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REVIEW

Glycogen storage diseases: An update

Ersin Gümüş, Hasan Ozen

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Abstract

Glycogen storage diseases (GSDs), also referred to as glycogenoses, are inherited metabolic disorders of glycogen metabolism caused by deficiency of enzymes or transporters involved in the synthesis or degradation of glycogen leading to aberrant storage and/or utilization. The overall estimated GSD incidence is 1 case per 20000-43000 live births. There are over 20 types of GSD including the subtypes. This heterogeneous group of rare diseases represents inborn errors of carbohydrate metabolism and are classified based on the deficient enzyme and affected tissues. GSDs primarily affect liver or muscle or both as glycogen is particularly abundant in these tissues. However, besides liver and skeletal muscle, depending on the affected enzyme and its expression in various tissues, multiorgan involvement including heart, kidney and/or brain may be seen. Although GSDs share similar clinical features to some extent, there is a wide spectrum of clinical phenotypes. Currently, the goal of treatment is to maintain glucose homeostasis by dietary management and the use of uncooked cornstarch. In addition to nutritional interventions, pharmacological treatment, physical and supportive therapies, enzyme replacement therapy (ERT) and organ transplantation are other treatment approaches for both disease manifestations and longterm complications. The lack of a specific therapy for GSDs has prompted efforts to develop new treatment strategies like gene therapy. Since early diagnosis and aggressive treatment are related to better prognosis, physicians should be aware of these conditions and include GSDs in the differential diagnosis of patients with relevant manifestations including fasting hypoglycemia, hepatomegaly, hypertransaminasemia, hyperlipidemia, exercise intolerance, muscle cramps/pain, rhabdomyolysis, and muscle weakness. Here, we aim to provide a comprehensive review of GSDs. This review provides general characteristics of all types of GSDs with a focus on those with liver involvement.

Key Words: Glycogen storage disease; Liver; Muscle; Hypoglycemia

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Core Tip: Glycogen storage diseases are multisystemic diseases that can present at any age. Primarily affected organs are liver and skeletal muscle, but heart, central nervous system, kidneys, intestines, and other organs may also be affected. As the initial presenting symptoms can occur in adulthood, it is a group of rare diseases that should be recognized and managed by not only pediatricians but also physicians taking care of adults.

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INTRODUCTION

Glycogen storage diseases (GSDs), also referred to as glycogenoses, are inherited metabolic disorders of glycogen metabolism caused by deficiency of enzymes or transporters involved in the synthesis or degradation of glycogen[1]. Disturbances in glycogen metabolism result in aberrant storage and/or utilization of glycogen. Both glycogen formation and breakdown involve several enzymatic reactions and are strictly dependent on hormone regulation (Figure 1)[2]. After a meal, insulin stimulates glycogen storage in muscle and liver by simultaneously promoting glycogen synthesis and inhibiting glycogen breakdown. During exercise or between meals, glucagon and cathecolamines inhibit glycogen synthesis while promoting glycogen breakdown[3]. Hepatic glycogen serves as a depot source of glucose to maintain euglycemia during fasting periods while glycogen in muscle provides glucose to produce necessary energy during high-intensity exertion.

GSDs are multisystemic diseases that can present at any age from the neonatal period to adulthood. The overall GSD incidence is approximately 1 case per 20000-43000 live births and 80% of hepatic GSDs are caused by types I, III, and IX[4,5]. This heterogeneous group of rare diseases represents inborn errors of carbohydrate metabolism and are classified based on the deficient enzyme and affected tissues (Table 1). GSDs primarily affect liver or muscle or both as glycogen is particularly abundant in these tissues. However, besides liver and skeletal muscle, depending on the affected enzyme and its expression in various tissues, multiorgan involvement including heart, kidney and/or brain may be seen[6]. Although GSDs share similar clinical features to some extent, there is a wide spectrum of clinical phenotypes. Hypoglycemia is the hallmark of hepatic GSDs. Hepatomegaly is also a cardinal manifestation of GSDs with liver involvement except for GSD-0. Muscle GSDs, on the other hand, may present with exercise intolerance, muscle cramps/pain, rhabdomyolysis, and muscle weakness and in the case of cardiac involvement, cardiomyopathy[7]. Since the initial presenting symptoms can occur in adulthood, it is a group of rare diseases that should be recognized and managed by not only pediatricians but also physicians taking care of adults. Being multisystemic diseases, GSDs are best managed by a cross-disciplinary approach to achieve good metabolic control, improve the quality of life of patients, and reduce morbidity and mortality[7]. It is recommended that a medical professional with expertise in treating such conditions (e.g., a metabolic disorders specialist, a biochemical geneticist, an endocrinologist, or a hepatologist) should lead and coordinate the patient's care together with a metabolic dietician. Nephrologists, hematologists, genetic counselors, cardiologists, gastroenterologists, neurologists, physical therapists, social workers, and transplant specialists may also be required in the management of a GSD depending on the specific manifestations, complications, and type of the disease. In this article, we aim to update the review published in 2007[1] based on new data and provide a comprehensive review of GSDs. This review provides general characteristics of all types of GSDs with a focus on those with liver involvement.

GSDS INVOLVING LIVER

GSD-0; glycogen synthase deficiency

There are two types of glycogen synthase (GYS) encoded at different genetic loci; muscle GYS (GYS1; 19q13.33) and liver GYS (GYS2; 12p12.1)[8]. In 1963, GSD-0 was initially reported as glycogen synthetase deficiency in the liver[9]. GSD-0 is distinct from other hepatic GSDs due to the marked decrease in liver glycogen content, thereby making its classification questionable as a genuine GSD. However, since the disease exhibits a phenotype like that of the classic glycogenoses due to unavailability of glycogen during periods of fasting, it is classified as a GSD. GSD-0 is an autosomal recessive genetic disease^[10]. The disease is caused by homozygous or compound heterozygous mutations in the GYS2 gene which was mapped to 12p12.2 in 1994[11]. Liver GYS, the hepatic isoform, is responsible for catalyzing the



