patients with COVID-19 have compromised gut microbiota, which has well-known immunomodulatory potential. Blood and stool samples were collected from 100 COVID-19 patients in a two-hospital cohort study. A significant alteration in gut microbial composition was found in COVID-19 patients compared to healthy individuals. Gut commensal microbiota with known immunomodulatory potential, such as *Bifidobacteria*, *Faecalibacterium prausnitzii*, and *Eubacterium rectale,* decreased in number and remained low even when the samples were collected until 30 d after recovery from COVID-19 infection. Moreover, blood markers, including AST, C-reactive protein, lactate dehydrogenase, and GGT, were elevated in this perturbed composition, which also showed stratification with disease severity[20]. Since the digestive and respiratory systems have an impact on each other *via* the gut-lung axis (common mucosal immune system), it is believed that improved GI ecology will have a positive impact on COVID-19 patients[73].

Although a small case series from China suggested that COVID-19 patients had less *Lactobacillus* and *Bifidobacteria* during microbial dysbiosis, no conclusive research linked the intestinal microbiota to COVID-19 at that time[74]. However, the SARS-CoV-2 receptor ACE2 was found to control intestinal microbial homeostasis *via* amino acids in a previously reported study[75]. Gut microbiota are known to produce SCFAs by fermentation. Predominant gut bacteria [*Ruminococcaceae* (*cluster IV*) and *Eubacterium* (*cluster XIVa*) in order *Clostridia* and phylum *Firmicutes*] produce SCFAs including acetate, propionate, and butyrate, which are frequently metabolized[76]. SCFAs that remain undigested further promote the formation of naïve CD4+ T cells, which mainly aid in controlling the level of lymphocytes in bone marrow and peripheral blood circulation (Figure 1). Consequently, gut microbial homeostasis is disturbed, ultimately compromising the immune system.

Increased levels of cytokines and inflammatory cells during COVID-19 infection are linked to sepsis and acute respiratory distress syndrome (ARDS). Inflammatory cytokines [IL-6, IL-8, IL-10, and tumor necrosis factor α (TNF-α)] were found to be high in number, leading to ARDS and multiple-organ dysfunction[77,78]. Butyric acid produced by intestinal bacteria is known to reduce cytokine storm[79]. Thus, the gut microbiota could help in reducing the prevalence of ARDS and sepsis, which are major mortality risks in COVID-19. Moreover, some researchers think that sepsis and abnormalities of the gut microbiota should be promoted together[80].

POTENTIAL THERAPEUTICS: PROBIOTICS AND PREBIOTICS

Considering the link between gut dysbiosis due to cytokine storm and COVID-19 severity, modulation of the gut microbiome holds great therapeutic potential for disease modification (Figure 2). However, there is currently no microbiota-directed therapy that has been shown to be effective in preventing the development or progression of COVID-19. Nevertheless, scientists are raising concerns about the health benefits and disease prevention properties of diet and gut microbiota during the course of infection. Growth of *Bifidobacterium* and *Lactobacillus* spp. in the human gut is promoted by plant-based fibers, which also help reduce harmful microbiota (*Clostridia*)[81]. Since microbial SCFAs are produced by the fermentation of dietary fibers and have anti-inflammatory effects, fiber intake can improve the host immune system[82]. When tested in mouse models, a fiber-rich diet promoting SCFA was found to increase immunity against allergic inflammation in the lungs, whereas a low-fiber diet with low SCFA levels increased allergic airway disease[83,84]. Studies have verified that the use of whole-grain fiber can reduce the mortality rate in various respiratory diseases[85].

Similarly, oral administration of probiotics alters the composition of the gut microbiota once it reaches the intestine[81]. Several studies have shown that the consumption of probiotics (beneficial bacteria) changes the local and systemic inflammatory balance, which in turn reduces respiratory infections and other extra-intestinal illnesses. When *Lactobacillus gasseri* SBT2055 was administered orally as a probiotic in mice, the inflammatory response against respiratory syncytial virus infection in the lungs was repressed. Levels of proinflammatory cytokines (IL-6, TNF-α, IL-1β, and chemokine ligand 2) significantly decreased and were maintained at equivalent levels compared to control mice [86]. Furthermore, the cellular immunity of 30 elderly volunteers was boosted when they took *Bifidobacterium lactis HN019*[87]. Similarly, placebo-controlled clinical trials using the probiotic *Bacillus subtilis* and *Enterococcus faecalis* were effective and safe ways to prevent ventilator-associated pneumonia and gastric colonization of potentially pathogenic microorganisms[88]. Probiotics seem to be among the most suitable, efficient, and potentially safe strategies if dysbiosis is indeed involved in the pathogenesis of severe COVID-19. In fact, the National Health Commission (China) suggested the use of probiotics for maintaining gut microbial homeostasis and preventing secondary bacterial infections[89].

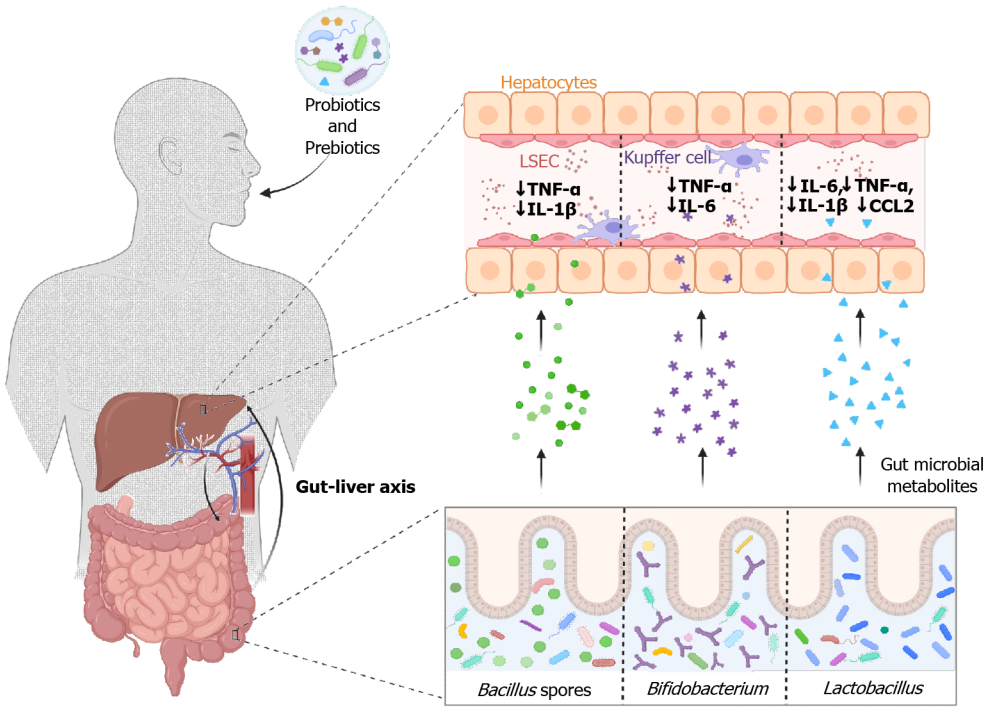

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Figure 2 Modulation of gut microbiota and its role in the gut-liver axis during coronavirus disease 2019. Probiotics and prebiotics could be used as potential therapeutics to lower coronavirus disease 2019 symptom severity by producing various bioactive metabolites, which are absorbed into the liver mainly *via* the hepatic portal vein, for regulation of hepatic function by reducing inflammatory cytokines. CCL2: Chemokine (C-C motif) ligand 2; IL-1β: Interleukin-1 beta; IL-6: Interleukin 6; LSEC: Liver sinusoidal endothelial cells; TNF-α: Tumor necrosis factor-α.

Since drug-induced liver damage is one of the major outcomes of COVID-19 infections, scientists are focusing on possible strategies to mitigate liver damage. As discussed, hepatotoxicity is most likely induced by chemical exposure, disrupted intestinal microbiome, gut mucosal barrier damage, and systematic immune activation. However, many preclinical studies have shown that prebiotic and probiotic supplementation could improve drug-induced liver injury[90]. Pretreatment with *Lactobacillus reuteri* DSM 17938 was performed on rats after undergoing a model of liver failure. Not only did it lower serum ALT, AST, GGT, IL-1, IL-2, IL-18, macrophage colony-stimulating factor, and macrophage inflammatory protein 3α levels, but it also improved histological abnormalities in the terminal ileum and liver caused by d-galactosamine[91].

Similarly, the hepatotoxic effect of acetaminophen was observed to be reduced using probiotic Mega Spore Biotic TM, which is a *Bacillus* spore-based probiotic. It also decreased proinflammatory cytokines such as TNF-α and L-1β and reduced hepatocyte necrosis[92]. Acetaminophen is a widely used antipyretic and has adverse effects in COVID-19 patients[93]. Moreover, the use of *Bifidobacterium adolescentis* CGMCC15058 in rats with liver failure was reported to have therapeutic effects with reduced levels of inflammatory liver cytokines such as TNF-α and IL-6[94]. Amplicon sequencing revealed the loss of potential SCFA-producing gut microbiota, such as ASV0AKS_*Oscillibacter*, ASV009F_*Anaerofustis*, ASV02YT_*Blautia*, ASV07LA_*Blautia*, and ASV0AM6_*Eubacterium hallii* in post-acute COVID-19 syndrome. These gut microbial species could be elevated using a high-fiber formula. Clinical parameters such as alkaline phosphatase, AST, ALT, albumin, and total bilirubin returned to normal levels after a high-fiber diet, leading to improved post-acute COVID-19 GI symptoms and liver function[95].

CONCLUSION

The gut microbiota play an important role in maintaining human health. Irrespective of the poor understanding of the connection between the gut and drug-induced hepatic injury mechanisms, the gut microbiota poses a significant role in liver protection through different pathways seems to be critical. The use of antiviral agents in SARS-CoV-2 patients results in gut dysbiosis that may predispose patients to severe COVID-19, as intestinal permeability and bacterial products spilling out enhanced by increased proinflammatory cytokine due to liver damage led to the severity of symptoms. It is therefore crucial that we explore potential preventive and therapeutic targets, such as probiotics and dietary interventions for gut rebiosis. Different probiotics using diverse prebiotics produce a variety of hepatic protective bioactive metabolites that could mitigate drug-induced liver damage during COVID-19. We

believe that the risk of drug-induced liver injury could be minimized by boosting hepatic function *via* rebuilding the dysbiotic intestinal environment with probiotics and prebiotics. The targeted intervention of gut microbiota may regulate the intestinal microbial community and thus manage liver injury. Moreover, we suggest well-planned experiments on animal models and clinical trials to understand the interactions between gut microbes and liver diseases to use this approach comprehensively.

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FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

Locked nucleic acid real-time polymerase chain reaction method identifying two polymorphisms of hepatitis B virus genotype C2 infections, rt269L and rt269I

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Abstract

BACKGROUND

The presence of two distinct hepatitis B virus (HBV) Pol RT polymorphisms, rt269L and rt269I, could contribute to the unique clinical or virological phenotype of HBV genotype C2. Therefore, a simple and sensitive method capable of identifying both types in chronic hepatitis B (CHB) patients infected with genotype C2 should be developed.

AIM

To develop a novel simple and sensitive locked nucleic acid (LNA)-real time-polymerase chain reaction (RT-PCR) method capable of identifying two rt269 types in CHB genotype C2 patients.

METHODS

We designed proper primer and probe sets for LNA-RT-PCR for the separation of rt269 types. Using synthesized DNAs of the wild type and variant forms, melting

temperature analysis, detection sensitivity, and endpoint genotyping for LNA-RT-PCR were performed. The developed LNA-RT-PCR method was applied to a total of 94 CHB patients of genotype C2 for the identification of two rt269 polymorphisms, and these results were compared with those obtained by a direct sequencing protocol.

RESULTS

The LNA-RT-PCR method could identify two rt269L and rt269I polymorphisms of three genotypes, two rt269L types ['L1' (WT) and 'L2'] and one rt269I type ('I') in single (63 samples, 72.4%) or mixed forms (24 samples, 27.6%) in 87 (92.6% sensitivity) of 94 samples from Korean CHB patients. When the results were compared with those obtained by the direct sequencing protocol, the LNA-RT-PCR method showed the same results in all but one of 87 positive detected samples (98.9% specificity).

CONCLUSION

The newly developed LNA-RT-PCR method could identify two rt269 polymorphisms, rt269L and rt269I, in CHB patients with genotype C2 infections. This method could be effectively used for the understanding of disease progression in genotype C2 endemic areas.

Key Words: Hepatitis B virus; Genotype C2; Polymerase; rt269; Locked nucleic acid-real time-polymerase chain reaction; Chronic hepatitis B

Core Tip: Hepatitis B virus (HBV) genotype C2 infections have distinct clinical or virological traits, including a higher risk of hepatocellular carcinoma, lower response rate to interferon or prolonged hepatitis B e antigen-positive phase. We recently reported that the presence of two HBV Pol RT polymorphisms, rt269L and rt269I, contributed to unique traits of HBV genotype C2. Here, instead of time- or labor-consuming direct sequencing, we developed a new locked nucleic acid (LNA)-real time-polymerase chain reaction (RT-PCR) method for the separation between rt269L (L1 and L2) and I type from Korean chronic hepatitis B patients of genotype C2. The newly developed LNA-RT-PCR could be effectively used for the understanding of epidemiology and disease progression in genotype C2 endemic areas.

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INTRODUCTION

Although vaccines and therapeutic agents are currently available against hepatitis B virus (HBV), HBV infection is still a high-risk global health issue. More than 350 million people are chronically infected, and approximately 786000 patients die annually worldwide due to HBV-related diseases, including cirrhosis and hepatocellular carcinoma (HCC)[1,2].

HBV belongs into hepadnaviridae and is an enveloped and partially double-stranded DNA virus. Its genome is approximately 3.2 kb in length and contains 4 overlapping open reading frames: Surface antigens (S), core proteins (C), polymerase (Pol), and X proteins (X)[3]. The HBV reverse transcriptase can lead to HBV mutations of higher frequency than that of other DNA viruses due to its lack of proofreading ability[4,5]. This results in the failure of antiviral therapy with nucleos(t)ide analogs and liver disease progression *via* persistent infections[5-9]. According to the criteria of an 8% divergence in HBV genome sequences, HBV has been grouped into 10 genotypes as A-J[10-12]. A number of studies on HBV genotypes have reported that they play significant roles in the development of different disease profiles during chronic hepatitis B (CHB) infection as well as distinct geographic and ethnic distributions[13,14]. Of note, genotype C, particularly C2, *vs* genotype B showed a higher HBV replication capacity and higher tendency of chronicity and more frequently developed into liver cirrhosis (LC) and HCC in CHB patients of HBV endemic Asian nations, such as China, Japan and South Korea[11,15-19]. In addition, incomplete response to interferon (IFN) therapy and higher levels of mutations were also reported in genotype C2 infections[18,20-22]. However, thus far, which factor can explain several

distinct characteristics in clinical and virological aspects found in genotype C2 infections remains elusive.

As one likely answer to this issue, we have recently reported that the presence of two HBV Pol RT polymorphisms, rt269L and rt269I, that are found only in HBV genotype C could affect viral phenotypes and clinical outcomes and cause worse responses to IFN therapy in genotype C2 infections. In particular, we showed that the wild rt269L type infection that is distinct in genotype C *vs* the rt269I type is more strongly related to higher HBV replication and hepatitis B e antigen (HBeAg) positive serostatus, which are two distinct traits of genotype C infections[23-25]. This suggests that the presence of RT polymorphisms, particularly the wild rt269L type, could at least partly contribute into clinical or virological traits that are distinct in genotype C infections. However, our previous study has limitations in exploring the distribution of rt269 polymorphisms in CHB patients due to use of a conventional nested polymerase chain reaction (PCR) based direct sequencing protocol, which could underestimate genuine HBV quasispecies in patient sera[23]. A locked nucleic acid (LNA) is a nucleic acid analog containing a methylene bridge that connects the 2'-oxygen of ribose with the 4'-carbon[26,27]. The real time PCR method using a LNA-based probe capable of improving the hybridization affinity for complementary sequences shows strong mismatch discriminatory power[28,29]. Therefore, without the application of nested PCR, it could discriminate HBV mutations from CHB patients with high sensitivity and specificity.

Therefore, in this study, for the first time, we sought to develop a novel simple and sensitive locked nucleotide probe (LNA probe)-based RT-PCR (LNA-RT-PCR) method that is capable of separating two different rt269 polymorphisms, the wild-type rt269L (CTC/A) and rt269I type (ATC), in CHB patients of genotype C2.

MATERIALS AND METHODS

Patient sera, HBV DNA extraction and genotyping

For this study, serum samples from 94 patients who visited Seoul National University Hospital (2005-2007), met the inclusion criteria of hepatitis B surface antigen (HBsAg) positivity and HBV DNA positivity (for more than 6 mo), and were lamivudine, adefovir dipivoxil, entecavir, telbivudine, tumor necrosis factor, and peg-IFN treatment-naïve were used. All patients had negative tests for hepatitis C virus, human immunodeficiency virus and markers for coexisting autoimmune liver disease and did not have an alcohol or drug addiction. HBV DNA was extracted from 200 μL of serum samples using the QIAamp DNA Blood Mini Kit (QIAGEN Inc, Hilden, Germany). To analyze the genotyping, a nested PCR-based sequencing protocol targeting partial HBsAg sequences was used as previously described [30]. This study was approved by Seoul National University Hospital (IRB-1012-131-346).

