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OPINION REVIEW

Percutaneous endoscopic gastrostomy – Too often? Too late? Who are the right patients for gastrostomy?

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Abstract

Percutaneous endoscopic gastrostomy is an established method to provide nutrition to patients with restricted oral uptake of fluids and calories. Here, we review the methods, indications and complications of this procedure. While gastrostomy can be safely and easily performed during gastroscopy, the right patients and timing for this intervention are not always chosen. Especially in patients with dementia, the indication for and timing of gastrostomies are often improper. In this patient group, clear data for enteral nutrition are lacking; however, some evidence suggests that patients with advanced dementia do not benefit, whereas patients with mild to moderate dementia might benefit from early enteral nutrition. Additionally, other patient groups with temporary or permanent restriction of oral uptake might be a useful target population for early enteral nutrition to maintain mobilization and muscle strength. We plead for a coordinated study program for these patient groups to identify suitable patients and the best timing for tube implantation.

Key words: Gastrostomy; Nutrition; Dementia; Percutaneous endoscopic gastrostomy; Oncologic diseases; Endoscopy; Neurodegenerative disorders

Core tip: Gastrostomy is an established method for enteral nutrition of patients, but according to our experience and clinical studies, the wrong patients are often supplied with tube feeding. In addition to patients with clear indications, patients with advanced dementia receive gastrostomies for long-term-feeding. More data are needed for indication and timing of tube implantation, not only in demented patients.

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INTRODUCTION

The method of percutaneous endosocopic gastrostomy (PEG) as a tool for enteral nutrition was first described in 1980 by Gauderer *et al*[1]. Since then, PEG has evolved as the method of choice in patients with apparent or imminent long-term restriction of oral nutrition. Gastrostomy is easy to install percutaneously using translucency during gastroscopy. The tube needs some care, which is largely standardized and, if necessary, can be easily removed by simple gastroscopy.

When a technique comes of age, it is time to review its current practice as well as the indications for and complications of this intervention. Is enteral nutrition indeed superior to parenteral nutrition? Are patients who receive a gastrostomy appropriately chosen for this intervention? Do we need more data to assess the usefulness of PEG in certain situations?

ENTERAL *VS* PARENTERAL NUTRITION

There is ample evidence from experimental and clinical studies that enteral nutrition (orally or *via* a tube) confers many positive effects in comparison to parenteral nutrition. These effects include preservation of the intestinal mucosal barrier, reduction of intestinal and other infections and improvement of the overall prognosis of patients with long-term artificial nutrition[2-7]. Additionally, parenteral nutrition requires administration of lipid formulations *via* a port system, which promotes port infections and septic complications. In a meta-analysis comprising almost 4000 patients who had undergone surgery for gastrointestinal (GI) tumors, parenteral nutrition was associated with a significantly higher rate of infectious and noninfectious complications[8]. In a very recent Japanese study, enteral nutrition *via* PEG was associated with a significantly longer survival (median survival of 317 *vs* 195 d) compared to parenteral nutrition in older patients with dysphagia[9]. Therefore, as far as it is technically and functionally feasible, enteral nutrition is preferable to parenteral nutrition. This is also emphasized by the ESPEN guideline for ethical aspects of artificial nutrition, which recommends enteral over parenteral nutrition in order "to support intestinal functions to the greatest possible extent"[10].

COMPLICATIONS AND TYPE OF ACCESS AND TUBE

Several large case series have investigated complication rates in PEG patients. Severe complications during or immediately after gastrostomy are rare (1.8%) and include bleeding, perforation and peritonitis[11]. Late complications occur in approximately 5% of patients and are mostly associated with nursing failures, leading to tube leakage or blockage, mucosal overgrowth of the retaining plate in the stomach ("buried bumper") or aspiration. Mild local infections at the tube insertion site have been reported in approximately 11% of cases[11,12] and require only local treatment. More recent studies have reported severe complications (acute and during feeding) in 3.8%–10% of PEG patients[13,14]. Patients with dementia did not have significantly more complications than those without dementia in one large study[15], but this remains controversial.

To ensure maximal effect of enteral nutrition *via* tube feeding, before gastrostomy, basic considerations are necessary for each individual case to check suitability of the patient and the clinical situation for this intervention (see Table 1). These considerations should also encompass alternative interventions such as metal stents or surgical procedures.

The pull method is the standard procedure for gastrostomy and tube implantation. Since 2000, a push-/introducer-PEG method has also been possible; this method is extremely attractive for patients with pharyngeal or esophageal tumor stenosis precluding gastroscopic access to the stomach[16]. However, in our clinical experience as well as according to existing data, whenever possible, the pull-PEG method should

Table 1 Basic considerations for percutaneous endoscopic gastrostomy implantation and typical access types

Basic considerations for PEG implantation	
Is oral nutrition - for whatever reason - so inadequate that intervention is justified?	
Is enteral nutrition likely to be necessary for at least 3 wk?	
Is the intestine distal to the access path functional?	
Are risk factors for complications absent?	
Is the anatomy suitable for PEG?	
Is compliance sufficient for PEG handling (feeding in (half) upright position, infection prophylaxis, mobilization of the PEG tube, *etc.*)?	
Typical access types	
Pull-PEG (Ponsky-Gauderer)	After diaphanoscopy, primary puncture with a trocar followed by pulling the tube with a thread through the esophagus
Push-/Introducer-PEG (Russell)	With diaphanoscopy, primary gastropexy followed by direct introduction of a balloon-fixed tube

PEG: Percutaneous endoscopic gastrostomy.

be preferred due to lower complication rates and better handling[17,18].

ACCEPTED INDICATIONS FOR GASTROSTOMY

Percutaneous endoscopic gastrostomy has been established as a treatment option for transient or permanent dysphagia due to neurologic disorders, *e.g.*, stroke[19,20]. In the same way, patients with oncological diseases of the mouth and throat as well as the esophagus can benefit from a temporary PEG tube during multimodal therapy, especially during radiotherapy. Ensuring adequate nutrition allows the therapy to be carried out in a timely manner and at full dose by preventing weight loss and, thus, ultimately improves patient prognosis[21] (Table 2).

DEMENTIA – THE MOST DOUBTFUL INDICATION FOR GASTROSTOMY

Patients with degenerative cerebral diseases, above all dementia, have increasingly received gastrostomies and represented in some studies and regions the largest group of tube feeded patients[22-24]. Given the lack of evidence for a benefit in this patient group, this issue generates debates already for decades. In a time with an increasing economic health burden, a necessity to improve the efficiency of health care in an aging society and health care workers often pressed for time, this development is understandable but must be viewed with great skepticism.

Frequently, the indication of gastrostomy is the result of an acute deterioration in the health state and/or expression of a state of emergency in caring for these patients. Occasionally, cultural or religious reasons also play a role when relatives do not approve limiting therapy, although the quality of life is already dramatically reduced, and the prognosis is limited. Sometimes, gastrostomy is advocated because people caring for the patient, including their physicians, are unable to cope with difficult nursing and medical situations.

Comfort feeding[25] is propagated as an alternative to artificial nutrition, but this approach requires more human resources, is very cost-intensive and probably cannot be executed in high numbers in today's care structures. From a practical point of view, it is understandable that gastrostomy is performed to keep processes and personnel structures within affordable limits in a nursing home, but this approach often does not meet the needs of the patient. Eventually, gastrostomy, as well as long-term tube feeding, carry similar risks as other interventional measures[26,27]; additionally, it may detain patients from the pleasures of tasting and of social contacts. Furthermore, advanced dementia patients tend to manipulate access points and tubes and thereby are prone to injure themselves. A risk-benefit analysis is therefore particularly important in any patient group and should be provided to the patient and/or his relatives.

The wish of supporting the nutrition of demented patients using tube feeding leads to a high rate of gastrostomies in patients with already advanced disease. Often these patients already suffer from progressive malnutrition and immobility. In many studies with demented patients, the complication rate of gastrostomy is unacceptably

Table 2 Accepted and data-supported indications for percutaneous endoscopic gastrostomy (for references see text)

Main disease groups	Diagnosis/reason for dysphagia
Cancer	Head and neck cancer
	Pharyngeal cancer
	Esophageal carcinoma
	Cancer with functional bowel obstruction (percutaneous endoscopic gastrostomy used as a decompression measure)
Neurodegenerative disorders	Stroke
	Amyotrophic lateral sclerosis
	Multiple sclerosis
	Severe brain damage from various reasons (trauma, persistent vegetative state, psychomental retardation, *etc*.)

high[28,29]. We and others think that this is more related to patient factors than an innate risk of the intervention[30]. This view is supported by data from studies showing that control patients (with no PEG) had a very similar or even worse mortality[29,31], and patients with only mild dementia had a significant higher benefit than those with advanced dementia[28].

We call this the PEG paradox – choosing the patients too late for the intervention leads to missing benefit and greater harm including higher morbidity and mortality.

A Cochrane systematic review conducted in 2009 did not find a single randomized controlled trial that investigated the benefits of tube feeding in patients with dementia[32]. Consequently, recent guidelines do not encourage gastrostomy in patients with advanced dementia[33], although clear and high-quality data in this clinical field are lacking. Table 3 shows the recent studies that examined the effects of tube feeding in patients with dementia[34-39]. Reviews and meta-analyses[40-42] mostly identified two severe problems of PEG studies in dementia patients. First, no randomized, prospective, properly controlled studies have been conducted. Most available studies have retrospective designs and suffer from a huge selection bias, and control groups are poor or unmatched. Second, in most studies, patients with dementia are not properly staged and are treated as a homogenous patient group. This prevents the identification of subgroups (*e.g*., patients with only mild to moderate dementia) that might benefit from enteral nutrition *via* tube feeding. Other problems include poor exclusion and inclusion criteria, inappropriate outcome measures and small sample sizes[42].

NON-NEUROLOGICAL PATIENT GROUPS WITH POSSIBLE BENEFIT

In our opinion and clinical experience, there are other patient groups in clinical medicine that could benefit significantly from early gastrostomy. Even though it is hardly supported by study data, patients with chronic pancreatitis and pronounced (postprandial) pain syndrome often benefit from tube feeding that prevents weight loss, maintains mobility and physical activity, and thus, improves their quality of life. In our clinical experience, pulmonary cachexia in chronic obstructive pulmonary disease (COPD) patients can also be either avoided or alleviated by early PEG application. Although COPD has been identified as a risk factor for early mortality in patients with a PEG tube for other indications[43], there is not a single study investigating the effect of early enteral nutrition in patients with COPD who manifest cachexia or are at risk for malnutrition. In many cancers, even cancer outside the GI tract such as lung, prostate and hematological tumors, malnutrition is frequent[44] (Table 4). Early and consistent enteral nutrition can enable timely and dose-appropriate chemotherapy and thus improve prognosis, since weight loss is one of the main risk factors for premature death in many cancers[45-47]. At least for the quality of life endpoint, this has already been shown in several studies[48], but proof for hard endpoints such as overall survival is currently lacking.

It is also conceivable that patients with other severe diseases (such as ulcerative reflux disease or severe eosinophilic esophagitis) may also benefit from gastrostomy,

Table 3 Studies of enteral nutrition with dementia patients in recent years

Ref.	Design	Number of patients with dementia	Main results	Study problems/Appraisal
Higaki *et al*[15], 2008	Retrospective cohort study	311 (143 with and 168 w/o dementia)	No significant differences in survival	No controls w/o PEG
Suzuki *et al*[28], 2012	Observational study	1353	Significantly more benefit in patients with early dementia	Endpoint "Level of independent living of demented elderly" not validated, no controls
Ticinesi *et al*[34], 2016	Observational study	184 (54 with PEG, 130 w/o PEG)	Survival with PEG significantly worse	Selection bias, no basic data for PEG-group *vs* non-PEG-group, patients with advanced dementia had better results compared to those with early dementia
Nunes *et al*[35], 2016	Retrospective observational study	46 (only CDR 2 and 3)	Low albumin, transferrin and cholesterol as predictors for poor survival	No controls
Cúrdia *et al*[36], 2017	Prospective cohort study, uncontrolled	26 (out of 60 in the whole cohort)	Significant decrease in hospitalization and visits to ER, > 50% healing of pressure ulcers	Only internal controls, no dementia grading
Ayman *et al*[37], 2017	Retrospective cohort	165, control group with PEG for other reasons	Significantly shorter survival in dementia patients	No dementia control group, no dementia rating
Gingold-Belfer *et al*[38], 2017	Retrospective Cohort, uncontrolled	189	Albumin level associated with longer survival (at baseline as well as during observation)	No control group, no dementia rating
Van Bruchem-Visser *et al*[39], 2019	Retrospective cohort	42 (out of 303 in the whole cohort), no controls w/o PEG	Survival with PEG significantly shorter in patients with dementia	Selection bias, no dementia rating, PEG-indication partly unclear

w/o: Without; ER: Emergency room; PEG: Percutaneous endoscopic gastrostomy.

even if they are young. However, supporting data are lacking. Therefore, physicians are often reluctant to consider gastrostomy in these otherwise healthy and, often, young patients. At present, such decisions must remain extremely individualized. To what extent an intermittent PEG system in this patient population can contribute to the maintenance of a certain body weight and, thus, help to avoid physical weakness should be the subject of future studies. Nevertheless, data regarding the prognosis of such patients with or without enteral nutrition are quite important and economically and individually relevant; for example, for employment biographies.

TIMING OF GASTROSTOMY

In the neurological field, gastrostomy also represents an important therapeutic option for patients with amyotrophic lateral sclerosis (ALS), depending on the overall situation and the preference of these patients[19], who are conscious until their death. Weight loss in these patients is present very often, even without dysphagia[49]. Recent data also indicate that the time of tube insertion should be advanced compared to the current approach[50]. Patients with ALS had a significant better survival if enteral nutrition was initiated before the presence of weight loss[49]. To date, this aspect of the "timing" of gastrostomy has been disregarded. Earlier continuous enteral nutrition has the potential to improve prognosis significantly and should be considered in future studies. "Early" in this respect would mean gastrostomy before the underlying disease (regardless whether neurological or non-neurological) has caused significant malnutrition and weight loss accompanied by catabolism or restricted mobility. Here, the GLIM criteria can play an important role (with the underlying disease as etiologic criterion and a clear cut anticipatory definition of the phenotypic criterion)[51]. Timing of the intervention by such criteria would improve the patient selection and reduce the complication rate. With early gastrostomy, the prevalence of low albumin, higher age and higher comorbidity (all risk factors for worse outcome[29]) would be lower in patients selected for this intervention.

This may close the circle of argumentation in the case of patients with dementia; much more than before, gastroenterologists must also learn to assess patients with

Table 4 Additional patient groups with a lack of data but potential benefit if the timing of gastrostomy is correct

Chronic pancreatitis
COPD with manifest or imminent undernutrition/cachexia
Severe eosinophilic esophagitis
Severe ulcerative reflux disease
Cancer with undernutrition syndrome
(Mild to) moderate dementia

COPD: Chronic obstructive pulmonary disease.

chronic degenerative cerebral diseases. These diseases will increase substantially during the next decades. In patients with very advanced stages of dementia with complete immobility, lack of speech production and contractures, a gastrostomy is probably more harm- than useful. However, patients with early or moderate dementia, for whom we have not thought about enteral feeding so far, could possibly benefit from tube feeding.

Early tube feeding could prevent the progressive immobility of dementia patients and, thus, preserve their quality of life for longer. Data regarding these patients are extremely scarce (see discussion above), but a few subgroup analyses as well as some studies with better defined patient groups support this view[28,36,52]. In a large Japanese study, the selection of patients with early or moderate dementia increased the proportion of patients with a benefit as measured by the level of independent living four times as compared to patients with advanced dementia[28].

However, in studies regarding nutritional support for dementia patients, no general benefits were obtained in cognitive tests[33]. Therefore, while dementia cannot be stopped, mobility and quality of life may be maintained longer. To date, due to this poor data situation, tube feeding and parenteral nutrition have only been recommended "to overcome a crisis situation" and "for a limited time" in the guidelines for this group of patients overall, and not at all or only as "very rare exception" for patients in late stages[33].

CONCLUSION

In our opinion, we must therefore pay attention to the following: Patients with dementia in very advanced stages should no longer be treated with artificial nutrition of any kind. We must explain this to the relatives and referring doctors. We must draw their attention to the data that suggest more and more severe complications in these patients than in less seriously ill patients as well as to the missing benefit for these patients. On the other hand, we may have to think about tube feeding at an earlier stage for patients at nutritional risk due to temporary or chronic restrictions of oral feeding. These patients should be made more consistently aware of the possibility of a gastrostomy before weight loss or even catabolism has occurred. This can affect younger, otherwise completely healthy patients as well as dementia patients in an earlier, still mobile stage.

In summary, while there may not necessarily be a current under- or over-utilization of PEG, there is a need to improve patient selection. To achieve this goal, we need more prospective randomized controlled studies to better define the indications for PEG in the patient groups and conditions outlined above.

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OPINION REVIEW

Can adiponectin have an additional effect on the regulation of food intake by inducing gastric motor changes?

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Abstract

The regulation of food intake is a complex mechanism, and the hypothalamus is the main central structure implicated. In particular, the arcuate nucleus appears to be the most critical area in the integration of multiple peripheral signals. Among these signals, those originating from the white adipose tissue and the gastrointestinal tract are known to be involved in the regulation of food intake. The present paper focuses on adiponectin, an adipokine secreted by white adipose tissue, which is reported to have a role in the control of feeding by acting centrally. The recent observation that adiponectin is also able to influence gastric motility raises the question of whether this action represents an additional peripheral mechanism that concurs with the central effects of the hormone on food intake. This possibility, which represents an emerging aspect correlating the central and peripheral effects of adiponectin in the hunger-satiety cycle, is discussed in the present paper.

Key words: Adipokines; Adiponectin; Adipose tissue; Food intake; Gastric motility; Satiety signals

Core tip: Central structures involved in the regulation of food intake receive and integrate signals from peripheral organs, including white adipose tissue. This paper summarizes the main findings on the influence of adiponectin, a pleiotropic hormone secreted by adipocytes, on food intake. In addition to its central actions, adiponectin has been recently reported to cause gastric motor changes known to be peripheral signals implicated in the hunger-satiety cycle. In this light, we discuss the potential role of adiponectin as a peripheral signal in the signaling network underlying hunger and satiety.

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INTRODUCTION

The regulation of food intake consists of complex signals that allow the maintenance of energy and nutrient balance. Several neurotransmitters that act at the central level are involved in energy homeostasis by regulating food intake and/or energy expenditure. A number of pathological conditions may lead to a loss of this control. This can be observed in obesity, various metabolic diseases or eating disorders, where the signaling systems that underlie appetite control are dysregulated and not yet fully clarified. Therefore, it is important to elucidate the role of any single actor in this extremely complex mechanism that could largely benefit the pathophysiology and treatment of obesity and eating disorders. However, due to the complexity of the physiological mechanisms underlying the regulation of food intake, we attempt to make a simplified synthesis of the main central structures and some of the main peripheral signals involved in the regulation of food intake to provide the reader with a better understanding of the topic discussed.

CENTRAL STRUCTURES INVOLVED IN THE REGULATION OF FOOD INTAKE

In brief, central nervous system structures, the hypothalamus in particular, are able to integrate signals of different nature through afferent and efferent pathways to and from the brainstem as well as peripheral organs[1]. The arcuate hypothalamic nucleus appears to be the most critical area in the integration of such signals, some of which are prevalently involved in the control of energy homeostasis and others related to the short-term regulation of food intake[2]. The arcuate hypothalamic nucleus contains neurons whose activation influences food intake by exerting anorexigenic or orexigenic effects. Particularly, the activation of neurons that express pro-opiomelanocortin (POMC), a precursor for many peptides, leads to a decrease in food intake. In contrast, the stimulation of adjacent neurons, which synthesize neuropeptide Y (NPY) and agouti-related peptide, is known to cause an increase in food intake. These two systems influence each other in opposite ways. The arcuate hypothalamic nucleus represents the main integrative center of signals coming either from other hypothalamic areas involved in feeding behavior (such as the paraventricular nucleus, the dorsomedial nucleus and the lateral nucleus) or from extra hypothalamic areas[3], such as the nucleus tractus solitaris. The latter receives signals from the periphery and transmits them to the hypothalamus.

PERIPHERAL SIGNALS INVOLVED IN THE REGULATION OF FOOD INTAKE: THE ROLE OF THE GASTROINTESTINAL TRACT AND ADIPOSE TISSUE

There are many signals of both neural and hormonal nature that originate from the periphery and are able to modulate feeding. In particular, the gastrointestinal tract is a source of signals able to influence food intake either through the release of gut hormones or through gastrointestinal motor changes[4]. The latter could modify the activity of the vagal afferent fibers, which send signals to the hypothalamic regions through the interposition of the nucleus tractus solitaris. Gut hormones, such as cholecystokinin, glucagon-like peptide-1, ghrelin and peptide tyrosine tyrosine, can reach the brain by entering the systemic circulation and may also send signals to the central structures by stimulating receptors present on vagal afferent neurons of the gut mucosa[5].

Gastrointestinal motor changes, particularly those related to the stomach, are recognized as key mediators of the hunger-satiety cycle. Gastric accommodation as well as gastric emptying indeed play a significant role in the regulation of stomach distension[6]. The latter has been shown to trigger stretch and tension, thus stimulating mechanosensitive receptors. These, in turn, transmit their information *via* the afferent

nerves[7,8] to several brain areas[9]. It is known that the tone of the gastric proximal portion decreases during food intake. This process of active relaxation is mediated by different parasympathetic reflex pathways, which lead to a decrease in contractile cholinergic input and an increase in nitric oxide release[10]. Several studies reported that the delay of gastric emptying, physiologically or artificially induced, and hence the dilation of the gastric wall are related to an increase in satiety feelings and fullness, leading to food intake termination[11-13]. Interestingly, obese people showed enhanced gastric emptying, whereas a subgroup of patients with anorexia nervosa had significantly delayed gastric emptying[14,15]. Moreover, several studies combining ultrasound and scintigraphy experiments found an inverse correlation between satiety and gastric distension/emptying[16-18].

Evidence exists that gastric distension-induced satiety can also be regulated by some anorexigenic gut hormones (*e.g.*, cholecystokinin and glucagon-like peptide-1), which induce gastric distension and inhibit gastric emptying (and at the same time increase gastric compliance). Such effects on gastric motility can contribute to their effects on inducing satiety[19].

In this regard, several hormones, not only from the gastrointestinal tract but also from other organs, such as white adipose tissue (WAT), have been reported to modulate appetite in humans[20]. WAT secretes adipokines and bioactive signaling molecules[21-23]; thus, it is a part of the endocrine signaling system. This is the reason why WAT cannot be thought of as an inactive organ but rather an endocrine organ. Adipose tissue has complex interactions with the brain and peripheral organs and plays an active role in energy homeostasis and several other processes[24,25]. For instance, it controls the hunger-satiety cycle by providing hormonal signals about energy stores to the brain *via* adipokines. Among them, in addition to leptin, whose effects have been extensively investigated, adiponectin has also been reported to have a role in regulating feeding behavior by sending signals to the hypothalamus[26].

ADIPONECTIN: CENTRAL ACTIONS IN THE REGULATION OF FOOD INTAKE AND EFFECTS ON GASTRIC MOTILITY

Among adipokines, adiponectin has recently attracted much attention because of its multiple peripheral actions: It seems to be implicated in several physiological conditions and to have a significant role in the maintenance of whole-body homeostasis[27]. Moreover, adiponectin has been reported to exert numerous beneficial effects as an antidiabetic, anti-atherosclerotic, antiapoptotic and anti-inflammatory agent in both animals and humans[28,29]. The hormone has also been shown to have a role in suppressing lipogenesis and gluconeogenesis and to increase fatty acid oxidation and energy consumption[30].

Adiponectin is one of the most concentrated hormones in plasma. However, despite its abundance, circulating adiponectin plasma concentrations change significantly[27] in a number of health and pathological conditions. In fact, a peculiar feature of adiponectin is the inverse correlation of its circulating concentrations with weight, waist circumference, body mass index and obesity[31,32]. In this regard, a significant increase in adiponectin concentration in adipose tissue and plasma has been described after caloric restriction or bariatric surgery in obese subjects[33,34]. However, the mechanisms underlying obesity-associated reductions in plasma adiponectin concentrations have not yet been elucidated in detail.

In addition to peripheral actions, adiponectin has also been reported to exert central actions in the regulation of food intake, but they are still controversial. Nonetheless, the presence of adiponectin receptors has been proven in different regions of the human brain[35,36], including the hypothalamic arcuate and lateral nuclei[37].

Adiponectin receptors (AdipoR1, AdipoR2, and T-cadherin) are indeed expressed in the brain as well as in the peripheral tissues and show different affinities for specific adiponectin isoforms, *i.e.*, trimeric, hexameric, and high-molecular-weight multimeric isoforms[38]. Posttranslational modifications are important for the determination of adiponectin functionality since different isoforms of adiponectin exhibit different biological activities. In particular, trimeric and hexameric forms are reported to be those mainly involved in the central regulation of food intake. Hexamers and high-molecular-weight adiponectin oligomers have been proposed to bind to the T-cadherin receptor in the brain, even if their interaction remains unclear[38,39]. Although AdipoR1 and AdipoR2 are both highly expressed in various brain areas, only hypothalamic AdipoR1 has been suggested to mediate adiponectin regulation of food intake and energy expenditure in mice[35]. A simplified scheme illustrating the main central and peripheral effects of adiponectin and the related receptors involved is reported in Figure 1.

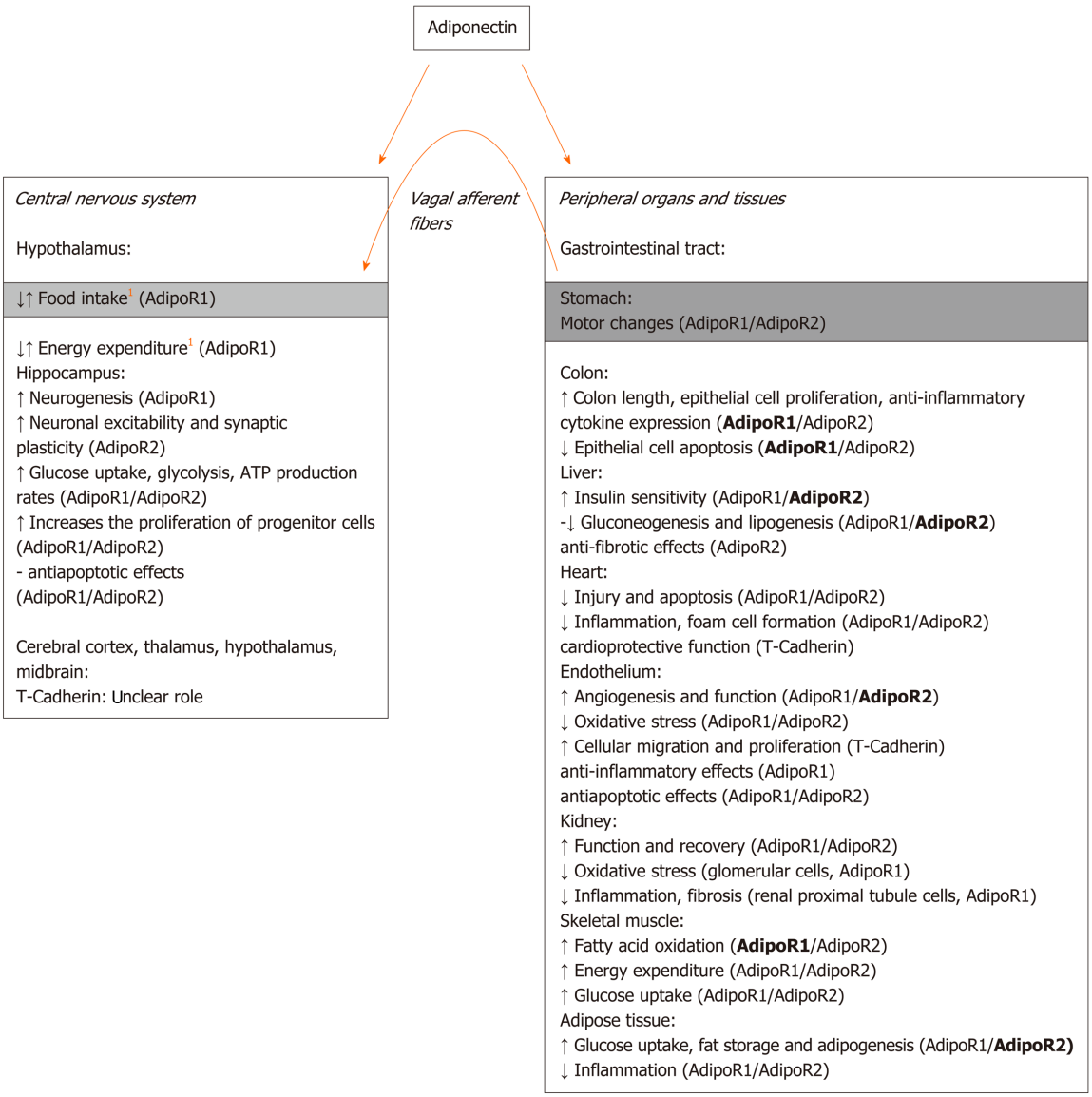

Figure 1 Simplified scheme summarizing some of the main adiponectin central and peripheral effects and the related receptors involved. The scheme is based on the current knowledge and illustrates our hypothesis (orange arrow) concerning an additional peripheral mechanism through which adiponectin may influence food intake at the central level. The receptor that seems to be mostly activated for the related effect is indicated in bold. [1]indicates controversial results.

Whether different isoforms of adiponectin enter the brain has long been a debated issue, but recent literature has reported that numerous adipokines are indeed able to pass through the blood-brain barrier and act right on the brain[40]. Specifically, adiponectin can cross the blood-brain barrier and can also be secreted locally in the brain tissue[41], likely leading to different biological effects[38,40]. In particular, some adiponectin isoforms (trimeric and hexameric) are indeed able to reach the brain and hypothalamic centers[40] to interact with the areas that control hunger and satiety. Although this is a very exciting finding, many other aspects remain to be solved, primarily whether adiponectin exerts anorexigenic or orexigenic effects.

Notwithstanding an early observation of Qi and coworkers[42], who reported no central effects of adiponectin on food intake in mice, numerous subsequent studies indeed refer to its ability to influence feeding by acting on hypothalamic nuclei. In this view, Kubota *et al*[35] observed that full-length adiponectin, intravenously injected in mice, enhanced adenosine monophosphate-activated protein kinase activity in the arcuate hypothalamus[43] *via* AdipoR1, thus promoting food intake during fasting. Contrasting results have been reported by Coope *et al*[26], who demonstrated that direct intracerebroventricular injection of adiponectin in fasted rats induced a dose-related reduction in food intake. This effect was abolished following the inhibition of AdipoR1 but not AdipoR2. Coope *et al*[26] proposed that the discrepancy between their results and those obtained by Kubota *et al*[35] could be related to the different protocols employed. In fact, the perfusion of the hormone through the jugular vein[35] did not

take into account that adiponectin may not be able to easily pass through the blood-brain barrier according to its different isoforms[5]. As a matter of fact, only the trimer and low-molecular-weight hexamer have been detected in the human cerebrospinal fluid, with the adiponectin trimer being the predominant oligomer[38]. This also gives a possible explanation for the lack of effects observed by Qi *et al*[42]. Indeed, a reduction in food intake has been recently observed following intracerebroventricular injection of adiponectin in rats[44], in agreement with the above-reported results by Coope and coworkers[26].

The effects of adiponectin on food intake and hypothalamic neuronal activity appear to also be related to different nutritional states and glucose plasma concentrations. In this view, electrophysiological experiments highlight that adiponectin inhibits NPY neurons and activates POMC neurons in a phosphoinositide-3-kinase-dependent and activated protein kinase-independent manner at various physiological glucose levels[45]. Two other recent studies[46,47] showed that intracerebroventricular injection of adiponectin in mice influences feeding and activates POMC neurons in relation to low or high glucose concentrations, whereas the inhibitory action on NPY neurons is glucose-independent. These results proposed an exciting new role for adiponectin as an attenuator of the effect of changes in the glucose level on POMC neuron activity and feeding behavior.

Moreover, many substances that act centrally to regulate food intake are also able to exert their effects at the gastrointestinal smooth muscle level. Among these, leptin is one of the best characterized[48]. However, notwithstanding the above-reported effects of adiponectin in the hunger-satiety cycle at the central level, no data were present in the literature about its possible peripheral effects on gastrointestinal motility until recently. The ability of the hormone to influence the mechanical responses in strips from the mouse gastric fundus has been recently reported, and the expression of both AdipoR1 and AdipoR2 receptors in these preparations has been revealed[49]. Particularly, a neuromodulatory action for the hormone has been suggested due to its ability to reduce the amplitude of the neurally induced contractile responses and to enhance that of the relaxant ones (likely through the involvement of the nitric oxide pathway). Interestingly, in addition to its neuromodulatory action, adiponectin is capable of inducing gastric motor changes through a direct relaxant effect on smooth muscle[49]. Likewise, electrophysiological investigations[50] indicate the ability of the hormone to hyperpolarize the resting membrane potential, suggesting a reduction of gastric smooth muscle cell excitability and thus an inhibitory action on excitation-contraction coupling.

CONCLUSION

All the above considerations indicate that adiponectin may have an additional effect on the regulation of food intake by inducing gastric motor changes. The depressant actions on the gastric muscle exerted by adiponectin may indeed be directed to facilitate relaxation, which may lead to gastric wall distension, known to be a peripheral satiety signal. Thus, the most attractive hypothesis is certainly that such adiponectin effects may be truly regarded as a reinforcing peripheral mechanism engaged by the hormone itself to concur with its central anorexigenic effects. Going a step further, we can speculate that the depressant actions of the hormone on gastric motility may also cause delayed gastric emptying, thus concurring with an increase in satiety feelings and fullness, leading to food intake termination. With this in mind, we can hypothesize the existence of a link between the depressant peripheral effects exerted by adiponectin at the gastric level and its central anorexigenic effects.

However, it must be remembered that most of the studies have been carried out in animals. Both central and peripheral effects of adiponectin in the regulation of food intake certainly deserve to be further investigated from a translational perspective. Dysfunction in generating signals or in the interpretation of these signals by the brain may indeed lead to obesity and/or eating disorders.

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REVIEW

Diet in neurogenic bowel management: A viewpoint on spinal cord injury

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Abstract

The aim of this review is to offer dietary advice for individuals with spinal cord injury (SCI) and neurogenic bowel dysfunction. With this in mind, we consider health conditions that are dependent on the level of lesion including skeletal muscle atrophy, autonomic dysreflexia and neurogenic bladder. In addition, SCI is often associated with a sedentary lifestyle, which increases risk for osteoporosis and diseases associated with chronic low-grade inflammation, including cardiovascular and chronic kidney diseases. The Mediterranean diet, along with exercise and dietary supplements, has been suggested as an anti-inflammatory intervention in individuals with SCI. However, individuals with chronic SCI have a daily intake of whole fruit, vegetables and whole grains lower than the recommended dietary allowance for the general population. Some studies have reported an increase in neurogenic bowel dysfunction symptoms after high fiber intake; therefore, this finding could explain the low consumption of plant foods. Low consumption of fibre induces dysbiosis, which is associated with both

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endotoxemia and inflammation. Dysbiosis can be reduced by exercise and diet in individuals with SCI. Therefore, to summarize our viewpoint, we developed a Mediterranean diet-based diet and exercise pyramid to integrate nutritional recommendations and exercise guidelines. Nutritional guidelines come from previously suggested recommendations for military veterans with disabilities and individuals with SCI, chronic kidney diseases, chronic pain and irritable bowel syndrome. We also considered the recent exercise guidelines and position stands for adults with SCI to improve muscle strength, flexibility and cardiorespiratory fitness and to obtain cardiometabolic benefits. Finally, dietary advice for Paralympic athletes is suggested.

Key words: Neurogenic bowel dysfunction; Body composition; Mediterranean diet; Food–drug interactions; Microbiota; Paralympic athletes

Core tip: Dietary advice for individuals with a spinal cord injury (SCI) and with neurogenic bowel dysfunction (NBD) must be carefully considered. This advice should include: (1) Energy and nitrogen balance; (2) Under/over-nutrition; (3) Comorbidities and polypharmacy; and (4) Micronutrient deficiency. Dysbiosis and low-grade inflammation, typical consequences of SCI, can be reduced by increasing both physical exercise and fibre intake, but particular fermentable carbohydrates and source of fiber, can sometimes increase NBD symptoms. Water intake is particularly important for NBD management and can be critical during and after exercise. Multi-disciplinary cardiovascular risk reduction programs, including tailored nutrition, strength, aerobic and flexibility training, and personalized dietary supplementation are recommended for individuals with SCI and NBD.

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INTRODUCTION

Spinal cord injury (SCI) may result in motor paralysis and sensory loss below the level of the lesion[1-5] and impaired function of the autonomic nervous system (ANS) originating from the spinal cord[6]. The physio-pathological consequences of the SCI are therefore manifold, including an injury level-dependent impaired function in thoracic and abdominal organs and their related tissues[6]. The lack of motor stimulation below the level of SCI induces changes that can result in neurogenic obesity via adipose tissue accumulation[7]. The latter is associated with the release of proinflammatory adipokines that lead to chronic low-grade inflammation[7,8]. Although both under-nutrition and over-nutrition can be observed in individuals with SCI[9], compared to the guidelines for general population, individuals with chronic SCI have lower fibre intake and greater energy intake relative to energy needs[10]. Among individuals with SCI, daily intakes of whole fruits, vegetables and whole grains were lower than recommended dietary allowances[11-13]. Appropriate nutritional recommendations[14] and exercise[7] for individuals with SCI could play an important role in decreasing the risk of cardiovascular disease (CVD) and cardiometabolic syndrome (CMS).

On the other hand, symptoms of bowel dysfunction, including constipation and faecal incontinence, have a high impact on quality of life of individuals with SCI[15]. It has been reported that many characteristics of abdominal pain among patients with SCI resemble those of able-bodied individuals with chronic idiopathic constipation[16]. Dysmotility and abdominal discomfort have been attributed to the ingestion of certain carbohydrates (CHO) in individuals with gastrointestinal disorders, including irritable bowel syndrome (IBS) and functional constipation[17]. In addition to dietary advice, lifestyle recommendations, including exercise, have been suggested for

patients with IBS[18], in particular in those with slow or uncoordinated transit[19]. It has been suggested that exercise may modulate intestinal permeability and motility, stool transit time and consistency[20].

In this review, considering the above-mentioned issues, we will discuss dietary management of neurogenic bowel dysfunction (NBD) among individuals with SCI and suggest a Mediterranean diet (Med-D)-based pyramid and nutritional advice for Paralympic athletes with SCI.

NEUROGENIC BOWEL DYSFUNCTION IN SPINAL CORD INJURY

NBD refers to bowel dysfunction consequent to the ANS injury and/or lack of central nervous system control (mainly from hypothalamus and brainstem)[21]. Although symptoms of both upper (esophageal[22]/gastroesophageal[23]) and lower (intestinal) gastrointestinal disorders can occur, the latter are highly prevalent in SCI and include constipation (prevalence ranging between 56% and 80%) and faecal incontinence (range 42%-75%)[21]. Colon dysfunction following SCI can be divided into two main types, depending on the level of the lesion: An upper motor neuron syndrome and a lower motor neuron syndrome[21]. Typically constipation and incontinence occur for injury above and below the thoracic (T)11/T12 region, respectively[24]. On the other hand, given that traumatic SCI usually occurs at the cervical or thoracic levels, the spinal defecation centre, located in the sacral spinal cord, is typically intact in these individuals[25].

In patients with motor complete SCI, Vallès *et al*[26] identified three different neuropathophysiological patterns. Pattern A is characterized by frequent constipation, moderate delay in colonic transit time (CTT) and the absence of anal relaxation during the defecation. Pattern B is characterized by defecatory difficulty, moderate delay in CTT, increased anal resistance during the defecation and preserved sacral reflexes; Pattern C is characterized by severe incontinence associated with severe delay in CTT and absence of sacral reflexes.

In a long (19-year, 1996-2015) follow up study on living with SCI, no significant changes in quality of life and faecal incontinence were found, whereas a higher prevalence (from 19% to 31%) of the population with SCI considered themselves to be constipated and increased with time the use of oral laxatives[27].

It has been suggested that constipation is a major cause of abdominal pain or discomfort after SCI, rather that neuropathic pain, with pain considered more intense and unpleasant among individuals with chronic idiopathic constipation compared to patients with SCI[16]. Furthermore, there was no association between neurological level of SCI and abdominal pain as 79% of individuals with cervical and thoracic SCI and 86% with lumbar SCI had pain[16].

Sensory stimuli below the injury level due to bowel management are among the common triggers for autonomic dysreflexia (AD), which is a complex syndrome characterized by sudden episodic high vasoconstriction-induced hypertension and resulting symptoms due to massive sympathetic ANS activity deriving from the lack of hypothalamus and brainstem control. AD generally occurs among individuals with SCI at or above the T6 level[6], above splanchnic sympathetic outflow.

Inskip *et al*[28] found that in a group of about 150 individuals with SCI at T7 or above, 74% experienced at least one AD symptom during bowel care including "goosebumps, spasticity, flushing and sweating" in about half population, along with general unwellness (43%) and headache (38%)". "Heart palpitations, irregular heartbeats, or a feeling of fluttering in the chest" globally considered as symptoms of arrhythmia consequent to AD were found in 45 of 141 (32%) individuals with SCI at or above T7[28]. Longer durations of bowel care due to SCI and more severe AD were associated with lower quality of life[28].

The NBD score has been identified as a condition-specific tool to assess quality of life as "subjective well-being" among individuals with SCI[29]. NBD score includes frequency of bowel movements (0-6 points), headache, perspiration or discomfort before or during defecation (0-2 points), tablets and drops against constipation (0-2 points each), time used for each defecation (0-7 points), frequency of digital stimulation or evacuation (0-6 points), frequency of faecal incontinence (0-13 points), medication against faecal incontinence (0-4 points), flatus incontinence (0-2 points) and perianal skin problems (0-3 points)[30]. According to Krogh *et al*[30] a severe NBD score is ≥ 14, moderate an NBD score of 10-13, minor an NBD score of 7-9 and very minor an NBD score of 0-6. Liu *et al*[31] reported that differences in NBD scores were dependent on both completeness and level of injury. Severe NBD was observed in 71.1% of individuals with American Spinal Injury Association (ASIA) classification A

(loss of motor and sensory function at S4-5 level), whereas lower percentages were found in patients with incomplete SCI (prevalence of 32.4%, 24.2% and 27.1% in ASIA classifications B, C and D, respectively)[31]. Furthermore, a higher percentage of individuals with cervical (48.2%) and thoracic (40.0%) SCI had severe NBD, compared to those with lumbar SCI (14.3%)[31]. Other factors, including use of laxatives and fibre intake, affect NBD score among individuals with traumatic SCI and minor to moderate bowel dysfunction (NBD Score: 8.01 ± 4.49), whereas neither the use of anticholinergics nor opioid agents were significantly associated with NBD[32]. In conclusion, living with SCI and NBD has an impact on dietary intake (amount or type of foods) and habit (time restricted or skip meal)[33], with potential effects on nutritional status.

DYSBIOSIS IN SPINAL CORD INJURY: POTENTIAL TARGET FOR FIBRE, DIETARY INTERVENTIONS AND EXERCISE TRAINING

Antibiotic treatment, due to recurrent infections, and altered CTT significantly alter the composition of gut microbiota in individuals with SCI[34]. The use of antibiotics decreases microbial diversity by 25% and increases the *Bacteroidetes*/*Firmicutes* ratio[35]. In the "Guidelines for Management of Neurogenic Bowel Dysfunction in Individuals with Central Neurological Conditions" of the Multidisciplinary Association of Spinal Cord Injured Professionals[36], it is concluded that there is some evidence that the use of probiotics may help to restore colonic flora after antibiotic treatment.

Several clinical studies have evaluated the intestinal microbiota among individuals with SCI and all have reported decreased *Firmicutes* compared to healthy controls[37-39]. Furthermore, the reduced microbial diversity in individuals with SCI[37,38] has been associated with unfavourable metabolic profiles[37]. At the phylum level, *Bacteroidetes* and *Firmicutes* were negatively[38] and positively correlated with high density lipoprotein cholesterol (HDL)[37], respectively, and *Firmicutes* were negatively correlated with serum glucose (GLU)[38]. Exercise[40,41] and Med-D[42] are known to improve metabolic (serum GLU and lipids) markers and cardiorespiratory fitness was reported to be significantly correlated to both microbial diversity[20,43,44] and *Firmicutes* to *Bacteroidetes* ratio in healthy adults[45]. Animal-based diets increased the abundance of *Bacteroides*[46,47] and decreased *Firmicutes* which metabolize dietary plant polysaccharides[47]. *Prevotella*[48] abundance was associated with long-term fibre intake, estimated using the Diet History Questionnaire[48]. In individuals with SCI, GLU was negatively correlated with *Prevotella*[38] and *Megamonas*[37,38] (more abundant in a healthy group than in groups with SCI)[37-39]. Although, *Megamonas* and *Prevotella* may have a positive effect on CHO metabolism, they were positively correlated with the NBD score, probably due to gas and short-chain fatty acids (SCFA), the end products of microbial fibre fermentation[38]. In IBS, discomfort, constipation and altered faecal consistency can be influenced by the presence of dietary fibre and fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAP)[18,49].

Reported mean fibre intake, ranging between 15.0 g/d[11] and 16.9-17.6 g/d[10], met the Academy of Nutrition and Dietetics Evidence Analysis Library minimum target range for individuals with SCI (which starts at a minimum of 15 g/d and increases up to 30 g/d as tolerated)[50]. Stoffel *et al*[51] reviewed studies on neurogenic bowel management and reported that a high-fibre diet prolonged constipation and increased CTT and NBD score in persons with SCI and suggested that clinicians may recommend a low-fibre diet to reduce CCT. In a recent systematic review, Yeung *et al*[52] concluded that consumption of about 15 g/d of dietary fibre could be beneficial in managing neurogenic bowel in SCI, whereas an increase in fibre intake from 25 g/d to 31 g/d increased CTT and evacuation time.

Increased stool consistency (looser stool with high transit time) was inversely related to microbiota diversity and positively associated with *Bacteroidetes*/*Firmicutes* ratio and increased CCT[46]. SCFA can increase levels of serotonin, triggering contraction of the smooth muscle in the gastrointestinal tract[46] and SCFA levels have been negatively correlated with CTT[53].

On the other hand, individuals with metabolic syndrome (Met-S) had reduced SCFA[54] and SFCA were positively correlated with adherence to the Med-D pattern[55]. Triglycerides (TG) in individuals with SCI were negatively correlated with *Eubacterium_rectale*, a butyrate-producing bacterial genus[37]. *Dialister* (decreased in SCI)[37,39] was negatively correlated with low density lipoprotein cholesterol and positively correlated with HDL.

Gungor *et al*[39] found that, compared to healthy controls, there was a preferential decrease in *Marvinbryantia* in the upper motor neuron group, whereas in the lower

motor neuron group, *Roseburia* was decreased[39]. Gungor *et al*[39] suggested that although the identity of the genera that are dysregulated in both groups may differ, the reduction of butyrate (a SCFA) production would be expected to be similar. High fat diets decrease butyrate levels and can induce gut dysbiosis, dysmotility and constipation[46]. On the other hand, physically fit individuals had increased abundances of key butyrate-producing taxa, including *Roseburia*[43].

SCFA are also involved in gut-brain signalling implicated in the regulation of metabolic and immune homeostasis[53]. Sympathetic noradrenergic nerves innervate the vasculature and tissue parenchyma of the gut-associated lymphoid tissues[56]. Norepinephrine released by sympathetic post-ganglionic nerve terminals binds to beta (β)-adrenergic receptors on innate and adaptive immune cells leading to suppression of their antimicrobial functions[56].

In pre-clinical SCI models, intestinal epithelial cell permeability is increased and is associated with enhanced bacterial translocation and endotoxemia [increased lipopolysaccharide (LPS)][56]. Data from animal models suggest that gut dysbiosis, LPS and gut-associated lymphoid tissues[57] lead to the chronic low-grade inflammation associated with Met-S after SCI[53,56]. LPS elicits strong inflammatory responses by binding to extracellular toll-like receptor 4. In mouse studies, gut dysbiosis, free fatty acids, and the activation of toll-like receptor 4 are associated with enteric neuronal death, damaged function of the enteric nervous system and delayed transit time[46]. In this context, LPS was negatively associated with adherence to the Med-D[55] and cardiorespiratory fitness was negatively correlated with LPS[43]. Therefore, both exercise and diet could reduce endotoxemia and inflammation induced by dysbiosis in individuals with SCI.

DIETARY ADVICE AND MEDITERRANEAN DIET-BASED PYRAMID FOR INDIVIDUALS WITH SPINAL CORD INJURY

Individuals with SCI experience significant skeletal muscle atrophy mainly below the level of injury[58], which may contribute to decreases in resting energy expenditure[59]. On the other hand, the immune system is an energy consumer and during chronic inflammation re-allocation of energy-rich fuels to the activated immune system, called "energy appeal reaction", can lead to inadequate energy regulation, muscle protein breakdown, low vitamin D levels, cachectic obesity, insulin resistance, dyslipidaemia, Met-S and inflammation-related anaemia and osteopenia[60,61].

The SCI-SCREEN, a nutritional screening model developed in a neurorehabilitation unit, incorporated the Lagerström's proposed body mass index (BMI) cut-offs for SCI patients, differentiating between individuals with paraplegia (estimated muscle mass reduction of 7.5%) and those with tetraplegia (estimated muscle mass reduction of 12.5%)[62]. On the other hand, the "Guidelines for Identification and Management of Cardiometabolic Risk after Spinal Cord Injury" of the Consortium for Spinal Cord Medicine expert panel suggested definitions of obesity as: > 22% of fat mass (FM) or BMI ≥ 22 kg/m^2, given that waist circumference is not a validated proxy for obesity in SCI[63].

Recommendations for energy intake in individuals with SCI in the subacute and community living phases were 22.7 kcal/kg and 27.9 kcal/kg of body mass (BM) for individuals with tetraplegia and paraplegia, respectively[50]. Recommendations for protein intakes did not differ between individuals with tetraplegia and paraplegia, amounting to 0.8-1 g/kg (of BM)[50]. Higher energy and protein recommendations were suggested for individuals with pressure ulcers[50] and for Paralympic Athletes[64]. Wheelchair athletes have been reported to have low energy availability, low protein consumption and CHO intakes near the lower limit of the recommended range (3.0-12.0 g/kg)[65]. This suggests that wheelchair athletes could be at risk of post-exercise ketosis. The latter (about 0.5-1.0 mM in response to 2 h of exercise performed in an overnight fasted state) depends on the intensity and duration of the exercise performed, as well as on the nutritional status and on the ketone body uptake by skeletal muscle, which are able to extract about 50% of circulating ketone bodies when concentrations are low (0.1-0.5 mM)[66]. In a case report, a 36-year-old man, wheelchair bound secondary to genetically confirmed spinal muscular atrophy, presented to an emergency department with epigastric pain and vomiting due to ketoacidosis, and reported decreased food intake in the preceding week in an attempt to lose weight, with a diet consisting mainly of proteins and vegetables with minimal CHO in the 48 h prior to his presentation[67]. The authors suggested that the severity of the ketosis, which seemed inconsistent with moderate starvation alone, could be due to other factors, including low muscle mass[67].

On the other hand, the sublesional muscle atrophy with SCI significantly reduces

the primary storage site for CHO, promoting increased circulating GLU and risk of type 2 diabetes[14]. The reduction in fat free mass (FFM) and the increase in FM are key features in people with SCI that could increase the risk for CMS[14]. In an environment of reduced mobility, energy needs are reduced, and there is an increased risk of obesity-related chronic diseases, making energy balance a critical and fundamental issue in SCI[68]. The spinal nutrition screening tool has been validated for patients with SCI to assess the malnutrition risk[9]. The spinal nutrition screening tool assesses eight criteria: History of recent weight loss, BMI, age, level of SCI, presence of comorbidities, skin condition, appetite and ability to eat[9]. These authors reported that patients with paraplegia were less likely to be malnourished (33.7%) than those with tetraplegia (66.1%) and readmitted patients had a lower incidence of under-nutrition (41.6%) than newly injured patients (50%)[9]. On the other hand, a 67% of individuals with SCI were at potential risk of over-nutrition (BMI > 22 kg/m^2)[9]. It has been reported that the components of CMS (abdominal obesity, hypertension, insulin resistance, and dyslipidaemia) are not equally weighted; sarcopenic obesity is a highly prevalent finding after SCI and appears to be the most relevant risk factor, followed by insulin resistance[63].

Among the dietary strategies there is the "adaptability of the diabetes prevention program" (DPP) for SCI, consisting of a 24-wk energy-restricted Med-D[14]. Preliminary data in men with chronic SCI who were obese and pre-diabetic, showed that a therapeutic lifestyle intervention including diet, exercise, patient education and professional support, resulted in BM reduction that exceeded the DPP criterion (7%). The intervention also demonstrated improvements in insulin resistance and lipid profile (HDL increase and TG decrease)[14]. A systematic review of dietary interventions in adults with SCI emphasized the potential of diet in conjunction with exercise in minimizing CVD risk[69], whereas the "Consortium for Spinal Cord Medicine" noted success in weight loss using the Med-D in the DPP[63]. Med-D has been suggested for Veterans with disabilities[70], patients with chronic kidney disease (CKD)[71] and individuals with chronic pain[72]. Furthermore, Allison *et al*[73], recently reported that in individuals with SCI, a 3-mo anti-inflammatory diet similar to the Med-D plus supplementation decreased fat intake and proinflammatory dietary components (trans fatty acids, caffeine and sodium) and increased protein intake and some nutrients with established anti-inflammatory properties, including vitamins A, C, and E, and omega (ω)-3 polyunsaturated fatty acids (PUFA), with no change in CHO or energy intake[73]. Significant reductions in the proinflammatory cytokines interferon-γ, interleukin-1β, and interleukin-6 were observed and several proinflammatory mediators were negatively correlated with anti-inflammatory nutrients, including vitamin A, carotenoids, ω-3 PUFA, and zinc, whereas the change in total calories as well as individual macronutrients were not shown to be significantly correlated with any inflammatory mediator[73]. Obesity in SCI results in a state of chronic low-grade inflammation primarily due to proinflammatory adipokines secreted from excess adipose tissue[7]. Accordingly, individuals with chronic motor complete SCI, severe lower extremity spasticity and lower FM had lower leptin and fasting GLU than patients with SCI with no or mild spasticity[74]. On the other hand, no differences in bone mineral density (BMD), low density lipoprotein cholesterol, HDL, TG and glycosylated haemoglobin were observed between the two groups[74]. Although it has been reported that protein intake is negatively associated with BMD of lumbar vertebrae in women with SCI[75], in a cross-sectional study, no significant relationships were found between BMD and intakes of protein, calcium, vitamins D and or serum 25(OH)D in individuals with chronic SCI (94% male, lesions from C1 to T12). However, significant associations were found between BMD at the femoral neck and lumbar spine with visceral adipose tissue, insulin and leptin[76]. Leptin enhances both arterial and venous thrombosis by promoting platelet adhesion, activation and aggregation[77] and it has been recently observed that some Paralympic athletes with SCI had a higher platelet-derived CVD risk rather than CMS risk[78]. Caffeine can increase platelet aggregation, which may be associated with an elevated risk of thrombosis[79]. Conversely, among natural dietary compounds and functional foods of the Med-D with antiplatelet activities (including ω-3 PUFA, olive oil, garlic, onions and tomatoes)[80], O'Kennedy *et al*[81] reported that a water-soluble tomato extract, having antiplatelet anti-angiotensin-converting enzyme and anti-inflammatory activities, became the first product in Europe to obtain an approved health claim[82,83].

Vitamins A, B5, B7, B9, D, E, potassium, and calcium are deficient compared to the USDA guidelines in individuals with SCI[10]. Many of these micronutrients are linked to CHO, lipid, and/or vascular dysfunction, whereas recommendations for pressure ulcer management suggest evaluation for vitamins A and C, zinc and iron[50].

It has been reported that level and completeness of lesion, injury duration, mobility, FM%, time spent outdoors and comorbidities were not associated with

plasma 25(OH)D, whereas in a multivariable model (adjusting for age, planned exercise, sex, race, wine consumption, and smoking status) supplement (but not dietary) intake was significantly associated with increased 25(OH)D[84]. Similarly, in a univariable model, stretching, range of motion, or physical therapy was not associated with higher 25(OH)D level, whereas other planned exercise was associated with higher 25(OH)D levels[84].

A systematic review emphasized the potential of diet in conjunction with exercise in minimizing CVD risk in SCI[69], whereas the Consortium for Spinal Cord Medicine did not recommend a single nutritional intervention but noted success in weight loss using the Med-D in the DPP[63]. Med-D has been suggested for Veterans with disability[70], patients with CKD[71] and individuals with chronic pain[72].

In a recent meta-analysis, 86% and 43%-74% of individuals with chronic SCI had excessive intake of CHO, and 43%-74% had excessive intake of proteins[10]. These authors[10] also reported a mean of 57 kcal/d from alcohol and pointed out that participants were also likely to underreport their true alcohol consumption given its effects on weight and health and the stigma that is often associated with alcohol consumption. In a study that included only overweight and obese individuals (BMI > 22 kg/m^2, mean 30.5 ± 6.33 kg/m^2 with a range from 22.44 kg/m^2 to 49.91 kg/m^2) with SCI (50% with paraplegia and 50% with tetraplegia), although participants' intakes were within the recommended range of amounts for all macronutrients, they were on the low end of the recommended range for protein and on the high end for total fat[11]. Furthermore, individuals with SCI report excess intake of added sugars, sodium and saturated fat and inadequate intake of healthy fats, seafood, plant protein, fruits, vegetables and whole grains, considering the 2015-2020 Dietary Guidelines for Americans[11]. Yeung *et al*[52] concluded that further studies are required because it is unclear from the available studies the extent to which confounding factors, such as age, gender, physical activity, level of injury, time since SCI and comorbidities affect outcomes. The prevalence of polypharmacy is expected to increase with age and concurrent urological management must be considered in individuals with SCI.

A cross-sectional analysis[85] showed that over 1 out of 3 Veterans with SCI had CKD and in a 14-year retrospective cohort study[86], individuals with SCI and CKD had a significantly shorter survival time (10.13 mo *vs* 10.97 mo), higher 1-year mortality (17.65% *vs* 8.54%), and higher risk of mortality than those with SCI but without CKD (adjusted hazard ratio, 2.25). It has been reported that the overall prevalence of CKD was 8.0% and 22.4%, by serum creatinine-based and cystatin-C-based estimated glomerular filtration rate, respectively, and was greater in individuals with neurogenic bladder (NB)[87]. However, serum creatinine was not able to detect the early deterioration of renal function in NB patients because they present muscle wasting due to disuse and/or denervation[87]. Comorbid diabetes, small bladder volume, recurrent urinary tract infection (UTI) and proteinuria were significantly associated with CKD in the multivariable analysis[87]. Key findings from the Committee of the "Joint SIU-ICUD Consultation on Urologic Management of the Spinal Cord Injured Patient" include: (1) Renal function deterioration can occur early but can also occur at later stages following SCI, and can be related to ageing, obstruction, stone disease (due to calcium mobilization from bones, reduced mobility and UTI, with urea-splitting organisms); (2) SCI patients have higher rates of prescribed medications from multiple high-risk classes (analgesic–narcotics, anticonvulsants, antidepressants and skeletal muscle relaxants); (3) Osteoporosis and complete sensory or motor injuries are associated with higher risk of fractures; and (4) Clinicians should screen SCI patients for malnutrition and obesity[88]. Patients with SCI most frequently used products to treat pain (68%), constipation (42%), muscle spasm (42%), hypertension (42%), and depression (37%). When including natural health products, vitamins and minerals, polypharmacy was present in 74% of patients with SCI[88]. In particular, NB can be observed in individuals with SCI treated with drugs metabolized by CYP3A4 (oxybutynin, solifenacin and darifenacin)[89]. Therefore, caution has been recommended when simultaneously consuming grapefruit juice during treatment with these drugs[89]. Other citrus fruit juices, cruciferous vegetables (broccoli, cabbage, and daikon radish sprouts), soy foods (soy milk, veggies slices, tofu, and roasted soy nuts), tea and cranberry and pomegranate juices can induce adverse food-drug interactions and should be avoided in individuals in treatment for comorbidity[70,90]. Individuals with SCI are at increased risk of developing symptomatic UTI[91]. The use of cranberries (particularly juice) is widely recommended to prevent and treat UTI[92], although evidence related to the prevention of UTI are inconsistent[93]. On the other hand, the European Food Safety Authority Panel on Food Additives and Nutrient Sources Added to Food, considered the possible association between the consumption of (-)-epigallocatechin-3-gallate (EGCG, the most relevant catechin in green tea) and hepatotoxicity[94]. Catechins from green

tea infusion (prepared in a traditional way and reconstituted drinks with an equivalent composition to traditional green tea infusions: EGCG from 90 mg to 300 mg), are in general considered to be safe[94]. However, doses equal or above 800 mg EGCG/d have been shown to induce a significant increase of serum transaminases[94]. Furthermore, human studies have reported that consumption of polyphenol-enriched oolong tea (750 mL for 10 d)[95] or a beverage containing black tea polyphenols (55 mg, 3 times/d for 10 d)[96] increased faecal lipid excretion. Catechins are among the antinutrient antioxidants that can have pharmacological effects[97], including the inhibition of lipase[98]. The pharmacological inhibition of lipase was associated with the excretion of the inflammatory marker faecal calprotectin in healthy individuals[99]. Spices to flavour dishes and other comfort food and beverages (sweet, cocoa, coffee, tea) should be limited and alcoholic drinks should be avoided by individuals with SCI[100], due to the potential interactions of phytochemicals and alcohol with drugs and/or the effect on bowel motility, water and energy balances.

Some foods and beverages, including alcohol, caffeine, tea, coffee, cola and chocolate, prunes, figs and sorbitol containing foods, can overstimulate bowel activity or draw excessive fluid into the colon resulting in very watery stools[36]. On the other hand, individuals with SCI and NBD often have an increase in CCT, resulting in excessive fluid reabsorption and the formation of hardened stools[50]. Therefore, the recommendations for fluid intake are 1/ml fluid per kcal of estimated energy needs plus 500 mL or 40 mL per kg BM plus 500 mL[50] (Figure 1).

For all the above-mentioned reasons, we propose a Med-D-based and exercise pyramid for individuals with SCI (Figure 1). This should be integrated with the previous indications for veterans[70], the available recommendations/suggestions for individuals with SCI with or without NBD[14,36,50,63,69,73], CKD[71], chronic pain[72] and IBS (including low FODMAP)[18], and the exercise guidelines for adults with SCI[101], along with the position statement on exercise and SCI of Exercise and sports science Australia[102]. This approach is likely to be effective in improving muscle strength, flexibility and cardiorespiratory fitness, and to obtain cardiometabolic benefits.

DIETARY ADVICE FOR PARALYMPIC ATHLETES

Applying sports nutrition plans is essential for athletes with an impairment and represents an aid when sport and exercise are practiced[103]. This is important as a component for both therapy and rehabilitation[104] in particular for individuals with SCI[101,105,106]. Paralympic athletes exhibit higher quality of life and health status associated with their opportunities to practice physical activities and to participate in sport competitions[107], and these opportunities increased considerably in recent decades[104,108]. Their levels of performance and physical fitness are increased vs. non-athletes with SCI, and many Paralympic athletes are now engaged in intense training sessions that require highly demanding nutritional needs. For example, Gerrish *et al*[109] reported mean energy intakes of elite Canadian and American athletes with SCI, categorized by level of injury that were above the recommendations for sedentary individuals with SCI [22.7 kcal/(kg·d) in tetraplegia and 27.9 kcal/(kg·d) in paraplegia, see Figure 1][50]. These Authors, indeed, found the following energy intakes among athletes with SCI: 26 ± 7.9 kcal/(kg·d), 28 ± 14 kcal/(kg·d), 36 ± 14 kcal/(kg·d)and 36 ± 16 kcal/(kg·d) for athletes with levels cervical, from T1 to T6, from T7 to T12 and lumbar, respectively[109].

The first step in providing nutritional advice for both health purposes and sports performance is to estimate daily energy requirements, an estimate that is often difficult to assess in Paralympic athletes. Grams *et al*[110], in wheelchair basketball athletes, calculated energy expenditures using values provided by Collins *et al*[111], Abel *et al*[112] and Bernardi *et al*[113], and found a high variability in energy intake relative to BM [ranging from 25 kcal/(kg·d) to 64 kcal/(kg·d)].

Existing equations that estimate energy expenditure at rest are based on able-bodied populations and include variables that are difficult to measure accurately in athletes with SCI, such as height or total BM[64]. The approach of Collins and collaborators is preferred in this context[111]. The daily energy requirement should be estimated by measuring the energy expenditure in basal conditions[111] and during actual sport activities[113] and training[114]. Simulations of training and competitions should be assessed on the field[114] and physiological profile of athletes determined in the laboratory[115], testing athletes repetitively to evaluate the effects of training[116]. In order to have a complete energy expenditure assessment, all other activities performed during the day which cannot be actually measured should be taken into account converting the data using at least three-days of activity. Various questionnaires for energy expenditure can be used, including Ainsworth's tables[117],

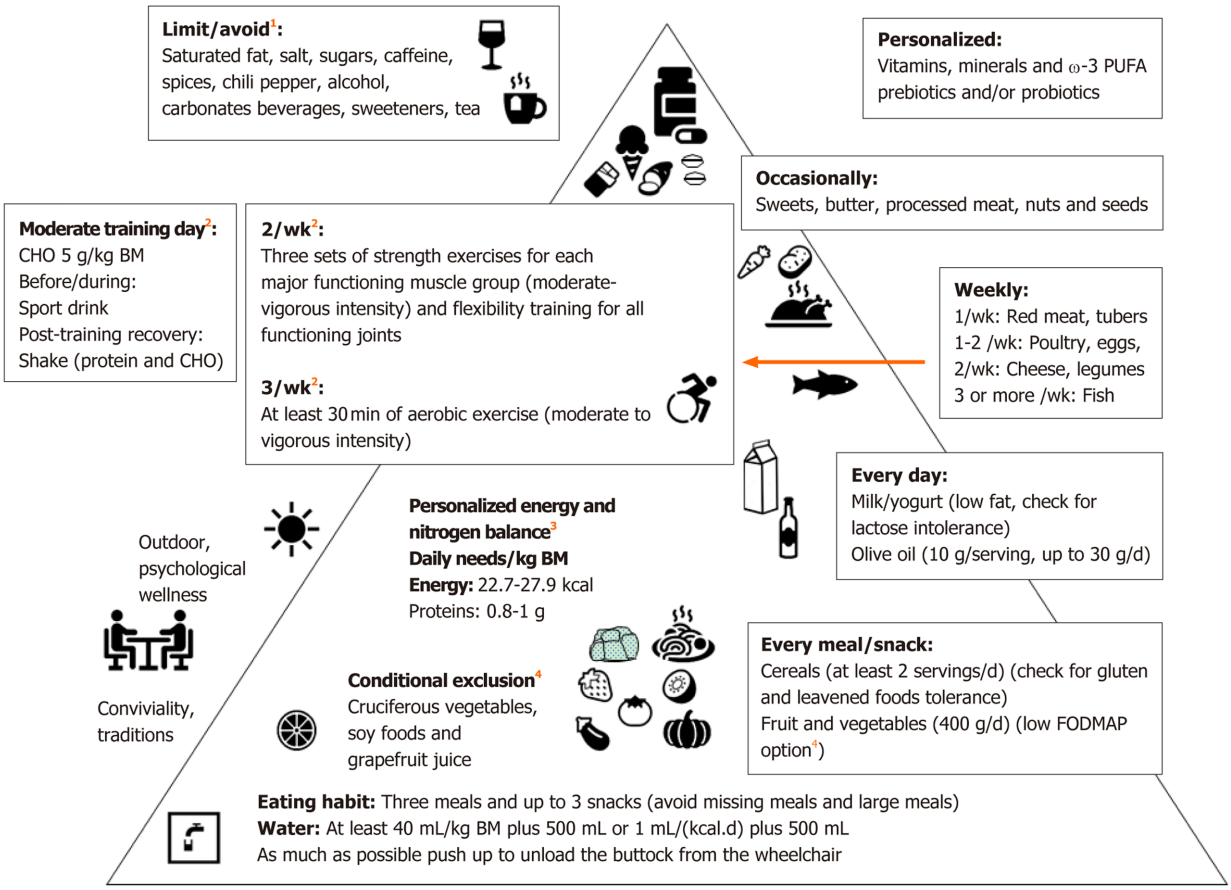

Figure 1 Mediterranean diet based and exercise pyramid for individuals with spinal cord injury. Dietary advice from the previous indications for veterans[70], individuals with spinal cord injury with or without neurogenic bowel dysfunction[14,36,50,63,69,73], chronic kidney disease[71], chronic pain[72] and irritable bowel syndrome[18]. [1]Limit/avoid: Potential undeliverable effects[11,36,79,94-96]; [2]Exercise recommendations, derived from the integration of two recent papers[101,102] devoted to adults with spinal cord injury, are aimed to improve muscle strength, cardiorespiratory fitness, flexibility and cardiometabolic health. As a general rule, the exercise intensity should progress with time from moderate to vigorous at a rate (weeks-months) dependent from the initial level of fitness of the individual. However, personalized training is deliverable associated with sport-type-targeted nutritional recommendations (including timing and supplementation) for Paralympic athletes[64]; [3]Personalized energy and nitrogen balance[50,64]: Ideal body mass 10%-15% and 5%-10% lower that for quadriplegia and paraplegia, respectively; Energy: 22.7 kcal/(kg·d) and 27.9 kcal/(kg·d) for quadriplegia and paraplegia, respectively and 30-40 kcal/(kg·d) in the presence of pressure ulcers; Proteins: Stage II pressure ulcers: 1.2-1.5 g/(kg·d), Stage III and IV pressure ulcers: 1.5-2.0 g/(kg·d); Paralympic athletes: 1.2-1.7 g/(kg·d); [4]Low fermentable oligosaccharides, disaccharides, monosaccharides, and polyols: Banana, blueberry, strawberry, grape, melon, kiwi, cucumber, eggplant, green beans, lettuce, spinach, chives, pumpkin, tomato, zucchini. Cranberry juice may be associated with reduced urinary tract infection but could induce food-drug interactions, which can be observed also with other foods and beverages (conditional exclusion)[70,90]. BM: Body mass; w-3 PUFA: Omega-3 polyunsaturated fatty acids; CHO: Carbohydrates; FODMAP: Fermentable oligosaccharides, disaccharides, monosaccharides, and polyols.

or by using accelerometers, the Physical Activity Scale for Individuals with Physical Disabilities and the Veterans Administration Physical Activity Questionnaire modified for SCI[118].

Paralympic athlete's diet must be appropriate for both the physical activity performed and the characteristics of the individual's body composition, monitored over time. Assessment of body composition of athletes with impairments is therefore crucial to evaluate the general health status and monitor adaptation to diet and training[119,120], but in individuals with a great loss of metabolically active tissue and an asymmetric distribution of fat above and below the spinal lesion, this assessment may not be accurate[103] and can make it difficult to identify small to moderate changes in physique traits[65].

In athletes with SCI, as already pointed out in sedentary individuals with SCI, a reduction in resting energy expenditure, which depends on the level of the spinal lesion and the consequent loss of actively controllable musculature, is often observed[111]. Male and female athletes with SCI generally have lower energy requirements compared to able-bodied athletes. Despite lower energy needs, Paralympic athletes may still be consuming too few calories[65,121,122]. Aside from taking into account the previously mentioned issues and respecting an appropriate energy balance[114], in general dietary recommendations for athletes with impairments should not differ from those for able-bodied athletes[64].

Depending on the type of sport practiced, *i.e.*, skill sports, power sports, intermittent (aerobic and anaerobic alternate metabolism) sports and endurance sports[123], because energy expenditure can vary widely[113], food intake and in particular energy requirements need to be tailored precisely to optimize performance. In particular, in endurance sports, such as Nordic sitting skiing, which requires a high energy expenditure, the competition strategy is similar, in spite of the long race duration (45-50 min) to that of an "all-out" exercise in which the highest speed is maintained from the start to the end[124]. Indeed, successful athletes need not only possess a high aerobic power but also a very high glycolytic capacity[125]. Diet and consequently GLU stores (glycogen) can become a major performance limiting factor of these competitive performances. An adequate CHO intake is essential to maintain training intensity, combat fatigue, protect immune function, sustain training adaptations and provide a key fuel for the brain and central nervous system[126-128]. Although some Paralympic athletes (such as Nordic sitting skiers) had mean FM% ranging from 14.1 (best performers) to 15.1 (others), athletes competing in power and intermittent sports, had FM% (20.9 in Alpine sitting skiers and 20.9 in Para Ice Hockey players)[125] similar to those of non-athletic able-bodied men[129]. In male individuals with a normal BMI (18.5-24.9 kg/m^2) and age between 20 and 79 years, Shea *et al*[129] reported that both medium (FM = 15.3%-20.7%) and high (FM ≥ 20.8%) percentages of FM groups, had a significantly greater proportion of metabolically abnormal phenotypes (12.7% and 26.6% respectively), compared to individuals with low (FM ≤ 15.2%) percentage of FM, in whom this prevalence was equal to 6.2%. From that, a possible low total FFM can expose Paralympic athletes with SCI to a risk of both post-exercise ketosis (due to low ketone bodies uptake by skeletal muscles)[66,67] and type 2 diabetes (due to reduction of muscle CHO storage)[14]. Therefore, in endurance and high energy expenditure intermittent sports, CHO in Paralympic athletes with SCI is an energy source more important than in able-bodied athletes. From the above-mentioned reasons, CHO with low glycaemic index (*i.e.*, Med-d cereals) should be preferred in the habitual diet. Adequate intake of sugars before and during exercise and sport should compensate for the low muscle glycogen stores (upper suggested range), whereas after exercise, GLU needs should be lower (lower suggested range) than able-bodied athletes. Before competition, boiled potatoes, which have a glycaemic index (78 ± 4) comparable to those of white bread (75 ± 2) and white rice boiled (73 ± 4) and higher than white spaghetti (49 ± 2)[130], could be a favourable GLU source. We therefore summarize the ACSM nutritional guidelines for able-bodied athletes[131] related to the CHO intake in table 1 that indicates the quantities (ranges) and timing of intake to provide high CHO availability for designated training or competition sessions according to athlete's BM and session characteristics. Unlike the Position statement of the International Society of Sports Nutrition regarding the timing of macronutrients in highly trained individuals, CHO re-feeding [1.2 g/(kg·h)] with a preference towards CHO sources that have a high (> 70) glycaemic index for rapid restoration of glycogen is required (< 4 h of recovery time)[132] must be evaluated on the light of possible co-morbidity in SCI[85,87,133].

Paralympic athletes' dietary protein intake necessary to support metabolic adaptations, repair, remodelling, and for protein turnover, generally ranges from 1.2 g/(kg·d) to 1.7 g/(kg·d)[64] (Table 1). In cases of energy restriction, sudden inactivity as occurs as a result of injury, or in athletes competing in weight lifting sports (where classes depends on BM range), elevated protein intakes typically range from 2.0 g/(kg·d) to 2.3 g/(kg·d)[64,126,131] for a limited period of time (2 wk), may be advantageous in preventing FFM loss[134,135]. Mettler *et al*[135], after a week of habitual diet (week 1, run-in) and a week of isocaloric diet with a macronutrient energy intake composition of 50% carbohydrate, 15% protein, and 35% fat (week 2), compared two energy-restricted diets: one with 15% protein [about 1 g/kg, control group (CP)] and one with 35% protein [about 2.3 g/kg, high-protein group (HP)] for 2 wk (weeks 3 and 4). Decreases in BM (-3.0 ± 0.4 kg and -1.5 ± 0.3 kg for the CP and HP, respectively) and lean BM (-1.6 ± 0.3 kg and -0.3 ± 0.3 kg) were larger in the CP compared to those in the HP and similar FM loss were observed[135]. However, urea was higher in the HP group (8.0 ± 0.4 mmol/L and 7.9 ± 0.5 mmol/L in week 3 and 4, respectively) compared to the CP group (5.3 ± 0.4 mmol/L and 5.4 ± 0.5 mmol/L in week 3 and 4, respectively)[135]. The authors concluded that because body composition data indicate that there was no protein accretion but still a slight loss of lean BM, some of the proteins must have been metabolized, causing increased urea values[135]. Considering the risk of CKD typical among individuals with SCI[85,87], protein intakes over 2.0 g/(kg·d) should not be recommended in Paralympic athletes with SCI, even for limited period of time.

Timing is also important for proteins (Table 1). For example, co-ingestion of CHO with protein during the recovery period resulted in improved net protein balance post-exercise. Ingesting protein (approximately 20 g to 30 g total protein, or

Table 1 Suggested intakes and timing of carbohydrate and protein for paralympics[64,131,132]

Nutrients	Food sources	Quantity	Timing
Carbohydrate	Pasta, rice, cereals, breads, legumes, potatoes, fruit, sugar	Light: Low intensity or skill-based activities: 3-5 g/(kg·d); Moderate: Exercise program (1 h/d): 5-7 g/(kg·d); High: Endurance program (1-3 h/d moderate to high-intensity exercise): 6-10 g/(kg·d); Very high: Extreme commitment (> 4-5 h/d moderate to high-intensity exercise): 8-12 g/(kg·d)	Before or during the exercise session, during recovery from a previous session; extended (> 60 min) bouts of high intensity (> 70% VO_{2max}): CHO at a rate of about 30-60 g of CHO/h in a 6%-8% CHO electrolyte solution (6-12 fluid ounces) every 10-15 min throughout the entire exercise bout
Proteins	Beef, fish, poultry, eggs, and dairy products (high biological value sources), legumes	From 1.2 g/(kg·d) to 1.7 g/(kg·d)	After exercise, co-ingestion of CHO with a high-protein recovery snack is recommended to help restore muscle glycogen effectively; Post-exercise ingestion (immediately to 2-h post) of high-quality protein sources stimulates robust increases in muscle protein synthesis; Protein-rich foods/fluids (such as milk or cheese) before bed promotes muscle protein synthesis overnight

CHO: Carbohydrate; VO_{2max}: Maximum oxygen uptake; >: Major.

approximately 10 g essential amino acids, especially from high biological value sources, like beef, fish, poultry, eggs, and dairy products[64] during exercise or the recovery period (post-exercise) led to increased whole body and muscle protein synthesis as well as improved nitrogen balance[136,137].

The IOS 2018[136,137] stated that protein supplements, usually low in CHO and providing 20-50 g protein per serving from high quality sources (whey, casein, milk, egg) or vegetables (*e.g.*, soy), may contain other ingredients, some of which are not evidence-based and may increase the risk of contamination. For these reasons, protein supplements should be taken with caution.

All athletes with SCI should be encouraged to hydrate adequately (2-2.5 L/d unless other indications)[138]. Indeed, athletes with SCI are sensitive to hydration problems, in particular wheelchair-bound athletes tend to reduce their intake of liquids to avoid the complexity associated with toilet hygiene[139]. Moreover, thermoregulatory function may be impaired in Paralympic athletes and individuals with SCI[6,107,140]. In hot conditions poor thermoregulation due to impaired sweat rate can lead to overheating which can be treated by hand cooling, foot cooling, ice vests and spray bottles[102,141,142]. The fluid plan that suits most able-bodied athletes and athletic events consist of an intake of 0.4 L/h to 0.8 L/h[131], although this should be customized to the athlete's tolerance, thermoregulatory function, and opportunities for drinking fluids. Monitoring changes in BM (before and after exercise) is an easy method to evaluate fluid needs[103,139]. Electrolyte replacement supplements (50-60 mM sodium, 10-20 mM potassium and low CHO 2-4 g/100 mL) can be used for rapid rehydration following large sodium losses during ultraendurance activities, and sport drinks (containing 5%-8% CHO, 10-35 mM sodium, 3-5 mM potassium) during exercise has been identified to be useful for post-exercise rehydration and CHO refuelling by the IOS 2018[136,137]. Although caffeine is among the few supplements (including buffering agents and nitrate) that have been shown to favourably affect performance[136,137], energy drinks containing caffeine should be avoided by athletes with SCI and NBD[36] (Figure 1).

