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REVIEW

Challenges in diagnosis of pancreatic cancer

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Abstract

Pancreatic cancer is a growing source of cancer related death, yet has poor survival rates which have not improved in the last few decades. Its high mortality rate is attributed to pancreatic cancer biology, difficulty in early diagnosis and the lack of standardised international guidelines in assessing suspicious pancreatic masses. This review aims to provide an update in the current state of play in pancreatic cancer diagnosis and to evaluate the benefits and limitations of available diagnostic technology. The main modalities discussed are imaging with computed tomography, magnetic resonance imaging, endoscopic ultrasound and positron emission tomography and tissue acquisition with fine needle aspiration. We also review the improvements in the techniques used for tissue acquisition and the opportunity for personalised cancer medicine. Screening of high risk individuals, promising biomarkers and common mimickers of pancreatic cancer are also explored, as well as suggestions for future research directions to allow for earlier detection of pancreatic cancer. Timely and accurate diagnosis of pancreatic cancer can lead to improvements in the current poor outcome of this disease.

Key words: Pancreatic cancer; Diagnosis; Challenges; Imaging; Biomarkers; Screening; Endoscopic ultrasound; Pitfalls

Core tip: Pancreatic cancer is becoming a leading cause of cancer related death in Western societies. Rapid and accurate diagnosis of a pancreatic mass is crucial for improving outcomes. Current practice utilises multi-detector computed tomography and/or magnetic resonance imaging, with a dedicated pancreas protocol as the initial modality. Endoscopic ultrasound is the preferred method to further evaluate pancreatic masses as it has more superior diagnostic accuracy and can provide tissue acquisition. Pitfalls in diagnosis of pancreatic

cancer are discussed, as careful recognition of these conditions is important. There are exciting developments of new diagnostic techniques that open the possibility of personalised cancer medicine.

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INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer related death in Western societies and is projected to be the second leading cause within a decade. It has an average annual incidence rate of 12.5 per 100000 population (which is 3% of all cancers) in America, but has a disproportionately high mortality, with an average annual death rate of 10.9 per 100000[1]. Pancreatic cancer is difficult to be diagnosed at an early stage, with the vast majority of cancers found to be already metastatic at the time of initial diagnosis. Only 9.7% of pancreatic cancer are at a local stage at time of diagnosis[2]. These poor survival rates have not changed significantly in nearly 40 years.

Ductal adenocarcinoma and its variants account for over 90% of pancreatic malignancies. Presenting features of this disease may include weight loss, jaundice, malabsorption, pain, dyspepsia and nausea; however, many patients are asymptomatic and no early warning signs of pancreatic cancer have been established.

Known risk factors for pancreatic cancer include cigarette smoking (relative risk increase of 2.5 times[3]), high body mass index and lack of physical activity[4], diabetes[5] and chronic pancreatitis[6]. Furthermore, there are also a number of inherited cancer syndromes linked to pancreatic cancer including Hereditary Breast and Ovarian Cancer Carriers of the BRCA1 or BRCA2 germline mutations, familial atypical multiple mole melanoma syndrome (FAMMM), Peutz-Jeghers syndrome, hereditary pancreatitis, Hereditary Nonpolyposis Colorectal Cancer (Lynch Syndrome) and familial pancreatic cancer. These higher risk groups may be a good target for screening and early diagnosis programs.

Surgical resection is the only curative treatment for pancreatic cancer. Unfortunately, because of late presentation, only 15% to 20% of patients are candidates for pancreatectomy. Furthermore, prognosis is poor, even after a complete resection. Five year survival after pancreaticoduodenectomy, or Whipples procedure, is approximately 21% for negative margin resections (R0) and 11% for microscopically positive margin resections (R1)[7]. Even in patients with negative margin resections with presumed curative intent, up to 71% can have disease recurrence[7].

The motivation for this research is the dismal outcomes for pancreatic cancer that have failed to significantly improve; it is this that is the key problem to be solved. The main focus of this review is to describe the current state of play in pancreatic cancer diagnosis. Rapid and accurate diagnosis of a pancreatic mass is crucial for improving outcomes. After evaluating the evidence underpinning all of the widely used modalities for diagnosis, we intend to make a comparison of these modalities and provide an evidence-based algorithm for diagnosis.

The main objective of this review was to evaluate and compare the suitability and accuracy of the current diagnostic modalities that exist for pancreatic cancer. We are currently lacking effective diagnostic and screening modalities to diagnose pancreatic cancer at an early, and therefore more likely curative stage. Therefore, it is valuable to have a thorough understanding of the currently available diagnostic technology, including its benefits and limitations, in order to provide direction for future research. Pitfalls and mimickers of pancreatic cancers, biomarkers and the current screening programs in high risks individuals will also be discussed.

LITERATURE SEARCH

A MEDLINE search was conducted using the following keywords and phrases: "pancreatic cancer, diagnosis, imaging, biomarkers, screening, endoscopic ultrasound, pitfalls", with a focus on more recently published research. In addition, we performed a manual review of the reference lists of the primary and review articles to ensure identification of all relevant articles. In particular, large scaled meta-analyses and systematic reviews were preferred.

RESULTS

Diagnosis relies on imaging modalities such as computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET) and endoscopic ultrasound (EUS) that are used along with tissue acquisition. Early detection is the only way of identifying small cancers and proceeding with curative surgery. We describe the different diagnostic modalities that currently exist, evidence underpinning their use and compare the benefits and disadvantages of each. Table 1 below provides a summary of our findings and Figure 1 shows a suggested algorithm based on our findings for the evaluation of a patient with pancreatic cancer.

CT SCANNING

Multi-detector computed tomography (MDCT) is the most widely available and best-validated tool for imaging patients with pancreatic adenocarcinoma. MDCT takes reproducible multi-planar imaging which provides good spatial resolution and attenuation between tumour and

Table 1 Benefits and limitations of pancreatic cancer diagnostic modalities

Diagnostic modalities	Advantages	Limitations
MDCT	Most commonly available	Nephrotoxicity
	Best validated	Radiation exposure
	Cheapest	
MRI	Superior imaging	Expensive
	Depiction of local pancreatic disease	Less available
	Iodine-free and no radiation	Contraindicated with some metal implants
EUS +/- FNA	Safe and less invasive	Less available in some countries
	High sensitivity	Operator dependent
	Able to detect small lesions	Inability to detect distant metastasis
	Able to take histological sample	
PET/CT	Metastatic disease detection	Expensive
	Clarification of equivocal CT findings	Less available
	Monitoring recurrence and response to adjuvant therapy	Radiation and contrast exposure

CT: Computed tomography; MDCT: Multi-detector computed tomography; MRI: Magnetic resonance imaging; EUS: Endoscopic ultrasound; FNA: Fine needle aspiration; PET: Positron emission tomography.

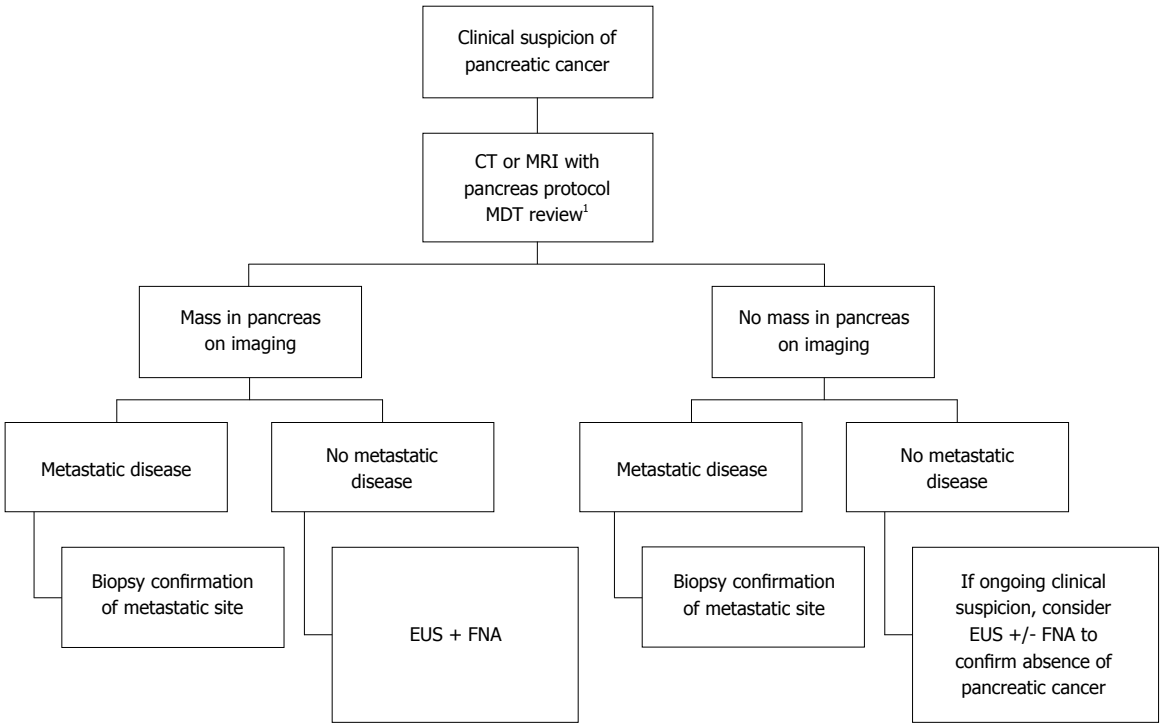

Figure 1 Algorithm for the evaluation of a patient that has clinical suspicion of pancreatic cancer. [1]Multi-disciplinary review should involve a panel including gastroenterologist, surgeon, medical and or radiation oncologist, diagnostic imaging and pathologist. CT: Computed tomography; MDCT: Multi-detector computed tomography; MRI: Magnetic resonance imaging; EUS: Endoscopic ultrasound; FNA: Fine needle aspiration.

background pancreatic parenchyma with wide anatomic coverage, and thus allowing comprehensive examination of local and distant disease in one single section[8].

Numerous international guidelines endorse the use of CT as the initial modality in diagnosis of suspected pancreatic cancer[9,10]. In particular, MDCT is best performed according to a dedicated pancreas protocol[10]. Despite some inter-institutional variability, the standard MDCT pancreas protocol is a helical type scan that takes interval images of 0.5 to 1 sub-millimetres, with two phases: pancreatic parenchymal phase at 40 to 50 seconds and portal venous phase at 65 to 70 seconds. The majority of modern scanners are 128 and 256 slice scanners. It includes the administration of both intravenous high iodine concentrated contrast, injected at a rate of 3 to 5 mL per second and ingestion of neutral oral contrast. The pancreatic phase is described as the intermediate between the arterial and hepatic phase where maximal enhancement of the pancreas is achieved to see the contrast between tumour and pancreatic parenchyma, as well as visualization of the peri-pancreatic arteries and veins[11]. The image is usually reconstructed in the following ways: (1) axial views at 2 to 5 mm thickness; (2) coronal and sagittal views with multi-planar reformats at 2 to 3 mm thickness; and (3) vascular evaluations with maximum intensity projections

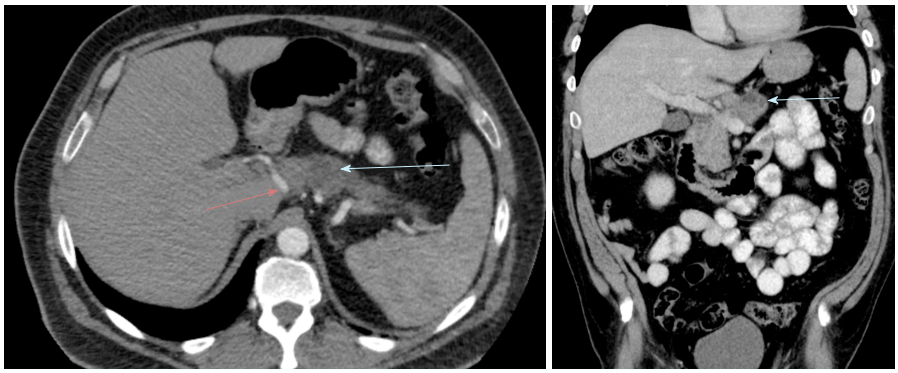

Figure 2 Axial and coronal plane view on computed tomography of a patient with a 2 cm mass in the body of pancreas (blue arrow), abutting splenic artery (red arrow).

or three dimensional (3D) volumetric thick sections.

Pancreatic cancer appears on CT as an ill-defined mass that enhances poorly compared to adjacent normal pancreatic tissue; thus appearing hypodense on arterial phase scans in 75% to 90% of cases, but may become isodense on delayed scans. Findings which may be predictive of pancreatic cancer include, from lowest to highest specificity: ductal dilatation (sensitivity 50% and specificity 78%), hypo-attenuation (sensitivity 75% and specificity 84%), ductal interruption (sensitivity 45% and specificity 82%), distal pancreatic atrophy (sensitivity 45% and specificity 96%), pancreatic contour anomalies (sensitivity 15% and specificity 92%), and common bile duct dilation (sensitivity 5% and specificity 92%)[12]. Figure 2 demonstrates two views on CT imaging of a pancreatic cancer which has abutted into the splenic artery.

When compared with other imaging modalities, CT performs well in the diagnosis of pancreatic cancer. A large meta-analysis comparing various imaging modalities for the diagnosis of pancreatic cancer found a combined sensitivity and specificity of 89% and 90% respectively for CT[13], which was equivalent to MRI. There has been reported improvement in the detection of pancreatic cancer with recent suggestions of sensitivities up to 96% for MDCT, secondary to acquisition of thin collimation images, improved spatial and temporal resolution and use of multi-planar reconstruction and 3D technique[14].

Multi-planar reconstruction on CT is important in tumour staging; providing selective visualization of important arterial and venous structures. This allows for precise visualization of the relationship of the primary tumour to the superior mesenteric artery (SMA), superior mesenteric vein (SMV) and coeliac axis thereby providing an assessment of vascular invasion and resectability. CT is able to distinguish abutment, encasement, narrowing, or occlusion of the portal vein/SMV at the confluence and allow the surgeon to determine if a venous reconstruction is technically feasible[14]. The accuracy of CT in assessment of vascular invasion is not strong, with the most recent studies showing a sensitivity of only 60% and specificity 94% when determining involvement of surrounding vessels[15]. The reason for favouring specificity over sensitivity for vascular invasion is to avoid denying surgery to patients with potentially resectable tumours[16]. Despite these values, consensus statements suggest that preoperative evaluation of surgical resectability be based on CT[17]. CT is also able to provide 3D reconstruction which can be very useful for pre-operative planning by the surgeon.

CT also plays an important role in predicting unresectability. If the tumour surrounds a vessel by more than 180 degrees and occlusion of the SMV/portal vein without surgical options of reconstruction, then it is deemed T4 disease and is unresectable[14]. Recent studies have demonstrated that CT's sensitivity for unresectable disease is between 52% to 91%, and specificities of 92% to 100%[18]. One study also showed that different generations of MDCT equipment did not impact these values[19].

CT also provides the benefit of diagnosing distant intra-abdominal and/or lung metastasis, which is important given that diagnosis of pancreatic cancer is often delayed. Findings of peritoneal carcinomatosis on CT include ascites, peritoneal thickening, contrast enhancement, nodular bowel wall thickening, and soft-tissue infiltration of the omentum[16].

Whilst overall a safe, non-invasive and relatively cheap test to perform, contrast CT is accompanied by the risk of nephrotoxicity from the iodine-contrast agent and as well as involving exposure to radiation. There is also individual variability in getting parenchyma enhancement due to technical factors such as the generation of CT scanner, contrast material volume and concentration and rate of injection, and patient factors such as age, weight and cardiac output[8]. Despite this, most centres still endorse the use of MDCT as the first line modality of choice for diagnosing pancreatic cancer and should not be substituted by other more advanced imaging modalities.

MRI

MRI of the pancreas works by evaluating the speed of the diffusion process by random translational mole-

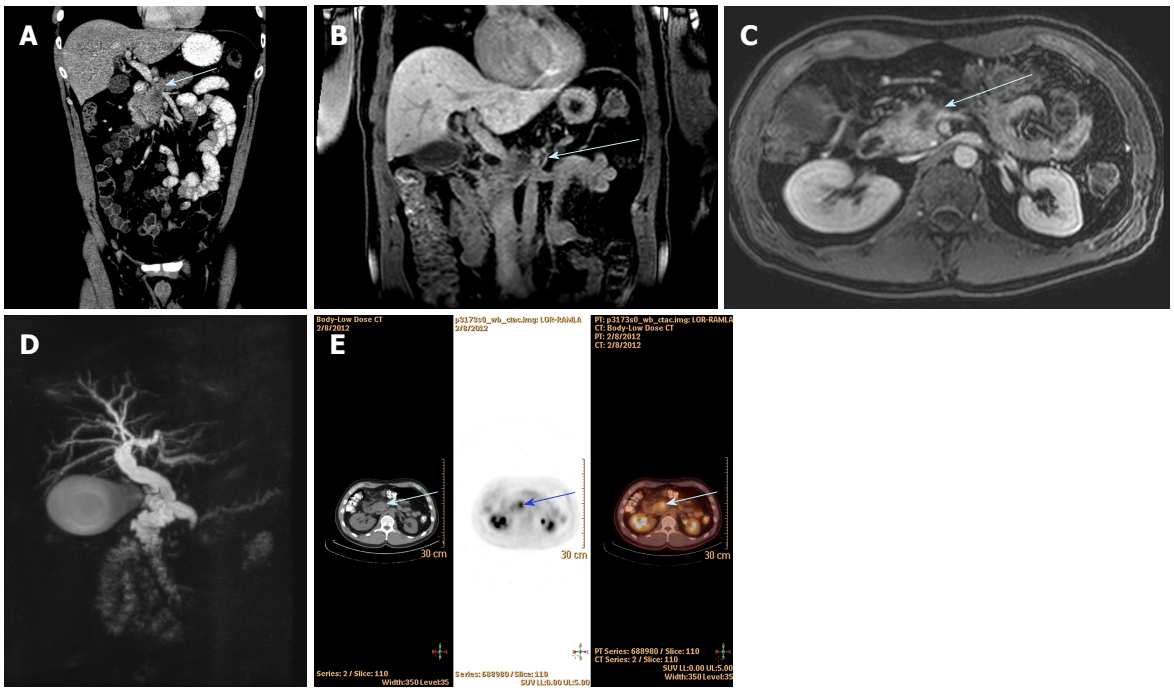

Figure 3 Multimodal imaging techniques utilised for a patient with 2.8 cm head of pancreas cancer (blue arrow) with portal vein and superior mesenteric vein invasion. A: Hypodense mass on coronal view on CT; B: T1-weighted coronal view on MRI; C: T1-weighted axial view on MRI; D: MRCP view with dilated CBD and PD (double duct sign); E: Axial view on PET CT imaging showing marked FDG avidity. CT: Computed tomography; MDCT: Multi-detector computed tomography; MRI: Magnetic resonance imaging; PET: Positron emission tomography; MRCP: Magnetic resonance cholangiopancreatography; FDG: Fluorodeoxyglucose.

cular motion which differs between extracellular and intracellular components of tissue, as well as differences in tissue cellularity and cell density[20]. The pancreas protocol for MRI includes several sequences: T2-weighted single-shot fast spin-echo (SSFSE), T1-weighted in-phase and opposed-phase gradient echo (GRE), T2-weighted fat-suppressed fast spin-echo (FSE), and diffusion-weighted imaging (DWI) all provide an axial plane with less than 6mm thick slices[21]. There is also the option to have pre- and dynamic post- IV contrast administration (gadolinium) 3D T1-weighted fat-suppressed gradient- echo (in pancreatic, portal venous, and equilibrium phases) which provides an axial plane but with the thinnest possible slices of 2 to 3 mm[21]. Pancreatic adenocarcinomas normally appear hypo-intense to normal pancreas on precontrast T1-weighted images and hypointense or isointense on post-contrast T1-weighted images[16], as seen in Figure 3.

MRI theoretically allows tumour detection at an earlier stage by providing a comprehensive analysis of the morphological changes of the pancreas parenchyma, as well as that of the pancreatic duct. Despite this, in meta analyses, MRI has only been shown to be equally sensitive and specific in diagnosing and staging pancreatic cancer as CT; with a combined sensitivity and specificity of 89% and 89% respectively[13]. This is likely due to the difficulty in demonstrating a significant benefit when the sensitivity and specificity of CT are already relatively high. For this reason, MRI is not widely used as the primary imaging modality in most centres due to issues of its cost and availability[9]. Most experts nevertheless acknowledge the added utility of MRI over CT in certain situations; including the main benefit in differentiating iso-attenuating pancreatic lesions and in characterization of indeterminate liver lesions identified at prior CT examinations[9]. MRI is also valuable in patients with impaired renal function or patients with sensitivities to iodinated contrast. Furthermore, other specific situations MRI seems to have an advantage over CT is in differentiating pancreatic tumours from mass-forming pancreatitis, for tumours less than 2 cm, in the presence of hypertrophied pancreatic head or focal fatty infiltration of the parenchyma[22]. In the authors' experience, MRI is often used as a second-line test when there is a high clinical suspicion of pancreatic tumour despite none being visible on CT.

EUS WITH FINE NEEDLE ASPIRATION

EUS is performed under sedation and involves an upper gastrointestinal endoscopic examination with the use of an echoendoscope. The echoendoscope transducer is positioned in the stomach, in direct proximity to the pancreas so that it enables detailed high-resolution imagines of the pancreas and surrounding vessels, lymph nodes and left lobe of the liver. EUS is a safe, well-tolerated procedure and has the added benefit of allowing fine needle aspiration to be performed in order to obtain a cytopathological diagnosis. It is particularly ideal for lesions less than 2 cm or when there is a clinical

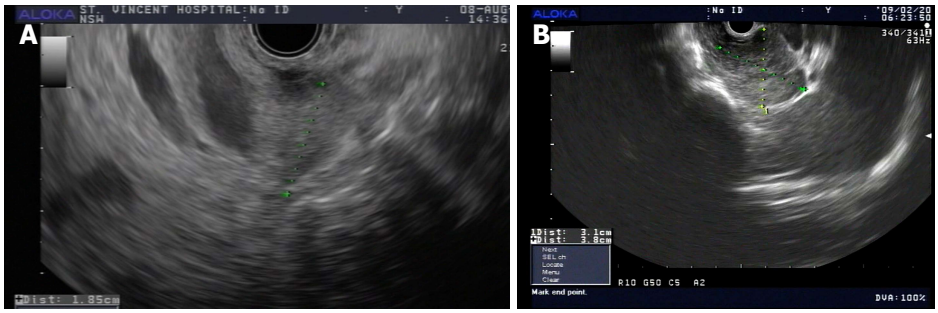

Figure 4 Endoscopic ultrasound images of (A) a small pancreatic adenocarcinoma in the head of the pancreas (1.8 cm) not seen on other modalities; and (B) a 3.1 cm pancreatic adenocarcinoma in the tail of the pancreas.

suspicion of pancreatic cancer but other modalities have failed to identify a mass and for obtaining a confirmatory biopsy. Figure 4 demonstrates the appearance of pancreatic cancers on EUS imagining.

In large meta-analyses EUS with fine needle aspiration (EUS-FNA) was found to be highly accurate in not only diagnosing malignancy but also in diagnosing the correct aetiology for solid pancreatic masses, with sensitivity of over 85% and specificity of 96%[23-26]. Longitudinal studies have also observed a significant increase in diagnostic accuracy over time, likely reflecting an increase in operator proficiency with experience and better visualisation with newer echoendoscopes. The increase in the diagnostic accuracy was seen from 1995-2000 to 2001-2010, with pooled sensitivity of 83.0% increasing to 87.8%, while the pooled specificity remained high at 96.6% and 95.6%[23]. EUS is also used as a reliable tool for local staging, as studies have shown a sensitivity and specificity of 72% and 90% respectively for T1-2 staging, 90% and 72% respectively for T3-T4 staging, and 87% and 92% respective for vascular invasion[27].

The evidence suggests that EUS may have distinct advantages in pancreatic cancer diagnosis when compared with other modalities. Comparative studies with CT have demonstrated the superiority of EUS in primary tumour detection and staging with the absence of a focal mass lesion on EUS reliably excluding pancreatic cancer irrespective of clinical presentation with a negative predictive value of 100%[28]. It has also been shown that the diagnostic accuracy of EUS when no identifiable mass was found on spiral CT was 92%[29]. In a recent meta-analysis, CT scan showed lower sensitivity than EUS for nodal staging (24% *vs* 58%) and vascular invasion (58% *vs* 86%); however, the specificities for nodal staging (88% *vs* 85%) and vascular invasion (95% *vs* 93%) were comparable in studies where both imaging techniques were performed[30]. EUS has its greatest benefit over CT and MRI for small pancreatic neoplasms (less than 2 cm), having a sensitivity of 94% compared with 69% for MDCT and 83% for MRI[31].

Still, perhaps the clearest demonstration of the benefits of EUS-FNA is its ability to obtain a tissue biopsy. Large meta-analyses have demonstrated superiority of EUS-FNA, with pooled sensitivity of more than 85% to 92% and pooled specificity of 94% to 100% in the diagnosis of pancreatic lesion[23-25,28]. EUS is also shown to be the best imaging modality for detecting vascular (especially portal vein) invasion, with a reported accuracy of 82%, compared with CT's accuracy of 79%[29]. The overall complication rate of EUS-FNA is very low 0.85%[32] (including infection, self-limiting pancreatitis) and if the tumour is in the head of pancreas, the needle tract will be part of the resected specimen thus minimising the risk of tumour seeding. Tumour seeding during EUS-FNA is a rare but important complication to be considered, with only a few case reports ever documented[33,34]. Apart from this, other major complications such as perforation, are extremely rare with a risk of 1:2500[24].

Fine needle aspiration technique

Different techniques in retrieving samples have been investigated for EUS including "fanning", "slow pull" and the "wet suction" technique (WEST). Randomised trials comparing "fanning", which involves sampling multiple areas within a lesion with each pass, with standard technique found that fanning was superior and fewer passes were required to establish the diagnosis[35]. There was however no difference in diagnostic accuracy, technical failure or complication rates[35]. As for the "slow pull" technique, where minimum negative pressure is provided by removing the stylet from the needle slowly and continuously, lower scores for contamination with blood were found, with a higher sensitivity of diagnosis of malignancy[36]. Lastly, the WEST technique, which involves flushing the needle with 5 mL of saline solution to replace the column of air within the lumen of needle to improve the quality of aspirate, also resulted in significantly better cellularity and specimen adequacy in cell blocks and specimen adequacy, but had no difference in the amount of blood contamination[37].

On-site cytopathologist

The presence of an on-site cytopathologist has a beneficial effect on the diagnostic yield of EUS FNA, by significantly lowering the number of inadequate samples, and increasing the diagnostic sensitivity and overall accuracy for malignancy[38,39]. Studies demonstrated the cost effectiveness of having an on-site cytopathologist where the same accuracy of 87% was achieved with

only 2.1 passes, compared to the 4 passes needed when real-time evaluation of specimens was not available[40].

Contrast-enhanced EUS

Contrast-enhanced EUS (CE-EUS) is a technique in which during the EUS, a second-generation low mechanical index microbubble ultrasound agent (UCA) is injected peripherally. Due to its small size (2 to 10 μm), it detects very slow flow and provides real time perfusion imaging without the burden of Doppler-related artefacts[41].

Observational studies have demonstrated more accurate characterization of solid pancreatic lesions seen on EUS by estimating their vascularity after injecting a contrast agent. It was also found that a hyper-enhancing lesion on CE-EUS was highly specific (more than 98%) for excluding adenocarcinoma, while a hypo-enhancing and hypo-echoic lesion was highly sensitive (more than 86%) for adenocarcinoma[41]. It also helps differentiate between a pancreatic adenocarcinoma (because of lower uptake of contrast, or hypoenhancement) and neuroendocrine tumours (NET), lymphoma, metastasis, and pseudo-papillary tumours that mimic cancer but show hyper-enhancement on CE-EUS. CE-EUS is beneficial in confirming that small pancreatic lesions are NET (hypervascular lesions with early arterial enhancement), characterisation of a mural nodule and malignant transformation of intrapapillary mucinous neoplasms[42] and in providing further information on solid masses in patients with chronic pancreatitis. There is also potential to utilise CE-EUS for targeted EUS FNA to improve the accuracy of biopsy by avoiding necrotic tissue and by selecting the most adequate target. There are minimal studies available assessing this and so this poses a potential topic for future research.

Despite these findings, CE-EUS is not yet widespread in all centres around the world. CE-EUS should not be used in patients with unstable angina and there is a small chance of an allergic reaction to the contrast.

EUS fine needle aspiration versus fine needle biopsy

There has been recent research looking into techniques to increase the amount of tissue acquisition to improve the diagnostic accuracy of samples. Fine-needle biopsy needles (EUS-FNB) have been designed in order to allow core biopsies with preserved architecture which would enable histological analysis, by shearing tissue from the target lesion. Initially, 19-gauge calibre needles were utilised but the mechanical friction caused by the torqued echoendoscope limited its use for evaluating pancreatic head masses[43]. Studies assessing Trucut needles showed that there was no significant difference between the diagnostic accuracy of 19-gauge Trucut needle and EUS-FNA needle, with a reported accuracy of 78% and 89% in one study[44]. However, there were more technical issues experienced with Trucut needle.

Newer 19-gauge, 22-gauge 25-gauge EUS needles with reverse bevel technology (Pro-core, Cook Medical; Winston Salem, NC, United States), Franseen type needles (Acquire, Boston Scientific, Marlborough, MA, United States) and fork-tip needles (Shark Core, Medtronic, Minneapolis, MN, United States) were developed to overcome the technical issues which allowed acceptable histological core samples and cytology aspirates, with diagnostic accuracies of more than 90%[45].

A study comparing 22-gauge FNA and FNB needles showed diagnostic cytologic specimens in 89.3% of patients and histologic specimens in 80% of patients with solid pancreatic mass lesions[43]. Similarly, a recent meta-analysis showed no significant difference in diagnostic adequacy (75.2% *vs* 89.0%), or diagnostic accuracy (85.8% *vs* 86.2%) between biopsy and aspiration needles[46]. Most recently, a small retrospective study showed better results with a 25-gauge core biopsy needle reporting a combined cytological and histologic sensitivities of 85%, specificities of 100% and accuracies of 86% with a single pass and minimal complications[47].

If the FNB needle design can be further improved and be routinely shown to provide diagnostic yields high enough to eliminate the need for on-site cytopathological evaluation, then this could also lead to a significant reduction in the costs of pancreatic cancer.

EUS elastography

EUS elastography measures tissue elasticity in real time using a dedicated software during an EUS examination. Elasticity is depicted using a colour map, where hard tissue is shown in dark blue, medium hard tissue in cyan, tissue with intermediate hardness in green, medium soft tissue in yellow and soft tissue as red. Pancreatic malignancy appears as a heterogenous blue predominant mass, whereas normal pancreas appear as homogeneous green and inflammatory pancreatic masses have a heterogeneous, green-predominant appearance[48]. The sensitivity and specificity of EUS elastography to differentiate benign from malignant pancreatic lesions has been reported as 92.3% and 80.0%, respectively, compared to 92.3% and 68.9%, respectively, for the conventional EUS B-mode images[49]. Elastography is mainly used in Europe. It does have limitations, as colour pattern provides a subjective determination and has intra-observer and inter-observer variability. Other studies reviewing elastography has not been strong and so more research is required to make conclusions regarding its benefits.

While elastography and CE-EUS provide additional benefits to standard EUS, the combination of elastography and CE-EUS does not significantly increase the diagnostic accuracy of either of the techniques performed alone[50]. Each modality has its benefits in selected cases.

PET

PET with F-18-fluorodeoxyglucose (18FDG) has no additional benefit in diagnosis of pancreatic cancer. However, a more recent triple phase enhanced 18FDG-

PET has been combined with CT to produce one fusion image, as seen in Figure 3. At this point in time, experts do not recommend PET/CT as a substitute for high-quality contrast-enhanced CT because its role is still being established[9]. Despite this, the research has been promising with the use of PET/CT for staging. A meta-analysis has shown that the pooled sensitivity of PET in diagnosis, in evaluating N staging and in liver metastasis were 91%, 64% and 67% respectively; and the corresponding specificities were 81%, 81% and 96% respectively[51]. These values are higher than CT alone. However, as a diagnostic tool, PET/CT performs similarly to CT alone and hence adds no benefit over the current primary diagnostic tools in diagnosing pancreatic cancer[52].

Though the value of PET/CT alone for diagnosing pancreatic cancer has not been shown to be better, some studies have investigated its combined use with other techniques. A meta-analysis has shown that the combination of PET/CT plus endoscopic ultrasonography is useful for suspected pancreatic cancer because of the high sensitivity of PET/CT and the high specificity of endoscopic ultrasonography[53]. While initially it was hoped that PET/CT will be able to differentiate between mass-forming chronic pancreatitis and pancreatic cancer, this is not the case due to considerable overlap between the Standardised Uptake Value (SUVmax) values of these two diseases[54]. FDG PET/CT has been shown to provide additional benefit in detecting distant metastasis, particularly bone metastasis[55].

PET/CT shows promising role in assessing tumour response to chemo-radiation therapy with the measurement of the change in SUV pre- and post- treatment, which could potentially serve as a trial for preoperative neoadjuvant therapies[56,57].

In conclusion, at the present stage, PET/CT has no role in routine diagnosis of pancreatic cancer but can be used as an adjuvant modality in selected cases.

ULTRASOUND AND ENDOSCOPIC RETROGRADE CHOLANGIOPANCREATOGRAPHY

The pancreas is a retroperitoneal organ and hence the sensitivity of transabdominal ultrasound is poor in detecting pancreatic cancer and is not used in diagnosis or staging of pancreatic cancer[58]. The sensitivity according to studies vary between 48% and 89% with lower specificity and accuracy, with variation in these rates with the size of the tumour and operator's level of experience[59].

Given the excellent modern imaging, Endoscopic retrograde cholangiopancreatography (ERCP) plays a less prominent role in diagnosis of pancreatic cancer[60], and is mainly used as a therapeutic modality due to potential complications such as pancreatitis and perforation[61]. ERCP remains an important modality to provide biliary drainage in obstructing head of the pancreas cancer and can provide biliary and pancreatic duct brushing cytology in patients with invasive pancreatic cancer[62]. Pancreatogram obtained during the ERCP can show pancreatic duct stenosis, obstruction, narrowing and abnormal branching of the main pancreatic duct, obstruction and encasement of the common bile duct. There are few studies that looked at ways to attain cytological samples during ERCP through the use of an endoscopic naso-pancreatic drainage (ENPD) tube which is placed in the main pancreatic duct to collect pancreatic juice repeatedly - a technique known as serial pancreatic-juice aspiration cytologic examination or "SPACE"[63]. Only small-scale studies have examined the use of this technique with relatively promising results[63-65], but more research is required prior to recommendation of its use.

BIOMARKERS

At present, there is no reliable diagnostic biomarker for pancreatic cancer. A number of potential tumour markers have been evaluated, but the most extensively studied for diagnosing pancreatic cancer is carbohydrate antigen 19-9 (CA 19-9). CA 19-9 is however expressed and shed in a number of pancreatic and hepatobiliary diseases, as well as other malignancies. CA 19-9 may be falsely positive in cases of biliary infection, inflammation, or obstruction (regardless of aetiology) and does not necessarily indicate cancer or advanced disease[66]. For these reasons, it performs poorly as a screening tool, with a low positive predictive value of 0.5% to 0.9%[66]. However, CA 19-9 does have a role as a prognostic marker and for monitoring for recurrence after resection[9]. It performs better in symptomatic patients, with a sensitivity and specificity of 79% to 81% and 82% to 90% respectively for the diagnosis of pancreatic cancer in this setting[67,68]; with a CA 19-9 serum level of 100 U/mL suggestive of unresectability or metastatic disease[67]. As well as its issues with specificity, CA 19-9 sensitivity is also suboptimal; for example, CA 19-9 may be undetectable in Lewis antigen-negative individuals and hence can be negative in patients with advanced cancer.

There are a number of potential pancreatic cancer biomarkers that are being investigated. in particular, serum macrophage inhibitory cytokine 1 (MIC-1) is a promising biomarker whose levels in the serum are typically elevated in patients with pancreatic adenocarcinoma. Though performing sub-optimally when used on its own, it has been shown to produce improved diagnostic accuracy when combined with CA 19-9[69]. Other studies have also studied single research biomarkers such as CECAM-1, Span-1, DUPAN-2, Alpha4GnT, PAM4, and combined biomarkers with CEA, CA 19-9, and CA 242[70,71], but none demonstrating sufficient diagnostic accuracy to be used as a screening test at this stage.

More recently, a combined panel of protein and microRNAs serum exome for pancreatic cancer have emerged as potential diagnostic tools with improved sensitivities and specificities but have yet to have testing

within larger cohorts[72]. There has also been early research reviewing the use of inorganic nanomaterials such as gold and carbon nanotubes which can be targeted towards specific pancreatic cancer cells, in early detection and diagnosis[73].