Synthesis of positive control DNAs for variants at the HBV rt269 codon

We prepared six positive control DNAs for L1 [CTC, wild type (WT)] and the variants I (ATC) and L2 (CTA) at the HBV rtL269I locus. The DNAs were synthesized based on the HBV C2 polymerase sequence by Integrated DNA Technologies, Inc. They were 473 bp long and included the three variant sequences, with one of the 'A/G' polymorphisms near the variant sequence (Figure 1, Supplementary Table 1). These were used for the development of the methods for the application of LNA real-time PCR to a rapid differential and quantitative identification of the WT and variants. We used these DNAs to intentionally mix DNA templates with WT control DNA and variant control DNA in different ratios in a range of amounts to mimic clinical samples. We also used positive controls for melting temperature (T_m) analysis, detection sensitivity, and endpoint genotyping and for the construction of quantification standard graphs for LNA-RT-PCR to estimate the quantity of HBV WT and variant DNA in clinical samples.

Primer and LNA probe design

Primers were designed using LightCycler Probe Design Software 2.0 (LC PDS 2.0) Version 1.0.R.36 (Roche). The primers were designed to have high melting temperatures (> 65 °C) and to be highly conserved in the target DNA region of HBV. We used LC PDS (version 2.0) software for the probe design and referred to the design guidelines of the LNA manufacturer (Integrated DNA Technologies). The potential presence of cross-complementarities among all the primers and LNA probes was checked by using LC PDS 2.0 software. The LNA probes were purchased from Integrated DNA Technologies, and primers were purchased from Macrogen.

RT-PCR

A LightCycler Version 96 system (Roche) was used for LNA-RT-PCR, and three channels were used for the experiment. An optimal reaction mixture was established for the sensitive and specific detection of target sequences. A 10-μL reaction mixture was prepared for each sample as follows: 1 μL PCR buffer for *Taq* (Ex *Taq* HS, Takara), 2 mmol/L $MgCl_2$, 0.2 mmol/L deoxynucleoside triphosphate mixture

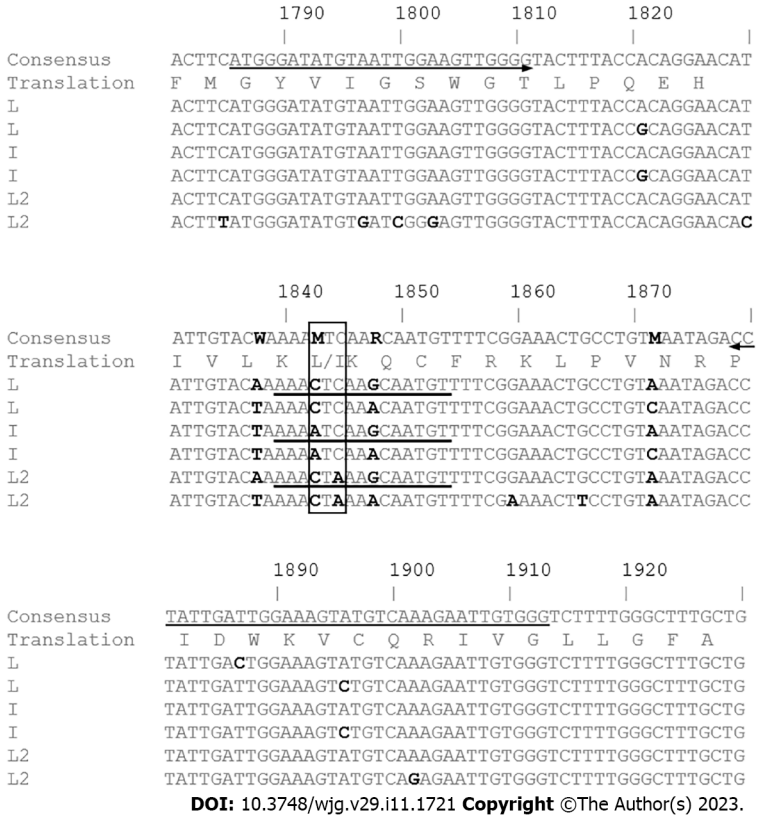

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Figure 1 Primer and locked nucleic acid probe positions designed for the detection of three genotypes of polymorphisms in the rt269 codon, 'L1', 'I' and 'L2'. Arrows indicate the primer positions. Underlines indicate the probe positions. The numbers designate the nucleotide position on the hepatitis B virus *P* gene sequence. Boldface bases denote the different bases. The box represents the codon and amino acid sequences of rtL269 variants. This single nucleotide difference is the basis of their discriminative identification by locked nucleic acid probes in this study. The amino acid sequence is shown as one-letter amino acid symbols.

(Takara), 0.2 μM forward primer, 0.8 μM reverse primer, 0.4 μM LNA FAM probe (L_CTC), 0.4 μM LNA Hex probe (I_ATC), 0.2 μM LNA Cy5 probe (L2_CTA), 0.25 u Ex *Taq* HS (Takara), 1 mg/mL bovine serum albumin (Ambion, ThermoFisher), 2 μL template DNA, and PCR-grade water (Roche). The cycling conditions were as follows, with default ramping speed rates if not specified: 60 s at 95 °C; four cycles of 10 s at 95 °C, 10 s at 58 °C, and 25 s at 72 °C with a 2.2 °C/s ramp; 46 cycles of 10 s at 95 °C, 10 s at 58 °C (with a single fluorescence acquisition), 25 s at 72 °C with a 2.2 °C/s ramp, and melting-curve analysis with 10 s at 95 °C, 60 s at 53 °C, and 1 s at 80 °C with a 0.08 °C/s ramp under continuous fluorescence acquisition at a rate of 4 readings/°C.

Identification of the WT and variant forms

Identification of the WT and variant forms 'I' and 'L2' at the rt269 codon in a sample was performed based on the three different LNA probe-specific T_m measurements at their own specified channels. To establish the diagnostic T_m range for the WT and variant forms, the control DNAs of the WT form, the variant forms and their mixtures at a variety of ratios were tested to observe melting peak formation and measure the specific T_m values for the WT and variant forms.

Construction of standard quantification curves

Six types of standard quantification curves for the WT and variant forms were generated with known amounts of positive control DNAs for their application to the estimation of the amount of the target DNAs in unknown samples. The standard curves were produced by duplicate LNA real-time PCR for each target DNA with known amounts (4.0E + 08 to 4.0E + 01 copies) of control DNAs. The R^2 correlation for all the standard curves was greater than 0.99. The limit of detection and limit of quantification of the WT and variant forms were determined among the series of diluted copies. These standard curves were applied to the quantification of DNA samples in a pure form and dominant type of variants in a mixed form.

Construction of standard genotyping plots to determine a dominant type in a mixture sample

To determine a dominant type of rtL269I variant in a mixture of a sample, standard genotyping plots were constructed using LNA real-time PCR with positive control DNA mixture sets in various ratios

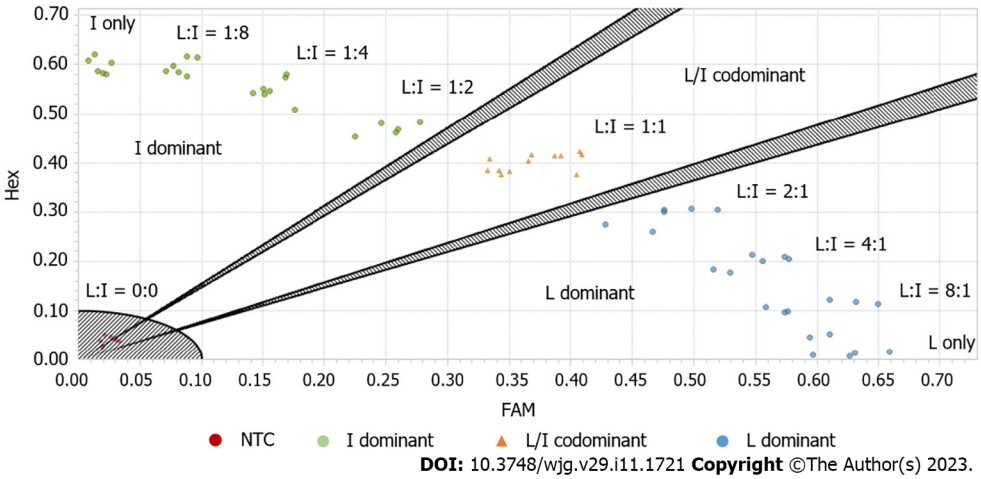

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Figure 2 Differentiation of dominant hepatitis B virus rtL269 genotype variants using an endpoint genotyping method (LC96 software). L plus I genotype mixtures were prepared with known amounts of the genotypes in various ratios. I-dominant mixtures were positioned closer to axis Hex with higher Hex-fluorescence values, whereas L-dominant ones were located closer to axis FAM with higher FAM-fluorescence values. NTC: Nontemplate control.

and the endpoint genotyping tool of LC 96 system software. These plots were based on the endpoint fluorescence (EPF) values at the two channels for comparison. These plots were applied to determine the dominant type in the clinical samples (Figure 2).

Application of LNA-RT-PCR to clinical samples

The DNA of a total of 94 human sera was tested for the identification of the WT and 'I' and 'L2' variant forms of the HBV RT gene by LNA-RT-PCR. The quantification cycle (Cq), EPF, and T_m produced by the WT- and variant-targeting LNA probes with sample DNA were measured. Identification of the WT and variant forms was determined by comparing their T_m values obtained from their specific channel (FAM for WT, Hex for 'I', and Cy5 for 'L2') with their diagnostic T_m ranges obtained from standard assays.

Comparison of LNA-RT-PCR and direct sequencing for identification of WT and variant DNA

A total of 94 clinical samples were tested for the comparison of the LNA-RT-PCR method and directing sequencing method in the accurate identification of the rt269 variant and WT DNA. Direct sequencing was performed using the same primer sets producing the 128-bp LNA-RT-PCR amplicon.

RESULTS

Primer and probe design for LNA-based RT-PCR

First, we investigated the full-length HBV reverse transcriptase sequences from 131 treatment-naïve Korean patients chronically infected with HBV genotype C2 (GenBank No CH patients (GenBank Nos: KX264864-KX264922) and HCC patients (GenBank Nos: KX264792-KX264863)[30]. SeqMan II software Version 5.03 (DNASTAR) was used to search for appropriate primer sequences for LNA-based RT-PCR that are highly conserved to first obtain the shortest possible amplification product of the rt269 codon for efficient PCR (Figure 1).

We found three distinct sequence types in the rt269 codon from 131 patients, two types in rt269L, CTC (designated L1) and CTA (designated L2), and one rt269I type, ATC (designated I). Therefore, we designed three different LNA probes for specific simultaneous detection in a single reaction of the 'L' (WT), 'I', and 'L2' variants of HBV. The sequences of primers and LNA probes are shown in Table 1 and Figure 1.

Determination of the diagnostic T_m range for the identification of the WT and variant forms

Identification of the three sequence types, "L1", "I" and "L2", was accomplished by LNA-RT-PCR melting curve analysis by observation of their melting peak formation and their specific T_m measurement at their specified channel (Table 2, Figure 3). LNA-RT-PCR with samples of WT control DNA (n = 68) in amounts ranging from 4.0E + 00 to 4.0E + 08 copies resulted in a 100% positive detection rate and 100% specificity. A distinct melting peak formation at the FAM channel in all the tested WT control DNA samples with T_ms of 62.4 ± 0.4 °C for 'L' and 58.0 ± 0.2 °C for 'L" was observed, but no significant melting peak formation at the other channels (Hex and Cy5) was observed. LNA-RT-PCR with samples of the 'I' positive control DNA (n = 76) also resulted in a 100% positive detection rate and 100% specificity. A distinct melting peak formation in the Hex channel, 60.2 ± 0.7 °C for 'I' and 56.6

Table 1 Primers and locked nucleic acid probes developed for the identification of the hepatitis B virus L/I/L2 variants by multiprobe locked nucleic acid real-time polymerase chain reaction

Primer/probe	Sequence (5' to 3')[1]	T_m (°C)[2]	Target	CH
Primers (product: 128 bp)				
Forward	ATGGGATATGTAATTGGAAGtTGGGG	65-67	HBV P gene	
Reverse	CCCACAATTCttTGACATACTTTCCAATCAATAGG	67-69	HBV P gene	
LNA Probes				
L_CTC	5′ 6-FAM-AAA+C+T+CAAR+CA+ATGT - 3′ IABkFQ	61-64	L (WT)	FAM
I_ATC	5′ HEX-AAA+A+T+CAAR+CAA+T+GT - 3′ IABkFQ	61-64	I	HEX
L2_CTA	5′ CY5-AAA+C+T+AAAR+CAA+T+GT - 3′ IABkFQ	60-63	L2	CY5

[1]Locked nucleic acid nucleotides are written +A, +C, +T or +G.

[2]Primer T_m was calculated by using LC PDS software version 2.0, and probe T_m was calculated by https://www.exiqon.com/ls/pages/exiqontmpredictiontool.aspx.

T_m: Melting temperature; CH: Channel; HBV: Hepatitis B virus; WT: Wild type; LNA: Locked nucleic acid.

± 0.2 °C for 'I'', was observed, but no significant melting peak formation in the other channels (FAM and Cy5) was observed. LNA-RT-PCR with samples of the 'L2' positive control DNA (n = 52) also resulted in a 100% positive detection rate and 100% specificity. A distinct melting peak formation at the Cy5 channel, 64.6 ± 0.1 °C for 'L2' and 61.2 ± 0.2 °C for 'L2'', was observed, but no significant melting peak formation at the other channels (FAM and Hex) was observed.

LNA-RT-PCR with samples (n = 320) of the 'L' WT positive control DNA plus 'I' control DNA or 'L2' plus 'I', mixed in different ratios (1:1, 1:2, 1:4, 1:8, 2:1, 4:1, and 8:1) in amounts ranging from 4.0E + 01 to 4.0E + 08 copies resulted in a nearly 100% positive detection rate (only three samples undetected in the smallest amount of DNA) for both the variant and WT DNA. A distinct melting peak formation at the FAM, Hex, and Cy5 channels in all the mixed DNA samples with detectable T_ms was observed. The measured T_ms were shifted slightly downward from the range of the T_ms measured only with nonmixed DNAs, as shown in Table 1. These slight changes did not affect the identification of the sequence types in the samples.

Application of LNA-RT-PCR to clinical samples and comparison with the results of the direct sequencing protocol

Of the 94 clinical samples tested by our LNA-RT-PCR method, 87 samples (92.6% sensitivity) were positively identified as 'L1' (WT), 'I', and 'L2' variants in single or mixed forms. Among the positively identified samples (n = 87), all samples produced a distinct melting peak or peaks with a T_m or T_ms within the diagnostic T_m range for the WT form "L1" or the two variant forms "I" and "L2". Of the 87 positively detected samples, 63 (72.4%) and 24 samples (27.6%) were identified either singly or in a mixed manner, respectively. Of the 63 samples identified singly, the prevalence of the 'L1' type, 'I' type and 'L2' type was 82.5% (n = 52), 12.7% (n = 8) and 4.8% (n = 3), respectively (Table 3). Of the 24 mixed form samples (27.6%), the prevalence of samples with almost the same ratio of L1 and I (codominant cases) was 29.2% (n = 7). The prevalence of L1 (L1 + I or L1 + L2) and I dominant (L1 + I) cases was 54.2% (n = 13) and 16.7% (n = 4), respectively. Given that the dominant cases included the respective exclusive cases, of the 87 positively detected samples, the prevalence of L, I and coinfection with L and I was 78.2% [n = 68, L1(65) + L2(3)], 13.8% (n = 12), and 8.0% (n = 7), respectively. PCR direct sequencing using the same primer set used in the LNA-RT-PCR method enabled the successful separation between the L1, L2 and I sequence types in all 94 clinical samples (100% sensitivity). Comparison between results obtained by both direct sequencing and LNA-RT-PCR protocols showed that of the 87 samples identified by LNA-RT-PCR, all (86 samples, 98.9% specificity) but one sample (SNU3-479) produced completely identical results between the two protocols (Figure 4). A mismatched sample was identified as I dominant (L1:I = 1:4) by LNA and exclusive I type by the direct sequencing protocol. The distinct results between both protocols may be due to the difference in sensitivity between the protocols. All seven samples not detected by the LNA-RT-PCR method were demonstrated to have mutations in their respective probe binding sequences by a direct sequencing protocol, which could interfere with normal LNA-RT-PCR (Table 4).

Table 2 Measurement of melting temperatures of the L/I/L2 variants by multiprobe locked nucleic acid real-time polymerase chain reaction

Genotype of positive control DNA [copies, (4.0E + 00)-(4.0E + 08)]	Target sequence[1]	Measured T_m (°C) in channel[2]								
		Min[3]	Max[3]	mean ± SD (detection)[3]	Min[4]	Max[4]	mean ± SD (detection)[4]	Min[5]	Max[5]	mean ± SD (detection)[5]
L (n = 34)	AAA**CTC**AAGCAATGTT	61.6	63.1	**62.4 ± 0.4** (34, 100%)	-	-	- (0, 0%)	55.2	56.2	55.8 ± 0.2 (26, 76.4%)
L′ (n = 34)	AAA**CTC**AAACAATGTT	57.8	58.7	**58.0 ± 0.2** (34, 100%)	-	-	- (0, 0%)	-	-	- (0, 0%)
I (n = 42)	AAA**ATC**AAGCAATGTT	-	-	- (0, 0%)	59.0	61.6	**60.2 ± 0.7** (42, 100%)	-	-	- (0, 0%)
I′ (n = 34)	AAA**ATC**AAACAATGTT	-	-	- (0, 0%)	56.3	57.1	**56.6 ± 0.2** (34, 100%)	-	-	- (0, 0%)
L2 (n = 34)	AAA**CTA**AAGCAATGTT	-	-	- (0, 0%)	-	-	- (0, 0%)	64.4	64.8	**64.6 ± 0.1** (26, 100%)
L2′ (n = 18)	AAA**CTA**AAACAATGTT	-	-	- (0, 0%)	-	-	- (0, 0%)	60.9	61.4	**61.2 ± 0.2** (18, 100%)

[1]Bold, target codon; underline, A/G polymorphism.
[2]Bold, target specific melting temperature.
[3]FAM.
[4]Hex.
[5]Cy5.
Bold words represent genotype-specific T_ms. T_m: Melting temperature; -: No significant melting temperature; SD: Standard deviation.