Nitrates are popular supplements for prolonged submaximal exercise and high-intensity, intermittent, short-duration efforts. Increasing nitric oxide and enhances muscle blood flow, but potential risks for gastrointestinal upset in susceptible athletes have been suggested[136,137]. High nitrate-containing foods include leafy green and root vegetables, including spinach, rocket salad, celery, and beetroot[136,137]. It has been suggested that beetroot juice (among functional beverages) may improve performance[143,144]. However, the positive effects of beetroot juice seen in young individuals were not observed in older adults[145]. In a recent randomized (placebo-controlled) cross-over study in upper body trained able-bodied individuals and Para-cyclists with SCI (lesion level between C4 and L4), Para-cyclists showed higher nitrate concentrations after beetroot juice, whereas no differences were found in performance in either group[146]. Although a meta-analysis reported nitrate-independent blood pressure lowering effects of beetroot juice[147] and we found only a case report of

interaction with methotrexate[148], supplementation in patients with in CKD requires further studies[149]. Furthermore, caution with chronic use of beetroot juice to enhance sports performances has been suggested due to increases in carcinogenic N-nitroso compounds in urine after consumption[150].

In general, an analysis of a Paralympic athlete's sweat loss and dietary intakes is recommended to evaluate fluids, carbohydrate, protein, iron, and vitamin D status to detect nutrient insufficiencies that greatly impact athletic performance[64]. Indeed, Paralympic athletes often fail to meet the recommended dietary allowances for vitamin D, vitamin E, pantothenic acid, magnesium, potassium, iron (females), calcium (females), vitamin A (males) and folate (males)[151]. Many Paralympic athletes are at higher risk of low bone density and osteoporosis; therefore, it is important to optimize all the nutrients and factors that support bone health. Calcium-rich foods such as dairy products, fish with soft bones, and calcium-fortified foods should be encouraged, as athletes with impairments are often found to consume insufficient calcium[64]. But since Paralympic athletes are also at high risk of being deficient in vitamin D due to the nature of their health conditions or inadequate micronutrient intakes, and vitamin D can impact morphology and functionality of human skeletal muscles, testing for vitamin D status is highly recommended. In addition, promotion of safe exposure to the sunlight and vitamin D–rich food sources, such as oily fish and eggs is recommended[64]. In addition, supplementation should be provided in cases of deficiency.

Commonly consumed supplements by Paralympic athletes are vitamin D, protein powder, sport bars, and sport drinks[151,152]. In a recent study, elite athletes with SCI improved handgrip strength after a supplementation protocol based on initial 25(OH)D concentrations, whereas no change in 20-m wheelchair sprint performance was observed[153]. Vitamin D is among the nutraceuticals with moderate evidence of efficacy for immune health in athletes, along with vitamin C and probiotics[136,137]. However, according to the "IOC Consensus Statement: Dietary Supplements and the High-Performance Athlete" (IOC 2018) careful monitoring is necessary to avoid its toxicity[136,137].

The dietary recommendations, in addition to taking into account the requirements related to training and sport type, require careful consideration of the specific characteristics and physiopathology of the athlete to accommodate the unique issues of individual athletes regarding health status, nutrient needs, performance goals, physique characteristics (*i.e.*, age, gender, stage of development, body composition), practical challenges, food preferences, and environmental conditions. Similar to able-bodied athletes there is no "one size fits all" approach. Nutrition plans need to be personalized to the individual athlete to take into account the nature of the health condition and the consequent impairment and its impact on functional capacity, the use of medications, and any coexisting medical conditions and responses to various strategies. Special nutrition recommendations are needed since athletes with impairments and in particular those with SCI are at major risk of medical complications, including low bone density and osteoporosis, epithelial wound and pressure ulcers, urolithiasis and UTI, and chronic constipation.

DISCUSSION

Bowel function, as codified by the International Classification of Functioning, Disability and Health is frequently compromised after SCI. Among International Classification of Functioning, Disability and Health ICF domains, the greatest impact of NBD is on personal and environmental factors, with 45.3% reporting need of assistance, 45.3% in emotional health and 46.9% in loss of privacy[154].

Relationships among inflammation, fatigue pain and behaviour aspects have been discussed[155]. Although a 3-mo anti-inflammatory diet with increased intake of vitamins A, C, and E, and ω-3 PUFA and reductions in trans fatty acids, caffeine and sodium, reduced chronic inflammation in individuals with SCI[73], barriers to adhering to this diet have been reported[156]. Among the reasons for low compliance are the reported increase in NBD Score after high fibre intake[51,52]. On the other hand, exercise training, reducing constipation and restoring eubiosis, can improve quality of life in individuals with SCI and NBD. Moreover, exercise improved semen quality[106] and upper limb aerobic training increased aerobic fitness[116] and reduced inflammatory cytokines[8] and oxidative stress[105]. Different sport activities have different effects on physical fitness components[113,125]. In the "Veterans Exercise Testing Study", Myers *et al*[157] demonstrated that fitness is inversely related to overall health care costs among veterans.

Paralympic athletes, like individuals with SCI, had low diet quality, in terms of

fruits, vegetables, legumes and cereals and there is a need for nutrition education for this population[158]. Due to a reported low nutritional knowledge[159], personalized nutrition and education related to different macro and micronutrients requirements compared to able-bodied athletes is recommended. Furthermore, mixed (outdoors and indoors) training programs[160] could improve vitamin D status[161]. Concerning sport-related muscle pain[162], we included in our Med-D-based and exercise pyramid (Figure 1) the suggestions from the Med-D for individuals with chronic pain[72].

CONCLUSION

The present review suggests dietary advice for individuals with SCI and NBD and underscores that multidisciplinary risk reduction programs, including dietary advice[114] and exercise[118], are recommended, as depicted in the Med-D-based and exercise pyramid for individuals with SCI (Figure 1). Finally, we agree with the recently suggested systematic control needed to re-adapt nutritional programs for wheelchair athletes[163].

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REVIEW

Role of gut microbiota-immunity axis in patients undergoing surgery for colorectal cancer: Focus on short and long-term outcomes

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Abstract

Human body is colonized by a huge amount of microorganisms mostly located in the gastrointestinal tract. These dynamic communities, the environment and their metabolites constitute the microbiota. Growing data suggests a causal role of a dysbiotic microbiota in several pathologies, such as metabolic and neurological disorders, immunity dysregulations and cancer, especially the well-studied colorectal cancer development. However, many were preclinical studies and a complete knowledge of the pathogenetic mechanisms in humans is still absent. The gut microbiota can exert direct or indirect effects in different phases of colorectal cancer genesis. For example, *Fusobacterium nucleatum* promotes cancer through cellular proliferation and some strains of *Escherichia coli* and *Bacteroides fragilis* produce genotoxins. However, dysbiosis may also cause a pro-inflammatory state and the stimulation of a Th17 response with IL-17 and IL-22 secretion that have a pro-oncogenic activity, as demonstrated for *Fusobacterium nucleatum*. Microbiota has a crucial role in several stages of postoperative course; dysbiosis in fact seems related with surgical site infections and *Enterococcus faecalis* (and other collagenase-producers microbes) are suggested as a cause of anastomotic leak. Consequently, unbalanced presence of some species, together with altered immune response may also have a prognostic role. Microbiota has also a substantial role in effectiveness of chemotherapy, chemoresistance and in the related side effects. In other words, a complete knowledge of the fine pathological mechanisms of gut microbiota may provide a wide range of new diagnostic tools other than therapeutic targets in the light of tailored medicine.

Key words: Intestinal microbiota; Colorectal cancer; Chemo-resistance; Therapeutic strategies

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Core tip: Microbiome and immunity sciences are fields in rapid evolution gaining growing attention. The gut microbiota-immunity interplay seems to have a very important role in all the different phases of colorectal cancer process from oncogenesis to treatment and prognosis. However, many aspects have been studied only in experimental models and many theories must still be proved in humans. Providing the actual state of art of this interplay on the different steps involved in colorectal cancer, new multidisciplinary studies in humans according to this perspective may be drafted with the purpose of widening the possibilities of treatment against this frequently diagnosed pathology.

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INTRODUCTION

A large and diversified group of microorganisms comprehending bacteria, viruses and fungi normally populates intestinal mucosa, such as every other epithelial surfaces. These microbes, together with their metabolic products and their local microenvironment compose the so-called microbiota[1,2]. Although most of microbiota strains are not cultivable, recent technologies of genomic sequencing, proteomics and metagenomic analysis of DNA and RNA allowed the initial identification of this population of microorganisms, along with their metabolic production and signal pathways[1-4].

Nevertheless, these commensal microbes are normally symbiotic and the immune system has established various tolerance mechanisms[2]; but, in specific conditions, the equilibrium break of microbiota-immunity axis can be responsible for several pathologies[2]. The hypothesis that a microorganism could be the cause of a surgical disease started with the discovery of the *Helicobacter pylori* role in the genesis of peptic ulcer. However, since the prevalence of this infection is much more higher than the incidence of peptic ulcer and since peptic ulcer may present without this infection, *Helicobacter pylori* was considered a "not necessary" nor "sufficient" agent to cause this pathology[1].

Similarly, the potential pathogenetic role of gut microbiota (GM) alteration in the initiation and progression of colorectal cancer (CRC) has been recently discussed[5]. For this purpose, the microorganisms may have a direct causal role or act perturbing the local immune response[2]. However, this complex relation is still far from being completely understood: The microbiota is dynamic, varying on hourly basis and the "current" microbiota of every person is the result of the individual past exposure to external agents, making the task to draft general conclusions even more challenging[3].

Several prognostic factors for CRC, for both short-term postoperative outcomes and long-term oncological outcomes, have undoubtedly been recognized[6], but, new potential prognostic factors have been proposed along the years and, in particular, the potential prognostic role of the microbiota is attracting much attention[3]. However, differences in microbiota may be at least a part of the cause of different outcomes achieved in a group of patients treated with the same protocols[3].

Although surgical resection is the cornerstone in the CRC management, whenever technically feasible, chemotherapy has a complementary role in advanced stages of disease. Relationship between chemo-resistance and intestinal microbiota has been advocated[5] but the fine mechanisms still remain unknown. Since chemo-resistance reduces the survival expectancy, the understanding of the causes of this phenomenon would be extremely important[5].

The aim of this review is a summary of the actual state of art on a developing research field: The interplay between microbiota and inflammatory/immune response applied on patients undergoing surgery for colorectal cancer, which is a pathology with high incidence and not negligible morbidity and mortality rates. Microbiota-based approach may provide a wide and quite revolutionary range of possibilities to interfere with the different phases of CRC management. Particular attention was set on postoperative outcomes in order to provide inspiration for

further studies and for new potential strategies for the treatment, but also for the prevention of colorectal cancer.

GUT MICROBIOTA-IMMUNITY AXIS IN HEALTH

Advent of new technologies in metagenomic field and mass spectrometry pushed the investigators to analyze the possibility of the existence of both "health-promoting" and "disease-promoting" ecosystem of microorganisms[1]. Comprising almost 99% of the total amount of human-associated microbial mass, thousands of different species of commensal bacteria are required for a healthy gastrointestinal tract[2,7,8]. These microorganisms are members of different domains comprehending Bacteria, Archaea and Eukarya while the four most represented phyla of bacteria are Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria[2]. In particular, about 90% of gut bacteria belongs to Bacteroidetes and Firmicutes[8,9]. The gut microbiota help in several host tasks such as in digestion of complex foods (*e.g.*, pectins), vitamins production and metabolism of glycans and fats[9]. Nevertheless, they have a role in protection against external pathogens or toxic compounds[2,7,8].

A strong relationship between intestinal microbiota and immunity system has been described[1,2,5,10]. The innate immune system associated with the mucosal surfaces accounts for approximately 80% of the active immune system and the great majority of them are located in the gastrointestinal tract[2].

In addition, the microbiota largely contributes to the development of the lymphoid tissue[11] and it can modulate host immune system, both innate and adaptive[12]. Intestinal microbiota interacts with the immune response elements of the whole body through dendritic cells or through the stimulation of epithelial receptors, even in absence of bacterial translocation[1]. Consequently, intestinal microbes may also have either a negative or a positive effect on the immunity[11]. The Figure 1 represents a simplified summary of these interactions.

INTESTINAL MICROBIOTA AND IMMUNITY DYSREGULATION IN COLORECTAL CANCER

The colorectal cancer is the third most frequent cancer worldwide[13] and there is a probability 4%-5% of a having a CRC in the life span[9]. Various risk factors for CRC have already been described such as life and dietary style or some comorbidities (*e.g.*, ulcerous colitis or other conditions) leading to a persistent and prolonged colon inflammation[14,15]. Some bacteria with pro-inflammatory activities may modify the permeability of the intestinal mucosa easing the translocation of pathogens and their toxins[2,16]. Furthermore, protracted inflammation causes prolonged oxidative stress that may be responsible for DNA damages[17], as demonstrated for *Escherichia coli* in animal models[18].

Several papers have already highlighted a potential role of intestinal dysbiosis in the initiation and progression of human CRC[14] taking advantage of previously published studies on animal models[19,20]. Dysbiosis is defined as (1) The abnormal and predominant presence of pathogens in an environment or (2) Alterations of the considered normal proportion of the different specimens composing the microbiota[1]. This new "ecosystem" is also called pathobiome[21]. Moreover, the modifications within the microbiota related with a particular disease may take place at every taxonomic level, from the phylum to species making the discovery of these modifications and of their causal effect, an extremely challenging task[4].

Three different pathogenetic models have been proposed. According to the "alfa-bugs" model, some species (*e.g.*, enterotoxigenic *Bacteroides fragilis*-ETBF) may have direct pro-oncogenic effect acting against both immune system and protective microbial species[22]. The "bacterial driver-passenger" model suggests that some "driver bacteria" promote cancer development through DNA damage. Subsequently, as consequence of new selective pressures, "passenger bacteria" replace them having protective or cancer-promoting activities. The results of this new balance will determine tumor progression or tissue healing[23]. To note that, according to this model, microbes responsible for tumor initiation may be absent during the subsequent phases[24]. In the "keystone pathogen" model, some poorly represented pathogens have the unbalanced ability to alter the equilibrium within the normal microbiota causing a dysbiosis[25]. These theories are depicted in Figure 2.

Alterations in microbiota composition have been found in samples from normal colorectal mucosa, feces and tumor specimen in patients affected by CRC[5,26-28]. Interestingly, in a case-control study of Flemer, significative differences in microbiota

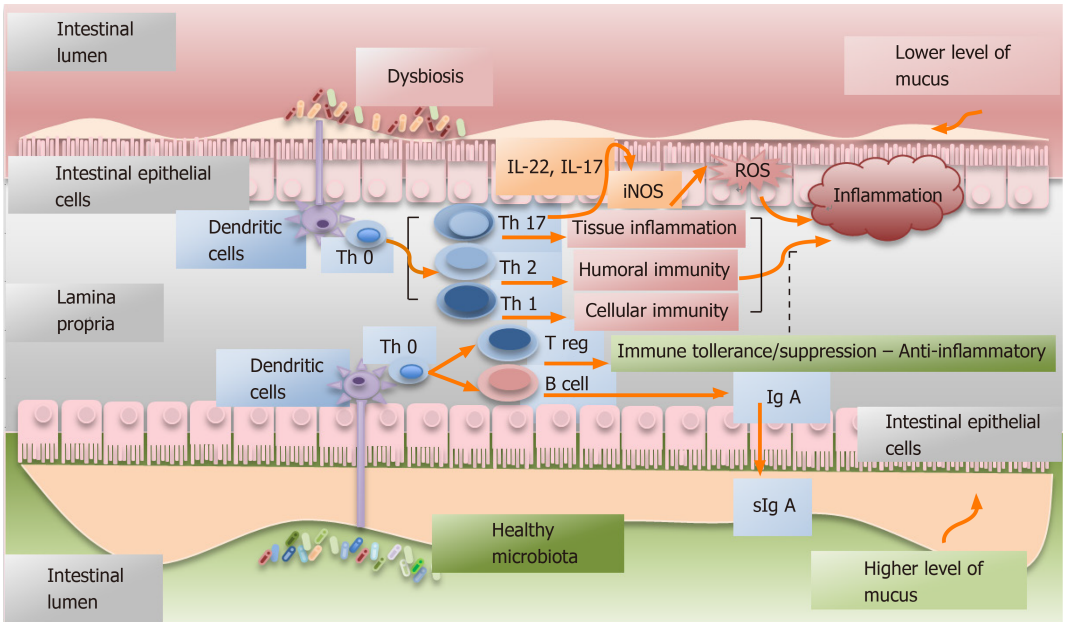

Figure 1 Simplified graphic depicting the interactions between the healthy or dysbiotic microbiota and immune system. Dysbiosis is defined as the abnormal and predominant presence of pathogens in an environment or as alterations of the considered normal proportion of the different specimens composing the microbiota[1]. The microbiota largely contributes to the development of the lymphoid tissue[11] and it can modulate host immune system (innate and adaptive)[12]. Intestinal microbiota interacts with the immune response elements through dendritic cells. In dysbiosis, a Th17-type of immune response may be activated with consequent production of IL-17 and IL-22, both having a pro-inflammatory and pro-tumoral effect[2]. Furthermore, IL-22 can favor the expression of inducible nitric oxide synthase and the subsequently production of oxygen reactive species that are linked to cancer promotion[45].

composition were found between healthy volunteers and people affected by intestinal polyps[28], suggesting GM alteration in a very early stage of disease. In CRC patients, higher presence of some microorganisms (*e.g.*, *Fusobacterium*, *Enterococcus faecalis*, Staphylococcaceae or Coriobacteridae) has been reported in previously published papers together with a lower presence of other microbes including Enterobacteria, *Bifidobacterium*, *Lactobacillus* and *Treponema*[29,30]. Coherently with the well-known different behavior of the right or left colon cancer, different GM modifications have been found in proximal and distal CRC[28]. Microbiota composition found in right colonic cancer was more similar to that found in control group with lower activation of Th17 response[28]. However, further clinical implications are still missing[28].

In particular, an association between *Fusobacterium nucleatum* (*F. nucleatum*) and CRC has been proposed and a suggested pathogenic mechanism involves the activation of β-catenin signal pathway causing cellular proliferation (as consequence of the bindings between FadA and E-cadherin, located on the cells of the intestinal epithelium)[31]. The *F. nucleatum* resulted much more represented in CRC patients when compared with healthy people[5,17]. Furthermore, its presence seems related with high-level of instability of microsatellites (MSI)[32,33] and with a CpG island methylator phenotype[34]. Nevertheless, the number of *F. nucleatum* and of *Bacteroides fragilis* (*B. fragilis*) (both in stool sample and tumor tissue) seems to increase along with the progression from adenoma to adenocarcinoma[35-37].

Similarly, a relation between the population number *of B. fragilis* and *Escherichia coli* (*E. Coli*) (adherent-invasive ones) and tumor size has been reported[38]. Possible pathogenetic mechanisms include bacterial production of toxins, known as genotoxins, able to generate damage to DNA[39]. Examples of these toxins are *B. fragilis* toxin, the cytolethal distending toxin or colibactin toxin produced by polyketide synthase (pks) positive *E. coli* or the cytotoxic necrotizing factor 1[24,39]. Higher genes' expression of *B. fragilis* toxin and colibactin toxin has been found in patients affected by familial adenomatous polyposis (FAP) when compared with healthy people[40].

Furthermore, some microbial metabolites derived from alimentary intake may result genotoxic and cytotoxic[41]. *Clostrudium*, *Bacteroides* and *E. coli* have been reported to have this capacity[23]. In addition to direct promoting effects, intestinal microbiota may interfere in cancer proliferation through the interplay with the immune response. *F. nucleatum* resulted associated with lower level of $CD3^+$ T cells[32,33], increased production of TNF-α, IL-6, IL-12 and IL-17 (all having a pro-tumoral effect), upregulation of myeloid-derived cells, and indirect suppression of $CD4^+$ T cells activity[9,17,31]. Nonetheless, Fap2 protein produced by this microorganism is able to prevent the antitumor effect of NK cells and other T cells binding with

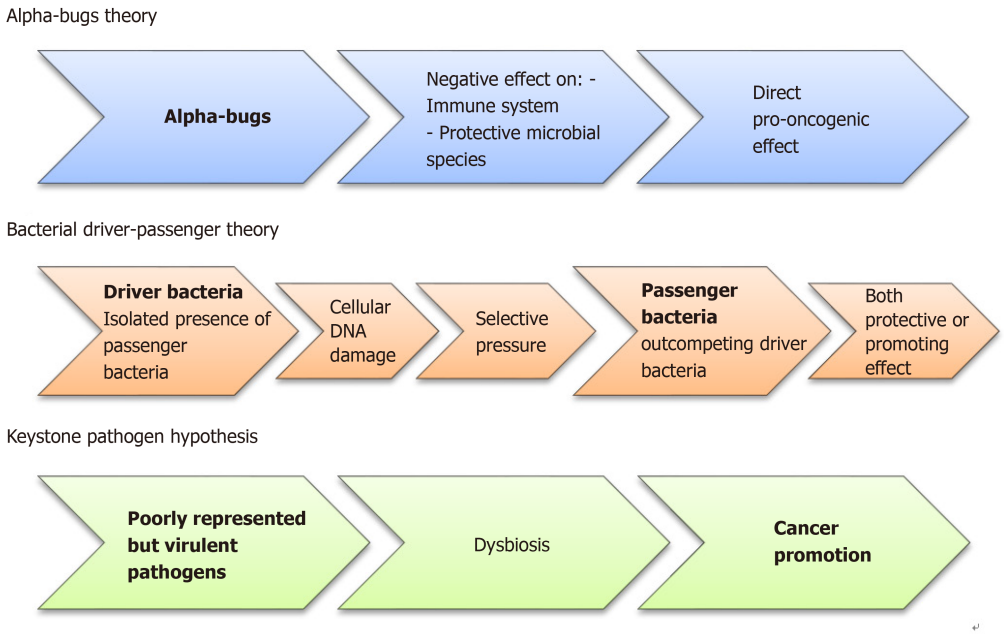

Figure 2 Diagram showing the three pathogenetic models involved in colorectal cancer initiation and promotion. Currently, to explain the colorectal cancer development, three different pathogenetic models have been suggested. According to the "alfa-bugs" model, some species (*e.g.*, *Bacteroides fragilis*) may have direct pro-oncogenic effect acting against both immune system and protective microbial species[22]. The "bacterial driver-passenger" model suggests that some "driver bacteria" promote cancer development through DNA damage. Subsequently, the "passenger bacteria" replace them having protective or cancer-promoting activities. The results of this new balance will determine tumor progression or tissue healing[23]. Finally, in the "keystone pathogen" model, some poorly represented pathogens have the unbalanced ability to alter the equilibrium within the normal microbiota causing a dysbiosis[25].

inhibitory receptors[32,42].

In animal models, *B. fragilis* toxin can activate the signal transducer and transcription-3 (STAT3) pathway that is related with a specific Th17 differentiation. On the contrary, inhibition of IL-17 and IL-23 with antibodies has an antitumor action[39]. Furthermore, in presence of ETBF, regulatory T cells, which are usually related with an antitumor effect, seem to promote cancer progression through Th17 expansion[43]. Nonetheless, *B. fragilis* can promote inflammatory response inducing the signaling NF-κB pathway[44].

Some species of *Clostridium* (segmented filamentous bacteria) are other microbes able to activate Th17 producing IL-17 and IL-22 with a pro-inflammatory and pro-tumor effect[2]. Furthermore, IL-22 can favor the expression of inducible nitric oxide synthase (iNOS) and subsequently, the production of oxygen reactive species that are linked to cancer promotion[45]. High levels of IL-23 have also been found in human CRC and they seem able to activate Th17 response with further production of IL-17 e IL-22[46]. Other promoting cancer cytokines are IL-6 and 1[17]. Further specific details will not be object of this review and can be found elsewhere[17].

Despite the crucial role of inflammation in CRC development, use of non-steroidal anti-inflammatory drugs is not routinely indicated due to their potentially severe side effects[9]. The Figure 3 shows some examples of suggested mechanisms. On the contrary, some microorganisms seem to have a direct protective effect against tumor growth, for example, those producing short-chain fatty acid (*e.g.*, butyrate or acetate)[5]. Accordingly with previously published data, *Bifidobacterium* seems able to inhibit tumor progression reducing the infection rate from enteropathic microorganism and decreasing the production of bile products[47,48]. Moreover, some microbes may exhibit an anticancer activity through the interaction with immune system. This positive effect is related with the phagocyte stimulation, the enhancement of NK cytotoxicity and an incremented production of immunoglobulins, including IgA (that contributes to the mucosal barrier activity)[10,11]. Evidences from experimental studies suggest that *Bifidobacterium* may also favors and antitumor immune response, inhibiting NF-κB signaling pathway[17,49]. Similarly, *Faecalibacterium prausnitzii* may have a positive effect through the induction of IL-10 secretion and the modulation of Treg response[24]. IL-10 is able to control the proliferation of Th17 cells stopping cancer progression[2,50]. Furthermore, IL-10 downregulates TNF-α production and iNOS expression[17].

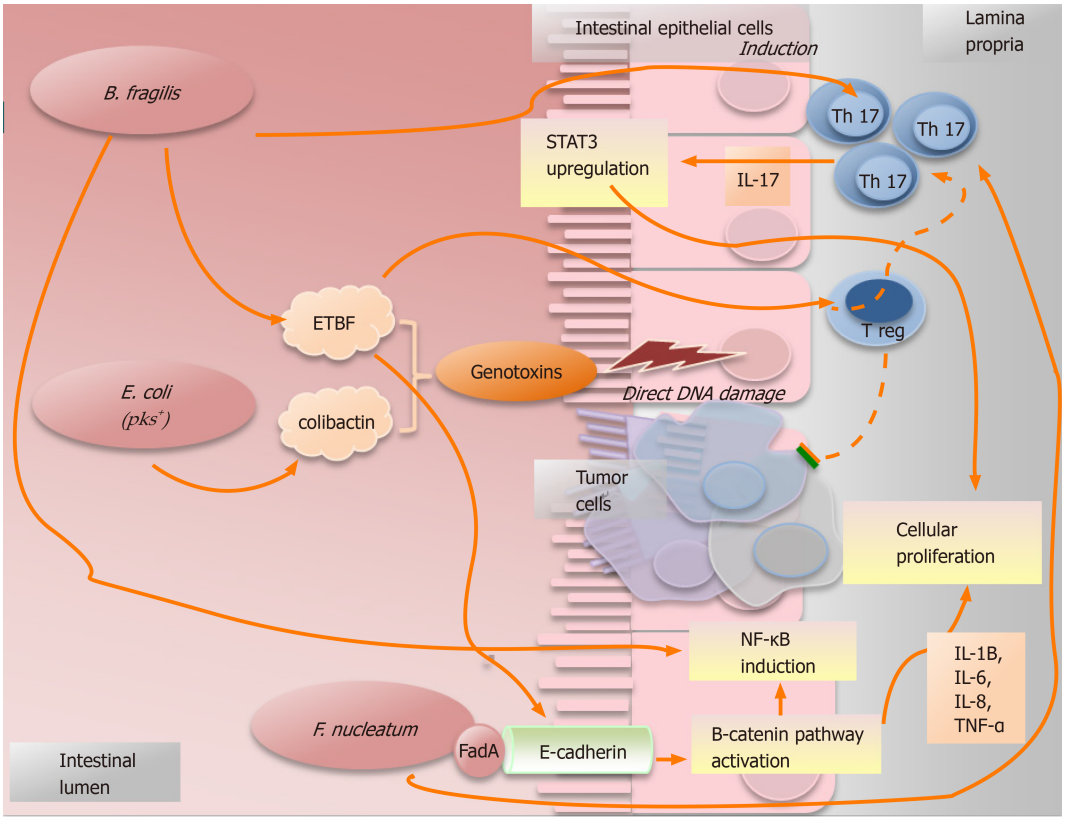

Figure 3 Simplified picture illustrating some examples of how microorganisms promote the cancer. *Bacteroides fragilis* can cause the induction of Th17-type immune response with upregulation of signal transducer and transcription-3. Moreover, some subtypes of *Bacteroides fragilis* can secrete the toxin EBTF that can cause cancer in different ways: (1) Through direct DNA damage; (2) By Treg cells, which in presence of EBTF, seem to promote cancer progression through Th17 expansion[43]; and (3) Through the stimulation of the cleavage of E-cadherin which causes cellular proliferation and intestinal barrier breakage[23]. Nonetheless, *B. fragilis* can promote an inflammatory response inducing the signaling NF-κB pathway[44]. The particular group of polyketide synthase (pks) positive *Escherichia coli* (*E. coli* pks$^+$) maintained a genomic island called "pks". These bacteria can produce the genotoxin "colibactin", that is able to induce direct DNA damage and, consequently, to increase the frequency of gene mutations[24,39]. *Fusobacterium nucleatum* can activate the β-catenin signal pathway causing cellular proliferation as consequence of the bindings between the bacterial adhesin FadA and E-cadherin which is located on the cells of the intestinal epithelium[31]. Furthermore, it is related to an increased production of some pro-cancer cytokines, such as TNF-α, IL-6, IL-12 and IL-17. STAT3: Signal transducer and transcription-3; ETBF: Enterotoxigenic *Bacteroides fragilis*; *E. coli*: *Escherichia coli*; *F. nucleatum*: *Fusobacterium nucleatum*.

SURGICAL TREATMENT OF CRC: GUT MICROBIOTA-IMMUNITY AXIS IN SHORT AND LONG-TERM OUTCOMES

Surgical stress determined by treatments before and after surgery (including bowel preparation, antibiotic exposure, proton-pump inhibitors' administration and fasting) and the operation for CRC itself seem to reduce the GM biodiversity. Consequently, the balance within the intestinal microbiota and its environment results altered[51].

Deng *et al*[5] reported a reduction of Bacteroidetes and Firmicutes and an increase of Proteobacteria in patients surgically treated for CRC when compared to healthy volunteers. However, although this study is focused in evaluating the microbiota in fecal samples from 4 different groups (healthy controls, CRC patients, CRC patients operated and CRC patients treated with chemotherapy), the sample size is very small (5 patients operated within a total of 69 people involved). However, similar studies on patients after surgery for CRC confirmed a reduction of obligate anaerobes including several species of *Clostridium*, *Bacteroides* and *Prevotella* together with a reduction of *Bifidobacterium*. On the contrary, *Enterococcus*, *Staphylococcus* and *Pseudomonas* resulted enriched after surgical treatment[52].

Sze *et al*[53] evaluated GM changes before and after surgery in patients treated for adenomas, advanced adenomas or carcinomas. Carcinoma group had the major significant variation in microbial composition before and after surgical treatment and, interestingly, microbiota after surgery resulted quite similar to healthy people. Consequently, microbial alteration found during the follow-up may be considered as a potential biomarker for tumor recurrence and may be used to stratify the recurrence risk[53]. However, the real impact of these findings remains unknown and definitive conclusions cannot be drawn. The Table 1 summarizes the GM changes in healthy people and in patients with CRC or surgically treated.

Table 1 Composition of gut microbiota in healthy people, in patients with colorectal cancer and after colorectal cancer surgery

Healthy	Colorectal cancer	Post-operation
Bacteroidetes[5]	↑ Staphylococcaceae[28-30]	↑ Proteobacteria[5], ↑ Pseudomonas aeruginosa
Firmicutes[5], ↑ Lachnospiraceae ↑ *Clostridium*	↑ Coriobacteridae[28-30], ↑ *Fusobacterium nucleatum*[5,32,33]	↓ Bacteroidetes[52], ↓ *Bacteroides ovatus*, ↓ *Prevotella*
↑ *Bifidobacterium*[52], ↓ *Fusobacterium nucleatum*[5,17]	↑ *Enterococcus faecalis*[28-30], ↑ *Bacteroides fragilis*[35-38] ↑ *Escherichia coli* pks$^+$[38]	↓ Firmicutes[52], ↓ *Clostridium* but, ↑ *Enterococcus*, ↑ *Staphylococcus*
	↓ *Bacteroides ovatus*[37]	↓ *Bifidobacterium*[52]
	↓ *Bifidobacterium*[28-30]	
	↓ *Lactobacillus*[28-30]	
	↓ *Treponema*[28-30]	

↑: Higher abundance; ↓: Lower abundance; pks$^+$: Polyketide synthase positive.

Short-term outcomes

The well-known better outcomes following bloodless interventions or postoperative course not requiring an antibiotic therapy may also be related with a better preservation of the pre-existing microbiota equilibrium[1]. Nevertheless, the good results obtained following the application of the enhanced recovery after surgery programs may also be related to a virtuous interaction with the microbiota, as well[1]. Obviously, the ability of the single individual's microbiota to "refaunate" is of pivotal importance and many "molecular-level" aspects remain unevaluated. Consequently, further investigations according with these new perspectives are needed. Nonetheless, the analysis of the different outcomes within a group treated according to a specific protocol may become more interesting than the comparison between application or not of the protocol itselfs[1,3].

Postoperative infections

In general surgery and especially in colorectal surgery, the infection rate is still high (about 15%), being a common readmission cause with an increase in the costs, as well[3]. Obviously, intraoperative direct contamination is an unquestionable risk factor for infections. However, after elective surgery, during which the level of contamination is low, surgical site infections (SSIs) do exist and *Staphylococcus aureus* strains (which belong to cutaneous resident flora) represent the most frequently found species[3]. Furthermore, cultures from surgical site at the surgery-end are often poor efficient in predicting a future SSIs and, eventually, the related causal pathogens. This may have several explanations: (1) The actual instruments are not able to detect the whole composition or (2) Other still unknown mechanisms may also have a causal role[3]. According to the proposed "Trojan Horse hypothesis", some low-abundant pathogens including methicillin-resistant *Staphylococcus aureus* or *E. faecalis* are carried on by macrophages or neutrophils from the gut to distant sites such as surgical wound[3,54].

Nonetheless, in patients considered at high risk for SSIs, these pathogens may have a particular high virulence that resulted no more balanced by the other microorganisms after surgical insult[3,54]. Prolonged postoperative fasting, not adequately compensated with enteral nutrition, may alter the GM composition causing a higher surgical site infection rate[55]. Opioid drugs seem to both directly inhibit immune system and cause microbiota changes, favoring a greater virulence of the microbes[1,56,57]. On the contrary, the use of competitive opioids antagonists seems related to a reduction of morbidity rates after colorectal surgery[58]. Consequently, reducing preoperative fasting and decreasing the use of opioids should be encouraged.

Finally, the intestinal microbiota may also have a role in wound healing[57,59]. A possible mechanism is related to the fermentation of lactic acid from specific species of the intestinal microbiota that enables the production of the neuropeptide oxytocin through a stimulation of the vagus nerve. This peptide allows the recruitment of T cells for an enhanced healing of surgical incision[59].

Anastomotic leak

Anastomotic leak (AL) is the most feared surgery-related complication. It is defined as a spillage of intestinal material outside the sutured bowel at the anastomotic site, caused by a defect of intestinal wall. Depending on its severity, it may be related to further complications (including death) and its management may range from

observation to surgical treatments[60]. Anastomotic leak rates are reported to range between 1% and 19%[60]. The well-known risk factors for intestinal AL are: Tension between the intestinal edges, reduced blood supply to the viscera and technical errors.

However, surgery causes inflammation and inflammatory response may induce gut dysbiosis. Consequently, a causal role of intestinal pathogens has been reported. In 1954, Cohn conducted a study on dogs demonstrating that colon decontamination totally avoided anastomotic leak and resulted able to reverse colonic ischemia[61]. More recently, the causal role of some microorganisms including *Enterococcus faecalis* and *Pseudomonas aeruginosa*, has been suggested[1,62]. In animal models, these species can cause the leak of the anastomosis producing high level of collagenase (matrix metalloproteinase-MMP), mostly MMP-2 and MMP-9[62]. Abundance of these species has been found in humans who had surgery complicated by anastomotic leak[1,62] and copious presence of *E. faecalis* seems to persist into the colon despite bowel preparation[52]. Moreover, in animal models, morphine administration has been shown to increase the presence of more adhesive *E. faecalis* within anastomotic tissue. Consequently, there is greater collagenase production favoring higher anastomotic leak rates[63].

Anyhow, collagenase-producing bacteria seem to be necessary but insufficient in causing an anastomotic leak[3]. The other conditions needed to cause an anastomotic leak are: (1) The microbiota unbalancing (with protective microbes reduced enough to unleash pathogens); (2) Inflammatory response from anastomotic tissue is also required to make the pathogens sense and respond to a such modified environment, and (3) Pathogens must be virulent enough to overcome host defenses[3]. Low rectal resections and neoadjuvant radiotherapy are independently associated with a higher anastomotic leak rate. In patients undergoing neoadjuvant radiotherapy, interplay between radiation and GM alteration has also been advocated as a cause for higher AL rate. Radiotherapy seems to promote higher levels of virulent anaerobes in the treated site[21]. On the contrary, there are commensal GM bacteria, which have a pivotal role in defending epithelial cells of the intestine from apoptosis induced by radiations[64].

Even in this condition, microbiota seems to have both a protective and harmful role. Further studies in humans should be performed in order to assess which are health-promoting or noxious species. Again, the global result of complex modifications within this environment is more important than the single pathogen itself confirming the Koch's postulates[1,65]. The simple analysis of the mere presence of a pathogen in a stool sample may seem quite reductive to achieve a complete understanding of a multifactorial event[3].

Although some recent and apparently countercurrent studies suggesting that oral antibiosis and complete mechanic bowel preparation allow the reduction of postoperative morbidity[66], a definitive response whether and, above all, how bowel preparation and/or antibiotic therapy resulted in a modified rate of the anastomosis leakage is still lacking and level 1 evidence are still missing[1,67]. Finally, independently from dysbiosis, a causal inflammation role in the AL at the anastomotic site has been advocated[68]. Involved innate immune response, mostly composed of neutrophils, may exacerbate hypoxia in damaged tissues, consuming oxygen during oxidative burst[69]. In emphasis, the use of non-steroidal anti-inflammatory drugs may seem intriguing; however, they are not routinely indicated, essentially for their relation with higher bleeding rate.

Other short-term outcomes

Ileus is any reduction of the normal propulsive activity of the bowel. After surgery, a transient impairment of the peristalsis always happens for few days. For postoperative ileus, undeniable causal factors are the manipulation of the bowel together with an excessive use of opioid drugs[3]. Nonetheless, experimental studies with animal models revealed a potential role of the interplay microbiota-immune system in postoperative ileus[70]. A potential pathogenetic mechanism involves a peristaltic dysfunction, caused by intestinal nervous system response to intestinal macrophages activated by the microbiota[71]. A recent study reported a reduction of postoperative ileus rate after administration of oral non-absorbable antibiotics[66]. Similarly, preoperative oral intake of probiotics (*Lactobacillus* and *Bifidobacterium*) seems to enhance the return to the normal bowel function[51]. However, a deeper understanding of this relation is required to draft further studies[1].

In prolonged postoperative ileus, high levels of IL-6 and leucocyte bowel infiltration have been found, suggesting also a direct role of inflammation. These data are coherent with the reduced duration of postoperative ileus after minimally invasive surgery[68]. Finally, in animal models, it was also reported a possible causal GM role in the formation of postoperative adhesions[70].

Long-term outcomes: Intestinal microbiota and immunity as prognostic factors

Recurrence rate after curative CRC surgery is reported to be up to 40% and the great majority of recurrence appears within 3 years from operation[6,72]. Local recurrence, mostly perianastomotic in the extraluminal space, shows a reported rate ranging between 1% and 23%[21,73]. A higher incidence of local recurrence rate has been observed after anastomotic leak. We can hypothesize different explanations: (1) A delayed start of adjuvant therapy; (2) Implant of exfoliated tumor cells that are invariably persistent in the colon after resection[21]; and (3) A process of metachronous initiation of a new tumor and new tumor promotion by a persistent inflammatory status[74]. The last two possible mechanisms, together with the consequent reduction of oral intakes, longer hospital stay (with major probabilities of nosocomial infections) and aggravated surgical stress, have a strong relation with the GM alterations[21]. As previously stated, anastomotic leak is related with the presence of microbes producing MMPs[1,62]. Nevertheless, preoperative high serum level of MMP-2 and MMP-9 has been reported to be an independent marker of a worse oncological outcome[75]. Increased proteolysis is also typical of more invasive tumors and it is not only related with anastomotic leak[75]. Moreover, MMP-9 presence in tumor sample was found in the 85% of patients with high serum level of MMP-9[75].

F. nucleatum and enterotoxigenic *B. fragilis* seem related with more advanced CRC stages, lower rate of disease-free survival and, consequently, they appear to be responsible for a worse prognosis[33,76,77].

Kosumi and colleagues evaluated the potential prognostic influence of *Bifidobacterium* in a wide cohort of 1313 patients affected by CRC[78]. The *Bifidobacterium* presence in tumoral tissue was found in 30% of this cohort. Although previously published data suggested a protective role of this microorganism in CRC[47-49], no significant association was found between *Bifidobacterium* number and survival rate. Multiple hypotheses may explain this finding: (1) Bifidobacteria produce lactic acid that, in high level, may boost tumor growth reducing immune response activation; (2) Bifidobacteria can also produce acetate that is utilized by tumor tissue; and (3) Bifidobacteria may have different roles in healthy and tumoral tissue in which these microbes may act together with other species (*e.g.*, *Fusobacterium*). Nonetheless, the number of these microorganisms in CRC tissue resulted associated with the extent of signet ring cells[78].

Expression levels of different cytokines seems also related to tumor prognosis[17]. High IL-17 levels predict an adverse prognosis with a rapid development of metastasis[79]. Similarly, high level of let-7a microRNA family are related with lower presence of $CD3^+$ and $CD45RO^+$ which significantly correlated with higher cancer-related mortality[80]. On the contrary, high density of $CD45RO^+$ cells within tumor tissue has been proposed as an independent positive prognostic biomarker and it is related with longer survival also independently from MSI status[81]. According to these findings, traditional prognostic systems including the TNM classification system of the American Joint Committee on Cancer and Union International Cancer Control appear to be no longer sufficient to estimate patients' oncological outcomes[82]. From the joint effort of 14 centres with a proven expertise located in 13 countries of North America, Europe and Asia, a consensus Immunoscore was created. It resulted from the assessment by digital pathology of the density of specific T-cells ($CD3^+$ and $CD8^+$) in the tumor sample and in the infiltrating margins. In order to assess the prognostic role of the inflammatory infiltrate in tumoral tissue from primary resected CRC, the resulting score was tested and validated in a study including 2681 patients[82]. Inter-observer reproducibility of this score resulted high. Immunoscore revealed to be so powerful in the prognostic stratification of the patients (in terms of time to recurrence, disease-free and overall survival rates) to result more reliable than the TNM classification system suggesting the necessity to create a unique and integrated classification system[82]. Moreover, it seems also a survival predictor stronger than the MSI status[83]. Overall, the highest was the Immunoscore, the lowest resulted the recurrence rate at 3 years from surgery ranging from 5% in patients having high Immunoscore to 26% in patients having low Immunoscore. Five-years overall survival rates were 82%, 77% and 62% in high, intermediate and low Immunoscore, respectively. Similar results were confirmed even after adjustment for the principals known potential prognostic factors (*e.g.*, demographic characteristics, TNM stage, MicroSatellite Instability status). Ultimately, this score may represent a valuable tool for tailored adjuvant chemotherapy[82].

GUT MICROBIOTA-IMMUNITY INTERPLAY IN PATIENTS UNDERGOING CHEMOTHERAPY AFTER CRC SURGERY

CRC is mostly treated with cytotoxic drugs including 5-fluorouracil, capecitabine and/or platinum-based agents[21]. However, different chemotherapy regimens exist and are actually administered according to tumor stage, patient's general conditions and mutational status. Details that are more specific will not be object of this review.

Microbiota may interfere with chemotherapy through different ways: Modification of the GM composition, metabolisms of xenobiotics and modulation of the immune response[84,85]. Consequently, the so-called "pharmacomicrobiomics" is increasingly attracting attention[85]. Unequivocally, these treatments alter the composition of intestinal microbiota. In the previously cited paper of Deng *et al*[5], the demonstrated reduction of *Bacteroides* (mostly *B. ovatus*) in surgically treated patients is higher when compared with the reduction of this microorganism in the patients treated only with chemotherapy. Therefore, surgery seems more effective than chemotherapy against this potential pathogen[5]. Nevertheless, other species such as *Veillonella dispar* or higher abundance of *Prevotella copri* and *Bacteroides plebeius* were found in only chemotherapy-treated patients[5]. However, a relation between these quantitative differences and the potential clinical impact has to be further analyzed.

Intestinal microbiota is responsible for xenobiotic metabolism. According to experimental results, a different clinical response to the treatments with fluoropyrimidines seems related also with GM modifications other than with genetic polymorphisms[86]. Previous papers have already emphasized the possible relation between intestinal microbiota and chemoresistance in CRC patients[5]. *F. nucleatum* has a causal role in chemoresistance *via* the activation of the autophagy pathway. In particular, autophagosome formation is activated in the CRC cells with the production of their related proteins[87]. Consequently, the detection of copious presence of this species may represent a prognostic biomarker for chemoresistance, suggesting a probable modification in the administered chemotherapy[87]. High levels of circulating IL-22 have also been described as associated with chemo-resistance[88]. Nevertheless, high levels of regulatory T cells create an immunosuppressive environment reducing the efficacy of the host antitumor immune system and of the antiblastic therapy[89].

Chemotherapy treatments often cause chemotoxicity and the symptoms are mostly diarrhea, nausea, mucositis and hand-foot syndrome. Dysbiosis caused by chemotherapy administration has a causal role in colitis and diarrhea[85]. Anaerobic species are usually reduced together with an inferior production of butyrate. Butyrate is a short-chain fatty acid (SCFA) responsible for the trophysm of the intestinal mucosa. Nevertheless, this SCFA has an antitumoral action blocking cellular replication, promoting apoptosis[85], stimulating the IL-10 production and inhibiting the NF-κB activation[11]. Butyrate is also implicated in mucosal barrier efficacy through higher production of mucus[11].

Finally, the microbiota appears also to have a direct causal role in chemotoxicity. For example, irinotecan-induced mucositis is the consequence of the reactivation of its liver metabolite from intestinal bacterial β-glucuronidases[85,90]. There are several isoforms of β-glucuronidases, related to different toxicity levels[90]. On the contrary, the preservation of commensal microbiota, able to modulate tumoral micro-environment, has been reported to be pivotal in an optimal response to chemotherapy with oxaliplatin[91]. Nevertheless, a favorable gut microbiota may also have a role in the reduction of these side effects[9].

The identification of specific microbes as predictor of chemotherapy response together with a complete understanding of the mechanisms causing chemotoxicity may allow a tailored therapy with the reduction of side effects[92].

FUTURES PERSPECTIVES: INNOVATIVE STRATEGIES FOR CRC PREVENTION AND TREATMENT

Gut microbiota have a causal role in all the CRC steps, from its initiation to response to chemotherapy. Therefore, new approaches microbiota-based may add further possibilities against this tumor. Nevertheless, high expertise in "bacterial management" is required.

Role of diet in cancer prevention

Since an inappropriate diet represents a major risk factor for CRC initiation, a diet rich in fibers and vegetables, especially from cruciferous family, may aid in maintaining a healthy microbiota. Nevertheless, specific supplementary nutrients may help from the

cancer prevention to the avoidance of anastomotic leak after surgery[1,21]. Probiotic and prebiotic consumption may positively modulate metabolic activity of the gut microbiota with a lower production of carcinogenic compounds[41]. Moreover, according to the results of *in vitro* studies, some microbes are able to bind genotoxic compounds to their cellular wall[93].

SCFAs are the results of bacterial fermentation of complex carbohydrates. Conjugated linoleic acid has a suppressive action over cellular proliferation and favors cellular apoptosis in a dose-dependent proportion[11]. It is produced by several species including *Lactobacillus casei* and *L. acidophilus*[94]. Dietary supplementation with nutrients able to increase the production of butyrate or other SCFAs may help in maintaining a healthy and balanced microbiota[1]. *Lactobacillus, Bifidobacterium* and *Faecalibacterium prausnitzii* may be used as probiotic with the same purposes together with the maintenance of an active immunosurveillance[11,95]. It has also been reported that some probiotics may change the equilibrium between Th1 and Th2 cells stimulating production of anti-inflammatory cytokines and suppressing the release of pro-inflammatory cytokines [11].

Surely, a positive regulation of the gut microbiota and its metabolic activities through the diet appears to be very interesting but certainly a challenging approach[2]. Since diet is only one of the multiple recognized risk factors for CRC, specific large-scale and life-long studies on humans are extremely difficult even to draft other than to interpret, indeed. Similarly, since some microbes have been reported to have both a protective and harmful role, an excessive introduction of supplement with probiotics may have additional negative effects and the limit in a population will probably remain unknown.

New diagnostic biomarkers

Colonoscopy is a very important examination in the CRC management, allowing to locate the tumor into the colon and to obtain a preoperative histological diagnosis of malignancies. However, patient's discomfort more than the possibility of complications may induce several patients to postpone the exam, leading to a late diagnosis. Consequently, the research of new, less invasive diagnostic biomarkers seems very important. High serum levels of MMP-2 and MMP-9 have been proposed as biomarker for CCR and they seem to relate with particular cancer aggressiveness. In a small sample study including 32 CRC patients and 11 controls (benign disease), MMP-2 and MMP-9 resulted more reliable than traditional serum markers such as CEA[75]. Analysis of microbes from saliva samples has been suggested as a non-invasive new biomarker of CRC assuming the existence of a correlation in GM composition between mouth and gut[96]. However, our pilot study failed in finding a significative difference of microbial saliva composition, comparing healthy controls with patients affected by CRC[27].

As previously stated, microbial composition varies together with the progression healthy-adenoma-adenocarcinoma[35-37,53]. On healthy volunteers, a great majority of the phylum Firmicutes has been found[27] and the genera *Clostridium*, family Lachnospiraceae, may represent a biomarker in stool sample for healthy people[5]. On the contrary, the identification in stool samples of higher presence of *Fusobacterium nucleatum* in CRC patients when compared with healthy volunteers suggests its possible role as a novel diagnostic biomarker[5,97]. Zackular *et al*[37] conducted a study to demonstrate the potential as a screening tool of fecal modified microbiota. Data from 90 people (30 healthy, 30 with adenomas, 30 with adenocarcinomas) were analyzed. The authors confirmed that the fecal microbial panel was more important than a single microbe suggesting CRC as polymicrobial disease and they found a significative difference between healthy and adenoma group.

However, further large-scale, case-control studies of multidisciplinary teams are still required to confirm these findings and to obtain more information about bacterial species at strain level. Nevertheless, the eventual correlation between microbiota and other not modifiable characteristics of the patients (*e.g.*, sex or age) should be evaluated, as well.

Perioperative management

In perioperative settings, enhanced recovery after surgery programs should be encouraged. A prolonged fasting of 6-12 h may profoundly alter GM composition and it seems no more justifiable. On the contrary, supplementary food containing microbes able to ferment acid lactic may allow an enhanced healing of the surgical wound[1]. Nonetheless, in animal models, oral supplementation with non-absorbable phosphate usually lacking after surgery, reduced bacterial related anastomotic leak[98]. Antibiotic therapy should be carefully administered and, when required, for the shortest needed period. Opioid drugs should be avoided and other methods of analgesia encouraged. Bowel preparation should be modified in order to try to

eliminate only, or mostly, virulent agents maintaining a helpful biodiversity[67].

The discovery of unevenness of species (*i.e.*, *V. dispar*) in chemotherapy-treated patients still misses a clinical correlation[5]. Hopefully, the recognition of a such potential biomarker may represent a supplementary target in chemotherapy modulation. Unraveling at least a part of the mechanisms of chemoresistance may provide new strategies for chemotherapy optimization[5]. Similarly, the discovery of favorable or unfavorable microbiota for chemoresistance and side effects may help in tailoring chemotherapeutic regimens[85,92]. Immune system has both protective and potential harmful role so the research of strategies to enhance positive action together with the reduction of negative effects should be encouraged. Nevertheless, a deeper understanding of immune-related cancer progression may provide new target for immunotherapy in CRC[17,80].

CONCLUSION

In conclusion, microbiota-based approaches may have a huge impact on CRC initiation, progression and treatments. However, most of the previously published studies are performed on animal models or in small groups of humans. Moreover, most of them are only "quantitative" and a correlation with the clinical impact of the findings is still lacking. Finally, and above all, CRC and its "history" is multifactorial so the evaluation of the impact of modification in gut microbiota composition on CRC management is challenging but very intriguing.

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REVIEW

Metabolomics profile in gastrointestinal cancers: Update and future perspectives

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Abstract

Despite recent progress in diagnosis and therapy, gastrointestinal (GI) cancers remain one of the most important causes of death with a poor prognosis due to late diagnosis. Serum tumor markers and detection of occult blood in the stool are the current tests used in the clinic of GI cancers; however, these tests are not useful as diagnostic screening since they have low specificity and low sensitivity. Considering that one of the hallmarks of cancer is dysregulated metabolism and metabolomics is an optimal approach to illustrate the metabolic mechanisms that belong to living systems, is now clear that this -omics could open a new way to study cancer. In the last years, nuclear magnetic resonance (NMR) metabolomics has demonstrated to be an optimal approach for diseases' diagnosis nevertheless a few studies focus on the NMR capability to find new biomarkers for early diagnosis of GI cancers. For these reasons in this review, we will give an update on the status of NMR metabolomic studies for the diagnosis and development of GI cancers using biological fluids.

Key words: Metabolomics; Nuclear magnetic resonance spectroscopy; Pancreatic cancer; Gastric cancer; Colorectal cancer; Biological fluids

Core tip: Searching for new tumor biomarkers is essential for the early diagnosis of gastrointestinal tumors. Biofluids could give important data, reducing the need for invasive screening and nuclear magnetic resonance-based metabolomics is an optimal approach to understand metabolic dynamics in biofluids.

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INTRODUCTION

The continuous increase of the global population, associated with the extended life expectancy, made the cancer one of the main causes of death[1], and for many countries, a very heavy health burden. Despite numerous advances in diagnosis and therapy, gastrointestinal (GI) cancers remain some of the most aggressive cancers for both men and women, as we have previously reported[2]. In particular, the most aggressive types of GI cancers are pancreatic cancer (PC), gastric cancer (GC) and colorectal cancer (CRC). Furthermore, gastric and CRC are respectively in third and fifth place by incidence and even in second and third as regards mortality in both sexes[3]. The GC is one of the most malignant cancers worldwide with a very high rate in Asia[4]. Unfortunately, most cases of GC are diagnosed in the advanced stages with consequent poor prognosis[5]. The epidemiological and molecular feature of GC differ according to the histological type and cancer location. Currently there are several methods for diagnosing GC; however, there are no standardized guidelines[6]. Regarding CRC, it is one of the most diagnosed neoplasms in the world, both among men and women, and is the third most common malignancy[7]. 5-year survival can reach 90% if the tumor is diagnosed at an early stage and is localized, but survival decreases significantly if the tumor is diagnosed late and is spread to other organs[8]. To date, the fecal occult blood and the serum tumor-associated markers are the test commonly used in the clinic; however, the lack of sensitivity and specificity of these markers limits their application in the CRC diagnosis[9,10]. Lastly, PC is one of the most aggressive cancers, with 5-year survival rates of only 5%. PC is currently ranked as the fourth leading cause of cancer-related deaths in the United States and is estimated to be the second leading cause of such fatalities by the year 2020[7]. Mortality is mainly due to late diagnosis because the PC symptoms (such as nausea, weight loss, weariness, abdominal pain) are not disease-specific[11,12]. PC is diagnosed by resonance, computed tomography, endoscopic retrograde cholangiopancreatography and endoscopic ultrasound[13].

For the clinical monitoring of GI cancer, carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 are used as serological tumor-associated markers as well as detection of occult blood in the stool. However, these tests are not currently useful as diagnostic screening as they have low specificity and low sensitivity[14]; but, for the previously reported reasons, the effectiveness of the anti-GI cancers' treatments depends on an early diagnosis. Based on clinical characteristics, different models for various cancers have been developed to assess the causal risk, however although the results may seem significant at the population level, they have a low predictive value when considering the individual patient[15].

A growing number of studies have suggested that the GI metabolites regulate pathogen infection in the different intestinal sections, through genome-based analysis of bacteria and especially by high-throughput metabolomics[16-18]. Many metabolites affect the cell adhesion and biofilm formation; for example, the D-amino acids produced by *Bacillus subtilis* prevent biofilm formation[19]. In addition, recent studies have revealed a major role of metabolites in the regulation of the immune system taking part in the modulation of the adaptive immune cell development, in particular T lymphocytes[20], which have a crucial role in the genesis of the above-mentioned GI tumors. As we have reported in previous studies, the PC as well as gastric and CRC, show an altered specific immune response characterized by a decreased number of effective T cells[21-23].

One of the hallmarks of cancer is dysregulated metabolism, during which cancer cells show increased glucose uptake and produce lactate. This process is named the "Warburg effect", but how and why cancer cells reprogram their metabolic state is not well understood. Several metabolic changes associated with cancer can be linked to cellular growth; in fact, the biosynthesis of lipids, proteins, nucleic acids are required for tumor formation and survival. In most cases the expression of oncogenes or the loss of tumor suppressors lead to changes in the metabolism, by expression, activity or flow of the main metabolic pathways. Numerous components of glucose and glutamine (Gln) metabolism have emerged as important regulators of cancer

metabolism. Considering the importance of metabolic changes in the development and cancer prognosis, the metabolomics represents a fundamental –omics' study, as it can be used to evaluate (assess) the alterations of the main metabolites[14]. It is now clear that the metabolic characteristics of cancer cells change with the disease progression[24,25] and typical metabolic changes include deregulated absorption of amino acids and glucose, increased nitrogen demand and increased use of anabolic metabolic pathways[26]. This metabolic reprogramming can be useful for the diagnosis of tumors at an early stage and biological fluids could give important information, reducing the need for invasive screening. Urine and blood are easily accessible matrix that could be used to identify possible biomarkers associated with cancer risk, presence and prognosis, using nuclear magnetic resonance (NMR) analysis[27]. Blood passes through every organ of the human body, acting as a transport for secreted/excreted molecules (in response to physiological stimuli or stress), while urine contains molecules eliminated by renal filtration[28,29]. Furthermore, there are a lot of evidence suggesting that microbial metabolism by gut microbiota produces a variety of compounds, including fatty acids, indole and vitamin K, many of which have toxic effects on the lumen and contribute to the GI carcinogenesis, especially for CRC. In addition, there are many evidence (essentially in experimental models) that suggest a role of the intestinal microbiome in the PC carcinogenesis. Finally, a growing number of microbiome researchers are recognizing that considerable information could be gained by using a more integrative approach that also includes comprehensive fecal metabolite analysis. Feces contain many molecules that reflect nutrient ingestion, digestion and absorption by gut bacteria and GI tract. The dry fecal matter consists of bacterial biomass (25%-54%), exfoliated colonic epithelial cells, undigested food residues (fiber, protein, DNA, mucopolysaccharides, *etc*.) and small molecules or metabolites such as sugars, organic acids, and amino acids. These small molecules compose fecal metabolome.

There is an increasing interest to use metabolomic based approaches to investigate cancer metabolism. The two majors' instrumental metabolomic techniques are NMR and mass spectrometry (MS). The advantages of these two techniques are intrinsically different. MS platform provides sensitivity and selectivity for metabolomics research, while NMR provides a very high reproducibility, it is quantitative and requires minimal steps for sample preparation allowing to avoid separation or derivatization[30]. Due to the possible impact that NMR-based metabolomics performed using easily accessible biofluids could have on the standard clinical practice of cancer diagnosis, prognosis and risk evaluation, this review aims to be a comprehensive overview of the literature available to date in this restricted, but promising field. Conversely, the use of MS based techniques or the metabolomic analysis of cells, tissues and animal models is reported elsewhere[31,32]. Interestingly, while *e.g*., breast cancer has been extensively investigated using NMR-based metabolomics of systemic biofluids (especially for relapse risk prediction)[33], for GI cancers this field still appears in its infancy. In this review, we will give an update on the current status of NMR metabolomics' studies for the diagnosis of GI cancers, discussing the suitability of the different biological samples used and the future perspectives for this analytical approach (Figure 1).

METABOLIC ALTERATIONS IN GI CANCERS

Warburg effect is a shift from adenosine triphosphate (ATP) synthesis by oxidative phosphorylation to ATP generation through glycolysis, also in aerobic condition[34]. Tumor cells obtained a large amount of their energy from aerobic glycolysis, converting glucose to lactate instead of metabolizing it in the mitochondria through oxidative phosphorylation. Therefore, in terms of ATP production per glucose molecule consumed, glycolysis is less efficient than oxidative phosphorylation. This metabolic change forces tumor cells to demand a large glucose amount to satisfy their increased energy, biosynthesis and redox needs. In details, lactate accumulation elicits acidic microenvironment, protective for cancer. The presence of lactic acid induces, in cancer cells, the expression of glycolytic enzymes such as 6-phosphofructokinase 1 (PFK1) to: (1) Increase the ATP provisions; (2) Escape from the cell apoptosis mechanism; and (3) Promote angiogenetic mechanisms, providing in this way a comfortable microenvironment for cancer development and metastasis[35]. Abnormal glucose metabolism in GC, with high levels in serum of 3-hydroxypropionic acid and pyruvic acid, may be involved to tumor proliferation leading to aggressive cancer cell proliferation, which needed a large ATP amount, causing, in turn, abnormal levels of intermediate glucose metabolism[36,37].

In PC, abnormal metabolism depends by cellular factors on the anomalous activity

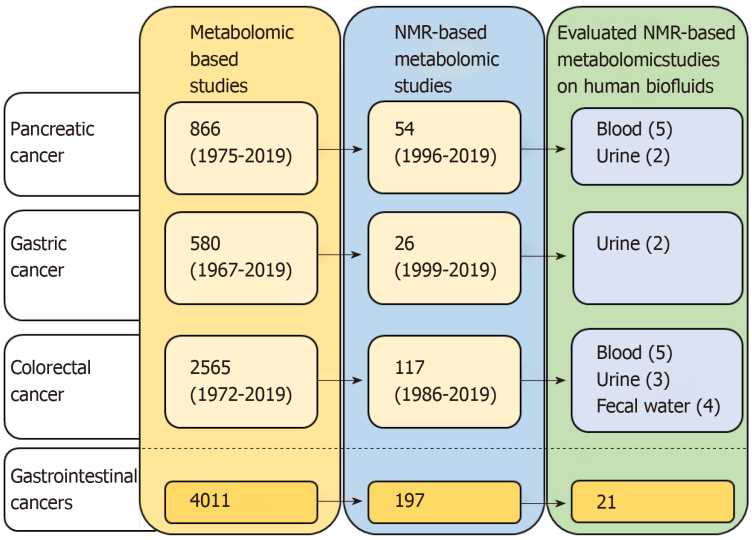

Figure 1 Selection of discussed nuclear magnetic resonance-based metabolomics review. The figure shows the study workflow. First, we searched for metabolomic-based studies, then we limited our research to nuclear magnetic resonance-based metabolomic studies and finally we only selected 21 nuclear magnetic resonance-based metabolomic studies on human bio-fluids, in particular blood, urine and fecal water. NMR: Nuclear magnetic resonance.

of some oncogenes that change the physiological nutrients consumption[38]. In addition, the metabolic reorganization is carried out by the activation of alterations in genes and oncogenic signaling pathways. In fact, some studies show that mutations of *K-RAS* and other oncogenes (and tumor suppressors) represent the key that leads to an acceleration of PC growth by reprogramming directly cellular metabolism[39,40]. It is now assured that the *K-RAS* gene has a crucial role in PC glucose metabolism. An excessive glucose uptake and overexpression of glycolytic enzymes, including type 1 glucose transporter, hexokinase 1/2, phosphofructokinase and lactate dehydrogenase A[41,42] characterize the PC. For all these reasons, lactate is an important "performer" for tumor-connective tissue and energy trade in cellular compartments around cancer microenvironment. In addition, the acidity of the microenvironment helps to repress immune system by promoting chronic inflammation and by suppressing the adaptive immune response[43] leading by T cells.

Also, in CRC, in response to hypoxia, the expression of the glucose transporter 1 is upregulated in neoplastic cells, inducing the enzymes that metabolize glycogen, including glycogen synthase and glycogen phosphorylase. However, altered glycogen metabolism and its potential impact on the CRC biology remain poorly understood[44]. Increasing evidence demonstrated that Gln is an important metabolic substrate and energy source for tumor cells that need Gln for their growth and survival, a dependence called "glutamine addiction". Recent studies have shown that some cancer cells use Gln to carry out the metabolic processes linked to the cell proliferation and to maintain amino acid levels of the tricarboxylic acid (TCA) cycle, exosamine, nucleotides and other molecules[45,46]. Finally, a new study reports an alternative pathway in the Gln metabolism adopted by PC cells and essential for tumor growth. Usually cells use glutamate dehydrogenase to convert glutamate derived from Gln to α-ketoglutarate in mitochondria in order to use it in the TCA cycle[47,48]. However, the PC feeds the TCA cycles through another pathway, so that the aspartate derived from Gln arrives in the cytoplasm and so, transformed into oxaloacetate *via* aspartatetransaminase (GOT1). The oxaloacetate is then converted into pyruvate at the end of the cycle to increase the ratio between NADPH/NADP + and facilitate the maintenance of the redox state[40].

The growth of cancer cells is strongly based on the possibility of exploiting more autonomous proliferative signaling pathways[49]. PC cells depend strictly on these reactions; in fact, the Gln deprivation or the deactivation of enzymes of this chain of reactions causes an increase in reactive oxygen species and a reduction in reduced glutathione. Finally, the inhibition of the enzymes linked to this cycle reduces the PC growth both *in vitro* and *in vivo*[50].

Lastly, the fatty acid synthase (FASN) is overexpressed in various tumors, showing an important role in cancer onset and progression. Several *in vitro* studies have documented that elevated lipogenesis is correlated with poor prognosis in different tumor types[51]. Lipogenesis is also involved in signal transduction of tumor cells and

is increased in tumor tissue and associated with tumor prognosis. Moreover, FASN reduction can promote apoptosis in tumor cells, inhibiting tumor growth and metastasis. Previous studies have demonstrated that FASN is overexpressed in cancer tissue and serum of GC patients. In fact, usually the tumors present enzymes supporting the production of a large number of lipids for the survival and proliferation of neoplastic cells. The tumors need more lipids as energy sources compared to normal cells. Acetyl-CoA carboxylase, the rate-limiting enzyme for the synthesis of fatty acids, if blocked, inhibits the growth and apoptosis of breast, lung and colon cancer cells[52-55].

METABOLOMICS OF BIO-FLUIDS

Metabolomics is an optimal approach to describe the metabolic dynamics that reflect the response of living systems to pathophysiological stimuli, genetic modifications and environment factors. Indeed, the comprehensive evaluation of metabolites, the low molecular weight organic molecules involved in all biochemical processes as substrates or products with different specific functions, is fundamental to observe and measure the response of the organism to diverse conditions. In recent years, metabolomics has been widely applied to investigate cancer metabolism. Current platforms for metabolomics are NMR and gas, liquid chromatography, ultra-high-pressure liquid chromatography [GC, liver cancer (LC) and UPLC] and more recently capillary electrophoresis, usually hyphenated to MS. The applications of NMR spectroscopy are not limited to liquid and solid samples but extend to intact tissue samples with the use of high-resolution magic angle spinning NMR spectroscopy. NMR is more reproducible, and it not requires laborintensive sample manipulations like fractionation to get quantitative results, while MS is more sensitive than NMR (10^{-12} mol/L *vs* 10^{-6} mol/L). Indeed, these two analytical platforms do have a complementary nature[56,57]. One dimensional (1D) proton (^{1}H) NMR approaches are mostly applied in metabolomics studies, offering several advantages thanks to the natural abundance of the ^{1}H isotope (99.9%) and fast experiments under controlled temperature, giving the possibility to exploit the behavior of many molecules reducing the possibility of sample denaturation. 1D NOESY, CPMG (Carr–Purcell–Meiboom–Gill)[58] and ^{1}H diffusion-edited are the most used pulse sequences in metabolomics studies, which permit namely the observation of both low- and relatively high-molecular weight molecules, the low-molecular-weight compounds selectively, and the selective observation of macromolecular components present in the sample. However, some authors prefer to remove the macromolecular components *via* centrifugation. Platforms for the automatic screening of compounds are always under developing, the B.I. platform (Bruker IVDr, Bruker BioSpin) for analysis and quantification lipids ad lipid subfractions in blood samples.

Ultimately, there are different pre-analytical procedures (which also include the storage of the samples) that could affect the sample and it should be taken into account when comparing the findings of different studies[59,60]. Optimal standard operating procedures for pre-analytical handling of blood and urine for metabolomics' studies and biobanks are well reported by Bernini *et al*[61]. Often 2D spectra for metabolomics studies are required to assign new metabolites, for doubtful cases and metabolite quantification. 2D experiments generally require much longer acquisition times than the standard 1D pulse sequences; therefore, in defining the total acquisition time, one should also consider the stability of the sample under the selected experimental conditions.

Another important aspect of the metabolomic workflow is the use of appropriate statistics to analyze multiparametric data. Most common methods for metabolomic normalization, multivariate and univariate statistical techniques are well reviewed by Vignoli *et al*[56].

NMR based metabolomics has widely demonstrated to be an optimal strategy for diseases' diagnosis[62-65], classification and prognosis[66] and as previously reported we will give an update on the current status of NMR-based metabolomic studies using biological fluids for the diagnosis of GI cancers. In Table 1 we have summarized the different NMR studies evaluated and discussed. Biological samples, are extremely valuable as direct reporters of the diseased region. However, systemic biofluids, such as urine or blood (serum and plasma), but also fecal water, have a slight biochemical correlation with a diseased organ or apparatus, but present two main advantages: The simple, noninvasive or minimally invasive collection, and the ability to reflect the overall response of the patient to the pathological status. On the other hand, urine, blood and fecal water are largely variable in the chemical composition and in number of metabolites, with urine and fecal metabolites being heavily influenced by lifestyle

factors such as food and liquid intake, while blood samples show a better-defined and stable metabolome (Figure 2). Detecting and characterizing cancer-associated biomarkers by metabolomics analysis of bio-fluids could make easy and minimally invasive (reducing the collection of tissue biopsies) the diagnostic approach, representing a valid opportunity of success in the early cancer detection. Altered metabolites in the three different biofluid (blood, urine and stool) identified in 21 NMR-based metabolomic studies, were extracted and summarized respectively in Tables 2-4. A total of 46 metabolites were extracted to be significantly altered in blood samples (serum/plasma), 64 in urine and 28 in fecal water of patients with GI cancers, compared to healthy subjects (or controls). Finally, ten publications report the use of NMR metabolomics to study GI cancers on blood samples, seven are based on urine samples and four on stool samples.

Blood samples

Serum samples are commonly used in clinic to test the presence of tumor markers such as carcinoembryonic antigen and the carbohydrate antigen 19-9, however these tests have good sensitivity but poor specificity due to the presence of false positive results given by other non-neoplastic condition. Thus, the relevance to develop alternative screening tools improving the early detection and fine defining the cancer classification. Blood metabolomics has demonstrated the potential to help GC diagnosis. OuYang *et al*[67], based on a small cohort of 17 PC patients and 23 healthy subjects showed that entire ^{1}H-NMR serum spectra could be used to discriminate the two groups using principal component analysis (unsupervised multivariate statistical approach), identifying altered levels of 3hydroxybutyrate and lactate (Table 2) in PC patients. These alterations were also detected by Zhang *et al*[68] in plasma samples of PC patients (n = 19) in parallel to lower levels of citrate, low-density lipoprotein, high-density lipoprotein, valine, lysine, leucine, isoleucine, histidine, glutamine, glutamate, alanine, and higher levels of NAG (N-acetyl glycoproteins), very-low-density lipoprotein, lipid glyceryl, dimethylamine and acetone (Table 2) compared to healthy subjects. Moreover, the authors identified differences in the plasma metabolomic profile of PC patients also compared to chronic pancreatitis patients (n = 20). More recently, Michálková *et al*[69], compared the plasma samples of 10 PC patients with ten healthy controls, obtaining an impressive discrimination accuracy (94%). However, this study is based on a very limited sample population and the absence of patients being treated is not specified in the exclusion criteria, which could influence the model accuracy.

Bathe *et al*[70], in a well-designed study, demonstrated the possibility to distinguish PC (n = 56) from benign pancreatic conditions (benign masses and chronic pancreatitis) and patients with gallstone disease (n = 43), matched by age, jaundice and incidence of diabetes, by NMR metabolomic analysis of serum (AUROC 0.83). Another more recent paper[71] of the same group reported on a bigger monocentric cohort (n = 157) the difference in the metabolomic profile of malignant and benign pancreatic and periampullary lesions using ^{1}H-NMR and GC-MS. Indeed, it represents an important finding, since, in the clinic, is not always possible to distinguish PC from other non-pancreatic adenocarcinomas such as periampullary adenocarcinomas, especially when located near to the pancreas' head. McConnell *et al*[71], used both ^{1}H-NMR and GC-MS to analyzed the metabolomic profile of serum samples of PC patients. Interestingly, comparing the accuracies for the discrimination among patients and controls obtained using the two approaches, it emerges that NMR-based models are more accurate than GC and then NMR-GC combined models (^{1}H-NMR dataset: Average of 14 metabolites, AUROC 0.74; GC-MS dataset: Average of 18 metabolites, AUROC 0.62; combined CG-MS/^{1}H-NMR datasets: Average of 20 metabolites, AUROC 0.66). A similar approach was proposed by Farshidfar *et al*[72] that using metabolomic data obtained from both ^{1}H-NMR and GC-MS platforms, discriminated serum samples of patients with liver-limited metastasis from local (stage II and III) CRC (NMR AUROC 0.88, GC-MS AUROC 0.87) or extrahepatic metastasis patients (NMR AUROC 0.72, GC-MS AUROC 0.90). In a multicentric study with ^{1}H-NMR metabolomic, Bertini *et al*[73] correctly discriminated the serum profile of metastatic CRC patients (regardless of chemotherapy) from healthy subjects (96.7% accuracy). In addition, the authors used the metabolomic profile as an independent predictor of overall survival (OS) obtaining a hazard ratio of 3.37. In particular, short OS patients were characterized by lower serum level of creatine, lipid (-C=C-CH2-C=C-), lipid (-CH=CH-) and valine and higher levels of lipid (-CH2-OCOR) and NAG. Gu *et al*[74] used serum samples to investigate differential metabolomic profile between CRC patients (n = 40), colorectal polyp patients (n = 32) and healthy controls (n = 38). Patients with colon polyps are at high risk for the development of colon cancer, and compared to the metabolism of healthy controls, they found that the major abnormal metabolic pathways were the pyruvate metabolism, glycerolipid metabolism, Gln and

Table 1 List of evaluated studies

Type of tumor	Ref.	Type of biofluid	Sample size	Mono- or multi-centric study	Cohort allocation	NMR (MHz)	Acquisition temperature and pulse sequences
PC	[70]	S	99 (56 PC; 43 control patients: Benign pancreatic masses, pancreatitis and gallstone disease)	Mono	Calgary, Canada	600	298 K, 1D Noesy: 1024 sc; 2D TOCSY, HSQC
PC	[67]	S	30 (17 PC; 23 HS)	Mono	Fuzhou, China	500	298 K, CPMG: 256 sc
PC	[68]	P	59 (19 PC; 20 chronic pancreatitis; 20 HS)	Mono	Xi'an, China	600	298 K, 1D Noesy: 64 sc
PC	[71]	S	157 (122 PC/periampullary cancer; 35 benign pancreatic/periampullary disease)	Mono	Calgary, Canada	600	298 K, 1D Noesy: 1024 sc; 2D TOCSY, HSQC
PC	[69]	P	20 (10 PC; 10 HS)	Mono	Prague, Czech Republic	500	298 K, CPMG: 128 sc
CRC	[87]	S	57 (38 CRC; 19 HS)	Mono	Birmingham, United Kingdom	800	CPMG: 128 sc; TOCSY: 32 sc; hadamard-TOCSY: 8 scasc
CRC	[73]	S	297 (153 mCRC; 139 HS)	Multi	Denmark	600	310 K, CPMG: 64 sc; JRES: 1 sc
CRC	[72]	S	112 (42 lCRC; 45 liver metast.; 25 extrahepatic metast.)	Mono	Calgary, Canada	600	298 K, 1D Noesy: 1024 sc; 2D TOCSY, HSQC
CRC	[75]	P	70 (40 CRC; 30 liver metastases from CRC)	Multi	Hamburg, Germany	600	300 K, 1D Noesy, CPMG and Diff: 64 sc each
CRC	[74]	S	110 (40 CRC; 32 colorectal polyp patients; 38 HS)	Mono	Xiamen, China	600	298 K, CPMG: 256 sc
PC	[76]	U	87(33 PDAC; 54 HS)	Mono	Verona, Italy	600	300 K, 1D Noesy 32 sc; JRES and HSQC
PC	[77]	U	89 (32 PDAC; 32 benign; 25 HS)	Mono	Alberta, Canada	600	298 K, 1D Noesy: 32 sc
GC	[81]	U	145 (75 GC; 81 HS)	Mono	Seoul, Korea	600	298 K, 1D Noesy: 64 sc
GC	[78]	U	123 (43 GC, 40 benign gastric disease, 40 HS)	Multi	Alberta, Canada	600	298 K, 1D Noesy: 128 sc
CRC	[68]	U	113 (55 CRC; 18 EC; 40 HS)	Mono	Guangdong, China	400	1D Noesy: 256 sc
CRC	[82]	U	62 CRC	Mono	Alberta, Canada	600	298 K, 1D Noesy: 32 sc
CRC	[80]	U	248 (92 CRC; 156 HS)	Mono	Seoul, Korea	500	NA
CRC	[83]	ST	33 (21 CRC; 11 HS)	Mono	Valencia, Spain	600	283 K, CPMG: 256 sc; 2D TOCSY, HSQC
CRC	[84]	ST	100 (68 CRC; 32 HS)	Mono	Guangdong, China	400	298 K, 1D Noesy: 64 sc
CRC	[86]	ST	99 (50 CRC; 49 HS)	Mono	United Kingdom	600	1D Noesy 2816 sc; 2D COSY, HSQC and HMBC
CRC	[85]	ST	140 (70 CRC; 70 HS)	Mono	Guangdong, China	400	298 K, 1D Noesy: 64 sc

S: Serum, P: Plasma, U: Urine, ST: Stool; PC: Pancreatic cancer; HS: Healthy subjects; CRC: Colorectal cancer; EC: Esophageal cancer; GC: Gastric cancer; lCRC: Locoregional; mCRC: Metastatic colorectal cancer; sc: Number of scans; NA: Not available information.

glutamate metabolism, and alanine, aspartate and glutamate metabolism. Moreover, they distinguished the metabolomic profile of CRC patients from that of colorectal polyposis (AUROC 0.727). Ghini *et al*[75] raised a very important point about the sample collection by quantifying the effect of preoperative anesthesia on the plasma metabolomic profile of patients with CRC or CRC and LC metastasis. The collection of plasma sample during the preoperative anesthesia is a common procedure that should be avoided in standard metabolomic studies. The authors demonstrated that if compared before the anesthesia CRC *vs* LC can be distinguished using CPMG spectra with an overall classification accuracy of 76.5%, while comparing samples collected during the anesthesia the discrimination accuracy of CRC *vs* LC rose to 90.4%. The increased discrimination was attributed to the authors to the different pharmaceutical treatments administered to CRC patients respect to LC patients.

Urine samples

Some NMR-based metabolomic studies are focused on the characterization of the tumor profile in urine samples sometimes considering heterogeneous group of cases (*e.g.*, patients with different cancer stage, metastatic patients, patients with also other cancer types, *etc*.). If not properly considered, these factors represent important confounding elements.