SCREENING PROGRAMS

Pancreatic cancer screening is not feasible in the general population due to the low incidence of pancreatic cancer and lack of a cheap, easy and accurate screening test. However, approximately 5% to 10% of pancreatic cancers are due to a known genetic mutation and/or have familial aggregation. As pancreatic cancer patients become symptomatic later in the course of the disease, early detection programs have been developed in asymptomatic people at high risk of pancreatic cancer (individuals with a 5% or more lifetime risk of pancreatic cancer). The high-risk groups include familial pancreatic cancer (members of a family with at least 2 first degree relatives with pancreatic cancer) and inherited pancreatic syndromes including Peutz-Jeghers syndrome (lifetime risk of pancreatic cancer 36%), familial atypical multiple mole melanoma syndrome (lifetime risk 17%), hereditary pancreatitis (lifetime risk 49%), PALB2 mutation, known BRCA2 carrier with a first degree with pancreatic cancer, Lynch syndrome with a first degree with pancreatic cancer[74]. In these high risk groups, the International Cancer of the Pancreas (CAPS) Consortium recommends starting screening at age 50, with yearly surveillance if no pancreatic lesions are detected at baseline assessment[75]. EUS and MRI are the imaging modalities of choice for screening as they have sufficient sensitivities and specificities to detect small lesions (or early cancer) and do not carry the risks of radiation exposure. In these high risk groups, the overall yield for detecting premalignant and malignant lesions using EUS is 20% and using MRI/MRCP is 14%[76]. EUS performs better for small solid lesions and MRI for cystic lesions. The current data from prospective observational studies indicate that the diagnostic yield of neoplastic pancreatic lesions varies significantly, depending if pre-cancerous lesions (such as cysts, branch duct IPMN, main duct IPMN) are included or not in the analysis, the screening modality and the target population, being between 5% to 43%, whereas the detection rate for pancreatic cancer is 2%. These data are consistent with the findings from a recent systematic review of 542 high-risk individuals screened[76]. Currently, screening programs are recommended to be conducted only by experienced clinicians in a research setting with prospective data collection and close international collaboration.

PERILS, PITFALLS AND SUBTLETIES IN THE DIAGNOSIS OF PANCREATIC CANCER

With the use of multimodal imaging techniques and tissue acquisition as described above, a definitive diagnosis of pancreatic cancer can be made in the majority of patients when suspicion arises. Nevertheless, there are a number of situations where diagnostic findings are difficult to distinguish from other benign conditions affecting the pancreas. Accurate diagnosis in these settings is crucial given the disparate therapeutic implications, and generally relies on identifying and recognising radiological or endoscopic subtleties, emphasising the importance of close collaboration with expert centres.

Focal chronic pancreatitis

Focal chronic pancreatitis is a common mimicker of pancreatic cancer. It can form a focal mass and subsequently can cause pancreatic and biliary ductal obstruction which may be indistinguishable in appearance to that caused by ductal adenocarcinoma[14]. Standard imaging techniques including CT, MRI can be inconclusive to distinguish the two in selected cases. Depending on the degree of inflammation and fibrosis, CE-EUS and elastography could help distinguish between pancreatic adenocarcinoma and pseudotumoural chronic pancreatitis. In these cases, EUS guided biopsy is important and very close monitoring is recommended in biopsy negative cases.

Autoimmune pancreatitis

Autoimmune pancreatitis clinically can present in a similar fashion to pancreatic cancer; both most often occurring in older persons typically aged over 60 years and presenting as painless jaundice, new-onset diabetes mellitus, and raised levels of serum tumour markers[77]. Serum IgG4 is frequently increased in autoimmune pancreatitis, but occasionally can be mildly raised in 4% to 7% of pancreatic cancers[78]. However, the specificity of IgG4 to autoimmune pancreatitis is strong, especially when the serum IgG4 level is significantly raised to at least twice the upper limit of normal[79]. Typical CT findings for autoimmune pancreatitis include a smooth, diffusely enlarged homogenous gland with delayed enhancement and capsule-like rim[78] as seen in Figure 5. However, autoimmune pancreatitis can also appear as a mass on CT if there is focal involvement[14]. PET/CT with 18FDG has been shown to help differentiate these two diseases, with diffuse pancreatic uptake of FDG and concomitant uptake by salivary glands more suggestive of autoimmune pancreatitis[80]. Histopathologic evidence from a biopsy via EUS-FNB can produce the most definitive confirmation by demonstrating typical features of autoimmune pancreatitis such as lympho-plasmacytic sclerosing pancreatitis, abundant IgG4 positive cells, idiopathic duct centric pancreatitis and/or granulocyte epithelial lesion in the pancreatic duct[81]. IgG4 staining of the ampulla biopsy is also suggestive of autoimmune pancreatitis. Finally, autoimmune pancreatitis is usually sensitive to treatment with steroids, so a positive therapeutic trial can be helpful in excluding pancreatic cancer in equivocal cases[77].

Solid pseudopapillary neoplasm of the pancreas

Solid pseudopapillary neoplasm (SPN) is a rare indolent

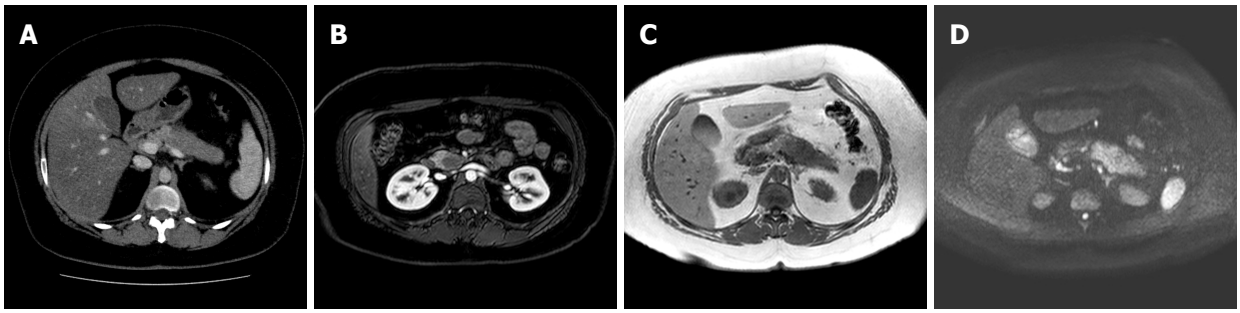

Figure 5 Multimodal imaging techniques demonstrating autoimmune pancreatitis in a patient. A: Diffuse enlargement "sausage shape" of the tail axial view on CT; B: Head of the pancreas axial view in arterial phase on MRI; C: Tail of the pancreas T1-weighted axial view on MRI; D: Homogenous restricted diffusion on DWI axial view MRI. CT: Computed tomography; MRI: Magnetic resonance imaging; DWI: Diffusion-weighted imaging.

neoplasm that has a low malignant potential and can be cured with resection but can be difficult to differentiate radiologically from pancreatic adenocarcinoma[82]. SPN usually occurs in younger women and is located in the tail of the pancreas. MRI is better than CT in detecting this tumour, with typical findings of an encapsulated mass with solid and cystic components, as well as haemorrhage without an obvious internal septum[81]. The typical EUS appearance is of mixed solid cystic lesion with a median tumour size of 4.2 cm but sometimes it can present as a solid mass. The diagnostic yield of CT alone is 23%, EUS is 41% with a combined diagnostic yield of 52%. EUS FNA significantly increased the diagnostic yield to 82%[83].

It is also important to not incorrectly diagnose adenocarcinoma in patients with SPN as there is a 5-year survival rate of 96.9% post resection for SPN regardless of the size of the tumour[82].

Annular pancreas

Annular pancreas is a rare congenital migratory abnormality, with a reported incidence rate of up to 1 in 1000, and is due to incomplete rotation of the ventral anlage around the duodenum that leads to the pancreas encircling the second part of the duodenum[84]. These patients are asymptomatic and it is usually an incidental finding on CT or MRI. An experienced radiologist should be able to distinguish an annular pancreas from a pancreatic mass, as a normal enhancing pancreas and pancreatic duct encircling the second part of the duodenum.

Pancreatic lipomatosis

Sometimes fatty replacement of the anterior portion of the pancreatic head is seen in diabetes, obese or elderly people. This can mimic a hypodense mass on CT, however an MRI with in and out phases can exclude the presence of a true mass by showing the presence of intracellular fat[85].

PERSPECTIVES AND FUTURE DIRECTIONS

Future research should focus on improving outcomes in pancreatic cancer through the development of new diagnostic techniques with higher diagnostic accuracy. We should also aim to develop better tools to assess risk for developing cancer thereby facilitating better targeting of screening programs and better selection of patients for surgery.

Apart from those already discussed, examples of other promising novel diagnostic techniques that are under research include needle based confocal laser endomicroscopy (n-CLE), where a probe is passed through a 19-gauge EUS needle for real-time visualization of the tissue at the microscopic level in the pancreatic cysts, thus providing an optical biopsy[86]. Similarly, probe based confocal laser endomicroscopy (p-CLE) can be used during an ERCP for indeterminate pancreato-biliary stricture[87].

An ideal FNB needle design has not been found yet. A recent study showed that fork tip needle had a higher histologic yield than bevel needle but further studies are needed to compare all types of FNB needles[88]. Obtaining adequate histological samples of the tumour during the EUS is very attractive, as it can lead to enough samples for DNA extraction, comprehensive whole exome sequencing and next generation sequencing (NGS) of the pancreatic tumour. A large amount of DNA will facilitate preoperative genomic profiling and chemotherapy testing and will play a role in individualised cancer treatment.

Mutation of the KRAS oncogene is present in 75% to 95% of pancreatic cancer tissues. Combining EUS-FNA cytology with *KRAS* mutation analysis on the biopsy material can increase the pancreatic cancer accuracy from 85% to 94%[89]. This study shows promising results particularly as EUS-FNB needles will continue to improve and more material is obtained during the biopsy.

Detection of *TP53* mutations in secretin-stimulated pancreatic juice samples collected from the duodenum of the patients with high grade dysplasia and pancreatic cancer[90] opens a new area of future research in diagnosis and potential screening for early pancreatic cancer.

EUS guided sampling of portal venous blood for circulating tumour cells may enhance the ability to detect occult metastatic disease, allowing improved patient selection for surgery[91]. Advances in these fields will be most beneficial in improving the outcomes of patients with pancreatic cancer.

Whole genome sequencing of pancreatic adenocarcinoma has found chromosomal rearrangements leading to gene disruption and new candidate drivers in pancreatic carcinogenesis[92]. The development of focus panel testing for pancreatic cancer is already underway which will potentially allow tumour subtyping, and may aid in the development of tumour specific targeted therapies[93]. These and other advances in genetic understanding, including the identification of several microRNAs involved in regulation of aberrant cell replication, render them potential biomarkers for diagnosis and prognosis[94].

CONCLUSION

While the early diagnosis of pancreatic cancer remains challenging, improvements in diagnostic technology and methodologies in the last decade will hopefully translate into improved outcomes. Screening of high-risk individuals using EUS and/or MRI is recommended and shows promise in early detection. In patients with suspected pancreatic cancer we propose the use of CT or MRI as first-line investigations, with the choice between the two being determined by cost, availability and local expertise. Such cross-sectional imaging modalities remain the gold standard for staging, both of the primary lesion and detection of distal metastases. EUS has become a powerful diagnostic modality and should be used in adjunct, being superior in the detection of small lesions and having the ability to obtain a tissue diagnosis. While research is ongoing, at present there is no role for the use of any routine biomarker in the diagnosis of pancreatic cancer. Atypical cases can occur and differentiation of malignant from benign pancreatic lesions can be challenging; in these cases, opinion from a radiologist with pancreato-biliary expertise should be sought.

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REVIEW

Biliary strictures complicating living donor liver transplantation: Problems, novel insights and solutions

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Abstract

Biliary stricture complicating living donor liver transplantation (LDLT) is a relatively common complication, occurring in most transplant centres across the world. Cases of biliary strictures are more common in LDLT than in deceased donor liver transplantation. Endoscopic management is the mainstay for biliary strictures complicating LDLT and includes endoscopic retrograde cholangiography, sphincterotomy and stent placement (with or without balloon dilatation). The efficacy and safety profiles as well as outcomes of endoscopic management of biliary strictures complicating LDLT is an area that needs to be viewed in isolation, owing to its unique set of problems and attending complications; as such, it merits a tailored approach, which is yet to be well established. The diagnostic criteria applied to these strictures are not uniform and are over-reliant on imaging studies showing an anastomotic narrowing. It has to be kept in mind that in the setting of LDLT, a subjective anastomotic narrowing is present in most cases due to a mismatch in ductal diameters. However, whether this narrowing results in a functionally significant narrowing is a question that needs further study. In addition, wide variation in the endotherapy protocols practised in most centres makes it difficult to interpret the results and hampers our understanding of this topic. The outcome definition for endotherapy is also heterogenous and needs to be standardised to allow for comparison of data in this regard and establish a clinical practice guideline. There have been multiple studies in this area in the last 2 years, with novel findings that have provided solutions to some of these issues. This review endeavours to incorporate these new findings into the wider understanding of endotherapy for biliary strictures complicating LDLT, with specific emphasis on diagnosis of strictures in the LDLT

setting, endotherapy protocols and outcome definitions. An attempt is made to present the best management options currently available as well as directions for future research in the area.

Key words: Biliary strictures; Endoscopic management; Stenting; Self-expanding metal stents; Living donor liver transplantation

Core tip: Multiple lacunae exist in our current understanding of biliary strictures complicating living donor liver transplantation (LDLT). Although endoscopic management is regarded the mainstay of treatment, results are variable with multiple determinants of success. The definition of these strictures itself may need to be re-examined, from a mere narrowing on imaging studies to a more comprehensive approach which can signify functional impedance to bile flow. This review outlines the current practices of management and endeavours to incorporate novel concepts, such as functionally significant obstructions, endoscopic protocols and outcome definitions, into the wider understanding of endoscopic management for biliary strictures complicating LDLT.

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INTRODUCTION

Biliary stricture complicating living donor liver transplantation (LDLT) is a relatively common complication, occurring in most transplant centres across the world. Improvements in technique, better postoperative care, surgical expertise and immunosuppressive medications have resulted in a decreased incidence of biliary complications over the years[1-3]. The Roux en Y hepaticojejunostomy has given way to duct-to-duct anastomosis (DD) which is the preferred mode of biliary reconstruction in most cases[4-8]. DD is technically easy, enables rapid gastrointestinal recovery, has a lower risk of cholangitis and maintains physiological choledochoenteric continuity[2]. Moreover, DD allows an easy endoscopic access to the anastomosis, enabling endotherapy in most cases. Thus, surgical management of biliary strictures following LDLT has slowly been replaced by endoscopic retrograde cholangiography (ERC) with stent placement, which emerged as the first-line therapy during the last few decades[1-3,9-11].

Compared to deceased donor liver transplantation (DDLT), the biliary anastomosis in LDLT is more peripheral, smaller and complex[12,13], resulting in tortuous and angulated strictures, especially due to hypertrophy of the transplanted liver[14]. As a result, the efficacy and safety profiles as well as the outcomes of endoscopic management of biliary strictures complicating LDLT represents an area that needs to be viewed in isolation, owing to its unique set of problems and attending complications; it merits a tailored approach, which is yet to be well established.

This review focuses on the pathogenesis, morphology and diagnosis of the endoscopic management of biliary strictures complicating LDLT, as well as some unique problems associated with it. An attempt is made to present the best current management options available as well as rational perspectives on future research in the area.

BILE DUCT STRICTURES: LDLT *vs* DDLT

LDLT as a treatment for end-stage liver disease is especially popular in most Asian countries, owing to a difficulty in organ procurement from deceased donors[15-17]. However, DDLT constitutes the majority of transplants in the West, being performed within the framework of a uniform, systematic organ allocation programme along with better organ procurement from deceased donors, that ensures shorter waiting periods and allows for the use of DDLT as a standard-of-care for end-stage liver disease. As is apparent, this is predicated on a higher number of deceased donors, early referral for liver transplantation, requisite infrastructure and specialised man-power for fair and efficient organ allotment; a lack thereof is palpable in Asia, paving the way for robust LDLT programmes, especially in far eastern countries like those in Japan, Hong Kong, China and Korea[17-19].

While the principles of endoscopic therapy for biliary strictures complicating DDLT are similar to those that occur with LDLT, there seems to be some differences in the incidence, morphology and type of strictures, which can be summarised as follows.

Incidence

Bile duct strictures are the most common biliary complication after liver transplantation, accounting for approximately 40% of all biliary complications[7,12,20-23]. In general, biliary strictures have been reported to occur in up to 5% of DDLT cases[24,25]. On the other hand, those of LDLT have been reported to occur more frequently: 7.3%-60.0% in right-lobe grafts[4,12,26-28] and 24% in left lateral segment grafts[29]. In a study by Gomez *et al*[30], biliary strictures occurred more frequently following LDLT than in DDLT [10/30 (33.3%) *vs* 27/357 (7.6%), respectively].

Morphology

Owing to the use of a smaller graft in LDLT (as compared

Table 1 Incidence of biliary strictures with median time of onset after living donor liver transplantation

Study	Year	Total number, *n*	Biliary strictures, %	Median time of onset after LT in mo
Yazumi *et al*[13]	2006	273	27	6.2
Seo *et al*[43]	2009	239	12	8.6
Chang *et al*[22]	2010	353	32	-
Hsieh *et al*[11]	2013	110	37	2.1
Wadhawan *et al*[93]	2013	338	10	3
Ranjan *et al*[94]	2016	305	3	12
Rao *et al*[31]	2017	458	10	-

LT: Liver transplantation.

to the whole liver in DDLT), the calibre of the bile ducts used for the anastomosis is smaller, often having a disparity between the recipient and donor ducts. Surgical anastomosis in these cases demand technical expertise and experience in working with small ducts in order to create an anastomosis that is free of tension and has an adequate luminal diameter to enable free flow of bile. As a result, strictures developing in this situation will have a unique morphology and sometimes bizarre configurations. Attempts have been made to classify these strictures based on cholangiographic appearance, size and number of ducts, all of which may have bearing on the outcome of endoscopic management[31].

Aetiology and type of stricture

Posttransplant biliary strictures can be classified as anastomotic strictures (AS) and nonanastomotic strictures (NAS). AS is more common in LDLT patients than in DDLT patients. This system of classification as AS or NAS is extremely useful as clinical outcomes and treatment modalities of the two types are somewhat different[1]. AS account for about 80% of biliary strictures after LDLT[32]. Factors contributing to the development of AS include technical problems of the biliary anastomosis, ischemia or bile leaks[33]. Although the effect of ischemia is far more pronounced in NAS, it might also play a significant role in AS. AS usually present later in the course of the posttransplant recovery[34], with a median interval of 5-8 mo after the liver transplantation[20,35,36]. It usually is a single, sometimes tortuous stricture, at or within 5 mm of the anastomotic site[33].

NAS, on the other hand, usually results from hepatic artery injury or thrombosis, causing irreversible biliary fibrosis due to ischemia[1]. Other causes which have been implicated include long cold ischemia time or ABO type incompatibility[33,37]. Of all biliary strictures after liver transplantations, NAS accounts for only 10%-25%[11,13,22]. In a Japanese study, NAS accounted for only 6% of all biliary strictures and occurred in 5 of the 273 LDLT patients (2%)[13]. In another study with 339 patients undergoing right lobe LDLT, only 11 patients (10%) developed NAS, while 121 patients (36%) developed AS, accounting for more than 90% of the biliary strictures in that patient cohort[22]. The Mayo Clinic Hospital in Arizona reported only 3 of 110 LDLT patients (3%) developing ischemic-type strictures[11].

BILIARY STRICTURES COMPLICATING LDLT

Incidence and pathophysiology

Biliary complications continue to be the most common complication after LDLT, having a reported incidence of 20%-43%, with biliary strictures accounting for 3%-40% of cases. Most biliary strictures present in the first year following the liver transplantation[9,22,36,38,39] (Table 1).

With increasing experience in LDLT, in most Asian countries there has been a decrease in overall incidence of biliary complications, from 30% in the initial years to around 15%-25% in recent years[3,6,20,25,31,40]. High-volume LDLT centres in Tokyo and Hong Kong, having extensive experience in management of biliary strictures, report a relatively steady rate of biliary strictures, of 20%-25%[8,41]. In an elegant study by Morioka *et al*[21] from the Kyoto University, LDLT recipients were divided into three time cohorts which differed with regard to case experience. They found that while the rate of bile leak significantly decreased with experience, there were no differences in the development of biliary strictures among the three groups (19%, 28% and 26% in the three groups; $P = 0.290$). In a similar study from the University of Toronto, Shah and colleagues[20] did not find any difference in the rate of biliary strictures after chronological stratification of their patient cohort into the first 65 cases and the next 65 cases. However, a recent study by Kim *et al*[36] did show that patients who underwent more recent procedures were less likely to have biliary strictures, suggesting a small but important role of surgical expertise on the risk of biliary strictures.

There seems to be several possible factors other than poor surgical expertise that result in bile duct injury and subsequent strictures. Among these, an important cause of strictures is postoperative bile leak that causes local inflammation and fibrosis[23,38,42,43]. Other factors identified include older donor age[20], preoperative model of end-stage liver disease score[40,43], multiple biliary ducts[43], graft cold ischemia time[41,44], bile duct diameter[44,45], acute cellular rejection[41] and hepatic artery thrombosis (HAT). As evident, most of these factors lead to ischemic injury of the bile duct, eventually culminating in strictures. While ischemia is a major factor in NAS, it also plays a major role in the development of

AS. This is because the bile duct epithelium is particularly vulnerable to ischemia, as compared to hepatocytes or vascular endothelium[46-48]. Local ischemic insults can also arise from devascularisation at the time of hilar dissection. Moreover, oxygen supply to the biliary tract is solely supplied by the hepatic artery. Therefore, HAT can lead to severe ischemic stress on the bile duct, resulting in fibrosis and strictures. HAT is a major determinant of NAS[49] and complex, long AS[46] that are usually difficult to manage endoscopically. In addition, technical factors like the number of biliary anastomosis, discrepancy in ductal diameters, unavailability of biliary microsurgical modalities, and aberrant biliary anatomy have been found to be significant risk factors for the development of biliary strictures[1,46,50,51] Moreover, endoscopic management is particularly affected in patients with multiple biliary anastomoses[31].

Multiple causative factors with intersecting and often overlapping pathophysiological pathways make it impossible to implicate a single factor that can predictably lead to stricture formation. A useful approach would be to aggregate individual factors as a composite score for prognostication, early diagnosis and treatment.

Diagnostic challenges

The most common clinical presentation of biliary strictures complicating LDLT is a cholestatic pattern of liver function test results, with or without symptoms of obstructive jaundice, such as icterus or pruritus. However, other causes of cholestatic jaundice, such as graft rejection (acute or chronic), recurrence of primary disease (hepatitis B/C), fibrosing cholestatic hepatitis C, sepsis or drug-induced cholestasis[46], make the diagnosis of biliary strictures particularly challenging[52]. A high index of suspicion is warranted in such patients, especially since they rarely have abdominal pain due to hepatic denervation and immunosuppressants[53-55]. The diagnostic protocol practised in most centres involves a composite approach with clinical features, laboratory evaluation, imaging studies and liver biopsy.

Among the factors detected by laboratory tests, bilirubin, alkaline phosphatase, gamma-glutamyl transferase and the transaminases are assessed initially. However, these tests are nonspecific, with limited diagnostic role for differentiating a biliary cause from a hepatocellular aetiology for the symptoms. Liver biopsy is extremely valuable to differentiate biliary strictures from acute cellular rejection, but since it is an invasive procedure with possible complications, it should be reserved for cases with a high degree of suspicion for rejection.

Imaging studies

Currently, the diagnosis of biliary strictures depends heavily on cross-sectional imaging, which classically demonstrates an anastomotic narrowing. Initial imaging with ultrasonography of the abdomen with Doppler study is carried out to examine the intrahepatic biliary radicles, along with vascular patency. However, the sensitivity of ultrasonography to detect biliary dilatation in the posttransplant patient is as low as 38%-66%, thereby rendering it unreliable as a sole screening modality for the diagnosis of biliary strictures[56-58]. A scan by hepatobiliary iminodiacetic acid scintigraphy is excellent for detecting bile leaks[59]; however, its role in detecting biliary strictures is poor, with a sensitivity of around 60%[59,60]. Computed tomography is useful for detection of fluid collections and non-biliary lesions, having a higher spatial resolution than magnetic resonance imaging[61]. However, it is grossly inadequate for the diagnosis of biliary strictures, unless used in conjunction with the injection of biliary contrast agents, like iodipamide meglumine. These contrast agents have been found to be unsafe, with higher incidence of allergic reactions; as such, the use of computed tomography for biliary strictures has fallen out of favour in most centres.

Magnetic resonance cholangiography (MRC) has emerged as a reliable noninvasive tool to detect biliary strictures complicating LDLT. The presence of bile within the ducts allows for accurate delineation of the biliary tree, which can highlight the anastomotic stricture with diagnostic sensitivity and specificity in excess of 90%[62-64]. MRC can provide detailed imaging of the entire biliary tract and is especially useful for complex and intrahepatic strictures. Moreover, it can provide a roadmap for therapeutic interventions. MRC has a high negative predictive value that prevents unnecessary procedures[65,66]. The limitations of MRC include lack of interventional capability and high cost as a routine diagnostic modality. It has also been found to have a high rate of false positives.

Most LDLT recipients with DD can show a narrowing of the anastomotic site in the absence of a 'functionally significant obstruction' (FSO). Here, FSO is defined as a critical obstruction at the anastomotic site that results in a clinically significant impedance to bile flow, evidenced by clinical symptoms and biochemical alterations of cholestasis. In our centre, a unique set of metrics are applied in the MRC to indicate FSO. While most literature alludes to a dilated proximal duct and a narrowing as pathognomonic of a stricture[3], we have found it useful to use the ratio of the proximal duct diameter to the recipient ductal diameter to signify FSO. This approach is based on the hypothesis that a FSO should cause impedance of bile flow that will result in the hold-up of bile above the stricture, causing dilatation and higher diameter on MRC, along with collapse of the recipient bile duct below the anastomosis, due to lack of bile flow across the stricture resulting in a smaller ductal diameter on the MRC. The resultant ratio may provide valuable clues as to the severity of obstruction at the anastomosis and enable a singular, reproducible definition of biliary strictures that can potentially dictate further treatment with better outcomes. The management approach practised in our centre using

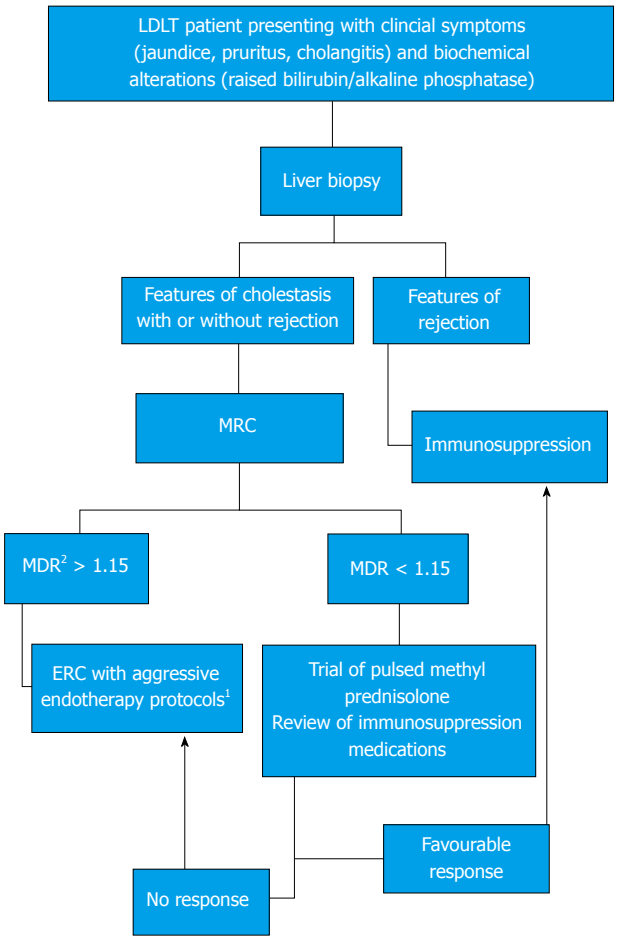

Figure 1 Management algorithm used in our centre for the treatment of biliary strictures. [1]Aggressive endotherapy protocols: Multiple ERC with stricture dilatation and gradual upsizing of stents done every 3 mo for a minimum period of 1 yr; [2]MDR = MRC ductal ratio calculated as maximum diameter of the recipient hepatic duct divided by the maximum diameter of the donor bile duct as seen on an MRC done at the time of presentation with clinical symptoms and biochemical alterations. The cut-off of 1.15 was computed after an internal review of our patient data showed a sensitivity and specificity of > 90% for the diagnosis of a functionally significant stricture (unpublished data). ERC: Endoscopic retrograde cholangiography; LDLT: Living donor liver transplant; MRC: Magnetic resonance cholangiography.

this method of diagnosing biliary strictures complicating LDLT is shown in Figure 1. However, prospective trials in multicentric populations are required before widespread application of this definition in the management protocol for biliary strictures.

Endoscopic management (endotherapy)

Endoscopic management (endotherapy) is usually the first line of treatment for biliary strictures complicating LDLT and includes ERC with biliary sphincterotomy, balloon dilatation and stent placement[11,30,31]. Endotherapy is the mainstay of treatment and is usually the only avenue outside of surgery used in a majority of patients. However, endotherapy in LDLT recipients may be difficult because of complex strictures with multiple ductal anastomoses in bizarre configurations[1]. It is a point of contention whether balloon dilation in isolation or balloon dilation with stent placement is superior for the treatment of biliary strictures. A higher complication rate notwithstanding, stent placement after balloon dilation should be the standard of care in these patients[67,68]. Moreover, endotherapy has the advantage over percutaneous transhepatic cholangiography because it enables the placement of multiple large-calibre stents, and is more physiological and less invasive.

Who will benefit from endotherapy?

Various studies have tried to identify factors influencing the outcome of endoscopic management in postLDLT biliary strictures (Table 2). The detection of these factors would help in streamlining the decision-making process and ideal patient selection for endotherapy. Donor characteristics, and pretransplant and posttransplant factors have been found to influence the endoscopic outcomes[9,22,31,43,69]. Chang *et al*[22] reported an overall success rate of only 48%, especially for patients with NAS (P = 0.016) and strictures associated with posttransplant hepatic artery stenosis/thrombosis (P = 0.016). Kim *et al*[70] reported that only the interval between LDLT and the first ERC had an effect on the outcome with endotherapy and, contrary to other reports, that longer stricture duration had a better outcome (P = 0.041). Swan neck deformity in right-lobe LDLT was also seen as a risk factor for difficult endoscopic procedure as well as poorer outcomes. Finally, bile leaks were found to be one of the most important risk factors for stricture formation as well as poorer stricture resolution with endotherapy[70]. Kato *et al*[31] reported a 51% overall rate of stricture resolution in postLDLT biliary strictures with DD and noted bile leaks as the major cause of stent failure[9]. Our previous study showed HAT, multiple biliary anastomosis and older donor age to be significant predictors of poor outcome to endotherapy.

A paucity of conclusive evidence in the background of heterogenous patient populations and endoscopic management protocols make it difficult to identify a subgroup of patients who will respond to endotherapy in a predictable fashion. In this regard, uniform criteria for diagnosis of biliary strictures as FSO may aid in identifying patients with a significant stricture amenable to endotherapy, an approach that needs further validation.

When to intervene?

The timing of intervention has been an area that has lacked adequate validation. Usually, ERC in the first few weeks after LT is considered dangerous, due to the risk of damage to the anastomosis. This, however, is more applicable for bile leaks, as opposed to strictures since the development of strictures usually takes a long time. It is important to note that strictures that develop in the first 6 mo are more likely to respond to endotherapy than strictures that present late[20,69].

A subgroup of patients that merit further study in-

cludes those who present with asymptomatic biochemical alterations, liver biopsy inconclusive of rejection and imaging studies showing an anastomotic narrowing. The question of whether to wait it out or subject the patient to aggressive endotherapy protocols is a particularly harrowing one. On the one hand, there is considerable data which have demonstrated that a delay in treatment may be a significant determinant of poor response to endotherapy[71,72]. In a study by Buxbaum *et al*[69] it was noted that a longer waiting time for ERCP was related with a greater likelihood of failure with endotherapy. However, the quantitative finding did not reach statistical significance. In our previous report, we found a similar inverse relationship between time to ERC and endoscopic success, but again the finding was not statistically significant[69]. However, in the study by Shah *et al*[20], a similar patient subgroup was kept under follow-up with close observation and most were found to be stricture-free during the long-term follow-up.

It would seem plausible that a delay in ERC with stent placement should lead to a suboptimal success owing to establishment and organisation of the fibrosis; however, the identification of strictures that merit treatment, as opposed to those that can be managed conservatively, warrants further study. Again, in this regard, the concept of FSO alluded to earlier may provide some valuable insights. Yet, larger studies exploring the accurate definition of FSO are needed before any recommendations are made, and current practice of early ERC with stent placement after balloon dilation should be continued until proven otherwise.

Protocol for endoscopic management of strictures

Ideal endoscopic management protocols include ERC with endoscopic sphincterotomy, balloon dilatation and stent placement. Repeated ERC at 2- to 3-mo intervals with up-sizing of the stent or the use of multiple stents with or without repeated balloon dilation for at least 1 year is the most preferred protocol across transplant centres globally[2,3]. However, the need for balloon dilation of the strictures prior to the placement of endoprosthesis is not universally accepted, with some endoscopists opting for stent placement alone in the first ERC, followed by balloon dilations in subsequent ERC. Yet, there is no data that suggests superiority of this method over both balloon dilation and stent placement at the first ERC.

In 2003, Park *et al*[73] described an alternate protocol wherein patients without a significant narrowing on ERC are dilated with a balloon after sphincterotomy and a 5F nasobiliary catheter is placed above the stricture. This has many theoretical advantages in that it is easy to perform a cholangiogram to assess improvement of the stricture and obtain bile samples for culture; thus, occlusion of the drain is readily diagnosed and does not necessitate a repeat endoscopy to remove the catheter[73]. However, it is not a widespread endoscopic protocol because of reduced patient acceptance with no uniform data on therapeutic superiority. An ideal approach to this decision should involve careful consideration of the time interval between the ERC and liver transplantation, the ductal diameters and length of the stricture before coming to a decision on the use of both balloon dilation and stent therapy.

The need for endoscopic sphincterotomy has also been examined, with a few authors proposing stent placement across an intact sphincter of Oddi to reduce the incidence of cholangitis and pancreatitis[13,74]. However, there is no data corroborating this perceived risk of cholangitis. Moreover, we have found that the incidence of postERC pancreatitis is lower in liver transplantation patients than in the general patient population - a finding that was also observed in a multicentric study by Law and colleagues[75]. This can be due to as yet unidentified effects of immunosuppressants on the inflammatory cascade that drives post ERC pancreatitis, representing an area that needs further study.

The major disadvantage of endoscopic therapy is the necessity for frequent procedures required for stent exchanges, which may increase complications in addition to increased cost of care. Stent exchanges are usually performed every 2-3 mo to avoid stent occlusion and cholangitis. Most of the studies in this regard have been on DDLT patients, but it seems reasonable to apply the same protocol in LDLT patients. Few studies[76] have performed stent exchanges and dilation every 2 wk and shown a superior success rate. Tabibian *et al*[77] showed that a stent therapy protocol wherein stent exchanges are performed only once the patient is symptomatic is superior to protocol-driven procedures. However, it would seem pragmatic to exchange the stents every 3 mo for a minimum of four exchanges over a 1 year period and give a stent-free trial provided the last cholangiogram shows stricture resolution.

Some Japanese groups advocate the placement of 'inside stents', whereby the plastic stent is completely in the bile duct with an intact sphincter, reducing the duodenobiliary reflux and consequently preventing cholangitis[13,14,74,78]. This is postulated to improve stent patency rates, with fewer ERC requirements and lower incidence of cholangitis as it prevents duodenobiliary reflux. The University of Kyoto reported use of a similar strategy, with modified Amsterdam stents placed inside the bile duct in 94/118 LDLT patients (80%) and yielding a stricture resolution rate of 68%[74]. Tsujino and colleagues[2] at the University of Tokyo, in as-yet unpublished data, have alluded to their experience in the area with 63 LDLT recipients who underwent inside stent placement. The median interval for stent exchange was as high as 161 d, with cumulative risk of stent dysfunction being 12.3% at 6 mo and 18.1% at 12 mo[2].

Another promising approach is the use of the self-expanding metal stent (SEMS). SEMS was introduced to overcome the need for repeated procedures in

Table 2 Therapeutic efficacy and factors affecting outcome of endotherapy in biliary strictures complicating living donor liver transplantation

Study	Year	Biliary strictures, *n* (%)	Median duration of stent therapy	Overall stricture resolution, %	Factors that affected endoscopic outcome
Zoepf *et al*[68]	2005	7 (7.78)	4 mo	100	
Yazumi *et al*[13]	2006	75 (27.5)	8.9 mo	33	
Gomez *et al*[30]	2009	10 (33.34)		20	
Seo *et al*[43]	2009	29 (12.1)	24.1 ± 12.7 wk	65	
Chang *et al*[22]	2010	113 (32)		48	NAS, HAT, Pretransplant TACE
Hsieh *et al*[11]	2013	41 (37.3)	5.3 mo	79	
Wadhawan *et al*[93]	2013	35 (10.4)		91	
Ranjan *et al*[94]	2016	10 (3.28)	4 mo	70	
Rao *et al*[31]	2017	41 (10.2)	6 mo	48	Donor age, multiple biliary anastomoses, duration of stent therapy
Kato *et al*[9]	2009	41 (42.7)	14.5 mo	85	Stricture length, bile leak

HAT: Hepatic artery thrombosis; NAS: Nonanastomotic strictures; TACE: Transarterial chemoembolization.