DISCUSSION

LNA-based RT-PCR assays have been widely applied to viral single-nucleotide polymorphism analysis as well as simple viral detection in clinical settings instead of the less sensitive traditional RT-PCR or nested RT-PCR assays prone that are to cross-contamination[31,32]. In particular, it has recently been reported that this method could successfully identify YMDD mutations of HBV from Korean patients with chronic HBV infections[30,33]. In the present study, we developed an LNA-RT-PCR assay using melting curve analysis for the identification of two polymorphisms within codon 269 of HBV Pol, rt269L and rt269I (three genotypes, rt269L1, rt269L2 and rt269I), with the advantages of easy performance and a low likelihood of cross-contamination. The clinical application of the LNA-RT-PCR assay was also compared in parallel with a direct sequencing protocol using clinical samples. Our data showed that the LNA-RT-PCR assay can separate the two polymorphisms in the rt269 codon of HBV Pol in clinical specimens with high sensitivity (92.6%, 87/94 samples) and specificity (98.9%, 86/87 samples) (Table 3). Of note, this assay can determine an almost exact ratio between two types within specimens from mixed cases (23/24 cases), suggesting its feasibility in the analysis of quasispecies distribution in mixed samples (Table 3, Figure 4).

Our LNA-based RT-PCR assays showed that the WT 'L1' type (n = 65, 74.7%) was found at the highest frequency in our cohort, followed by the 'I' type (n = 12, 13.8%) and 'L2' type (n = 3, 3.4%) (Table 3). This finding suggests that the 'L1' type is responsible for the majority of HBV infections in South Korea and that the WT form is prevalent in genotype C2 infections. Additionally, these findings suggest that the I type may be a variant of L1 rather than an independent polymorphism. Indeed, our

Table 3 Rates of positive detection of the hepatitis B virus L/I/L2 variants in a total of 94 clinical samples by locked nucleic acid real-time polymerase chain reaction

Type of detection	No. of samples	Percentage
Clinical samples	94	100
Single	63	67.0
L	55	58.5
I	8	8.5
Mixed	24	24.5
L + I (1:1)	7	7.4
L dominant	13	4.3
I dominant	4	12.8
Unidentified	7	7.4
Inconsistent with direct sequencing	1	1.1

Table 4 Samples which cannot be identified by locked nucleic acid real-time polymerase chain reaction assay

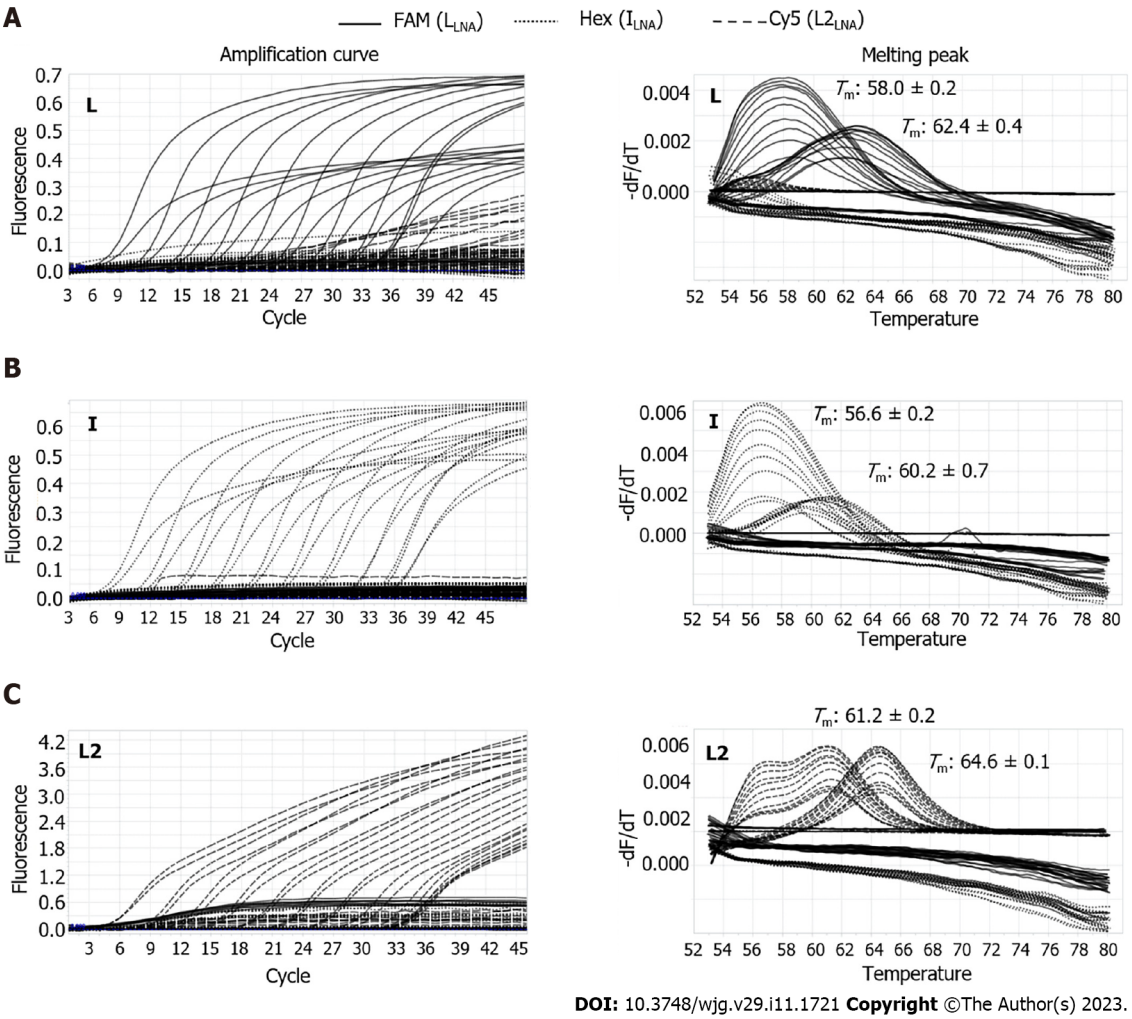
No.	Patients	Direct sequencing (AAACTCAARCAATGT)	Type	LNA-RT-PCR
1	SNU3 30 HCC	AAAATCAAGCA**C**TGT	I	Not detected
2	SNU3 70 HCC	AAAAT**T**AAGCAATGT	I	Not detected
3	SNU3 82 CH	AAAATCAAAC**T**ATGT	I	Not detected
4	SNU3 123 HCC	AAACT**T**AAGCAATGT	L	Not detected
5	SNU3 31 CH	AAAATC**C**AGCAATGT	I	Not detected
6	SNU3 355 LC	AAAAT**T**AAGCAATG	I	Not detected
7	SNU3 388 LC	AAACT**T**AAGCAATGT	L	Not detected

Bases in bold indicate the different ones from the target probe sequence. LNA-RT-PCR: Locked nucleic acid real-time polymerase chain reaction; HCC: Hepatocellular carcinoma; CH: Chronic hepatitis; LC: Liver cirrhosis.

previous study based on a direct sequencing protocol also showed that the 'L1' type *vs* the 'I' type is more closely related to higher HBV replication, higher HBsAg levels and HBeAg positive serostatus [23], suggesting that the majority of the 'L1' type infections in our cohort may be due to its enhanced viral infectivity. Therefore, it is tempting to speculate that the 'L1' type uniquely found in genotype C2 infections may contribute to some distinct traits of the genotype C2 infections, including an enhanced duration of the HBeAg-positive stage[34-36], higher infectivity[37,38] and a higher prevalence of occult infection *via* vertical transmission[33,39,40]. Since our LNA-based RT-PCR assays can identify L1 of higher infectivity and other variants (L2 or I type) related to disease progression from large serum samples without time-consuming or labor intensive sequencing procedures, it could help in the management or treatment of chronic patients in genotype C2 endemic nations, including China, Japan and South Korea.

In 7 (7.4%) of the 94 samples, despite successful amplification, our LNA-based RT-PCR assays failed to separate the two polymorphisms in the rt269 codon (Table 4). Comparison with the direct sequencing protocol revealed that all seven samples amplified but not identified by LNA-based RT-PCR assays had one more mismatch mutation that was different from the probe binding sequences. This was enough to interfere with normal detection due to the lower meting temperature than the respective probe. Therefore, in the samples amplified but not identified by our LNA-based RT-PCR assays, a further direct sequencing protocol should be recommended for the identification of the two polymorphisms.

A total of 24 (27.6%) of the 87 positively detected samples were identified in a mixed manner, and L1, in most cases of mixed infections, was dominant or codominant over I or L2. These findings further support our hypothesis that I or L2 may be a variant of the L1 type rather than an independent polymorphism. However, to clarify whether mixed infection in a patient is due to simple mutation of L1 to L2 or I type or superinfection of another type, further quasispecies analysis should be investigated in the future.



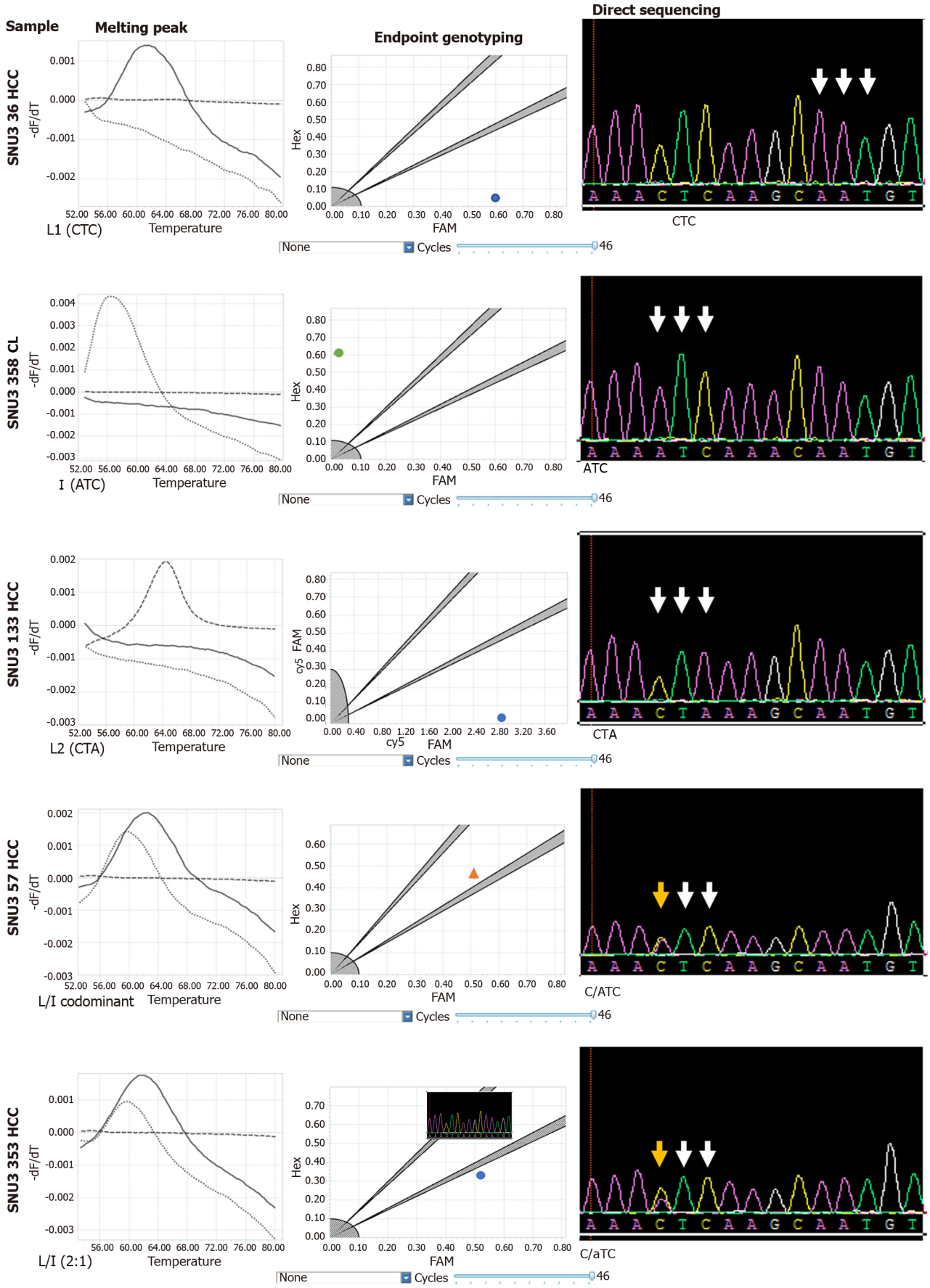
DOI: 10.3748/wjg.v29.i11.1721

Figure 3 Multiprobe locked nucleic acid real-time polymerase chain reaction for discrimination among three types of polymorphisms in the rt269 codon. Amplification curves are shown on the left, and melting peaks are shown on the right. A: With L1 wild-type DNA templates, L1-type specific signals in the FAM channel (solid) were detected, showing their dominant amplification and distinct melting temperatures (T_m), with minimal cross signals of amplification and melting peaks generated by weak cross hybridizations of the other probes (I and L2), which were differentiated from the T_m values for I and L2 detection; B: For I variant-type DNA templates, I-type specific signals in the Hex channel (dotted) were detected, showing their exclusive amplifications and distinct T_m values, with no cross signals; C: For L2 variant-type DNA templates, amplification curves showed weak cross signals, but melting peaks were distinct with no cross signals.

The limitation of this study is that all the samples included were obtained from patients at the initial stage of drug use and are from one medical institution. To determine the exact clinical significance of L1, L2 and I infections or mixed infections in genotype C2-infected chronic patients, our LNA-based RT-PCR assays should be applied to a larger population-based cohort of multicenter registries in future studies.

CONCLUSION

In conclusion, our data showed that the LNA-RT-PCR method developed in this study can successfully identify two different polymorphisms, rt269L (L1 and L2) and rt269I, in the rt269 codon of HBV Pol from CHB patients with genotype C2 infections. The wildtype 'L1' form is more prevalent than the rt269I form in Korean CHB patients with genotype C2 infections, which is possibly due to its higher infectivity. Therefore, our LNA-RT-PCR method enables the separation of rt269 types and could be effectively used for a deeper understanding of epidemiology and disease progression in genotype C2 endemic areas.


Sample
Melting peak
Endpoint genotyping
Direct sequencing
SNU3 36 HCC
L1 (CTC)
Temperature
-dF/dT
Hex
FAM
CTC
None
Cycles
46
SNU3 358 CL
I (ATC)
ATC
SNU3 133 HCC
L2 (CTA)
cy5
CTA
SNU3 57 HCC
L/I codominant
C/ATC
SNU3 353 HCC
L/I (2:1)
C/aTC

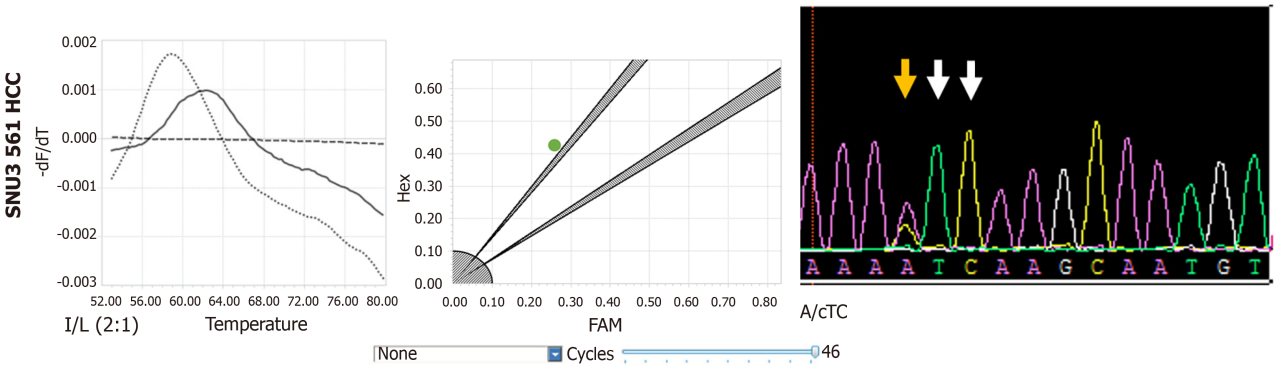


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Figure 4 Confirmation of multiprobe locked nucleic acid real-time polymerase chain reaction identification results of hepatitis B virus rtL269 variants by direct sequencing. Nucleotide bases are shown in the parentheses. Lowercase letters represent the base present in a lower amount relative to the dominant variant. Bold indicates the dominant amino acids and bases. Arrows represent the codon sequence positions for leucine or isoleucine; yellow, mixed bases.

ARTICLE HIGHLIGHTS

Research background

Hepatitis B virus (HBV) genotype C infections has distinct clinical or virological traits including higher risk of hepatocellular carcinoma, lower response rate to interferon or prolonged hepatitis B e antigen-positive phase. As a likely answer to this issue, we have recently reported that the presence of two HBV Pol RT polymorphisms, rt269L and rt269I could contribute to unique traits of HBV genotype C.

Research motivation

For the identification between two rt269 types from chronic patients of genotype C2 endemic areas instead of time or labor consuming direct sequencing protocol, we sought to develop a novel simple and sensitive locked nucleotide probe based real-time polymerase chain reaction (LNA-RT-PCR) method capable of separating two rt269 types, rt269L type encoding leucine, 'L' (L1: CTC, L2: CTA) and rt269I type encoding isoleucine (ATC) from chronic hepatitis B (CHB) genotype C2 patients.

Research objectives

To develop a novel simple and sensitive LNA-RT-PCR method capable of identifying two rt269 types in CHB genotype C2 patients.