Napoli *et al*[76] proposed a characteristic urinary metabolomics signature of pancreatic ductal adenocarcinoma (PDAC) in a male cohort. However, the selected PDAC group is very heterogeneous, including 12 patients with liver metastasis, 4 diabetic patients and 3 pancreatitis. These effects, together with gender effect, were not examined in the study. Other studies suggested urine as an excellent bio-fluid to monitor the effect of treatment on patients or for the identification of benign form reducing the need for invasive intervention. Davis *et al*[77] demonstrated that using urine sample is possible to discriminate PDAC patients (n = 32) from 25 healthy controls (AUROC of 0.988) and from 32 with benign pancreatic disease (AUROC 0.95) with optimal accuracies. They also evaluate the effect of complete surgical resection on metabolomic profile demonstrating a recovering tendency towards the normal profile. The main study criticism is the limited sample size. The results should be validated on a larger cohort. Similarly, urine profile has been investigated by Chan *et al*[78] in a multicentric study to discriminate a group of GC patients (GC, n = 43) from patients with benign gastric diseases (n = 40), such as gastritis, ulcer, portal hypertensive gastropathy, gastro-oesophageal reflux disease and polyps, and from a group of healthy subjects (n = 40). The study described a characteristic GC profile compared to benign gastric disease subjects and HS. However, despite being one of the few multicentric studies, important confounding factors, such as the presence of patients under neoadjuvant and adjuvant therapy in GC group, and the presence of *Helicobacter pylori* positive patients, are not considered. The possibility to use urine profile for the diagnosis of early stage cancer would be a great opportunity and Wang *et al*[79] showed a characteristic urinary metabolomic fingerprint of stage I and stage II CRC patients (stage I/II *vs* stage III/IV: R2Y = 0.41; Q2 = 0.45). Moreover, in this study authors identified both urinary metabolomic differences in early stage CRC samples respect to esophageal cancer, suggesting that upper and lower GI cancers have different metabolomic profiles, and both overlapping metabolites attributable to shared tumorigenesis pathways (disturbed gut microflora and urea metabolism) associated to tumor cells proliferation/growth. However, Wang *et al*[79] did not focus their research on the characterization of the metabolites that differentiate stage I/II from stage III/IV. A recent study[80] proposed the urine NMR metabolomics as a diagnostic method for pre-invasive CRC patients. The authors evaluated the metabolomic profile of advanced adenoma and stage 0 CRC, revealing a high predictive accuracy in the diagnosis of early colorectal neoplasia patients (CRN *vs* healthy subjects: Specificity 96.2% and sensitivity 95%). However, case and control groups are not sex matched and the groups used for the comparison are numerically unbalanced; thus, the results obtained should be validated on a larger and balanced court of CRN samples. Urine samples have been studied in GCs research also to evaluate the effect of the treatment on patients. Follow-up urine samples, have been analyzed by Jung *et al*[81] to investigate alterations of urinary markers in GC patients underwent curative surgery. The authors showed that the urinary metabolomic profile has a high predictive value for low- and high-stage GC. Moreover, through the analysis of matched tumour and normal stomach tissues, they found results consistent with those obtained from the urine profile, evidencing an up-regulation of lipid

Table 2 Panel of altered metabolites' levels identified in blood samples of gastrointestinal cancer patients *vs* healthy controls

	S			P			S			
	PC[70]	PC[67]	PC[71]	PC[68]	PC[69]	CRC[75]	CRC[67]	CRC[73]	CRC[72]	CRC[74]
2-aminobutyrate									↓[2]	
2-hydroxyisovalerate									↓	
2-oxoglutarate									↑[1]	
3-hydroxybutyrate	↑	↓		↓	↑	↑	↑	↑		
3-hydroxyisovalerate		↓								
Acetate						↓	↑	↑		
Acetoacetate						↑	↑			
Acetone	↑			↑						
Alanine			↓	↓	↑			↓		↓
Arginine						↑				
Asparagine	↓									
Beta-alanine									↓	
Citrate				↓		↑		↓		↓
Creatine	↓		↓					↓	↑	
Creatinine		↑								
Ethanol	↓									
Formate								↑	↓	
Glucose										↑
Glutamate	↑		↑	↓					↓	↑
Glutamine	↓		↓	↓	↑			↓	↑	↓
Glycerol								↑	↑	
HDL				↓						
Histidine				↓					↓	
Hypoxanthine									↑	
Isobutyrate									↓	
Isoleucine		↑		↓					↑	
Isopropanol			↑							
Lactate		↓		↓	↑		↑	↓		
LDL				↓						
Leucine		↑						↓		↓
Lysine	↓		↓	↓						
Mannose	↑		↑						↑	
Myo-inositol			↑							
N-acetyl glycoproteins				↑				↑		
O-phosphocholine									↑	
Ornithine			↓							
Phenylalanine	↑		↑					↑		
Proline			↓					↑		↓
Pyruvate							↑	↓		
Serine										↑
Threonine	↓		↓							
TMAO		↓								
Tyrosine						↓		↓		↓
Urea			↑							
Valine				↓	↑			↓		↓
VLDL				↑						

[1]↑Higher metabolite levels in gastrointestinal cancers.
[2]↓Lower metabolite levels in gastrointestinal cancers. HDL: High-density lipoprotein; LDL: Low-density lipoprotein; TMAO: Trimetlylamine oxide; VLDL: Very-low-density lipoprotein; CRC: Colorectal cancer; PC: Pancreatic cancer.

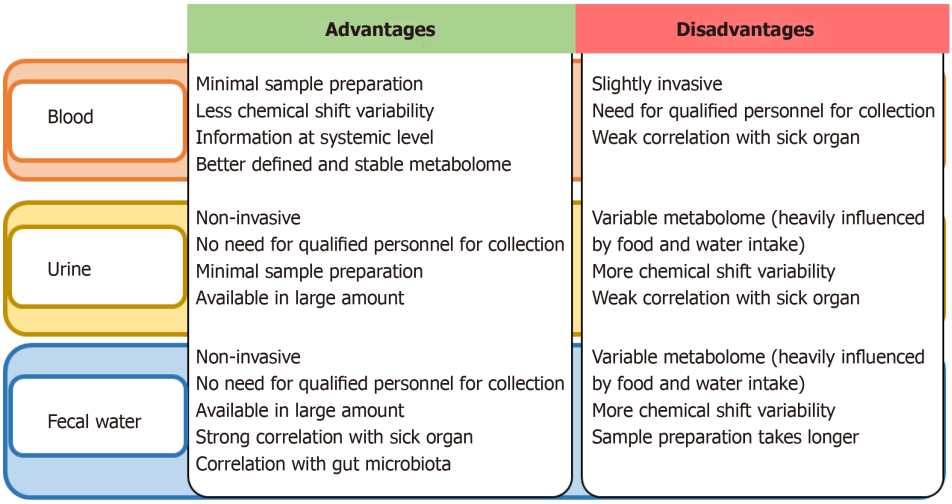

Figure 2 Summary of the advantages and disadvantages of various bio-fluids. The figure shows the advantages and disadvantages linked to the use of the three bio-fluids take into consideration for human nuclear magnetic resonance analyses.

oxidation-related metabolites and amino acids in GC patients (Table 3). Even if limited by a small sample size, Dykstra *et al*[82] published an interesting NMR-based metabolomic study on the topic of personalized medicine in CRC patients. In this retrospective study, authors developed predictor model that is moderately accurate in predicting treatment delay, which depends on reactions to chemotherapy, other medical condition and patient choice. Therefore, the possibility to develop a method capable in predicting it could be important to help clinicians planning for future procedures.

Urinary metabolomic could represent a good non-invasive alternative to determine tumor-associated perturbations and despite the good results of these mentioned retrospective studies, new NMR metabolomic based prospective studies should be performed. The latter, if validated with independent and larger cohorts, could demonstrate the importance of NMR metabolomic for the diagnosis of GI cancers using urine samples. Indeed, urine metabolomic analysis could be easily implemented to be used as wide scale population screening. However, in clinics, the biggest drawback of urine metabolomics' profile is the variability of the samples, due to different host (*e.g.*, lifestyle and diet) and environmental factors and finally the pathophysiological status of the patients. More attention should be paid during the experimental design to control these variability factors.

Fecal water

Despite the rising approval of fecal metabolomics, so far there isn't a standardized method to collect, prepare and analyze fecal samples. This deficiency of standardization is intensifying by the fact that this type of matrix is a semi-solid mixture of endogenous and exogenous components, so a quite complicated sample preparation for metabolomic analyses is required. In addition, fecal metabolite analysis has never been examined through a systematic review or a systematic study, differently from urine, serum, plasma, cerebrospinal fluid, and saliva biofluids. Currently 4 studies take into consideration the metabolic analysis with NMR to quantify the concentration of metabolites in fecal extracts in the CRC, while there are even no works investigating the stool metabolic composition of GC and PC patients. The first study conducted by Monleón *et al*[83] on CRC using a ^{1}H-NMR on a small cohort of 11 controls and 21 CRC patients showed that fecal water extracts have an abundance of small metabolites such as lactate, glucose and amino acids. The spectra exhibited high variability because of the lack of dietary control. Nevertheless, multivariate analysis showed significant differences between the two groups. Similar results were obtained in two different study of Lin *et al*[84,85]. In the first study they investigated the NMR-based fecal metabolomics fingerprinting as predictors of earlier diagnosis in different stages of CRC. In particular, their findings revealed that the fecal metabolic profiles of healthy subjects can be well discriminated from those of even early stage (stage I/II) CRC patients. Moreover, the levels of glucose, lactate, SCFAs, glutamate and succinate at stage I/II differed significantly from those at stage III and IV, giving important molecular information about the staging of CRC. In their second study a total of 70 CRC patients and 70 healthy subjects were enrolled, to rough out the paralleled metabolites of CRC biopsy and the near non neoplastic

Table 3 List of altered metabolites' levels identified in urine samples of gastrointestinal cancer patients *vs* to healthy controls

	U					
	PC[76]	PC[77]	CRC[79]	CRC[80]	GC[78]	GC[81]
1-methylnicotinamide		↑[1]			↓[2]	↓
2-furoylglycine					↑	
2-hydroxyisobutyrate		↑				
2-oxobutyrate						↑
2-phenylacetamide	↑					
3-aminoisobutyrate				↑		↑
3-hydroxyisovalerate	↓			↑		
4-hydroxyphenylacetate		↑				↑
4-pyridoxate		↑				
Acetate						↑
Acetoacetate	↑		↑			
Acetone		↑				↑
Acetylated compounds	↑					
Alanine			↓	↑	↑	↑
Aminobutyrate		↑				
Arginine						↑
Ascorbate				↓		
Asparagine			↓			
Betaine						↑
Choline		↑	↓			
Cis-aconitate		↑	↑			
Citrate	↓			↓		
Creatinine	↓		↓	↓		
Cysteine			↓			
Dimethylamine		↑			↓	
Dimethyl sulfone			↓			
Formate					↑	↑
Fucose		↑				
Glucose	↑	↑				
Glutamine			↑			
Glycerol				↓		
Glycine	↓					↑
Glycolate						↑
Guanido-acetate			↑			
Hippurate	↓		↓	↓		
Histidine						↑
Homocysteine			↑			
Hypoxanthine		↑				↓
Indoxyl sulfate						↑
Isocitrate			↓			
Lactate						↑
Leucine	↑					↑
Mannitol						↑
Methanol		↓				
Methionine						↑
Methylamine			↓			
N-acetyl serotonin					↑	
N-methyl hydantoin						↑
O-acetyl carnitine		↑				↑
Phenylacetyl glycine						↑

Phenylalanine			↓			↑
Putrescine						↑
Succinate						↑
Sucrose					↑	
Taurine		↑		↑		↑
Threonine				↓		
Threonine		↑				
TMAO		↑				
Trans-aconitate		↑	↑		↑	
Trigonelline	↓	↓				
Tryptophan		↑				
Tyrosine						↑
Urea				↑		
Valine						↑
Xylose		↑				

[1]↑Higher metabolite levels in gastrointestinal cancers.
[2]↓Lower metabolite levels in gastrointestinal cancers. U: Urine; TMAO: Trimetlylamine oxide; CRC: Colorectal cancer; PC: Pancreatic cancer.

tissues pre- and postoperative fecal samples from the same patients. This work unveiled distinct and discriminatory metabolites across both matrices of CRC patients, but in particular fecal acetate demonstrated the highest diagnostic performance for discriminating CRC from healthy subjects. In the study of Le Gall *et al*[86], presented a list of fecal metabolites expressed in concentration units among 50 CRC patients and 49 controls. Their results showed that there are significant alterations in the metabolite composition of fecal extracts from patients with CRC compared to controls.

DISCUSSION

Early stage GI cancers usually present no symptoms, so are diagnosed at advanced stages with a consequence of poor prognosis. The discovery of predictive biomarkers might lead to early diagnosis with increase in the quality and length of patients' lives. Therefore, the development of low-cost and non-invasive diagnostic techniques is necessary to reduce unfavorable prognosis and medical expenses. In this review, we have reported the results of a series of studies, focusing our survey to NMR-based metabolomic applications in biological fluids, for the discovery of biomarker candidates for GI cancers. The main reason for restricting our analysis to this particular topic stems from the fact that NMR analysis of biofluids is a high throughput, robust, quantitative and reproducible technique that perfectly fits with the concept of large-scale non-invasive population screening. NMR spectra can be easily obtained in a matter of minutes (from 10 to 30 for common fluid samples), and without the need of complex sample pre-treatments. NMR thus offers the possibility to obtain an untargeted and unbiased snapshot of the sample composition, and, with the possibility of quantifying multiple compounds simultaneously, it could become a reference clinical tool for the study of complex biofluids. However, in order to realize in adequate way this approach, it is necessary to standardize both the pre-analytical and the analytical procedures employed for: (1) Sample collection; (2) Handling, transportation; (3)Preparation; and (4) Instrumental analysis. In fact, all these steps could affect the composition of the samples and consequently the analysis' results. The technical specifications for the pre-analytical processes for metabolomics in urine, venous blood serum and plasma have been published by CEN (CEN/TS 16945:2016), following the evidence reported by Bernini *et al*[61]. Unfortunately, these recommendations are still not universally applied. Looking more carefully to the methods employed by the 21 studies considered here, it clearly appears that they are implemented using a variety of machines (spanning from 400 to 800 MHz, being the 600 MHz the most represented), pulse sequences (1D noesy, CPMG, 2D spectra), and number of scans. Therefore, a not perfect match of the results obtained is expected. Furthermore, and more importantly, the cohorts' composition, even for the same cancer kind, is not exactly the same: *i.e.*, for PC, in some papers the patients are compared with healthy controls, in some other with patients with benign masses or

Table 4 Panel of altered metabolites' levels identified in fecal water samples of gastrointestinal cancer patients versus healthy controls

	Fw			
	CRC[83]	CRC[84]	CRC[86]	CRC[85]
4-aminohippurate			↓[2]	
Acetate	↓	↓		↓
Alanine		↑[1]	↓	↑
Beta-alanine			↓	
Butyrate	↓	↓		↓
Cholate			↓	
Deoxycholate			↓	
Galactose			↓	
Glucose		↓	↓	
Glutamate		↑		↑
Glutamine		↓	↓	
Glycerol			↓	
Hexose-phosphate			↑	
Isobutyrate			↑	
Isoleucine		↑	↓	↑
Isovalerate			↑	
Lactate		↑		↑
Leucine	↑	↑		↑
Litho deoxycholate			↓	
Methanol			↓	
Ornithine			↓	
Phenylacetate				
Proline	↑	↑		
Propionate		↓		↓
Succinate		↑		↑
Taurine			↓	
Valine		↑		↑
Xylose			↓	

[1]↑Higher metabolite levels in gastrointestinal cancers.
[2]↓Lower metabolite levels in gastrointestinal cancers. Fw: Fecal water; CRC: Colorectal cancer.

with other diseases. The same is true for CRC (where the controls are healthy subjects or patients with polyposis) and for GC (where the controls are healthy subjects and patients with benign lesions). In any case, the main limitation that emerges from the analysis of the selected papers is the small sample size generally employed. The larger study[73] involves 297 participants (153 CRC and 139 HS), the smallest[69] only 20 participants (10 PC and 10 HS), with the others ranging from few tens to a bit more than one hundred participants. Consequently, the statistical power is quite limited, with a not negligible probability of spurious or not reproducible results. Moreover, almost all the studies are monocentric with cohorts recruited in Asia (China), North America (Canada), and Europe (Denmark, Czech Republic, Spain). Only two papers[75,78] involved multicentric cohorts. The consequence is that, due to the known influence of dietary habits and genetic background on the metabolic profile, the results could be not immediately compared due to the broad geographical distribution of the study cohorts. However, all these limitations can be considered also a strength: If from these different study designs and cohorts emerge some similar results or trends, they can be considered robust enough to be further investigated as candidate biomarkers for GI cancers.

In other words, carefully looking at the results of the 21 evaluated studies, we can identify some common alterations (Figure 3).

Three studies on blood reported that 3-hydroxybutyric acid was present in higher amount in CRC cancer[73,75,87] while results on PC are discordant. 3-Hydroxybutyrate is a ketone body and one of its main functions is to provide acetoacetyl-CoA and acetyl-

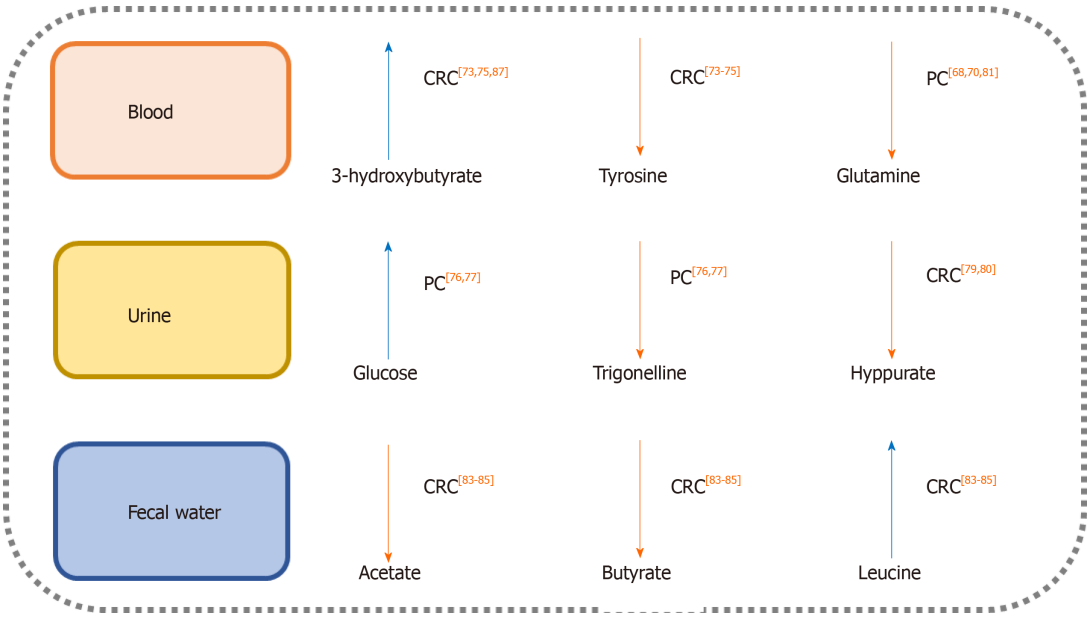

Figure 3 Most significant metabolites identified in the 21 studies analyzed. We have summarized in the outline the most significant metabolites identified in blood, urine and fecal water in the 21 evaluated studies. The green and the red arrows indicate respectively the increase or decrease of the metabolite detected in colorectal cancer or pancreatic cancer in the corresponding references. CRC: Colorectal cancer; PC: Pancreatic cancer.

CoA for the synthesis of cholesterol and lipids. 3-hydroxybutyrate amount in blood increases as oxidation levels increase. Its overexpression could lead to an enhanced lipogenesis promoting tumor growth. Moreover, tyrosine levels in blood samples were concordantly lower in three CRC based studies[73-75]. Several other published studies report the same results; however, the tyrosine[88,89] role has not yet been clarified.

Instead, for what concern the five reported PC cancer studies, only Gln level in blood was found concordant in more than two studies[68,70,81]. Gln plays an important role in regulating redox homeostasis[90]. Cancer cells show an increase Gln demand as the result of a shift from glucose oxidation to "Warburg effect".

Compared to blood, urine has the main advantages of being non-invasive and available in large amounts. Nevertheless, its natural abundance of metabolites and its high variability among patients makes it difficult bio-fluids to analyze. Among all the mentioned studies, it does not emerge a common biomarker(s) describing a metabolic alteration due to GI. This could be ascribed firstly to the sample variability and secondly to the different tumors and disease stage. Several other factors such as gender, age, hormonal status, diet, or physical activity should be taken into account. Moreover, in some cases it is important to evaluate the effect of patients' comorbidities, such as diabetes in the PC, where it may be either a risk factor or a symptom.

Higher glucose level in urine of PC patients, identified by Napoli *et al*[76] and Davis *et al*[77] could be due to the presence of diabetic patients in the group of cases, which were not excluded from both studies.

Trigonelline too, was identified in lower amount in PC by both studies. Its presence may be related to particular dietary products (*e.g.*, coffee, tea, *etc.*) but may also arise from endogenous niacin methylation. However, some of the identified variations in urine could also be attributed to the interaction of the host with the gut-microflora such as lower hippurate levels, identified in urine of CRC patients by Wang *et al*[79] and Kim *et al*[80], supporting what previously seen that CRC is associated with an altered intestinal microbial composition[91].

Fecal water extract, like urine, can be an interesting bio-specimens due to its non-invasive collection. However, at present there are not studies in literature considering the NMR analysis of these bio-fluids in PC and GC patients. Three of the four studies that we have analyzed[83-85] find common alteration of 3 metabolites: Acetate, butyrate, and leucine. Acetate and butyrate are short chain fatty acids (SCFAs), a microbial-derived metabolite, normally produced by the gut bacteria. SCFAs are absorbed by the intestinal epithelium and used as energy sources for intestinal barrier protection[92,93]. The depletion of SCFAs, especially butyrate, in feces could suggest that there has been an intestinal dysbiosis in CRC patients and consequently an alteration in bacterial products[94-96]. Acetate is a precursor molecule for endogenous cholesterol

and can be transformed to acetyl-CoA for lipid biosynthesis. This data confirms that the shift to lipogenesis is a typical change of cancer metabolism. Acetate is probably the most discriminative metabolites of SCFAs in the three studies, and in particular Lin and his group find a link between acetate levels in CRC feces and glucose and myo-inositol levels in colorectal tumor tissues. Significant depletions of glucose and myo-inositol in CRC tissues and decrease of acetate levels in feces could be indicative of an increased energy demand by cancer cells for their growth. Compared to healthy subjects an increase of leucine is reported in the feces of CRC patients, this could be due to the epithelium inflammation that leads to malabsorption of nutrients[97]. However, the amino acid metabolic profile is often very varied as there is no dietary control in patients.

CONCLUSION

Summarizing the results of the selected studies, we can conclude that NMR analysis of bio-fluids could be a high throughput, quantitative and reproducible test that fully fits with the concept of large-scale non-invasive population screening for the GI cancers. To date, there is small the number of studies exploring this opportunity and focusing only on the patients with CRC and in addition using a restricted number of patients and the same country. Therefore, future perspectives are to plan multicentric studies involving a high number of patients and evaluating not only CRC patients but also patients with pancreatic or GC. In addition, a crucial point will have to be the evaluation of interfering factors such as gender, age, hormonal status, diet, physical activity and especially comorbidities and the metabolites associated with gut microbiota, such as the SCFAs. Finally, despite the restricted number of the studies using the stool for the metabolic NMR analysis, we think that the fecal water samples could be an interesting and cheap bio-fluid to explore for future applications.

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REVIEW

Nervous mechanisms of restraint water-immersion stress-induced gastric mucosal lesion

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Abstract

Stress-induced gastric mucosal lesion (SGML) is one of the most common visceral complications after trauma. Exploring the nervous mechanisms of SGML has become a research hotspot. Restraint water-immersion stress (RWIS) can induce GML and has been widely used to elucidate the nervous mechanisms of SGML. It is believed that RWIS-induced GML is mainly caused by the enhanced activity of vagal parasympathetic nerves. Many central nuclei, such as the dorsal motor nucleus of the vagus, nucleus of the solitary tract, supraoptic nucleus and paraventricular nucleus of the hypothalamus, mediodorsal nucleus of the thalamus, central nucleus of the amygdala and medial prefrontal cortex, are involved in the formation of SGML in varying degrees. Neurotransmitters/neuromodulators, such as nitric oxide, hydrogen sulfide, vasoactive intestinal peptide, calcitonin gene-related peptide, substance P, enkephalin, 5-hydroxytryptamine, acetylcholine, catecholamine, glutamate, γ-aminobutyric acid, oxytocin and arginine vasopressin, can participate in the regulation of stress. However, inconsistent and even contradictory results have been obtained regarding the actual roles of each nucleus in the nervous mechanism of RWIS-induced GML, such as the involvement of different nuclei with the time of RWIS, the different levels of involvement of the sub-regions of the same nucleus, and the diverse signalling molecules, remain to be further elucidated.

Key words: Restraint water-immersion stress; Stress-induced gastric mucosal lesion; Central mechanism; Peripheral mechanism; Neurotransmitter/neuromodulator; Pathway

Core tip: The nervous mechanisms of gastric mucosal lesion in rats subjected to restraint water-immersion stress were investigated. Abnormal regulation of the enteric nervous system, mainly due to the enhanced activity of the parasympathetic nervous system, can

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induce gastrointestinal dysfunction. The central nucleus such as dorsal motor nucleus of the vagus, nucleus ambiguous, nucleus of the solitary tract, paraventricular nucleus, supraoptic nucleus, mediodorsal nucleus of the thalamus, central nucleus of the amygdala and medial prefrontal cortex are all involved in the formation of stress-induced gastric mucosal lesion. Nitric oxide, 5-hydroxytryptamine, hydrogen sulfide, calcitonin gene-related peptide, vasoactive intestinal peptide, acetylcholine, catecholamine, glutamate, γ-aminobutyric acid, oxytocin and arginine vasopressin may be involved in the physiological process.

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INTRODUCTION

Stress is defined as a disordered state of homeostasis caused by internal or external noxious stimuli, and the gastrointestinal tract is the main organ that responds to stress[1,2]. Stress-induced gastric mucosal lesion (SGML) is an acute GML mainly characterized by inflammatory erosion and gastrointestinal bleeding, which has a high mortality rate and is one of the most common visceral complications following trauma[3-5]. Thus, exploring the mechanisms that underlie the occurrence and development of SGML as well as the identification of more effective, safer and more affordable drugs have become one of the research hotspots in modern biology.

There are nerve plexuses in the gastrointestinal tract, with great independence in food digestion, nutrient uptake and waste removal, while the central nervous system (CNS) provides external nerves that regulate and control these functions[6]. Neural control of the gut is hierarchic with four basic levels of integrative organization. The first level is the enteric nervous system (ENS) that behaves like a local minibrain; the second level is in the prevertebral sympathetic ganglia; the third and fourth levels are within the CNS. Sympathetic and parasympathetic signals to the digestive tract exist at level 3, and represent the final common pathways for information outflow from the CNS to the gut. The fourth level includes higher brain centers that provide input for integrative functions at level 3[7,8]. The nervous mechanisms of SGML have been explored by many scholars from all over the world using methods such as basic physiology, electrophysiology, immunocytochemistry and pharmacology on the basis of neurotransmitters (neuromodulators), receptors, agonists and blockers[9-18]. In this study, the nervous mechanism of restraint water-immersion stress (RWIS)-induced gastric dysfunction was investigated, in order to provide ideas to further reveal the nervous mechanism underlying the occurrence and development of SGML.

GMLS INDUCED BY RWIS

RWIS was first discovered by Takagi, and has been widely used to elucidate the mechanism of SGML and screen for potential therapeutic drugs[19-21]. RWIS is a powerful multiple stress model that integrates psychological factors (*e.g.*, fear, anger, anxiety and despair) and physiological factors *(e.g.*, hunger, struggle and cold water). During RWIS, the anesthetized rats were fixed on a wooden board in the supine position, and then placed in cold water at 21 ± 1 °C below the sternum xiphoid in an "erect position" after resuscitation (Figure 1A). RWIS can irritate the rats, and leads to a decline in body temperature, gastric dysfunction and dotted or strip-like ulcers on the gastric mucosa within a few hours after induction (Figure 1B). In addition, RWIS can increase the gastric ulcer index values[22] in a time-dependent manner (Figure 1C).

PERIPHERAL NERVOUS MECHANISM OF RWIS-INDUCED GML

ENS

ENS consists of both submucosal plexuses and intermuscular plexuses. The

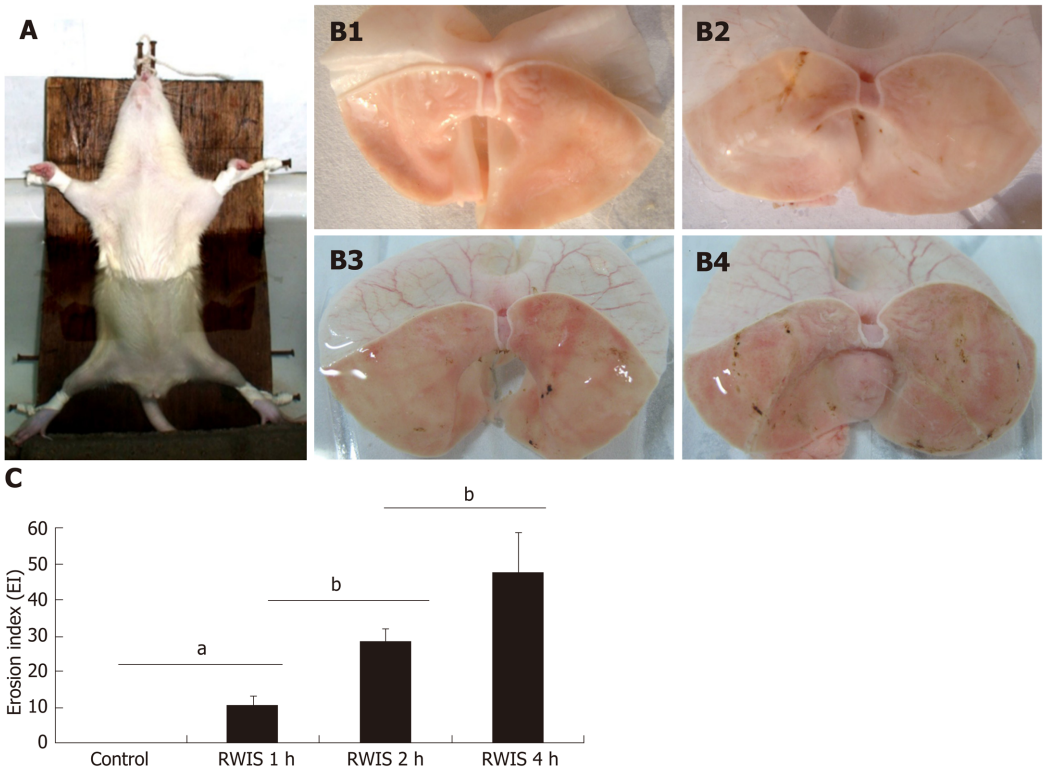

Figure 1 Effects of restraint water-immersion stress on gastric mucosal lesions in rats. A: The animal model of restraint water-immersion stress (RWIS). B: Gastric mucosal damage induced by RWIS (B1: control; B2: 1-h RWIS; B3: 2-h RWIS; B4: 4-h RWIS). C: Effects of RWIS on the rates of gastric erosion. n = 5; mean ± SE; $^{a}P < 0.05$, $^{b}P < 0.01$. RWIS: Restraint water-immersion stress.

submucosal plexuses are located in the submucosal muscles, which mainly regulate gastrointestinal mucosa contraction and glandular secretion in the lamina propria. To regulate the movement of gastrointestinal smooth muscles, glandular secretion, gastrointestinal blood flow, intestinal epithelial substance transport, gastrointestinal immune response and inflammatory process, the intermuscular plexuses distributed throughout the entire digestive tract between longitudinal muscles and circular muscles can form a network with sensory neurons, intermediate neurons and motor neurons *via* synapses. Moreover, the ENS neurons are interconnected to form an independent nervous system similar to the brain and spinal cord, which is also known as the "gut brain" due to a high degree of independence as well as the largest and most complex autonomic nervous system in the peripheral nerves. At the same time, ENS has a large number of afferent nerves derived from the CNS, such as the vagus nerve and spinal nerve, both of which can interact and serve as the gut-brain axis[23]. Abnormal regulation of ENS can induce gastrointestinal dysfunction, by elevating the amplitudes and index values of gastric motility (gastric hyperkinesia), decreasing gastric mucosal blood flow (GMBF) and gastric mucus secretion, increasing the production of gastric acid and proliferation of gastric mucosal cells[14,24-27]. Huang *et al*[15-17] examined the molecular signaling pathways related to the maintenance of gastrointestinal tract homeostasis and gastric mucosal barrier integrity, as well as the energy-related proteins that retain the levels of intracellular adenosine triphosphate (ATP). However, the exact functions of these molecules in SGML need to be further evaluated using molecular biology techniques.

SGML-related neurotransmitters/neuromodulators in ENS

Similar to the CNS, there are many types of neurons in the ENS, which differ from each other not only in morphology and structure, but also in neurotransmitter diversity. Apart from acetylcholine (ACh) and norepinephrine (NE), numerous endogenous substances, such as substance P (SP), vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP), ATP, 5-hydroxytryptamine (5-HT) and nitric oxide (NO), have recently been found to play important roles as neurotransmitters in the gastrointestinal neural network. These neurotransmitters are also termed the non-adrenergic non-cholinergic (NANC) nerves. Gastrointestinal excitatory motor neurons release excitatory transmitters, such as ACh and SP, thus promoting gastrointestinal smooth muscle contraction and glandular secretion. On the contrary, inhibitory motor

neurons release inhibitory transmitters, such as VIP and NO, thus suppressing gastrointestinal smooth muscle contraction and glandular secretion[28]. All these gastrointestinal excitatory and inhibitory motor neurons can interact with each other under a complex and delicate balance. If this balance is broken, gastrointestinal dysfunction may be induced.

NO: NO is an active and unstable inorganic gaseous molecule secreted by NANC nerves in the ENS, which serves as a major inhibitory neurotransmitter in the gastrointestinal tract. Nitric oxide synthase (NOS) is a key rate-limiting enzyme in NO production, and NOS assessment can indirectly determine the changes in NO, which can be classified into eNOS, nNOS and iNOS. eNOS is mainly distributed in vascular endothelial cells, and the synthesized NO is mainly used to regulate GMBF and promote gastric mucosal repair. nNOS is mainly distributed in cells within the intermuscular and submucosal plexuses, endocrine cells and vascular endothelial cells, and the synthesized NO interacts with gastrointestinal hormones (VIP and ATP) to jointly regulate gastrointestinal motility[29]. iNOS is highly expressed upon the stimulation of endotoxins and cytokines, which produces a large amount of NO within a short period of time, and ultimately results in cell damage. Previous studies have demonstrated that NO can inhibit gastric acid secretion and neutrophil adhesion, improve gastric mucosal blood circulation and eliminate oxygen free radicals, thereby protecting the gastric mucosa from injury[30,31]. It was reported by Nishiad *et al*[31] that the expression level of iNOS increased significantly in the gastric mucosa of RWIS rats, while that of eNOS reduced significantly, indicating that the changes in iNOS and eNOS activities in the gastric mucosa are closely related to the incidence of GML. In SGML, L-NAME (NOS inhibitor) can decrease the production of NO, thus exacerbating acute GML and inhibiting the healing process of chronic gastric ulcers, while L-Arg (NO precursor) can obviously prevent the injury[32-34]. Wei *et al*[35] found that NO is involved in RWIS, and can promote the SGML healing process.

The mechanisms of NO in protecting gastric mucosa are as follows: (1) NO can reduce vascular permeability, inhibit platelet adhesion and aggregation in gastric mucosal vascular endothelium, and prevent thrombosis. (2) Under physiological conditions, gastric mucosal vascular endothelium synthesizes NO, which in turn regulates vascular smooth muscle tension and maintains GMBF. (3) In acute GML, NO increases GMBF by dilating the mucosal blood vessels, thus promoting gastric mucosal repair[33]. In addition, the secretion of gastric acid can also be inhibited by NO. Upon the reaction of stimulus against gastric mucosa, enterochromaffin cells and mastocytes can release histamine to stimulate parietal cells for gastric acid production, thus aggravating the mucosal lesion. In addition, endogenous NO can inhibit the stimulation of histamine through parietal cells, thus reducing gastric acid secretion and protecting gastric mucosa. It has been found that, through *in vivo* and *in vitro* experiments, the NO donor FK409 and sodium nitroprusside can markedly suppress the gastrin-induced increase in histamine release and gastric acid secretion in rats, and L-NAME further increases gastric acid secretion[30]. Gastric mucous cells promote NO synthesis by expressing high-level NOS, and enhance the mucous barrier through the NO effects of promoting mucin synthesis and secretion. Based on the findings of previous experiments, RWIS-induced GMLs can weaken the synthesis and secretion of gastric mucus by reducing nNOS activity, while the NO donor L-Arg can increase nNOS activity and mucus secretion[36].

5-HT: 5-HT is an important neurotransmitter that widely exists in the brain and digestive tract. It has been estimated that 90% of 5-HT is synthesized and secreted by enterochromaffin cells in the gastrointestinal tract[37]. There are four types of 5-HT receptors, of which 5-HT3R and 5-HT4R are the two receptor subtypes most closely related to gastrointestinal function. 5-HT3R is mainly found within the neurons of myenteric plexuses in the stomach and colon[38], while 5-HT4R is mainly distributed within the neurons of myenteric plexuses in the ascending colon, duodenum and gastric smooth muscles, as well as the intestinal submucosa[39]. Both 5-HT3R and 5-HT4R are involved in gastrointestinal motility, gastric acid and mucus secretion, and regulation of local mucosal blood flow. Due to the absence of internal and external nerve fibers in the mucous epithelium, it is a transepithelial phenomenon that ENS senses and reacts to chemical stimulus in the enteric cavity, which may be partly mediated by enterochromaffin cells. Upon response to stimulus, these cells may release 5-HT, and subsequently activate the submucosal primary afferent nerve fibers through 5-HT3R distributed on the vagal afferent nerve fibers, and transmit intestinal information to the center, thereby regulating local excitement and inhibition[40]. Serotonin reuptake transporter (SERT) is a type of translocator responsible for re-uptake of 5-HT from the synaptic cleft, which can rapidly eliminate 5-HT from the

synapse and reduce 5-HT concentration in the intestinal tract and tissue space. Thus, pharmacological blockage of SERT function can obviously decrease gastrointestinal motility[41]. Chen *et al*[42] reported that, in SERT-knockout mice, watery diarrhea is likely to be caused by the enhanced action of the 5-HT signaling pathway, and slow transit constipation may be caused by the insensitivity of 5-HT receptors due to excessive 5-HT production. In addition, Morita *et al*[43] showed that 5-HT3R and 5-HT4R antagonists can inhibit the antral motility during the interdigestive period (phase III) and colonic motility in dogs. The above data indicate that the 5-HT signaling system is involved in the sensory and motor functions of the gastrointestinal tract. Apart from its motor and sensory functions in the gastrointestinal tract[44], 5-HT inhibits gastric acidity by increasing the synthesis of mucus[45]. It has been reported that 5-HT simulates prostaglandin (PG) synthesis by enhancing the activity of the cyclooxygenase pathway, which in turn stimulates mucosal blood flow and contributes to the secretion of mucus along with bicarbonate. Therefore, the 5-HT signaling system plays a vital role in the regulation of gastrointestinal motility, secretion and visceral sensation. An abnormal 5-HT signaling pathway may trigger gastrointestinal endocrine disorders, leading to a variety of gastrointestinal diseases. In addition, indomethacin-induced intestinal lesions in mice can be prevented by pretreatment with p-chlorophenylalanine (a 5-HT synthesis inhibitor). The administration of the 5-HT4 receptor agonist (mosapride) or 5-HT3 receptor antagonists (*e.g.*, ondansetron and ramosetron) can dose-dependently reduce the severity of indomethacin-induced intestinal lesions, whereas a high dose of GR113808 (a 5-HT4 receptor antagonist) significantly aggravated these lesions. These findings suggest that endogenous 5-HT exerts a dual role in the pathogenesis of indomethacin-induced intestinal lesions: Pro-ulcerogenic action *via* 5-HT3 receptors and anti-ulcerogenic action *via* 5-HT4 receptors[46]. Additionally, the cold-restraint stress significantly increased mean ulcer index values in gastric tissue, while a decrease in enterochromaffin cell count was observed with a concomitant decrease in 5-HT content and adherent mucosal thickness. Pretreatment with Aegle marmelos reduced mean ulcer index values, and increased enterochromaffin cell count, 5-HT content and adherent mucosal thickness in ulcerated gastric tissue, suggesting that the high enterochromaffin cell count and 5-HT levels exert protective effects against cold-restraint stress-induced gastric mucosal injury[47].

Hydrogen sulfide: Hydrogen sulfide (H_2S) is a new type of endogenous gaseous signaling molecule, and many tissues in the body can catalyze L-cysteine to H_2S *via* cystathionine-synthase (CBS) and cystathionine-γ-lyase (CSE). In recent years, it was found that H_2S is involved in various physiological and pathological processes in the body, which in turn regulates the motility, resists the inflammation, affects the visceral sensitivity, and promotes glandular secretion. Specifically, its regulation of digestive tract motility is mainly manifested as the suppression of intestinal motility. Exogenous administration of sodium hydrosulfide (NaHS; a H_2S donor) can enhance the secretion of colon mucosal and submucosal chloride in guinea pigs in a concentration-dependent manner, and this secretion can be inhibited by H_2S CSE and CBS blockers[48]. Krueger *et al*[49] found that H_2S promotes intestinal secretion *via* activation of transient receptor potential vanilloid-1 (TRPV1) receptor and the release of SP, thus activating cholinergic neurons. In addition, Dhaese *et al*[50] found that NaHS can relax the gastric fundus smooth muscles of mice in a concentration-dependent manner, which can be weakened by myosin light chain phosphatase (MLCP) inhibitors, instead of ATP-sensitive potassium channel (KATP) blockers. This indicates that H_2S-induced dilation of gastric fundus smooth muscles is realized, at least partly, by activating MLCP, but is not related to KATP.

H_2S is able to protect the gastrointestinal tract and promote the repair of GML. Nonsteroidal anti-inflammatory drugs (NSAIDs) can markedly down-regulate the expression of CSE in gastric mucosa and reduce the synthesis of endogenous H_2S. However, NaHS can be used to decrease the synthesis of tumor necrosis factor-alpha (TNF-α), intercellular adhesion molecule-1 and lymphocyte function associated antigen-1, reduce the adhesion of white blood cells to mesenteric vessels, increase GMBF and suppress GML caused by NSAIDs[51]. Both exogenous administration and endogenous release of H_2S are gastroprotective against cold restraint stress-induced gastric injury. It has been reported that NaHS attenuates the ulcer index by reducing gastric acid output, mucosal carbonyl content, pepsin activity and ROS generation. Moreover, H_2S also reduces TNF-α level and myeloperoidase activity[52]. Magierowski *et al*[53] demonstrated that treatment with NaHS plays an important physiological role in gastric mucosal protection against stress-induced lesions. The protective effect of NaHS is often accompanied by an enhancement in gastric microcirculation, possibly mediated by a significant local increase in the gastric mucosal production of H_2S. Furthermore, the mechanism of H_2S-induced gastroprotection may involve activation

of the endogenous prostaglandin/cyclooxygenase (PG/COX) system, increase biosynthesis of prostaglandin E2 (PGE2), afferent sensory fibers release of CGRP *via* VR-1 receptors and an anti-inflammatory effect resulting in the inhibition of pro-inflammatory cytokines (*e.g.*, TNF-α).

CGRP: CGRP is the main transmitter of capsaicin-sensitive sensory nerves, with a wide variety of functions, which is widely distributed throughout the cardiovascular, gastrointestinal and respiratory systems and commonly recognized as a protector of gastric mucosa. CGRP is the most effective vasodilator discovered to date, and it can improve blood flow through two mechanisms. (1) CGRP binds to receptors on endothelial cells and up-regulates NOS to produce NO, thereby relaxing the vascular smooth muscles. (2) CGRP directly binds to receptors on vascular smooth muscle cells and increases GMBF by activating KATP channels without involving the endothelium[54]. In addition, CGRP also exerts anti-apoptotic, anti-platelet aggregation, anti-oxidation, anti-proliferation and anti-aging properties. Previous findings have shown that CGRP can significantly inhibit gastric acid secretion[55]. Following craniocerebral injury and severe burn, the concentrations of hydrogen ions in gastric juice may be elevated. It has also been found that excess gastric acid production is related to neuroendocrine disorders, and a decrease in CGRP secretion is caused by nerve center and hypothalamic injury[56]. The gastric acid secretion in mice can be facilitated by an intracerebroventricular injection of thyrotropin-releasing hormone, but inhibited by CGRP. A large amount of experimental data has proved that noxious stimuli, such as GML, can trigger the sensory neurons to release CGRP, activate intermediate neurons and motor neurons. Besides, it also directly acts on smooth muscles and inhibits gastrointestinal motility, whose mechanisms are as follows: (1) The longitudinal muscles and circular muscles of the intestine are directly dilated. And (2) The NANC inhibitory neurons are stimulated, and other inhibitory neurotransmitters are released, such as NO and VIP, thereby inhibiting gastrointestinal motility[57].

CGRP can help to improve local gastric microcirculation and protect against stress- or drug-induced GML by increasing gastrointestinal blood flow and regulating gastrointestinal motility. A previous study demonstrated that CGRP down-regulation is related to the pathogenesis of gastric ulcers[58]. After stimulation, capsaicin-sensitive sensory nerve fibers may release CGRP, and then CGRP increases the levels of prostacyclin and PGE2 in gastric mucosa, thereby inhibiting the activation of neutrophils and degranulation of mastocytes, reducing the secretion of inflammatory mediators (*e.g.*, histamine), and alleviating gastrointestinal inflammation[59]. In a mouse model of ischemia-reperfusion injury, intraperitoneal treatment with CGRP significantly reduced gastric mucosal edema, hemorrhage, apoptosis, mucosal separation and inflammatory cell infiltration[60]. RWIS-induced gastric ulcers were inversely correlated with the decrease in CGRP-like immunoreactivity observed in the whole thickness of the stomach corpus. Systemic administration of CGRP exhibited a significant decrease in the lesion index of RWIS-induced ulcers, and such protection was inhibited by the functional ablation of afferent neurons induced by capsaicin pretreatment. These findings suggest that endogenous CGRP plays a defensive role in RWIS-induced ulcers[61].

VIP: As one of the most important peptide neurotransmitters, VIP is widely distributed in the circulatory, immune, reproductive and digestive systems, as well as the central and peripheral nervous systems. VIP possesses dual functions in the body, and acts as both a gastrointestinal hormone and a neuropeptide, and has been considered a type of brain-gut peptide. VIP is mainly produced by the central and peripheral nervous systems, released by the parasympathetic postganglionic fibers and coexists with ACh, which plays a regulatory role in local mucosal immunoregulation. In the digestive system, VIP is predominantly distributed in the endocrine cells of gastrointestinal mucosa as well as the submucosal plexuses and smooth muscle layer. Moreover, VIP regulates gastrointestinal absorption, inhibits gastric acid secretion, protects the gastrointestinal mucosa from acid-induced damage, and promotes the secretion of water, electrolytes, pancreatic juice and intestinal juice in the intestine. Additionally, VIP is able to induce smooth muscle relaxation and exert a potent vasodilator effect[62]. The regulatory effect of VIP on gastrointestinal smooth muscle motility is closely related to the main inhibitory neurotransmitter NO, in which VIP can promote NO synthesis, relax circular muscles, inhibit gastric motility and reduce gastric tightness.

In addition, VIP exerts an impact on the integrity of the gastrointestinal mucous membrane barrier. For instance, VIP can induce the secretion of water and bicarbonate in the pancreas, thus promoting the formation and repair of the gastrointestinal mucous membrane barrier. Moreover, VIP can stimulate the

production of intestinal juice, and inhibit the secretion of gastric acid and gastrin, thereby protecting the gastric mucosa, suppressing the occurrence of gastric and duodenal ulcers, and promoting the repair of ulcers[63]. In ethanol-induced GML, the content of VIP decreases obviously in the gastric mucosa, by reducing the release of NO and increasing the production of endothelin[64]. It has been reported that cold-restraint stress induces gastric lesions and mast cell degranulation, and exacerbates lipid peroxidation in gastric tissue. VIP can prevent stress-induced ulcers and mast cell degranulation, and protect gastric tissue from lipid peroxidation. When VIP was administered after stress-induced ulcers, it was demonstrated to be therapeutically beneficial, suggesting that VIP is valuable for the prevention of gastric mucosal damage induced by cold-restraint stress[65]. Recent studies have shown that VIP significantly suppressed cold-restraint stress gastric lesions and markedly decreased the content of histamine in the tissue. Histamine plays an important role in the development of gastric ulcers, and mast cell-derived histamine might be essential during this process. The mechanisms of the action of VIP on gastric tissue histamine levels can be explained by its inhibitory effect on the release of gastrin hormone, mast cells and enterochromaffin-like cells[66].

CENTRAL MECHANISM OF RWIS-INDUCED GML

Some studies have found that RWIS leads to the elevation of blood corticosterone and adrenocorticotropic hormone levels in rats, and their levels in plasma also gradually rise over a prolonged period of stress[67]. This seems to indicate that the activity of the hypothalamic-pituitary-adrenal (HPA) axis is enhanced during RWIS. However, severing the subphrenic vagus nerves or consuming atropine can significantly alleviate and even cure RWIS-induced GML, but removing the pituitary glands and adrenal glands or administering phenoxybenzamine (adrenergic α-receptor blocker) has little impact on RWIS-induced GML, gastric hyperkinesia and RWIS-induced gastric acid secretion. This suggests that the HPA axis does not play a major role in RWIS-induced GML, and the peripheral nervous mechanism of RWIS-induced GML is mainly through the enhanced parasympathetic activity[14-16]. Therefore, the nervous mechanism of RWIS-induced gastrointestinal dysfunction in rats is mainly the "enhanced activity of parasympathetic nervous system", rather than the traditional ideas of the "enhanced activity of sympathetic-adrenal medulla system" and "HPA axis".

SGML-related central nuclei

Medullary gastrointestinal center: In recent years, the mechanisms of the primary central-brainstem loop in regulating gastric function have been clarified using electrophysiological and immunohistochemical techniques. Parasympathetic control is dominant in the nervous regulation of gastric function. The parasympathetic nerves that control the stomach and intestines are from the dorsal motor nucleus of vagus (DMV) and nucleus ambiguous (NA) of the medulla oblongata (both of which are the initial nuclei of parasympathetic preganglionic fibers and essential visceral motor nuclei). The nucleus of the solitary tract (NTS), located at the dorsolateral side of the DMV, is an important primary visceral sensory nucleus; while area postrema (AP), located at the dorsomedial side of the NTS, has a chemosensory function. Eventually, a dorsal vagal complex (DVC) is formed. The DVC and NA are the lower centers of visceral parasympathetic nerves that integrate both sensory afference and visceral efference, which are also the primary centers for the regulation of gastric function[68,69].

The DVC and vagal efferent play an outstanding role in the regulation of gastric mucosal resistance to injury. However, the role of the vagal nerve is likely to be dual, as it can mediate both mucosal damaging and protective effects. It has been demonstrated that thyrotropin-releasing hormone (TRH) acting centrally in the DVC can stimulate gastric acid and pepsin secretion, induce gastric emptying and trigger ulceration *via* activation of parasympathetic outflow to the stomach[70]. Similarly, activation of the dorsal motor nucleus of vagus by RX-77368 (a TRH analogue) through an intracisternal injection at high dose could promote the formation of GML. Furthermore, electrical stimulation of the vagus has been found to induce GML and mast cell degranulation[71]. In addition, TRH, or its analogue RX-77368, injected at the dorsal motor nucleus of vagus, has been reported to protect the gastric mucosa against ethanol injury through stimulation of vagal cholinergic pathways, as both vagotomy and atropine can reverse the gastroprotective effects[72,73]. Biochemical and pharmacological studies have demonstrated that the mechanisms of vagal-mediated gastroprotective effects may be due to the activation of vagal cholinergic pathways, secretion of gastric PG and production of NO[74]. Our previous study revealed that

RWIS induced significant gastric mucosal damage and activated astrocytes by increasing the levels of glial fibrillary acidic protein and neurons, as indicated by Fos expression in the DMV and NTS. Intracerebroventricular administration of the astroglial toxin L-α-aminoadipate could alleviate RWIS-induced gastric mucosal damage. Immunohistochemistry results showed that L-α-aminoadipate decreased the activation of both astrocytes and neurons induced by RWIS. These findings provide strong evidence that astrocytic and neuronal activation in the DMV and NTS may be closely related to RWIS-induced gastric mucosal damage[75].

With the expression of immediate early genes (*e.g.*, c-Fos) as an index, Zhang and colleagues[18] evaluated the activities of neurons in the medullary gastrointestinal center at different time periods of stress using an immunohistochemistry-based technique. They found that c-Fos was highly expressed in DVC and NA at different time periods of RWIS (30, 60, 120 and 180 min), and its expression intensity was highest in the DMV, followed by the NA and NTS. The above findings preliminarily reveal the temporal-spatial rule of nuclear activity in the primary center that regulates gastrointestinal function in rats during RWIS, and further confirm that RWIS-induced gastric dysfunction is mainly caused by the enhanced parasympathetic activity. In other words, there is a neural circuit ("medullary gastrointestinal center-gastrointestinal wall plexus loop") between the medulla oblongata and the gastrointestinal tract. Under RWIS, the information of gastrointestinal motility is transmitted as follows: Information → vagal afferent nerves → NTS → DMV/NA, while those of medullary efferents are disseminated as follows: DMV/NA → vagal efferent nerves → gastrointestinal wall plexuses, thereby causing gastric hyperkinesia, increasing gastric acid secretion and reducing gastric mucus secretion, and ultimately leads to GML and fecal impaction.

However, in the case of electrical stimulation of NTS in normal rats, gastric motility may be inhibited, and a possible reason for this is that excitement of the NTS activates inhibitory neurons in the DMV, thus suppressing gastric motility through a non-cholinergic neural pathway[68,76]. In addition, gastric motility was significantly inhibited when electrical or chemical stimulation induced neuronal excitation in the NA and DMV, indicating that excitation of the NA and DMV also exerts an inhibitory effect on gastric motility[77]. However, the above findings are contradictory to the immunohistochemical results showing that the expression levels of c-Fos in the NA, DMV and NTS were obviously enhanced in RWIS-induced rats. This is probably due to the fact that the activity of the higher center (*e.g.*, anterior hypothalamus) eliminates the inhibition of medullary visceral centers on the stomach during RWIS, thereby causing gastric hyperkinesia and increasing gastric acid secretion.

Anterior hypothalamus: The hypothalamus region is generally divided into the anterior hypothalamus and posterior hypothalamus. With regard to the regulation of visceral function, the anterior hypothalamus may act as the parasympathetic center, while the posterior hypothalamus serves as the sympathetic center. At different time points of RWIS (30, 60, 120, and 180 min), Fos is expressed in different degrees in the supraoptic nucleus (SON), paraventricular nucleus (PVN) and suprachiasmatic nucleus (SCN) in the anterior hypothalamus, most noticeably in the PVN and SON[78]. Chemical or electrical stimulation of the PVN can markedly aggravate gastric ulcers in RWIS rats, whereas electrical damage of the PVN can alleviate ulcers[79]. Bilateral vagotomy can suppress GML triggered by electrical stimulation of the PVN[80,81], indicating that PVN stimulation-induced GML is mediated by parasympathetic pathways. Lu *et al*[82] found that chemical (L-Glu) or electrical stimulation of the SON could promote gastric acid secretion. These results further confirm the above-mentioned statements that the nervous mechanism of RWIS-induced gastrointestinal dysfunction is mainly through the "enhanced activity of parasympathetic nervous system". Therefore, the excessive activities of the SON and PVN in the anterior hypothalamus may be one of the central mechanisms underlying stress-induced gastrointestinal dysfunction.

Central nucleus of the amygdala: The amygdaloid complex is an important subcortical nucleus in the limbic system, which is involved in the regulation of mood, emotion and visceral activities related to emotional stimulation, and has thereby attracted considerable attention[83,84]. The central nucleus of the amygdala (CEA) is an important nucleus of the amygdaloid complex, which is closely associated with the autonomic responses to stress and maintenance of gastric mucosal integrity[85-88]. At different time points (30, 60, 120 and 180 min) of stress, the expression level of Fos was significantly enhanced in the CEA, and was second only to that in the anterior hypothalamus[89]. According to previous anatomical studies, CEA has complex fiber connections with the NTS and DMV in the medullary gastrointestinal center[90,91]. It has also been found in physiological studies that electrical stimulation of different

regions in the CEA can promote the secretion of gastric acid[92], enhance or decrease gastric motility[93,94], induce gastric ulcers[95], and alter the activity of neurons in NTS and DMV in the medullary gastrointestinal center[90,91]. Moreover, electrical stimulation of CEA-induced vagus nerve-mediated gastric ulcers, demonstrates that the CEA may play a role similar to the hypothalamus. One possible reason is that its neuronal activity diminishes the inhibitory effect of the medullary gastrointestinal center on the gastrointestinal tract, but further research is needed to verify this hypothesis.

Medial prefrontal cortex: The medial prefrontal cortex (mPFC), the highest-level association cortex in mammals, plays a key role in many advanced brain functions. RWIS includes psychological stress and physiological stress. According to the immunohistochemical findings, the expression levels of Fos were relatively high in the prelimbic cortex (PL) and infralimbic cortex (IL) of the mPFC in RWIS rats, in addition to the DMV, NTS, NA, SON and PVN. In both IL + PL-sham-operated and IL + PL bilateral-lesioned groups, exposure to RWIS for 4 h could affect the curves of gastric motility by changing from high-frequency, short-term and low-amplitude fast waves to low-frequency, long-term and high-amplitude slow waves, which represent an obvious sign of gastric hypermotility. Synaptic plasticity can maintain the stability of neural circuits, and the brain can adapt to changes in both internal and external environments through neural plasticity changes in synaptic structure and function when coping with stress. Compared to the control group, the concentration of postsynaptic density protein 95 (PSD95) decreased in the mPFC of the RWIS 4-h group[96]. From the dendritic spine staining results, it was found that the number of dendritic spines per unit length (10 μm) in the 1-, 2- and 4-h RWIS groups was significantly lower than that in the control group. Moreover, according to electron microscopic observations of PFC synapses, the synapses displayed an unclear outline, and there were more perforated synapses and more vacuolated mitochondria observed in the 4-h RWIS group, indicating that oxidative stress causes certain damage in mitochondria. In addition, the number of synapses per unit area, number of presynaptic vesicles and thickness of the PSD in the 4-h RWIS group were obviously decreased compared to those in the control group. From the results of proteomic analysis, it was observed that there were 129 differentially expressed proteins (88 up-regulated and 41 down-regulated) between the control group and the 4-h RWIS group. According to the GO analysis, 22% (29) of the differentially expressed proteins were directly related to nervous system functions, including synaptic plasticity (6), axon morphology and growth (12), neurodevelopment and apoptosis (10), and neural signal transmission (1), while some were associated with synaptic vesicle circulation and toxicological metabolism. These findings indicate that the mPFC is involved in the regulation of RWIS-induced gastric dysfunction, but its exact role requires further study.

Mediodorsal nucleus of the thalamus: Using Fos, SYN and SYN-I as indices, it has been demonstrated that the PFC is involved in the responses to RWIS. However, gastric motility was not significantly affected following an injection of sodium L-glutamate, electrical stimulation of PL or IL of the PFC, suggesting that other nuclei are required to transmit RWIS signals into the PFC. After electrophoresis of the PFC, no labeled nerve cell bodies were observed in the PVN or SON, but were found in the mediodorsal nucleus of the thalamus (MD), indicating that the MD may emit fibers to the PFC.

MD is the largest subnucleus in the medial thalamic nucleus group, as well as the only thalamic nucleus with a fiber connection to the PFC. We also found that the expression level of Fos was significantly upregulated in the MD of RWIS rats compared to that in the control group, and the expression levels of SYN and SYN-I in the 1-h RWIS group were significantly higher than those in the control group, indicating that MD is involved in RWIS. As has been previously reported in the literature, the MD exhibits complex efferent and afferent fiber connections with the PFC, hypothalamus and brain stem, thus forming multiple neural circuits[97]. In addition, the MD is a higher-order thalamic relay nucleus that forms the cortex-thalamus-cortex circuit, which serves as an important integration site of visceral and physical activities[98]. A recent study reported that the density of dendritic spines on the dendritic shaft of neurons in the MD was markedly decreased in the 1-h RWIS group compared to that in the control group[99]. Electrophysiological findings showed that the firing rate of MD neurons was significantly reduced, the mean interspike interval was significantly prolonged and the burst rate gradually declined in awake rats during RWIS, suggesting that RWIS exerts an inhibitory effect on the neurons in the MD. Quantitative proteomic analysis revealed that a total of 65 dysregulated proteins were identified, which are mainly involved in the signaling pathways

associated with neurological diseases. Moreover, 31 upregulated proteins were primarily related to cell division, while 34 downregulated proteins were related to neuronal morphogenesis and neurotransmitter regulation. Furthermore, glycogen synthase kinase-3 beta might be related to the central mechanism through which RWIS gives rise to stress-induced gastric ulcers.

Central neurotransmitters/neuromodulators in SGML

ACh: ACh is the first discovered, classical neurotransmitter that is distributed throughout the motor and sensory systems, brainstem reticular structure, limbic system and cerebral cortex. ACh has been shown to affect the sensory, motor, learning, memory and other functions in the CNS, especially excitation function. Continuous intravenous injection of ACh can cause severe GML in rats[100]. Moreover, injecting ACh into the NTS can significantly enhance the protective effect of electroacupuncture against GML, while injecting atropine can weaken this effect, suggesting that the effect of ACh is mediated by the cholinergic M receptor[101]. In addition, injecting low-dose ACh into the lateral ventricle evidently strengthens the aggravating effect of electrical stimulation of the PVN on SGML, which is also mediated by the M receptor[79]. We studied and proved that the excessive activity of cholinergic neurons in the middle segment of the DMV and NA is one of the primary central mechanisms responsible for RWIS-induced gastric dysfunction.

Catecholamine: Catecholamine (CA) is a collective term for NE, epinephrine (E) and dopamine (DA), and is one of the classic neurotransmitters abundantly distributed in the central and peripheral nervous systems. Numerous morphological and electrophysiological studies have confirmed that NE and DA are important endogenous inhibitory neurotransmitters that protect the integrity of gastric mucosa during stress[102,103]. A micro-injection of NE into the lateral ventricle can reduce the aggravating effect of electrical stimulation of the PVN on SGML in a dose-dependent manner, which is mediated by β-adrenergic receptors[79]. DA antagonists applied in the center may worsen SGML, while DA agonists can relieve SGML[104]. Moreover, after 6-OHDA is injected into the ventral tegmental area to deplete endogenous DA, or haloperidol (a DA receptor antagonist) is injected into the NA, the protective effect of neurotensin against RWIS-induced GML is obviously inhibited[105]. Zhao *et al*[106] found that catecholaminergic neurons in the nucleus of the medullary visceral center participate in the regulation of RWIS-induced GML, whereas catecholaminergic neurons in the nucleus of the anterior hypothalamus are rarely or not involved. Therefore, the neurons responsible for RWIS are not located in the anterior hypothalamus, but instead the neuronal activity in the nucleus may be regulated by medullary catecholaminergic neurons.

Glutamate: Glutamate (Glu) is the most abundant and widely distributed neurotransmitter in the brain and CNS, and conveys peripheral nociceptive information to the center. It has been closely associated with rapid excitatory synaptic transmission, neuronal development and death, synaptic plasticity and the incidence of some neurological diseases[107-110]. Yao *et al*[111] found that the contents of excitatory amino acids (Asp, Glu and Gln) in the brain tissues of rats were significantly higher in the 5-h RWIS group than those in the control group. Micro-injection of L-Glu into the middle segment of rat DMV could enhance vagus nerve-mediated gastric motility, while injection of L-Glu into the final segment of the DMV and micro-injection of L-Glu into rat NTS could inhibit such gastric motility[10,112]. The amount of H^+ in the secreted gastric juice of rats injected with Glu into the AP evidently rises compared to that in rats injected with normal saline, and this effect is mediated by the vagus nerve. Krowicki *et al*[113] injected L-Glu into the NA of rats, and the results showed that the peak of intragastric pressure and contraction of pyloric smooth muscles were greatly increased. Furthermore, we found that micro-injection of L-Glu into rat AP, right NA, DMV and NTS might suppress gastric motility in a dose-dependent manner.

γ-aminobutyric acid: γ-aminobutyric acid (GABA) is one of the major inhibitory neurotransmitters in the CNS, and is widely distributed in various regions of the brain, and has been associated with higher brain functions (*e.g.*, attention, working memory, mood and motorial inhibition). Shi *et al*[114] found that gastric ulcers were significantly relieved after injecting GABA and pentobarbital sodium (a GABAA receptor agonist) into the lateral ventricle, and they were aggravated after injecting bicuculline (a GABAA receptor blocker). This indicates that GABAA receptor mediates the inhibitory effect of GABA on RWIS-induced GML.

Oxytocin: Oxytocin (OT) is a neuropeptide composed of 9 amino acids, which is mainly synthesized in the magnocellular part of the hypothalamic SON and PVN[115]. When an OT receptor (OTR) blocker was injected into the animal DMV, gastric

secretion was suppressed and motility was enhanced. When OT was injected into the DMV, the opposite results were obtained[116]. The injection of OT into the lateral ventricle significantly relieved GML induced by cold restraint stress or subcutaneous injection of mercaptoethylamine[117]. RWIS could induce the expression levels of Fos in 34% and 28% of OT neurons residing in the PVN and SON of rats, indicating that RWIS activates OT neurons in both the PVN and SON[118]. It has been reported that OTR neurons are almost uniformly distributed in the start-final segments of the nucleus of the medullary gastrointestinal center. Moreover, the number of Fos+OTR-immunoreactive neurons (Fos+OTR-IR) in the 1-h RWIS group was obviously increased compared to that in the control group, and the proportions of Fos+OTR-IR neurons in Fos-IR neurons were approximately 58% and 45% in the DMV and NTS, respectively, in the 1-h RWIS group, indicating that the activities of neurons in the DMV and NTS are regulated by OTR-mediated OT[118].

Arginine vasopressin: Arginine vasopressin (AVP) is also a peptide hormone mainly secreted by large-cell neuroendocrine cells of the PVN and SON in the hypothalamus. In addition to the PVN and SON, AVP neurons are also distributed in the SCN, peripheral hypothalamic area, lateral hypothalamic area, posterior hypothalamic area and the CEA. RWIS can induce the expression levels of Fos in AVP neurons to 40% and 53% in the PVN and SON, respectively, in rats, indicating that RWIS activates the AVP neurons in both the PVN and SON[118]. Similar to OTR, AVP neurons (V1bR) are almost uniformly distributed in the start-final segments of the nucleus of the medullary gastrointestinal center. Moreover, the number of Fos + OTR-positive neurons in the 1-h RWIS group was obviously increased compared to that in the control group, and the proportions of Fos + V1bR immunoreactive positive neurons in Fos-positive neurons in the DMV and NTS were approximately 72% and 52%, respectively, in the 1-h RWIS group, indicating that the activities of neurons in the DMV and NTS are regulated by V1bR-mediated AVP[118]. Micro-injection of AVP into the DMV in rats can significantly inhibit gastric mobility and promote gastric acid secretion. Following micro-injection of AVP into the DMV, gastric motility was significantly inhibited. Furthermore, the inhibitory effect of AVP on gastric motility could be completely blocked by both SR49059 (a specific AVP receptor antagonist) and hexamethonium (a specific neuronal nicotinic cholinergic receptor antagonist). These data indicate that AVP inhibits gastric motility by acting on the specific AVP receptor in the DMV, with the potential involvement of parasympathetic preganglionic cholinergic fibers[119].

In addition, the types of neurotransmitters/neuromodulators in the medullary gastrointestinal center in response to RWIS have been studied and it was found that SP, enkephalin, H_2S and other neurotransmitters/neuromodulators are involved in the regulation of RWIS-induced gastric dysfunction[120,121].

Central nervous pathways of SGML

Some scholars have summarized the regulatory pathways of gastrointestinal function under normal physiological conditions[6,122,123], and the major nuclei involved[124]. According to these studies, several hypotheses have been put forward, and a preliminary consensus has been reached by domestic and foreign scholars on the peripheral and central nervous mechanisms of RWIS-induced gastrointestinal dysfunction. It was shown that the sensory information from the gastrointestinal tract and other internal organs are transmitted to the NTS through visceral sensory fibers in the vagus nerve, integrated by NTS secondary neurons in the brain stem, and then conveyed to relevant nuclei, such as the DMV, *via* neurotransmitters (*e.g.*, Glu). DMV regulates the gastric motility through parasympathetic nerves, and parasympathetic neurons control the gastric function *via* two different pathways. (1) The M receptor is activated to enhance gastric motility and secretion *via* the cholinergic excitatory pathway. And (2) Gastric function is inhibited mainly by releasing NO or vasopressin (VP) *via* activating the NANC pathway (Figure 2). With regard to the advanced central nervous mechanism of RWIS-induced gastrointestinal dysfunction, the potential advanced central nervous regulatory pathway of gastrointestinal function in rats under RWIS is hypothesized based on the previous works of our research group and relevant scholars (Figure 2). The MD receives the advanced neural activity information from the PFC (possibly IL) *via* the cortical-thalamic pathway, integrates such information with that from subcortical structures (*e.g.*, the hypothalamus and medulla oblongata), and then feeds the integrated information back to the PFC *via* the thalamic-cortical pathway. At the same time, there is a two-way fiber connection between the IL and CEA, and the final integrated information from the PFC is directly or indirectly fed back to the medullary gastrointestinal center through the CEA, thereby inducing gastric dysfunction.

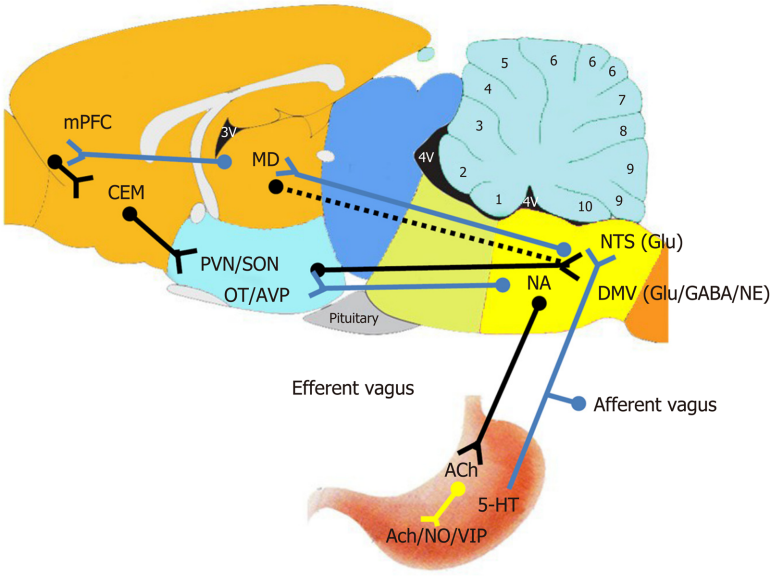

Figure 2 Schematic diagram of the central nervous pathways of restraint water-immersion stress-regulated gastrointestinal function. Solid line: The pathways that have been determined; Dashed line: The pathways that remain to be further verified; Blue line segment: Upward; Green line segment: Downward. mPFC: Medial prefrontal cortex; CEA: Central nucleus of the amygdala; MD: Mediodorsal nucleus of the thalamus; DMV: Dorsal motor nucleus of vagus; NA: Nucleus ambiguous; NTS: Nucleus of the solitary tract; VIP: Vasoactive intestinal peptide; 5-HT: 5-hydroxytryptamine; NO: Nitric oxide; ACh: Acetylcholine; NE: Norepinephrine; GABA: γ-aminiobutyric acid; Glu: Glutamate; PVN: Paraventricular nucleus; SON: Supraoptic nucleus; OT: Oxytocin; AVP: Arginine vasopressin.

CONCLUSION

To sum up, the nervous mechanism of RWIS-induced gastrointestinal dysfunction in rats is mainly the "enhanced activity of parasympathetic nervous system", rather than the traditional ideas of the "enhanced activity of sympathetic-adrenal medulla system" and "HPA axis", and multiple nuclei in the brain, characterized by multi-stage and autonomous regulation.

The nuclei in the brain interact with each other either cooperatively or antagonistically to form a complex information network. As a result, the same nucleus may exert inhibitory and excitatory effects on gastric function. For instance, the DMV not only inhibits gastric motility, but also promotes gastric acid secretion. Hence, the role of a certain nucleus has not been specifically defined by scholars in the discussion of multiple formation mechanisms of SGML. Therefore, further research is needed to resolve this uncertainty.

In addition, the levels of nuclei involvement varied with the time of stress. At different time points (30, 60, 120 and 180 min) of RWIS in rats, the expression levels of c-Fos were markedly different in the DMV, NTS and NA, and the highest expression level was observed in the DMV, followed by NTS and NA. Moreover, the different sub-regions of the same nucleus also exhibit different levels of involvement[18]. Notably, the expression levels of c-Fos differed significantly in the hypothalamic SON, PVN, SCN, CEA and mPFC, and its expression intensities from high to low were as follows: SON, SCN and PVN at 30 min; SON, PVN, SCN and mPFC at 60 min; SON, SCN, CEA and PVN at 120 min; SON, PVN and CEA at 180 min[98]. Thus, the temporal-spatial relation of nuclear activity in RWIS-induced GML remains to be elucidated further.

SGML is the result of the combined action of the central and peripheral nervous mechanisms, which involves a wide variety of signaling molecules as well as a complex information network that leads to GML under RWIS. The signaling molecules, such as SP, have dual effects on gastric function. Therefore, these signaling molecules may not only promote or inhibit the gastric function, but also directly act on a single site in the stomach, which require more in-depth studies in the near future.

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MINIREVIEWS

Treatment of gastrointestinal bleeding in left ventricular assist devices: A comprehensive review

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Abstract

Left ventricular assist devices (LVAD) are increasingly become common as life prolonging therapy in patients with advanced heart failure. Current devices are now used as definitive treatment in some patients given the improved durability of continuous flow pumps. Unfortunately, continuous flow LVADs are fraught with complications such as gastrointestinal (GI) bleeding that are primarily attributed to the formation of arteriovenous malformations. With frequent GI bleeding, antiplatelet and anticoagulation therapies are usually discontinued increasing the risk of life-threatening events. Small bowel bleeds account for 15% as the source and patients often undergo multiple endoscopic procedures. Treatment strategies include resuscitative measures and endoscopic therapies. Medical treatment is with octreotide. Novel treatment options include thalidomide, angiotensin converting enzyme inhibitors/angiotensin II receptor blockers, estrogen-based hormonal therapies, doxycycline, desmopressin and bevacizumab. Current research has explored the mechanism of frequent GI bleeds in this population, including destruction of von Willebrand factor, upregulation of tissue factor, vascular endothelial growth factor, tumor necrosis factor-α, tumor growth factor-β, and angiopoetin-2, and downregulation of angiopoetin-1. In addition, healthcare resource utilization is only increasing in this patient population with higher admissions, readmissions, blood product utilization, and endoscopy. While some of the novel endoscopic and medical therapies for LVAD bleeds are still in their development stages, these tools will yet be crucial as the number of LVAD placements will likely only increase in the coming years.

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Core tip: Left ventricular assist devices are becoming increasingly common as life-prolonging therapy in advanced heart failure. However, left ventricular assist devices have shown high rates of gastrointestinal bleeding with 18%-40% of patients having episodes of bleed. Arteriovenous malformations are primarily responsible, which can be both challenging to control and cause many patients to discontinue essential anti-platelet and anti-coagulation therapies. Small bowel lesions are common in this population, frequently requiring small bowel endoscopic evaluation. For refractory cases, medical management is required including octreotide, thalidomide, angiotensin converting enzyme inhibitors/angiotensin II receptor blockers, estrogen-based therapies, desmopressin, doxycycline or bevacizumab to prevent further gastrointestinal bleeding.

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INTRODUCTION

Left Ventricular Assist Devices (LVAD) are becoming increasingly common for end-stage heart failure with 20000+ devices placed a year in over 180 hospitals around the United States and growing[1]. These devices are used either as a bridge to heart transplantation or as definitive, destination therapy, which owe their success in part to continuous flow technology which improves pump durability. Unfortunately, current devices appear to increase the incidence of gastrointestinal (GI) bleeding, which is seen in 18-40% of patients that have received an LVAD[2-7]. This is a major contributor to increased morbidity and mortality, where there is a 5-fold increased risk for readmission due to GI bleeding[8]. These episodes lead to recurrent hospitalizations, increased lengths of stay, costs, blood transfusions, and time off anticoagulation and antiplatelet therapy, which significantly increases the risk of pump thrombosis and stroke[8]. Furthermore, as many of these patients are being bridged to transplant, the increased number of blood transfusions may lead to higher antibody production (allosensitization), thus limiting the donor pool and prolonging the time to transplant. In this review we aim to summarize current literature addressing the management of GI bleeding in patients with LVADs.

ETIOLOGY AND PATHOPHYSIOLOGY

Among all GI bleeding in patients with LVADs, 47% of the episodes originated from the upper GI tract, 22% are from the lower GI tract, 15% are from the midgut (ligament of Treitz to the ileocecal valve), and nearly 19% remain unknown[5]. Arteriovenous malformations (AVM) account for 29%-44% of GI bleeding in this population, and mainly are found in the midgut[5,9]. These lesions can be notoriously hard to treat, as they are frequently multiple lesions throughout the GI tract and bleed intermittently leading to unremarkable endoscopic evaluations[5]. Other common lesions include gastritis (22%), peptic ulcer disease (13%), diverticular hemorrhage (6%), colonic polyps (5%), and colitis (4%), with the remaining causes unknown[10].

Both *in vitro* and *in vivo* studies have investigated the etiology of increased AVMs in LVAD patients, which has led to the development of current therapeutics. Biomarkers studied include von Willebrand factor (vWF), tumor growth factor-β (TGF-β), tissue factor (TF), vascular endothelial growth factor (VEGF), tumor necrosis factor-α (TNF-α), and angiopoietin 1 and 2. Von Willebrand factor (vWF) is significantly broken down in LVAD patients. According to Bartoli *et al*[11], there is a "2-hit hypothesis". The first hit includes the degradation of vWF causing acquired vWF deficiency (due to sheer stress creating protein unraveling, making vWF susceptible to

ADAMTS-13 breakdown), contributing to reduced interaction of vWF-platelet and vWF-collagen. The second hit involves these smaller vWF causing upregulation of angiogenesis and AVM formation in LVAD patients[11]. TNF-α has been reported to induce pericyte apoptosis, TF and angiopoietin-2 expression, and vascular instability leading to increased risk of AVM-related bleeds[12]. Likewise VEGF and TGF-β have been similarly upregulated in LVAD patients and implicated in these bleeds[3,5,12,13]. Angiopoietin-1, which normally is associated with vascular stability, is downregulated in patients with LVAD[3]. These factors affecting AVM formation and stability lead to an increased risk of GI bleeding and dictate many medical therapies that will be discussed in this review (Figure 1).

Acute GI bleeding management

Medical management: Gastrointestinal bleeding is a significant issue in LVAD patients leading to the discontinuation of antiplatelet and anticoagulation therapy. Initial management for acute GI bleeding include IV fluid resuscitation, electrolyte replacement, packed red blood cell transfusion to hemoglobin goals of 7-9 g/dL, and discontinuation of antiplatelet (acetylsalicylic acid (ASA) and P2Y12 inhibitors) and anticoagulation (Coumadin) medications[14-16]. After an acute episode, antiplatelet drugs may be restarted and anticoagulation may be rechallenged with either the same International Normalized Ratio (INR) goal (typically 1.5-3.5) or at a reduced goal[17,18]. Those patients with a high frequency of GI bleeding may have both antiplatelet and anticoagulation medications discontinued for an extended period of time, thus imparting significant risk for LVAD thrombosis.

While present day continuous flow devices have several advantages over the older pulsatile pumps, they have been implicated in the higher risk for the formation of AVMs and increased GI bleeding. It is thought that continuous flow LVADs lead to intestinal hypoperfusion, local hypoxia, vascular dilation, and AVM formation[19]. One technique that can be instituted to reduce GI bleeding is reducing the pump speed under ECHO guidance to increase pulsating flow while ensuring adequate LV off-loading[5]. One study looking at the factors of GI bleeds in LVAD patients found decreases in GI bleeding rates with only small decreases in pump speed (HeartMate II 9560 rpm *vs* 9490 rpm, $P < 0.001$; HeartWare 2949 rpm *vs* 2710 rpm, $P < 0.001$)[20].

Other conservative management strategies for active GI bleeding and prevention of future episodes include prophylactic proton pump inhibitor (PPI) use and reversal of anticoagulation. PPIs are often prescribed to LVAD patients, especially when they have had prior episodes of GI bleeding. However, the risk reduction of further GI bleeding is marginal as the majority of bleeding are from AVMs. PPI use was similar between those that did have GI bleeding versus those that did not in a study observing 101 LVAD patients ($P = 0.47$)[18].

Reversal of anticoagulation is typically done with a combination of vitamin K (oral or IV), fresh frozen plasma (FFP) and/or prothrombin complex concentrate (PCC). There is currently a lack of high-quality, prospective large-scale studies providing recommendations on specific regimens and dosings. However, one review article on anticoagulation reversal has suggested using IV vitamin K and PCC in hemodynamically significant bleeding[21]. Since FFPs require crossmatching, time for thawing, increased volume per unit administered in a fluid sensitive population, and have a higher risk of transfusion-related reactions, PCC is preferred for reversal of anticoagulation[21]. Ultimately, there remains high variability of dosages used throughout the literature, and differing indications for reversal of anticoagulation which often remains largely at the discretion of the treating provider. Following the reversal of anticoagulation, varying rates of thromboembolic events have been reported. While older studies utilizing recombinant activated factor VII at "high-doses" (30-70 μg/kg) revealed thromboembolic event rate of 36.7%[22], more recent literature quote rates of 2.9% (1 of 38) and suggest that larger doses of reversal agents can still have low rates of thromboembolism[4].