Table 1 Overview of glycogen storage diseases

GSD type (eponym)	OMIM#	Defective enzyme or transporter	Gene/inheritance	Gene location	Primary tissue involvement	Distinctive features
GSD-0	GSD-0a: 240600	Liver glycogen synthase	GYS2/AR	12p12.1	Liver	No hepatomegaly. Postprandial hyperglycemia, glycosuria, and hyperlact- atemia. Extremely low amount of glycogen in liver tissue
	GSD-0b: 611556	Muscle glycogen synthase	GYS1/AR	19q13.33	Muscle	Cardiac involvement, risk of sudden cardiac arrest
GSD-I (von Gierke)	GSD-Ia: 232200	Glucose-6-phosphatase	G6PC/AR	17q.21	Liver	Coagulopathy, anemia, osteopenia, osteoporosis, renal dysfunction, HA, HCC
	GSD-Ib: 232220	Glucose-6-phosphatase transporter	SLC37A4/AR	11q23.3	Liver	Neutropenia, neutrophil dysfunction, recurrent infections, oral and intestinal mucosal ulcers, IBD, autoimmunity
GSD-II (Pompe)	232300	Acid α-glucosidase	GAA/AR	17q25.3	Muscle	Cardiomyopathy, infantile-onset form. Muscle weakness, late-onset form
Danon disease (formerly GSD- IIb)	300257	Lysosome-associated membrane protein-2	LAMP2/XLD	Xq24	Muscle	Skeletal and cardiac myopathy, arrhythmia, intellectual disability
GSD of heart	600858	AMP-activated protein kinase, γ-2 regulatory subunit	PRKAG2/AD	7q36.1	Muscle	Severe ventricular hypertrophy. Electrocardio- graphic preexcitation and conduction system disease. Premature sudden cardiac death (< 40 yr)
GSD of heart, lethal congenital	261740	AMP-activated protein kinase, γ-2 noncatalytic subunit	PRKAG2/AD	7q36.1	Muscle	Some mutations (R531Q, R384T) cause more severe phenotype. Fetal onset, extreme cardio- megaly, death in infancy
GSD-III (Cori/Forbes)	IIIa/IIIb: 232400	Glycogen debrancher enzyme	AGL/AR	1p21.2	IIIa: Liver + muscle; IIIb: Liver	Liver fibrosis, cirrhosis, HA, HCC (as a complication of cirrhosis). IIIa: Elevated CK, motor developmental delay, myopathy, cardiomyopathy
GSD-IV (Andersen)	232500	Glycogen branching enzyme	GBE1/AR	3p12.2	Liver	Classical hepatic form (rapidly progressive liver disease, HSM, cirrhosis, HCC). Non- progressive hepatic form. Neuromuscular presentation (perinatal, congenital, childhood and adult forms). Myopathy, cardiomyopathy, neuropathy, CNS involvement, APBD. Amylopectin aggregations in liver
GSD-V (McArdle)	232600	Muscle glycogen phosphorylase	PYGM/AR	11q13.1	Muscle	Exercise intolerance, muscle cramps, rhabdomyolysis, myoglobinuria, "second wind" phenomenon
GSD-VI (Hers)	232700	Liver glycogen phosphorylase	PYGL/AR	14q22.1	Liver	Phenotypic variability (overlap with GSD-IX). Severe hepatic involvement reported. Mild hypotonia and cardiopathy reported. Excessive glycogen accumulation with structurally normal glycogen in liver tissue. Enzyme deficiency in erythrocytes, leukocytes
GSD-VII (Tarui)	232800	Muscle phosphofruc- tokinase	PFKM/AR	12q13.11	Muscle	Exertional myopathy, exercise intolerance, muscle cramps, hemolytic anemia. Rapidly progressive infantile form (multisystem involvement, seizures, cardiomyopathy)
GSD-IX	GSD-IXa1 (XLG-1): 306000	Phosphorylase kinase, α -subunit, liver	PHKA2/XLR	Xp22.13	Liver	The most common subtype. Symptomatic female carriers due to X chromosome inactivation. Clinical symptoms and laboratory abnormalities gradually disappear with age. Severe phenotypes reported
	GSD-IXb: 261750	Phosphorylase kinase, β -subunit	PHKB/AR	16q12.1	Liver	Marked accumulation of glycogen in both liver and muscle. Muscle symptoms are generally mild or absent
	GSD-IXc: 613027	Phosphorylase kinase, γ-subunit	PHKG2/AR	16p11.2	Liver	More severe phenotype with increased risk for liver fibrosis and cirrhosis
	GSD-IXd: 300559	Phosphorylase kinase, α -subunit, muscle	PHKA1/XLR	Xq13.1	Muscle	Muscle weakness and muscle cramps during exercise. Mostly in adults



GSD-X	261670	Muscle phosphogly- cerate mutase	PGAM2/AR	7p13	Muscle	Exercise intolerance, muscle cramps and pain, rhabdomyolysis, myoglobinuria
Fanconi-Bickel syndrome (formerly GSD- XI)	227810	Glucose transporter 2	SLC2A2/AR	3q26.2	Liver	Hepatorenal involvement. Proximal renal tubular dysfunction. Osteoporosis/rickets. Different patterns of dysglycemia. Postprandial hyperglycemia and hypergalactosemia
GSD-XI	612933	Lactate dehydrogenase A	LDHA/AR	11p15.1	Muscle	Exertional myoglobinuria, easy fatigability, exercise induced myalgia, erythem- atosquamous skin lesions on the extensor surfaces of the extremities
GSD-XII	611881	Fructose-1,6- bisphosphate aldolase A	ALDOA/AR	16p11.2	Muscle	Rhabdomyolysis induced by fever and/or exercise, hemolytic anemia with or without myopathy or cognitive dysfunction
GSD-XIII	612932	Enolase 3 (β-enolase)	ENO3/AR	17p13.2	Muscle	Exercise intolerance, exercise induced myalgia, muscle weakness
GSD-XV	613507	Glycogenin-1	GYG1/AR	3q24	Muscle	Ventricular arrhythmogenic cardiomyopathy, progressive muscle weakness

GSD: Glycogen storage disease; HA: Hepatic adenoma; HCC: Hepatocellular carcinoma; AR: Autosomal recessive; XLR: X-linked recessive; XLD: X-linked dominant; CK: Creatinine kinase; CNS: Central nervous system; APBD: Adult polyglucosan body disease: IBD: Inflammatory bowel disease.



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Figure 1 Simplified pathway of glycogen synthesis and degradation in hepatocytes. Glucose and glycogen convert into one another via synthesis or degradation (glycogenolysis) through various steps. The liver plays a central role in maintaining normoglycemia. During the fasting state, the liver maintains glucose homeostasis via a metabolic shift from synthesizing glycogen to endogenous glucose production by glycogenolysis and gluconeogenesis. Specific enzyme or transporter defects in these pathways are associated with clinical and biochemical manifestations including hepatomegaly, hypoglycemia, hyperlipidemia, hypertriglyceridemia, hyperlactatemia, and hyperuricemia. GSD: Glycogen storage disease; UDP-Glucose: Uridine diphosphate glucose; Glucose-1-P: Glucose 1phosphate; Glucose-6-P: Glucose-6-phosphate; Acetyl-CoA: Acetyl coenzyme A; TCA: Tricarboxylic acid.

> rate-limiting step in hepatic glycogen synthesis. GYS deficiency in liver leads to a marked reduction in hepatic glycogen stores. The inability to synthase glycogen inevitably leads to conversion of dietary carbohydrate to lactate rather than being stored as glycogen in the liver. Postprandial hyperglycemia, glycosuria, and lactic acidemia are replaced by ketotic hypoglycemia during fasting[12]. There is often ketosis after a routine overnight fast.

> There are wide phenotypical variations[13]. Fasting hypoglycemia usually manifests in late infancy when overnight feedings are discontinued. Hypoglycemia typically occurs early in the morning prior to having breakfast. Hypoglycemia is responsible for the symptoms observed in GSD-0, which encompasses lethargy, pallor, nausea, vomiting, and, in some cases, seizures. Although some children may display developmental delay, most are neurologically normal. Some patients may remain asymptomatic or experience only mild symptoms[14]. Notably, liver enlargement is not a feature of GSD-0. GSD-0 is the only hepatic GSD that is not typically associated with hepatomegaly[15]. Short

stature and osteopenia are frequently observed in GSD-0, but other long-term complications commonly seen in other GSDs have not been documented [16]. Hyperglycemia and glycosuria are rare presentations in GSD-0 but may pose diagnostic difficulties when observed[17]. Postprandial hyperglycemia and glycosuria when taken together with a normal sized liver may mistakenly indicate early stages of diabetes. GSD-0 is underdiagnosed due to the lack of physical findings and milder phenotype[16,18].

Symptoms in GSD-0 are rapidly alleviated by frequent intake of protein-rich meals and bedtime consumption of uncooked cornstarch (UCCS), a slow-release glucose source. The preservation of gluconeogenesis and fatty acid oxidation pathways explains the less severe clinical course of GSD-0 compared to other types of hepatic GSDs. Increased protein intake during meals provides necessary substrates for gluconeogenesis and shows a protective effect against overweight/obesity and insulin resistance[19]. Extended periods of fasting can result in severe hyperketonemia and elevated plasma free fatty acid levels, which in turn leads to the inhibition of alanine release from skeletal muscle causing a reduction in the availability of gluconeogenic substrates, thereby exacerbating hypoglycemia [16]. While fasting is associated with hypoglycemia, hyperketonemia, and low alanine concentrations, feeding causes hyperglycemia and hyperlactatemia. Simple carbohydrates should be limited, and lowglycemic-index complex carbohydrates should be included in the diet to minimize postprandial hyperglycemia and hyperlactatemia. Patients are generally fed more frequently during the daytime to prevent hypoglycemia.

