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Contents

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Contents

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Weekly Volume 27 Number 45 December 7, 2021

Contents

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EDITORIAL

Orosomucoid in liver diseases

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Abstract

In this editorial, the roles of orosomucoid (ORM) in the diagnoses and follow-up assessments of both nonneoplastic diseases and liver tumors are discussed with respect to the publication by Zhu *et al* presented in the previous issue of *World Journal of Gastroenterology* (2020; 26(8): 840-817). ORM, or alpha-1 acid glycoprotein (AGP), is an acute-phase protein that constitutes 1% to 3% of plasma proteins in humans and is mainly synthesized in the liver. ORM exists in serum as two variants: ORM1 and ORM2. Although the variants share 89.6% sequence identity and have similar biological properties, ORM1 constitutes the main component of serum ORM. An interesting feature of ORM is that its biological effects differ according to variations in glycosylation patterns. This variable feature makes ORM an attractive target for diagnosing and monitoring many diseases, including those of the liver. Recent findings suggest that a sharp decrease in ORM level is an important marker for HBV-associated acute liver failure (ALF), and ORM1 plays an important role in liver regeneration. In viral hepatitis, increases in both ORM and its fucosylated forms and the correlation of these increases with fibrosis progression suggest that this glycoprotein can be used with other markers as a noninvasive method in the follow-up assessment of diseases. In addition, similar findings regarding the level of the asialylated form of ORM, called asialo-AGP (AsAGP), have been reported in a follow-up assessment of fibrosis in chronic liver disease. An increase in ORM in serum has also been shown to improve hepatocellular carcinoma (HCC) diagnosis performance when combined with other markers. In addition, determination of the ORM level has been useful in the diagnosis of HCC with AFP concentrations less than 500 ng/mL. For monitoring patients with AFP-negative HCC, a unique trifucosylated tetra-antennary glycan of ORM may also be used as a new potential marker. The fact that there are very few studies investigating the expression of this glycoprotein and its variants in liver tissues constitutes a potential limitation, especially in terms of revealing all the effects of ORM on carcinogenesis and tumor behavior. Current findings indicate that ORM2 expression is decreased in tumors, and this is related to the aggressive course of the disease. Parallel to this finding, in HCC cell lines, ORM2 decreases HCC cell migration and invasion, supporting reports of its tumor suppressor role. In conclusion, the levels of ORM and its different glycosylated

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variants are promising additional biomarkers for identifying ALF, for monitoring fibrosis in viral hepatitis, and for diagnosing early HCC. Although there is evidence that the loss of ORM2 expression in HCC is associated with poor prognosis, further studies are needed to support these findings. Additionally, investigations of ORM expression in borderline dysplastic nodules and hepatocellular adenomas, which pose diagnostic problems in the differential diagnosis of HCC, especially in biopsy samples, may shed light on whether ORM can be used in histopathological differential diagnosis.

Key Words: Orosomucoid; Alpha-1-acid glycoprotein; Viral hepatitis; cirrhosis; Hepatocellular carcinoma; Downregulation

Core Tip: Orosomucoid (ORM) has been suggested as a noninvasive marker in the diagnosis and follow-up of liver diseases. Currently, the results support the hypothesis that ORM can be used together with other markers to diagnose acute liver failure, monitor the development of cirrhosis, and detect early hepatocellular carcinoma (HCC). Although its role in carcinogenesis has not been entirely determined, the fact that decreased ORM2 expression is associated with carcinogenesis and poor prognosis warrants further study with the aim of better understanding the role of ORM in tumor behavior. The use of ORM expression to distinguish HCC from other neoplastic lesions and its role in differential diagnosis await investigation.

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INTRODUCTION

Orosomucoid (ORM), also known as alpha-1 acid glycoprotein (AGP), is an unusual protein with a carbohydrate content of 45% and a very low pI (2.8-3.8) that was identified more than a century ago[1]. Its variation in molecular weight between 37 and 54 kDa is closely related to the differences in its glycosylation content. Although the level of ORM varies according to species, it constitutes 1% to 3% of plasma proteins in humans[2]. This level is slightly higher for men than for women[3]. It is mainly synthesized in the liver; hence, it is also considered a hepatokine. However, smaller amounts can also be produced by breast epithelial cells, type II alveolar epithelial cells, endothelial cells, granulocytes, monocytes, and macrophages[2].

As the name implies, ORM is mainly composed of polypeptide chains and carbohydrate segments. The structures of genes encoding the polypeptide chain of ORM can vary within a broad spectrum. It is located in a cluster of 3 neighboring genes located on the long arm of the 9th chromosome in humans[4]. These neighboring genes are known as AGP-A, AGP-B, and AGP-B'. Among these, AGP-A, which has 3 different alleles (ORM1 * S, ORM1 * F, and ORM1 * F2), encodes ORM-1, and the distribution in the frequency of these alleles in humans may differ according to geographical region[3,5]. AGP-B and AGP-B' encode ORM2. Therefore, ORM exists in human serum as two variants: ORM1 and ORM2[4]. Notably, most individuals carry a mixture of these variants. Although these variants are reported to share 89.6% sequence identity and similar biological properties, ORM1 is the main component of serum ORM, as it is present in plasma at a fivefold higher concentration than ORM2[6,7]. ORM contains 5 N-linked glycans linked to the polypeptide structure, each of which can be a bi-, tri-, or tetra-antennary glycan and can exhibit varying degrees of fucosylation and sialylation and branching[8]. These different glycosylation sites change the protein's biological properties.

ORM is one of the major acute-phase proteins in humans, and its levels increase 2-fold to 6-fold in plasma in most disease states, including inflammation and cancer. Although the biological role of ORM remains unclear, it can regulate immunity and

play roles in both pro- and anti-inflammatory responses, can bind to endogenous ligands such as steroids, and has the ability to bind and transport large numbers of basic and neutral lipophilic drugs[2,8]. Recent studies have shown evidence that it can affect muscle tissue through glycogen metabolism, act as an adipokine in adipose tissue, and be involved in bile metabolism[9-11]. The general properties of ORM are summarized in Table 1.

Because ORM levels vary in many diseases and since their biological effects depend on the glycosylation pattern, this glycoprotein is an attractive target for use in diagnosis and treatment[3]. Accordingly, many current studies are exploring different organs, including the liver, with the aim of using ORM as a potential biomarker for the diagnosis and monitoring of diseases and of revealing its roles in carcinogenesis and tumor behavior.

In this article, the role of ORM in the diagnosis and follow-up of liver diseases is presented.

ORM IN ACUTE LIVER DAMAGE AND REGENERATION

Loss of liver function caused by viral hepatitis, cirrhosis, alcohol, and drug-induced liver damage is a life-threatening condition. Therefore, many studies have been conducted to better understand liver regeneration mechanisms, mainly to shed light on possible clinical applications, such as in the treatment of acute liver failure (ALF), which is a lethal disease characterized by sudden hepatic metabolic and immunological function loss. Liver regeneration is a complicated but coordinated multistep process that is mediated by the integration of multiple factors. Among these factors, proliferation plays a crucial role in the initiation of regeneration, as suggested by a large amount of data on the activation of the cell cycle and the proliferation of quiescent hepatocytes[12,13]. Therefore, eliminating arrest-promoting mechanisms affects the growth of differentiated hepatocytes; in other words, proliferation plays a key role in overcoming liver failure. Although some mitogens, such as growth factors, have been shown to affect proliferation, in recent years, paracrine mediators and nonmitogenic cytokines, including ORM1, have also been shown to participate in the control of this process in a coordinated manner[14]. Indeed, ORM1 expression increases during regeneration following liver resection in both humans and mice[15].

Moreover, the knockdown of ORM1 downregulates the signaling pathways controlling chromatin replication, supporting the notion that ORM1 plays a role in the cell proliferation involved in liver regeneration[16]. This finding is also in line with previous findings related to STAT-3, which is one of the transcription factors involved in liver injury and is associated with proliferation, that show that ORM synthesis is induced in liver injury due to the use of drugs that cause oxidative stress[17,18]. In proteomic analyses, it has been suggested that the sharp decrease in serum ORM levels in HBV-induced ALF (HBV-ALF) patients, as indicated by comparisons between liver tissue samples obtained from HBV-ALF and healthy individuals, may be a valuable biomarker in the diagnosis of ALF in patients with chronic liver disease[19]. Parallel to these findings, the results from a recent study on HBV-ALF showed the downregulation of four genes involved in the immune response and the complement and coagulation cascades, including ORM1 and ORM2; this suggests that both can be potential treatment targets for ALF[20]. However, the tissue expression level of ORM in the liver of patients with HBV-ALF was found to be 4.595-fold higher than that in the liver of healthy patients[21]. This finding is partially explained by the hypothesis that blood ORM accumulates in the liver in response to ALF.

Although further studies that involve a large number of patients and analyze both the blood and tissue levels in the same patients are needed to clarify the role of ORM in the diagnosis and treatment of ALF, current evidence indicates that ORM can be a useful marker in the diagnosis of this lethal disease.

ORM AND NONNEOPLASTIC LIVER DISEASES

As an acute-phase reactant, the ORM level in plasma, which is normally between 4% and 6%, can vary in many inflammatory diseases, including that of the liver. Recently, Oguz *et al*[22] showed that patients with HCV hepatitis have higher ORM levels than healthy individuals. Moreover, evidence has shown that the ORM level fluctuates with fibrosis progression and increases with the development of cirrhosis. Regarding the treatment of HCV hepatitis, no significant difference was found in the responding

Table 1 A brief overview of the general properties of orosomucoid protein

General properties of orosomucoid protein		
Chromosome location	9	
Genes	AGP-A	AGP-B and AGP-B′
Product	ORM1	ORM2
Alleles	ORM1*S, ORM*F, ORM*F2	Monomorphic, except in Japan
Structure		
Polypeptide chain	Single, 183 amino acids with disulfide bonds; There are 22 amino acid differences between ORM 1 and ORM2	
Carbohydrate parts	Five N-linked potential glycosylation sites: Sialic acid, neutral hexoses, mannose, fructose, galactose and hexosamine. Alterations in fucosylation, sialylation, and branching affect its biological properties	
Synthesis	Predominantly by hepatocytes and parenchymal cells. Extrahepatic secretion is rare (breast, endothelial cells, and tumor cells)	
Secretion		
Inflammatory mediators	Glucocorticoids, TNF-α, Interleukins: 1, 6, 8, 11	
Exogenous factors	Phenobarbital, Rifampicin, Retinoic acid, Macrolides	
Biological activities		
Acute-phase reactant	Concentration is elevated 1-10 times during several pathological conditions. Infection, inflammation, tumor, surgery, tissue injury, sepsis, and necrosis	
Immunomodulation	Inhibit leukocyte rolling/adhesion and migration and lymphocyte proliferation. Vitamin D-mediated macrophage deactivation. Agalacto/asialo derivative suppresses the immune response. ORM1 contributes to both anti- and proinflammatory signals to mediate mechanisms activated by the acute-phase response	
Transporting protein	Drug-binding and transporting in the serum. The existence of two forms in the blood also has an influence the binding affinity. ORM1 binds warfarin, prazosin, imatinib, quinidine, and dipyridamole. ORM2 binds methadone, disopyramide, propafenone, and amitriptyline	
Endothelial functions	Maintain the barrier function of capillaries. Regulate injury-induced angiogenesis. Enhance blood-brain barrier functional integrity. Beneficial effect on the glomerular barrier	
Metabolism	ORM1 increases glucose uptake activity in adipocytes. A potential biomarker in distinguishing obese women with metabolic syndrome from those without metabolic disturbances	

ORM: Orosomucoid; TNF-α: Tumor necrosis factor alpha.

group and the nonresponding group, suggesting that ORM levels can only be used as adjuvants to monitor early treatment response. Although not significant, the remarkable increase observed in the early phase of treatment has been attributed to the association of ORM with IFN. In contrast to these findings, another study found that the ORM level in HCV hepatitis was lower than that in healthy subjects and was significantly increased with fibrosis progression[23]. It has been suggested that this decrease may be due to HCV proteins suppressing C3 synthesis. Additionally, variations in the level of ORM were associated with neither necroinflammatory activity nor viral genotype.

High levels of ORM have also been reported in HBV hepatitis and associated cirrhosis[19-21]. In contrast, ORM levels were observed to be within normal limits in NASH and chronic alcoholic liver disease[24,25].

Since the glycosylation pattern of ORM can be modified throughout diseases, these alterations were analyzed for any correlation with the severity of liver diseases to determine the use of ORM as a surrogate marker of fibrosis. In recent years, the fucosylated form of ORM has been observed at a higher level in patients with both HBV and HCV hepatitis than in healthy individuals[26-29]. Moreover, it was emphasized that the fucosylated ORM might be useful in monitoring fibrosis because of it increased with the progression of fibrosis toward cirrhosis[27]. Another interesting finding indicates that during this progression, the increase in ORM fucosylation is associated with a concordant decrease in sialylation. These results support the idea that its glycosylation is modified by the severity of fibrosis and might be useful in disease monitoring[30].

In light of the accumulated data on the modification of the sialylation content of ORM, a few recent studies have been performed to evaluate the diagnostic performance of asialo-AGP (AsAGP) in the detection of cirrhosis in patients with chronic

liver disease. Increasing serum levels of AsAGP were correlated with the degree of fibrosis, and this level was highest in cirrhosis[31,32]. There have also been findings indicating that an increased level of AsAGP shows an inverse correlation with albumin but a positive correlation with the stage of fibrosis, and it may be a positive predictor of cirrhosis[31]. However, in these studies, similar results were not found in comparisons with liver stiffness, a noninvasive method for detecting fibrosis. Thus, further studies are warranted.

ORM IN LIVER TUMORS

Hepatocellular carcinoma (HCC) continues to be one of the major causes of cancer deaths worldwide due to its lack of specific clinical findings at the early stages and the lack of efficient screening methods[33]. To prevent HCC from being diagnosed in advanced stages where treatment options are limited, many efforts have been made to identify new diagnostic and screening methods. For this purpose, several tumor markers have been proposed for use in HCC diagnosis. Because evidence has established that ORM is associated with carcinogenesis and behavior in many organs, changes in the ORM serum level in HCC have been investigated as diagnostic markers. The results of many studies have indicated that the ORM level is increased in patients with HCC and is significantly higher than that in patients with chronic hepatitis and cirrhosis[34-37]. ORM has also been shown to improve HCC diagnostic performance when combined with other markers, such as des-g-carboxy prothrombin (DCP)[38].

Furthermore, determination of the ORM level has been observed to be useful in the diagnosis of HCC in which the AFP values are less than 500 ng/mL[39]. In light of these data, it can be concluded that monitoring ORM levels together with AFP and/or other biomarker levels may be useful in the early detection of HCC. Additionally, it has recently been shown that the combination of urinary ORM-1 levels with urinary AFP levels has a high sensitivity (85%) in the diagnosis of HCC and that this noninvasive method can also be used[40].

Few studies have evaluated the role of ORM in the evolution and prognosis of cholangiocarcinoma (CCC). An experimental study showed that the ORM2 Level increases before tumor onset and tends to be upregulated during tumor progression [41]. The levels of ORM2 were also investigated in patients with CCA. The sensitivity and specificity of ORM2 in distinguishing CCC patients from healthy individuals were 92.86% and 73.68%, respectively[42].

It has been proposed that alterations in the glycosylation pattern of ORM can be used to detect the progression and metastasis of many types of cancer[43]. Indeed, the aberrant glycosylation of ORM in liver cancer progression has received considerable attention in biomarker studies. Previous studies revealed that although there was an increase in the ORM levels of patients with liver cirrhosis and HCC compared to healthy controls, different degrees of fucosylation may distinguish HCC cases from cirrhosis cases[44-47]. Performing an elegant study, Liang *et al*[48] demonstrated a unique trifucosylated tetra-antennary glycan of ORM predominantly identified in HCCs. However, this glycan was absent in both healthy subjects and the majority of cirrhosis patients, as determined by matrix-assisted laser desorption ionization-mass spectrometry, providing a new potential marker for monitoring AFP-negative HCC patients. Although the level of fucosylated ORM differs significantly in advanced stages, determining whether these markers can be used to determine HCC behavior requires further studies in large series.

Similar to nonneoplastic diseases of the liver, the efficacy of AsAGP in the diagnosis of HCC has been investigated in some studies. The AsAGP level is higher in cirrhosis and HCC[49,50]. Kim *et al*[50] revealed that these increases in both cirrhosis and HCC might be related to damaged asialoglycoprotein receptors on the hepatic cell surface, as demonstrated in human and animal studies. The release of extra neuraminidase into the circulation during cellular transformation and the production of incomplete asialoglycoproteins in hepatic cells are also hypothesized to explain the increase in AsAGP in these patients.

In liver diseases, the vast majority of studies addressing ORM were performed in body fluids. However, there are very few studies that investigated ORM expression at the tissue level that have yielded significant results. Although ORM1 and ORM2 are mostly observed in liver tumors compared to other organ cancers, their expression levels are significantly decreased compared with those in neighboring liver tissue, suggesting that ORM genes are downregulated in liver tumors[37,51,52]. The down-

regulation of ORM2 inversely correlates with intrahepatic metastasis and histological grade; in other words, ORM2 is negatively correlated with aggressive tumor behavior [51]. Parallel to these findings, studies using HCC cell lines showed that ORM2 decreased HCC cell migration and invasion, supporting its tumor suppressor role[52]. In addition, Zhu *et al*[52] observed that the prognosis of patients with low ORM2 expression was worse than that of patients with higher ORM2 expression. Therefore, it has been suggested that ORM2 may be a new prognostic factor for liver cancer patients. Moreover, in this study, the inverse association between downregulated ORM2 expression and pathways involved in hepatocarcinogenesis, such as the G2/M checkpoint, E2F target signaling, Wnt/β-catenin, and hedgehog signaling pathways, also supported the use of ORM2 as a marker of liver cancer[51]. The observation of the involvement of ORM2 in tumor-associated macrophage infiltration and the T-cell mediated checkpoint in liver tumor tissue also suggested that its downregulation may be another marker for efficiently predicting the need to apply immune checkpoint therapy. It should be noted that since none of the three current guidelines on the management of HCC include the use of ORM in diagnosis, further studies are needed before recommending its use as a diagnostic marker[53-55]. Moreover, these findings should also be supported by further studies in not only HCC but also CCC. In addition to the relationship of ORM with the immune checkpoint, its relationships with other pathways that may be potential therapeutic targets need to be investigated more comprehensively. The role of ORM expression in differential diagnosis, especially in biopsy samples, to distinguish other neoplastic lesions, such as borderline dysplastic nodules and adenomas, from HCC has not been reported. Furthermore, considering that metastatic tumors of the liver are more common than primary tumors, the role of ORM in these tumors is unknown.

CONCLUSION

In addition to being an acute-phase reactant, ORM is a potential biomarker that can be used with other liver markers for the diagnosis of ALF in the follow-up assessment of fibrosis in viral hepatitis. Similarly, it can be used together with other noninvasive methods to detect early stages of HCC. Recent studies suggest that ORM expression may be useful in determining the behavior of HCC and tumor progression.

However, in HCC, the relationship of ORM with other pathways targeted for treatment in addition to immune checkpoint pathways should be clarified.

Furthermore, the relationship between metastatic tumors and ORM expression, if any, and its role in the histopathological differential diagnosis of HCC require further investigation.

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FRONTIER

Novel frontiers of agents for bowel cleansing for colonoscopy

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Abstract

The incidence of colorectal cancer (CRC) is characterized by rapid declines in the wake of widespread screening. Colonoscopy is the gold standard for CRC screening, but its accuracy is related to high quality of bowel preparation (BP). In this review, we aimed to summarized the current strategy to increase bowel cleansing before colonoscopy. Newly bowel cleansing agents were developed with the same efficacy of previous agent but requiring less amount of liquid to improve patients' acceptability. The role of the diet before colonoscopy was also changed, as well the contribution of educational intervention and the use of adjunctive drugs to improve patients' tolerance and/or quality of BP. The review also described BP in special situations, as lower gastrointestinal bleeding, elderly people, patients with chronic kidney disease, patients with inflammatory bowel disease, patients with congestive heart failure, inpatient, patient with previous bowel resection, pregnant/lactating patients. The review underlined the quality of BP should be described using a validate scale in colonoscopy report and it explored the available scales. Finally, the review explored the possible contribution of bowel cleansing in post-colonoscopy syndrome that can be related by a transient alteration of gut microbiota. Moreover, the study underlined several points needed to further investigations.

Key Words: Colonoscopy; Bowel preparation; Cleansing agents; Polyethylene glycol; Adequate cleansing; Constipation

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Core Tip: Colonoscopy is the best modality for colorectal cancer (CRC) screening, preventing death from CRC through removal of adenomatous polyps and early detection of CRC. The accuracy of colonoscopy is related to quality of bowel preparation (BP). International guidelines underlined the methods to improve BP. In this review, we aimed to summarize the current strategy to increase bowel cleansing before colonoscopy.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in both genders[1] with an incidence characterized by rapid declines in the wake of widespread screening. Colonoscopy is considered the gold standard for CRC screening. Colonoscopy with removal of adenomatous polyps prevents death from CRC[2].

To perform screening colonoscopy, the high quality of endoscopic procedure is mandatory. An adequate bowel preparation (BP) is one of the most important factors, ensuring a high accuracy of procedure, thought an optimal visualization of colonic mucosa increasing adenoma detection rate (ADR)[3-5]. ADR is defined as the percentage of screening colonoscopies in which one or more conventional adenomas are detected[6]. ADR is inversely associated with the risks of interval and lower long-term CRC incidence and mortality[7,8].

Moreover, an inadequate BP is associate to prolonged procedures, higher cost leaded to repeat colonoscopy (longer hospital stay and no cost/efficacy of screening program), lower cecal intubation rates, higher risk of electrocautery and unsatisfactory patient experience with an increased likelihood of repeat procedure.

Despite European Society of Gastrointestinal Endoscopy (ESGE) Guidelines recommended a minimum of 90% procedure with adequate BP, with a target of > 95% [9], suboptimal BP is still encountered in clinical practice[10].

Other review papers regarding bowel cleansing were published[11,12]. However, new evidences change same feature of bowel cleansing process.

The aim of the present review is to describe the current literature regarding BP options, in order to explore factors that can be improved.

BP QUALITY SCALE

ESGE guidelines recommend recording the BP quality using a validated scale[9]. Validity refers to how well the scale measures what it is aimed to assess. For BP, validity could be assessed by comparison of different scales or with another parameter of colonoscopy quality. Another essential attribute of a scale is the reliability that indicates the reproducibility of the results in the same operator (intrarater reliability) or between different endoscopist (interrater reliability).

Several scales were proposed in the last decades to describe the quality of BP of colonoscopy.

The first one was the Aronchick Scale[13] and it is still one of the most commonly used validated BP quality scales in clinical trials and clinical practice.

The quality of the preparation is described as the percentage of entire colonic mucosa covered by stool, before washing or suctioning, ranging from 1 (excellent) to 5 (inadequate). No study has evaluated a threshold to define adequate the BP described by Aronchick Scale.

Validity was not evaluated in clinical studies, while inter-observer reliability was assessed in one study (coefficient was 0.77 in the total colon)[14].

The second developed scale was the Ottawa Bowel Preparation Quality Scale (OBPQS)[14]. This scale is composed by two separate scores. One score is assigned

according to global fluid quantity in the entire colon, from 0 (small amount of fluid) to 2 (large amount of fluid). The second score quantifies the visibility of three separate colon segments (right colon, mid colon and rectosigmoid colon) and also the amount of washing or suctioning required achieving optimal visualization and it ranges from 0 to 4. The total score is obtained by adding the score of each segment and total colon fluid score, ranging from 0 (excellent) to 14 (poor), before washing or suctioning. In one study, the value of at least 8 was proven to be an optimal cut-off value to define inadequate BP because of the inability to detect a 5 mm polyp[15].

The validity was also demonstrated in two study comparing OBPQS with visual analogue scale[16] and with Boston Bowel Preparation Scale (BBPS)[17].

The study of Martinato *et al*[16] detected also a good agreement between nurses and physicians, [r = 0.6010 (95%CI for r 0.4877 to 0.6944)]. One prospective study demonstrated the high interobserver agreement and reliability of OBPQS compared to Aronchick Scale[14] with no statistically significant differences between segment evaluations. Intra-observer reliability and clinical relevance were not evaluated.

BBPS described the colonic mucosa that can be evaluated. The advantages of this scale are multiple. First of all, it is a numeric score ranging from 0 (unprepared colon mucosa) to 9 (entire mucosa well seen) for the entire colon, avoiding the use of qualitative and subjective terms. Second vantage is that a score is assigned for each colonic segment (right colon, transverse, left colon-each one from 0 to 3), allowing a detailed description of BP. Third, the score is assigned after washing and suctioning as recommended by United States Multi-Society Task Force on Colorectal Cancer[18]. Finally, the validity and the reliability of this score has been evaluated in several studies.

The validity of the score was proven in several studies, demonstrating the association with polyp detection rate, insertion and withdrawn times, needed to repeat colonoscopy for inadequate BP. Lai *et al*[19], including 633 screening colonoscopies (22 clinicians), found an association with BBPS ≥ 5, higher polyp-detection rate, an inversely correlation with BBPS and insertion and withdrawal times, and an inverse relation between BBPS and the need to repeat colonoscopy for an inadequate preparation. The latest inverse correlation was confirmed in the study of Calderwood *et al*[20] and in the study of Kim *et al*[21] that also confirmed a correlation with polyp detection rate (PDR). Calderwood conducted a second study with a very large sample size (74 endoscopists performed 2516 colonoscopies) finding that a total score of ≥ 6 and score of ≥ 2 for each segment is the definition of adequate BP[22]. The best cut-off of 2 in each segment as definition of adequate BP is proven also by Clark *et al*[23].

The reliability was determinate in different studies demonstrating a good interobserver agreement, quantified as intraclass correlation coefficient or weighted kappa (ranging between 0.67-0.93)[19-21,24,25]. Indeed, a good intraobserver agreement were found in three different studies (weighted kappa = 0.77; 95%CI: 0.66-0.87[19]; weighted kappa = 0.78; 95%CI: 0.73-0.84)[20] and weighted kappa = 0.67; 95%CI: 0.51-0.84)[24].

The results of these large and very well conducted studies corroborating the validity and the reliability of BBPS, allowed to suggest the routine use of BBPS in the clinical practice as proposed by Parmar *et al*[26].

Promising data come from artificial intelligence, as recently described by Zhou *et al* [27]. They developed a deep convolution neural network called ENDOANGEL to assign BBPS, with a 91.9% of accuracy. In the unique study on this topic, so further data are needed to support the routinely use of this system.

Bubbles scale

None of the previous scales provided an adequate evaluation of presence of bubbles that can impact on mucosa evaluation. This inadequacy affects also the strength of the conclusions of two recent meta-analyses reporting a benefit of added oral simethicone to increase BP[28,29].

The amount of foam/bubble interfering with colonic visualization was also measured in different studies regarding BP[30-42].

Parente *et al*[30] evaluated the presence of bubble in terms of the overall impact on mucosal visualization, as excellent (clear imaging, no or minimal amount of bubbles or foam that can be easily removed), fair (modest amount of bubbles and foam that can be cleared, with some waste of time) and insufficient (a large amount of foam and bubbles that reduces significantly the clear visualization of the mucosa) in each bowel segment.

A Bowel Bubble Scale, a four-point scoring system (0, no bubbles; 1, minimal or occasional bubbles; 2, moderate or obviously present; and 3, severe or many bubbles that vision is obscured) was developed by McNally *et al*[32] and used by Guo *et al*[31] and Yuanchao *et al*[33].

Another intraluminal Bubbles Scale was used in studies performed by Matro *et al* [34] graded 4 segments of the colon (cecum, right colon and hepatic flexure, transverse colon and splenic flexure, and colon distal to the splenic flexure) and each colon segment was graded using a 3-point scale (A = no/minimal bubbles, B = moderate bubbles/interfere with detecting a 5 mm polyp, and C = severe bubbles/interfere with detecting a 10 mm polyp).

Repici *et al*[35] measured the bubble score according the overall mucosal visibility using a 3-grading scale from grade 0 (optimal) to grade 2 (insufficient), the same scale was used by Spada *et al*[38] to asses mucosal visibility.

Yoo *et al*[36,37] used a scale assigned the bubble score in accordance with the degree of obscuration by bubbles, bile, or debris from 0 (severe obscuration) to 3 (no obscuration), applied also by Zhang *et al*[40].

A revised version of this scale was adopted by Rishi *et al*[39], who assigned the score (from 1 to 4) according the percent circumference of colonic mucosa clear of all bubbles/foam, not divided between segments of the colon.

Movareji *et al*[41] used a bubble scale used adapted from the one previously described by Sudduth *et al*[42], evaluating the entire colon by adding each individual segment score (from 0, no or minimal bubbles, to 3, bubbles filling the entire lumen).

In the two latest studies, the authors failed to validate and establish the reliability of the colon bubble scales. In particular, the interobserver agreement for bubble scale score was moderate (kappa = 0.537[41], kappa = 0.4024[39]).

Recently a new scale, named Colon Endoscopic Bubble Scale (CEBuS) was developed and its reliability was determined in a multicentre prospective observational study[43]. The scale CEBuS ranged from 0 (no or minimal bubbles, covering < 5% of the surface) to 2 (bubbles covering > 50%). A high intraobserver reliability [kappa 0.82 (95%CI: 0.75-0.88) *vs* 0.86 (95%CI: 0.85-0.88)] and high interobserver agreement [ICC 0.83 (0.73-0.89) *vs* 0.90 (0.86-0.94)] were reported in both experts group and mix expert/non-expert group. These encouraging preliminary results needed to be confirmed with a larger study.

CLEANSING AGENTS FOR BP

Four-liter high-volume polyethylene glycol (PEG)-based preparations were the first formulations introduced for bowel cleansing prior to colonoscopy. These isosmotic solutions provide rates of adequate BP > 90%[44-47] , without producing relevant fluid shifts or electrolyte imbalances[11]. Despite high efficacy and safety, the large volume of liquids and poor solution taste may decrease patients' compliance to the assumption of these preparations[12].

PEG-based and non-PEG-based low-volume solutions have been developed in order to reduce the total volume of preparation and improve patients' acceptability. The hyperosmotic 2 L PEG-based agents (containing PEG plus ascorbate, citrate, or bisacodyl) showed similar efficacy in bowel cleansing with higher patients' tolerability and willingness to repeat the preparation compared to high-volume PEG-based solutions in meta-analyses[44,45] and randomized trials[38,48-51]. Additionally, comparable adenoma detection rates were found between 2 L PEG plus ascorbate and 4 L PEG solutions[49,50]. A recently developed low-volume solution of 1 L PEG plus ascorbate had similar quality of BP, adenoma detection rate, and safety profile compared to 2 L PEG plus ascorbate in a randomized trial[52]. This preparation showed higher rate of adequate colon cleansing compared to 4 L high-volume PEG (84.3% *vs* 77.4%, P = 0.039) in hospitalized patients, with no differences in electrolyte imbalances, creatinine and haematocrit[53]. However, these results are based on a post-hoc analysis of an observational study. The non-PEG-based hyperosmotic low-volume preparations include magnesium citrate with sodium picosulfate, oral sulfate solution (*i.e.* trisulfate), and oral sodium phosphate. As the PEG-based low-volume solutions, these formulations showed non-inferiority in terms of efficacy and better safety profile as well as patients' tolerability compared to 4 LPEG[54-58].

On these bases, current ESGE guidelines recommend low-volume PEG-based and non-PEG-based solutions as alternatives of equal efficacy to high-volume PEG-based formulation for routine BP, with the exception of oral sodium phosphate for the relevant risk of kidney injury[59]. However, safety concerns have been raised on hyperosmotic low-volume agents in patients at risk for hydroelectrolyte imbalances, such as those suffering from severe renal insufficiency or congestive heart failure. Moreover, ascorbate-containing solutions are contraindicated in people with phenylketonuria or glucose-6-phosphate dehydrogenase deficiency[60]. Thus, the

choice of the adequate preparation for bowel cleansing prior to colonoscopy should be individualized, especially in specific categories of patients at high risk of adverse events.

TIMING OF BP

Timing of consuming BP is highly important. The last dose of BP should be started in the 5 h before colonoscopy and ended 2 h before the scheduled time of the procedure [59]. This recommendation is translate in clinical practice in two different timing for colonoscopy of the morning and colonoscopy of the afternoon.

For morning colonoscopy, both American and European Guidelines strongly recommend split-dose regimens[59,61,62].

Split-dose regimen is defined as assuming half of the BP the day before the colonoscopy and half on the day of the colonoscopy. Several evidences provided the superiority of split dose regimens over a day-before preparation to achieve a better colon cleaning, regardless the cleansing agent[48,63-69]. Moreover, the split-dose preparation showed better patient tolerability and higher proportion of patients willing to repeat the regimen[47,70].

Effectiveness of colonoscopy is highly dependent on the quality of BP. Different observational studies and also a recent meta-analysis found that split dose preparations increase adenoma detection rate[69,71-75]. The meta-analysis demonstrated also an increase rate of advance adenomas and sessile serrated polyps in split-dose regimen, including seven trials comparing split-dose *vs* day-before BP regimens. No differences in the same variables were found comparing spilt-dose and same-day BPs [75]. Another meta-analysis did not confirm the increase of ADR with split dose regimen, but it included only 4 randomized controlled trials (RCTs)[70] with moderate overall quality of evidence.

For afternoon colonoscopy, the same-day BP is recommended[59].

Considering studies including higher number of colonoscopies scheduled in the afternoon, same-day BP showed similar rate of adequate bowel cleaning, with no difference in tolerability and patient willingness to repeat it, comparing to split-dose regimens. The ADR was similar for the two regimens as showed by two different meta-analysis[75,76]. Moreover, patients in same-day regimens reported better sleep quality (OR 0.44, 95%CI: 0.24-0.82)[77].

Instead, the same-day regimen showed a significantly lower quality of BP considering studied including only morning colonoscopies[78], or lower patient tolerability and compliance[79,80], with lower willingness to repeat the same preparation in the future[79].

DIET BEFORE COLONOSCOPY

Diet restriction has traditionally been recommended before colonoscopy because it can reduce the amount of stools in the intestines, but adherence is low. The European and American Societies of Gastrointestinal Endoscopy actually recommended the use of a low residue diet (LRD) for colonoscopy defined as a diet with a total fiber intake inferior of 10 g/day[59,81].

Two meta-analysis[82,83] including studies comparing LRD with clear liquid diet (CLD) on the day before colonoscopy examination, found a significantly higher odds of tolerability and willingness to repeat preparation with no differences in adequate BPs or adverse effects.

In the last year two new meta-analysis comparing LRD *vs* CLD for BP before colonoscopy were published[84,85].

Zhang *et al*[84] performed a systematic literature search until September 2019 and they included twenty RCTs. Adequacy of bowel cleansing and polyps detection rate were similar in both groups (P = 0.79 and P = 0.68 respectively). There were significantly fewer adverse events in individuals in LRD group: nausea (P = 0.02) vomiting (P = 0.04), hunger (P < 0.001), and headache (P = 0.02). In addition, significantly more individuals in the LRD group found it easy to complete the diet (P = 0.01) and showed willingness to repeat it (P = 0.005).

Chen *et al*[85] included 16 studies and found a significantly better tolerability and willingness to repeat intestinal preparation in patients with LRD compared with CLD (both P < 0.05), but no differences with adequate intestinal preparations, detected polyp or overall adverse reactions.

These latest evidences showed that LRD is a promising approach for BP before colonoscopy with comparable adequacy of BP with that of CLD.

A recent study of Gimeno-Garcia *et al*[86] aimed to assess if a 3 d LRD is better regarding bowel cleansing than a single day LRD regimen, concluded that there is not a concrete advantage.

Recently, Avalos *et al*[87] performed a meta-analysis of randomized trials comparing BP outcomes between a LRD or regular diet (RD) compared with a CLD. Twelve RCTs, grouped patients taking a LRD (8 RCTs) or a RD (4 RCTs) and compared them to patients taking a CLD. In the 7 high-quality studies included, they no found differences in BP quality among the LRD/RD and CLD groups (RR 0.98; 95%CI: 0.93-1.04). Tolerability and willingness to repeat were better in the liberalized diet arm. There was no significant difference in the adenoma detection rate, whereas hunger was more common in the CLD group (RR 1.93, 95%CI: 1.13-3.3)[87]. Further studies are needed to confirm other findings, Table 1.

ADJUNCTIVE DRUGS

Various adjuvant drugs have been added to standard BP regimens to increase quality of BP by direct action (as simethicone) or by the improving of patient experience.

Simethicone

Simethicone is an antifoaming agent using to reduce excessive gas, abdominal discomfort, and bubble formation in the gastrointestinal tract.

Several RCTs have investigated the effect of oral simethicone on bowel cleansing.

Since 2011, four meta-analyses were conducted. The first one[88] included 7 RCTs (714 patients) comparing purgative plus Simethicone with purgative alone for colonoscopy. The air bubbles were significantly decreased, while no difference in adequate colon preparation was found.

The role of added oral simethicone on ADR was investigated in meta-analysis of Pan *et al*[89]. Such meta-analysis included 6 RCTs (1855 patients) and found an increase of ADR in simethicone group. Different result was found by another meta-analysis[28], including 12 randomized controlled studies (6003 participants) that found no difference in ADR between the groups with or without simethicone.

The last meta-analysis by Moolla *et al*[29] aimed to determine the effect that simethicone has on bowel cleanliness, ADR and tolerability, and included 16 RCTs (5630 patients) using PEG for bowel agent cleaning. Authors found an increase rate of adequate BP in PEG cohort with simethicone compared with PEG alone (OR 1.48), considering all 16 RCTs.

This finding was confirmed in three subgroup analysis: (1) Excluding RCT with bisacodyl or with different volume preparation; (2) Including only preparation with PEG 2 L; and (3) PEG single dosing the day before. On the other hand, considering patients with split dose regimen, no difference was found in adequate bowel colonoscopy rate between PEG group and PEG + simethicone group.

Regarding ADR, no difference was found considering all studies evaluating ADR (7 studies). However, ADR was significantly higher increase in simethicone group and in the subgroup analysis considering single-dosing preparations (3 RCTs). Moreover, the authors found an increase of bloating in PEG alone group, while no differences were found in the incidence of nausea, vomiting and abdominal pain.

Currently, ESGE guidelines suggest the use of oral simethicone for BP[59].

Instead, the routine use of simethicone through the working channel is advised against by ESGE guidelines[90], due to evidence that simethicone may contribute to biofilm formation in the endoscope working channel, reducing reprocessing effectiveness[91].

Recently,vmulti-society guideline[92] underlined factors associated to simethicone persistence in the endoscope channel. The first is the concentration, the second is the modality of delivering. So, when simethicone is needed, the guideline suggested the use of lowest concentration (less the 5%) and the smallest volume needed avoiding the simethicone delivering *via* water bottle/irrigation jet channel.

Similar recommendations were reported by Gastroenterological Society of Australia [93], despite allowing the administration of simethicone the endoscope irrigation channel.

Both guidelines recommended a strict adherence to manufactures' instruction for each passage of simethicone use (way for simethicone administration, cleaning and disinfection of the scope).