Research methods

We designed appropriate primer and probe sets for LNA-RT-PCR for the separation of rt269 types. The developed LNA-RT-PCR method was applied to a total of 94 CHB patients of genotype C2 for the identification of two rt269 polymorphisms, and these results were compared with those obtained by a direct sequencing protocol.

Research results

The LNA-RT-PCR method could identify two rt269L and rt269I polymorphisms of three genotypes, two rt269L types ['L1' (WT) and 'L2'] and one rt269I type ('I') in single (63 samples, 72.4%) or mixed forms (24 samples, 27.6%) in 87 (92.6% sensitivity) of 94 samples from Korean CHB patients.

Research conclusions

The newly developed LNA-RT-PCR method could identify two rt269 polymorphisms, rt269L and rt269I, in CHB patients with genotype C2 infections. This method could be effectively used for the understanding of disease progression in genotype C2 endemic areas.

Research perspectives

The newly developed LNA-RT-PCR method could identify three rt269 types, L1, L2 and I from CHB patients of genotype C2 with high-sensitivity and specificity. It could play a relevant role in the clinical management of CHB patients of genotype C2 infection.

FOOTNOTES

Author contributions: Kim K and Kim BJ contributed to study conception and design, and designed and performed experiments; Choe WH contributed to collection of clinical data; Kim K, Choi YM, Kim DH, Jang J, Choe WH, and Kim BJ contributed to data acquisition, data analysis and interpretation; Kim K, Choi YM, Choe WH, and Kim BJ contributed to writing of article, editing, reviewing and final approval of article.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Risk factors predict microscopic extranodal tumor deposits in advanced stage III colon cancer patients

Yi-Han Jhuang, Yu-Ching Chou, Yu-Chun Lin, Je-Ming Hu, Ta-Wei Pu, Chao-Yang Chen

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Abstract

BACKGROUND

Colorectal cancer is a frequent cause of cancer-related mortality in patients with lymph node or distant metastases. Pericolonic tumor deposits (TDs) are considered prognostically distinct from lymph node metastases.

AIM

To investigate risk factors for extranodal TDs in stage III colon cancer.

METHODS

This was a retrospective cohort study. We selected 155 individuals diagnosed with stage III colon cancer from the database of the Cancer Registry of the Tri-Service General Hospital. The patients were allocated into the groups with/without N1c. Multivariate Cox regression analysis and Kaplan-Meier method were done. The primary outcomes investigate the association between the covariates and extranodal TDs, and prognostic significance of the covariates regarding the survival.

RESULTS

There were 136 individuals in the non-N1c group and 19 individuals in the N1c group. Patients with lymphovascular invasion (LVI) had a higher risk of TDs.

Overall survival rates of patients with and without LVI were 6.64 years and 8.61 years, respectively (P = 0.027). The N1c patients without LVI had higher overall survival than those who with LVI (7.73 years *vs* 4.42 years, P = 0.010).

CONCLUSION

Patients having stage III colon cancer with LVI have a higher probability of having TDs than those with stage III colon cancer without LVI. Stage III colon cancer patients with TDs and LVI could have poor prognosis and outcome.

Key Words: Colon cancer; Tumor deposits; Lymphovascular invasion; Risk factor

Core Tip: Tumor deposits has been associated with poor outcome in patient with colorectal cancer. In our study, we investigated the risk factors predicting extranodal tumor deposits in stage III colorectal cancer patients according to the new American Joint Committee on Cancer TNM staging and helped pathologist not to miss the subgroup of N1c patients. Sincerely, we look forward to more robust therapeutic approach and closer survivorship planning for this subgroup of high-risk stage III colon cancer patients in the future.

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INTRODUCTION

Colorectal cancer (CRC) is the fourth most common cancer and the fifth most frequent cause of cancer-related mortalities worldwide[1]. The International Union Against Cancer/American Joint Committee on Cancer (AJCC) TNM classification has been utilized for cancer staging[2] and its function has aimed to determine patient care and treatment, as well as to predict cancer prognosis. The TNM staging system is revised every few years as knowledge of cancer continually expands[3].

A pericolonic tumor deposit (TD) is a perineural, peri- or intra-vascular tumor extending beyond the muscularis mucosae. It is different from lymph node metastases and should not be contemplated its prognostic value[4-7]. The disease-free survival influence of even small pericolonic TDs is significant[8], recommending that pericolonic TDs of all volumes should be contemplated clinically important[7,9,10].

The TNM classification of pericolonic TDs as lymph node metastases or sporadic tumor extensions is perhaps inaccurate. The quantity and greatest dimension of pericolonic TDs should be stated separately from lymph node metastases. In the seventh edition of the AJCC TNM staging system, TD was classified as pN1c in stage III colon cancer patients without lymph node metastasis[11].

Extranodal deposits are a different form of metastatic disease in patients with CRC. The relationship with vascular invasion and earlier development of metastases probably implies that a significant information of extranodal deposits may represent blood-borne spread. Some researchers have indicated worse prognosis of patients with TDs and have claimed that TDs should be categorized as M classification[6,9].

In this single-institution retrospective study, we investigate the risk factors predicting extranodal TDs in stage III CRC patients according to the new (eighth edition) AJCC TNM staging. In regard to possible poor prognosis of stage III CRC patient with factors predicting extranodal TDs, adjust the adjuvant chemotherapy regimens with target therapy or immunotherapy might be considered.

MATERIALS AND METHODS

Study design and population

All data were obtained retrospectively from the Cancer Registry database of the Tri-Service General Hospital (TSGH), Taipei, Taiwan. The cancer registry of the TSGH included 4067 patients with CRC from 2010 to 2016. The inclusion criteria were described as follows: (1) All the patients received colonoscopy with tumor biopsy and pathology proved malignancy; and (2) abdomen computed tomography or whole body positron emission tomography scan showed no distant metastasis. All patients received regular postoperative follow-up at our colorectal outpatient department. The exclusion

criteria were described as follows: (1) Lack of comprehensive data in long-term follow-up; (2) death within 30 d of surgery; (3) local excision or uncertain procedure; (4) synchronous tumors; and (5) rectal cancer or non-stage III colon cancer patients (Figure 1). A total of 155 individuals diagnosed with stage III colon cancer from a single institution of a medical center were enrolled. All patients signed informed consents, and the Institutional Review Board (IRB) of TSGH permitted our study (TSGHIRB No. C202005173). Our study was conducted only for medical research and was in accordance with the Declaration of Helsinki.

The definition of N1c

TDs, also called extranodal TDs, were defined as tumor cells in the pericolic or perirectal fat tissue without proof of residual lymph node tissue in the relevant lymphatic system of the primary tumor site [6]. According to the latest TNM 8th staging system announced in 2016, aiming to eliminate any lesion with identifiable structures pointing towards LN metastasis, extramural venous invasion or perineural invasion[12]. The hematoxylin eosin (HE) staining of extra-nodal TDs (N1c group) showed that there are nodules made up of tumor cells found in the structures near the colon that do not seem to be lymph nodes (Figure 2A). The HE staining of non-TDs (non-N1c group) showed that tumor cells revealed in regional lymph nodes (Figure 2B). A list of potential microscopic features that may be valuable to aid in the difference between tumor deposit and a positive lymph node was compiled and sent out for ranking with the virtual slides. The virtual slides were sent to three pathologists with a certain interest in gastrointestinal pathology for review. Each pathologist was asked to render an opinion of either tumor deposit or lymph node metastasis for each slide.

Covariates

The covariates comprised age, gender, body mass index (BMI), cigarettes smoking and alcohol consumption habits, hypertension (HTN), diabetes mellitus (DM), neutrophil-to-lymphocyte ratio (NLR)[13], carcinoembryonic antigen (CEA), CA 19-9, and tumor characteristics. The tumor characteristics included tumor location (right and left), T stage, mean tumor size, tumor type (ulcerative and polypoid), tumor grade (well, moderate, and poorly differentiated), epidermal growth factor receptor (EGFR) expression, and lymphovascular invasion (LVI). LVI was defined as tumor cells invading the lymphatic or blood vessels microscopically[14]. In our study, all specimens had been fixed immediately in formalin solution. For definite diagnosis, two pathologists resected our specimens for routine pathological examination independently by hematoxylin and eosin staining (H&E staining) and immunohistochemistry based on the eighth edition of the AJCC TNM system and the World Health Organization (WHO) criteria.

Statistical analysis

All data were analyzed using SPSS Statistics software (IBM Corp., Released 2016. IBM SPSS Statistics for MAC version 24.0. Armonk, NY, United States). Student's *t*-test was used to analyze quantitative variables in terms of the mean with SD. The chi-square test was used to analyze the qualitative variables in terms of frequency and percentage. Multivariate Cox regression analysis was performed to investigate the association between the covariates and extranodal TDs. The Kaplan-Meier method was used to calculate the overall survival and disease-free survival rates. Statistical significance was set at $P < 0.05$.

RESULTS

Baseline characteristics

We included 155 individuals with stage III colon cancer in our study; their clinicopathological characteristics are shown in Table 1. There were 136 (88%) individuals in the non-N1c group and 19 (12%) individuals in the N1c group. The mean age of the N1c and non-N1c groups was 66.8 years and 65.2 years, respectively. N1c was observed mainly in male patients. The other characteristics showed no significant difference between the N1c and non-N1c groups, except for LVI ($P = 0.049$).

Risk factors of predicting extranodal TDs (N1c)

We investigated the association between the covariates and extranodal TDs using multivariate analysis (Table 2). There was no significant relationship between N1c and other confounding factors, such as age (HR = 1.03, 95%CI = 0.99-1.08), BMI (HR = 1.03, 95%CI = 0.68-1.04), HTN (HR = 0.87, 95%CI = 0.21-3.85), type II DM (HR = 0.69, 95%CI = 0.19-2.52), NLR (HR = 1.03, 95%CI = 0.92-1.15), CEA (HR = 1.00, 95%CI = 1.00-1.01), CA 19-9 (HR = 1.02, 95%CI = 0.99-1.04), and tumor characteristics. Notably, in stage III colon cancer, male patients (HR = 6.16, 95%CI = 1.24-30.10) with LVI (HR = 4.62, 95%CI = 1.17-18.33) had a higher risk of extranodal TDs.

Table 1 Baseline characteristics of N1c group and non-N1c group, *n* (%)

Item	N1c	Non-N1c	*P* value
Sample size (*n*)	19	136	
Gender			0.08
Male	13 (68.4)	64 (47.1)	
Female	6 (31.6)	72 (52.9)	
Mean age (yr)	66.79	65.17	0.09
BMI (kg/m^2)	22.77	23.41	0.15
Habit of smoking	8 (42.1)	46 (33.8)	0.48
Habit of alcoholic drinking	4 (21.1)	13 (9.6)	0.13
Hypertension	6 (31.6)	46 (33.8)	0.85
Diabetes mellitus	4 (21.1)	29 (21.3)	0.98
Neutrophil to Lymphocyte ratio	4.33	4.62	0.42
CEA (mg/dL)	12.83	18.23	0.62
CA 19-9 (mg/dL)	23.61	29.14	0.20
Tumor characteristics			
Location			0.27
Right colon	6 (9)	61 (91)	
Left colon	13 (14.8)	75 (85.2)	
T stage			0.90
T1	1 (5.3)	5 (3.7)	
T2	1 (5.3)	12 (8.8)	
T3	16 (84.2)	110 (80.9)	
T4a	0 (0)	4 (2.9)	
T4b	1 (5.3)	5 (3.7)	
Mean tumor size (cm)	4.24	4.8	0.98
Tumor type			0.26
Polypoid	8 (42.1)	76 (55.9)	
Ulcerative	11 (57.9)	60 (44.1)	
Tumor grade			0.62
Well	0	5 (100)	
Moderate	16 (13.3)	104 (86.7)	
Poor	3 (10.0)	27 (90.0)	
EGFR	17 (89.5)	117 (86.0)	0.68
Lymphovascular invasion	3 (15.8)	53 (39.0)	0.049

95%CI: 95% confidence interval; BMI: Body mass index; NLR: Neutrophil to lymphocyte ratio; CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigen 19-9.

Overall survival

The overall survival rates of patients with and without LVI were 6.64 years and 8.61 years, respectively (P = 0.027, Figure 3A), indicating that patients without LVI had higher overall survival than those with LVI. Furthermore, we divided the patients into N1c and non-N1c groups for subgroup analysis in regards to the impact of LVI on overall survival. In the non-N1c group, the overall survival rates of the patients with and without LVI were 6.91 years and 8.56 years, respectively (P = 0.13, Figure 3B), whereas the overall survival rates of the individuals with and without LVI in the N1c group were 4.42 years and 7.73 years, respectively (P = 0.01, Figure 3C).

Table 2 N1c group *vs* non-N1c group by multivariable cox regression

Item	Multivariable logistic regression		
	Exp (B)	95%CI	*P* value
Gender			
Female	Reference		
Male	4.62	(1.17-18.33)	0.030
Age	1.03	(0.99-1.08)	0.190
BMI	0.84	(0.68-1.04)	0.110
Habit of smoking	0.92	(0.27-3.07)	0.890
Habit of alcoholic drinking	1.41	(0.29-6.81)	0.670
Hypertension	0.87	(0.21-3.85)	0.850
Diabetes mellitus	0.69	(0.19-2.52)	0.570
Neutrophil to Lymphocyte ratio	1.03	(0.92-1.15)	0.660
CEA	1.00	(1.00-1.01)	0.770
CA 19-9	1.02	(0.99-1.04)	0.200
Tumor characteristics			
Location			
Right colon	Reference		
Left colon	1.67	(0.45-6.21)	0.440
T stage			
Tumor size	0.99	(0.96-1.02)	0.550
Tumor type			
Polypoid	Reference		
Ulcerative	1.75	(0.62-4.92)	0.290
Tumor grade	0.51	(0.12-2.21)	0.370
EGFR	1.05	(0.20-5.48)	0.950
Lymphovascular invasion	6.16	(1.24-30.10)	0.027

95%CI: 95% confidence interval; BMI: Body mass index; NLR: Neutrophil to lymphocyte ratio; CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigen 19-9.

Disease-free survival

The disease-free survival rates of patients with and without LVI were 5.92 years and 8.16 years, respectively (P = 0.005, Figure 4A), indicating that patients without LVI had higher disease-free survival than those with LVI. As before, subgroup analysis of LVI was performed by patient grouping into N1c and non-N1c groups. The disease-free survival rates of the patients in the non-N1c group with and without LVI were 5.98 years and 8.18 years, respectively (P = 0.013, Figure 4B). However, in the N1c group, the disease-free survival rates were 4.42 years and 7.56 years, respectively, for patients with and without LVI (P = 0.097, Figure 4C).

DISCUSSION

Patients with stage III colon cancer are contemplated to have a clinically significant hazard of distant metastasis after surgical resection. However, individuals with stage III colon cancer have a spectrum of risk of progressive disease. Beside tumor stage, the NCCN identifies LVI, TDs, and perineural invasion as histopathological characters related with patient survival[15]. In addition, TDs have been comprehensively studied in colon cancer, with most studies representing that they are an essential prognostic variable[15]. Some studies have even stated that TDs and tumor budding are the only histological variables that individually predict tumor recurrence in stage III colon cancer and should be comprised

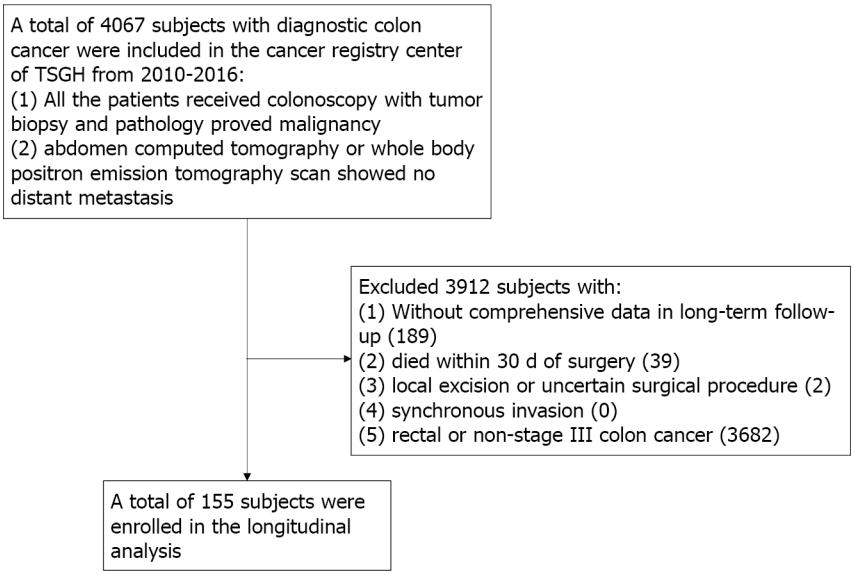

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Figure 1 Description of the study flowchart.

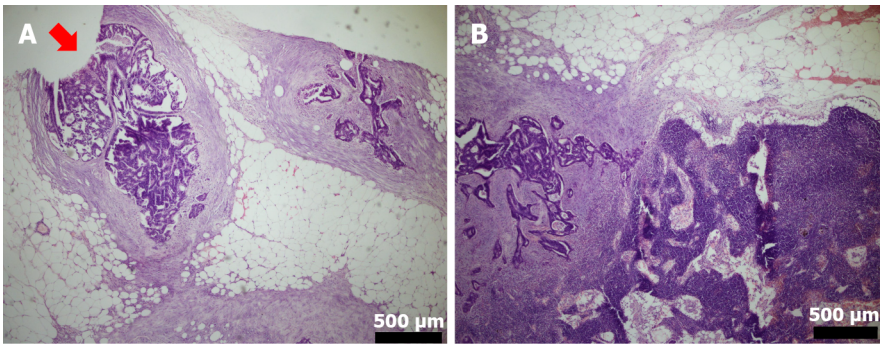

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Figure 2 Hematoxylin-eosin staining. A: The hematoxylin-eosin (HE) staining of N1c; B: The HE staining of non-N1c.

as part of a regular comprehensive pathological risk appraisal. TDs are defined as a discrete focus of tumor within the lymph node drainage area of the primary carcinoma with no distinguishable lymph node[15].