The administration of glucose or galactose to patients with GSD-0 results in elevated levels of serum lactate and lipids and can be used as a diagnostic test[2,17]. Traditional methods of diagnosis, such as liver biopsy to confirm extremely low hepatic glycogen levels and low to absent GYS activity, have been replaced by non-invasive mutation analysis of the *GYS2* gene.

Browner et al[20] discovered that muscle GYS, which is distinct from liver GYS, is expressed in both muscle and heart. The defect may be inherited or acquired. Enzyme activity is decreased in patients with type 2 diabetes. Muscle GYS deficiency causes cardiomyopathy and exercise intolerance in affected patients[8]. Histologic examination of muscle shows lack of glycogen and mitochondrial proliferation.

GSD-I; von Gierke disease; hepatorenal glycogenosis

The disease was first described by Gierke^[21] in 1929 based on autopsy results showing excessive glycogen storage in the livers and kidneys of two patients. In 1952, Cori and Cori[22] discovered the deficiency of glucose-6-phosphatase (G6Pase) as the causative defect in patients with similar disease phenotype. After more than two decades, in 1978, Narisawa et al[23] described the deficiency of glucose-6-phosphate translocase (G6PT), the transporter protein of G6Pase complex (G6PC).

The deficiency of either G6Pase or G6PT activity causes GSD type I (GSD-I). The G6PT/G6PC functions as a multicomponent system and is responsible for glucose production by catalyzing the terminal step of both the glycogenolysis and gluconeogenesis pathways[24]. G6PT translocates G6P into the endoplasmic reticulum, wherein G6Pase converts G6P into free glucose and inorganic phosphate [25]. Two major subtypes of GSD-I are defined according to which part of the complex is defective. Deficiency of the catalytic subunit of G6Pase causes GSD-Ia while deficiency of G6PT activity results in GSD-Ib. Approximately 80% of cases with GSD-I are type Ia while the remaining 20% are type Ib. The presence of further subtypes (GSD-Ic and GSD-Id) is controversial. The majority, if not all, of typical cases of GSD-I are attributed to mutations in the genes encoding G6Pase and G6PT. Additionally, it has been noted that only two subtypes of GSD-I (namely, GSD-Ia and GSD-Ib) have been confirmed in clinical practice, and the existence of other forms of GSD-I requires further substantiation. Because both glycogenolysis and gluconeogenesis are affected due to inability in converting G6P to free glucose the main metabolic derangement of both subtypes is fasting hypoglycemia.

GSD-I is inherited in an autosomal recessive manner. The overall incidence of the disease is approximately 1:100000[26]. The estimated carrier rate in the general population is 1:150. The disease may be more prevalent in people of Ashkenazi Jewish (c.247C>T), Mexican-Hispanic (c.379_380dupTA) and Japanese heritage (c.648G>T) due to the increased frequency of mentioned pathogenic variants. The carrier frequency for the c.247C>T variant among Ashkenazi Jews has been reported to be as high as 1:63[27].

GSD-la; G6Pase deficiency

In 1952, Cori and Cori[22] identified the first specific enzyme deficiency associated with an inherited disorder through demonstration of G6Pase deficiency. Subsequently, in 1995, the gene that encodes the catalytic subunit of the G6PC was identified on chromosome 17q21[28]. Later, its molecular and biochemical characteristics were described in detail^[29].

While some neonates may exhibit severe hypoglycemia and lactic acidosis, infants who do not receive any treatment typically present between 3-6 mo of age (at a median age of 6 mo) coinciding with prolonged feeding intervals, increased sleeping time through the night or onset of an intercurrent illness disrupting normal patterns of feeding[30]. The onset of symptoms can be soon after birth, and episodes typically remain unresponsive to glucagon therapy. Symptoms mainly include difficulties with feeding, tremors, pallor, excessive sweating, hyperventilation, cyanosis, apnea, irritability, seizures, somnolence, and cerebral edema/dysfunction, with exacerbations typically occurring in the morning or prior to feedings. Severe episodes of ketotic hypoglycemia, if untreated, may eventually lead to coma and



sudden infant death[31]. Older infants may exhibit certain physical characteristics, such as doll-like facies with full cheeks and relatively thin extremities along with frequent lethargy, difficulty in waking from sleep, tremors, an insatiable appetite, growth retardation, and a prominent abdomen resulting from pronounced enlargement of the liver and kidneys. In some cases, xanthomas may appear on extensor surfaces, such as the elbows, knees, or buttocks. During an infection, symptoms of severe hypoglycemia are more prevalent owing to diminished appetite and/or gastrointestinal symptoms (e.g., vomiting and diarrhea) both preventing adequate oral intake. Delayed motor development can be seen but cognitive development is generally normal unless there is cerebral damage due to prolonged or recurrent neuroglycopenia[1,32].

Impaired platelet function, especially in individuals with inadequate metabolic control predisposes patients to nose bleeding[33]. Diminished glucose uptake into platelets due to chronic hypoglycemia and subsequent intracellular ATP deficiency have been proposed as potential causes of platelet dysfunction in GSD-Ia[34]. Additionally, decreased plasma concentration of von Willebrand factor antigen indicating an acquired von Willebrand disease was reported for patients with GSD-Ia[35]. In addition, epistaxis, easy bruising, menorrhagia, intrahepatic adenoma hemorrhage, and excessive bleeding during surgical procedures can also occur[36,37].

Patients with GSD-I have hypovitaminosis D despite adequate supplementation[38]. Low bone mineral density is a long-term complication of GSD-I particularly in those with poor metabolic control [39,40]. Osteoporosis may arise as a consequence of poor nutrition, chronic lactic acidosis and hypogonadism[41]. Anemia is a common complication in both subtypes of GSD-I with a reported prevalence ranging from 17% to 60% across different age groups[30]. The etiology of anemia in GSD-I is complex and involves various factors, including the restrictive nature of the diet, altered iron absorption due to excessive intake of UCCS, chronic lactic acidosis, chronic kidney disease, bleeding diathesis, chronic illness, suboptimal metabolic control, hepatic adenomas, and inflammatory bowel disease. The prevalence and pathophysiology appear to differ in individuals with GSD-Ia and those with GSD-Ib. A multicenter study involving 202 subjects with GSD-I (Ia/Ib: 163/39 subjects) showed that anemia is more common in patients with GSD-Ib compared to GSD-Ia (71.8% vs 41.7%, respectively). In addition, the prevalence of severe anemia is also increased in GSD-Ib in comparison to patients with GSD-Ia (41% vs 4.9%, respectively)[42]. Severe anemia in GSD-Ia appears to be related to large hepatic adenomas, while in GSD-Ib it is often associated with enterocolitis^[42]. Development of severe anemia during the course of the disease warrants further evaluation for hepatic adenomas and inflammatory bowel disease in GSD-Ia and GSD-Ib, respectively.

Patients diagnosed with GSD-Ia or GSD-Ib may experience intermittent diarrhea which seems to deteriorate with age[43]. Diarrhea was reported in 35% of the GSD-Ia and in 55% of the GSD-Ib patients [30]. However, the cause of diarrhea remains unknown. Intolerance to UCCS and inflammatory bowel disease are possible causes of diarrhea in this population. Inflammatory bowel disease is a well characterized feature in individuals with GSD-Ib. Neutropenia and impaired neutrophil function are the underlying causes of inflammatory bowel disease in GSD-Ib[44]. However, inflammatory bowel disease was also recently reported in adult patients with GSD-Ia as a new, long-term complication of the disease [45]. The prevalence of symptomatic inflammatory bowel disease in adults with GSD-Ia also seems to be higher than the general population [45]. The authors speculated that inflammatory bowel disease in GSD-Ia may be caused by chronic UCCS therapy, which could be altering the microbiota of the gastrointestinal tract leading to inflammation. More recently, very early onset inflammatory bowel disease was reported in a child with GSD-Ia at the age of 42 mo[46].