Endoscopic therapy: A treatment algorithm is shown in Figure 2. Endoscopic evaluation and treatment is frequently performed in LVAD patients given their high rates of GI bleeding. Most patients will receive an esophagogastroduodenoscopy (EGD) and/or colonoscopy on the initial presentation with concerns for GI bleeding. If these evaluations are unremarkable, the next step would be to consider a repeat EGD, given the high rates of upper GI bleeds, with possible push enteroscopy to explore small bowel sources, and repeat a colonoscopy if the initial colonoscopy prep was suboptimal[7,16].

Push enteroscopy allows for evaluation of the small bowel; most commonly to the proximal jejunum. Marsano *et al*[23] found that EGD had high rates of detection (45%), however many required repeat endoscopy at a rate of 1.8 endoscopes per patient in their study. On repeat endoscopy the majority underwent push enteroscopy with a

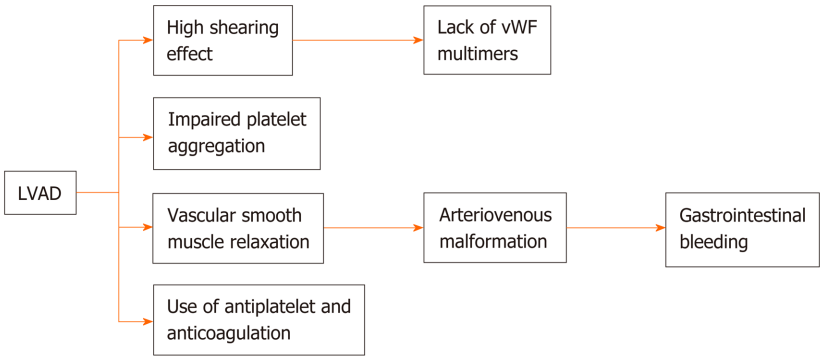

Figure 1 Pathophysiology of gastrointestinal bleeding in left ventricular assist device patients. LVAD: Left ventricular assist device; vWF: von Willebrand factor.

high small bowel detection rate of 80% in repeat bleeding episodes with 25% of their patients having jejunal lesions[23].

A significant portion of LVAD-related GI bleeding come from the midgut/unknown origins, which makes evaluation of the small bowel important in this population. While there may be some conflicting data that balloon (single or double) enteroscopy is superior, however, according to the American Society of Gastrointestinal Endoscopy (ASGE) guidelines, it is still recommended to proceed with video capsule endoscopy (VCE) as first line evaluation of the small bowel[15]. This is a safe technique with no electromagnetic interference to LVADs or implantable cardioverter-defibrillators (ICD) devices[24]. One study showed initial VCE had a diagnostic yield of 58.5% versus proceeding with double balloon enteroscopy first that had a yield of 41.5%[25]. Not only does VCE have a greater diagnostic yield, but it also is helpful in deciding the subsequent endoscopic approach based on the presumed location of the bleed. Further, there is an increased overall yield of 7% with double balloon enteroscopy if done after VCE[25].

Double balloon enteroscopy (DBE) allows clinicians to use a "push-and-pull" technique to visualize the small bowel. In the realm of obscure GI bleeding, the diagnostic yield of this technique was found to be 56% (95%CI: 48.9%-62.1%) in one large meta-analysis[26]. Specifically in LVAD patients with GI bleeding, the diagnostic yield with DBE was 69%[27]. In this study, 10 patients underwent 13 DBEs for 11 episodes of melena and 2 episodes of hematochezia. Mid-bowel lesions were found 56% of the times, while 33% of lesions were in the proximal bowel, and 11% were in both proximal and mid-bowel. The most common lesions identified were AVMs, consisting of 44% of the discovered lesions.

AVMs are often found in LVAD patients and are commonly treated with contact or non-contact thermal therapy [argon plasma coagulation (APC)] given the large, diffuse nature of these lesions[16]. One study showed that APC treatment of colonic AVMs could significantly decrease blood transfusions and increase overall hemoglobin in a 16-mo follow-up[28]. However, the upper/midgut predominance of AVMs in LVAD patients versus the classic right colonic location of AVMs in non-LVAD patient may change the nature of these AVMs. In addition, the tendency of increased anticoagulation in LVAD patients may cause APC to be less effective in LVAD patients, and may even provoke future bleeding episodes as APC can cause ulceration[29]. Clinicians should carefully consider the use of APC in LVAD patients, and may benefit from alternative methods to control bleed endoscopically or with the medical therapies discussed below. According to the European Society of Gastroenterology (ESGE), there is not enough evidence to prescribe treatment recommendations for AVM lesions at this time[14].

Apart from thermal therapy, other endoscopic treatment options for AVMs and other sources of GI bleeding found in LVAD patients include epinephrine injection, electrocautery, endoclips, band ligation, acrylic glue and hemostatic spray/powder[15,16,30,31]. With large bleeding ulcers, it is recommended to approach these lesions with "dual therapy", typically with epinephrine and another modality[14]. Hemostatic spray is currently not seen as "conventional" therapy and used in setting of "salvage" therapy[24,30]. One study looking at TC-325, hemostatic powder, showed a 90.7% primary hemostasis rate with a rebleeding rate of 26.1%. It was, however, found to be less effective in patients on anticoagulation, like LVAD patients, with a hemostasis rate of 63%[30].

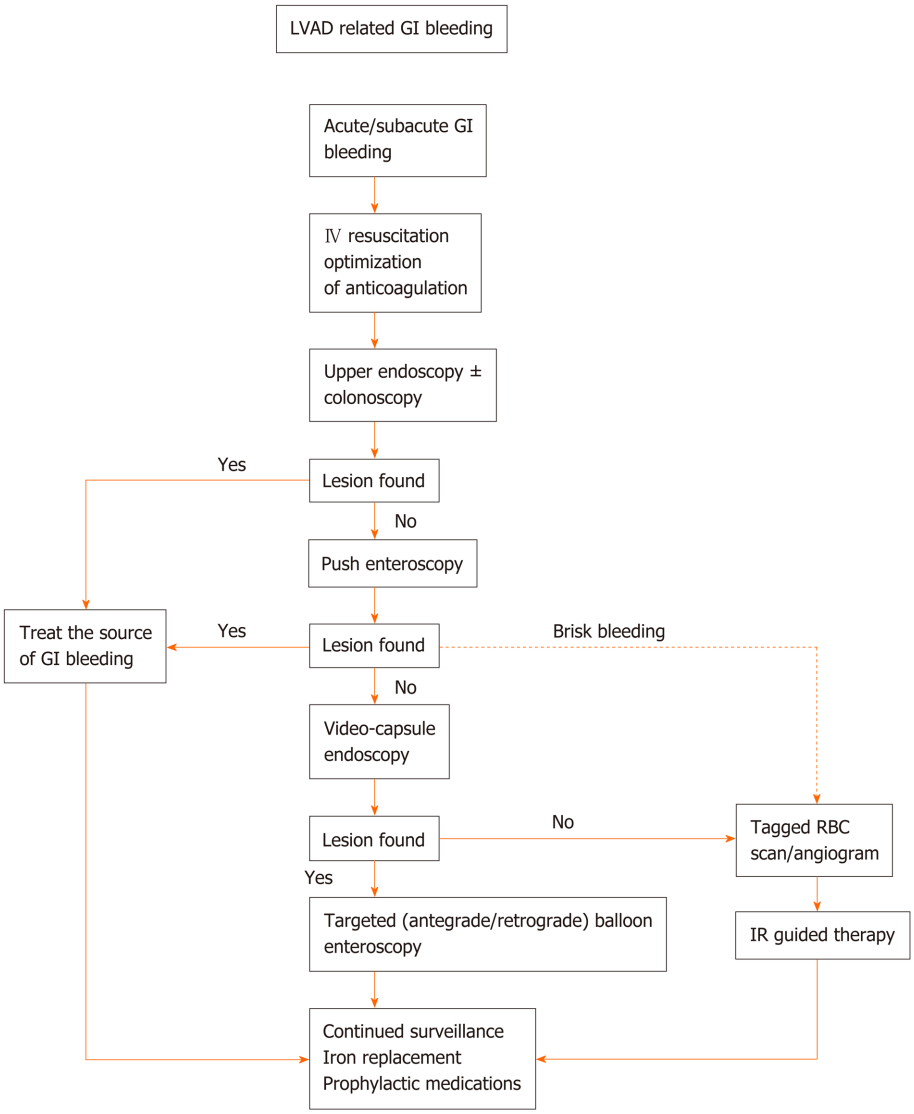

Figure 2 Treatment algorithm for left ventricular assist device-related gastrointestinal bleeding. Angiogram and tagged RBC scan are two common imaging modality to elucidate locations of bleed for further intervention. Iron deficiency is a common with chronic hemolysis in left ventricular assist device patients. Iron replacement can be initiated after checking iron studies. LVAD: Left ventricular assist device; GI: Gastrointestinal.

Prophylactic medications: Arteriovenous malformation targeted medical therapy

The diffuse nature AVM are responsible for many cases of bleeding/rebleeding in LVAD patients; up to 29%-44%. Endoscopy may be able to identify an acutely bleeding lesion, but one bleeding AVM lesion usually portends others along the GI tract that may bleed in the future, as well as the formation of new AVMs. Therefore, medical therapy targeted at the driving factors of these lesions have been used to reduce the episodes of rebleeding and ultimately decrease the number of hospitalizations, endoscopic procedures and blood transfusions.

Octreotide: After conservative medical therapy is tried and failed, a common first agent that is used to reduce recurrent GI bleeding in LVAD patients is octreotide, a somatostatin analogue. It is believed that octreotide reduces bleeding rates by decreasing portal venous pressures due to splenic vasodilation, enhancing platelet aggregation, and inhibiting of angiogenesis in the GI tract through downregulation of VEGF and suppression of digestive enzymes[5,32]. Octreotide is currently dosed 50 to 100 μg subcutaneous (SQ) twice daily (BID) or octreotide long-acting release (LAR) 20 to 30 mg intramuscularly (IM) monthly. A study observing the effects of octreotide LAR 20 mg IM monthly in LVAD patients showed a significant reduction of bleeding events in their 26 patient cohort (20 Heartmate II, 5 HVAD, and 1 HeartAssist). The average number of bleeding episodes pre-treatment was 3 ± 2.4 per year in this group with improvement to 0.7 ± 1.3 per year after initiation of monthly octreotide. 43% of the cohort was free of further bleeding episodes in follow-up[13]. Furthermore, AVM

lesions were found 44% of the times on endoscopy prior to octreotide therapy, but only 28% of the times afterwards.[13] Long acting 20 mg depot octreotide has also been studied in a prophylactic setting started within 1 mo of LVAD implant in a phase I trial of 10 patients that prevented any GI bleeding episodes within the first four months when otherwise typical rates are 39% within the first 2 mo after implantation[32,33].

Octreotide is seen currently as the first line agent, but does have some potential side effects that need to be discussed with patients. These side effects include diarrhea, abdominal pain, nausea, vomiting, gallstone formation, glucose abnormalities, pruritis, hypothyroidism, headaches and dizziness[34].

Thalidomide: Thalidomide has been used for several indications over the years since it was initially synthesized in the 1950s, including for morning sickness, sedation, and Kaposi sarcoma treatment. LVAD patients have benefited from thalidomide for its anti-angiogenesis effect through its ability to downregulate VEGF and basic fibroblastic growth factor (bFGF)[35]. This has been shown to reduce the rate of recurrent AVM-related bleeds. In a study that tested thalidomide 100 mg daily versus iron supplementation daily (control) in non-LVAD patient for four months showed a 50% or greater reduction in the rate of GI bleeding in 71.4% of patients on thalidomide versus only 3.7% in the control group over a one year follow-up. 46.4% of patients in thalidomide arm had complete cessation of further bleeds versus 0% in the control arm[35]. Recently, a 2019 study of 17 LVAD patients receiving thalidomide showed favorable results with a reduction of GI bleeding from an average 4.6 episodes per year to 0.4 episodes per year. Additionally, blood transfusions reduced from 36.1 to 0.9 units of transfusions per year. In this study 50 mg of thalidomide was used daily or twice daily with uptitration to 100 mg twice daily with bleeding episodes[6].

However, there are some notable shortcomings with thalidomide. Thalidomide has been shown to have a relatively high adverse event rate; ranging from 58-71%[6,36]. The more common adverse events encountered were dizziness, neuropathy, fatigue, constipation, transaminitis, and somnolence. There does seem to be dose-dependent response in adverse effects with 87.5% of patients improving with lower doses of thalidomide. Additionally, access and cost are both seemingly issues. Thalidomide is generally cost-prohibitive if not covered by insurance, and prior authorization is typically needed; delaying drug initiation. In Namdaran *et al*[6], bridging with octreotide was used on average for 11 d while approval was received and could be used as a workaround. Finally, both prescriber and pharmacist need to be approved by the THALOMID Risk Evaluation and Mitigation Strategy program to prescribe the thalidomide, which limit the number of providers that can offer this therapy.

Angiotensin converting enzyme inhibitors/angiotensin II receptor blockers: Angiotensin converting enzyme inhibitors (ACEi) and angiotensin II receptor blockers (ARBs) are common cardiac medications that appear to have a benefit in preventing recurrent GI bleeding in LVAD patients. Houston *et al*[3] looked at 131 LVAD patients and recorded whether or not they were on an ACEi or ARB for at least 30 days with LVAD support. This study found that of the 31 patients that did not receive an ACEi or an ARB, 48% had a GI bleeding episode with 29% of those being related to AVMs; while only 24% of those that received an ACEi or ARB had GI bleeding and only 9% were related to AVMs. Ultimately, an OR 0.29 for all causes of GI bleed, along with an OR 0.23 for AVM bleeds were found. It is thought the mechanism mainly centers around the downregulation of TGF-β, which normally upregulates VEGF. In addition, these agents create a blockade of angiotensin-II from acting upon angiotensin I receptors, causing a downregulation of the angiogenesis.

Their study also looked at dosing of these medications and failed to show a dose-dependence on its ability to reduce AVM-related bleeds[3]. The effect of ACEi/ARB on AVM lesions seem to be separate to its blood pressure effect as beta-blockers and calcium channel blockers independently tested in LVAD patients did not produce the same results in decreasing the number of GI bleeds that ACEi/ARBs were able to. Additional studies are needed to validate the effect of ACEi/ARBs in this population.

Estrogen-based hormone therapy: Hormone therapies, either estrogen-only or estrogen in combination with progesterone, have been studied in the prevention of AVM bleeds with conflicting results. One multicentered, randomized, controlled trial studied 72 non-LVAD patients that used ethinylestradiol 0.01 mg daily and norethisterone 2 mg daily versus placebo for at least a year. This trial did not show a significant difference between the two arms. Treatment failure rate was 39% *vs* 46% between hormone therapy and control, respectively. Likewise, the number of bleeding episodes, transfusion requirements, severe adverse events and mortality did not differ significantly between groups[37]. Contrarily, isolated case reports have evaluated

individual LVAD patients with remarkable results reducing frequent GI bleeds through the use of estrogen-based therapies[35,38]. Overall, the benefits of hormone therapy are theorized to be caused by improved vascular endothelium integrity and reduced bleeding times[5]. The literature available for hormone therapy in LVAD patients is very sparse. Furthermore, the concerns for thromboembolism with estrogen-based therapies will likely give pause to many practitioners in this already pro-thrombotic population without further large-scaled studies.

Doxycycline: Doxycycline is a relatively inexpensive medication that is widely used in both the short-term setting to treat pneumonia to chronic management in inflammatory acne. Bartoli *et al*[11] conducted an *in vitro* study looking at the prospect of using doxycycline to reduce LVAD bleeds by stabilizing ADAMTS-13[11]. High shear stress was added to human plasma samples with high doses of doxycycline (0.6 to 20 mg/dL). vWF degradation was greatly diminished with 10 out of the 11 degradation fragments reduced from baseline by 12% ± 2% when doxycycline was added. It was found that doxycycline reduced the plasma and recombinant forms of ADAMTS-13 activity in these suprashear stress environments. Additionally, vWF:collagen binding was increased with doxycycline and resulted in overall improved platelet aggregation without hyperactivating platelets to increase thrombosis formation. This showed promising *in vitro* results, but dosages of doxycycline that were effective ranged from 5-20 mg/dL, which far exceeds the 0.3 to 0.5 mg/dL most patients receive today. While this is below toxic limits of 2000 mg/kg in rat models, further testing will surely need to occur before this therapy becomes mainstream. To date, there is sparse other data on doxycycline use in LVAD patients.

Desmopressin: Desmopressin is a vasopressin analog that is currently approved for use in patients with either hemophilia A or mild-to-moderate von Willebrand disease (type I), and may also have its use in LVAD patients to reduce GI bleeding[39]. There are isolated case reports of achieving control of GI bleeding secondary to LVAD[2]. vWF multimers are in gross deficit in LVAD patients, as noted in this review. Desmopressin has the ability to increase the amount of vWF by 150%-200% at the 150 mcg dose[2]. Although, there are potential adverse effects of hyponatremia and fluid retention with desmopressin, which can be detrimental to patients with poor right-sided cardiac function and may require intensified oral diuretic regimens[2]. Overall, this medication still shows promise. Again the literature is sparse and larger studies are needed.

Bevacizumab: Preliminary results from a single institutional study show remarkable results from bevacizumab, a humanized monoclonal antibody against VEGF, in lowering the rate of GI bleeding in refractory cases[40]. This study evaluated 5 patients with LVADs and showed annual decreases in blood product usage from 45.8 to 6.0 units, reduced hospitalizations per year from 5.6 to 1.7 and an annual reduction in endoscopy from 10.6 to 2.3 procedures. Bevacizumab was well tolerated without side effects in the study participants. As with other novel agents, further studies are necessary.

CONCLUSION

GI bleeding is a frequent complication of LVAD placement due to the continuous flow LVAD devices causing dysregulation of angiogenesis-related biomarkers and sheer stress. Conservative methods such as reducing anticoagulation doses and discontinuing antiplatelet and anticoagulation therapies are unsafe and not successful in most instances. The AVMs are frequently located in the midgut and pose challenges with routine endoscopy. Video capsule endoscopy has a high diagnostic yield and should be performed after unremarkable EGD and colonoscopy in most cases. Afterwards, prompt push enteroscopy, or DBE is recommended to treat lesions before they stop bleeding, as well as to reduce the number of blood products transfused in LVAD patients. Octreotide is a commonly used medication in GI bleeding and is the first line agent for those with refractory bleeding. ACEi/ARB may have secondary benefits in reducing AVM-related bleeding. Some case studies indicate the role of estrogen-based hormone therapies, doxycycline, and desmopressin. Other medications with significant side effects need to be evaluated in larger studies, including thalidomide and bevacizumab. With the increase of durable LVAD placements each year, we will manage more frequent instances of GI bleeding, and it will be important to continue to refine diagnostic and therapeutic methods to treat and prevent these episodes.

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MINIREVIEWS

Role of γδT cells in liver diseases and its relationship with intestinal microbiota

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Abstract

γδT cells are unconventional T lymphocytes that bridge innate and adaptive immunity. Based on the composition of T cell receptor and the cytokines produced, γδT cells can be divided into diverse subsets that may be present at different locations, including the liver, epithelial layer of the gut, the dermis and so on. Many of these cells perform specific functions in liver diseases, such as viral hepatitis, autoimmune liver diseases, non-alcoholic fatty liver disease, liver cirrhosis and liver cancers. In this review, we discuss the distribution, subsets, functions of γδT cells and the relationship between the microbiota and γδT cells in common hepatic diseases. As γδT cells have been used to cure hematological and solid tumors, we are interested in γδT cell-based immunotherapies to treat liver diseases.

Key words: γδT cells; Liver diseases; Viral hepatitis; Autoimmune liver disease; Non-alcoholic fatty liver disease; Liver cirrhosis; Liver cancer; Intestinal microbiota

Core tip: γδT cells are unconventional T lymphocytes that bridge innate and adaptive immunity. These cells are enriched in epithelial layers of different tissues and the peripheral blood. They have been used in hematological and solid tumors for immunotherapies. The effects of γδT cells in liver diseases depend on subsets, mechanisms and different stages of diseases. We herein discuss the distribution, subsets, functions of γδT cells and the relationship between the microbiota and γδT cells in common hepatic diseases.

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INTRODUCTION

The liver receives approximately 1.5 L of blood from the portal vein and hepatic artery[1]. The liver is the major location of immune responses, metabolic activities and the clearance of toxins[2]. It acts as a sentinel, guarding against pathogen components such as lipopolysaccharide from the gut, and is enriched in innate immune cells, including Kupffer cells, natural killer (NK) cells and natural killer T (NKT) cells and adaptive immune cells, such as T lymphocytes, as well as the cytokines they secrete[3]. In dysregulated hepatic immune conditions, patients die from infections that occur in end-stage liver diseases, such as acute and chronic liver failure (ACLF), liver cirrhosis and liver cancer. Moreover, tumor cells escape immune surveillance, leading to metastasis. γδT cells account for a tiny proportion of immune cells in peripheral blood; however, they are abundant in the human liver. These cells are both protective and pathogenic in different liver diseases[4]. In recent years, γδT cells have attracted increasing attention. In this review, we will explore the properties and roles of γδT cells in the peripheral blood and liver in diverse hepatic diseases (Table 1).

γδT CELLS

γδT cells develop before other T cells in all vertebrates. They serve as neonatal protectors when the functions of αβT cells are impaired and act as antigen presenting cells[5]. γδT cells are 15%-25% of the T lymphocyte population and 3%-5% of total lymphocytes in the liver; however, the highest frequency of γδT cells is seen in the gut mucosa[6]. The frequency of γδT cells in the liver outnumbers that in the peripheral blood[1]. γδT cells are unconventional T lymphocytes with unique properties that bridge the innate and adaptive immunity. They express γδT cell receptor (TCR) and do not require antigen presentation with the help of major histocompatibility complex[7]. These cells recognize major histocompatibility complex class I chain-related antigens A and B (MICA and MICB) and nonpeptide metabolites of isoprenoid biosynthesis[7]. γδT cells are also activated by cytokines without TCR stimulation, which allows them to act earlier than αβT cells.

Human γδT cells can be divided into three groups according to their δ chain expression[7]. Approximately 56.4% of hepatic γδT cells express Vδ2+ chains, whereas approximately 8.9% of hepatic γδT cells express Vδ1+ chains[8]. The Vδ1+ chain usually combines with Vγ2,γ3,γ4,γ5 and γ8 chains[9], and the abovementioned subsets are mainly located in the epithelial layer of the gut, dermis, liver and spleen to maintain the integrity of epithelial tissue[9,10]. The CD1 family members are the ligands for Vδ1 T cells. Moreover, intestinal epithelial Vδ1 recognizes MICA or MICB through the TCR and NKG2D[9]. Vδ1 T cells usually proliferate during intracellular infections, fungal infections, viral infections and celiac disease[1]. Vδ2 T cells exclusively pair with the Vγ9 chain (also termed Vγ9Vδ2 T cells), which are mainly present in the peripheral blood and make up over 90% of peripheral circulating γδT cells[1] and 1%-5% of circulating T cells[7]. Vδ2 T cells usually expand during microbial infections[1]. Vδ2+ T cells can be divided into Vγ9+Vδ2+ and Vγ9-Vδ2+ subsets[11]. The ligands for Vγ9Vδ2 T cells are phosphoantigens on microbes and transformed cells. Intermediate metabolites of microbial isoprenoid biosynthesis, (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate, an isopentenyl pyrophosphate that is produced *via* the mevalonate pathway by transformed cells, activate these cells in a TCR-dependent manner. F1-ATPase expressed by tumor cells and butyrophilin3A1 are antigen-recognition molecules essential to Vγ9Vδ2 T cells activation[9]. Toll-like receptors (TLRs) and natural killer receptors coordinate with the TCR to stimulate Vγ9Vδ2 T cells. For example, pathogen-associated molecular patterns activate Vγ9Vδ2 T cells through TLRs and induce cytokines and chemokines. Vγ9Vδ2 T cells can also recognize MICA/MICB and UL-16 binding protein through NKG2D. DNAM-1, a kind of natural killer receptor that recognizes nectin-like-5, also participates in Vγ9Vδ2 T cells activation. Toxic shock syndrome toxin and staphylococcal enterotoxins are

Table 1 The roles of γδT cells in liver diseases

Liver diseases	Mechanisms of γδT cells	Functions
Viral hepatitis	Production of IFN-γ, TNF-α; Expression of CD107a; Downregulation of IL-17, IL-22	Amelioration
	Exhaustion of CD8+T cells; Production of IL-17 and TNF-α	Aggravation
Liver bacterial, fungal and parasitic infections	Production of IL-9, IL-17, TNF-α	Protection
Autoimmune liver diseases	Production of IL-10 and granzymes; Expression of CD39, CD73, NKG2D; Downregulation of NKT cells	Amelioration
	Expression of IL-17; Apoptosis of Tregs	Aggravation
Nonalcoholic fatty liver disease	Expression of IL-17	Aggravation
Liver fibrosis and cirrhosis	Expression of IL-17	Aggravation
	Apoptosis of HSC	Amelioration
Liver tumors	Expression of Ia and LFA-1, CD56 and CD161; Production of IFN-γ, perforin	Anti-tumor

IFN-γ: Interferon-γ; TNF-α: Tumor necrosis factor-α; IL-17: Interleukin-17; NKT: Natural killer T; HSC: Hepatic stellate cells.

superantigens that are involved in Vγ9Vδ2 T cell activation[9]. Zoledronate is used to stimulate Vγ9Vδ2 T cells as an immunotherapy against solid tumors and is receiving increasing attention[1]. Activated Vγ9Vδ2 T cells not only play an important role in cytotoxicity and promoting inflammatory processes, but also induce differentiation and maturation of innate immune cells *via* chemoattractant cytokine ligand 3 (CCL3), CCL4 and chemokine (C-X-C motif) ligand 10 (CXCL10)[1,12]. The third group of γδT cells are Vδ3 T cells, which are approximately 0.2% of circulating T cells. These cells are rich in the liver in healthy individuals and in patients with chronic viral infections, such as cytomegalovirus (CMV) and HIV, and leukemias[9]. Some Vδ3 T cells recognize glycolipids presented by CD1d[1].

Based on the diverse cytokines produced by γδT cells[6], they can be divided into different functional subsets through stimulation. Differentiation requires transcription factors such as T-bet and eomesodermin for interferon-γ (IFN-γ) expression and retinoic acid-related orphan receptor and Runx1 for interleukin-17 (IL-17) expression[13-15]. The IFN-γ-producing subset express the Vδ1 or Vγ9Vδ2 chains[6]. Qureshi *et al*[16] support the observations that γδT cells and NK cells are the producers of IFN-γ in the early immune response, which is followed by the cellular immune response. γδT cells also act as T regulatory cells (termed γδTreg cells). These cells inhibit peripheral blood mononuclear cell proliferation[9]. Approximately 70%-90% of γδT cells express CD27, and 10%-30% of γδT cells are $CD27^-$[15]. IL-17-secreting γδT cells, also called γδT17 cells, are mainly located in lymphoid organs and peripheral tissues[17,18]. γδT17 cells are $CD27^-$ but express C-C motif receptor 6 (CCR6) and CD25[15,19]. γδT17 cells play a pathogenic role in infection and autoimmune diseases. Scavenger receptor $SCART2^{high}$ γδT cells belong to a new subset of activated γδT17 cells[20] and appear under noninflammatory conditions.

γδT CELLS IN THE IMMUNE SYSTEM

As a kind of unique population, γδT cells act as a bridge between innate and adaptive immunity. Their roles in immune responses depend on many aspects, such as the existing locations, the stimuli used to activate and the period of responses[21]. Their pleiotropy, such as Th1 and Th2 phenotypes, is determined by specific stimuli and cytokines in the microenvironment, and is exhibited at different stages of immune responses[22,23]. Most of the γδT cells are Th1 phenotype. During the early stage of innate immune response, γδT cells sense the stressed epithelial cells or dendritic cells (DCs), then recruit innate cells, including neutrophils and macrophages, by producing IL-17 and CCL2, respectively[24]. During the middle stage of enhanced adaptive response, the interaction between γδT cells and DCs is intensive, leading to the proliferation and polarization of γδT cells and maturation of DCs[24,25]. γδT cells regulate B cells to produce a large number of immunoglobulins in the absence of αβT cells. In addition, human Vγ9Vδ2 T cells act as antigen presenting cells and present antigens to CD4+T cells and CD8+T cells, initiating adaptive responses[22,26]. Whereas, γδT cells play the opposite roles and kill macrophages and αβT cells and promote

tissue repair by producing IL-10 during the later stage[21,24].

γδT cells are also involved in antitumor immune responses. The activated cells exert cytotoxic effects by secreting perforin, granzymes, IFN-γ, tumor necrosis factor-α (TNF-α), *etc*. γδT cells influence DCs and αβT cells which regulate the immune responses in killing tumor cells. The combined regimens consisting of zoledronic acid, IL-2 and Vγ9Vδ2 T cells have shown promising effects in clinical trials[27].

γδT CELLS IN LIVER DISEASES

γδT cells in hepatitis B/C and other hepatic virus infections

Hepatitis B virus (HBV) infection is one of the major liver diseases in Asian people. Approximately 90% of infants with acute infection gradually develop chronic hepatitis B (CHB). Only about 5% of adults develop CHB, and the remaining adults are acutely infected[28]. Among HBV carriers, γδT cells prevented concanavalin A-induced hepatitis in HBV-transgenic mice, indicating that γδT cells can be used to ameliorate liver injury in HBV carriers[29]. Moreover, Jia *et al*[30] showed that the percentage of γδT cells in peripheral blood was low, whereas the percentage of intrahepatic γδT cells in the inflamed lobular area was high. The proportion of γδT cells in blood was inversely correlated with acute hepatitis B severity. Circulating γδT cells in acute hepatitis B with an activated memory TemRA phenotype exhibited enhanced cytotoxicity against HBV by producing IFN-γ and TNF-α[30]. A study showed that the number of γδT cells, especially Vδ2 T cells, in the liver and peripheral blood of patients with CHB infection decreased during disease progression and during pegylated IFN-α treatment. Vδ2 T cells have increased production of TNF-α and increased expression of CD107a which enhanced the cytotoxicity of these cells in the case of IFN-α therapy[31]. The number of Vδ2 T cells in immune-activated (IA) patients was lower than that in immune-tolerant or healthy subjects and was negatively correlated with disease severity. These cells downregulated IL-17 and IL-22 production in the microenvironment and ameliorated liver injury in IA patients with CHB[32]. Nevertheless, a previous study showed that in an HBV-carrier mouse model, liver γδT cells facilitated HBV-associated tolerance by indirectly inducing antiviral CD8+T cells exhaustion through myeloid-derived suppressor cells infiltration in the liver in an IL-17-dependent manner[33]. Moreover, inhibiting the NKG2A-HLA-E pathway-mediated CD8+T cells response prevented γδT cells from inhibiting HBV replication in CHB patients, while the frequency of γδT cells was negatively correlated with HBeAg seroconversion[32,34]. In addition, Chen *et al*[35] showed that γδT cells in the blood of HBV-ACLF patients that produced higher amounts of IL-17 and TNF-α were significantly decreased compared to those of patients with CHB and healthy controls. This finding indicated that γδT cells may take part in the pathogenesis of HBV-ACLF due to their inflammatory and cytotoxic properties. Thus, the functions of γδT cells during HBV infection depend on the cell subsets and different stages of disease.

With regard to hepatitis C virus (HCV) infection, approximately 75%-80% of adults who are acutely infected develop chronic hepatitis C infection[36]. γδT cells, especially Vδ1 T cells with an effector phenotype, accumulated in the liver of patients with chronic HCV infection and HIV/HCV-co-infected patients. These Vδ1 T cells that originated from the peripheral blood homed to the HCV-infected liver, showed a Th1-cytokine-secreting pattern and led to liver necroinflammation[10,37,38]. Whereas, Vγ9Vδ2 T cells were decreased in the peripheral blood of these patients[39]. Tseng *et al*[37] showed that γδT cells from these patients can be stimulated and produced IFN-γ and TNF-α by a cytokine cocktail *in vitro* and are highly cytotoxic to primary hepatocytes, suggesting a pathogenic role for γδT cells in HCV infection. Moreover, γδT cells isolated from liver tissue with viral infection expanded exclusively in the liver but not in peripheral blood[37]. Therefore, γδT cells display pathogenic function in HCV-infected individuals.

Lu *et al*[40] demonstrated that liver TCR γδ+ CD4-CD8- (double negative, DN) T cells with an activated phenotype of CD25-CD28-CD69+ were markedly increased in murine hepatitis virus strain 3 infection and were activated to produce TNF-α, IFN-γ, IL-17A and IL-2. These cells were cytotoxic to murine hepatitis virus strain 3-infected hepatocytes *via* the TNF-α pathway, indicating the critical role of TCR γδ+DN T cells in viral clearance. Ajuebor *et al*[41] showed that γδT cells accelerated acute liver injury, which was infected with adenovirus expressing the *Escherichia coli LacZ* gene in a CXCL9-CXCR3-dependent mechanism. The reduced level of IFN-γ and CXCL9 due to the lack of γδT cells and hepatocytes, respectively, may contribute to alleviation of liver injury.

γδT cells in liver bacterial, fungal and parasitic infections

γδT cells were the main producers of IL-17 during *Schistosoma japonicum* infection in the liver, and they were the first line of defense before T helper cell 17 (Th17) reacted. IL-17 contributed to granulomatous inflammation and fibrosis, which were reduced by an anti-IL-17 monoclonal antibody[42]. Li *et al*[43] showed that IL-9-producing γδT cells played a part in *Schistosoma japonicum* infection in C57BL/6 mouse. Mice that were deficient in γδT cells and infected with *Listeria monocytogenes* developed liver injury due to TNF-α produced by CD8+T cells. This liver pathology was reversed by infusing Vδ4+ γδT cells that secrete IL-10, controlling the proliferation of CD8+ T cells and reducing TNF-α production. Thus, γδT cells protected liver tissue by regulating pathogen-stimulated CD8+ T cells. γδT cells maintained CD8+ T cell homeostasis[44]. A study showed that γδT cells were important early in infections, whereas αβT cells played a part later in infection. IL-17 produced by Vδ4+ γδT cells protected against infection. In parasitic infections, γδT cells were also protective.

γδT cells in autoimmune liver diseases

Autoimmune liver diseases are chronic liver diseases caused by immune dysfunction. They consist of autoimmune hepatitis (AIH), primary biliary cholangitis, primary sclerosing cholangitis (PSC) and overlap syndrome. AIH is characterized by interface hepatitis, increased transaminase and immunoglobulin G and various autoantibodies[45]. Primary biliary cholangitis is characterized by destruction of intrahepatic bile ducts and cholestasis. Without proper treatment it will advance to end-stage liver disease[46]. PSC is distinguished by biliary inflammation and fibrosis. The most common symptoms are hepatomegaly and splenomegaly[47]. γδT cells have been shown to play immunoregulatory roles in different studies[48], such as in a mouse model of adriamycin-induced nephropathy[49], and in pulmonary fibrosis[50]. The number of γδT cells was markedly increased in the blood and portal vein and bile duct proliferation areas in patients with PSC and AIH[51]. Peripheral Vδ1 T cells that produce IFN-γ and granzyme B were the main subtype, whereas Vδ2 T cells were low in AIH patients. In a scurfy transfer model, $CD62L^{lo}CD44^{hi}$ γδT cells in $TCR\alpha^{-/-}$ mice prevented multi-organ autoimmune diseases before transfer. These cells produced the immunosuppressive cytokine IL-10 and granzymes, and highly expressed CD39 and CD73, which resembled the pattern of ectoenzyme-mediated degradation of ATP to adenosine that was seen in Foxp3+ Tregs cells[52]. γδT cells also exerted suppressive functions *via* NKG2D, which is expressed by NK cells, NKT cells and γδT cells. γδT17 cells acted as protectors in autoimmune liver diseases. IL-17A produced by Vγ4+ γδT cells prevented concanavalin A-induced fulminant hepatitis by downregulating NKT cells and IFN-γ production[6]. However, γδT17 cells also played an opposite role in the immunopathology of liver diseases[1]. IL-17 produced by these cells, rather than Th17 cells, contributed to hepatic inflammation in a mouse model of biliary atresia (BA) and in BA patients[5]. The pathogenesis of BA is not fully understood; however, experiments show that the autoimmune process may be involved. Neutralization of IL-17 alleviated the severity of liver inflammation in BA and ultimately protects against liver fibrosis[5]. γδT cells also induced Treg cell apoptosis and reversed Treg cell functions to promote effector T cells activities and lead to autoimmune liver diseases[48]. The functions of γδT cells vary from situation to situation. These cells serve as promising immunotherapies in clinical trials for cancer, and whether γδT cells can be used in autoimmune liver disease treatment remains to be elucidated.

γδT cells in non-alcoholic fatty liver disease

Nonalcoholic fatty liver disease (NAFLD) is one of the metabolic diseases which includes different stages, ranging from hepatic steatosis to steatohepatitis. NAFLD results from multiple factors, and hepatic γδT17 cells that exacerbate steatohepatitis are one of the important mechanisms[53,54]. The γδT cells recruited to the liver were mainly γδT17 cells, which aggravated NAFLD by regulating CD4+T cells[55]. Although He *et al*[56] only mentioned that the expression of IL-17 by Th17 cells declined, hepatic γδT cells also decreased in lentiviral vectors encoding pre-Mir-26a-treated mice after high fat diet feeding, suggesting an improvement in NAFLD. We hypothesize that IL-17 secreted by γδT cells may also play a part in the pathogenesis of NAFLD. Therefore, γδT17 cells promote NAFLD progression.

γδT cells in liver fibrosis and cirrhosis

Liver cirrhosis is the outcome of many chronic liver diseases, such as HBV infection in Asia and HCV infection and alcohol abuse in developed countries. The process involves necroinflammation, activation and accumulation of hepatic stellate cells (HSC) and fibrosis[57]. γδT cells are a primary cell type found in the portal area of liver cirrhosis patients[58]. These cells produce IL-17, which facilitated fibrosis progression by activating HSC and Kupffer cells[59,60]. In mice infected with *Schistosoma japonicum*,

Zheng *et al*[61] found that γδT cells recruited neutrophils to the liver and caused liver fibrosis by producing IL-17A. Wang *et al*[62] also demonstrated that the HMGB1-TLR4-IL23-IL17 axis between macrophages and γδT cells exacerbated liver inflammation. In the presence of IL-23 and IL-1β, the interaction between exosomes and TLR3 in HSCs promoted increased production of IL-17A by γδT cells, which activated HSCs and exacerbated liver fibrosis at an early stage[63]. Ni *et al*[64] argued that enhanced expression of IL-17A may occur directly from exosomes. Moreover, TLR4 but not TLR3 is expressed in HSCs. A previous study showed that TLR3 is protective against liver fibrosis[65]. However, another study showed that CCR6-expressing γδT17 cells in the injured liver hamper liver inflammation and fibrosis by the induction of HSC apoptosis through Fas/Fas-ligand (FasL) interactions[66]. In addition, γδT cells, especially IFN-γ-producing γδT cells, also exert protective functions against liver fibrosis by killing activated HSCs in a NKp46-, TRAIL- and Fas-ligand-dependent manner directly or indirectly by promoting NK cell-associated cytotoxicity against HSCs[67]. Therefore, the opposite effects of γδT cells on liver fibrosis and cirrhosis are associated with the underlying mechanisms.

γδT cells in liver tumors

Liver cancers are one of the most common cancers worldwide. Many chronic liver diseases, such as HBV and HCV infection, lead to the pathogenesis of liver cancers[68]. Viey *et al*[69] showed that γδT cells had the ability to infiltrate tumors. Patients with hepatocellular carcinoma (HCC) had an increased number of CCR2-expressing Vδ1+ γδT cells in the liver[8,70]. γδT cells expressed lymphocyte-activation markers Ia and LFA-1, indicating an activated status in hepatic tumor-bearing subjects[71]. The expression of CD56 and CD161 also increased, revealing cytotoxicity against hepatic tumors[72]. γδT cells lysed hepatoma cells and markedly reduced hepatic tumor cells activity *in vitro*[73]. Vδ1+ γδT cells produced IFN-γ and exerted cytotoxic effects[74]. CMV-stimulated Vδ1+ γδT cells inhibited primary HT-29 colonic cancer and metastatic foci such as in the liver compared to those of control mice[75,76]. Activated ($CD44^{high}$) Vγ4+ γδT cells also participated in tumor immune surveillance by secreting increased IFN-γ and perforin in $TCR\delta^{-/-}$ mice due to the high level of eomesodermin in Vγ4+ γδT cells, suggesting a protective role in the tumor immune response[77]. Thus, Vγ4+ γδT cells might be a novel therapy in liver diseases[78]. Recently, it was demonstrated that the ratio of peritumoral HSC to γδT cells can be a valuable predictor of the prognosis of HCC after resection and was always positively correlated with tumor progression. In this study, γδT cells inhibited the behavior of progressive HCC[79]. Another study showed that in the HCC group who sequentially used radiofrequency ablation/cellular immunotherapy (CIT), the outcome was efficient and safe compared to that of the group using radiofrequency ablation alone; specifically, NK cells and γδT cells had robust cytotoxicity and may prevent the recurrence of HCC[80]. Qian *et al*[81] found that the combination of cellular immunotherapy not only favored the progression-free survival of HCV-positive HCC patients but also affected long-lasting viral control. Bispecific antibodies such as MT110 boosted the antitumoral effect of γδT cells. Gustafsson *et al*[82] reasonably hypothesized that the presentation of γδT cells and TAA to CD8+ cytotoxic T cells may lead to enhanced killing of tumor cells with the help of activated CD4+ helper T cells. Therefore, γδT cells play an antitumor role in liver cancers[6,83].

RELATIONSHIPS BETWEEN LIVER/INTESTINAL γδT CELLS AND INTESTINAL MICROBIOTA

The microbiota plays an important role in maintaining hepatic γδT17 cells homeostasis. The mechanism underlying the abovementioned phenomenon could be attributed to lipid antigens, components of intestinal microbiota, that were presented by hepatocyte CD1d *via* the portal vein, which activated hepatic γδT cells and produced IL-17A. Activated γδT17 cells have been identified to have pro-inflammation and anti-infection abilities, aggravating liver disease[84,85]. For example, the quantity of microbiota affected γδT17 cells in the liver, thus accelerating the development of NAFLD[2]. In addition, during cholestatic liver diseases the increased intestinal permeability allowed bacterial translocation to the liver, especially *Lactobacillus gasseri*. Accordingly, hepatic γδT cells responded to the microbial stimulus to secrete IL-17A, exacerbating the cholestatic liver diseases[86]. Hepatic γδT17 cells showed an active and mature status by expressing $CD44^{high}CD62L^{-}$. A study performed on lung cancer demonstrated that the number of γδT cells in the liver decreased in the absence of commensal microbiota. Li *et al*[2] demonstrated that *Escherichia coli* alone could restore hepatic γδT17 cells in a dose-dependent manner.

Moreover, supplementation of γδT cells and IL-17A restored immune surveillance in antibiotic-treated mice[87].

γδT cells make up 10%-30% of CD3+ T cells in the intestine of healthy subjects[11]. γδT cells are important in maintaining homeostasis of the intestinal barrier in order to kill pathogens and prevent bacteria from translocating to the liver. The commensal microbiota residing on the epithelial surface of the gastrointestinal tract modulated intestinal mucosal γδT cells[88]. These microbiota induced-γδT cells exert their roles in many pathological processes in the initial stage or in the later stage[85]. For example, IL-17A produced by γδT cells affected intestinal immunity. In addition, some studies suggested that IL-17A was protective and maintained barrier functions by regulating the tight junction protein occludin in a DSS-induced colitis model[89]. A recent study suggested that *Lactobacillus breves* DM9218 directly stimulated γδT17 cells by expressing TLR2 in the colon, leading to beneficial effects on colitis[88]. Another study also suggested that the bacterial consortium markedly stimulated the proliferation of γδT17 cells in the colonic lamina propria. Specific beneficial microorganisms, such as *Bifidobacterium* and *Bacillus spp*, promoted TLR2 expression on γδT cells, which led to enhancement of barrier functions[90]. *In vitro*, only the *Bacillus* strains but not *Bifidobacterium* promoted TLR2 and IL-17 expression. However, bacteria constituting the families *Prevotellaceae*, *Rhodospirillaceae*, and *Flavobacteriaceae* were inversely related to γδT17 cells in the intestine. Bacteria in the family *Bifidobacteriaceae* were positively correlated with γδT17 cells[90]. The communication between intestinal epithelial cells (IEC) and γδ intraepithelial lymphocytes (IEL) relied on microbiota and served to maintain homeostasis of intestinal immunity. γδIEL depended on IL-15 produced by IEC which were stimulated by microbiota to sustain their presence and functions. In turn, γδIEL promoted IEC functions, such as maintenance of the epithelial barrier and lysis of invasive pathogens. Moreover, microbial localization impacted the biological behaviors of γδIEL, for instance, enhanced cytotoxicity against deleterious bacteria[85]. Microbiota was also involved in the relationship between γδT cells and cutaneous carcinogenesis. Microbial infection resulted in Vδ2-γδT cells residing in tissue epithelial layers. The Vδ2-γδT cells proliferated and secreted IFN-γ upon encountering antigens, leading to cancer cell death. On the other hand, the anti-tumor subset could be transformed into the pro-tumor subset with the help of IL-23. Due to a larger intercellular space and subsequent bacterial translocation, γδT17 cells expanded, causing tumorigenesis. IL-17 also inhibited effector T cells through myeloid-derived suppressor cells indirectly[85].

CONCLUSION

In brief, the effects of γδT cells in liver disease depend on subsets, mechanisms and different stages of diseases. γδT cells not only show cytotoxicity against viral hepatitis but also exacerbate CHB and HBV-ACLF. These cells also play an antitumor role in liver cancers. This minority of cells is mainly used to treat hematological and solid tumors through the production of IFN-γ, TNF, cytotoxic granules, as well as the functions of γδT17 cells. γδT cells that function as carriers for chimeric antigen receptors are being explored due to their reduced risk of side effects. T cells that are engineered with defined γδTCRs are outstanding in cancer treatment. The functions of γδT cells have also been important in various virus infections, especially CMV. We should emphasize their beneficial roles and engineer this type of immune cell in adoptive immunotherapies, such as targeting specific receptors and expanding cytotoxic anticancer immune cells, and then apply these immune treatments not only for liver diseases but also other systemic diseases.

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ORIGINAL ARTICLE

Basic Study

Serum outperforms plasma in small extracellular vesicle microRNA biomarker studies of adenocarcinoma of the esophagus

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Abstract

BACKGROUND

Circulating microRNAs (miRNAs) are potential biomarkers for many diseases. However, they can originate from non-disease specific sources, such as blood cells, and compromise the investigations for miRNA biomarkers. While small extracellular vesicles (sEVs) have been suggested to provide a purer source of circulating miRNAs for biomarkers discovery, the most suitable blood sample for sEV miRNA biomarker studies has not been defined.

AIM

To compare the miRNA profiles between matched serum and plasma sEV preparations to determine their suitability for biomarker studies.

METHODS

Matched serum and plasma samples were obtained from 10 healthy controls and 10 patients with esophageal adenocarcinoma. sEV isolates were prepared from serum and plasma using ExoQuick™ and quantified using NanoSight. RNA was extracted from sEV preparations with the miRNeasy Serum/Plasma kit and profiled using the Taqman Openarray qPCR. The overall miRNA content and the

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expression of specific miRNAs of reported vesicular and non-vesicular origins were compared between serum and plasma sEV preparations. The diagnostic performance of a previously identified multi-miRNA biomarker panel for esophageal adenocarcinoma was also compared.

RESULTS

The overall miRNA content was higher in plasma sEV preparations (480 miRNAs) and contained 97.5% of the miRNAs found in the serum sEV preparations (412 miRNAs).The expression of commonly expressed miRNAs was highly correlated (Spearman's R = 0.87, $P <$ 0.0001) between the plasma and serum sEV preparations, but was consistently higher in the plasma sEV preparations. Specific blood-cell miRNAs (hsa-miR-223-3p, hsa-miR-451a, miR-19b-3p, hsa-miR-17-5p, hsa-miR-30b-5p, hsa-miR-106a-5p, hsa-miR-150-5p and hsa-miR-92a-3p) were expressed at 2.7 to 9.6 fold higher levels in the plasma sEV preparations compared to serum sEV preparations ($P < 0.05$). In plasma sEV preparations, the percentage of protein-associated miRNAs expressed at relatively higher levels (Ct 20-25) was greater than serum sEV preparations (50% *vs* 31%). While the percentage of vesicle-associated miRNAs expressed at relatively higher levels was greater in the serum sEV preparations than plasma sEV preparations (70% *vs* 44%). A 5-miRNA biomarker panel produced a higher cross validated accuracy for discriminating patients with esophageal adenocarcinoma from healthy controls using serum sEV preparations compared with plasma sEV preparations (AUROC 0.80 *vs* 0.54, $P < 0.05$).

CONCLUSION

Although plasma sEV preparations contained more miRNAs than serum sEV preparations, they also contained more miRNAs from non-vesicle origins. Serum appears to be more suitable than plasma for sEV miRNAs biomarkers studies.

Key words: Biomarkers; Exosomes; Extracellular vesicles; Circulating microRNA; MicroRNAs; Plasma; Serum; Blood cells; Real-time polymerase chain reaction; Adenocarcinoma of esophagus

Core tip: Current evidence suggests that circulating small extracellular vesicles (sEVs) function as delivery cargo shuttles for various molecules. MicroRNAs are small non-coding RNAs with important roles in the regulation of gene expression, are often dysregulated in diseases, and are relatively stable in the circulation. MicroRNAs circulating in sEVs are consequently considered as highly suitable candidates for use as non-invasive biomarkers. Extracellular vesicle preparations derived from serum and plasma are recognised to be enriched in sEVs, but not purely comprised of them. Most circulating sEV microRNA biomarker studies have used plasma, but here we show that sEVs isolated from serum are less contaminated with blood cell and protein-associated microRNAs.

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INTRODUCTION

MicroRNAs (miRNAs) are small non-coding RNA molecules (21-23 nucleotides) that can regulate gene expression *via* various mechanisms including repression of messenger RNA translation. miRNAs are important regulators because a single miRNA can target multiple genes. Furthermore, specific miRNA expression signatures have been shown to be tissue-specific[1], and disease-specific[2-4]. miRNAs

are found in a range of body fluids such as serum, plasma, whole blood, urine and saliva. These circulating miRNAs are highly stable in different conditions (*e.g.*, temperature, pH and storage period) and can be easily measured. For these reasons, circulating miRNAs have garnered significant research interest as potential biomarkers for diagnostic, prognostic and treatment prediction purposes.

However, there are many challenges in the process of biomarker discovery to clinical practice for circulating miRNAs. Various factors can influence the quality and outcome of biomarker studies, which include the choice of sample, processing conditions, biomarker detection and analysis methods[5,6]. There is also increasing awareness about the multiple origins of specific circulating miRNAs and the implications this has on how we should evaluate and interpret miRNAs biomarkers studies[7-10]. A study by Pritchard *et al*[8] highlighted that a large proportion of circulating miRNA cancer biomarkers identified in the literature overlapped with those that have been reported to be highly expressed in blood cells. This has raised concerns on factors such as hemolysis and whether different types of blood samples used in miRNA studies may vary in their content of miRNAs originating from blood-cells.

Small extracellular microvesicles (sEVs), are considered to be a more stable and disease-specific source of circulating miRNAs for biomarker development[11]. In cancers, circulating miRNAs encapsulated in sEVs have been shown to have critical functional roles such as regulating disease progression, metastasis and sensitivity to specific drugs[12]. Circulating miRNAs can also be found complexed with the Argonaute2 (Ago2) protein, which functions to protect the miRNAs against RNases and enhance their stability in the circulation[13,14]. Although these protein-associated circulating miRNAs have been found to be present in larger quantities than sEV miRNAs, their functional roles in disease pathogenesis and potential utility as biomarkers have not been investigated. Thus far, the focus remains on circulating sEV miRNAs as preferred candidates for biomarkers development and protocol-related studies[11,15-17].

A crucial step in the development of robust circulating miRNAs biomarkers is to determine which blood sample is optimum for the study. This is a challenging question to address due to the multiple origins of circulating miRNAs and experimental factors that can influence miRNA levels. Previous studies have endeavoured to address this question by comparing circulating miRNA profiles, mostly of cell-free miRNAs across different blood samples, and have reported inconsistent results[10,11,16,18,19]. There are only limited studies that have comprehensively investigated and reported sEV miRNAs profiles between different blood samples[11,13]. In this study, we compared miRNA profiles between matched serum and plasma sEV preparations, collected from healthy controls and patients with esophageal adenocarcinoma, for the presence of reported specific vesicular and non-vesicular miRNAs. We also compared the performance of a previously identified multi-biomarker panel (comprising of 5 sEV miRNA ratios)[20], between serum and plasma sEV preparations, to discriminate patients with esophageal adenocarcinoma from the healthy individuals.

MATERIALS AND METHODS

Patient recruitment and sample collection

Individuals visiting Flinders Medical Centre (Adelaide, South Australia) and the Royal Adelaide Hospital (Adelaide, South Australia) for endoscopy procedures and management of esophageal cancer were recruited for a biomarker research study. Ethical approval was obtained from the Southern Adelaide Clinical Human Research Ethics Committee and the Royal Adelaide Hospital Research Committee. All individuals provided written informed consent for blood and personal data collection for research purposes. The study was conducted in accordance with the Declaration of Helsinki's (2008) statement for the ethical principles for medical research involving human subjects.

Blood samples from 10 healthy controls (median age 56.5 ± 10) and 10 patients with locally advanced esophageal adenocarcinoma (median age 59.5 ± 7) were used. The individuals were previously part of a larger biomarker study for esophageal adenocarcinoma[20]. The "healthy controls" all underwent endoscopy with biopsies and were not identified as having Barrett's esophagus, gastroesophageal reflux disease, or cancer. Only individuals with no endoscopic or histological abnormality were included in the control group. Matched serum and plasma samples from each individual was collected at the same time prior to their endoscopy procedure. Blood was collected from the patients with cancer prior to any treatment. Collection was

performed with 8 mL Z Serum Separator Clot Activator tubes Vacuette® (cat# 455078) and 9 mL K3E K3EDTA tubes Vacuette® (cat# 455036) respectively.

Blood processing and extracellular vesicle isolation

All blood samples were left at room temperature for a period of 16-24 h before processing with a standardised protocol established in our laboratory. Serum was collected *via* centrifugation of blood at 650 *g* for 15 min and stored as 1 mL aliquots at -80 °C for later use. Plasma was collected *via* centrifugation at 650 *g* for 15 min to separate the plasma supernatant from the red blood cells and buffy coat containing white blood cells. The top clear layer of plasma supernatant was transferred to a fresh 10 mL tube (Techno-Plas Pty Ltd., Australia; cat# S9716-V06) for a second centrifugation at 650 *g* for 15 min and the supernatant was stored as 1 mL aliquots at -80 °C for later use.

For extracellular vesicle isolation, aliquots (1 mL) of the matched serum and plasma from the 10 healthy controls and 10 patients with esophageal adenocarcinoma were retrieved from -80 °C and quick thawed. The aliquots were centrifuged at 16000 *g* at 4 °C for 30 min to exclude large microparticles. Two hundred and fifty microliter supernatants from each sample was processed with the ExoQuick™ kit (System Biosciences, CA, United States; EXOQ20A-1) according to the manufacturer's protocol. All samples were incubated with ExoQuick™ at 4 °C for 16 h. The extracellular vesicle pellet isolated from each sample was resuspended with 50 μL phosphate buffered saline.

Size distribution and quantification of extracellular vesicles

The size and concentration of extracellular vesicles isolated from each sample was measured using a NanoSight LM10 Nanoparticle Analysis System and Nanoparticle Tracking Analysis Software (NanoSight Ltd., Malvern, United Kingdom). One microliter of vesicle suspension was serially diluted in pre-filtered phosphate buffered saline to a dilution factor of 1:3200 for the NanoSight measurement. This dilution factor was determined in the laboratory to achieve an average particle concentration range of 10^8-10^9/mL for our samples, which is the optimal measurement range recommended by the manufacturer's protocol. The diluted sample was injected into the NanoSight instrument sample inlet port and a 60 s video were captured for measurement. The measurements were performed in triplicate for each diluted sample by re-injecting the same sample into the sample inlet port. Average particle size and concentration for each sample was evaluated using the batch-processing settings within the NTA software.

Extracellular vesicle miRNA extraction and profiling

The miRNeasy Serum/Plasma kit (QIAGEN, #217184) was used according to the manufacturer's protocol. After the addition of 500 μL QIAzol Lysis reagent to each vesicle pellet, 5 μL (0.1 picomole) of each of the synthetic RNA molecules ath-miR-159a and cel-miR-54 were added (Shanghai Genepharma Co. Ltd.). The final RNA elution from each sample was performed with 24 μL of RNase-free ultrapure water.

The Taqman® OpenArray® Human microRNA panel (Life technologies, #4461104) was used to profile the expression of 758 miRNAs. The detailed steps for the miRNA profiling were as previously described[20]. The profiling was performed using the Biotrove OpenArray NT cycler at the Flinders Genomics Facility (Flinders University, South Australia). The Realtime PCR Statminer® software (v4.5, Integromics) was used to assess the miRNA expression as cycle threshold (Ct) value per assay. The relative miRNA expression was calculated as $2^{(40-Ct)}$. The data has been submitted to the Gene Expression Omnibus website (GSE142855).

Statistical analysis

Wilcoxon signed-rank test was used to investigate the pairwise differences between the matched serum and plasma samples of individual. This included comparisons on the particle concentrations, number of miRNAs detected and relative expression of specific miRNA. Correlation was assessed using the Spearman's rank correlation coefficient. The diagnostic accuracy of a previously identified 5-miRNA ratio panel[20] was determined using leave-one-out cross-validation and receiver-operating characteristics (ROC) curve analysis. Statistical significance was defined by a P value < 0.05. Statistical analyses were performed using Stata software version 13.1 (StataCorp, College station, TX, United States) and IBM® SPSS® Statistics software version 25.

RESULTS

Particle yield

The Nanosight system was used to compare the profiles of particles isolated from the matched serum and plasma of healthy individuals. The main population of particles isolated from serum and plasma were similar in size, at 97.7 ± 3.3 nm and 93.1 ± 3.1 nm respectively (Figure 1A). The range of particle sizes detected in the samples, including those from the cancer patients (Supplementary Figure 1A), were consistent with the reported sizes of exosomes (30-150 nm)[16,21,22]. To be consistent with the Minimal Information for Studies of Extracellular Vesicles 2018 guidelines, we refer here to the majority particle population in the preparations as "small extracellular vesicles (sEVs)" , while noting that a minor population of "medium-large extracellular vesicles (m/lEVs)" were also detected[23]. The average concentration of particles from healthy controls was 1.2-fold higher in the serum sEV preparations compared to the matched plasma sEV preparations (Wilcoxon signed-rank test, $P = 0.047$) (Figure 1B). However, there was no statistical difference in the yield of particles in the matched serum sEV preparations and plasma sEV preparations from the cancer patients (Wilcoxon signed-rank test, $P = 0.56$) (Supplementary Figure 1B).

miRNA content in serum and plasma sEV preparations

The number of miRNAs detected was greater in plasma sEV preparations than serum sEV preparations, either for those detected in each sample (total detectable), or for those detected in all samples (Figure 2A). In plasma sEV preparations, 480 miRNAs were detected, and 45.4% (218 miRNAs) of these were robustly expressed in all samples. In serum sEV preparations, 412 miRNAs were detected and 31.1% (128 miRNAs) of these were robustly expressed in all samples. Pairwise comparison of the number of total miRNAs that were detectable was consistently higher in the plasma sEV preparations (Wilcoxon signed-rank test, $P = 0.005$) (Figure 2B). The number of miRNAs unique to plasma sEV preparations was also greater than the number of miRNAs unique to serum sEV preparations (108 *vs* 40). Furthermore, a large proportion of the miRNAs unique to plasma sEV preparations were expressed in at least 50% of the cohort (at least 5 out of 10 samples) (Supplementary Table 1). While for miRNAs unique to serum sEV preparations, only 1 miRNA (hsa-miR-1233) was expressed in at least 50% of the cohort.

The majority of the miRNAs detected in serum sEV preparations were also detected in plasma sEV preparations. 372 miRNAs were commonly expressed between serum and plasma sEV preparations, which represented 90.3% of the total miRNA content in serum sEV preparations. Of these, 118 miRNAs were commonly expressed in all serum and plasma sEV preparations. The relative expression of the 372 commonly expressed miRNAs was significantly correlated (Spearman's R = 0.87, $P < 0.0001$) (Figure 2C). There was a stronger correlation among the common 118 miRNAs expressed in all serum and plasma sEV preparations (Spearman's R = 0.92, $P < 0.0001$) (Figure 2D). Similar observations of the overall miRNA content were found in the matched serum and plasma sEV preparations from the patients with esophageal adenocarcinoma (Supplementary Figure 2), although the overall number of miRNAs were higher in the sEV preparations from the cancer patients compared to healthy individuals.

Highly expressed miRNAs in serum and plasma sEV preparations

The top 20 most abundant miRNAs expressed in the serum and plasma sEV preparations were compared (Table 1). 16 out of 20 of the most abundant miRNAs were common between serum and plasma sEV preparations. However, the expression levels of the 16 common miRNAs were 2 to 11-fold higher in the plasma sEV preparations compared to serum sEV preparations (Wilcoxon signed-rank test, $P < 0.05$) (Figure 3). Of the 20 most highly expressed miRNAs in plasma sEV preparations, hsa-miR-484, hsa-miR-130a-3p, hsa-miR-30c-5p and hsa-miR-221-3p were not detected in serum sEV preparations. Of the 20 miRNAs that were highly expressed in serum sEV preparations, hsa-miR-1274b, RNU6-1, hsa-miR-517a-3p and hsa-miR-25-3p were not detected in plasma sEV preparations.

Presence of blood-cell specific miRNAs

The presence of miRNAs reported by Wang *et al*[10], 2012, and Pritchard *et al*[8], 2012, to be highly expressed or uniquely expressed in blood cells was examined in our serum and plasma derived sEV preparations. Both Wang *et al*[10] and Pritchard *et al*[8] identified hsa-miR-223-3p and hsa-miR-451a as highly abundant in blood cells. Wang *et al*[10] reported 27 miRNAs that were uniquely expressed in blood cells. Pritchard *et al*[8] 2012 reported 44 additional miRNAs that were highly expressed in blood cells.

Both hsa-miR-223-3p and hsa-miR-451a were found to be among the top 20 most

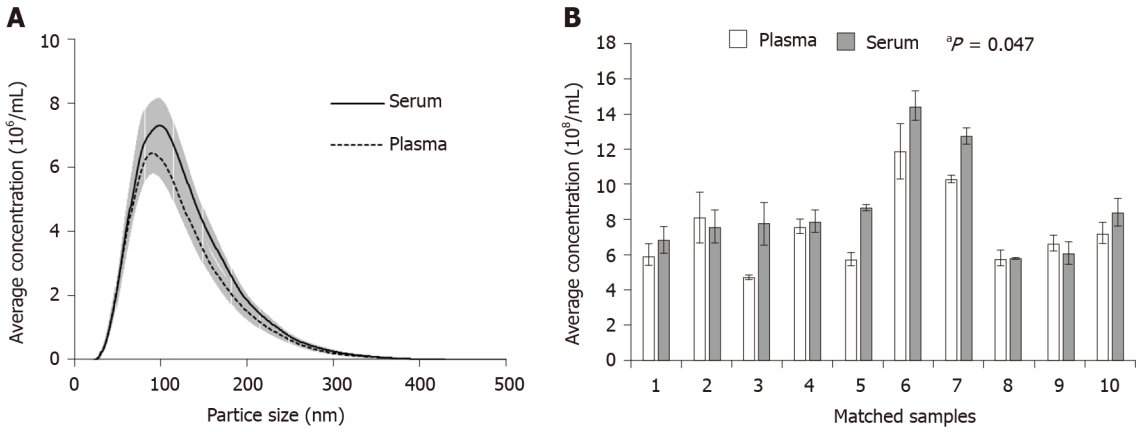

Figure 1 NanoSight measurements of isolated vesicles from matched serum and plasma samples. A: The overall size distribution of particles (SEM indicated by shaded areas) was similar between the matched serum (n = 10) and plasma samples (n = 10); B: Pairwise comparison of the average concentration (± SEM) of particles demonstrated higher particle yields in serum (Wilcoxon signed-rank test, ^aP = 0.047).

highly expressed miRNAs in our serum and plasma sEV preparations (Figure 3). Compared to serum sEV preparations, hsa-miR-223-3p was expressed at 9.6-fold higher in plasma sEV preparations (Wilcoxon signed-rank test, P = 0.0051), while hsa-miR-451a was expressed at 2.5-fold higher in plasma sEV preparations (Wilcoxon signed-rank test, P = 0.01). An additional 6 blood-cell miRNAs were identified in the top 20 most highly expressed miRNAs as consistently expressed at higher levels in plasma sEV preparations compared to serum sEV preparations (2.7 to 5.6 fold; hsa-miR-19b-3p, hsa-miR-17-5p, hsa-miR-30b-5p, hsa-miR-106a-5p, hsa-miR-150-5p and hsa-miR-92a-3p; Figure 3). In addition, we identified 4 blood-cell miRNAs (hsa-miR-98-5p, hsa-miR-30d-3p, hsa-miR-146b-3p and hsa-miR-19b-1-5p) that were robustly expressed in at least 50% of the plasma sEV preparations, that were not expressed in the serum sEV preparations (Supplementary Table 1).

Presence of reported vesicular miRNAs and protein-associated miRNAs

The presence of unique vesicular miRNAs, whole blood miRNAs (blood-cell miRNAs) and cell-free miRNAs (protein-associated miRNAs) reported by Cheng *et al*[11] were compared in our plasma and serum sEV preparations (Figure 4). Overall, we detected 12 of Cheng *et al*[11]'s unique vesicular miRNAs in our serum sEV preparations, and 14 of Cheng *et al*[11]'s unique vesicular miRNAs in our plasma sEV preparations (Figure 4). Smaller numbers of Cheng *et al*[11]'s unique whole blood miRNAs and cell-free miRNAs were detected in our plasma and serum sEV preparations (Figure 4). Several of these unique miRNAs were detected in only a small number of serum or plasma sEV preparations. We therefore identified those that were more reliably and robustly expressed in at least 50% of samples. In serum derived preparations, 6 unique vesicular miRNAs and only 1 unique whole blood miRNA were robustly expressed. In comparison, there were more unique vesicular miRNAs (11 miRNAs) and cell-free miRNAs (5 miRNAs) robustly expressed in plasma derived preparations. These observations were consistent in the matched samples from patients with esophageal adenocarcinoma (Supplementary Table 2).

We next evaluated for the presence of vesicle-associated miRNAs and protein-associated miRNAs reported by Arroyo *et al*[13] (Figure 5). The list of miRNAs that were assessable on the TaqMan OpenArray platform are provided in Supplementary Tables 3 and 4. To investigate the relative expression levels of these miRNAs in serum and plasma sEV preparations, we partitioned them into the following 5 bins using their qPCR Ct's: bin-1, not detected; bin-2, 40 > Ct ≥ 30; bin-3, 30 > Ct ≥ 25; bin-4, 25 > Ct ≥ 20; bin-5, Ct < 20. The percentage of the total number of vesicle-associated miRNAs, and protein-associated miRNAs, was then determined for each bin, for serum sEV preparations and for plasma sEV preparations.

We found that serum sEV preparations had a greater percentage of vesicle-associated miRNAs expressed at relatively high levels (Ct's < 25) than plasma sEV preparations (60% *vs* 44%), whereas plasma sEV preparations had a greater percentage of protein-associated miRNAs expressed at relatively high levels than serum sEV preparations (62% *vs* 31%; Figure 5). Plasma sEV preparations also had a greater percentage, than serum sEV preparations, of protein-associated miRNAs expressed at very high levels (Ct's < 20; 22% *vs* 0%), and plasma sEV preparations had a higher percentage of undetected vesicle-associated miRNAs than serum sEV

Table 1 Top 20 abundant microRNAs expressed in plasma and serum small extracellular vesicle preparations

Rank	Plasma miRNA	Relative levels ± SD (× 10^5)	Serum miRNA	Relative levels ± SD (× 10^5)
1	hsa-miR-223-3p[1]	521.3 ± 310.6	hsa-miR-92a-3p[1]	100.2 ± 178.0
2	hsa-miR-92a-3p[1]	268.7 ± 188.5	hsa-miR-223-3p[1]	53.8 ± 24.8
3	hsa-miR-20a-5p[1]	89.8 ± 38.3	hsa-miR-451a[1]	17.6 ± 22.1
4	hsa-miR-19b-3p[1]	83.2 ± 40.1	hsa-miR-19b-3p[1]	14.7 ± 15.8
5	hsa-miR-24-3p[1]	79.0 ± 44.7	hsa-miR-20a-5p[1]	14.6 ± 14.3
6	hsa-miR-30a-5p[1]	54.8 ± 31.1	hsa-miR-30a-5p[1]	9.4 ± 11.0
7	hsa-miR-451a[1]	43.3 ± 22.0	hsa-miR-320a-3p[1]	9.3 ± 13.5
8	hsa-miR-320a-3p[1]	40.1 ± 17.5	hsa-miR-328-3p[1]	9.1 ± 3.7
9	hsa-miR-484	36.7 ± 23.4	hsa-miR-24-3p[1]	7.2 ± 3.2
10	hsa-miR-17-5p[1]	27.2 ± 12.6	hsa-miR-1274b	6.4 ± 3.9
11	hsa-miR-106a-5p[1]	26.0 ± 11.7	RNU6-1	5.5 ± 4.0
12	hsa-miR-16-5p[1]	21.2 ± 20.8	hsa-miR-106a-5p[1]	5.4 ± 4.2
13	hsa-miR-328-3p[1]	19.2 ± 5.4	hsa-miR-16-5p[1]	5.1 ± 8.8
14	hsa-miR-130a-3p	17.5 ± 7.2	hsa-miR-17-5p[1]	5.0 ± 3.9
15	hsa-miR-30c-5p	17.1 ± 11.6	hsa-miR-150-5p[1]	4.0 ± 2.1
16	hsa-miR-146a-5p[1]	16.2 ± 12.0	hsa-miR-517a-3p	2.8 ± 2.4
17	hsa-miR-221-3p	13.4 ± 7.2	hsa-miR-30b-5p[1]	2.1 ± 1.2
18	hsa-miR-150-5p[1]	13.3 ± 8.5	hsa-miR-146a-5p[1]	1.8 ± 1.1
19	has-hsa-miR-30b-5p[1]	11.5 ± 6.6	hsa-miR-191-5p[1]	1.7 ± 0.9
20	hsa-miR-191-5p[1]	10.6 ± 5.9	hsa-miR-25-3p	1.6 ± 2.3

[1]miRNAs were common between serum and plasma.

preparations (33% *vs* 10%). We observed similar distributions of vesicle-associated and protein-associated miRNAs in serum and plasma sEV preparations from patients with esophageal adenocarcinoma (Supplementary Figure 3). Overall these results indicated that serum sEV preparations contained higher levels of vesicle associated miRNAs, and lower levels of protein associated miRNAs, compared with plasma sEV preparations.

Diagnostic performance of multi-biomarker panel in serum and plasma

To investigate whether the above observed differences in proportions of non-vesicular to vesicular miRNAs in serum and plasma sEV preparations may influence outcomes of biomarker studies, we compared the diagnostic performance of a previously identified multi-biomarker panel[20] in the matched sEV preparations from serum and plasma samples (Figure 6). The multi-biomarker panel consisted of 5 specific miRNA ratios (RNU6-1/hsa-miR-16-5p, hsa-miR-25-3p/hsa-miR-320a, hsa-let-7e-5p/hsa-miR-15b-5p, hsa-miR-30a-5p/hsa-miR-324-5p, hsa-miR-17-5p/hsa-miR-194-5p) that discriminated esophageal adenocarcinoma patients from healthy controls and non-dysplastic Barrett's esophagus[20]. When assessed in the serum sEV preparations, the multi-biomarker panel achieved a good prediction accuracy (AUROC = 0.95) and remained robust in leave-one-out cross validation (AUROC = 0.90). When assessed in the matched plasma sEV preparations, the multi-biomarker panel was less accurate in predicting which patients had esophageal adenocarcinoma (AUROC = 0.80) and performed considerably worse in leave-one-out cross validation (AUROC = 0.54).

DISCUSSION

To date, there have only been limited studies investigating sEV miRNA profiles concurrently in serum and plasma sEV preparations to determine their suitability for biomarker studies[11,16]. Based on our overall study findings, we observed significant differences in the proportion of reported sEV associated miRNAs between serum and plasma sEV preparations. Our results suggest that there is a greater concern for potential contamination of non-vesicular miRNAs in the plasma sEV preparations, and that this may influence biomarker studies. Therefore, we propose serum to be the preferred choice over plasma for future sEV miRNA biomarkers studies.

Under our specific study conditions, we isolated similar sEV yields yet overall

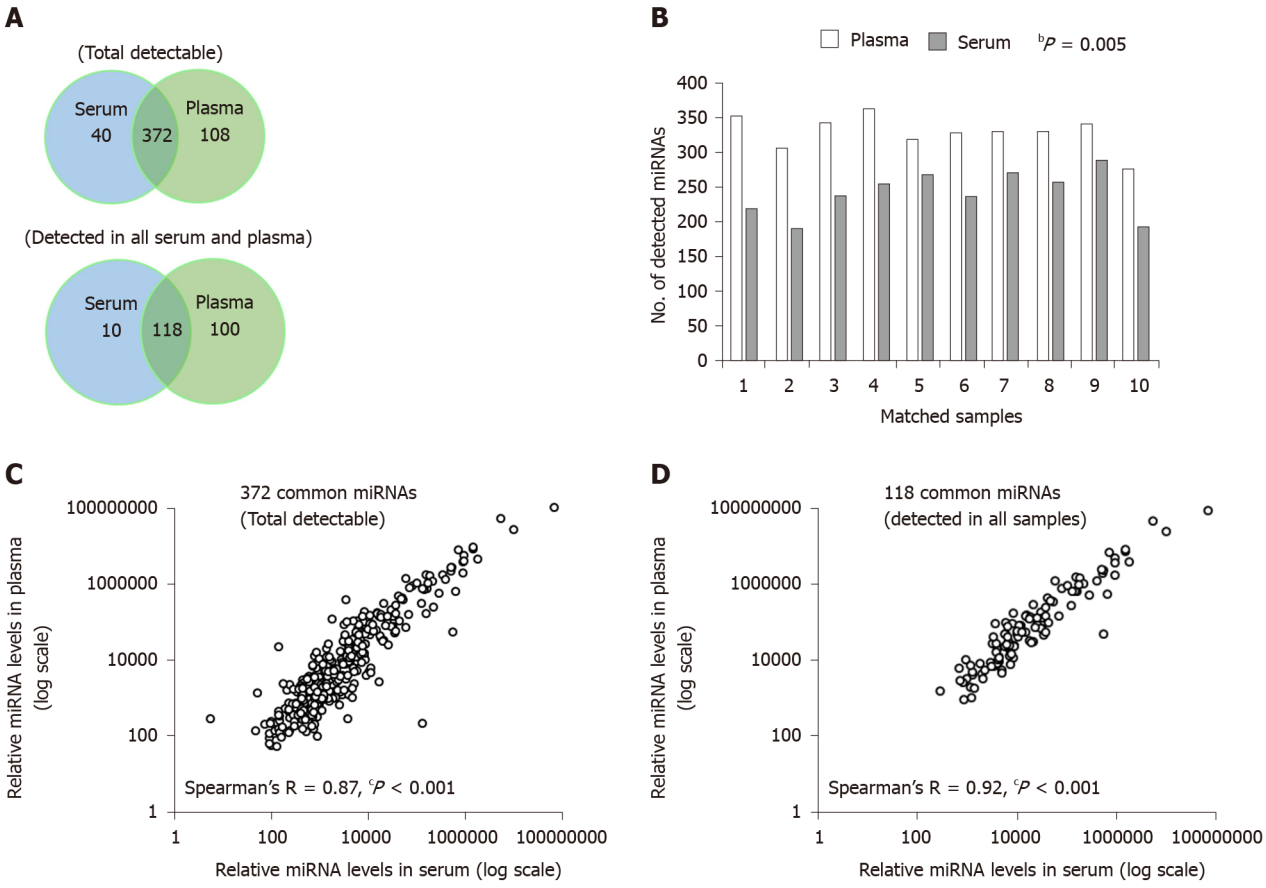

Figure 2 Comparison of the microRNA content between serum and plasma small extracellular vesicles. A: The number of total detectable microRNA (miRNAs) (top Venn diagram), and the number of miRNAs detected in all serum or plasma samples (bottom Venn diagram), were higher in plasma; B: Pairwise comparison of the number of total detectable miRNAs was significantly higher in the plasma (Wilcoxon signed-rank test, $^{b}P = 0.005$); C: Correlation of the average relative expression of the 372 common total detectable miRNAs (Spearman's R = 0.87, $^{c}P < 0.001$); D: Correlation of the average relative expression of the 118 common miRNAs detected in all serum or plasma samples (Spearman's R = 0.92, $^{c}P < 0.001$). miRNA: MicroRNA.

higher miRNA content in plasma compared to serum sEV preparations. These findings are in contrast with previous studies that reported an overall higher miRNA content in serum sEV preparations compared to plasma sEV preparations[11,16]. In Cheng *et al*[11], next generation sequencing was used to profile a larger number of miRNAs than our TaqMan OpenArray platform. However, the study only utilised matched samples from 3 healthy individuals and used different methods than us for sEV isolation. The number of miRNAs detected was also marginally higher in the serum sEV preparations compared to plasma sEV preparations (412 *vs* 386 miRNAs). Although Ding *et al*[16] used the same sEV isolation and quantification techniques as us, the blood processing and miRNA detection methods were different to our study. The authors reported higher sEV yield, higher albumin contamination, larger microvesicles and higher number of miRNAs detected in the serum compared to plasma sEV preparations[16]. However, blood samples used for the sEV quantification and sEV miRNA profiles were derived from different individuals (5 and 20 healthy individuals respectively). Despite the disparities among these studies, the evidence that miRNA profiles differ between matched serum and plasma sEV preparations is consistent.

Possible explanations for the different miRNA profiles of sEV preparations from serum and plasma might include factors that impact upon the amount of protein-bound (non-vesicular) miRNAs present, and/or upon the sEVs produced from blood cells. These factors could include the different tubes with different additives that are used for producing plasma compared to serum, and that the production of serum involves blood clotting while the production of plasma specifically avoids this by using anti-coagulants. Blood clot formation involves trapping an array of proteins into the clot mesh, and this results in a significantly lower protein content in serum than in plasma[24], which may directly contribute towards an overall depletion of protein-bound miRNAs in serum compared to plasma. Besides Ago2 protein, high density lipoprotein (HDL) is another type of protein based vehicle for peripherally circulating miRNAs in plasma[25]. Of particular interest, it has been reported that HDL is trapped

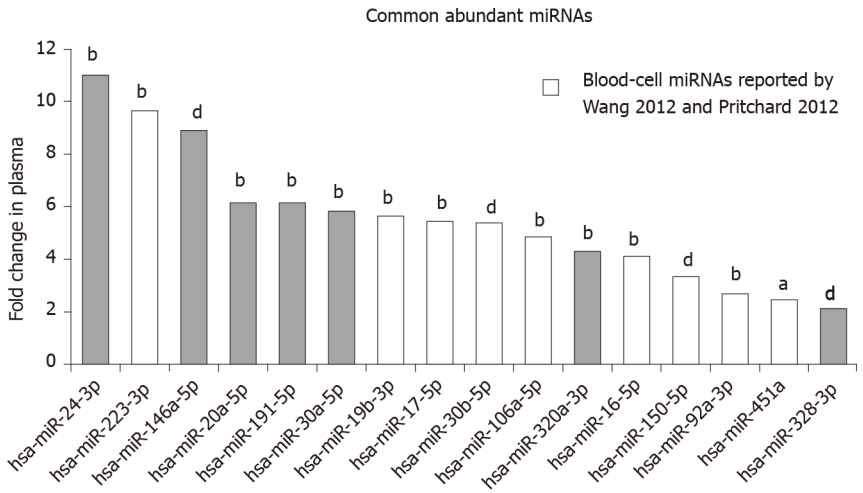

Figure 3 Fold difference in abundance of the common most abundant microRNAs in plasma compared with serum small extracellular vesicles preparations. The fold change is calculated as the relative expression in the plasma divided by the relative expression in the serum. All common abundant microRNAs, including those previously reported as blood-cell microRNAs by Wang *et al*[10] and Pritchard *et al*[8], were significantly higher in the plasma than serum. (Wilcoxon signed-rank test, $^{b}P = 0.0051$; $^{d}P = 0.007$; $^{a}P = 0.01$).

in the mesh that forms during clotting[26]. Therefore, it is possible that HDL-bound miRNAs are trapped in the clot, thereby resulting in lower numbers of HDL-bound miRNAs in serum than plasma. The method that we used for preparing sEVs involves the precipitation of membrane particles, and methods based upon this technique are known to result in co-precipitation of lipoproteins[27]. Taken together, it is possible that our serum sEV preparations contain less HDL-bound miRNAs than our plasma sEV preparations, and this may explain the lower overall miRNA abundance in our serum sEV preparations, as well as the tendency for our serum sEV preparations to contain a higher proportion of sEV associated miRNAs than our plasma sEV preparations. Different blood collection tube components have been shown to interfere with clinical chemistry assays in different ways[24]. The blood collection tubes used for plasma preparation in our study contained EDTA as the anti-coagulant. It has been reported that EDTA results in platelet activation, which increases the release of microvesicles, including sEVs, from platelets[28,29]. This might suggest that our plasma preparations would be more biased, than our serum sEV preparations, towards containing platelet derived / activated platelet sEV derived miRNAs. Consistent with this possibility, the top two miRNAs that were most heavily biased towards our plasma derived preparations were hsa-miR-223-3p, which is the most abundant miRNA in platelets, and hsa-miR-24-3p, which is a biomarker for platelet activation[30]. Another possibility is that the different blood tube components in the tubes used for preparing serum and plasma have different impacts upon the number of sEVs produced from blood cells, and / or the miRNA expression in blood cells, which translates into differences in the miRNA composition of sEVs derived from them[31]. It is also possible that the differences in blood tube components might impact upon the specific blood cell miRNAs that are sorted into sEVs[31].