Table 1 Low fiber diet on the day preceding colonoscopy

Type of food	Allow	Avoid
Milk and milk products	Skim or low-fat milk; Buttermilk; Low-fat cheeses; Low-fat ice cream; Sherbet; Yogurt without seeds, berries, rinds or nuts	Yogurt with seeds, berries, rinds or nuts
Vegetables	Any well-cooked vegetables without seeds (*e.g.*, carrots, pumpkin); Lettuce; Potatoes without skin; Strained vegetable juice	All raw vegetables, except lettuce; Broccoli; Brussels sprouts; Cabbage and sauerkraut; Cauliflower; Corn; Fried vegetables; Greens (mustard, turnip, collards); Mushrooms; Okra; Onions; Peppers; Potato skins
Meats and other protein foods	Eggs; Smooth nut butters; Tofu; Tender, well-cooked meat, poultry and fish	Chunky nut butters; Legumes; Nuts or seeds; Tough or chewy cuts of meat
Grains	Bread, bagels, rolls, crackers, pasta and cereals made from white or refined flour (*e.g.*, crispy rice cereal and cornflakes); Cooked cereals (farina and creamy rice); White rice	Brown rice and wild rice; Cereals made from whole grains; Grain products made with seeds or nuts; Whole-wheat or whole-grain breads, rolls, crackers or pasta
Fruits	Fruit juice without pulp (except prune juice); Most canned, soft and pureed fruit without skin (except pineapple); Peeled apple; Ripe banana or melons	All raw fruits except peeled apple, ripe bananas and melon; Canned berries, canned cherries; Dried fruits, including raisins; Prunes and prune juice
Beverages	Coffee, tea, chamomile; Sports drinks; Water	
Condiments	Ketchup and mustard; Margarine, butter, oils, mayonnaise, sour cream and salad dressing; Plain gravies; Spices, cooked herbs, bouillon, broth, and soups made with allowed vegetables; Sugar, clear jelly, honey and syrup	

Agents improving patient experience

To increase the quality of BPs, several adjuncts were evaluated. All of them act through the increasing of tolerability and palatability of bowel cleaning agents.

Four studies evaluated the role of drinks different from water. One study evaluated the BP, the palatability and the adverse effects of Coca-Cola (Coke) Zero as solvent for PEG comparing with water. The authors found a better quality of BP and palatability in Coke group, with no difference in rate of adverse events neither in PDR[94]. The palatability is also increased with orange juice intake before drinking 2 L of PEG plus ascorbic acid[95], while no differences were found in quality of BP. Also, pineapple juice was tested to increase palatability of BP. In one single randomized study[96], patients were assigned to one of the following regimens: 4 L PEG or 2 L PEG or 2 L PEG plus 1 L of pineapple juice. The third group had better quality of bowel cleansing in the right side and in transverse colon, but no difference in tolerability.

A prospective, randomized controlled recent study of Hao *et al*[97] aimed to evaluate the effectiveness and safety of concomitant use of green tea (GT) with 2 L PEG in BP for colonoscopy. Adding GT increased the compliance, reduced adverse events with comparable bowel cleanliness in BP.

Five studies evaluated the role of tablets and gum chewing in the BP. The study of Lan *et al*[98] compared two groups of patients received 2 L PEG alone or plus citrus reticulata peel in form of "buccal tablet" eaten between drinks. The second group had higher acceptable taste, lower rate of swallowing difficulty and adverse events with no differences in quality of colonic cleansing. Three randomized studies evaluation the contribution of gum chewing[99-101] and in all of them, patients' tolerability was better in gum chewing group than the other group. In one study[99], better quality was reached in gum chewing group, no difference was found in the other two studies.

The menthol candy drops[102] were used in one randomized study demonstrating the better grade preparation in candy drops-added group, with no difference in side effects.

A recent systematic review and meta-analysis was performed including 6 single-blind RCTs (1187 patients)[103]. The included adjuncts were citrus reticulata peel, orange juice, menthol candy drops, simethicone, Coke Zero and sugar-free chewing gum. The study concluded that the adjunct improved palatability and willingness to repeat BP, with fewer side effects as bloating, vomiting, but no difference in nausea or abdominal pain. Moreover, the rate of adequate BP was higher in the adjunct group.

EDUCATIONAL INTERVENTION

In the effort to improve BP, several methods emphasizing the importance of BP quality and the instructions for BP were evaluated. Different methods were tested, including

pictures, cartoon visual aids, booklets, video, instructions by the nurse, short message service, smartphone applications were evaluated separately with conflicting results.

Seven meta-analyses[104-110] were conducted to compare the adequacy of BP in patients who received enhanced instructions and patients who received standard ones. All of them demonstrated that enhanced instructions are useful to improve the quality of BP, and in the same time to increased ADR.

So, both European and United States guidelines suggested the enhanced instruction before colonoscopy[18,59].

SPECIFIC CATEGORIES OF PATIENTS

Lower gastrointestinal bleeding

Colonoscopy has an important role for optimal management of acute lower gastrointestinal bleeding (LGIB), with diagnostic and therapeutic potential[111].

Colonoscopy should be performed after hemodynamic stabilization. Moreover, adequate colon cleansing is crucial to achieve before performing colonoscopy for LGIB, because of the increasing risk of perforation, and major risk of missed bleeding mucosal lesions in poorly prepped colon and properly evaluation of the entire mucosa [112].

However, cleansing the colon from stool, clots and blood is difficult to accomplish [111].

According to the latest European guidelines, preparation for colonoscopy should include 4-6 L of a polyethylene glycol solution or the equivalent, administered over 3-4 h until the rectal effluent is clear. A nasogastric tube can be placed to facilitate colon preparation in intolerant to oral intake patients. Prokinetic/anti-emetic agent immediately prior to initiating the colon preparation may reduce nausea and facilitate gastric emptying[59].

Although colonoscopy has several advantages in the management of LGIB (identification of bleeding sources, multiple therapeutic options, definitive diagnosis, reduction of hospital length of stay and safety), it also has several disadvantages (need for colon preparation and sedation, experienced staff and endoscopy facilities, low prevalence of stigmata of hemorrhage, invasive nature, and rare but serious complications)[111].

A higher risk of urgent colonoscopy adverse events may occur in elderly patients with comorbidities or on antithrombotic therapy[113,114]. BP may increase the risk of vomiting, aspiration pneumonia a volume overload[111].

Niikura *et al*[115] in a retrospective review investigated adverse events and hemodynamic instability during BP and colonoscopy in hospitalized patients with acute LGIB. They showed that during BP, the 9% of LGIB patients experienced an adverse event. None of them experienced volume overload, aspiration pneumonia or loss of consciousness; however, 7% had hypotension and 2% vomited. There were no significant differences in the five BP-related adverse events between LGIB and non-GIB patients.

The use of lower volume or alternative colon preparation solutions in LGIB patients is not well defined, only preliminary data are available and seems encouraging[116].

The American College of Gastroenterology, ESGE and British Society of Gastroenterology recommends against un-prepped colonoscopy in the setting of acute LGIB[59, 117,118].

A prospective pilot study of Repaka *et al*[119] in severe LGIB subjects reported the feasibility and safety of unprepared hydroflush colonoscopy that combined three 1-L tap water enemas, a water-jet pump irrigation system, and a mechanical suction device to cleanse the colon. Cecal intubation was performed in 69.2% of patients and definitive bleeding sources of 38.5% of patients were detected. However, localization of diverticular bleeding, can be difficult in the setting of residual blood and stool and poor visualization may also increase the risk of perforation.

A recent single-center study performed on elderly patients with severe LGIB investigated the efficacy, safety and outcomes of unprepared polyethylene glycol-flush retrograde colon cleansing colonoscopy[120]. In this study cecal intubation was 100%, the rate of definitive bleeding sources was 90.9%. They concluded that this approach was safe, effective and reduced the time of hospital stay, therefore further data are necessary.

Although, the international guidelines recommend BP of this cohort of patients, the best modality to achieve the cleaning of the colon is still an open problem.

Chronic kidney disease and hemodialysis

The assessment of renal function is a key point in the choice of the most adequate and safe bowel cleansing agent prior to colonoscopy, since the assumption of hyperosmotic solutions may lead to dehydration and electrolyte imbalances in people with pre-existing chronic renal disease[59]. Although the relevance of the issue, high quality evidence on different preparations for this high-risk population is lacking, with available data deriving from observational studies. Lee *et al*[121] found no difference in electrolytes or estimated glomerular filtration rate (eGFR) between 4 L PEG and 2 L PEG plus ascorbate in patients with an eGFR < 60 mL/min before colonoscopy. A transient > 30% rise in creatinine levels was recorded in 7.5% and 11.5% of high-volume and low-volume group, respectively (P > 0.05). In a similar population, Russman *et al*[122] showed that oral sodium phosphate was associated with a 12.6 (95%CI: 1.5-106.5) times increased risk of renal function worsening compared to 4 L PEG. Frazzoni *et al*[53] compared 1 L PEG plus ascorbate with 4 L PEG, including 52 patients with chronic kidney disease, showing no different shift in serum electrolytes levels and creatinine. Considering these results, PEG-based preparations may be a safe choice in people with pre-existing mild to moderate chronic kidney disease (eGFR ranging from 89 to 30 mL/min), whereas current international guidelines do not recommend hyperosmotic low-volume PEG-based agents in people with severe renal insufficiency (eGFR < 30 mL/min) for the high risk of electrolyte imbalances. However, high quality randomized trials are needed to better clarify the safety profile of PEG-based solutions in the setting of chronic kidney disease. On opposite, the use of non-PEG-based low volume preparations should be avoided in this population due to possible magnesium toxicity or acute phosphate nephropathy[53,123].

Some warnings have been raised on the safety of bowel cleansing agent administration in people on haemodialysis[124]. Indeed, potential intravascular depletion following bowel cleansing agent intake may lead to hypotension and thrombosis of the arteriovenous fistula. Moreover, the association of BP assumption and hemodialysis treatment may cause severe hypovolaemia. Additionally, high-volume PEG-based solutions may produce fluid overload in these anuric patients. Despite these relevant concerns, there is currently no high quality evidence on the safety of the different formulations of bowel cleansing agents in this population, which has been systematically excluded from randomized trials. Only two studies explored the efficacy and safety of PEG-based preparations prior to colonoscopy in patients with pre-existing chronic kidney disease, including a cohort of people receiving hemodialysis[121,125]. The authors found no significant variation in serum electrolyte levels after the assumption of PEG-based formulations. However, these studies have a retrospective observational design and enrolled a total of 37 patients on hemodialysis. On these bases, specific recommendations on the use of bowel cleansing agents in this at-risk population are not provided. The first randomized trial comparing the efficacy and safety of 4 L PEG *vs* 2 L PEG plus citrate prior to colonoscopy in people receiving hemodialysis is currently ongoing (NCT04709770).

Inflammatory bowel disease

Adequate BP is crucial to assess disease activity in patients with inflammatory bowel disease (IBD). Moreover, the widespread promotion of dye-based and virtual chromoendoscopy as appropriate diagnostic techniques for neoplasia surveillance in this population at high risk of CRC further emphasizes the relevance of achieving high quality BP[126-128].

Evidence from randomized trials showed comparable efficacy between high-volume and low-volume PEG-based solutions in people with IBD. Manes *et al*[129] found no significant difference in adequate bowel cleansing between 4 L PEG and 2 L PEG plus bisacodyl in 216 patients with ulcerative colitis (75.0% *vs* 81.5%, respectively). Kim *et al*[130] demonstrated comparable rates of satisfactory BP between 4-liter PEG and 2-liter PEG plus ascorbate (96.2% *vs* 92.9%; P = 0.68) in a cohort of 109 participants with ulcerative colitis. Similarly, Kato *et al*[131] showed the non-inferiority of 2 L PEG plus ascorbate in terms of bowel cleansing compared to 4 L PEG in 70 patients with ulcerative colitis or Crohn's disease. In these trials low-volume formulations had higher patients' tolerability and willingness to repeat the preparation than 4 L PEG. Based on these results, both high-volume and low-volume PEG-based BPs are recommended in patients with IBD before colonoscopy[59], although low-volume agents may be a more advisable choice in people undergoing a considerable number of colonoscopies during their lifetime[132]. Conversely, low-volume non-PEG-based preparations should be avoided in this population, since they may cause mucosal alterations mimicking IBD[18,59]. Lawrance *et al*[133] showed a 10-fold higher rate of

preparation-induced mucosal inflammation with magnesium citrate plus sodium picosulfate and sodium phosphate compared to 4 L PEG in a randomized trial enrolling 634 participants without pre-existing or suspected IBD. Sodium phosphate-related inflammatory abnormalities were detected in 3.3% of patients in a prospective observational study including 730 participants without previous diagnosis of IBD and not using non-steroidal anti-inflammatory drugs[134].

Inpatient

Previous evidence underlined that inpatient status is one of the associated factors with inadequate BP[135-138]. In this cohort of patients, the percentage of colonoscopy with adequate preparation is between 50% and 75%[135,139,140], thus increasing the hospital length and costs[138].

It is crucial to identify predictive factors associated with inadequate BP in this cohort of patients, and in the same time, to found the best bowel cleansing agent.

The explanation could be the worse American Society of Anesthesiologists status in inpatient setting[141] prolonged immobility and the use of concomitant drugs that can impair bowel motility[142], as opiate drug[139].

The multicenter observational study of Fuccio *et al*[143] identified the factors associated with a more proper colon cleansing (physicians' meetings to optimize BP, written and oral instructions to patients, admission to gastroenterology unit, split-dose regimens, a 1 L polyethylene glycol-based bowel purge, and 75% or more intake of BP). The authors, also, found factors associated to an increased risk of inadequate colon cleansing(bedridden status, constipation, diabetes mellitus, use of anti-psychotic drugs, and 7 or more days of hospitalization).

Considering the modifiable factors, Gkolfakis *et al*[140] evaluated the role of education interventions to increase adequate BP in a recent meta-analysis. In the six included studies, the adequacy was achieved in 77% (62%-91%) of patients with education interventions *vs* 50% (32%-68%) of patients with no intervention. However, this strategy is not enough to reach to 90% of adequate colonoscopy as required by ESGE guidelines.

Regarding the choice of BP, only one study was aimed to assess the role of low volume PEG solution in inpatient cohort. In a retrospective post-hoc propensity matching score analysis of a previously prospective observational study, Frazzoni *et al*[53] found a higher rate of adequate bowel cleansing in group prepared with 1 L-PEG plus ascorbate hyperosmolar preparation than patients with the 4 L-PEG preparation. A specifically designed study is needed to better investigate the efficacy and the safety of low volume bowel agent in this setting of patients.

Elderly people

Patients with more than 65 years require special attention during the BP before colonoscopy, due to fragile equilibrium and/or increase incidence of concomitant diseases. Large volume of BP has a better risk profile, causing less electrolyte abnormalities and low risk of dehydration, but requires a high patient's compliance. Low volume cleaning agents with magnesium citrate or bisacodyl or sodium phosphate should be avoided in this fragile category of patients, due to an increased risk of electrolyte unbalance, ischemic colitis and renal function impairment, respectively [144-146].

Only two RCTs were specifically designed to evaluate BP in elderly people. Jung *et al*[48] enrolled 230 patients aged > 65 years with normal renal function and electrolytes, randomly assigned to one of 3 arms (single-dose 4 L-PEG on the day before colonoscopy; split-dose 4 L-PEG; or split-dose 2 L-PEGA). The rate of adverse events did not differ among the 3 groups, however, patients in 2 L-PEGA group had higher willingness to repeat the same preparation than other groups. The second study[57] evaluated the efficacy safety and efficacy, safety, and acceptability of the oral sulfate solution (OSS) preparation, comparing to 4 L–PEG, in elderly patients. This RCT, enrolling 193 patients, concluded that OSS with a split-dose regimen has greater acceptability and comparable efficacy in bowel cleansing compared to 4 L PEG.

So, despite low evidence, ESGE guidelines[59] suggested the use of PEG solution in elderly patients, and ASGE guidelines[147] recommended to avoid sodium phosphate preparations in these patients.

Congestive heart failure

People with congestive heart failure are at high risk of electrolyte imbalances following the intake of BPs. Indeed, this clinical condition is associated with a decrease in renal blood flow and eGFR. This may lead to acute phosphate nephropathy, due to the reduction in phosphate excretion, or hyponatraemia, linked to hypovolaemia and

high-volume water assumption[124]. Despite the substantial lack of evidence on the efficacy and safety of BPs in this population, high-volume isotonic PEG-based solutions may represent the most adequate option for their reduced risk of causing electrolyte imbalances and fluid shifts[59,124]. Low-volume PEG-based solutions may be an alternative approach due to the reduction in the total volume of liquid intake, although they are currently not recommended in patients with significant congestive cardiac failure (New York Heart Association class III or IV) for the potential harms linked to the osmotically active components included in the formulations[59]. Regardless of the preparation used, strict monitoring is advocated when PEG-based bowel cleansing agents are administered in people with congestive heart failure. On the other hand, low-volume non-PEG based solutions should be avoided in patients with congestive cardiac failure, especially oral sodium phosphate for the risk of causing acute phosphate nephropathy[59,124].

Randomized trials comparing high-volume *vs* low-volume PEG-based preparations in people with congestive heart failure are needed to assess the efficacy and safety of these agents and inform clinical decisions.

Patients with constipation

Constipation is a common gastrointestinal disorder in the community with a global prevalence of 12%–17%[148]. It has been found that constipation exists in 11.9%–17.5% of patients undergoing colonoscopy[137,149] and it is considered one of the risk factors for inadequate BP[137,149-151].

Chen *et al*[152] investigated the efficacy, tolerance, and safety of oral sodium phosphate compared with PEG in patients with chronic constipation and demonstrated that oral sodium phosphate provides better quality BP, despite a smaller amount of intestinal air bubbles than standard 4-L PEG.

Another study of Pereyra *et al*[153] compared the efficacy of different doses of sodium phosphate (NaP) and PEG alone or with bisacodyl for colonic cleansing in constipated and non-constipated patients. In constipated patients the combination of NaP plus bisacodyl presented higher rates of satisfactory colonic cleansing than PEG (95% *vs* 66%; $P = 0.03$).

Although NaP has been shown to be effective in BP of patients with constipation, its use may be causally related to serious organ toxicity (*i.e.* renal damage and permanent renal failure). Therefore, its routine use is not recommended[59].

Despite the low quality of the evidence, additional bowel purgatives are often considered in patients with chronic constipation[59].

An Italian RCT[30] compared bowel cleansing efficacy, tolerability and acceptability of 2 L polyethylene-glycolcitrate-simethicone (PEG-CS) plus 2 d bisacodyl (reinforced regimen) *vs* 4 L PEG in patients with chronic constipation undergoing colonoscopy. There was no statistically significant difference in bowel-cleansing efficacy between the enhanced regimen 2 L PEG-CS plus 2-d bisacodyl and split-dose 4 L PEG in patients with chronic constipation. However, the low-volume PEG preparation containing simethicone showed greater patient acceptability and compliance and was associated with a reduced amount of foam and bubbles over the colonic mucosa.

In a study of Lu *et al*[154] 90 patients with constipation were enrolled and randomly divided into study group (lactulose oral solution and polyethylene glycol electrolyte powder), and control group (polyethylene glycol electrolyte powder only) with 45 patients in each group. Cleansing was significantly better in the study group than in the control group ($P < 0.05$).

One nonrandomized study of Kunz *et al*[155], including 372 patients, compared the effectiveness of high-volume (4 L) PEG solution with low-volume (2 L) PEG solution with ascorbate in constipated and non-constipated adults: no statistically significant difference between the two group was found.

A prospective, randomized, investigator-blinded trial[156] randomized 227 patients with constipation into three groups; enema before purgative use, enema after purgative use, and no enema. The authors found a statistically significant better colon cleansing in the female patients in the enema before purgative group and they concluded that use of enemas before purgatives in patients with constipation significantly improves adequacy of right colon cleansing.

In a multicenter, retrospective cohort study of Yoshida *et al*[157], the efficacy of short duration of polyethylene glycol plus electrolytes (PEG + E Movicol) in improving BP with highly concentrated PEG for colonoscopy in patients with chronic constipation was analyzed. Two or four sachets of PEG + E were prescribed for 1 wk before colonoscopy. They found an improvement rate of BP of 72.6%, regardless of gender, age, and underlying diseases. Also, insertion time and pain score were improved.

Recently, Dang *et al*[158] performed a systematic review of the literature aimed to determine the ideal BP regiment for patients with chronic constipation. Patients receiving NaP had a higher chance of a successful BP than patients receiving PEG (P = 0.003). So, they concluded that, in chronically constipated patients undergoing colonoscopy, the use of NaP may result in superior colonic cleanliness when compared to PEG, however quality of evidence was low. In summary, evidence that would allow recommendation of a special regimen or supplemental treatment for BP in patients with chronic constipation is still lacking. Further studies are needed to establish patient-specific colonoscopy preparation protocols, indeed ESGE does not suggest any specific BP in patients with constipation[59].

Patient with previous bowel resection

Patients with previous bowel resection for neoplasia need to undergo a strictly follow up with colonoscopy to detect anastomotic recurrence and prevent metachronous lesion[159]. A good BP is extremely important in these cohorts of patients. Unfortunately, the history of colorectal surgery is a risk factor for inadequate colon preparation. In fact, the study performed by Lim *et al*[160] is the first one demonstrating that the percentage of inadequate BP is higher in the resection group (gastric or colonic resection) than in the control group. This data was confirmed by Pontone *et al*[161] using the same cleaning agent (4 L PEG).

However, other evidence did not support this data. Indeed, In Yoo *et al*[162] did not found a statistically significant difference in adequate cleansing between patients with colonic resection and control group. So, if the patients with colonic resection are a category of patients "hard to prepare" is still debated.

Moreover, the right bowel cleaning agent is still an open problem for this cohort. In a study by Yoo *et al*[162], the BP was performed using two types of agents (2 L and 4 L). In the resection group, the univariate analysis showed a better bowel cleansing in patients who received 2 L of PEG-Asc (1 L at 8:00 PM the day before the colonoscopy, the second 1 L 5 h before the procedure).

A specifically designed study to assess the better cleaning agent in this cohort of patients was performed by Mussetto *et al*[163]. The authors did not find any difference in adequate BP between patients with prior colorectal resection using low volume *vs* high volume preparation; however, the first preparation was better tolerated. The authors demonstrated as well a greater efficacy of low volume preparation in the right colon. However, this finding needs to be taken with caution because the study was not adequately sized and powered to specifically assess this issue. So, a larger study is needed to investigate the better cleaning agent for these patients.

Recently, a prospective, single-center, randomized controlled, endoscopist-blinded study was performed aiming to compare morning-only 2 L PEG group or a split-dose 4 L PEG in patients with previous colorectal surgery for CRC[164]. Adequate BP rate and patients' satisfaction were higher in the 4 L PEG group than in the other one.

No significant differences were found in PDR, ADR, patient compliance, tolerance, willingness to repeat the preparation or difficulty of the BP process.

Pregnant/lactating patients

Colonoscopy should be performed only if is strongly indicated in pregnant/breast-feeding women. According to ESGE guidelines there are insufficient evidence to determine for or against the use of specific regimens. PEG regimens may be preferred and tap water enemas may be considered for sigmoidoscopy[59].

Limited information is available about the safety of bowel cleansing agents during pregnancy. The systemic absorption of PEG is minimal and abdominal bloating and gas symptoms are infrequent. However, polyethylene glycol solutions have not been studied during pregnancy. Sodium phosphate solutions should be avoided during pregnancy because of it may cause fluid and electrolyte disturbance and may be associated with the risk of phosphate nephropathy. In addition, newborns may have bone demineralization and bone growth failure because of maternal phosphate overload. BP with phosphate enemas before flexible sigmoidoscopy may be safe, but has not been studied in pregnancy; instead, sigmoidoscopy with tap water enemas may be sufficient.

Therefore, flexible sigmoidoscopy with tap water enemas is preferred instead of colonoscopy[165,166]. To our knowledge, no study in the publicly available literature has yet reported the safety profiles of the various BP agents/regimens in lactating women. Interrupting breastfeeding during and after BP with cathartic agents or application of a tap water enema for sigmoidoscopy it would seem the more careful choice[167].

BP AND POST-COLONOSCOPY SYNDROME

Post-colonoscopy syndrome is a condition characterized by persistent abdominal pain, discomfort and bloating after the procedure. In more than 30% of patients, the symptoms affected the normal activity and became persistent for at least 48 h after the procedure[168].

It is more common in females and when the procedural time is long, and may be predicted by conscious sedation and irritable bowel syndrome diagnosis[169].

In this regard, it has been speculated that a transient alteration of gut microbiota, induced by bowel cleansing, could partially concur to its pathogenesis.

It is easily hypothesizable that a profound cleansing induced by ingestion of a purgative solution rich in minerals and PEG may induce a change in microbiota composition. Several studies have tried to address this issue.

In a study on ten adult patients receiving a 4 L-PEG solution, after one month a reduction in *Firmicutes* and an increase in *Proteobacteria* was observed; in particular gamma-proteobacteria were 2.5 times more abundant. At family level, an increase of *Enterobacteriaceae* and a suppression of *Lactobacillaceae* was recorded; overall, authors concluded that this profile change was hallmarked by a reduction of beneficial species [170]. In another study conducted on a pediatric population of 31 children receiving sodium picosulphate, magnesium citrate and senna, a lower diversity in microbial communities was observed after preparation, with increased *Faecalibacterium* and decreased *Ruminococcus*, *Escherichia*, *Pseudobutyrivibrio* and *Subdoligranum*[171]. Chen *et al*[172] enrolled twenty male overweight adults undergoing bowel cleansing with water and sodium phosphate and checked microbiota composition 28 d after the procedure. They identified two different microbiota phenotypes at baseline: *Bacteroides* -dominant and *Prevotella*-dominant. In the first group, preparation induced *Bulleida* appearance, while in the second one an increase in *Akkermansia* was noted. Interesting, authors underlined that both *Bulleida* and *Akkermansia* are associated with type 2 diabetes and obesity.

It is a debated topic whether the change in microbiota composition is transient. Mai *et al*[173] have demonstrated that these alterations may persist for several weeks.

On the other hand, a study[174] conducted on 23 healthy adults receiving 2 L-PEG and ascorbate showed a 31-fold reduction of microbiota load. However, within 14 d, normalization of such imbalance was observed. Interestingly, a single dose (instead of split preparation) implied more profound changes with increase of *Proteobacteria* and *Fusobacteria*. Additionally, it was demonstrated that the preparation increased pH, thus lowering species producing short chain fatty acids and reducing mucous layer. Someone has speculated that in this study the reversion to microbiota normality could have been justified by the fact that only young patients have been enrolled, thus prompting the need of studies on a more variegated population[175].

However, some studies did not find radical difference in taxonomic abundance after BP[176]. For example, in a Japanese study[177] on eight young adults receiving sodium picosulphate and sennosides, it was observed only a transient modification with increase of *Streptococcus* that reverted after 14 d. However, in this study, a more evident change in microbiota-derived metabolites was found, with increase in alanine, carnitine, choline and others. Similarly, O'Brien *et al*[178] recruited 15 adults who were given 2 L-PEG plus bisacodyl, and, after 3 mo, only four patients did not return to pre-colonoscopy microbiota state.

Only one study was specifically aimed to evaluate the microbiota composition in post-colonoscopy syndrome. In a South Korean study[179], 24 patients underwent colonoscopy after 2 L-PEG plus ascorbate bowel cleansing with evaluation of microbiota composition. Five out of 24 experienced abdominal pain, discomfort, distension, constipation or diarrhea after the endoscopy. It was found that these patients had a high ratio *Firmicutes*/*Bacteroidetes* compared to those without post-colonoscopy syndrome. Moreover, they exhibited a higher alpha diversity, which progressively improved after the colonoscopy, paralleling the regression of symptoms.

Two RCT studies evaluated the role of probiotic administration after colonoscopy in the resolution of bloating, abdominal pain and altered bowel function post colonoscopy. In the first one probiotic group had a lower number of pain day after colonoscopy performed with air insufflation[180]. The same group did not found significant difference in post-procedural discomfort, bloating nor time to return of normal bowel function between probiotic and placebo groups, after colonoscopies performed with CO_2 insufflation[181].

Therefore, despite the evidences are scarce and worth of investigation in the future, these researches could represent a hint about the involvement of microbiota, BP and insufflation in pathogenesis of minor complications after colonoscopy.

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FRONTIER

Chronic rejection after liver transplantation: Opening the Pandora's box

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Abstract

Chronic rejection (CR) of liver allografts causes damage to intrahepatic vessels and bile ducts and may lead to graft failure after liver transplantation. Although its prevalence has declined steadily with the introduction of potent immunosuppressive therapy, CR still represents an important cause of graft injury, which might be irreversible, leading to graft loss requiring re-transplantation. To date, we still do not fully appreciate the mechanisms underlying this process. In addition to T cell-mediated CR, which was initially the only recognized type of CR, recently a new form of liver allograft CR, antibody-mediated CR, has been identified. This has indeed opened an era of thriving research and renewed interest in the field. Liver biopsy is needed for a definitive diagnosis of CR, but current research is aiming to identify new non-invasive tools for predicting patients at risk for CR after liver transplantation. Moreover, the minimization or withdrawal of immunosuppressive therapy might influence the establishment of subclinical CR-related injury, which should not be disregarded. Therapies for CR may only be effective in the "early" phases, and a tailored management of the immunosuppression regimen is essential for preventing irreversible liver damage. Herein, we provide an overview of the current knowledge and research on CR, focusing on early detection, identification of non-invasive biomarkers, immunosuppressive management, re-transplantation and future perspectives of CR.

Key Words: Liver transplantation; Chronic rejection; Immunosuppression; T cell-mediated rejection; Antibody-mediated rejection; Donor-specific antibody; Re-transplantation; Graft loss; Complications; Outcomes

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Core Tip: Chronic rejection (CR) still represents a cause of graft loss after liver transplantation. Recent advances in understanding the pathways leading to CR, through a T cell-mediated or antibody mediated injury, are opening new strategies for its management. Early detection of CR, tailored immunosuppressive regimen and strict monitoring are essential to prevent graft loss rejection-related requiring re-transplantation. The current perspectives aim to identify non-invasive biomarkers predicting patients at risk for CR in order to prevent irreversible liver damage by adequate immunosuppressive regimen and improving long-term outcomes after liver transplantation.

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INTRODUCTION

Chronic rejection (CR) of a liver allograft is an immunologically mediated insult to the parenchyma, resulting in damage to vessels and bile ducts[1]. The prevalence of CR after liver transplantation (LT) has been declining over the last years, mainly due to the introduction of new potent immunosuppressive (IS) regimens. However, when CR occurs, it may be successfully treated only in the early phases, while irreversible injury may lead to graft loss requiring re-transplantation[1]. Despite CR being a post-transplantation complication that has been recognized for a long time, its pathogenesis, as well as its clinical implications and treatment, are fields that still require full elucidation.

CR is the consequence of the activation of the host's immune system against the liver allograft and can occur through two general mechanisms: (1) T cell-mediated and (2) Antibody-mediated pathways[1,2]. T cell-mediated chronic rejection (TCMCR) is a long-known complication of LT. Antibody-mediated chronic rejection (AMCR) instead has been observed initially for heart, kidney and pancreas transplants. Yet, it was thought to spare liver allografts, because the liver is a relatively immune-privileged organ and is commonly not targeted by antibodies. Today, it is known that AMCR affects liver allografts as well, albeit rarely, and with not-so-clear clinical consequences. Nonetheless, the condition can evolve to graft failure and death. Therefore, it is of paramount importance to progress in the prevention, early diagnosis, and specific management of CR.

IS drugs play a crucial role in influencing the onset of CR. After LT, the "adequate" IS is considered as this which maintains a viable graft and healthy patients, balancing the risk of rejection (maintaining the target drugs' blood trough levels) and the risk of IS-associated side effects. Yet, different strategies of IS regimens are currently adopted depending of a large number of variables (such as recipient's renal function, cardiovascular disease, risk of infections and tumours, recurrence of underling liver disease). Nevertheless, there is not a unique definition for the optimal IS regimen since it should be tailored to each recipient.

In this narrative review, we highlight the current knowledge and hint to future perspectives on CR in LT, which today represents a growing field for the long-term outcomes of LT recipients.

IMMUNE PRIVILEGES OF THE LIVER ALLOGRAFT

The liver allograft is uniquely immunologically privileged organ and can be transplanted successfully despite positive crossmatch, irrespective of HLA matching. LT requires less amount of induction therapy and maintenance IS compared to other organs, and features of CR rates are low even when IS regimens are minimized or absent.

The mechanisms underlying LT tolerance have not been fully elucidated yet, but appear to be related to particular characteristics of the liver micro-environment. Firstly, the liver contains both innate immune cells expressing undetectable levels of major histocompatibility complex antigens and a large population of migratory immune cells, which have immunoregulatory activity inducing its privileged status[1, 2]. Secondly, the liver has evolved to clear high concentrations of bacterial antigens gut-derived coming from the portal circulation, without inducing useless and potentially harmful immune activation. In particular, with high intrahepatic antigen load, T-cells activated in the liver can induce to a dysfunctional response or to a regulatory phenotype. This may be due to high concentrations of interleukin 10 produced by Kupffer cells or due to antigen presentation by sinusoidal endothelial cells or stellate cells, which have poor costimulatory capacity. Therefore, the intravascular compartment of the liver (composed by sinusoidal antigen presenting cells and endothelial cells, Kupffer cells and dendritic cells) is also recognized as an anatomical structure that supports the induction of tolerance.

DEFINITION OF CHRONIC REJECTION

The diagnosis of CR is made on the basis of clinical, laboratory, histologic and radiological criteria (Table 1); among these, the histological recognition of CR is a fundamental step in diagnosis.

The most widely accepted histologic criteria for the diagnosis of CR are those proposed by the Banff Working Group, an international expert panel, which are periodically refined and updated[1,2].

T cell-mediated chronic rejection

The criteria for TCMCR include the presence of three features: Bile duct atrophy/pyknosis affecting the majority of bile ducts, bile duct loss in more than 50% of portal tracts and foam cell obliterative arteriopathy[2]. The latter feature is considered pathognomonic. Yet unfortunately, it is rarely found in needle biopsy specimens, while it has traditionally been observed in lost allografts at re-transplantation or autopsy. Therefore, the diagnosis relies mainly on the detection of bile duct atrophy and bile duct loss. Both of these features, instead, are rather unspecific, often making CR a diagnosis of exclusion, which requires a thorough exclusion of other causes, including arterial stenosis or biliary strictures, drug-mediated injury and cytomegalovirus infection. An important point in the differential diagnosis is the general absence of "ductular reactions" in TCMCR specimens, in contrast to what is common in other biliary diseases. Small arterial branches may also be missing in TCMCR, making the identification of portal tracts difficult, as well as a distinction between bile ducts and ductular reactions. Staining for cytokeratin may be helpful, as well as epithelial membrane antigen, which preferentially stains bile ducts, as opposed to ductules[3].

Pathologists have also developed TCMCR grading criteria, which are particularly useful, as they correlate with the reversibility of the condition and with prognosis[2]. TCMCR is distinguished into early and late stages according to the Banff schema[2], as summarized in Table 2. Histologically, the most important characteristic in the differentiation between early and late CR is the loss of bile ducts, which occurs in less than 50% of portal tracts (with associated degenerative changes in other ducts), early rejection and greater than 50% in late rejection. Other diagnostic criteria are bridging perivenular fibrosis and small arterial loss, which have all been correlated with a high rate of graft failure. The staging distinction of TCMCR is clinically important; early CR is potentially reversible, whereas late-stage CR is generally irreversible.

Antibody-mediated chronic rejection

The existence of AMCR has been elusive and a strict definition has only been attained in recent years (Table 1)[4]. This has been mainly explored through studies on suboptimally immunosuppressed patients, in those undergoing IS weaning and in the paediatric population, as most primary paediatric diseases requiring LT are not immune-mediated and do not recur[5]. The development of AMCR seems to be multifactorial. It has been proposed that a first independent liver insult (*i.e.*, acute rejection and low immunosuppression) may be necessary to precipitate AMCR by inducing up-regulation of class II HLA molecules and consequently rendering the liver vulnerable to donor-specific antibody (DSA)-mediated damage.

Table 1 Histological definition and clinical features of chronic rejection

	T cell-mediated chronic rejection	Antibody-mediated chronic rejection
Histological definition (according to the 2016 Banff Group [1])	Presence of bile duct atrophy/pyknosis affecting the majority of bile ducts; OR Bile duct loss in more than 50% of the portal tracts; OR Foam cell obliterative arteriopathy	At least mild mononuclear portal and/or perivenular inflammation with interface and/or perivenular necroinflammatory activity; AND At least moderate portal/periportal, sinusoidal or perivenular fibrosis; AND Positive C4d staining in at least 10% of the portal tracts; AND Circulating DSAs in serum samples collected within 3 months of biopsy; AND Other causes have reasonably been excluded
Incidence	2%-5%	Unknown
Risk factors	(1) History of T cell-mediated *acute* rejection episodes; (2) Autoimmune aetiology of the primary liver disease; (3) Non-compliance with IS therapy; (4) Cyclosporine-based IS regimens as opposed to tacrolimus-based regimens; (5) Previous re-transplantation for rejection; (6) Donor/recipient gender mismatch; and (7) Donor age greater than 40	(1) Donor-specific antibodies (especially *de novo* anti-HLA class II antigens); (2) Inadequate IS (cyclosporine regimens or low CNI concentrations); (3) MELD score > 15; (4) Young age at transplantation; and (5) Re-transplantation
Clinical implications	15%-20% graft loss	Increased fibrosis and graft failure in an unknown percentage of patients

CNI: Calcineurin inhibitors; DSA: Donor-specific antibody; IS: Immunosuppressive; HLA: Human leukocyte antigen; MELD: Mayo End-Stage Liver Disease.

Table 2 Histological features of early and late chronic T cell-mediated rejection according to the Banff schema[2]

Structure	Early CR	Late CR
Small bile ducts (< 60 μm)	(1) Degenerative changes involving the majority of ducts: Eosinophilic transformation of the cytoplasm; Increased nucleus: Cytoplasm ratio; nuclear hyperchromasia; uneven nuclear spacing; ducts only partially lined by biliary epithelial cells; and (2) Bile duct loss in < 50% of the portal tracts	(1) Degenerative changes in remaining bile ducts; and (2) Loss in > 50% of the portal tracts
Terminal hepatic venules and zone 3 hepatocytes	(1) Intimal/luminal inflammation; (2) Lytic zone 3 necrosis and inflammation; and (3) Mild perivenular fibrosis	(1) Focal obliteration; (2) Variable inflammation; and (3) Severe (bridging) fibrosis
Portal tract hepatic arterioles	Occasional loss involving < 25% of the portal tracts	Loss involving > 25% of the portal tracts
Other	So-called "transition" hepatitis with spotty necrosis of hepatocytes	Sinusoidal foam cell accumulation and marked cholestasis
Large perihilar hepatic artery branches	Intimal inflammation and focal foam cell deposition without luminal compromise	(1) Luminal narrowing by subintimal foam cells; and (2) Fibrointimal proliferation
Large perihilar bile ducts	Inflammation damage and focal foam cell deposition	Mural fibrosis

CR: Chronic rejection.