Table 3 Role of self-expanding metal stent in the management of post living donor liver transplantation biliary strictures

Study	Year	Patients who underwent cSEMS, *n*	Duration of stent therapy	Stricture resolution, %	Complications, %
Kaffes *et al*[87]	2014	10	12 wk	100	10
Jang *et al*[89]	2017	35	3.2 ± 1.4 mo	82.9	14.3
Kao *et al*[88][1]	2013	200	3 mo	80-95	16
Rao *et al*[2]	2018	4	8 mo		75

[1]This was a meta-analysis comparing SEMS with multiple plastic stent, and both DDLT and LDLT patients were included; [2]Unpublished data. cSEMS: Covered Self-expanding metal stent; DDLT: Dead donor liver transplantation; SEMS: Self-expanding metal stent.

conjunction with providing better and faster stricture resolution in these patients, owing to the larger diameter (30F) of the stents. The initial use of uncovered SEMS proved to be a disaster because of high rates of complications due to stent ingrowth and over-growth causing stent occlusion, formation of biliary sludge and stones within the stents, and more importantly vascular complications in the long term[79-82]. Covered (c)SEMS was subsequently introduced and seemed to counter most of the disadvantages of the uncovered SEMS (Table 3). The ease of endoscopic removal of cSEMS makes them an ideal treatment modality for benign biliary strictures.

Randomised controlled trials in DDLT patients comparing SEMS with multiple plastic stents have shown they are similar with respect to stricture resolution, but SEMS required fewer interventions compared to plastic stents[83,84]. A recent meta-analysis also showed no superiority of SEMS over multiple plastic stenting, but supported the use of SEMS owing to shorter treatment time and fewer procedures[85]. Very few reports have explored the role of SEMS in LDLT patients[86-88]. In an elegant study by Jang *et al*[89], a short fully-covered SEMS with a waist in the centre to prevent migration (Kaffes stent by Taewoong Medical, Seoul, South Korea) (Figure 2) was used in 35 LDLT patients with refractory strictures. The cases showed an excellent response rate, with 83% stricture resolution and 6% migration[89]. However, the safety and efficacy of SEMS in LDLT is yet to be understood. At our centre, cSEMS was deployed in 4 LDLT recipients with refractory biliary strictures. However, stent occlusion due to sludge and stones was seen in 3/4 patients (75%) (unpublished data), necessitating additional plastic stent placement inside the cSEMS.

The placement of cSEMS in high anastomotic strictures may also lead to blockage of secondary branches of the bile duct and subsequent biliary stasis in those segments. Tsujino and colleagues[71] at the University of Tokyo advocate the placement of inside stents along with covered SEMS to counter this problem, and this approach seems to hold promise pending future studies. A particularly distressing complication of cSEMS is stent migration, which is reported in 16%-33% of cases[83,88]. The rate of stent migration seems to be higher in LDLT patients than other patients with benign biliary strictures[90]. Finally, the placement of cSEMS may aid duodenobiliary reflux due to disruption of the sphincter mechanism and possibly result in ascending cholangitis.

Endoscopic management: Outcome

In order to discuss therapeutic efficacy, a uniform definition of biliary strictures has to be established. Most studies have used multiple criteria for endoscopic success, and these include symptomatic and/or biochemical improvement, imaging studies showing resolution of stricture, and absence of recurrence of cholestasis requi-

Figure 2 Fully-covered modified self-expanding metal stent used in the management of biliary strictures complicating Living donor liver transplantation. The Kaffes stent (Taewoong Medical, Seoul, South Korea) is shown.

ring endoscopic/percutaneous interventions[9,14,31,69,91]. It would seem beneficial to study these patients at two time points - one assessing the response after a protocol-based endotherapy practice for a minimum of 1 year, and a more long-term evaluation to assess rate of recurrence and need for repeat interventions. Both these treatment endpoint scans provide valuable insights on the course of AS and will help in establishing a uniform treatment protocol and follow-up.

In the study by Buxbaum *et al*[69], long-term success was evaluated as a treatment goal and a 68% success rate was found. In our previous report, stricture resolution at the end of 1 year was only 48%[31]. This low rate of stricture resolution may be due to a larger proportion of patients who had multiple biliary anastomoses, older donor age and/or a nonuniform endoscopy protocol applied for management. Future studies likely need to look at a composite outcome, combined with better patient selection criteria, which can yield uniform and predictable results of therapeutic efficacy for endotherapy.

In the event of technical failure of ERC, which is defined as inability to cross the stricture with a guidewire, a percutaneous transhepatic cholangiography with biliary drainage *via* a percutaneous catheter can be performed[70,73]. It is usually successful, especially in patients with cholangitis who need urgent biliary drainage. Percutaneous transhepatic cholangiography with drainage is still a second-line therapy, as it is invasive and carries risk of complications like bleeding, pseudoaneurysm of the hepatic artery, bile leaks, infection, arterioportal fistula and portal vein thrombosis[45,92]. Patient compliance is also poor, and it is usually combined with an ERC in a rendezvous procedure at our centre. Using this technique, we have been able to get across the stricture in most patients. All our patients who have undergone the rendezvous procedure had subsequent successful ERC with stent exchanges, and documented resolution of stricture. We found that it is safe and precludes surgery in most patients with a primary endoscopic failure.

NOVEL INSIGHTS AND AREAS OF FUTURE RESEARCH

As in any scientific work, the formulation of the problem is paramount for the development of a solution which more often than not is obvious to a trained clinician. To that end, the definition of biliary strictures in most studies is not uniform and needs to be re-examined. In the study by Buxbaum and colleagues[69], cholangiographic appearance of narrowing in a patient with altered liver functions were treated with endoscopy. In the study conducted at the University of Toronto, however, only symptomatic alterations of liver function tests in conjunction with a narrowing on imaging were considered for endoscopic management[20]. In our study on LDLT recipients, we also included asymptomatic patients with biochemical and imaging features of biliary obstruction[31]. This nonuniform definition is reflected in the varying success rates of endotherapy reported from these three studies (68%, 81% and 48% respectively).

A stricture that responds to endoscopic therapy should be regarded as a 'true' stricture, or more appropriately a FSO. This may provide a multidimensional definition of biliary strictures that has the potential to streamline patients into mutually exclusive treatment pathways and ensure therapeutic efficacy. The ratio between donor duct diameter and the recipient ductal diameter on the MRC in conjunction with a subjective assessment of contrast drainage across the stricture during the ERC to indirectly signify the functional impedance to bile flow of the stricture has been found to be useful in our centre, having a superior therapeutic efficacy of 83% in preliminary findings (unpublished data). This approach to seek out an obstruction that is functionally significant in a symptomatic patient may yield a patient group who will be predictably responsive to endotherapy.

However, it is important to understand that endotherapy may not be the answer for all strictures across the board. As Abraham H Maslow remarked - 'If the only tool you have is a hammer, it is tempting to treat everything as if it were a nail'; it is important to realise that certain patients with biliary strictures of an unfavourable morphology will not respond as well to endotherapy. The correct identification of the patients who will respond to endotherapy is the need of the hour. The development of innovative modalities, newer stent designs and endoscopic accessories needs to propel future work in this area, especially for those patients who are refractory to stent therapy.

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MINIREVIEWS

Role of osteoprotegerin/receptor activator of nuclear factor kappa B/receptor activator of nuclear factor kappa B ligand axis in nonalcoholic fatty liver disease

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Abstract

Concomitantly with the increase in the prevalences of overweight/obesity, nonalcoholic fatty liver disease (NAFLD) has worldwide become the main cause of chronic liver disease in both adults and children. Patients with fatty liver display features of metabolic syndrome (MetS), like insulin resistance (IR), glucose intolerance, hypertension and dyslipidemia. Recently, epidemiological studies have linked obesity, MetS, and NAFLD to decreased bone mineral density and osteoporosis, highlighting an intricate interplay among bone, adipose tissue, and liver. Osteoprotegerin (OPG), an important symbol of the receptor activator of nuclear factor-B ligand/receptor activator of nuclear factor kappa B/OPG system activation, typically considered for its role in bone metabolism, may also play critical roles in the initiation and perpetuation of obesity-related comorbidities. Clinical data have indicated that OPG concentrations are associated with hypertension, left ventricular hypertrophy, vascular calcification, endothelial dysfunction, and severity of liver damage in chronic hepatitis C. Nonetheless, the relationship between circulating OPG and IR as a key feature of MetS as well as between OPG and NAFLD remains uncertain. Thus, the aims of the present review are to provide the existent knowledge on these associations and to discuss briefly the underlying mechanisms linking OPG and NAFLD.

Key words: Nonalcoholic fatty liver disease; Insulin resistance; Metabolic syndrome; Osteoprotegerin; Receptor activator of nuclear factor kappa B; Receptor

activator of nuclear factor kappa B ligand

Core tip: Recently, epidemiological studies have linked obesity, metabolic syndrome, and nonalcoholic fatty liver disease (NAFLD) to decreased bone mineral density and osteoporosis, highlighting an intricate interplay among bone, adipose tissue, and liver. Osteoprotegerin (OPG), an important symbol of the receptor activator of nuclear factor-B ligand/receptor activator of nuclear factor kappa B/OPG axis activation, has recently been suggested to have critical roles in the initiation and perpetuation of obesity-related comorbidities including NAFLD. The available studies have reported either positive or negative associations between OPG and NAFLD. Thus, more research is needed to clarify its role in this liver disease.

Pacifico L, Andreoli GM, D'Avanzo M, De Mitri D, Pierimarchi P. Role of osteoprotegerin/receptor activator of nuclear factor kappa B/receptor activator of nuclear factor kappa B ligand axis in nonalcoholic fatty liver disease. *World J Gastroenterol* 2018; 24(19): 2073-2082 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i19/2073.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i19.2073

INTRODUCTION

Concomitantly with the increase in the prevalences of overweight/obesity, nonalcoholic fatty liver disease (NAFLD) has worldwide become the main cause of chronic liver disease in both adults and children[1,2]. NAFLD implies accumulation of lipids within hepatocytes, with a spectrum ranging from simple steatosis to steatohepatitis (NASH), progressive to cirrhosis[3-5]. Although patients with NAFLD have a high risk of mortality from liver complications, the primary cause of mortality in such patients is cardiovascular disease (CVD)[6]. Indeed, NAFLD may be considered in adults as well as in children a multisystem disease affecting several extra-hepatic organs and involving a range of extra-hepatic chronic diseases, in particular type 2 diabetes, CVD, and chronic renal disease[7-11]. These diseases have the same underlying pathophysiological features associated with metabolic syndrome (MetS), including insulin resistance (IR), chronic systemic inflammation and hyperlipidemia. Recently, epidemiological studies have linked obesity, MetS, and NAFLD to decreased bone mineral density (BMD) and osteoporosis, highlighting an intricate interplay among bone, adipose tissue, and liver[12-14]. With regard to this, the association between NAFLD and decreased BMD has been also reported in the pediatric obese population[15,16].

Osteoprotegerin (OPG), an important symbol of the receptor activator of nuclear factor-B ligand (RANKL)/receptor activator of nuclear factor kappa B (RANK)/OPG axis activation, has recently been highlighted as an important factor of the biochemical mechanisms underlying the association between MetS and CVD[17-20]. Tumor necrosis factor (TNF) superfamily molecules, namely, RANKL, its receptor (RANK), and its soluble (decoy) receptor, OPG, mediate interactions (RANKL-OPG axis) that exert multiple actions on bone metabolism, endocrine functions, and the immune system[21-23]. The RANKL-OPG axis is typically considered for its role in bone metabolism, but proinflammatory cytokines [*e.g.,* interleukin (IL)-1b, IL-6, and TNF-α] that are regulated by the RANKL-OPG axis in mediating bone resorption in osteoporosis, may also play critical roles in the initiation and perpetuation of obesity-related comorbidities[21-23]. There is arising evidence that RANKL/RANK/OPG system participate in the pathogenesis of atherosclerosis and CVD by expanding the detrimental actions of inflammation and multiple risk factors including dyslipidemia, endothelial dysfunction, type 2 diabetes, and high blood pressure[20].

Clinical data have displayed that circulating OPG concentrations are associated with hypertension and left ventricular hypertrophy in the general population, with vascular calcification and altered endothelial function in subjects with and without diabetes, and with severity of liver damage in patients with chronic hepatitis C[22-25]. Moreover, epidemiological studies have shown that OPG concentrations may predict morbidity and mortality from CVD[26]. Nonetheless, still the association of circulating OPG with IR as a key feature of MetS as well as of OPG with NAFLD remains uncertain. Thus, the aims of the present review are to provide the existent knowledge on these associations and to discuss briefly the possible underlying mechanisms linking OPG and NAFLD. We searched in MEDLINE and EMBASE databases utilizing the words "OPG", "RANKL", "RANK", "IR", "MetS", and "NAFLD" individually and in combination to recruit all published articles from 1990 to 2018.

OPG/RANK/RANKL SYSTEM

OPG, first recognized in 1997, is a cytokine belonging to the superfamily of TNF receptor[27-30]. It has been termed OPG for its protective role in bone. The OPG gene discovered and cloned in 1998 is a single -copy gene localized on chromosome 8 (8q24) consisting of five exons over 29 kilobases[31]. Fom a biochemical aspect, OPG is a glycoprotein with a primary structure of 401 aminoacids and a molecular weight of 60 kilodaltons. OPG has seven structural domains, which actuate its biological functions in specific manners[32]. The amino terminal domains one to four, containing plenty of cysteine, impart osteclastogenesis inhibitory characteristics. Domains five and six at the carboxy terminal end include apoptosis-mediating death domain homologous regions. Domain seven encloses a heparin-binding region along with a free cysteine residue required for disulfide bond formation

and dimerization. In fact, further to its monomeric structure, OPG may be completed at the cys-400 residue in the heparin binding domain to constitute a disulphide-linked dimer[32]. Before being secreted as monomeric and dimeric forms, the twenty-one aminoacid's signal peptide of OPG is split from the N-terminal achieving a 380 aminoacid's mature OPG protein. Therefore, as long as the OPG monomer is biologically active, OPG homodimer molecule is more active and its production is necessary to generate complete biological activity *in vitro* and *in vivo*. This is because the homodimer form possesses higher affinity for the RANKL ectodomain than the OPG monomer. RANKL and TNF-related apoptosis-inducing ligand (TRAIL) bind to OPG with similar affinities[32].

OPG is highly expressed in various organs and tissues including osteoblasts, lungs, cardiac tissue, renal tissue, hepatic tissue, spleen, thymus, prostate, ovary, small intestine, thyroid, lymphnodes, trachea, adrenal gland, testis, and bone marrow, endothelial cells and vascular smooth cells, while it is encountered at very low levels in brain, placenta, and skeletal muscle[23,26,33]. OPG has also been discovered by means of immunohistochemistry in atherosclerotic plaques of aortas and coronary arteries. Furthermore, OPG expression has recently been demonstrated in human adipose tissue[34].

RANK, an additional member of the TNF receptor superfamily, is expressed on the surface of hematopoietic precursor cells and mediates signaling that activates osteoclastogenesis[35]. Its ligand RANKL is typically expressed on osteoblast/stromal cell surfaces. RANKL is also encountered in stimulated T-lymphocytes, lymph nodes, thymus, mammary gland, lungs, spleen and bone marrow. It is a transmembrane protein, however, in the blood is also present a soluble form (sRANKL). sRANKL seems to derive from cleavage of membrane RANKL or to be produced by T-lymphocytes. Membrane-bound RANKL or sRANKL binds to RANK through interaction with specific molecules such as TNF receptor-associated factor (TRAF) proteins. The most important role of TRAFs in RANK-RANKL signaling is the stimulation of NF-kBs as well as mitogen-activated protein kinases and interferon-regulatory molecules. TRAF proteins may also take part to chronic inflammatory state and infection[36].

ROLE OF OPG/RANK/RANKL SYSTEM IN BONE AND OTHER TISSUES

The wide variety of cells and tissues in major organ systems such as the skeletal, vascular, and immune systems as well as other systems producing OPG, RANKL, and RANK support their role in the function of these organs (Figure 1). Typically, the OPG/RANK/RANKL axis regulates remodeling of bone as well as differentiation and activation of osteoclasts, and thus, the crucial equilibrium between formation and resorption of bone. RANKL binds to RANK on osteoprogenitor cells and controls osteoclastogenesis and bone resorption. OPG acting as a soluble decoy receptor, negatively regulates this interaction and competes with RANK, preventing RANKL-RANK interactions.

While OPG is expressed in the vessels of healthy mice, RANK and RANKL are not detected in the arteries of healthy adult mice. In contrast, RANKL and RANK have been discovered in the calcified arteries of $OPG^{-/-}$ mice and RANK expression occurred simultaneously with the appearance of multinuclear osteoclast-like cells[37]. These findings suggest that vascular OPG protects against RANK/RANKL induced osteoclast formation. In humans, RANKL and RANK are often undetected in the non-diseased vessel, while OPG is expressed in normal arteries. However, early as well as advanced human atherosclerotic lesions of carotid arteries and abdominal aortas manifest both RANKL and OPG immunoreactivity and mRNA expression[38-40].

Immune cells express OPG, RANKL, and RANK and these are believed to regulate inflammatory and immune responses[41-43]. Binding of RANKL to RANK augments dentritic cells' survival, enhances the immunostimulatory capacity of dentritic cells, and modulates activated T-cells. In particular, RANKL/RANK signaling in the immune system controls the development of thymocyte-mediated medulla, and the development of self-tolerance in T cells as well as the number of regulatory T cells (Treg). RANKL also regulates the production of proinflammatory cytokines in macrophages[41]. An important function of OPG in the immune system is related to the cytotoxic ligand TRAIL, a potent activator of apoptosis. Binding of OPG to TRAIL inhibits cell apoptosis[44].

Yet, OPG, RANKL, and RANK have been demonstrated to be expressed in normal brain of rodents. Notably, in normal brain, RANKL/RANK signaling has been related to fever and body temperature control. The stimulation of RANKL/RANK signaling obtained by the deletion of OPG or the administration of RANKL has been demonstrated to prevent the exacerbation of infart volume as well as cerebral edema through the inhibition of the production of pro-inflammatory cytokines[45].

The multiple actions of OPG/RANKL/RANK axis, including modulation of cell survival, mineralization and inflammation suggest a potential role as mediator of metabolic complications including insulin resistance, type 2 diabetes, MetS and NAFLD.

CLINICAL STUDIES

Insulin resistance

NAFLD is strictly associated with IR, which is also a main determinant in the pathogenesis of type 2 diabetes and MetS. Even if investigators agree that IR is determined by alterations in intracellular insulin signaling, various causes have been suggested to explain by what means such insulin signaling alterations originate in NAFLD. Inflammation, activation of endoplasmic reticulum stress pathways, and deposit of lipids in hepatocytes have all been proposed to determine IR in NAFLD[46,47].

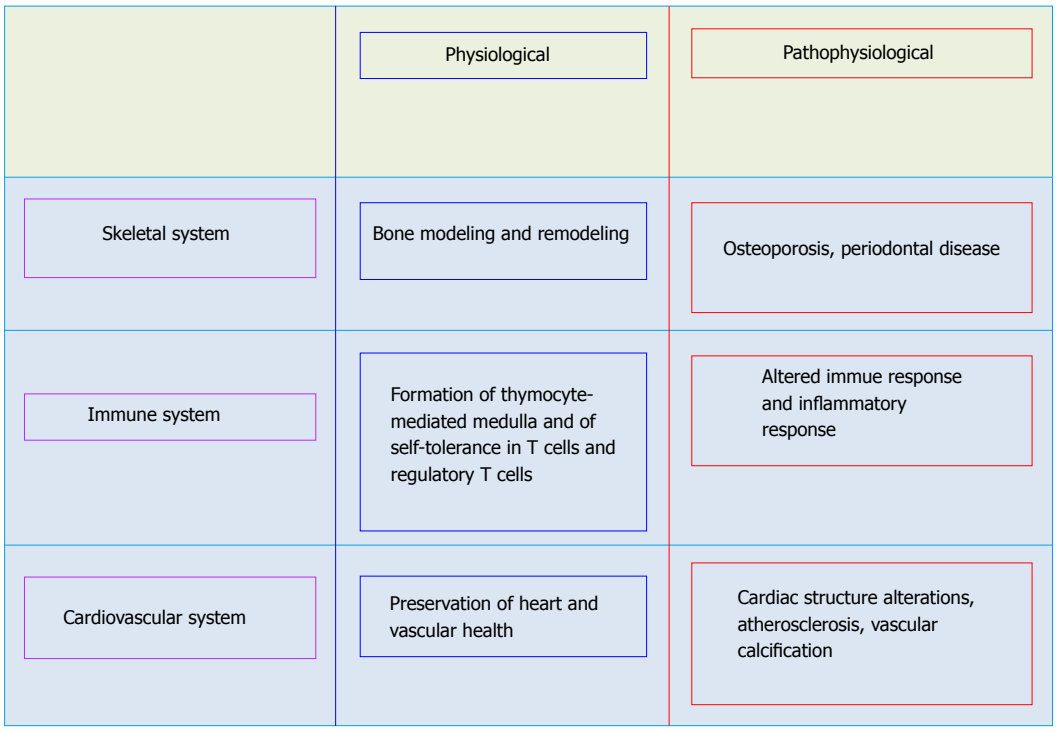

Figure 1 Role of osteoprotegerin/ receptor activator of nuclear factor-B ligand/receptor activator of nuclear factor kappa B axis in physiological and pathophysiological conditions.

Numerous studies have reported on the association between OPG and IR with contrastant results[48-62]. In a cohort of 106 subjects with obesity, including eighteen with type 2 diabetes, Gannage-Yared *et al*[48] demonstrated a positive relationship between OPG and IR as evaluated by the homeostasis model assessment for IR (HOMA-IR). In a cross-sectional study, Yaturu *et al*[49] demonstrated that OPG was significantly associated with insulin levels and IR as well as with C-reactive protein (CRP) and TNF-α in patients affected by type 2 diabetes, most likely reflecting the proinflammatory state in this population. Pepene *et al*[50] reported a positive association of OPG with HOMA-IR in a cohort of women with polycystic ovary syndrome. Akinci *et al*[51] found that women with a history of gestational diabetes mellitus developing MetS showed increased OPG values compared to women who did not fulfill MetS criteria. Yet, these authors showed that OPG concentrations were associated with markers of IR, with carotid intima-media thickness (IMT) and with subclinical inflammation. Suliburska *et al*[52] found that HOMA-IR values and OPG values were significantly increased in obese adolescents than in the control group. A significant positive correlation between OPG and IR was found. In a large population of individuals with normal glucose tolerance (n = 599), with impaired glucose tolerance (n = 730) and with newly diagnosed diabetes (n = 327), respectively, Niu *et al*[53] demonstrated that elevated circulating OPG levels were independently related to impaired glucose regulation and a higher risk of microalbuminuria. Bilgir *et al*[54] found that circulating OPG and sRANKL values were significantly increased in prediabetic patients than in control individuals. There was a positive relationship between sRANKL and OPG. Yet, sRANKL was positively associated with body mass index (BMI), HOMA-IR, and inflammatory markers such as high-sensitivity CRP. Duan *et al*[55] demonstrated that circulating OPG concentrations were increased in Chinese postmenopausal women with diabetes and prediabetes. Moreover, serum OPG levels showed significant correlation with IR. Mashavi *et al*[56] showed that OPG values were significantly increased in postmenopausal women affected by osteoporosis and impaired glucose metabolism (including impaired glucose tolerance and type 2 diabetes) than women with normal glucose tolerance. OPG concentrations were independently associated with IR as evaluated by HOMA-IR. Recently, Daniele *et al*[57] found that high OPG concentrations were correlated with increased endogenous glucose production (primarily reflecting liver glucose production) and hepatic IR in individuals with impaired glucose regulation, supporting the possibility that OPG could have a role in glucose homeostasis derangement that usually precede overt type 2 diabetes.

There have also been some studies demonstrating a negative relationship between OPG and IR, though they were predominantly based on healthy populations. In a healthy population (exhibiting normal glucose tolerance and exercise stress tests, thus excluding hyperglycemia and ischemic heart disease, respectively), Ashley *et al*[58] found that OPG correlated inversely with HOMA-IR, and suggested that high IR in healthy subjects is associated with low levels of circulating OPG. In a subsequent

study, these authors showed that OPG was higher in patients with abnormal glucose tolerance compared to normoglycemic healthy subjects[59]. Nonethless, OPG did not correlate with the severity of IR as evaluated by HOMA-IR either on univariate or multiple linear regression, suggesting that OPG elevation in these individuals may be due to other factors. In agreement with these findings, Ugur-Altun and colleagues[60,61] in two separate studies - the former involving obese patients without diabetes *vs* lean healthy subjects, the latter healthy young women - found a negative relationship between OPG and IR. Ayina Ayina *et al*[62] demonstrated that HOMA-IR was inversely associated with OPG values in women with obesity, meaning that elevated OPG concentrations may be expression of high insulin sensitivity.

The heterogeneity of the results of the studies on the association between OPG and IR might reflect differences in the population included in terms of gender, age, ethnic background, and, importantly, in terms of metabolic-associated diseases. Indeed, a positive relationship has been found in studies that involved individuals with high levels of IR, such as those affected by type 2 diabetes and a previous history of gestational diabetes, while a negative relationship in those that involved healthy subjects. It should be acknowledged that elevated circulating OPG has emerged as a strong, independent predictor of CVD[63,64]. In particular, plasma OPG is considered a marker of vascular calcifications[65], a feature often seen in patients with impaired glucose homeostasis[66] and recently shown to involve insulin actions[67]. OPG concentrations in patients affected by obesity and type 2 diabetes may thus reflect the presence of CVD.

Metabolic syndrome

Scant and contrastant literature is available on the association between OPG and MetS. Initial studies found no correlation between OPG and MetS[68,69]. In particular, in a cohort of elderly Lebanese men, Gannage-Yared *et al*[68] found no significant difference in OPG concentrations between individuals with and without MetS. Similar findings were reported by Nabipour *et al*[69] in a population-based sample of postmenopausal women. In subsequent studies, however, an association between OPG and MetS has been reported. In individuals with peripheral artery disease, circulating concentrations of OPG were raised in obese patients with MetS[70]. Akinci *et al*[51] found that women with a history of gestational diabetes mellitus developing the MetS showed increased OPG levels than women who did not fulfill the MetS criteria. These findings were previously reported by the same authors in a sample of 128 women with previous gestational diabetes and 67 age-matched controls. OPG values were associated with obesity, IR, and carotid IMT[71].

Recently, Pérez de Ciriza *et al*[72] demonstrated that patients with MetS had significantly elevated OPG concentrations than those without the syndrome. Of note, OPG values significantly and positively correlated with the number of cardiovascular risk factors. In addition, OPG expression in adipose tissue was endorsed, and MetS patients expressed elevated OPG mRNA values compared to those without. Bernardi *et al*[73] demonstrated that circulating OPG was higher in patients with MetS compared to controls. In high-fat diet fed C57BL6 mice, they also found that OPG was elevated, and that OPG administration promoted systemic and adipose tissue proinflammatory changes resembling those observed in HDF fed mice. Finally, in patients with type 2 diabetes, Tavintharan *et al*[74] found OPG to be a significant predictor of MetS also after adjustment for age, sex, ethnic origin, glucose levels, and microvascular complications.

The variation of the results of the studies on the association between MetS and OPG may be in part explained by differences in the population included in terms of gender, age and associated diseases, and importantly, in diagnostic criteria utilized.

NAFLD

There are few studies on the relationship between OPG and NAFLD, with either positive or negative associations having being described[75-80] (Table 1). In a cross-sectional study, Yilmaz *et al*[75] first reported that OPG levels were significantly decreased in patients with definite and borderline NASH than in subjects with simple liver steatosis. The authors also found a negative relationship between OPG and HOMA-IR, and between OPG and serum transaminases values. Thus, low OPG concentration in subjects affected by NAFLD may reflect the effects of IR, as well as the occurrence of severe liver necroinflammation. Yang *et al*[76] tested the accuracy of non-invasive biological markers for identification of NASH, including OPG, in 179 patients with biopsy-proven NAFLD (training group) and 91 age- and sex-matched healthy controls. Further 63 subjects with NAFLD were separately included as validation group. Serum levels of OPG decreased progressively from controls to patients with NAFLD but without NASH, and reached the lowest levels in patients with NASH. Sensitivity and specificity of OPG for assessing NASH were 81.30% and 74.60%, respectively. In a case-control study involving 746 patients affected by type 2 diabetes (of whom 367 with ultrasound-diagnosed NAFLD), Niu *et al*[77] demonstrated that the OPG concentrations were significantly decreased in patients with NAFLD compared to patients without liver involvement. The subjects in the lowest OPG quartile were at higher risk for NAFLD. Finally, Erol *et al*[78] evaluated the association of OPG concentrations with obesity, IR, and NAFLD in children and adolescents. OPG concentrations in the youth with obesity were significantly decreased than in controls. Among obese youths, those with high fasting insulin and high HOMA-IR values displayed significantly lower OPG values. Patients with

Table 1 Studies assessing the association between osteoprotegerin and nonalcoholic fatty liver disease

Ref.	Study design	Population	Findings
Yilmaz *et al*[75], 2010	Cross-sectional study	56 adult patients with histological-proven definite NASH; 26 with borderline NASH; 17 with simple fatty liver; and 58 healthy controls without evidence of liver disease (normal results on liver function tests and normal liver ultrasound).	OPG levels were significantly decreased in patients with definite NASH and borderline NASH than in controls. No significant differences were found between patients with simple fatty liver and controls.
Ayaz *et al*[79], 2014	Case-control study	60 adult patients with ultrasound-proven NAFLD and 30 control subjects.	OPG levels were significantly increased in patients with NAFLD compared to control subjects.
Yang *et al*[76], 2015	Cross-sectional study	179 patients with biopsy-proven NAFLD (training group) and 91 age- and gender-matched healthy subjects. 63 other NAFLD patients were separately collected as validation group.	Serum levels of OPG decreased in a stepwise fashion in controls, non-NASH NAFLD patients and NASH patients.
Monseu *et al*[80], 2016	Cross-sectional study	314 adult subjects with at least one criterion for metabolic syndrome.	OPG levels were positively associated with both liver markers (such as alanine aminotransferase, gamma-glutamyl transferase and ferritin levels) and increased liver fat content as assessed by magnetic resonance imaging.
Niu *et al*[77], 2016	Case-control study	746 adult patients with type 2 diabetes, of whom 367 with ultrasound-proven NAFLD.	OPG levels were significantly decreased in patients with NAFLD compared to those without NAFLD. Participants in the lowest OPG quartile had a significantly increased risk for NAFLD (OR = 3.49, 95%CI: 1.86-6.94).
Erol *et al*[78], 2016	Cross-sectional study	107 children with obesity of whom 62 had ultrasound-proven NAFLD and 37 control subjects.	OPG levels in the obese group were significantly lower than in controls. Among obese youths, those with high fasting insulin and high HOMA-IR values had significantly lower OPG levels. Patients with hepatic steatosis had lower OPG concentrations than those without liver involvement, although they did not reach statistical significance.

NASH: Non-alcoholic steatohepatitis; OPG: Osteoprotegerin; NAFLD: Nonalcoholic fatty liver disease; HOMA-IR: Homeostasis model assessment for insulin resistance.

hepatic steatosis had lower OPG concentrations than those without liver involvement, although they did not reach statistical significance. In contrast, Ayaz *et al*[79] demostrated that patients with NAFLD diagnosed *via* ultrasonography had OPG levels significantly higher compared to controls. Monseu *et al*[80] determined the association between OPG and visceral adipose tissue and liver fat content as measured by magnetic resonance imaging, as well as other markers of the MetS in dysmetabolic adults. OPG levels were positively correlated with visceral fat liver and liver fat content, as well as liver markers such as alanine aminotransferase and HOMA-IR index.

Some points must be considered when interpreting the results of the few aforementioned studies. First, half of them have included a small sample size. Second, the clinical heterogeneity of patients' population enrolled in the studies. Third, methodologic heterogeneity in defining the reference standard. In fact, liver disease was differently evaluated, with the majority of the studies utilizing ultrasonography that is known to be unable to assess severity of liver disease such as NASH.

BIOLOGICAL ROLE OF OPG IN NAFLD

OPG acting like a decoy receptor for TRAIL and RANKL neutralizes their biological actions. Of note, TRAIL is a relevant inductor of apoptosis in hepatocyte cells[81]. Because enhanced hepatocyte apoptosis has a key role in the progression of liver disease, that is from simple steatosis to NASH[82], it is tempting to suppose that the decrease in circulating concentrations of OPG in NAFLD, observed in the majority of the studies, might be responsible for alterations in the mechanisms protecting against hepatocyte apoptosis. Notably, accumulation of OPG is closely related to reduced apoptosis in several cell types[81,83]. These findings may imply that OPG exert a common defensive effect on the pathophysiologic derangements responsible for NAFLD through at least two different mechanisms: The first mechanism involves IR, while the second is based on protection of hepatocytes from cell death by apoptosis. Nonethless, the exact mechanisms responsible for the decrease of OPG in subjects with NAFLD and NASH need additional studies.

ANIMAL DATA

The development of transgenic technologies in mice has led to advances in knowledge of the role of OPG/RANKL/RANK system in bone metabolism and cardiometabolic functions. Concerning cardiometabolic disorders, Hao *et al*[84] showed that $OPG^{-/-}$ mice exhibited a significant increase in systolic blood pressure since early stages of life, and that this rise was in parallel with the osteoporotic change in these mice. $OPG^{-/-}$ mice also

presented a higher heart weight/body weight ratio than age-matched wild-type mice, indicating that OPG plays an important role in the preservation of cardiac structure. Kiechl *et al*[85] developed hepatocyte-specific RANK knockout ($RANK^{LKO}$) mice and compared them with wild-type mice. While $RANK^{WT}$ mice experienced insulin resistance after 4 wk of a high-fat diet (NFD), $RANK^{LKO}$ mice did not. A very recent study demonstrated that mice lacking β-catenin in osteoblasts exhibit during the postnatal period reduced bone mass, increased glucose level, reduced insulin production, reduced fat accumulation and increased energy expenditure. OPG overexpression normalized not only the reduced bone mass but also the reduced fat accumulation and increased energy expenditure[86].

CONCLUSION

Contention still exists on the exact role of OPG/RANKL/RANK system in IR and NAFLD. The available studies have reported either positive or negative associations between OPG and IR as well as between OPG and NAFLD. As previously outlined, possible explanations of the discordant results may be related to differences in the study population in terms of gender, age, ethnic background, and, importantly, in terms of cardiometabolic-associated diseases. Interestingly, OPG seems to have a dichotomous role in humans, as suggested in CVD. In healthy subjects, the proatherogenic and antiatherogenic effects are being held in a fine balance, while in the presence of persistent risk factors the proatherogenic pathway becomes predominant. Moreover, there are differences between human and animal studies. Observational studies in human subjects show that circulating OPG concentrations are associated positively with severity and progression of coronary artery disease, atherosclerosis, and vascular calcification whereas animal studies support a protective role for OPG[87]. Future studies are necessary to clarify the role of OPG in NAFLD.

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MINIREVIEWS

Mediterranean diet and nonalcoholic fatty liver disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is emerging as the most common chronic liver disease, and is characterized by a wide spectrum of fat-liver disorders that can result in severe liver disease and cirrhosis. Inflammation and oxidative stress are the major risk factors involved in the pathogenesis of NAFLD. Currently, there is no consensus concerning the pharmacological treatment of NAFLD. However, lifestyle interventions based on exercise and a balanced diet for quality and quantity, are considered the cornerstone of NAFLD management. Mediterranean diet (MD), rich in polyunsaturated fats, polyphenols, vitamins and carotenoids, with their anti-inflammatory and anti-oxidant effects, has been suggested to be effective in preventing cardiovascular risk factors. In adults, MD has also been demonstrated to be efficacious in reducing the risk of metabolic syndrome. However, few studies are available on the effects of the MD in both adult and pediatric subjects with NAFLD. Thus, the aims of the present narrative review are to analyze the current clinical evidence on the impact of MD in patients with NAFLD, and to summarize the main mechanisms of action of MD components on this condition.

Key words: Mediterranean diet; Children; Nonalcoholic fatty liver disease; Adults

Core tip: Lifestyle interventions based on exercise and a balanced diet, are considered the cornerstone of nonalcoholic fatty liver disease (NAFLD) management. The Mediterranean diet (MD), low in saturated fats and animal protein, high in antioxidants and fibers, and with an adequate omega-3 to omega-6 fatty balance, has been suggested to be effective in NAFLD. Although the results from the available studies are encouraging, there is still need of trials with larger sample size, along with the standardization of the criteria to evaluate

adherence to the diet, before including the MD as a therapeutic dietary pattern in NAFLD.

Anania C, Perla FM, Olivero F, Pacifico L, Chiesa C. Mediterranean diet and nonalcoholic fatty liver disease. *World J Gastroenterol* 2018; 24(19): 2083-2094 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i19/2083.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i19.2083

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver disease[1,2]. It represents a wide range in liver damage that may lead to severe liver disease such as cirrhosis and hepatocellular carcinoma[3]. Adults as well as children with fatty liver display abnormal glucose and lipid metabolism. Therefore, NAFLD is now considered an important component of the metabolic syndrome (MetS)[4]. The mechanism of liver injury in NAFLD is considered to be a "multiple-hit process". The first "hit" leads to an increase in liver fat, while the next multiple factors lead to inflammation[5]. Indeed, the early manifestation of NAFLD is triglyceride accumulation in the liver associated with insulin resistance, which is considerably affected by factors such as hyperenergetic diets, sedentary lifestyle, and genetic susceptibility. Fat accumulation in the liver is associated with lipotoxic hepatocellular injury due to elevated free fatty acids, free cholesterol and other lipid metabolites. Thus, mitochondrial dysfunction with oxidative stress and endoplasmic reticulum stress-associated mechanisms are activated[6].