One of the most significant differences between the miRNA profiles of our serum and plasma sEV preparations was the higher level of expression of reported blood cell miRNAs in plasma sEV preparations. In previous studies, circulating cell-free hsa-miR-451a, hsa-miR-16-5p and hsa-miR-223-3p are among the most common miRNAs assessed as indicators of haemolysis or blood cells contamination[10,18,32-35]. Although we found several reported blood cell miRNAs, including hsa-miR-451a, hsa-miR-16-5p and hsa-miR-223-3p, to be abundant in both plasma and serum sEV preparations, they were all more highly expressed in plasma sEV preparations. The presence of higher blood cell contamination in plasma sEV preparations was further supported by the observation that several reported blood-cell miRNAs were uniquely expressed only in our plasma derived samples, and a greater number of unique cell-free miRNAs, reported by Cheng 2014, were detected in the plasma derived samples.

Although the overall miRNA content was higher in our plasma sEV preparations, the concern was the high abundance of miRNAs in plasma sEV preparations that were reported to be from non-vesicular origins. Contrary to the consistently high miRNA content in plasma compared to serum derived sEV preparations, we observed a larger percentage of highly expressed vesicle-associated miRNAs in the serum sEV

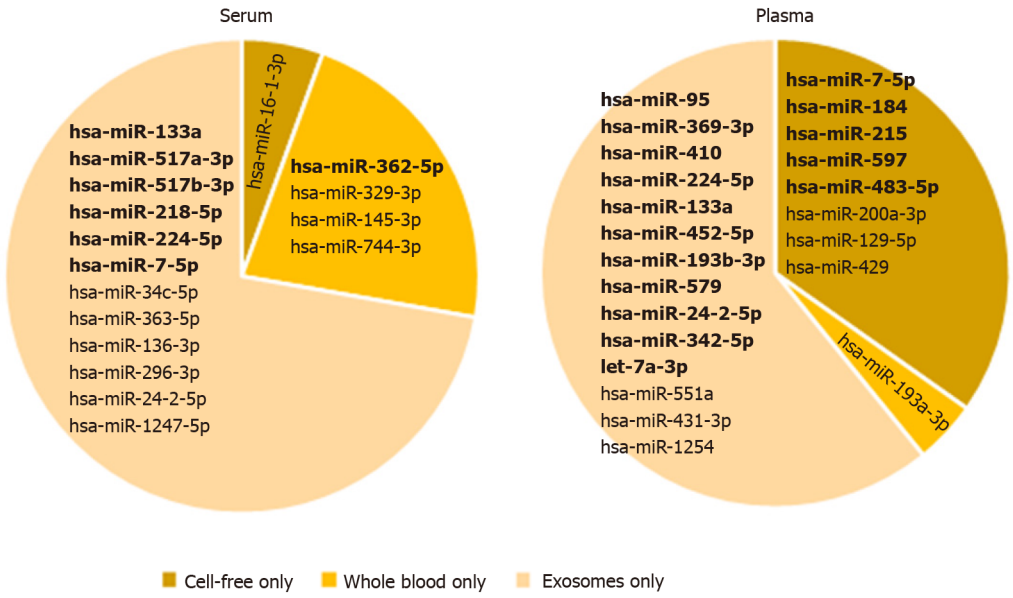

Figure 4 Presence of microRNAs reported to be uniquely expressed in whole blood, cell-free, or small extracellular vesicles, in serum and plasma. The lists of unique miRNAs were derived from Cheng *et al*[11]. miRNAs detected in at least 50% of each sample type are presented in bold.

preparations, but a larger percentage of highly expressed protein-associated miRNAs in the matched plasma sEV preparations. We acknowledge that this conclusion is reliant on the findings of a single study (Arroyo 2011) and will require further validation. However, unlike blood-cell miRNAs, specific miRNAs that are highly expressed or uniquely expressed in sEVs or in protein-complexes with Ago2 are not as well-established. We identified only two studies, Cheng 2014[11], and Arroyo 2011[13], comprehensively reporting specific sEV miRNA profiles and protein-associated miRNA profiles in serum and plasma. Interestingly, RNU6-1, which has been reported to be enriched in sEVs, was also found to be abundant in our serum sEV preparations (top 20 most highly expressed miRNAs) but not in our plasma sEV preparations[36-39]. Altogether, these findings suggest that although a large proportion of miRNAs were consistently more highly expressed in plasma sEV preparations compared to serum sEV preparations, we identified a subset of miRNA candidates reported to be of sEV origin to be more highly expressed in serum sEV preparations.

Taking all these findings together, serum appears preferable to plasma for sEV miRNA biomarkers studies. As a proof-of-concept, we evaluated the diagnostic performance of a multi-biomarker panel to discriminate esophageal adenocarcinoma patients from the healthy controls in this study cohort when assessed in the serum sEV preparations compared to plasma sEV preparations. The diagnostic accuracy of the biomarker panel had higher cross validated prediction accuracy when assessed in the serum sEV preparations than in plasma sEV preparations. However, we recognise that our study findings are based on a small sample size and are specific to our study conditions, and further work is necessary to validate these findings. In particular, there is currently limited understanding on circulating miRNAs in protein-complexes, and a need to consider their role in future sEV miRNAs studies.

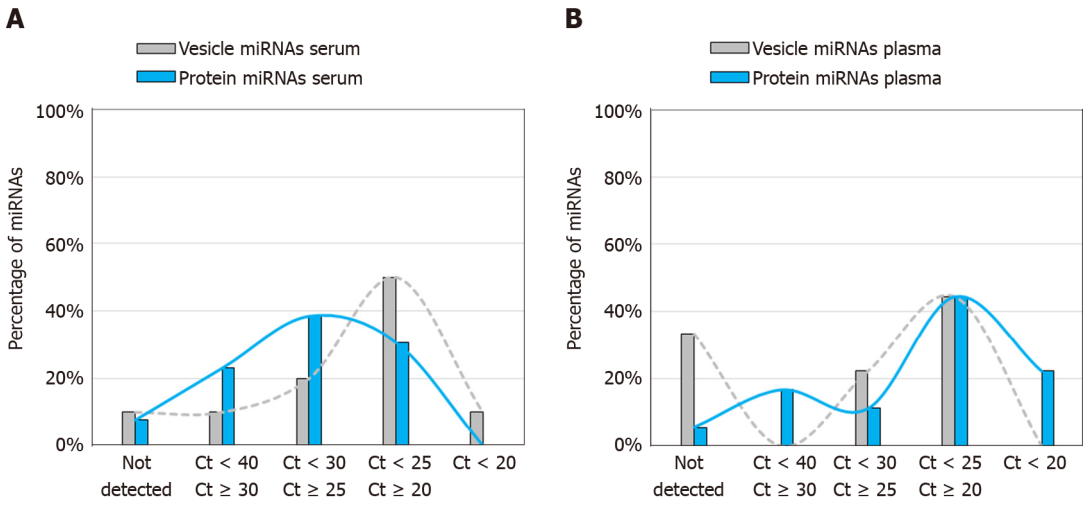

Figure 5 Percentage of vesicle-associated (grey) and protein-associated (blue) microRNAs expressed at levels within the indicated cycle threshold range in small extracellular vesicle preparations from healthy controls. A: Serum; B: Plasma. The list of vesicle-associated and protein-associated miRNAs assessed in the serum and plasma were derived from Arroyo *et al*[13]. The bar graphs represent the percentage of microRNAs (miRNAs) within each cycle threshold range out of the total vesicle-associated miRNAs or protein-associated miRNAs assessed respectively in each sample type. Smoothed lines were added to aid visualisation of the trends.

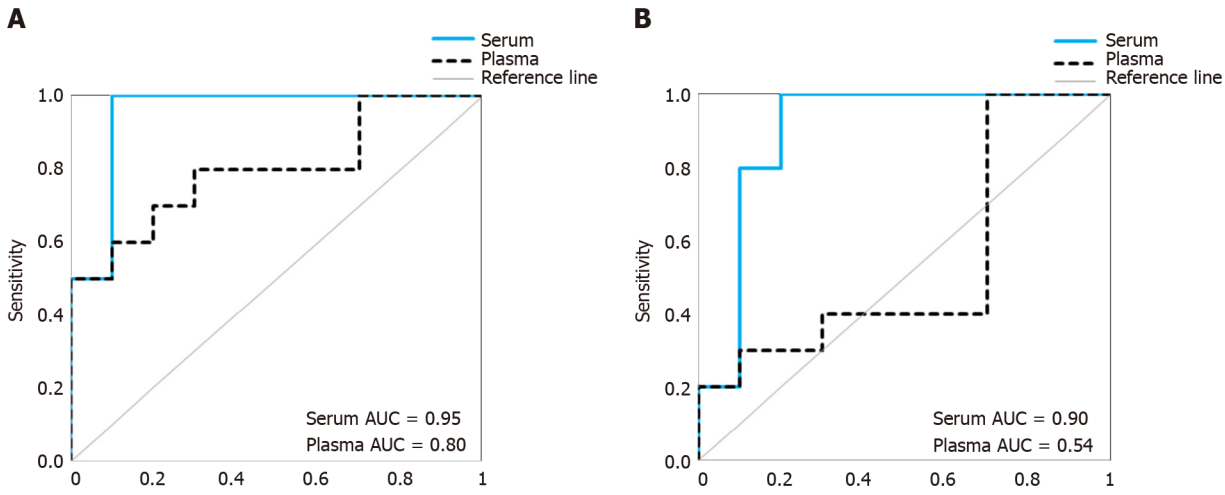

Figure 6 Comparison of the diagnostic performance of a previously identified 5-microRNA ratio biomarker panel (Chiam *et al*[20]) for detecting esophageal adecarcinoma. A: The diagnostic accuracy of the biomarker panel was assessed by receiver-operating characteristics curve analysis. The area under the curve was greater in the serum than the plasma; B: The biomarker panel was assessed by leave-one-out-cross validation. The serum small extracellular vesicle preparations produced greater diagnostic accuracy than the plasma small extracellular vesicle preparations. Healthy individuals, *n* = 10; esophageal adenocarcinoma, *n* = 10. AUC: Area under the curve.

ARTICLE HIGHLIGHTS

Research background

Small extracellular vesicles (sEVs), including exosomes, are shed from tumors into the blood circulation. These circulating sEVs are an excellent source of microRNA (miRNA) biomarkers for cancer research. Blood serum and blood plasma both contain sEVs, however at present there is no consensus on which of these two blood sample types is most useful for biomarker analysis.

Research motivation

Extracellular vesicle preparations derived from serum and plasma are known to be enriched in sEVs, but they also contain significant amounts of non-vesicle associated miRNAs derived from sources such as blood cells and protein-bound miRNAs. These non-vesicles associated miRNAs could interfere with cancer biomarker discovery. Our study was motivated by the need to determine which blood sample contains the least amount of non-vesicle associated miRNAs. This knowledge has the potential to improve cancer biomarker discovery and translation.

Research objectives

We sought to compare the miRNA profiles between serum and plasma sEV preparations to determine their suitability for biomarker studies. We also sought to compare the diagnostic performance of these two sample types using a previously established multi-miRNA biomarker panel for esophageal adenocarcinoma.

Research methods
Matched serum and plasma samples from 10 healthy controls and 10 patients with esophageal adenocarcinoma were used for this study. sEVs were isolated with using Exoquick™. RNA extracted from the vesicles was profiled using the Taqman Openarray qPCR.

Research results
The overall miRNA content was higher in plasma sEV preparations (480 miRNAs) and contained 97.5% of the miRNAs found in the serum sEV preparations (412 miRNAs). The expression of commonly expressed miRNAs was highly correlated (Spearman's R = 0.87, $P <$ 0.0001) between the plasma and serum sEV preparations but was consistently higher in the plasma sEV preparations. Specific blood-cell miRNAs (hsa-miR-223-3p, hsa-miR-451a, miR-19b-3p, hsa-miR-17-5p, hsa-miR-30b-5p, hsa-miR-106a-5p, hsa-miR-150-5p and hsa-miR-92a-3p) were expressed at 2.7 to 9.6 fold higher levels in the plasma sEV preparations compared to serum sEV preparations ($P < 0.05$). In plasma sEV preparations, the percentage of protein-associated miRNAs expressed at relatively higher levels (cycle threshold 20-25) was greater than serum sEV preparations (50% *vs* 31%). While the percentage of vesicle-associated miRNAs expressed at relatively higher levels was greater in the serum sEV preparations than plasma sEV preparations (70% *vs* 44%). A 5-miRNA biomarker panel produced a higher cross validated accuracy for discriminating patients with esophageal adenocarcinoma from healthy controls using serum sEV preparations compared with plasma sEV preparations (AUROC 0.80 *vs* 0.54, $P < 0.05$).

Research conclusions
Although plasma sEV preparations contained more miRNAs than serum sEV preparations, they also contained more miRNAs from non-vesicle origins.

Research perspectives
Serum appears to be more suitable than plasma for sEV miRNAs biomarkers studies. Future studies on sEV associated cancer biomarkers may benefit from using serum as the sample type for analysis.

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ORIGINAL ARTICLE

Basic Study

Conservation and variability of hepatitis B core at different chronic hepatitis stages

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Abstract

BACKGROUND

Since it is currently not possible to eradicate hepatitis B virus (HBV) infection with existing treatments, research continues to uncover new therapeutic strategies. HBV core protein, encoded by the HBV core gene (*HBC*), intervenes in both structural and functional processes, and is a key protein in the HBV life cycle. For this reason, both the protein and the gene could be valuable targets for new therapeutic and diagnostic strategies. Moreover, alterations in the protein sequence could serve as potential markers of disease progression.

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AIM
To detect, by next-generation sequencing, *HBC* hyper-conserved regions that could potentially be prognostic factors and targets for new therapies.

METHODS
Thirty-eight of 45 patients with chronic HBV initially selected were included and grouped according to liver disease stage [chronic hepatitis B infection without liver damage (CHB, n = 16), liver cirrhosis (LC, n = 5), and hepatocellular carcinoma (HCC, n = 17)]. HBV DNA was extracted from patients' plasma. A region between nucleotide (nt) 1863 and 2483, which includes *HBC*, was amplified and analyzed by next-generation sequencing (Illumina MiSeq platform). Sequences were genotyped by distance-based discriminant analysis. General and intergroup nt and amino acid (aa) conservation was determined by sliding window analysis. The presence of nt insertion and deletions and/or aa substitutions in the different groups was determined by aligning the sequences with genotype-specific consensus sequences.

RESULTS
Three nt (nt 1900-1929, 2249-2284, 2364-2398) and 2 aa (aa 117-120, 159-167) hyper-conserved regions were shared by all the clinical groups. All groups showed a similar pattern of conservation, except for five nt regions (nt 1946-1992, 2060-2095, 2145-2175, 2230-2250, 2270-2293) and one aa region (aa 140-160), where CHB and LC, respectively, were less conserved (P < 0.05). Some group-specific conserved regions were also observed at both nt (2306-2334 in CHB and 1935-1976 and 2402-2435 in LC) and aa (between aa 98-103 in CHB and 28-30 and 51-54 in LC) levels. No differences in insertion and deletions frequencies were observed. An aa substitution (P79Q) was observed in the HCC group with a median (interquartile range) frequency of 15.82 (0-78.88) *vs* 0 (0-0) in the other groups (P < 0.05 *vs* CHB group).

CONCLUSION
The differentially conserved *HBC* and HBV core protein regions and the P79Q substitution could be involved in disease progression. The hyper-conserved regions detected could be targets for future therapeutic and diagnostic strategies.

Key words: Hepatitis B virus; Hepatitis B core gene; Next-generation sequencing; Genetic conservation; Amino acid substitution; Gene therapy; Small interfering RNA

Core tip: New tools for hepatitis B virus infection treatment and follow-up are needed. Hepatitis B virus core protein has a key role in viral replication and persistence. Analysis of viral quasispecies by next-generation sequencing can identify conserved regions in viral genes or proteins that may serve as targets for new therapeutic and diagnostic strategies. Moreover, it may help identify prognostic markers of liver disease progression. Here, we detected hyper-conserved nucleotide and amino acid regions regardless of the clinical stage. Moreover, we observed several group-specific conserved and variable regions and an amino acid substitution that could be indicative of different disease progression.

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INTRODUCTION

Hepatitis B virus (HBV) is a small virus with a specific tropism for the liver. It belongs

to the *Hepadnaviridae* family. Despite the existence of effective preventive vaccines, an estimated 257 million people worldwide live with chronic HBV infection and more than 880000 people die every year of HBV-related complications such as liver cirrhosis (LC) and hepatocellular carcinoma (HCC)[1].

HBV is an enveloped virus equipped with 3.2 kb of partially double-stranded circular DNA produced by the reverse transcription of an RNA intermediate known as pregenomic RNA[2]. This ribonucleic intermediate is produced from a viral DNA molecule that interacts with cellular (histone and non-histone) and viral proteins, forming a "mini-chromosome" known as covalently closed circular DNA (cccDNA) that remains in hepatocyte nuclei for the rest of the cell's life[3]. Although current antiviral therapy can control viral replication, it is not capable of interfering with the formation or persistence of cccDNA, rendering HBV infection eradication impossible. This mini-chromosome could even be a source of HBV reactivation after clinical resolution and HBsAg seroclearance[4]. Due to persistent infection, up to 1% of Caucasian patients with noncirrhotic chronic HBV infection have been found to develop HCC[5].

Gene therapy has emerged as one of the most promising strategies for blocking disease progression, and results from studies investigating the potential of small interfering RNA (siRNA) systems as adjuvant therapy are encouraging[6]. SiRNA is a double-stranded noncoding RNA [with an optimal length of 21 nucleotides (nt)] that interacts with target messenger RNA, promoting its degradation and silencing of the gene[7].

HBV reverse transcriptase lacks 3' to 5' proofreading activity, which leads to viral genome variability comparable to that observed in an RNA virus[8]. This genetic variability is further increased by inter- and intra-genotype recombination events[9]. In short, HBV circulates as a complex mixture of closely related genetic variants (haplotypes) known as quasispecies[10].

The HBV core protein (HBc) [encoded by the HBV core gene (*HBC*) from the PreCore/Core open reading frame (ORF)] is essential for viral replication. It is a structural 21-kDa protein that self-assembles to create dimers that assemble in hexamers forming the icosahedral viral capsid[11,12]. It has 183 amino acids (aa) (185 for genotype A) with a N-terminal domain and a C-terminal domain (CTD) connected through a linker region. The N-terminal domain ranges from aa position 1 to 149 (including the linker region aa 140 to 149) and constitutes the α helix-rich assembly domain[13]. The CTD is shorter (aa 150 to 183, or 185 for genotype A) and constitutes the functional domain[13]. The CTD allows HBc to intervene in a multitude of processes such as subcellular traffic, viral genome release, capsid assembly and transport, RNA metabolism, and viral pregenomic RNA reverse transcription[14]. Considering just how essential this protein is for viral replication, it could be an optimal target for gene therapy. Moreover, mutations in HBc may have different roles in liver disease progression, positioning them as potentially useful prognostic genetic markers.

Next-generation sequencing (NGS) is a highly sensitive technique for studying viral quasispecies; it is capable of detecting highly conserved regions of the HBV genome, regardless of genome or clinical stage[9]. Moreover, it supports the identification and quantitative determination[15] of specific variants that could be used as markers to predict prognosis and treatment response in patients with HBV infection.

The aim of this study was to apply NGS to analyse HBc conservation and variability at the nt and aa levels in patients with different stages of chronic HBV infection in order to identify hyper-conserved regions of the *HBC* gene that could be a target for gene therapy and to determine possible prognostic factors of disease progression

MATERIALS AND METHODS

Patients and samples

The study was reviewed and approved by the Clinical Research Ethics Committee of Hospital Universitari Vall d'Hebron (PR(AG)146/2020). No animals were used.

Forty-five patients with chronic HBV infection were recruited from members of the general population seen at the outpatient clinic at Vall d'Hebron University Hospital in Barcelona, Spain. They tested negative for hepatitis D virus, hepatitis C virus, and human immunodeficiency virus, and had a viral load > 3 log IU/mL, which is the limit of polymerase chain reaction (PCR) amplification sensitivity. HBV serological markers such as the surface antigen (HBsAg), the e antigen (HBeAg), and anti-HBe antibodies were tested using commercial chemiluminescent assays on a COBAS 8000 analyzer (Roche Diagnostics, Rotkreuz, Switzerland). HBV DNA was quantified by

real-time PCR with a detection limit of 10 IU/mL (COBAS 6800, Roche Diagnostics). Patients were divided into 3 clinical groups according to liver disease stage determined by biopsy or diagnostic imaging in line with the EASL guidelines[16]: Chronic HBV infection without liver damage (CHB group), chronic HBV infection with liver cirrhosis (LC group), and chronic HBV infection with hepatocellular carcinoma (HCC group).

HBC gene amplification and NGS

HBV DNA was extracted from 200 μL of serum using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The region of interest was amplified through a 3-step nested PCR protocol (Figure 1). The first step (PCR1) covered a large region between nt 1774-2930 that includes the *HBC* gene (nt 1901-2464 for genotype A and 1901-2458 for other genotypes). As the Illumina MiSeq platform (Illumina, San Diego, CA, United States) allows read lengths of up to 600 bp, the following amplification steps were performed by dividing *HBC* into 2 amplicons (amplicon 1 = nt 1863-2317 and amplicon 2 = nt 2205-2483), which overlapped in a 112 nt-long portion (PCR2). The M13-tail, added in step 2, was used for the last step (PCR3), which introduced a 10 nt-long sample-specific multiplex identifier. All the PCR steps were performed using high-fidelity Pfu Ultra II DNA polymerase (Stratagene, Agilent Technologies, Santa Clara, CA, United States). The primers and protocols are reported in Table 1.

The final PCR products were purified with Agencourt AMPure XP magnetic beads (Beckman Coulter, Beverly, LA, United States) and their quality verified using the Agilent 2200 TapeStation System and D1000 ScreenTape kit (Agilent Technologies, Waldbronn, Germany).

Purified amplicons were quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific-Life Technologies, Austin, TX, United States) and pooled to guarantee that the 2 amplicons for each patient were adequately represented in the analysis (2.5x for amplicon 1 and 1x for amplicon 2, due to their different lengths). The amplicon pool was sequenced by NGS on the Illumina MiSeq platform.

The reads obtained underwent an in-house bioinformatics filtering procedure based on R scripts[17], as previously described by our group[18]. For each amplicon, a group of unique sequences (haplotypes) forming the viral quasispecies was obtained. All sequences that did not match in the overlapping 112-nt region between amplicon 1 and 2 were discarded.

The bioinformatics methods used in this study were reviewed by Mercedes Guerrero-Murillo from the Microbiology Department at Vall d'Hebron Hospital (Barcelona, Spain) and by Dr. Josep Gregori from the Liver Disease Viral Hepatitis Laboratory at Vall d'Hebron Hospital (Barcelona, Spain), CIBERehd research group, and Roche Diagnostics SL.

Genotyping of the haplotypes

The amplicons from each patient were aligned with the same region of the respective amplicons extracted from 106 full-length HBV genome sequences representative of genotypes A to J obtained from the NCBI GenBank (Supplementary Table 1). Genotyping was conducted by applying distance-based discriminant analysis (DB rule)[19,20], which considers the inter- and intra-class variability of all genotypes. Genetic distances were computed according to the Kimura-80 model[21].

Conservation and mutation analysis

Sequence conservation at nt and aa levels was determined by calculating the information content (IC) of each position in a multiple alignment of all haplotypes detected with a frequency > 0.25.

This analysis calculates the mean IC for windows of 25 nt (or 10 aa), starting from the first position in the multiple alignment and moving forward in steps of 1[9]. The hyper-conserved regions were detected by aligning all haplotypes, regardless of clinical stage. Differences in sequence conservation between the groups were determined by comparing IC values.

To identify specific nt insertions and deletions (indels) and aa substitutions that could discriminate between the groups, haplotype sequences were aligned with their genotype-specific consensus sequence. Consensus was obtained by aligning the sequences of the subgenotypes of interest extracted from the 106 full-length HBV genome sequences. Polymorphisms were identified by aligning haplotype sequences with a population consensus sequence and discarded.

Statistical analysis

Sequence conservation differences between the groups in the sliding windows were analysed using the Wilcoxon–Mann–Whitney test. Frequencies of aa changes detected

Table 1 Primer design and polymerase chain reaction protocols for each amplified region

	PCR	Primer	Primer sequence (5'-> 3')	Amplified region	Protocol
1st step	PCR1	Forward	TAGGAGGCTGTAGGCATA	1774-2930	95 °C 5 min; (95°C 20 s, 49 °C 20 s, 72 °C 15 s) × 35 cycles; 72 °C 3 min
		Reverse	GGAAAGAATCCCAGAGG		
2nd step	PCR2 A.1	Forward A.1	**GTTGTAAAACGACGGCCAGT**TTCAAGCCTCCAAGCTGT	1863-2317	95 °C 2 min; (95 °C 20 s, 58 °C 20 s, 72 °C 15s) × 35 cycles; 72 °C 3 min
		Reverse A.1	**CACAGGAAACAGCTATGACC**GATAGGGGCATTTGGTGGTCT		
	PCR2 A.2	Forward1 A.2	**GTTGTAAAACGACGGCCAGT**GGTTTCATATTTCTTGCC	2205-2483	95 °C 2 min; (95 °C 20 s, 50 °C 20 s, 72 °C 15 s) × 35 cycles; 72 °C 3 min
		Forward2 A.2	**GTTGTAAAACGACGGCCAGT**GGTTTCACATTTCCTGTC		
		Forward3 A.2	**GTTGTAAAACGACGGCCAGT**GGTTTCACATTTCTTGTC		
		Forward4 A.2	**GTTGTAAAACGACGGCCAGT**GGTTTCACATTTCCTGCC		
		Reverse A.2	**CACAGGAAACAGCTATGACC**TCCCACCTTATGAGTCCAAG		
3rd step	PCR3	Forward (specific per sample)	**GTTGTAAAACGACGGCCAGT**+specific 10 nt MID		95 °C 2 min; (95 °C 20 s, 60 °C 20 s, 72 °C 15 s) × 20 cycles; 72 °C 3 min
		Reverse (specific per sample)	**CACAGGAAACAGCTATGACC**+specific 10 nt MID		

Bold nucleotides indicate the M13 sequence. Forward primers in PCR2-A2 were multiplexed at the same concentration to cover all HBV genotypes. The protocols of amplification are reported. A.1: Amplicon 1; A.2: Amplicon 2; PCR: Polymerase chain reaction; MID: Multiplex identifier.

were compared with the Kruskal-Wallis test and described as median and interquartile range (IQR). All analyses were performed in R version 3.2.3. $P < 0.05$ was considered significant.

RESULTS

Patients characteristics and NGS results

Of the 45 patients with chronic hepatitis initially included in the study, 38 passed the sequencing quality filters and had correctly overlapping amplicons 1 and 2. After application of the quality filters, a median (IQR) of 133156.5 (85961.25-605212) and 66571 (25958.5-2301225) sequences per patient were obtained respectively for amplicon 1 and amplicon 2. NGS data were submitted to the GenBank SRA database (BioProject accession number PRJNA625435; BioSample accession numbers are reported in Supplementary Table 2). In the clinical groups, there were 16 patients with CHB, 5 with LC, and 17 with HCC. The clinical and viral characteristics (including genotypes) are reported in Table 2.

Sequence conservation at the nt level

Sequence conservation was studied by applying a sliding window analysis to the entire *HBC* sequence overlapping the 2 amplicons at the common 112 nt-long portion. No differences in IC were observed on analyzing the sequences by haplotype considering or not their relative frequency (Figure 2A). Considering the IC of all the nt-sequence haplotypes obtained (regardless of clinical group), we identified 3 hyper-conserved regions (nt 1900-1929, 2249-2284, and 2364-2398, Figure 2B). Most of the nt positions within these regions yielded the maximum IC value of 2 bits (100% conservation).

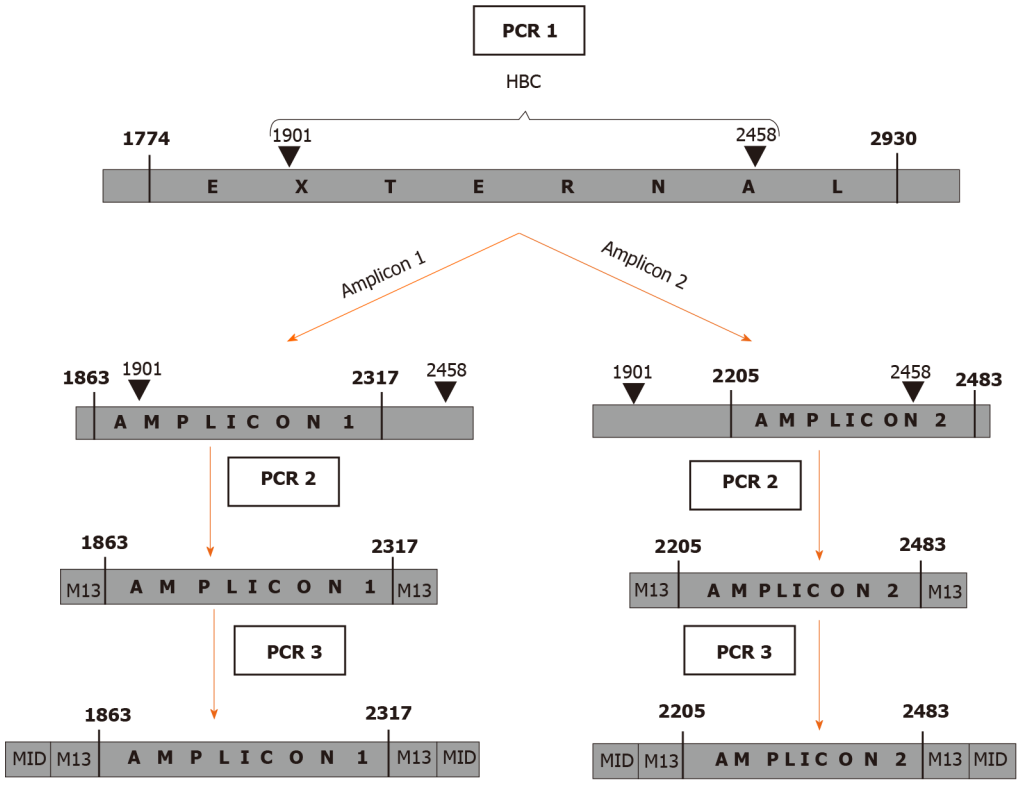

Figure 1 Schematic summary of the 3 amplification steps. In the first amplification step (PCR1), a large region was amplified. In the following step (PCR2), the region was divided into 2 amplicons that overlapped in a 112 nucleotide-long portion. In the third step (PCR 3) a sample identifier (MID) was added. PCR: Polymerase chain reaction; MID: Multiplex identifier.

On comparing the IC of each clinical group by haplotype, the HCC and LC groups showed similar conservation patterns; CHB was notably associated with the lowest level of conservation, mainly evident in 5 regions: nt 1946-1992, 2060-2095, 2145-2175, 2230-2250, and 2270-2293 ($P < 0.05$, Figure 3A). Three group-specific conserved regions were detected: 1 in the CHB group (nt 2306-2334) and 2 in the LC group (nt 1935-1976 and 2402-2435; Figure 3B). Most of the nt positions within these regions yielded the maximum IC value of 2 bits (100% conservation).

Sequence conservation at the aa level

The aa sequences of the haplotypes were translated from their respective nt sequences using the *HBC* reading frame.

Sliding window analysis of the aa haplotypes of the 38 patients by haplotype and haplotype frequency (Figure 4A) showed that the HBc protein was highly conserved throughout its sequence except for the central region (between aa 50 and 100), where conservation was slightly decreased. Two common hyper-conserved regions were detected: 1 between aa 117-120 and 1 between aa 159-167 (Figure 4B). All the aa in these regions had a conservation of around 100% (4.32 bits).

On analyzing aa conservation by haplotype in relation to clinical stage, the 3 groups showed a similar pattern, except for a region between aa 140 and 160, which was less conserved in the LC group compared with the CHB and HCC groups ($P < 0.05$, Figure 5A). Again, 3 group-specific conserved aa regions were detected: 1 in the CHB group (aa 98-103) and 2 in the LC group (aa 28-30 and 51-54, Figure 5B). All the aa in these regions had a conservation of around 100% (4.32 bits).

nt indels and aa changes

nt indels and aa changes were identified by aligning the patients' haplotypes with their genotype-specific consensus sequence.

In the CHB group, 8/16 patients had indels in *HBC*, *vs* 2/17 in the HCC group and 1/5 in the LC group. The indels consisted of the insertion or deletion of one nt at positions 1951 or 2085 (a thymine in 1951 and a guanine in 2085; Table 3). In all cases, a truncated HBc protein was produced. However, due to the limited number of patients, no statistical differences were observed on comparing the frequencies between the groups.

On analysing the presence of aa changes, we identified the aa substitution P79Q

Table 2 Main clinical and viral characteristics of hepatitis B virus-infected patients enrolled in the study

Median [IQR]	CHB (*n* = 16)	HCC (*n* = 17)	LC (*n* = 5)	*P* value
Age	38.5 [33.5-46.5]	67 [58-69]	56 [48-66]	0.002
Viral load (log IU/mL)	6.8 [5.7-8.0]	5.5 [4.7-6.7]	5.7 [4.8-6.2]	NS
ALT	56.5 [41.25-180.5]	70 [47-212]	46 [43-79]	NS
AST	56 [34.75-124]	120.5 [59-163.5]	66.45 [48.675-84.225]	NS
Platelets (10^9/L)	183 [161.5-226]	136 [98.5-255]	81.5 [61.25-101.75]	NS
Proportion				
Gender (male)	11/16	15/17	3/5	
HBeAg (positive)	8/16	3/17	0/5	
Genotype, % (*n*)				
A	18.8 (3)	5.9 (1)	20.0 (1)	
C	37.5 (6)	5.9 (1)	20.0 (1)	
D	25.0 (4)	64.7 (11)	40.0 (2)	
D/A	(0)	5.9 (1)	0.0 (0)	
D/E	6.3 (1)	11.8 (2)	0.0 (0)	
E	6.3 (1)	0.0 (0)	20.0 (1)	
F	6.3 (1)	5.9 (1)	0.0 (0)	

D/E and D/A indicate mixtures of the 2 genotypes. The frequency of each genotype within the clinical groups is reported as percentage (%) and number of patients (*n*). CHB: Chronic hepatitis B infection without liver damage; HCC: Hepatocellular carcinoma; LC: Liver cirrhosis; ALT: Alanine aminotransferase (normal value < 40 IU/mL); AST: Aspartate aminotransferase (normal value < 40 IU/mL); IQR: Interquartile range; NS: No-statistical *P* value.

(proline to glutamine) in the HCC group with a median (IQR) frequency of 15.82 (0-78.9) *vs* (0-0) in the CHB group ($P < 0.05$) and 0 (0-0) in the LC group (Figure 6).

DISCUSSION

The HBc protein, encoded by the *HBC* gene, is a key element in viral replication and disease progression and is involved in both structural and functional processes. Studying gene and protein sequences in patients with different clinical stages of HBV infection could provide important information on the pathogenic role of this protein. Moreover, the identification of hyper-conserved regions at both nt and aa levels could help develop new therapeutic approaches, including gene therapy. In this study, we used NGS to analyse *HBC* quasispecies in a group of patients with chronic HBV infection stratified by liver disease stage.

First, we studied quasispecies conservation to search for hyper-conserved nt and aa regions regardless of clinical stage or viral genotype. Current treatment based on nucleos(t)ide inhibitors does not affect cccDNA levels or transcriptional activity and therefore cannot eliminate HBV infection. This viral mini-chromosome supports the continuous expression of viral antigens that possibly contribute to disease progression, even in the presence of drug-induced viral suppression[22].

New therapeutic approaches are thus required to control HBV expression, and the targeted delivery of siRNA is one of the most promising approaches under investigation[23]. Several siRNAs are currently being tested against X and S ORFs. A study conducted in chimpanzees showed that multiple injections of ARB-1467 (a mixture of 3 interfering RNAs targeting both X and S ORFs[24]) led to a 90% reduction in HBsAg levels and a 50% reduction in cccDNA within 28 d of treatment[25]. None of the molecules currently available, however, target *HBC*, which considering its role in viral replication could be a valuable target for siRNA-based therapies.

In this study, we analysed quasispecies conservation of the entire *HBC* gene in patients infected by different HBV genotypes and with different clinical stages of disease in order to identify hyper-conserved regions that might be useful for pangenotypic and panclinical RNA silencing strategies. On analyzing nt conservation for the group of 38 patients, we detected 3 shared hyper-conserved regions, namely the start codon of *HBC* expression (nt 1900-1929), a portion with 2 CD8 epitopes (HLA-A24 and A3303) (nt 2249-2284)[26], and an arginine-rich portion of the CTD (nt 2364-2398). All 3 sequences could be valuables targets for a new gene silencing

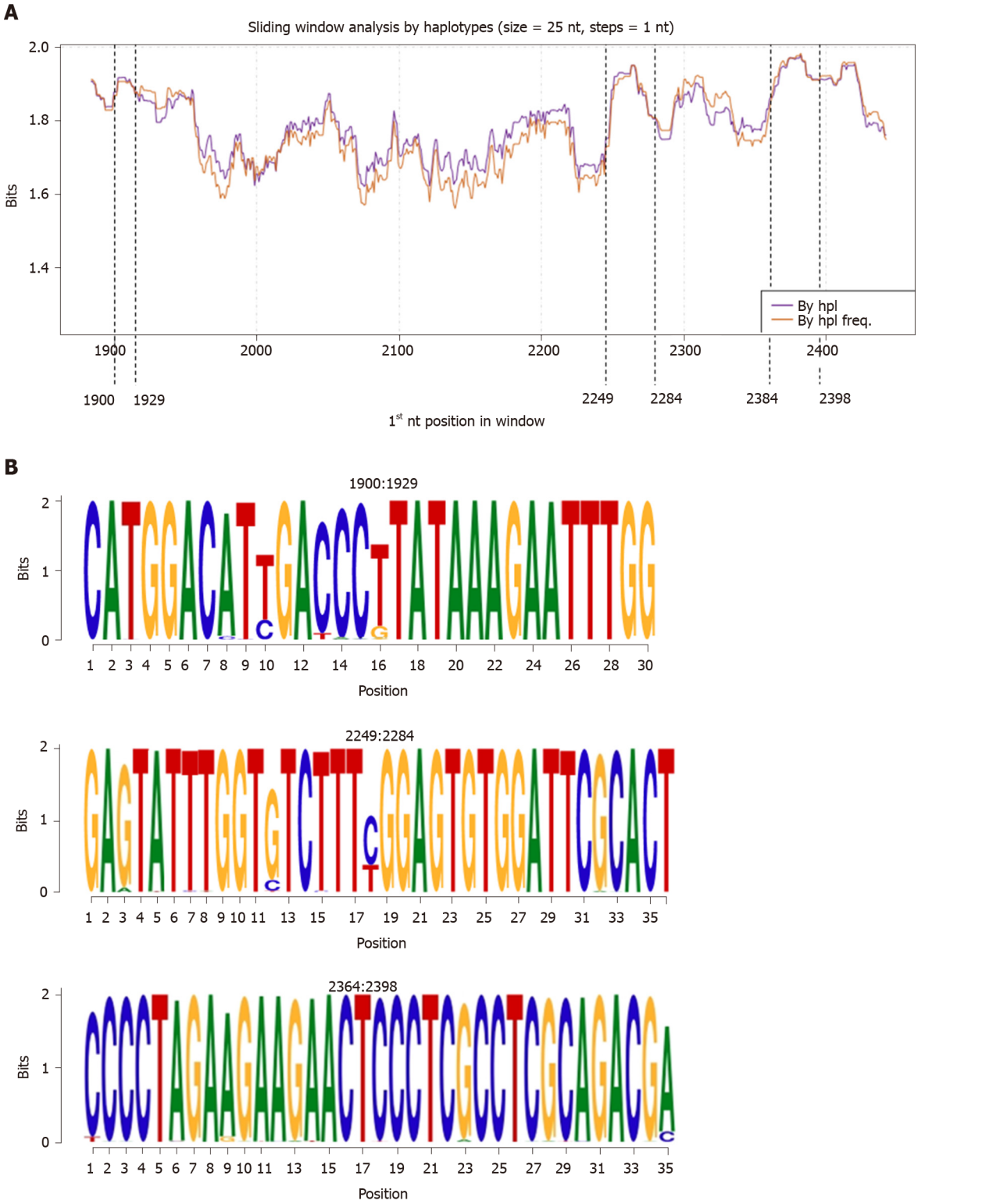

Figure 2 Information content analysis at nucleotide level. A: Sliding window analysis of Hepatitis B core gene performed by aligning the quasispecies haplotypes for all 38 patients with and without considering their relative frequency. Each point on the graph represents the mean information content (in bits) of the 25-nucleotides windows, with forward displacement of 1 nucleotide step between windows. The purple line shows the analysis by haplotype (By hpl), which is the mean information content obtained from the multiple alignments of all quasispecies haplotypes. The orange line represents the analysis by haplotype frequency (By hpl freq), which is the mean information content from the multiple alignments of all the patients' quasispecies haplotypes considering their relative frequency. The dashed lines indicate the 3 common hyper-conserved regions observed, with reporting of their positions. B: Representation of detected hyper-conserved regions as sequence logos (with reporting of nucleotide positions). The relative sizes of the letters in each stack indicate their relative frequencies at each position within the multiple alignments of nucleotide haplotypes. The total height of each stack of letters depicts the information content of each nucleotide position, measured in bits (Y-axis): from minimum (0) to maximum conservation (2). By hpl: Analysis by haplotype; By hpl freq: Analysis by haplotype frequency; nt: Nucleotide.

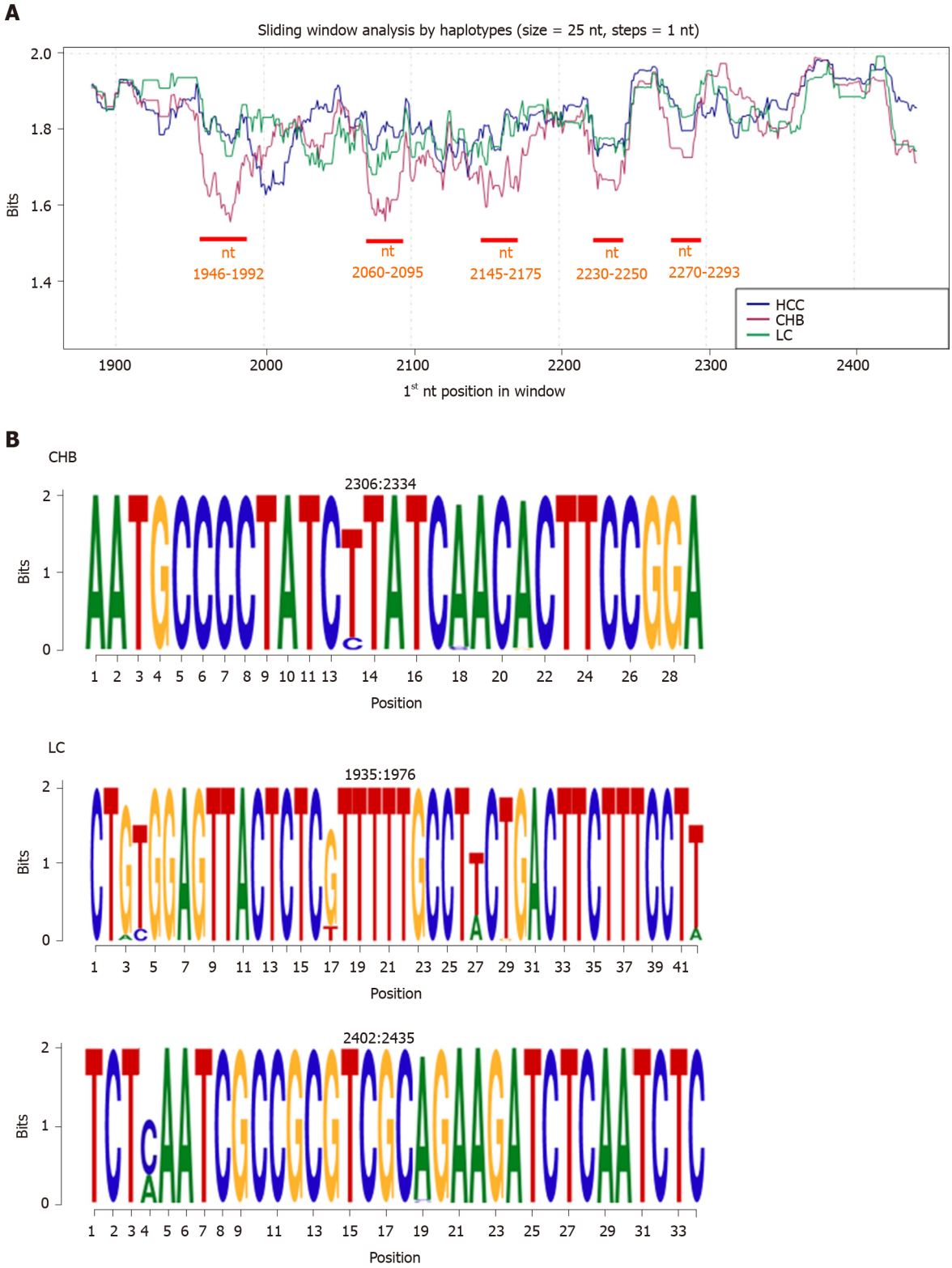

Figure 3 Information content analysis at nucleotide level by clinical stage group. A: By-haplotype sliding window analysis of the Hepatitis B core gene according to different clinical groups (HCC in blue, CHB in red, and LC in green). The portions and positions where CHB showed lower levels of conservation than the others ($P < 0.05$) are shown in red. B: Representation of the information content of CHB- and LC-specific conserved nucleotide regions as sequence logos. Positions are reported at the top of each logo. CHB: Chronic hepatitis B infection without liver damage; HCC: Hepatocellular carcinoma; LC: Liver cirrhosis; nt: Nucleotide; *P*: *P* value.

strategy.

At the aa level, we observed 2 common hyper-conserved regions (aa 117-120 and 159-167), which fell into the second and third hyper-conserved nt portions (nt 2249-2284 and 2364-2398 respectively). The CTD plays a key role in HBc function. It contains the 4 arginine-rich domains (RRR aa 150-152, RRR aa 157-159, RRRR aa 164-

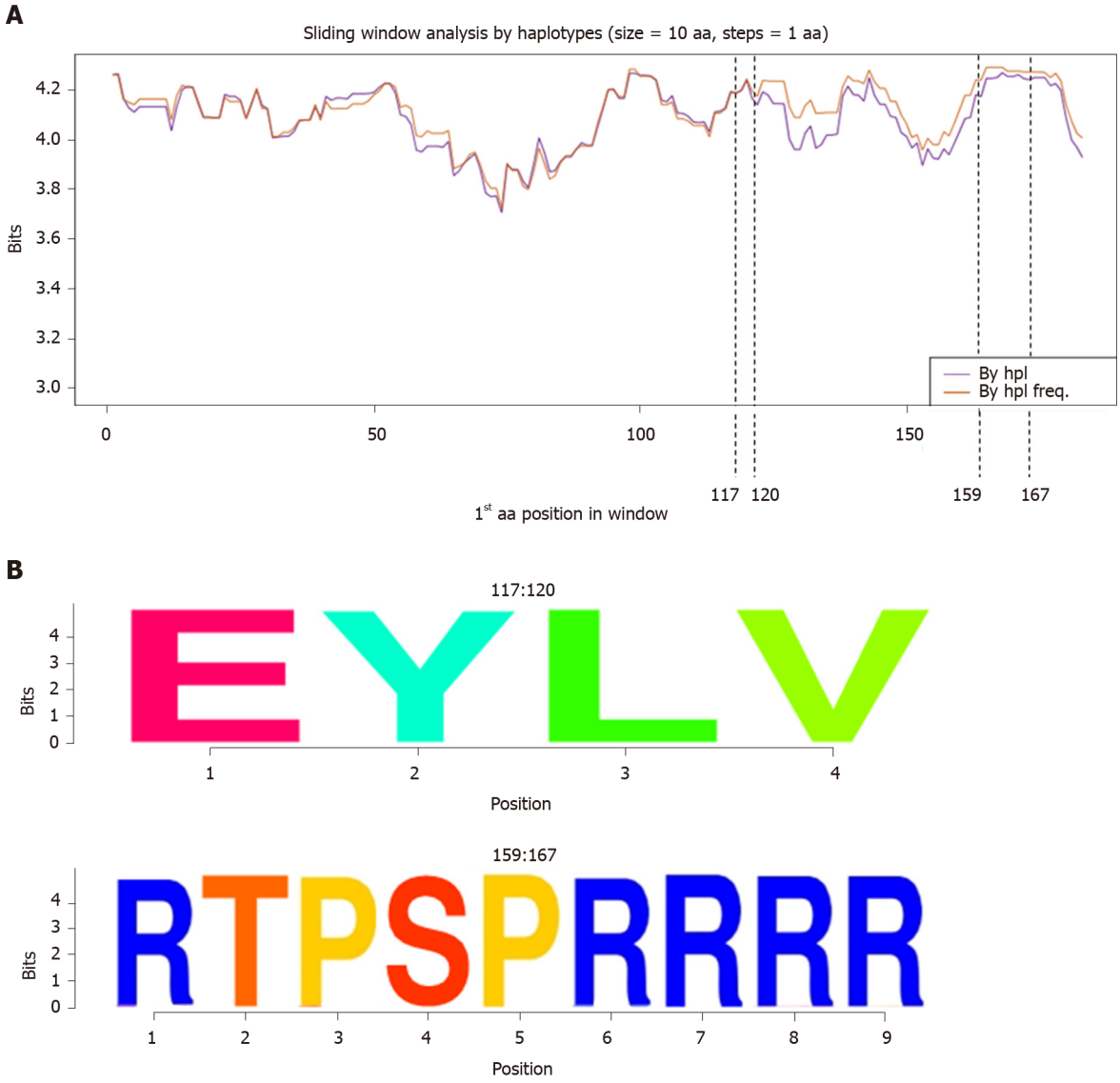

Figure 4 Information content analysis at amino acid level. A: Sliding window analysis of the Hepatitis B core protein sequence for all 38 patients with and without consideration of relative frequency. Each point on the graph is the result of the mean information content (in bits) of the 10-amino acid in size windows, with forward displacement between them of 1 amino acid step. The purple line represents the information content of all the quasispecies haplotypes (By hpl) whereas the orange line indicates the information content considering haplotype frequency (By hpl freq). The dashed lines show the 2 common amino acid hyper-conserved regions observed, with reporting of their positions. B: Representation of amino acid hyper-conserved regions detected as sequence logos (with reporting of amino acid positions). The relative sizes of the letters in each stack indicate their relative frequencies at each position within the multiple alignments of amino acid haplotypes. The total height of each stack depicts the information content of each amino acid position, measured in bits (Y-axis); range: 0 bits (0% conservation) to 4.32 bits (100% conservation). By hpl: Analysis by haplotype; By hpl freq: Analysis by haplotype frequency; aa: Amino acid.

167, and RRRR aa 172-175) that guarantee adequate protein subcellular localization acting as nuclear or cytoplasmic localization signals[27]. The second hyper-conserved aa region (aa 159-167) included one of these arginine-rich domains.

The high degree of sequence conservation observed in HBc may be indicative of its importance in protein function, positioning it as a possible target for diagnostic and therapeutic strategies. Recent studies have defined HBV core-related antigen (HBcrAg, which consists of HBc, HBeAg, and HBV p22 protein) as a promising serological viral marker, particularly for patients with low viral loads, such as treated patients[28] and patients with chronic HBeAg-negative infection[29]. This potential marker, however, has some limitations related to its high limits of detection (2 log IU/mL) and quantification (3-7 log IU/mL). The hyper-conserved regions observed in our study could be used as targets to improve HBc detection technology.

Aptamers are emerging as a promising diagnostic and therapeutic option for different diseases[30]. These molecules consist of single-strand DNA or RNA with high affinity and specificity and no toxicity or immunogenicity[31]. *In vitro* testing of an aptamer generated using the matrix domain of HBV (located in the large surface protein L and related to the nucleocapsid envelope) resulted in a 50% decrease in

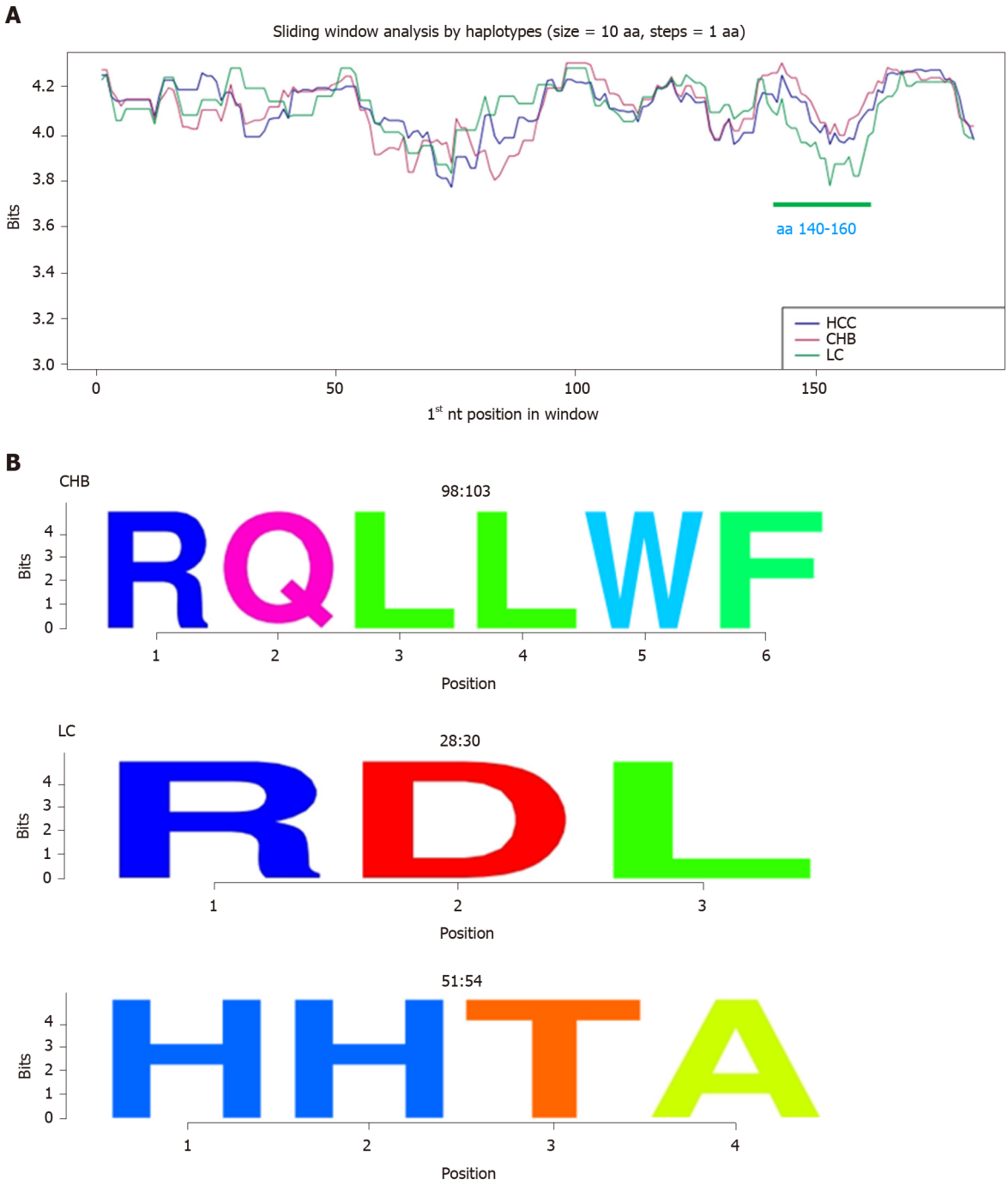

Figure 5 Information content analysis at amino acid level by clinical group. A: Sliding window analysis of the Hepatitis B core protein by haplotype between the different clinical groups (HCC in blue, CHB in red, and LC in green). The green horizontal line corresponds to the region where LC group is less conserved compared to the CHB and HCC groups ($P < 0.05$). B: Representation of CHB- and LC-specific conserved amino acid regions as sequence logos. Positions are reported at the top of each logo. CHB: Chronic hepatitis B infection without liver damage; HCC: Hepatocellular carcinoma; LC: Liver cirrhosis; aa: Amino acid; *P*: *P* value.

HBV titre in treated cell supernatants[32]. In another study, an aptamer targeting *HBC* resulted in a reduction in extracellular HBV DNA by interfering with nucleocapsid assembly[31]. Again, the hyper-conserved regions detected in our study could be novel targets for aptamer-based strategies that might work independently of clinical stage or HBV genotype. They could be also used to elaborate a new HBV detection system, as has been done with hepatitis C virus[33] and syncytial viruses[34].

On analyzing nt and aa conservation in relation to clinical stage of HBV infection, all 3 groups showed similar patterns at the aa level, although the HBV quasispecies in the LC group was slightly less conserved (mainly between aa 140-160). At the nt level, conservation was lower in the CHB group than in the other 2 groups, largely in the 5 regions between nt 1946-1992, 2060-2095, 2145-2175, 2230-2250, and 2270-2293. This finding could be consistent with the high replication rate of HBV during this clinical stage. Moreover, the first variable region (nt 1946-1992) includes three CD8 HLA

Table 3 Relative frequencies of nucleotide insertions/deletions detected

Clinical stage (*n*/total)	Patient	Relative frequency (% of mutated haplotypes)	
		1951 (1 nt: T)	2085 (1 nt: G)
CHB (8/16)	1	8.36 (8.7)	
	2		17.12 (40)
	3		3.19 (5)
	4	0.37 (5.9)	
	9	2.02 (8.82)	
	10		1.34 (50)
	12		1.04 (10)
	13	2.79 (22.22)	
HCC (2/17)	28		0.78 (4)
	33		2.42 (4.8)
LC (1/5)	34		17.42 (19.2)

The table shows the relative frequency of insertions/deletions, together with the percentage (%) of mutated haplotypes per patient. Only patients carrying these mutations were included in the table. CHB: Chronic hepatitis B infection without liver damage; HCC: Hepatocellular carcinoma; LC: Liver cirrhosis; T: Thymine; G: Guanine; nt: Nucleotide.

epitopes (epitopes B5101, B3501, and B0702 at nt positions 1958-1982)[26], suggesting an attempt at immune evasion. Although the CHB group had the lowest levels of sequence conservation, we detected 2 group-specific conserved regions: aa 98-103 and nt 2306-2334. The nt region included the first 5 aa of the linker region, suggesting thus an important role for this region, which is involved in capsid assembly[35,36] and viral DNA synthesis[36]. In the LC group we detected 2 exclusively conserved nt regions (nt 1935-1976 and 2402-2435, which would translate respectively to aa 11-25 and 167-178) and 2 exclusively conserved aa regions (aa 28-30 and 51-54). The first related regions (nt 1935-1976 and aa 28-30) included portions of HBc (aa 14-18 and aa 23-39 respectively) that are involved in capsid assembly and envelopment and virion production[37], highlighting the importance of these functions in LC. The second LC-specific nt region (nt 2402-2435) contained an arginine-rich domain of the CTD when translated.

The identification of group-specific conserved regions suggests different evolutionary histories that may have different effects on disease progression. Further studies, however, are needed to prove the association between these regions and different clinical stages and to investigate their role in liver disease progression.

Considering the risk and severity of disease progression, identification of prognostic factors would be of great help. A number of studies have focused on detecting aa changes possibly related to different clinical stages. The mutations T1753C and A1762T/G1764A (K130M/V131I in HBx) of basal core promoter, for example, were identified as possible prognostic markers for HCC[38,39], while HBc aa mutations F24Y, E64D, E77Q, A80I/T/V, L116I, and E180A were linked to the development of cirrhosis and HCC[40]. In our study, one of the aa changes detected, P79Q, was exclusively observed in the HCC group. Mutations at this position have been found to be slightly associated with tumour relapse after resection[41]. More *in vitro* studies are required to investigate the role of the P79Q mutation in liver disease progression.

One limitation of our study is that we were not able to include large numbers of patients with different stages of liver disease due to the limits of PCR detection. This was particularly evident in the LC group, which was very small. Larger samples are needed to confirm our results. Moreover, although the Illumina MiSeq platform offers long read lengths, they are not sufficient to cover the entire *HBC* gene, making it necessary to divide it into 2 partially overlapping amplicons. Nonetheless, these 2 fragments were treated as independent samples during sequencing and subsequently analysed as such.

In summary, we have identified a number of nt and aa hyper-conserved regions that could be valuable targets for new therapeutic and diagnostic strategies. The role of group-specific conserved regions in liver disease progression requires further analysis. The P79Q substitution could be a possible prognostic factor for HCC. *In vitro* studies, however, are required to determine whether this change might affect viral

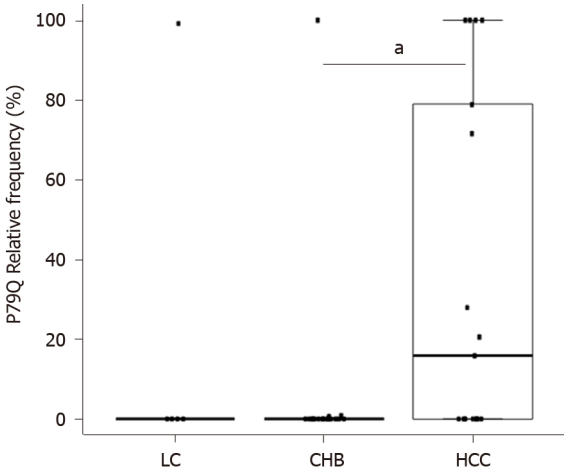

Figure 6 Relative frequency of P79Q substitution in the 3 clinical groups. Each dot represents a patient. The Bonferroni-corrected *P* value was calculated by Kruskal-Wallis test with posthoc Dunn multiple comparison test. ($^{a}P < 0.05$). CHB: Chronic hepatitis B infection without liver damage; HCC: Hepatocellular carcinoma; LC: Liver cirrhosis; *P*: *P* value; P79Q: Proline to glutamine in position 79.

replication and to investigate associations between cellular damage and onset of HCC.

ARTICLE HIGHLIGHTS

Research background

Despite the existence of effective preventive vaccines, an estimated 257 million people worldwide live with chronic hepatitus B virus (HBV) infection and more than 880000 people die due to the development of liver cirrhosis and/or hepatocellular carcinoma. Although infection can be controlled with existing treatment, eradication is currently impossible due to the persistence of covalently closed circular DNA in hepatocyte nuclei that acts as a template for viral expression. New therapeutic approaches are needed, and gene therapy has been proposed as one of the most promising options. HBV core protein [encoded by the HBV core gene (*HBC*)] is a structural protein with functional activity that has a key role in viral replication and disease progression. Accordingly, it could be a potential target for new therapeutic and diagnostic strategies, and its variability could be a valuable prognostic factor for disease progression.

Research motivation

As eradication of HBV infection is currently unachievable, new therapeutic strategies are necessary. Moreover, current treatments cannot interfere with the expression of viral proteins that can favor disease progression. Gene therapy based on silencing RNA is one of the most promising therapeutic approaches currently under investigation. The identification of hyper-conserved regions in key viral genes and proteins (such as *HBC*) is essential to orchestrate an effective strategy regardless of clinical stage or viral genotype.

Research objectives

This study aimed to identify, by next-generation sequencing, hyper-conserved regions in *HBC* quasispecies of patients with different clinical stages of chronic HBV infection that could be a valuable target for gene therapy. Considering the essential role of the *HBC* gene and its encoded protein HBV core protein in HBV infection, changes in gene and protein conservation in specific clinical groups could be determining factors in disease progression and hence serve as prognostic factors for clinical follow-up.

Research methods

The *HBC* gene was amplified by a 3-nested PCR protocol and later sequenced by next-generation sequencing (MiSeq, Illumina, United States) in 38 HBV-monoinfected chronic patients [16 with chronic hepatitis B infection without liver damages (CHB group), 5 with liver cirrhosis (LC group) and 17 with hepatocellular carcinoma (HCC group)]. Quasispecies sequences were genotyped by distance-based discriminant analysis, and general and intergroup nucleotide (nt) and amino acid (aa) conservation was determined by sliding window analysis. The presence of nt insertion and deletions and/or aa substitutions in the different groups was determined by aligning the sequences with a genotype-specific consensus sequence.

Research results

Three nt (nt 1900-1929, 2249-2284, 2364-2398) and two aa (aa 117-120, 159-167) hyper-conserved regions shared by all the clinical groups were identified. By comparing gene and protein conservation between the different clinical groups, a similar pattern of conservation was observed, although CHB showed five nt less conserved regions (nt 1946-1992, 2060-2095, 2145-

2175, 2230-2250, 2270-2293) and LC one aa less conserved region (between aa 140 and 160). Moreover, some group-specific conserved regions were detected at both nt (nt 2306-2334 in CHB and 1935-1976 and 2402-2435 in LC) and aa (aa 98-103 in CHB and 28-30 and 51-54 in LC) levels. No differences in indel frequency were observed between the clinical groups. Contrarily, we identified an aa substitution (P79Q) that was more frequent in HCC [median (interquartile range) frequency of 15.82 (0-78.9) *vs* 0 (0-0) for the other groups; $P < 0.05$ *vs* the CHB group].

Research conclusions

We have identified a number of nt and aa regions that were highly conserved in the presence of different viral genotypes and clinical stages. These could be valuable targets for future pangenotypic and panclinical therapeutic and diagnostic strategies. The different clinically related conserved regions and the P79Q aa substitution could potentially be used as prognostic factors for disease progression.

Research perspectives

Our findings could guide the creation of a new gene therapy strategy based on RNA silencing. In-depth analysis of group-specific conserved or variable regions and their role in disease progression is needed. Further *in vitro* studies are required to determine whether the P79Q aa substitution might affect viral replication and to investigate associations between cell damage and onset of HCC.

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ORIGINAL ARTICLE

Basic Study

Sleeve gastrectomy ameliorates endothelial function and prevents lung cancer by normalizing endothelin-1 axis in obese and diabetic rats

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Abstract

BACKGROUND

Previous evidence has implied that obesity is an independent risk factor for developing cancer. Being closely related to obesity, type 2 diabetes mellitus provides a suitable environment for the formation and metastasis of tumors through multiple pathways. Although bariatric surgeries are effective in preventing and lowering the risk of various types of cancer, the underlying mechanisms of this effect are not clearly elucidated.

AIM

To uncover the role and effect of sleeve gastrectomy (SG) in preventing lung cancer in obese and diabetic rats.

METHODS

SG was performed on obese and diabetic Wistar rats, and the postoperative transcriptional and translational alterations of the endothelin-1 (ET-1) axis in the lungs were compared to sham-operated obese and diabetic rats and age-matched healthy controls to assess the improvements in endothelial function and risk of developing lung cancer at the postoperative 4th, 8th, and 12th weeks. The risk was

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Data sharing statement: Research data of the current study is available at the Mendeley Data Depository, under: Ruzi, Rishat; Xiong, Yacheng; Li, Jianwen; Zhong, Mingwei; Xu, Qian; Yan, Zhibo; Zhu, Jiankang; Cheng, Yugang; Hu, Sanyuan; Zhang, Guangyong (2020), "Data for: Sleeve Gastrectomy Ameliorates Endothelial Function and Prevents Lung Cancer by Normalizing Endothelin-1 Axis and Reduces DNA damage in Obese and Diabetic Rats", Mendeley Data, v3. http://dx.doi.org/10.17632/vj5469pmv6.3

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also evaluated using nuclear phosphorylation of H2A histone family member X as a marker of DNA damage (double-strand break).

RESULTS
Compared to obese and diabetic sham-operated rats, SG brought a significant reduction to body weight, food intake, and fasting blood glucose while improving oral glucose tolerance and insulin sensitivity. In addition, ameliorated levels of gene and protein expression in the ET-1 axis as well as reduced DNA damage indicated improved endothelial function and a lower risk of developing lung cancer after the surgery.

CONCLUSION
Apart from eliminating metabolic disorders, SG improves endothelial function and plays a protective role in preventing lung cancer *via* normalized ET-1 axis and reduced DNA damage.

Key words: Sleeve gastrectomy; Lung cancer; Endothelin-1 axis; Endothelial dysfunction; DNA damage; Obesity

Core tip: To explore the potential mechanism of bariatric surgery to reduce the risk of cancer, sleeve gastrectomy (SG) was performed on obese and diabetic rats. As a result, with a disrupted endothelin-1 axis, sham-operated subjects manifested deteriorated endothelial function and an increased risk of developing cancer compared to the healthy controls. However, far more than improving glycometabolism, SG reversed these negative effects by normalizing the endothelin-1 axis and reducing DNA damage, which contributed to the effects of SG to ameliorate endothelial function and prevent lung cancer.

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INTRODUCTION

Emerging evidence implies that obesity is an independent risk factor for multiple types of cancer[1], and type 2 diabetes mellitus (T2DM), an obesity-related concomitant disease, can favor a suitable environment for tumor formation, progression, and metastasis by influencing several biochemical and physiological factors, such as adipokines, inflammatory mediators, and altered microbiome[2]. Endothelial dysfunction (ED) is a complication of both obesity[3] and T2DM[4], which refers to a proinflammatory and prothrombotic state[5], and is capable of impairing blood vessels, causing cardiovascular disease and end-organ damage. Fundamentally speaking, the anomaly of endothelins (ETs) is caused by the disrupted ET axis. ETs are a group of proteins comprising three 21-amino acid peptides (ET-1, ET-2, and ET-3), two distinct rhodopsin-like G protein-coupled receptor subtypes (ET-A and ET-B), and ET-converting enzymes (ECEs), which all jointly facilitate the generation of biologically active ETs[6]. Previous research has indicated that the ET axis effects numerous signaling pathways involved in the mediation of apoptosis and growth in different cells[7]. In cancer cells, the ET axis activates autocrine/paracrine feedback loops, promoting the development and progression of tumors[8], such as lung cancer[9], *via* several different mechanisms[10].

Among the commonly known isoforms of ETs, ET-1 is the most clinically relevant which has the highest expression[11] and makes a significant contribution to ED in many ways[5]. ET-1 is originally secreted by endothelial cells through both constitutive and regulated (or rapid release) pathways[12], of which the downstream effects are regulated by ET-A and ET-B[6]. ET-A is mainly expressed in vascular smooth muscle

cells, while ET-B is expressed in endothelial cells, vascular smooth muscle cells, macrophages, and platelets[13]. Several processes activated by the non-linear signaling network of extracellular binding of ET-1 to ET-A, for instance, cell proliferation, apoptosis, cell invasion and metastasis, angiogenesis, osteogenesis, and nociception[14-16], are involved in normal cell function as well as the development and progression of cancer. Hence, due to an organic abundance in both ET-A and ET-B, ET-1 plays various roles in mediating pulmonary carcinogenesis[17-19], and ED is a predictor of the diagnosis and prognosis of lung cancer[20]. Meanwhile, as a common but sometimes fatal lesion in normal cells, DNA damage can lead to canceration, where the double-strand break is deemed the most harmful type, resulting in the general and instant DNA damage response in cells and the phosphorylation of Ser-139 of the H2A histone family member X (H2AX) at the site of DNA damage (γ-H2AX foci)[21]. Hence, marking nuclear γ-H2AX in a certain cell population is considered a brilliant way of recognizing early DNA damage, which has been frequently applied in cancer studies[22-24].

Bariatric surgeries are universally performed as an effective treatment for severe obesity, T2DM, and other related comorbidities[25]. Besides, whether in clinical trials or animal studies, they have been shown to prevent or lower the risk of cancers[26-29], including lung cancer[30]. Unfortunately, compared to these apparent effects, little is known about the underlying mechanisms of lowered cancer risk after bariatric surgeries. Thus, based on the scarcity of relevant studies and the negative impacts of obesity and T2DM on endothelial function, tumor development, and the essential role of ET-1 axis in tumor pathogenesis of the lung, we speculated that there may be a correlation between the ET-1 axis with ameliorated endothelial function and lowered risk of cancer following bariatric surgeries. Herein, using an obese and diabetic rat model induced with a high-fat diet and streptozotocin, we investigated the potential effect of sleeve gastrectomy (SG) in improving endothelial function and lowering the risk of carcinogenesis by normalizing the ET-1 axis and ameliorating DNA damage.

MATERIALS AND METHODS

Animals and study design

The protocol of the current study is shown in Figure 1. Seventy male Wistar rats (200 g average body weight; SPF Biotechnology Co., Ltd., Beijing, China) were housed in independent ventilated cages under constant ambient temperature (24-26 °C) and humidity (50%-60%) in a 12-h light/dark cycle. The study protocol was approved by the Institutional Animal Care and Use Committee of The First Affiliated Hospital of Shandong First Medical University (China), and every effort was made to minimize the pain and discomfort of the study subjects. And in accordance with the 3R principle of humane experimental technique[31] to minimize the number of animals used, five animals were included in each sub-group that was divided by the postoperative time points (every 4 wk, see below), and to meet this sample size, animals were unequally and randomly divided into the following three groups based on their differences in postoperative mortality estimated during preliminary experiment (data not shown).

Group 1 representing control (C; n = 15): Animals were given a standard diet (15% of calories as fat; Laboratory Animal Center of Shandong University, Shandong, China) during the whole protocol, with no intervention made.

Group 2 representing sham-operated control (SH; n = 22): Animals were fed a high-fat diet (40% fat, 42% carbohydrate, and 18% protein, as a total percentage of calories; Xietong Pharmaceutical Bio-engineering Co., Ltd., Jiangsu, China) for 8 wk and then received intraperitoneal injection of streptozotocin at a dose of 30 mg/kg body weight (0.01 mol/L citrate buffer, pH 4.5; Sigma-Aldrich, St. Louis, MO, United States) to induce hyperglycemia, followed by a sham surgery 1 wk later. In this group, two rats were excluded due to unaltered blood glucose after injection [fasting blood glucose (FBG) < 11.1 mmol/L for 3 d in a row]. Ultimately, 20 rats were qualified for further processing.

Group 3 representing SG-operated (n = 33): T2DM was induced exactly the same as the SH group, followed by SG at 1 wk after injection. For this group, three rats were excluded according to the same criteria as above.

Surgical procedures

Operations were performed following overnight fasting, with anesthesia being achieved by applying gaseous anesthesia (2% isoflurane). SG was performed as previously described[32]. Briefly, it involved the following steps: (1) A 4-cm midline epigastric incision was made to identify the structures; (2) The gastric omentum was

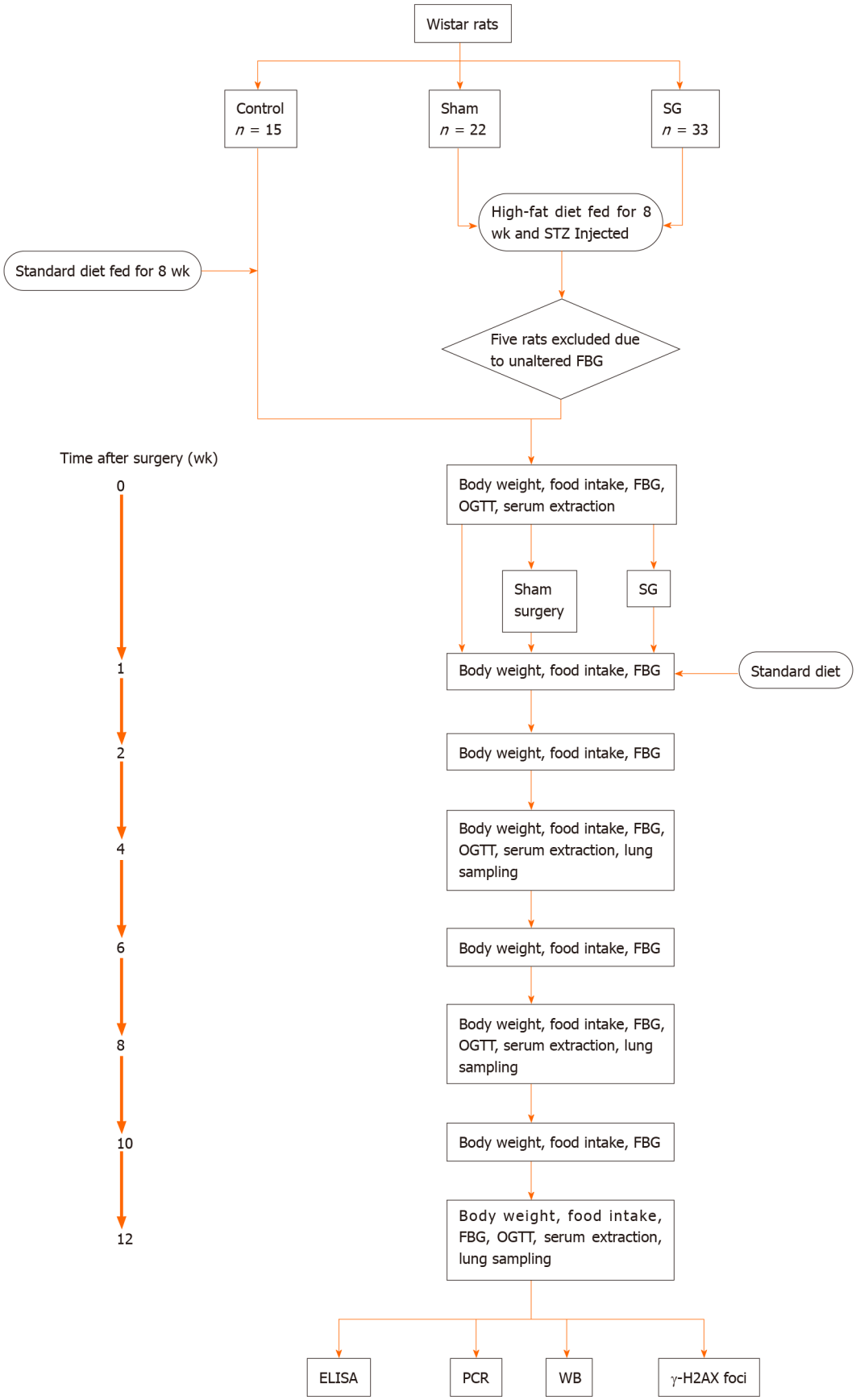

Figure 1 Flow chart of the study protocol. ELISA: Enzyme-linked immunosorbent assay; FBG: Fasting blood glucose; OGTT: Oral glucose tolerance test; PCR: Polymerase chain reaction; SG: Sleeve gastrectomy; STZ: Streptozotocin; WB: Western blot; γ-H2AX foci: H2A histone family member X focus assay.

dissected and the gastric cardium was disclosed; (3) The short gastric vessels, related gastroepiploic vessels, and branches of the left gastric vessels in the greater curvature were ligated and transected using 7-0 silk suture (Ningbo Cheng-He Microsurgical Instruments Factory, Zhejiang, China); (4) The gastric fundus and a large portion of the gastric body were removed; and (5) The residual stomach was closed using 5-0 silk suture (Ningbo Cheng-He Microsurgical Instruments Factory).