The difficulty in identifying this condition is due to the lack of specific histologic findings. According to the 2016 Banff conference report, AMCR is histologically defined as inflammation with an interface of necroinflammatory activity, moderate fibrosis and evidence of C4d-positive staining in at least 10% of the portal tracts in a patient with circulating human leukocyte antigen (HLA) DSAs in serum samples collected within 3 mo of biopsy and when other causes have reasonably been excluded [1].

Finally, a combination of the two types of rejection (AMCR and TCMCR) is also possible. Suspicion of combined AMCR should be raised when late TCMCR presents with severe fibrosis and/or is resistant to therapy. In these cases, C4d staining and DSA dosing should be performed.

For the time being, a definitive diagnosis of CR can only be made with histologic confirmation and therefore requires a liver biopsy, which is a fairly invasive procedure. Therefore, identification of non-invasive biomarkers to predict or identify patients with CR is highly desirable and has become the object of investigation. However, at present, no non-invasive tests have been identified for the diagnosis of CR, which hence, still remains a histological diagnosis.

INCIDENCE AND RISK FACTORS FOR CHRONIC REJECTION

Nowadays, CR is relatively uncommon in liver allografts, and its incidence has declined vertiginously in the last decades due to improved IS, especially after the introduction of tacrolimus (TAC)-based IS regimens. Incidence rates of 15%-20% documented in the first LT series have now been minimized to 2%-5% in the adult population after a median of 5 years, while it has been recorded in up to 16% of the paediatric population[6,7]. These figures primarily refer to TCMCR, as the incidence of AMCR today remains unknown. In fact, AMCR develops in a fraction of patients with persistent or *de novo* DSAs, which themselves account for around 15% of recipients.

After LT, the incidence of CR is significantly lower compared to other solid organ transplants, such as heart (25%-60%), combined kidney and pancreas (20%-40%), pancreas alone (30%-70%) and lung (28%-45%) transplantation. Notably, in contrast to different organ transplants, the risk of CR is highest during the first year and seems to decrease thereafter[6].

Risk factors for the development of TCMCR have been extensively studied. The most important and consistently reported risk factor is the number and severity of preceding T cell-mediated acute rejection episodes (Figure 1)[1,2]. Other frequently cited risk factors include autoimmune aetiology of the primary liver disease, non-compliance with IS therapy, cyclosporine-based IS therapy (as opposed to TAC-based regimens), previous re-transplantation for rejection, donor/recipient gender mismatch and donor age greater than 40 years[6,7]. The risk factors themselves seem to be influenced by specific IS regimens, with patients on TAC exhibiting loss of association with most risk factors, with the exception of acute rejection episodes[6]. A newly identified risk factor is donor/recipient mismatch in the minor histocompatibility antigen glutathione S-transferase T1.

DSAs are considered a risk factor for the development of AMCR, as they are a necessary, although insufficient, requirement for the diagnosis of AMCR[8]. Other risk factors for AMCR include possibly inadequate IS therapy [cyclosporine regimens or low concentrations of calcineurin inhibitors (CNIs)], recipient's Mayo End-Stage Liver Disease (MELD) score > 15, young age at transplantation and re-transplantation.

CLINICAL IMPLICATIONS OF CHRONIC REJECTION

TCMCR can develop at any time after LT and no specific time parameter exists, although most cases occur more than 90 d after LT. It can manifest as a spectrum of disease severity, ranging from mild and inconsequential alterations in blood tests to liver failure and death. The most typical presentation is that of a cholestatic-pattern in liver function tests (LFTs), with the preferential elevation of gamma glutamyl-transpeptidase and alkaline phosphatase, in a patient with previous episodes of acute T cell-mediated rejection. At other times, this scenario may develop as a consequence of untreated or therapy-refractory acute rejection. Rarely, it may evolve insidiously with worsening of LFTs in patients without previous acute rejection.

Early TCMCR can also be found in protocol biopsies without clinical or laboratory alterations, which is a rare event in adults, but found in up to 20% of cases of paediatric LT[9,10]. The clinical implication of this last situation is uncertain. Symptoms are rarely present and unspecific, such as fatigue, abdominal pain and fever. Biochemical dysfunction may regress or further evolve into progressive jaundice and other signs heralding liver failure, such as coagulopathy and malnutrition.

Progression or regression of TCMCR can be predicted based on the histological grading and timing of occurrence[2,9]. Early TCMCR can be reversible with prompt adoption of an adequate therapy. On the other hand, late TCMCR is associated with a much higher chance of evolution into liver failure. Deterioration may be rapid and allograft failure may typically occur within the first year[6]. TCMCR occurring more than 1 year after LT is usually seen in inadequately immunosuppressed patients, either as a result of non-compliance, intolerable side effects or failed IS weaning attempts. Overall, around 15%-20% of patients with TCMCR will ultimately face graft loss and require re-transplantation.

The clinical implication of AMCR is particularly controversial. Its existence has been debated for a long time, yet in the last years, there is mounting evidence that AMCR can portend poor graft outcomes after LT.

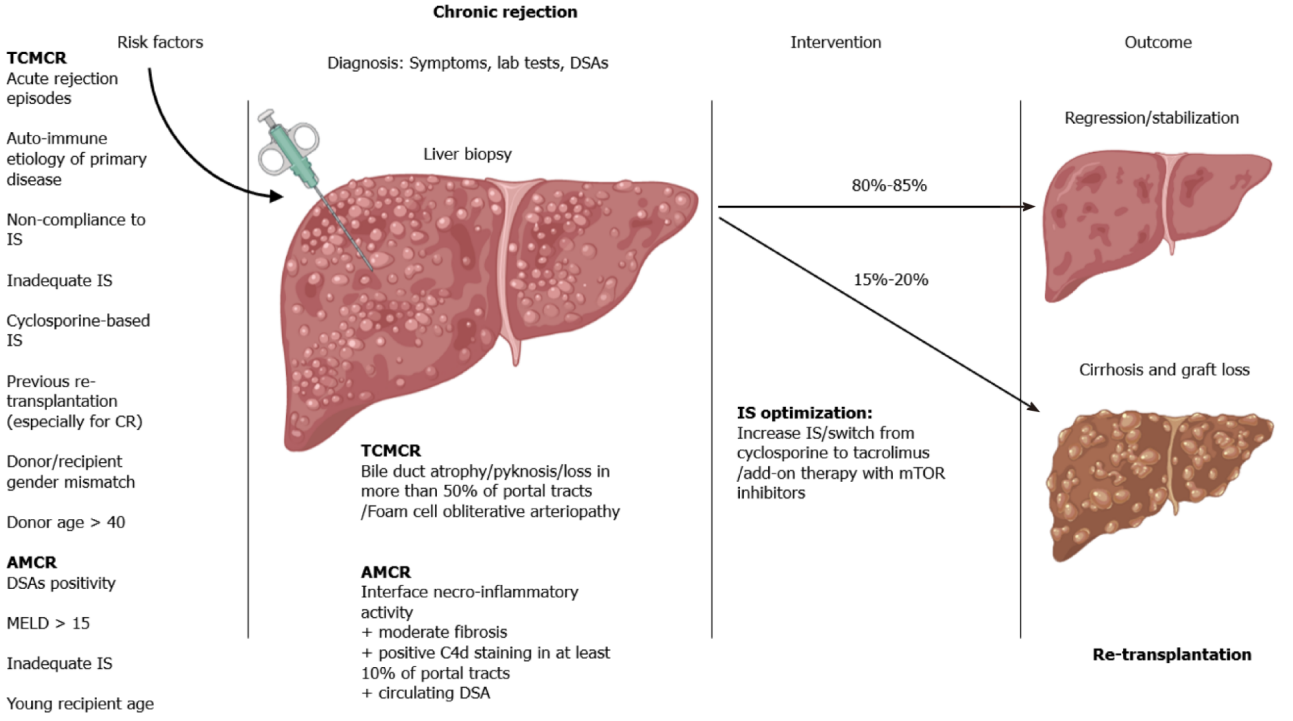

Figure 1 Chronic rejection in a nutshell.

ROLE OF DONOR SPECIFIC ANTIBODIES

DSAs can either be existent before LT (preformed), and may disappear or persist, or they can be generated *de novo* after LT. Generally, preformed DSAs are more closely correlated to acute rejection, while *de novo* DSAs are more tied to AMCR[8,11]. *De novo* DSAs may appear in 0.4%-8% of patients 1 year after LT. O'Leary *et al*[4] showed that the prevalence of *de novo* DSAs was 62% in patients with CR and 38% in patients without CR (P = 0.047). In the same study, the difference was even more significant within the first year after LT, where DSAs were found in 44% and 13% of patients with and without CR, respectively (P = 0.004)[4]. The positivity of DSAs have been associated with higher mortality after LT. On the other hand, recently Feng *et al*[5] published the long-term results of IS withdrawal in 12 paediatric patients and reported the absence of fibrosis or inflammation in protocol biopsies, despite the presence of DSAs in nine recipients.

In this scenario, nowadays, a major goal in the field of transplant immunology is to understand the role of serum DSAs in LT recipients with normal LFTs. Recently, Höfer *et al*[12] correlated DSA testing with liver allograft histological findings in patients with normal graft function, aiming to screen for subclinical rejection/fibrosis in a prospective biopsy-based program. Their results indicated that DSA positivity was associated with greater graft inflammation [odds ratio (OR): 5.4] and fibrosis (OR: 4.2) and to histological criteria that excluded the possibility of the minimization of immunosuppression (in 92%-97% of cases). On the other hand, DSA seronegativity could predict the absence of fibrosis in 87%-99% of cases. Therefore, the authors suggest that DSA testing can help to preselect patients for IS reduction in case of DSA negativity, while DSA positivity should prompt further studies using elastography or liver biopsy for the assessment of subclinical graft injury. In the same German study, another non-invasive biomarker, namely cytokeratin-18 cell death marker (M65), was investigated for liver damage; however, it had no diagnostic value for the detection of subclinical liver graft injury. Interestingly, studies have suggested that DSA seropositivity can also accelerate the progression of fibrosis in patients with hepatitis C virus (HCV) recurrence[13].

These data indicate that serum DSA monitoring in post-liver transplant follow-up may be highly helpful, especially in patients in whom IS minimization is attempted, as in those with long-term graft dysfunction.

In 2017, the Consensus on Managing Modifiable Risk in Transplantation (COMMIT) Group, an expert panel of transplant clinicians who provide practical recommendations for the identification and management of modifiable risk factors to maximize

graft and patient survival after transplantation, recommended testing for serum DSAs, when available, in all patients with a well-functioning graft at 1, 5 and 10 years after transplantation. According to the COMMIT Group, DSA positivity should warrant protocol graft biopsies or, at least, the use of non-invasive testing for fibrosis[14]. However, these recommendations are based on a low level of evidence from the currently available literature. Moreover, despite there being growing evidence for the possible benefit of measuring serum DSAs in LT, routine screening for DSAs is not yet available in all transplant centres.

All these data suggest that DSA testing should be implemented both in adult and paediatric LT recipients during long-term follow-up to better understand their role in the post-transplant liver injury process.

IMMUNOSUPPRESSION AND CHRONIC REJECTION

Judicious management of immunosuppression is crucial for the long-term outcomes and quality of life of LT recipients. The perfect management of IS regimens rests on a thin line, with rejection-mediated graft damage on one side and drug-related side effects on the other. The adverse effects of immunosuppression should never be underestimated and may include the development of kidney injury, metabolic syndrome, neurological complications, malignancies, cytomegalovirus and other opportunistic infections. Furthermore, the aetiology of primary liver dysfunction should always be kept in mind as immunosuppression may have impact on its recurrence. Recurrence of hepatocellular carcinoma (HCC) or unresolved HCV infection are favoured by immunosuppression, while autoimmune aetiologies, such as autoimmune hepatitis, primary sclerosing cholangitis or primary biliary cirrhosis, often warrant an increase of IS therapy. Therefore, it is clear that IS management is highly complex and must be tailored to the particular patient, taking into consideration a great number of variables.

Generally, after solid organ transplantation, the IS regimen is manipulated mainly for drug-related toxicity or for oncologic prevention (*de novo* or recurrent tumours) and different strategies can be adopted, such as: (1) Switch to another IS regimen; (2) IS minimization; or (3) IS withdrawal. However, all these actions may affect the development of CR, a possibility which should not be disregarded. In the following paragraphs, we will analyse how IS management might influence the establishment of CR-related injury.

Standard immunosuppressive regimens

TAC-based IS therapy is currently the most commonly used regimen after LT, as there is evidence that TAC decreases the chance of graft failure. Moreover, there is consistent proof in the literature that TAC-based IS regimens also decrease the risk of CR[6].

To prevent graft rejection, initial TAC trough levels should be kept between 6 and 10 ng/mL. A large meta-analysis has demonstrated that levels < 10 ng/mL guarantee no increase in rejection episodes, while halving the incidence of nephrotoxicity, compared to levels > 10 ng/mL. The first month after transplantation, the TAC dosage may be decreased with a target of 4-8 ng/mL. Thus, while initial TAC target levels are largely endorsed in the literature, there is no consensus on the optimal level in the long term. The COMMIT Group states that "lower" TAC levels may be acceptable in combination therapy, when TAC-related toxicity is an issue[14]. Also, the International Liver Transplant Society (ILTS) suggests that, from year 1 after LT onward, TAC trough levels can be dropped to 3 ng/mL; furthermore, after the fifth year, keeping drug levels "above detectable" is fine as long as graft function is optimal[15].

TAC is often initially used with other IS drugs, the most common of which are corticosteroids and mycophenolate mofetil (MMF). During the first 90 d after LT, steroids should be gradually tapered, leading to suspension after the third month. When aiming for TAC monotherapy, higher trough levels should be maintained, while dual therapy permits lower levels to be acceptable. Indeed, in a retrospective study aiming to assess CR in LT recipients receiving low-dose CNI monotherapy, TAC levels < 5 ng/mL were not associated with silent CR on protocol liver biopsies[16].

When TAC needs to be reduced or suspended due to adverse events, the guidelines recommend adjunct or substitution with MMF or other IS agents, such as mammalian target of rapamycin (mTOR) inhibitors (everolimus and sirolimus), to keep a low threshold for suspicion of rejection[15].

Nephrotoxicity is the major side effect of TAC requiring dose adjustments of the drug. When nephrotoxicity is anticipated to be an issue before transplantation, induction with basiliximab plus MMF and delayed TAC introduction may be used to decrease renal impairment in the first weeks, as demonstrated by the DIAMOND study[17]. In this multicentre prospective randomized trial, delayed initiation of high-dose TAC significantly reduced renal function impairment compared with immediate post-transplant administration, while the risk of rejection was similar[17].

For later appearing nephrotoxicity, renal-sparing regimens are often used, with most being common alternative agents to CNIs. Several studies have shown that switching from TAC to mTOR inhibitor-based regimens, such as everolimus monotherapy, significantly improved renal function after LT. Everolimus significantly increases the estimated glomerular filtration rate (amounting to 5.6 mL/min), with a low incidence of histologically proven rejection (5%) and side effects[18]. In contrast, for sirolimus, a randomized trial comparing *ab initio* standard TAC with low-dose TAC plus sirolimus found an excess of graft loss mortality in the combination therapy arm, which was due to hepatic artery/portal vein thrombosis or sepsis.

TAC-based IS regimens are usually also later replaced with mTOR inhibitor regimens in patients transplanted for HCC. In fact, TAC trough levels above 10 ng/mL during the first 30 d post-LT seem to increase the rate of HCC recurrence; hence, mTOR inhibitors, which have an anti-proliferative effect, may offer protection from tumour recurrence, maintaining the same risk of post-transplant graft rejection.

Immunosuppressive minimization

As rejection is considered a relatively uncommon cause of graft failure after LT, current guidelines suggest to employ IS minimization strategies in an effort to limit the morbidity associated with excessive immunosuppression, which represents the main cause of late mortality after LT[14]. In fact, IS minimization has been recognized to be feasible and is nowadays incorporated in existing recommendations[14,19]. However, IS minimization protocols need to be rigorous in order to avoid the risk of favouring acute and chronic rejection[19].

First, IS minimization strategies should be entertained after the third post-transplantation month, in patients with stable laboratory exams for a minimum of 4 wk and under tight control. Liver allograft biopsy is not required prior to IS minimization, but any sign of rejection, including rising LFTs, should prompt returning to previous IS doses and performing an eventual biopsy, even in the setting of a clinically-well patient. As clinical concordance in whether a patient needs biopsy is quite low among transplant clinicians, a model to predict acute rejection on biopsy has been proposed[20]; yet, no specific model for anticipating CR has been developed yet.

However, in LT recipients under an IS minimization regimen, insufficient TAC serum concentrations have been linked with AMCR. In a retrospective cohort of 749 adult LT recipients, Kaneku *et al*[21] reported that *de novo* serum DSA formation (incidence rate: 8%) was favoured by low CNI levels (TAC < 3 ng/mL or cyclosporine < 75 ng/mL) and by the use of cyclosporine in place of TAC, while it was inversely related to age > 60 years old and a MELD score > 15[21]. Patients with *de novo* DSAs were found at significantly higher risk for graft loss and death. Hence, serum DSA monitoring in patients who are candidates for IS minimization may be useful. Accordingly, the ILTS guidelines encourage clinicians to screen for serum DSAs before minimization attempts and to consider biopsy if strong DSA positivity is detected[15].

Additionally, an association between variability in TAC levels and late CR has been implied, especially in paediatric LT recipients. The largest of these studies included a heterogeneous population (n = 144) of children (age: 8-18 years) transplanted with different solid organ grafts (liver, kidney, heart or lung), where high variability in TAC blood levels increased the risk of late CR and graft loss, hampering the generalization of results to adult liver grafts[22]. Therefore, until new evidence emerges, excess variability in TAC levels should probably be best avoided.

Immunosuppressive withdrawal

Nonetheless, many studies have gone further and investigated the possibility of IS withdrawal, a unique chance offered to LT recipients[23]. The aim of IS withdrawal is to avoid long-term IS-related side effects, which influence patient/graft survival, as well as the quality of life of LT recipients. Overall, among IS withdrawal trials, CR had a very low incidence, accounting for 0%-3%[24].

Initial pioneering studies demonstrated the feasibility and safety of IS discontinuation after LT, but they lacked sample size assessments, clear withdrawal protocols, histopathologic surveillance and long-term follow up[25]. In these studies, acute rejection episodes could generally be managed successfully, and only three cases

of CR were reported, two of which culminated in graft loss and re-transplantation.

A prospective trial in 102 patients found that 42 (41.17%) LT recipients could be weaned off completely and did not suffer rejection, as assessed by protocol liver biopsies[22]; moreover, the time elapsed from transplantation was the key variable influencing the success of IS weaning, with high chances 10 years from transplantation, while success was rare when 3-5 years from LT had elapsed and was achieved only in patients > 50 years of age.

Recently, Jucaud *et al*[26] described the prevalence and impact of *de novo* DSAs during a multicentre IS withdrawal trial in adult LT recipients. Patients who were IS-free had a high (66.7%) prevalence of *de novo* DSAs, in particular for HLA-I. Yet, these *de novo* DSAs did not seem to be deleterious to the graft. However, their significance in the long term is still unknown, as well as their possible association with CR in patients who achieved a successful and sustained suspension of IS therapy.

Nowadays, around 20% of LT recipients can ultimately be weaned off IS therapy completely[24]. Yet, IS withdrawal is not a common practice and should only be attempted in the controlled setting of a clinical trial. There is accumulating evidence that under-immunosuppression may lead to *de novo* DSA formation, and in turn, DSA formation may lead to AMCR or mixed CR and eventually to graft loss[10,24]. Therefore, further IS weaning studies should include protocol biopsies and DSA testing[24].

TREATMENT OF CHRONIC REJECTION

Immunosuppressive management

The treatment of LT recipients who have developed CR is difficult and not entirely defined, as in general, there is still a low level of evidence. Thus, most recommendations are only based on expert opinions[14,15].

Generally speaking, patients with TCMCR may benefit from increased IS blood levels. Nonetheless, the increase in dosage should be gradual. High variability in TAC concentrations should be avoided, especially when graft function is impaired, as this may increase mortality through over-IS-related events[14]. Therefore, TAC dosage modifications should be carried out progressively and with special caution in patients with liver dysfunction[14].

Despite there being limited evidence to support a specific IS regimen for CR, patients taking a cyclosporine-based IS regimen should be switched to a TAC-based one[6]. The conversion from cyclosporine to TAC is associated to a 70% response rate and graft survival; moreover, an "early" conversion to TAC, before bilirubin levels exceed 10 mg/dL, seems to be fundamental to achieve good outcomes[6].

In a recent report, 23 patients with biopsy-proven CR were treated with a "rescue therapy" consisting of addition of an mTOR inhibitor (either everolimus or sirolimus) to the baseline IS regimen[27]. In 12 patients (52%), this strategy was successful and reversed CR, while the other patients had a poor outcome and most of them required re-transplantation. Although evidence is scarce and further data are needed, these preliminary data suggest that mTOR inhibitor add-on therapy may be valuable in treating TCMCR, and its potential benefits should be further explored in this setting.

While acute antibody-mediated rejection has different potential treatment strategies, most of which are transposed from the kidney transplantation experience, none of these is indicated for AMCR. So far, the treatment of AMCR remains an unmet clinical need, with no study to date on the subject, to the best of our knowledge.

In case of a strong positivity for serum DSAs is detected (MFI > 5000), irrespective of the class of anti-HLA antibodies, and the histological pattern is consistent with AMCR, the COMMIT Group suggested to reinforce the baseline IS regimen by increasing the CNI trough level (if tolerated) or by the introduction of MMF or other agents in patients receiving CNI monotherapy[14]. Moreover, if the histological pattern is suggestive of *de novo* autoimmune hepatitis with positive DSAs, the addition of corticosteroids is recommended. In all cases, strict follow-up and evaluation of therapeutic changes need to be based on repeated liver biopsies and DSA/MFI monitoring. Yet, with the new recent international definition of AMCR and the evidence that its incidence is expanding, a new field of study has been opened, and it is likely that the treatment of AMCR will be the object of many investigations in the near future.

In summary, despite the lack of evidence based on randomized trials, so far literature data suggest that when CR is diagnosed early (before the development of significant ductopenia, perivenular fibrosis and obliterative arteriopathy), CR is

potentially reversible by increasing or changing the IS regimen.

Improved histology and resolution of liver function abnormalities can be seen in successfully treated patients. In contrast, late CR is usually unresponsive to increased IS therapy and generally requires re-transplantation.

Re-transplantation

When CR is severe enough to compromise graft function, the chances of regression are quite low and most patients will ultimately face graft loss. In such cases of advanced CR, the only chance for survival is therefore re-transplantation.

In the early series, CR represented the most frequent indication for re-transplantation. Yet, this rate has significantly decreased after the introduction of CNIs. In a recent multicentre study analysing data from the European Liver Transplant Registry, including LT performed from 1988 to 2016, CR was found to be the cause of around 16% of all re-transplants[28]. Furthermore, according to the Clinical Practice Guidelines in Liver Transplantation of the European Association for the Study of the Liver, the rate of graft loss due to ductopenic CR has significantly decreased in recent years to less than 2%[29].

On the other hand, re-transplantation is a technically challenging procedure with significantly worse outcomes than primary LT. The technical difficulty mainly lies in a more complex dissection, with a large percentage of patients necessitating vascular and biliary reconstructions[30-32]. In analyses of the UNOS database, the 1-year risk of graft failure was 37.8% and patient survival at 1, 3 and 5 years was 67%, 60% and 53%, respectively, which is far below the corresponding rates for primary LT[30]. In fact, to avoid futility of liver re-transplantation, many studies have proposed stringent predictive scores be used to select appropriate candidates[33].

Among patients candidate for liver re-transplantation, most deaths are due to sepsis and are directly related to the MELD score, time from the primary LT (liver re-transplantation within 1 year from the first LT has the highest mortality), and warm ischaemic time. Other studies have found a correlation with donor age above 60, recipient age above 50, bilirubin levels and intraoperative transfusions[34]. Moreover, MELD scores higher than 30 are associated with prohibitive mortality risks (42% at 1 year and 21% at 5 years)[33-34]. However, no increased postoperative mortality has been reported in liver re-transplantation for CR, as opposed to other indications[33]. IS in re-transplantation for CR remains an issue as to date there is no data to guide choice of therapeutic regimens. Induction therapy with antibodies such as basiliximab is reasonable yet controversial. In particular, some studies suggest lower rates of acute rejection episodes while others do not confirm such results[35-37].

FUTURE PERSPECTIVES

The future of CR prevention, diagnosis and management holds promise. The recent recognition of AMCR in liver allografts has opened a whole new area of research. One of the main objectives would be to recognize patients at high risk for rejection. Many studies are currently aiming to identify useful biomarkers for rejection. Yet, translation of the results into clinical practice is still lagging behind. Despite new promising biomarkers, such as intracellular IFN-γ and IL-2, being proposed to select patients at higher risk for acute rejection, no biomarker has yet been recognized to identify CR [38].

Another area of interest is the definition of non-invasive methodologies for the diagnosis of CR. Lutz *et al*[39] have shown how liver ultrasound, with calculation of the right hepatic vein resistance index, and transient elastography have a high correlation with graft fibrosis. Unfortunately, although fibrosis is indeed a component of CR, it is not specific; however, detection of significant fibrosis may represent a valuable tool to select asymptomatic patients to be biopsied.

Another potential benefit of identifying non-invasive biomarkers for CR would be to drive a tailored IS regimen according to the individual patients risk to develop CR [38]. For example, they could predict a response or adverse effects of diverse drugs in the individual patient. Nowadays, as a similar experience is growing in kidney transplant recipients in this field, we foresee that it will also occur for LT recipients.

Finally, rejection prevention would be the ultimate goal. Schlegel *et al*[40] have demonstrated in a mouse model that graft preconditioning with hypothermic oxygenated perfusion can induce immune tolerance and prevent graft failure, even in the absence of IS therapy. Their results suggest that one day operational tolerance could be "induced" rather than depend strictly on the so far unpredictable graft-host

interaction, reducing or abolishing the risk of CR.

CONCLUSION

CR of liver allografts has become a relatively rare entity due to stronger IS regimens, but it still represents a diagnostic and management challenge to the transplant clinician. In the last decade, the definition of antibody-mediated rejection has opened the way for an entire new research field in the study of CR, effectively unleashing a Pandora's box. The next few years hold promise to improve CR management by the identification of non-invasive biomarkers predicting patients at risk for CR, which could hopefully be prevented by the use of tailored IS regimens, thus no longer becoming an issue after LT.

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OPINION REVIEW

Humans have intestinal bacteria that degrade the plant cell walls in herbivores

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Abstract

The cell walls of plants are mainly made of cellulose and contain a large number of calories. However, the main component, cellulose, is an indigestible plant fiber that is thought to be difficult for humans to use as energy. Herbivores acquire energy through the degradation of cell wall-derived dietary fiber by microorganisms in the digestive tract. Herbivores, especially horses, have a highly developed cecum and large intestine, and plants are fermented for their efficient use with the help of microorganisms. Humans also have an intestinal tract with a wide lumen on the proximal side of the large intestine, in which fermentation occurs. The digestive process of horses is similar to that of humans, and many of the intestinal bacteria found in horses that degrade plants are also found in humans. Therefore, it is thought that humans also obtain a certain amount of energy from cell wall-derived dietary fiber. However, the intake of dietary fiber by modern humans is low; thus, the amount of calories derived from indigestible plant fiber is considered to be very low. Cellulose in the plant cell wall is often accompanied by hemicellulose, pectin, lignin, suberin, and other materials. These materials are hard to degrade, and cellulose is therefore difficult for animals to utilize. If the cell wall can be degraded to some extent by cooking, it is thought that humans can obtain calories from cell wall-derived dietary fiber. If humans can use the calories from the cell wall for their diet, it may compensate for human food shortages.

Key Words: Intestinal flora; Human; Herbivore; Indigestible plant fiber; Cell wall; Calorie

Core Tip: The plant cell wall is mainly composed of cellulose and contains a high number of calories. However, it is classified as an indigestible dietary fiber, and its

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energy utilization is difficult. Many of the intestinal bacteria found in herbivorous horses that degrade plants are found in humans. Therefore, it is thought that humans can also utilize plant cell walls for energy to some extent. If cell wall-derived dietary fiber can be cooked to make it easier for humans to use, it may compensate for human food shortages.

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INTRODUCTION

Cell walls support the structure of plants and are mainly composed of cellulose. Cellulose contains a large amount of potential energy, although only the method of binding glucose molecules makes it different from starch. Therefore, it is beneficial for organisms to use cell walls as energy. However, with the exception of a few multicellular organisms, such as termites, the main organisms that are capable of digesting the cell wall with their own cellulases are microorganisms, and most higher organisms cannot digest the cell wall without the assistance of microorganisms.

Some bacterial groups have enzymes that degrade cell wall components through fermentation, and many herbivores use this fermentation ability in the digestive tract to obtain energy from plant cell walls. Plant digestion is divided into various methods according to the evolution of animals. Many herbivores have a large fermenting organ in their digestive tract, but there are also herbivores, such as giant pandas that eat bamboo as their staple food, that have a digestive tract similar to that of carnivorous animals[1]. Therefore, herbivores do not necessarily need fermenting organs.

Humans are classified as omnivorous animals and eat plants. Plants consumed by humans are utilized *via* the decomposition and absorption of easily available plant cell contents. Most components of the cell wall are classified as indigestible insoluble dietary fibers and are thought to contribute little to the energy of humans. However, in the human intestinal flora, there are many bacterial species that degrade the cell wall that are also found in the gastrointestinal flora of herbivorous animals. Therefore, intestinal bacteria can ferment cell wall components that are classified as insoluble dietary fibers. It is thought that the components degraded by bacteria can be utilized by humans.

The utilization of energy derived from cell walls can improve the increasing eating habits of humans. In this paper, we examine the similarities in intestinal bacteria between humans and herbivores and summarize the findings on the utilization of energy derived from plant cell walls by humans.

CELL WALL DIGESTION

Plant cell walls consist of two layers. The outer layer is formed first and is called the primary cell wall. The primary cell wall is mainly composed of cellulose. Cellulose has a strong molecular bond with glucose, and crystalline cellulose is very hard and wiry. Hemicellulose and pectin are bound to hard wire-like cellulose. Therefore, cellulose is considered to be difficult to degrade. In the secondary cell wall formed at the site where plant growth has stopped, lignin binds to cellulose to increase its strength. Lignin is a polymer that can have completely different structures depending on the plant species and the growing environment. This lignin binds to cellulose and interferes with cellulase action[2]. Furthermore, it is very difficult to degrade cell walls with attached waxy suberin. Cork is rich in suberin and lignin; however, the fact that cork retains its morphology for a long period of time suggests that it is difficult to degrade, even by bacteria.

Moreover, the cell wall is made of hard cellulose solidified with other molecules to further increase its strength. For this reason, a large amount of effort is required to degrade the cell wall. Thus, most organisms do not degrade the cell wall themselves and instead utilize a method to mainly consume cell contents that are easily absorbed.

For example, although butterfly or moth larvae actively eat the leaves of plants, there are few bacteria in their gastrointestinal tracts. They chew the leaves and absorb the cytoplasm in them, and the cell walls are excreted from the body. In other words, except for a few kinds of insects, such as termites, it is difficult even for insects to utilize the cell wall.

If organisms consume a large number of plants, then enough of the contents of the cells can be ingested. However, plant abundance is not always sufficient. Large amounts of plants are required, especially for large herbivorous organisms. Herbivores are animals that use bacteria to degrade plant cell walls to efficiently ingest and digest plants. If the cell wall can be converted into energy, efficient energy intake can be achieved with fewer plants. Herbivores have bacteria in their gastrointestinal tracts that can degrade cell walls. There are several ways in which bacteria degrade the cell wall and absorb cell wall components.

DIGESTION OF PLANTS BY HERBIVORES

Herbivorous mammals can be broadly divided into three types according to digestion. Type 1 herbivores have a large fermenting organ in front of the stomach, and this type includes cows and goats. Type 2 herbivores have a large intestine and cecum with a large volume in which fermentation occurs, and this type includes rabbits and horses. This group is further divided into two subtypes: a subtype that conducts coprophagia, such as rabbits (2a), and a subtype that does not conduct coprophagia, such as horses (2b). Type 3 herbivores, such as giant pandas, have a digestive tract that is not much different from that of carnivorous animals. This type of herbivore does not have a large fermenting organ. The features of each digestion type are briefly described below.

Type 1 has a large fermenting organ in front of the stomach

Cows are known to have four stomachs. The rumen (1st stomach) is a very large organ where bacteria ferment food. It produces alcohols and volatile fatty acids, such as acetic acid, propionic acid and butyric acid, which are then absorbed. Fermentative bacteria increase exponentially at this site. The reticulum (2nd stomach) is located at the base of the esophagus, near the entrance to the rumen, and is a ruminant motor organ that returns poorly chewed food to the mouth. The ruminant returns the food to the mouth where it is chewed again before being sent to the rumen. Well-degraded food passes through the reticulum and is sent to the omasum (3rd stomach). The omasum is located on the anal side of the reticulum and has a file-like mucosa that provides the final mechanical crushing of food. The first to third stomachs are thought to be derived from the esophagus. The final stomach, the abomasum (4th stomach), produces gastric acid. Here, the food is chemically degraded, and the increased bacterial cells are destroyed by gastric acid. Then, in the small intestine, both the gastric residue and the degraded bacterial cell components are digested and absorbed.

Type 2 uses the cecum and the large intestine as large fermenting organs

Type 2a herbivores conduct coprophagia: In rabbits, food that is consumed orally passes through the stomach and into the small intestine, similar to the process in humans. First, the stomach absorbs nutrients that can be digested and absorbed. Indigestible polysaccharides, mainly cell walls, are degraded and fermented by bacteria in the dilated cecum and the oral side of the large intestine. The absorbable nutrients such as short-chain fatty acids (SCFAs), mainly volatile fatty acids, are absorbed instantly. Nutrients that are difficult to absorb by the large intestine, such as vitamins synthesized by intestinal bacteria, pass through the stomach again by coprophagia and are absorbed by the small intestine.

Type 2b herbivores do not conduct coprophagia: In horses, food that is consumed orally passes through the stomach and into the small intestine, similar to the process in rabbits. First, the stomach absorbs nutrients that can be digested and absorbed. Indigestible substances are degraded and fermented by bacteria in the cecum and oral side of the dilated large intestine to absorb nutrients. However, since coprophagia is not normally conducted, the efficiency of decomposition and absorption of nutrients is considered lower than that of rabbits and other animals engaging in coprophagia. This type of gastrointestinal tract is long, but it is similar to that in humans. Since the analysis of horse intestinal flora has been sufficiently advanced, we compare horse and human intestinal bacteria in the subsequent section.

Type 3 does not have a large fermenting organ

Giant pandas are herbivores that eat bamboo as their staple food; however, their digestive tract is said to be similar to that of carnivores. The intestinal bacteria found in giant pandas have been reported to be distinct[3].

THE INTESTINAL BACTERIA OF HORSES COMPARED TO THOSE OF HUMANS

Humans do not have a large cecum like horses, although the ascending colon to transverse colon is wide, and fermentation by intestinal bacteria occurs in this area. In humans, bacteria that are ingested orally are killed by gastric acid (pH: 1.5-2.0), which is more acidic than that in horses (pH: 4.4)[4]. Thus, the number of intestinal bacteria is very small on the oral side of the small intestine. However, intestinal bacteria increase rapidly from the proximal end to the distal end of the small intestinal tract. Moreover, gut bacteria progress from aerobic bacteria to anaerobic bacteria with increasing distance in the small intestine. By the ileocecal valve, the bacterial count increases to 10^7-10^9/mL and eventually increases to approximately 10^{10}-10^{12}/mL in the colon[5]. It is estimated that this flora contains 500-1000 different species of bacteria[6], with nearly 80% belonging to the two bacterial phyla *Bacteroidetes* and *Firmicutes*[7].

Table 1 shows a modified report of the horse gut microbiota compiled by Kauter *et al*[8]. This table summarizes whether microorganisms found in the intestinal tract of horses are also found in humans. The gram-negative bacilli *Bacteroides thetaiotaomicron* and *B. ovatus*, which metabolize complex dietary polysaccharides, have been found in the human intestine and have been reported to be involved in the degradation of plant cell walls[9]. The genus *Bacteroides* has been reported to increase in abundance in the large intestines of horses that consume increased proportions of grass[10]. *Bacteroides* is also a cell wall-degrading bacterium that accounts for a large proportion of the intestinal flora in both humans and horses[7]. In addition, some bacterial species reported in humans have been identified as cell wall-degrading bacteria. Therefore, these bacteria were added to create a table. From this table, it can be seen that many species of horse bacteria are also present in the human colonic flora. In other words, the intestinal bacteria of humans can degrade plant cell walls and synthesize SCFAs, including volatile fatty acids. These SCFAs can be absorbed by the large intestine[11]. Horses can obtain energy from this system. This raises the question of how much energy humans obtain from the cell wall.

CALORIES DERIVED FROM THE CELL WALL IN HUMANS

In general, a reference for the current caloric contribution of food was determined by S ánchez-Peña *et al*[12]. This reference includes the inputs of carbohydrates 4 kcal/g, lipids 7 kcal/g, and proteins 4 kcal/g, and the input of alcohols 7 kcal/g was recently added. In recent years, it has been recommended to display these values in joules, *i.e.*, carbohydrates 17 kJ/g, lipids 37 kJ/g, proteins 17 kJ/g, and alcohols 29 kJ/g. One calorie is approximately 4.186 J, and there is a slight difference between the cal display and the J display. The standards are set by the national institutions of each country. There are differences between countries regarding items, but there are no differences in the basic amount of heat. The Food and Agriculture Organization has proposed adding dietary fiber to the calorie calculation, and 2 kcal/g and 8 kJ/g have been adopted in countries that add dietary fiber calories to the calculation[13]. Dietary fiber has fewer calories than carbohydrates because watery dietary fiber is calculated as calories. However, insoluble dietary fiber, which is the main component of plant cell walls, is not used in calorie calculations.