A notable finding among the majority of patients with this condition during childhood is growth retardation, while short stature is commonly observed in affected adults [5,47,48]. In the absence of effective treatment, a range of long-term complications may arise in individuals with GSD-I, including delayed puberty, liver adenomas, hepatocellular carcinoma, renal dysfunction, chronic kidney disease, chronic renal failure, urolithiasis, arterial and pulmonary hypertension, osteopenia/osteoporosis, polycystic ovary syndrome, and gout. Cognitive delay and epilepsy due to repeated or severe hypoglycemic events may occur[31]. Hyperlipidemia may cause xanthomas, pancreatitis, and cholelithiasis[30,49]. Acute pancreatitis may develop secondary to very high serum triglycerides in GSD-I and necessitate plasmapheresis^[50].

Systemic metabolic perturbations and glycogen deposition in the kidneys result in glomerular and proximal and distal renal tubular injury. Renal manifestations may occur in childhood but often are not noticed without proper diagnostic work-up. The prevalence of renal involvement tends to rise as patients age[51]. Glomerular hyperfiltration, whose underlying mechanism is not yet fully understood, is typically the initial manifestation of renal involvement. Possible etiologies have been suggested including activation of the renin-angiotensin system, persistent oxidative stress, profibrotic cytokines such as transforming growth factor- β , and changes in energy reserves of renal tubular epithelial cells[52-54]. Glomerular hyperfiltration then progresses to microalbuminuria, proteinuria, glomerular scarring and interstitial fibrosis, and end-stage renal disease in adult patients [48,55]. Hypercalciuria and hypocitraturia due to proximal and distal tubular dysfunction cause nephrocalcinosis and/or urolithiasis 56, 57]. This may increase the risk of urinary tract infections causing further renal parenchymal damage. Hypertension and hematuria are other findings [55,56]. Systemic hypertension may develop early in childhood but is seen more often in adults with GSD-I[58]. Renal cysts have also been described in



individuals with GSD-I[59]. Gout can develop due to persistent hyperuricemia as gouty attacks, gouty tophi, and kidney stones.

In GSD-Ia patients, various types of liver lesions, including hepatic adenoma, hepatocellular carcinoma, hepatoblastoma, focal fatty infiltration, focal fatty sparing, peliosis hepatis, and focal nodular hyperplasia have been reported, with hepatic adenomas being the most prevalent among them[37]. The prevalence of hepatic adenomas was reported to vary between 22% to 75%, and they usually manifest during or after puberty, particularly in the second or third decade of life. The median age of adenoma presentation is 15 years[30]. Although the prevalence of hepatic adenomas increases with age in GSD-I, they may be seen in younger children[60]. Progression in size and/or number of hepatic adenomas occurs in half of patients[30]. Inadequate metabolic control appears to play a central role in hepatic adenoma formation. The degree of hyperlipidemia is associated with development of hepatic adenomas [61]. However, the pathophysiological mechanisms are yet to be fully understood and factors other than metabolic control may also be responsible for adenoma formation. In a recent study by Cho et al[62], in addition to mitochondrial dysfunction and metabolic alterations caused by G6Pase deficiency, persistent autophagy impairment and activation of multiple tumor-promoting pathways were reported as contributing factors to hepatic adenoma/hepatocellular carcinoma development in GSD-I. Chromosomal and genetic alterations may also play a role in hepatocellular carcinoma associated with GSD-I[63]. Hepatic adenomas have the potential to transform into hepatocellular carcinoma over an extended period, with reports of malignant transformation occurring as long as 28 years after initial diagnosis[64,65]. A rapid increase in size or number of adenomas is associated with an increased risk of adenoma to hepatocellular carcinoma transformation and should be evaluated carefully.

The link between GSD-I and risk for cardiovascular disease is controversial. Although GSD-Ia patients have elevated levels of triglycerides, very low density lipoprotein and low density lipoprotein, the occurrence of endothelial vascular dysfunction and atherosclerosis is uncommon. It has been suggested that the increased serum levels of apoE may offset the elevated risk of atherosclerosis associated with dyslipidemia[66]. Moreover, the reduced von Willebrand factor antigen and density of individual oligomers found in 60% of GSD-Ia patients may also contribute to protection against vascular complications[35]. In addition, an increase in serum levels of antioxidative factors may contribute as a protective mechanism[67,68]. There are conflicting data regarding whether patients with GSD-I are at increased risk for atherosclerosis[69,70]. Pulmonary hypertension is a rare long-term complication of GSD-I with few cases reported. Patients with a concomitant predisposing condition for pulmonary arterial hypertension are at increased risk[37].

The main neurological impact of GSD is related to hypoglycemia. Patients with GSD-I may suffer from brain damage, which may be caused by recurrent severe hypoglycemia^[71]. Studies have found a significant correlation between the frequency of hospital admissions for hypoglycemia and abnormalities in both performance ability tests and brainstem auditory evoked potentials. In addition, electroencephalography abnormalities were found to be correlated with dietary compliance. The magnetic resonance imaging abnormalities observed in GSD-I patients were the dilatation of occipital horns and/or hyperintensity of subcortical white matter in the occipital lobes[71]. Brain imaging abnormalities were more frequent among GSD-I patients with early symptom onset, frequent and longer hospital admissions, and poor metabolic control including elevated levels of uric acid, lactate, and triglyceride[32,72].

Some females may have polycystic ovaries and irregular menstrual cycles with normal fertility^[73]. Women with GSD-Ia may have pregnancies and deliveries without complications^[74]. In consideration of the risk of development of hepatic adenomas in GSD-I patients, estrogen-containing contraceptives should be avoided whenever possible [75]. In addition to hypoglycemia, the most prominent laboratory abnormalities observed in patients with GSD-I include lactic acidosis, hyperlipidemia (especially hypertriglyceridemia but also hypercholesterolemia), and hyperuricemia (Figure 1). Mild elevation in transaminase levels is usually detected [30]. Ultrasonographic examination may reveal enlarged kidneys in affected patients of all ages. Serum biotinidase activity is increased in GSD-Ia patients [76-79]. Biotinidase activity was reported to be positively correlated with hypertriglyceridemia in subjects with GSD-I while severe fibrosis and cirrhosis were related to reduced enzyme activity[80]. There may also be hypercalciuria^[5]. There is little or no increase in blood glucose concentration in response to administration of glucagon and this may even lead to worsening of the metabolic acidosis. Histopathological examination of the liver in patients with GSD-Ia typically reveals a mosaic pattern with pale-staining and swollen hepatocytes. Other observed features include steatosis and nuclear hyperglycogenation. Periodic acid-Schiff (PAS)-positive and diastase sensitive glycogen is evenly dispersed throughout the cytoplasm. Glycogen accumulation may be within the normal range or exhibit only a mild increase. While fibrosis is not as prominent in GSD-I as in GSD types III, IV, and VI, it may still be present in some affected individuals [5,81-83]. GSD-Ia is usually suspected based on a set of clinical (e.g., hepatomegaly) and biochemical features (e.g., hypoglycemia, lactic acidosis, hypercholesterolemia, hypertriglyceridemia, and hyperuricemia). The definitive diagnosis is confirmed by a mutation analysis or a liver biopsy and an enzyme assay. If a liver biopsy is performed, diagnosis can be confirmed by measuring G6Pase enzyme activity on a liver biopsy specimen; however, it should be kept in mind that measurement of G6Pase enzyme activity will not detect GSD-Ib. When the specific mutation in the index case is known, prenatal diagnosis *via* chorionic villus sampling can be performed for GSD-I[84].