Laparotomy was performed on the SH rats to expose the stomach, esophagus, and small intestine. No other intervention was made. Furthermore, operative time was prolonged to induce a degree of comparable anesthetic stress that experienced by the SG rats.

For postoperative care, mixed tap water (4 g of white granulated sugar, 0.25 g of cefuroxime, and 0.4 g of ibuprofen dissolved in 500 mL of tap water) was given to rats in the SH and SG groups at 24 h after the operation for 3 d. Then, approximately 7 g of Total Nutrition Formula for Dietary Fiber (Nutren Fibre; Nestlé Health Science, Epalinges, Switzerland) was dissolved in 500 mL tap water and given for another 3 d. From postoperative day 7, all operated animals were given free access to a standard diet and tap water until the end of the protocol, same as the C group as abovementioned.

All C rats survived until the end of the protocol. Two rats in the SH group died of hyperglycemia during the postoperative period of 3 mo. Eighteen rats in the SG group survived, while the other 12 died of the following causes: Gastric leakage (n = 3), infection (n = 3), intestinal obstruction (n = 4), and unknown cause (n = 2; inconclusive on autopsy).

In all, 15, 18, and 18 rats survived in the C, SH, and SG groups, respectively, and to favor an equal sample size for each group, five rats in each group were randomly euthanatized by applying an overdose of 10% chloral hydrate (5 mL/kg) every 4 wk after operation. Hence, 15 rats of each group were included in this study. Body weight and food intake were monitored before the surgery and weekly in the first 2 wk after surgery, and then once in every 2 wk until the end of the protocol at the 12th week after the operation. Blood samples were collected before the surgery and mensal euthanasia, followed by tissue sampling and preparation for further testing.

Blood chemistry

FBG level was measured with a glucometer (Roche OneTouch Ultra®; LifeScan, Johnson and Johnson, Milpitas, CA, United States) at the abovementioned time intervals, same as measurements of body weight and food intake. Blood was collected from the tail vein in unconscious rats and samples were centrifuged at 3000 rpm for 8 min; the serum was then extracted and stored at -80 °C. Serum insulin was measured with ELISA kits (CUSABIO, Hubei Province, China). Furthermore, the homeostasis model assessment of insulin resistance (Homa-IR) was used to estimate the degree of insulin resistance, which was calculated with the following formula: Homa-IR = fasting serum insulin (mIU/L) × FBG (mmol/L)/22.5[33].

Oral glucose tolerance test

Oral glucose tolerance test (OGTT) was performed before the operations and at 4, 8, and 12 wk after surgery. After an overnight fast, rats were administered with 1 g/kg glucose by oral gavage. Blood glucose was measured in conscious rats at 0, 10, 30, 60, and 120 min after the gavage. Area under the curve for OGTT was calculated by the trapezoidal method.

Quantitative real-time polymerase chain reaction

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, United States). The cDNA was synthesized using random primers (Servicebio, Hubei, China) and Revert Aid First Strand cDNA Synthesis Kit (#K1622; Thermo Fisher Scientific, Waltham, MA, United States). Quantitative real-time polymerase chain reaction (PCR) was performed on a Cycler System (StepOne Plus; Applied Biosystems Inc., Foster City, CA, United States), with the analyses being performed using the FastStart Universal SYBR Green Master (Servicebio) in a total PCR reaction containing 25 μL of quantitative PCR Mix, 2.0 μL of 7.5 μmol/L primers, 2.5 μL of reverse transcription product, and 8.0 μL of double-distilled H_2O. The specific sequences of the primers for analyzed genes are shown in Table 1.

Western blot analysis

Lung tissues were homogenized and centrifuged at 12000 rpm for 10 min. Supernatants were extracted and the protein concentration was determined with a BCA kit (G2026; Servicebio). Protein solution was added into the loading buffer (G2013; Servicebio) in a ratio of 4:1 and denatured in boiling water for 15 min. Samples were loaded on 5% SDS-PAGE gels (Servicebio), then separated by

Table 1 Specific sequences of primers used in polymerase chain reaction analysis

Gene	Primer sequence, 5'-3'	GenBank No.	Length, bp	Annealing temperature, °C
GAPDH	F: CTGGAGAAACCTGCCAAGTATG	NM_017008.4	138	60
	R: GGTGGAAGAATGGGAGTTGCT			
ET-1	F: TCTGCCACCTGGACATCATCTG	NM_012548.2	205	60
	R: CTTTGGGCTCGGAGTTCTTTGT			
ET-A	F: ACCGCCATTGAAATTGTCTCC	NM_012550.2	173	60
	R: AGCCACCAGTCCTTCACGTCTT			
ET-B	F: CCAAAGACTGGTGGCTGTTCA	XM_006252431.3	183	60
	R: CAAACACGAGGACCAGGCAG			
ECE-1	F: CACAACCAAGCCATCATTAAGC	NM_053596.2	275	60
	R: TTGGAGTCGGCACTGACATAGA			

F: Forward; R: Reverse.

electrophoresis and transferred onto polyvinylidene fluoride membranes (Millipore, Burlington, MA, United States). After being blocked in 5% fat-free milk for 1 h, membranes were incubated overnight with the following primary antibodies: ET-1 (ab117757; Abcam, Cambridge, United Kingdom), ET-A (ab85163; Abcam), ET-B (ab262694; Abcam), ECE-1 (sc-376017; Santa Cruz Biotechnology, Dallas, TX, United States), and GAPDH (ab9485; Abcam). The next day, the membranes were incubated in horseradish peroxidase-conjugated secondary antibodies, which were diluted 3000 times with Tris-buffered saline Tween-20. Bands were visualized with ECL solution (Servicebio) and the band intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, MA, United States).

Immunohistochemical analysis of γ-H2AX

Paraffin sections (5 μm) of the lungs were deparaffinized, rehydrated in a solution series (xylene 15 min thrice, absolute ethyl alcohol 5 min twice, 85% ethanol 5 min once, and 75% ethanol 5 min once), and washed with distilled water. Then, they were put into citrate antigen retrieval solution (pH 6.0), followed by a 3% hydrogen peroxide solution and incubation in darkness at room temperature for 25 min. Afterwards, the slides were placed in phosphate-buffered saline (pH 7.4) and washed thrice on the decolorization shaker (5 min each) to block the endogenous peroxidase activity after deparaffinization and antigen retrieval. Anti-histone γ-H2AX antibody (ChIP grade, 1:2000, ab20669; Abcam) was used and an appropriate elite horseradish peroxidase-diaminobenzidine system was applied to reveal antibody. The sections were counterstained with hematoxylin. Finally, the sections were dehydrated through an alcohol and xylene gradient and sealed with neutral gum.

When completed, the sections were made into electronic slides using the Pannoramic Digital Slide Scanners (Pannoramic DESK, P-MIDI, P250 and P1000; 3DHISTECH Ltd., Budapest, Hungary). Then, a slide representing each animal was randomly selected and covered with FOV rectangles in CaseViewer v2.0 (3DHISTECH Ltd.), and five fields at× 800 magnification from different rectangles were chosen for subsequent image analysis using the ImageJ software for semi-quantitative analysis. A γ-H2AX-positive nucleus (*i.e.*, a double-strand break-damaged nucleus within a cell population counterstained by hematoxylin) was easily distinguishable when a homogeneous brown precipitate was observed covering partial or entire nucleus compared to the undamaged nucleus, which was colored blue. Levels of DNA damage in each group at different time points are expressed as the average percentage of γ-H2AX positive (brown-stained) to negative (blue-stained) nuclei of the sections.

Statistical analysis

Results were analyzed using two-way ANOVA with Tukey's multiple comparison tests (GraphPad Prism 8.3; GraphPad, La Jolla, CA, United States). P values < 0.05 were considered significant. Data are expressed as the mean ± SE of the mean.

RESULTS

Metabolic parameters

SG brought a significant improvement to the metabolic disorder (Figure 2). An increase in both body weight and food intake was noted in all the three groups, where C rats had a steady increase but the other two groups (SH and SG) showed a decline during the early postoperative stage (Figure 2A and B, Tables 2 and 3). In terms of the glycemic change (Figure 2C-F, Tables 4-7), the FBG of the C group stayed nearly unaltered, which was verified by OGTT, with a slight increase being noted. Similarly, despite the elevation in the fasting serum insulin level, the Homa-IR of the C animals did not change much, either. The SH group, in contrast, showed a continual FBG increase, which was further reflected by OGTT. Moreover, although the fasting insulin levels of the C group were not significantly different from those of the other two groups at most of the time points, the SH animals demonstrated an aggravated insulin resistance. On the other hand, the rapid reduction in body weight and in food intake was accompanied by the FBG returning to normal in the SG rats; the results of OGTT were nearly the same as in C rats. However, from the middle and late postoperative periods, the FBG of some SG rats began to rise. The fasting serum insulin levels of the SG animals increased after surgery but were not significantly different from those of the other groups. Nevertheless, similar to the results of FBG and OGTT, Homa-IR of the SG rats increased with age but was still much lower than that of the SH group. These results indicated that SG improved glycometabolism and insulin sensitivity in obese and diabetic rats with a rapid weight loss and reduction in food intake.

Gene expression of ET-1 axis

The mRNA expression of genes of the ET-1 axis are shown in Figure 3 and Tables 8-11. On the whole, C rats manifested steady expression in these genes, where an increase was notable in SH animals, even though the differences between the other two groups were not so conspicuous throughout the postoperative period. SG showed an effect of lowering, in other words, normalizing, the overexpressed genes. For *ET-1*, the SG group had lower expression than the C group at postoperative weeks 4 and 8, which was significantly lower than that in SH rats at the 8th and 12th wk after surgery. For *ET-A*, both SH and SG animals had an elevation at the last time point, yet a significant difference existed only between the C and SH animals. Similarly, with an obvious rise in *ET-B* expression at postoperative week 12, the SH group had significantly higher expression than the other two. As for *ECE-1*, an elevation was seen in both the SH and SG groups over time, with the former being significantly higher than the latter at the first time point. The C group was opposite, having much lower expression than the SH group at postoperative weeks 4 and 12. Although not entirely capable of offsetting the gap between C and SH animals at all time intervals, the SG group had normalized abnormalities in the ET-1 axis, indeed.

Protein expression of ET-1 axis

Results of protein expression of the ET-1 axis were not absolutely consistent with those from PCR tests (Figure 4 and Tables 12-15). In terms of the expression of both ET-1 and ET-A, a decrease was seen in the C group, which was opposite in the SG group. The SH group, however, was inconstant in the expression of ET-1 and the same as that in the SG group for ET-A. For both ET-1 and ET-A, the C group had lower expression, which was directionally opposite in the SG group; the SH group, however, was inconstant for the former but the same as in the SG group for the latter. The C group showed nearly steady or slightly increased expression in ET-B and ECE-1 as the animals aged, and the SG group showed gradually decreasing expression in these two proteins. The SH group showed continually increasing expression in ET-B and a maintained high level of ECE-1 expression.

DNA damage

The representative images of the γ-H2AX assay are shown in Figure 5. These images provide an intuitionistic comparison among groups, where it can be seen that there were more γ-H2AX-positive nuclei (red arrows) in the SH group than in the other two groups (Figure 5A). Furthermore, the semi-quantification analysis confirmed that the SH rats had more γ-H2AX-positive nuclei compared to the other two groups (Figure 5B and Table 16), indicating that cells in obese and diabetic rat lungs suffer from much more severe DNA damage than those in healthy and SG-operated rats.

DISCUSSION

Our rat model-based study demonstrated elevated expression of genes and proteins of the ET-1 axis and increased DNA damage in obese and diabetic lungs, indicating a deteriorated endothelial function and a greater risk of cancer development. However,

Table 2 Comparisons of body weight (g) among groups

Time after surgery (wk)				*P* value		
	C	SH	SG	C *vs* SH	C *vs* SG	SH *vs* SG
0	401.7 ± 13.0	507.5 ± 20.4	516.6 ± 20.1	< 0.0001	< 0.0001	0.4768
1	421.5 ± 13.9	464.1 ± 21.0	433.0 ± 25.6	< 0.0001	0.3071	0.0003
2	448.8 ± 13.0	480.8 ± 21.6	419.5 ± 27.6	0.0002	0.0006	< 0.0001
4	481.7 ± 13.9	515.3 ± 23.1	437.5 ± 27.0	< 0.0001	< 0.0001	< 0.0001
6	513.8 ± 14.8	555.5 ± 19.9	460.1 ± 30.2	< 0.0001	< 0.0001	< 0.0001
8	540.2 ± 16.1	580.9 ± 19.1	484.1 ± 30.2	< 0.0001	< 0.0001	< 0.0001
10	560.4 ± 10.6	595.2 ± 15.4	510.6 ± 36.5	0.0284	0.0008	< 0.0001
12	578.6 ± 12.1	602.0 ± 11.2	541.4 ± 35.4	0.1953	0.0174	< 0.0001

Data are expressed as the mean ± SD. C: Control; SH: Sham; SG: Sleeve gastrectomy.

all these alterations were able to be ameliorated by SG, a marvelous treatment for metabolic disorder that is competent in preventing or lowering the risk of various types of cancer as well.

Being crucial for maintaining vascular homeostasis, the endothelium modulates blood flow, nutrient delivery, vascular smooth muscle cell proliferation and migration, fibrinolysis, coagulation, inflammation, and platelet and leukocyte adherence[34]. In contrast, ED, a common complication of both obesity and T2DM, is one of the concrete manifestations of this homeostasis being destroyed, which ultimately results in hypertension, thrombosis, and various cardiovascular diseases[35]. So far, it has been known that adipose tissue inflammation, nitric oxide bioavailability, insulin resistance, and oxidized low-density lipoprotein are the main contributors to obesity-related ED[35]. Under the diabetic condition, ED is mainly caused by hyperglycemia, insulin resistance, abnormal cell growth factors, and vasoactive substances[36]. With its elevated release from adipose tissue, ET-1 exerts an anti-insulin effect by reducing the expression of insulin receptor, insulin receptor substrate-1, and phosphodiesterase-3B, while increasing the expression of ET-B[37]. As a result, more fat deposition is induced by fatty acids released from the visceral adipose tissue because of the insulin resistance[38]. Combined with the dramatically increased protein expression of ET-A, ET-1 leads to more lipolysis *via* the activation of ET-A in adipose tissue[35]. On the other hand, the balance between endothelial-derived relaxation and contraction factors is disrupted in T2DM where the vasodilator factors (*i.e.*, nitric oxide, prostacyclin, and endothelial-derived hyperpolarizing factor) and vasoconstricting factors (*i.e.*, ET-1, angiotensin II, and prostaglandin) are inordinate[36]. ET-1, as one of the serum markers of ED, can be transcriptionally increased by enhanced glycosylated end-products (known commonly as AGEs) in patients with poorly controlled blood glucose[39]. Therefore, ET-1 is considered a predictive factor for diabetic complications[40].

Regarding the close relation of the ET axis with cancer development, former studies have stated that cancers can be divided into three types based upon the expression level of endothelin receptors[41], and lung cancer belongs to the third type based upon its characteristic overexpression of ET-1, ET-A, and ET-B altogether[42], which is in line with our findings. The overexpression of ET-A in cancer has been shown to be associated with invasive biological behavior[43], while the expression of ET-B has been linked to lymphoid infiltration[44]. Considering the important role of the ET-1 axis in carcinogenesis, it has been targeted in pharmacotherapy for cancers, such as ECE inhibition and antagonism of ET-A and ET-B[45]. Although a noncancerous animal model was used, and the similar ET-1 alteration profile may not lead to cancer in these obese and diabetic animals, it is still a perfect example of obesity and T2DM providing a suitable environment for tumor formation. Further comparisons between the SH and SG groups of rats suggested that SG normalized the ET-1 activity profoundly, just as it did in reducing body weight and stabilizing glycometabolism. Consistent with this, it was reported in a clinical trial that SG can reduce plasma ET-1 levels[46]. Moreover, it was suggested by another study, which made a comparison to normalized blood glucose, that weight loss is much more important in the regulation of circulating levels of ET-1 in morbidly obese subjects[47], which also explains why the T2DM in some SG animals seemed to relapse in the middle and late periods after surgery while they still showed positive changes in the ET-1 axis.

DNA damage is a parameter of evaluating the risk of developing cancer, and it

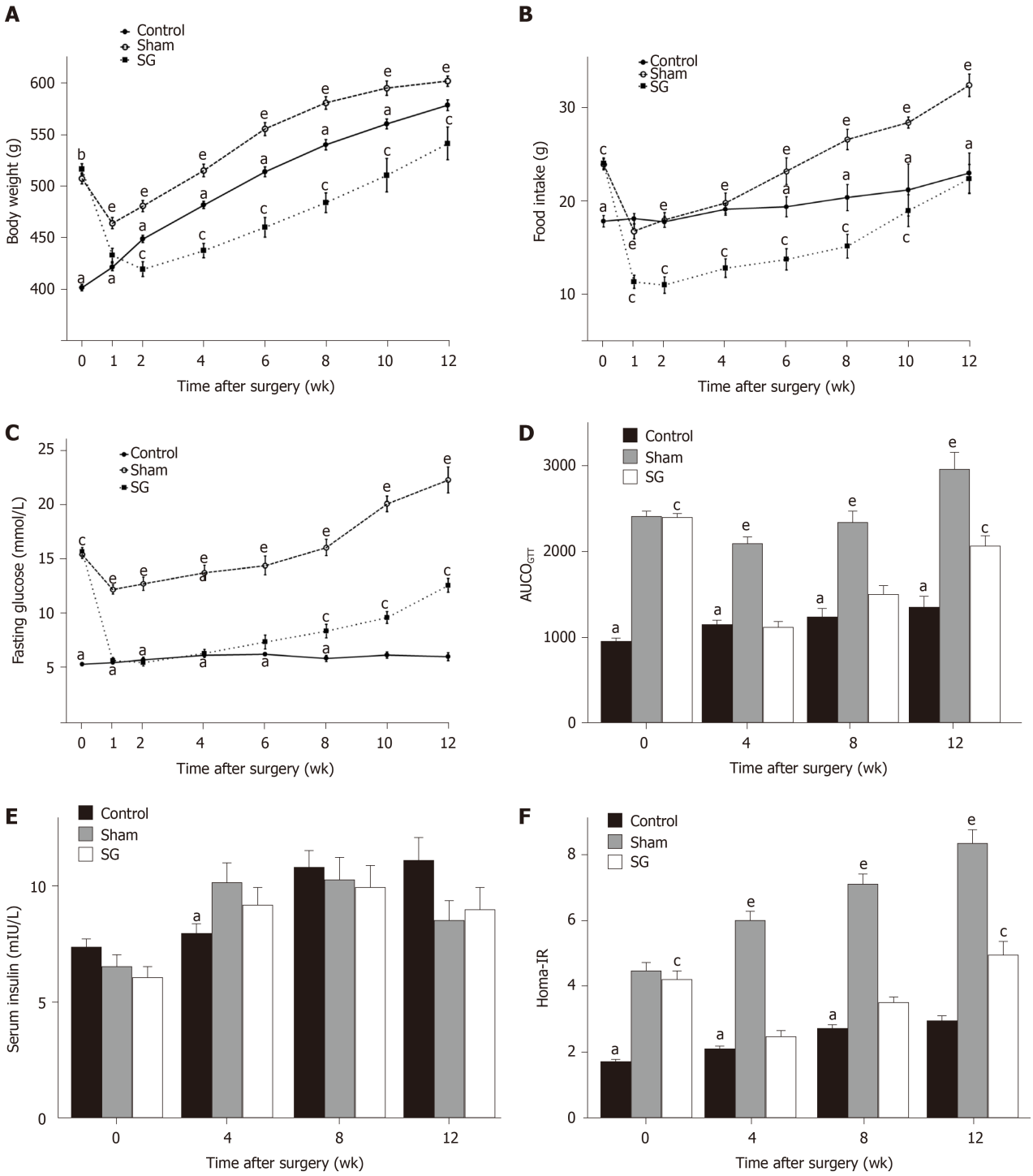

Figure 2 Comparisons of metabolic parameters among groups. A: Body weight; B: Food intake; C: Fasting glucose level; D: Area under the curve of oral glucose tolerance test; E: Fasting serum insulin level; F: Homeostasis model assessment of insulin resistance compared to sham-operated and control rats. [a]*P* < 0.05: Control *vs* sham; [c]*P* < 0.05: Control *vs* sleeve gastrectomy; [e]*P* < 0.05: Sham *vs* sleeve gastrectomy. AUC_{OGTT}: Area under the curve of oral glucose tolerance test; Homa-IR: Homeostasis model assessment of insulin resistance; SG: Sleeve gastrectomy.

affects DNA replication, leading to mutations, and lowers cell metabolism and survival[48]. In obesity, DNA damage is closely related to chronic inflammation, reactive oxygen species, oxidative stress, and cytokines[49]. Meanwhile, the activation of certain oncogenes induced by DNA damage can cause diabetes[50,51]. And as introduced, diabetes is correlated with DNA damage, mutation, and canceration[52]. Since γ-H2AX is a regular marker of DNA damage response, its level can reflect the risk of canceration. A higher level of γ-H2AX was found in precancerous tissues, when compared to that in tumors in a lung cancer rat model induced by chronic inflammation[53]. Likewise, high level γ-H2AX is also related to oxidative stress and type 1 DM in adolescents[54]. While obesity and diabetes injure the lungs by causing

Table 3 Comparisons of food intake (g/d) among groups

Time after surgery (wk)				*P* value		
	C	SH	SG	C *vs* SH	C *vs* SG	SH *vs* SG
0	17.9 ± 2.4	23.9 ± 2.2	24.1 ± 2.2	< 0.0001	< 0.0001	0.9932
1	18.1 ± 2.2	16.8 ± 3.2	11.4 ± 2.7	0.5071	< 0.0001	< 0.0001
2	17.8 ± 2.4	18.0 ± 3.1	11.1 ± 3.4	0.9848	< 0.0001	< 0.0001
4	19.1 ± 2.4	19.8 ± 4.1	12.9 ± 3.8	0.8433	< 0.0001	< 0.0001
6	19.4 ± 3.4	23.2 ± 4.5	13.8 ± 3.6	0.0274	0.0005	< 0.0001
8	20.4 ± 4.5	26.6 ± 3.5	15.2 ± 4.0	< 0.0001	0.0014	< 0.0001
10	21.2 ± 6.2	28.4 ± 1.3	19.0 ± 3.8	0.0018	0.5398	< 0.0001
12	23.0 ± 4.8	32.4 ± 2.7	22.4 ± 3.4	< 0.0001	0.9550	< 0.0001

C: Control; SH: Sham; SG: Sleeve gastrectomy.

DNA lesions in nuclei as previously described[55,56], the SG rats in our study presented much more cells with a normal morphology. In line, recent studies have found that bariatric surgeries are effective in reducing DNA damage indeed, in both humans[57,58] and animals[59]. Based on the possible mechanism of the DNA damage caused by obesity and diabetes, we can assume that SG exhibited a positive role in preventing cancer by eliminating the harmful factors that contribute to the genetic damage.

In summary, obesity and T2DM significantly increase the risk of multiple diseases and cancer, in which, disruption in the ET-1 axis leads to ED and an increased risk of developing lung cancer. Nevertheless, with a profound effect of reducing body weight and improving glycometabolism and insulin sensitivity, SG improves endothelial function and exerts a protective role in preventing lung cancer by normalizing the ET-1 axis and weakening the effects of DNA damage. In the context of the current scarcity of basic research covering both cancer and bariatric surgeries, an attempt was made in the current study to provide new perspectives and lines of evidence for the role of SG in preventing cancer. We believe that these findings will deepen our understanding of anticancer effects of bariatric surgeries and broaden the theoretical basis for the evaluation of the surgical effect of SG, especially in supporting the clinical findings of bariatric surgery for lowering the risk of lung cancer. However, more future studies are warranted to uncover the other potential mechanisms of such effects.

Table 4 Comparisons of fasting blood glucose (mmol/L) among groups

Time after surgery (wk)	C	SH	SG	*P* value		
				C *vs* SH	C *vs* SG	SH *vs* SG
0	5.3 ± 0.7	15.4 ± 1.5	15.7 ± 1.3	< 0.0001	< 0.0001	0.8776
1	5.5 ± 0.7	12.2 ± 1.6	5.7 ± 1.1	< 0.0001	0.9423	< 0.0001
2	5.7 ± 1.0	12.7 ± 2.3	5.5 ± 1.2	< 0.0001	0.8982	< 0.0001
4	6.2 ± 1.0	13.8 ± 2.7	6.3 ± 1.4	< 0.0001	0.9534	< 0.0001
6	6.3 ± 0.6	14.4 ± 2.7	7.4 ± 2.0	< 0.0001	0.2619	< 0.0001
8	5.9 ± 0.9	16.1 ± 2.4	8.4 ± 1.9	< 0.0001	0.0017	< 0.0001
10	6.2 ± 0.7	20.1 ± 1.6	9.6 ± 1.3	< 0.0001	0.0023	< 0.0001
12	6.0 ± 0.8	22.3 ± 2.7	12.6 ± 1.5	< 0.0001	< 0.0001	< 0.0001

C: Control; SH: Sham; SG: Sleeve gastrectomy.

Table 5 Comparisons of areas under curve of oral glucose tolerance test among groups

Time after surgery (wk)	C	SH	SG	*P* value		
				C *vs* SH	C *vs* SG	SH *vs* SG
0	956 ± 123	2419 ± 197	2407 ± 147	< 0.0001	< 0.0001	0.9907
4	1148 ± 214	2101 ± 293	1125 ± 226	< 0.0001	0.9651	< 0.0001
8	1241 ± 316	2346 ± 408	1507 ± 314	< 0.0001	0.0591	< 0.0001
12	1353 ± 282	2967 ± 421	2075 ± 247	< 0.0001	< 0.0001	< 0.0001

C: Control; SH: Sham; SG: Sleeve gastrectomy.

Table 6 Comparisons of fasting serum insulin levels (mIU/L) among groups

Time after surgery (wk)	C	SH	SG	*P* value		
				C *vs* SH	C *vs* SG	SH *vs* SG
0	7.385 ± 1.359	8.243 ± 2.349	6.104 ± 1.734	0.6134	0.2858	0.8341
4	7.989 ± 1.542	8.238 ± 1.597	9.212 ± 2.835	0.0263	0.3189	0.4710
8	10.808 ± 2.309	10.348 ± 1.898	9.946 ± 3.054	0.8770	0.6826	0.9362
12	11.146 ± 2.173	7.734 ± 1.525	9.042 ± 2.005	0.1864	0.3240	0.9439

C: Control; SH: Sham; SG: Sleeve gastrectomy.

Table 7 Comparisons of insulin resistance (mIU × mmol/L^2) among groups

Time after surgery (wk)	C	SH	SG	*P* value		
				C *vs* SH	C *vs* SG	SH *vs* SG
0	1.721 ± 0.157	4.474 ± 1.078	4.216 ± 1.061	< 0.0001	< 0.0001	0.6563
4	2.136 ± 0.234	5.988 ± 1.227	2.503 ± 0.667	< 0.0001	0.4289	< 0.0001
8	2.744 ± 0.309	7.116 ± 0.934	3.525 ± 0.656	< 0.0001	0.0811	< 0.0001
12	2.944 ± 0.365	8.341 ± 0.941	4.979 ± 0.833	< 0.0001	0.0003	< 0.0001

C: Control; SH: Sham; SG: Sleeve gastrectomy.

Table 8 Comparisons of endothelin-1 mRNA expression among groups

Time after surgery (wk)				*P* value		
	C	SH	SG	C *vs* SH	C *vs* SG	SH *vs* SG
4	1.51 ± 1.21	2.33 ± 1.08	1.31 ± 0.39	0.3293	0.9316	0.1828
8	1.11 ± 0.41	2.88 ± 0.80	0.87 ± 0.39	0.0100	0.9085	0.0033
12	1.56 ± 0.86	3.07 ± 1.55	1.65 ± 0.63	0.0318	0.9878	0.0449

C: Control; SH: Sham; SG: Sleeve gastrectomy.

Table 9 Comparisons of endothelin receptor A mRNA expression among groups

Time after surgery (wk)				*P* value		
	C	SH	SG	C *vs* SH	C *vs* SG	SH *vs* SG
4	1.17 ± 0.77	1.77 ± 0.88	1.09 ± 0.59	0.4552	0.9859	0.3669
8	1.57 ± 0.42	1.73 ± 0.55	1.21 ± 0.62	0.9460	0.7454	0.5501
12	1.61 ± 0.97	2.92 ± 1.03	2.71 ± 1.01	0.0334	0.0859	0.9047

C: Control; SH: Sham; SG: Sleeve gastrectomy.

Table 10 Comparisons of endothelin receptor B mRNA expression among groups

Time after surgery (wk)				*P* value		
	C	SH	SG	C *vs* SH	C *vs* SG	SH *vs* SG
4	1.06 ± 0.40	0.96 ± 0.27	0.94 ± 0.58	0.9581	0.9393	0.9981
8	0.98 ± 0.65	1.45 ± 0.62	1.07 ± 0.68	0.3994	0.9666	0.5442
12	1.00 ± 0.53	2.08 ± 0.84	1.17 ± 0.37	0.0141	0.8937	0.0419

C: Control; SH: Sham; SG: Sleeve gastrectomy.

Table 11 Comparisons of endothelin-converting enzyme-1 mRNA expression among groups

Time after surgery (wk)				*P* value		
	C	SH	SG	C *vs* SH	C *vs* SG	SH *vs* SG
4	1.11 ± 0.61	2.24 ± 0.55	0.99 ± 0.39	0.0191	0.9534	0.0090
8	1.40 ± 0.21	2.35 ± 1.00	1.52 ± 0.57	0.0550	0.9526	0.1031
12	1.32 ± 0.81	2.60 ± 0.73	1.65 ± 0.35	0.0073	0.6881	0.0552

C: Control; SH: Sham; SG: Sleeve gastrectomy.

Table 12 Comparisons of relative endothelin-1 protein expression among groups

Time after surgery (wk)				*P* value		
	C	SH	SG	C *vs* SH	C *vs* SG	SH *vs* SG
4	0.95 ± 0.11	1.10 ± 0.14	0.69 ± 0.10	0.3229	0.0354	0.0007
8	0.68 ± 0.10	1.79 ± 0.25	1.02 ± 0.12	< 0.0001	0.0056	< 0.0001
12	0.60 ± 0.06	1.64 ± 0.20	1.36 ± 0.24	< 0.0001	< 0.0001	0.0229

C: Control; SH: Sham; SG: Sleeve gastrectomy.

Table 13 Comparisons of relative endothelin receptor A protein expression among groups

Time after surgery (wk)				*P* value		
	C	SH	SG	C *vs* SH	C *vs* SG	SH *vs* SG
4	0.87 ± 0.17	0.82 ± 0.13	0.61 ± 0.08	0.7836	0.0036	0.0207
8	0.72 ± 0.16	0.97 ± 0.13	0.82 ± 0.08	0.006	0.44	0.1108
12	0.50 ± 0.09	1.17 ± 0.11	1.03 ± 0.09	< 0.0001	< 0.0001	0.1867

C: Control; SH: Sham; SG: Sleeve gastrectomy.

Table 14 Comparisons of relative endothelin receptor B protein expression among groups

Time after surgery (wk)				*P* value		
	C	SH	SG	C *vs* SH	C *vs* SG	SH *vs* SG
4	1.06 ± 0.11	1.29 ± 0.16	1.26 ± 0.20	0.0575	0.095	0.9697
8	0.54 ± 0.11	1.71 ± 0.16	1.03 ± 0.14	< 0.0001	< 0.0001	< 0.0001
12	1.14 ± 0.08	2.28 ± 0.19	1.00 ± 0.16	< 0.0001	0.2966	< 0.0001

C: Control; SH: Sham; SG: Sleeve gastrectomy.

Table 15 Comparisons of relative endothelin-converting enzyme-1 protein expression among groups

Time after surgery (wk)				*P* value		
	C	SH	SG	C *vs* SH	C *vs* SG	SH *vs* SG
4	0.13 ± 0.03	0.36 ± 0.06	0.39 ± 0.02	< 0.0001	< 0.0001	0.5966
8	0.15 ± 0.04	0.25 ± 0.05	0.24 ± 0.04	0.0123	0.0125	> 0.9999
12	0.19 ± 0.05	0.37 ± 0.06	0.22 ± 0.06	< 0.0001	0.6298	< 0.0001

C: Control; SH: Sham; SG: Sleeve gastrectomy.

Table 16 Comparisons of semi-quantified DNA damage levels (percentage of γ-H2AX-positive cells) among groups

Time after surgery (wk)				*P* value		
	C	SH	SG	C *vs* SH	C *vs* SG	SH *vs* SG
4	0.83 ± 0.43	3.06 ± 0.47	1.15 ± 0.31	0.0009	0.835	0.0046
8	1.71 ± 0.63	4.73 ± 1.04	1.73 ± 0.72	< 0.0001	0.999	< 0.0001
12	1.86 ± 0.37	6.70 ± 1.80	2.75 ± 1.09	< 0.0001	0.2672	< 0.0001

C: Control; SH: Sham; SG: Sleeve gastrectomy.

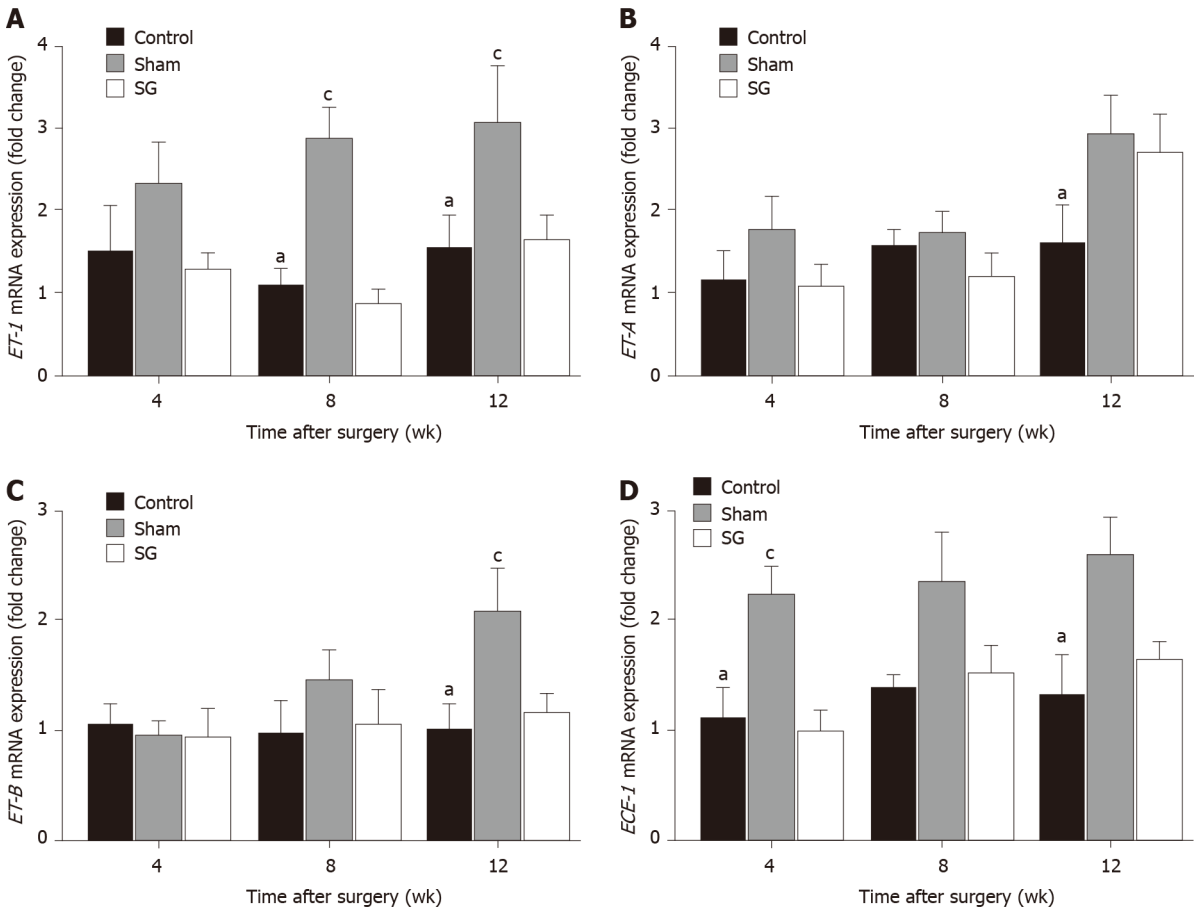

Figure 3 Expression of genes of the endothelin-1 axis. [a]*P* < 0.05: Control *vs* sham; [c]*P* < 0.05: Sham *vs* sleeve gastrectomy. SG: Sleeve gastrectomy.

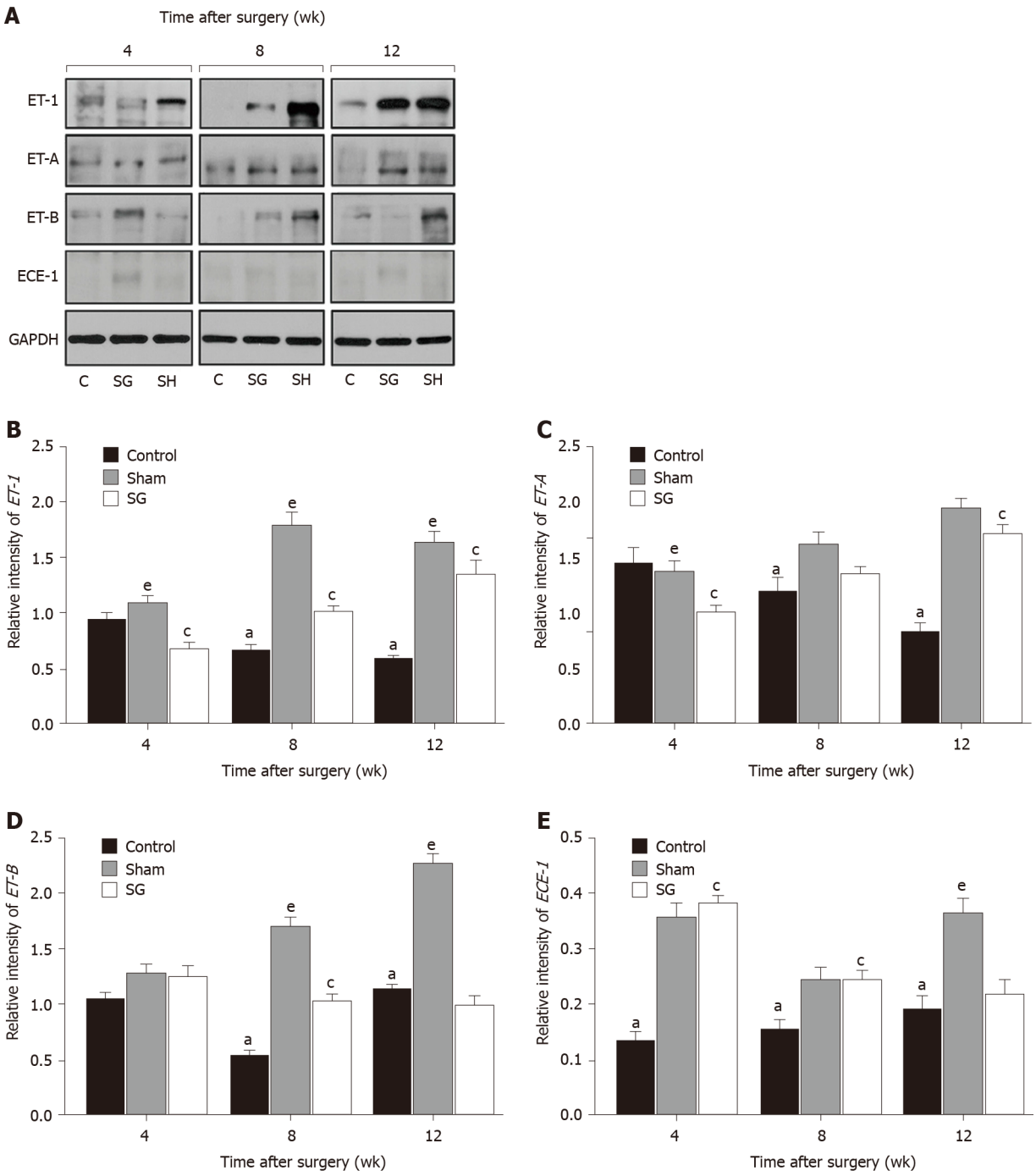

Figure 4 Protein expression of the endothelin-1 axis. A: Immunoblotting of the proteins; B-E: Relative band intensity. $^{a}P < 0.05$: Control *vs* sham; $^{c}P < 0.05$: Control *vs* sleeve gastrectomy; $^{e}P < 0.05$: Sham *vs* SG. ET: Endothelin; ECE: ET-converting enzyme; SG: Sleeve gastrectomy.

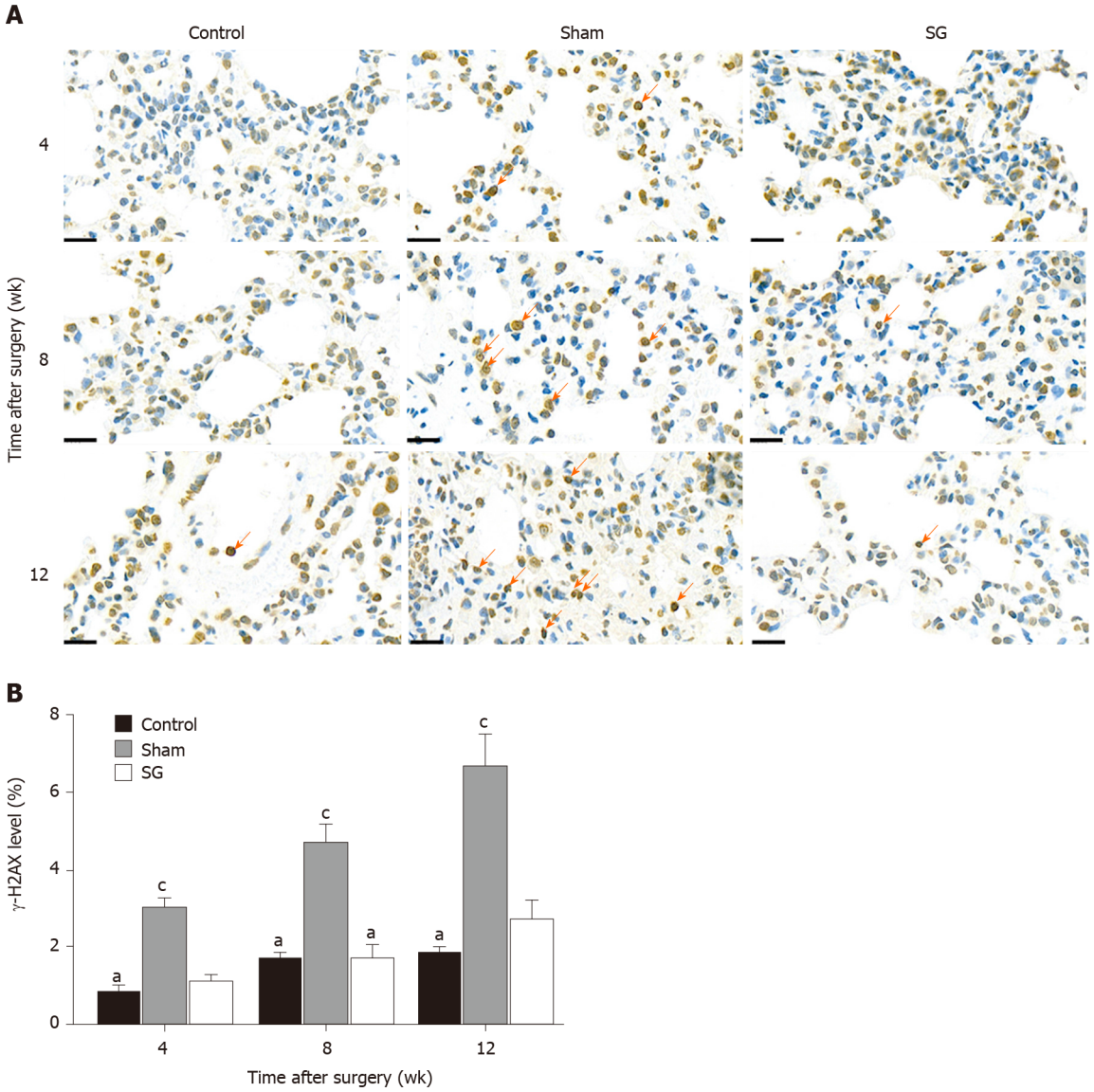

Figure 5 Immunohistochemical assessment of DNA damage using the γ-H2AX foci assay. A: Representative images of γ-H2AX-positive cells (red arrows) at different time points (bar = 20 μm); B: Semi-quantification of the level of DNA damage. $^{a}P < 0.05$: Control *vs* sham; $^{c}P < 0.05$: Sham *vs* sleeve gastrectomy. SG: sleeve gastrectomy.

ARTICLE HIGHLIGHTS

Research background

Although it is stated that bariatric surgeries are capable of preventing numerous types of cardiovascular diseases and cancer, the basic studies are still warranted to explain such roles by revealing the possible mechanisms. Herein, an attempt was made to answer these questions in an innovative way by investigating one of the important regulation systems in the body – the endothelin-1 (ET-1) axis.

Research motivation

Providing evidence for the lowered risk of cardiovascular disease and cancer will not only enlighten the multifunctional effects of sleeve gastrectomy (SG), which is deemed to be "metabolic", but also broaden and deepen our understanding of related fields and help improve the acceptance of SG as well.

Research objectives

In the current study, an ameliorated status of the ET-1 axis was confirmed, indicating the improvement in endothelial function and decline in the risk of lung cancer. Plus, the latter was also identified by the decreased level of DNA damage. Collectively, these findings are bound to attract and inspire future researchers to look for the clues of the benefits brought by bariatric surgeries that extend beyond the known metabolic improvements.

Research methods
SG and sham surgery were performed to clarify the effect induced on the ET-1 axis. In order to determine the real baseline of the parameters, healthy controls were also included. Moreover, the γ-H2AX foci assay was applied to provide stronger evidence of SG in preventing lung cancer, which was not an approach used commonly in previous research related to bariatric surgeries.

Research results
The results indicated that SG improved endothelial function and prevented lung cancer by normalizing the ET-1 axis, providing animal-based findings and suggesting new perspectives for the clinical field. The potential mechanisms of how SG effects the ET-1 axis and whether it is a direct impact or achieved by moderating metabolic disorder, however, need to be explored further.

Research conclusions
Beyond reducing body weight and improving both glycometabolism and insulin sensitivity, SG improves endothelial function and exerts a protective role in preventing lung cancer by normalizing the ET-1 axis and lessening DNA damage. These findings may present a new theoretical basis for clinical implications of SG.

Research perspectives
We believe that looking for more lines of evidence which expand our knowledge beyond the metabolic impact of bariatric surgeries is important, particularly because it will illuminate and illustrate the whole picture of the therapeutic effects of these procedures. Undoubtedly, factors that change under metabolic disorder and contribute to canceration, are the entry points and keys to the answer.

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ORIGINAL ARTICLE

Clinical and Translational Research

Clinicopathological features of early gastric cancers arising in *Helicobacter pylori* uninfected patients

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Abstract

BACKGROUND

Persistent *Helicobacter pylori* (*H. pylori*) infection causes chronic inflammation, atrophy of the gastric mucosa, and a high risk of developing gastric cancer. In recent years, awareness of eradication therapy has increased in Japan. As *H. pylori* infections decrease, the proportion of gastric cancers arising from *H. pylori* uninfected gastric mucosa will increase. The emergence of gastric cancer arising in *H. pylori* uninfected patients though rarely reported, is a concern to be addressed and needs elucidation of its clinicopathological features.

AIM

To evaluate the clinicopathological features of early gastric cancer in *H. pylori*-uninfected patients.

METHODS

A total of 2462 patients with 3375 instances of early gastric cancers that were treated by endoscopic submucosal dissection were enrolled in our study between May 2000 and September 2019. Of these, 30 lesions in 30 patients were diagnosed as *H. pylori*-uninfected gastric cancer (HpUIGC). We defined a patient as *H. pylori*-uninfected using the following three criteria: (1) The patient did not receive treatment for *H. pylori*, which was determined by investigating medical records

statement: The study protocol was approved by the Ethics Committee of Yokohama City University Medical Center Hospital (Approval number: D1602024).

Informed consent statement: Patients were not required to provide informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

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and conducting patient interviews; (2) Lack of endoscopic atrophy; and (3) The patient was negative for *H. pylori* after being tested at least twice using various diagnostic methods, including serum anti-*H. pylori*-IgG antibody, urease breath test, rapid urease test, and microscopic examination.

RESULTS

The frequency of HpUIGC was 1.2% (30/2462) for the patients in our study. The study included 19 males and 11 females with a mean age of 59 years. The location of the stomach lesions was divided into three sections; upper third (U), middle third (M), lower third (L). Of the 30 lesions, 15 were U, 1 was M, and 14 were L. Morphologically, 17 lesions were protruded and flat elevated type (0-I, 0-IIa, 0-IIa + IIc), and 13 lesions were flat and depressed type (0-IIb, 0-IIc). The median tumor diameter was 8 mm (range 2-98 mm). Histological analysis revealed that 22 lesions (73.3%) were differentiated type.The HpUIGC lesions were classified into fundic gland type adenocarcinoma (7 cases), foveolar type well-differentiated adenocarcinoma (8 cases), intestinal phenotype adenocarcinoma (7 cases), and pure signet-ring cell carcinoma (8 cases). Among 30 HpUIGCs, 24 lesions (80%) were limited to the mucosa; wherein, the remaining 6 lesions showed submucosal invasion. One of the submucosal invasive lesions showed more than 500 μm invasion. The mucin phenotype analysis identified 7 HpUIGC with intestinal phenotype and 23 with gastric phenotype.

CONCLUSION

We elucidated the clinicopathological characteristics of HpUIGC, revealing recognition not only undifferentiated-type but also differentiated-type. In addition, intestinal phenotype tumors were also observed and could be an important tip.

Key words: Early gastric cancer; Helicobacter pylori; Un-infection; Negative; Clinicopathological features; Endoscopic submucosal dissection; Mucins; Phenotype

Core tip: Chronic *Helicobacter pylori* (*H. pylori*) infection is a major risk factor for gastric cancer. Historically, gastric cancers in Japan were related to *H. pylori* infection, and the frequency of *H. pylori* uninfected gastric cancer (HpUIGC) was very rare. However, the rarity of gastric cancer in *H. pylori* negative patients may be partly owing to underreporting, and the mechanisms behind the development and progression of this type of gastric cancer must be elucidated. This study elucidated the clinicopathological features of *H. pylori* uninfected gastric cancer from 30 gastric cancer patients. Differentiated-type gastric cancers without submucosal invasion were most prominent.

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INTRODUCTION

Most gastric cancers involve *Helicobacter pylori* (*H. pylori*) infection in their development, and in 1994 *H. pylori* was certified as a "definite carcinogen" for gastric cancer development[1,2]. *H. pylori* infection results in inflammation, atrophy of the gastric mucosa, and intestinal metaplasia; when *H. pylori* infection becomes chronic there is a high-risk gastric cancer[3].

In recent years, awareness of eradication therapy has increased in Japan, thus reducing the rate of *H. pylori* infection, especially in the young people due to the improvement of sanitary environment and expanding the indication of eradication[4]. As *H. pylori* infections decrease, the proportion of gastric cancers arising from *H. pylori*

uninfected gastric mucosa will increase[5]. However, at the moment, *H. pylori*-uninfected gastric cancer (HpUIGC) is very rare as compared to *H. pylori*-positive gastric cancer (HpPGC). The definition is not yet well established, and its frequency is reported to differ from 0.4 to 5.4%[6-11]. Previous studies have reported that the undifferentiated-type of HpUIGC is more frequently observed than differentiated-type[7-9]. However, in recent years, differentiated-type gastric cancers such as oxyntic glands adenoma/adenocarcinoma and foveolar type adenoma/adenocarcinoma, even including HpUIGC, were reported[12,13]. Few studies have investigated HpUIGC and its clinicopathological features have not been sufficiently documented. Therefore, elucidation of characteristics of early-stage HpUIGC is essential. We evaluated the characteristics of HpUIGC treated with endoscopic submucosal dissection (ESD) focusing on pathological and endoscopic features.

MATERIALS AND METHODS

Determination of H. pylori-uninfection status

Three criteria were used to determine whether a patient was *H. pylori*-uninfected: (1) No medical history of *H. pylori* eradication therapy, which was determined by investigating the patients' medical records and conducting patient interviews; (2) Lack of endoscopic atrophy, patients with C-0 atrophy were selected as HpUIGC[14]. As a supplementary finding, we referenced the endoscopic findings of the Kyoto classification score, including RAC (regular arrangement of collecting venule)[15,16]. The endoscopic findings were subsequently verified by three skilled endoscopists (KH, CS, and SM). And (3) laboratory examination that included serum anti-*H. pylori*-IgG antibody, Urease breath test (UBT), Rapid urease test (RUT), and microscopic examination[17]. If a test was negative for *H. pylori* by two or more examinations this was considered *H. pylori* uninfected[18,19]. Among the HpUIGC patients, the presence or absence of pathological atrophy was evaluated using the updated Sydney system in the background mucosa of ESD specimens[20]. Tumors satisfying all the three conditions described above were identified as HpUIGC.

Patients

Between May 2000 and September 2019, a total of 2569 patients with 3477 gastric cancers were treated by endoscopic submucosal dissection (ESD) at Yokohama City University Medical Center. Of these patients, 2462 consecutive patients with 3370 gastric cancers were assessed for *H. pylori* status and enrolled in this study. The remaining 107 patients included 87 patients with cancer in their gastric remnants, 16 with cancer in their gastric tubes, 4 with neuroendocrine tumors, were excluded. Of the 3370 gastric cancers, 30 gastric cancers satisfied the three criteria outlined above and were classified as HpUIGCs.

Characterization of clinicopathological features of the HpUIGCs

We investigated the frequency and features of HpUIGC. Clinicopathological features including age, sex, location, macroscopic type, histological type, tumor size, depth of invasion, presence or absence of lymphovascular invasion, and treatment outcome were evaluated. The location of the gastric lesions was categorized based on stomach location: upper third (U), middle third (M), and lower third (L). The histological type was identified as differentiated or undifferentiated according to the 15th edition of the Japanese classification of gastric cancer[21]. The differentiated type was further classified into well-differentiated (tub1), moderately differentiated (tub2), or, papillary (pap) adenocarcinoma. The undifferentiated type was classified as poorly differentiated (por) or signet-ring cell (sig) adenocarcinoma. HpUIGC was further categorized into four types based on their histopathological features (1) Fundic gland adenocarcinoma, (2) Foveolar-type adenocarcinoma, (3) Intestinal phenotype adenocarcinoma, and (4) Pure signet-ring cell carcinoma. Finally, the 30 cases of HpUIGC were evaluated for their mucin phenotypes and endoscopic features.

Indications of ESD

Indications of gastric ESD were determined according to the gastric cancer treatment guidelines of the Japanese Gastric Cancer Association (JGCA). Briefly, the indication criteria were defined as differentiated-type mucosal gastric cancer lesions without ulcers [UL (-)] regardless of size, differentiated-type mucosal gastric cancer lesions ≤ 3 cm in size with ulcers [UL (+)], undifferentiated-type mucosal gastric cancer lesions ≤ 2 cm in size without ulceration [UL (-)], and confirming no evidence of lymph node metastasis (LNM), and distant metastasis by preoperative computed tomography[22].

Endoscopic submucosal dissection

All lesions were treated by ESD. The gastric ESDs were performed as previously described[23,24]. Briefly, after marking approximately 5 mm around the borders of the lesion, circumferential incision and submucosal dissection were made using an IT-knife2 (Olympus Medical Systems Corporation, Tokyo, Japan) or Dual knife (Olympus Medical Systems Corporation, Tokyo, Japan). Hyaluronic acid and/or glycerol were used as the submucosal injecting solution.

Histopathological investigation

The resected specimens were fixed with 10% buffered formalin immediately after the procedure. To reliably evaluate the deepest part of the lesion and the horizontal margin, it was cut into thin sections (2-3 mm) parallel to the oral side to the anal side[21]. The resected specimens were embedded in paraffin and mounted on slides then subjected to hematoxylin and eosin staining and immunohistochemistry. Specimen size, tumor size, macroscopic type, and the depth of invasion were measured in accordance with the Japanese Gastric Cancer Treatment Guideline 2014 (Ver. 4)[22], describing the. Treatment was deemed curative when all of the following conditions were fulfilled: en bloc resection, negative horizontal margin (HM0), negative vertical margin (VM0), and no lymphovascular infiltration [ly(-), v(-)]. In histologically differentiated-type tumors with pT1a UL(-) regardless of tumor size, pT1a UL(+) with tumor size ≤ 3 cm, histologically of differentiated type, and pT1b (SM1, 500 microns from the muscularis mucosae) with tumor size ≤ 3 cm were judged curative. In histologically undifferentiated-type, pT1a, UL(-) with tumor size ≤ 2 cm was also considered as curative. Resections that does not satisfy any of the above criteria were considered non-curative.

Immunohistochemistry and histological classification

Immunohistochemical staining of the 30 HpUIGCs was performed in representative sections taken from the tumor at its largest diameter. Mucin phenotype was evaluated using MUC2, MUC5AC, MUC6, CD10, CDX2, PG-I, and H+/K+ -ATPase markers. We used monoclonal antibodies against the following markers: Mucin 5AC (MUC5AC) as a marker for gastric foveolar cells, MUC6 as a marker for gastric mucous neck cells and pyloric glands, MUC2 as a marker for intestinal goblet cells, CD10 as a marker for small intestinal brush border, CDX2 as a marker for epithelial intestinal differentiation. The tumors that showed differentiation to the fundus gland were immunochemically stained for definitive diagnosis of the oxyntic tumor; PG-I was used as a marker for chief cells, and proton pump/H+/K+-ATPase alpha subunit as a marker for parietal cells. MUC2, MUC5AC, MUC6, CD10, PG-I, H+/K+-ATPase reactivity was considered significantly positive when > 10% of tumor cells were stained. Cases with < 10% positive cells were regarded as unaffected. The mucin phenotype was divided into (1) gastric phenotype, (2) intestinal phenotype, and (3) gastric and mixed intestinal phenotype. The gastric and mixed intestinal phenotype was subdivided into gastric phenotype dominant or intestinal phenotype dominant[25,26].

RESULTS

A total of 2462 consecutive patients with 3370 c gastric cancers (3132 early gastric cancer lesions and 238 adenomas) were enrolled. In total, 30 lesions form 30 patients (1.2%) were classified HpUIGC. The clinicopathological features of HpUIGC are shown in Table 1. The study included 19 males and 11 females with a mean age of 59 years. Of the 30 lesions, 15 were U, one was M and 14 were L. Morphologically, 17 lesions were protruded and flat elevated type (0-I, 0-IIa, 0-IIa+IIc), and 13 lesions were flat and depressed type (0-IIb, 0-IIc). Tumor diameter ranged from 2 mm to 98 mm, with a median diameter of 8 mm. Histopathologically, 22 lesions (73.3%) were identified as differentiated-type and eight lesions (26.7%) as undifferentiated-type. All of the undifferentiated lesions tested were signet-ring cell carcinomas. Tumor invasion in 24 lesions (80%) was limited to the mucosa, while the remaining 6 lesions showed submucosal invasion. One of the lesions invaded the submucosal layer to a depth of 500 μm (SM2). Outcomes for the HpUIGC patients were positive, all received successful en bloc resections, free from tumor margin. The curative resection rate was 96.3%. Details of 30 HpUNGCs are shown in Table 2.

Histological and endoscopic features of HpUINGC

Histologically the HpUIGC lesions were classified into fundic gland type adenocarcinoma (7 cases), foveolar type well-differentiated adenocarcinoma (8 cases), intestinal phenotype adenocarcinoma (7 cases), and pure signet-ring cell carcinoma (8 cases). The histological and endoscopic findings of different types of lesions are

Table 1 Clinicopathological features of *Helicobacter pylori* uninfected early gastric cancers

	***n* = 30**
mean age ± SD (yr)	59 ± 9
Gender, *n*	
Male	19 (63.3%)
Female	11 (46.7%)
Location, *n*	
Upper part	15 (50%)
Middle part	1 (3.3%)
Lower part	14 (46.7%)
Morphology, *n*	
Protruded /flat	17 (56.7%)
Depressed	13 (43.3%)
Mean tumor diameter (range)	8 (2-98 mm)
Depth of invasion, *n*	
M	24 (80%)
SM1	5 (16.7%)
SM2	1 (3.3%)
Histological type, *n*	
Differentiated type	22 (73.3%)
Undifferentiated type	8 (26.7%)
Histological classification	
Fundic grand type adenocarcinoma	7 (23.3%)
Foveolar type well differentiated adenocarcinoma	8 (26.7%)
Intestinal phenotype adenocarcinoma	7 (23.3%)
Pure signet ring call carcinoma	8 (26.7%)
Ulcerative finding, *n*	
(-)	30 (100%)
(+)	0 (0%)
En-bloc resection, *n*	96.7%
R0+curative resection (cura A)	93.3%
Complication	
Perforation	6.7%
Delayed perforation	0%
Delayed bleeding	0%

explained below.

Fundic gland type adenocarcinoma (Figure 1): Histopathological finding: HE staining showed the presence of tumor cells mimicking fundic glands at the bottom of the mucosa, and the surface of the tumor was covered with non-cancerous epithelium. Immunohistologically, the fundic gland cancer cells were positive for PG-I and MUC6, and part of the tumor expressed H+/K+-ATPase. Six of the seven lesions showed submucosal invasion, while one of them showed SM2 invasion (distance from muscularis mucosae was 780 μm). None of the lesions revealed lymphovascular invasion. Endoscopic finding: All seven lesions were located in the upper part of the stomach and were recognized as small protrusions. With white light imaging, a yellowish-white tumor covered with non-cancerous epithelium was observed in submucosal tumors (SMTs). Magnified narrow-band imaging (ME-NBI) revealed dilated branched vessels and intervening part on the lesion's surface.

Foveolar type well-differentiated adenocarcinoma (Figure 2): Histopathological finding: Seven of the foveolar type gastric cancers were composed of dysplastic columnar cells with clear cytoplasm and showed villous or papillary structures mimicking foveolar epithelium. On the surface of the mucosa, the expanded glands composed of non-tumor cells pushed up the cancerous epithelium. Tumor existed only on the surface, and atypia was recognized as low-grade well-differentiated adenocarcinoma. None of the lesions showed submucosal invasion, although the

Table 2 Case series of *Helicobacter pylori* uninfected early gastric cancer in present study

Case	Classification	Sex	Age	Location	Morphology	Size	Depth	Histology	v/ly	RUT	UBT	HP-IgG
1	Fundic	m	55	U	0-IIc	6	sm1	tub1	0		+	+
2	Fundic	m	80	U	0-IIc	12	sm1	tub2 > tub1	0		+	+
3	Fundic	f	65	U	0-IIa	6	sm1	tub1,	0		+	+
4	Fundic	f	65	U	0-IIa	10	m	tub1	0	+	+	+
5	Fundic	m	69	U	0-IIa + IIc	8	sm1	tub1,	0		+	+
6	Fundic	m	62	U	0-IIa	13	sm2	tub1	0		+	+
7	Fundic	m	67	U	0-I	6	sm1	tub1	0		+	+
8	Foveolar	f	34	U	0-IIa	35	m	tub1	0		+	+
9	Foveolar	f	66	U	0-IIa	33	m	pap	0	+	+	+
10	Foveolar	m	63	U	0-IIa	55	m	tub1	0		+	+
11	Foveolar	f	69	U	0-IIa	98	m	tub1	0		+	+
12	Foveolar	f	51	U	0-IIa	28	m	tub1	0		+	+
13	Foveolar	m	72	U	0-IIa	63	m	tub1	0		+	+
14	Foveolar	m	64	U	0-IIa	42	m	tub1 > pap	0		+	+
15	Foveolar	f	50	U	0-IIa	2	m	tub1	0		+	+
16	Intestinal	m	66	L	0-IIc	9	m	tub1	0		+	+
17	Intestinal	m	49	L	0-IIc	5	m	tub1	0	+	+	+
18	Intestinal	f	65	L	0-IIa	3	m	tub1	0	+	+	
19	Intestinal	f	61	L	0-IIc	5	m	tub1	0		+	+
20	Intestinal	f	43	L	0-IIc	3	m	tub1	0		+	+
21	Intestinal	m	48	L	0-IIa	7	m	tub1	0		+	+
22	Intestinal	f	52	L	0-IIa	5	m	tub1	0		+	+
23	Sig	m	58	L	0-IIc	4	m	sig	0	+	+	
24	Sig	m	55	L	0-IIc	5	m	sig	0	+	+	+
25	Sig	m	65	M	0-IIc	5	m	sig	0		+	+
26	Sig	m	49	L	0-IIc	13	m	sig	0		+	+
27	Sig	m	53	L	0-IIc	4	m	sig	0		+	+
28	Sig	m	45	L	0-IIc	8	m	sig	0		+	+
29	Sig	m	46	L	0-IIb	12	m	sig	0		+	+
30	Sig	m	84	L	0-IIa	2	m	sig	0		+	+

Fundic: Fundic grand type adenocarcinoma; Foveolar: Foveolar type well differentiated adenocarcinoma; Intestinal: Intestinal phenotype adenocarcinoma; Sig: Pure signet ring cell carcinoma; v/ly: Lyphovascular invasion; RUT: Rapid urease test; UBT: Urease breath test.

tumor size was large (mean diameter 37.3 ± 18.3 mm). All foveolar type gastric cancers were positive for MUC5AC, but negative for PG-I, MUC2, and CD10 were negative. MUC6 was positive for expanded non-cancerous glands in the middle to the bottom layer of the mucosa. No lymphovascular invasion was observed in any of the eight gastric cancers.Endoscopic finding: All lesions were located in the upper part of the stomach. Seven of eight foveolar-type well-differentiated adenocarcinomas were observed as laterally spreading elevated lesions with whitish color such as an intestinal-type adenoma, and one lesion showed raspberry-like appearance [13]. ME-NBI showed a papillary or villous shaped fine mucosal pattern with intra-structural irregular vessels in all lesions. One of the foveolar type well-differentiated adenocarcinomas was recognized as a small protrusion with a raspberry-like appearance in the greater curvature of the upper part of the stomach. Although this lesion resembled a hyperplastic polyp, tumor cell atypia revealed well-differentiated adenocarcinoma.

Intestinal phenotype adenocarcinoma (Figure 3): Histopathological finding: All tumors showed well-differentiated adenocarcinoma characterized by tubular structures lined by tall columnar cells with hyperchromatic, pencillate, and pseudostratified nuclei. Luminal borders were sharp with a brush border. Goblet cells were positive for MUC2 and the brush border of intestinal absorptive epithelial cells was positive for CD10. The surface epithelium was focally positive for MUC5AC, and deeper glands were focally positive for MUC6. These lesions were classified as the intestinal-type dominant mucin phenotype. Endoscopic finding: The white light

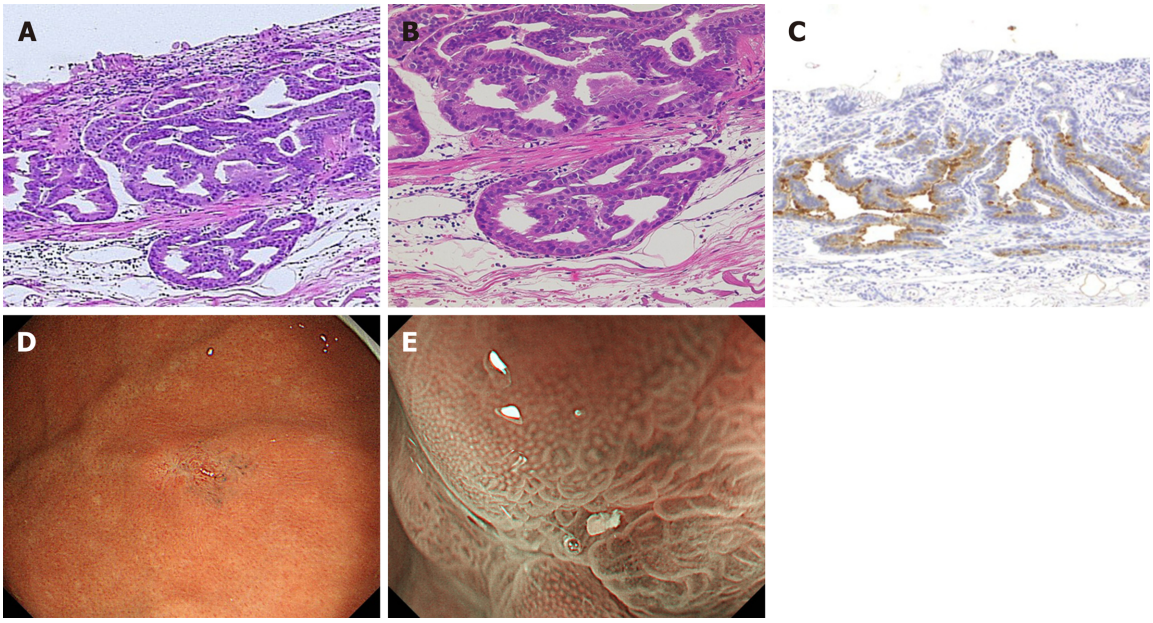

Figure 1 Fundic gland type adenocarcinoma. A: Cancer cells with enlarged nuclei formed irregular glands. The surface of the tumor was covered with non-cancerous epithelium, hematoxylin and eosin, original magnification 4 ×; B: The tumor invaded into the submucosal layer in a state that kept the muscularis mucosa. The distance from muscularis mucosa was 75 μm, hematoxylin and eosin, original magnification 40 ×; C: PG-I stains showed focal positive for cancer cells; D: The submucosal tumor like a small protrusion on the greater curvature of the upper gastric body, white light endoscopy; E: The surface of the tumor was covered with normal epithelium, magnifying endoscopy with narrow-band imaging.

image revealed a 0-IIa+IIc-type lesion mimicking verrucosa with a red tone, approximately 5 mm in size, and all the lesions were found in the gastric antrum. Unlike conventional verrucosa, which frequently occurs in the antrum, it was characterized by only one or two humps. An irregular microvascular pattern and irregular microsurface pattern with a demarcation line were observed in the recessed area with ME-NBI.

Pure signet-ring cell carcinoma (Figure 4): Histopathological finding: Pure signet-ring cell carcinoma existed in the proliferative zone to the surface layer of mucosa. Most of the lesions, cancer cells were limited to the proliferative zone, and the surface layer of mucosa was found to be covered with non-cancerous epithelium. Endoscopic finding: In the lower part of the stomach, mainly in the antrum, discolored and slightly depressed lesions were observed with a white light image. In six of these, the tumor size was less than 10 mm. Typical features such as corkscrew-like vessels[27], could not be observed.

Mucin phenotype of H. pylori-uninfected gastric cancers

All HpUIGC lesions were evaluated for their mucin phenotype (Table 3). The fundic gland adenocarcinoma, foveolar type well-differentiated adenocarcinoma, and pure signet-ring cell adenocarcinoma revealed gastric phenotype or gastric phenotype dominant, whereas intestinal phenotype adenocarcinoma showed intestinal phenotype dominant.

Long-term outcomes

We investigated long-term outcomes of 30 HpUIGC cases with a median 30-mo (ranged 10-138 mo) observation period. Neither gastric cancer mortality nor death from other diseases was observed; therefore, both overall survival and disease-free survival were 100%. Metachronous gastric cancer was not observed during patient follow-up.

DISCUSSION

H. pylori infection causes chronic inflammation and atrophy of the gastric mucosa and often results in gastric cancer. Since 1994, *H. pylori* has been recognized as a "definite carcinogen", contributing to the development of gastric cancer[1-2]. A prospective study reported that *H. pylori* eradication therapy suppressed two-thirds of metachronous gastric cancer[28]. As a result, since 2010, the Japanese insurance system has allowed

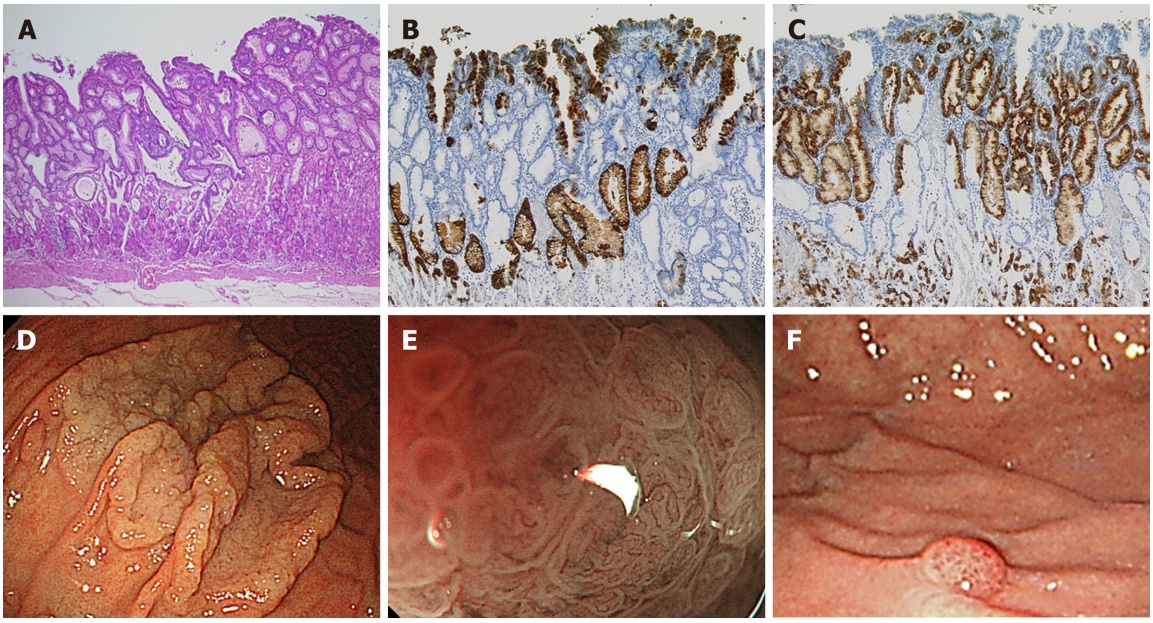

Figure 2 Foveolar type adenocarcinoma. A: The villous structure composed of dysplastic columnar cells with clear cytoplasm. Hematoxylin and eosin, original magnification 4 ×; B: MUC5AC was positive for cancer; C: MUC6 was negative for cancer but positive for expanded noncancerous glands in the middle layer of mucosa; D: Whitish laterally spreading elevated lesion was seen at the greater curvature of the upper gastric body, white light endoscopy; E: A papillary or villous like fine mucosal pattern with intra-structural irregular vessels in all lesions, one lesion showed raspberry-like appearance magnifying endoscopy with narrow-band imaging; F: One lesion showed raspberry-like appearance.

patients who have undergone endoscopic resection to receive *H.pylori* eradication therapy[17], and in the current Japan eradication therapy for *H. pylori* is insurance adaptation to all *H. pylori* infection patients. Improved sanitation has significantly reduced the rate of new *H. pylori* infections and *H. pylori* infection rate among young adults is reported to be decreasing yearly[4,5].

The frequency of *H. pylori*-negative gastric cancers is low[6-11]; however, this number is expected to increase, and the frequency of HpUIGC may increase proportionately. Currently, HpUIGC is still rarely reported so far, and the frequency varies considerably from 0.66% - 14% of gastric cancers[6-11,29-31]. The variation in this range may be owing to differences in the definition of *H. pylori* uninfected status in previous reports.

H. pylori detection methods possess high sensitivity and specificity and are usually divided into invasive (endoscopic based) and noninvasive methods. Invasive diagnostic tests include endoscopic imaging, histology, RUT, culture, and molecular methods. Non-invasive diagnostic tests include UBT, stool antigen test, serological, and molecular examinations. The accuracy of *H. pylori* infection diagnosis varies depending on the test. The sensitivity and specificity of UBT, serum anti-HP-IgG antibody, and RUT are 95% and 95%, 91%-100%, and 50%-91%, and 85%-95% and 95%-100%, respectively. However, some tests may produce false negatives owing to Proton Pomp Inhibitor (PPI) or patient factors, including past antibiotic use. To confirm *H. pylori* un-infected status, it is necessary to prove multiple tests[17,32-34]. In Japan, combination diagnostic testing showed the occurrence of gastric cancer ranged from 0.42% to 0.66% in patients without *H. pylori* infection[6,7,9]. Even if a patient is currently negative for *H. pylori* tests, there is a possibility of past infection; therefore, assessment of gastric atrophy is necessary to distinguish determine if there was a past infection. We emphasized endoscopic findings revealing C-0 atrophy The ESD specimen was confirmed to have no evidence of histological atrophy and inflammation in the background mucosa using the updated Sydney system[20]. In this study, the combination of two or more *H. pylori* tests (serum antibody, UBT, RUT, *etc*.) based on the Japanese society for *H. pylori* research guidelines in combination with no history of *H. pylori* eradication therapy were used to confirm *H. pylori* un-infection. As a result, HpUIGC was diagnosed in 30 of 2462 cases (1.2%). This was similar to previous reports. The average age of patients in our study was 59 years old; however, the males were, on average, older than the females[6-11].

Undifferentiated-type adenocarcinomas were more common than differentiated-type adenocarcinoma, and pure signet-ring cell carcinomas appeared at a rate similar to previous reports[5,7]. Unlike previous reports, most of the lesions (22/30 lesions) were the differentiated type. The eight undifferentiated-type adenocarcinomas were

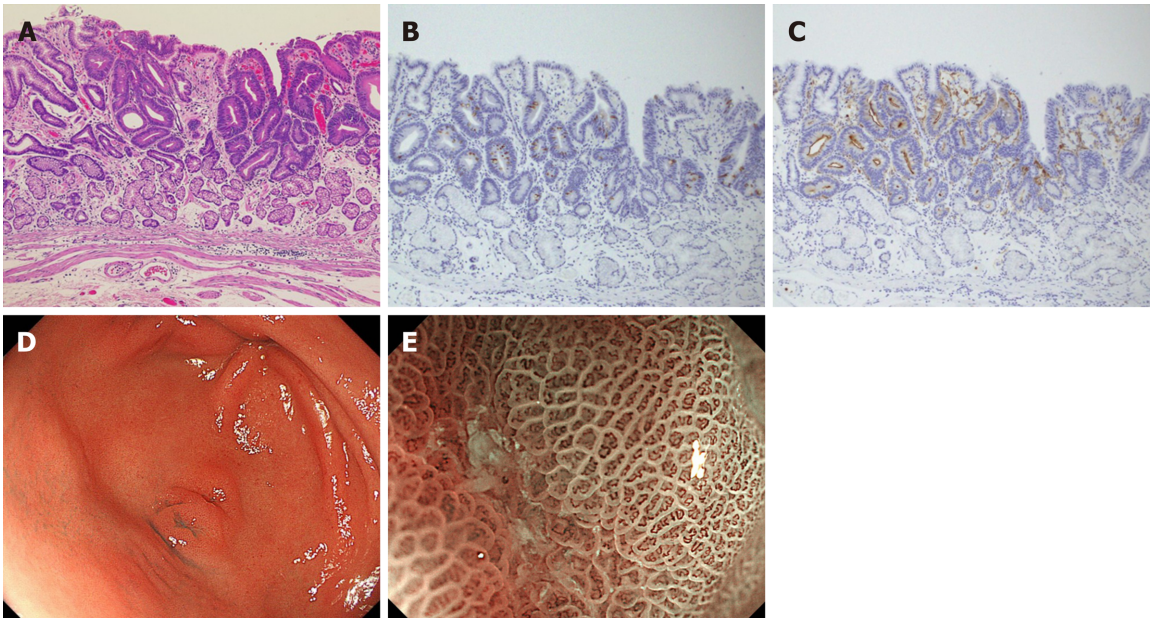

Figure 3 Intestinal-type adenocarcinoma. A: The tubular structures lined by tall columnar cells with hyperchromatic, pencillate, and pseudostratified nuclei, hematoxylin and eosin, original magnification 4 ×; B: MUC2 was weakly positive for cancer cells; C: CD10 was positive for cancer cells; D: 0-IIa+IIc type tumor mimicking verrucosa on the gastric antrum, white light endoscopy; E: An irregular microvascular pattern and irregular microsurface pattern with a demarcation line were observed just in the recessed area, magnifying endoscopy with narrow-band imaging.

tiny pure signet-ring cell carcinomas that were confined to the proliferative zone and did not contain poorly differentiated type components. Signet-ring HpUIGCs were easily recognized owing to their lack of atrophy as minute discolored depressed lesions.