Obesity is considered a key player in the development of NAFLD, and the majority of patients with NAFLD are either obese or overweight. However, NAFLD has been reported also in lean subjects. "Lean" NAFLD represents subpopulation of patients with fatty liver and normal BMI. These patients are usually insulin resistant and have low HDL-C and higher triglyceride concentrations when compared to lean healthy controls[7]. Visceral obesity (as opposed to general obesity), insulin resistance, high fructose and high cholesterol intake are the most prevalent risk factors for lean NAFLD, although genetic factors (*e.g.,* Palatin-like phospholipase domain -containing 3 and Transmembrane 6 superfamily member 2 gene variants) may have an important role.

NAFLD diagnosis requires proof of steatosis, which relies on imaging techniques in clinical practice. Liver biopsy remains the gold standard to address such diagnosis and is the only valid method for differentiating NASH from simple steatosis, however it is neither feasible nor ethical to perform liver biopsy as a tool in all putative patients. Noninvasive imaging techniques, such as ultrasound (US), computed tomography (CT), magnetic resonance imaging (MRI), and proton magnetic resonance spectroscopy (MRS), can also identify fatty infiltration of the liver[8-10]. US is perhaps the most practical way to assess hepatic steatosis, due to its relatively low cost, availability, and safety. A major limitation of this operator-dependent technique is its limited sensitivitiy and specificity for diagnosing and quantifying hepatic steatosis. MRS is considered the non-invasive reference standard in the assessment of liver steatosis, because it is able to measure the real concentration of triglycerides within the hepatocytes. However, MRS is too time consuming for routine clinical practice, and requires a skilled operator to correctly perform the examination, process the data, and interpret the results. MRI has shown greater promise for the quantitative assessment of hepatic steatosis in adults and children. Until recently, the most widely used method was based on the modified Dixon technique[8]. This imaging method is reliable in the absence of magnetic field non-homogeneity and iron deposition. Recent improvement in MRI have provided measurement of the proton density fat-fraction [(PDFF): The fraction of the liver proton density attributable to liver fat],which is a inherent property of tissue and a direct measure of liver fat content. MRI-PDFF is accurate, precise, and reliable for quantifying liver steatosis having been validated against liver biopsy in both adults and children[9,10].

Currently, there is no agreement with respect to the pharmacological treatment of NAFLD. However, lifestyle interventions based on exercise and a balanced diet for quality and quantity, are considered the cornerstone of NAFLD management[11]. Mediterranean diet (MD), which is characterized by a significant amount of fibers, polyunsaturated fats and antioxidants, has been suggested to decrease the risk of cardiovascular diseases (CVD). In adults, MD has also been demonstrated to be efficacious in reducing the risk of MetS[12-15]. However, few studies are available on the effects of MD in both adults and children with NAFLD. Thus, the present narrative review aims to present an analysis of the available literature on the effects of the MD in patients with NAFLD, and to summarize the main mechanisms of action of MD components on this condition. To identify relevant studies, a systematic literature search on MEDLINE and EMBASE databases was conducted using the following keywords: "Mediterranean diet", "nonalcoholic fatty liver disease", "hepatic steatosis", "steatohepatitis". All searches were limited to studies published in English language

DIET IN NAFLD TREATMENT

Results of studies regarding pharmacological options for treatment of NAFLD are inconclusive[11]. At the moment the best treatment to manage NAFLD is lifestyle intervention to achieve weight loss[11]. A 7% to 10% body weight reduction after energy restriction and/or regular physical activity is associated with histological improvement, resolution of liver fat, necroinflammation and fibrosis[16,17]. Though weight loss is considered the

Table 1 Traditional Mediterranean diet components

Components	Consumption	Rich in
Fresh fruits	Daily, 3 servings	Vitamin C, polyphenols, carotenoids, fibers
Vegetables	Daily, 6 servings	Vitamin C, polyphenols, ω-3-PUFA, carotenoids, fibers
Olive oil	Daily[1]	MUFA, polyphenols
Unrefined cereals	Daily, 8 servings	Polyphenols, fibers
Nuts	Weekly	Polyphenols, ω-3-PUFA, fibers
Legumes	Weekly, ⩾ 3 servings	Polyphenols, fibers
Fish	Weekly, 5-6 servings	ω-3-PUFA
Red wine	Weekly, ⩾ 7 glasses	Polyphenols

[1]As the main added lipid.

most effective treatment in NAFLD, some diets that involve excessive and/or rapid weight loss (*e.g*, very low carbohydrate, high fat diets) may actually cause or exacerbate the disease, inducing insulin resistance[18,19]. As weight reduction is a consequence of physical activity and a 'healthy diet', dietary habits rather than weight loss *per se* may improve NAFLD[18]. Dietary treatment to achieve weight loss must have not only quantitative but also qualitative characteristics. Most studies conclude that energy restriction alone is not enough to treat NAFLD[20], and that the composition of the diet, with modulation of both macro and micronutrients, is crucial[21]. Therefore, a balanced nutrition and a moderate weight loss can now be considered as the best therapeutic approach in NAFLD. According to international guidelines, the first step for treating NAFLD is to limit the intake of calories, of fats (saturated fatty acids, trans fatty acids), and of fructose and, conversely, to increase the intake of lean protein, fibers, and n-3 polyunsaturated fatty acid (PUFA)[15]. Indeed, MD appears as a useful dietary option to produce weight loss followed by concomitant metabolic benefit for NAFLD.

MEDITERRANEAN DIET

MD is a nutritional model which has its origins in the States surrounding the Mediterranean Sea. It was therefore traditionally used by the populations living in these regions. Although MD pattern may vary among countries and regions owing to cultural, ethnic, religious and agricultural differences, the common MD pattern consists of eating primarily unrefined cereals, vegetables and fresh fruit, olive oil, and nuts; eating fish, white meat and legumes in moderation; limiting red meat, processed meats and sweets; and drinking red wine in moderation (Table 1). Therefore, the main characteristics of MD are beneficial fatty acid profile consisting of a low consumption of saturated fat and cholesterol, and, conversely, of a high consumption of monounsaturated fatty acid (MUFA) with a balanced PUFA omega-6 to omega-3 ratio, along with a high content of complex carbohydrates and fibers. Ancel Keys, who conducted large multinational studies in the 1950s-1980s[22-24], first reported a lower mortality rate from CVD and cancer among people living in Greece - as well as in certain parts of Italy and the former Yugoslavia - in comparison to other populations. Afterwards, other studies have confirmed these findings, recognizing MD as a healthy and useful diet for reducing the risk of CVD and cancer[25-28] as well as of obesity and type 2 diabetes[29]. Yet, MD has been proposed as a longevity determinant in these populations[30]. Many studies suggest that the protective effects of MD may be due mostly to the anti-inflammatory and anti-oxidant properties of its components. In particular, the capacity of MD to reduce the risk of development and progression of NAFLD has been attributed to the nutraceutical effect of bioactive compounds and phytochemicals with antioxidant and anti-inflammatory capacity such as fibers, monounsaturated and omega-3 fatty acids and phytosterols[31,32]. NAFLD is associated with visceral obesity, insulin resistance, dyslipidemia, and chronic inflammation all of which are features of Mets. MD may improve NAFLD by modulating the presence of these conditions. In particular, the antioxidant and anti-inflammatory effects as well as the lipid-lowering effects and gut-microbiota-mediated production of metabolites are the principal mechanisms by which MD can influence metabolic health as well as NAFLD.

CLINICAL STUDIES ON MEDITERRANEAN DIET IN NAFLD PATIENTS

Cross sectional studies

Recently, researchers have focused on the possible association between MD and NAFLD. Data from cross sectional studies suggest that MD components have a beneficial effect on NAFLD[33]. As such, the EASL-EASD-EASO clinical Practice Guidelines have recently encouraged MD as a lifestyle choice for treating the disease[16]. The available studies are presented in Table 1[34-40]. Kontogianni *et al*[34] were the first to explore the potential impact of MD on NAFLD and its severity in 73 overweight/obese adult patients, of whom 34 had liver biopsies. They found that the MD score was inversely associated to serum alanine aminotransferase (ALT) and insulin concentrations as well as to histological characteristics of severe steatosis. A higher adherence to MD (as determined by MedDietScore) was not followed by a lower likelihood of having NAFLD, even after adjustment for abdominal fat level. However, it was associated with a less severe liver disease[34]. Indeed, patients with nonalcoholic steatohepatitis (NASH) were less likely to adhere to MD (P = 0.004) versus patients without NASH. Limitations of the study are the cross-sectional design which enables to establish a casual relation; the small sample size; and patients' selection

criteria (elevated ALT, and ultrasound diagnosis of fatty liver and its severity). Similarly, in a study including 82 adult subjects with biopsy-proven NAFLD, Aller *et al*[35] demonstrated that patients with greater adherence to MD (as determined by the 14-item MD assessment tool) were less likely to present histological features of severe steatosis and NASH, as well as to have severe insulin resistance. In a population-based study involving 797 apparently healthy Chinese adults, Chan *et al*[36] evaluated the relationship between two diet-quality scores [Diet Quality Index-International (DQI-I) and MD score] in subjects with (n = 220) and without (n = 577) NAFLD [as established by proton-magnetic resonance spectroscopy (^{1}H-MRS)]. DQI-I, but not the MD score, was significantly related to the NAFLD prevalence, and this association was stronger in overweight/obese versus normal weight subjects. Lack of an association between MD and NAFLD prevalence can be explained by the fact that the intake of certain foods such as milk and milk products, olive oil, wine and nuts was lower in this study cohort than in the traditional MD[36]. Although the study by Chan *et al*[36] included a relatively large sample size and the diagnosis of NAFLD was achieved by ^{1}H-MRS, its major limitation is represented by lack of adjustment in the analysis of lifestyle factors such as physical activity. Recently, Trovato *et al*[37] in a study involving 1199 overweight/obese adult patients [with (n = 532) and without (n = 667) ultrasound-diagnosed hepatic steatosis] found that NAFLD patients were less likely to be adherent to MD. Notably, poor MD adherence strongly predicted the occurrence of NAFLD, independently of body mass index (BMI), homeostatic model assessment of insulin resistance (HOMA-IR), and physical activity score. Very recently, Baratta *et al*[38] showed that MD adherence was inversely related to NAFLD prevalence (as assessed by ultrasound) in a large cohort of overweight/obese adults with cardio-metabolic risk. Subjects with intermediate to high adherence to MD were less likely to have NAFLD and more likely to improve cardio-metabolic features[38]. Again, limitations of the last two studies include their cross-sectional study design; lack of a normal weight control group; and use of ultrasound for diagnosing NAFLD.

In children (Table 2), there are only two studies on the association between NAFLD and MD[39,40]. Cakir *et al*[39] first analyzed in obese youths the association between MD adherence [as assessed by the Mediterranean Diet Quality Index (KIDMED)] and NAFLD (as diagnosed by ultrasound and/or elevated ALT levels, as well as by exclusion of other causes of fatty liver disease). The authors evaluated overweight/obese children with (n = 106) and without (n = 21) NAFLD, as well as children (n = 54) with normal BMI and without known chronic disease. Subjects with a low MD adherence were more likely to present with a higher BMI, though no correlation was found with other parameters including steatosis severity. Limitations of the study are the cross-sectional design; the small sample size; assessment of fatty liver severity by ultrasound; and failure to include physical activity level[39]. Very recently, Della Corte *et al*[40] analyzed the adherence to MD (as assessed by the KIDMED score) in 243 overweight/obese youths with and without NAFLD. Of these, 100 underwent liver biopsy. Poor adherence to MD was related to severity of liver damage as well as to higher levels of C-reactive protein (CRP), insulin and HOMA-IR values, homeostatic model assessment of β cell function and blood pressure levels, thus suggesting increased inflammatory potential of unhealthy diets[40]. Lack of a normal weight control group as well as failure to adjust for confounding variables are major limitations of this study.

CLINICAL STUDIES ON MEDITERRANEAN DIET IN NAFLD PATIENTS

Longitudinal studies

Longitudinal studies are available, to our knowledge, only in adult patients (Table 3)[33,41-46]. Fraser *et al*[41] in a quasi-randomized trial evaluated the effect of three different dietary interventions [the 2003 recommended American Diabetes Association diet; a low glycemic index (LGI) diet; and a modified MD] on ALT concentrations in 259 individuals with obesity and type 2 diabetes. Food-energy intake was similar across all three diets,but diet profiles differed in fat and carbohydrate components. The lowest ALT level at 6 and 12 mo of follow-up was achieved after MD intervention, independently of weight loss, HOMA-IR or triacylglycerol values[41]. In a very small, randomized, cross-over intervention trial involving 12 non-diabetic patients with biopsy-diagnosed NAFLD, Rayan *et al*[42] compared MD to an isoenergetic standard low fat-high carbohydrate diet. After 6 wk of treatment, patients experienced after MD intervention a 38% reduction in liver steatosis (as assessed by ^{1}H-MRS) and improvement of insulin sensitivity compared to patients on low-fat, high-carbohydrate diet, independently of weight loss or waist circumference changes[42]. In a randomized,controlled study involving adult subjects wth type 2 diabetes, Bozzetto *et al*[43] evaluated the effects of an isoenergetic MUFA diet versus a diet higher in carbohydrate and fiber. They found that the hepatic fat content (as measured by ^{1}H-MRS before and after 8 wk of intervention) significantly decreased with MUFA diet, independently of exercise. Subsequently, in a single arm trial including 90 overweight NAFLD patients, Trovato *et al*[44] evaluated the Bright Liver Score at baseline and 1, 3, and 6 mo after MD intervention. Over the 6-mo period, adherence to MD resulted in a significant reduction of liver fat content, independently of other lifestyle changes[44]. In a 6-mo randomized controlled study, Abenavoli *et al*[45] compared three groups of overweight patients with ultrasound-diagnosed NAFLD who received either MD alone (n = 10), or MD supplemented with the Reasil complex including silybin (an extract of *Silybum marianum* commonly known as

Table 2 Cross sectional studies on the association between Mediterranean diet and non-alcoholic fatty liver disease

Authors, year, country[ref.]	Patient population	NAFLD Diagnosis	Adherence to MD	Comment
Kontogianni, 2014, Greece[34]	73 overweight/obese adult patients with NAFLD *vs* 58 age-, gender-, and BMI-matched controls with normal liver ultrasound/ liver chemistry	Patients who met all the following criteria: abnormal ALT and/or GGT; ultrasound evidence of hepatic steatosis and/or compatible liver histology; and no other cause of liver steatosis	Adherence to MD (as estimated by MedDietScore) did not differ significantly between patients and controls	Higher adherence to MD was not associated with lower likelihood of having NAFLD (even after adjustment with abdominal fat level). However, it was associated with lesser degree of insulin resistance and less severe liver disease among patients with NAFLD
Aller, 2015, Spain[35]	82 adult patients with NAFLD (of whom 56 had NASH, and 26 non-NASH; 35 had steatosis grade 1, and 47 steatosis grades 2 and 3)	Liver biopsy in all 82 patients	Higher adherence to MD (as estimated by the 14-item MD assessment tool) was higher in patients with low grade of steatosis than in those with high grade, in patients without NASH than in those with NASH, and in patients without liver fibrosis than in those with liver fibrosis	In the logistic regression analysis,one unit of the 14-item MD assessment tool was associated with a lower likelihood of having NASH (OR = 0.43) and steatosis (OR = 0.42)
Chan, 2015, Hong Kong[36]	797 apparently healthy Chinese adults (332 male, 465 female) of whom 220 (27.6%) had diagnosis of fatty liver	^{1}H MRS was performed to measure IHTG. Fatty liver was defined as IHTG $\geqslant$ 5%	Subjects with fatty liver showed lower gender-adjusted MD score than those without fatty liver	Multivariate adjusted regression analyses showed an inverse association between MD score and prevalence of fatty liver, which approached the level of significance
Trovato, 2016, Italy[37]	1199 overweight/ obese adult patients with (n = 532) and without (n = 667) hepatic steatosis	Hepatic steatosis and its severity were assessed by ultrasound	Greater prevalence of overweight/ obesity (as assessed by BMI) and insulin resistance (as assessed by HOMA-IR), sedentary life habits, increased TG and HDL-C, greater use of Western diet food, as well as poor adherence to MD (as assessed by 1-wk recall questionnaire) were found in patients with hepatic steatosis *vs* those without it	Multiple regression analysis, weighted by years of age, displayed BMI, HOMA-IR and adherence to MD as the most powerful predictors of hepatic steatosis severity
Baratta, 2017, Italy[38]	584 overweight/obese adult patients with $\geqslant$ 1 CVD risk factor	Ultrasound evaluation	57 (9.8%) patients had low MD adherence (as estimated by Med-Diet questionnaire), while 436 (74.6%) and 91(15.6%) had, respectively, intermediate and high MD adherence. NAFLD prevalence significantly decreased from subjects with low to high adherence to MD (from 96.5% to 71.4%, P < 0.001)	In a multiple logistic regression analysis, MD adherence (intermediate *vs* low OR = 0.115; P = 0.041; high *vs* low OR: 0.093; P = 0.030) were independently associated with NAFLD
Cakir, 2016, Turkey[39]	Overweight/obese children with (n = 106, Group 1) and without (n = 21, Group 2) hepatic steatosis; and children with normal BMI and without known chronic disease (n = 54, Group 3)	Assessment of hepatic steatosis and its severity by ultrasound	Prevalence of a low level of MD adherence (as established by KIDMED index score) was significantly higher in Group 1 children compared to those belonging to Groups 2 or 3	The level of adherence to MD was negatively correlated with BMI, but no significant correlation was found with ALT, total body fat, TG, and HOMA-IR. No significant difference in the level of MD adherence was found between patients with hepatic steatosis grade1 and those with grades 2 and 3
Della Corte, 2017, Italy[40]	4 subgroups of overweight/obese children: with and without fatty liver; with and without NASH.	Among the 243 study children, ultrasound identified and excluded fatty liver in 66 and 77, respectively. The remaining 100 underwent liver biopsy identifying and excluding NASH in 53 and 47, respectively	Prevalence of a low level of adherence to MD (as estimated by KIDMED score) was significantly higher in patients with NASH compared to those without NASH as well as to those with and without fatty liver (100% *vs* 28.8% *vs* 37.9% *vs* 9.1%; P = 0.01)	Poor adherence to MD was associated to severe liver damage, with a negative correlation with NAFLD activity score and fibrotic stage

ALT: Alanine aminotransferase; BMI: Body mass index; CVD: Cardiovascular disease; GGT: Gamma-glutamyl transferase; ^{1}H MRS: Proton magnetic resonance spectroscopy; HOMA-IR: Homeostasis model assessment of insulin resistance; IHTG: Intrahepatic triglyceride content; MD: Mediterranean diet; NAFLD: Non-Alcoholic Fatty Liver; NASH: Non-Alcoholic Steatohepatitis; OR: Odds ratio; TG: Triglycerides.

milk thistle), phosphatidylcholine and vitamin E (n = 10), or no pharmacological and nutritional treatment (n = 30) . After 6 mo of follow-up, MD either alone or in association with the Realsil complex resulted in significant improvement in fat accumulation as well as in BMI, waist circumference, total cholesterol,

Table 3 Longitudinal studies on the effects of Mediterranean diet on non-alcoholic fatty liver disease in adult patients

Authors, year, country[ref.]	Study design	Patient population	Intervention (duration, type, number of patients)	Liver outcome	Other outcomes
Fraser, 2008, Israel[41]	An open label, parallel design, quasi-randomized (allocation by alternation) controlled trial	Overweight /obese patients with T2DM	3 groups at 6/12 mo: 1. ADA diet, *n* = 64/54; 2. Low GI diet, *n* = 73/64; 3. Modified MD, *n* = 64/61. Energy contents similar in all three diets	ALT levels significantly decreased at 6 and 12 mo in modified MD *vs* low GI or ADA diets, independently of waist to hip ratio, BMI, HOMA and triacylglycerol values	
Bozzetto, 2012, Italy[43]	Randomized, controlled, parallel-group design	36 overweight /obese patients with T2DM	8 wk, 4 groups: 1. High-CHO/ high-fiber/ low GI diet, *n* = 9; 2. MUFA diet, *n* = 8; 3. High-CHO/ high-fiber/ low GI diet + exercise, *n* = 10; 4. MUFA diet + exercise, *n* = 9.	Liver fat (as measured by ^{1}H MRS) decreased more in groups 2 (-25%) and 4 (-29%) than in groups 1 (-4%) or 3 (-6%). Two-way repeated-measures ANOVA showed a significant effect on liver fat content for MUFA diet, independently of exercise. There were no significant ALT and AST changes in all groups.	At the end of intervention, there were no significant changes in body weight,WC, as well as in glucose, total cholesterol, LDL-C, HDL-C, TG, and HOMA-IR values from baseline in all groups
Ryan, 2013, Australia[42]	A randomized, controlled, cross-over study	12 non-diabetic patients with a biopsy-proven NAFLD at baseline	A cross-over 6-wk dietary intervention study comparing traditional MD *vs* low fat/high-CHO	MD group demonstrated a significant decrease in liver fat (as measured by ^{1}H MRS) compared to the low fat/ high-CHO group (39% *vs* 7%). ALT and GGT did not significantly decrease with either diet	At the end of intervention, no significant changes in body weight, WC, as well as in TG, and HDL-C in both groups. Peripheral insulin sensitivity improved only in the MD group. Systolic BP declined significantly in both groups, though to a lesser degree in the low fat/ high-CHO group
Trovato, 2015, Italy[44]	Single arm	Non-diabetic overweight/obese patients with ultrasound evaluation of liver fat changes from baseline	90 patients following intervention with MD alone for 1, 3, and 6 mo	Liver fat significantly decreased only after 6 mo of intervention. By a multiple linear regression model, changes in adherence to the MD and BMI were found to independently explain the variance of decrease of liver fat (R^2 = 0.519; P < 0.0001). No significant ALT changes were observed throughout the follow-up	Significant decrease of BMI followed by parallel increases of the MD adherence as well as of physical activity were observed from the first month of intervention. Significant decrease of HOMA-IR was observed only after 3 and 6 mo
Abenavoli, 2015, Italy[45]	Controlled randomized study	Overweight/obese patients with ultrasound evaluation of liver fat changes from baseline	6 mo, 3 groups: 1. Hypocaloric MD, *n* = 10; 2. Hypocaloric MD plus Realsil complex, *n* = 10; 3. No treatment, *n* = 10.	Compared to the group that did not undergo any treatment, MD either alone or associated with the Realsil complex led to significant improvement in liver steatosis	Compared to the group that did not undergo any treatment, those following the MD either alone or associated with the Realsil complex had improvement in BMI, WC, hip circumference, as well as in total cholesterol, and TG. Improvement in insulin sensitivity occurred only in patients receiving MD plus the Reasil complex
Misciagna, 2016, Italy[46]	Randomized, controlled, parallel-group design	A population almost composed of non-diabetic overweight/ obese patients (18 to 79 years old, without overt CVD) with ultrasound evaluation of liver fat at baseline and follow-up	3 and 6 mo, 2 groups: 1. MD with low GI, *n* = 44; 2. Control diet(based on INRAN guidelines), *n* = 46	MD with low GI was associated until 55 yr of age, in both men and women, with a more intense reduction in liver fat than a control diet, at both the 3rd and 6th month	Six months after intervention, in both groups, the number of obese patients decreased while the number of overweight subjects increased. Lower TG and glucosemia were found at 6 mo in both groups

Gelli, 2017, Italy[33]	Single arm	46 (11 normal weight; 35 overweight/obese) subjects (42 with ⩾ 1 MetS component; 4 with T2DM) with ultrasound evaluation of liver fat at baseline and follow-up	All patients followed intervention with MD alone for 6 mo	At end-intervention, the percentage of patients with hepatic steatosis grade ⩾ 2 was reduced from 93% to 48%; mean AST, ALT, GGT decreased significantly	At end of intervention, of the 35 overweight/obese patients, 12 showed ⩾ 7% weight reduction while 7 achieved normal weight; mean serum total cholesterol, HDL-C, AST, TG, glucose concentrations, and HOMA-IR values significantly improved

ADA: American Diabetes association; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; BP: Blood pressure; CHO: Carbohydrates; GGT: Gamma-glutamyl transferase; GI: Glycemic index; ^{1}H MRS: Proton magnetic resonance spectroscopy; HDL-C: High density lipoprotein-cholesterol; HOMA-IR: Homeostasis model assessment of insulin resistance; INRAN: Italian National Research Institute for Foods and Nutrition; LDL-C: Low density lipoprotein-cholesterol; MD: Mediterranean diet; MetS: Metabolic syndrome; MUFA: Monounsaturated fatty acid; T2DM: Type 2 diabetes mellitus; TG: Triglycerides; WC: Waist circumference.

triglyceride and insulin resistance values[45]. In a randomized controlled study, Misciagna *et al*[46] compared two groups of non-diabetic overweight-obese patients with moderate/severe ultrasound-diagnosed NAFLD who followed, respectively, a control diet (based on the Italian National Research Institute guidelines) and a low glycemic Index Mediterranean Diet (LGIMD). Compared to the control diet, LGIMD resulted in a major reduction of liver fat at both 3th and 6th month[46]. Finally, very recently, in a single arm, observational study, Gelli *et al*[33] treated with MD 46 normal weight (n = 11) or overweight/obese (n = 35) patients with NAFLD. They determined liver enzymes, metabolic parameters, CVD risk indexes, and ultrasound-based NAFLD severity. At the end of treatment, the proportion of patients with liver steatosis grade ⩾ 2 was reduced from 93% to 48%. Also, metabolic parameters and liver enzymes decreased significantly[33].

Several points need be considered when interpreting the results of the aforementioned studies. First, they were based on high-risk populations, therefore not representative of the general population. Second, most of them were based on a small sample size. Notably, none of the studies provided information on how sample size was calculated and how participants were randomly assigned to the intervention groups. As matter of fact, there may be synergistic and antagonistic interactions among food components of MD that may be difficult to detect unless very large samples are used. Third, MD includes a variety of eating patterns and, therefore, a wide range in assessment score items. As such, using a score for assessment of adherence to a dietary pattern is limited by subjectivity, leading therefore to a great variability in interpretation of study results. Fourth, the majority of studies utilized ultrasonography that is known to be highly operator-dependent, and to have limited repeatability and reproducibility. In addition, ultrasonography has shown low accuracy in assessing severity of liver disease including presence and extent of fibrosis[47]. Fifth, most studies failed to take into account total energy intake. Finally, most studies failed to adjust for potential confounders including physical activity, and socioeconomic and cultural levels, which might have influenced lifestyle habits of the population studied.

BIOLOGICAL MACHANISMS OF MEDITERRANEAN DIET

Anti-inflammatory and antioxidant effects of MD components

MD is based on compounds, such as polyphenols, vitamins and other biomolecules that have anti-inflammatory and antioxidant effects. This seems to be relevant, since inflammation and oxidative stress play a central role in the pathogenesis of NAFLD/NASH.

Polyphenols are present in whole-grain cereals, vegetables and fresh fruits, olive oil, nuts and red wine. They are a heterogenic group of bioactive compounds, including several hydro-soluble antioxidants, characterized by a phenolic structure[48]. Based on their chemical structure, there are two categories of polyphenols: flavonoid polyphenols, and the non-flavonoid polyphenols[49].

Flavonoids are polyphenolic compounds that are ubiquitously found[50] and provide much of the flavor and color to fruits and vegetables. They have hepato-protective effects in view of their antioxidant and anti-inflammatory potential[49,51-53]. Among non-flavonoids, resveratrol, a stilbene polyphenol content in red wine, has been shown to exert hepato-protective activity by affecting the three interacting components of homeostasis such as the vessel, the blood platelets and the clotting and the fibrinolytic system of plasma[54,55]. Vitamins, which are significant components of MD, can also be considered dietary antioxidants. They reduce cellular stress and, in this way, they have a pivotal role in preventing NAFLD progression. Vitamin E has been shown to improve histological features of NASH[56-59]. Vitamin D has immunomodulatory, anti-inflammatory and anti-fibrotic properties while vitamin D supplementation has been demonstrated to ameliorate NAFLD histopathology[60,61]. When incubated with isolated rat liver, vitamin C has been shown to decrease levels of mitochondrial reactive oxygen species generation, and to increase the levels of antioxidant enzymes and the activity of the electron transport chain[62].

Carotenoids are also part of MD; they comprise a class of natural fat-soluble pigments acting as antioxidants,

which are found in several fruits and vegetables[63]. Among them, lycopene has been investigated as a potential protective agent in NAFLD in view of its potent antioxidant effects[64]. Studies in lycopene-fed rats have shown that lycopene has a preventive effect on experimental NASH by reducing steatosis and inflammation as well as oxidative stress[65].

Lipid-lowering effect of MD components

The beneficial effects of MD on the hepatic lipid metabolism and, consequently, on NAFLD prevention, is influenced primarily by its fatty acid composition which is characterized by high MUFA content with a balanced PUFA omega-6 to omega-3 ratio due to the abundance of vegetables, legumes, nuts, olive oil and fish (instead of red meats)[66]. It has been proved that MUFA intake may prevent the development of NAFLD by improving plasma lipid levels, reducing body fat accumulation and decreasing postprandial adiponectin expression[67,68]. PUFA regulate three major transcriptional factors controlling multiple pathways involved in hepatic carbohydrate and lipid metabolism. PUFA activation of hepatic peroxisome proliferator-activated alpha (PPARα) enhances fatty acid oxidation, while PUFA suppression of sterol regulatory element binding protein-1 (SREBP-1) and of carbohydrate regulatory element binding protein (ChREBP)/Max-like factor X (MLX) results in the inhibition of glycolysis and of *de-novo* lipogenesis. As such, PUFA promote a shift in metabolism toward fatty acid oxidation and away from fatty acid synthesis and storage, and may positively affect NAFLD[69,70]. In addition to improvement in steatosis, PUFA may induce an independent, anti-inflammatory effect *via* suppression of tumor necrosis factor and interleukin-6, responsible for the inflammation occurring in NASH[71]. Opposite health effects have been found regarding the role of n-6 PUFA on NAFLD. N-6 PUFA, such as linoleic acid may have a pro-inflammatory role due to their direct relation with the production of arachidonic acid (AA). AA is metabolized to give rise to the eicosanoid family of inflammatory mediators (*e.g*. prostaglandins, leukotrienes and related metabolites), and through these to regulate the production of inflammatory cytokines[72]. Excessive amounts of omega-6 PUFA and a very high omega-6 to omega-3 ratio have been involved in the pathogenesis of many diseases, including CVD, cancer, and inflammatory and autoimmune diseases[73]. A proportionally high intake of n-6 PUFA is considered pro-inflammatory and possibly associated with an increased risk of MetS. Therefore, not only PUFA intake but also the n-6 PUFA to n-3 PUFA ratio is relevant.

Several studies have shown that a reduced intake of saturated fat is associated with a reduction of plasma concentrations of total cholesterol, very low density lipoprotein (LDL)-cholesterol and triglycerides[74].

MD can also contribute to lowering plasma cholesterol by high consumption of water-soluble fibers which are found in large concentration in some MD components, mainly beans, vegetables and fruits and whole-grain cereals. Water-soluble fibers have been shown to increase the rate of bile excretion therefore reducing serum total and LDL cholesterol[75].

GUT MICROBIOTA AND MD COMPONENTS

The liver is closely connected to the gut as it receives about 70% of its blood supply directly from the intestine *via* the portal vein. Therefore, it is one of the organs mostly exposed to gut-derived toxic products, such as bacteria and bacterial derivates. This cross-talking between the intestine and the liver is known as the "gut-liver axis" and has been linked to liver pathologies, including NAFLD. The relationship between NAFLD and altered microbiota is mainly supported by studies on animal models[76,77].There are limited data in humans[78,79]. Gut microbiota plays a crucial role in the complex pathogenesis of NAFLD through a variety of mechanisms such as predisposition to obesity, induction of insulin resistance as well as of liver inflammation, and alteration of choline metabolism[80]. Other mechanisms include increased microbiome-modulated metabolites such as bile acids, short chain fatty acids, lipopolysaccharides as well as dysbiosis-induced intestinal barrier dysfunction[81]. Many different factors may influence microbiota composition, including age, comorbid conditions, host genotype and exposure to antibiotics, and dietary habits[82]. Diet largely influences gut microbiota and its products[83]. Specific dietary factors, such as macronutrient composition (*e.g.* increased protein intake), food type (*e.g.* glycemic index or load) or the presence of specific bioactive compounds (omega-3 fatty acids, fibers or polyphenols) have been shown to influence the diversity and functionality of the gut microbiota[84]. Also protein, insoluble fibers and fat content have important effects on gut microbiota structure, function, and its secretion of metabolites that modulate immune function and multiple metabolic and inflammatory pathways[85-87]. Therefore, MD may have a significant impact on the composition and diversity of the microbiota. As MD is characterized by a high dietary fiber intake, it promotes beneficial modification of the gut microbiota with decreased *Firmicutes* and increased *Bacteroides*, which have been shown to ameliorate obesity, inflammation and related metabolic alterations. Polyphenols contained in MD induce an increase in *Bifidobacteria*, associated with various metabolic benefits such as plasma cholesterol reduction and a decrease of C-reactive protein (CRP)[88]. Gut microbial production of trimethylamine N-oxide from dietary choline and l-carnitine enhances the risk of developing CVD in both animals and humans, independently of CVD risk factors[89]. MD benefits on the gut microbiota could also be the consequence of a low content of choline and l-carnitine in MD diet.

CONCLUSION

MD, low in saturated fats and animal protein, high in antioxidants, fiber and MUFA, and with an adequate omega-3 to omega-6 fatty balance, represents an healthy dietary pattern, which has been shown to decrease CVD, MetS, and type 2 diabetes. Although MD seems particularly attractive for its potential to improve liver status, literature concerning the efficacy of this dietary pattern in patients with NAFLD is still limited to few cross-sectional as well as to few longitudinal studies with certain limitations. In particular, longitudinal studies have included small sample size, short-term follow-up, different designs, different time points of data collection, and above all poor methodology for reporting the trial or diagnosing the liver outcome and its associated comorbidities, anyone of which or any combination of which may limit the generalizability of study results. There is room for adequate randomized dietary intervention trials comparing MD with a control diet in a large sample of the general population, along with a validation of the MD indexes in the heterogeneous patient population with NAFLD.

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ORIGINAL ARTICLE

Basic Study

Detection of hyper-conserved regions in hepatitis B virus X gene potentially useful for gene therapy

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Abstract

AIM

To detect hyper-conserved regions in the hepatitis B virus (HBV) X gene (*HBX*) 5′ region that could be candidates for gene therapy.

METHODS

The study included 27 chronic hepatitis B treatment-naive patients in various clinical stages (from chronic infection to cirrhosis and hepatocellular carcinoma, both HBeAg-negative and HBeAg-positive), and infected with HBV genotypes A-F and H. In a serum sample from each patient with viremia > 3.5 log IU/mL, the *HBX* 5′ end region [nucleotide (nt) 1255-1611] was PCR-amplified and submitted to next-generation sequencing (NGS). We assessed genotype variants by phylogenetic analysis, and evaluated conservation of this region by calculating the information content of each nucleotide position in a multiple alignment of all unique sequences (haplotypes) obtained by NGS. Conservation at the HBx protein amino acid (aa) level was also analyzed.

RESULTS

NGS yielded 1333069 sequences from the 27 samples, with a median of 4578 sequences/sample (2487-9279, IQR 2817). In 14/27 patients (51.8%), phylogenetic analysis of viral nucleotide haplotypes showed a complex mixture of genotypic variants. Analysis of the information content in the haplotype multiple alignments detected 2 hyper-conserved nucleotide regions, one in the *HBX* upstream non-coding region (nt 1255-1286) and the other in the 5′ end coding region (nt 1519-1603). This last region coded for a conserved amino acid region (aa 63-76) that partially overlaps a Kunitz-like domain.

CONCLUSION

Two hyper-conserved regions detected in the *HBX* 5′ end may be of value for targeted gene therapy, regardless of the patients' clinical stage or HBV genotype.

Key words: Hepatitis B virus; Hepatitis B X gene; Hepatitis B X protein; Gene therapy; Next-generation sequencing; HBV conserved regions; Small interference RNA

Core tip: Hepatitis B virus (HBV) is not cured with classic treatments, and liver disease can progress by persistence and expression of covalently-closed circular DNA. Gene therapy with small interference RNA may be an effective approach to ensure inhibition of viral expression and disease progression, and hepatitis B virus X gene (*HBX*) transcripts could be optimal targets for this therapy. This study includes patients with different HBV genotypes and clinical stages to cover many clinical and virological situations. Using next-generation sequencing, we found two hyper-conserved *HBX* regions, candidates for small interference RNA therapy, which could enable pan-genotypic inhibition of HBV expression, regardless of the patients' disease status.

González C, Tabernero D, Cortese MF, Gregori J, Casillas R, Riveiro-Barciela M, Godoy C, Sopena S, Rando A, Yll M, Lopez-Martinez R, Quer J, Esteban R, Buti M, Rodríguez-Frías F. Detection of hyper-conserved regions in hepatitis B virus X gene potentially useful for gene therapy. *World J Gastroenterol* 2018; 24(19): 2095-2107 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i19/2095.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i19.2095

INTRODUCTION

Despite the efficacy of preventive vaccines, an estimated 257 million people are living with chronic hepatitis B virus infection (CHB) and more than 880000 people die each year of hepatitis B virus (HBV)-related complications such as cirrhosis and hepatocellular carcinoma (HCC) (WHO report, July 2017).