The mainstay of treatment is to prevent hypoglycemia by avoiding prolonged fasting[85]. Continuously providing a dietary supply of glucose during the day and night by frequent feedings, frequent ingestion of UCCS or nocturnal enteral tube feeding are possible feeding strategies. Infants and children should be fed frequently, not allowing fasting periods longer than 3-4 h. In adolescents and adults, fasting more than 5-6 h should be avoided. Small, frequent meals with balanced macronutrient content and use of UCCS are recommended. Continuous intragastric feeding through a nasogastric or gastrostomy tube can be used overnight allowing the patients to sleep through the night[37]. To ensure adequate glucose supply, a glucose infusion rate of 8-10 mg/kg/min should be maintained for infants, while a rate of 4-8 mg/kg/min is recommended for older children. UCCS can be introduced as early as 6-12 mo of age. For the administration of UCCS in GSD-I patients, the recommended dose is 1-1.5 g of UCCS per kilogram of ideal body weight every 3-4 h for young children and 1.5-2 g of UCCS per kilogram of body weight every 4-5 h for older children, adolescents, and adults[85]. Digestion of UCCS is slow, enabling a sustained release of glucose, thereby achieving a more stable glycemic profile over an extended duration, in contrast to other carbohydrate sources. The administration of UCCS has been shown to achieve adequate glycemia for a median duration of 4.25 h (ranging between 2.5-6 h)[86]. Glycosade®, a modified, waxy maize extended-release cornstarch, is available as a single-dose overnight treatment[87].

In GSD-I, intake of fructose and galactose, which cannot be metabolized to glucose via G6P, further contributes to the metabolic derangement. Lactose (galactose and glucose), fructose and sucrose (fructose and glucose) should be restricted in all age groups. Restricting the intake of fruits, vegetables, juices, and dairy products renders the diet inadequate. Micronutrients, vitamins, and minerals should be supplemented to avoid nutritional deficiencies. The recommended dietary plan is to provide 60%-70% of calories from complex carbohydrates, such as whole-grain breads, pastas, legumes, and rice, with a portion of the carbohydrates coming from cornstarch. Additionally, 10%-15% of calories should come from protein and 25%-30% from fat. Effective dietary management is essential to minimize the metabolic derangement associated with GSD-I and to reduce the development of long-term complications[37,85]. However, caution must be exercised to avoid overtreatment. Overtreatment with UCCS has many consequences including obesity, increased glycogen storage in the liver, worsening lactic acidosis, increased gastrointestinal disturbances, hyperinsulinemia, and insulin resistance[88].

If there is anemia, the causes must be evaluated (e.g., nutritional deficiencies, liver adenomas, enterocolitis, menorrhagia in females, and occult blood loss from the gastrointestinal tract) and appropriate treatment should be started. In the case of severe anemia, hepatic adenomas in GSD-Ia and enterocolitis in GSD-Ib should be investigated [42]. To prevent gout in the presence of hyperuricemia, allopurinol is typically administered at a dosage of 10 mg/kg/d, divided into three doses. If acidosis is present, indicated by a blood base excess of less than -5 mmol/L or a blood bicarbonate level below 20 mmol/L, bicarbonate or potassium citrate should be prescribed, with a recommended dose of 1 to 2 mmol/kg/d divided into four doses and 5 to 10 mEq every 8-12 h, respectively[85]. Angiotensin converting enzyme inhibitors or angiotensin receptor blockers should be used to delay the progression of renal damage[53, 89-91]. Evidence of hyperfiltration (sustained estimated glomerular filtration rate > 140 mL/min/1.73 m²), persistent microalbuminuria and frank proteinuria should prompt initiation of angiotensin converting enzyme inhibitors or angiotensin receptor blockers[37]. If serum triglyceride levels remain high despite optimizing dietary treatment, the administration of lipid-lowering drugs, such as 3hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors and fibrates, may be necessary to decrease the risk of atherosclerosis, cholelithiasis, and pancreatitis. For adults with persistently elevated cholesterol levels, statins may be considered as a treatment option[85]. The positive effect of mediumchain triglycerides on lowering serum cholesterol and triglyceride levels has been reported[92,93].

Recommendations regarding perioperative management of patients with GSD-I are available [37,94]. Close monitoring of blood glucose, electrolytes, and lactate levels is crucial during the peri-operative period. The patient should be admitted to the hospital 24 h before the surgery, continuous intravenous supply with 10% dextrose should be provided and continued until oral feeding is re-established. The administration of Ringer lactate solution should be avoided in GSD-I patients, as it may exacerbate lactic acidosis and worsen metabolic decompensation[37]. Bleeding time must be normalized before elective surgical interventions by 24-h continuous gastric drip feeding for one week or by intravenous glucose infusion over 24 to 48 h[85].

GSD-lb; G6PT deficiency

In 1968, after realizing that in vitro G6Pase activity was normal despite glucose not being released from G6P in vivo, a second subtype of GSD-I was identified [95]. In 1975, it was elucidated that a transport system specific to G6P exists and is responsible for transporting G6P from the cytoplasm to the endoplasmic reticulum[96]. The responsible gene, SLC37A4 (the solute carrier family 37 member 4), has been cloned and located on chromosome 11q23[97,98].

GSD-Ib is characterized by distinctive features such as recurrent infections, neutropenia, and neutrophil dysfunction, in addition to the clinical symptoms and findings observed in GSD-Ia. While not all GSD-Ib patients have neutropenia and neutrophil dysfunction, these conditions are common and predispose patients to severe infections and inflammatory bowel disease[44]. Patients with GSD-Ib may have normal neutrophil counts in the first year of life. G6PT gene, unlike G6Pase, is also expressed in



hematopoietic progenitor cells, which may be responsible for neutropenia and recurrent infections in GSD-Ib[99]. The neutrophil dysfunction in GSD-Ib includes both impaired motility and respiratory burst[100,101]. Impaired glucose transport across the cell membrane of polymorphonuclear leukocytes may be responsible for neutrophil dysfunction in GSD-Ib. Microsomal transport of G6P has a potential role in the antioxidant protection of neutrophils. Dysfunction of this transporter due to genetic defects in G6PT may impair cellular functions and induce apoptosis, contributing to the neutrophil dysfunction seen in GSD-Ib[102]. Some individuals with GSD-Ib do not develop neutropenia. It has been suggested that this could be due to residual transporter activity of some G6PT mutations[103]. GSD-Ib patients with neutropenia and neutrophil/monocyte dysfunction are at an increased risk for severe infectious complications due to impaired immune function. Young children with GSD-Ib may experience frequent otitis, gingivitis, periodontal disease, dental caries, and skin abscesses. Oral and genital ulcerations and intestinal mucosal ulcers may occur[43,104]. Individuals with GSD-Ib may experience recurrent episodes of diarrhea. The underlying cause of this symptom appears to be inflammation of the intestinal mucosa, as evidenced by elevated fecal α1-antitrypsin excretion and colonic inflammation in colonoscopic biopsies[44]. There is no established association between the specific genetic mutations causing GSD-Ib and the occurrence of neutropenia, bacterial infections, and other systemic complications in affected individuals^[105]. Patients with GSD-Ib may require liver transplantation. Although hypoglycemia, lactic acidosis and dyslipidemia improve after liver transplantation, neutropenia generally continues to be present as it is primarily attributable to an intrinsic defect in the neutrophils [106-108].

Another characteristic clinical finding of GSD-Ib is the occurrence of Crohn disease-like colitis[109, 110]. The enterocolitis observed in GSD-Ib patients has been found to have histological features similar to those seen in inflammatory bowel disease/Crohn disease, characterized by transmural inflammatory changes and the formation of granulomas[111]. Accompanying findings and symptoms include fever, diarrhea, and perioral and anal ulcers. Interestingly, the severity of the primary disorder does not appear to be correlated with the occurrence or severity of intestinal symptoms [109,110]. Manifestations of inflammatory bowel disease may improve with granulocyte colony-stimulating factor (G-CSF) treatment[112]. Enteral nutrition with a polymeric formula enriched in the anti-inflammatory cytokine transforming growth factor- β is recommended as a first-line treatment of digestive complications in GSD-Ib[113]. Inflammatory bowel disease may require treatment with anti-inflammatory and immunosuppressive medications[113]. Successful treatment of inflammatory bowel disease with biologics including infliximab and adalimumab in GSD-Ib patients refractory to conventional treatment has been reported [114,115].