TDs are defeined as extramural focal aggregates of cancer cells located in the peritumoral fatty tissue (either mesocolon or mesorectum), which have no continuity with the main tumor mass and are not associated with a lymph node[16]. However, there is still a debate about what TDs really are, as they usually share different morphologies that make their origin unclear[16]. Some studies believe that TDs simply represent a stage of the LVI and/or perineural invasion process during which malignant cells begin to proliferate, giving rise to distinct nodules of cancer[16] that have to be distinguished from the involved lymph nodes. Other studies believe that TDs either represent a sporadic tumor spread, a totally replaced lymph node (LN), venous invasion with extravascular extension, and/or less commonly, a small vessel or perineural invasion[17]. TDs are generally present in about 4.5%-45.0% of CRC patients[16], while their incidence looks to be greater in advanced and/or metastatic tumors[16]. Jin *et al*[17] demonstrated that about 10% of CRCs have TDs, and 2.5% of colon cancers and 3.3% of rectal cancers have TDs without positive LNs.

Interobserver variability exists among pathologists in interpreting TDs[17]. It is clear that the determination of TD remains subjective, and no single criterion or group of criteria are comprehensively used or agreed upon. However, knowledge of the potential challenges and possible solutions may help reduce interobserver variability. In our study, we used the multivariable Cox regression model to analyze characteristics, including age, sex, comorbidities, tumor location, tumor staging, and tumor markers, of the CRC patients. We showed that LVI could predict CRC patients with N1c component, and this could allow pathologists to pay more attention to this subgroup of patients.

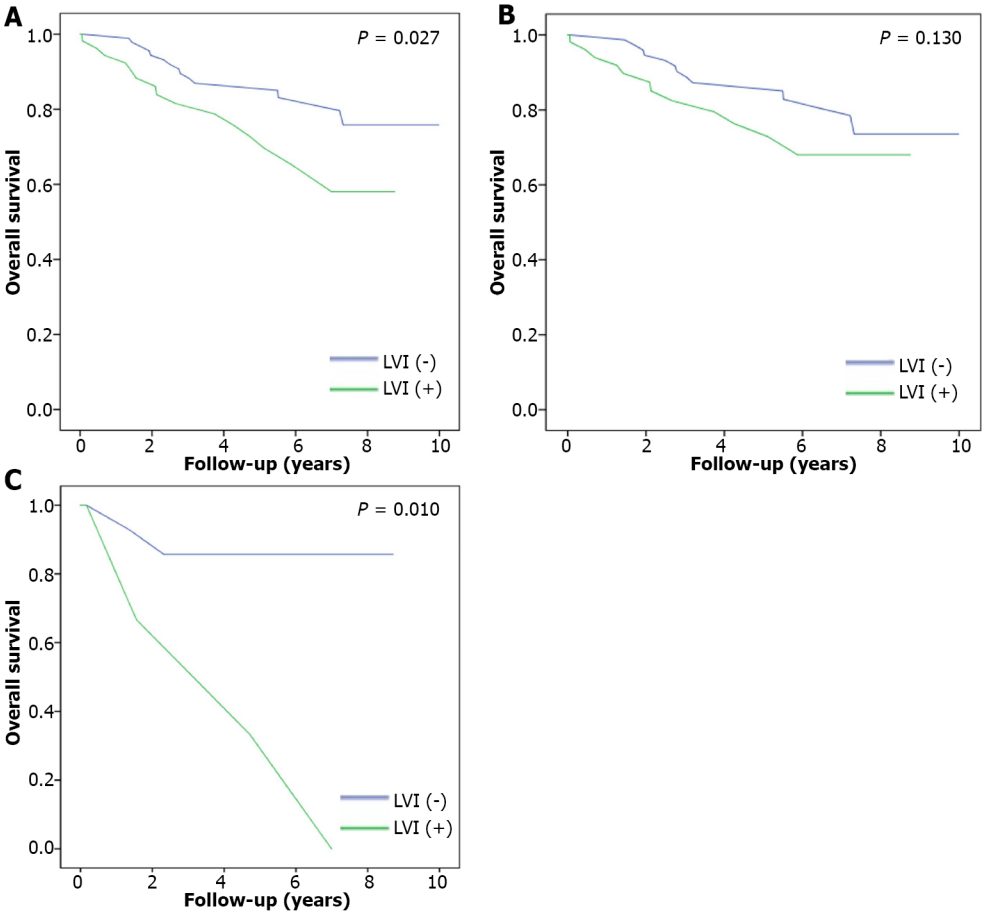

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Figure 3 Overall survival. A: The overall survival of the patient with/without lymphovascular invasion (LVI); B: The overall survival of the non-N1c patient with/without LVI; C: The overall survival of the N1c patient with/without LVI.

LVI positivity, characterized by the extension of tumor cells into lymphatic and/or blood vessels, has long been recognized as a probable indicator of lymph node metastasis, prognostic indicator, and predictor of patient outcomes. Many studies have investigated the presence of LVI in CRC and have determined it to be a strong stage-independent prognostic marker[18]. Patients with LVI usually have a higher chance of disease progression and poorer prognosis[18]. On the other hand, in recent years, TDs have become a hotspot in colon cancer study. In the seventh and eighth editions of the AJCC staging system, TDs were included in the nodal staging[19]. TD patients without regional lymph node metastasis were correlated with other high-risk characters, because there was more LV and perineural invasion in this group. This finding correlates with histopathologic results in other studies because TDs were revealed to be of perineural origin in 77% of cases, intravascular origin in 83% of cases, and a combined perineural, perivascular, and intravascular origin in 40%[20].

In a recent systematic review and meta-analysis of stage I-IV CRC, TDs were always related with worse overall survival and disease-free survival[20]. One study stated that the survival curves of all patients in stages I-III with TDs were more similar to the survival curves of the stage IV than stage III patients, and patients with TDs in stages I-III showed similar mortality rates as stage IV patients[21]. Another up-to-date study has indicated that the presence of TD in individuals with stage III colon cancer is related with a 2.2-fold increased risk of developing disease recurrence[22]. In our study, the results reported that LVI could predict TDs in patients with stage III colon cancer. The subgroup of N1c stage III colon cancer patients with LVI showed poor prognosis regarding overall survival, while the non-N1c subgroup patients showed no significant difference.

Despite showing that the predicted risk factor of LVI makes the prognostic significance of TDs in stage III colon cancer patients more promising, there are still some limitations to the study. This is a retrospective study of observational data using a small sample of patients, the prevalence of N1c in colon cancer could as low as 1.59% in the previous study[23], which might result in statistical bias with inconsistent results between overall and disease-free survival. Further prospective studies with more patients involved might address our result more promising. There might also be systematic differences in the pathological evaluation of the surgical specimens, which may have biased the outcomes.

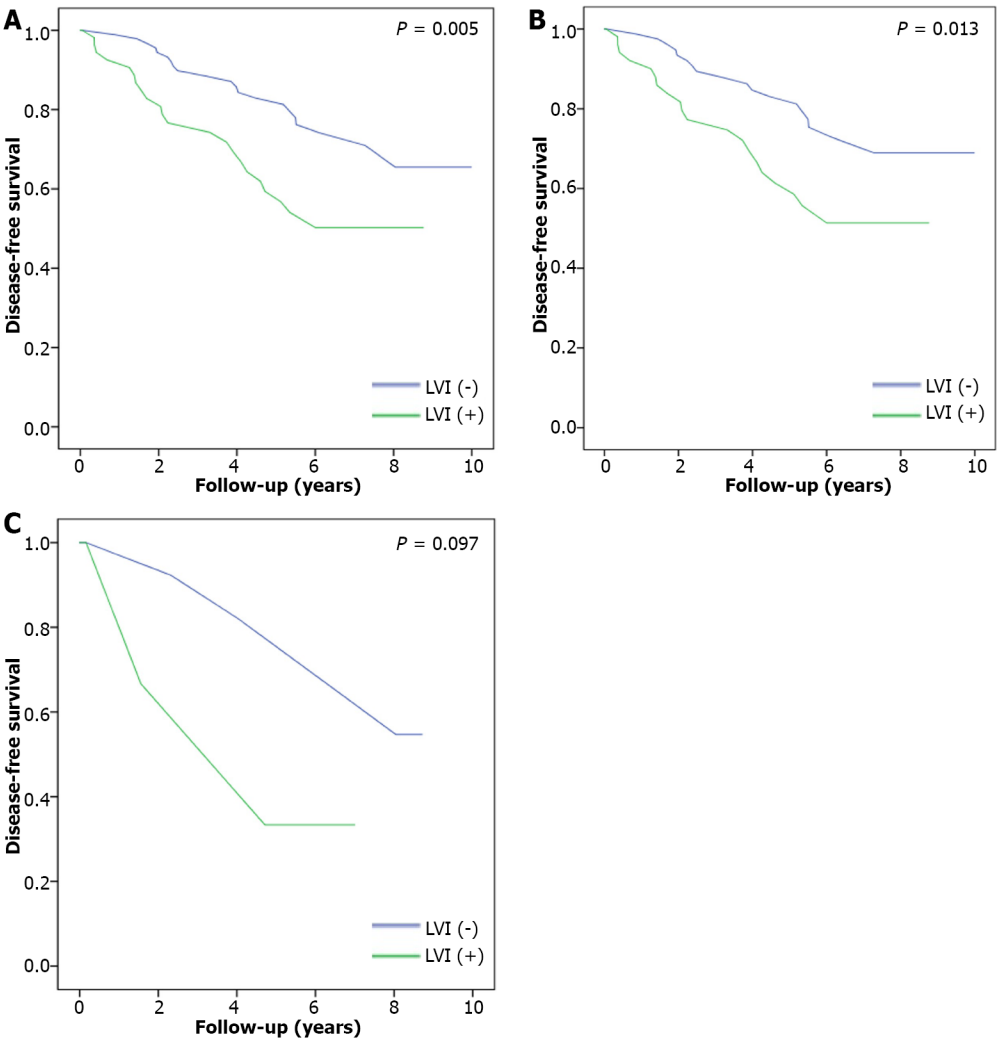

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Figure 4 Disease-free survival. A: The disease-free survival of the patients with/without lymphovascular invasion (LVI); B: The disease-free survival of the non-N1c patient with/without LVI; C: The disease-free survival of the N1c patient with/without LVI.

CONCLUSION

Patients with stage III colon cancer with LVI might be more likely to have TDs. Interobserver variability among pathologists and the multidisciplinary committee might at times influence consistent interpretation and reporting, and the frequent association between co-occurrence of TDs and LVI may postulate extra insight into the nature and derivation of TDs. Pathologist should not miss these subgroups of N1c patients, because TDs in combination with LVI could predict poor patient outcomes. Greater attention must be paid to the subject of TD positivity and prompt suitable risk stratification by considering a more robust therapeutic approach and closer survivorship planning for this subgroup of high-risk stage III colon cancer patients, who might be undertreated and require adjustment of adjuvant chemotherapy regimens. Amendment in the delivery of proper care to these patients may increase survival and should be an object of future quality ambition.

ARTICLE HIGHLIGHTS

Research background

In the American Joint Committee on Cancer (AJCC) TNM staging system, tumor deposit (TD) was classified as pN1c in stage III colon cancer patients without lymph node metastasis, but extranodal deposits are a distinct form of metastatic disease in patients with colon cancer in some studies.

Research motivation

To conduct a retrospective study to investigate risk factors for extranodal TDs in stage III colon cancer.

Research objectives
We used SPSS Statistics software. Student's *t*-test and the chi-square test were utilized to investigate quantitative variables and qualitative variables. Multivariate Cox regression analysis was performed to investigate the association between the covariates and extranodal TDs. The Kaplan-Meier method was utilized to analyze the overall survival and disease-free survival rates.

Research methods
We selected 155 patients diagnosed with stage III colon cancer from the database of the Cancer Registry of the Tri-Service General Hospital retrospectively. The patients were categorized into the groups with/without N1c. Multivariate Cox regression analysis and Kaplan-Meier method were done. The primary outcomes investigate the association between the covariates and extranodal TDs, and prognostic significance of the covariates regarding the survival.

Research results
Patients with lymphovascular invasion (LVI) had a higher risk of TDs. Overall survival rates of patients with and without LVI were 6.64 years and 8.61 years, respectively. The N1c patients without LVI had higher overall survival than those who with LVI.

Research conclusions
Stage III colon cancer patients with TDs and LVI could have poor prognosis and outcome.

Research perspectives
Greater attention must be paid to the issue of TD. Amendment in the delivery of proper care to these patients may increase survival and should be a target of future quality ambition.

FOOTNOTES

Author contributions: Jhuang YH contributed to conceptualization, data curation, formal analysis, investigation, methodology, validation, and writing original draft; Chou YC contributed to methodology, software and supervision; Lin YC, Hu JM, and Pu TW contributed to data collection; Chen CY contributed to supervision, validation, review and editing.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board (IRB) of TSGH (Approval No. C202005173).

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

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

Data sharing statement: Data can be acquired from the corresponding author.

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ORIGINAL ARTICLE

Retrospective Study

Outcomes of ABO-incompatible liver transplantation in end-stage liver disease patients co-infected with hepatitis B and human immunodeficiency virus

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Abstract

BACKGROUND

Human immunodeficiency virus (HIV)-positive patients coinfected with hepatitis B virus (HBV) are eligible for liver transplantation (LT) in Africa and Southeast Asia, particularly China. However, the outcome of HIV-HBV coinfected patients referred for ABO-incompatible LT (ABOi-LT) is unknown.

AIM

To clarify the outcome of ABOi-LT for HIV-HBV coinfected patients with end-stage liver disease (ESLD).

METHODS

We report on two Chinese HIV-HBV coinfected patients with ESLD who underwent A to O brain-dead donor LT and reviewed the literature on HIV-HBV coinfected patients treated with ABO-compatible LT. The pretransplantation HIV viral load was undetectable, with no active opportunistic infections. Induction therapy consisted of two sessions of plasmapheresis and a single dose of

rituximab in two split doses, followed by an intraoperative regimen of intravenous immunoglobulin, methylprednisolone, and basiliximab. Post-transplant maintenance immunosuppressive agents consisted of tacrolimus and mycophenolate mofetil, and prednisone.

RESULTS

At the intermediate-term follow-up, patients showed undetectable HIV viral load, CD4(+) T cell counts greater than 150 cells/μL, no HBV recurrence, and stable liver function. A liver allograft biopsy showed no evidence of acute cellular rejection. Both patients survived at 36-42 mo of follow-up.

CONCLUSION

This is the first report of ABOi-LT in HIV-HBV recipients with good intermediate-term outcomes, suggesting that ABOi-LT may be feasible and safe for HIV-HBV coinfected patients with ESLD.

Key Words: ABO incompatibility liver transplantation; Human immunodeficiency virus; Hepatitis B virus; End-stage liver disease; Immunosuppression

Core Tip: The outcome of human immunodeficiency virus (HIV)-hepatitis B virus (HBV) coinfected patients referred for ABO-incompatible liver transplantation (LT) (ABOi-LT) is unknown. We report on two Chinese HIV-HBV coinfected patients with end-stage liver disease (ESLD) who underwent A to O brain-dead donor LT and reviewed the literature on HIV-HBV coinfected patients treated with ABO-compatible LT. At intermediate-term follow-up, patients showed undetectable HIV viral load, CD4(+) T cell counts greater than 150 cells/μL, no HBV recurrence, and stable liver function. Both patients survived at 36-42 mo of follow-up. This is the first report of ABOi-LT in HIV-HBV recipients with good intermediate-term outcomes, suggesting that ABOi-LT may be feasible and safe for HIV-HBV coinfected patients with ESLD.

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INTRODUCTION

The recent introduction of highly active antiretroviral therapy (HAART) has dramatically changed the natural history of human immunodeficiency virus (HIV) infection[1]. Both the mortality rate and the incidence of acquired immune deficiency syndrome (AIDS) due to HIV infection have decreased with effective suppression of viral replication and prophylaxis against opportunistic infections[2]. However, HIV-infected patients are frequently coinfected with hepatitis B virus (HBV) since both viruses share similar modes of transmission, resulting in an increased risk of developing chronic liver disease[3,4]. After the dramatic improvement in the survival of HIV-infected patients with HAART, hepatitis cirrhosis and its complications have replaced opportunistic infections as the leading cause of mortality in the HIV-HBV coinfected patient[5]. In addition, almost all of the antiretroviral agents are metabolized in the liver. Patients with hepatic metabolic impairment cannot use these agents, accounting for the increased mortality associated with AIDS[6]. Accordingly, end-stage liver disease (ESLD) accounts for up to 50% of deaths in HIV-infected patients[7,8].

It has long been thought that HIV is a contraindication to liver transplantation (LT) in the pre-HAART era since immunosuppression can reportedly aggravate HIV infection and complications[9]. Case reports have shown nearly 25% AIDS-related mortality in HIV patients 6 mo after transplantation [10,11]. However, an increasing body of evidence suggests comparable survival rates between HIV-positive and HIV-negative recipients after LT in the HAART era[12-14]. These results suggest that HIV infection should not be a contraindication to LT, provided the underlying HIV disease is under control. Recent studies have shown that common indicators of controlled HIV disease-infected patient pretransplantation include an HIV viral load < 200 copies/mm^3, a CD4(+) T cell count greater than 200 cells/μL, and the absence of active opportunistic infections for at least 6 mo[15].