HBV is an enveloped DNA virus with partially double-stranded circular DNA. HBV replication requires RNA intermediate and the activity of a reverse transcriptase. This implies a high probability that genetic mutations will occur, as the reverse transcriptase lacks 3′ to 5′ proofreading activity, leading to a viral mutation rate of 10^{-4} to 10^{-5} substitutions/site/year, similar to that observed for RNA viruses[1]. Inter- and intra-genotype recombination events can further increase HBV variability[2]. Hence, HBV circulates as a complex mixture of genetic variants, known as a quasispecies[3], that enables the virus to escape from the host's immune system, antiviral treatment, and vaccination, thereby promoting progression to CHB. Furthermore, the mutational profile is closely associated with HBV genotype, and the genotype is associated with differing effectiveness of the treatments used and outcomes of the infection[4,5].

The main therapeutic approach for HBV infection is based on inhibition of the viral polymerase by the action of nucleotide analogues, whose goal is to improve the patients' quality of life and prolong survival by preventing progression of the disease[6]. However, HBV cannot be completely eradicated with these drugs because the viral intermediate known as covalently closed circular DNA (cccDNA) can persist within the nucleus of HBV-infected liver cells. cccDNA interacts

with histone and non-histone proteins, including viral proteins such as the core and X protein (HBx), and forms a minichromosome that permits transcription of *HBV* genes[7], including pregenomic RNA, the precursor of *de novo* viral DNA genomes. Because cccDNA persists, it constitutes a viral reservoir that could promote reactivation of the infection after treatment interruption[8]. Within this challenging scenario, research has been aimed at deeply investigating the host-virus interactions to better understand the mechanisms that establish persistent HBV infection and to find new therapeutic targets that can cure it.

In this line, new treatment approaches are currently under development[9], with gene therapy being a promising option. Homing endonucleases, such as zinc-finger endonucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and RNA-guided clustered regulatory interspaced short palindromic repeats associated with the Cas endonuclease family (CRISPR/Cas), can cleave selected sequences in cccDNA, resulting in disruption of the gene due to nonspecific DNA repair with consequent elimination of the viral minichromosome[10,11]. However, systematic random integration of the viral genome in the host genome could represent a strong limitation to this strategy. Indeed, the activity of this "molecular scissors", although sequence-specific, could entail a potential risk of damage for the human genes close to the viral site of integration.

Another promising gene therapy consists in silencing specific genes at the post-transcriptional level through a sequence-specific interaction between an mRNA target and small interfering RNA (siRNA)[12]. With this approach, various regions of the viral mRNA sequence can be targeted, including non-coding regions, without affecting the host DNA. Although these therapies show promise, the high variability of HBV and the association between this variability and the patients' clinical outcome suggests that it may be important to find a highly conserved target to guarantee their efficacy.

A good candidate for targeted gene therapy could be the HBx protein, encoded by the HBV X gene (*HBX*). This pleiotropic and multifunctional protein trans-activates the expression of the viral genes. Together with the HBV core protein (HBc), HBx attaches to the cccDNA structure and is crucial for HBV replication[7]. In addition, this protein interacts with several cell signaling pathways and genes, thus affecting many cellular activities[13-15]. Due to its wide range of activity, HBx plays a key role in the pathogenesis of HBV infection and disease progression, and is strongly associated with HCC. Hence, it could be an optimal target for a hypothetical curative therapy for HBV infection.

The *HBX* gene, nucleotides (nt) 1374-1838, contains important regulatory elements[16,17]. The coded protein is comprised of 2 domains. The N-terminal domain [amino acid (aa) 1-50, encoded by the 5' end of the gene] acts as negative regulator of the HBx transactivation function, which resides in the C-terminal domain (aa 51-154, encoded by the 3' end). Interestingly, a significant presence of multiple variants with deletions and/or insertions (indels) has been found in the 3' end of *HBX*[18-20]. Considering this variability, the 3' coding region of the X gene would be ruled out as a possible therapeutic candidate[21]. However, the conservation at 5' end of *HBX* and its potential for use as a gene therapy target remains unexplored. To silence *HBX* at the post-transcriptional level, the non-coding region included in *HBX* transcripts, upstream of the coding region, should also be considered. The *HBX* gene is located near the co-terminal 3' end; hence, all HBV mRNAs produced during the infection include this sequence (Figure 1). Consequently, by targeting *HBX* transcripts at the coding or non-coding level, interference with expression of all the viral proteins could be achieved.

The aim of this study was to determine the conservation of a region of the HBV genome encompassing the *HBX* 5' coding region and upstream non-coding region (included in all HBV transcripts) in samples from HBV-infected patients in various clinical stages and with different viral genotypes. The ultimate objective was to find hyper-conserved regions that might be feasible targets for gene therapy, which could be used whatever the patient's clinical status or HBV genotype.

MATERIALS AND METHODS

Patients and samples

From a cohort of 46 well-characterized CHB patients attending the outpatient clinic of Vall d'Hebron University Hospital (Barcelona, Spain), we selected a group of 27 patients in various clinical stages and with different viral genotypes. The samples included were 17 from HBeAg-negative patients (3 with chronic infection and14 chronic hepatitis, 2 of them with cirrhosis and 1 with HCC), and 10 from HBeAg positive (2 with chronic infection and 8 with chronic hepatitis, 3 of them with cirrhosis and 2 with HCC, characterized according to the latest EASL guidelines[6]), infected with several HBV genotypes: 5 A, 1 B, 7 C, 8 D, 2 E, 3 F, 1 H (Table 1).

All 27 patients were treatment-naïve, tested negative for hepatitis D virus (HDV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV), and had a serum sample with viremia levels > 3.5 logIU/mL, the sensitivity limit of the PCR to amplify the studied region (described below). The study was approved by the Ethics Committee of Vall d'Hebron Research Institute, and all patients signed a consent form to participate.

Serological and virological determinations

HBV serological markers (HBsAg, HBeAg, and anti-HBe) and anti-HCV antibodies were tested using commercial chemiluminescent assays on a COBAS 8000 analyzer (Roche Diagnostics, Rotkreuz, Switzerland). Antibodies against HDV were tested using the HDV Ab kit (Dia.Pro

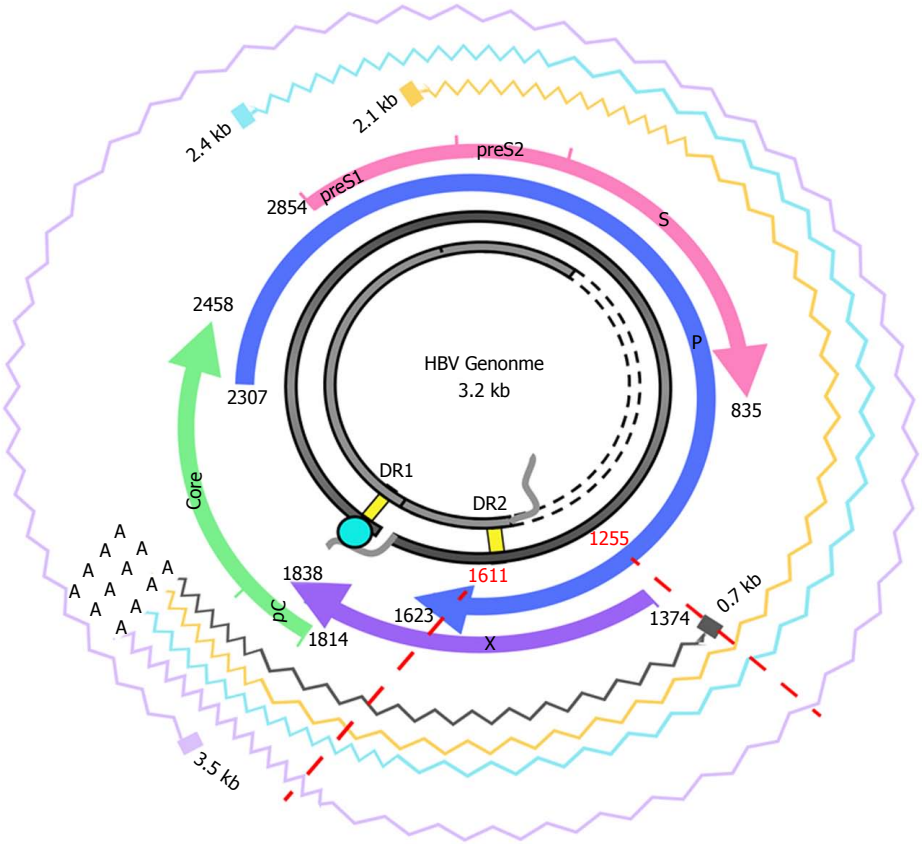

Figure 1 Hepatitis B virus genome and transcripts. The figure shows the HBV genome (in grey), with the DR1 and DR2 (direct repeat) regions, necessary for viral DNA synthesis. The viral ORFs are highlighted with colored arrows, and nucleotide positions are reported. Wavy lines show the various HBV transcripts: the 3.5-kb transcript, corresponding to the pregenomic RNA, which is translated to the core and polymerase and later subjected to reverse-transcription in the viral capsid, or to the precore/core transcript, which is translated to the precore protein; the 2.4-kb and 2.1-kb transcripts, which are translated, respectively, to large and medium/small HBsAg); and the 0.7-kB transcript, which is translated to the HBx protein. The region analyzed in this study and its corresponding nt positions are indicated by red dashed lines. Note that the region of interest is included in all the viral transcripts. HBV: Hepatitis B virus; ORF: Open reading frame.

Diagnostic Bioprobes, Sesto San Giovanni, Italy), and anti-HIV antibodies were tested by the Liaison XL murex HIV Ab/Ag kit (DiaSorin, Saluggia, Italy). HBV-DNA was quantified by real-time PCR with a detection limit of 10 IU/mL (COBAS 6800, Roche Diagnostics). HBV genotypes in the region of interest were determined by Sanger sequencing and by phylogenetic analysis with the same regions extracted from 102 full-length HBV genome sequences representative of HBV genotypes A to H, obtained from GenBank (Supplementary Table 1 and Supplementary Figure 1).

Amplification of the region of interest

In this study we analyzed a portion of the HBX gene encompassing *HBX* gene encompassed nt 1255 to nt 1611, a region included in the 5′ end of all the viral transcripts. It covered a non-coding upstream region (nt 1255-1373) and the 5′end of the *HBX* coding region (nts 1374-1611), encoding aa 1 to 79 of HBx.

HBV DNA was extracted from 500 μL of serum with the QIAamp UltraSens Virus Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Molecular amplification was performed by nested PCR. The first PCR round used primers carrying the universal adaptor M13 (underlined sequence) in their 5′ end (forward 5′-GTTGTAAAACGACGGCCAGT ATGCGTGGAACCTTTGTGGCT-*3′* and reverse 5′-CACAGGAAACAGCTATGACCATGGGCGTTCACGGT GGTCT-3′) using the following protocol: 95 ℃ for 2 min, followed by 30 cycles of 95 ℃ for 15 s, 60 ℃ for 20 s, and 72 ℃ for 15 s, and finally, 72 ℃ for 3 min. The second PCR round was performed using the primers: forward 5′-CGTATCGCCTCCCTCGCGCCATCAG-MID-GTTGTAAAACGACGGCCAGT-3′ and reverse 5′-CTATGCGCCTTGCCAGCCCGCTCAG-MID-CACAGGA AACAGCTATGACC-3′. These primers included the 2 adaptors for the ultra-deep pyrosequencing system at their 5′ ends, followed by a unique identifier multiplex identifier sequence (MID), which enabled grouping the sequences for each sample/patient, and the same M13 universal adaptor sequences as those used in the first PCR in the 3′ ends. This second amplification protocol comprised one denaturation step of 95 ℃ for 2 min, followed by 20 cycles of 95 ℃ for 15 s, 60 ℃ for 20 s, and 72 ℃ for 15 s, and finally, 72 ℃ for 3 min. All PCR steps were performed using high-fidelity Pfu Ultra II DNA polymerase (Stratagene, Agilent Technologies, Santa Clara, United States). The final PCR products (amplicons)

Table 1 Main clinical and virological characteristics of the hepatitis B virus infected patients enrolled

Patient	Age	Sex	Origin	Clinical stage	HBeAg	HBV DNA (log IU/mL)	ALT (IU/L)	Genotype[1]
1	27	M	Sub-Saharan	Chronic hepatitis	Negative	7.8	170	E
2	31	M	Asian	Chronic hepatitis	Negative	6.6	103	C
3	51	M	Caucasian	Chronic hepatitis	Negative	6.6	262	D
4	28	F	Caucasian	Chronic hepatitis	Negative	7.9	126	D
5	47	F	Caucasian	Chronic hepatitis	Negative	6.3	170	D
6	37	F	Caucasian	Chronic hepatitis	Negative	4.5	53	D
7	37	M	Caucasian	Chronic hepatitis	Negative	4.5	33	F
8	38	M	Caucasian	Chronic hepatitis	Negative	5.0	29	D
9	46	M	Caucasian	Chronic hepatitis	Negative	5.8	88	F
10	46	F	Caucasian	Chronic hepatitis	Negative	5.3	23	H
11	71	F	Caucasian	Chronic hepatitis	Negative	6.2	87	F
12	51	M	Asian	Chronic hepatitis	Negative	5.7	435	C
13	52	M	Caucasian	Chronic infection	Negative	4.4	18	A
14	40	F	Caucasian	Chronic infection	Negative	4.2	29	D
15	33	M	Asian	Chronic infection	Negative	4.3	25	D
16	63	M	Hispanic	Cirrhosis	Negative	4.0	16	A
17	53	M	Caucasian	Cirrhosis/HCC	Negative	3.7	36	A
18	35	M	Sub-Saharan	Chronic hepatitis	Positive	5.7	36	E
19	37	M	Caucasian	Chronic hepatitis	Positive	8.4	32	C
20	45	M	Caucasian	Chronic hepatitis	Positive	5.6	35	A
21	29	F	Asian	Chronic hepatitis	Positive	6.9	355	B
22	28	M	Asian	Chronic hepatitis	Positive	> 8.0	341	C
23	28	M	Asian	Chronic infection	Positive	8.7	24	C
24	28	F	Asian	Chronic infection	Positive	8.8	22	C
25	55	F	Caucasian	Cirrhosis	Positive	5.4	73.9	A
26	82	F	Caucasian	Cirrhosis/HCC	Positive	4.8	24	C
27	64	M	Caucasian	Cirrhosis/HCC	Positive	6.3	45	D

[1]Genotype determined by Sanger sequencing of the X region (same region as was analyzed by next-generation sequencing). ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; M: Male; F: Female; HBV: Hepatitis B virus.

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

Next-generation sequencing and sequence quality control

Purified DNA from each sample was quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific - Life Technologies, Austin, United States), and a pool was formed in which each amplicon was adequately represented in the analysis. The pool was sequenced by next-generation sequencing (NGS) based on ultra-deep pyrosequencing (UDPS) on the GS-Junior or GS FLX platforms (454 Life sciences-Roche, Branford, United States), following the manufacturer's protocol. The two platforms are reported to be interchangeable[22].

The sequences (reads) obtained after UDPS underwent an in-house bioinformatics filtering procedure, based on scripts developed in R language[23], as previously described by our group[22]. Briefly, the sequences were assigned to each patient (demultiplexed) according to their specific MID, and primers were trimmed. After a general quality filter step, reads with the same nt sequence were collapsed into haplotypes (unique sequences covering the full amplicon observed on the clean set of sequences). Only haplotypes common to the forward and reverse strands and present in abundances of at least 0.1% were accepted; their final frequencies were calculated as the sum of reads observed in each strand. Finally, haplotypes with abundances below 0.25% were excluded.

To analyze the aa sequence of HBx, all individual nt haplotypes from each patient were translated into aa sequences in the *HBX* gene open reading frame (ORF), which was translated from frame 2. In the fragment analyzed (nt 1255-1611) this ORF expanded from nt 1374 to 1611, encoding aa 1 to 79 of the HBx protein. The upstream sequence was not translated, as it corresponded to a non-coding region whose sequence is included in the *HBX* transcripts. Once translated, identical aa sequences were recollapsed into aa haplotypes and their frequencies were updated.

Genotyping of the region haplotypes

The genotype of the nt haplotypes obtained by UDPS was determined by discriminant analysis with the same regions extracted from the 102 full-length patterns used for Sanger sequencing (Supplementary Table 1 and Supplementary Figure 1). We determined the maximum genetic distances between sequences from the same HBV genotype in this region and the minimum genetic distances between sequences from different genotypes,

in order to set a sequence identity threshold: sequences with an identity above this threshold were clustered together. Genotyping of each cluster centroid was done by distance-based discriminant analysis (DB rule)[24,25], which takes into account the inter- and intra-class variability of all genotypes. Genetic distances were computed according to the Kimura-80 model[26].

Conservation analysis

Sequence conservation was determined by calculating the information content (IC) of each position in a multiple alignment of all the different sequences found in the patients. This analysis, based on Shannon's uncertainty, was done for a multiple alignment of nt and aa sequences, and is defined as[27]:

$$ICj\ (\text{nt}) = \log_2(4) - \sum_{i=1}^{4} pij\ \log_2(pij)$$

$$ICj\ (\text{aa}) = \log_2(20) - \sum_{i=1}^{20} pij\ \log_2(pij)$$

where *j* stands for the *j-th* position in the alignment, *i* runs over the 4 nucleotides (or over the 20 aa), and p_{ij} is the frequency of the *i-th* nucleotide (or aa) in the *j-th* alignment position. IC ranges from 0, indicating maximum uncertainty or variability, to $\log_2 4$ (*i.e.*, 2 bits) for nt or $\log_2 20$ (*i.e.*, 4.32 bits) for aa, indicating maximum information or conservation.

When considering variability in human genetics, a mutation is commonly considered fixed if it is found in at least 1% of the population[28]. However, in viral quasispecies, variants can be present at any abundance in a patient, and the limit for defining a fixed mutation has not yet been established. Taking that into account, we considered two scenarios providing limiting values in our analysis. In the first scenario, we only included the most abundant nucleotide at each position in each patient (consensus approach). The IC values computed in this way would be the upper limit of conservation. In the second scenario, we included all variants in the haplotypes from each patient that were present at abundance greater than 0.25% (quasispecies approach). The IC values computed in this way would be the lower limit of conservation.

Sliding window analysis was then carried out to locate the fragment of at least 25 nt or 10 aa (which corresponds to the length of a possible target for siRNA therapy) with the highest IC within the multiple alignments. This analysis uses windows of 25 nt (or 10 aa) starting from the first position in the multiple alignments and moves forward in steps of 1 (nt or aa). For each window, the analysis computes the mean IC of each position within the window. In addition, the results are represented as sequence logos created using the R language package motifStack[27].

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

RESULTS

Analysis of the NGS sequences obtained and genotyping results

After applying the quality filters, 1333069 sequences were obtained from the 27 serum samples, yielding a median (IQR) of 4578 (2478-9279) sequences per patient.

In the region from nt 1255 to 1611 extracted from the 102 full-length HBV genome sequences from GenBank, analysis of the maximum genetic distance within the same genotype (data not shown) resulted in a sequence identity threshold of 96%. Therefore, for each patient, haplotypes with a sequence identity > 96% were clustered together and were considered to belong to the same HBV genotype. Results of the phylogenetic analysis of master sequences from each cluster in each patient and the 102 GenBank patterns are shown in Table 2. Genotype D nt haplotypes were the most frequent in our patients, followed by genotypes C, A, E, F, B, and H. None of the patients included showed genotype G haplotypes. Moreover, in 14/27 cases (51.8%), some haplotypes were found corresponding to different genotypes than those previously identified by Sanger sequencing, thus yielding a complex mixture of genotypic variants.

Conservation of the HBX nucleotide sequence in the region of interest

The region of interest was studied in multiple nt alignments of the entire quasispecies in order to highlight the most highly conserved regions. Sliding windows analysis was implemented in two scenarios: using the consensus approach (n = 27 sequences) and using the quasispecies approach (n = 720 sequences). Of note, the relative frequency of each haplotype was not considered in the multiple alignments, so that the conservation results would not be influenced by haplotype fitness. As no differences were seen when the analyses by the 2 approaches were superimposed (2 highly conserved regions with a mean IC near 2 bits were observed in both; Figure 2), the results reported below all refer to the analysis in the quasispecies scenario.

The first hyper-conserved region identified was between nt 1255 and 1286 (23 nt in length) (Figure 3A). Most of the nucleotide positions showed high conservation, yielding IC values near 2 bits (100% maximum conservation), with the exception of position 1272 which showed an IC between 1.6 and 1.8 bits (80%-90% maximum conservation) and positions 1258 and 1284, with an IC between 1.4 and 1.6 bits (70%-80% maximum conservation).

The second hyper-conserved region consisted of 3 conserved nt fragments (1519-1543, 1545-1573, and 1575-1603: 25, 29, and 29 nt in length, respectively)

Table 2 Results of genotyping of nucleotide haplotypes obtained in each patient, extracted by next-generation sequencing based on ultra-deep pyrosequencing analysis %

Patient	A	B	C	D	E	F	H
1	0	0	0	0	100	0	0
2	0	0	100	0	0	0	0
3	7.1	0	0	92.9	0	0	0
4	0	0	0	100	0	0	0
5	0	0	0	100	0	0	0
6	1.7	0	0	98.3	0	0	0
7	0	0	0.3	51.3	0	48.4	0
8	7.1	0	0	92.9	0	0	0
9	0	0	0.3	7	0	92.7	0
10	0.9	0	0	50.9	0	0	48.2
11	0	0	0.3	7	0	92.7	0
12	95.1	0	4.4	0.5	0	0	0
13	0	0	100	0	0	0	0
14	46.6	0	8.2	33.2	0	12	0
15	89.7	0	4.4	8	0	1.5	0
16	0	0	0	100	0	0	0
17	0	0	0	100	0	0	0
18	0	0	0	0	100	0	0
19	0	0	95.3	3,6	0.9	0	0
20	100	0	0	0	0	0	0
21	0	99.6	0.4	0	0	0	0
22	0	0	100	0	0	0	0
23	0	0	87.8	12.2	0	0	0
24	0	0	100	0	0	0	0
25	97.9	0	0	2.1	0	0	0
26	0	0	100	0	0	0	0
27	0	0	0	100	0	0	0

%A to %H indicates the percentage of nucleotide haplotypes from each patient, classified as HBV genotype A, B, C, D, E, F or H. HBV: Hepatitis B virus.

spanning a region between nt 1519 and 1603 (85 nt). Five of these 85 nt positions (5.9%) showed an IC below 1.8 bits: positions 1527, 1557, 1589, and 1602 between 1.6 and 1.8 bits, and position 1524 between 1.4 and 1.6 bits (Figure 2B).

Conservation of the HBx amino acid sequence

To further confirm the nt conservation found, we also analyzed aa conservation in the same 2 scenarios considered for nt variants (n = 27 sequences for the consensus and n = 330 for the quasispecies approach). As was seen with the nt sequences, there were no difference when the 2 analyses (quasispecies *vs* consensus) were superimposed (Figure 4), which highlighted a single highly conserved region. Again, the results reported refer to the analysis using the quasispecies approach.

One highly conserved region was identified between aa 63 and 76 (13 aa), which included a portion of a Kunitz-like domain (Figure 5). All aa showed conservation near 4 bits (100% maximum conservation). This region in the HBx protein corresponded to the hyper-conserved nt sequence between positions 1563 and 1602. The first hyper-conserved nt region observed (nt 1255-1286) was not taken into account in this analysis, as it corresponded to a non-coding region and therefore, was not translated into aa.

DISCUSSION

Although classic nucleotide analogue-based therapies can effectively control HBV infection, eradication of the virus is not achieved because of persistence of the viral minichromosome, cccDNA. Furthermore, even though HBV replication can be inhibited by drug treatment, production of viral antigens may be maintained, and this could lead to progression of the disease[29]. To overcome this challenge, new therapeutic approaches are needed, and gene therapy has emerged as an interesting option.

Ramanan *et al*[30] proposed a gene therapy based on CRISPR/Cas9 to specifically target a conserved region in HBV cccDNA. These authors reported an anti-HBV effect both *in vitro* and *in vivo,* together with inhibition of *de novo* infection in HepG2-hNTCP cells. However, in HBV infection, the viral genome may be inserted in the host genome. Hence, it is possible that a molecular scissors strategy, such as the CRISPR/Cas9 approach, might imply a risk of affecting the host genome in the regions of viral genome insertion.

With the siRNA approach, viral replication could be hampered and disease progression limited by direct interference with the viral messengers. As has been seen in both cell and mouse models[12,31-33], this interfering RNA regulates the expression of specific viral genes by promoting cleavage of targeted mRNAs, thus

Figure 2 Sliding window analysis of the nucleotide region of interest in the *hepatitis B virus X* gene (nt 1255-1611). Each point on the graph is the result of the mean information content (IC, bits) of the windows 25-nt in size, with displacement between them in 1-nt steps. The purple line represents the mean IC from the multiple alignments of all haplotypes (in abundance > 0.25%) in the quasispecies (QS) of all patients (n = 720), whereas the blue line shows the mean IC obtained from the multiple alignments of the consensus obtained for each patient (n = 27).

inhibiting HBV replication. Specifically, siRNA promotes target mRNA cleavage in a sequence-specific manner through the RNA-induced silencing complex (RISC)[34].

Definition of an extremely conserved region in an optimal HBV genomic region, such as the *HBX* gene, could be very useful for siRNA-based gene therapy strategies, and some authors have investigated this concept. In a recent study using predictive software, Thongthae *et al*[33] estimated potential siRNA target sites in the *HBX* gene (positions: 1317-1337, 1357-1377, and 1644-1664) from an HBV genotype A sequence. These were later tested *in vitro*, and a reduction in HBV expression was observed. In another effort, the Arbutus Biopharma Corporation recently published a phase-two study in this line. An siRNA was used as treatment for patients with chronic HBV infection, and the preliminary data indicated that the therapy was well tolerated and led to a significant reduction in HBsAg levels[35,36].

HBX is located near the co-terminal 3' end of all the HBV mRNAs, which implies that interference at this level could abrogate the production of all the viral antigens. In addition, the *HBX* gene encodes a protein, HBx, which plays a key role in the HBV viral cycle. However, previous data reported by our group[37] and supported in other studies[17,38-40] have described considerable variability in the HBx transactivating C-terminal domain (encoded by the 3' end of the gene), with multiple insertions and deletions. Because of this variability, this region would not be considered an appropriate gene therapy target.

In light of the importance of the HBx protein for viral replication, it would be reasonable to posit that the gene encoding this protein would have a conserved region. On that basis and after excluding the 3' end region, we focused our study on the 5' end region of *HBX* and its upstream non-coding region (nt 1255-1611). For a gene therapy to be effective in a broad range of conditions, the target sequence should remain conserved in a wide spectrum of clinical and virological situations. Hence, we analyzed samples from a heterogeneous group of 27 HBV-infected patients (in different clinical stages of HBV infection and with different viral genotypes) to seek a conserved target sequence over this spectrum. Two hyper-conserved regions were found. The first was located between nucleotides 1255 and 1286 in the non-coding region. Of note, *HBX* transcripts initiate at several different sites (between nt 1250-1350)[41], which means that this conserved region might be not present in all of them, but would likely be present in the other viral transcripts. The second hyper-conserved region was located between nucleotides 1519 and 1603, within the coding region.

Conserved regions in this portion of the HBV gene have been reported previously. Karinova *et al*[42] observed two conserved regions in the S and X ORF of the HBV genotype A genome. These authors found that a CRISPR/Cas9 molecular scissor directed to this conserved region in *HBX* was able to modify both episomal cccDNA and chromosomally-integrated HBV DNA in reporter cell lines, thereby interfering with HBV replication and with *de novo* infection of hepatoma cell lines. In addition, with the use of predictive software, Thongthae *et al*[33] estimated some potential siRNA targets in the *HBX* gene (including the non-coding region identified here) in a single viral sequence, and reported the efficacy of this approach in an *in vitro* study. The value of the present study is that conservation of the regions examined was directly substantiated by sequencing analysis of patient samples, taking into account different HBV genotypes and different clinical stages of the infection. Furthermore, the nucleotide conservation documented here was supported by detection of a conserved region in the HBx protein sequence between aa 63 and 76, which is encoded by nt 1563-1602 (within the second hyper-conserved region).

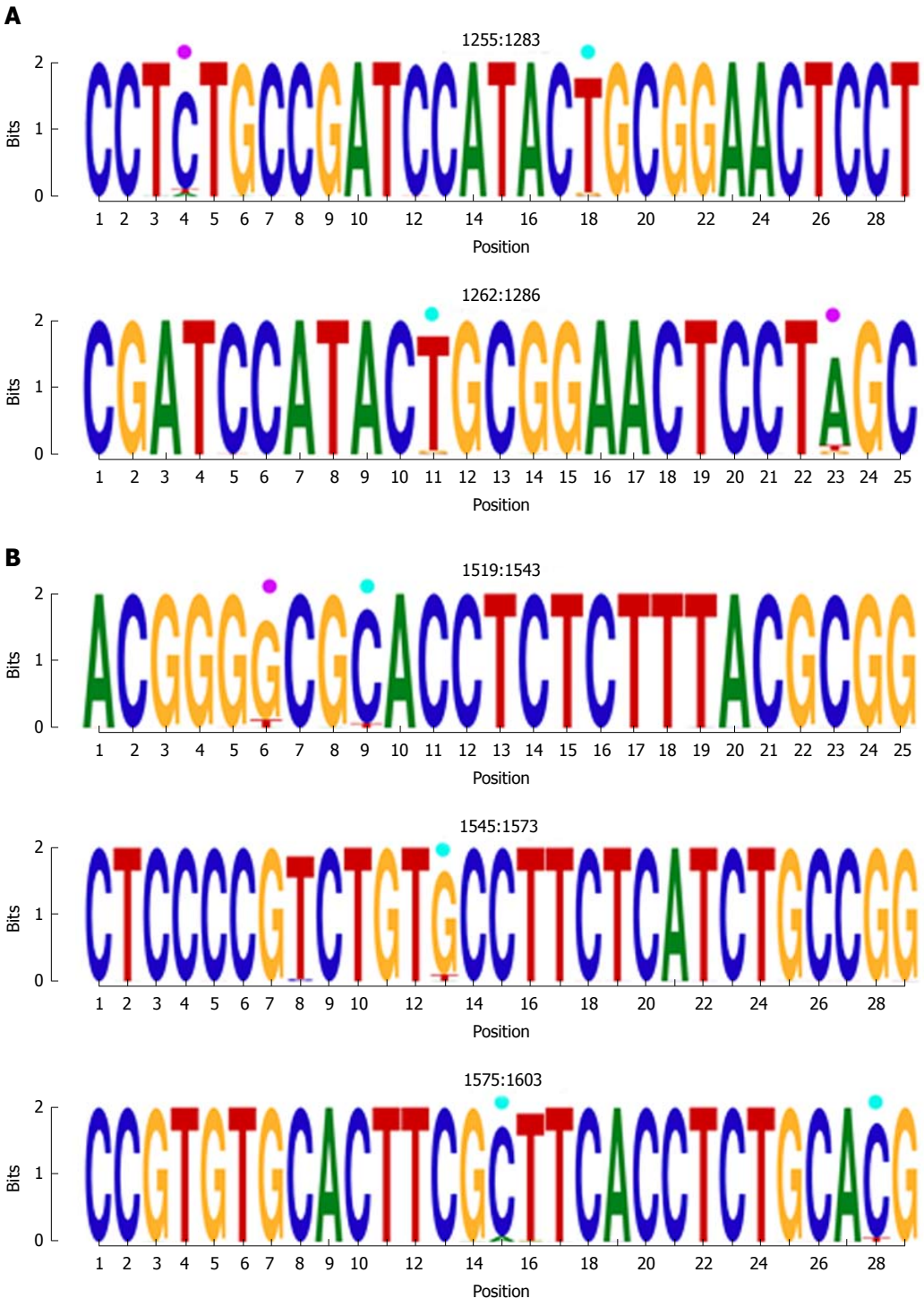

Figure 3 Representation by sequence logos of the information content of the most conserved regions detected in the multiple alignment of all nucleotide haplotypes obtained (quasispecies), nts 1255-1286 and nts 1519-1603. The relative sizes of the letters in each stack of nt sequence logos indicate their relative frequencies at each position within the multiple alignments of nt haplotypes. The total height of each stack of letters depicts the IC of each nt position, measured in bits (Y-axis): 0 bits being the minimum and 2, the maximum conservation. Nucleotide positions with an IC between 1.6 and 1.8 (80%-90% of maximum conservation) are indicated by light blue circles and those with an IC between 1.4 and 1.6 bits (70%-80% of maximum conservation) by pink circles. IC: Information content.

Of note, this fragment includes some aa from one of the HBx Kunitz-like domains (aa 58-70)[43], which are able to inhibit the function of cellular degrading enzymes, such as proteases[44]. This suggests that this portion of the HBx protein may be conserved to preserve the integrity of the protein, protecting it from undesired degradation.

As a limitation of the study, we should mention the relatively small sample size. From the initial group of 46 well-characterized treatment-naïve CHB patients available, only those with viremia levels high enough to amplify the HBV genome region of interest by our PCR technique could be included. Furthermore, we wished to

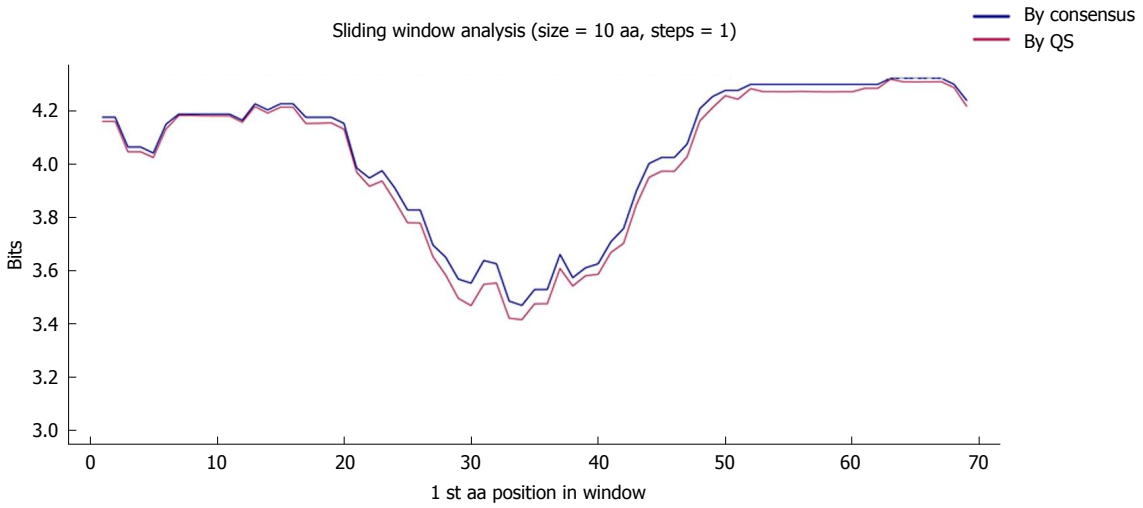

Figure 4 Sliding window of the coded the core and X protein amino acid sequence (aa 1-79). Each point on the graph is the result of the mean information content (IC, bits) between windows of 10-aa size with displacement between them in 1-aa steps. The purple line represents the mean IC from the multiple alignments of all haplotypes (in abundance > 0.25%) in the quasispecies (QS) of all patients (n = 330), whereas the blue line shows the mean IC obtained from the multiple alignments of the consensus obtained for each patient (n = 27).

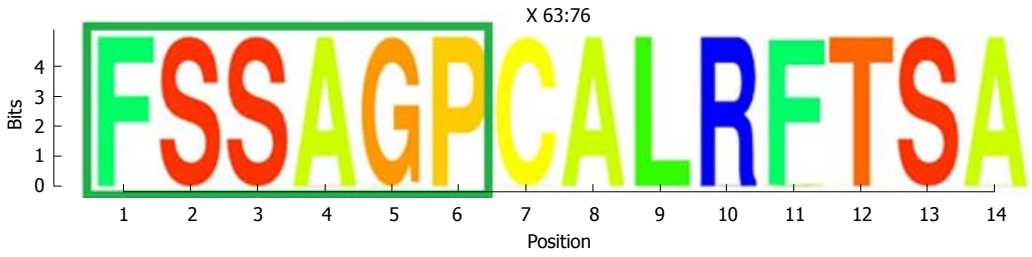

Figure 5 Representation by sequence logos of the information content of the conserved region in the the core and X protein amino acid sequence (aa 63-76). The relative sizes of the letters in each stack in the sequence logos indicate their relative frequencies at each position within the multiple alignments of aa haplotypes obtained. The total height of each stack of letters depicts the IC of each aa position, measured in bits (Y-axis): 0 bits being the minimum and 4.32 bits the maximum conservation. Amino acids belonging to the Kunitz-like domain portion are framed in green. IC: Information content.

have a representation of various clinical stages of HBV infection and most HBV genotypes (A to F and H), which yielded a sample of 27 patients. Larger samples should be analyzed in future studies to confirm conservation of the regions investigated. We also have to point out that the NGS technology used in this study (GS-Junior platform, 454/Roche) has been discontinued by the supplier; nonetheless, the protocol described here can be adapted to currently available platforms, such as the Illumina MiSeq (San Diego, United States). Finally, *in vitro* functional studies should be performed to test the potential usefulness of the 2 hyper-conserved domains described here as targets for siRNA-based antiviral gene therapy.