GSD-Ib is characterized by an increased risk for developing autoimmune disorders like thyroid autoimmunity and myasthenia gravis[116]. GSD-Ib patients have a higher likelihood of developing thyroid autoimmunity and hypothyroidism, while GSD-Ia patients show little indication of thyroid pathologies[117,118]. Based on the slightly elevated levels of thyrotropin, even in patients with overt hypothyroidism, it could be postulated that there is concomitant damage occurring at the hypothalamus or pituitary gland[118]. Recently, predisposition to autoimmunity in GSD-Ib patients was linked with a profound defect in conventional T cells and regulatory T cells caused by defective engagement of glycolysis in T cells due to G6PT deficiency[119]. Although a rare outcome of GSD-Ib, patients may develop terminal kidney disease, which may necessitate kidney transplantation[106].

Nutritional management of GSD-Ib is similar to that of GSD-Ia. Neutropenic patients with GSD-Ib should be treated with G-CSF. G-CSF therapy may normalize the number of neutrophils and restore myeloid functions[120-122]. The implementation of a combined therapeutic approach including both dietary management and G-CSF treatment improves the prognosis of patients by significantly mitigating metabolic and myeloid abnormalities. G-CSF administration is associated with not only an elevation of peripheral neutrophil counts, but also a reduction in the incidence of febrile episodes and infections, as well as improvement in enterocolitis in patients with GSD-Ib[123]. In conjunction with other therapies (aminosalicylates, mesalamine, and corticosteroids), G-CSF ameliorates inflammatory bowel disease symptoms[124]. To prevent complications such as splenomegaly, hypersplenism, hepatomegaly, and bone pain, it is recommended that the lowest effective dose of G-CSF is used. Caution must be exercised regarding the development of splenomegaly and myeloid malignancy [124,125]. Vitamin E has been reported to be effective in reducing the frequency of infections and improving neutropenia [126].

Liver transplantation is the ultimate therapy for hepatic metabolic disease related to GSD-I. There is no possibility of the recurrence of GSD-I within the allograft. Liver transplantation is warranted in various situations, such as hepatic adenomas with a high risk of malignant transformation, rapid progression in size and/or number of hepatic adenomas, development of hepatocellular carcinoma, poor metabolic control despite medical therapy, and growth failure[127]. Liver transplantation corrects all liver related biochemical abnormalities including hypoglycemia, lactic acidosis, hyperuricemia, and hyperlipidemia, but its potential to reverse and/or prevent renal disease remains uncertain[107,128-130]. Recently, an unusual post-transplant finding of two siblings with persistent hyperuricemia requiring allopurinol treatment has been reported[131]. Moreover, chronic renal failure is a well-known complication that may arise as a consequence of liver transplantation in individuals with GSD-Ia, and progression to renal failure within a few years of transplantation was reported [128]. It is uncertain

whether post-transplantation renal failure is related to disease progression, toxicity from immunosuppressants used after liver transplantation, a secondary reaction to poor metabolic control, or a combination of these factors. Renal transplantation in GSD-I, on the other hand, corrects only renal abnormalities[132]. Conflicting results have been reported in different studies regarding whether catchup growth is achieved or not following liver transplantation in children with GSD-I[133,134].

Despite improved survival and growth, long-term complications of GSD-I like progressive renal failure and development of hepatic adenomas do not respond completely to dietary treatment. Although liver transplantation corrects metabolic derangement and improves the quality of life of these patients, it is not without complications[128]. These findings suggest that novel therapeutic approaches with higher success and lower complication rates are warranted. A recent advance in the treatment of neutropenia and neutrophil dysfunction in individuals with GSD-Ib is repurposing empagliflozin, a sodium-glucose co-transporter-2 (SGLT2) inhibitor that is approved to treat type 2 diabetes in adults, to improve neutrophil number and function. A study conducted by Veiga-Da-Cunha et al[135] revealed the crucial function of glucose-6-phosphate transporter in neutrophils, which clarifies the pathophysiology of neutropenia in GSD-Ib patients. In addition to G6P, G6PT transports the G6P structural analog 1,5anhydroglucitol-6-phosphate (1,5AG6P). Neutrophils lacking G6PT activity cannot transport 1,5AG6P from the cytosol into the endoplasmic reticulum, where it is normally dephosphorylated by G6PC3, a phosphatase in the membrane of the endoplasmic reticulum. Cytosolic accumulation of 1,5AG6P inhibits glucose phosphorylation by hexokinases that catalyzes the first step of glycolysis. As glycolysis is the sole energy source for mature neutrophils, depletion of intracellular G6P leads to a deficit in energy production which in turn results in neutrophil dysfunction and subsequent apoptosis. Empagliflozin inhibits renal SGLT2 leading to increased urinary excretion of 1,5AG. This leads to a reduction in the concentration of 1,5AG in the blood, thereby decreasing the cellular accumulation of toxic 1,5AG6P in neutrophils[136]. Following the first report of successful repurposing of empagliflozin to treat neutropenia and neutrophil dysfunction in 4 patients with GSD-Ib, several case reports and case series have shown beneficial effects of this treatment approach on neutrophil number and function, inflammatory bowel disease, recurrent infections[137-139], oral and urogenital mucosal lesions, skin abscesses, anemia, wound healing, and dose reduction or even cessation of G-CSF therapy in GSD-Ib patients[140-144]. A recent international multicenter study examining the clinical experience of 112 patients with GSD-Ib treated with empagliflozin reported improvements in neutrophil counts in the majority of patients, leading to the cessation of regular G-CSF injections in 55% of the participants[145]. Despite a favorable safety profile in patients with GSD-Ib, there is a risk of hypoglycemia with SGLT2 inhibitors. A low dose at treatment initiation with careful titration to optimal dosing is recommended [141]. Growing evidence suggests that empagliflozin is a candidate for first-line treatment of neutropenia and neutrophil dysfunction related symptoms in GSD-Ib patients.

Another promising novel therapeutic strategy is gene therapy by using recombinant adeno-associated virus vectors. The use of a viral vector to administer G6Pase and hepatocyte transplantation are being investigated as potential treatments for GSD-I. Various animal models have shown an increase in hepatic G6Pase and G6PT activity, as well as improvements in metabolic parameters[146-150]. Multiple approaches have been explored for the integration of the G6Pase transgene into the host genome[151, 152]. The successful correction of metabolic imbalances in animal models through gene therapy shows promising potential for future applications of gene therapy in humans. A phase I/II clinical trial using a recombinant adeno-associated virus vector expressing a codon-optimized human G6Pase-α or G6PC for treatment of human GSD-Ia (NCT 03517085) has just been completed and the results are pending.

GSD-III; Cori disease; Forbes disease; limit dextrinosis; amylo-1,6-glucosidase deficiency; glycogen debrancher deficiency

Glycogen debrancher enzyme has two independent catalytic activities; alpha-glucanotransferase and amylo-1,6-glucosidase, with the two catalytic sites being separated on the same polypeptide. Both catalytic activities are required for complete debranching enzyme activity [153]. Deficient activity of these catalytic sites results in accumulation of glycogen with short outer chains, previously defined as limit-dextrins. Deficiency in glycogen debranching enzyme due to biallelic pathogenic variants in the AGL gene results in the harmful accumulation of abnormal glycogen in hepatocytes. The AGL gene was mapped to the chromosomal locus 1p21, and its nucleotide sequence was determined, revealing the existence of multiple tissue-specific isoforms [154,155]. GSD-III is inherited in an autosomal recessive manner.