We further considered how much dietary fiber actually becomes calories. We examined the extent to which dietary fiber becomes calories. Dietary fiber that is fermented is generally considered to be soluble dietary fiber. However, since bacteria that degrade insoluble dietary fiber, such as cellulose, also exist in humans, as described above, insoluble dietary fiber is slightly degraded by fermentation in the intestinal tract of humans. The caloric content of cellulose was reported to be 4.16 kcal/g[14]. However, it is estimated that there will be large individual differences in the acquisition of calories based on not only whether the cellulose is raw or cooked but also the influence of cell wall strength, the intestinal flora and intestinal residence time. Elia and Cummings[15] suggested that 70% of dietary fiber is fermented. A total of

Table 1 Enterobacteria that break down fibers derived from cell walls

Family	Genus		Species	Putative effects	Horse		Human	
						Ref.		Ref.
Acidaminococcaceae	*Phascolarctobacterium*	spp.		Fiber fermenters	+	[21]	+	[22]
Bacteroidaceae	*Bacteroides*	spp.		Plant wall degradation	+	[7]	+	[7]
			ovatus	Polysaccharide decomposition; Plant wall degradation	N/A		+	[9]
			thetaiotaomicron	Complex polysaccharide decomposition	N/A		+	[9]
Clostridiaceae	*Clostridium*	spp.		Cellulolytic, fibrolytic	+	[8]	+	[23]
Eubacteriaceae	*Eubacterium*	spp.		Cellulolytic, fibrolytic	+	[8]	+	[23]
Fibrobacteraceae	*Fibrobacter*	spp.		Plant wall degradation	+	[7]	N/A	
			intestinalis	Plant wall degradation	+	[24]	N/A	
Lachnospiraceae	*Butyrivibrio*	spp.		Cellulolytic, fibrolytic	+	[8]	+	[25]
	Blautia	spp.		Fiber fermenters	+	[26]	+	[27]
Prevotellaceae	*Prevotella*	spp.		Fiber fermenters	+	[26]	+	
			copri	Fiber fermenters	N/A		+	[28]
Ruminococcaceae	*Ruminococcus*	spp.		Cellulolytic, fibrolytic bacteria	+	[8]	+	
			albus	Plant wall degradation	+	[8]	+	[29]
			bromii	Plant wall degradation	N/A		+	[30]
			champanellensis sp.nov.	Plant wall degradation	N/A		+	[31]
			flavefaciens	Plant wall degradation	+	[32]	+	[33]
			sp.nov.	Plant wall degradation	N/A		+	[34]

Based on the intestinal flora of horses compiled by Kauter *et al*[8], we selected microorganisms that decompose cell walls and summarized whether they exist in the human large intestine. Several bacterial species reported in humans as cell wall-degrading bacteria are added to the Table. N/A: Not available.

51.0% of the energy initially present is lost through feces, and 3.5% is lost through H_2 and CH_4 gaseous products. It is assumed that the remaining 45.5% is energy that can be absorbed and metabolized by human tissues[15]. Based on this calculation, the calories of dietary fiber were set to approximately 2 kcal/g and 8 kJ/g.

In the 1980s, when it was thought that cellulose could not be digested by humans, tests were conducted on humans using radioactive isotopes to examine the degradation of cellulose. ^{14}C-cellulose was given to each of 10 healthy subjects, and stool and breath samples were collected for 7 d. A wide range of variations in ^{14}C recovery were recorded, with 57% in feces, 16% in respiration as $^{14}CO_2$. The total recovery rate of ^{14}C was reported to be 73%[16]. Some of the $^{14}CO_2$ recovered by respiration was metabolized by intestinal bacteria and absorbed through the large intestinal mucosa. The remaining 27% did not recover and may have been incorporated into the body. At the time, it was suspected that the study did not use pure cellulose but used cellulose containing starch. However, it has since been proven that cellulose is degraded by gut bacteria. Therefore, this study is considered to provide valuable human data. The wide range of variations in ^{14}C recovery may be related to differences in intestinal bacteria and different lengths of stay in the gastrointestinal tract. This study showed that 27%-43% of cellulose could have been used by humans. As mentioned above, the actual cell wall is made of more than indigestible cellulose alone, and it is estimated that the actual amount utilized by humans is below this value. Therefore, it is understandable that the cell wall, which is classified as water-insoluble dietary fiber, is not added to the caloric calculation.

Ninety to ninety-five percent of the SCFAs produced in the large intestine by the fermentation of dietary fiber, including watery dietary fiber, are absorbed by the large intestine[17]. It has been reported that SCFAs absorbed in the colon contribute 6% to

10% of the total energy requirement of humans and that this contribution is probably increased in humans who consume more fiber[18,19]. Currently, the United States Food and Drug Administration (FDA) has set the recommended daily intake (RDA) of fiber at 38 g/d and 25 g/d for healthy men and women, respectively, aged 19-50 years. However, most Americans are reportedly not consuming the recommended dietary fiber intake. In other words, even if the encouraged fiber intake is met and is completely converted into energy, it amounts to approximately 150-100 kcal. Therefore, the proportion of dietary fiber in the total amount of energy in the current diet is minimal [20].

CONCLUSION

Water-insoluble dietary fiber derived from plant cell walls is considered to be indigestible dietary fiber, and it is difficult to ferment and degrade. However, many species of bacteria present in the intestines of herbivores are also found in the digestive tract of humans. Therefore, it is possible that the dietary fiber derived from plant cell walls is degraded to some extent, even in the digestive tract of humans, and used as energy. Since the amount of dietary fiber in the total food intake is minimal, the calorie intake is minimal. The main reason why it is difficult to degrade dietary fiber derived from plant cell walls is that cellulose, which is difficult to degrade, is in a firmly solidified state integrated with hemicellulose, pectin, lignin, suberin, and other components. If cellulose can be separated from these other components and degraded, it is possible that plant cell walls can contribute more calories in humans than when cellulose is combined with the other cell wall components. The cell wall contains a large amount of potential energy. Thus, if the amount of energy utilized from the cell wall by cell wall-degrading bacteria can be increased, the food situation in food-deficient areas can be expected to improve. Currently, bioethanol is being developed, and its dietary use in the degradation of cell walls is anticipated.

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MINIREVIEWS

Gut microbiome in allogeneic hematopoietic stem cell transplantation and specific changes associated with acute graft *vs* host disease

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Abstract

Allogeneic hematopoietic stem cell transplantation (aHSCT) is a standard validated therapy for patients suffering from malignant and nonmalignant hematological diseases. However, aHSCT procedures are limited by potentially life-threatening complications, and one of the most serious complications is acute graft-versus-host disease (GVHD). During the last decades, DNA sequencing technologies were used to investigate relationship between composition or function of the gut microbiome and disease states. Even if it remains unclear whether these microbiome alterations are causative or secondary to the presence of the disease, they may be useful for diagnosis, prevention and therapy in aHSCT recipients. Here, we summarized the most recent findings of the association between human gut microbiome changes and acute GVHD in patients receiving aHSCT.

Key Words: Gut microbiome; DNA sequencing technologies; Allogeneic hematopoietic stem cell transplantation; Transplants; Acute graft *vs* host disease; Biomarkers; Composition; Function

Core Tip: This review reports the compositional and functional changes in gut microbiome of allogeneic hematopoietic stem cell transplantation recipients associated with acute graft-versus-host disease that could serve a biomarker for diagnosis and

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prevention in patients receiving allogeneic hematopoietic stem cell transplantation.

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INTRODUCTION

In human health, DNA sequencing technologies, including 16S rRNA gene-based amplicon sequence analysis and whole genome shotgun metagenomic analysis were used to discover how exogenous and intrinsic host factors influence gut microbiome composition[1,2]. In large cohorts, scientists investigated the impact of factors such as lifestyle, dietary information, anthropometrics and drugs on the gut microbiome communities[3,4]. They found that age, gender, dietary factors and intrinsic parameters were highly correlated with composition and function of the gut microbiome. They also observed that several drug categories, such as antibiotics, proton-pump, metformin, statins, and laxatives, had a strong effect on the gut microbiome. On the other hand, gut microbiome can affect the bioavailability of oral drugs[5]. Thus, microorganisms can impact drug absorption and metabolism, that may explain, in part, inter-individual heterogeneity in drug response and disposition[6].

In addition, DNA sequencing technologies were used to investigate associations between composition or function of the gut microbiome and various disease states. Studies performed cross sectional comparisons between subjects with and without disease, and reported changes in the gut microbiome of patients with inflammatory bowel diseases, colorectal cancer, diabetes, obesity and metabolic disease, or autoimmune diseases compared to controls[7]. Even if it remains unclear whether these microbiome alterations are causative or secondary to the presence of the disease, gut microbiome could be viewed as a diagnostic biomarker[8].

Allogeneic hematopoietic stem cell transplantation (aHSCT) is a standard validated therapy, which every year allows an increasing number of patients suffering from malignant and nonmalignant hematological diseases, to benefit from an HSC transplant. The donor can be related (called geno-identical if HLA matched or haploidentical if sharing only one haplotype) or unrelated (called pheno-identical if HLA matched and including cord blood). However, aHSCT procedures are limited by potentially life-threatening complications, and one of the most serious complications is acute graft-versus-host disease (GVHD). GVHD occurs when immune competent T cells in the donated tissue recognize the recipient as foreign[9], that induces tissue damages in target organs of the host[10], including skin, liver and the gut, and typically occurs 3 to 6 wk after transplantation in nearly 60% of related donor transplants and 80% of unrelated donor transplants[11]. Hahn *et al*[12] assessed acute GVHD risk factors in 1960 adults after HLA-identical sibling myeloablative transplant in 226 centers worldwide. They found that cyclophosphamide and total-body irradiation, blood cell *vs* bone marrow grafts in patients age 18 to 39 years, recipient age 40 and older, chronic myeloid leukemia, Karnofsky performance score less than 90, and recipient/donor cytomegalovirus-seronegative were independent factors significantly associated with grade 2 to 4 acute GVHD[12].

Based on preclinical data demonstrating reduced acute GVHD severity in germ-free or antibiotic-treated mice (*i.e.*, gut microbiome with reduced diversity and richness), the gut microbiome was suggested to play a pivotal role in the pathogenesis of acute GVHD following aHSCT[13]. These findings are in contradiction with the common paradigm that loss of microbiome diversity is associated with diseases[14]. Most recent studies, based on microbiome sequencing, however, reported that higher diversity of intestinal microbiota at the time of neutrophil engraftment was associated with lower mortality in patients undergoing allogeneic hematopoietic-cell transplantation[15].

Here, we summarized the most recent findings of the association between human gut microbiome changes and graft *vs* host disease of the intestinal tract in patients receiving aHSCT.

ALTERATIONS OF THE GUT MICROBIOME DURING aHSCT

Several studies reported the profound alteration of the gut microbiota during the aHSCT procedure, as summarized in Table 1. Following various conditioning regimens, both myeloablative or of reduced-intensity, a recent 16S rRNA gene-based amplicon sequence analysis that profiled 8767 fecal samples from 1362 patients undergoing aHSCT at four different centers observed a significant gut microbiota disruption characterized by loss of diversity and domination by single taxa[15], defined as occupation of at least 30% of the gut microbiota by a single predominating bacterial taxon. They also identified an association between lower intestinal diversity and higher risks of transplantation-related death. Moreover, samples collected prior transplantation also showed evidence of microbiome disruption, and lower diversity before transplantation was associated with poor survival. In another study, mortality outcomes were significantly worse in patients with lower intestinal diversity, with an overall survival at 3 years of 36% in low gut microbiome diversity patients compared to 67% in high diversity groups. Overall, low diversity showed a strong effect on mortality after multivariate adjustment for other clinical predictors. Furthermore, in subjects with lower diversity, the gut microbiota was generally dominated by a single bacterial genus, including *Enterococcus*, *Streptococcus*, Enterobacteriaceae (*Escherichia* and *Kluyvera*), and *Lactobacillus*[16].

Taur *et al*[16] analyzed a total of 439 fecal specimens obtained from 94 patients during their transplant hospitalization and reported that over the course of aHSCT, gut microbiome diversity index decreased and remained low until the end of the observation period (Day 35 post-transplant). Patients also demonstrated significant changes in gut microbiome composition, and in most patients, the microbial composition became dominated by a single bacterial taxon: *Enterococcus* (40% of the patients), Streptococcus (37%) and the phylum Proteobacteria (13%). Interestingly, they demonstrated that patients with enterococcal domination in the gut had a 9-fold increased risk of Vancomycin Resistant *Enterococcus* bacteremia, and intestinal domination by Proteobacteria increased the risk of bacteremia with aerobic gram-negative bacilli 5-fold[17]. In a recent study using metagenomic shotgun, able to achieve bacterial identification to species and strain level, Ilett *et al*[10] reported that, in addition to a significant loss of gut microbiota diversity, post-aHSCT samples were enriched in *Staphylococcus*, *Eggerthella*, *Streptococcus*, *Enterococcus* and *Lactobacillus* compared to pre-aHSCT samples, and several samples were dominated by a single micro-organism [*Enterococcus* (n = 41 of 112) or *Streptococcus* (n = 10 of 112)]. At species level, they observed an enrichment of *Enterococcus faecium*, *Lactobacillus delbrieckii*, *Staphylococcus epidermidis*, and *Streptococcus thermophilus* in the post-aHSCT samples compared to pre-aHSCT samples[10].

In patients receiving intensive chemotherapy regimen used as myeloablative conditioning treatment to prepare patients for aHSCT, gut microbiota after chemotherapy exhibited significant decrease in Firmicutes and Actinobacteria, and significant increase in Proteobacteria compared to samples collected before chemotherapy. At the genus level, gut microbiota after chemotherapy was significantly depleted in *Ruminococcus*, *Oscillospira*, *Blautia*, *Lachnospira*, *Roseburia*, *Dorea*, *Coprococcus*, *Anaerostipes*, *Clostridium*, *Collinsella*, *Adlercreutzia* and *Bifidobacterium*, and with significant increase in *Citrobacter*, *Klebsiella*, *Enterococcus*, *Megasphaera* and *Parabacteroides* compared with samples collected before chemotherapy. In addition, functional composition assessed using PICRUSt[18] revealed that following conditioning regimen, patients had reduced capacity for nucleotide metabolism, energy metabolism, metabolism of cofactors and vitamins, and increased capacity for glycan metabolism, signal transduction and xenobiotics biodegradation[19].

Thus, overall, aHSCT procedure is associated with a loss of gut microbiota diversity, a decrease in micro-organisms associated with health-promoting effects[20], and with a significant increase or domination by potentially pathobionts (Figure 1).

ALTERATIONS OF THE DIVERSITY OF THE GUT MICROBIOME AND ACUTE GVHD

Studies reported that decreased gut microbiota diversity and richness was associated with the onset of acute intestinal GVHD. Jenq *et al*[21], in 2015, reported in a cohort of 115 patients receiving aHSCT, that increased bacterial diversity was associated with reduced GVHD-related mortality[21]. Moreover, in their recent 16S rRNA gene-based amplicon sequence analysis, Peled *et al*[15] found that higher intestinal diversity was

Table 1 Gut microbiome diversity, composition and function changes in gut microbiota during allogeneic hematopoietic stem cell transplantation procedure

Ref.	Sequencing technology	Change in diversity	Changes in composition	Change in functions
Peled *et al* [15]	16S rRNA gene-based amplicon sequence	Loss of diversity	Gut microbiota dominate by single taxa (occupation of at least 30% of the gut microbiota by a single predominating bacterial taxon), including *Enterococcus*, *Streptococcus*, Enterobacteriaceae (*Escherichia* and *Kluyvera*), and *Lactobacillus*	
Taur *et al* [16]	16S rRNA gene-based amplicon sequence	Decrease of the gut microbiome diversity index	Gut microbiota composition frequently dominated by a single bacterial taxon, including *Enterococcus*, *Streptococcus* or Proteobacteria	
Ilett *et al*[10]	Metagenomic shotgun	Loss of gut microbiota diversity	Post-aHSCT samples enriched in *Staphylococcus*, *Eggerthella*, *Streptococcus*, *Enterococcus* and *Lactobacillus* compared to pre-aHSCT samples, &t species level enrichment in *Enterococcus faecium*, *Lactobacillus delbrieckii*, *Staphylococcus epidermidis*, and *Streptococcus thermophilus*	
Montassier *et al*[19]	16S rRNA gene-based amplicon sequence	Loss of gut microbiota diversity	Decreases in abundances of Firmicutes and Actinobacteria, and significant increases in abundances of Proteobacteria	Reduced capacity for nucleotide metabolism, energy metabolism, metabolism of cofactors and vitamins, and increased capacity for glycan metabolism, signal transduction and xenobiotics biodegradation

HSCT: Hematopoietic stem cell transplantation.

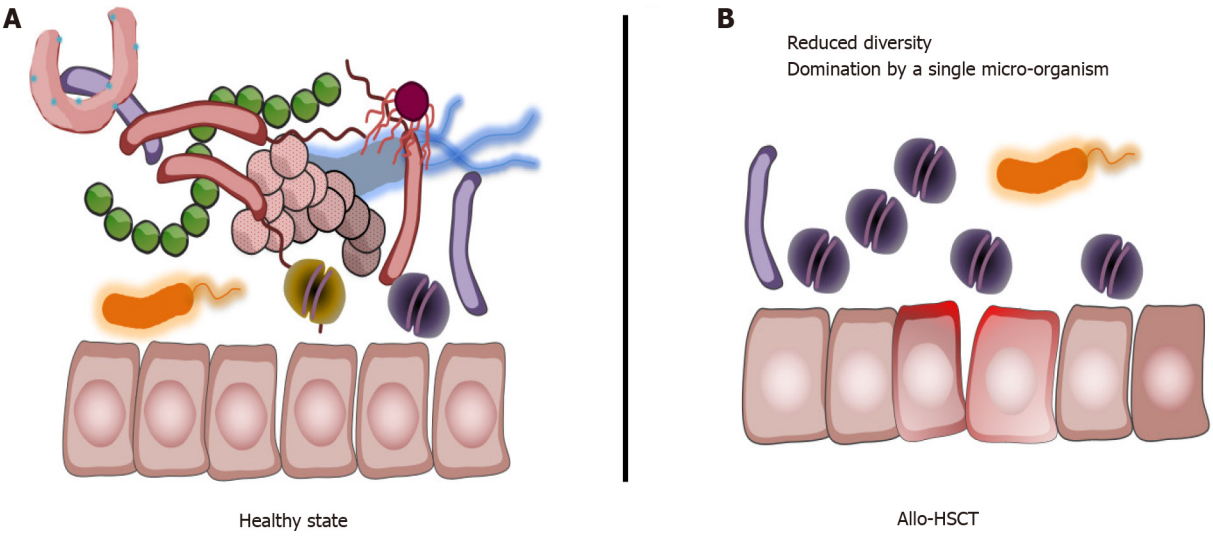

Figure 1 Gut microbiota changes during the allogeneic hematopoietic stem cell transplantation procedure. A: Healthy state with diverse and rich gut microbiota; B: Following conditioning regimen, gut microbiome alterations are marked by a drastic loss of diversity, a significant decrease in micro-organisms associated with health-promoting effects, and with a significant increase or domination, defined as occupation of at least 30% of the microbiota by a single predominating bacterial taxon, by potentially pathobionts. Allo-HSCT: Allogeneic hematopoietic stem cell transplantation.

associated with decreased risk of deaths attributable to GVHD (17 GVHD-related deaths among 244 patients in the higher-diversity group *vs* 26 such deaths among 184 patients in the lower-diversity group; hazard ratio, 0.49; 95%CI: 0.26-0.90)[15].

In a cohort of 44 patients, in which 16 (36%) experienced acute intestinal GVHD (median time to diagnosis: 53 d), Galloway-Peña *et al*[22] found that lower Shannon diversity index, popular diversity index in the ecological literature, of fecal samples collected at the time of engraftment was significantly associated with increased incidence of acute GVHD[22]. Golob *et al*[23] also found that diversity was statistically significantly lower in patients with acute GVHD when compared to those with no acute GVHD[23]. In another cohort of 57 patients, Liu *et al*[24] reported that decreased gut microbiota recipients' diversity was not associated with decreased risk of aGVHD. However, they found that high gut microbiota donor diversity was associated with

decreased risk of acute GVHD[24]. In a cohort of 70 patients, Payen *et al*[25] reported that patients with severe aGVHD had reduced gut microbiota diversity at disease onset, whereas patients with mild aGVHD had gut microbiota diversity more similar to those of controls[25].

Ilett *et al*[10] applied shotgun metagenomic sequencing to study a large cohort of adults (n = 150) undergoing aHSCT. Among them, 36 developed acute GVHD (median time to development: 34 d (interquartile range, 26-50 d post-aHSCT). They did not find significant association between diversity measures and acute GVHD in samples collected from the pre-aHSCT period. However, in samples collected in the early post-aHSCT period, patients who later developed acute GVHD had a significantly lower gene richness compared with those who did not develop acute GVHD[10].

Altogether, these findings clearly associate decreased diversity and richness of the gut microbiota, known to be associated with enhanced inflammation and impaired immunity[26], to onset of acute GVHD (Table 2).

COMPOSITION CHANGES IN GUT MICROBIOME AND ACUTE GVHD

Using a 16S rRNA-based sequencing analysis, Jenq *et al*[21] reported that genus *Blautia* were most significantly associated with reduced GVHD-related mortality, whereas genus *Veillonella*, was associated with increased GVHD-related mortality[21].

Holler *et al*[27] reported that post-transplant samples were increased in enterococci, and that the increase was more pronounced in patients developing acute GVHD compared to patients who did not develop aGVDH. They confirmed this trend using enterococcal polymerase chain reaction (PCR) and observed a predominance of *Enterococcus faecium* and *Enterococcus faecalis* in samples collected in patients who developed acute GVHD. Moreover, post-transplant samples collected in patients with acute GVHD were significantly depleted in Clostridia and *Eubacterium rectale*[27]. Stein-Thoeringer *et al*[28] also reported that fecal domination by *Enterococcus* (*i.e.*, relative genus abundance ≥ 30% in samples collected during early post-transplant period) was associated with increased risk of acute GVHD, and increased GVHD-related mortality [28]. Importantly, in a in gnotobiotic model, the authors demonstrated that *Enterococcus* growth is dependent on disaccharide lactose, and that dietary lactose depletion attenuates *Enterococcus* outgrowth and reduces the severity of GVHD[28].

In their cohort of 44 patients, Galloway-Peña *et al*[22] reported that only one taxon at the time of engraftment, *Coriobacteriia*, a class of Gram-positive bacteria within the Actinobacteria phylum, was negatively correlated with the incidence of aGVHD[22]. In another cohort of 107 patients, Doki *et al*[29] reported patients who developed acute GVHD exhibited a significantly higher abundance of phylum Firmicutes than patients who did not develop aGVHD[29].

Golob *et al*[23] found in a cohort of 66 patients, that in samples collected at neutrophil recovery post-HCT, the presence of Actinobacteria and Firmicutes was positively correlated with subsequent acute GVHD, whereas Lachnospiraceae were negatively correlated. In detail, *Butyricicoccus*, *Bacteroides luti*, *Bacteroides thetaiotaomicron*, *Bacteroides ovatus*, and *Bacteroides caccae* were negatively correlated with subsequent acute severe GVHD, while *Rothia mucilaginosa*, *Solobacterium moorei*, *Veillonella parvula*, and *Bacteroides dorei* were positively correlated[23]. Payen *et al*[25] reported that *Lachnoclostridium*, *Blautia*, *Sellimonas*, *Anaerostipes*, *Faecalibacterium*, *Flavonifractor*, *Erysipelatoclostridium* and *Lactococcus* were negatively associated with subsequent acute severe GVHD, whereas *Prevotella* and *Stenotrophomonas* were considered positive biomarkers of severe aGVHD. Moreover, using qPCR, they observed a significant depletion of the *Blautia coccoides* group (cluster XIVa) in patients with aGVHD compared with controls and patients with no aGVHD[25].

Based on a shotgun metagenomic sequencing analysis in 150 patients, Ilett *et al*[10] found that no bacteria were associated with acute GVHD in samples collected during the pre-aHSCT period. In samples collected during the early post-aHSCT period, they found that *Blautia*, *Akkermansia*, and *Campylobacter*, as well as the specific species *Akkermansia muciniphila*, *Blautia obeum*, *Blautia hydrogenotropica*, and *Blautia hansenii* were all significantly associated with reduced risk of a GVHD[10]. Still using shotgun metagenomic sequencing analysis, Turner assessed samples collected in nine patients with aGVHD and treated with standard-of-care high-dose steroids. Three of these patients were steroid-refractory, whereas six had a response. They showed that *Dorea longicatena* was associated with response to high-dose steroids treatment whereas *Akkermansia muciniphila* was associated with refractoriness. They also reported that maintenance of a stable *Dorea/Akkermansia* ratio predicted steroid response, whereas a

Table 2 Gut microbiome diversity, composition and function changes in patients receiving allogeneic hematopoietic stem cell transplantation procedure and developing acute graft versus host disease

Ref.	Sequencing technology	Change in diversity	Changes in composition	Change in functions
Ilett *et al*[10] (150 patients)	Shotgun metagenomic	Decrease in gene richness during the early post-aHSCT period in patients with aGVHD	Decrease in *Akkermansia muciniphila*, *Blautia obeum*, *Blautia hydrogenotropica*, and *Blautia hansenii* during the early post-aHSCT period in patients with aGVHD	Increase in toxin named PetZ, that triggers bacterial autolysis in pathological bacteria during the pre-aHSCT and the early post-aHSCT period in patients with aGVHD
Holler *et al*[27] (31 patients)	16S rRNA V3 sequencing		Increase in enterococci in patients who subsequently developed acute GVHD	
Galloway-Peña *et al*[22] (44 patients)	16S rRNA V4 sequencing	Lower Shannon diversity index in fecal samples collected at the time of engraftment in patients with subsequent aGVHD	*Coriobacteriia* negatively correlated with the incidence of acute GVHD	Fecal metabolites (Fecal indole and butyrate levels determined using liquid chromatography tandem mass spectrometry) associated with acute GVHD
Liu *et al*[24] (57 patients)	16S rRNA V4 sequencing	High gut microbiota donor diversity associated with decreased risk of aGVHD in recipient		
Doki *et al*[29] (107 patients)	16S rRNA V4 sequencing		Higher abundance of phylum Firmicutes in samples collected before aHSCT in patients with acute GVHD	
Golob *et al*[23] (66 patients)	16S rRNA V3-V4 sequencing	Diversity significantly lower in patients with GVHD	*Butyricicoccus*, *Bacteroides luti*, *Bacteroides thetaiotaomicron*, *Bacteroides ovatus*, and *Bacteroides caccae* negatively correlated with subsequent acute GVHD, *Rothia mucilaginosa*, *Solobacterium moorei*, *Veillonella parvula*, and *Bacteroides dorei* positively correlated with subsequent onset of GVHD	
Michonneau *et al*[31] (99 patients)	Metabolomics			Significant decrease in tryptophan metabolites, including microbiota-produced compounds, such as 3-indoxyl sulfate, indoleacetate, indoleacetylglutamine, and indolepropionate in patients with aGVHD
Payen *et al*[25] (70 patients)	16S rRNA V3-V4 sequencing	Decreased diversity in acute GVHD	*Lachnoclostridium*, *Blautia*, *Sellimonas*, *Anaerostipes*, *Faecalibacterium*, *Flavonifractor*, *Erysipelatoclostridium* and *Lactococcus* negatively associated with subsequent acute GVHD	

HSCT: Hematopoietic stem cell transplantation; GVHD: Graft-versus-host disease.

decline in this ratio preceded refractory disease. Importantly, Shono *et al*[30] previously demonstrated in a mouse model an increased in *Akkermansia muciniphila*, a commensal bacterium with mucus-degrading capabilities, raising the possibility that mucus degradation may contribute to murine GVHD[30].

Further studies are needed to confirm or not the controversial role of *Akkermansia* in acute GVHD. Moreover, some species of the genus *Blautia* should be investigated as a potential biomarker: High relative abundance at the time of engraftment being protective against GVHD, while low relative abundance could be considered a risk factor for secondary development of GVHD.

FUNCTION CHANGES IN GUT MICROBIOME AND ACUTE GVHD

The functional alterations of the intestinal microbiome in patients receiving aHSCT are currently poorly described because the majority of studies have used 16S ribosomal RNA gene-based technics, which boil down to providing bacterial taxonomy only at the genus level, but are ineffective in obtaining functional information. In a cohort of 44 patients, Galloway-Peña *et al*[22] reported that fecal metabolites (fecal indole and butyrate levels) were not associated with aGVHD[22]. In another study, Michonneau *et al*[31] reported that aGVHD was characterized by specific metabolomics changes in

two cohorts of patients (n = 99). They found that bile acids, plasmalogens, tryptophan, and arginine metabolites were the main contributors involved, especially the aryl hydrocarbon receptor ligand 3-indoxyl sulfate. In addition to host-derived metabolites, they also identified significant variation in microbiota-derived indole compounds, especially in aryl hydrocarbon receptor ligands. The authors suggested that allogeneic immune response during aGVHD might be influenced by bile acids and by the decreased production of aryl hydrocarbon receptor ligands by gut microbiome that could limit indoleamine 2,3-dioxygenase induction and influence allogeneic T cell reactivity[31]. To assess if altered composition of the gut microbiota may result in an altered metabolome, which potentially disrupts functionalities at the onset of aGVHD, Payen *et al*[25] quantified the Short Chain Fatty Acids (SCFA) content in fecal samples with measurement of total SCFAs and acetate, propionate, and butyrate. They found that the fecal amount of all SCFAs was drastically diminished at aGVHD onset. In detail, they observed that total SCFAs, acetate and butyrate respectively decreased by 80%, 75% and 95% in severe aGVHD patients as compared with controls[25]. Importantly, in a mouse model, Mathewson *et al*[32] demonstrated that butyrate restoration improved intestinal epithelial cells junctional integrity and mitigated aGVHD[32].

Based on a shotgun metagenomic sequencing analysis, Ilett *et al*[10] found that a total of 1267 and 1289 genes were present in significantly different amounts among those who developed aGVHD *vs* those who did not develop aGVHD during pre-HSCT and early post-aHSCT, respectively. Of these genes, 24 overlapped between the 2 time periods, all being significantly higher in abundance among those who did not develop aGVHD. They pointed out that 1 gene (O2.CD1-0-PT_GL0039283) had a 3-log fold-change in both periods and is known to function as a toxin named Petz, that triggers bacterial autolysis in pathological bacteria[10].

CONCLUSION

The investigations described in this mini review provide an understanding of the role of the gut microbiome in the pathophysiology of aGVHD in patients receiving aHSCT. Observational studies have shown that a decrease in diversity of the gut microbiome and specific species or metabolic pathways were associated with aGVHD. These specific changes could serve as biomarkers for diagnosis and prevention in patients receiving aHSCT. Moreover, functional alterations of the gut microbiome during aHSCT should be more investigated so that modulations of the gut microbiome could be tested to prevent this potentially life-threatening complication.

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ORIGINAL ARTICLE

Clinical and Translational Research

MicroRNAs expression influence in ulcerative colitis and Crohn's disease: A pilot study for the identification of diagnostic biomarkers

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Abstract

BACKGROUND

Inflammatory bowel disease (IBD) comprises two distinct diseases, Crohn's disease (CD) and ulcerative colitis (UC), both of which are chronic, relapsing inflammatory disorders of the gastrointestinal tract with a mostly unknown etiology. The incidence and prevalence of IBD are continually increasing, indicating the need for further studies to investigate the genetic determinants of these diseases. Since microRNAs (miRNAs) regulate protein translation *via* complementary binding to mRNA, discovering differentially expressed miRNAs (DE) in UC or CD patients could be important for diagnostic biomarker identification, assisting in the appropriate disease differentiation progressing the understanding of IBD pathogenesis.

AIM

To determine the miRNA expression profile in UC and CD patients and the potential pathophysiological contributions of differentially expressed miRNA.

METHODS

A total of 20 formalin-fixed paraffin-embedded colonic samples were collected

authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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from the Pathology Department of Botucatu Medical School at São Paulo State University (Unesp). The diagnosis of UC or CD was based on clinical, endoscopic, radiologic, and histological criteria and confirmed by histopathological analysis at the time of selection. The TaqMan™ Array Human MicroRNA A+B Cards Set v3.0 (Applied Biosystems™) platform was used to analyze 754 miRNAs. Targets of DE-miRNAs were predicted using miRNA Data Integration Portal (mirDIP) and the miRNA Target Interaction database (MiRTarBase). All statistical analyses were conducted using GraphPad Prism software. Parametric and nonparametric data were analyzed using *t*-tests and Mann-Whitney *U* tests, respectively.

RESULTS
The results showed that of the 754 miRNAs that were initially evaluated, 643 miRNAs were found to be expressed in at least five of the patients who were diagnosed with either CD or UC; the remaining 111 miRNAs were not considered to be expressed in these patients. The expression levels of 28 miRNAs were significantly different between the CD and UC patients ($P \leq 0.05$); 13 miRNAs demonstrated a fold-change in expression level greater than 1. Five miRNAs with a downregulated expression were selected for enrichment analysis. The miRNAs whose expression levels were significantly lower in UC patients than in CD patients were enriched in certain signaling pathways that were mostly correlated with cancer-related processes and respective biomarkers.

CONCLUSION
MiRNAs could be used to differentiate UC from CD, and differently expressed miRNAs could help explain the distinct pathophysiology of each disease.

Key Words: Crohn's disease; Ulcerative colitis; Inflammatory bowel disease; miRNA; Differential diagnosis; Biomarker

Core Tip: This study identified 27 microRNAs (miRNAs) with significantly different expression levels between Crohn's disease (CD) and ulcerative colitis (UC) patients. Five miRNAs whose expression levels were significantly lower in the UC patients than in the CD patients were selected for enrichment analysis, which revealed enrichment in certain signaling pathways that were mostly associated with cancer-related processes. The characterization and comparison of the differentially expressed miRNAs in this study could lead to novel diagnostic biomarkers to differentiate UC from CD. These markers might also predict prognosis, help elucidate the distinct pathophysiology of each disease, and lead to novel therapeutic target identification.

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC), the two main phenotypes of inflammatory bowel disease (IBD), are chronic, relapsing inflammatory disorders of the gastrointestinal (GI) tract whose etiologies remain unknown[1,2]. Although its pathophysiological mechanisms are unclear, IBD has been linked to an inappropriate and exacerbated immune response to commensal bacteria in genetically susceptible hosts, as well as with environmental factors, defects of the epithelial barrier, and dysregulation of the innate and adaptive immune responses at the level of the intestinal mucosa[1,2].

IBD affects 6 to 8 million people worldwide, with a prevalence of 84.3 people (79.2-89.9) per 100000 in 2017 and a mortality rate of 0.51[3]. The incremental increase in the incidence and prevalence of IBD globally is indicative of the need for further population-based genetic studies of those affected by these diseases. Differences in miRNA expression between these two diseases could lead to the identification and validation of diagnostic biomarkers, facilitating differential diagnosis and improving the understanding of IBD pathogenesis[4].

MiRNAs are a class of small (18-25 nucleotides in length), endogenous, non-coding, single-stranded RNA molecules that can negatively regulate target gene expression at the post-transcriptional level through binding to the 3′untranslated regions of the target mRNAs and promoting mRNA degradation or translational repression[4]. Overall, there is evidence that miRNAs contribute to the regulation of at least one-third of all protein-coding mRNAs, including those involved in the development, metabolism and cell cycle control[5]. Although studies have shown that miRNA plays an important role in both CD and UC and that they are differentially expressed in these disease states, there may have been potential confounding factors that were not considered. For example, the patients in these studies were treated with various medication classes, such as immunosuppressants, corticosteroids, and/or aminosalicylates[5-8]. Therefore, the aim of the present study was to investigate miRNA expression patterns in the tissues of treatment-naïve CD and UC patients to assess changes in miRNAs without the confounding influence of pharmacological agents.

MATERIALS AND METHODS

Ethics statement

The study was approved by the Botucatu Medical School Research Ethics Committee (protocol No. 71379417.2.0000.5411).

Sample collection

A total of twenty formalin-fixed paraffin-embedded (FFPE) colonic samples were collected from patients in the Pathology Department of Botucatu Medical School at São Paulo State University (Unesp). IBD diagnosis was based on clinical, endoscopic, radiologic, and histological criteria[9]. After sample selection, the diagnoses of UC or CD were confirmed by histopathological analysis of the biopsied colonic samples collected from each patient during a diagnostic colonoscopy; therefore, none of the patients received any medication at the time of the examination.

RNA isolation, reverse transcription, pre-amplification, and qRT-PCR analyses

Total RNA from each colon biopsy was isolated using MagMAX™ FFPE DNA/RNA Ultra Kit (Thermo Fisher Scientific, Massachusetts, EUA). For reverse transcription, Megaplex™ Reverse Transcription Primer Pools and Taqman miRNA reverse transcription kits (Thermo Fisher Scientific) were used. Briefly, 3 μL of RNA (1-350 ng) were reverse-transcribed by combining the Megaplex RT Primer (Thermo Fisher Scientific) with the TaqMan® miRNA Reverse Transcription Kit (Thermo Fisher Scientific). A volume of 2.5 μL of the reverse transcription product was used for pre-amplification with Megaplex™ PreAmp Primers (Thermo Fisher Scientific) and TaqMan® PreAmp Master Mix (Thermo Fisher Scientific). For qRT-PCR, 9 μL of the pre-amplified product was mixed with 441 μL of H_2O and 450 μL of TaqMan® Universal PCR Master Mix II without uracil-N-glycosylase (Thermo Fisher Scientific). Subsequently, 100 μL of each sample was loaded into each fill reservoir of the TaqMan microfluidic array cards. Real-time-PCR analysis was performed using a QuantStudio™ Real-Time PCR System (with TaqMan® Array Block) (Thermo Fisher Scientific) with universal cycling conditions [95°C/10 min, then (95°C/15 s, 60°C/60 s) for 40 cycles]. The TaqMan miRNA array output data (.sds files) were uploaded to the ThermoFisher Cloud App (https://www.thermofisher.com/mysso/LoginDisplay) and analyzed *via* the relative quantification (ΔΔCt) method using defined threshold settings for each miRNA. The geometric means of the threshold cycle (Ct) values of three small nucleolar RNAs (snRNAs), RNU44, RNU48, and U6, were used as the endogenous controls. Briefly, the change in quantification cycle (ΔCq) value was calculated as:

Cq (miRNA of interest) - mean Cq (endogenous control).

The ΔΔCq was subsequently calculated as:

ΔCq (miRNA of interest) - mean of ΔCq (miRNA of interest in the reference group - CD).

The relative quantification (Rq or gene expression fold-change) was calculated as $2^{-(\Delta\Delta Cq)}$. Subsequently, the FC was calculated using the Rq and the FC values, and *P* values were log2- and log10-transformed, respectively, and plotted as a volcano plot, displaying the -log 10(*P* value) *vs* the log2 (FC) for each target in the UC group relative to that in the CD group. The volcano plot was generated using an online server (https://paolo.shinyapps.io/ShinyVolcanoPlot/) after defining the statistical cutoffs as a log2 fold-change > 1 and a *P* value < 0.05. A heat map was created using a web tool (http://www.heatmapper.ca/expression/).

Target prediction and gene enrichment analysis

The targets of the DE miRNAs were predicted using miRNA Data Integration Portal (mirDIP) (http://ophid.utoronto.ca/mirDIP/index.jsp)[10,11] and the miRNA Target Interaction database (MirTarBase) (http://miRTarBase.mbc.nctu.edu.tw/)[12]. The combined gene list of each miRNA was uploaded to the Enrichr database (http://amp.pharm.mssm.edu/Enrichr/), a web server for comprehensive gene set enrichment analysis[13,14]. Duplicate susceptibility genes were excluded prior to analysis. The CD and UC susceptibility genes were identified from the National Human Genome Research Institute-European Bioinformatics Institute (NHGRI-EBI) Catalog of human genome-wide association studies (GWAS)[15].