In summary, this study, performed in serum samples from HBV patients infected by different viral genotypes and in different clinical stages, identified regions in the *HBX* gene with high levels of conservation in all these circumstances. We found 2 hyper-conserved regions, the first in the non-coding region of *HBX* transcripts, and the second in the *HBX* coding region, which was conserved at both the nt and aa level. These hyper-conserved regions could be candidates for targeted gene therapies such as the siRNA approach. Of particular interest, because of the co-terminal localization of the *HBX* gene, a siRNA system designed to target these regions could interfere with expression of all the HBV viral transcripts.

ARTICLE HIGHLIGHTS

Research background

Hepatitis B virus (HBV) infection can be controlled with current treatments, but cure is not achieved due to persistence of covalently closed circular DNA (cccDNA) in the nuclei of infected hepatocytes. This minichromosome forms a viral reservoir that is a source of residual viral replication and expression of viral proteins; thus, it has a key role in liver disease progression. To surmount this circumstance, new anti-HBV therapeutic approaches are under development, with gene therapy being a promising option. Among these approaches, small interference RNA (siRNA) can be used to silence specific genes at the post-transcriptional level through a sequence-specific interaction with target mRNAs, resulting in inhibition of viral protein expression. Among all the HBV proteins, Hepatitis B X protein (HBx), coded by the HBV X gene (*HBX*), is a determining factor in the infection. It regulates cccDNA expression and interacts with several cellular pathways, facilitating liver disease progression. Of particular note, because of its location near the co-terminal 3' end, all HBV transcripts include the *HBX* sequence. Hence, it could be a valuable target for a hypothetical curative treatment based on gene therapy. In this sense, identification of hyper-conserved regions within *HBX* is needed to define a new gene therapy system that would be effective whatever the patient's clinical stage or HBV genotype.

Research motivation

Although antiviral therapy can suppress viral replication, the risk of liver disease progression and development of hepatocellular carcinoma (HCC) remains due to cccDNA-related expression of viral antigens. Interference with expression of the viral proteins could be helpful to limit progression of the disease, and siRNAs would be valid tools in this sense. To design an effective siRNA, an appropriate target must be found. The *HBX* sequence is included in all the viral transcripts due to its co-terminal localization in the viral genome. siRNAs targeting hyper-conserved regions of this gene would interfere with expression of all the viral proteins. Furthermore, as these regions are conserved in the spectrum of clinical disease phases and viral genotypes, it would be a valid therapeutic approach for a wide range of situations. This could profoundly limit the risk of HCC, particularly in patients with low viremia due to antiretroviral efficacy.

Research objectives

Considering the essential role of HBx in viral infection and its potential utility as target for gene therapy, the aim of this study was to identify hyper-conserved regions within the HBV genome encompassing the *HBX* 5' coding region and the upstream non-coding region (included in all HBV transcripts) in samples from HBV-infected patients in various clinical stages and with different viral genotypes. The regions identified might be feasible targets for a gene therapy able to inhibit viral protein expression in a wide spectrum of clinical and virological circumstances, thus limiting liver disease progression and the risk of HCC.

Research methods

The study included 27 treatment-naïve chronic hepatitis B monoinfected patients in different clinical stages and with several HBV genotypes (from A-F and H). A serum sample from each patient with viremia > 3.5 log IU/mL was analyzed. The *HBX* 5' end region [nucleotide (nt) 1255-1611] was PCR-amplified and later analyzed using next-generation sequencing (NGS). The sequences (reads) obtained after sequencing underwent an in-house bioinformatics filtering procedure, and haplotypes with a relative frequency ≥ 0.25% were maintained in the analysis. Haplotypes were genotyped by discriminant analysis with the same regions extracted from the 102 full-length patterns. Conservation of the quasispecies sequences was determined by calculating the information content (IC), based on Shannon's uncertainty, of each position in a multiple alignment of all different sequences found in the patients. Sliding window analysis was then carried out to locate the fragment of at least 25 nt or 10 aa (which corresponds to the length of a possible target for siRNA therapy) with the highest IC within the multiple alignments, moving forward in steps of 1 (nt or aa). This method enables detection of conserved regions within the 5' *HBX* gene by directly analyzing the viral quasispecies obtained with NGS.

Research results

After applying the quality filter, 1333069 haplotype sequences were obtained. Genotyping analysis highlighted a complex mixture of HBV genotypes. By studying the nt conservation, we identified two hyper-conserved nucleotide regions in *HBX*. The first one, between nt 1255 and 1286, corresponded to a non-coding region, whereas the second one, consisting of 3 conserved fragments (spanning an overall portion between 1519 and 1603), coincided with a coding region. Of note, the fragment between nt 1563 and 1602 was also conserved at the amino acid level, identifying a region between residues 63 and 76, which included a portion of a Kunitz-like domain. These results highlight new potential targets for gene therapy, mainly based on siRNA. Of note, *in vitro* and *in vivo* functional studies of the specific siRNAs should be performed to test their potential usefulness for therapy.

Research conclusions

Gene therapy represents a highly promising therapeutic tool to achieve a cure against HBV infection. Several sequence-specific treatment systems are currently in development, and identification of conserved sequences would provide useful therapeutic targets. Detection of a target present in all the clinical disease stages and HBV genotypes could lead to development of a therapy that would be effective in a wide range of situations. Considering the key role of HBx in viral infection and disease progression, we focused the study on analyzing conservation of the *HBX* gene. Of note, considering the high variability previously observed in the 3'end of *HBX*, we speculated that the 5' end could be a better subject for study. Moreover, thanks to the co-terminality of this viral gene, a siRNA targeting this gene could interfere with all the viral transcripts. Here, we investigated conservation of a portion of the HBV genome encompassing the *HBX* 5' coding region and upstream non-coding region, both of which are included in all HBV transcripts. By NGS analysis, we identified two hyper-conserved regions in our region of interest in serum samples from HBV patients with different clinical and virological characteristics. This new therapeutic tool could have relevant applicability in clinical practice. Together with inhibition of the expression of one of the main viral proteins involved in HBV replication and disease progression, it could block the expression of the other viral antigens, thus profoundly interfering with disease evolution and the appearance of HCC. Furthermore, the NGS method developed here could be used to find other hyper-conserved regions within the HBV genome that could be potential targets for gene therapy based on siRNA.

Research perspectives

This study describes a method that can be used to find other conserved sequences in the HBV genome, making it a starting point in the search for other possible targets for gene therapy. Here, the hyper-conserved regions were found by directly analyzing the viral quasispecies sequences obtained using NGS. These regions can then be used to produce siRNA molecules for *in vitro* and *in vivo* testing of antiviral activity.

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ORIGINAL ARTICLE

Prospective Study

Decreasing recurrent bowel obstructions, improving quality of life with physiotherapy: Controlled study

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Abstract

AIM

To compare (1) quality of life and (2) rate of recurrent small bowel obstructions (SBO) for patients treated with novel manual physiotherapy *vs* no treatment.

METHODS

One hundred and three subjects (age 19-89) with a history of recurrent adhesive SBO were treated with a manual physiotherapy called the Clear Passage Approach (CPA) which focused on decreasing adhesive crosslinking in abdominopelvic viscera. Pre- and post-therapy data measured recurring obstructions and quality of life, using a validated test sent 90 d after therapy. Results were compared to 136 untreated control subjects who underwent the same measurements for subjects who did not receive any therapy, which is the normal course for patients with recurring SBO. Comparison of the groups allowed us to assess changes when the physiotherapy was added as an adjunct treatment for patients with recurring SBO.

RESULTS

Despite histories of more prior hospitalizations, obstructions, surgeries, and years impacted by bowel issues, the 103 CPA-treated subjects reported a significantly lower rate of repeat SBO than 136 untreated controls (total obstructions $P = 0.0003$; partial obstructions $P = 0.0076$). Subjects treated with the therapy demonstrated significant improvements in five of six total domains in the validated Small Bowel Obstruction Questionnaire (SBO-Q). Domains of diet, pain, gastrointestinal symptoms, quality of life (QOL) and pain severity when compared to post CPA treatment were significantly improved ($P < 0.0001$). The medication domain was not changed in the CPA treated group ($P = 0.176$).

CONCLUSION

CPA physical therapy was effective for patients with adhesive SBO with significantly lower recurrence rate, improvement in reported symptoms and overall quality of life of subjects.

Key words: Clear passage approach; Manual therapy; Physical therapy; Small bowel obstructions; Adhesions; Physiotherapy; Alternative therapy

Core tip: A manual soft tissue physical therapy protocol is an effective low risk preventative treatment option for patients who suffer recurrent adhesive small bowel obstructions. It increased the quality of life of subjects by decreasing pain, decreasing recurrent obstructions, improving diet and increasing bodily function. Because the therapy was performed in an outpatient setting, it eliminated the need for hospitalization and the risk and increased cost of surgery.

Rice AD, Patterson K, Reed ED, Wurn BF, Robles K, Klingenberg B, Weinstock LB, Pratt JS, King CR, Wurn LJ. Decreasing recurrent bowel obstructions, improving quality of life with physiotherapy: Controlled study. *World J Gastroenterol* 2018; 24(19): 2108-2119 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i19/2108.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i19.2108

INTRODUCTION

Adhesive small bowel obstruction (SBO) is a common life-threatening complication of surgery or abdominal trauma in both pediatric and adult populations, typically caused by adhesions that form as a normal part of the healing process[1-3]. As a side effect of the inflammatory response required for healing to occur, adhesions form in tissues at and near the surgical repair within hours, due to the presence of collagen and scar tissue mediators.

Surgery is frequently cited as the primary cause of adhesive bowel obstruction; repeat surgery increases the risk of adhesion formation[4]. The financial cost per patient for adhesiolysis and bowel resection was $65955 and $114175, respectively, with 30-d hospital readmission rates of 12.3% and 18.1% in the United States in 2010[5]. The average hospital stay for cases of SBO surgery was 14.2 d, and for abdominal adhesiolysis was 8.4 d[5].

The present cost of a one-week course of CPA physical therapy is under $7000 in the United States (and is comparable in the United Kingdom). The therapy is performed in an outpatient setting; no hospitalization or surgery is required.

A large previously published clinical study that followed adult subjects 10 years post-surgery found that more than 30% of the surgical small bowel resection subjects underwent additional surgery due to adhesions within the study time frame[6]. In a prospective analysis of patients undergoing laparotomy who had previously had one or more abdominal operations, 93% (195/210) were found to have adhesions attributable to their prior surgery[1]. Patients with mesh inserted during surgery have been identified as having an increase in adhesion formation, leading to increased complexity of future surgical procedures[7-9].

Thus, adhesion related disease causes significant surgical efforts, hospital resources and comprises major expenditures each year. Additionally, there is a considerable negative impact on the patients' quality of life (QOL).

In the absence of bowel ischemia, strangulation or perforation, current guidelines for the management of SBO are gastric decompression with suction, full strength gastrographin (in some institutions), and IV hydration for the first 24-72 h to see if the obstruction will resolve without surgery[10]. While sometimes effective in treating the current obstruction, this approach does not address

the internal adhesions or the risk of subsequent bowel obstructions[11,12]. The only treatment currently available to reverse adhesive bowel obstructions is surgery, which frequently causes new adhesions. A number of surgical techniques, modifications, and adhesion preventing medications and barriers have been investigated, but no solution has been shown to significantly prevent the formation of adhesions to date[13-19]. Thus, any therapy that decreases the risk of bowel obstruction in the absence of surgery is of significant importance.

Manual physical therapy (mPT), a method of physiotherapy and rehabilitation, is used to treat patients with a wide variety of adhesive conditions including burns, adhesive capsulitis, radiculopathy, pain, infertility, and lessening of scars[20-30]. This therapy has shown promise in preventing adhesion formation in animal models[31,32]. The Clear Passage Approach (CPA), a specialized mPT regimen, hypothesized to deform the adhesions that cause SBO episodes, has been demonstrated as effective in decreasing adhesions, pain, and improving QOL in subjects with recurrent SBO in case control and efficacy studies[33-35]. In pilot studies, independent radiologic reports showed that the therapy cleared bowel stricture and obstruction, obviating the need for planned laparotomy for adhesiolysis and SBO[36,33-35].

Surgery is often necessary in cases of bowel obstruction complicated by ischemia or caused by hernia or cancer, however surgery has not been shown to improve quality of life (QOL), reduce pain or recurrence rate in patients with adhesive SBO. Further, except as shown in pilot studies of CPA therapy, there is currently no other treatment available to patients with known adhesions who are currently not obstructed to reduce recurrence, pain or need for further surgery. In this study, we report on the use of the CPA, a manual physical therapy protocol, to treat abdominal and pelvic adhesions that cause SBO, and to improve the quality of life (QOL) of study subjects when compared to untreated subjects in a control arm.

Aim and hypothesis

The aim of the study is twofold: (1) To determine whether a manual physiotherapy can lower the rate of repeat SBO in patients who have undergone prior adhesive bowel obstructions and surgeries, and (2) to examine whether the therapy can improve the quality of life of these patients, using a validated test.

The hypothesis is that the manual physiotherapy treatment can decrease SBO recurrence and improve the self-reported quality of life in subjects with a history of adhesive SBO, compared to untreated control subjects.

MATERIALS AND METHODS

Eligibility criteria

Subjects in the retrospective CPA treatment arm were selected for inclusion in the study based upon medical history and completion of both a pre-treatment and post-treatment questionnaire. Only adult subjects were included in this study. Exclusion criteria included: BMI > 36, active infection, abnormal ovarian cysts, surgery within the last 90 d, and bleeding disorders. Each subject was provided with a written informed consent as is standard for the clinical practice. Subjects with a recent history of cancer were excluded from this study. A total of 103 subjects were treated at one of five private physical therapy clinics affiliated with Clear Passage between November 2012 and October 2015. All CPA therapists and affiliates completed 80 h practical training and evaluation course prior to becoming certified in the Clear Passage Approach. All subjects with follow up were eligible and included in the analysis.

Subjects in the control arm were recruited *via* website and online advertisements for the study. Inclusion criteria included: adult subjects who had experienced a partial or total bowel obstruction within the last two years. Exclusion criteria included: Cancer within the last 5 years, chemotherapy or radiation treatments in the last 10 years, current pregnancy, or prior treatment at Clear Passage. All study related activities were performed *via* the NIH Assessment Center (www.assessmentcenter.net) including screening, informed consent and all data collection. A total of 460 subjects were screened, with 281 subjects eligible and 260 completing the initial questionnaire; 6 subjects were removed from the control study due to having previously received CPA treatment, 117 subjects were lost to follow up, 1 subject was removed due to less than 50% of the follow up questionnaire being completed. A total of 136 subjects completed both questionnaires and were included in the analysis. The study diagram is shown in Figure 1. This study was approved by MaGil Institutional Review Board.

Study design

This was a non-randomized controlled study comparing subjects with a history of SBO that underwent CPA treatment to that of untreated control subjects. The CPA treatment group included a retrospective chart review of subjects treated for adhesive disease with a history of SBO that had undergone standard clinic follow up. All subjects included in the treatment arm received treatment within private outpatient physical therapy clinics by therapists certified in the CPA. Subjects in a prospective untreated control arm, including those not treated with the CPA, were recruited for participation in a survey based observational data collection.

Outcomes were measured *via* an observational manner using the validated paper based SBO Questionnaire (SBO-Q) using previously established quartiles[37]. The SBO-Q was developed and validated to be used as a patient reported tool to quantify the experiences of patients with recurrent SBOs. The questionnaire is comprised of five domains and a pain rating section. Each domain measures a distinct aspect of QOL. The Diet domain assesses the diet of the subject from inability to tolerate any liquid to a normal solid food diet. The Pain domain reported pain throughout the body, with oral intake of food or liquid, and with bowel function.

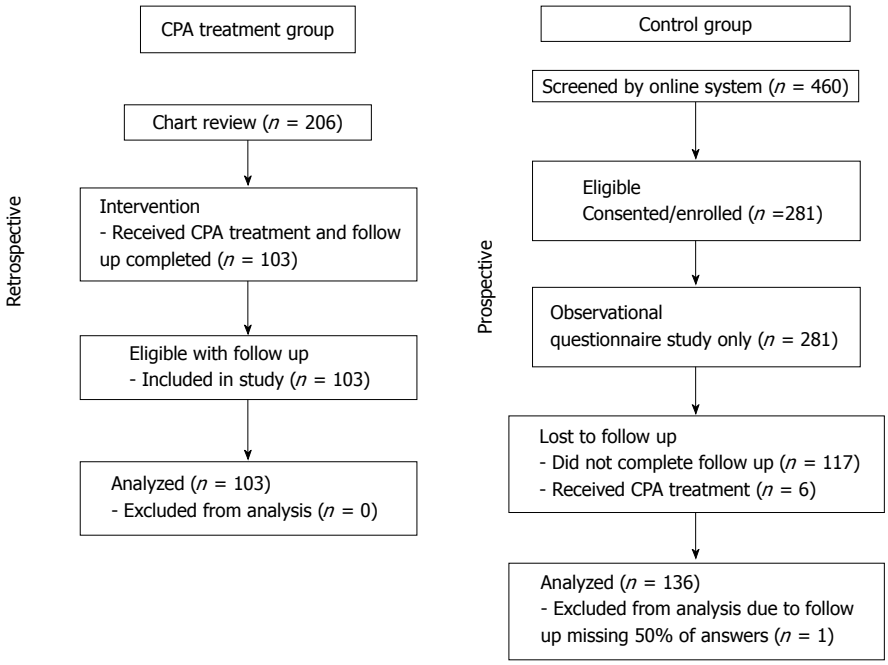

Figure 1 Study diagram.

The Gastrointestinal (GI) Symptom domain quantifies symptoms often associated with SBO such as nausea, emesis, bowel spasm and constipation. The Medication domain reports the frequency of medications required to maintain bowel function. The QOL domain quantifies the amount of time off work, social activities and overall concern of the patient regarding their bowel function. These tend to be major lifetime concerns for many people who experience recurring bowel obstructions.

Subjects provided previous medical history and records; no radiologic evaluations were performed as a part of the study. Questionnaires were completed prior to treatment for the control measure. The post-treatment questionnaires were sent to the subjects 90 d post-treatment, with completion at 78-140 d post-treatment for the CPA treatment arm and 90-115 d for the control arm subjects.

Sample size calculation

For evaluating the power of our study, we made some simplifying assumptions. We assumed that differences in the 6 domain scores before and after treatment follow a normal distribution. For all but two of the SBO-Q domains we assumed that the mean difference was zero (*i.e.*, no treatment effect), but for two domains we assumed that the treatment lowered the average domain score by 1 point. We assumed that the standard deviation of each difference was 2.5 points. We then simulated the power by looking at the proportion of times that both multiplicities adjusted two-sided *P*-values for the two domains where we assumed a treatment effect is less than 5%. When selecting a sample size of 100, this was the case in 82% of our simulations. Therefore, 100 patients in the pre- and post-treatment comparison result in at least 80% power to detect a significant difference of 1 point in the mean domain score of two domains. All computations were made in the statistical software R[38].

Treatment

Subjects treated with the CPA received manual therapy to areas of the body identified by the treating therapists as showing decrease mobility or function during the evaluation *via* palpation and range of motion tests. All subjects were evaluated from head to toe. In the case of these subjects the focus of the therapy was to the abdominopelvic viscera that demonstrated decreased mobility, hypothesized to be due to adhesions formed after surgery, trauma, or other tissue damage, therefore leading to SBO episodes. The majority of the therapy focused on treating abdominopelvic viscera that demonstrated decreased mobility with palpation. Treatment was administered in multiple hour sessions, which included up to four hours a day over the course of 5 d with the typical subject completing 20 h of treatment. The average number of treatment hours per subject was 22.6 ± 8.5 h with a range of 10-74 h; 75 of 103 subjects in CPA treatment group received 20 h of treatment.

In order to decrease adhesions, the CPA uses techniques from a variety of manual modalities to treat the subject in an individualized manner, focusing on each subjects' areas of restriction and concern with the goal of deforming adhesions and increasing the mobility of adhered tissues and organs, working from the most superficial tissues to those deeper in the body. The therapy accomplishes this by the use of various site-specific pressures across restrictive bands of adhered tissues and structures within the abdomen and pelvis, using a bimanual deep massage. The intent of the therapy is to detach and reverse the crosslinking that

Table 1 Demographics

Characteristics	Control	CPA treatment	*P* value
Age, yr			
Median	33.5	57	< 0.001
Range	19-71	19-89	
Sex			
Male	58	33	0.0853
Female	78	70	
Race			
White	80	90	
Black/African American	39	1	
American Indian/Alaska Native	1	2	
Asian	3	2	
Native Hawaiian/Pacific Islander	0	0	
Other	11	0	
Unknown	2	8	
Ethnicity			
Hispanic or Latino	12	1	
Not Hispanic or Latino	108	91	
Unknown	16	11	
Marital Status			
Married/long term relationship	47	69	
Single	63	16	
Divorced/widowed	25	11	
Unknown	1	7	

CPA: Clear Passage Approach.

binds collagenous fibers together to form adhesions. The therapists work progressively deeper from the most superficial tissues restoring mobility *via* myofascial release[39]. Adhesions within and between organs and interstitial spaces within the viscera were addressed using the Wurn technique[29,34] which is a bimanual soft tissue manipulation that can include a slow stretch to adhered areas within the abdomen and pelvis, previously shown to return patency to occluded fallopian tubes. The intent of this technique is to detach crosslinks that bind collagen fibers, thus shearing apart adhesions manually, in order to return the tissues to a more mobile, less adhered state. Toward the end of deep tissue work, CPA certified therapists may use visceral manipulation to increase organ motility[39]. The amount of force and time the force was applied to each area has the potential to be significant but was maintained within the tolerance of the subject in accordance with guidelines of the American Physical Therapy Association. Detailed clinical treatment records were maintained throughout the course of therapy.

Subject monitoring

Subjects enrolled in the control arm of the study were not monitored as this was an observational web-based study arm. Subjects included in the treatment arm were monitored daily for changes in pain, diet, bowel habits and overall well-being during their treatment as is standard practice. Adverse events were monitored by the treating therapists during the course of treatment and reviewed during the chart review process. There were no adverse events reported in the CPA treated group within the clinical data, or by follow up.

Disease scoring

The overall degree of adhesive disease was determined using a clinic generated disease scoring table that takes into account all qualitative and quantitative measures performed during the initial evaluation and post treatment discharge assessment. The degree of impact on a subject's quality of life for range of motion was determined using multiple resources, as well as the extensive experience of the therapists. Multiple other standard physiotherapy tests were also performed and included in the assessment for disease score[40-44]. Supplemental Table 1 contains the disease scoring protocol in which the score is expressed as a decimal in which 0 represents no disease or impact on overall function and 1 is severely impacted.

Statistical analysis

We used χ^2 tests and *t*-tests for the analysis comparing the demographic and medical variables between the treated and control group.

For analyzing the multivariate responses from the SBO-Q we used the minimum *P*-value based on a paired *t*-statistic for each survey question as the test statistic for the overall and the domain hypotheses[45]. The raw and multiplicity adjusted *P*-values displayed in tables refer to testing the null hypothesis that the mean difference between the initial and follow-up survey equals 0. Since there are 6 domains for which we tested for a mean difference, we used multiplicity adjusted *P*-values to adjust the raw *P*-values and account for the fact that we are testing 6 hypotheses simultaneously. For a particular domain, a significant difference in the mean domain score can be concluded when the corresponding multiplicity adjusted domain *P*-value is less than 0.05.

Table 2 Medical history

	Control	CPA treatment	*P* value
Number of previous surgeries			
0	4	4	< 0.001
1-2	89	16	
3-5	39	34	
> 6	3	49	
unknown	1	0	
Number of prior partial bowel obstructions in the last 3 yr			< 0.001
0	3	24	
1-10	132	70	
11-20	1	8	
> 20	0	1	
Number of prior total bowel obstructions			0.4894
0	4	53	
1	47	29	
2	46	11	
3	27	3	
4 or more	12	7	
Number of years impact on life due to bowel issues			
Average (SD)	5.5 (7.2)	10.7 (11.2)	< 0.0001

CPA: Clear Passage Approach.

Using the multiplicity adjusted *P*-values (computed *via* the Bonferroni correction) will control the overall (familywise) error rate at 5%. Similarly, the multiplicity adjusted *P*-values for each survey question account for the fact that we are testing 37 hypotheses comparing mean differences simultaneously, one for each question. For any given survey question, a multiplicity adjusted *P*-value less than 0.05 implies a significant difference in the mean score, controlling the overall rate of Type I errors at 5% when testing these 37 hypotheses simultaneously.

The statistical package R (R Foundation for Statistical Computing, Vienna, Austria) was used for all statistical analyses.

RESULTS

A total of 103 CPA treated subjects and 136 untreated control subjects were included in the analysis. Demographics for study subjects are located in Table 1 and number of prior surgeries and bowel obstructions are located in Table 2. Subjects in the CPA treatment group were significantly older, and the total numbers of prior surgeries, partial SBOs, and years impacted with bowel symptoms were much higher than that of the control group. There were 46.2% male subjects in the control group and 32% male subjects in the CPA treated group ($P = 0.0853$). The average age of subjects in the control group was 36.4 ± 12.5 years and 54.5 ± 15.7 years for the CPA treated group ($P < 0.001$); the average number of prior surgeries in the control is 2.07 ± 1.29 as compared to 4.56 ± 2.86 for the CPA treated ($P = 0.0001$). The average number of prior partial SBOs in the control group was 1.88 ± 1.60 and 3.97 ± 5.09 in the CPA treated group ($P = 0.0001$). The number of total SBO was 2.17 ± 1.85 for the control group and 1.68 ± 7.95 for the CPA treated group ($P = 0.4894$). The number of years the subjects reported bowel symptoms impacted their lives was 5.5 ± 7.2 for the control group and 10.7 ± 11.2 for the CPA treated group ($P < 0.001$).

Rates of recurrent SBO

All subjects at the 90-d follow up questionnaire were queried regarding the numbers of bowel obstructions, hospitalization, and surgery for SBO experienced. There were differences between the groups, with the CPA treated group reporting fewer total number of obstructions, hospitalizations and surgeries. A total of 124 control and 103 CPA treated subjects responded to the questions. 21.77% of the control group subjects and 8.74% of the CPA group subjects experienced a partial bowel obstruction between the two questionnaires ($P = 0.0076$), 14.52% of control group subjects and 0.97% of CPA group subjects reported a total bowel obstruction ($P = 0.0003$), 5.65% of control group subjects and 1.94% of CPA group subjects reported undergoing surgery for a bowel obstruction ($P = 0.1548$). The differences between untreated and treated patients at follow up would naturally extrapolate into a sizable decrease in cost, due to avoiding repeat SBO-related hospitalizations and surgeries.

SBO-Q analysis

For both the control and CPA group we tested the null hypothesis that for each of the 6 domains the mean domain score the first time the survey was administered was equal to the mean domain score the second time the survey was administered 90 d later. The alternative hypothesis was that there was a significant difference in the mean domain score between the initial and follow-up administration of the survey for at least one domain. Missing data from any question/subject was removed from the overall analysis.

For the control group data, despite the fact that no intervention took place between the two times the survey

Table 3 Average difference (before/after) in Small Bowel Obstruction Questionnaire scores and *P*-values for each domain and question for the control group

Domain question	Difference mean, St. Dev		Raw *P*-value	Multiplicity adjusted *P*-value	Lower confidence bound	Upper confidence bound
Diet	-0.06	0.93	0.425	1.000	-0.28	0.15
Liquid	-0.09	1.08	0.342	1.000	-0.39	0.21
Soft	-0.20	1.20	0.055	1.000	-0.54	0.14
Solid	-0.11	1.48	0.388	1.000	-0.53	0.31
Anything	0.13	1.35	0.256	1.000	-0.25	0.51
Pain[1]	0.17	0.61	0.002	0.013	0.02	0.31
General[2]	0.50	1.23	0.000	0.000	0.15	0.85
Upper GI	0.00	1.11	1.000	1.000	-0.31	0.31
Lower GI[2]	0.35	1.14	0.000	0.016	0.03	0.67
BM[2]	0.50	0.96	0.000	0.000	0.23	0.77
Head_neck	0.01	1.05	0.935	1.000	-0.29	0.30
Migrane	0.05	1.18	0.612	1.000	-0.28	0.38
Coccyx	-0.13	1.07	0.150	1.000	-0.43	0.17
Eating[2]	0.35	1.24	0.001	0.045	0.00	0.70
Drinking	0.02	1.10	0.815	1.000	-0.29	0.33
Back	0.01	1.11	0.877	1.000	-0.30	0.33
GI symptoms[1]	0.18	0.58	0.000	0.003	0.05	0.32
Nausea	0.29	1.22	0.006	0.215	-0.05	0.64
Vomit	-0.04	0.88	0.627	1.000	-0.28	0.21
GI_spasm	-0.01	0.97	0.860	1.000	-0.29	0.26
Constipation	0.06	0.90	0.448	1.000	-0.19	0.31
Diarrhea	0.26	1.14	0.010	0.354	-0.06	0.58
BS_JLM	0.04	0.96	0.592	1.000	-0.22	0.31
Gas_bloat_dist[2]	0.39	1.32	0.001	0.028	0.02	0.76
Inc_sounds[2]	0.41	1.14	0.000	0.002	0.09	0.73
No_BM	0.07	1.10	0.437	1.000	-0.24	0.38
Ab_BM	0.01	1.22	0.889	1.000	-0.33	0.36
Eat_bloat[2]	0.45	1.51	0.001	0.025	0.03	0.88
Medication	-0.03	1.39	0.804	1.000	-0.35	0.29
Meds	-0.03	1.39	0.804	1.000	-0.42	0.36
Quality of life	0.10	0.77	0.126	0.759	-0.08	0.28
Off_work	0.13	1.19	0.218	1.000	-0.21	0.46
Off_social	0.10	1.42	0.396	1.000	-0.30	0.50
Off_sex	0.12	1.37	0.313	1.000	-0.27	0.51
Off_daily_function	0.25	1.38	0.036	1.000	-0.14	0.64
Off_eat_out	0.34	1.23	0.002	0.059	-0.01	0.69
Massage_worry	-0.31	1.19	0.003	0.104	-0.65	0.02
Worry	0.06	1.07	0.521	1.000	-0.24	0.36
Pain severity	0.23	1.46	0.064	0.382	-0.10	0.57
Duration_pain	0.33	1.24	0.003	0.105	-0.02	0.68
Recent_max_pain	0.24	2.62	0.295	1.000	-0.50	0.97
Recent_min_pain	0.35	2.17	0.064	1.000	-0.26	0.96
Recent_avg_pain	0.02	1.89	0.892	1.000	-0.51	0.55

[1]Denotes domains for which average scores (over all questions within a domain) are significantly different between the initial and the follow-up survey, controlling the familywise error rate (over the 6 domains) at 5%. [2]Denotes questions for which scores are significantly different between the initial and the follow-up survey, controlling the familywise error rate (over the 37 questions included in the SBO-Q) at 5%. SBO-Q: Small Bowel Obstruction Questionnaire.

was administered, Table 3 shows a significant difference (multiplicity adjusted $P < 0.05$) in mean domain scores for the Pain and GI Symptoms domain. However, the lower bounds of a corresponding confidence interval for the mean difference in domain scores are close to zero (0.02 and 0.05, respectively), indicating that, while statistically significant, the actual improvement may be very small.

A further analysis of the individual survey questions shows that within the Pain domain, General, Lower GI, BM and Eating pain improved significantly between the initial and follow-up survey. However, note the small lower bounds for the corresponding confidence intervals for the mean difference, indicating very small changes except perhaps for BM and General pain. The significant improvement in those two could reflect a time-effect, the perceived pain lessening over time. Similarly, the three significant differences for individual questions in the GI Symptoms domains all have very small lower bounds of corresponding confidence intervals. For all other domains and all other individual survey questions within domains no significant differences emerged (multiplicity adjusted *P*-values > 0.05). The diet domain had multiplicity adjusted $P = 0.425$, the Medication domain $P = 0.804$, and the QOL domain $P = 0.126$, all indicating no major changes in symptoms over the three-month period studied.

For the CPA group, Table 4 shows significant differences for all domains (multiplicity adjusted *P*-value < 0.05) except for the Medication domain (multiplicity adjusted

Table 4 Average difference (before/after) in Small Bowel Obstruction Questionnaire scores and *P*-values for each domain and question for the Clear Passage Approach treatment group

Domain question	Change mean, St. Dev		Raw *P*-value	Multiplicity adjusted *P*-value	Lower confidence bound	Upper confidence bound
Diet[1]	0.77	1.03	0.000	0.000	0.49	1.04
Liquid[2]	0.49	1.19	0.000	0.003	0.10	0.87
Soft[2]	0.71	1.38	0.000	0.000	0.26	1.16
Solid[2]	0.91	1.70	0.000	0.000	0.35	1.47
Anything[2]	0.98	1.66	0.000	0.000	0.44	1.53
Pain[1]	0.52	0.73	0.000	0.000	0.33	0.72
General[2]	0.88	1.41	0.000	0.000	0.43	1.34
Upper GI[2]	0.85	1.34	0.000	0.000	0.41	1.29
Lower GI[2]	0.58	1.49	0.000	0.005	0.10	1.07
BM[2]	0.49	1.08	0.000	0.001	0.13	0.85
Head_neck	0.39	1.39	0.005	0.201	-0.06	0.85
Migrane	0.11	0.69	0.117	1.000	-0.12	0.34
Coccyx	0.16	0.75	0.035	1.000	-0.09	0.41
Eating[2]	0.68	1.51	0.000	0.001	0.18	1.17
Drinking[2]	0.46	1.26	0.000	0.013	0.05	0.87
Back[2]	0.59	1.29	0.000	0.000	0.17	1.01
GI symptoms[1]	0.44	0.68	0.000	0.000	0.26	0.62
Nausea[2]	0.53	1.13	0.000	0.000	0.16	0.90
Vomit	0.17	0.58	0.003	0.113	-0.02	0.36
GI_spasm	0.20	0.89	0.026	0.979	-0.09	0.49
Constipation[2]	0.54	1.48	0.000	0.013	0.06	1.03
Diarrhea[2]	0.41	1.19	0.001	0.028	0.02	0.79
BS_JLM	0.06	1.01	0.559	1.000	-0.27	0.39
Gas_bloat_dist[2]	0.72	1.46	0.000	0.000	0.24	1.19
Inc_sounds	0.37	1.35	0.008	0.286	-0.08	0.81
No_BM[2]	0.56	1.36	0.000	0.003	0.11	1.01
Ab_BM[2]	0.63	1.54	0.000	0.003	0.13	1.14
Eat_bloat[2]	0.71	1.39	0.000	0.000	0.26	1.17
Medication	0.40	1.84	0.029	0.176	-0.09	0.89
Meds	0.40	1.84	0.029	1.000	-0.20	1.00
Quality of life[1]	0.78	0.85	0.000	0.000	0.55	1.01
Off_work[2]	0.42	0.91	0.000	0.000	0.12	0.72
Off_social[2]	0.78	1.29	0.000	0.000	0.36	1.21
Off_sex[2]	0.61	1.45	0.000	0.003	0.12	1.10
Off_daily_function[2]	0.99	1.40	0.000	0.000	0.53	1.45
Off_eat_out[2]	0.64	1.38	0.000	0.000	0.19	1.09
Massage_worry[2]	0.75	1.82	0.000	0.003	0.15	1.34
Worry[2]	1.32	1.64	0.000	0.000	0.78	1.85
Pain Severity[1]	1.51	1.66	0.000	0.000	1.07	1.95
Duration_pain[2]	1.21	1.86	0.000	0.000	0.61	1.82
Recent_max_pain[2]	2.70	3.26	0.000	0.000	1.62	3.78
Recent_min_pain[2]	0.65	1.82	0.001	0.023	0.04	1.25
Recent_avg_pain[2]	1.20	1.97	0.000	0.000	0.55	1.86

[1]Denotes domains for which average scores (over all questions within a domain) are significantly different between the initial and the follow-up survey, controlling the familywise error rate (over the 6 domains) at 5%. [2]Denotes questions for which scores are significantly different between the initial and the follow-up survey, controlling the familywise error rate (over the 37 questions) at 5%.

P-value = 0.176). The domain confidence intervals indicate that the mean difference in the average Diet and Quality of Life score falls somewhere in between 0.5 and 1.0 on the 5-point scale, which is a rather large average effect. (For comparison, in the control group, no significant differences were reported for these two domains). Further, the difference in the average Pain Severity score was significant, ranging from 1.07 to 1.95. (For the control group, there was no significant difference.)

Overall, in the CPA group 29 out of 37 survey questions showed a significant difference (in fact, improvement), compared to just 7 in the control group. Judging by the confidence intervals (CIs), some of the strongest improvements were seen in the Pain, QOL, and Diet domains. In the Pain Domain, notable improvements occurred for the questions "Experience of pain in general" (CI: 0.43-1.34), "Experience pain in the upper GI region" (CI: 0.41-1.29), "Recent Maximum Pain" (CI: 1.62-3.78) and "Duration of Pain" (CI: 0.61-1.82).