GSD-III makes up about 24% of all GSDs, and its estimated incidence is approximately 1 case per 83000 live births in Europe, and 1 in 100000 live births in North America[156]. Certain populations have an increased prevalence due to a founder effect. The highest known GSD-III prevalence occurs in Inuit population in Nunavik (about 1:2500, c.4456delT variant), the Faroese population of the Faroe Islands (about 1:3600, c.1222C>T variant) and North African Jews from Israel (about 1:5400, c.4456delT variant) [156-158]. There is currently limited evidence supporting a correlation between disease severity and pathogenic variants in the AGL gene, except for specific exon 3 variants (c.18_19delGA and c.16C>T) which have been found to be associated with GSD-IIIb (liver involvement only). It was suggested that in muscle isoforms of the AGL gene, alternative exon or translation initiation may not require exon 3,



thereby resulting in normal enzyme activity in the muscle tissues of patients with GSD-IIIb who harbor an exon 3 deletion[159,160]. Recent evidence suggests that the presence of frameshift, nonsense, and splice site variants may lead to severe phenotypes. Differences in tissue expression of the deficient enzyme is responsible for the phenotypic variability observed in GSD-III patients[153].

GSD-III is characterized by heterogeneous involvement of the liver, skeletal muscle, and cardiac muscle, leading to variable clinical presentations. Various subtypes are defined by the extent of tissue involvement. Two major subtypes of GSD-III have been identified. GSD-IIIa affects both the liver and the muscle (skeletal and cardiac) and is the most prevalent subtype accounting for approximately 85% of cases. Meanwhile, GSD-IIIb primarily affects only the liver and comprises approximately 15% of all GSD-III cases[48,159]. In a limited number of cases, it has been demonstrated that there is a selective loss of either glucosidase activity (resulting in muscle involvement, referred to as GSD-IIIc) or transferase activity (resulting in both muscle and liver involvement, referred to as GSD-IIId)[161,162].

Hepatomegaly, ketotic hypoglycemia, growth retardation and dyslipidemia (hypertriglyceridemia) are the dominant features of hepatic involvement in infancy and childhood. As gluconeogenesis is intact in GSD-III, fasting hypoglycemia tends to be milder than that seen in GSD-I. During infancy, serum hepatic transaminases are markedly elevated. Uric acid and lactate concentrations are relatively normal [163]. Symptoms and laboratory findings related with liver involvement often improve with age and usually disappear after puberty [164,165]. However, liver disease can also be progressive resulting in liver fibrosis, cirrhosis, hepatic failure, and end-stage liver disease[107,165]. Hepatic fibrosis may occur as early as 1 year of age[166]. Overt liver cirrhosis is not common and occurs rarely [153,165]. Hepatocellular carcinoma can develop as a long-term complication of liver cirrhosis, rather than transformation of an adenoma to carcinoma, as seen in GSD-I[167,168]. The prevalence of hepatic adenomas has been reported to range from 4% to as high as 25% [169]. A recent descriptive, retrospective, international, multi-center cohort study revealed that the overall prevalence of severe hepatic complications (hepatic cirrhosis, hepatic adenomas and/or hepatocellular carcinoma) was 11% [170]. Liver transplantation for cirrhosis and/or hepatocellular carcinoma have been reported [107,168]. Children with failure to thrive often catch-up in height in adulthood with optimized, individualized dietary management.

Muscle symptoms associated with GSD-III can manifest concurrently with liver disease or long after hepatic disorders or even after the resolution of hepatic symptoms during childhood. An elevation in creatine kinase (CK) level is observed in 81% to 94% of cases with muscle involvement, serving as a useful indicator of muscle pathology[171]. Nonetheless, a normal CK level does not entirely exclude the possibility of an underlying muscular disease[172,173]. The median age of onset of CK elevation was reported to be 10 years[170]. Although muscle involvement becomes clinically more obvious later in life, mild muscle weakness on physical examination, motor developmental delay (delayed sitting, delayed standing upright, delayed onset of walking), exercise intolerance, and hypotonia were reported in the majority of pediatric patients with GSD-III[174-176]. Muscle weakness and wasting may slowly progress and become severe by the third or fourth decade of life[165,173].

In a subset of adult patients with GSD-III, muscle symptoms can present in the absence of any clinical or previous evidence of liver dysfunction[165,177]. Muscle weakness, although minimal during childhood, is slowly progressive in nature and may become the predominant feature with significant permanent muscle weakness in adults with type IIIa disease[171]. Although myopathy generally progresses slowly and is not severely debilitating, some patients may have severe muscle involvement leading to loss of ambulation[170]. Myopathy can be proximal, distal, or more generalized. Exercise intolerance with muscle fatigue, cramps and pain are evident in more than half of patients[170,174,175]. Bulbar or respiratory dysfunctions are rarely seen in GSD-III patients while no clinical involvement of facial or ocular muscles has been described in the literature[178].

Cardiac involvement in GSD-III is variable. Cardiac involvement is present in most patients, with varying degrees of severity ranging from ventricular hypertrophy detected on electrocardiography to clinically apparent cardiomegaly^[179]. Left ventricular hypertrophy, right ventricular hypertrophy, interventricular septal hypertrophy, QT prolongation, sinus tachycardia, and pulmonary hypertension were among electrocardiographic and/or echocardiographic findings of cardiac involvement[170,180]. According to International Study on Glycogen Storage Disease data presented by Sentner et al [170], 58% of patients with GSD-IIIa showed cardiac hypertrophy mostly presented by electrocardiographic and/ or echocardiographic signs of left ventricular hypertrophy. In the same cohort, only 15% of all patients developed hypertrophic cardiomyopathy. Mogahed et al [175] reported that cardiac muscle involvement is less common and mostly subclinical in the pediatric age group. However, a more recent study reported that 91% of patients showed cardiac involvement at a median age of 2.6 years, 86% of cases being under 2 years of age[181]. Moreover, 56% of the patients presented with a symptomatic cardiomyopathy at some point during the follow-up period indicating a more severe cardiac phenotype especially in those on a diet with insufficient caloric and protein intake and suboptimal UCCS treatment [181]. Cardiomyopathy usually presents with asymptomatic left ventricular hypertrophy but can progress to hypertrophic cardiomyopathy with decreased left ventricular function and/or arrhythmias, severe cardiac dysfunction, or congestive heart failure[181,182]. Sudden death has occasionally been reported^[183].

Patients with GSD-III may exhibit facial abnormalities such as indistinct philtral pillars, bow-shaped lips with a thin vermillion border, a depressed nasal bridge and a broad upturned nasal tip, and deepset eyes, particularly in younger patients[184]. Some individuals with GSD-III may have an increased risk of developing osteoporosis with reduced bone mineral density which, in part, may be due to suboptimal nutrition, the effects of metabolic abnormalities and muscle weakness^[41,185,186]. Bone fractures due to osteopenia and osteoporosis were reported in patients with GSD-III[170]. Polycystic ovary disease has been reported in women with GSD-III with no significant effect on fertility [187]. Type 2 diabetes may occur during the course of the disease in adulthood [188]. Michon *et al* [189] reported global cognitive impairment in adult GSD-III patients as an underlying cause of psychological and attention deficits seen in this patient group.

Liver histology shows uniform distension of hepatocytes secondary to glycogen accumulation. There is often septal formation, periportal and reticular fibrosis, fine microsteatosis, and less frequently, micronodular cirrhosis without inflammation or interface hepatitis. Skeletal muscle shows subsarcolemmal glycogen accumulation[12]. The diagnosis of GSD-III is made by identification of biallelic AGL pathogenic variants on molecular genetic testing. If the diagnosis cannot be established by genetic analysis, demonstrating enzyme deficiency in peripheral leukocytes or erythrocytes, cultured skin fibroblasts or in the liver or muscle tissue samples is necessary.