Unusual neoplastic changes in ME-NBI were owing to tumor cells only existing in the proliferative zone of the mucosa, and the surface layer being covered with non-cancerous epithelium. This is the reason why pathological findings do not show the typical corkscrew pattern[35]. Immunochemically, tumor cells showed a gastric phenotype. Reportedly, signet-ring cell carcinoma of the intestinal phenotype infiltrate from the proliferative zone of the mucosa into the deep mucosal layer, and infiltrate into the submucosa individually while maintaining the muscularis mucosa, and sometimes progressing to scirrhous gastric cancer. Gastric phenotype signet-ring cell carcinomas that progress from the proliferative zone to the surface layer of the mucosa, have a lower potential of being malignant potential than the intestinal phenotype[36,37].

Previous reports on the differentiated type of HpUIGC have mostly identified fundic gland type adenocarcinomas and gastric phenotype gastric cancer with low-grade atypia. However, with the small number of reports, it is difficult to identify the consistent clinicopathological features of HpUIGC. To the best of our knowledge, the present study reports the largest number of HpUIGC cases, 30, that had been evaluated for both endoscopic and pathological findings. It is notable that, in addition to pure signet-ring cell carcinoma and fundic gland type adenocarcinomas, which were often seen in previous reports, our study also included gastric phenotype low-grade adenocarcinoma, foveolar type adenocarcinoma, and intestinal-type adenocarcinoma. Additionally, our endoscopic findings and histopathological observations varied from those typically found in HpPGC.

The occurrence of fundic gland type adenocarcinomas has been reported next to the undifferentiated type in previous reports of HpUIGC[38,39]. This tumor has a gastric phenotype and low-grade adenocarcinoma occurring in the fundic gland on the middle layer of mucosa or just above the muscularis mucosae. The tumor is covered with non-cancerous epithelium; therefore, it demonstrates a submucosal tumor-like (SMT-like) morphology and sometimes infiltrates the submucosa. Similar to previous research, all cases in the present study showed SMT-like morphology, of which six cases (85.7%) showed frequent submucosal invasion (SM1: 5, SM2: 1) without lymphovascular invasion[12,39,40]. The only case where a tumor invaded into SM2 received additional surgical treatment as per the guidelines[22]. Proximal gastrectomy was selected, and no lymph node metastasis was observed in the resected specimen.

The foveolar type adenocarcinoma mainly showed low-grade atypia adenocarcinoma with a tendency to differentiate into the foveolar epithelium. Since there

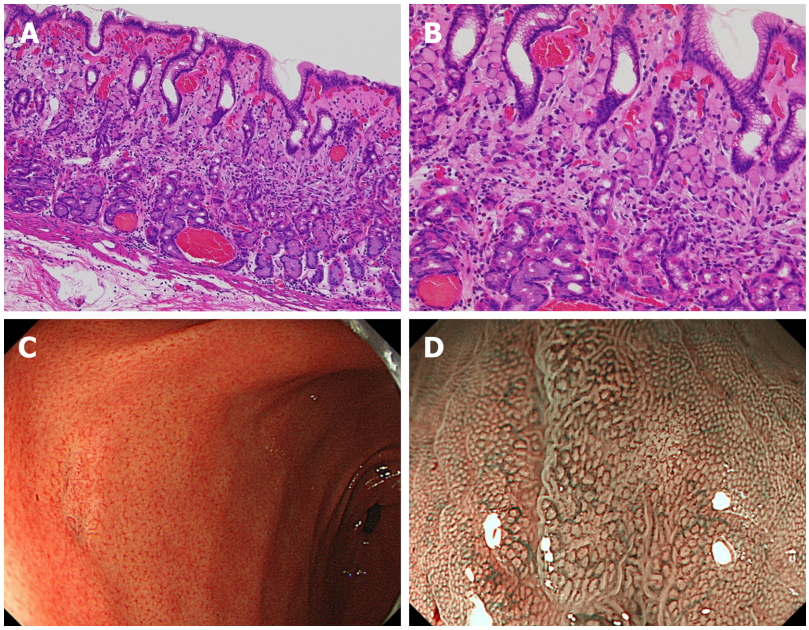

Figure 4 Pure signet-ring cell carcinoma. A, B: Pure signet ring cell carcinoma were observed proliferative zone to the surface layer of mucosa, hematoxylin and eosin, A: Original magnification 4 ×; B: High magnification 40 ×; C: Discolored slightly depressed lesion on the gastric antrum, white light endoscopy; D: The surface of the tumor was covered with normal mucosa, magnifying endoscopy with narrow-band imaging

are few reports on HpUIGC differentiated cancer other than fundic gland type adenocarcinoma[11], the characteristics have not been clarified. This tumor has been classified as dysplasia/adenoma in the West[41]; however, in Japan, only the typical pyloric gland adenoma is classified as adenoma, and other fundic gland types and foveolar type are often treated as an adenocarcinoma even if it is non-invasive. Therefore, we classified them as foveolar-type adenocarcinomas. This type of tumor had unique histological findings such as MUC6 positive cell proliferation beneath the superficial dysplasia/well-differentiated adenocarcinoma *in situ* components[42]. We found MUC5AC positive tumor cells derived from the foveolar epithelium and a MUC6 positive cystic expanded gland in the middle layer of the mucosa, so flat or protruded macroscopic type was defined as a characteristic.

Whether this MUC6-positive cell was cancerous or noncancerous is still controversial. However, we determined that MUC6 positive cells were non-cancerous since the junction between MUC5AC positive cancer cells and MUC6 positive cells was clear, and no cell atypia was found in MUC6 positive cells with low KI-67 index. This tumor showed discolored flat elevation with a granular structure similar to intestinal adenoma as a colonic lateral spreading tumor (LST)[43]. However, this tumor was seen in the greater curvature of the fornix in the upper third of the stomach, not in the lower part of the stomach where intestinal-type adenomas occur frequently.

Although the intestinal phenotype of HpUIGC is rare and only a few cases were seen in case reports[44-47], it is essential to recognize that there are not a few intestinal phenotype adenocarcinomas among HpUIGCs. In this study, 7 verrucous-like tumors found in the antrum predominantly showed the intestinal phenotype, and to the best of our knowledge, this is the first report that revealed the endoscopic and the pathological features of this kind of tumor. In intestinal phenotype cancers, as the tumor grows, the gastric phenotype of the background mucosa gradually changes to the intestinal phenotype of the tumor and is eventually replaced or considered to be null. In this study, the tumor showed mixed gastrointestinal phenotype as the intestinal phenotype adenocarcinoma was very small, and the gastric phenotype in the background remained. Intestinal phenotype adenocarcinoma is characterized by a macroscopic type resembling a single verrucous found in the antrum. It is desirable to perform the endoscopy screening with ME-NBI in *H. pylori* uninfected patients to identify this tumor. It is reported that gastric epithelial cells might be generated by stem cells and progenitor cells located in the isthmus[48]. The types of stem cells in the isthmus are different between corpus and antrum, and the only antrum contains intestinal stem cells. Considering these observations, the intestinal-type adenocarcinoma we observed in the current study could be generated from the intestinal stem cells and, thus, detected only in the antrum.

In recent years, NBI diagnosis has become indispensable in the diagnosis of gastric

Table 3 Mucus phenotype of *Helicobacter pylori* uninfected early gastric cancers

	MUC5AC	MUC6	CD10	MUC2
Fundic grand type adenocarcinoma	++	++	-	-
Foveolar type well differentiated adenocarcinoma	++	-	-	-
Intestinal phenotype adenocarcinoma	-~+	-~+	++	+~++
Pure signet ring cell carcinoma	++	++	-	-

cancer. In Japan, Yao's VS (microvascular architecture and microsurface structure) classification system is widely cited[49]. Further, the classification of Yokoyama *et al*[35] is useful because it can aid in identifying early gastric cancers in NBI images. However, in this HpUIGC series, there are many cases that cannot be characterized by the conventional NBI classification of gastric cancer. For example, in fundic gland type adenocarcinoma, the demarcation line is not clear because the tumor is covered with non-neoplastic foveolar epithelium so that the surface microstructure pattern is regular and typical NBI findings of differentiated type adenocarcinoma such as fine network pattern or intralobular loop pattern could not be confirmed. In addition, pure signet-ring cell carcinoma does not exhibit a typical corkscrew pattern as mentioned above. These cases classified in accordance with the NBI classification system. Therefore, HpUIGC may not conform to the conventional NBI classification system.

The mucin phenotype of gastric cancer is related to the growth, and biological malignancy of the tumor, and especially the differentiated-type gastric cancer with gastric phenotype may mix undifferentiated comportment as growth. Therefore, the biological malignancy of the differentiated-type gastric cancer with the gastric phenotype was considered higher than the intestinal-type[50-52]. However, in recent years, among the differentiated-type gastric cancer with the gastric phenotype, the existence of tumors with low-grade atypia such as extremely well-differentiated adenocarcinoma has been clarified. The gastric phenotype of differentiated adenocarcinoma is often difficult to distinguish from normal mucosa or regenerative epithelium because of its low-grade atypia[53]. Among HpUIGCs, the gastric phenotype is predominant in most of the undifferentiated and differentiated adenocarcinomas developed from the fundic gland area[9,29].

The etiology of gastric cancers, excluding *H. pylori* infection, is known to be associated with several factors including lifestyle, viral infection, autoimmune disorders, and germline mutations, but the main causal factor of HpUIGC remains unclear[54,55]. Bile acid reflux into the remnant stomach after gastrectomy is considered a risk factor for carcinogenesis, particularly, after Billroth II reconstruction[56]. Similarly, intestinal metaplasia caused by bile acid exposure might pose a risk of carcinogenesis in the stomach without *H. pylori* infection. In order to definitively determine the gastric cancer risk factors, further case studies and genetic analyses are needed. Although long-term outcomes of the present study were favorable, the observation period was short.

The study design and small sample number may be limitations to our study. A single-center retrospective cohort study and a small number of *H. pylori*-uninfected gastric cancer patients may not be representative of the broader population. In addition, although two or more tests were used to confirm *H. pylori* infection status the following the guidelines confirmed negative, but because the study period is long, the second limitation is that the types of tests are not unified.

The present study elucidated the clinicopathological features of HpUIGC, which is very rare. Herein, we classified HpUIGC into four categories according to histopathology and studied their endoscopic and pathological characteristics. To the best of our knowledge, we are the first to report that differentiated-type gastric cancers can possess the gastric and intestinal phenotype. HpUIGC malignancy is low; however, because the carcinogenic mechanism is unclear and further studies are required.

ARTICLE HIGHLIGHTS

Research background

In recent years, awareness of eradication therapy has increased in Japan. As *Helicobacter pylori*(*H. pylori*) infections decrease, the proportion of gastric cancers arising from *H. pylori* uninfected gastric mucosa will increase. The emergence of gastric cancer arising in *H. pylori* uninfected patients though rarely reported, is a concern to be addressed and needs elucidation of its clinicopathological features.

Research motivation
Previously, *H. pylori*-uninfected gastric cancer including case report such as undifferentiated gastric cancer or fundic gland-type gastric cancer was reported. However, due to the rare frequency, there was very few reports. In the future, *H.pylori*-uninfected gastric cancer may increase relatively; therefore, importance of clarifying the clinicopathological features of those is desired. In this study, we experienced 30 cases of *H. pylori*-uninfected early gastric cancer and could classify histopathological features of these.

Research objectives
To clarify clinicopathological feature of *H. pylori*-uninfected gastric cancer (HpUNGC) treated by endoscopic submucosal dissection (ESD).

Research methods
This study is retrospective study. A total of 2462 patients with 3375 instances of early gastric cancers that were treated by ESD were enrolled in our study between May 2000 and September 2019. We defined a patient as *H. pylori*-uninfected using the following three criteria; i) the patient did not receive treatment for *H. pylori*, which was determined by investigating medical records and conducting patient interviews, ii) lack of endoscopic atrophy, and iii) the patient was negative for *H. pylori* after being tested at least twice using various diagnostic methods, including serum anti-*H. pylori*-IgG antibody, urease breath test, rapid urease test, and microscopic examination.

Research results
Of these, 30 lesions in 30 patients were diagnosed as HpUIGC. Histologically 30 HpUIGC lesions were classified into 4 types (fundic gland type adenocarcinoma, foveolar type well-differentiated adenocarcinoma, intestinal phenotype adenocarcinoma, and pure signet-ring cell carcinoma). Unlike previous reports, most of the lesions (22/30 lesions) were the differentiated type.

Research conclusions
In this study, we classified 30 HpUIGCs into 4 types histologically. Unlike previous reports, there were more differentiated cancers than undifferentiated cancers. Although the most of HpUIGC showed gastric phenotype, it is essential to recognize that there are not a few intestinal phenotype adenocarcinomas among HpUIGCs. HpUIGC is very rare, among which, histologically high incidence of undifferentiated adenocarcinoma. Besides undifferentiated adenocarcinoma and gastric fundic gland type adenocarcinoma, there is another HpUIGC having different histopathological features. HpUIGC may show various type of histopathological features. Histologically, HpUIGC is classified into at least 4 types (fundic gland type adenocarcinoma, foveolar type well-differentiated adenocarcinoma, intestinal phenotype adenocarcinoma, and pure signet-ring cell carcinoma). To the best of our knowledge, the present study reports the largest number of HpUIGC cases that had been evaluated for both endoscopic and pathological findings. To recognize clinicopathological feature of HpUIGC will be helpful for early detection of HpUIGC in the future clinical practice.

Research perspectives
To recognize the various clinicopathological features of HpUIGC is useful for clinical diagnosis in the future. Because HpUIGC is rare frequency, we consider multicenter clinical trial for case collection to elucidate more detail of the clinicopathological characteristics of HpUIGC. Multicenter observational trial is the best method for the future research.

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ORIGINAL ARTICLE

Case Control Study

Anhedonia and functional dyspepsia in obese patients: Relationship with binge eating behaviour

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Abstract

BACKGROUND

Obese patients (Ob) with a binge eating disorders (BED) behavior pattern have a higher prevalence of postprandial distress syndrome (PDS) compared to Ob without a BED behavior pattern, while an increase of PDS has been described in Ob after sleeve gastrectomy (SG). Hedonic response to a meal is dissociable from satiation in healthy subjects. Anhedonia is the lowered ability to experience pleasure. There are no studies investigating the presence of anhedonia in Ob with and without SG and its relationship to PDS symptoms.

AIM

To assess the relationship among anhedonia, BED and upper gastrointestinal symptoms in two group of morbidly Ob with and without SG.

METHODS

Eighty-one Ob without SG, 45 Ob with SG and 55 healthy controls (HC) were studied. All subjects fulfilled the binge eating scale (BES) to investigate BED, the validated 14 items Snaith-Hamilton pleasure scale (SHAPS) to assess Anhedonia as well as the Beck Depression Inventory-II (BDI II) and State Trait Anxiety Inventory (STAI) questionnaires to screen for depression and anxiety. All patients underwent a standardized questionnaire investigating the intensity-frequency scores (0-6) of upper gastrointestinal symptoms and were diagnosed for the presence of functional dyspepsia (FD) and its subtypes according to ROME IV criteria.

RESULTS

Ob without SG who were positive for BED had a 4.7 higher risk of FD compared to Ob without SG who were negative for BED (OR: 4.7; 95.0%CI 1.23-18.24; P = 0.02). STAI-Y2 scores were significantly higher in Ob without SG positive for BED

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(42.2 ± 1.5 *vs* Ob negative for BED: 39.6 ± 1 .0, $P = 0.04$), while SHAPS scores and BDI II did not differ in the two groups (1.16 ± 1.30 *vs* 0.89 ± 1.02, $P = 0.49$). A lower prevalence of BED (BES > 17: 11.4% *vs* 40.7%, $P = 0.001$) and BDI-II (6.8 ± 1.2 *vs* 13.8 ± 1.9, $P = 0.005$) was reported in Ob with SG than Ob without SG, on the contrary total mean scores of STAI-Y1 and STAI-Y2 were significantly higher in Ob with SG than Ob without SG. Thirty-five percent of Ob with SG fulfilled the diagnosis of FD. SHAPS mean scores and the prevalence of anhedonia did not differ among the two groups (18.2 *vs* 8.1%, $P = 0.2$). Fifty-four percent of Ob with SG achieved surgical success excess weight loss > 50%. Excess weight loss was negatively related to SHAPS total mean scores [adjusted B: -7. 099 (95%CI: -13.91 to -0.29), $P = 0.04$].

CONCLUSION

Ob without SG showed a higher prevalence of PDS, mood disorders and anxiety when positive for BE behavior compared to those negative for BE behavior, whereas no differences were found in SHAPS score. Ob with SG showed a higher prevalence of PDS compared to Ob without SG. Concerning psychological aspect, BED and depression are less frequent in the Ob with SG, while both state and trait anxiety are significantly higher. Moreover, the more an Ob with SG is anhedonic, less surgical success was achieved.

Key words: Morbid obesity; Functional dyspepsia; Postprandial distress syndrome; Epigastric pain syndrome; Anhedonia; Binge eating disorders; Sleeve gastrectomy

Core tip: Binge eating disorders (BED) co-occur with mood disorders and anxiety, whereas the relationship with anhedonia in obese patients undergoing sleeve gastrectomy (SG) is not known. We studied two group of morbidly obese patients with and without SG to assess the relationships among anhedonia, BED and functional dyspepsia. Our results suggest that a more regular screening for functional dyspepsia in SG candidates might help to disclose the presence of BED that may jeopardize postsurgical outcomes. Although anhedonia was not associated with BED in this study, worse surgical outcome was observed in patients with anhedonia independent of early satiety and postprandial fullness.

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INTRODUCTION

Obesity is increasing in industrialized countries[1]. Bariatric surgery (BS) for weight loss has emerged as an effective treatment of morbid obesity (Class II and III) since it accomplishes sustained weight loss, reduces obesity-related comorbidities and improves quality of life[2-4]. Among bariatric procedures, sleeve gastrectomy (SG) has increased in popularity and in 2014 became the most performed procedure in the world[5]. The association between obesity and some psychopathological features, specifically binge eating disorder (BED) is frequently present prior to surgery[6,7]. BED is characterized by recurrent episodes of binge eating and diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-V)[8]. It has been previously demonstrated that BED occurs in a subset ranging from 27% to 47% in patients with severe obesity undergoing BS[9] and may jeopardize postsurgical outcomes[10,11]. Obese patients (Ob) with BE behavior pattern have a higher prevalence of postprandial distress syndrome (PDS), a subtype of functional dyspepsia (FD) according to Rome III criteria[12] and, an increase of PDS has been described in Ob after SG[13]. FD is a syndrome characterized by the presence of chronic or recurrent symptoms invariably referred to the epigastrium, if no structural and/or biochemical

alterations are detectable. The effect of specific patterns of eating behavior such as BED on the development of FD symptoms has not yet been completely defined[14]. From a physiological perspective, excessive intake of food over a relatively short time could potentially overcome the functional accommodation and emptying, contributing to the genesis of gastrointestinal symptoms in obese individuals[15]. Conversely, after SG the new onset of PDS might be linked to the decreased gastric capacity and altered duodenal sensitivity[13]. In obesity BED co-occur with a variety of psychiatric disorders, especially mood (49%), anxiety (41%) and substance use disorders (22%)[16]. Anhedonia, defined negatively as lack of and/or decreased capacity to experience pleasure[8], is considered one of the most indicative symptoms of mood disorders and depression[14,15]. It has been recently demonstrated that the presence of anhedonia, regardless of whether the participant had received a diagnosis of major depression, was associated with uncontrolled, emotional and binge eating. Moreover, weight loss was greater among participants without anhedonia[17].

We hypothesized that a vicious circle, aided by an eating disorder (ED) behavior and the presence of anhedonia may perpetuate upper gastrointestinal (GI) symptoms prior to surgery[18-20] and, eventually, influence the outcome of BS. Therefore, the objective of this study was to evaluate the relationship among anhedonia, BED and upper GI in morbidly Ob with and without SG.

MATERIALS AND METHODS

Participants

Eighty-one Ob without SG and 45 Ob with SG, similar for age and sex, were recruited from an outpatient clinic devoted to surgical therapy of obesity and related disorders at San Giovanni Bosco Hospital, Naples, Italy and run by surgeons and gastroenterologists trained in the field. The study was approved by the Ethical Committee of the ASL Napoli Centro. Informed consent was obtained from all patients.

The inclusion criteria were Caucasian females and males between the ages of 18 and 75 years having the ability to understand and the willingness to comply with the study procedures. Exclusion criteria were serious, unstable medical condition, major psychiatric disorders, previous history of drug or alcohol abuse, previous major abdominal surgery with the exception of laparoscopic SG, laparoscopic cholecystectomy and appendectomy and pregnant women.

Demographic characteristics (gender, age, smoking habits, school degree), anthropometric data [weight, height and body mass index (BMI)], and prevalence of comorbidities, such as, hypertension, dyslipidemia, type II diabetes mellitus, and respiratory diseases were individually collected. In Ob undergoing SG excess weight loss (EWL) was calculated[21] and the surgical success was defined as EWL > 50%[22]. Fifty-five healthy controls (HC) were enrolled from friends of obese patients and hospital staff as the control group. Inclusion criteria for HC were Caucasian adults, aged between 18 to 75 years without upper gastrointestinal complaints. Exclusion criteria were similar to that for Ob. Demographic characteristics (gender, age, smoking habits, school degree) and anthropometric data (weight, height and BMI) were collected for each HC.

Upper gastrointestinal symptoms

All patients were evaluated using a standardized questionnaire that assesses upper GI symptoms such as dysphagia for solids, dysphagia for liquids, regurgitation, heartburn, non-cardiac chest pain, cough, belching, nausea, bloating, early satiation, epigastric fullness, epigastric pain and epigastric burning with a scoring frequency from 0 to 3 (0 = absent, 1 = 2 d/wk; 2 = 3-5 d/wk; and 3 = 6 or 7 d/wk) and intensity from 0 to 3 (0 = absent; 1 = not very bothersome, not interfering with daily activities; 2 = bothersome, but not interfering with daily activities; and 3 = interfering with daily activities). A frequency-intensity score from 0 up to a maximum of 6 was obtained for each symptom[12].

Anhedonia

All participants were evaluated by the Snaith–Hamilton pleasure scale (SHAPS). The SHAPS is a self-assessment tool used to measure hedonic experience or positive patient value. It is composed of 14 items that cover four domains of pleasure: Interests/pastimes, social interaction, sensory experiences and food/drink. Higher scores represent a reduction of hedonic tone. A score of three or more allows a categorical definition of anhedonia[23,24]. An Italian version of the SHAPS has been previously validated and has been used in routine clinical practice and research[25].

Binge eating behaviour

All participants were evaluated by the binge eating scale (BES), a specific questionnaire originally created to investigate binge-eating behavior in obese patients. The BES measures the behavioral aspects of binge eating, as well as the feelings and thoughts associated with such behavior[26]. It is a self-administered questionnaire composed of 16 multiple choice items: Eight items that describe behavioral manifestations (for example, eating fast or consuming large amounts of food) and eight items on associated feelings and cognitions (for example, fear of not stopping eating)[27]. Each item has a response range from 0 to 3 points (0 = no severity of the BES symptoms, 3 = serious problems of the BES symptoms). Based on the BES total score, individuals can be categorized into three groups according to established cut scores of binge eating severity[28]. A frequent convention is to use the BES as a screening measure to classify all participants with scores greater than or equal to 17 as "binge eaters"[29,30].

Depression

All participants filled in the Beck Depression Inventory-II (BDI-II) scale. The BDI-II is a self-report questionnaire that is integrated into routine clinical practice (as screening tools) in large managed-care organizations. BDI-II follows the criteria for depression listed in the fourth edition of the DSM. The test consists of twenty-one questions that not only assess the presence of depression, but also the severity of depression as well[31]. Each question has a set of at least four possible responses ranging in intensity, with a value of 0 to 3 is assigned for each answer and the total score compared to a key to determine severity. The standard cut-off scores are as follows: 0-9: Indicates minimal depression; 10–18: Indicates mild depression; 19–29: Indicates moderate depression; 30-63: Indicates severe depression[32]. For this study we considered a cut-off for this questionnaire of 14, considering patients positive with a score higher than 14 and negative with a score lower than or equal to 14. An Italian version of the BDI-II was previously validated[33,34].

Anxiety

The state trait anxiety inventory (STAI) is one of the most widely used self-report measures for the presence and grade of anxiety. The questionnaires contain 20 items, with responses related to terms of intensity (from "almost never" to "almost always"). The items are grouped into two axes, which permit a distinction between existing anxiety (STAI-Y1) and predisposition to an anxious reaction as a personality characteristic (STAI-Y2). A score ≥ 40 is the cut-off value for both scales[35].

Functional dyspepsia criteria

All Ob were investigated for the presence of FD according to ROME IV criteria[36], after the exclusion of any organic disease. A complete physical examination, blood tests, upper GI endoscopy and additional tests were performed when indicated[37]. The scores were calculated for the 4 cardinal symptoms pragmatically described by the Rome IV Committee such as postprandial fullness, early satiation, epigastric pain, and epigastric burning (Table 1). The spectrum of FD includes patients suffering from the diagnostic categories of PDS which is characterized by meal-induced dyspeptic symptoms, epigastric pain syndrome (EPS) which refers to epigastric pain or epigastric burning that does not occur exclusively postprandially, can occur during fasting, and can be even improved by meal ingestion, and overlapping PDS and EPS, which is characterized by meal induced dyspeptic symptoms and epigastric pain or burning.

Outcome parameters

The primary outcomes were to evaluate upper GI symptoms, the level of anhedonia and BED in Ob with and without SG. Secondary outcomes were the evaluation of the coexistence of BED with depressive mood and anxiety as well as the relationship of anhedonia level with the outcome of BS.

Statistical analysis

The data are expressed in frequencies and percentages for qualitative variables, as mean ± standard error (mean ± SE) for quantitative ones unless otherwise indicated. Significance was expressed at $P < 0.05$ level. When appropriate, a χ^2 test for categorical data and analysis of variance for continuous data were used. We then performed a subgroup analysis to test the risk of having PDS according to the presence or absence of BED using a logistic model. Finally, using a regression analysis, we tested how much the EWL changed according to the level of anhedonia and to the intensity-frequency scores of PDS symptoms. The SPSS for Windows version 15.0 statistical package (SPSS Inc, Chicago, IL, United States) was used for statistical analysis.

Table 1 Diagnostic criteria for functional dyspepsia[37]

Functional dyspepsia		
One or more of the following:	And	No evidence of structural disease (including at upper endoscopy) that is likely to explain the symptoms
Bothersome postprandial fullness		
Bothersome early satiation		
Bothersome epigastric pain		
Bothersome epigastric burning		
Post Prandial distress syndrome		Epigastric pain syndrome
must include one or both of the following at least 3 d/w:		Must include at least 1 of the following symptoms at least 1 day/ w:
Bothersome postprandial fullness (*i.e*, strict enough to impact on usual activities)		Bothersome epigastric pain (*i.e*, severe enough to impact on usual activities)
Bothersome early satiation (*i.e*, severe enough to prevent finishing a regular-size meal)		Bothersome epigastric burning (*i.e*, severe enough to impact on usual activities)

RESULTS

Obese patients without SG

Demographic characteristics, anthropometric data, and prevalence of comorbidities in Ob without SG and HC are shown in Table 2. Thirty-three/81 (40.7%) of Ob without SG *vs* 3/55 (5.5%) of HC reported a patient a pathological score for BED (> 17) at BES ($P < 0.001$).

STAI-Y1 and STAI-Y2 total scores were not significantly different in OB without SG and HC. Thirty nine percent of Ob without SG showed a pathological score at Beck Depression Inventory-II (BDI II) (> 14) and BDI II scores were significantly higher in Ob without SG compared to HC (Table 2). SHAPS mean scores did not differ among the two groups (Table 2) and the prevalence of anhedonia, according to DSM-V (score > 3), was similar in Ob without SG and HC (8.1% *vs* 6.2%, $P = 0.69$).

Frequency-intensity scores for selected upper GI symptoms such as dysphagia for solids, dysphagia for liquids, regurgitation, heartburn, non-cardiac chest pain, cough, belching, nausea, early satiation, epigastric fullness, epigastric pain and epigastric burning in Ob without SG are illustrated in Figure 1. According to the presence or absence of BED, the frequency intensity scores of the studied upper GI symptoms (dysphagia for solids, dysphagia for liquids, regurgitation, heartburn, non-cardiac chest pain, cough, belching, nausea, bloating) did not differ between Ob without SG who were positive or negative for BED (Table 3).

The prevalence of FD, according to Rome IV criteria, was significantly higher in Ob without SG who were positive for BED compared to those who were negative for BED (36.7% *vs* 10.5%, $P = 0.01$). Specifically, except for two patients who complained of EPS symptoms, all the remaining Ob fulfilled the diagnostic criteria for PDS. Ob without SG who were positive for BED showed 4.7 higher risk of having FD, independent of age and gender (OR: 4.7; 95.0%CI: 1.23-18.24; $P = 0.02$) compared to Ob without SG who were negative for BED. Figure 2 describes the frequency-intensity score of the 4 cardinal FD symptoms in Ob without SG who were positive or negative for BED.

Mean SHAPS scores and the prevalence of anhedonia, according to DSM-V (score > 3) did not differ in Ob without SG positive or negative for BED (1.16 ± 1.30 *vs* 0.89 ± 1.02, $P = 0.49$ and 10.5 *vs* 5.6%, $P = 0.58$). Also, BDI II score was similar in the two groups (10.3 ± 2.6 *vs* 16.7 ± 2.7, $P = 0.09$). Although mean STAI-Y1 scores were not significantly different among the two groups ($P = 0.43$), mean STAI-Y2 scores were significantly higher in Ob without SG positive for BED (42.2 ± 1.5 *vs* 39.6 ± 1.0, $P = 0.04$).

Obese patients with SG

Obese patients having undergone SG (Ob with SG) were similar for age and sex to Ob without SG (Table 4). Fifty-four percent of Ob with SG achieved surgical success (EWL > 50%). A significantly lower frequency of pathological score for BED (> 17) at BES was reported in Ob with SG than Ob without SG (11.4% *vs* 40.7%, $P = 0.001$). SHAPS mean scores shown in Table 4 and the prevalence of anhedonia, according to DSM-V (score > 3) (18.2 *vs* 8.1%, $P = 0.2$) did not differ among the two groups. Total mean BES, BDI II, STAI-Y1 and STAI-Y2 in Ob with SG compared to those without SG are reported in Table 5. Frequency-intensity scores of other upper GI symptoms such

Table 2 Demographic characteristics, anthropometric data, and prevalence of comorbidities in obese patients without Sleeve Gastrectomy and healthy control

	Ob without SG (*n* = 81)	HC (*n* = 55)
Gender (M/F)	23/58	18/37
Age (yr)	36.5 ± 1.3	36.7 ± 1.2
Weight (kg)	122.1 ± 3.2	64 ± 2.5[b]
BMI (kg/m^2)	44.4 ± 0.9	23.2 ± 0.6[b]
Ethnic origin (Caucasian)	100	100
Smoking	27.7	14.3
Number of cigarettes per day	22.5 ± 3.7	11 ± 1.0
Diabetes	8.5	0
Hypertension	19.1	7.1
Dyslipidemia	25.5	14.3
Respiratory diseases	42.6	0[b]
Musculoskeletal disorders	19.1	0
BES	15.5 ± 0.9	5.4 ± 0.6[b]
STAI-Y1	39.7 ± 0.9	41.1 ± 1.7
STAI-Y2	40.1 ± 0.9	41.2 ± 1.6
BDI 2	13.8 ± 1.9	5.8 ± 0.5[b]
SHAPS	1.0 ± 0.2	0.8 ± 0.1

Data are expressed as percentage (%) or as mean ± SE.
[b]$P < 0.01$ *vs* HC. M: Male; F: Female; BMI: Body mass index; BES: Binge eating disorder; SHAPS: Snaith-Hamilton pleasure scale; BDI II: Beck depression inventory-II; STAI: State trait anxiety inventory; HC: Healthy control; Ob: Obese patients; SG: Sleeve gastrectomy.

as dysphagia for solids, dysphagia for liquids, regurgitation, heartburn, non-cardiac chest pain, cough, belching, nausea, bloating was not significantly different among the two groups (Table 6).

Thirty-five percent of Ob with SG fulfilled the diagnosis of FD, in particular PDS, according to Rome IV criteria; frequency-intensity scores of early satiation and postprandial fullness were significantly higher in Ob with SG compared to those without SG (Figure 3). In Ob with SG accordingly to the presence or absence of BED the frequency-intensity scores of early satiation and postprandial fullness did not differ (2.0 ± 1.4 BED positive *vs* 2.28 ± 0.4 BED negative). In Ob with SG the regression analysis showed that EWL is negatively related to SHAPS total mean scores [adjusted B -7. 099 (95%CI: -13.909 to -0.290), $P = 0.04$] independent of early satiation and postprandial fullness.

DISCUSSION

In this study two groups of Ob were evaluated. In the group without SG a higher prevalence of BED, depression and anxiety disorders but not anhedonia was observed in comparison with HC. Moreover, Ob without SG fulfilling BED criteria showed more anxiety and a higher risk of having FD. The other group of Ob with SG showed a turnabout of BED, depression and anxiety levels and a marked increase in PDS prevalence compared to Ob without SG. In addition, a higher excess weight loss was significantly associated with a lower anhedonia level.

This is, to the best of our knowledge, the first study to assess the relationships among psychiatric comorbidity and upper GI symptoms in Ob with and without SG. Depression was repeatedly associated with an overconsumption of food[38,39] and BED[40]. The presence of anhedonia, irrespective of whether the participant had received a diagnosis of major depression or dysthymia, was associated with uncontrolled, emotional eating[17]. Many studies claim that the prevalence of anxiety disorders is higher than the general population for all ED[41] as well as trait anxiety is associated with increased rates of compensatory behaviors, binge eating, and body dissatisfaction[42]. BED correlates with an increased BMI[6,7] and is a common findings in Ob undergoing BS and influences their outcome. It is already known that the frequency of FD according to Rome III criteria was similar in Ob and control subjects, while Ob with coexisting BE behavior have a higher prevalence of PDS[12]. In this

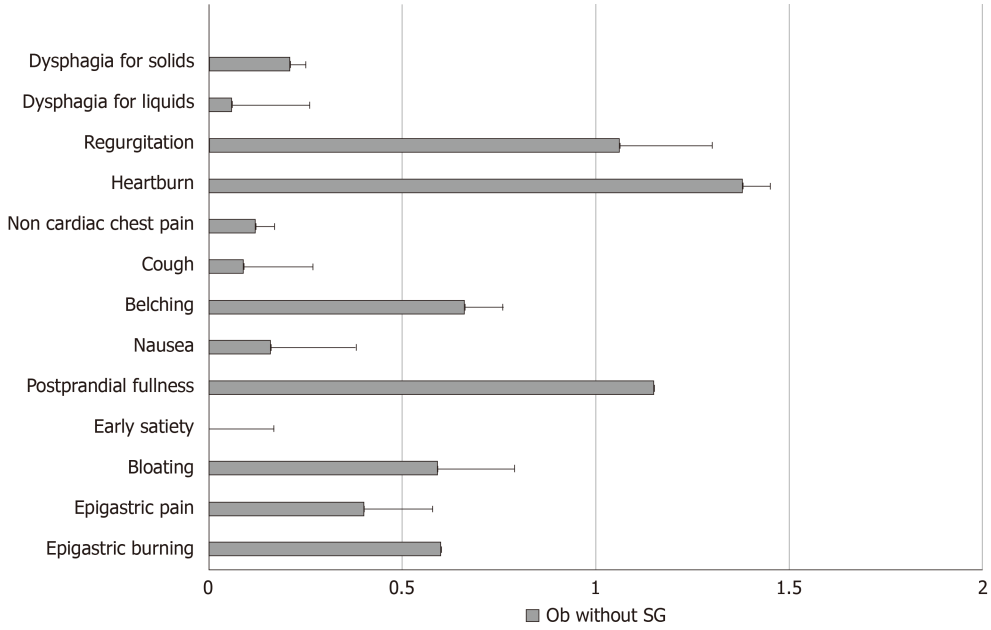

Figure 1 Frequency-intensity scores for selected upper gastrointestinal symptoms in obese patients without sleeve gastrectomy. Ob: Obese patients; SG: Sleeve gastrectomy.

study we confirmed this result with the new Rome IV criteria as well as the finding that early satiation is not a feature of Ob without SG. This finding is consistent with the datum that satiation signals that inhibit ingestion are reduced with increased BMI[43]. Conversely, the increase in frequency and severity of postprandial fullness in Ob without SG that fulfilled BED criteria might be explained by an excessive food intake over a relatively short time that could potentially impair the functional accommodation and the gastric emptying[12].

Our study cannot establish the direction of effect of these associations with regard to obesity, depression, anhedonia, anxiety, eating behavior and upper GI symptoms because of the design; however, our results suggest that in Ob without SG, the presence of BED might be a dominant player in determining FD. On the other side, concerning the psychological aspect, Ob with SG showed a reduced depression severity but an increased level of anxiety compared to Ob without SG; however, the level of anhedonia did not differ between the two Ob groups. It has been previously demonstrated that bariatric surgery might decrease depression and mental health issues post-surgery[44]. Higher rates of post-surgical depression are associated with less weight loss success[44]. Furthermore, our results demonstrated for the first time that EWL after SG was negatively related to anhedonia. In other words, the more a subject is anhedonic, less surgical success was achieved. A previous study has already suggested that weight loss was greater among participants without anhedonia that participated to a weight loss counseling intervention healthy program[17]. The prevalence of BED was lower in Ob with SG compared to Ob without SG. Since BED might negatively affect surgical outcome, it is possible that Ob with BED did not undergo BS[10,11].

In Ob with SG early satiation and postprandial fullness are more frequent than in Ob without SG, while epigastric pain and epigastric burning as well as other upper GI symptoms have the same frequency in both Ob groups. It is noteworthy that early satiation was absent in patients without SG; conversely, the reduction in gastric capacity following SG leads to a new onset of this symptom. Hence, our results suggest that in Ob with SG the increase of PDS symptoms is independent from the presence of BED.

Our data confirm a key role of epigastric symptoms in determining calorie intake in health, obesity, and dyspepsia. The complex and reciprocal interplay between biological, psychological, and social factors, rather than from linear monocausal etiopathogenetic processes is a common characteristic of both functional dyspepsia and obesity. For example, it is well known that individual assessment of obesity and development of counseling should include not only clinical factors such as body weight and dietary intake, but also an assessment of psychological factors such as psychological eating behavior traits. Rome IV criteria that are based on the biopsychosocial approach could appeared unnecessary in the daily care of these Ob

Table 3 Frequency intensity scores of the studied gastrointestinal symptoms such as dysphagia for solids, dysphagia for liquids, regurgitation, heartburn, non-cardiac chest pain, cough, belching, nausea, bloating in obese patients without sleeve gastrectomy who were positive or negative for binge eating disorder

	Ob without SG positive for BED (*n* = 33)	Ob without SG negative for BED (*n* = 48)	*p*
Dysphagia for solids	0.34 ± 0.19	0.10 ± 0.07	0.20
Dysphagia for liquids	0	0.10 ± 0.07	0.22
Regurgitation	0.68 ± 0.24	1.33 ± 0.29	0.11
Heartburn	1.28 ± 0.39	1.45 ± 0.30	0.72
Non cardiac chest pain	0.21 ± 0.15	0.05 ± 0.05	0.28
Cough	0.14 ± 0.10	0.05 ± 0.05	0.40
Belching	0.72 ± 0.28	0.62 ± 0.22	0.76
Nausea	0.14 ± 0.10	0.18 ± 0.16	0.84
Bloating	1.52 ± 0.33	0.85 ± 0.26	0.11

Data were expressed as mean ± SE. BED: Binge eating disorders; Ob: Obese patients; SG: Sleeve gastrectomy.

but still can serve as a useful guide to help understand through symptoms the complexity of the disease and to capture also other comorbidity of the clinical condition of Ob optimizing the treatment. In fact, regarding the management a physician who acknowledges the reality of the patient's complaints, engages in an effective physician–patient interaction, and reduces symptom severity and health care seeking. Conversely, simply managing symptoms such as postprandial fullness may lead to perform unnecessary diagnostic studies to rule out pathologic disease and is likely to promote a vicious cycle of symptom anxiety and health care seeking[45]. Another strength of the present study is that our findings offer a screenshot of the Italian obese population.

There are some limitations in this study. One is the relatively small sample size and the heterogeneity of patients that might have limited the ability to generalize the findings to wider clinical samples. Another limitation is the design of the study that weakens our findings as compared to results from a prospective longitudinal study that are needed to establish a causal relationship among anhedonia, BED and upper GI symptoms. Moreover, all data were collected using self-reported questionnaires that might reflect the participant's own perspective. However, the simplicity of the questionnaire makes it somewhat easy for the participants to give accurate information questionnaires and they were validated in Italian language.

In conclusion, the results of the current study suggest that a more regular screening of PDS symptoms accordingly to Rome Criteria when evaluating obese patients before bariatric surgery might help to disclose the presence of BED that may jeopardize postsurgical outcomes. Although anhedonia was not associated with BED in this study, worse surgical outcome was observed in patients with anhedonia independent of the higher intensity-frequency scores of early satiation and postprandial fullness. An individual assessment of psychological factors such anhedonia should be incorporated into tailoring future treatment interventions in patients with unfavorable surgical outcome. Further research is urgently required to understand the pathophysiological interactions between anhedonia, BED and the onset of upper GI symptoms in morbidly obese patients pre and post bariatric surgery.

Table 4 Demographic characteristics, anthropometric data, and prevalence of comorbidities in obese patients with sleeve gastrectomy

	Ob with SG (*n* = 45)
Gender (M/F)	7/38
Age (yr)	38.36 ± 1.6
Weight (kg)	88.47 ± 3.2
BMI (kg/m^2)	32.74 ± 1.0
WL	29.61 ± 2.2
EWL (%)	53.41 ± 3.45
Months since the operation	25.67 ± 5.14
Ethnic origin (Caucasian %)	45
Smoking (%)	35.3%
Number of cigarettes per day	13.17 ± 3.5
Diabetes (%)	9.1%
Hypertension (%)	18.2%
Dyslipidemia (%)	36.4%
Respiratory diseases (%)	54.5%
Musculoskeletal disorders (%)	45.5%

Data are expressed as percentage (%) or as mean ± SE. M: Male; F: Female; BMI: Body mass index; WL: Weight loss; EWL: Excess weight loss; Ob: Obese patients; SG: Sleeve gastrectomy.

Table 5 Total mean scores at questionnaires in obese patients without sleeve gastrectomy and with sleeve gastrectomy

	Ob without SG	Ob with SG
BES	15.5 ± 0.9	9.4 ± 1.1[b]
STAI-Y1	39.7 ± 0.9	44.7 ± 1.1[b]
STAI-Y2	40.1 ± 0.9	44.7 ± 1.3[b]
BDI II	13.8 ± 1.9	6.8 ± 1.2[b]
SHAPS	1.0 ± 0.2	1.1 ± 0.3

Data are expressed as mean ± SE.
[b]$P < 0.01$ *vs* OB with SG. BES: Binge eating disorder; SHAPS: Snaith-Hamilton pleasure scale; BDI II: Beck depression inventory-II; STAI: State trait anxiety inventory; Ob: Obese patients; SG: Sleeve gastrectomy.

Table 6 Upper gastrointestinal symptoms in obese without sleeve gastrectomy and obese with sleeve gastrectomy

	Ob without SG	Ob with SG
Dysphagia for solids	0.21 ± 0.09	0.15 ± 0.15
Dysphagia for liquids	0.06 ± 0.04	0.15 ± 0.15
Regurgitation	1.06 ± 0.2	1.38 ± 0.34
Heartburn	1.38 ± 0.23	0.96 ± 0.33
Non cardiac chest pain	0.12 ± 0.07	0.38 ± 0.22
Cough	0.09 ± 0.05	0.15 ± 0.15
Belching	0.66 ± 0.17	0.46 ± 0.20
Nausea	0.16 ± 0.09	0.42 ± 0.26
Bloating	1.13 ± 0.21	0.23 ± 0.17[b]

[b]$P < 0.01$ *vs* Ob with SG. Ob: Obese patients; SG: Sleeve gastrectomy.

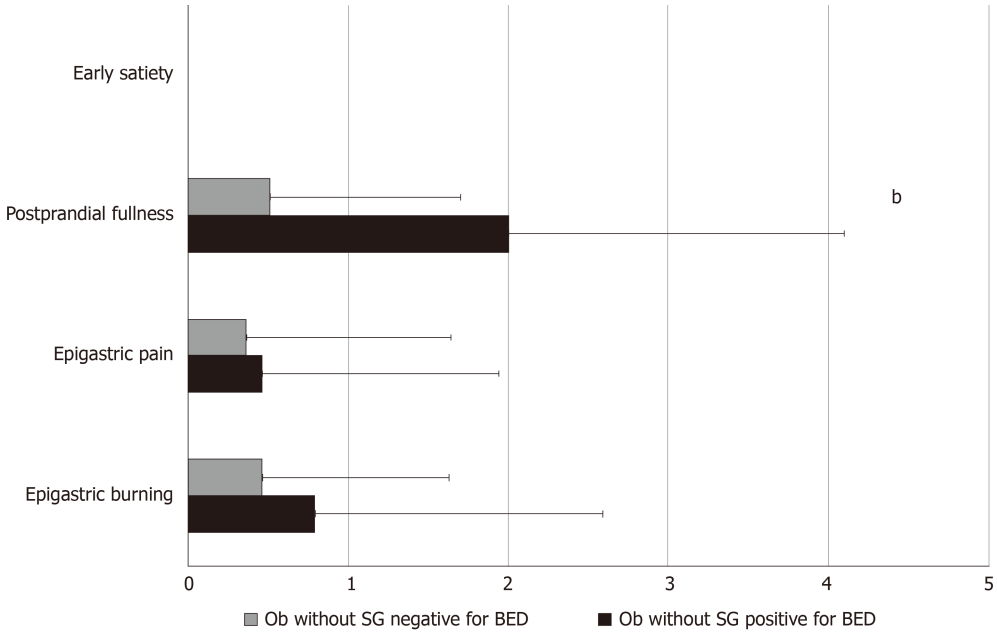

Figure 2 Frequency-intensity scores (m ± SE) of the 4 cardinal symptoms: early satiation, postprandial fullness, epigastric pain, and epigastric burning in obese patients without sleeve gastrectomy who were positive or negative for Binge eating disorder. Ob: Obese patients; SG: Sleeve gastrectomy; BED: Binge eating disorder.

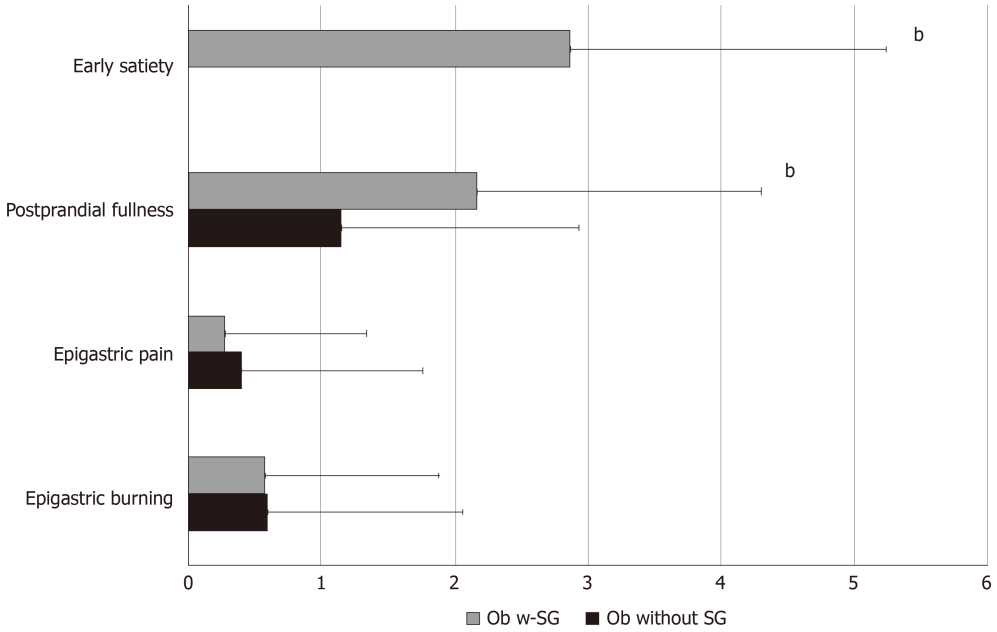

Figure 3 The frequency-intensity scores (m ± SE) of the 4 cardinal symptoms: Early satiation, postprandial fullness, epigastric pain, and epigastric burning in the studied obese patients without sleeve gastrectomy and obese patients with sleeve gastrectomy. Ob: Obese patients; SG: Sleeve gastrectomy.

ARTICLE HIGHLIGHTS

Research background

Obesity is increasing in industrialized countries. Among bariatric procedures for weight loss sleeve gastrectomy (SG) has emerged as an effective treatment of morbid obesity. The association between obesity and some psychopathological features, specifically binge eating disorder (BED) is frequent. Anhedonia was associated with uncontrolled, emotional and binge eating. Weight loss was greater in obese patients (Ob) without anhedonia. Ob with BED have a higher prevalence of postprandial distress syndrome (PDS), a subtype of functional dyspepsia (FD) according to Rome III criteria and, an increase of PDS has been described in Ob after SG.

Research motivation

The effect of specific patterns of eating behavior such as BED and on the development of FD symptoms has not yet been completely defined in Ob with and without SG. There are no studies investigating the presence of anhedonia in Ob with and without SG and its relationship to PDS symptoms.

Research objectives
In this study we aimed to assess the relationship among anhedonia, BED and upper gastrointestinal symptoms in two group of morbidly Ob with and without SG.

Research methods
Ob without SG, Ob with SG and healthy controls (HC) the binge eating scale (BES**)** to investigate BED, the validated 14 items Snaith-Hamilton pleasure scale (SHAPS) to assess anhedonia, the Beck Depression Inventory-II (BDI II) and state trait anxiety inventory (STAI) questionnaires to screen for depression and anxiety. They were diagnosed for the presence of functional dyspepsia (FD) and its subtypes according to ROME IV criteria.

Research results
Ob without SG who were positive for BED had a 4.7 higher risk of FD and a higher STAI-Y2 scores than Ob negative for BED, while SHAPS scores and BDI II did not differ between the two groups. Ob with SG showed a higher prevalence of PDS and STAI-Y1 and STAI-Y2 scores compared to Ob without SG. Conversely, Ob with SG had a lower prevalence of BED and BDI-II than Ob without SG. Excess weight loss was negatively related to SHAPS total mean scores [adjusted B – 7. 099 (95%CI: -13.91- -0.29), $P = 0.04$].

Research conclusions
Ob without SG showed a higher prevalence of PDS, mood disorders and anxiety when positive for BE behavior. Ob with SG showed a higher prevalence of PDS compared to Ob without SG. Concerning psychological aspect, BED and depression are less frequent in the Ob with SG, while both state and trait anxiety are significantly higher. Moreover, the more an Ob with SG is anhedonic, less surgical success was achieved.

Research perspectives
A more regular screening of PDS symptoms accordingly to Rome IV Criteria before bariatric surgery might help to disclose the presence of BED. An individual assessment of psychological factors such anhedonia should be incorporated into tailoring future treatment interventions in patients with unfavorable surgical outcome. Further research is urgently required to understand the pathophysiological interactions between anhedonia, BED and the onset of upper GI symptoms in morbidly obese patients pre and post bariatric surgery.

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ORIGINAL ARTICLE

Retrospective Study

Decreased of BAFF-R expression and B cells maturation in patients with hepatitis B virus-related hepatocellular carcinoma

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Abstract

BACKGROUND

Recent evidence has indicated the role of B cells and B cell-activating factor (BAFF) in the development of hepatocellular carcinoma (HCC).

AIM

To characterize circulating BAFF receptor expression and B cell subpopulations in patients with hepatitis B virus (HBV)-related HCC.

METHODS

Peripheral blood samples collected from 41 patients with chronic HBV infection (25 patients without HCC and 16 patients with HCC) and 9 healthy controls were assessed for BAFF receptors [BAFF-R(B cell-activating factor receptor), transmembrane activator and cyclophilin ligand interactor, B-cell maturation antigen] and B cell subpopulations by multicolor flow cytometry.

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RESULTS

The frequency of BAFF-R expressing B cells to total B cells was significantly lower in patients with HCC (3.39% ± 2.12%) compared with the non-HCC group (5.37% ± 1.90%) and healthy controls (6.23% ± 2.32%), whereas there was no difference in transmembrane activator and cyclophilin ligand interactor and B-cell maturation antigen. The frequencies of $CD27^{+}IgD^{+}$ memory B cells, $CD27^{+}IgD^{-}$ class-switched memory B cells and plasmablasts were significantly lower in the patients with HCC compared to patients without HCC (1.23 ± 1.17 *vs* 3.09 ± 1.55, $P = 0.001$, 0.60 ± 0.44 *vs* 1.69 ± 0.86, $P < 0.0001$ and 0.16 ± 0.12 *vs* 0.37 ± 0.30, $P = 0.014$, respectively). However, the ratio of naïve and transitional B cell did not differ significantly between the three groups. In addition, decreased BAFF-R expression on B cells was significantly correlated with large tumor size and advanced tumor stage.

CONCLUSION

Our data demonstrated BAFF-R expression was reduced in B cells that involved with the frequencies of B cells maturation in patients with HCC. The depletion of BAFF-R might play an important role in the development of HCC in patients with chronic HBV infection.

Key words: B cells; Hepatitis B virus; Hepatocellular carcinoma; B cell-activating factor receptor

Core tip: This study explored the expression of B cell-activating factor receptor (BAFF-R) and B cell subpopulations in patients with hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC). Our data showed that BAFF-R expression was significantly lower in patient with HCC compared with non-HCC and healthy controls. In addition, the frequencies of $CD27^{+}IgD^{+}$ memory B cells, $CD27^{+}IgD^{-}$ class-switched memory B cells and plasmablasts were significantly lower in the patients with HCC compared to patients without HCC. In addition, decreased BAFF-R expression was significantly correlated with tumor size and tumor stage. This study is the first report suggesting that the depletion of BAFF-R in B cells might be responsible for B cell maturation in patients with HBV-related HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) represents one of the most common malignant tumors worldwide, especially in East and Southeast Asian countries, where hepatitis B virus (HBV) infection is highly prevalent[1]. In Thailand, the incidence of liver cancer is approximately 38.6 and 17.2 per 100000 person-years, respectively and at least 50%-60% of all HCC cases are attributable to chronic HBV infection[2]. Hepatocarcinogenesis is a complex multistep process involving the persistence of liver damage and inflammation, which results in a distinctive sequence of chronic hepatitis, cirrhosis and finally HCC development[3]. As chronic inflammation is a crucial driver of disease progression, accumulative evidence has indicated that immune-mediated host-virus interactions play an essential role in the development of HBV-related HCC[4]. While the importance of T-cell immunity has been well documented, the role of B cells in HCC progression is not yet completely understood and needs further investigation.

B cells are classically known to stimulate immune response *via* the activation of T cells and antibody production[5]. The activation of specific B cells results in the

proliferation and development of memory B cells and antibody-producing plasma cells, which are beneficial to neutralize and control active viral replication. The survival of B cells depends on the expression of a functional B cell receptor and signals from B cell-activating factor (BAFF), a member of the tumor necrosis factor (TNF) superfamily[6]. This cytokine binds to three receptors expressed on B cells including BAFF receptor (BAFF-R), transmembrane activator and cyclophilin ligand interactor (TACI) and B-cell maturation antigen (BCMA). In general, BAFF-R activates downstream pathways that regulate survival and maturation of B cells, TACI induces immunoglobulin (Ig) class switching, whereas BCMA promotes plasma B cells survival[7]. Human B cells are comprised of distinct phenotypic and functional subpopulations characterized based on different developmental stages such as transitional, naïve, memory B cells and plasmablasts. Since BAFF receptors and B cell subsets play diverse but crucial roles in modulating B cell function, analysis of their expression and subpopulation frequencies could provide more insights into the immunological characteristics of B cell selection in patients with HCC.

Recent evidence has suggested that B cells exhibit dual biological effects in promoting and inhibiting the development and progression of several cancers[8]. Regarding HCC, a previous study showed that increased percentage of circulating B cells was found in individuals with advanced HCC compared with early tumor staging[9]. Recently, it was also demonstrated that tumor infiltrating B cells was associated with disease prognosis in patients with HBV-related HCC[10]. Moreover, the close proximity and interaction of tumor-infiltrating T cells and B cells suggested an increased immune activation that might contribute to better prognosis in patients with HCC[11]. In addition, we recently reported that plasma BAFF levels significantly increased in patients with HBV-related HCC compared with the non-HCC group and healthy controls[12]. Together, these data suggest that B cells and BAFF may play an important role in HCC development and progression in patients with chronic HBV infection. So far, the phenotypes of circulating B-cell subtypes in patients with HBV-related HCC have not been well characterized.

In the present study, we aimed to compare the expression of BAFF receptors and the distribution of B cell subsets in the peripheral blood of patients with HBV-related HCC compared to individuals without HCC and healthy controls. Our data showed that BAFF-R expression was significantly reduced in B cells that involved with the frequencies of B cells maturation in patients with HCC. Interestingly, decreased BAFF-R expression was significantly associated with progressive HCC including large tumor size and advanced cancer stage.

MATERIALS AND METHODS

Patients

A total of 50 participants including 41 patients with chronic HBV infection (25 without HCC and 16 with HCC) and 9 healthy individuals were recruited from King Chulalongkorn Memorial Hospital and blood donors at National Blood Centre Thai Red Cross Society, Bangkok, Thailand, respectively. The diagnosis of chronic HBV infection was confirmed by the presence of serum hepatitis B s antigen (HBsAg) at least 6 mo. Patients with co-infection with hepatitis C virus (HCV) and/or human immunodeficiency virus (HIV) were not included in this study. In addition, patients with evidence of other malignancies or autoimmune disorders were excluded.

Patients in the HCC group were diagnosed on the basis of typical imaging studies and/or histopathology (fine needle aspiration, core liver biopsy or surgical resection) according to the standard guideline[13]. Diagnostic criteria of HCC by imaging studies were based on findings of focal hepatic lesions with hyperattenuation at the arterial phase, hypoattenuation at the portal phase in dynamic CT or MRI. The clinical parameters of patients with HCC at initial diagnosis were collected, which included sex, age, liver function tests, serum alpha-fetoprotein (AFP) level and HCC staging classified by the Barcelona Clinic Liver Cancer (BCLC) system[14].

The study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB no. 438/60) and participants had provided written informed consent. The study followed the Helsinki Declaration and Good Clinical Practice guidelines.

Flow cytometry staining

All blood samples were collected in sodium heparin tube and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation using Percoll PLUS density gradient media (GE Healthcare, Philadelphia, Pennsylvania, PA, USA) at 1500 rpm for 30 min at room temperature.

PBMCs were washed twice with Hank's balanced salt solution (HBSS) (GIBCO, New York, NY, USA) with 5% fetal bovine serum (FBS) and 100 U/mL penicillin and 100 μg/mL streptomycin. Total lymphocytes were counted and stored at liquid nitrogen before analysis.

Cryopreserved PBMCs from each patient were defrosted and added in complete in RPMI1640 Medium (GIBCO, New York, NY, USA) supplement with 10% FBS. At least 200000 cells were stained with combinations of antibodies including anti-CD19-PE, anti-CD38-FITC, anti-IgD-PerCP-Cy5.5, anti-CD27 Alexa Fluor 700, anti-CD24 PE-CyTM7 (BD Biosciences, San Jose, CA, USA), anti-CD268 (BAFFR)-APC, anti-CD267 (TACI)-APC and anti-CD269 (BCMA)-APC (BioLegend, San Diego, CA, USA). The cells were re-suspended in the buffer before performed flow cytometry using BD LSR II Flow Cytometry Analyzer (BD Biosciences, San Jose, CA, USA). Flow cytometry data were analyzed with Flowjo software version 10 (Tree star, Ashland, Oregon, USA).

Assays of plasma BAFF levels and HBV markers

Plasma BAFF levels were determined by ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Qualitative hepatitis B s antigen (HBsAg) was measured by commercially available enzyme-linked immunosorbent assays (Abbott Laboratories, Chicago, IL). Serum HBV DNA levels were quantified by Abbott Real Time HBV assay (Abbott Laboratories).

Statistical Analysis

Statistical analysis was performed by using SPSS version 22 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism v5.0 (GraphPad Software, San Diego, CA). Values were presented as mean ± standard deviation (SD), and percentages as appropriate. The statistical significance among groups was assessed by using the Kruskal-Wallis test, followed by multiple comparison test. The correlation data were analyzed by Spearman's rank test. The P value less than 0.05 was considered statistically significant.

RESULTS

Demographic and clinical characteristics of patients

Baseline clinical characteristics of 9 healthy controls and 41 patients with chronic HBV infection (25 patients without HCC and 16 patients with HCC) are summarized in Table 1. Patients in each group were significantly older than healthy controls (P < 0.001). However, there was no difference in mean age distribution among groups of patients. Compared with the non-HCC group, patients with HCC had significantly lower mean hematocrit, platelet counts and serum albumin. However, there was no significant difference among groups in terms of other baseline clinical parameters.

Plasma BAFF levels and BAFF receptor expression

Patients with HCC had significantly higher mean plasma BAFF levels compared with those without HCC (1208.8 ± 386.1 *vs* 914.8 ± 159.3 pg/mL; P = 0.042) (Table 1), which confirmed our recent report[12]. Percentages of total B cells in peripheral blood among the studied groups were not different, although there was a trend of higher total B cells in the HCC group compared with the non-HCC group (20.09% ± 10.37% *vs* 15.03% ± 4.09%, P = 0.079, Supplement Figure S1). The expression of BAFF receptors including BAFF-R, TACI and BCMA were further investigated (Figure 1 and 2). We found that there was a significantly decreased in percentage of BAFF-R expressing B cells in the HCC group (3.39% ± 2.12%) compared with the non-HCC group (5.37% ± 1.90%; P = 0.004) and healthy controls (6.23% ± 2.32%; P = 0.006). However, the percentage of BAFF-R expressing B cells was similar between the non-HCC group and controls (P = 0.28). In addition, the frequencies of TACI and BCMA expressing B cells were not significantly altered among the studied groups.

Analysis of B cell subpopulations

An in-depth analysis of B cell subpopulations was further analyzed in patients with or without HCC and in healthy controls. B cell population characteristics and gating strategy are shown in Figure 1. Overall, the absolute number of CD19+ B cells was not significantly different between groups. However, the frequency of IgD memory B cells (CD27+IgD+) were significant reduced in patients with HCC compared with the non-HCC individuals (1.23% ± 1.17% *vs* 3.09% ± 1.56%; P = 0.001) (Figure 3A and B). A significantly lower number of class-switched memory B cells (CD27+IgD-) in patients with HCC (0.60% ± 0.44%) was also observed when compared with patients without HCC (1.69% ± 0.86%; P < 0.001) and healthy controls (1.45% ± 0.81%; P = 0.019)

Table 1 Demographic and clinical characteristics of patients and healthy controls

	Healthy controls (*n* = 9)	Patients without HCC (*n* = 25)	Patients with HCC (*n* = 16)	*P* value
Sex (males)	4 (44.4%)	12 (48.0%)	13 (81.3%)	0.072
Age, yr	27.6 (25-34)	42.8 (20-77)	60.8 (34-77)	< 0.001[c]
Hemoglobin (g/dL)		13.3 ± 1.5	12.1 ± 1.9	0.082
Hematocrit (%)		41.4 ± 4.1	36.7 ± 5.7	0.008[b]
Platelets count (10^3/μL)		246.4 ± 56.7	180.1 ± 80.8	0.007[b]
White cell count (10^3/μL)		6.5 ± 2.2	6.1 ± 2.0	0.59
INR		1.0 ± 0.1	1.1 ± 0.2	0.34
AST (U/L)		48.9 ± 42.2	80.0 ± 65.0	0.07
ALT (U/L)		75.8 ± 83.1	56.9 ± 32.3	0.407
ALP (mg/L)		59.3 ± 14.3	177.5 ± 118.0	0.066
Total bilirubin (mg/dL)		0.7 ± 0.1	0.8 ± 0.4	0.757
Albumin (g/dL)		4.5 ± 0.3	3.4 ± 0.6	0.001[b]
AFP (IU/mL)		2.4 ± 2.2	4810.2 ± 10236.1	0.531
$\log_{10}$ HBV DNA (IU/mL)		5.4 ± 2.6	3.0 ± 1.9	0.066
BAFF (pg/mL)		914.8 ± 159.3	1208.8 ± 386.1	0.042[a]
BCLC stage				
A-B			12 (75.0%)	
C			4 (25.0%)	

Data expressed as mean ± SD or *n* (%) as appropriate;
[a]*P* < 0.05;
[b]*P* < 0.01;
[c]*P* < 0.001. INR: International normalized ratio; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; AFP: Alpha-fetoprotein; BAFF: B cell-activating factor; BCLC: Barcelona clinic liver cancer.

(Figure 3A and C). Moreover, the frequency of plasmablasts was significantly decreased in the HCC group compared with the non-HCC group (0.16% ± 0.12% *vs* 0.37% ± 0.30%; *P* = 0.014) (Figure 4A). However, no significant difference between groups was found regarding the frequencies of naïve and transitional B cells (Figure 3A, D and 4B).

Clinical correlation of BAFF-R

The clinical correlations of BAFF-R expression were further investigated in patients with HCC. Our results showed that BAFF-R had positive correlation with hematocrit (r = 0.550, *P* = 0.029), white cell count (r = 0.518, *P* = 0.042) and serum albumin (r = 0.604, *P* = 0.032). In contrast, BAFF-R exhibited negative correlation with patient's age (r = -0.533, *P* = 0.033), serum AFP (r = -0.557, *P* = 0.034) and tumor size (r = -0.542, *P* = 0.032) (Figure 5A-E). BAFF-R did not significantly correlate with other clinical parameters including platelet counts, international normalized ratio (INR), total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and HBV DNA level. Regarding tumor staging, BAFF-R expression in patients in advanced BCLC stage (stage C) was significantly lower than that of patients with earlier tumor stages (stages A and B) (1.61% ± 0.71% *vs* 4.73% ± 3.14%, *P* = 0.007) (Figure 5F).

DISCUSSION

Chronic HBV infection is a complex infectious disorder characterized by immune-mediated liver inflammation, which results in repeated destruction and regeneration of hepatocytes, and ultimately leads to the development of HCC[3]. Overall, HBV-related HCC is a leading cause of cancer death in Thailand and other Asian countries, with a very poor prognosis because of its aggressiveness and high recurrence rates[15]. As a result, a better understanding regarding the molecular mechanisms and progression of HCC is highly needed. Among several immunological factors associated with hepatocarcinogenesis, it is likely that B cells and BAFF may contribute to disease progression and HCC development in patients with chronic HBV infection[10]. In this context, we previously demonstrated that higher expression of plasma BAFF was linked to disease severity and overall survival in HBV-related

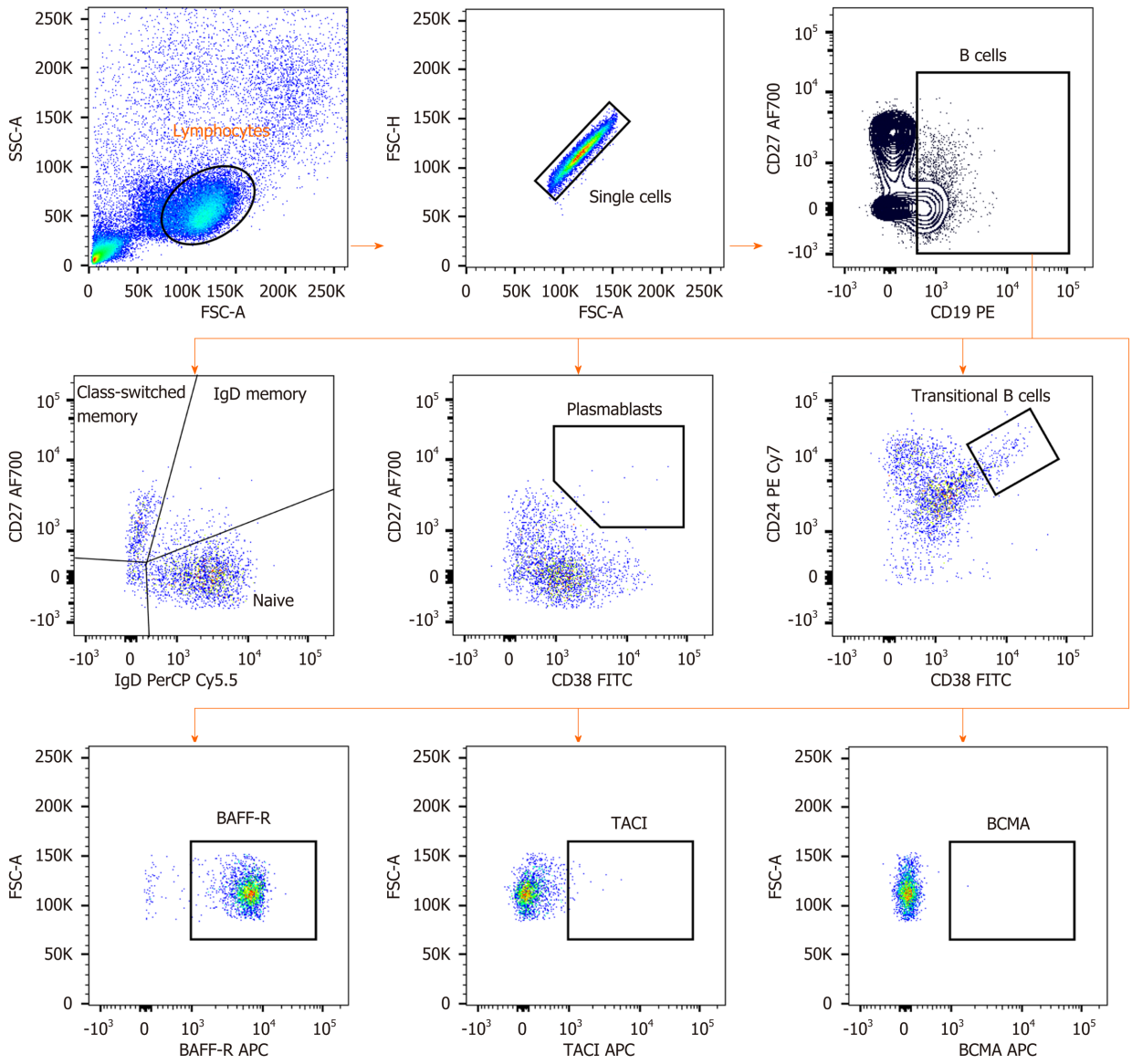

Figure 1 Representative plot shows the gating strategy for identification of B cell subsets. (1) Firstly, lymphocyte population was initially determined from forward scatter and side scatter plot. (2) Positive for CD19 was selected from CD19 *vs* CD27 plot. (3) Naive B cells, IgD and class-switched memory B cells were then identified from IgD *vs* CD27 plot. And (4) Plasmablasts and transitional B cells were selected from CD38 *vs* CD27 and CD38 *vs* CD24, respectively. Moreover, B cell-activating factor receptors were gated on CD19 positive cells. FSC: Forward scatter; SSC: Side scatter; BAFF-R: B cell-activating factor receptors; BCMA: B-cell maturation antigen; TACI: Transmembrane activator and cyclophilin ligand interactor.

HCC[12]. However, the role of BAFF receptors and distribution of circulating B-cell subtypes in HCC remains unclear.

In the present study, we explored the expression of BAFF receptors including BAFF-R, TACI and BCMA, which are expressed almost exclusively on B cells[7]. Interestingly, we found that the percentage of B cells expressing BAFF-R was significantly decreased in patients with HBV-related HCC compared with HBV patients without HCC and healthy controls. However, frequencies of TACI and BCMA on B cells were not significantly altered among groups. Thus, these results might indicate that BAFF-R but not TACI and BCMA play an important role in HCC development in patients with chronic HBV infection. In fact, BAFF-R is clearly a key receptor involved in the successful survival and maturation of B cells[16]. Previous studies have demonstrated that BAFF-R is important not only in B cell development, but also is the major mediator of BAFF-dependent co-stimulatory responses in peripheral B and T cells[17]. Similarly to the study of BAFF-R-deficient mice, BAFF-R is crucial for the development of B cells up to the stage of IgM+ immature/transitional B cells but cannot complete maturation in the spleen[18]. Moreover, BAFF-R is considered to be the most important receptor due to its critical role in regulatory B cells (Bregs) survival[19]. As previously demonstrated, targeting of BAFF-R in patients with

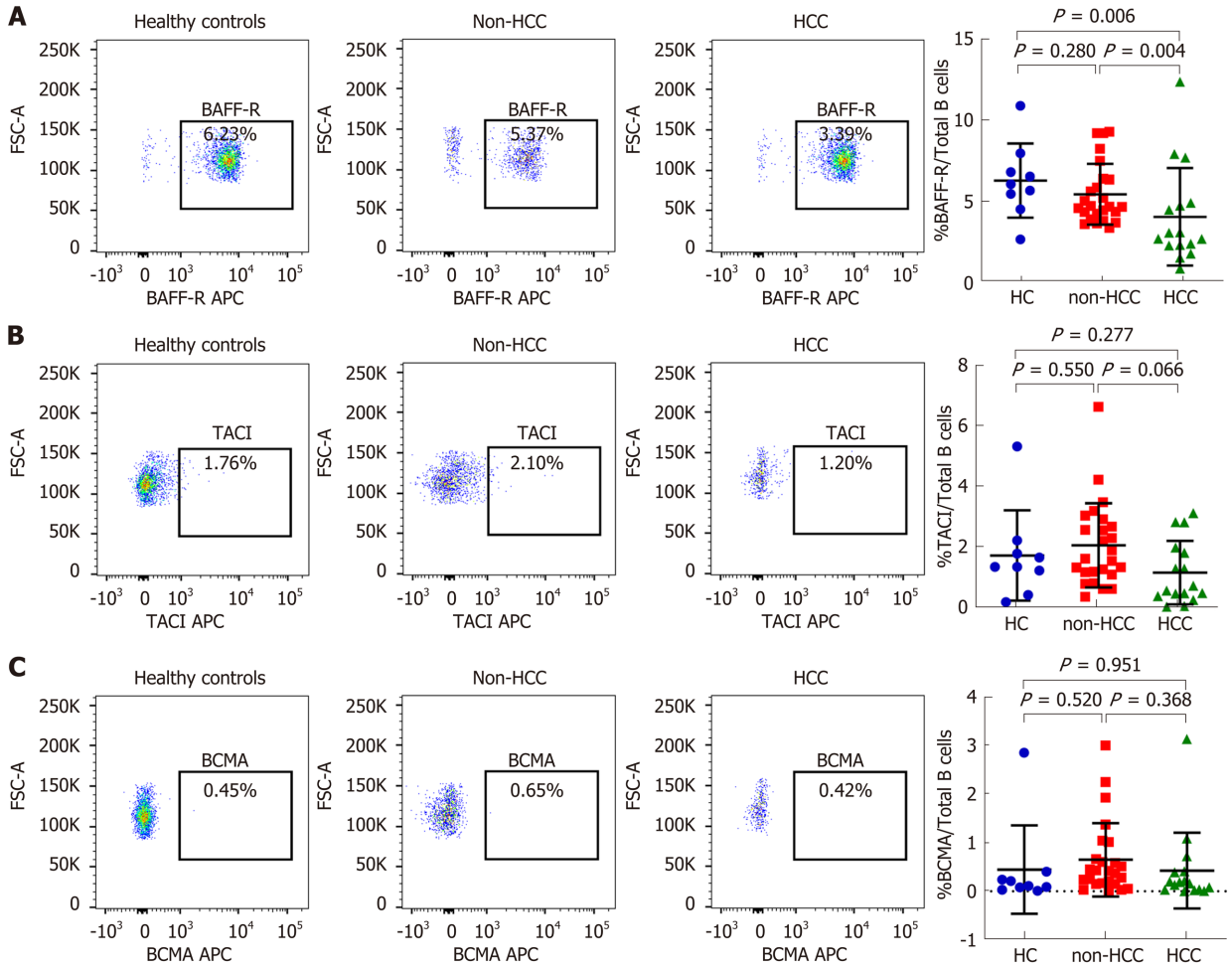

Figure 2 The frequency of B cell-activating factor receptors expression was significantly lower in patients with hepatocellular carcinoma compared with the other groups. Representative fluorescence activated cell sorting and statistic plots of A: B cell-activating factor receptors; B: Transmembrane activator and cyclophilin ligand interactor; C: B-cell maturation antigen. TACI: Transmembrane activator and cyclophilin ligand interactor; BCMA: B-cell maturation antigen; BAFF-R: B cell-activating factor receptors; HCC: Hepatocellular carcinoma; HC: Healthy controls.

precursor B-lineage acute lymphoblastic leukemia (B-ALL) could significantly impact the survival and basal proliferation of leukemia B-cell precursors[20]. Moreover, downregulation of circulating BAFF-R was shown to be associated with disease activity in patients with in patients with autoimmune disorders[21]. In this study, we also demonstrated that decreased BAFF-R expression on B cells was significantly correlated with tumor size and more advanced BCLC stages, indicating a contribution of BAFF-R to the disease progression of HBV-related HCC.

Normal B cell development and survival can promote an effective immune response to clear pathogens, whereas abnormalities in B cells differentiation and activation leads to the disruption of B cell homeostasis[5]. Accordingly, circulating B cell subset frequencies were also determined in this study. We found that the frequencies of CD27+IgD+ memory B cells, CD27+IgD- class-switched memory B cells and plasmablasts were significantly lower in patients with HCC compared to the non-HCC group. In contrast, number of naïve B cells did not differ significantly among groups. These findings were consistent with a previous report demonstrating that decreased of CD27+ memory B cells was found in patients with HCC but no differences in CD27- naïve and total B cells[22]. Moreover, a similar result was observed in patients with HCV-related HCC in which CD27+ memory B cells, and more specifically CD27+IgM+ memory B cells, were markedly less frequent in patients with HCC independent of HCV infection[23]. Memory B cells are long-lived and could capture and present the antigen to MHC molecules and then modulate CD4+ and CD8+ T cells immune response[24]. They also could potentially activate other immune cells such as dendritic cells and macrophages by producing pro-inflammatory cytokines. Therefore, it is possible that the decrease in total memory B cells and plasmablast in HBV-related HCC might reflect the defect of generating protective immunity against HBV in these individuals. In a model of lung cancer, other cell

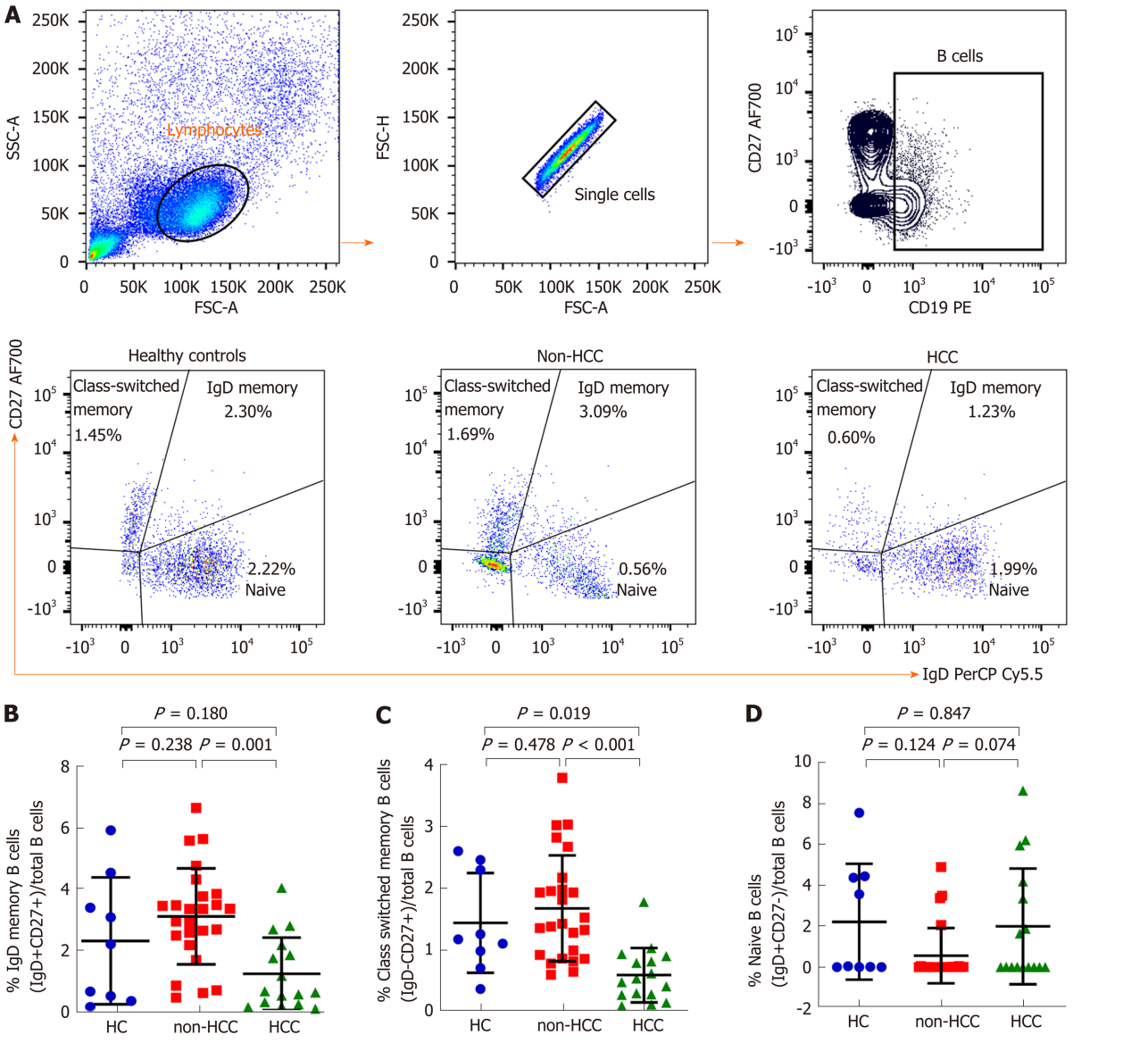

Figure 3 Class-switched and IgD memory B cells were significantly lower in patients with hepatocellular carcinoma compared with the non-hepatocellular carcinoma group. A: Gating strategy and relative of; B: IgD memory B cells; C: Class switched memory B cells; D: Naïve B cells in healthy controls, patients without hepatocellular carcinoma (Non-HCC) and patients with hepatocellular carcinoma. HCC: Hepatocellular carcinoma; HC: Healthy controls.

types, such as myeloid-derived suppressor cells can al so impair B cells function through the secretion of IL-17, which is associated with decrease of antibody production[25]. In addition to anti-tumor capacity, B cells can also modulate T cell immune responses *via* the function of Bregs[26]. Here, we did not directly measure Bregs population but instead investigated the CD19+CD24hiCD38hi transitional B cells, which have predominant Breg in this subset[27]. In this context, we did not observe any significant difference of the transitional B cells among the studied groups.