Patients who have undergone the significant trauma of SBO accompanied by long hospital stays (average 14.2 d[5]), followed by invasive surgeries and recovery from those events have significant "QOL" concerns afterwards. With regard to the QOL domain, some noteworthy large differences between the initial and follow-up survey were observed for the question "How often the subject worries about another SBO" (CI: 0.78-1.85), "How often the subject was unable to perform daily functions" (CI: 0.53-1.45) and "How often the subject was unable to attend social events", (CI: 0.36-1.21). Related to QOL

Table 5 Quartile assignment for each domain of the Small Bowel Obstruction Questionnaire for all groups and questionnaire time points

	Baseline					90 d/90 d post treatment				
	Diet	Pain	GI	QOL	Medication	Diet	Pain	GI	QOL	Medication
Quartiles for control subjects										
Normal	44	16	24	4	100	34	24	26	12	100
Mild	54	76	79	37	27	61	80	86	22	24
Moderate	24	41	29	59	9	30	29	22	78	12
Severe	14	3	4	36	0	11	3	2	24	0
Quartiles for CPA treated subjects										
Normal	24	47	50	22	71	52	74	74	60	82
Mild	21	27	34	39	7	26	22	20	26	3
Moderate	35	24	15	27	25	16	6	8	9	18
Severe	23	5	4	15	0	9	1	1	8	0

but analyzed under the Diet domain, the question about "The ability to eat anything" also showed some large improvements (CI: 0.44-1.53).

When the total number of subjects per SBO-Q domain are assessed for both questionnaires for the quartiles indicating the degree of impact (normal, mild, moderate and severe) it was observed that the general trend demonstrated more subjects reporting experiences corresponding to no impact or normal for the quartiles in the CPA treatment group than the control group (Table 5). The significance observed in the individual question score analysis is also observed in this representation of the data, showing the clinical significance of these changes in the CPA treated group.

Trunk range of motion

All CPA treated subjects underwent a comprehensive physical therapy initial evaluation that included measurements for range of motion (ROM). It has been previously demonstrated that a significant improvement in ROM in the trunk was observed in patients with a history of SBO and may be a positive predictor for outcome. Included in these measurements are: trunk flexion; trunk extension; left and right side bending; left and right trunk rotation[40,42–44].

Every subject who presented with decreased ROM demonstrated improvement for at least one of the measurements presented in Table 6. Furthermore, 31.47% of total measurements were within the normal range after CPA treatment compared to 19.74% before treatment. Although not all subjects had a normal ROM for all measures after treatment, all subjects demonstrated overall improvements.

Statistical analysis of changes showed a significant increase in the mean ROM after treatment compared to before for all six measures (multiplicity adjusted *P*-values < 0.05).

Patients with a history of SBO and surgery often presented with a kyphotic posture; we surmised this was due to the pull of abdominal adhesions, creating a forward flexion of the trunk. The improvement in trunk extension following CPA treatment suggests that adhesions in the abdomen and pelvis that prevented the subject from bending backwards or standing upright prior to therapy diminished enough to show significant improvement in trunk extension following the manual treatment. The improvements in side bending and rotation also suggest that decreasing adhesions in the abdomen and pelvis allowed improved trunk ROM. These findings indicate that improvements in trunk ROM measurements may be suitable as a predictor for degree of adhesion deformation post CPA treatment.

Disease scoring

The observations made by the therapists at the initial evaluation and after CPA treatment were utilized to generate an objective numerical value for analysis of the overall state of the adhesive disease of the treated subjects. This numerical score takes into account the entire patient - assessed from head to toe, and includes ROM, organ motility, standard physical therapy tests, posture and tissue response with a range from 0 (no disease or deficiencies) to 1.0 (maximum disease) (supplemental Table 1). A total of 101 subjects in the CPA treated group were included in the assessment; results showed significant improvement in the full body disease score. Prior to CPA treatment the average disease score was 0.65 ± 0.11 (range 0.37-0.95) compared to post CPA treatment 0.29 ± 0.10 (range 0.1-0.53) ($P < 0.001$). This measure correlates well with both the ROM measures (Table 6 and the changes observed in the quartiles post CPA treatment in Table 5).

DISCUSSION

It is widely accepted that the adhesions that cause SBO and symptomology in subjects are often caused due to prior abdominal or pelvic surgery[10,46]. This study is the first controlled study of a specific physical therapy protocol known as the Clear Passage Approach (CPA) in subjects with a history of SBO. The results of this study are very similar to those reported in an earlier uncontrolled efficacy study and case reports using the CPA to treat subjects with recurring SBO[35]. In all measures, the subjects treated with the CPA experienced significant improvements as compared to experiences

Table 6 Range of motion averages in Clear Passage Approach treated subjects prior to and post Clear Passage Approach treatment

Range of motion measure (normal)	Pretreatment (n = 103) mean (SD)	Post treatment (n = 103) mean (SD)	Raw P value	Multiplicity adjusted P value	Lower confidence bound	Upper confidence bound
	Number with normal ROM	Number with normal ROM				
Flexion (80)	76.9 (19.4)	82.5 (15.1)	< 0.001	0.010	-10.4	-0.7
	48	74				
Extension (25)	25.2 (13.7)	29.9 (11.8)	< 0.001	< 0.001	-7.8	-1.5
	45	77				
Left side bending (45)	38.0 (10.3)	42.6 (8.7)	< 0.001	< 0.001	-7.2	-2.1
	37	59				
Right side bending (45)	38.8 (12.2)	43.4 (8.7)	< 0.001	< 0.001	-7.4	-1.8
	47	62				
Left rotation (45)	37.0 (14.8)	44.2 (14.4)	< 0.001	< 0.001	-10.4	-4
	23	47				
Right rotation (45)	39.3 (13.6)	45.5 (12.3)	< 0.001	< 0.001	-9.5	-2.9
	44	70				

ROM: Range of motion.

before CPA treatment and when compared to untreated controls. Manual physical therapy can provide adjunct therapy for known adhesions where there is no other available therapy proven to decrease recurrent SBO or to treat pain associated with adhesive disease. Unlike surgical procedures, adhesions do not appear to occur after treatment with the CPA[28].

Because treatment group participants had more complex histories and symptoms, their improvements likely had more of an impact on daily function than subjects in the control group. The results from this study suggest that the CPA can be used to treat adhesions and scar adherence safely in the recurrent SBO subject population, demonstrating significant improvements in overall pain, severity of pain, QOL and number of episodes of SBO as compared to untreated subjects. Further, subjects who were previously concerned about having another SBO episode reported a significant decrease in that concern three months after treatment. In addition, the cost savings from using a relatively inexpensive outpatient treatment in place of surgery to prevent future obstruction, hospitalization and surgery is worthy of consideration.

Based upon changes in range of motion and disease scores, it is inferred that tissue and organ mobility was improved as the subjects demonstrated an increased range of motion in active movement tests. Improvements in range of motion allowed subjects to perform daily tasks more easily and contributed positively to their overall QOL.

The limitations of this study include the lack of randomization and sham treatment group. A sham treatment is challenging to accomplish in a study such as this where the treatment is 20 h of manual therapy over the course of one week. Indirect measures used to assess adhesions in the subjects including improvement in self-reported symptoms; ROM and objective disease scoring were used as outcome measures. While these measures are indirect, all correlated well with the post CPA treatment experiences and the decreased number of SBOs and surgeries in the treatment group, compared to the control group.

Today when a patient is discharged after non-surgical (or surgical) management of adhesive SBO, there is no therapy that can be prescribed to help reduce recurrence. Further, the quality of life for patients who have undergone SBO, with or without surgery, is often greatly compromised. Repeat surgery for SBO is widely regarded as a primary cause of recurring SBO. This technique appears to delay or obviate surgery for many patients who have undergone the trauma of SBO, at a fraction of the cost of surgery. It offers a therapy where none is currently available. The CPA therapy appears to be a viable conservative option for patients who previously were told that there was nothing that they could do to reduce their chances of recurrent SBO.

ARTICLE HIGHLIGHTS

Research background

Manual physiotherapy (mPT), called "manual physical therapy" in the United States, has been used to treat a wide variety of adhesive conditions including burns, adhesive capsulitis, radiculopathy, pain and the lessening of scars. In pilot studies, independent radiologic reports showed that the Clear Passage Approach (CPA), a specialized mPT performed in an outpatient setting, cleared bowel stricture and obstruction, obviating the need for planned surgical adhesiolysis and bowel resection. In case control and efficacy studies, CPA demonstrated effectiveness decreasing adhesions and pain, and improving quality of life (QOL) in subjects with recurrent small bowel obstruction (SBO).

Surgery is often cited as a primary cause of recurrent SBO due to the formation of post-operative adhesions. If a non-invasive outpatient therapy can decrease recurrent SBO and reduce the need for additional surgeries, it can improve quality of life for patients, with lower risk and decreased cost.

Research motivation

Adhesions that form after surgery present major problems for physicians and their patients. Surgeons note that adhesion barriers and gels are not always effective at preventing recurrent adhesions. The opportunity to delay or prevent post-surgical adhesions that can cause pain and recurring bowel obstruction is profound for patients. Many people live in fear that another major surgery or death could occur at any time, due to a recurrent obstruction.

Research objectives

The study has two main objectives: (1) To determine whether a manual physiotherapy can lower the rate of repeat SBO in patients who have undergone prior adhesive bowel obstructions and surgeries; and (2) to examine whether the therapy can improve the quality of life of these patients, using a validated test.

Research methods

This is a controlled phase two study in which 103 subjects with a history of recurrent adhesive SBO were treated with a manual physical therapy called the CPA. The focus of the therapy was to decrease adhesive crosslinking in abdominopelvic viscera. Pre- and post-therapy data measured recurring obstructions and quality of life using a validated test sent 90 d after therapy. Results were compared to 136 untreated control subjects who underwent the same measurements, but who did not receive any therapy. Until this method was developed, physical therapy has never been investigated as a course to treat recurring SBO.

Research results

Despite histories of more prior hospitalizations, obstructions, surgeries, and years impacted by bowel issues, the 103 CPA-treated subjects reported a significantly lower rate of repeat SBO than 136 untreated controls (total obstructions $P = 0.0003$; partial obstructions $P = 0.0076$). Subjects treated with the therapy demonstrated significant improvements in five of six total domains in the validated Small Bowel Obstruction Questionnaire (SBO-Q). Domains of diet, pain, gastrointestinal (GI) symptoms, quality of life (QOL) and pain severity when compared to post CPA treatment were significantly improved ($P < 0.0001$). The medication domain was not changed in the CPA treated group ($P = 0.176$).

Numerous studies examine the use of mPT to decrease adhesions and pain, and to improve function for conditions in various parts of the body. Pilot studies that examine the use of CPA include independent radiographs of cleared bowel obstruction. In a 10-year retrospective study of using CPA to treat adhesion-related female infertility ($n = 1392$), CPA opened blocked fallopian tubes in 60.85% (143/235) of women diagnosed with total tubal occlusion. In a recent study, mPT was shown to disrupt bowel adhesions in a rat model. This is the first controlled study of using a manual physiotherapy to decrease adhesions in the bowel.

The availability of the therapy is presently limited to private outpatient clinics in the United States and United Kingdom where therapists have been fully trained and certified in the CPA.

Research conclusions

A manual physiotherapy significantly improved quality of life and significantly decreased the rate of re-occlusion for patients with a history of SBO. Performed in an outpatient setting, the non-invasive therapy significantly reduced repeat obstructions. In addition, the physical therapy has a much lower risk and cost than hospitalization or surgery.

The study proposes that manual physiotherapy, which is commonly used to decrease adhesions in a variety of conditions, may be useful to decrease adhesions in the bowel. As such, it may delay or prevent recurring SBO.

This study noted that: (1) Post-surgical adhesions are frequently cited as the primary cause of SBO; (2) average costs in the United States for adhesiolysis and SBO are $65955 and $114175, respectively; (3) average hospital stays for adhesiolysis and SBO are 8.4 d and 14.2 d, respectively; (4) costs for a novel non-surgical physiotherapy to address adhesions and SBO are less than $7000; (5) Subjects treated with the therapy reported a significantly lower rate of repeat SBO than the untreated controls (total obstructions $P = 0.0003$; partial obstructions $P = 0.0076$); (6) subjects treated with the therapy demonstrated significant improvements in five of six total domains in the validated SBO-Q; (7) statistical analysis showed a significant increase in all six measures of trunk range of motion (flexion, extension, left and right side bending, left and right rotation.)

A manual physiotherapy significantly decreased the rate of recurrent small bowel obstruction (SBO) and improved quality of life for patients with a history of prior SBO. Based on the decreased number of recurring obstructions and the measurable improvements in trunk range of motion, this manual physiotherapy can significantly improve outcomes and quality of life for patients who undergo abdominal or pelvic surgery to treat adhesions or SBO.

This study proposes the use of a specialized manual physiotherapy, the CPA, to delay or prevent recurring SBO. This study confirmed and quantified hypotheses from earlier pilot studies that CPA could increase QOL, delay or prevent recurring SBO, and delay or obviate the need for additional surgery for patients with recurring SBO, with less risk and a lower cost than the present model. The major implication for clinical practice is that physicians, who are often stymied by the frequent recurrence of adhesions and SBO following abdominal surgery or resection, now have a less risky and less costly alternative to repeat surgery.

Research perspectives

There is a place for a multi-disciplinary approach to a vexing problem for surgeons - the recurrence of adhesions and SBO following abdominal surgery or pelvic surgery. We hope to be able to quantify further the degree to which CPA can decrease the recurrence of SBO, and the need for repeat surgeries. A prospective controlled study with closely matched study groups could be performed with the CPA method *vs* sham physiotherapy.

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META-ANALYSIS

Prognostic impact of the red cell distribution width in esophageal cancer patients: A systematic review and meta-analysis

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Abstract

AIM

To clarify the previous discrepant conclusions, we performed a meta-analysis to evaluate the prognostic value of red cell distribution width (RDW) in esophageal cancer (EC).

METHODS

We searched the PubMed, EMBASE, Web of Science and Cochrane Library databases to identify clinical studies, followed by using STATA version 12.0 for statistical

analysis. Studies that met the following criteria were considered eligible: (1) Studies including EC patients who underwent radical esophagectomy; (2) studies including patients with localized disease without distant metastasis; (3) studies including patients without pre-operative neoadjuvant therapy; (4) studies including patients without previous antiinflammatory therapies and with available preoperative laboratory outcomes; (5) studies reporting association between the preoperative RDW and overall survival (OS)/disease-free survival (DFS)/cancer-specific survival (CSS); and (6) studies published in English.

RESULTS

A total of six articles, published between 2015 and 2017, fulfilled the selection criteria in the end. Statistical analysis showed that RDW was not associated with the prognosis of EC patients, irrespective of OS/CSS [hazard ratio (HR) = 1.27, 95% confidence interval (CI): 0.97-1.57, P = 0.000] or DFS (HR = 1.42, 95%CI: 0.96-1.88, P = 0.000). Subgroup analysis indicated that elevated RDW was significantly associated with worse OS/CSS of EC patients when RDW > 13% (HR = 1.45, 95%CI: 1.13-1.76, P = 0.000), when the patient number $\leqslant$ 400 (HR = 1.45, 95%CI: 1.13-1.76, P = 0.000) and when the study type was retrospective (HR = 1.42, 95%CI : 1.16-1.69, P = 0.000).

CONCLUSION

Contrary to our general understanding, this meta-analysis revealed that RDW cannot serve as an indicator of poor prognosis in patients with EC. However, it may still be a useful predictor of unfavorable prognosis using an appropriate cut-off value.

Key words: Red cell distribution width; Prognostic impact; Systematic review; Meta-analysis

Core tip: Red cell distribution width (RDW) has been established as a prognostic factor for cancer patients. In consideration of esophageal cancer (EC), many articles have concluded that RDW is correlated with poor prognosis. However, recent studies have indicated that elevated RDW harbors no prognostic value for EC, which might, instead, be a favorable prognostic factor for EC patients. No consensus is available in the previous literature concerning whether elevated RDW is a negative or favorable prognostic factor for EC patients. To this end, for the first time, this systematic review and meta-analysis was performed to evaluate the prognostic value of RDW in EC.

Xu WY, Yang XB, Wang WQ, Bai Y, Long JY, Lin JZ, Xiong JP, Zheng YC, He XD, Zhao HT, Sang XT. Prognostic impact of the red cell distribution width in esophageal cancer patients: A systematic review and meta-analysis. *World J Gastroenterol* 2018; 24(19): 2120-2129 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i19/2120.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i19.2120

INTRODUCTION

Esophageal cancer (EC) is one of the most common digestive malignancies worldwide[1,2], ranking as the fourth leading cause of cancer-related death[3] and leading to approximately 400000 deaths in 2012[4]. The incidence of EC varies widely across different countries and regions[5]. According to the latest national statistics, EC is the fourth most common malignant tumor type in China[1], with cancer-related morbidity and mortality rates of 11.1% and 13.3%, respectively, in 2015, which has emerged as a severe public health problem, particularly in some high-risk rural areas[6]. However, in 2012, the cancer-related morbidity and mortality rates of EC (3.41% and 2.98%, respectively) were lower in North America and Europe than those in China[4].

EC is a highly aggressive digestive malignancy characterized by rapid growth and early metastasis. The rate of distant metastasis in EC patients is as high as 20%-30% at the time of initial diagnosis[7]. Despite the progress in radical resection and adjuvant therapy (radiation and chemotherapy), the 5-year overall survival (OS) rate of patients with EC remains approximately 20% in China[8], emphasizing an urgent need to detect effective prognostic biomarkers which could guide personalized therapeutic strategy for EC patients[9,10].

In recent years, accumulating evidence has shown that systemic inflammatory responses are closely associated with tumor initiation, progression and invasion[11-14]. Therefore, a variety of inflammatory indicators have been explored to assess their potential prognostic roles in various cancers. One such marker is the red blood cell distribution width (RDW), which is defined as the coefficient of variation in the red blood cell size. An elevated RDW indicates anisocytosis, which is considered as the basis for the clinical diagnosis of iron deficiency anemia[15]. However, fluctuations in the RDW have recently been reported as involved in many other pathophysiological conditions. For example, an elevated RDW is strongly associated with chronic inflammation, poor nutritional status, and age-associated diseases, which is indicative of changes in erythropoiesis. In addition, a number of studies have demonstrated a significant correlation between RDW and conventional inflammatory parameters, such as the C-reactive protein, interleukin-6 and tumor necrosis factor-α levels and erythrocyte sedimentation rate. Cancer has been revealed to be associated with chronic inflammation, and the latter is a key determinant of disease progression and survival in various cancers[16,17]. In 2007, Felker *et al*[18] found that RDW could serve as an independent predictor of morbidity and mortality in heart failure. Recent studies have revealed that RDW is associated with prognosis in several types of cancer, such as lung cancer[19], prostate

Table 1 PubMed search strategy

Number	Search items
#1	Esophageal Neoplasm.ti,ab
#2	esophagus neoplasm.ti,ab
#3	esophagus neoplasms.ti,ab
#4	cancer of esophagus.ti,ab
#5	cancer of the esophagus.ti,ab
#6	esophagus cancer.ti,ab
#7	esophagus cancers.ti,ab
#8	esophageal cancer.ti,ab
#9	esophageal cancers.ti,ab
#10	esophageal squamous cell cancer.ti,ab
#11	ESCC.ti,ab
#12	esophageal adenocarcinoma.ti,ab
#13	or #1- #12
#14	red cell distribution. ti,ab
#15	RDW.ti,ab
#16	or #14- #15
#17	#13 and #16

cancer[20] and EC[21].

RDW is an important complete blood count parameter that is routinely monitored in cancer patients. Several studies conducted in recent years[21-26] have investigated the relationship between EC prognosis and RDW due to the easy accessibility of obtaining blood samples and the low cost of analyzing RDW. However, the results of these studies show some discrepancies, which could be attributed to differences in the study design and relatively small sample sizes. To this end, in the present study, a meta-analysis was performed to identify the correlation between RDW and survival in EC patients.

MATERIALS AND METHODS

This meta-analysis was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines.

Data sources and search strategies

A systematic review of studies that evaluated the prognostic value of RDW in EC patients was performed. Four databases were electronically searched: Medline (host: OVID) from 1946 to April 2017; EMBASE (host: OVID) from 1974 to April 2017; and Web of Science and Cochrane Database of Systematic Reviews from 2005 to June 2017. The search terms used in our study were "RDW", "red cell distribution width with esophageal neoplasm", "esophagus neoplasm", "esophagus neoplasms", "cancer of esophagus", "cancer of the esophagus", "esophagus cancer", "esophagus cancers", "esophageal cancer", "esophageal cancers", "esophageal squamous cell cancer" (ESCC), and "esophageal adenocarcinoma". Both free text and MeSH terms were used as keywords. The search strategy used for the PubMed database is shown in Table 1, and the presented search strategy was also used for the other electronic databases.

Study selection

The search was performed by two investigators (Xu and Wang) who also evaluated the titles and abstracts of all candidate articles. Full-text was reviewed when the articles could not be categorized based on the title and abstract. Articles were included and excluded in accordance with the corresponding criteria defined in this study. Any disputes during the selection period were discussed with and resolved by a third investigator (Xiong). A flowchart demonstrating the details of the study selection according to the PRISMA guidelines is shown in Figure 1.

Inclusion and exclusion criteria

Studies that met the following criteria were considered eligible: (1) A study of EC patients who underwent radical esophagectomy; (2) a study of patients with localized disease without distant metastasis; (3) a study of patients without preoperative neoadjuvant therapy; (4) a study of patients without previous antiinflammatory therapies and with available preoperative laboratory outcomes; (5) a study of the association between the preoperative RDW and OS/disease-free survival (DFS)/cancer-specific survival (CSS); and (6) a complete paper published in English. Studies that met the following criteria were excluded: (1) Letters, case reports, reviews or preclinical studies; (2) studies describing a repeated analysis or duplicate data; (3) studies lacking key information for further analysis; and (4) nonhuman studies.

Data extraction

We used predesigned extraction forms for data collection. The following information was extracted from each study: first author's name, year of publication, country of the patients, research type, number of male/female patients included in the study, pathological types, RDW cut-off value, hazard ratio (HR) of elevated RDW for OS, CSS and DFS with 95% confidence interval (CI) and *P*-value. Assuming that most of the deaths were related to cancer, in the case of unavailability of OS information, data for CSS were extracted. HRs from multivariable analyses were extracted when available; otherwise, HRs from univariable analyses were extracted or estimated from Kaplan-Meier survival curves as described by Parmar and colleagues[27]. HRs for subgroups were compared defined by different markers.

Data synthesis and statistical analyses

HRs and the corresponding 95%CIs were directly obtained from each publication. If the values were not directly reported, the values were calculated according to the method described by Parmar and colleagues[27]. The meta-analysis was performed with STATA software version 12.0 (STATA Corporation, College Station, TX, United States) to combine the HRs with 95%CIs for categorical data and the weighted mean difference or standardized mean difference with 95%CIs for continuous data. All statistical tests were bilateral, and a *P* value < 0.05 was considered as statistical significance. If the data were not suitable for pooling, a systematic narrative synthesis of the information was performed,

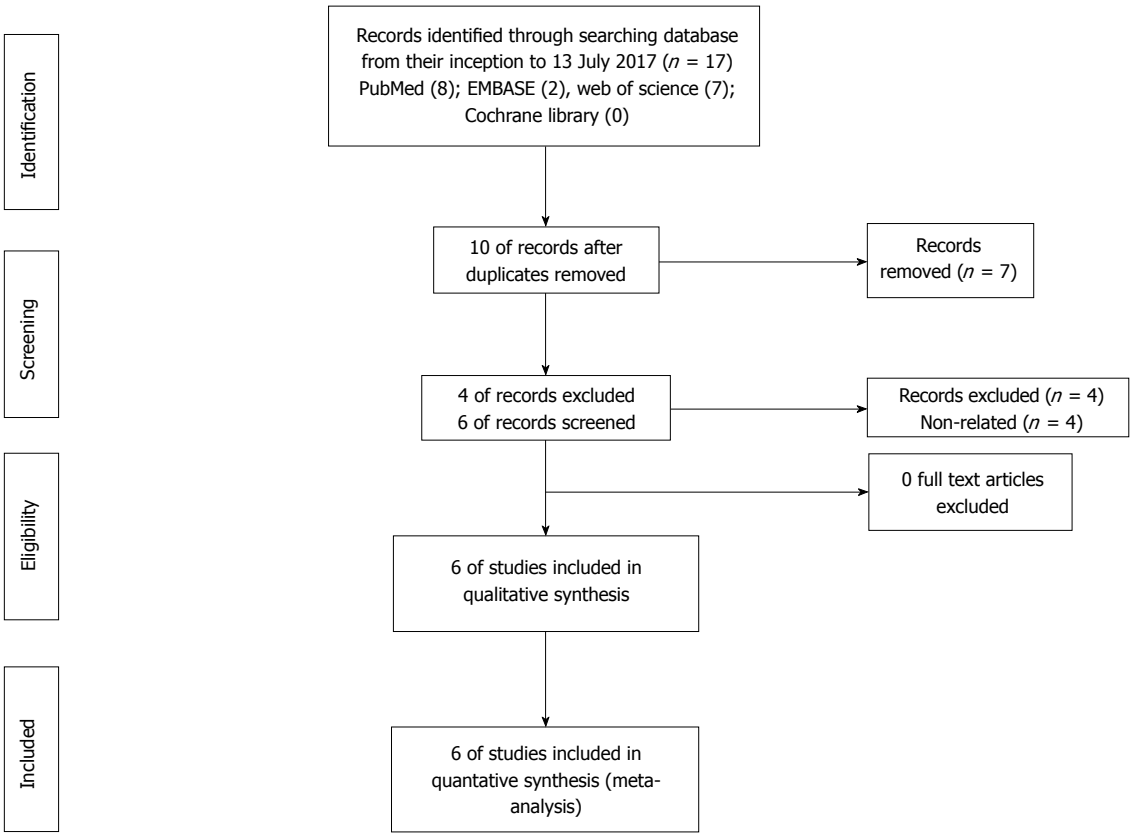

Figure 1 Methodological flow diagram of the meta-analysis.

which was presented in the text to understand and to summarize the findings as well as characteristics of the included studies.

Heterogeneity analysis

The heterogeneity of the pooled results was assessed through Cochran's Q test and Higgins *I*-squared statistic. Significant heterogeneity was identified by $P < 0.05$ and/or $I^2 > 50\%$, and the random-effects model (DerSimonian-Laird method) was used to combine the data. Otherwise, the fixed-effects model (Mantel-Haenszel method) was employed. To explore the potential source of heterogeneity among studies, subgroup analyses were performed according to various variables, such as the RDW cut-off value, the patient number in each study, and the study type and quality.

Assessment of study quality

The quality of the included studies was assessed by using the Newcastle-Ottawa quality scale (NOS)[28]. Three aspects, namely, selection, comparability and outcomes, were assessed on this scale, which had a maximum score of 9. Studies with scores $\geqslant 7$ were considered to be of high quality.

Sensitivity analysis

If significant heterogeneity was observed, a sensitivity analysis was performed after data extraction and subgroup analyses. This sensitivity analysis, which included the sequential omission of each study using the "metaninf" STATA command, aimed to validate the findings of this meta-analysis.

Assessment of publication bias

Begg's funnel plot and Egger's linear regression test were performed to evaluate publication bias, and a *P* value of < 0.05 was considered statistically significant.

RESULTS

Search results and study characteristics

Initially, 17 studies were selected from the electronic databases, and 10 studies remained after the removal of duplicates. After reading the titles and/or abstracts, four unrelated studies were excluded, and six full-text articles[21-26] were further assessed. None of these studies were excluded after thorough review of full-text. These six studies[21-26], which included 3826 patients, were included in this meta-analysis. The detailed search method and a flowchart representing the selection process are shown in Figure 1. These studies included five retrospective studies and one prospective study. The sample sizes varied from 144 to 2396, with a median value of 638. All six studies were conducted in Asian countries. The cut-off values for the RDW ranged from 12.2% to 15.3%. All six studies reported a correlation between RDW and OS/CSS, and two of the studies also investigated the association between RDW and DFS.

Table 2 Main characteristics of included studies in meta-analysis

Order number	Author	Year of publication	Country	Research type	Patients number	Male	Female	Pathological types	RDW, cut-off value, %	CSS/OS/ PFS/DFS	HR, U	LCI, U	UCI, U	*P*-value, U	HR, M	LCI, M	UCI, M	*P*-value, M	NOS score
1	Chen *et al*	2015	China	Retrospective	277	240	37	ESCC	14.5	CSS	1.719	1.268	2.331	< 0.001	1.396	1.022	1.908	0.036	6
2	Wan *et al*	2016	China	Retrospective	179	150	29	ESCC (133), EAC (46)	15	OS	3.087	1.85	5.152	< 0.001	1.895	1.023	3.508	0.042	7
2	Wan *et al*	2016	China	Retrospective	179	150	29	ESCC (133), EAC (46)	15	DFS	3.208	1.922	5.353	< 0.001	1.907	1.02	3.565	0.043	7
3	Hirahara *et al*	2016	Japan	Retrospective	144	129	15	ESCC	15.3	CSS	2.332	1.304	4.19	0.005	1.684	0.929	3.071	0.03	6
4	Sun *et al*	2016	China	Retrospective	362	268	94	ESCC	13.6	OS	1.381	0.946	2.016	0.094					5
5	Zhang *et al*	2016	China	Retrospective	468	376	92	ESCC	12.2	OS	1.505	1.068	2.122	0.02	1.356	0.948	1.94	0.095	7
5	Zhang *et al*	2016	China	Retrospective	468	376	92	ESCC	12.2	DFS	1.474	1.046	2.077	0.027	1.349	0.943	1.929	0.101	7
6	Hu *et al*	2017	China	Prospective	2396	1822	574	ESCC	12.90 (men)	OS	0.85	0.76	0.94	0.002	0.84	0.75	0.93	0.001	8
6	Hu *et al*	2017	China	Prospective	2396	1822	574	ESCC	12.70 (women)	OS	1.02	0.89	1.18	0.73	1.01	0.88	1.17	0.996	8
6	Hu *et al*	2017	China	Prospective	2396	1822	574	ESCC		OS					0.92	0.75	1.08	0.051	8

With the exception of one study, the NOS scores of all the other studies were > 5. The general characteristics of the six included studies are summarized in Table 2.

Impact of the RDW on OS and DFS in EC

The HRs and 95%CIs from the six studies involving 3826 patients were extracted and then pooled. The pooled results showed that the RDW was not associated with OS or CSS (HR = 1.27, 95%CI: 0.97-1.57, P = 0.000; Figure 2), with significant heterogeneity among the six studies (I^2 = 53.6%, P = 0.056; Figure 2); thus, the random-effects model was adopted for further analyses. The correlation between the RDW and DFS in EC patients were further investigated based on the pooled HRs and 95% CIs from two studies comprising 647 patients. As a result, RDW was not associated with DFS (HR = 1.42, 95%CI: 0.96-1.88, P = 0.000; Supplementary Figure 1), and no heterogeneity was observed (I^2 = 0.0%, P = 0.423; Supplementary Figure 1). In consideration of the significant heterogeneity of the pooled results regarding the effect of the RDW on OS/CSS, subgroup analyses, sensitivity analyses and Begg's funnel plot and Egger's linear regression analyses were conducted to further identify the heterogeneity source.

Sensitivity analyses was carried out by sequentially omitting each study to investigate its influence on results, indicating that the study conducted by Hu *et al*[26] was the primary source of heterogeneity (Figure 3). After exclusion of this study, the heterogeneity was effectively reduced or eliminated (I^2 = 0.0%, P = 0.926; Supplementary Figure 2). Surprisingly, the corresponding pooled HR varied with the inclusion (HR = 1.27, 95%CI: 0.97-1.57, P = 0.000; Figure 2) and omission of this study (HR = 1.42, 95%CI: 1.16-1.69, P = 0.000; Supplementary Figure 2). After reviewing the six studies included in our meta-analysis, we found that the study conducted by Hu *et al*[26] was the only prospective study, whereas the other five studies were retrospective ones. The sensitivity analyses indicated that the study type might be a source of heterogeneity. Therefore, we performed a subgroup analysis based on the study type (Supplementary Figure 3) and found that a high RDW was significantly associated with poor OS/CSS in patients with EC in the subgroup of retrospective studies.

To further explore other sources of heterogeneity, we performed a subgroup analysis based on the RDW cut-off values (⩽ 13% or > 13%). For RDW cut-off value > 13%, heterogeneity was effectively reduced or eliminated after exclusion of the study in which the cut-off value was ⩽ 13%, (I^2 = 0.0%, P = 0.850; Figure 4), and the corresponding pooled HR was increased (HR = 1.45, 95%CI: 1.13-1.76, P = 0.000; Figure 4). This finding indicated that the RDW cut-off value might be another source of heterogeneity. Thus, at an RDW cut-off value > 13%, a high RDW is significantly associated with poor OS/CSS in patients with EC.

Furthermore, when the subgroups were stratified by patient number (⩽ 400 or > 400), the heterogeneity was effectively reduced or eliminated after excluding the studies with > 400 patients (I^2 = 0.0%, P = 0.850; Supplementary Figure 4), and the corresponding pooled HR was increased when the patient number was ⩽ 400 (HR = 1.45, 95%CI: 1.13-1.76, P = 0.000; Supplementary Figure 4). Hence, the subgroup analyses indicated that the patient number might also be a source of heterogeneity. For studies

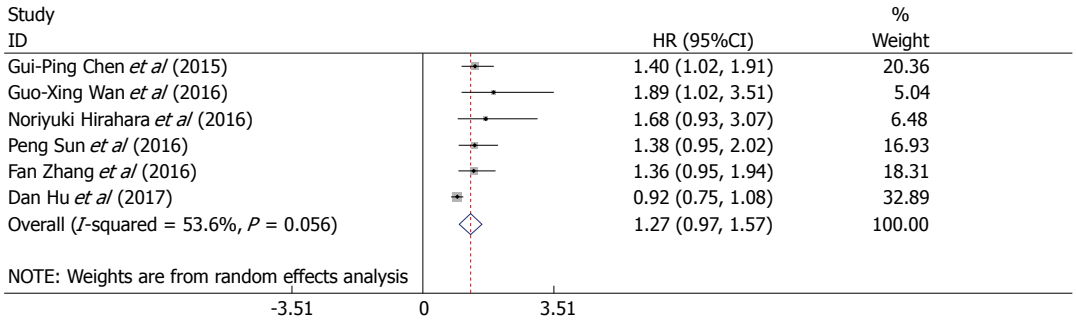

Figure 2 Forest plots of studies evaluating HR with 95%CI of red cell distribution width for overall survival in esophageal cancer patients. CI: Confidence interval; HR: Hazard ratio.

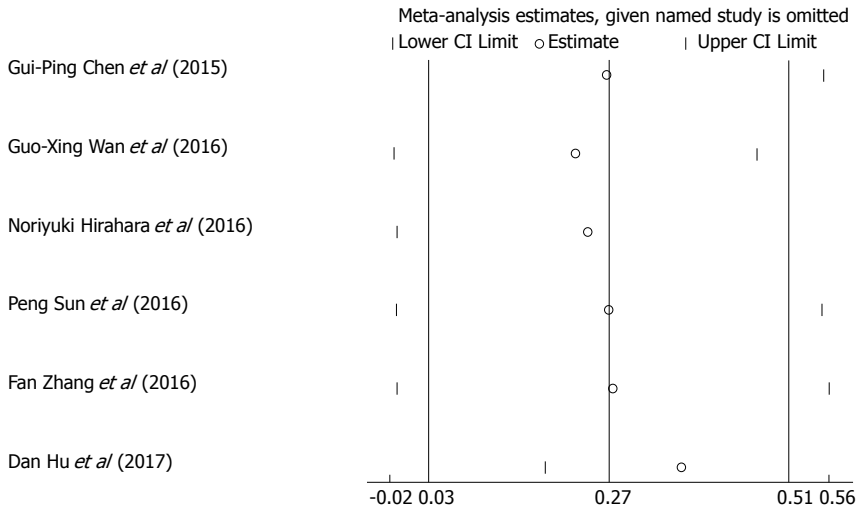

Figure 3 Effect of individual studies on the pooled HR for red cell distribution width and overall survival of esophageal cancer patients. HR: Hazard ratio; RDW: Red cell distribution width.

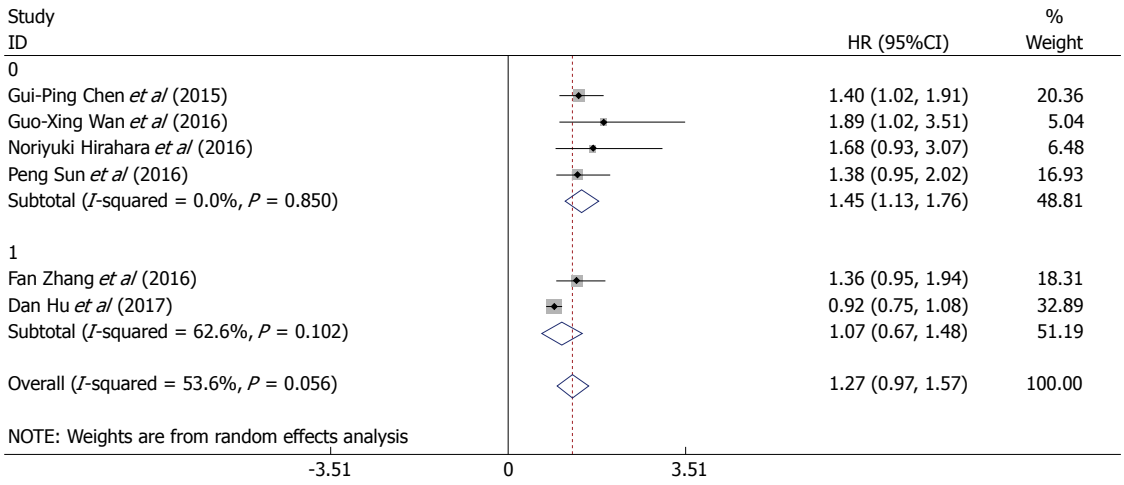

Figure 4 Forest plots of RDW > 13% *vs* RDW ⩽ 13% evaluating HR with 95%CI of red cell distribution width for overall survival in esophageal cancer patients. CI: Confidence interval; HR: Hazard ratio; RDW: Red cell distribution width. 0: RDW > 13%; 1: RDW ⩽ 13%.

with patient number ⩽ 400, a high RDW was significantly associated with poor OS/CSS in patients with EC.