Statistical analysis

The statistical methods of this study were reviewed by Ana E. V. Quaglio from the Laboratory of Phytomedicines, Pharmacology, and Biotechnology (PhytoPharmaTec), Department of Biophysics and Pharmacology, São Paulo State University (Unesp). All statistical analyses were conducted using GraphPad Prism (GraphPad Software, San Diego, CA, United States). To determine if data is from a Gaussian distribution were performed D'Agostino-Person normality test. Parametric and non-parametric data were analyzed using unpaired and two-tailed *t*-tests and Mann-Whitney *U* tests, respectively. Statistical significance was considered for $P \leq 0.05$.

RESULTS

This study included 20 patients with IBD (10 with UC and 10 with CD), with a mean age at the time of diagnosis of 36.1 and 31.6 years for those with UC and CD, respectively. In terms of sex, 40% of the UC and 30% of the CD patients were male, and 60% and 70% of the UC and CD patients were female, respectively. Other characteristics of the patients are presented in Table 1.

In this study, 377 miRNAs and four controls were analyzed in each plate, totalizing 754 miRNAs of interest. Of these, 643 miRNAs were expressed in at least five patients diagnosed with UC or CD, whereas 111 miRNAs were not considered to be expressed in these patients. The expression levels of 27 miRNAs significantly differed between the CD and UC patients. Relative to the expression levels in CD patients, the five miRNAs that were downregulated in UC patients with a fold-change greater than 1 were selected for the subsequent enrichment analysis: miR-192-3p/5p, miR-378a-3p/5p, and miR-429 (Figure 1).

Target prediction and gene enrichment analysis of miRNAs

To assess the potential functions of miRNAs in UC or CD, the targets of the differentially expressed miRNAs (Supplementary Table 1) were predicted using an online database. Relative to the expression levels in CD patients, the miRNAs that were decreased in UC patients were those that were enriched in signaling pathways, such as forkhead box protein O (FOXO), transforming growth factor-beta (TGF-β), and mitogen-activated protein kinase (MAPK), as well as in pathways associated with cancer, particularly colorectal cancer (CRC) (Table 2).

Several of the miRNA gene targets have been previously flagged as CD or UC susceptibility genes. Using the IBD susceptibility gene list from the 2019 GWAS[17], the miRNA targets that had already been linked to one of the two diseases were identified. There are 619 and 474 genes that have been proposed as CD and UC susceptibility genes, respectively. Of the predicted targets of the miRNAs that were downregulated, 24 and 11 overlapped with the proposed CD and UC susceptibility genes, respectively. A total of 328 genes were common to both diseases, and 54 were common to the two diseases and the predicted targets (Figure 2 and Supplementary Table 2).

Table 1 Demographic characteristics and clinical features of inflammatory bowel disease patients

	Ulcerative colitis	Crohn's disease
Number of patients	10	10
Age at onset (yr, mean)	36.1 ± 18.31	31.6 ± 14.6
Sex, (%)		
Male	40	30
Female	60	70
Race, (%)		
Caucasian	100	90
Non-caucasian	0	10
Alcoholism, (%)	10	20
Smoking, (%)	20	10
Family history of IBD, (%)	10	0
Site of UC, (%)		
Proctitis	20	-
Left-sided	30	-
Extensive	50	-
Site of CD, (%)		
Ileal	-	20
Colonic	-	20
Ileocolonic	-	60

IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease.

DISCUSSION

MiRNAs are short single-stranded, post-transcriptional regulatory RNAs that are involved in major cellular processes. The mature miRNA species may be derived from either the 5′ or 3′ arms of the precursor duplex and are referred to as the miRNA-5p and -3p species, respectively. Initially, it was believed that only one of these strands was functional and that the other strand, known as miRNA*, was destined for degradation[16]. However, recent reports have indicated that both the miRNA and miRNA* species often co-exist, and both are functional[16,17]. Based on this finding, the miRNA-5p and -3p nomenclature are used solely based on the 5′- or 3′-arm derivation of the miRNA species[16]. This study described, for the first time, the co-existence of miR-192-3p and miR-192-5p and, miR-378a-3p and miR-378a-5p in the colonic samples of IBD patients. In addition to miR-429, the expression levels of both miRNAs were decreased in UC compared with CD patients.

Most of the altered pathways identified following the enrichment analysis are related to cancer processes, alterations in the extracellular matrix (ECM), inflammatory mediators like TGF-β, members of the heat shock protein (HSP) family, and MAPKs. The decrease in the expression of miRNAs related to all these pathways leads to dysregulation. Proteoglycans are important components of the ECM that have multiple functions depending on both their protein and carbohydrate constituents[18]. Heparan sulphate proteoglycans on the cell membrane may play diverse roles in cancer-related processes, acting as either inhibitors or promoters of tumor progression depending on the tumor type and stage of progression[19]. In CRC, for example, the cell surface proteoglycan syndecan-2 (SDC2 gene) is upregulated, leading to increased cell migration[20]. Moreover, high SDC2 expression was shown to be related to tumorigenic behaviors mediated through the regulation of cell adhesion, proliferation, and migration[21]. Recently, chronic inflammatory hypoxia-mediated SD2 expression was reported to be correlated with CRC development[21]. In addition, acute inflammation could also induce SDC2 expression predominantly in the proximal colon, indicating its potential as a biomarker for acute colonic inflammation[22].

Table 2 Gene enrichment analysis of decreased microRNAs in ulcerative colitis patients compared with Crohn's disease patients

Pathway	*P* value	FDR
Proteoglycans in cancer	2.27E-11	6.99E-09
FoxO signaling pathway	2.37E-11	3.64E-09
Pathways in cancer	7.49E-11	7.69E-09
Colorectal cancer	1.25E-07	5.51E-06
TGF-β signaling pathway	1.47E-07	4.53E-06
Signaling pathways regulating pluripotency of stem cells	4.72E-07	1.32E-05
Autophagy	5.58E-07	1.32E-05
ErbB signaling pathway	9.95E-07	2.19E-05
mTOR signaling pathway	3.70E-06	7.61E-05
MAPK signaling pathway	1.66E-05	2.22E-04

FoxO: Forkhead box protein O; TGF: Transforming growth factor-β; ErbB: Erythroblastic leukemia viral oncogene homolog; mTOR: Mammalian target of rapamycin; MAPK: Mitogen-activated protein kinase; FDR: False discovery rate-adjusted.

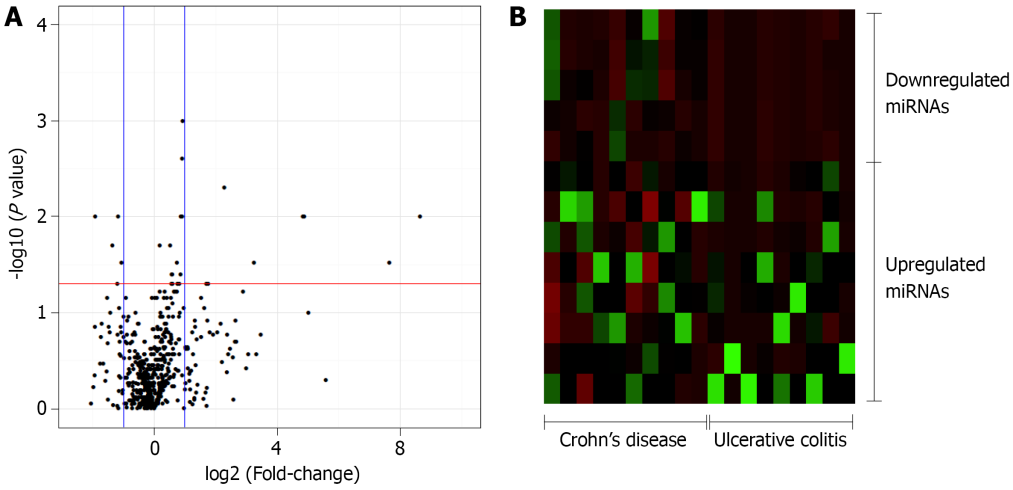

Figure 1 Volcano plot and heat map of the patients. A: Volcano plot of the 754-microRNA (miRNA) analyzed in inflammatory bowel disease patients. Control group: Crohn's disease (CD). Threshold x: fold change = 1; Threshold y: $P \leq 0.05$; B: Heat map of the 13 significantly different miRNA between the CD and ulcerative colitis patients with a fold-change in expression level greater than 1 relatively to the expression levels in CD patients (8 upregulated and 5 downregulated). miRNA: MicroRNA.

In the present study, SDC2 was predicted to be regulated by miR-429, which was proven to be related to cancer progression and metastasis, likely due to dysregulated SDC2 expression, increasing cell migration and proliferation. It has been reported that miR-429 becomes downregulated in esophageal squamous carcinoma cells, and its expression could predict poor prognosis for patients[23]. Moreover, they showed that miR-429 inhibited cellular proliferation through the nuclear factor kappa B (NF-κB) pathway and inhibited cell migration-mediated epithelial-mesenchymal transition (EMT) processes[23].

In nasopharyngeal carcinoma, miR-429 functions as a tumor suppressor by downregulating talin-1 (TLN1), a protein that enhances the migration and invasion of various carcinomas[24]. This miRNA is also related to an antimetastatic function, as it can regulate the metastasis of hepatocellular carcinoma by directly targeting Crk-like protein, which is involved in processes related to EMT, as well as the progression, development, invasion, metastasis, and apoptosis of a variety of cancers[25]. Most functional studies have reported that miR-429 plays an oncogenic role in CRC, and its expression is downregulated as the disease progresses[26,27]. In studies involving a dextran sodium sulphate-induced model of intestinal inflammation, miR-429 was downregulated[28], corroborating the findings of the present study. Others have

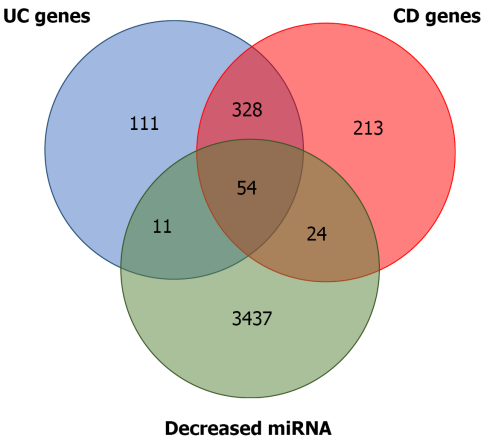

Figure 2 Overlap between predicted microRNA targets for microRNA differentially expressed and ulcerative colitis and Crohn's disease susceptibility genes. Detailed information is provided in Supplementary material 1. miRNA: MicroRNA; UC: Ulcerative colitis; CD: Crohn's disease.

reported that miR-429 modulates mucin secretion in human CRC cells and mouse colon tissues *via* upregulation of myristoylated alanine-rich protein kinase C substrate expression confirming that miR-429 is a candidate for intestinal anti-inflammatory therapy in human UC[28].

Another family of proteins with an important correlation with miRNA function is the HSP family. HSPs comprise several classes of constitutively active and/or stress-induced molecular chaperones that assist in proper polypeptide folding, the refolding of denatured proteins, protein transport, and stabilization of native protein structures, and numerous HSPs are overexpressed in inflamed tissues[29]. A recent review by Hoter & Naim (2019)[30] that analyzed the roles of HSPs in IBD found accumulating evidence linking the upregulation of HSP90 and/or HSP70 expression to the pathogenesis of IBD in the intestinal mucosa of patients with UC at the time of diagnosis, with the expression levels decreasing following the initiation of pharmacological therapy. Similar results were reported in a pre-clinical model of intestinal inflammation in which the colonic expression of HSP70 increased following the induction of intestinal inflammation[31]. Several authors have reported that HSPs are subject to post-transcriptional regulation by miRNAs[32]. The selected downregulated miRNAs found in the UC patients in the present study are predicted to regulate several members of the HSP70 family, such as HSPA1B and HSPA2, as well as HSP90B1, a member of the HSP90 family that plays critical roles in the folding of proteins such as Toll-like receptors and integrins[33], suggesting that the decrease in these miRNAs may have been related to the increased levels of the altered HSPs observed in the UC patients. Dysregulation on HSP70/90 Levels has also been related to intestinal inflammation associated with CRC. For example, Hoter & Naim (2019)[30] found that HSP90 was highly expressed and was implicated in the progression from UC to UC-associated CRC. Furthermore, HSP90 inhibition has been actively investigated as a treatment for gastrointestinal and CRC arising from IBD progression.

MiR-378a-3p/5p, two of the miRNAs correlated with HSP downregulation, have also been shown to be altered during intestinal inflammation[34]. The involvement of miR-378 in IBD patients has also been reported[35-37], indicating an upregulation in UC or a downregulation in CD. These differences may have resulted from the small number of patients included in these previous studies or the use of pharmacological agents by the participants, which could reflect treatment rather than disease effects. The patients in the present study were treatment naïve; therefore, the differences in expression resulted from the disease itself. A recent study conducted by Dubois-Camacho *et al*[38] in 2019, corroborates the present findings, as they showed that a decrease in miR-378a expression in UC patients was associated with higher levels of interleukin 33 and tumor necrosis factor-alpha (TNF-α), and that miR-378a expression levels increased following treatment with an anti-TNF-α monoclonal antibody[38].

In CRC, the overexpression of miR-378 inhibits the proliferation of colon cancer cells *in vitro* by inducing apoptosis and preventing migration and invasion[39]. For example, miR-378a also alleviated the malignant phenotypes of colon cancer cells by inhibiting the Wnt/β-catenin signaling pathway[39]. In addition, CRC patients with low miR-378a expression experienced a shorter survival time than those with high miR-378a expression, indicating that miR-378a may serve as an important diagnostic biomarker[40]. Collectively, it is possible to assume that the decrease in miR-378a-

3p/5p levels during inflammation and the later progression to CRC can increase HSP90 Levels. Thus, increased HSP90 and decreased miR-378a-3p/5p levels could act as important biomarkers and potential targets of pharmacological interventions for UC and UC-related CRC.

Among the downregulated miRNAs observed in IBD patients, miR-192-3p/5p, which was first cloned in 2003[41], is a tumor-related miRNA, as its dysregulation has been reported in several types of cancer[42]. Overexpression of miR-192 has been shown to induce apoptosis in bladder cancer cells and arrest breast cancer cells' growth[42,43]. Moreover, in CRC, miR-192 regulates the enzyme dihydrofolate reductase and cellular proliferation through the p53 tumor suppressor network, decreasing cancer progression and metastases[42,43]. Decreased levels of miR-192 are also related to IBD. For example, Wu *et al*[6] reported a link between chronic IBD and the altered expression of certain miRNAs, demonstrating that miR-192 was downregulated in the colonic epithelial cells of active UC patients which corroborates the findings of the present study.

An inverse correlation exists between the expression of miR-192 and MPI-2α, an epithelial cell-expressed chemokine previously implicated in IBD[6]. In addition, miR-192 was shown to be induced by TGF-β, suggesting that miR-192 plays a key role in inflammatory, fibrotic processes[6]. Moreover, other putative miR-192 targets identified in the enrichment analysis include mediators of inflammation and fibrosis, such as nucleotide-binding oligomerization domain-containing protein 2, TNF receptor-associated factor-interacting protein 3, 4, and 5, as well as matrix metalloproteinase 16 and 20 (Supplementary material 1). Changes in miR-192 expression have also been shown in a 2,4,6-trinitrobenzenesulfonic acid-induced intestinal inflammation model[44]. In that elegant study, the EMT was activated as a result of intestinal inflammation, along with a simultaneous increase in the early growth response protein 1 and fibroblast growth factor 2 expression levels (based on mesenchymal markers); on the other hand, miR-192 expression was decreased[44].

Conversely, elevated levels of miR-192 are associated with tumor suppression and cell proliferation[45-49]. Ji *et al*[45] demonstrated that miR-192 suppresses the growth of bladder cancer cells *via* targeting Yin Yang 1, a transcription factor that plays an important role in regulating development and cell proliferation. Similarly, Flammang *et al*[48], in a study involving pancreatic ductal adenocarcinoma, described miR-192 as a marker with prognostic value, as increased miRNA expression exerted a suppressive effect. In CRC, miR-192 also acts as a tumor suppressor, inhibiting CRC invasion[46, 47]. Furthermore, Huang *et al*[49] found that certain metabolites of normal intestinal microflora were capable of upregulating miR-192 expression, suppressing the proliferation of colon cancer cells through a decrease in bone morphogenic protein type 2 receptor levels; thus, cell proliferation, migration, and invasion ability are diminished, and the rate of cellular apoptosis is improved[49]. Collectively, these data indicate a potential role of miR-192-3p/5p as a biomarker for UC activity and a target for pharmacological treatment.

These results provide information that may explain the differences in CRC and mortality rates between UC and CD patients. Two cohort studies from Olén *et al*[50,51] (2020a; 2020b) compared CRC mortality and incidence in UC and CD patients. In the first study, which evaluated UC patients, the authors found that UC patients had a 36% higher incidence of CRC than that of reference individuals [hazard ratio (HR) = 1.66][51]. Among CD patients, the incidence was 21.9% higher than that of the reference group (HR = 1.4)[50]. These findings demonstrated a tendency toward a higher probability of developing CRC in UC compared with CD, which was associated with higher mortality[50,51]. The lower CRC incidence in CD could be explained by the early removal of this portion of the intestine[52]. Nevertheless, there was differential expression of these selected miRNAs between those with CD and UC. The decreased expression levels of miR-378a-3p/5p, miR-429, and miR-192-3p/5p in UC patients could represent a possible mechanism responsible for increasing their likelihood of developing CRC.

Although the overlap between DE-miRNAs targets and IBD susceptibility genes (Figure 2; Supplementary Table 1) suggested some involvement with the disease onset, progression and future cancer development, the data are preliminary and the sample size is small, so further studies are required to better comprehension of miRNA role in IBD pathogenesis. Future studies should aim to address some of the limitations by increasing the number of patients and investigating the potential links between these miRNAs and pathological UC and UC-associated CRC mechanisms.

CONCLUSION

In conclusion, this study highlighted the potential involvement of miR-192-3p, miR-192-5p, miR-378a-3p, miR-378a-5p and miR-429 as players in IBD pathology, UC differentiation and UC-associated CRC, indicating the potential use of these miRNAs as specific biomarkers for UC. Moreover, these miRNAs could be also useful as biomarkers for UC-associated CRC. Since samples of treatment-naïve patients were used, the distinct expression found in this study was a result from the disease itself with no medication effect. This way, the DE-miRNAs may represent a new pharmacological target for UC treatment.

ARTICLE HIGHLIGHTS

Research background

Inflammatory bowel disease (IBD) is a chronic and relapsing disorder of the gastrointestinal tract including two distinct phenotypes, ulcerative colitis (UC) and Crohn's disease (CD). IBD pathophysiological mechanisms are unclear.

Research motivation

IBD affects 6 to 8 million people worldwide and the incremental increase in the incidence and prevalence globally is indicative of the need for population-based genetic studies including microRNAs (miRNAs) expression profiles.

Research objectives

The present study aimed to investigate the miRNA expression patterns in tissue of treatment-naïve CD and UC patients and the potential pathophysiological contributions of differentially expressed (DE) miRNA in IBD.

Research methods

A total of 20 formalin-fixed paraffin embedded colonic samples were used in a TaqMan™ Array Human MicroRNA (Applied Biosystems™) platform aiming to analyze 754 miRNAs. After that, targets of DE-miRNAs were predicted using miRNa data integration portal (miRDIP) and the miRNA target interaction database (miRTarBase).

Research results

A total of 643 miRNAs were found to be expressed in both diseases but only 13 miRNAs were significantly different between the CD and UC patients ($P \leq 0.05$; fold-change > 1). The miRNAs whose expression levels were significantly lower in UC patients than in CD patients (miR-192-3p/5p, miR-378a-3p/5p and miR-429) were enriched in signaling pathways that were mostly correlated with cancer-related processes and respective biomarkers.

Research conclusions

Ulcerative colitis and Crohn's disease presented distinct patterns of miRNA expression that could be useful as new pharmacological targets besides acts as biomarkers for UC-associated CRC.

Research perspectives

New studies should be done with the DE-miRNAs using a large number of patients aiming to confirm the differences found in this pilot study. With this confirmation, the DE-miRNAs will be able to be used as pharmacological targets and differential markers for each disease.

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ORIGINAL ARTICLE

Observational Study

Multimodality management of gallbladder cancer can lead to a better outcome: Experience from a tertiary care oncology centre in North India

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Abstract

BACKGROUND

Surgical resection is a treatment of choice for gallbladder cancer (GBC) patients but only 10% of patients have a resectable disease at presentation. Even after surgical resection, overall survival (OS) has been poor due to high rates of recurrence. Combination of surgery and systemic therapy can improve outcomes in this aggressive disease.

AIM

To summarize our single-center experience with multimodality management of resectable GBC patients.

METHODS

Data of all patients undergoing surgery for suspected GBC from January 2012 to December 2018 was retrieved from a prospectively maintained electronic database. Information extracted included demographics, operative and perioperative details, histopathology, neoadjuvant/adjuvant therapy, follow-up, and recurrence. To know the factors associated with recurrence and OS, univariate and multivariate analysis was done using log rank test and cox proportional hazard analysis for categorical and continuous variables, respectively. Multivariate analysis was done using multiple regression analysis.

RESULTS

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Of 274 patients with GBC taken up for surgical resection, 172 (62.7%) were female and the median age was 56 years. On exploration, 102 patients were found to have a metastatic or unresectable disease (distant metastasis in 66 and locally unresectable in 34). Of 172 patients who finally underwent surgery, 93 (54%) underwent wedge resection followed by anatomical segment IVb/V resection in 66 (38.4%) and modified extended right hepatectomy in 12 (7%) patients. The postoperative mortality at 90 d was 4.6%. During a median follow-up period of 20 mo, 71 (41.2%) patients developed recurrence. Estimated 1-, 3-, and 5-years OS rates were 86.5%, 56%, and 43.5%, respectively. Estimated 1- and 3-year disease free survival (DFS) rates were 75% and 49.2%, respectively. On multivariate analysis, inferior OS was seen with pT3/T4 tumor (P = 0.0001), perineural invasion (P = 0.0096), and R+ resection (P = 0.0125). However, only pT3/T4 tumors were associated with a poor DFS (P < 0.0001).

CONCLUSION
Multimodality treatment significantly improves the 5-year survival rate of patients with GBC up to 43%. R+ resection, higher T stage, and perineural invasion adversely affect the outcome and should be considered for systemic therapy in addition to surgery to optimize the outcomes. Multimodality treatment of GBC has potential to improve the survival of GBC patients.

Key Words: Gallbladder cancer; Multimodality; Surgical resection; Adjuvant; Chemotherapy; Chemoradiotherapy

Core Tip: Gallbladder cancer (GBC) is an aggressive malignancy with only 10% of cases amenable to resection at presentation and a dismal overall 5-year survival rate of 5%-13% after curative surgery. Recently, several experts have recommended that multimodality treatment, including neoadjuvant and adjuvant therapies, can improve survival. In this study, we share our experience with multimodality approach in GBC. Five-year overall survival was approaching 50%, and therefore we suggest that such approach can improve survival in this aggressive malignancy.

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INTRODUCTION

Gallbladder cancer (GBC) is the most common and most aggressive malignant disease of the biliary tract. A distinct geographical variability has been observed in the prevalence of GBC. Countries like India, Pakistan, Chile, Korea, and Japan have reported a higher prevalence as compared to the Western world. The highest incidence has been reported in regions like Delhi, India (21.5/100000), La Paz, Bolivia (15.5/100000), South Karachi, Pakistan (13.8/100000), and Quito, Ecuador (12.9/100000)[1,2].

Surgical resection is the treatment of choice but only 10% of patients have a resectable disease at presentation. Even after surgical resection, overall survival (OS) has been poor due to high rates of recurrence[2]. Recently, there has been an increased interest in multimodality treatment including both neoadjuvant and adjuvant therapy to improve outcomes. Although there are no randomized trials on the issue but improved outcomes have recently been reported using multimodality treatment. A recent expert consensus statement on GBC recommended that all patients with clinical T3–4 N+ disease should be considered for neoadjuvant chemotherapy (NACT) trials [3]. After curative resection, patients with T2 or higher and N+ disease should undergo adjuvant systemic chemotherapy or chemoradiotherapy (CRT). Adjuvant CRT should

be used in patients with positive margins after resection[3]. With advancements in surgical approach and systemic therapy, multimodality approach has a potential to obtain favorable outcomes in this aggressive disease[4].

We have adapted various aspects of the multimodality approach for GBC in the last decade. In this study, we aimed to analyze our outcomes for multimodality management of GBC.

MATERIALS AND METHODS

Patients

Institutional review board approval was taken for waiver of informed consent (RGCIRC/IRB-BHR/48/2020). All patients undergoing surgery for suspected gall bladder cancer from January 2012 to December 2018 were included. Data containing demographics, operative and perioperative details, histopathology, and neoadjuvant/adjuvant therapy was retrieved from a prospectively maintained electronic database. Follow-up data was collected from the database as well as telephonically.

Preoperative evaluation

All patients with suspicion of GBC were evaluated by contrast-enhanced computed tomography (CECT) of the abdomen and pelvis. CEA and CA19-9 were routinely measured in all cases. Since April 2015, all patients who had a resectable disease on CECT underwent an additional 18-FDG positron emission tomography (PET) scan to rule out distant metastasis as a part of the study to evaluate the role of PET scan in GBC[5]. All patients who presented with jaundice underwent magnetic resonance cholangiopancreatography to confirm the level of obstruction and biliary drainage procedure as indicated. Patients who underwent laparoscopic cholecystectomy outside for a benign disease and were found to have to have GBC on histopathology were defined as incidental GBC. They were evaluated similarly except that they were excluded from PET scan study due to possible high false positivity rate in view of ongoing inflammation at the postoperative site. Patients with locally advanced diseases were considered for NACT after discussion in multidisciplinary board. The following criteria were used to select patients for neoadjuvant therapy in primary GBC patients: (1) T4 lesion involving two or more adjacent organs or the hepatic hilum; (2) Extensive hepatic infiltration which required major liver resection (> 2 segments); (3) N2 disease (AJCC 7th); (4) Bulky regional nodes (> 3 cm in short axis); and (5) During waiting period after portal vein embolization.

The main aim for NACT was to select good tumor biology patients and improve R0 resection rate. Incidental cases were referred for NACT if they have a history of bile spillage in index surgery. Neoadjuvant CRT was not done in any patient.

The most commonly used regimen for NACT was gemcitabine (1000 mg/m^2 intravenously over 30-60 min) on days 1 and 8, and cisplatin (75 mg/m^2 intravenously over 2 h) on day 1, every 21 d. In case of renal compromise, carboplatin was used. After three cycles, patients were reassessed for response using PET-computed tomography (CT) and CECT of the abdomen and pelvis. Due to the retrospective nature of this study, the type and duration of chemotherapy were not controlled and were decided by the team of medical oncologists.

Data collection was done in concordance with ethical guidelines of Declaration of Helsinki. All patients provided informed consent prior to any intervention, chemotherapy, or surgery.

Surgical treatment

All patients underwent staging laparoscopy to rule out distant metastases. This was followed by exploratory laparotomy and inter-aortocaval (IAC) lymph node sampling for frozen section. Definitive procedure was generally abandoned if IAC nodes were positive for malignancy except for select cases. Resectable primary GBC underwent radical cholecystectomy which included *en bloc* resection of the gallbladder with a non-anatomical liver wedge (2 cm liver margin) or segment IVB/V resection with regional lymphadenectomy including retropancreatic lymph nodes (station 13) and common hepatic artery nodes (station 8) along with all the soft tissue around and in between hilar structures (station 12). In the initial period, the decision between non-anatomical wedge and segment IVB/V was taken by operative surgeon intraoperatively, but since 2014, all patients were part of a randomized controlled trial (RCT) comparing wedge resection and segment IVB/V resection for GBC (CTRI/2018/05/014324). Selected cases with extensive liver involvement or infiltration into right portal structures

underwent modified extended right hepatectomy (*en bloc* resection of the gallbladder along with segments V, VI, VII, VIII, and IVB) with regional lymphadenectomy. We did not perform hepato-pancreatoduodenectomy or vascular resections for GBC at our centre. Port sites were resected for all patients with incidental GBC before 2016, but it is not done routinely now. Common bile duct resection and adjacent organ (colon/stomach/duodenum) resections were performed only when necessary to achieve R0 status. All intraoperative and perioperative data was recorded. Postoperative complications were recorded and graded according to the Clavien-Dindo classification[6]. Histopathological data for all patients were retrieved and staging was done as per AJCC 8th classification[7].

All patients were discussed in multidisciplinary meetings for planning adjuvant therapy. Since January 2015, all T2/node positive GBC patients were included in an institutional RCT comparing adjuvant chemotherapy and CRT after radical cholecystectomy (R0 resection) [CTRI/2018/01/011296]. The patients randomized to chemotherapy were given single agent gemcitabine 1 gm/m^2 on days 1, 8, and 21 in each cycle for six cycles starting 3 wk after surgery. Chemo-radiation group received external beam radiation therapy (50.4 Gy, 1.8 Gy for 28 fractions). Radiation area included gallbladder fossa, tumor bed, and adjacent liver and regional nodes. Chemotherapy included injection of 5-FU 750 mg/m^2 on days 1-5 and on last days of radiotherapy in a concurrent fashion. All patients who received NACT completed a total of six cycles of perioperative chemotherapy. Patients with R1 resection received radiation therapy in addition to chemotherapy.

Follow-up

All patients were kept on regular follow-up, every 3 mo for first 2 years, and every 6 mo for next 3 years. At each visit, physical examination and tumor marker (CA19-9 and CEA) measurement were done. CECT of whole abdomen was done every 6 mo and those with suspicious or equivocal findings underwent PET-CT followed by histological confirmation of recurrence. All patients with recurrence were counselled for palliative therapy.

Statistical analysis

Demographic and preoperative data was given for all patients, including those who were found to have an unresectable/metastatic disease intraoperatively. But these patients were excluded from final analysis. Categorical variables are described using counts/percentages and the mean/median was used for continuous variables. OS and disease free survival (DFS) were calculated using Kaplan-Meier curves. OS was calculated from the date of diagnosis to death or last follow-up and DFS was calculated from the date of surgery to recurrence of disease. To know the factors associated with recurrence and OS, univariate and multivariate analysis was done using log rank test and cox proportional hazard analysis for categorical and continuous variables, respectively. Multivariate analysis was done using multiple regression analysis. The statistical review of the study was performed by a biomedical statistician.

RESULTS

Patient characteristics

From January 2012 to December 2018, a total of 298 patients were taken up for surgery for a suspected GB malignancy. Out of these, 22 patients were found to have benign disease on final histopathology and 2 had neuroendocrine tumors of the gallbladder, so they were excluded from final analysis (Figure 1). Among 274 patients with a confirmed histopathological diagnosis of GBC, 172 (62.7%) were female and the median age was 56 (range, 28-80) years. The most common presenting symptom was abdominal pain (80.7%), followed by jaundice (8.1%), non-specific symptoms (5.5%), dyspepsia, weight loss, loss of appetite, and fever. Ninety-six (35%) patients had incidental presentation and the median time interval between cholecystectomy and radical surgery was 30 (range, 11-175) d. Cholelithiasis was seen in 173 (63.1%) cases. Although CEA and CA19-9 levels were not available in some patients, CEA was raised in 57/174 (32.8%) and CA19-9 was raised in 94/209 (45%) cases.

Neoadjuvant therapy

Twenty-seven percent (75/274) of all patients received NACT. Out of the 75 patients,

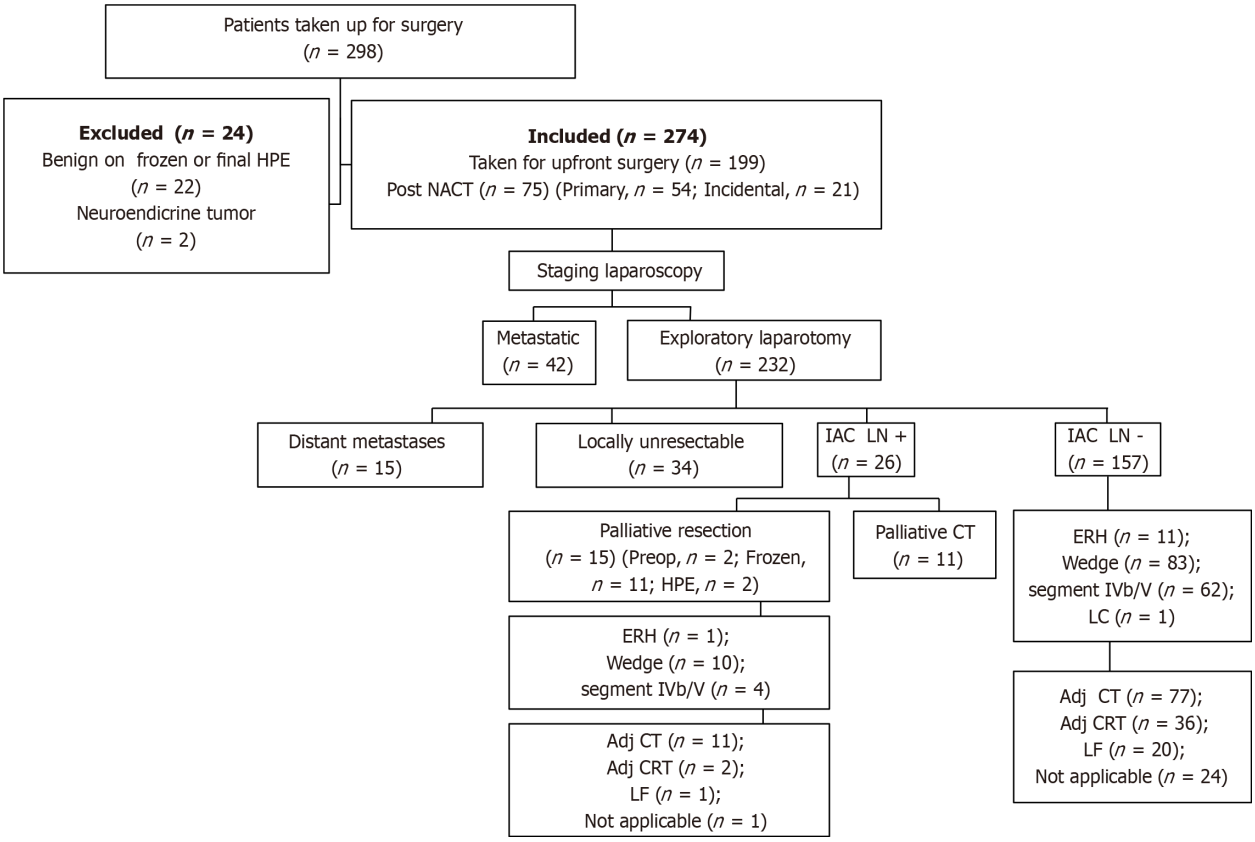

Figure 1 Details of suspected gallbladder cancer patients taken up for surgical exploration. NACT: Neoadjuvant chemotherapy; IAC LN: Inter-aortocaval lymph node; CT: Chemotherapy; LC: Laparoscopic cholecystectomy; LF: Lost to follow-up; CRT: Chemoradiotherapy; HPE: Histopathological examination; ERH: Extended right hepatectomy.

21 had incidental presentation and the rest 54 were non-incidental. Fifty-seven percent (43/75) of patients who received NACT could undergo curative resection and the rest 43% (32/75) were found to have an either metastatic or locally unresectable disease on exploration. Of 43 patients who successfully underwent surgery, 29 received gemcitabine with cisplatin, 12 received gemcitabine with carboplatin, and 2 received gemcitabine only. After NACT, 37 patients underwent radical cholecystectomy (22 had wedge liver resection, and 15 underwent anatomical segment IVb/V resection) and 6 had modified extended right hepatectomy.

Surgery

On exploration, 102 (staging laparoscopy, 42; laparotomy, 60) patients were found to have a metastatic or unresectable disease. Distant metastasis was seen in 66 patients (peritoneum, 40; liver, 15; IAC nodes, 11) and 34 had a locally unresectable disease on exploration. Two patients who were planned for major hepatectomy were found to have liver cirrhosis and surgery was abandoned. Of 172 patients who finally underwent surgical resection, 93 (54%) underwent wedge resection followed by anatomical segment IVb/V resection in 66 (38.4%) and modified extended right hepatectomy in 12 (7%) patients. One patient underwent laparoscopic cholecystectomy but was found to have T1a disease on final histopathology. Adjacent organ resection was done in 66 patients (CBD, 31; colon, 11; stomach/duodenum, 13; and multiple organs, 11). Median blood loss was 200 (range, 50-2000) mL and median duration of surgery was 270 (range, 120-540) min.

Morbidity and mortality

The postoperative mortality at 90 d was 4.6% (8/172), and the most common cause of death was bile leak and subsequent sepsis (n = 3) followed by postoperative liver failure (n = 2), acute myocardial infarction (n = 2), and ARDS (n = 1). Overall morbidity rate was 30.8% (53/172) but clinically significant complications (Clavien-Dindo grade III or more) were seen in only 12.2% (21/172) of cases. Median hospital stay was 9 (range, 3-54) d.

Histopathology

Histopathological details are described in Table 1. The most common histological diagnosis was adenocarcinoma seen in 160/172 (93%) cases. All patients were staged according to the AJCC 8th TNM classification. The majority of patients had a T2/T3 (83%) disease and 55/172 (32%) had a node positive disease. Median number of lymph nodes resected was 9 (range, 1-25). On final staging, the maximum number of patients had a stage III disease (III, 73; II, 45; IV, 33; I, 21).

Adjuvant therapy

Excluding the patients who had a stage I (n = 21) disease on final histopathology, 151 patients were eligible for adjuvant therapy. Approximately 86% (126/147) of patients received adjuvant therapy. Out of these, 88 received chemotherapy only and 38 received CRT. Ninety-seven percent of patients in the radiotherapy group (37/38) and 90.9% (80/88) patients in the chemotherapy group completed the intended treatment. Overall, 117 out of 126 (92.8%) patients completed the adjuvant therapy.

Follow-up and survival

During a median follow-up period of 20 mo, 71 (41.2%) patients developed recurrence. In the majority of them, recurrence was seen at a distant site (47/71, 66.2%) followed by loco-regional failure in 18/71 (25.4%) and at multiple sites in 6 (8.4%). The most common site of distant metastases was the peritoneum (n = 22) followed by the liver (n = 15), distant nodes (n = 9), and lung (n = 1). Median DFS and OS were not reached in our study. However, median OS for stage III and stage IV patients was 27.1 mo and 19.6 mo, respectively. Median DFS for stage III and stage IV patients was 24 mo and 13 mo, respectively. Estimated 1-, 3-, and 5-year OS rates were 86.5%, 56%, and 43.5%, respectively. Estimated 1- and 3-year DFS rates were 75% and 49.2%, respectively. Stagewise OS and DFS are shown in Figure 2. On log rank test, they correlated significantly.

Factors affecting survival

On univariate analysis, inferior OS and DFS were associated with upfront presentation (non-incidental), positive resection margin, lymph node involvement, higher T stage (T3 or T4), and lymphovascular and perineural invasion (PNI) (Table 2). Neoadjuvant therapy was given in advanced cases, hence the cohort was associated with a poor outcome. However, on multivariate analysis, inferior OS was seen with pT3/T4 tumour (P = 0.0001), PNI (P = 0.0096), and R+ resection (P = 0.0125). On multivariate analysis, only pT3/T4 tumors were associated with a poor DFS (P < 0.0001). Also, association of R+ resection with early recurrence was approaching the level of significance (P = 0.0513).