Interestingly, different finding regarding the distribution of B cell subpopulations was observed in patients with colorectal cancer, in which higher percentages of memory and plasma B cells were detected in peripheral blood[28]. In addition, increased numbers of circulating switched memory cells and plasmablasts were observed in other solid tumors including urinary bladder cancer, malignant melanoma, pancreatic cancer and prostate cancer[29]. Moreover, there was evidence that antibodies produced by B cells play a role in the cancer progression by initiating chronic inflammation[30]. Taken together, it is likely that role of B cell substes might not be similar among different cancer types. Therefore, categorizing B cell subpopulations in each cancer type is important not only for a better understanding the mechanisms by which immune cell subsets affect tumor biology but also for designing a successful treatment using immunotherapeutic approaches.

Overall, our findings indicated that BAFF-R expressing B cells had a decreased dependency on the maturation of B cells in patients with HCC compared with the

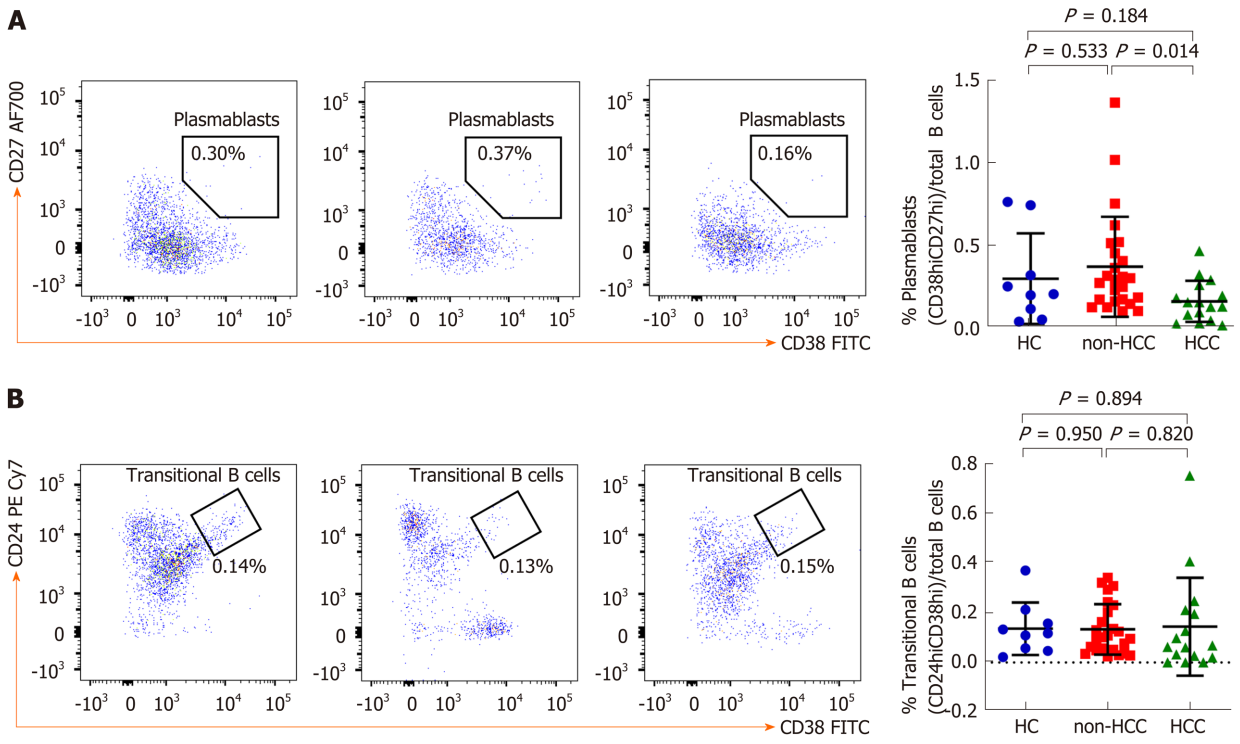

Figure 4 Lower frequency of plasmablasts in patients with hepatocellular carcinoma compared with non-hepatocellular carcinoma. Representative and statistic plots of A: Plasmablasts; B: Transitional B cells in healthy controls, patients without hepatocellular carcinoma (Non-HCC) and patients with hepatocellular carcinoma. HCC: Hepatocellular carcinoma; HC: Healthy controls.

non-HCC groups. It is therefore possible that down-regulation of BAFF-R could constitute resistance to the biological actions of BAFF and as such might, in part, be responsible for the elevated plasma BAFF concentrations in patients with HCC. On the other hand, it might be possible that chronic elevated BAFF levels in patients with HCC could lead to a downregulation of BAFF-R expression, as previously observed in patients with autoimmune disorders[21]. At this point, however, the underlying mechanism by which BAFF and BAFF receptors involve in HCC development is not clear and needed to be further elucidated. In this perspective, the design of therapeutic target of BAFF or BAFF-R might also be important for patients with HCC in the future.

Despite our interesting findings on the role of B cells and their ligand/receptors in disease progression in patients with HBV-related HCC, there were some limitations in this study. First, the study was retrospective and there might have many possible confounding factors, such as age and sex. Second, the sample sizes of patients with or without HCC were relatively small and a replicate study with a larger number of patients is needed to verify these observations and would provide further insights into the role of BAFF, as well as BAFF receptors and B cell response in HBV-related HCC. Moreover, further studies also need to validate these observations in patients with HCC regardless of underlying etiologies and to elucidate the mechanistic roles of B cell-mediated immune response in hepatocarcinogenesis.

In summary, we found the expression of BAFF-R was reduced in B cells that might reflect the diferent frequency of B cells maturation in patients with HCC. The memory B cells especially CD27+IgD- class-switched memory B cells and plasmablasts were significantly decreased whereas naïve and transitional B cells were not different in patients with HCC as compared with the non-HCC group and healthy controls. Additionally, decreased BAFF-R expression on B cells was significantly correlated with tumor size and more advanced BCLC stages. Together, these data indicate that abnormalities of B cell development according to the expression of BAFF-R is contributable to the development and progression of HBV-related HCC.

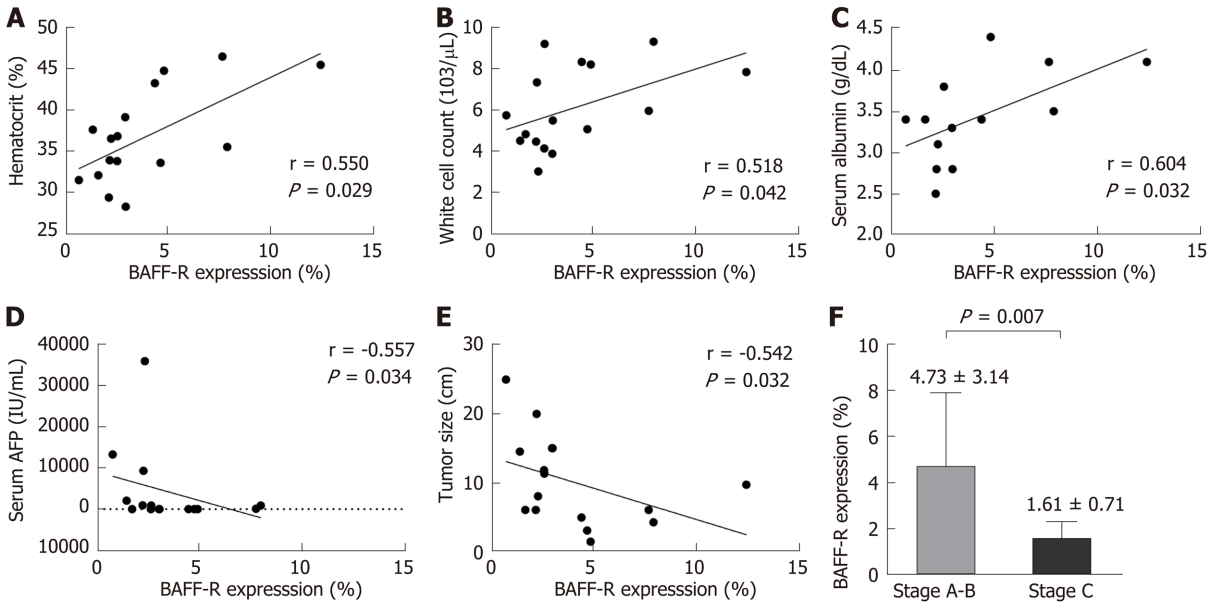

Figure 5 B cell-activating factor expression and clinical correlation in patients with hepatocellular carcinoma. A: Hematocrit; B: White cell count; C: Serum albumin; D: Serum alpha-fetoprotein; E: Tumor size; F: Tumor staging. BAFF-R: B cell-activating factor receptors; AFP: Alpha-fetoprotein.

ARTICLE HIGHLIGHTS

Research background

Recently, B cell-activating factor (BAFF) receptors and B cell subsets play diverse but crucial roles in modulating B cell function. Therefore, analysis of their expression and subpopulation frequencies could provide more insights into the immunological characteristics of B cell selection in patients with hepatocellular carcinoma (HCC).

Research motivation

To evaluate the association of BAFF receptors on B cell subsets according to clinical outcome of patients with chronic HBV infection.

Research objectives

This study aimed to compare the expression of BAFF receptors and the distribution of B cell subsets in the peripheral blood of patients with HBV-related HCC compared to individuals without HCC and healthy controls.

Research methods

Peripheral blood samples collected from chronic HBV infected patients with or without HCC and healthy controls were assessed for BAFF receptors [BAFF-R(B cell-activating factor receptor), transmembrane activator and cyclophilin ligand interactor, B-cell maturation antigen] and B cell subpopulations by multicolor flow cytometry.

Research results

The frequency of BAFF-R expression on B cells was significantly decreased in HBV-related HCC compared with HBV patients without HCC and healthy controls. In addition, the frequency of CD27+IgD+ memory B cells, CD27+IgD- class switched memory B cells and plasmablasts were significantly lower in patients with HCC compared to the non-HCC group. However, the frequency of naïve and transitional B cell did not differ significantly among groups. Moreover, decreased BAFF-R expression on B cells was significantly correlated with tumor size and more advanced Barcelona Clinic Liver Cancer stages.

Research conclusions

The expression of BAFF-R on B cells was significantly decreased that involved in the different frequencies of B cells maturation in patients with HCC.

Research perspectives

Larger samples and the mechanistic roles of B cell-mediated immune response in hepatocarcinogenesis are needed to further validate.

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ORIGINAL ARTICLE

Prospective Study

Role of dynamic perfusion magnetic resonance imaging in patients with local advanced rectal cancer

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Abstract

BACKGROUND

The management of rectal cancer patients is mainly based on the use of the magnetic resonance imaging (MRI) technique as a diagnostic tool for both staging and restaging. After treatment, to date, the evaluation of complete response is based on the histopathology assessment by using different tumor regression grade (TRG) features (*e.g.*, Dworak or Mandard classifications). While from the radiological point of view, the main attention for the prediction of a complete response after chemotherapy treatment focuses on MRI and the potential role of diffusion-weighted images and perfusion imaging represented by dynamic-contrast enhanced MRI. The main aim is to find a reliable tool to predict tumor response in comparison to histopathologic findings.

AIM

To investigate the value of dynamic contrast-enhanced perfusion-MRI parameters in the evaluation of the healthy rectal wall and tumor response to chemo-radiation therapy in patients with local advanced rectal cancer with histopathologic correlation.

METHODS

Twenty-eight patients with biopsy-proven rectal adenocarcinoma who underwent a dynamic contrast-enhanced MR study performed on a 1.5T MRI system (Achieva, Philips), before (MR1) and after chemoradiation therapy (MR2), were enrolled in this study. The protocol included T1 gadolinium enhanced THRIVE sequences acquired on axial planes. A dedicated workstation was used to generate color permeability maps. Region of interest was manually drawn on tumor tissue and normal rectal wall, hence the following parameters were calculated and statistically analyzed: Relative arterial enhancement (RAE),

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relative venous enhancement (RVE), relative late enhancement (RLE), maximum enhancement (ME), time to peak and area under the curve (AUC). Perfusion parameters were related to pathologic TRG (Mandard's criteria; TRG1 = complete regression, TRG5 = no regression).

RESULTS

Ten tumors (36%) showed complete or subtotal regression (TRG1-2) at histology and classified as responders; 18 tumors (64%) were classified as non-responders (TRG3-5). Perfusion MRI parameters were significantly higher in the tumor tissue than in the healthy tissue in MR1 ($P < 0.05$). At baseline (MR1), no significant difference in perfusion parameters was found between responders and non-responders. After chemo-radiation therapy, at MR2, responders showed significantly ($P < 0.05$) lower perfusion values [RAE (%) 54 ± 20; RVE (%) 73 ± 24; RLE (%): 82 ± 29; ME (%): 904 ± 429] compared to non-responders [RAE (%): 129 ± 45; RVE (%): 154 ± 39; RLE (%): 164 ± 35; ME (%): 1714 ± 427]. Moreover, in responders group perfusion values decreased significantly at MR2 [RAE (%): 54 ± 20; RVE (%): 73 ± 24; RLE (%): 82 ± 29; ME (%): 904 ± 429] compared to the corresponding perfusion values at MR1 [RAE (%): 115 ± 21; RVE (%): 119 ± 21; RLE (%): 111 ± 74; ME (%): 1060 ± 325]; ($P < 0.05$). Concerning the time-intensity curves, the AUC at MR2 showed significant difference ($P = 0.03$) between responders and non-responders [AUC ($mm^2 \times 10^{-3}$) 121 ± 50 *vs* 258 ± 86], with lower AUC values of the tumor tissue in responders compared to non-responders. In non-responders, there were no significant differences between perfusion values at MR1 and MR2.

CONCLUSION

Dynamic contrast perfusion-MRI analysis represents a complementary diagnostic tool for identifying vascularity characteristics of tumor tissue in local advanced rectal cancer, useful in the assessment of treatment response.

Key words: Rectal neoplasm; Chemotherapy; Radiotherapy; Tumor staging; Treatment response; Magnetic resonance imaging

Core tip: The management of rectal cancer has changed in the past years. The possibility to achieve a complete response after chemo-radiotherapy and the results of non-surgical management of the disease ("watch and wait" approach) highlighted the need to obtain an accurate assessment of complete response. In this setting, dynamic-contrast-enhanced magnetic resonance imaging, which provides valuable information about the degree of angiogenesis, is considered a promising functional tool. This study aimed to assess dynamic-contrast enhanced-parameters differences between the tumor tissue and the healthy rectal wall, both before and after chemo-radiotherapy, and to find a possible correlation with pathological findings and TNM stage.

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INTRODUCTION

The incidence of rectal cancer in the European Union is 125000 per year, accounting for 35% of total colorectal cancer incidence, and it is predicted to increase further in both genders[1]. In the past 20 years, the management of this tumor has changed due to the introduction of magnetic resonance imaging (MRI) primarily as a diagnostic tool for staging and restaging, and secondarily as an implement in decision making after neoadjuvant therapies or surgical procedures (total mesorectal excision)[2]. Moreover, the possibility to achieve a complete response (CR) after the chemo-radiotherapy

(CRT) in up to 30%-40% of patients and the results of non-surgical management of the disease (called "watch and wait" approach)[3] revival the discussion in the research field to obtain an accurate clinical assessment of CR before surgery. To date, the evaluation of CR is based on the histopathology assessment by using different tumor regression grade (TRG) features (*e.g.*, Dworak or Mandard classifications)[4-6]. From the radiological point of view, the main attention for the prediction of a CR after CRT focuses on MRI and the potential role of diffusion-weighted images and perfusion imaging represented by dynamic-contrast enhanced MRI (DCE-MRI). The main aim is to find a reliable tool to predict tumor response in comparison to histopathologic findings (such as TNM stage and TRG scale). MRI with T2 weighted and diffusion-weighted images is mandatory[7] and it is the first-choice examination for primary staging and restaging after CRT to decide the appropriate management of rectal cancer patients. When considering "watchful and waiting" as a treatment option after CRT, it is necessary to correlate the restaging MRI findings with clinical and endoscopic examination[3,7,8]. In this setting, DCE-MRI is considered a promising functional research implement[7]. This technique allows us to assess the vascularity of the tumor and can provide valuable information about tumor aggressiveness and the degree of angiogenesis in staging and restaging[9,10]. Since angiogenesis is a key factor in the growth and dissemination of colorectal cancer, the characterization of the angiogenic status of the tumor could allow for a more targeted approach to treatment[11]. The presence of hypoxic areas inside the tumor may influence the outcome of CRT[12]. due to the reduction of uptake and retention of chemotherapeutic agents within the pathological tissue[12]. These factors depend on tumor perfusion and chemotherapeutic agent extravasation through the vessel wall. Therefore, the ability to measure tumor vessels and tumor microenvironment before therapy may provide critical information on the selection of the most effective treatment strategy[13]. Moreover, vascular changes in the tumor may occur during CRT, and they may be an expression of tumor response or indicate the persistence of viable tumor cells before surgery. All semi-quantitative parameters [*e.g.*, area under the curve (AUC), maximum signal intensity or peak enhancement ratio, wash-in slope, mean transit time], are extracted directly from time-signal intensity curves created by specific software from the dynamic sequences. Semi-quantitative parameters are less time-consuming in comparison with semi-qualitative ones[14], but are affected by the acquisition systems so that comparison and quantification of these parameters can be difficult[15,16]: The real concentration of contrast agent in tissues is not estimated[17] due to a non-linear relation between signal-intensity change and contrast-medium concentration[11]. Only one study[18] have been assessed the correlation between semi-quantitative DCE-MRI parameters in rectal cancer and healthy rectal wall in the same group of patients. The purpose of this study is to investigate the value of DCE-MRI parameters in the evaluation of the response to CRT, by having histology as the reference standard, in patients with local advanced rectal cancer. In particular, we investigated whether a difference exists in DCE-parameters between the tumor tissue and in the healthy rectal wall, both before and after CRT, and a possible correlation with pathological findings expressed as Mandard's TRG and TNM stage.

MATERIALS AND METHODS

Patients

The study was ethically approved by the San Gerardo Hospital Ethics Committee and informed consent was obtained from all patients.

One-hundred-ninety consecutive patients with a biopsy-proven diagnosis of rectal adenocarcinoma from 2006 to 2018, who underwent MRI examination of lower abdomen at our department, were enrolled.

The inclusion criteria were: (1) Biopsy-proven adenocarcinoma; (2) MRI of lower abdomen for rectal cancer staging (MR1) performed with standard protocol and DCE-sequences; (3) Neoadjuvant preoperative CRT; (4) MRI of the lower abdomen after CRT (MR2) performed with standard protocol and DCE-sequences; and (5) Excision of the primary rectal cancer and following histopathological examination, resulting in the definition of the ypTNM grade and TRG sec. Mandard. Exclusion criteria were: (1) Surgery performed without previous neoadjuvant therapy; (2) No ypTNM or TRG assessment; (3) No MRI study performed at our radiology department/or MRI study performed without DCE sequences; and (4) Suboptimal DCE sequences not useful for software analysis.

MRI protocol

All MRI examinations were performed on a 1.5-T system (Achieva Plus; Philips, The

Netherlands) with multi-channel phased-array body coil. After a planning scan, axial T2 weighted turbo spin-echo (T2WI-TSE) images covering the entire length of the rectum were acquired and used to plan high-resolution scans. Scan protocol included axial TSE T1 weighted sequences (slice thickness: 3 mm; slice: 20; gap: 3 mm; TR: 612 ms; TE: 14 ms; flip angle: 90°; FOV: 180; RFOV: 85; matrix: 272 × 320; NSA: 4; time: 4.43 min); sagittal TSE T2 sequence (slice thickness: 3 mm; slice: 32; gap: 0 mm; TR: 5501 ms; TE: 85 ms; flip angle: 90°; FOV: 220; RFOV: 105; matrix: 276 × 200; NSA: 4; time: 4.40 min); orientated axial TSE T2 sequence (slice thickness: 3.5 mm; slice: 18; gap: 3.5 mm; TR: 4750 ms; TE: 120 ms; flip angle: 90°; FOV: 180; RFOV: 85; matrix: 256 × 256; NSA: 4; time: 3.05 min); orientated coronal TSE T2 sequence (slice thickness: 3 mm; slice: 20; gap: 0.5 mm; TR: 5058 ms; TE: 125 ms; flip angle: 90°; FOV: 180; RFOV: 100; matrix: 256 × 256; NSA: 4; time: 3.47 min). The orientated axial and coronal oblique images were performed orthogonal and parallel, respectively, to the long axis of the rectal tumor. Diffusion-weighted images with background body-signal suppression using a Multi-slice Spin Echo Eco-planar Single Shot (SE-EPI-SSh) sequence were included in the standard protocol (b-value 0 and 1000 s/mm2; slice thickness: 6 mm; slice: 12; gap: 6 mm; TR: 3000 ms; TE: 74 ms; flip angle: 90°; b-value: 0 and 700 s/mm2; FOV: 380; RFOV: 80; matrix: 240 × 256; NSA: 4; time: 1.30 min; SENSE factor: 1.5). Multiphase DCE-MRI was performed using ten consecutive acquisitions of 3D T1-weighted fat-suppressed spoiled recalled-echo sequences (THRIVE) on the axial plane (number of slices: 50; flip angle: 10°; slice thickness: 1mm; TR: 4.1 ms; TE: 1.97 ms; NSA: 2; matrix: 272 × 320; FOV: 180; time: 4.43). The dynamic study was obtained before, during, and after the intravenous injection of 0.1 mL/kg gadoxetic acid (1.0 mol Gadovist, Bayer, Leverkusen, Germany), with a flow rate of 1.5 mL/s and followed by a 30-mL saline flush at the same rate. The volume of the contrast agent was calculated based on the patient's body weight (0.1 mL per kg body weight). The arterial phase MRI was initiated immediately after the visual detection of contrast material at descending aorta by using a real-time bolus displayed method; the venous phase was performed with a fixed image delay of 80 s, followed by delayed coronal imaging at 4 min after contrast agent injection.

All sequences were obtained in free breathing. The total examination time was approximately 30 min. Luminal distention was achieved with the rectal administration of a small amount of sonography transmission gel (80 mL) to distend the rectal lumen. No intestinal cleansing nor spasmolytic drugs were administered.

MRI analysis

DCE raw data sets were uploaded on a dedicated workstation (Intellispace Portal; Philips Medical Systems), with a dedicated perfusion software (T1 Perfusion Package, Philips Medical Systems) that generated functional perfusion maps displayed in a color scale ranging from blue to red, considering blue the lowest range of the display. Functional color maps were analyzed in combination with contrast-enhanced images, both for staging (MR1) and restaging (MR2) images. A single radiologist, with more than 10 years of experience in the pelvis and rectal MRI, blinded to the histopathology results, manually drawn 3 different freehand regions of interest (ROI) on the rectal tumor on three consecutive slides, avoiding including the lumen and the mesorectal fat in the delineation. Other 3 different freehand ROIs were drawn on the normal rectal wall. The software also generated time-intensity curves and calculate perfusion parameters of tumor tissue and the healthy rectal wall (Figure 1). On the MR2 images, ROIs were manually drawn based on visual analysis of focal thickness within the location of the primary tumor bed; when no remaining thickness was observed the ROIs were drawn on MR2-T2W images considering the location of the tumor on MR1 images, to identify the presence of residual fibrotic tissue in the tumor bed. The size of the ROI of one section was not less than 10 voxels. For each ROI, located both in the tumor tissue and in the healthy rectal wall, the following quantitative parameters were calculated: Relative arterial enhancement (RAE, %), relative venous enhancement (RVE, %), relative late enhancement (RLE, %), maximum enhancement (ME, %), time to peak (TTP, s) and AUC (mm^2). RAE, RVE, and RLE represent the highest values percentage of intensity signal of contrast material concentration in the three different enhancement phases (arterial, venous, and delayed phase). The relative enhancement (RE, %), derived from the signal enhancement of a pixel of certain dynamic relative to that same pixel in the reference dynamic. The reference dynamic is normally the first, pre-contrast dynamic. The reference dynamic can be set to another dynamic, where I(D) stands for pixel intensity of current dynamic and I(Dref) stands for pixel intensity of reference dynamic created with the following formula: RE = [ID /I(Dref)-1] × 100, where I(D) stands for pixel intensity of current dynamic and I (Dref) stands for pixel intensity of reference dynamic.

ME represents the highest absolute values of signal intensity at the analyzed tissue level, and TTP corresponds to the time need to reach the maximum value of contrast

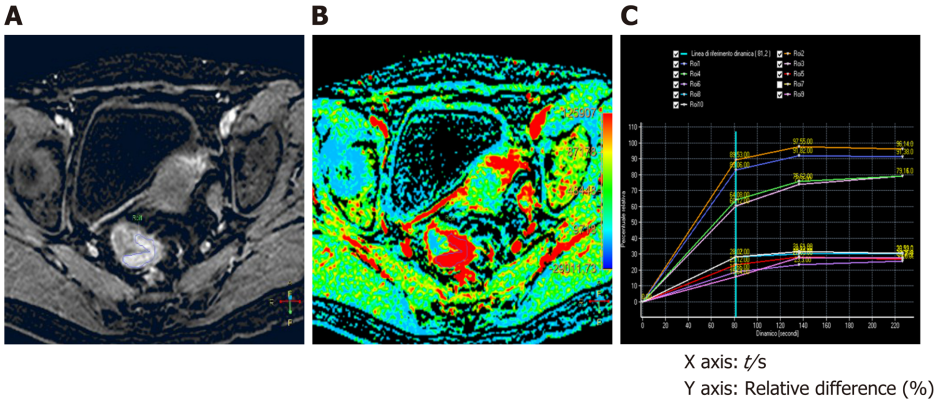

Figure 1 Staging dynamic-contrast enhanced magnetic resonance imaging (MR1) performed with 3D-T1 THRIVE images; the corresponding color map and time-intensity curves. A: The T1 THRIVE image shows an example of freehand delineation of the tumor during an MRI study performed for staging purpose (the tumor was a T3N0); B: The related color map was created by the software, and the delineation of the tumor on the T1 THRIVE was automatically reported also on the color map; C: The time-intensity curves table show the results of the delineations on the tumor (which correspond to the highest curves) and on the healthy rectal wall.

material concentration. The AUC represents the value of the area under the time-intensity curve generated for a selected ROI by the perfusion software.

Statistical analysis

Imaging-derived data were statistically analyzed by using IBM SPSS 21 (SPSS Incorporated, Chicago, Illinois, United States) and R software (R Foundation for Statistical Computing, Vienna, Austria https://www.Rproject.org/). Linear regression curves have been used to assess the relation of pre- and post-treatment perfusion values to the TRG classes. Wilcoxon test was used to verify data correlation before and after CRT. Mann-Whitney test for unpaired data was used to verify statistically significant differences for each parameter among tumor tissue and the healthy rectal wall, before and after CRT.

RESULTS

One-hundred-sixty-two patients did not meet the inclusion criteria, mainly because of the absence of DCE-sequences and a final group of 28 patients was enrolled in this study (men: 14, women: 14; mean age: 69 ± 19 years). Four patients were staged as cT3N0, 16 patients as cT3N1, 2 patients as cT3N0, and 6 patients as cT3N2. All of them completed CRT protocol with a total radiation dose of 50.4 Gy, in 28 fractions and 5-fluorouracil or 5-fluorouracil plus oxaliplatinum. MRI restaging (MR2) was performed 8-10 wk after the completion of CRT and then all patients underwent surgery (10-12 wk after the completion of CRT). After surgery 6 patients were ypT0N0, 8 patients were ypT2N0, 8 patients were ypT3N0, 6 patients were ypT3N1. Moreover, according to Mandard's TRG[6]: 6 patients were TRG1 and 4 patients were TRG2 (these patients, 36%, were considered as responders). Eighteen patients (64%) were TRG3 and were classified as non-responders.

Results of perfusion parameters at baseline (MR1)

At the baseline (MR1) perfusion MRI parameters were significantly higher in the tumor tissue compared to the healthy tissue ($P < 0.05$) (Table 1). These results were also confirmed from the visual assessment of the time-intensity curves (Figure 1). When considering the healthy rectal wall, no differences in the perfusion parameters were observed between responders and non-responders' group before CRT (MR1) (Table 2). When considering the tumor tissue at MR1, there was also no significant difference in the perfusion parameters between responders and non-responders (Table 3). All the perfusion parameters in the tumor tissue before CRT did not show any correlation with the ypT stage histology after surgery.

Results of perfusion parameters after chemoradiotherapy (MR2)

After CRT (MR2) no differences in the perfusion parameters were observed between responders and non-responders group concerning the healthy rectal wall (Table 2). Moreover, in the healthy rectal wall, perfusion parameters were not modified after CRT in comparison to corresponding values at the baseline (MR1) (Table 2). At MR2, perfusion values of the rectal residual tumor in responders were significantly lower (*P*

Table 1 Differences in terms of perfusion parameters in the healthy rectal wall and the tumor tissue, determines through the dynamic contrast enhanced magnetic resonance imaging study for staging rectal cancer (MR1)

DCE-MRI parameters	Healthy rectal wall	Rectal tumor	*P* value
RAE (mean ± SD)	57.5 ± 41.5	100 ± 38	0.005
RVE (mean ± SD)	75.5 ± 43	119.5 ± 26.5	0.005
RLE (mean ± SD)	82 ± 38.5	114 ± 22.5	0.005
ME (mean ± SD)	807 ± 227.5	1143.5 ± 348	0.004
TTP (mean ± SD)	217 ± 79	185 ± 41	0.040
AUC (mean ± SD)	114388.5 ± 46237.5	186197 ± 41042.5	0.005

DCE-MRI: Dynamic-contrast enhanced magnetic resonance imaging; RAE: Relative arterial enhancement; RVE: Relative venous enhancement; RLE: Relative late enhancement; ME: Maximum enhancement; TTP: Time to peak; AUC: Area under the curve.

< 0.05) [RAE (%): 54 ± 20; RVE (%) 73 ± 24; RLE (%): 82 ± 29; ME (%): 904 ± 429] compared to those ones of non-responders (RAE (%): 129 ± 45; RVE (%):154 ± 39; RLE (%): 164 ± 35; ME (%):1714 ± 427) (Table 4). Concerning the time-intensity curves, the AUC at MR2 showed a significant difference (P = 0.03) between responders and non-responders [AUC ($mm^2 \times 10^{-3}$) 121 ± 50 *vs* 258 ± 86], with lower AUC values of the tumor in responders compared to non-responders. In the responders group, perfusion values in the tumor decreased significantly at MR2 [RAE (%): 54 ± 20; RVE (%): 73 ± 24; RLE (%): 82 ± 29; ME (%): 904 ± 429] compared to the corresponding perfusion values at MR1 [RAE (%): 115 ± 21; RVE (%): 119 ± 21; RLE (%): 111 ± 74; ME(%): 1060 ± 325); (P < 0.05) (Table 4). No significant differences in the TTP values were found between responders and non-responders at MR2 concerning the rectal tumor. After CRT, at MR2, differences in perfusion parameters between the residual tumor tissue and the healthy tissue were still present, but in responder patients, the perfusion parameters of the residual tumor tended to be closer to the perfusion parameters of the healthy rectal wall, with similar time-intensity curves (Figures 2 and 3). A strong correlation between RAE and RVE in the tumor tissue after CRT (R2 = 0.96; P = 0.0001) was also found (Figure 4). Perfusion parameters of the residual tumor at MR2 in non-responders were higher in comparison to the corresponding healthy rectal wall at MR2 with an increasing difference in the time-intensity curve shape (Figures 3 and 5). Moreover, in non-responders, no reduction of the perfusion parameters in the tumor was observed at MR2 in comparison to the MR1 values. All perfusion parameters in the tumor tissue after CRT didn't show any correlation with the ypT stage histology after surgery.

DISCUSSION

DCE-MRI is one of the most recent functional implementations in the MR spectrum of imaging in breast imaging and prostate cancer to identify malignant tumors based on specific enhancement patterns[19,20]. In rectal cancer, it is considered a promising research tool[7] for the assessment of treatment response after CRT. With this technique, the vascularity of the tumor can be assessed, and it can provide valuable information about tumor aggressiveness and the degree of angiogenesis in both staging and restaging[9,10]. There is also evidence that DCE-MRI can help predict and assess response to neoadjuvant treatment[21,22]. DCE-MRI, using low-molecular-weight (< 1 kDa) gadolinium-based paramagnetic contrast media, is an imaging technique where T1-weighted sequences are rapidly repeated before, during and after intravenous contrast injection to study signal intensity changes induced by the path of the contrast bolus through tissues[9]. From a DCE-MRI study, time-intensity curves are usually created using specific software and they can be analyzed with three different approaches: Qualitative (by the visual analysis of the time-intensity curves), or with quantitative and semi-quantitative parameters[22,23]. All semi-quantitative parameters are extracted directly from time-signal intensity curves. These parameters, less time-consuming[14] in comparison with a quantitative approach, require less complicated software algorithms, are easier to obtain and reproduce than quantitative parameters[9], and are more reliable to be used in clinical practice. Up to 8%-30% of patients with local advanced rectal cancer treated with CRT achieve a pathological CR confirmed at histology after surgery[3]. The possibility to assess a CR to neoadjuvant

Table 2 Differences in terms of perfusion parameters between responders and non-responders, in the healthy rectal wall, determines through a dynamic contrast enhanced magnetic resonance imaging study during staging (MR1) and restaging (MR2) of rectal cancer after chemo-radiotherapy

DCE-MRI parameters	Responders			Non-responders		
	MR1	MR2	*P* value	MR1	MR2	*P* value
RAE (mean ± SD)	62 ± 33	51 ± 8	0.77	53 ± 50	63 ± 33	0.47
RVE (mean ± SD)	70 ± 40	60 ± 14	0.73	81 ± 46	69 ± 42	0.68
RLE (mean ± SD)	72 ± 35	76 ± 10	0.49	92 ± 42	63 ± 52	0.61
ME (mean ± SD)	662 ± 98	882 ± 251	0.11	952 ± 357	847 ± 358	0.87
TTP (mean ± SD)	230 ± 61	223 ± 32	0.64	204 ± 97	151 ± 56	0.05
AUC (mean ± SD)	120261 ± 46709	114347 ± 32930	0.74	108516 ± 45766	114187 ± 28584	0.99

DCE-MRI: Dynamic-contrast enhanced magnetic resonance imaging; RAE: Relative arterial enhancement; RVE: Relative venous enhancement; RLE: Relative late enhancement; ME: Maximum enhancement; TTP: Time to peak; AUC: Area under the curve.

CRT before surgery could potentially modify the management of rectal cancer treatment and lead to the new treatment approach, which is the "wait and see" or "watchful and wait"[3]. Different studies addressed the potential role of quantitative and semi-quantitative DCE-MRI parameters in predicting the response to therapy in primary rectal cancer with conflicting results[18,23-25]. Unfortunately, only a few studies investigated the potential of semi-quantitative parameters in the assessment of tumor characteristic with controversial results, demonstrating that tumors that better respond to CRT have lower values of TTP, AUC[24,25] or have a different degree of correlation with angiogenetic markers or micro-vessel density[9,26]. Our results are in line with those achieved by Shen *et al*[27] and Krishan *et al*[18] that compared perfusion parameters obtained from DCE-MRI in patients with rectal cancer to a control group of healthy patients[27] or the healthy rectal wall in the same group of the patient[18]. After CRT, there was a significant difference between responders and non-responders concerning all the RE parameters. In particular, patients that responded to treatment had lower absolute perfusion value after CRT than non-responders, in line with Petrillo *et al*[23,24] and Krishan *et al*[18], where maximum signal difference decreased in responders patients when compared to non-responders. Moreover, in line with the literature[9,18,25] the AUC was lower in responder patients in comparison to non-responders. The relation of semi-quantitative parameters and vascular changes in the tumor tissue is confirmed by the fact that no changes in these parameters were found after CRT when we analyzed the healthy tissue. The delineation and the assessment of perfusion values in the healthy tissue allowed to indirectly assess if changes in the perfusion parameters were due to post-radiation inflammation after CRT or to real changes in vascular characteristics. Interestingly all the perfusion parameters of the tumor tended to increase at MR2 in non-responders in comparison to the baseline values at MR1, while no increase in the perfusion value was observed after CRT in the same group of non-responder patients when considering the healthy rectal wall. Krishan *et al*[18] also demonstrated that in responder patients, rectal perfusion became similar to the adjacent normal rectal wall; their results correlated with normalization of perfusion parameters in tumor tissue with the healthy rectal wall. No robust evidence has been found for the value of DCE parameters as a measure of tumor aggressiveness, measured as the TNM stage. Although we found a difference between perfusion parameters in benign tissue and malignant tissue, we did not find any correlation between pre and post-treatment semi-quantitative DCE-parameters and pathological T stage after CRT. This could be due to the small sample of patients since other studies[24,25] showed lower semi-quantitative value for more aggressive tumors.

This study has some limitations. The main one is due to the small number of patients. Second, we did not consider the wash-in slope as previous authors[9,10,25], due to the different acquisition phases and software employed. Third, is the lack of pixel correlation to histology so that the delineated parameters are not exactly related to the histological specimen. We tried to overcome these limitations by drawing multiple ROIs and including in the analysis also the healthy rectal wall; a volumetric evaluation or a prospective study should be performed to obtain more reliable data.

In conclusion, Dynamic contrast perfusion-MRI parameters represent a complementary diagnostic tool in identifying vascularity characteristics of local advanced rectal cancer since they have been demonstrated to be related to the vascular changes which occur in the tumor in comparison to the healthy tissue. Moreover, DCE-MRI parameters can express the changes occurred in the tumor that

Table 3 Differences in terms of perfusion parameters between responders and non-responders, in the tumor tissue, determines through a dynamic contrast enhanced magnetic resonance imaging study for staging rectal cancer (MR1)

DCE-MRI Parameters	Responders	Non-responders	*P* value
RAE (mean ± SD)	115 ± 21	85 ± 55	0.27
RVE (mean ± SD)	119 ± 21	120 ± 32	0.94
RLE (mean ± SD)	111 ± 14	117 ± 31	0.72
ME (mean ± SD)	1060 ± 325	1227 ± 371	0.52
TTP (mean ± SD)	196 ± 12	175 ± 70	0.51
AUC (mean ± SD)	203361 ± 14347	169033 ± 67738	0.28

DCE-MRI: Dynamic-contrast enhanced magnetic resonance imaging; RAE: Relative arterial enhancement; RVE: Relative venous enhancement; RLE: Relative late enhancement; ME: Maximum enhancement; TTP: Time to peak; AUC: Area under the curve.

respond to the neoadjuvant treatment and are more reproducible than quantitative parameters.

Table 4 Differences in terms of perfusion parameters between responders and non-responders in the tumor tissue, determines through a dynamic contrast enhanced magnetic resonance imaging study for rectal cancer restaging (MR2) after chemo-radiotherapy

DCE-MRI parameters	Responders	Non-responders	*P* value
RAE (mean ± SD)	54 ± 20	129 ± 45	0.02
RVE (mean ± SD)	73 ± 24	154 ± 39	< 0.05
RLE (mean ± SD)	82 ± 29	164 ± 35	< 0.05
ME (mean ± SD)	904 ± 429	1714 ± 427	0.03
TTP (mean ± SD)	218 ± 18	196 ± 23	0.15
AUC (mean ± SD)	120894 ± 50495	258022 ± 86469	0.02

DCE-MRI: Dynamic-contrast enhanced magnetic resonance imaging; RAE: Relative arterial enhancement; RVE: Relative venous enhancement; RLE: Relative late enhancement; ME: Maximum enhancement; TTP: Time to peak; AUC: Area under the curve.

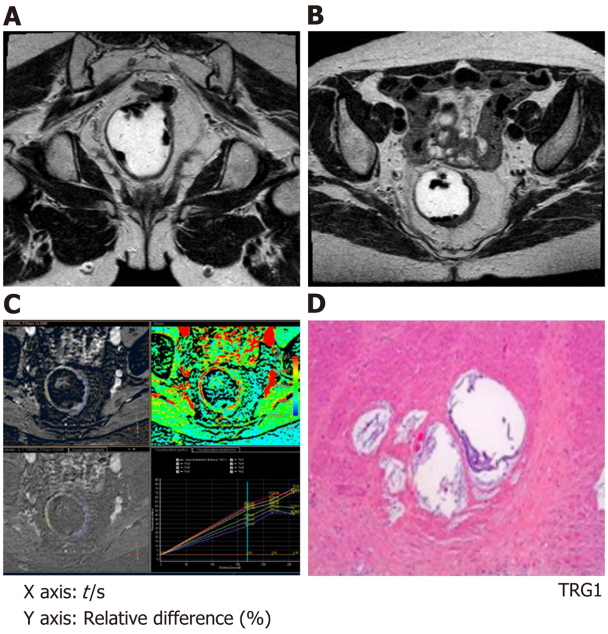

Figure 2 Magnetic resonance imaging study performed for rectal cancer restaging after chemo-radiotherapy with standard T2 weighted sequences and dynamic-contrast enhanced magnetic resonance imaging with 3D-T1 THRIVE images; the corresponding color map and time-intensity curves. A and B: The T2 weighted sequences show a slight thickness in the rectal wall on the left side (from 2 to 5 o'clock) that corresponds to the residual tumor bed; C: The dynamic-contrast enhanced-study show the delineation of the tumor and the time-intensity curves show, with similar curves for the tumor and the healthy rectal wall; D: At histology this patient was classified as a tumor regression grade 1.

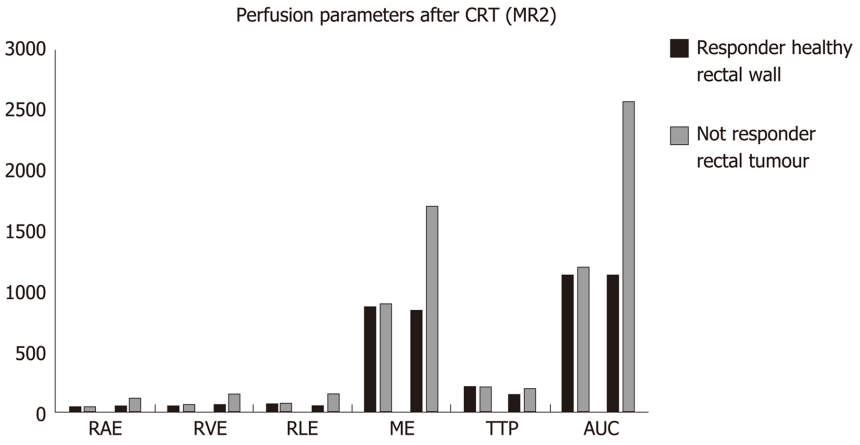

Figure 3 Differences in terms of perfusion parameters between responders and non-responders, in the tumor tissue and the healthy rectal wall, determines through a dynamic contrast enhanced magnetic resonance imaging study for rectal cancer restaging after chemo-radiotherapy. CRT: Chemo-radiotherapy.

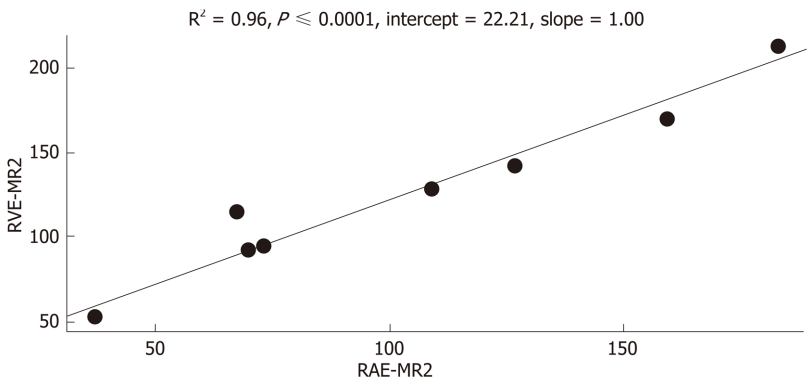

Figure 4 Correlation between the relative arterial enhancement and the relative venous enhancement at MR2.

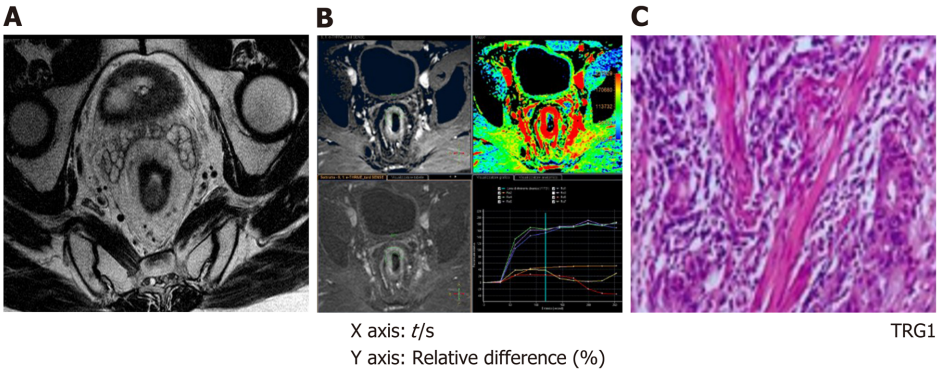

Figure 5 Dynamic-contrast enhanced magnetic resonance imaging study performed for restaging of rectal cancer after chemoradiation therapy with 3D-T1 THRIVE and T2 weighted sequences; the corresponding color map and time-intensity curves. A: The T2 weighted sequences show a slightly hypointense thickness in the rectal wall on the anterior side (from 11 to 2 o'clock) that corresponds to the fibrotic residual tumor bed; B: The dynamic-contrast enhanced-study performed after chemo-radiotherapy show that on the T1 THRIVE sequence some tissue thickness is present (12 o'clock) and the corresponding time-intensity curves show a significant difference between the healthy rectal wall and the tumor; C: At histology was classified as a tumor regression grade 3.

ARTICLE HIGHLIGHTS

Research background

The management of rectal cancer patients is mainly based on the use of the magnetic resonance imaging (MRI) technique as a diagnostic tool for both staging and restaging. After treatment, to date, the evaluation of complete response is based on the histopathology assessment by using different tumor regression grade features (*e.g.*, Dworak or Mandard classifications). While from the radiological point of view, the main attention for the prediction of a complete response after chemo-radiotherapy (CRT) focuses on MRI and the potential role of diffusion-weighted images and perfusion imaging represented by dynamic-contrast enhanced MRI (DCE-MRI). The main aim is to find a reliable tool to predict tumor response in comparison to histopathologic findings.

Research motivation

DCE-MRI is considered an important tool in the assessment of rectal adenocarcinoma because it permits to obtain important information about tumor vascularization quantitatively.

Research objectives

This study investigated the diagnostic role of DCE-MRI, in patients with rectal carcinoma who underwent chemoradiation therapy, as a complementary functional diagnostic tool in the assessment of the response of patients with rectal cancer after CRT, in comparison with standard histopathological analysis.

Research methods

This is an analysis of the evaluation of the response of patients with biopsy-proven rectal adenocarcinoma after chemoradiation therapy, who underwent DCE-MRI.

Research results

Semiquantitative perfusion parameters demonstrated higher values in the tumor tissue than in the healthy tissue. More important, after chemoradiation therapy, those patients that responded to therapy showed lower perfusion values in residual tumor tissue than those who did not

respond.

Research conclusions

Dynamic contrast MRI represents a complementary diagnostic tool in identifying vascularity characteristics of local advanced rectal cancer, before and after chemoradiation treatment.

Research perspectives

To strengthen our results, further studies should include a wider cohort of patients, by using pathological correlation as a gold standard.

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SYSTEMATIC REVIEWS

Association between non-alcoholic fatty liver disease and obstructive sleep apnea

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Abstract

BACKGROUND

Non-alcoholic fatty liver disease (NAFLD) is an emerging liver disease and currently the most common cause of incidental abnormal liver tests. The pathogenesis of NAFLD is multifactorial and many mechanisms that cause fatty liver infiltration, inflammation, oxidative stress and progressive fibrosis have been proposed. Obstructive sleep apnea (OSA) may be linked with the pathogenesis and the severity of NAFLD.

AIM

To study the association between NAFLD and OSA considering also the efficacy of continuous positive airway pressure (CPAP) treatment.

METHODS

A PubMed search was conducted using the terms "non-alcoholic fatty liver disease AND (obstructive sleep apnea OR obstructive sleep disorders OR sleep apnea)". Research was limited to title/abstract of articles published in English in the last 5 years; animal and child studies, case reports, commentaries, letters, editorials and meeting abstracts were not considered. Data were extracted on a standardized data collection table which included: First author, publication year, country, study design, number of patients involved, diagnosis and severity of OSA, diagnosis of NAFLD, patient characteristics, results of the study.

RESULTS

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In total, 132 articles were initially retrieved on PubMed search and 77 in the last five years. After removal of irrelevant studies, 13 articles were included in the qualitative analysis. There was a total of 2753 participants across all the studies with a mean age between 42 and 58 years. The proportion of males ranged from 21% to 87.9% and the mean body mass index ranged from 24.0 to 49.9 kg/m^2. The results of this review showed an increased prevalence of NAFLD in patients with diagnosis of OSA, even in the absence of coexisting comorbidities such as obesity or metabolic syndrome. Furthermore, the severity of NAFLD is associated with the increase in OSA severity. Effective CPAP treatment, although not always decisive, may stabilize or slow NAFLD progression with benefits on metabolic and cardiovascular functions.

CONCLUSION
In NAFLD patients, although asymptomatic, it is recommended to systematically perform polysomnography in order to early and better treat them before the development of potentially life threatening systemic dysfunctions.

Key words: Continuous positive air pressure; Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Obstructive sleep apnea; Obstructive sleep disorders; Sleep apnea

Core tip: The development of non-alcoholic fatty liver disease (NAFLD) seems to be closely associated with obstructive sleep apnea (OSA) even in the absence of coexisting comorbidities such as obesity or metabolic syndrome. Furthermore, the severity of NAFLD is associated with the increase in OSA severity. Effective continuous positive airway pressure therapy for OSA may improve serum aminotransferase levels and liver steatosis. As clinicians, our aim should be to screen OSA patients for NAFLD and vice versa those with NAFLD for OSA in order to early and better treat them before the development of potentially life threatening systemic dysfunctions.

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INTRODUCTION

Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is an emerging liver disease in Western countries[1,2] and currently the most common cause of incidental abnormal liver tests. Fatty liver includes a wide spectrum of histologic alterations. Simple steatosis generally represents a benign condition following a non-progressive clinical course. On the contrary, a subset of patients with non-alcoholic steatohepatitis (NASH), in particular those with a more severe fibrosis, are at higher risk for progressing to liver disease complications such as decompensated cirrhosis, liver cancer, and liver mortality[3]. NASH is projected to eventually overtake the hepatitis C virus and alcoholic liver disease as the leading cause of liver transplant[4].

However, the association of liver steatosis with a number of common metabolic conditions and cardiovascular risk factors has been also extensively reported. Indeed, it appears that in NAFLD the increased mortality of patients is primarily a result of cardiovascular diseases and, to a lesser extent, to liver related diseases[5,6]. In fact, patients with NAFLD show early signs of atherosclerosis, such as increased carotid artery intima-media thickness[7], coronary artery calcification[8] and endothelial dysfunction[9].

The pathogenesis of NAFLD is multifactorial and many mechanisms that cause fatty liver infiltration, inflammation, oxidative stress and progressive fibrosis have been proposed. Insulin resistance, the key feature of the metabolic syndrome (MetS),

is considered to play a central role in the first stages of fatty liver infiltration[10,11]. However, whether insulin resistance and hyperinsulinemia are components of MetS promoting fatty liver or whether NAFLD itself induces chronic hyperinsulinemia by impaired insulin degradation is still under debate. Chronic oxidative stress is a major player triggering the progression of simple steatosis to NASH as the result of an imbalance between pro-oxidant and anti-oxidant chemicals that lead to liver cell damage[12,13].

Finally, several lines of evidence clearly indicated that also genetic factors may predispose to NAFLD and among the others a variant located at the PNPLA3 gene (I148M) appears to show the strongest effect[14,15].

Obstructive sleep apnea

Obstructive sleep apnea (OSA) is a breathing disorder characterized by narrowing of the upper airway during sleep which compromise the normal ventilation[16]. The most common symptoms of OSA are excessive daytime sleepiness, fragmented sleep, snoring, fatigue and impairments in cognitive functions[17,18].

The prevalence of OSA is estimated to be 4% in the general population increasing up to 40% in some disease-specific populations, such as in patients suffering from metabolic syndrome[19], obesity[20], diabetes mellitus[21], arterial hypertension[22], cardiovascular disease[23], chronic kidney disease[24] and non-alcoholic fatty liver disease[25]. Furthermore, the prevalence of OSA increases with age, race and world region[26].

In these clinical settings, OSA is still underdiagnosed; probably the atypical presentation, the lack of data on the criteria for identifying the disorder and the lack of awareness of this entity among clinicians are important reasons.

The polysomnography (PSG) is the gold standard for the diagnosis of OSA[16]. The severity of OSA is defined by an apnea-hypopnea index (AHI) ≥ 5 and < 15 events/h as mild, ≥ 15 and < 30 events/h as moderate, and ≥ 30 events/h as severe[27].

Since continuous positive airway pressure (CPAP) can eliminate upper airway narrowing during sleep improving sleep fragmentation, daytime symptoms and quality of life[28,29], it remains the gold standard treatment for the clinical management of OSA.

OSA and chronic intermittent hypoxia may be linked with the pathogenesis and the severity of NAFLD[30]. Several studies indicate that OSA is a well-established independent factor of insulin resistance, which may predispose to the development and the progression of liver steatosis[31-33]. However, to clarify the independent effects of OSA on the development and progression of NAFLD in literature data is challenging due to the numerous cardiovascular and metabolic comorbidities which often coexist.

The aim of this review is to provide a more comprehensive overview of the association between NAFLD and OSA considering also the efficacy of CPAP treatment.

MATERIALS AND METHODS

Literature search, data selection and extraction

A PubMed search was conducted using the terms "non-alcoholic fatty liver disease AND (obstructive sleep apnea OR obstructive sleep disorders OR sleep apnea)". Research was limited to title/abstract of articles published in English in the last 5 years; animal and child studies, case reports, commentaries, letters, editorials and meeting abstracts were not considered. Review articles were examined to identify studies that were potentially eligible for inclusion.

Only potentially relevant studies underwent full-text review. Data were extracted on a standardized data collection table which included: First author, publication year, country, study design, number of patients involved, diagnosis and severity of OSA, diagnosis of NAFLD, patient characteristics, results of the study.

RESULTS

Study flow

A flow chart of the search for relevant studies is presented in Figure 1. In total, 132 articles were initially retrieved on PubMed search and 77 in the last five years. After removal of irrelevant studies and exclusion according to title, language and abstract (n = 67), 18 articles were selected for full-text review. A further 5 articles were excluded for the following reasons: 1 did not have a clear description of OSA

diagnosis, 2 had an inaccurate diagnosis of OSA and 2 did not have a clear description of patients enrollment and evaluation. Finally, 13 articles were included in the qualitative analysis.

Study characteristics

A summary of the 13 relevant studies is reported in Table 1. There was a total of 2753 participants across all the studies with a mean age between 42 and 58 years. The proportion of males ranged from 21% to 87.9% and the mean body mass index (BMI) ranged from 24.0 to 49.9 kg/m^2.

All the studies used PSG and AHI to diagnose OSA, according to the American Academy of Sleep Medicine (AASM) Clinical Practice Guideline[16], except for two that used cardio-respiratory polygraphy[34,35]. In 7 studies NAFLD was diagnosed by abdominal ultrasound, in 4 studies by abdominal computed tomography[36], fatty liver index (FLI)[37], aspartate aminotransferase (AST) to platelet ratio index (APRI)[38] and elastography[39], whereas only two studies used the gold standard liver biopsy[34,40].

The exclusion criteria considered in the relevant studies were as follows: Patients who had been previously diagnosed with or treated for OSA, patients with other sleep disorders or other chronic liver disease besides NAFLD; patients who were infected with hepatitis B and/or C virus; patients with excessive alcohol consumption; patients with current use of hepatotoxic drugs; patients who had any acute or chronic inflammatory disease, coronary heart disease, chronic obstructive pulmonary disease, and/or any solid organ failure or transplantation. Furthermore, diabetes mellitus represented an exclusion criterion in three studies[39,41,42].

DISCUSSION

Increased prevalence of NAFLD in patients with diagnosis of OSA

The results of this review showed an increased prevalence of NAFLD in patients with diagnosis of OSA. Therefore, hypoxia should be considered to have a key role in the pathogenesis of NAFLD.

The pathogenesis of NAFLD is commonly described as a two-hit model. The first hit is characterized by an increased intrahepatocytes triglyceride accumulation from adipose tissue lipolysis due to obesity and insulin resistance. The second hit is characterized by lipotoxic metabolite production, liver inflammation and steatosis progression due to oxidative stress, lipid peroxidation, mitochondrial dysfunction and some gene polymorphisms[43,44]. In OSA hypoxic environment, there is an increased adipose tissue lipolysis, oxidative stress, inflammation and liver fibrosis[45].

OSA is a well-established risk factor for hypertension, renal failure, obesity, insulin resistance, diabetes mellitus, MetS, liver steatosis and cardiovascular diseases[31-33,46,47]. However, to clarify the effects of OSA on the development and progression of NAFLD is challenging due to the several comorbidities which common coexist and are independently associated with systemic inflammation[48].

Bhatt *et al*[42] reported significantly higher levels of interleukin-6, leptin, macrophage migration inhibitory factor, high-sensitive C-reactive protein and tumor necrosis factor alpha, and significantly lower serum adiponectin levels in obese patients with OSA and NAFLD compared to the other groups, as a consequence of nocturnal hypoxia. All these inflammatory biomarkers seem to have an important pathophysiological role in the development of early metabolic and cardiovascular dysfunctions. Therefore, NAFLD represents an additional risk for systemic inflammation in patients with OSA. Furthermore, Agrawal *et al*[49] described a prevalence of 91.3% of NAFLD in a small group of patients with OSA and abdominal obesity whereas Qi *et al*[50] found a prevalence of 64% in 149 non-obese OSA patients.

Association between NAFLD and OSA in the absence of coexisting comorbidities

Furthermore, the results of this review showed that the association between OSA and NAFLD seems to be independent of coexisting comorbidities such as visceral fat or MetS. Yu *et al*[36] showed an association between OSA and NAFLD independently from visceral fat level in subjects with mean BMI of 24.7 kg/m^2, particularly in those with short sleep duration or excessive daytime sleepiness. Benotti *et al*[40] reported that, in patients with OSA without MetS, as the severity of AHI and hypoxia increased, the prevalence of more severe NAFLD significantly increased as well. However, the exact mechanisms involved in this association in the absence of visceral fat and MetS is still unclear. Certainly the effects of chronic intermittent hypoxia on liver may involve increased lipogenesis, formation of reactive oxygen species and proinflammatory cytokines which cause lipid peroxidation and hepatocyte injury[45]. Therefore, lipid metabolism, inflammation and OSA hypoxic environment may be of

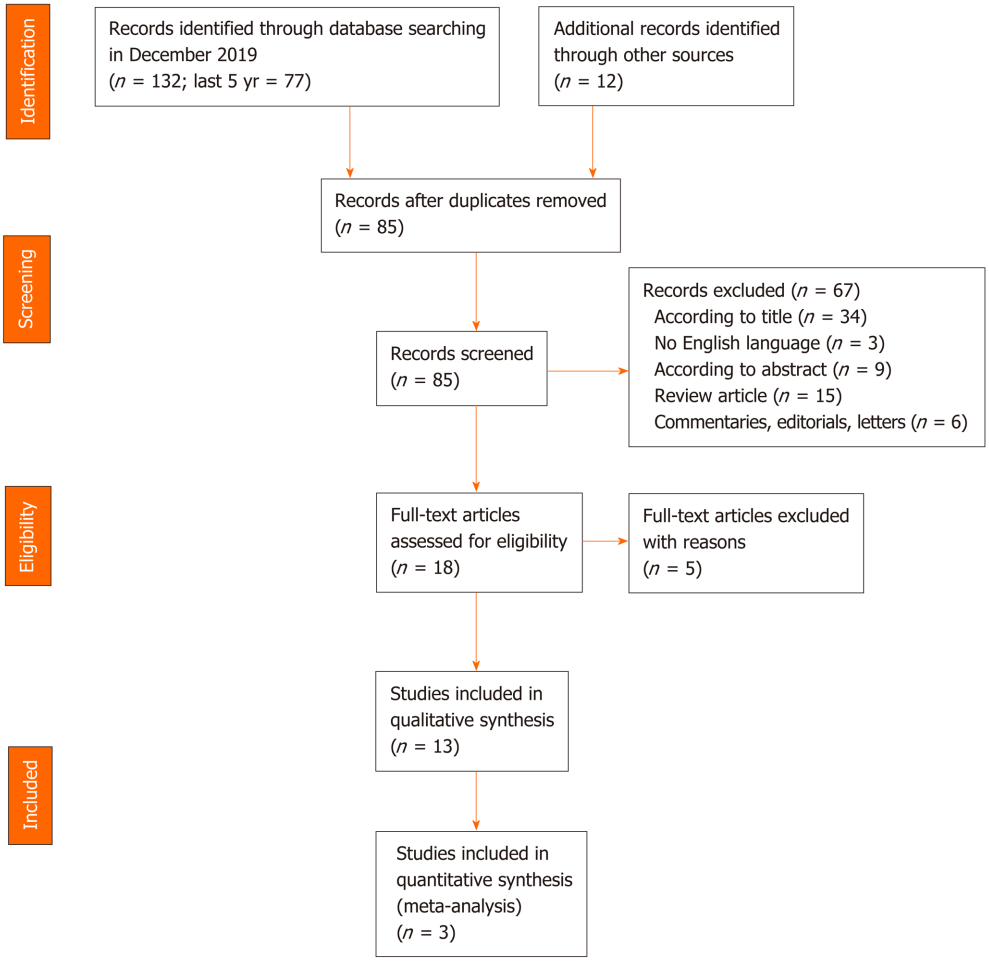

Figure 1 Flow chart of the search for relevant studies.

key importance in reducing the risk of NAFLD in OSA patients.

The severity of NAFLD is associated with the increase in OSA severity

Another important result of this review is that the severity of NAFLD is associated with the increase in OSA severity. Cakmak *et al*[51] found a significant association between the increase in NAFLD development and severity and the lowest oxygen saturation. Similarly, Petta *et al*[34] showed an association between the severity of liver damage with high risk of OSA and lower oxygen saturation. Arisoy *et al*[41] observed that BMI and hepatosteatosis grade progressively and significantly increased from patients without OSA to those with severe OSA. Chen *et al*[37] found a positive association between the severity of OSA and NAFLD. In particular, the prevalence of NAFLD was 20.4% in patients with AHI < 15 whereas it reached 52.1% in patients with AHI ≥ 15. Trzepizur *et al*[39] demonstrated an association between increasing OSA severity and liver fibrosis; patients with severe OSA and metabolic comorbidities are at higher risk of significant liver disease and advanced liver fibrosis.

Effective CPAP treatment may stabilize or slow NAFLD progression

The gold standard for the clinical management of OSA is CPAP treatment. Effective CPAP therapy for OSA may improve AST/alanine aminotransferase (ALT) levels[38,52] and liver steatosis[35]. Chen *et al*[52] showed a statistically significant increase in liver steatosis and serum aminotransferases with increasing OSA severity, and a significant decrease in both ALT and AST levels just after 3 mo of CPAP treatment. Kim *et al*[38] showed a favorable dose-response association between the severity of OSA and the improvement in serum aminotransferase levels and the regression of hepatic fibrosis after 6 mo of CPAP treatment; these findings correlated with the degree of adherence and were independent from the severity of obesity. Buttacavoli *et al*[35] described a significant improvement in hepatic steatosis after 6-12 mo of therapy with CPAP. Since in these studies the treatment with CPAP was relatively short, it was difficult to state definite and clear conclusions. However, some other longitudinal studies showed that 1 to 3 years CPAP therapy improved and reversed liver steatosis[35,53].

Table 1 Summary of the 13 included studies

Ref.	Study design	Number of patients	Diagnosis and severity of OSA	Diagnosis of NAFLD	Patient characteristics	Results
Agrawal[49], 2015 (India)	Prospective	23 (3 mild OSA, 5 mode rate OSA, 15 severe OSA)	- No OSA, AHI < 5; - Mild OSA, 5-14.9;	Abdominal ultrasound	Consecutive patients with diagnosis of OSA and abdominal obesity	- The prevalence of NAFLD in patients with OSA was 91.3% - AHI was an independent predictor of significant fibrosis
			- Moderate OSA, 15-30; - Severe OSA, > 30		Mean age: 46; Mean BMI: 32.2; Males: 78%	- No differences in the prevalence of NAFLD, raised transaminase levels and fibrosis according to the severity of OSA
Cakmak[51], 2015 (Turkey)	Retrospective	137 (118 OSA: -19 mild, - 39 moderate, - 60 severe, 19 no OSA)	- No OSA, AHI < 5; - Mild OSA, 5-14;	Abdominal ultrasound	All consecutive patients referred to a sleep laboratory due to sleep apnea symptoms	- Severity of NAFLD increased as AHI increased and lowest SpO_2, mean nocturnal SpO_2 levels decreased
			- Moderate OSA, 15-29; - Severe OSA, ≥ 30		Mean age: 55.7; Mean BMI: 34.5 (OSA), 33.2 (no OSA); Males: 44.5%	- There was a strong association between NAFLD severity and a decrease in lowest SpO_2 levels - Strong association between elevated liver enzymes and increase in nocturnal hypoxia severity in OSA patients
Petta[34], 2015 (Italy)	Cross-sectional	50 (25 OSA, 25 no OSA)	- No OSA, AHI < 5; - OSA, AHI ≥ 5	Liver biopsy	Consecutive patients with biopsy-proven NAFLD who underwent cardio-respiratory polygraphy Mean age: 53; Mean BMI: 33.5 (OSA), 29.0 (no OSA); Males: 58%	- Significant fibrosis was independently associated with mean nocturnal oxygen saturation < 95% in patients with NAFLD and OSA
Yu[36], 2015 (South Korea)	Cross-sectional	621 (286 OSA, 335 no OSA)	- No OSA, AHI < 5;	Abdominal CT scan	Subjects who examined the PSG and abdominal CT	- Patients with OSA were significantly older and had significantly higher BMI than those without OSA
			- OSA, AHI ≥ 5		Mean age: 56.6; Mean BMI: 24.7; Males: 57.2%	- The prevalence of NAFLD was 34% among patients with OSA and 21% among patients without OSA - Association between OSA and NAFLD independent of the visceral fat level in relatively lean individuals - This association was particularly strong in participants with excessive daytime sleepiness or short sleep duration regardless of visceral fat level

Arısoy[41], 2016 (Turkey)	Case-control	176 (52 mild, 34 moderate, 48 severe, 42 no OSA)	- No OSA, AHI < 5; - Mild OSA, 5-14; - Moderate OSA, 15-29; - Severe OSA, ≥ 30	Abdominal ultrasound	Subjects referred to a sleep center with clinical suspicion of OSA Mean age: 45.1 (no OSA), 42.9 (mild), 47.6 (moderate), 47.0 (severe); Mean BMI: 28.3 (no OSA), 30.1 (mild), 34.1 (moderate), 32.7 (severe); Males: 73.9%	- Hepatosteatosis grade, ALT and AST levels, BMI differed significantly among the groups - BMI and hepatosteatosis grade increased progressively and significantly from no OSA to severe OSA - Average desaturation and BMI were the parameters with the greatest independent effects on hepatosteatosis in the subjects with OSA
Benotti[40], 2016 (United States)	Retrospective	362 (115 mild, 80 moderate, 74 severe, 93 no OSA)	- No OSA, AHI < 5; - Mild OSA, 5-14; - Moderate OSA, 15-29; - Severe OSA, ≥ 30	Liver biopsy	Bariatric surgery candidates with clinical suspicion of OSA Mean age: 46.2; Mean BMI: 49.9; Males: 21%	- OSA severity was associated with NAFLD liver histology only in patients without metabolic syndrome
Buttacavoli[35], 2016 (Italy)	Observational	15	- Severe OSA, AHI ≥ 30	Abdominal ultrasound and elastography	Consecutive severe OSA patients at baseline and after 6-12 mo of CPAP treatment Mean age: 49.3; Mean BMI: 35.4; Males: 86.7%	- Most patients at diagnosis had severe liver steatosis (87%) - During follow-up, steatosis significantly improved in six patients without concurrent changes in the BMI range in the entire sample - No correlation was found between steatosis score and BMI at baseline, although a positive relationship between these variables was evident during CPAP treatment
Chen[37], 2016 (China)	Cross-sectional	319 (Group 1: 187 OSA with FLI < 60; Group 2: 132 OSA with FLI ≥ 60)	- No OSA, AHI < 5; - Mild OSA, 5-14.9; - Moderate OSA, 15-30; - Severe OSA, > 30	Fatty liver index (FLI) ≥ 60	All consecutive patients referred to a sleep center and diagnosed with OSA Mean age: 46.8 (Group 1), 42.3 (Group 2); Mean BMI: 24.5(Group 1), 28.5 (Group 2); Males: 79%	- Participants with a FLI ≥ 60 tended to be significantly fatter and had higher transaminase levels and severe PSG parameters of sleep apnea - Severity of OSA was independently associated with prevalence of NAFLD (52.1% in patients with AHI ≥ 15 *vs*. 20.4% in patients with AHI < 15)
Qi[50], 2016 (China)	Cross-sectional	175 (149 OSA: - 96 NAFLD, - 53 no NAFLD, 26 no OSA: - 10 NAFLD, - 16 no NAFLD)	- No OSA, AHI < 5; - Mild OSA, 5-14.9; - Moderate OSA, 15-29.9; - Severe OSA, > 30	Abdominal ultrasound	All consecutive non-obese patients referred to a sleep laboratory with clinical suspicion of OSA Mean age: 52.9 (OSA and NAFLD); Mean BMI: 24.0; Males: 87.9% (OSA), 77.3% (no OSA)	- Prevalence of NAFLD in OSA patients was 64% - BMI, lowest SpO_2, and triglycerides may be risk factors for promoting NAFLD in OSA patients

Chen[52], 2018 (China)	Observational	160 (42 moderate OSA, 88 severe OSA, 30 controls)	- No OSA, AHI < 5; - Moderate OSA, 5-30; - Severe OSA, ≥ 30	Abdominal ultrasound	All consecutive patients referred to a sleep laboratory with clinical suspicion of OSA Mean age: 42.6; Mean BMI: 28.0; Males: 86.9%	- Prevalence of liver steatosis was 64% among the groups; 59.5% and 81.8% in patients with moderate and severe OSA respectively - Increasing OSA severity was associated with higher BMI, waist circumference and neck circumference - ALT, AST and liver steatosis score increased significantly with an increase in OSA severity - OSA severity was independently associated with liver steatosis and elevation of serum aminotransferases, but not with liver fibrosis - Serum aminotransferase, as a biomarker of liver injury, decreased in OSA patients after 3 months of CPAP treatment
Kim[38], 2018 (United States)	Retrospective	351 (73 mild OSA, 102 moderate OSA, 176 severe OSA)	- No OSA, AHI < 5; - Mild OSA, 5-14.9; - Moderate OSA, 15-30; - Severe OSA, > 30	Suspected NAFLD was diagnosed if serum ALT > 30 U/L for men and > 19 U/L for women; Advanced fibrosis was identified by the AST to platelet ratio index (APRI) score	CPAP-treated OSA adult patients who had available serum ALT data before (within 3 months) and after (within 6 months) CPAP treatment Mean age: 57.6; Mean BMI: 32.2; Males: 59.3%	- The prevalence of suspected NAFLD was higher (90.3%) among patients with moderate to severe OSA versus among those with mild OSA (86.3%) - Fibrosis was correlated with OSA severity (7.6% for mild OSA versus 12.0% moderate OSA versus 19.7% for severe OSA) - There was a dose-response relationship between OSA severity and improvement in ALT and AST levels and APRI score after CPAP treatment, correlating with adherence status and without differences in the obesity severity status
Trzepizur[39], 2018 (France)	Cross-sectional	124 (34 mild, 38 moderate, 52 severe)	- No OSA, AHI < 5; - Mild OSA, 5-14.9; - Moderate OSA, 15-29.9;	Elastography	Patients with at least one criterion for metabolic syndrome with diagnosis of OSA Mean age: 52.4; Mean BMI: 29.9; Males: 65.6%	- Prevalence of advanced liver fibrosis was 12% - Increasing OSA severity was associated with BMI, waist circumference, ODI, percentage of sleep time with SpO_2 < 90% and higher proportions of male patients with metabolic syndrome

			- Severe OSA, ≥ 30			- Increasing OSA severity was also associated with higher LSM values with a marked increase between mild-to-moderate OSA and severe OSA - Patients with severe OSA and metabolic comorbidities are at higher risk of significant liver disease (LSM ≥ 7.3 kPa) and advanced liver fibrosis (LSM ≥ 9.6 kPa) - AHI and ODI were the factors with the strongest independent association with LSM
Bhatt[42], 2019 (India)	Case-control	240 (124 OSA and NAFLD, 47 OSA without NAFLD, 44 NAFLD without OSA, 25 no OSA and no NAFLD)	- No OSA, AHI < 5;	Abdominal ultrasound	Overweight/obese subjects (BMI > 23 kg/m^2)	- Mean values of AST, ALT and BMI were significantly higher in OSA with NAFLD group as compared to the other groups
			- OSA, AHI ≥ 5		Mean age: 44.8 (OSA and NAFLD); Mean BMI: 33.3 (OSA and NAFLD); Males: 55.0%	- Inflammatory markers showed a significant correlation in the OSA and NAFLD group - OSA and NAFLD operate as an independent contributors to the increased systemic inflammation that occurs in overweight/obese subjects

ALT: Alanine aminotransferase; AHI: Apnea-hypopnea index; APRI: Aspartate aminotransferase to platelet ratio index; AST: Aspartate aminotransferase; BMI: Body mass index; CPAP: Continuous positive airway pressure; CT: Computed tomography; FLI: Fatty liver index; LSM: Liver stiffness measurement; NAFLD: Non-alcoholic fatty liver disease; ODI: Oxygen desaturation index; OSA: Obstructive sleep apnea; PSG: Polysomnography; SpO_2: Oxygen saturation.

In conclusion, the development of NAFLD seems to be closely associated with OSA even in the absence of coexisting comorbidities such as obesity or MetS. These findings suggest that even relatively lean patients with OSA should be referred to hepatologists for specific management. As clinicians, our aim should be to screen OSA patients for NAFLD and vice versa those with NAFLD for OSA. Therefore, it is of great importance to set up a strong collaboration between gastroenterology and sleep medicine, in which internal medicine, cardiology and nephrology should have a key role. Furthermore, in NAFLD patients, although asymptomatic, it is recommended to systematically perform PSG in order to early and better treat them before the development of potentially life threatening systemic dysfunctions. Effective CPAP treatment, although not always decisive, may stabilize or slow NAFLD progression with benefits on metabolic and cardiovascular functions.

ARTICLE HIGHLIGHTS

Research background

The pathogenesis of non-alcoholic fatty liver disease (NAFLD) is multifactorial and is commonly described as a two-hit model. The first hit is characterized by an increased triglyceride accumulation in the hepatocytes due to obesity and insulin resistance. The second hit is characterized by lipotoxic metabolite production, liver inflammation and steatosis progression due to oxidative stress, lipid peroxidation, mitochondrial dysfunction and some gene polymorphisms. In obstructive sleep apnea (OSA) hypoxic environment, there is an increased adipose tissue lipolysis, oxidative stress, inflammation and liver fibrosis. OSA is a well-established independent factor of insulin resistance, which may predispose to the development and the progression of liver steatosis. However, to clarify the effects of OSA on the development and progression of NAFLD is challenging due to the several comorbidities which common coexist and are independently associated with systemic inflammation.

Research motivation

NAFLD is an emerging liver disease. The increased mortality of patients with NAFLD is primarily a result of cardiovascular diseases and, to a lesser extent, to liver related diseases. OSA is still underdiagnosed; its prevalence is estimated to be 4% in the general population increasing up to 40% in some disease-specific populations, such as in patients suffering from cardiovascular disease or metabolic syndrome. Probably the atypical presentation, the lack of data on the criteria for identifying OSA and the lack of awareness of this entity among clinicians are important reasons. Since OSA may be linked with the pathogenesis and the severity of NAFLD, it is very important to early and better diagnose and treat OSA in NAFLD patients, in which numerous cardiovascular and metabolic comorbidities often coexist.

Research objectives

The aim of this systematic review is to provide a more comprehensive overview of the association between NAFLD and OSA considering also the efficacy of the gold standard treatment for the clinical management of OSA, the continuous positive airway pressure (CPAP) treatment.

Research methods

A PubMed search limited to the last 5 years was conducted using the terms "non-alcoholic fatty liver disease AND (obstructive sleep apnea OR obstructive sleep disorders OR sleep apnea)". We did not consider animal and child studies, case reports, commentaries, letters, editorials and meeting abstracts.

Research results

Initially, a total of 132 articles were retrieved on PubMed search and 77 in the last 5 years. After removal of irrelevant studies, 13 articles were included in the qualitative analysis. 2753 participants with a mean age between 42 and 58 years were included across all the studies. The proportion of males ranged from 21% to 87.9% and the mean body mass index ranged from 24.0 to 49.9 kg/m^2. The results of this systematic review showed an increased prevalence of NAFLD in patients with OSA, even in the absence of coexisting comorbidities such as obesity or metabolic syndrome. Furthermore, the severity of NAFLD is associated with the increase in OSA severity. Effective CPAP treatment may stabilize or slow NAFLD progression with benefits on metabolic and cardiovascular functions.

Research conclusions

NAFLD seems to be closely associated with OSA even in the absence of coexisting comorbidities such as obesity or MetS. Hypoxia should be considered to have a key role in the pathogenesis of NAFLD. Therefore, all OSA patients, even relatively lean, should be referred to hepatologists for specific management and all NAFLD patients, even if asymptomatic, should be screened for OSA. Effective CPAP treatment, although not always decisive, may stabilize or slow NAFLD progression with benefits on metabolic and cardiovascular functions. The systematic use of polysomnography in NAFLD patients, although asymptomatic, will help clinicians to early diagnose OSA and better treat it before the development of potentially life threatening systemic dysfunctions.

Research perspectives

The association between NAFLD and OSA has been reviewed. A strong collaboration between gastroenterology and sleep medicine will have a key role in the management of these two conditions. Future research is needed to validate the efficacy of CPAP treatment on liver steatosis with longer longitudinal studies.

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