Finally, when the subgroups were stratified by NOS scores (⩽ 6 or > 6), heterogeneity was effectively reduced or eliminated after omitting the studies with NOS scores > 6 (I^2 = 0.0%, *P* = 0.876; Supplementary Figure 5), and the corresponding pooled HR was increased (HR = 1.42, 95%CI: 1.09-1.74, *P* = 0.000; Supplementary Figure 5). Thus, the study quality might be another source of heterogeneity.

Publication bias

Begg's funnel plot and Egger's linear regression analyses were performed to estimate the potential publication bias

in the present meta-analysis. The *P* values regarding OS/CSS were 0.133 (Begg's test; Supplementary Figure 6) and 0.005 (Egger's test; Supplementary Figure 7). Due to the small sample sizes of the included studies, there was significant publication bias in our study, which was also demonstrated by the funnel plot (Funnel plot; Supplementary Figure 8). Therefore, publication bias might be another source of heterogeneity.

DISCUSSION

To the best of our knowledge, this study constitutes the first meta-analysis investigating the prognostic value of the RDW in EC. The most notable finding is that RDW is not associated with the prognosis of EC patients, including both OS/CSS and DFS. This novel finding is inconsistent with previous conclusions regarding the prognostic value of RDW in EC. Significant heterogeneity was observed across the included studies. After investigating the source of this heterogeneity by subgroup and sensitivity analyses, we derived four major conclusions. (1) In the retrospective studies, an elevated RDW was associated with poor OS/CSS, which did not affect DFS. (2) In the included prospective study, an elevated RDW was associated with a favorable prognosis in male ESCC patients, which is contradictory to the conclusions of most previous studies on cancers[20,29-32]. Additionally, RDW was not associated with prognosis in female patients. (3) When the patient number was $\leqslant$ 400, an elevated RDW was associated with poor OS/CSS, but this prognostic correlation was not observed when the number of patients was > 400. And, finally, (4) an elevated RDW was significantly associated with poor OS/CSS when the RDW cut-off value was > 13%, but this association was not observed when the cut-off value was $\leqslant$ 13%.

The role of the RDW is being increasingly appreciated due to its close correlation with the risks of cardiovascular diseases and systematic inflammation[33,34]. Previous studies have identified RDW as an accurate predictor of inflammation in hepatitis B infection, mortality due to acute pancreatitis, and activity of inflammatory bowel disease[34-36]. Moreover, an elevated RDW has been found to be a risk factor and progression indicator in multiple malignancies[19,31,37,38].

In the last 2 to 3 years, studies concerning the correlation between RDW and EC prognosis have become increasingly prominent. However, the conclusions of these studies are varied and sometimes even conflicting. Four small-scale retrospective studies[21-24] included in this meta-analysis concluded that an elevated RDW was significantly associated with worse OS in EC patients. In addition, one intermediate-scale retrospective study[25] concluded that the RDW was not associated with OS at all. Moreover, one large-scale prospective study[26] concluded that an elevated RDW was associated with better OS in male but not female EC patients. Considering that men are three to four times more likely to suffer from EC than women[7], this finding harbors important clinical implications in guiding therapeutic strategies for EC.

There are several reasons for the discrepant conclusions from diverse studies. Four small-scale retrospective studies[21-24] demonstrated that an elevated RDW was a predictor of unfavorable prognosis in EC patients, and the underlying mechanism might be one of the following. First, since Rudolf Virchow noted the presence of leucocytes within tumor tissues approximately 150 years ago and suggested that cancer might be initiated as a result of chronic inflammation[11,39], numerous preclinical and population-based studies have verified his observation. Inflammation might contribute to increased RDW levels by not only impairing iron metabolism but also inhibiting the production of or response to erythropoietin or by reducing red blood cell survival[40,41]. Second, chronic inflammation has also been associated with poor response to chemotherapy[42]. Third, RDW has been found to be correlated with malnutrition, which is an independent risk factor for nosocomial infections associated with poor therapeutic response, an increased rate of treatment-related toxicity, reduced survival rates, and poor quality of life[43,44].

In contrast, one large-scale prospective study[26] concluded that an elevated RDW was a positive predictor of prognosis in male EC patients, however the actual mechanism remains largely undefined. This finding is consistent with the results of another cohort study[45] with data from 26709 nondiabetic adults with more than 14 years of follow-up, which indicated that a low RDW was significantly associated with an increased incidence of diabetes mellitus independent of traditional risk factors. The underlying mechanism might be as follows. Aerobic glycolysis has been proposed as a hallmark of cancer, and the acidic environment caused by aerobic glycolysis is a necessary component of carcinogenesis[46]. Due to the significant association between a low RDW and an increased incidence of diabetes mellitus, it is reasonable to speculate that an elevated RDW might be a surrogate indicator of improved glucose metabolism, which is a key factor for prolonged survival in EC patients. Nevertheless, further clinical evidence and preclinical experiments are warranted to support and verify the accurate mechanism and to identify the real prognostic significance of the RDW in EC.

However, when the data from female patients were included in the present study, the RDW was not found to be associated with OS (HR = 0.92, 95%CI: 0.75-1.08, *P* = 0.000; Supplementary Figure 9), which is consistent with the conclusions of an intermediate-scale retrospective study[25] included in this meta-analysis. Further analyses revealed that the two above studies have some common characteristics-the sample size was relative large (468 *vs* 2396) and the RDW cut-off values were 13%. This finding is consistent with the results of a study[47] conducted in 2012, which revealed that the RDW was elevated (> 14.8%) in 31.6% of benign biliary obstruction cases and 68.4% of malignant biliary stricture cases, whereas the RDW was reduced (< 14.8%) in

72.9% of benign cases and 27.1% of malignant cases (these differences were statistically significant, $P < 0.001$). Therefore, an RDW cut-off of 14.8% was the most suitable for predicting a malignant biliary stricture, with a sensitivity of 72% and a specificity of 69% (area under the curve = 0.755, 95%CI: 0.649-0.810). The conclusions of the two studies might be partly attributed to the lower RDW cut-off value. Thus, more large-scale studies exploring the actual relationship between RDW and the prognosis of EC patients are urgently needed in the future. Furthermore, it is necessary to establish a reasonable method for identifying the appropriate RDW cut-off value for predicting the prognosis of EC patients.

However, in contrast to the findings obtained from male EC patients, an elevated RDW was not associated with the prognosis of female EC patients[26]. Despite the prospective nature of the study demonstrating these results, we cannot neglect a potential correlation between the RDW and sex due to the large sample size in that study. However, more studies are needed to investigate and confirm this correlation and to explore the underlying mechanisms.

There are certain limitations that should be acknowledged in this meta-analysis. First, most of the studies included in this meta-analysis were retrospective in nature, and the numbers of patients in these retrospective studies were relatively small. Only one study was prospective, which might prevent generalization of the results. Second, this meta-analysis was performed based on the pooled HRs and 95%CIs from eligible studies, rather than detailed individual information. We were unable to exclude uncontrolled or unmeasured risk factors from the original studies, which might have confounded the true association, resulting in potential bias. Third, the cut-off values for the RDW varied across the included studies due to differences in the study populations and experimental methods. Although the patients were divided into RDW-high and RDW-low populations, the stratification might change depending on the cut-off values. Therefore, a standard and uniform cut-off value is needed to accurately define high *versus* low RDW. Fourth, all of the included articles were in English, most of the included studies included a small number of patients, and potential publication bias cannot be neglected. Thus, more large-scale, well-designed and high-quality prospective studies are required to elucidate the precise mechanisms linking the RDW to survival in EC patients.

In conclusion, an elevated RDW is not associated with the prognosis of EC patients, including both OS/CSS and DFS. This finding is contrary to previous knowledge regarding the prognostic value of the RDW in malignant tumors, particularly in EC. However, when the RDW cut-off value is > 13%, the patient number is $\leqslant$ 400, and the study type is retrospective, an elevated RDW is indeed significantly associated with worse OS/CSS in EC patients.

ARTICLE HIGHLIGHTS

Research background

Esophageal cancer (EC) was the eighth most common cancer globally, with about half of all cases occurring in China. Prominent symptoms usually do not appear until the cancer has infiltrated over 60% of the circumference of the esophageal tube, by which time the tumor is already in an advanced stage and the prognosis generally tends to be fairly poor. Therefore, finding a simple and effective prognostic indicator is particularly urgent for individualized treatment of EC patients. Recently, red blood cell distribution width (RDW), as an important complete blood count parameter which has a close correlation with cancer-related inflammation, has been investigated as an important prognostic factor for EC patients in more and more studies, but the conclusions of these studies have not been consistent. Therefore, we conducted this meta-analysis to explore and verify the real role of RDW in the prognosis of patients with EC.

Research motivation

We systematically reviewed the existing studies regarding the role of RDW in the prognosis of EC patients and performed a meta-analysis with the extracted data to clarify the real impact of RDW on the outcomes of the EC patients. Identifying the real role of RDW in the prognosis of patients with EC and the defects existing in the previous and current studies can guide the future researchers to conduct more well-designed related studies on this topic and the upstream or downstream research related to RDW.

Research objectives

The main objectives of this article were to perform a meta-analysis of the data provided in these studies with inconsistent conclusions about the prognostic effect of RDW on EC patients, and to verify the real impact of RDW on the prognosis of EC patients by increasing the sample size. In the end, we could determine whether we need to conduct further studies on this topic according to the conclusion of this systematic review and meta-analysis.

Research methods

First, we searched four related electronic databases (PubMed, EMBASE, Web of Science and Cochrane Library) using the identified MESH terms, and finally identified six studies which met the standards based on the inclusion and exclusion criteria of the selected literature, then we assessed the quality of the included studies according to Newcastle-Ottawa quality scale. Second, we used the electronic EXCEL table to collect the data from the included studies that we needed and utilized statistical software (STATA version 12.0) to conduct statistical analysis of the related data. Third, we performed the sensitivity analysis, subgroup analysis, Begg's funnel plot and Egger's linear regression test to explore the potential source of heterogeneity among studies, to find the influencing factors that affect the role of RDW in the prognosis of EC patients and point out the directions for further related research in the future.

Different from the traditional review, we used meta-analysis methods to synthesize data and perform statistical analysis to the relevant literature and quantify the effect of RDW on the prognosis of EC patients. Moreover, in addition to sensitivity analysis and subgroup analyses to find sources of heterogeneity, we also used the Begg's funnel plot and Egger's linear regression test to quantify publication bias rather than just using the traditional funnel plot for qualitative analysis. These were the characteristics and indicate the novelty of the research methods used in our study.

Research results

This systematic review and meta-analysis indicated that elevated RDW was not an independent risk factor for the worse outcome of EC patients overall, whether it's for overall survival/cancer-specific survival [hazard ratio (HR) = 1.27, 95% confidence interval (CI): 0.97-1.57, $P = 0.000$] or disease-free survival (HR = 1.42, 95% CI: 0.96-1.88, $P = 0.000$). The prognostic value of RDW in patients with EC is only reflected in the retrospective study (HR = 1.42, 95%CI: 1.16-1.69, $P = 0.000$) of small samples (sample size $\leqslant$ 400, HR = 1.45, 95% CI = 1.13-1.76, $P = 0.000$) currently, and there is a need to choose the appropriate RDW cutoff value (RDW > 13%, HR = 1.45, 95%CI: 1.13-1.76, $P = 0.000$) as a prerequisite. Therefore, the actual effect of RDW on the prognosis

of EC patients needs further prospective multicenter large-sample studies to be validated in the future.

Research conclusions

Different from the traditional viewpoints, our systematic and meta-analysis demonstrated that RDW had no correlation with the prognosis of EC patients, no matter favorable or unfavorable. Therefore, such traditional theories and assumptions, that cancer-related inflammation leads to an increased RDW in the blood, and elevated RDW in turn suggests the occurrence of cancer, were challenged and questioned by the results of our meta-analysis. At the same time, it also suggests that we could perform the meta-analysis to statistically analyze the inconsistent result data of different types of small-sample studies and achieve a conclusion that is completely different from our previous understanding. This leads to the emergence of new theories and assumptions and provides direction for our future research design and potential mechanism research. Our systematic reviews and meta-analysis suggest that we should be more cautious and rational to see the impact of increased RDW on the prognosis of EC patients in our future clinical work.

Research perspectives

From our study, we could learn that we can't blindly believe in traditional ideas that already exist. When the opinions of previous studies are inconsistent and chaotic, we should use statistical methods to perform statistical clustering analysis on various data, and draw a scientific conclusion to guide our clinical work and indicate the future research direction. Moreover, through the systematic analysis of the previous research, we should carry out more multicenter, large-sample prospective studies in the future to overcome the defects of the current research in the study design to further verify the role of RDW in the prognosis of EC patients. In addition, we also need to conduct further basic experiments based on the results of such above-mentioned optimized research to uncover its underlying mechanisms.

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CASE REPORT

Pressurized intraperitoneal aerosol chemotheprapy after misdiagnosed gastric cancer: Case report and review of the literature

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Informed consent statement: Written informed consent from patient involved in this study was received prior to study inclusion. All details that might disclose the identity of the subjects under study were omitted or anonymized.

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Abstract

We report the first application of pressurized intraperitoneal aerosol chemotherapy (PIPAC) as a rescue therapy before palliative D2 gastrectomy combined with liver metastasectomy performed in a 49-year-old woman with peritoneal carcinomatosis who was primarily diagnosed with and underwent surgery for a Krukenberg tumor. The PIPAC procedure was performed with the use of cisplatin at 7.5 mg/m^2 and doxorubicin at 1.5 mg/m^2 for 30 min at 37 ℃. Eight weeks after the PIPAC procedure, the patient underwent open classic D2 gastrectomy with the creation of a Roux-en-Y anastomosis (RNY) combined with liver metastasectomy. The patient underwent the classic protocol for chemotherapy combined with Xeloda. The patient felt better and returned to her daily activities. Multicenter data should be gathered to confirm the usefulness of PIPAC as a rescue or neoadjuvant supportive therapy in a very select group of patients who have been recently qualified to undergo classic chemotherapy or standard

oncologic surgical procedures.

Key words: Peritoneal carcinomatosis; Pressurized intraperitoneal aerosol chemotherapy; Neoadjuvant therapy; Gastric cancer; Krukenberg tumor

Core tip: The Krukenberg tumor (KT) is very often misdiagnosed as primary ovarian cancer and may be occasionally diagnosed during a clinical work-up. The fast implementation of effective treatment is always necessary. This case might contribute to future confirmation of the usefulness of pressurized intraperitoneal aerosol chemotherapy as a rescue or neoadjuvant, supportive form of therapy in a very select group of patients. This clinical development might be particularly important for patients with a KT presentation of gastric cancer who have been recently qualified to undergo classic chemotherapy or standard oncologic surgical procedures.

Nowacki M, Grzanka D, Zegarski W. Pressurized intraperitoneal aerosol chemotheprapy after misdiagnosed gastric cancer: Case report and review of the literature. *World J Gastroenterol* 2018; 24(19): 2130-2136 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i19/2130.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i19.2130

INTRODUCTION

Gastric cancer (GC) is one of the leading causes of cancer-related death worldwide, despite the fact that knowledge about its etiology, diagnostics, systemic chemotherapy and surgical techniques have significantly developed and improved during the last three decades[1-4]. The most problematic issue concerning the clinical management of GC is diagnosis at an advanced, and often metastasized, stage[5]. This circumstance is explained by the fact that local symptoms occur only at an advanced stage. Apart from extensive local tumor growth rendering the patient ineligible for curative resection, peritoneal carcinomatosis, which is synchronous in approximately 10% of patients, limits therapeutic options in this subset of patients[6-11].

Another metastatic site, other than the peritoneum, is the ovaries; tumors at these sites are referred to as Krukenberg tumors (KTs). These tumors are very often misdiagnosed as primary ovarian cancer and may be occasionally diagnosed during a clinical work-up for abdominal tenderness in the lower abdomen[12,13]. The KT is described mainly as a rare metastatic tumor of the ovary that originates from the gastrointestinal tract (stomach - 76%; colorectum - 4%, biliary system - 3%; appendix - 3%) but can also originate from breast (4%) and from other miscellaneous sites such as the pancreas, uterus, cervix, or urinary bladder[14,15]. KT is considered a late-stage disease, and despite growing clinical knowledge, there are still many controversies regarding standardized treatment protocols for this subset of patients[16]. To date, there are no universally accepted and recommended prognostic factors for KT treatment that indicate the superiority of one particular surgical algorithm or chemotherapeutic regimen over another[17,18].

We report the first case in the recent and past literature of the application of pressurized intraperitoneal aerosol chemotherapy (PIPAC) as neoadjuvant therapy before palliative D2 gastrectomy combined with liver metastasectomy performed in a patient who was with primarily diagnosed and underwent surgery for a KT.

Table 1 Coexisting diseases - data from the patient's medical history report

Diabetes - Type 1
Hypothyroidism
Paroxysmal atrial fibrilation
Hypertension
Rheumatoid arthritis

CASE REPORT

A 49-year-old woman with several co-morbidities (Table 1) was admitted in February 2017 to another hospital with ascites and abdominal masses. Abdominal ultrasound showed bilateral ovarian tumors, ascites and suspicion of peritoneal metastases, suggesting locally advanced ovarian cancer. An exploratory laparotomy was performed by the gynecology team, confirming the presence of two ovarian masses (left: 8 cm × 6 cm, right 12 cm × 8 cm), diffuse peritoneal metastasis (peritoneal carcinomatosis index - PCI = 19) and ascites (volume = 120 mL). Bilateral hysterectomy was performed in combination with bilateral adnexectomy, omentectomy, appendectomy and pelvic lymphadenectomy. The intraoperative situs was evaluated by the gastrointestinal surgeon who confirmed that complete cytoreduction was not possible.

Recovery was uneventful. Histology revealed signet ring tumor cells arranged singly, in cords or in nests within cellular ovarian stroma (Figure 1A and B). Additional staining revealed CK7(+), CK20(-), CDX2(+) and CA125(-) status, suggesting a primary tumor originating from the upper gastrointestinal (GI-) tract. Postoperative upper GI endoscopy showed a mucin-positive, poorly differentiated adenocarcinoma located in the antral mucosa (Figure 1C and D). Staging was completed with an abdominal CT scan that showed a superficially located metastasis in liver segment 5 (Figure 2).

The patient was referred in April 2017 to our tertiary center for further therapy.

At admission, the patient had reduced general condition (ECOG - 2) and in reduced nutritional status

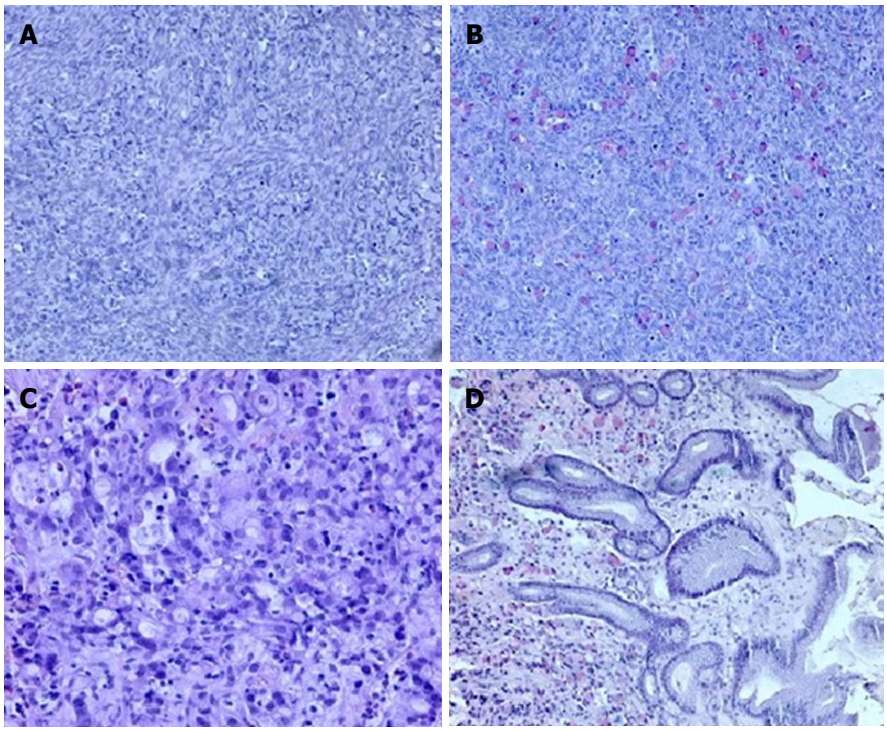

Figure 1 Histological ovary assay. A: Signet ring of tumor cell infiltration within the ovarian stroma (10 ×, HE); B: Mucicarmine staining highlights the presence of mucin in the cytoplasm (10 ×). Histopathologic evaluation of antral mucosa showing (C) poorly differentiated carcinoma infiltration with signet ring cells (20 ×, HE) and (D) mucicarmine staining of mucin-positive cells in the gastric mucosa (10 ×).

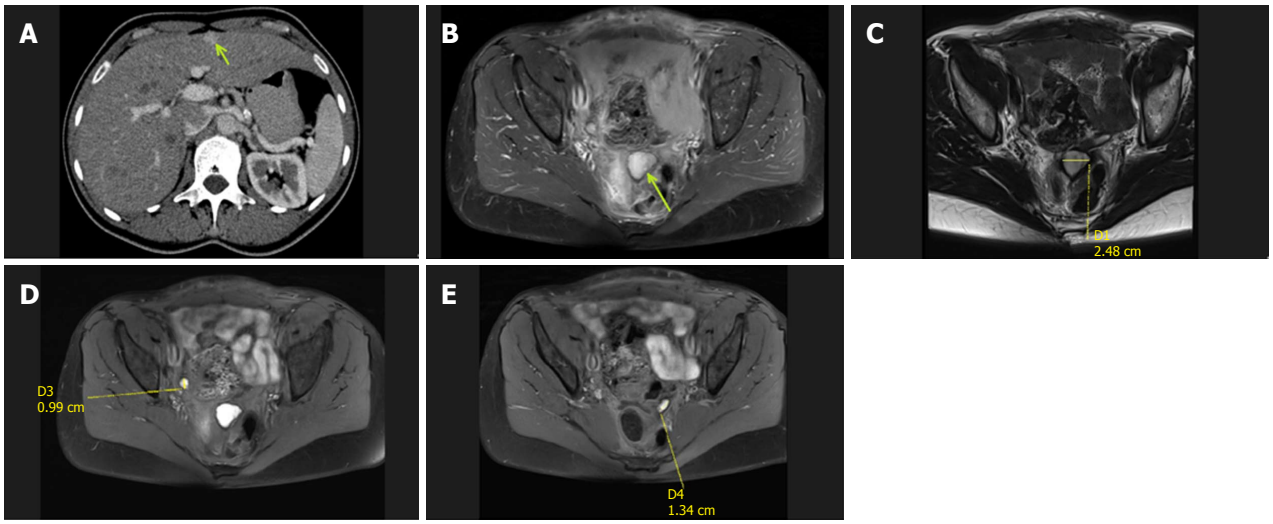

Figure 2 Abdominal computed tomography and abdominopelvic magnetic resonance scans. A: Post-contrast computed tomography image in the portal venous phase showing a hyperintense enhancing lesion in segment V (isodense in the native phase) of the liver, which was diagnosed as a superficially located suspicious metastatic lesion; B-E: Abdominopelvic magnetic resonance scans with evident masses and suspicious nodules.

(BMI of 18.37 and weight loss identified in the nutritional anamnesis). Laboratory tests were within normal limits. The patient was presented to the multidisciplinary tumor board, and a systemic combination chemotherapy combined with intraperitoneal chemotherapy with cisplatin and doxorubicin (as a pressurized aerosol, PIPAC C/D) was recommended, with palliative intent. The patient gave voluntary and informed consent to the planned treatment, and the study was performed in accordance with the precepts established by the declaration of Helsinki.

The PIPAC procedure was first performed in May 2017, according to standard protocols described by Hubner *et al*[19]. Shortly thereafter, after a 12 mmHg capnoperitoneum has been established, two trocars were inserted, and a staging laparoscopy was performed. After confirmation of the tightness of the abdomen, a solution of low-dose cisplatin (7.5 mg/m^2 BSA) and doxorubicin (1.5 mg/m^2 BSA) diluted in 200 mL of saline solution was aerosolized at a pressure of 12 mmHg and a temperature

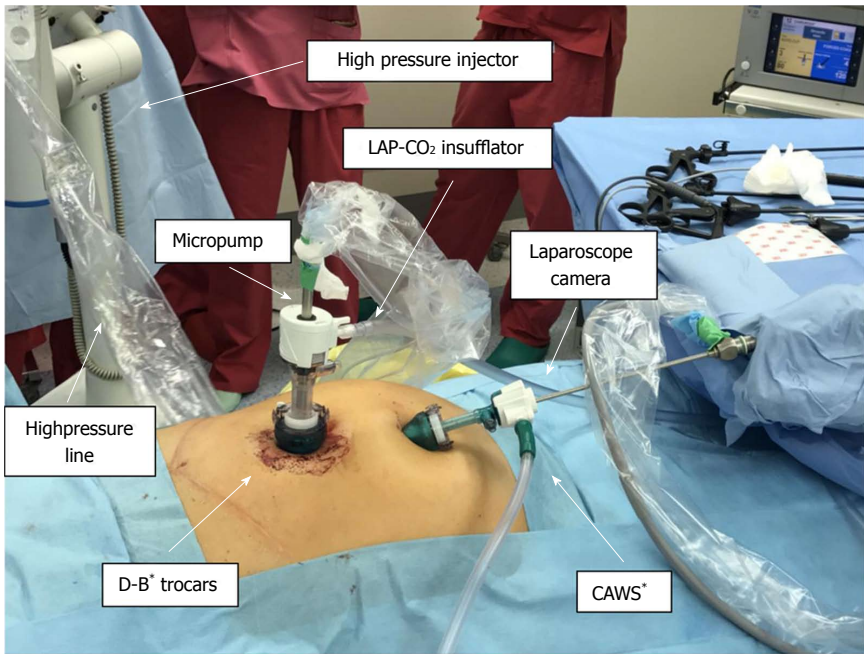

Figure 3 Schematic presentation of the pressurized intraperitoneal aerosol chemotherapy procedure. The procedure is performed under general anesthesia and based on standard diagnostic laparoscopy procedures. Two small incisions are always made to obtain surgical access using two double-balloon secured trocars (D-B* - double-balloon secured trocars). The first one is used for the laparoscopic camera and is connected to a closed aerosol waste system (CAWS*). The second one connected to the CO_2 insufflator is for the micropump nebulizer used for delivering chemotherapy under pressure *via* a high-pressure line.

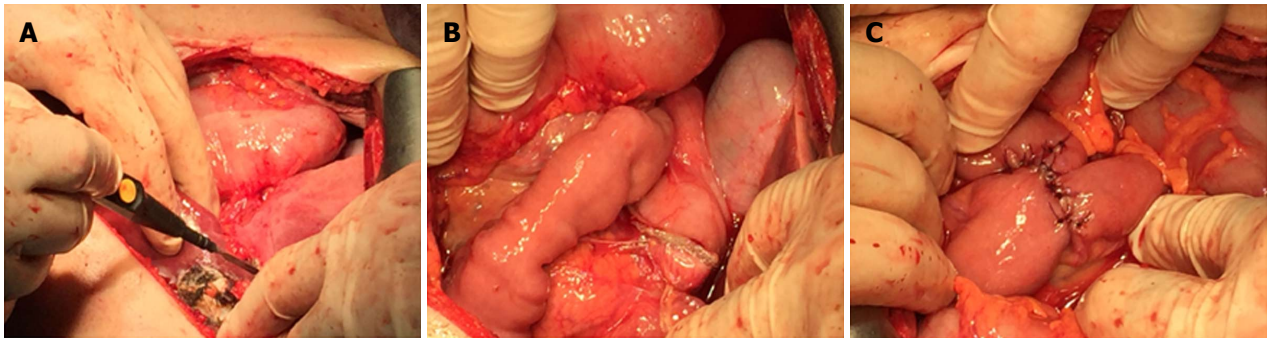

Figure 4 Palliative open D2 gastrectomy combined with liver metastasectomy. A: Liver metastasectomy procedure involving removal of the metastatic lesion combined with parenchyma coagulation; B: Open gastrectomy procedure showing the staple line after resection; C: Creation of a Roux-en-Y anastomosis (RNY) using sutures.

of 37 ℃ into the abdomen using a CE-certified nebulizer (Capnopen®, Capnomed, Villingendorf, Germany). After 30 min of application, the toxic aerosol was safely removed via a closed aerosol waste system (CAWS). The diagram displaying the PIPAC procedure is presented in Figure 3. The PIPAC procedure was tolerated very well, and no postoperative complications were noted.

Eight weeks after the PIPAC procedure, an exploratory laparotomy was performed. This time period has been suggested in the literature as the optimal time period between the next surgical intervention and each PIPAC surgery[20]. Macroscopically, a 3-cm tumor was palpated in the gastric body, infiltrating the gastric serosa, and no diffuse peritoneal metastasis were found anymore during a detailed standard surgical intraoperative PC lesion assessment, so complete cytoreduction (CC-0 according to Sugarbaker) appeared feasible. Therefore, curative gastrectomy, D2 lymphadenectomy and atypical liver resection were performed (Figure 4). Histopathologic evaluation demonstrated a poorly differentiated carcinoma of the stomach, which was ypT3N2 (4/40) and high grade (G3) (Figure 5). The liver metastasis had a diameter of 2.5 cm. All the resection margins were tumor-free, so the procedure was considered (potentially) curative. The postoperative course was uneventful. Four months after surgery, the patient was completely recovered and had returned to her daily activities (ECOG = 1; BMI = 20.23). Postoperative adjuvant chemotherapy with Xeloda was recommended. Narrow follow-up examinations (abdominal CT scan) will be performed.

DISCUSSION

The diagnosis of a Krukenberg tumor of gastric cancer

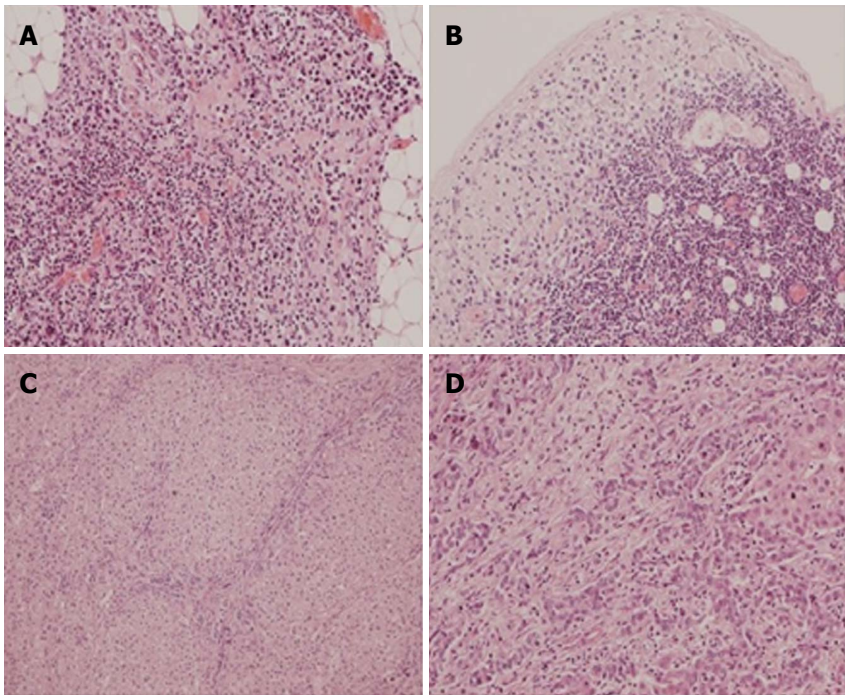

Figure 5 Histopathologic evaluation performed after open D2 gastrectomy combined with liver metastasectomy. A: Tumor microfocus infiltrations in the peritoneal adipose tissue in the vicinity of the distal surgical margin (obj. 20 ×, HE); B: Metastatic foci in the subcapsular region of the lymph node (obj. 20 ×, HE); C, D: Liver metastasis of gastric carcinoma (obj. 10 ×, 20 ×, HE).

origin is associated with a poorer prognosis than other types of primary origin KTs and is significantly more clinically problematic[21]. Several papers focus on different treatment options in patients with KT and mainly report on two specific issues: (1) The role of gastrectomy and metastasectomy under different clinical conditions; and (2) the assessment of the effectiveness and superiority of surgical and chemotherapy interventions[22,23]. The main problem in such descriptions is related to the fact that in large number of papers, the analyzed material concerned data only until the time of KT diagnosis[24].

In this paper, we present the case of 49-year-old PC patient with a high PCI index who was diagnosed primarily with KT due to an intrapathology assay in which bilateral hysterectomy combined with removal of the uterine appendages, omentectomy, appendectomy and pelvic lymphadenectomy was performed. The optimal treatment in such cases has still not been fully described in schematic recommendations and guidelines regarding the role of gastrectomy[25]. In recent and past literature, the beneficial outcomes of palliative gastrectomy have been presented[26]. Thus, because of the young age and good performance status of our patient along with the nonacceptance of standard intravenous forms of therapies with a parallel allowance for an intraperitoneal form of drug delivery, we elected the aforementioned treatment protocol consisting of "neoadjuvant" PIPAC followed by D2 gastrectomy. During our literature review we found information about some cases in which cytoreductive surgeries were performed in combination with hyperthermic intraperitoneal chemotherapy (CRS+HIPEC) procedures with good clinical effects that improved the survival of GC patients[27-29]. In our case, such an intervention was not possible due to the very aggressive CRS surgery and the patient's disqualification by medical oncologists. We also found interesting data about a novel and promising intraoperative drug delivery technique - pressurized intraperitoneal aerosol chemotherapy - that has also been used as a neoadjuvant therapy before cytoreductive surgery and hyperthermic intraperitoneal chemotherapy in different clinical cases[30]. In the recent and past literature, the effectiveness of repetitive PIPAC has also been documented for irresectable PC originating from pancreatic or ovarian cancer with a median histologically proven regression rate of up to 50%[20,30-32]. In this case, our MDT recommended PIPAC as a bridge (sandwich) therapy before a possible more definitive CRS+HIPEC procedure with palliative D2 gastrectomy without other cytoreduction and final qualification for standard chemotherapy protocols, according to the current international guidelines, until these therapies are personally acceptable to the patient.

During the clinical work-up, we encountered another problematic clinical issue regarding the patients metastatic lesion in the liver, which would normally render a patient ineligible for PIPAC treatment[33]. Fortunately, the metastatic lesion in our patient was located superficially, and after PIPAC was performed, no complications were reported.

In our opinion, in the future, additional clinical studies

should be performed in multiple centers to confirm the usefulness of PIPAC as a rescue or neoadjuvant, supportive form of therapy in a very select group of patients. This case might contribute to future confirmation of the usefulness of PIPAC as a rescue or neoadjuvant, supportive form of therapy in a very select group of patients. This clinical development might be particularly important for patients with a KT presentation of gastric cancer who have been recently qualified to undergo classic chemotherapy or standard oncologic surgical procedures.

ARTICLE HIGHLIGHTS

Case characteristics

A 49-year-old female patient with reduced general condition and nutritional status (low BMI and weight loss in the nutritional anamnesis) was admitted after bilateral hysterectomy with a diagnosis of diffuse peritoneal carcinomatosis and several co-morbidities.

Clinical diagnosis

The final clinical diagnosis was made by upper gastrointestinal (GI) endoscopy combined with a pathological assay that showed a mucin-positive, poorly differentiated adenocarcinoma located in the gastric antral mucosa.

Differential diagnosis

The differential diagnosis included severe peritoneal carcinomatosis and primary origin cancer with a particular emphasis on ovarian cancer.

Laboratory diagnosis

Despite the patient's reduced general condition and nutritional status, all of the performed laboratory tests were within normal limits.

Imaging diagnosis

The CT scan performed during hospitalization in our department showed an additional superficially located metastasis in liver segment 5.

Pathological diagnosis

In this case, staining revealed CK7(+), CK20(-), CDX2(+) and CA125(-) status, suggesting a primary tumor originating from the upper GI- tract. A postoperative upper GI endoscopy showed a mucin-positive, poorly differentiated adenocarcinoma located in the gastric antral mucosa.

Treatment

The PIPAC procedure was based on the administration of a solution of low-dose cisplatin (7.5 mg/m^2 BSA) and doxorubicin (1.5 mg/m^2) BSA diluted in 200 mL of saline solution aerosolized at a pressure of 12 mmHg and a temperature of 37 ℃ into the abdomen using a CE-certified nebulizer as neoadjuvant therapy before palliative D2 gastrectomy combined with liver metastasectomy.

Related reports

Very few cases of spontaneous regression of an intra-abdominal inflammatory myofibroblastic tumor have been reported in the literature. The clinical and pathological characteristics of inflammatory myofibroblastic tumors remain unclear, and the treatment is controversial.

Term explanation

The acronym PIPAC describes pressurized intraperitoneal aerosol chemotherapy (PIPAC).

Experiences and lessons

This case might contribute to future confirmation of the usefulness of PIPAC as a rescue or neoadjuvant, supportive form of therapy in a very select group of patients. This clinical development might be particularly important for patients with a KT presentation of gastric cancer who have been recently qualified to undergo classic chemotherapy or standard oncologic surgical procedures.

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