Impact of adjuvant therapy on overall outcome

In our study, 147 patients were advised to receive adjuvant therapy, out of which 117 patients completed the adjuvant therapy (adjuvant group) whereas 30 patients did not take/complete adjuvant therapy (non-adjuvant group). These two groups were comparable in baseline characteristics except for a higher incidence of post-cholecystectomy GBC in the adjuvant group (Table 3).

Estimated median OS for the adjuvant group and non-adjuvant group was 49.9 mo and 28.5 mo, respectively; however, the difference was not significant (P = 0.21). Estimated median DFS was 30.6 mo and 17.7 mo for the adjuvant and non-adjuvant group, respectively (P = 0.14) (Figure 3).

DISCUSSION

According to GLOBOCAN 2018 data, GBC accounts for 1.2% of all cancer diagnoses worldwide with a median survival of less than a year in advanced cases[8]. It is an aggressive malignancy with usually late presentation with an overall estimated 5-year survival rate of 5%-13%[9-11]. Radical surgery is the mainstay of treatment but survival with surgery alone is dismal in locally advanced cases[10].

Presentation is usually a decade late in Western patients as compared to those in our series[12], which can be attributed to endemicity of GBC in Indian subcontinent which has higher composition of younger population. It is diagnosed either incidentally (where cholecystectomy is performed for benign conditions) or mostly in advanced stage where patients present with cachexia with or without jaundice.

Table 1 Histopathological and perioperative details of resectable gallbladder patients (*n* = 172)

Patient characteristic	*n* (%)
Type of surgery	
Wedge resection	93 (54)
Anatomical segment IVb/V resection	66 (38.4)
Modified extended right hepatectomy	12 (7)
Lap cholecystectomy	1 (0.6)
Histology	
Adenocarcinoma	160 (93)
Adenosquamous	9 (5.2)
Carcinosarcoma	2 (1.2)
Squamous	1 (0.6)
Histological grade	
Well differentiated	33 (19.2)
Moderately differentiated	116 (67.4)
Poorly differentiated	23 (13.4)
pT stage	
T1	25 (14.5)
T2	70 (40.7)
T3	73 (42.4)
T4	4 (2.4)
pN stage	
N0	117 (68)
N+	55 (32)
LVI positive	54 (31.4)
PNI positive	56 (32.5)
IAC positive	15 (8.7)
R0/R1 resection	
R0	161 (93.6)
R1	11 (6.4) [liver (*n* = 4), cystic duct (*n* = 4), bile duct (*n* = 3)]
Final stage (AJCC 8th)	
I	21 (12.2)
II	45 (26.2)
III	73 (42.4)
IV	33 (19.2)
Postoperative morbidity	
Overall	53 (30.8)
Clavien-Dindo grade III & above	21 (12.2)
Bile leak	14 (8.1)
Clinically significant	5 (2.9)
90 d mortality	8 (4.6)
Adjuvant therapy	117/147 (79.6)
Chemotherapy only	80

Chemoradiotherapy	37
Advised but not taken/incomplete	30
Not indicated	25

AJCC: American Joint Committee on Cancer; IAC: Inter-aortocaval lymphnode; LVI: Lymphovascular invasion; PNI: Perineural invasion.

Table 2 Association between patient and disease characteristics with outcomes

	Overall survival			Disease free survival		
Factor	**HR**	**95%CI**	***P* value**	**HR**	**95%CI**	***P* value**
Sex						
Female	0.92	[0.56-1.51]	0.76	0.98	[0.61-1.6]	0.96
Male						
Jaundice						
Yes	0.9	[0.37-2.16]	0.81	1.19	[0.48-2.93]	0.67
No						
Incidental						
Yes	0.24	[0.07-0.81]	0.001	0.54	[0.34-0.86]	0.01
No						
Neoadjuvant therapy						
Yes	2.2	[1.27-3.81]	< 0.001	2.91	[1.64-5.16]	< 0.001
No						
Resection						
R+	4.08	[1.22-13.64]	< 0.001	4.13	[1.22-13.9]	< 0.001
R0						
Lymph node status						
Positive	1.91	[1.13-3.25]	0.006	2.44	[1.4-4.17]	0.001
Negative						
T stage						
T1a-T2						
T3-T4	5.01	[3.06-8.18]	< 0.001	4.21	[2.55-6.94]	< 0.001
LVI						
Yes	2.08	[1.22-3.5]	0.001	2.12	[1.23-3.64]	0.001
No						
PNI						
Yes	3.06	[1.75-5.37]	< 0.001	2.54	[1.45-4.45]	< 0.001
No						
Poorly differentiated						
Yes						
No	1.75	[0.81-3.7]	0.07	1.43	[0.66-3.08]	0.28

HR: Hazard ratio; CI: Confidence interval; LVI: Lymphovascular invasion; PNI: Perineural invasion.

Cholelithiasis has been associated with GBC in several studies with a prevalence of stones in approximately 70%-88% of cases of GBC[13,14]. Our study showed the absence of gallstones in approximate one-third of cases, which might be explained by

Table 3 Clinicopathological characteristics of patients who received adjuvant therapy *vs* those who did not

Patient characteristic	Adjuvant group (*n* = 117), *n* (%)	Non-adjuvant Group (*n* = 30), *n* (%)	*P* value
Age, yr (mean)	54.5	60	0.01
Sex (M:F)	47:70	10:20	0.63
Incidental GBC	55 (47)	7 (23.3)	0.03
Neoadjuvant therapy	33 (28.2)	7 (23.3)	0.76
Type of surgery			0.71
Wedge resection	62 (53)	16 (53.3)	
Anatomical segment IVb/V resection	48 (41)	11 (36.7)	
Modified extended right hepatectomy	7 (6)	3(10)	
Histology			0.91
Adenocarcinoma	108 (92.3)	28 (93.3)	
Adenosquamous	7(6)	2 (6.7)	
Carcinosarcoma	1	0	
Squamous	1	0	
Histological grade			0.12
Well differentiated	21 (18)	2 (6.7)	
Moderately differentiated	82 (70.1)	21 (70)	
Poorly differentiated	14 (11.9)	7 (23.3)	
pT stage			0.56
T1	6 (5.1)	2 (6.7)	
T2	57 (48.7)	11 (36.7)	
T3	52 (44.4)	17 (56.6)	
T4	2 (1.7)	0	
pN stage			0.47
N0	77 (65.8)	17 (56.7)	
N+	40 (34.2)	13 (43.3)	
LVI positive	37 (31.6)	13 (43.3)	0.32
PNI positive	40 (34.2)	12 (40)	0.7
R0/R1resection			0.84
R0	109 (93.2)	27 (90)	
R1	8 (6.8)	3 (10)	
Final stage (AJCC 8th)			0.79
I	3 (2.6)	1 (3.3)	
II	36 (30.8)	8 (26.7)	
III	56 (47.9)	13 (43.3)	
IV	22 (18.1)	8 (26.7)	

AJCC: American Joint Committee on Cancer; GBC: Gallbladder cancer; LVI: Lymphovascular invasion; PNI: Perineural invasion.

environmental and genetic predisposition of the study population to GBC.

Factors affecting survival

Incidental detection of GBC after cholecystectomy usually confers a favorable prognosis as the malignancy is usually detected in early stage[15,16]. Non-incidental cases are more likely to have advanced T stage, high-grade tumors, lymphovascular

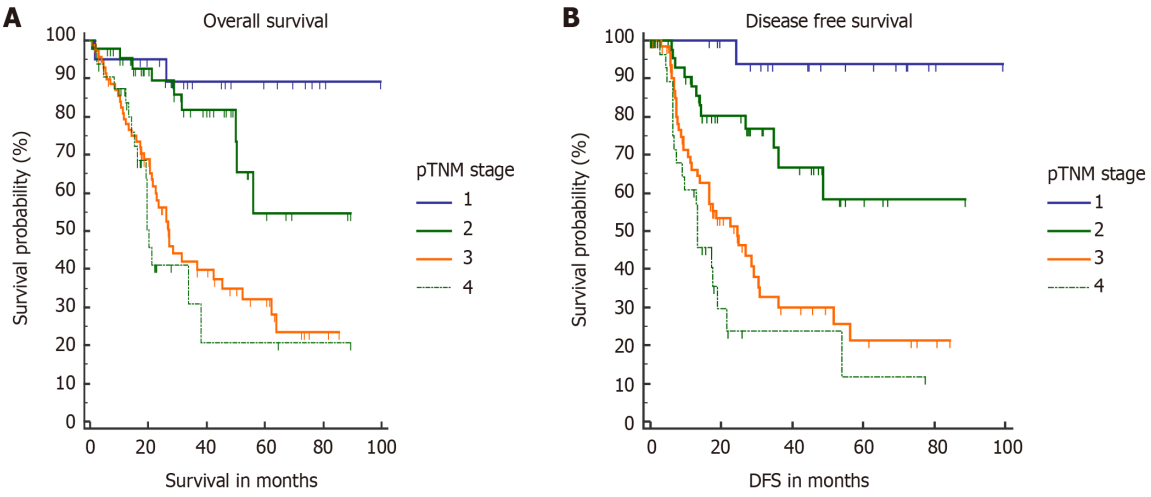

Figure 2 Stagewise overall survival and disease free survival. A: Stagewise overall survival; B: Stagewise disease free survival. DFS: Disease free survival.

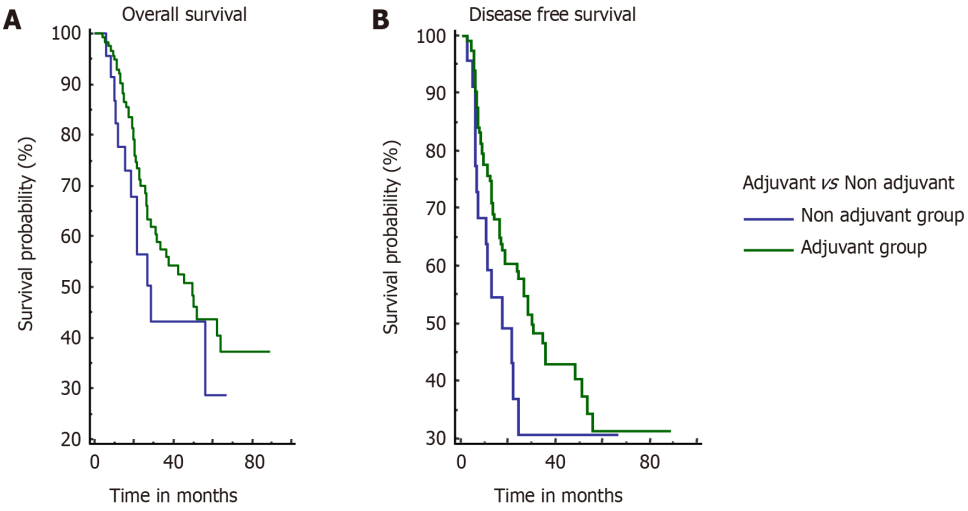

Figure 3 Comparison of overall survival and disease free survival of patients who received adjuvant therapy *vs* those who did not. A: Overall survival; B: Disease free survival.

invasion, positive lymph nodes, and R2 resection[16]. Optimal timing of completion of radical cholecystectomy is still debatable. Early surgery may lead to higher morbidity due to recent inflammation and adhesions and is also associated with a higher rate of unresectability due to breach of tumor and dissemination with seeding of tumor cells in the peritoneal cavity during index surgery[17]. Recently, a multi-institutional study showed a better survival when re-resection was performed between 4-8 wk from the index surgery although the retrospective and observational nature of the study casts apprehension over its universal application[18]. However, it is pertinent to give importance to bile spillage during index surgery, residual disease, and tumor biology rather than relying solely on the time interval[10,19]. In the present study, median time interval between index and redo surgery was 4 wk. Surgery was usually delayed with administration of NACT if there was any evidence of bile spillage. Future outcome also correlates well with the presence of residual disease on final exploration. Risk factors for finding residual disease include T3 tumors, PNI, and lymphovascular invasion[20]. Even half of the patients with incidental T1b/T2 GBC have residual disease on re-exploration and subsequently have a poor outcome[21]. However, higher T stage and poorly differentiated tumors have shown a high probability of residual disease at redo surgery[22]. In our study, 39.7% (29/73) of patients with incidental GBC were found to have residual disease at re-exploration. Incidental cases were found to have a significantly better survival on univariate analysis but not on multivariate analysis. This might be due to a smaller sample size of incidental cases in view of its lesser prevalence as compared to Western studies (Table 4). Also the

Table 4 Comparison of the present study with other studies on multimodality management of gallbladder cancer

Parameter	Our study	Patkar *et al*[4], 2018	Creasy *et al*[15], 2019
Sample size	274	400	437
Patients who underwent complete resection	172	320	255
Unresectable	102	80	182
Major liver resection, (%)	Yes (7)	No	Yes (24.3)
Incidental GBC, (%)	35	40	60.7
R1 resection, *n* (%)	11/172 (6.4)	10/320 (3.1)	15/255 (5.9)
Neoadjuvant in resectable group, *n* (%)	43/172 (25)	83/320 (25.9)	16/255 (6.3)
Final stage III/IV, *n* (%)	106/172 (61.6)	232/400 (58)	306/437 (70)
LN positivity, *n* (%)	56/172 (32)	98/320 (30.62)	NA
Residual disease in incidental cases, *n* (%)	29/73 (39.7)	68/160 (42.5)	172/276 (62.3)
Adjuvant therapy, *n* (%)	117/147 (79.6)	206/320 (64.4)	78/255 (30.7)
Recurrence, *n* (%)	71/172 (41.2)	98/320 (30.6)	NA
Dying of disease, *n* (%)	69/172 (40.1)	45/320 (14)	149/255 (58.4)
Estimated 3 yr OS, (%)	56	64	NA
Estimated 5 yr OS, (%)	43.5	NA	43 (only survivors)
Estimated 3 yr DFS, (%)	49.2	49	36

DFS: Disease free survival; GBC: Gallbladder cancer; LN: Lymph node; NA: Not available; OS: Overall survival.

number of truly incidental GBC (pT1) was much higher in Western studies as compared to our series (pT1 = 16.4%)[23].

Curative surgery with R0 resection improves the survival of GBC patients. The tendency of GBC to have early systemic dissemination often rules out radical surgery. A recently published study from our centre showed that routine application of 18-FDG PET changed management in approximately one-fourth of all resectable primary GBC patients and in one-third of locally advanced cases due to detection of unsuspected distant metastasis[5]. Similarly, routine application of staging laparoscopy before surgical exploration prevented non-therapeutic laparotomy in 23% of overall GBC patients with higher yield in locally advanced cases[24]. We universally applied staging laparoscopy in GBC patients before proceeding with curative surgery. It prevented laparotomy in 15.3% (42/274) of cases and helped in not only preventing surgical morbidity but also leading to quick commencement of palliative treatment. Staging laparoscopy is now routinely recommended prior to laparotomy for all suspected or proven GBC cases[3].

For non–metastatic GBC, standard surgical treatment is radical cholecystectomy which includes non-anatomical wedge or segment IVb/V resection with locoregional lymphadenectomy. Adjacent organ resection or major hepatectomy may be necessary to achieve negative margins. R0 resection was one of the major factors that significantly affected OS survival in our series. R1 resection was associated with a higher risk of death (hazard ratio [HR] = 4.08, 95%CI: 1.22-13.64, $P < 0.001$) and recurrence (HR = 4.13, 95%CI: 1.22-13.9, $P < 0.001$). All the patients with positive microscopic margin had a stage III or IV disease. Median OS in patients with R1 resection was significantly poor (19.6 *vs* 56.1 mo) (Figure 4). Patkar *et al*[4] also showed an inferior survival after R1 resection (17 *vs* 71 mo). It seems logical to give neoadjuvant treatment to avoid R1 resection in cases where tumor is close to resection margins on imaging, which is mostly in stages III and IV disease.

T stage is an important determinant of final outcome of GBC patients[25,26]. Increasing T stage is also associated with a higher probability of lymph nodal involvement and PNI[4,27]. Higher T stage (pT3/T4) was the only factor which negatively impacted both OS and DFS in our study. Median OS in pT3/T4 tumors was 21.5 mo (Figure 5).

PNI is acknowledged as a poor pathological factor with inferior outcome[26,28]. PNI is more frequently found in proximal tumors (tumors located in GB neck and

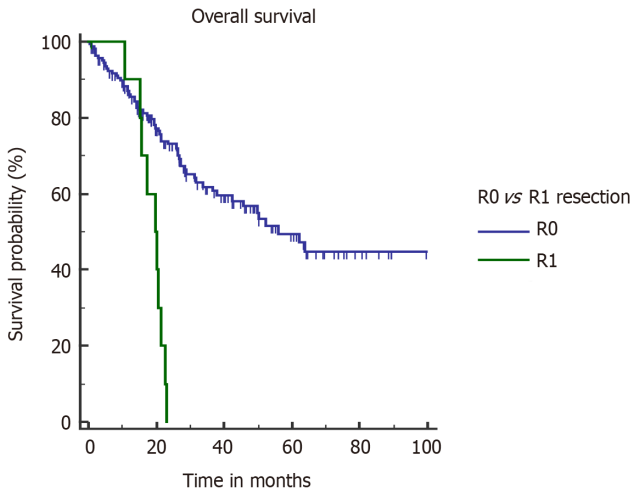

Figure 4 Survival curves of patients with R0 resection compared to R1 resection cases.

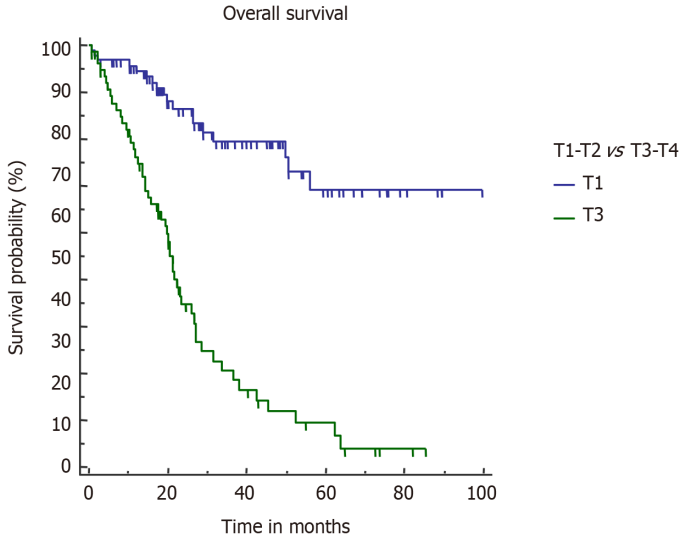

Figure 5 Survival curves of patients with pT1/T2 tumors compared to those with pT3/T4 tumors.

cystic duct) and with higher T stage[27]. PNI positive patients are shown to have significantly lower OS and DFS[26-28]. In our study, on multivariate analysis, PNI adversely affected OS (median OS, 21.3 mo) in PNI positive patients (Figure 6). Median OS was not reached in the PNI negative cohort. None of the patients with stage I disease was found to have PNI positivity, which correlates with the results of a recent study[27]. However, almost half of combined stage III/IV patients had PNI (48/106).

In past, various studies have reported about the adverse impact of node positivity on survival[4,15,29]. From the AJCC 8th edition, N classification of GBC was modified with more emphasis laid on the number rather than the location of involved nodes. Suspicious or confirmed involvement of lymph nodes is also one of the indications for neoadjuvant therapy[30]. In our study, 32% of operated patients had pathological involvement of lymph nodes but it did not affect survival or recurrence on multivariate analysis. LN sampling was adequate, with a median LN harvest of 9. Seventy-three percent of node positive patients completed intended adjuvant therapy. This might explain partly why lymph node positivity did not affect survival and recurrence in the present study.

Multimodality treatment

Chemotherapy is used as an adjunct to surgery in several settings of GBC: (1) As adjuvant therapy after surgical resection, with or without radiation to minimize recurrence; and (2) As neoadjuvant therapy in locally advanced GBC to downstage

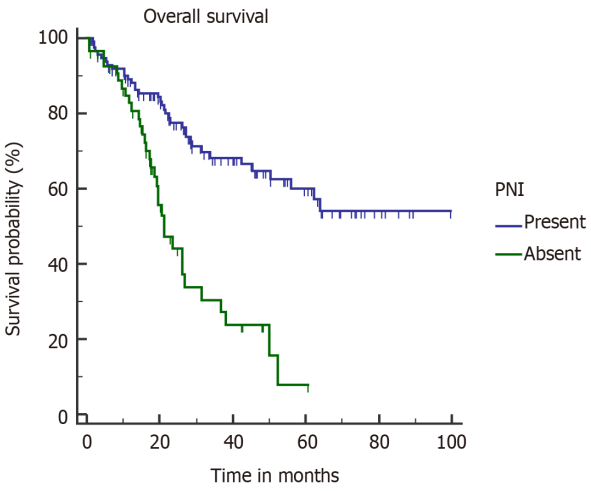

Figure 6 Survival curves of patients with perineural invasion compared to those without. PNI: Perineural invasion.

disease and select good biology tumors for surgery. Due to the rarity of GBC in the West, the data is often clubbed with other biliary malignancies, which leads to heterogeneity of data and hampers their applicability to GBC. Recently published studies from high volume centres have highlighted the need of multimodality management of GBC patients for further improvement in outcomes (Table 4).

Neoadjuvant chemotherapy

Neoadjuvant therapy for GBC is still not standardized in terms of indications, regimen, and duration. Institutions follow their own protocols based on the local data in the absence of randomized trials. The most suitable cases for implementation of NACT in GBC would be incidental GBC patients with residual mass on imaging or evidence of bile spillage during index surgery or locally advanced GBC where R0 resection is not feasible. Locally advanced GBC usually refers to T3 tumors with extensive liver involvement, T4 tumors, or those with any T stage and nodal involvement on imaging.

No randomized control trial has been conducted till date to test the efficacy of neoadjuvant therapy in GBC. A recent systematic analysis reviewed eight studies, out of which five were from India and only two were prospective studies. This calls attention to the paucity of the literature on neoadjuvant therapy for GBC[31]. The median OS for locally advanced cases that undergo curative resection following neoadjuvant therapy is found to be significantly better than that of patients who did not have surgery following neoadjuvant therapy[30,31]. In one of the largest studies, on a retrospective review of 160 patients, Chaudhari *et al*[30] reported a response rate of 52% with surgery feasible in 41% of cases. In another study from the same centre, 74% of patients who received neoadjuvant therapy could undergo R0 resection[4]. In a study from the West, Creasy *et al*[15] showed a median survival of 50 mo in locally advanced GBC patients who underwent surgery after preoperative gemcitabine based chemotherapy. In our study, the neoadjuvant therapy cohort had a poor survival due to the advanced nature of the disease in this subclass. However, 57% of patients with locally advanced disease initially could undergo surgery after NACT. Improvement in chemotherapeutic drug regimen with possible addition of targeted therapy might further improve resectability rate in future.

Adjuvant chemotherapy

Even after R0 resection, 30%-70% of patients develop recurrence over the time[4,15,32]. On analysis, 41% (71/172) of our patients developed recurrence after surgery, out of which 2/3 relapsed at distant sites. Higher rate of distant relapse in spite of R0 resection emphasizes on the need of inclusion of novel systemic therapies for further improvement in outcome and survival.

In contrast to neoadjuvant therapy, adjuvant therapy has been tested in the RCT setting with mixed results. In a meta-analysis by Ma *et al*[33], patients with positive lymph nodes, R1 resection, and non-stage I, benefited most from administration of adjuvant chemotherapy. Recently, several studies have highlighted various chemotherapy drug combinations with promising results after surgery. In the ABC-02 trial, 410 patients with advanced or metastatic biliary malignancy (36% cases were

GBC) were randomized to receive gemcitabine + cisplatin or cisplatin alone. The results demonstrated significant improvements in OS (11.7 *vs* 8.1 mo, $P < 0.001$) with the combination regimen[34]. Another French study (PRODIGE-12/ACCORD-18) evaluated 196 patients with biliary malignancy after surgical resection, out of which only 20% of patients had GBC. The trial randomized patients to receive gemcitabine + oxaliplatin or observation alone. The study found no survival benefit in the chemotherapy group. The study was criticized for including a lower proportion of high-risk patients (R1 resection and node positive patients) who can derive maximum benefit from adjuvant therapy[35].

More recently, in a study from UK (BILCAP trial), patients with biliary malignancies were randomized to receive either adjuvant capecitabine or observation alone after surgery. A total of 447 patients were included in the study, out of which only 18% were GBC cases. This study clearly demonstrated the benefit of adjuvant therapy in improving the OS and decreasing the recurrence rate during the first 2 years after surgery. However, in this study, there were issues with quality of surgery performed as 54% of cases had positive microscopic margins and also 38% had node positive disease which is a subclass that derives maximum benefit from adjuvant therapy[36].

Adjuvant chemoradiotherapy

In view of a 25%-68% rate of recurrence in loco-regional basin, researchers have been advocating administration of adjuvant CRT[4,37]. In a study from the United States (SWOG0809 trial), 79 patients with biliary tract cancer were analyzed after receiving adjuvant capecitabine and gemcitabine followed by radiotherapy and concurrent capecitabine. GBC comprised 32% of the study population. The local recurrence at 2 years was 11% with a median OS of 35 mo. In spite of the lack of a control group, this study provided clinicians with a well-supported regimen[38]. However, Fareed *et al* [39] found no survival benefit with adjuvant chemoradiation in resected GBC patients. In a recent multi-institutional analysis, resected GBC patients with high-risk features such as T3/T4 tumor, lymph node positivity, and R1 resection were found to derive maximum benefit after adjuvant therapy[40]. In present times, the data is still insufficient to conclusively advocate adjuvant chemotherapy over chemoradiation in node negative R0 resected patients. However, adjuvant chemoradiation is unanimously considered to be the treatment of choice in patients with R1/2 resection margins[3].

In the absence of standard clinical guidelines, in the current study, all patients with T stage ≥ 2 and/or positive lymph node were advised to receive adjuvant therapy. Three–fourth of all our patients received adjuvant therapy. Estimated 5-year OS rate was 43.5%, which is comparable to that observed in the MSKCC study[15] (Table 4). Historically, the 5-year OS rate after aggressive resection for GBC was 16%[41]. Even after all the advancements in surgical technique and perioperative care, the median survival for patients with stage I-III disease was 12.9 mo and 5.8 mo for those presenting with stage IV disease in the absence of multimodality treatment at MSKCC in 2008 with improvement in survival after increase in administration of systemic therapy[15,42]. Our study showed a better median survival for stage III and IV cases with multimodality treatment (27 mo and 20 mo, respectively). When comparing early stage disease (stages I and II) with locally advanced stage GBC (stages III and IV), the former had a significantly better survival (73.1 *vs* 41.4 mo, respectively, $P < 0.0001$), which emphasizes on the need for better chemotherapeutic regimen as well as uniform application of systemic therapy in the adjuvant setting.

Our study is one of the largest studies worldwide reporting improved outcomes following multimodality treatment in surgically resected patients. In wake of the scarcity of data on multimodality management of GBC, our study highlights the feasibility of better outcomes with proper utilization of systemic therapy with surgery to obtain optimum results. Correlation between specific chemotherapy regimens and survival is beyond the scope of this study due to its retrospective nature. Despite inherent limitations with potential biases, our study stresses on the urgent need for conducting randomized trials to form consensus on tackling an aggressive disease like GBC. In future, addition of genomic profiling-guided targeted therapy may potentially improve the survival and personalize the therapy of GBC patients.

CONCLUSION

GBC is an aggressive malignancy which warrants equally aggressive measures to provide patients with a meaningful survival. With addition of systemic therapy to

curative surgery, the 5-year survival rate in our study was 43%. R+ resection, higher T stage, and PNI adversely affected the outcome. Patients with higher stage (III/IV), nodal involvement, and high-risk features should be considered for systemic therapy in addition to surgery to optimize the outcomes. Multimodality treatment of GBC has a potential to improve the survival of these patients.

ARTICLE HIGHLIGHTS

Research background

Gallbladder cancer (GBC) is an aggressive biliary tract cancer with only 10% of cases amenable to resection at presentation with a dismal overall 5-year survival of less than 15% after surgery. Even after surgical resection, overall survival (OS) has been poor due to high rates of recurrence. With advancements in surgical approach and systemic therapy, multimodality approach has a potential to obtain favorable outcomes in this aggressive disease; however, there is a paucity of data in the literature for its uniform application.

Research motivation

In the management of patients with GBC, adoption of a multimodality approach should be considered.

Research objectives

The research purpose was to share our experience and give an overview on multimodality management of GBC patients.

Research methods

All the data of patients undergoing surgery for suspected GBC from January 2012 to December 2018 was retrieved from a prospectively maintained electronic database and analyzed.

Research results

Multimodality treatment significantly improved the 5-year survival of patient with GBC. Microscopically positive resection margin, higher T stage, and perineural invasion adversely affected the outcome.

Research conclusions

Gallbladder cancer has a favorable survival when treated with multimodality approach. Patients with high-risk features may particularly benefit from this approach

Research perspectives

Multimodality treatment of GBC has a potential to improve the survival of GBC patients.

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ORIGINAL ARTICLE

Observational Study

In-hospital mortality of hepatorenal syndrome in the United States: Nationwide inpatient sample

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Abstract

BACKGROUND

Hepatorenal syndrome (HRS) is a life-threatening condition among patients with advanced liver disease. Data trends specific to hospital mortality and hospital admission resource utilization for HRS remain limited.

AIM

To assess the temporal trend in mortality and identify the predictors for mortality among hospital admissions for HRS in the United States.

METHODS

We used the National Inpatient Sample database to identify an unweighted sample of 4938 hospital admissions for HRS from 2005 to 2014 (weighted sample of 23973 admissions). The primary outcomes were temporal trends in mortality as well as predictors for hospital mortality. We estimated odds ratios from multi-level mixed effect logistic regression to identify patient characteristics and treatments associated with hospital mortality.

RESULTS

Overall hospital mortality was 32%. Hospital mortality decreased from 44% in 2005 to 24% in 2014 ($P < 0.001$), while there was an increase in the rate of liver transplantation ($P = 0.02$), renal replacement therapy ($P < 0.001$), length of hospital stay ($P < 0.001$), and hospitalization cost ($P < 0.001$). On multivariable analysis, older age, alcohol use, coagulopathy, neurological disorder, and need for mechanical ventilation predicted higher hospital mortality, whereas liver transplantation, transjugular intrahepatic portosystemic shunt, and abdominal paracentesis were associated with lower hospital mortality.

CONCLUSION

Although there was an increase in resource utilizations, hospital mortality among patients admitted for HRS significantly improved. Several predictors for hospital mortality were identified.

Key Words: Hepatorenal syndrome; Liver transplantation; Mortality; Nationwide; Big data; Hospitalization; Outcomes; Predictors

Core Tip: In this study, we utilized the national inpatient sample database to assess the temporal trend in mortality and identify predictors for mortality among hospital admissions for hepatorenal syndrome in the United States. We demonstrated that the overall hospital mortality was 32%. Hospital mortality decreased from 44% in 2005 to 24% in 2014. There was an increase in the rate of liver transplantation, renal replacement therapy, length of hospital stay, and hospitalization cost.

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INTRODUCTION

Hepatorenal syndrome (HRS) is a serious complication of cirrhosis with an incidence as high as 32% among patients with advanced liver disease[1-7]. Previous studies have consistently demonstrated high morbidity, mortality, and resource utilizations[1,8-17]. Several factors have been associated with poor outcomes, including high model for end stage liver disease (MELD) score[18], degree of acute kidney injury (AKI)[11,19], extrahepatic organ failure[20], and sepsis[18,21].

In recent decades, there have been significant advances in knowledge, treatment, and optimal management of patients with HRS[1,7-17,22-24]. While terlipressin, a synthetic vasopressin analog with predominant vasopressin 1A receptor effect[25], has been used to treat HRS in many Asian and European countries, it is currently not yet available in the United States for the treatment of HRS[1,26]. Thus, currently available treatment options for HRS in the United States include albumin volume expansion, octreotide with or without midodrine, and intravenous cardiovascular medications like vasopressin and norepinephrine[1]. Nevertheless, there have been improvements in the overall care for patients with HRS, including liver transplantation and renal replacement therapy. In addition, several studies have suggested the use of transjugular intrahepatic portosystemic shunt (TIPS) for patients with HRS[27-29]. However, data specific to HRS, hospital mortality trends, and hospital admission resource utilization remain limited.

In this study, we aimed to evaluate patient characteristics, in-hospital treatments, mortality, and resource utilization during hospital admissions for HRS in the United States. We also assessed the temporal trend in mortality and identified the predictors for mortality.

MATERIALS AND METHODS

Study population

We conducted a retrospective cohort study of hospital admissions for HRS from 2005 to 2014 in the national inpatient sample (NIS) database. The detail of the NIS database was previously described[30]. We identified hospital admission with a primary discharge diagnosis using the international classification of disease-9 (ICD-9) diagnosis code of 572.4. The Mayo Clinic institutional review board approved this study (IRB number 21-007353 and date of approval; July 27, 2021) and exempted the need for informed consent because the data in NIS database was publicly available and de-identified.

Data collection

We abstracted patient and hospital characteristics, procedures, outcomes, and resource utilization from the database (Supplementary Table 1). Patient characteristics included age, sex, race, etiology of liver disease, medical comorbidity based on Elixhauser index [31], and admission day. Hospital characteristics included hospital size, ownership, location, teaching status, and region. Procedures included renal replacement therapy, liver transplantation, TIPS, abdominal paracentesis, and mechanical ventilation. Outcomes included hospital mortality, resource utilization, including length of hospital stay, and hospitalization cost. Since this study used data over 10 different calendar years, we adjusted hospitalization costs for inflation using the consumer price index and converted them to 2014 United States dollar equivalents.

Statistical analysis

The NIS database contains hospitalization data from a stratified sample of 20% of hospitals in the United States. As such, we used discharge weight provided by the Healthcare Cost and Utilization (HCUP) to estimate the total number of hospital admissions for HRS. We used descriptive statistics to summarize patient and hospital characteristics, procedures, outcomes, and resource use of HRS admission. We fitted logistic regression model for hospital mortality and liver transplantation, and standard least square linear regression for length of hospital stay, and hospitalization cost, using calendar years as the independent variable to assess the annual trend from 2005 through 2014. We estimated adjusted odds ratio (OR) for hospital mortality from multivariable multi-level mixed effect logistic regression, employing hospital identification number as random effect with patients-level characteristics clustered within hospital-level characteristics. We performed all statistical analyses using STATA, version 15 (StataCorp LP, College Station, TX, United States).

RESULTS

Patient characteristics, in-hospital treatments, outcomes, and resource use in hospital admission for HRS

There were 4938 hospital admissions with HRS as the primary diagnosis in the unweighted sample and 23973 admissions in the weighted sample. Table 1 shows patient and hospital characteristics of hospital admissions for HRS. The mean age was 58.8 ± 12.3 years, and the majority of patients were males (63%). Alcohol-related liver disease (46%) and viral hepatitis (25%) were the most common liver disease etiologies. Most patients were admitted to large urban teaching hospitals. Of those patients admitted for HRS, 21% received renal replacement therapy and 2% underwent liver transplant during their hospitalization. During this 10-year period, there was a 32% mortality observed for HRS admissions. The mean length of hospital stay was 8.8 d and the mean hospitalization cost was 73731 United States dollars.

Trends in hospital mortality, liver transplantation, length of stay, hospitalization cost in hospital admission for HRS

Table 2 showed the annual trend in hospital mortality, liver transplantation, length of hospital stay, and hospitalization cost in HRS admissions from 2005 to 2014.

There was a decreasing trend in hospital mortality from 44% in 2005 to 24% in 2014 among hospital admissions for HRS in the United States (OR: 0.92, 95%CI: 0.90-0.94 *per* year; $P < 0.001$) (Figure 1A).

Meanwhile, there was an increase in the rate of liver transplantation (OR: 1.11, 95%CI: 1.02-1.20 *per* year; $P = 0.02$) (Figure 1B) and renal replacement therapy (OR: 1.05, 95%CI: 1.02-1.08 *per* year; $P < 0.001$) (Figure 1C) performed in hospitalization for HRS.

There was an increasing trend in mean length of hospital stay (coefficient estimate 0.2 d *per* year; $P < 0.001$) (Figure 1D) and hospitalization cost (coefficient estimate 5778 United States dollars *per* year; $P < 0.001$) (Figure 1E) among hospitalization for HRS during 10-year period from 2005 to 2014.

Predictors for hospital mortality

In multivariable analysis (Table 3), older age (OR: 1.45 for 40-59 years, 1.77 for 60-79 years, 2.12 for ≥ 80 years, compared to 18-39 years; all $P < 0.001$), alcohol use (OR: 1.35; $P < 0.001$), coagulopathy (OR: 1.15; $P = 0.001$), and presence of a neurological disorder (OR: 1.38; $P < 0.001$) predicted higher hospital mortality.

Need for mechanical ventilation (OR: 9.24; $P < 0.001$) was associated with higher mortality, whereas liver transplantation (OR: 0.15; $P < 0.001$) and TIPS (OR: 0.23; $P < 0.001$), and abdominal paracentesis (OR: 0.48; $P < 0.001$) were associated with lower hospital mortality. Renal replacement therapy was not significantly associated with mortality risk.

DISCUSSION

In this study based on a large United States database of hospitalizations, the mortality rate for hospitalized patients with HRS decreased by approximately 50% during the 10-year study period. During the same period, there was a 2-fold increase in the incidence of HRS patients receiving a liver transplant and the incidence of in-hospital renal replacement therapy increased by 60%. Notably, there were also increase in length of hospital stay and a 2-fold increase in the estimated hospital cost, which is likely related to higher utilization of healthcare resources. This highlights the high economic burden of chronic liver disease in the United States[32,33].