ABO-incompatible LT (ABOi-LT) is considered to be a high-risk procedure, compared to ABO-compatible LT, associated with a higher rate of antibody-mediated rejection, biliary complications, hepatic artery thrombosis, and mortality[16]. Hence, ABO-incompatible liver grafts have been used as a rescue option. Advances in the treatment strategies for ABOi-LT[17] include plasmapheresis, intravenous immunoglobulin (IVIG), splenectomy, rituximab, antilymphocyte antibodies, and immunosuppressant medications have improved the post-LT outcomes. The past decade has witnessed an increase in ABOi-LT procedures with increasing success. No significant difference between rejection and allograft survival at 1, 3, and 5 years after transplantation was found in a United Network of Organ Sharing Database analysis between 1990 and 2010 that compared ABOi liver transplants with ABO-compatible transplants[18].

Notably, the China Liver Transplant Registry does not prohibit HIV patients from receiving organs. With the introduction and effectiveness of HAART therapy, the outcomes of HIV-positive recipients with ESLD are reportedly similar to HIV-negative recipients after LT[19]. However, the incompatibility between the ABO blood group and living organ donation severely limits the transplantation opportunities for this patient population[20]. Besides, LT in HIV patients using ABOi organs brings additional complexity and difficulty to this already intricate patient population. Therefore, assessing the practicability of ABOi-LT in HIV-positive recipients is essential. To our knowledge, ABOi-LT in an HIV-HBV coinfected recipient with ESLD has hitherto not been documented in the literature. Here, we report on two cases and review major clinical and research issues related to HIV-HBV coinfected patients treated with ABO-compatible LT. We present the following article in accordance with the AME Case Series reporting checklist.

MATERIALS AND METHODS

From January 2019 to December 2021, 7 patients with HIV infection underwent LT in our LT center, including 2 patients with ABOi-LT. These 2 patients underwent ABOi-LT between April 2019 and December 2019, and their clinical data were extracted from our database. Both patients received HAART and anti-HBV therapy before transplantation and presented undetectable HIV RNA and HBV DNA levels, while the CD4(+) T cell count of one patient was less than 100 cells/μL. The surgical technique of ABOi-LT was a modified piggyback technique with triangulation of the hepatic veins. The vena cava anastomosis was completed with three separate continuous sutures, first completing the right side of the triangle. Subsequently, we released the vena cava blood flow to reduce the cold ischemia time. Next, we successively performed portal vein and hepatic artery vascular anastomosis. Bile duct reconstruction was performed by end-to-end anastomosis (continuous for the posterior wall and interrupted for the anterior wall). The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This retrospective study was a single-center consecutive case series analysis approved by the Ethics Committee of the Third People's Hospital of Shenzhen (No. 2022-038-02). Written informed consent was obtained from all participants.

To the best of our knowledge, no cases of ABO incompatibility involving HIV-HBV coinfection recipients have been reported in the literature. Little information is available on HIV-HBV coinfected patients that undergo LT and methods to improve survival and reduce complications. We systematically searched for all patients diagnosed with HIV who underwent LT from 1995 to 2022. Search terms were (HIV or human immunodeficiency virus) AND (HBV or hepatitis B virus) AND (liver or hepatic) AND (transplantation or transplant) in Embase, MEDLINE, and PubMed. When data were missing, we contacted the study authors for additional information.

RESULTS

Clinical data of patients

Case 1: A 61-year-old HIV-positive Chinese man with grade IV hepatic encephalopathy and hepatorenal syndrome secondary to decompensated HBV cirrhosis was referred for LT. He was considered a good candidate for LT because he had undetectable HIV RNA levels for at least 5 years, the CD4(+) T cell count was 42 cell/μL, and he received lamivudine and efavirenz as part of his Reverse Transcriptase inhibitor-based HAART therapy regimen for approximately 6 years. The model for ESLD (MELD) score was 40. However, given the absence of a suitable ABO-compatible liver donor and the patient's critical condition requiring urgent LT, a man with blood type A+ was used as the donor (O+ recipient, A+ donor). The recipient's latest anti-A titers were 1:128 for immunoglobulin G (IgG) and 1:32 for immunoglobulin M (IgM) before transplantation.

The decision was made to proceed with LT on April 4, 2019. Induction therapy consisted of two sessions of plasmapheresis, and rituximab 375 mg/m^2 in split doses was administered intravenously

before surgery. IVIG 400 mg/kg, rituximab 375 mg/m^2 in split doses, and methylprednisolone 0.5 g were administered intravenously during the operation; and basiliximab 20 mg was administered intravenously prior to the release of circulation in the graft.

Following transplantation, the patient was started on methylprednisolone for 7 d and switched to prednisone 48 mg daily and tapered to 8 mg daily over 4 wk. Maintenance immunosuppressive agents included tacrolimus and mycophenolate mofetil. Initially, 1 mg tacrolimus twice daily was started on postoperative day (POD) 1 and titrated to achieve a trough of 12 to 15 ng/mL during the first 2 postoperative weeks. The tacrolimus dose was adjusted to maintain a concentration of 8 to 12 ng/mL from 2 wk to 1 mo after transplantation, 6 to 8 ng/mL within 1 to 6 mo after transplantation, and then 4 to 6 ng/mL for long-term follow-up in our institution. Mycophenolate mofetil was started on POD4 and adjusted reasonably according to the postoperative liver function, infection index, white blood cell count, and drug concentration. IVIG 400 mg/kg was given daily for 7 d, then every other day for two more sessions. In addition, the second dose of basiliximab 20 mg was given on POD4. HAART therapy consisting of dolutegravir and lamivudine was restarted on POD1. Post-transplant anti-HBV therapy included tenofovir alafenamide and monthly infusions of high-dose hepatitis B immune globulin (HBIG). Given the presence of HIV-associated immunodeficiency and antirejection medication use, infection prophylaxis is critical. The patient was treated with antibiotics, ganciclovir, and antifungal combination therapy.

Due to a high preoperative anti-A IgM/IgG titer, the anti-A IgM/IgG titer, CD4(+) T lymphocyte count, postoperative rejection, and the risk of opportunistic infection after LT were dynamically monitored. From POD1, ABO blood group antibody titers and T lymphocyte subsets were detected in the blood collected from the patient every other day. Postoperative monitoring showed that anti-A IgG/IgM decreased from 1:128/1:32 to 1:32/1:16 at 2 wk postoperatively (Figure 1A). There were no problems with postoperative infection and HIV management; the patient received post-transplant HAART and anti-HBV therapy with CD4(+) T cell counts ranging from 60 to 148 cells/μL with undetectable HIV RNA and HBV DNA levels. Allograft ultrasound showed normal blood vessels and biliary tract. Serum transaminase peaked at 458 U/L on POD1, then began to trend down. However, graft function worsened on POD14. Mild elevation of the hepatic enzymes [total bilirubin (Tbil): 97 μmol/L; γ-glutamyltransferase: 255 U/L; aspartate aminotransferase: 39 U/L; alanine aminotransferase (ALT): 61 U/L] was observed (Figure 1B). The IgG and IgM titers remained stable (1:32 and 1:16, respectively). The follow-up liver allograft biopsy showed mild acute cellular rejection, with a Banff rejection activity index score of 4 (Figure 2A). After the patient received intravenous pulse steroid therapy (80 mg of methylprednisolone for 3 d, then tapered), liver enzymes decreased and subsequently remained in the normal range. The patient was discharged on POD37 without any infection and exhibited normal liver function. On subsequent clinical follow-up, normal hepatic enzymes were maintained. His latest CD4(+) T cell count was 159 cells/μL on June 6, 2022. A follow-up liver biopsy 1 year after transplantation revealed no evidence of graft rejection. More than 3 years after LT, the patient and graft function remained stable.

Case 2: A 46-year-old HIV-HBV coinfected Chinese man with acute-on-chronic liver failure was referred for LT. The preoperative Tbil was 320 μmol/L, and the international normalized ratio was 9.85. A multidisciplinary team discussion concluded that he was considered a good candidate for emergency LT with a MELD score of 40 and received a HAART therapy regimen for approximately 3 years with undetectable HIV RNA levels and a CD4(+) T cell count of 120 cells/μL. However, in the absence of suitable ABO-compatible liver donors, a man with blood type A+ was used as the donor (O+ recipient, A+ donor). The recipient's most recent anti-A titers for IgG and IgM were both 1:32 before transplantation. On November 19, 2019, the patient underwent ABOi-LT in our LT center. The induction treatment and maintenance immunosuppression scheme is shown in patient 1. HAART therapy was restarted on POD1 without changes. Given the immunodeficiency status of the patient treated with antirejection medication, the patient was treated with antibiotics, ganciclovir, and an antifungal combination therapy for infection prophylaxis.

The baseline anti-A IgG/IgM titers were both 1:32. Although the baseline anti-A titer was high and the CD4(+) T cell count was less than 200 cells/μL, an emergency ABOi-LT was successfully performed. The anti-A IgG/IgM titer, CD4(+) T cell count, postoperative rejection, and the risk of opportunistic infection after LT were dynamically monitored. Postoperatively the anti-A IgG/IgM titers decreased from 1:32/1:32 to 1:16/1:4 at 2 wk. Postoperative infection was not observed, and HAART and anti-HBV therapy were continued; the post-transplant CD4(+) T cell counts ranged from 62 to 494 cells/μL with undetectable HIV RNA and HBV DNA levels. The patient was finally discharged on POD63 with normal liver function, IgG and IgM titers of 1:8 and 1:4, respectively, and the absence of any complications (Figure 3A).

Deteriorating graft function was observed three months after LT, with relatively stable liver function (Figure 3B). The blood drug concentration of tacrolimus was 3 ng/mL, and the CD4(+) T-cell count was 390 cells/μL. A follow-up liver biopsy at 6 mo after transplantation revealed no evidence of graft rejection. Twenty-five months after LT, he was hospitalized due to abnormal hepatic enzymes and underwent a liver biopsy. The pathology report suggested chronic cholangitis with bile duct sclerosis and the possibility of early chronic rejection (Figure 2B). After the adjustment of the immunosup-

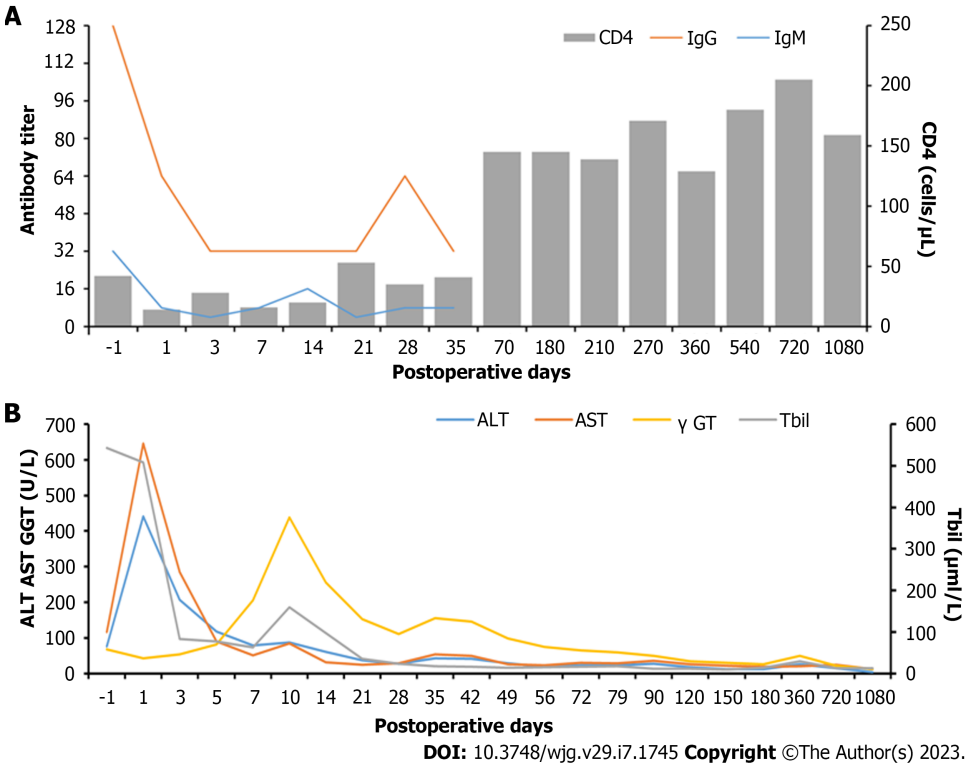

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Figure 1 Dynamic changes in immunological indicators and liver function in case 1. A: Trends of anti-A immunoglobulin M (IgM)/immunoglobulin G (IgG) titers and CD4(+) T cell counts over time; B: Trends of liver enzymes over time after liver transplantation. γGT: γ-glutamyltransferase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Tbil: Total bilirubin.

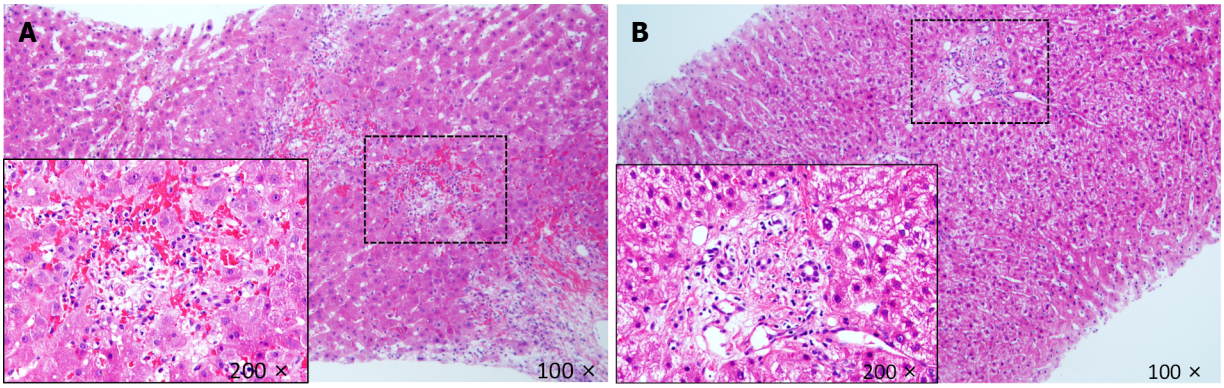

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Figure 2 Pathological results of liver allograft biopsy. A: Patient 1. The pathology results of liver allograft biopsy showed mild portal inflammation, mild venous endothelial inflammation, mild small bile duct inflammation and capillary bile duct cholestasis suggesting mild acute cellular rejection (Banff rejection activity index score, 4); B: Patient 2. The pathology results suggested chronic interlobular cholangitis with bile duct sclerosis and the possibility of early chronic rejection.

pressive drug regimen, the patient's liver function gradually stabilized. During the subsequent follow-up, the hepatic enzymes remained within the normal range with a CD4(+) T cell count of 467 cells/μL on February 7, 2022. Nearly 36 mo after LT, the patient and graft function remained stable.

Literature review

To date, there have been no reports of successful ABOi-LT in HIV-HBV coinfected patients. To understand the prognosis and risk of HIV complications in patients with simultaneous HBV infection after LT, we reviewed the literature for LT in HBV-HIV coinfected patients[12-15,21-27]. Eleven studies were screened, reporting the characteristics of 69 patients with HIV-HBV coinfection that underwent LT from 1995 to 2022 (Table 1). The etiology of liver disease was HBV-related cirrhosis (n = 62) and fulminant liver failure due to HBV (n = 7).

Table 1 Summary of outcomes pre- and post-liver transplantation in human immunodeficiency virus-hepatitis B virus coinfected patients

Ref.	Patients, *n*	Study period in yr	Pre-LT				Post-LT					
			Liver disease	CD4(+) T cell count as cells/μL	HIV-RNA as copies/mm^3	HBV-DNA as IU/mL	Median follow-up in mo	Latest CD4(+) T cell count as cells/μL	Latest HIV-RNA as copies/mm^3	Latest HBV-DNA as IU/mL	Rejections, *n*	Survival after LT in mo
Tateo *et al*[12]	13	1999-2007	HBV	173 (118-615)[1]	100% < 40	100% < 12	32 ± 5.2[2]	281 (10-810)[1]	100% < 40	100% < 12	2	100% 1 yr, 100% 3 yr, 100% 5 yr
Schreibman *et al*[27]	6	1999-2006	5 HBV, 1 ALF	100% > 100	100% < 200	66.7% < 12	60 (2-64)[1]	83.3% > 100	100% < 100	83.3% ND	1	66.7% 1 yr, 66.7% 3 yr, 66.7% 5 yr
Norris *et al*[25]	5	1995-2003	4 HBV, 1 ALF	187 (124-293)[1]	20% < 50	100% < 12	15 (6-65)[1]	467 (241-754)[1]	100% < 50	100% < 12	1	100% 1 yr, 100% 3 yr
Coffin *et al*[15]	22	2001-2007	21 HBV, 1 ALF	317 (38–1070)[1]	100% < 40	45.4% ND	42 (0.6-84)[1]	289 (48–744)[1]	NA	68% ND	5	85% 1 yr, 85% 3 yr
Vernadakis *et al*[1]	2	1996-2009	1 HBV, 1 ALF	219, 403	50% < 50	NA	3, 34	NA	NA	NA	0	3, 34
Neff *et al*[22]	4	1997-2001	2 HBV, 2 ALF	75% > 100	50% < 50	ND	21 (5-36)[1]	100% > 100	100% < 50	ND	3	100% 1 yr, 100% 3 yr
Anadol *et al*[23]	10	1997-2011	HBV	100% > 100	80% < 50	ND	NA	NA	ND	ND	1	80% 1 yr, 80% 3 yr, 80% 5 yr
Radecke *et al*[24]	1	1998-2001	HBV	196	380	NA	3	> 100	NA	NA	1	3
Schliefer *et al*[26]	1	1997-1999	ALF	477	< 80	NA	27	> 100	< 80	NA	0	27 (Alive)
Roland *et al*[21]	1	NA	HBV	439	ND	ND	20	305 to 700	ND	ND	0	20 (Alive)
Terrault *et al*[1]	4	2000-2002	HBV	175 (104-439)	100% < 75	ND	18, 25, 42, 48	315 (125-505)	ND	ND	0	100% 1 yr, 100% 3 yr

[1]Median (range);
[2]mean ± SD.
ALF: Acute liver failure; HBV: Hepatitis B virus; HIV: Human immunodeficiency virus; NA: Not available; ND: Not detectable; LT: Liver transplantation.