The marked improvement in the in-hospital mortality rate for HRS is likely reflective of changes in both medical and surgical management during the study period. Our study shows that there was an apparent increase in the number of liver transplants and renal replacement therapy around 2007 to 2008. This trend coincided with overall changes in clinical practice over the preceding years[34]. Although the unique pathophysiology of HRS has long been recognized as a functional renal failure occurring as a result of advanced liver disease[35], its treatment, including the initiation of in-hospital dialysis, and the role for liver transplantation have significantly evolved[36]. Historically, the initiation of renal replacement therapy in patients with HRS was felt to be controversial and futile. Increasing experience with liver transplantation in the setting of HRS as well as improved access to continuous renal

Table 1 Patient characteristics, in-hospital treatments, outcomes, and resource use in hospital admission for hepatorenal syndrome (mean ± SD)

	Unweighted, *n* (%)	Unweighted % ± SE	Weighted, *n* (%)	Weighted % ± SE
Total, *n* (%)	4938		23973	
Sex				
Male	3130	63.39 ± 0.68	15183	63.33 ± 0.31
Female	1808	36.61 ± 0.68	8790	36.67 ± 0.31
Age (yr)		58.8 ± 12.3		58.8 ± 12.3
18-39	266	5.39 ± 0.32	1299	5.42 ± 0.15
40-59	2461	49.84 ± 0.71	11933	49.77 ± 0.32
60-79	1927	39.02 ± 0.69	9365	39.06 ± 0.31
≥ 80	284	5.75 ± 0.33	1376	5.74 ± 0.15
Race				
White	3098	72.23 ± 0.68	15050	72.12 ± 0.31
Black	421	9.81 ± 0.45	2055	9.85 ± 0.21
Hispanic	511	11.91 ± 0.49	2495	11.95 ± 0.22
Asian/Pacific islander	81	1.89 ± 0.21	395	1.89 ± 0.09
Native American	57	1.33 ± 0.17	280	1.34 ± 0.07
Other	121	2.82 ± 0.25	593	2.84 ± 0.11
Admission day				
Weekday	3955	80.09 ± 0.57	19223	80.18 ± 0.26
Weekend	983	19.91 ± 0.57	4751	19.82 ± 0.26
Liver disease etiology				
Alcoholic liver disease	2249	45.54 ± 0.71	10935	45.61 ± 0.32
Viral hepatitis	1218	24.66 ± 0.61	5915	24.67 ± 0.28
Comorbidities				
Diabetes Mellitus	1260	25.52 ± 0.62	6132	25.58 ± 0.28
Hypertension	1937	39.23 ± 0.69	9437	39.36 ± 0.32
Fluid/electrolyte disorders	3548	71.85 ± 0.64	17233	71.88 ± 0.29
Coagulopathy	2115	42.83 ± 0.70	10286	42.90 ± 0.32
Anemia	1937	39.23 ± 0.69	9422	39.30 ± 0.31
Weight loss	872	17.66 ± 0.54	4255	17.75 ± 0.25
Cancer	658	13.32 ± 0.48	3198	13.34 ± 0.22
Congestive heart failure	630	12.76 ± 0.47	3042	12.69 ± 0.21
Chronic pulmonary disease	613	12.41 ± 0.47	2973	12.40 ± 0.21
Obesity	456	9.23 ± 0.41	2218	9.25 ± 0.19
Neurological disorders	234	4.74 ± 0.30	1147	4.78 ± 0.14
Pulmonary circulation disorders	176	3.56 ± 0.26	847	3.53 ± 0.12
Valvular disease	164	3.32 ± 0.25	795	3.32 ± 0.12
Peripheral vascular disorders	119	2.41 ± 0.22	587	2.45 ± 0.10
Depression	413	8.36 ± 0.39	2002	8.35 ± 0.18
HIV/AIDS	36	0.73 ± 0.12	173	0.72 ± 0.05
Substance use				

Smoking	583	11.81 ± 0.46	2825	11.78 ± 0.21
Alcohol	1930	39.08 ± 0.69	9394	39.19 ± 0.31
Drug use	209	4.23 ± 0.29	1023	4.27 ± 0.13
Bed size				
Small	611	12.30 ± 0.50	2898	12.09 ± 0.21
Medium	1210	24.41 ± 0.66	5905	24.63 ± 0.28
Large	3117	63.28 ± 0.74	15171	63.28 ± 0.31
Location/Teaching status				
Rural	651	13.89 ± 0.53	3167	13.21 ± 0.22
Urban, non-teaching	1723	36.76 ± 0.74	8320	34.70 ± 0.31
Urban, teaching	2564	49.35 ± 0.77	12487	52.08 ± 0.32
Hospital region				
Northeast	984	20.24 ± 0.62	4817	20.09 ± 0.26
Midwest	1122	23.03 ± 0.65	5406	22.55 ± 0.27
South	1699	34.03 ± 0.73	8261	34.46 ± 0.31
West	1133	22.70 ± 0.64	5489	22.90 ± 0.27
Medical procedures/interventions				
Renal replacement therapy	1018	20.61 ± 0.58	4929	20.56 ± 0.26
Paracentesis	2226	45.08 ± 0.71	10843	45.23 ± 0.32
Mechanical ventilation	499	10.10 ± 0.43	2412	10.06 ± 0.19
TIPS	46	0.93 ± 0.14	218	0.91 ± 0.06
Liver transplantation	85	1.68 ± 0.18	404	1.68 ± 0.08
LTA	66	1.34 ± 0.16	321	1.34 ± 0.07
SLKT	19	0.38 ± 0.09	93	0.39 ± 0.04
Outcomes				
Mortality	1573	31.90 ± 0.66	7616	31.81 ± 0.30
Length of hospital stay (d)		8.8 ± 10.9		8.8 ± 11.0
Hospitalization cost (United States $)		735701 ± 135526		73731 ± 135876

SE: Standard error; HIV/AIDS: Human immunodeficiency virus/acquired immunodeficiency syndrome; TIPS: Transjugular intrahepatic portosystemic shunt; LTA: Liver transplant alone; SLKT: Simultaneous liver-kidney transplantation.

replacement have resulted in a change in practice and a decrease in mortality[37].

In 2007-2008, multiple randomized control trials on terlipressin were published and have influenced the medical management HRS as well as patient outcomes[9-11,14,15]. Studies have shown potential beneficial effects of terlipressin, a potent selective splanchnic and extrarenal vasoconstrictor, on kidney function among patients with HRS[10,38,39]. Additionally, non-response to vasoconstrictors can also predict HRS mortality[40,41]. Unfortunately, as of 2020, the FDA has not yet approved the use of terlipressin for HRS in the United States. Results from the phase 3 trial terlipressin did not show any significant survival benefit and its use was associated with adverse events, such as respiratory failure[42,43]. Although terlipressin is currently not yet available in the United States[1,26], the observed findings of decreasing mortality trends for HRS in the Unites States are likely due to improvements in healthcare, increased access and acceptance of chronic intermittent hemodialysis for patients with liver disease as well as increased acceptance of liver transplantation for patients with acute decompensation[44].

In addition to liver transplantation, our study interestingly showed that TIPS and abdominal paracentesis were associated with lower hospital mortality among patients with HRS. Possible mechanisms underlying reduced mortality among patients who received paracentesis were that those who had abdominal paracentesis received more

Table 2 The annual trend in hospital mortality, liver transplantation, renal replacement therapy, length of hospital stay, hospitalization cost in hepatorenal syndrome admission from 2005 to 2014 (mean ± SD)

Year	Unweighted sample[1]	Weighted sample[1]	Hospital mortality weighted % ± SE	Liver transplantation weighted % ± SE	Renal replacement therapy weighted % ± SE	Length of stay (d)	Hospital cost (United States $)
Total	4931	23941	31.8 ± 0.7	1.7 ± 0.2	20.6 ± 0.6	8.8 ± 11.0	73731 ± 135876
2005	312	1471	43.8 ± 2.8	0.7 ± 0.5	13.8 ± 2.0	8.2 ± 8.6	42857 ± 67978
2006	330	1551	40.8 ± 2.7	0.6 ± 0.4	13.6 ± 1.9	7.2 ± 7.6	41841 ± 67254
2007	287	1358	36.7 ± 2.9	1.1 ± 0.6	17.0 ± 2.2	8.0 ± 8.9	49879 ± 77833
2008	367	1737	37.1 ± 2.5	1.9 ± 0.7	21.7 ± 2.2	8.6 ± 9.4	65419 ± 109901
2009	486	2363	31.1 ± 2.1	2.2 ± 0.7	20.8 ± 1.8	8.9 ± 11.3	71737 ± 123006
2010	610	2973	31.1 ± 1.9	0.9 ± 0.4	21.3 ± 1.7	9.3 ± 13.7	69778 ± 106971
2011	628	2934	30.3 ± 1.9	1.9 ± 0.5	23.3 ± 1.7	8.6 ± 9.6	82917 ± 154746
2012	603	3015	31.2 ± 1.9	2.0 ± 0.6	21.9 ± 1.7	9.1 ± 13.5	74951 ± 113671
2013	622	3110	28.3 ± 1.8	2.7 ± 0.7	21.4 ± 1.6	9.4 ± 12.1	95671 ± 210352
2014	686	3430	24.1 ± 1.6	1.7 ± 0.5	22.4 ± 1.6	9.0 ± 9.0	90829 ± 149495
P value			< 0.001	0.02	< 0.001	< 0.001	< 0.001

[1]Sample of hepatorenal syndrome patients having complete data on mortality status.
SE: Standard error.

aggressive treatments such as albumin and vasopressors, TIPS, and liver transplantation than those who received palliative care. Furthermore, abdominal paracentesis may have led to the diagnosis and treatment for spontaneous bacterial peritonitis[45]. The use of TIPS in patients with HRS remains controversial, although there is increasing data suggesting there may be benefit[24,29]. According to current best practice recommendations, the presence of HRS is not an absolute contraindication for TIPS and the presence of other indications, such as ascites, should guide decision making[29]. Specific to this topic, there is a clear need for additional randomized controlled trials, however, in the interim, there are an increasing number of small studies demonstrating positive outcomes in select HRS patients receiving TIPS[24,46,47]. Since mortality in patients with HRS undergoing TIPS is driven mainly by poor liver function it may be possible that there was a population selection bias and these patients had initially better liver function resulting in better survival.

Our study also showed several risk factors associated with in-patient mortality for HRS. These factors include advanced age, history of alcohol use, coagulopathy and presence of a neurological disorder. It is well known that older age, coagulopathy, and neurological disorder are associated with poor outcomes in patients with HRS[11,18-21]. Hepatic encephalopathy is known to be associated with mortality[48], and thus this could be the underlying reason for association between neurological disorder and increased in-patient mortality for HRS. Although specific knowledge regarding the duration and timing of alcohol use prior to hospitalization is a limitation of this dataset, active alcohol use is a known decompensating event that can result in AKI and HRS. It is also possible that recent alcohol use prevented certain patients from being suitable for liver transplantation. In this foreseeable scenario, initiation of renal replacement therapy has increasingly been used as a bridge to liver transplant eligibility and liver compensation.

There are several limitations in our study. The NIS is a hospitalized database. Thus, we did not evaluate the long-term outcomes of HRS following hospitalization. Although our study showed a decreasing trend of in-hospital mortality rates, it should not be generalized to the overall survival of patients with HRS. Estimates of in-hospital mortality do not include deaths that occur after discharge. The database did not contain MELD score, which predicted mortality in HRS patients[48]. In addition, treatment of HRS was not assessed in this study[40,41]. Data on medications including midodrine, octreotide, vasopressor, albumin infusion were not available in the database. Thus, we could not assess the effects of these agents and the response to

Table 3 Clinical characteristics associated with in-hospital mortality

Characteristics	Univariable analysis		Multivariable analysis	
	Unadjusted OR (95%CI)	*P* value	Adjusted OR (95%CI)	*P* value
Female sex	0.96 (0.85-1.09)	0.52	0.91 (0.78-1.07)	0.27
Age (yr)				
18-39	1 (ref)	-	1 (ref)	-
40-59	1.21 (0.91-1.60)	0.19	1.45 (1.28-1.64)	< 0.001
60-79	1.24 (0.93-1.65)	0.14	1.77 (1.68-1.87)	< 0.001
≥ 80	1.68 (1.17-2.42)	0.005	2.12 (1.51-3.00)	< 0.001
Race				
White	1 (ref)	-	1 (ref)	-
Black	1.38 (1.11-1.71)	0.003	1.26 (0.91-1.75)	0.16
Hispanic	1.05 (0.86-1.29)	0.61	1.12 (0.78-1.61)	0.53
Asian/Pacific Islander	1.44 (0.91-2.27)	0.12	1.30 (0.98-1.73)	0.07
Native American	1.04 (0.59-1.84)	0.88	1.11 (0.86-1.43)	0.43
Other	0.88 (0.59-1.32)	0.55	0.93 (0.48-1.81)	0.84
Weekend admission	1.14 (0.98-1.32)	0.08	1.05 (0.82-1.34)	0.69
Liver disease etiology				
Alcohol-related	0.90 (0.80-1.01)	0.08	0.85 (0.71-1.01)	0.06
Viral hepatitis	0.98 (0.86-1.13)	0.83	1.00 (0.81-1.24)	1.00
Comorbidities				
Smoking	0.96 (0.79-1.16)	0.66	1.15 (0.83-1.60)	0.40
Alcohol use	0.98 (0.87-1.11)	0.79	1.35 (1.26-1.45)	< 0.001
Drug use	0.84 (0.62-1.14)	0.26	0.77 (0.55-1.08)	0.13
HIV/AIDS	1.02 (0.51-2.07)	0.95	0.81 (0.58-1.13)	0.22
Autoimmune arthritis	1.10 (0.64-1.91)	0.73	1.14 (0.54-2.41)	0.72
Congestive heart failure	1.05 (0.88-1.26)	0.59	0.99 (0.87-1.12)	0.84
Chronic pulmonary disease	1.00 (0.84-1.21)	0.96	0.95 (0.78-1.16)	0.63
Coagulopathy	1.01 (0.90-1.15)	0.82	1.15 (1.16-1.25)	0.001
Diabetes mellitus	0.78 (0.67-0.89)	< 0.001	0.87 (0.73-1.04)	0.12
Hypertension	0.76 (0.67-0.86)	< 0.001	0.83 (0.70-1.01)	0.06
Lymphoma	1.42 (0.68-2.96)	0.35	1.53 (0.42-5.60)	0.52
Fluid/electrolyte disorders	0.85 (0.74-0.97)	0.02	0.87 (0.75-1.01)	0.07
Cancer	1.34 (1.13-1.59)	0.001	1.40 (0.88-2.23)	0.15
Neurological disorders	1.29 (0.98-1.70)	0.07	1.38 (1.21-1.58)	< 0.001
Obesity	0.87 (0.70-1.07)	0.20	0.92 (0.62-1.38)	0.70
Peripheral vascular disorders	0.78 (0.51-1.18)	0.23	0.78 (0.42-1.46)	0.44
Psychoses	0.80 (0.55-1.16)	0.24	0.93 (0.78-1.12)	0.44
Pulmonary circulation disorders	0.75 (0.53-1.05)	0.10	0.68 (0.43-1.08)	0.11
Valvular disease	0.75 (0.53-1.07)	0.12	1.01 (0.64-1.60)	0.96
Weight loss	0.91 (0.78-1.07)	0.27	1.05 (0.97-1.13)	0.21
Medical procedure				
Renal replacement therapy	0.98 (0.85-1.14)	0.81	0.92 (0.68-1.25)	0.59

Liver transplantation	0.33 (0.23-0.46)	< 0.001	0.15 (0.11-0.21)	< 0.001
TIPS	0.40 (0.18-0.90)	0.03	0.23 (0.12-0.43)	< 0.001
Paracentesis	0.46 (0.41-0.53)	< 0.001	0.48 (0.43-0.53)	< 0.001
Mechanical ventilation	6.97 (5.66-8.59)	< 0.001	9.24 (7.90-10.81)	< 0.001

HIV/AIDS: Human immunodeficiency virus/acquired immunodeficiency syndrome; TIPS: Transjugular intrahepatic portosystemic shunt; OR: Odds ratio; 95%CI: 95% confidence interval.

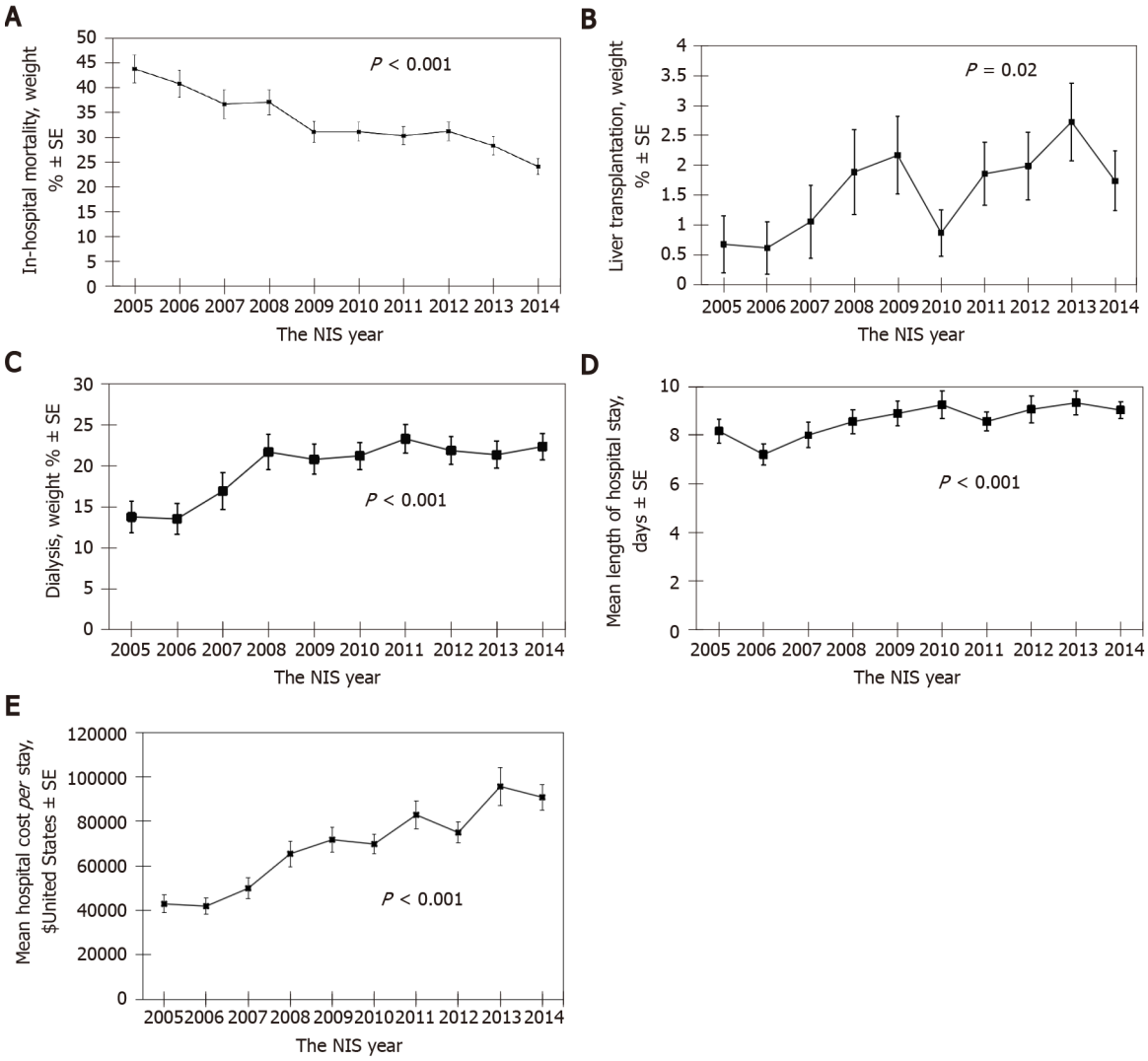

Figure 1 Data on admissions in the United States due to hepatorenal syndrome. A: Decreasing trend in hospital mortality among hospital admissions; B: Increase in the rate of liver transplantation among hospital admissions; C: Trend of renal replacement therapy among hospital admissions; D: Trend of mean length of hospital stay among hospital admissions; E: Trend of hospitalization cost among hospital admissions. NIS: National inpatient sample.

treatments on the outcomes of HRS. Lastly, HRS was identified by ICD-9 diagnosis code. Given definition of the HRS has changed over the years, these changes in definition may have affected the incidence of HRS in our study overtime.

CONCLUSION

In summary, our study showed a decreasing trend of in-hospital mortality rates in patients with HRS. These trends were likely related to advances in medicine, increased access and acceptance of renal replacement therapy, and increased utilization of liver transplantation which is the definitive treatment for HRS. Future studies are needed to

understand if these trends are impacted by other factors such as facility performance, patient care teams, health insurance reimbursement policies, or other factors.

ARTICLE HIGHLIGHTS

Research background

Hepatorenal syndrome (HRS) is a serious complication of cirrhosis, associated with high morbidity, mortality, and resource utilizations. In recent decades, there have been significant advances in knowledge, treatment and optimal management of patients with HRS.

Research motivation

There has been improvement in overall care for patients with HRS. Data on trends of hospital mortality and resource utilization in hospital admissions for HRS were limited.

Research objectives

We aimed to evaluate patient characteristics, in-hospital treatments, mortality, resource use among hospital admissions for HRS s in the United States. We also assessed the temporal trend in mortality and identified the predictors for mortality.

Research methods

We used the national inpatient sample database to identify unweighted sample of 4938 hospital admissions primarily for HRS from 2005 to 2014 (weighted sample of 23973 admissions). The primary outcome was the temporal trend in and predictors for hospital mortality. We estimated odds ratio from multi-level mixed effect logistic regression to identify patient characteristics and treatments associated with hospital mortality.

Research results

The overall hospital mortality was 32%. Hospital mortality decreased from 44% in 2005 to 24% in 2014 ($P < 0.001$), while there was an increase in the rate of liver transplantation ($P = 0.02$), renal replacement therapy ($P < 0.001$), length of hospital stay ($P < 0.001$), and hospitalization cost ($P < 0.001$). Multivariable analysis older age, alcohol abuse, coagulopathy, neurological disorder, and need for mechanical ventilation predicted higher hospital mortality, whereas liver transplantation, TIPs, and abdominal paracentesis were associated with lower hospital mortality.

Research conclusions

Although there was an increase in resource utilizations, hospital mortality among hospital admissions for HRS significantly improved.

Research perspectives

These trends were likely related to increased utilization of liver transplantation which is the definitive treatment for HRS. Future studies are needed to understand if these trends are impacted by other factors such as facility performance, patient care teams, health insurance reimbursement policies, or other factors.

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CASE REPORT

Clinical presentation of gastric Burkitt lymphoma presenting with paraplegia and acute pancreatitis: A case report

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Abstract

BACKGROUND

The incidence of gastric Burkitt lymphoma (BL), presenting as paraplegia and acute pancreatitis, is extremely low. BL is a great masquerader that presents in varied forms and in atypical locations, and it is prone to misdiagnosis and missed diagnosis. The prognosis of BL remains poor because of the difficulty in early diagnosis and the limited advances in chemotherapy.

CASE SUMMARY

A 53-year-old man was referred to our hospital from the local county hospital due to abdominal pain for two weeks and weakness in the lower extremities for one day. Magnetic resonance imaging of the abdomen and lumbar spine showed a swollen pancreas and gallbladder, with peripancreatic exudation and liquid collection, indicating acute pancreatitis and acute cholecystitis. Additionally, we observed abnormally thickened lesions of the gastric wall, multiple enlarged retroperitoneal lymph nodes and a well-demarcated, posterolateral extradural mass lesion between T9 and T12, with extension through the spinal foramen and definite bony destruction, suggesting metastasis in gastric malignancy. Subsequent whole-body positron emission tomography/computed tomography examination showed multifocal malignant lesions in the stomach, pancreas, gallbladder, bone, bilateral supraclavicular fossa, anterior mediastinum, bilateral axillary and retroperitoneal lymph nodes. Gastroduodenal endoscopy revealed primary BL with massive involvement of the gastric body and duodenum. The patient refused chemotherapeutic treatment and died one week later due to upper gastrointestinal hemorrhage. Afterward, we reviewed the characteristics of 11

patients with BL involving the stomach, pancreas or spinal cord.

CONCLUSION
Clinicians should be aware that BL can be the potential cause of acute pancreatitis or a rapidly progressive spinal tumor with accompanying paraplegia. For gastric BL, gastroscopy biopsies and pathology are necessary for a definite diagnosis.

Key Words: Burkitt lymphoma; Paraplegia; Acute pancreatitis; Case report

Core Tip: The incidence of Burkitt lymphoma (BL) is extremely low, and the clinical symptoms are atypical. The misdiagnosis rate is high, and the patient's prognosis is poor. The patient in this case was eventually diagnosed with BL involving the stomach, pancreas and vertebral column presenting with acute pancreatitis and neurological symptoms secondary to compression of the spinal cord. Chemotherapeutic treatment was refused by the patient, and he eventually died after one week due to upper gastrointestinal hemorrhage. This case reminds us that further transcriptomic and clinical studies are needed to explore desirable biomarkers for early BL. Eleven cases were reviewed with an emphasis on diagnostic criteria and treatment protocols. Clinicians need to raise awareness of BL and reduce misdiagnosis rates.

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INTRODUCTION

Burkitt lymphoma (BL) is a subgroup of high-grade non-Hodgkin's lymphoma (NHL) with an aggressive clinical course that was first described as a clinical entity in children in Central Africa by Denis Burkitt in 1958[1]. Clinically, patients with BL often present with solid tumors or large lymph nodes or symptoms similar to acute leukemia, and bone marrow invasion is present in more than 25% of cases[2]. BL has been classified into three subtypes according to the World Health Organization classification: Sporadic type, endemic type and immunodeficiency-associated type[3]. Endemic BL is most prevalent in children from equatorial Africa and New Guinea. Approximately 50% of endemic BL affects the jaw or kidneys. This endemic subtype could also occur in the distal ileum, cecum, greater omentum, ovaries and breasts. Nearly all cases are associated with Epstein-Barr virus[4]. Sporadic BL most commonly affects children[5] but represents less than 1% of NHL cases among adults[6]. Most sporadic BL occurs in the bowel, respiratory tract-associated lymphoid tissue and gut-associated lymphoid tissue. Immunodeficiency-associated BL is most frequently present in Human Immunodeficiency Virus-positive patients[4]. BL is highly sensitive to chemotherapy. Despite the long-term treatment-related sequelae of patients with BL treated with high-intensity chemotherapy regimens, patients who tolerate highly intensive combination chemotherapy regimens tend to have excellent oncologic outcomes. Currently, most treatment protocols for adult patients are based on pediatric clinical trials, and treatment-related toxicities remain a major barrier for those with advanced age. Hence, the overall prognosis for adult patients remains dismal[7]. Due to the rapid proliferation of BL, early diagnosis is essential for the effective treatment of BL. Until recently, BL was diagnosed mainly on the basis of clinical presentation, histopathological changes, morphology, immunophenotype and genotype. There have been few reports on adult patients with sporadic BL, especially adult patients with severe involvement of the stomach, pancreas and spinal cord. Herein, we report a case of gastric BL in an adult patient presenting with paraplegia and acute pancreatitis, along with a review of the literature.

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CASE PRESENTATION

Chief complaints

A 53-year-old male patient was admitted to the hospital with abdominal pain for two weeks and weakness in the lower extremities for one day.

History of present illness

This patient was admitted to the local hospital because of epigastric pain after alcohol consumption. He described the pain as intermittent, non-radiating and worsening with food consumption. The patient denied nausea, vomiting, constipation, fever or progressive weight loss. Based on abdominal pain, elevated levels of serum amylase, and findings of peripancreatic exudation and effusions by computed tomography (CT), the patient was diagnosed with acute pancreatitis. The patient was treated with antibiotics, proton pump inhibitors, fasting and short-term intravenous feeding and fluid therapy, and the abdominal pain was alleviated slightly. Unfortunately, on the 14th d of hospitalization, this patient developed a sudden onset of aconuresis and paraplegia. He was referred to our hospital for further examination.

History of past illness

The patient reported no remarkable history of past illness.

Personal and family history

There was no family history of malignant tumors.

Physical examination

The patient's vital signs were stable. No superficial lymphadenopathy was palpable. Regarding the pulmonary and cardiac examination, no obvious abnormality was observed. The abdomen was flat and soft. Physical examination revealed epigastric tenderness without rebound tenderness or Murphy's sign. No jaundice or palpable masses were observed. Neurologic examination revealed no abnormality in his cranial nerves. The muscle strength of the upper limbs was normal, while it was grade I in the lower limbs. Deep tendon reflexes in the affected limbs were diminished or absent. Bilateral Babinski signs were positive. Hypoesthesia beneath the T8 sensory dermatome was observed. Meningeal irritation signs were negative. He also showed bladder-urinary dysfunction.

Laboratory examinations

The auxiliary examination at admission showed that the white blood cell count was 14.68 × 10^9/L (normal range, 3.5 × 10^9/L - 9.5 × 10^9/L), RBC count was 3.95 × 10^9/L (normal range, 4.3 × 10^9/L - 5.8 × 10^9/L), HGB was 136.0 g/L (normal range, 130-175 g/L), PLT count was 324 × 10^9/L (normal range, 100 × 10^9/L-350 × 10^9/L), C-reactive protein was 34.64 mg/L (normal range, 0-6 mg/L), procalcitonin was 0.12 ng/mL (normal range, 0-0.05 ng/mL), serum amylase was 266 U/L (normal range, 0-125 U/L), lactic dehydrogenase (LDH) was 526 U/L (normal range, 71-231 U/L), and uric acid was 799 μmol/L (normal range, 71-231 μmol/L). Laboratory tests showed no abnormalities in liver function or electrolytes. His carbohydrate antigen 19-9 was 461.28 U/mL (normal range, 0-35 U/mL), and carbohydrate antigen 12-5 was 126.90 U/mL (normal range, 0-35 U/mL). Other tests revealed normal tumor marker levels, including carcino-embryonic antigen and alpha fetoprotein levels of 0.56 ng/mL (normal range, 0-5 ng/mL) and 2.6 ng/mL (normal range 0-8.1 ng/mL), respectively.

Imaging examinations

A CT scan at admission showed a swollen pancreas and gallbladder, with peripancreatic exudation and liquid collection, indicating a diagnosis of acute pancreatitis and acute cholecystitis. Magnetic resonance imaging (MRI) of the abdomen and lumbar spine at the 14th d after admission showed a swollen pancreas and gallbladder, with less peripancreatic exudation and liquid collection, indicating the remission of acute pancreatitis and acute cholecystitis. Additionally, MRI showed abnormally thickened lesions of the gastric wall, multiple enlarged retroperitoneal lymph nodes and a well-demarcated, posterolateral extradural mass lesion between T9 and T12, with extension through the spinal foramen and definite bony destruction (Figures 1 and 2). Whole-body positron emission tomography-CT (PET-CT) was then performed and showed multifocal malignant lesions in the stomach, pancreas, gallbladder, bone, bilateral supraclavicular fossa, anterior mediastinum, bilateral axillary and retroperitoneal

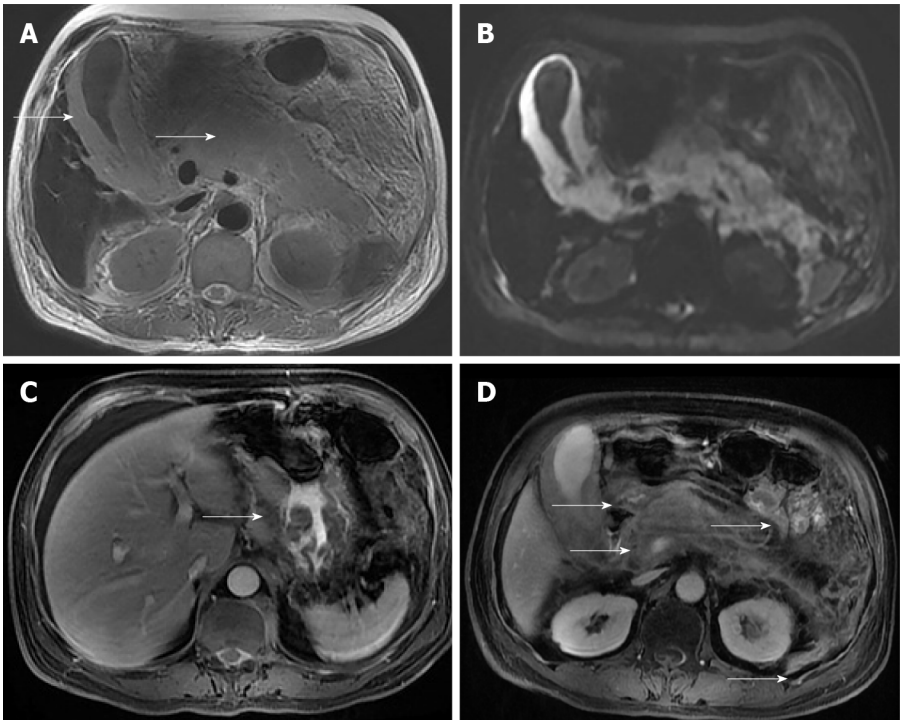

Figure 1 Magnetic resonance imaging of the abdomen at diagnosis. A: Axial T2-weighted magnetic resonance imaging (MRI) demonstrates homogeneous, hyperintense lesion in the whole pancreas and a markedly swollen gallbladder (arrows); B: Diffusion-weighted MRI shows abnormal hyperintensity in gall bladder wall and pancreas; C: Axial contrast-enhanced T1-weighted MRI shows the abnormal thickened lesions of the gastric wall (arrows), which display contrast enhancement in a × homogeneous fashion; D: Axial contrast-enhanced T1-weighted MRI shows the swollen gallbladder and multiple enlarged retroperitoneal lymph nodes (arrows), which display contrast enhancement in a homogeneous fashion.

lymph nodes (Figure 3), indicating multiple metastases of malignant tumors. Gastroduodenal endoscopy revealed massive involvement of the gastric body and duodenum with tumors (Figure 4). Histology and immunohistochemistry of gastric biopsies were suggestive of BL (Figure 5).

FINAL DIAGNOSIS

The histological findings, immunophenotype of the biopsies, and radiological findings were consistent with BL involving the stomach, pancreas and vertebral column. However, the primary lesion of BL is unclear. Because the patient had no symptoms of fever or weight loss, it was classified as group A. Due to the lack of bone marrow aspirate and trephine biopsy, we could not confirm the accuracy of the stage classification of BL in this case. Curiously, this patient presented with acute pancreatitis as the initial manifestation. One possible explanation for the presentation of acute pancreatitis is that the main pancreatic duct was obstructed by the substantial mass. Obstruction of the pancreatic orifice may impair the outflow of pancreatic juice and eventually induce pancreatitis.

TREATMENT

After admission to our department, this patient received short-term fasting, acid suppression, pancreatic enzyme suppression and fluid replacement for acute pancreatitis. Due to suspicion of necrotic pancreatitis, sulbactam sodium/cefoperazone sodium (3 g/d) was administered IV for one week.

Unfortunately, this patient developed sudden onset of aconuresis and paraplegia. According to the neurology consultation, acute myelitis was suspected. To inhibit the inflammatory response and block the antibodies, high doses of glucocorticoids and gamma globulin were applied for three days. Nevertheless, the efficacy of these treatments appeared poor. Concerning the high cost and potential side effects of these treatments, glucocorticoid and gamma globulin treatment was abandoned. Based on

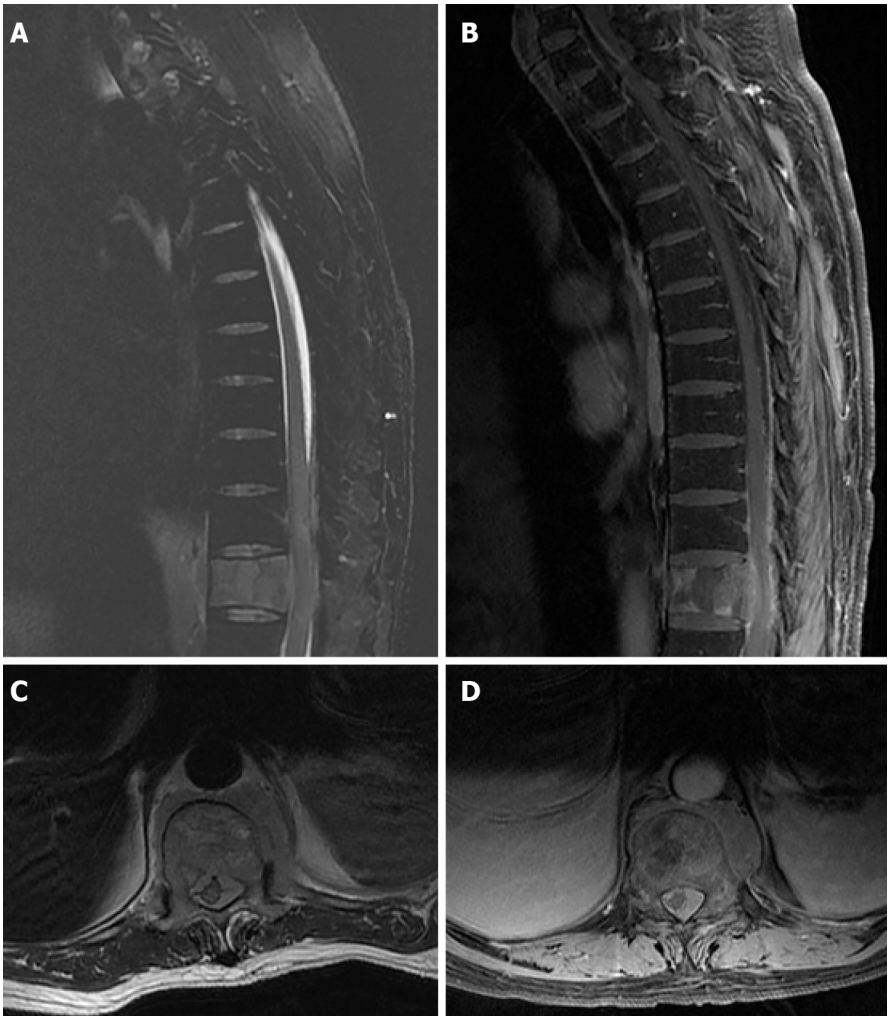

Figure 2 Magnetic resonance imaging of the thoracic and lumbar vertebrae at diagnosis. A: Sagittal T2-weighted magnetic resonance imaging (MRI) shows epidural mass at the centrum and left posterolateral aspect of the spinal cord at the T9 to T12 levels, resulting in severe cord compression; B: Sagittal contrast-enhanced T1-weighted MRI shows the lesions displaying contrast enhancement in a heterogeneous fashion; C: Axial T2-weighted MRI shows that epidural mass involves the centrum and left posterolateral aspect of the spinal cord; D: Axial contrast-enhanced T1-weighted MRI shows the lesions displaying contrast enhancement in a heterogeneous fashion.

the indication for further imaging tests, this patient was diagnosed with gastric BL *via* endoscopic biopsy. Accordingly, a chemotherapy combination of cyclophosphamide, adriamycin, vincristine and prednisolone (CHOP) was recommended for the patient, but he refused the chemotherapeutic treatment.

OUTCOME AND FOLLOW-UP

One week after diagnosis and refusal of chemotherapy, the patient died of upper gastro-intestinal hemorrhage.

DISCUSSION

The incidence of BL is extremely low, and the clinical symptoms are atypical. Thus, we need to raise awareness of BL and reduce the misdiagnosis rates. BL was first described in 1958 by a British surgeon named Denis Burkitt as a sarcoma involving the jaw in African children with characteristic symptoms[1]. There has been some improvement in the understanding of its epidemiological diagnosis and treatment in the ensuing half century. In this article, we report the 11th case of BL involving the stomach, pancreas and spinal cord diagnosed based on the radiological findings and immunophenotype of the biopsies. The clinical features of 10 previous cases of BL

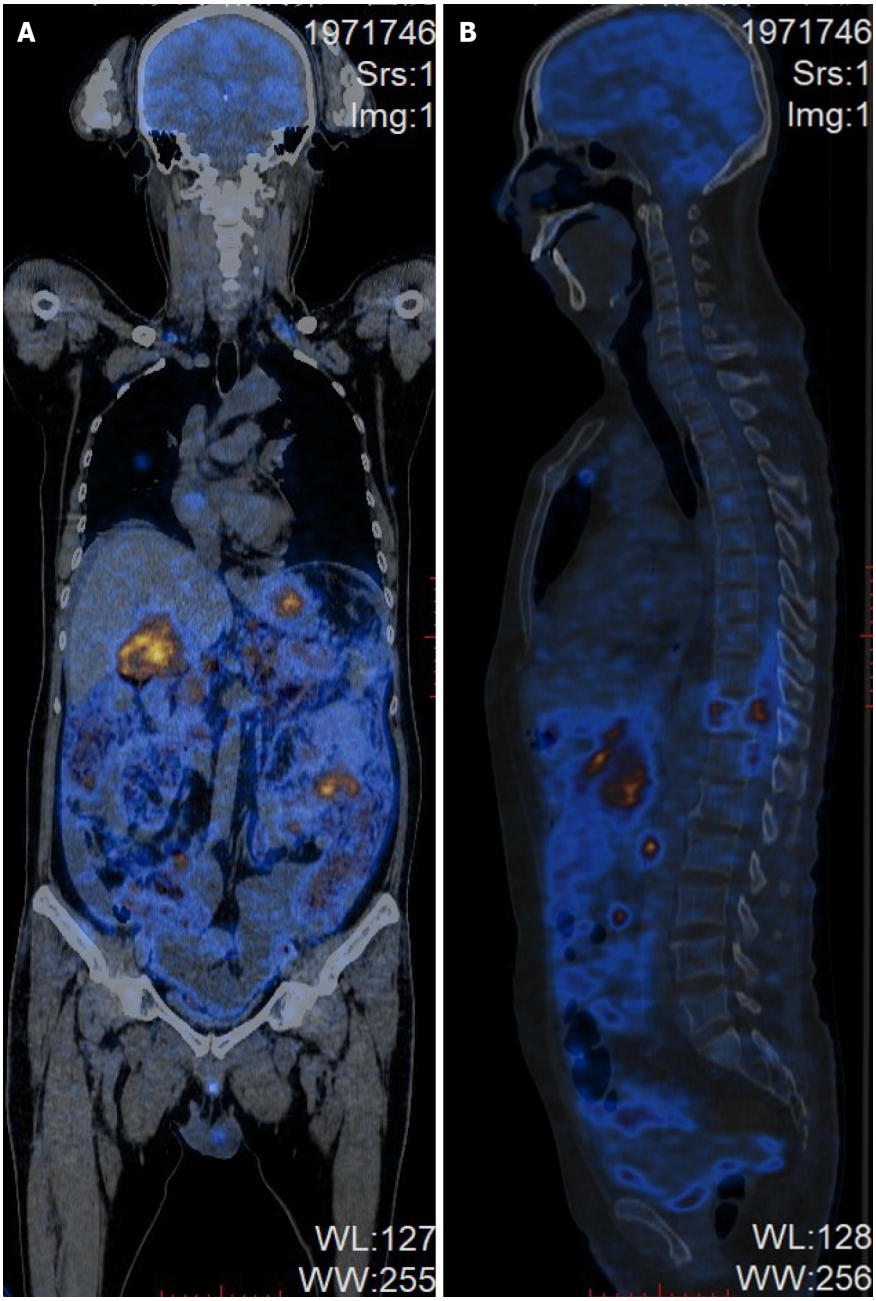

Figure 3 Positron emission tomography-computed tomography of the whole body at diagnosis. A: Coronal images; B: Sagittal images.

involving the stomach, pancreas or spinal cord are summarized in Table 1[8-17].

Among the previous cases, nine cases were reported in foreign countries, while only one patient came from China. From our review of the literature, a clear male predominance (70%) can be established. The ages of the patients range from 9 to 69 years, with a median age of 23 years. The initial symptoms, including abdominal distension, abdominal pain, lumbago, weakness in the lower extremities, fulminant hematemesis and progressive weight loss, are atypical. Regarding the detailed treatment protocols, seven patients received chemotherapy. Only one patient received palliative radiation treatment due to severe spinal cord involvement. Among the four patients who underwent surgical intervention, one patient underwent surgery for intraspinal decompression and mass separation, and the other three patients underwent distal or total gastrectomy. The outcome and follow-up of BL were reported in a total of eight cases. Regrettably, only a 9-year-old patient remained in clinical remission with completed chemotherapy, and no treatment-related sequelae 4 years were observed from initial diagnosis. Severe complications, including gastric perforation, sepsis and bacteremia, are always derived from intensive chemotherapy. Due to lymphoma recurrence or severe complications associated with chemotherapy, the other seven patients died within 6 mo of diagnosis.

Table 1 Clinical features of 10 previous cases of Burkitt lymphoma involving stomach, pancreas or spinal cord

Author	Age	Gender	Initial symptom	Affecting area	Biopsy area	Immunohistochemical studies	EB	HIV	Treatment	Prognosis
Kim *et al*[8]	69	Female	Low back pain radiating down to the right leg	Spinal cord at the L2 to L4 levels, intestine, live, bone and left supraclavicular lymph node	A posterolateral extradural mass lesion between L2 and L3	CD20 (+), CD79a (+), BCL-6 (+), CD10 (+), BCL-2 (-)	+	NA	NA	NA
Seo *et al*[9]	40	Male	Progressive pain and weakness in lower extremities	Spinal cord at the T2 to T4 levels, liver	An intraspinal extramedullary mass from T2 to T4, liver	CD20 (+), CD45RO (-)	NA	+	Chemotherapy and radiation therapy with HAART after surgery for intraspinal decompression and mass separation. Radiation	Died by massive pulmonary thromboembolism at 13 wk postoperatively
Chieng *et al*[10]	9	Male	Progressive pallor, peripheral oedema and respiratory distress	Stomach	Gastric body mass	CD20 (+), CD10 (+) and CD43 (+)	NA	NA	Induction chemotherapy with COP. Further chemotherapy included two courses of COPADAM followed by two courses of CYM and double intrathecal chemotherapy of methotrexate and hydrocortisone	Remains in clinical remission with complete resolution of the protein-losing enteropathy and no treatment related sequelae 4 yr from initial diagnosis
Bolandparvaz *et al*[11]	21	Male	Abdominal pain	Stomach	A huge mass in greater curvature of the stomach	NA	NA	NA	Total gastrectomy and roux-en-y esophagojejunostomy, chemotherapy was given for the patient 1 wk later without any other complication	NA
Gurzu *et al*[12]	60	Female	Fulminant hematemesis, recurring melena, epigastric pain, inappetence, and weight loss	Stomach	A huge mass in the antrum and posterior wall of the gastric body	CD20 (+), CD79a (+), BCL-6 (+), CD10 (+), Ki-67 (100%+), CD3 (-), CD5 (-), CD23 (-), TdT (-), bcl-2 (-), and Cyclin D1 (-)	-	NA	Distal gastrectomy	Died ten days after surgical intervention
Krugmann *et al*[13]	28	Male	Hematemesis and increasing abdominal pain	Stomach	A huge mass in the middle third of the stomach	CD20 (+), CD10 (+), BCL-6 (+), Ki-67 (95%+), CD3 (-), CD5 (-), CD23 (-), Cyclin D1 (-), BCL-2 (-) and TdT (-)	-	NA	Billroth-II surgical resection	Died due to lymphoma recurrence four months after onset
Liao *et al*[14]	26	Male	Fulminant hematemesis, abdominal pain	Stomach	A mass in the body and antrum of the stomach	CD20 (+), CD10 (+), BCL-6 (+), MUM-1 (-), CD30 (-)	NA	NA	Induction chemotherapy with two courses of R-ECHOP. Further chemotherapy included two courses of R-hyper CVAD followed by five courses of intrathecal prophylactic injection of chemotherapy drugs	Lymphoma recurrence six months after onset
Sağlam *et al*[15]	20	Male	Weight loss, back pain, mandible numbness, night sweats, and poor exercise tolerance	The body of the pancreas	A mass in the body of the pancreas	NA	NA	NA	Doxorubicin based combination chemotherapy	Died from sepsis during the second month of chemotherapy
Nistala *et al*[16]	21	Male	Jaundice, increasing swelling in	The head of the pancreas, cystic duct,	The first and second parts of	CD20 (+), CD10 (+), BCL-6 (+), CD5 (-), Mib-1 (99%+)	NA	NA	Two cycles of CHOP followed by hyper CVAD regimen as	NA

			the epigastric region	portal vein and hepatic artery, duodenum	duodenum				definitive therapy	
Konjeti *et al* [17]	68	Female	Belching, abdominal bloating and weight loss	The head of the pancreas, central hepatic duct and portal vein	The pancreatic head mass	CD20 (+), CD10 (+), C-myc (+), BCL-6 (+), CD3 (-), TdT (-), BCL-2 (-), Ki-67 (> 90%+)	NA	NA	Two cycles of chemotherapy regimen consisting of etoposide, prednisone, vincristine (Oncovin), and doxorubicin hydrochloride (Hydroxydaunorubicin hydrochloride)	Die due to the sepsis and bacteremia

EB: Epstein-Barr virus; HIV: Human Immunodeficiency Virus; HAAART: Highly active antiretroviral therapy; COP: Cyclophosphamide, vincristine and prednisolone; COPADAM: Cyclophosphamide, vincristine, prednisone, cytarabine, doxorubicin and methotrexate; CYM: Cytarabine and methotrexate; R-ECHOP: Rituximab, etoposide, cyclophosphamide, doxorubicin, vincristine and prednisone; CVAD: Cyclophosphamide, vincristine, doxorubicin, dexamethasone; CHOP: Cyclophosphamide, doxorubicin, vincristine and prednisolone; NA: Not available.

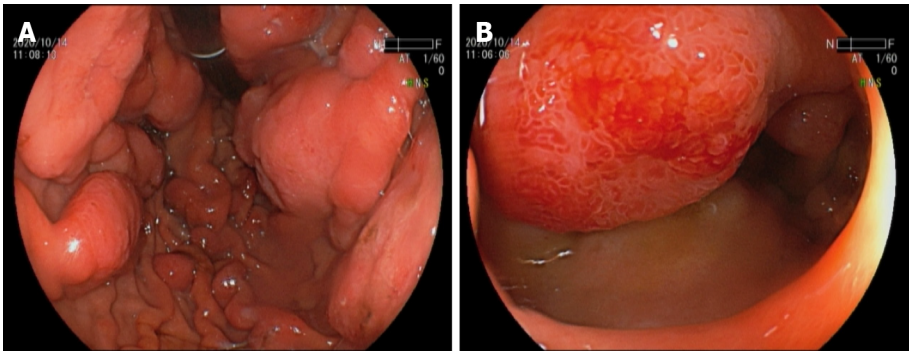

Figure 4 Gastric endoscopy. A: Multiple large (2 to 3 cm in diameter) raised ulcerated tumors involving both the greater and smaller curvatures of the gastric body; B: Numerous smaller tumors involving the anterior wall of the duodenal bulb and the second part of duodenum.

Three variants of BL have been described worldwide: Endemic, sporadic, and immunodeficiency-associated. Among the three subtypes, sporadic BL is regarded as the most common type[18]. The clinical features of BL are variable. In endemic BL, patients tend to present with jaw and other facial diseases. Cases of sporadic BL with an intraperitoneal mass as the initial manifestations are more common. Additionally, the clinical course of sporadic BL is usually aggressive, with frequent extranodal and central nervous system (CNS) involvement and an overall poor prognosis. BL with extranodal involvement usually occurs in the gastrointestinal tract (50%) and head and neck (25%). According to the statistics, CNS involvement is recognized in 13%-17% of all cases of BL[19]. This kind of cancer cell proliferates rather rapidly, with a doubling time of approximately 24 h, and the Ki-67 proliferation index tends to be 90%-100%. Clinically, a blood test usually reveals markedly elevated LDH and uric acid levels in the early stages, indicating a high tumor burden[20]. Herein, we report a case of gastric BL in an adult patient presenting with paraplegia and acute pancreatitis. Similarly, the auxiliary examination in this case also showed markedly elevated LDH and uric acid levels at admission. During hospitalization, this patient developed acute compression of the spinal cord. Abdominal CT at admission revealed no apparent abnormal findings except for the indication of acute pancreatitis. Unexpectedly, MRI of the abdomen and lumbar spine at the 14th d after admission indicated multisite metastasis in gastric malignancy, including in the pancreas, bone, bilateral supraclavicular fossa, anterior mediastinum, bilateral axillary and retroperitoneal lymph nodes. Finally, gastroduodenal endoscopy revealed massive involvement of the gastric body and duodenum with BL. Dawson's criteria are used to label primary gastrointestinal lymphoma, including absence of peripheral lymphadenopathy at the time of presentation, lack of mediastinal lymph node enlargement, normal total and differential white blood cell count, predominance of bowel lesion at the time of laparotomy with only lymph nodes obviously affected in the immediate vicinity and no lymphoma involved in the liver and spleen[21]. In this case, the patient had leukocytosis and multiple enlarged retroperitoneal lymph nodes and therefore did not fulfil the criteria. Hence, it was not a case of primary gastric lymphoma and the primary lesion of BL is unclear. This case reminds us that malignant tumors can originate from hematopoietic

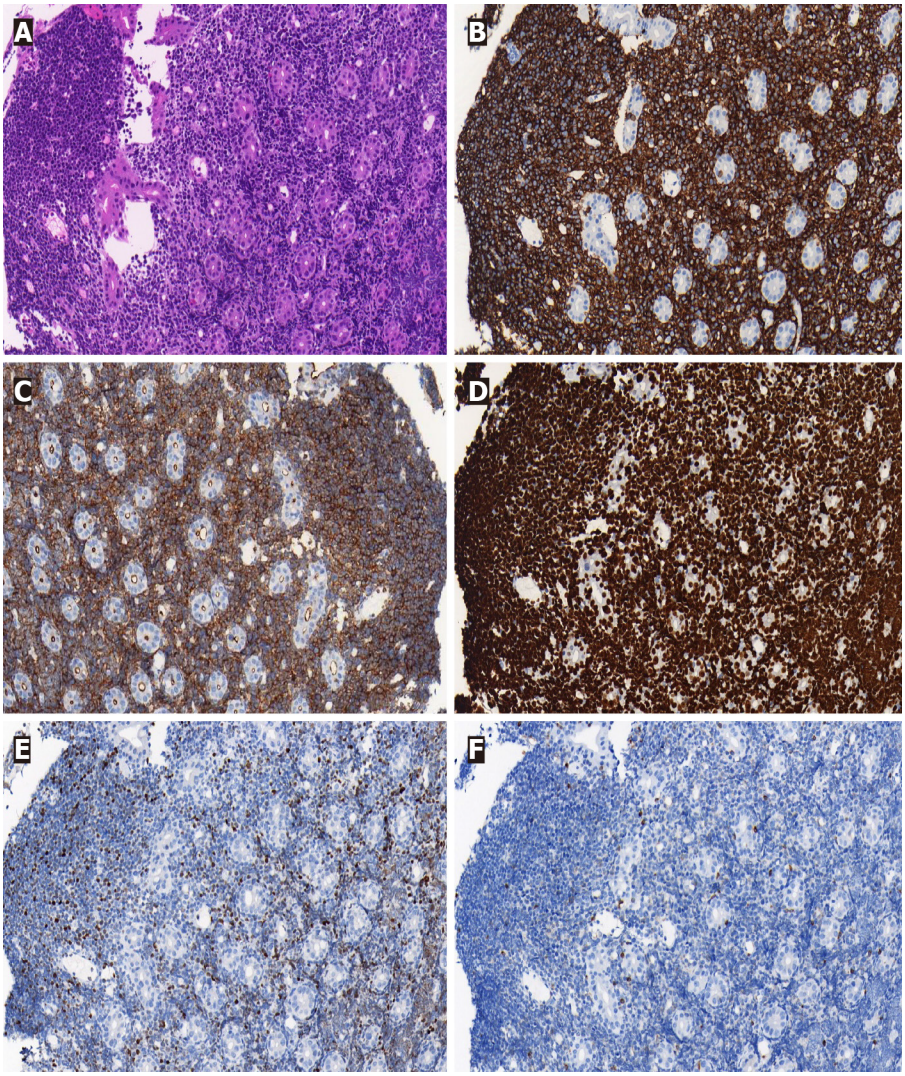

Figure 5 Histology and immunohistochemistry of gastric biopsies (× 200). A: Haematoxylin and eosin staining showed a characteristic "starry sky" appearance; B: Immunohistochemical staining was positive for CD20; C: Immunohistochemical staining was positive for CD10; D: Immunohistochemical staining was positive for Ki-67 (> 90% +); E: Immunohistochemical staining was positive for BCL-6; F: Immunohistochemical staining was negative for BCL-2.

malignancies, especially BL, and that this needs to be taken into consideration when there is abnormally rapid progression of the disease and when there are numerous affected areas or markedly elevated indicators of tumor burden.

Regarding the imaging evaluation of BL, CT scanning and three-dimensional reconstruction are more useful for accurately displaying bone destruction. When spinal cord involvement is suspected for clinical reasons, the preferred choice is MRI since it outperforms CT in depicting associated soft tissues. Additionally, diffusion-weighted MRI is a favorable diagnostic tool in oncologic imaging since it can reflect cellularity and proliferative activity in most malignancies. It is acknowledged that most malignancies are characterized by sustained proliferation, contributing to a high cellular density. More specifically, on diffusion-weighted MRI, BL demonstrates a markedly high signal intensity due to the relative restriction of water associated with high cellular density[22,23]. Since repeated serial imaging is essential for evaluating disease progression, MRI is also superior to CT due to its lack of ionizing radiation. For superior staging and assessment of the treatment response, PET/CT is a better choice since it can evaluate the functional status of abnormally hypermetabolic tissues throughout the whole body[24].

Histologically, the tumor cells of BL are medium-sized with an abundant, basophilic cytoplasm and display the typical "starry sky" pattern. The tumor cells are positive for BCL-6, CD19, CD20, CD22, CD10 and CD79a but negative for CD3, CD5, CD23 and TdT[25]. BL is characterized by the t (8; 14) (q24; q32) translocation of the c-myc and IgH genes, resulting in IgH-myc fusion, which can be detected by molecular analysis *via* fluorescence in situ hybridization. In our case, the tumor cells were negative for

creatine kinase and CD3, indicating that the tumor was not derived from the epithelium or T-cells. Additionally, the tumor cells were positive for CD20, CD79a, CD10, and BCL-6, suggesting germinal center-derived B cells. Combined with the high Ki67 index, the diagnosis of BL can be established.

Systemic chemotherapy is the preferred choice for the treatment of BL. Additionally, conventional radiotherapy, surgery, or a combination of both are recommended as the standard treatment unless severe compression of vital organs by lymphoma is observed[26]. Currently, most treatment protocols for adults are based on pediatric clinical trials. At present, most classical chemotherapy regimens show good efficacy and safety in children and relatively young patients. However, the prognosis of adult patients is poor due to their low response rate and severe treatment-related toxicity. Chemotherapy regimens, including CHOP, hyper-cyclophosphamide, vincristine, epirubicin, dexamethasone, etoposide, prednisone, vincristine, cyclophosphamide, epirubicin and cyclophosphamide, epirubicin, doxorubicin, vincristine, high-dose methotrexate/isophosphamide, cytarabine and etoposide, are still the backbone of therapeutic strategies for BL. Rituximab is an anti-CD20 chimeric antibody that acts by depleting CD20-positive B lymphocytes[27]. It has been reported that common chemotherapy regimens combined with rituximab can significantly improve the 3-year overall survival rate of BL patients (83% *vs* 70%)[28]. Treatment with prophylactic intrathecal methotrexate or cytarabine can lower the incidence of CNS relapse. Hence, it is regarded as a part of the first-line treatment option for BL[29,30]. In this case, the patient refused the chemotherapeutic treatment and died of upper gastrointestinal hemorrhage one week after diagnosis.

CONCLUSION

The incidence of BL is extremely low, and the clinical symptoms are atypical, contributing to the high misdiagnosis rate and poor prognosis. Clinically, malignant tumors originating in hematopoietic malignancies, especially BL, need to be taken into consideration if there is abnormally rapid progression of the disease and if there are numerous affected areas or markedly elevated indicators of tumor burden. CT or MRI could be an option for the detection of BL, while PET/CT is essential for the staging of BL. Histological assessment is indispensable for a definite diagnosis. Regarding the treatment of BL, chemotherapy is the preferred choice. Prophylactic intrathecal methotrexate or cytarabine is also recommended to lower the incidence of CNS relapse.

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LETTER TO THE EDITOR

SARS-CoV-2 infection in people with pre-existing liver disease: Further research is warranted

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Abstract

Patients with severe liver disease who have been infected with severe acute respiratory syndrome coronavirus-2 (coronavirus disease 2019) frequently develop acute respiratory distress syndrome and multiple organ failure, with a high mortality rate, as a result of the hyper-proinflammatory state known as the cytokine storm. Clinicians must recognize cytokine storms earlier to avoid intensive care admission and multi-organ damage, a critical life-threatening condition with prognostic and therapeutic implications

Key Words: Cytokine storm; Liver disease; Angiotensin-converting enzyme 2; Therapeutics; Inflammatory markers

Core Tip: Understanding the hepatic consequences of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection and its molecular mechanism has greatly evolved. Evidence suggests that coronavirus disease 2019 fatalities are primarily due to cytokine storm and abnormal immune function. Throughout the infection, interleukin-6, nuclear factor kappa B, and tumor necrosis factor-alpha are inflammatory cytokines released by SARS-CoV-2-infected macrophages and monocytes that cause acute liver injury. Anti-viral treatment with anti-inflammatory receptors, such as monoclonal antibodies, can be used to reduce the morbidity and mortality associated with SARS-CoV-2 infection.

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TO THE EDITOR

Recently we have seen a paper entitled "Impact of cytokine storm and systemic inflammation on liver impairment patients infected by SARS-CoV-2: Prospective therapeutic challenges" contributed by Ali *et al*[1] in your well-regarded journal "*World J Gastroenterology*"[1]. Regarding this paper, we would like to draw your attention to several valuable and interesting aspects. The current scenario is that the second wave of the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) [coronavirus disease 2019 (COVID-19)] pandemic is much more aggressive, with many more cases reported in various countries. As of April 2021, nearly 2.5 million deaths worldwide have been attributed to COVID-19. Based on the geographical distribution of the COVID-19 pandemic, it was found that in areas with a higher frequency, such as China, the rate of SARS-CoV-2 infected patients with liver impairment is also higher [2]. Most hospitalized COVID-19 patients have elevated liver biomarkers, primarily aminotransferase and bilirubin, which cause multi-organ failure[3,4]. This review paper by Ali *et al*[1] shows great public health interest. In their article, the authors elegantly described the impact of SARS-CoV-2 on hepatic impairment conditions. Besides, they focused on several current studies that indicated the role of the hyperinflammatory state that is known as "cytokine storm" concerning the angiotensin-converting enzyme 2 (ACE2) receptor as the main factor for the high rate of SARS-CoV-2 spreading and mortality and its putative therapies[5].

The SARS-CoV-2 directly enters the host cell through surface receptors and binds to ACE2[6]. ACE2 expression has been reported in different normal human organs, including the liver, where its expression is significantly low compared to the duodenum, kidney, and small intestine[7]. Accumulating evidence indicated the hepatic sharing of ACE2 after virus entry into the host cell. The underlying mechanisms of liver injury in COVID-19 patients are currently indistinguishable. However, human liver single-cell RNA-seq data indicated the co-expression of ACE2 and transmembrane serine protease 2 in liver progenitor cells, suggesting that the liver is the target of coronavirus disease[8].

Further, there is a 59.7% increase in ACE2 expression in cholangiocytes compared to 2.6% in hepatocytes, indicating that SARS-CoV-2 may directly bind to the ACE2 receptor, and the liver may be a good host for SARS-CoV-2[9,10]. Histological analysis of liver biopsies of COVID-19 patients revealed moderate microvascular steatosis, mild lobular, portal activity, and T cell overexpression, showing that the liver injury could have been caused by either SARS-CoV-2 infection or treatment[3,11]. A hospital-based study in China revealed elevated levels of proinflammatory cytokines, chemokines, and growth factors in COVID-19 patients compared to healthy adults[12,13]. Further, the patients with severe COVID-19 show hepatic dysfunction or liver disorders, including chronic liver disease, hepatitis viruses (types B, C, D, and E), hepatotropic virus infection, non-alcoholic fatty liver disease (NAFLD), and non-alcoholic steatohepatitis with elevated platelet, neutrophil, and lymphocyte counts, resulting in the worst outcomes from acute respiratory distress syndrome[14,15].

There is no consensus among researchers regarding liver damage in COVID-19 patients; some studies proposed the immediate cytopathic effect of the virus on hepatocytes or the biliary epithelium *via* ACE receptors[16,17]. Others postulated inflammatory and immune-mediated liver failure in patients with multiple organ damage[18]. However, hepatic inflammation involving cytokine activation was well-documented. A case study of COVID-19 patients demonstrated that the C-reactive protein (CRP) of 20 mg/L and a lymphocyte count of 1.1 109/L were independent risk factors for liver injury[19]. Kupffer cell activation is indeed a common finding in the liver of SARS-CoV-2 infected patients. Further, the altered macrophage polarization in SARS-CoV-2-infected patients with NAFLD suggests that SARS-CoV-2 has mechanisms to divert macrophage polarization in their preferred direction and increase the synthesis of inflammatory cytokines[18].

Regardless of the precise definition, the combinations of clinical manifestation and inflammatory markers (such as elevated plasma levels of CRP, lactate dehydrogenase, interleukin (IL)-6, IL-1, tumour necrosis factor-alpha (TNF)-α, and ferritin) could be used to define the "cytokine storm syndrome" in COVID-19 patients[20-22]. Besides this, treatment with anti-IL-6 receptor monoclonal antibodies (sarilumab and

tocilizumab), anti-IL-6 monoclonal antibodies (siltuximab), IL-1 inhibitors (Anakinra, Rilonacept, and Canakinumab), and TNF-α inhibitors (adalimumab, etanercept, and infliximab) showed promising results against SARS-CoV-2-induced cytokine storm[23-25]. In addition, corticosteroids that are known to alter the nuclear factor kappa B pathway central to the cytokine storm were used to manage the severe SARS and Middle East respiratory syndrome patients[26]. As a cytokine storm is a critical life-threatening condition and has prognostic and therapeutic implications, the clinicians must recognize cytokine storms earlier to avoid intensive care admission and multi-organ damage.

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LETTER TO THE EDITOR

Therapeutic potentials of fasudil in liver fibrosis

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Abstract

Fasudil has the potential to prevent liver fibrosis by activating natural killer cells and inhibiting the proliferation of hepatic stellate cells. Fasudil may be a promising clinical therapeutic drug for the prevention and treatment of liver fibrosis.

Key Words: Fasudil; Liver fibrosis; Natural killer cell; Hepatic stellate cell; Clinical therapeutic drug

Core Tip: This letter to the editor is to supplement the ongoing discussion on the therapeutic potentials of Fasudil in the treatment of hepatic fibrosis. Fasudil is potential for the treatment of liver fibrosis through activating natural killer cells and inhibiting the proliferation of hepatic stellate cells. Fasudil, a vasodilator used in clinical treatment of cerebral vasospasm, exhibits the protective and therapeutic effect on liver fibrosis.

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TO THE EDITOR

We read with great interest the recent basic study by Han *et al*[1], that reported Fasudil, a potent RhoA/ROCK inhibitor and vasodilator, prevents and treats liver fibrosis and liver injury. They found Fasudil alleviates thioacetamide (TAA)-induced liver fibrosis in mice. The anti-fibrotic phenotypic exhibition of Fasudil is impressive.

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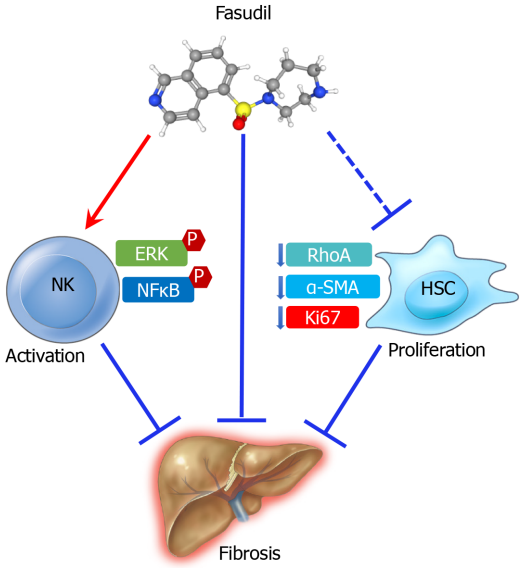

Figure 1 Schematic illustration depicting that fasudi prevents and treats liver fibrosis by activating natural killer cells and inhibiting hepatic stellate cells proliferation. NK: Natural killer; HSCs: Hepatic stellate cells.

Hepatic fibrosis is the formation of scar tissue in response to chronic liver damage, such as chronic hepatitis and hepatic steatosis[2]. Currently, there is no pharmacotherapy available approved by Food and Drug Administration (FDA) in the treatment of liver fibrosis[3]. Fasudil has been approved in Japan and China for the prevention of artery tightening and ischemia caused by cerebral vasospasm and pulmonary hypertension[4]. Due to its safety and efficacy, Fasudil might be a promising clinic agent for the prevention and treatment of liver fibrosis.

Hepatic stellate cells (HSCs) and natural killer (NK) cells play key roles in the pathogenesis of liver fibrosis. They isolated NK cells from mice treated with vehicle, TAA, or TAA and Fasudil and treated the NK-92 cells with different concentrations of Fasudil. These results showed that Fasudil robustly promotes NK cell activation. When discussing the effect of Fasudil on HSCs, they used human stellate cell line LX2 cells and observed that Fasudil directly induces apoptosis and inhibits the proliferation of LX2 cells. LX2 cell is indeed a model for the study of HSC activation. But to investigate HSCs activation, the model of primary HSCs subjected to culture activation and **L**X2 cells subjected to the stimulation of the potent profibrogenic cytokine transforming growth factor-beta 1 (TGF-β1) and then treated with the drugs under study are more widely accepted. Here, the authors proposed that Fasudil inhibited liver fibrosis by blocking HSCs activation by directly using the LX2 cells treated with Fasudil, which is far-fetched and hard to interpret. As primary HSCs are activated by prolonged culture, HSCs isolated from human or mouse livers and treated with the studied drug may be a more comprehensive approach to evaluate HSC activation.

Other studies also showed that Fasudil has anti-fibrotic phenotypic exhibition in rat models of hepatic fibrosis, such as Fasudil alleviated hepatic fibrosis in type 1 and 2 diabetic rats and carbon tetrachloride (CCl_4)-induced rat liver injury[5-7]. Combined with these studies, we proposed that Fasudil is potential for the treatment of liver fibrosis through multitargeted effects, as outlined in Figure 1. Taken together, Fasudi is a promising medication for the prevention and treatment of liver fibrosis.

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LETTER TO THE EDITOR

Diagnostic biomarkers for pancreatic cancer: An update

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Author contributions: Yang M and Zhang CY collected data, wrote, finalized the letter, and contributed equally.

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Abstract

Pancreatic ductal adenocarcinoma accounts for the primary type of pancreatic cancer (PC) with a 5-year survival rate of only about 10% in the United States. Early diagnosis will improve chances for curative treatment. To date, a broadly used serum marker for PC diagnosis is carbohydrate antigen 19-9, which is the only approved biomarker currently by the United States Food and Drug Administration. However, it has low specificity; therefore, development of novel biomarkers is urgently needed. Clinical trials are ongoing to evaluate candidate biomarkers for PC diagnosis, and the use of a multi-biomarker panel with current PC diagnostic biomarkers appears promising.

Key Words: Pancreatic ductal adenocarcinoma; Diagnosis; Biomarkers; Panel; Clinical trials

Core Tip: The development of ideal diagnostic biomarkers for pancreatic cancer (PC) is critically important for early diagnosis, large-scale screening, monitoring of therapeutic response, prediction of risk, and prognosis. So far, the only approved serum marker for PC diagnosis is carbohydrate antigen 19-9 (CA 19-9) in the United States; although, many potential biomarkers have been investigated. However, CA 19-9 has low sensitivity; hence, new solutions are needed. Herein, we summarize some of the ongoing clinical trials that aim to investigate the application of biomarkers in PC diagnosis.

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TO THE EDITOR

We read with great interest a review paper recently published by O'Neill and Stoita [1], reviewing diagnostic biomarkers currently applied in pancreatic cancer (PC). The biomarkers are from serum, urinary, pancreatic, salivary, biliary, and fecal sources and comprise many different types of molecules. For example, serum biomarkers include proteins of glycolipids, growth factors, cytokines, chemokines, adhesion molecules, non-coding RNAs (long non-coding RNAs and microRNAs), and liquid biopsy (exosomes, circulating tumor DNA or ctDNA, and circulating tumor cells or CTCs)[1].

Moreover, we agree with the authors' suggestion that early diagnosis of PC improves chances for curative treatment. PC comprises two main subtypes, including the more common exocrine cancers and less common endocrine cancers. Pancreatic ductal adenocarcinoma (PDAC) accounts for the primary type of PC, consisting of around 95% in exocrine cancers and about 90% in all PCs. The 5-year survival rate of PC is relatively low and was only 10% for all patients with PC in the United States from 2010 to 2016[2]. To date, the only approved serum marker for PC diagnosis is carbohydrate antigen 19-9 (CA 19-9) in the United States, even though it has low specificity[3]. However, CA 19-9 is a non-PC-specific marker, shown to increase in colorectal, liver, lung, and ovarian cancers, as well as desmoplastic fibroblastoma[4,5]. Because of the low specificity of CA 19-9, a multi-marker panel that combines some of the currently investigated biomarkers (with CA 19-9) can be used to improve the specificity and sensitivity of PC diagnosis. For example, a multi-biomarker panel with enzyme-linked immunosorbent assay using three potential biomarkers, leucine-rich alpha-2-glycoprotein 1, transthyretin, and CA 19-9, improved the diagnosis of PDAC in normal pancreas and benign pancreatic disease and other tumors[6]. Although a multi-biomarker panel provides a better approach for early PC diagnosis, some limitations, including cost, the requirement for large sample volumes, good technique and analytical performance, and practical feasibility, may impact their broad application[3,7,8].

In addition, many of the biomarkers discussed in the abovementioned paper, including extracellular matrix-associated proteins such as matrix metalloproteinase and tissue inhibitor of metalloproteinase 1, profibrotic factors such as transforming growth factor-beta, growth factors such as vascular endothelial growth factor, cell-cell interacting protein such as intercellular adhesion molecule 1, and microRNAs such as mi-R21, are not specific markers implicated in many other cancers and diseases[9-12]. Furthermore, germline mutations in genes such as cyclin-dependent kinase inhibitor 2A, tumor protein p53, serine/threonine kinase ATM, MutL homolog 1, and breast cancer 1 and 2 have been significantly associated with PC[13]. The authors also mentioned genetic factors associated with PC, such as *KRAS* in ctDNA and *KRAS* mutation in CTCs. Therefore, genetic mutation or inherited factors may be a predisposing factor for PC and should be considered during the diagnosis.

Finally, this letter summarizes the actively recruiting and completed clinical trials to evaluate diagnostic methods or biomarkers for PC (Table 1). The data were collected from the website https://clinicaltrials.gov (accessed on July 18, 2021) using the keywords biomarkers and PC. Overall, the specificity and sensitivity of PC diagnosis can be increased by using multiple marker panels in combination with CA 19-9 or with novel screened biomarkers. In addition, accuracy, cost-effectiveness, and ease of application together will ensure the broad application of any new diagnostic method.

Table 1 Clinical trials for pancreatic cancer with representative diagnostic biomarkers

Trial number	Biomarkers	Status	Year to complete	Results/Trial titles
NCT03311776	HA and PRO-C3	Completed	2035	Serum HA and PRO-C3 were prognostic for overall survival in patients with PC[14]
NCT04241367	ctDNA	Recruiting	2025	Verification of predictive biomarkers for pancreatic cancer treatment using multicenter liquid biopsy
NCT04143152	sTRA and CA 19-9	Recruiting	2023	Two biomarker panels with sTRA and CA 19-9 improved sensitivity and accuracy, compared to using only CA19-9 [15]
NCT03404661	Methylated DNA markers	Recruiting	2023	Optical and biochemical biomarkers in early pancreatic cancer significance: a prospective study
NCT04584996	CircRNAs	Recruiting	2023	Circular and non-coding RNAs as clinically useful biomarkers in pancreaticobiliary cancers
NCT04636788	Circulating exosomal small RNAs	Recruiting	2022	Diagnostic and prognostic values of EUS-FNA specimens and circulating exosomal small RNA in patients with pancreatic cancer
NCT03536793	Urinary tissue factor and Endo180	Recruiting	2022	Study of uTF and Endo180 as markers of early malignancy in cystic pancreatic lesions
NCT04549064	AREG	Recruiting	2021	Identification of AREG for the detection of pancreatic cancer by the biosensor
NCT03817866	Chromogranin A	Recruiting	2021	To validate the performance of Brahms Chromogranin A II Kryptor assay to monitor the course of disease in patients with well-defined gastroentero-pancreatic neuroendocrine tumors
NCT03214991	DNA	Unknown	2021	Circulating tumor DNA as a prognostic marker in patients with pancreatic cancer
NCT01664169	VEGF-A and VEGF-R2	Completed	2018	Validation of circulating biomarkers using the immunological multiparameter chip technology (IMPACT) platform on plasma specimens collected on CALGB 80303
NCT02974764	Circulating tumor cells	Completed	2018	Alterations in circulating tumor cells predicted the progression of pancreatic ductal adenocarcinoma, treatment response, and clinical outcomes[16]
NCT00674973	AREG, EGF, sHER2, TGF-α	Completed	2015	Exploratory analyses suggested that high AREG might predict progression-free survival in patients with pancreatic cancer treated with erlotinib[17]
NCT01675258	Four messenger RNA biomarkers (*KRAS*, *MBD3L2*, *ACRV1*, and *DPM1*) in salivary samples	Completed	2013	The logistic regression model using four biomarkers yielded an area under the curve value of 0.971 (cutoff 0.433) to detect resectable pancreatic cancer with 90.0% sensitivity and 95.0% specificity[18]
NCT00899158	Caspase-3 and pAkt in muscle, and urinary 3-MH	Completed	2008	Role of caspase-3, phosphatidylinositol-3 kinase, and 3-methylhistidine in the pathophysiology of skeletal muscle loss in weight-losing pancreas cancer patients

ACRV1: Acrosomal vesicle protein 1; AREG: Amphiregulin; DPM1: Dolichyl-phosphate mannosyltransferase subunit 1; EGF: Epidermal growth factor; circRNAs: Circular RNAs; ctDNA: Circulating tumor DNA; HA: Hyaluronan; MBD3L2: Methyl-CpG binding domain protein 3 like 2; pAkt: Phosphorylated Akt; PRO-C3: Propeptide of type III collagen; sHER2: Soluble human epidermal growth factor receptor 2; sTRA: Sialylated tumor-related antigen; TGF-α: Transforming growth factor-alpha; 3-MH: 3-methylhistidine.

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