Fifty-nine patients had HIV infection under control, with undetectable or low HIV viral loads and no previous AIDS events or opportunistic infections upon LT waiting list registration. At the time of transplantation, all but one patient had CD4(+) T cell counts above ≥ 100 cells/μL[22]. Polymerase chain reaction showed that 17.4% of patients (12/69) had detectable HBV DNA prior to transplant (among these, 10 patients received adefovir and/or entecavir therapy, and 2 patients received lamivudine

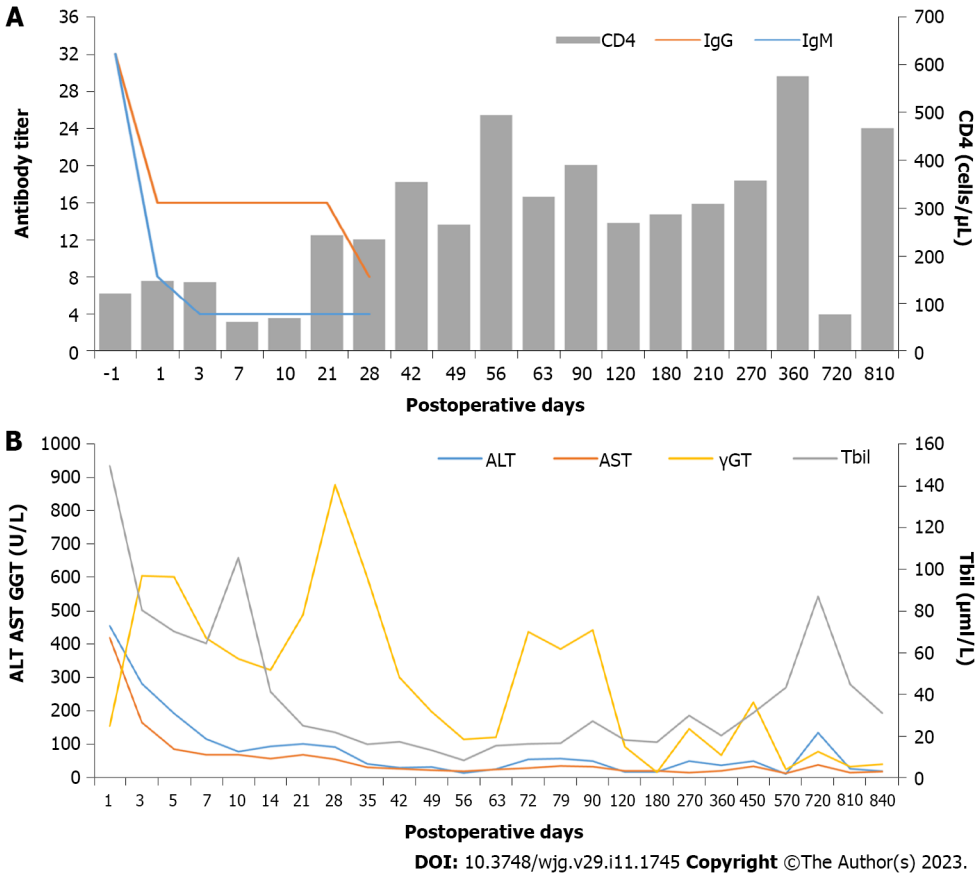

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Figure 3 Dynamic changes in immunological indicators and liver function of case 2. A: Trends of anti-A immunoglobulin M (IgM)/immunoglobulin G (IgG) titers and CD4(+) T cell counts over time; B: Trends of liver enzymes over time after liver transplantation. γGT: γ-glutamyltransferase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Tbil: Total bilirubin.

combined with tenofovir therapy).

All patients received HAART following LT and plasma HIV-RNA remained low to undetectable in all patients (one case presented with a viral load of 76 copies/mL) during follow-up. No viral breakthrough was observed. CD4(+) T cell counts were maintained at more than ≥ 100 cells/μL. All patients received dual immunoprophylaxis with hepatitis B immunoglobulin and anti-HBV medications. Eight patients tested positive for HBV DNA (among these, low-level HBV viremia was intermittently detected in 7 patients[15] but not associated with hepatitis B surface antigen detection or ALT elevation; for one patient[27] with a transiently positive HBV DNA, serum HBV DNA results were undetectable after tenofovir was added for antiviral therapy) (Table 1). 20.3% (14/69) of HIV-HBV coinfected patients were treated with high-dose prednisone or adjustment of immunosuppressive therapy after developing acute cellular rejection. However, one patient[23] died of hepatic artery thrombosis and graft failure due to rejection. The cumulative patient survival at one and three years in the HIV-HBV coinfected patients was 85.9% and 77.3%, respectively.

DISCUSSION

Current evidence suggests that in the era of HAART therapy, morbidity and mortality have declined in HIV-infected patients[28,29]. Mortality in this patient population is mainly attributed to comorbidities such as viral hepatitis infection, which is well-established to be associated with an increased prevalence of HIV-infected patients with ESLD. In recent years, studies have shown that the outcomes of HIV and non-HIV recipients were comparable, which has led to much controversy on transplantation in HIV patients[15]. Multiple centers have established protocols for HIV recipients, including an undetectable HIV RNA viral load and CD4(+) T cell counts greater than 200 cells/μL for sustained HAART therapy without other contraindications to LT. Overwhelming evidence[30-32] suggests promising outcomes for HAART-treated HIV-infected patients with maximally suppressed viral loads and no significant increase in opportunistic infections after LT.

Although LT is more common in selected HIV recipients, no studies have reported ABO incompatibility involving HIV-HBV coinfected recipients in the literature, while only 2 cases involving ABO-incompatible kidney transplantation have been reported in HIV recipients[33,34]. Our study provides the first documented cases of A to O incompatible LT in HIV-HBV coinfected recipients with ESLD. Ample evidence suggests that HIV-positive liver transplant recipients are prone to rejection due to immunosuppression or immune dysregulation from the virus itself[35-37]. Interestingly, the United Network of Organ Sharing Database[38] showed that the overall incidence of acute rejection in non-HIV recipients was 24.7% within 1 year after LT, which is similar to the incidence (20.3%) in our review of HIV-infected recipients. Regardless of the HIV infection status, acute rejection is common in ABOi transplantation, with an acute rejection rate of nearly 22%[18]. In our report, emergency ABOi-LT was successfully performed in HIV-HBV coinfected recipients that received induction therapy and adjunctive immunosuppressive regimens, including plasmapheresis, rituximab, basiliximab, methylprednisolone, and IVIG. Liver biopsy at mid-term follow-up did not show acute cellular rejection.

The two patients described in our study are the first cases of A to O brain-dead donor LT reported in the literature. The A-blood group is unique since it exhibits two phenotypes (A1 and A2) that harbor different immunogenicity. The A2 phenotype is characterized by reduced reactivity with anti-A isoagglutinin since it expresses fewer A epitopes on two of four possible core saccharide chains of the ABO antigen[39]. Besides, an increasing body of evidence[40,41] suggests that transplantation of A2 Liver grafts does not elevate anti-A titers after LT. Kluger *et al*[18] reported that blood group O recipients with A2 grafts exhibited no significant differences in rejection during the transplant hospitalization and at 12 mo postoperatively, with the same overall and graft survival rates as recipients with O grafts at 1, 5, and 10 years. The patient's preoperative anti-A IgG/IgM titers in case 1 were 1:128 and 1:32, respectively. No definite graft rejection was observed 3 years after LT because the donor's blood group was A2. Our center's protocol for A to O LT is based on baseline (pretransplantation) titer data. If the anti-IgG and the IgM titers are > 1:16, the preoperative management consists of two sessions of plasmapheresis, IVIG 400 mg/kg, and rituximab 375 mg/m^2 in split doses. In our article, both patients received this protocol. Postoperative anti-A titer monitoring in patient 1 showed that anti-A IgG/IgM decreased from 1:128/1:32 pre-LT to 1:32/1:16 at 2 wk. During the subsequent three years of clinical follow-up, the patient exhibited normal liver enzyme levels.

Preventing HBV recurrence and HIV progression has become a research hotspot in patients with HIV-HBV coinfection after LT. No consensus has been reached on the optimal anti-HBV therapy in patients with HIV-HBV coinfection. Anti-HIV drugs with anti-HBV activity include lamivudine, tenofovir, and emtricitabine. Drug therapy for HIV infection has important implications for preventing the recurrence of HBV infection. However, treating HBV infection in a coinfected patient with lamivudine or tenofovir alone can result in HIV resistance to these drugs, affecting anti-HIV treatment options in the future[42,43]. Accordingly, clinicians should be aware of the potential impact of nucleos/tide analogue selection on managing HIV-HBV coinfected recipients. Terrault *et al*[13] suggested that the combination therapy using nucleos(t)ide analogues and HBIG in the HIV-HBV coinfected recipient could effectively prevent post-transplantation HBV recurrence. In this report, the patient had excellent short outcomes after treatment with anti-HBV therapy, including tenofovir alafenamide and HBIG, and the HAART regimen consisting of dolutegravir and lamivudine. Subsequently, the two patients that underwent ABOi-LT with HIV were switched to albuvirtide and dolutegravir, well-recognized for their low hepatorenal toxicity and non-CYP450 enzyme inhibitors[44], reducing the impact of calcineurin inhibitor-type immunosuppressive drugs, which achieved intermediate-term excellent outcomes. Overall, it is essential to continuously monitor HIV RNA and HBV DNA levels and optimize anti-HIV and anti-HBV therapies to reduce postoperative complications and prolong survival in HBV-HIV coinfected recipients. Our HIV patients remained infection-free with good CD4(+) T cell count and stable liver function. Moreover, Albuvirtide and dolutegravir represent good options for HIV patients undergoing ABOi-LT.

Given that the number of HIV-infected patients with ESLD is expected to rise in the coming years, the same organ shortage issues that plague non-HIV patients will become increasingly severe for HIV patients[45]. To our knowledge, these two cases are the first reports of ABOi-LT in HIV-HBV coinfected patients with ESLD in the literature. In this study, the early course of ABOi-LT in HIV recipients was similar to that of ABO-compatible LT, without severe acute rejection. Desensitization regimens for ABOi-LT include rituximab, plasmapheresis, and IVIG. In previous studies[46,47] and our previous clinical practice, it has been observed that multiple doses of rituximab could increase the risk of infection, especially in immunodeficient HIV patients. Therefore, a sufficient dose and course of prophylactic antibiotics are crucial to prevent postoperative infection. Furthermore, the number of targeted B cells was significantly smaller in HIV transplant patients, and multiple doses of rituximab yielded no significant benefit for acute rejection or survival in transplant patients[48], suggesting that a single dose of rituximab may be sufficient. The desensitization protocol is usually initiated 2 to 3 d before transplantation. In these two cases, we administered a single dose of rituximab in two split doses, and two sessions of plasmapheresis were performed to reduce the anti-A titers with satisfactory results.

CONCLUSION

We successfully performed emergency ABOi-LT in HIV-HBV coinfected patients. Their intermediate-term outcomes are encouraging, with normal graft function. Thus, ABOi-LT may be safe and feasible in HIV-HBV coinfected patients with ESLD. We are cautiously optimistic that ABOi transplantation can be extended to other HIV-positive patients with ESLD.

ARTICLE HIGHLIGHTS

Research background

Human immunodeficiency virus (HIV)-positive patients coinfected with hepatitis B virus (HBV) are eligible for liver transplantation (LT) in Africa and Southeast Asia, particularly China. However, the outcome of HIV-HBV coinfected patients referred for ABO-incompatible LT (ABOi-LT) is unknown.

Research motivation

There have been no reports about the intermediate-term outcome of ABOi-LT in HIV-HBV coinfected recipients.

Research objectives

We sought to clarify the outcome of ABOi-LT for HIV-HBV coinfected patients with end-stage liver disease (ESLD).

Research methods

We report on two Chinese HIV-HBV coinfected patients with ESLD who underwent A to O brain-dead donor LT and reviewed the literature on HIV-HBV coinfected patients treated with ABO-compatible LT. Data of the pre- and post-transplantation were collected, including HIV viral load, CD4(+) T cell count, induction therapy methods, the immunosuppressive regimen and the clinical materials.

Research results

After follow-up for 36-42 mo, both patients survived with undetectable HIV viral load, CD4(+) T cell counts greater than 150 cells/μL, no HBV recurrence, and stable liver function. Liver biopsy showed no evidence of acute cellular rejection.

Research conclusions

This is the first study of ABOi-LT in HIV-HBV recipients with good intermediate-term outcomes, which suggests that ABOi-LT may be feasible and safe for HIV-HBV coinfected patients with ESLD.

Research perspectives

Due to the relatively small number of cases in the study, follow-up studies with large samples are still required.

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FOOTNOTES

Author contributions: Zhao D and Tang JX performed the conception and design; Zhao D contributed to the administrative support; Tang JX, Zhang KJ and Fang TS contributed to the provision of study materials or patients; Liang ZM, Yan X, Jin X and Zeng XC performed the collection and assembly of data; Weng RH and Xie LJ contributed to the data analysis and interpretation; All authors participated in the writing and final approval of the manuscript.

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CASE REPORT

Eosinophilic enteritis requiring differentiation from chronic enteropathy associated with *SLCO2A1* gene: A case report

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Abstract

BACKGROUND

Eosinophilic gastrointestinal disease (EGID) is a disorder characterized by infiltration of eosinophils causing mucosal damage and dysfunction of the gastrointestinal tract. The endoscopic findings of eosinophilic enteritis (EoN), an EGID variant, are nonspecific and occasionally difficult to diagnose. In contrast, chronic enteropathy associated with *SLCO2A1* (CEAS) is a chronic persistent small intestinal disorder characterized by endoscopic findings such as multiple oblique and circular ulcers.

CASE SUMMARY

We report the case of a 10-year-old boy who had suffered abdominal pain and fatigue for the preceding 6 mo. He was referred to our institute for investigation of suspected gastrointestinal bleeding because of severe anemia with hypoproteinemia and positive fecal human hemoglobin. The upper and lower gastrointestinal endoscopic findings were normal; however, double-balloon small bowel endoscopy showed multiple oblique and circular ulcers with discrete margins and mild constriction of the intestinal lumen in the ileum. The findings were highly consistent with CEAS, but urine prostaglandin metabolites were within normal limits, and no previously reported mutations in the *SLCO2A1* gene were identified. Histological evaluation demonstrated moderate to severe eosinophilic infiltration localized to the small intestine suggesting a diagnosis of EoN. Clinical remission was maintained with montelukast and a partial elemental diet, but emergent surgery for bowel obstruction due to small intestinal stenosis was performed two years after the initial treatment.

CONCLUSION

EoN should be considered in the differential diagnosis of CEAS-like small intestinal ulcerative lesions and normal urinary prostaglandin metabolite levels.

Key Words: Anemia; Chronic enteropathy associated with *SLCO2A1*; Double-balloon endoscopy; Eosinophilic gastrointestinal disease; Hypoproteinemia; Case report

Core Tip: Eosinophilic enteritis (EoN), a form of eosinophilic gastrointestinal disease localized to the small intestine, is extremely rare in children. The present pediatric case of EoN displayed multiple ulcerative lesions mimicking chronic enteropathy associated with *SLCO2A1* and bowel obstruction due to small intestinal stenosis. The diagnosis was confirmed by small intestinal biopsy using double-balloon enteroscopy and analysis of urine prostaglandin metabolites.

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INTRODUCTION

In eosinophilic gastrointestinal disease (EGID), tissue and functional disorders of the gastrointestinal tract are caused by inflammation due to abnormal infiltration of eosinophils within the gastrointestinal wall[1]. EGID can occur in any location between the esophagus and the colon, but localization to the small intestine is extremely rare[2]. The disease was re-classified from eosinophilic gastroenteritis (EGE) to eosinophilic enteritis (EoN) in 2022[3]. In addition, the nonspecific gastrointestinal endoscopic findings of EGID (edema, erythema, erosions, and ulcers) lead to difficulty in differentiating EGID from other digestive disorders[4].

In contrast, chronic enteropathy associated with *SLCO2A1* (CEAS) is a chronic persistent small bowel disease characterized by multiple oblique and circular ulcers with discrete margins in the ileum endoscopically. It is complicated by small intestinal obstruction due to ulcerative scarring and stenosis in the natural course[5].

Herein, we report a pediatric case of EoN involving multiple ulcerative lesions mimicking CEAS with diagnostic and therapeutic difficulties.

CASE PRESENTATION

Chief complaints

A 10-year-old Japanese boy presented to his family pediatrician with the complaints of easy fatigue and abdominal pain for 6 mo.

History of present illness

The patient presented to the family pediatrician with facial pallor and severe anemia (Hb: 2.9 g/dL) and was referred to his previous physician for admission. He then received red blood cell transfusion and iron supplementation. Further analysis also showed positive fecal human hemoglobin, indicating anemia due to gastrointestinal bleeding, and the patient was transferred to our institution for further evaluation.

History of past illness

The patient had no previous medical history.

Personal and family history

Prior to the patient's birth, the father had been treated with antibiotics for iron deficiency anemia caused by *Helicobacter pylori* infection.

Table 1 Laboratory findings on admission

	Laboratory data	Reference range
White blood cell count (/μL)	5300	4000-8000
Differential (percent)		
Neutrophils	2597 (49.0)	1800-4800
Lymphocytes	2067 (39.0)	1000-3600
Eosinophils	53 (1.0)	40-400
Hemoglobin (g/dL)	9.7	14-18
Hematocrit (%)	35.0	40-48
MCV (fL)	74.9	84-99
MCHC (g/dL)	27.7	32-36
Ferrum (μg/dL)	39	50-190
Ferritin (ng/mL)	23	30-400
Total protein (g/dL)	6.1	6.7-8.3
Albumin (g/dL)	2.9	3.9-4.9
Creatinine (mg/dL)	0.34	0.61-1.04
C-reactive protein (mg/dL)	0.1	< 0.3
Erythrocyte sedimentation rate (mm/h)	4	0-15
IgG (mg/dL)	665	870-1700
IgE (IU/mL)	515	0-173
Fecal human hemoglobin (ng/mL)	2018	< 50
Fecal calprotectin (μg/g)	510	< 50

MCV: Mean corpuscular volume; MCHC: Mean corpuscular hemoglobin concentration; IgM: Immunoglobulin M; IgE: Immunoglobulin E.

Physical examination

On physical examination, vital signs were as follows: Temperature, 36.8 °C; blood pressure, 99/60 mmHg; heart rate, 80 beats per min; respiratory rate, 18 breaths per min. His height was 122.7 cm (-1.63 standard deviation), and his weight was 24.1 kg (-0.92 standard deviation), with no significant growth disturbance on the growth curve and no other abnormal physical findings other than pale eyelid conjunctiva.

Laboratory examinations

Blood analysis demonstrated low levels of hemoglobin (9.7 g/dL) and albumin (2.9 g/dL), and fecal analysis showed elevated levels of human hemoglobin (2018 ng/mL) and calprotectin (510 μg/g). No elevation of inflammatory markers and no eosinophilia were observed (Table 1).

Imaging examinations

Upper and lower gastrointestinal endoscopy showed normal mucosal findings. Small intestinal capsule endoscopy was not performed because of the patency capsule retention in the stomach, and transanal double-balloon enteroscopy (DBE) was performed. DBE showed multiple oblique and circular ulcers with discrete margins at 70-100 cm proximal from the ileocecal valve with slight constriction of the small intestinal lumen (Figure 1).

Further diagnostic work-up

No histological abnormalities were identified on biopsies conducted by upper and lower gastrointestinal endoscopy. The ileal biopsy with DBE showed moderate to severe histological eosinophilic infiltration [maximum 80 eosinophils/high-power field (HPF)] and cryptitis within the mucosa (Figure 2). Of the urine prostaglandin metabolites that are elevated in CEAS, the levels in the present patient were as follows: Prostaglandin F2α metabolite, 3.2 (normal range: 3.0-4.0) ng/mg Cre; prostaglandin E2 metabolite, 2.09 (normal range: 2.0-3.0) ng/mg Cre; and prostaglandin D2 metabolite, 8.5 (normal range: 9.0-10) ng/mg Cre, all of which were within the normal ranges[6,7]. No previously

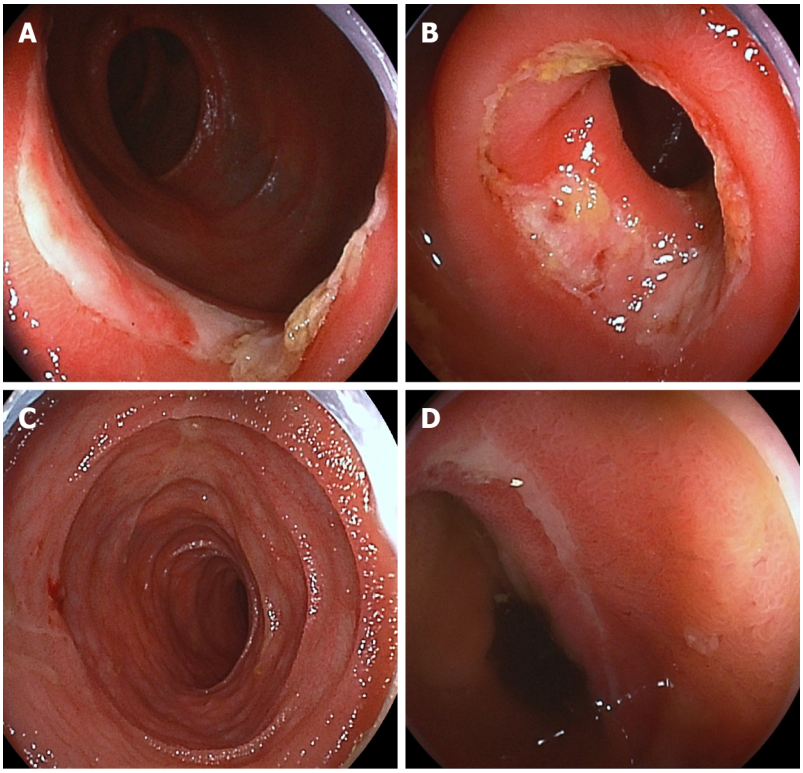

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Figure 1 Findings of initial double-balloon small intestinal endoscopy. A: Initial double-balloon enteroscopy (DBE) shows multiple oblique ulcers with discrete margins, 70-100 cm proximal to the ileal valve; B: The circular ulcers with slight constriction of the small intestinal lumen at initial DBE; C: Follow-up DBE performed 1 year later shows mucosal healing; D: The oblique ulcer and scars at 70 cm proximal to the ileal valve at follow-up DBE.

reported mutations in the *SLCO2A1* gene or in the targeted gene panels for very early-onset inflammatory bowel disease were identified[8].

FINAL DIAGNOSIS

The diagnostic findings and medical history indicated a final diagnosis of EoN.

TREATMENT

The patient was treated with montelukast (10 mg/d for a total of 26 mo), which reduced the frequency of abdominal pain. Partial elemental diet therapy (600 kcal/day for a total of 24 mo) was also implemented due to insufficient response of hypoalbuminemia and anemia[9]. Corticosteroids were not administered because the patient's family preferred that steroids be avoided.

OUTCOME AND FOLLOW-UP

The abdominal pain resolved completely 2 mo after the administration of montelukast and the partial elemental diet, and improvement of hemoglobin (11.2 g/dL) and hypoalbuminemia (3.5 g/dL) and normalization of fecal human hemoglobin (56 ng/mL) were observed after 4 mo. At follow-up of the small intestine by DBE performed 1 year later, mucosal healing was achieved, except for the oblique ulcer and scars at 70 cm proximal to the ileal valve, and no intestinal stenosis caused by the healing ulcer was observed (Figure 1). Eosinophilic infiltration had also disappeared on biopsy, suggesting histological remission. The patient was in clinical remission thereafter, but 2 years and 2 mo after the first visit, sudden bowel obstruction was induced by small intestinal stenosis, and emergent surgery was performed. The ileal macroscopic findings showed strictures at 40 cm and 44 cm proximal to the ileocecal valve, leading to ileal resection of the strictures and ileostomy (Figure 3). The histological findings of the resected specimen were of ulcer formation and peri-ulcer mucosal damage, suggesting intestinal stenosis in the process of ulcerative scarring. No significant granuloma or eosinophilic infilt-

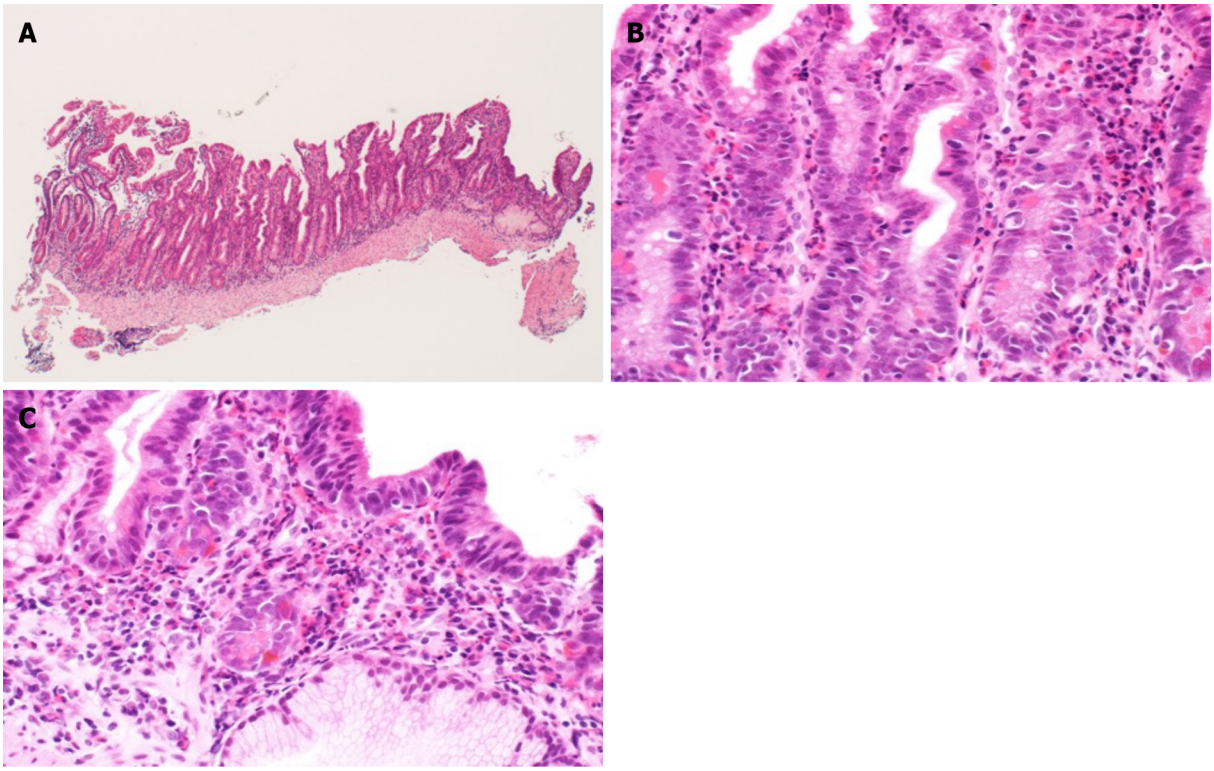

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Figure 2 Histopathological findings of ileal tissue obtained from double-balloon enteroscopy. A: Hematoxylin and eosin staining (× 100) shows mild villous atrophy; B: Hematoxylin and eosin staining (× 400) shows moderate to severe histological eosinophilic infiltration (maximum 80 eosinophils/high-power field); C: Hematoxylin and eosin staining (× 400) shows crypt destruction (cryptitis).

ration was observed (Figure 3). The patient's postoperative course was uneventful. Ileostomy closure was performed 2 mo later, and the patient is currently being followed on an outpatient basis. An ileal resection specimen obtained at ileostomy closure showed marked eosinophilic infiltration (> 50/HPF) in the subserosa (Figure 3).

DISCUSSION

The incidence of EGE in the United States is 2.5-30 cases per 100000 people, whereas the incidence in Japan is estimated to be 5.5 times higher[10]. EGE is usually difficult to diagnose because of the variety of gastrointestinal symptoms, as well as the extremely nonspecific findings of gastrointestinal endoscopy[11]. A case series that reported the small intestinal capsule endoscopic findings in 10 EGE cases found small intestinal lesions such as multiple erythematous lesions in 6 cases, erosions and ulcers in 5 cases, flattened or missing villi in 4 cases, and intestinal stenosis in 7 cases[9]. In that study, EGE was defined as EGID with extensive lesions extending from the stomach to the large intestine; however, only one pediatric and 6 adult cases of EoN localized to the small intestine have been reported (Table 2)[12-17]. These reports described various forms of ulcerative lesions and strictures in the small intestine, but all patients were diagnosed with EGE because of the difficulty of distinguishing EGE from CEAS based on the endoscopic images, as in the present case, and the patients had histologically significant eosinophilic infiltrates[12-17]. In contrast, the present case showed hypoproteinemia and iron deficiency anemia combined with multiple oblique and circular ileal ulcers, consistent with the diagnostic criteria for CEAS[18].

CEAS was first reported in Japan in 1968 as "nonspecific multiple ulcers of the small intestine"[5] and is caused by *SLCO2A1* germline variants encoding a prostaglandin transporter. The identification of hot spots of *SLCO2A1* variants is thus valuable for diagnosis but not currently included in the definitive diagnostic guidelines[5,7,18]. In addition, the clinical manifestations of CEAS are chronic and intractable nonspecific gastrointestinal symptoms comparable to EGID, and no effective treatment for these disorders has been established[18]. Ulcers of CEAS have been described as shallow oblique, circular, or longitudinal with discrete margins in case series of the endoscopic findings of CEAS[13,19]. CEAS-like diseases without *SLCO2A1* mutations have also been reported, including inherited eicosanoid metabolic disorders, inherited human cPLA2α deficiency, and cryptogenic multifocal ulcerous stenosing enteritis; histologically, however, these diseases show nonspecific inflammatory cell infiltration predominantly

Table 2 Summary of four previous cases of eosinophilic enteritis

Case	Age/sex	Location	Endoscopic findings				Laboratory findings	
			Multiple erythema	Erosions	Ulcer	Stricture	Anemia	Hypoproteinemia
1	62/F	Throughout	-	+	+	-	NR	NR
2	66/F	Throughout	-	+	+	-	NR	NR
3	48/M	Upper jejunum/ileum	-	-	+	-	-	-
4	2/M	Jejunum/proximal ileum	+	-	-	-	-	NR
5	70/F	Ileum	-	-	-	-	-	+
6	54/M	Ileum	-	-	-	+	-	-
7	68/M	Distal jejunum/proximal ileum	-	-	-	+	-	-

F: Female; M: Male; NR: No record.

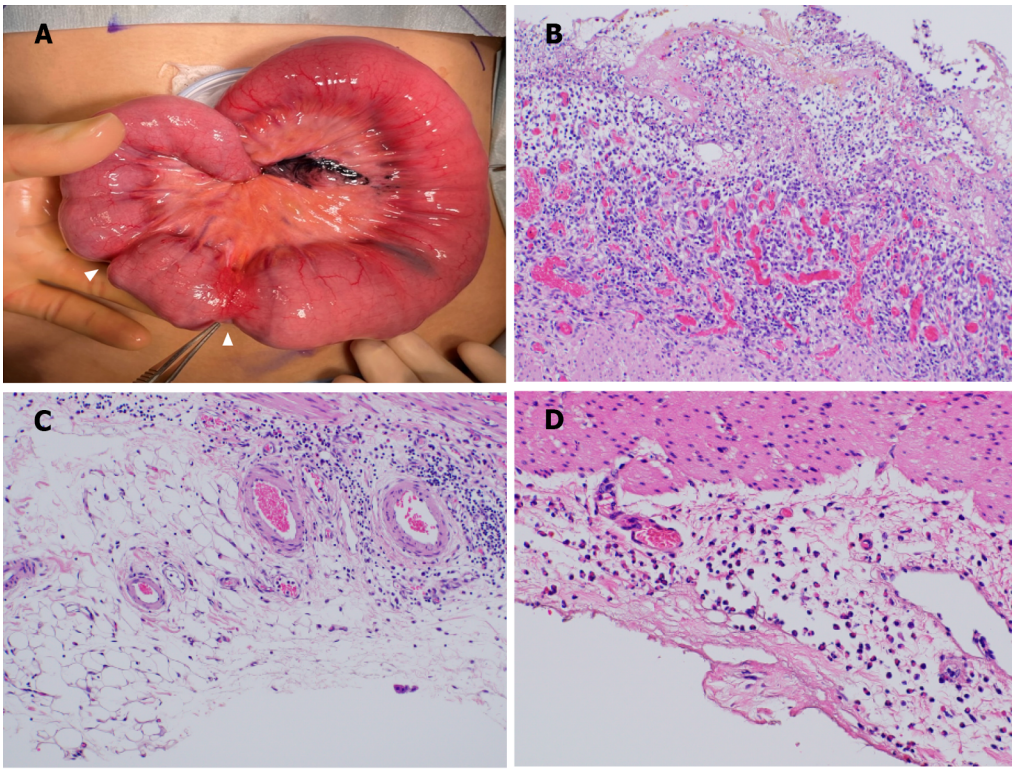

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Figure 3 Macroscopic and microscopic histological findings of the resected ileum. A: Macroscopically, strictures are observed 40 cm and 44 cm proximal to the ileocecal valve (arrowhead); B: Resected ileum shows ulcer formation and peri-ulcer mucosal damage histologically; C: No significant eosinophilic infiltration is observed transmurally; D: Resected ileum at the ileostomy closure shows marked eosinophilic infiltration (> 50/high-power field) in the subserosa.

by neutrophils, rather than eosinophils[20,21]. The present case thus meets the diagnostic criteria for CEAS, but the diagnosis of EoN was reasonable based on the histological findings and the therapeutic course and responsiveness.

The patient developed bowel obstruction induced by small bowel stricture during the clinical course. Most cases of CEAS manifest with small bowel stricture associated with the healing of ulcers and require long-term endoscopic follow-up and treatment, whereas few cases of small bowel stricture have been reported in EGID[16,17,22,23]. In particular, two EoN cases with stricture showed a transmural eosinophilic infiltration at the resected intestinal tract[16,17], suggesting that the eosinophilic infiltration in the muscle layer and serosa is a risk factor for intestinal stricture. However, identification of these by endoscopic mucosal biopsy may be extremely difficult. In the present case, the mucosal eosinophilic infiltration at the time of the small bowel resection was insignificant compared to that at the ileostomy closure, in which insufficient therapeutic agents were regularly administered, suggesting the treatment for EoN was unlikely to be inadequate. This fact indicates that periodic endoscopic follow-up with

consideration of the possibility of small bowel stricture would be required in EoN localized to the small bowel with CEAS-like ulcerative lesions.

CONCLUSION

EoN should be included in the differential diagnosis of patients who exhibit CEAS-like ulcerative lesions localized to the small intestine and have normal urinary prostaglandin metabolites. In addition, EoN with CEAS-like ulcerative lesions may require periodic endoscopic follow-up taking into account the potential complication of small bowel stricture.

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