A practice guideline was published by the American College of Medical Genetics and Genomics in 2010 providing recommendations on the diagnosis and management of the complications of GSD-III [176]. The mainstay of GSD-III treatment is dietary intervention, which aims to maintain normal blood glucose levels while balancing macronutrient and total caloric intake. This is achieved by the avoidance of fasting, frequent meals enriched in complex carbohydrates and use of UCCS. Continuous enteral feeding may be needed in some cases. Sucrose, fructose, and lactose are not contraindicated unlike GSD-I. UCCS can be used as early as the first year of life to prevent hypoglycemia. As an alternative, Glycosade®, an extended-release cornstarch, can also be used[87]. Caution must be exercised to avoid overtreating with cornstarch or carbohydrates, which may lead to excessive storage of glycogen in the liver and weight gain. In patients with myopathy, along with managing hypoglycemia, a high-protein diet is recommended as it prevents muscle protein breakdown during glucose deprivation, thereby preserving skeletal and cardiac muscle^[176]. A ketogenic diet (alone or in combination with high protein and ketone bodies) was also shown to ameliorate cardiomyopathy [190,191]. It has been shown that a high-fat, low-calorie and high-protein diet can reduce cardiomyopathy in individuals with GSD-III[192, 193]. The beneficial effects on cardiac or skeletal muscle function of these ketogenic or high-fat diets are possibly related to the increased ketone bodies or fats as fuel sources, or reduced glycogen accumulation through decreased carbohydrate intake. Whether long-term muscular, cardiac, or even liver complications can be prevented by these dietary approaches warrants further studies[194].

Liver transplantation corrects all liver related biochemical abnormalities but does not correct myopathy or cardiomyopathy [107,133,195]. Cirrhosis, liver dysfunction, and/or hepatocellular carcinoma are the main indications for liver transplantation. Detailed information about surveillance recommendations on hepatic, metabolic, musculoskeletal, cardiac, nutritional, and endocrine aspects of the disease can be found elsewhere [176]. Gene therapy and gene-based therapeutic approaches are in development.

GSD-IV; Andersen disease; brancher deficiency; amylopectinosis; glycogen branching enzyme deficiency

The disease was described by Andersen[196] in 1956 as "familial cirrhosis of the liver with storage of abnormal glycogen" and, in 1966, amylo-1,4 to 1,6-transglucosidase [glycogen branching enzyme (GBE)] deficiency was reported [197]. Branching of the chains is essential to pack a very large number of glycosyl units into a relatively soluble spherical molecule. Without GBE, abnormal glycogen with fewer branching points and longer outer chains resembling an amylopectin-like structure (polyglucosan) accumulates in various tissues including hepatocytes and myocytes[198]. The mapping of the GBE1 gene to chromosome 3p12.2 was first accomplished in 1993[199]. Notably, mutations in the same gene are also responsible for adult polyglucosan body disease. GSD-IV accounts for only 0.3% of all GSDs and follows an autosomal recessive inheritance pattern^[200]. This rare disorder has a prevalence of 1:600000 to 1:800000[201].

GSD-IV exhibits significant clinical heterogeneity and phenotypic variability, partly due to variations in tissue involvement, which may be influenced by the presence of tissue-specific isozymes [198,200]. The liver is the primary organ affected, with the classical hepatic form appearing normal at birth but progressing rapidly to cirrhosis in early life, leading to liver failure and death between 3 to 5 years of age[196]. Children with GSD-IV experience growth failure, hepatomegaly and/or splenomegaly, and cirrhosis within the first 18 mo of life. Besides the complications of progressive cirrhosis including portal hypertension, ascites and esophageal varices, the development of hepatocellular carcinoma was also reported[202]. In rare cases, the hepatic disease in GSD-IV may not progress or progress slowly[203]. Patients with the non-progressive hepatic form may present with hepatosplenomegaly and mildly elevated liver transaminases, and experience normal growth. Liver size and transaminase levels may return to normal[203]. Patients with the non-progressive hepatic form usually survive into adulthood.

GSD-IV can present with multiple system involvement, with the enzyme deficiency in both liver and muscle^[204]. This form of the disease can manifest as peripheral myopathy with or without cardiomyopathy, neuropathy, and liver cirrhosis. Onset of the disease can be from the neonatal period to adulthood[205]. The neuromuscular presentation can be divided into four groups based on age at onset [206]. In the perinatal (fetal) form, which can lead to hydrops fetalis and polyhydramnios, arthrogryposis develops due to akinesia[207]. Detection of cervical cystic hygroma during pregnancy may indicate the disease^[200]. Prenatal diagnosis can be performed by determining enzyme activity in cultured amniocytes or chorionic villi samples. Genetic studies can complement uncertain enzyme activity studies, such as equivocal results in prenatal fetal samples and in patients with higher levels of residual enzyme activity that overlap heterozygote levels^[208]. Mortality is unavoidable in the neonatal period. Liver cirrhosis or liver failure has not been reported. Severe hypotonia, hyporeflexia, cardiomyopathy, depressed respiration, and neuronal involvement are features of the congenital form of the disease[198,209-211]. Liver disease is not severe, and the child dies in early infancy due to other reasons. The childhood neuromuscular form may start at any age with either myopathy or cardiomyopathy [206, 212]. Presenting symptoms mainly include exercise intolerance, exertional dyspnea, and congestive heart failure in advanced stages. The disease can be confined to muscular tissue and serum CK level can be within the normal range. In the adult form, there is isolated myopathy or a multisystemic disease called adult polyglucosan body disease. Onset of symptoms can occur at any age during adulthood, usually after the age of 50, and may exhibit a resemblance to muscular dystrophies. Symptomatology includes progressive gait difficulty and proximal muscle weakness, which is more pronounced in the arms as compared to the legs. Both upper and lower motor neurons are affected in the disorder. The disease may manifest as pyramidal tetraparesis, peripheral neuropathy, early onset of neurogenic bladder, extrapyramidal symptoms, seizures, and cognitive dysfunction leading to dementia[210]. The diagnosis can be established by enzyme activity assay in erythrocytes[213]. Amylopectin-like inclusions are detected through ultrastructural examination of the central nervous system and skeletal muscle. These inclusions are intensely PAS-positive and diastase-resistant, both in neurons and muscular fibers [214]. Magnetic resonance imaging shows white matter abnormalities[215].

Liver biopsy can be diagnostic in patients with hepatic involvement[216]. The histopathological evaluation of the liver reveals abnormal hepatocellular glycogen deposits in the form of PAS-positive, diastase-resistant inclusions. Ultrastructural examination with electron microscopy reveals accumulation of fibrillar aggregations that are typical of amylopectin. Typically, enzyme deficiency can be documented through diagnostic assays performed on hepatocytes, leukocytes, erythrocytes, and fibroblasts. However, patients with cardioskeletal myopathy may exhibit normal leukocyte enzyme activity^[198]. The diagnosis of GSD-IV can be confirmed through histopathological examination, detection of enzyme deficiency, and mutation analysis of the GBE1 gene. Genetic confirmation is recommended whenever possible in patients with suspected GSD-IV to provide more data for genotype-phenotype correlations in this extremely rare disease. The genotype-phenotype correlation remains unclear for GSD-IV and the same genetic defect may cause different clinical presentations in unrelated patients[217]. Mutation analysis can also provide crucial diagnostic information in cases with equivocal results of biochemical analyses[218]. Mutations with significant preservation of enzyme activity may be related with milder (e.g., non-progressive hepatic form) and late-onset (e.g., adult polyglucosan body disease) phenotypes of the disease.

Hypoglycemia has traditionally been considered a late manifestation and generally develops due to hepatocellular dysfunction caused by progressive cirrhosis. At this stage of the disease, the biochemical profile of the patients is representative of what is observed in other causes of liver cirrhosis. However, a recent study has reported that fasting intolerance, as indicated by a thorough medical history, along with the presence of hypoglycemia and/or ketosis, can be observed in patients even in the absence of detectable liver injury or dysfunction based on biochemical or radiological assessment[201].

No specific dietary and pharmacological treatments are available for GSD-IV. There is a lack of established guidelines based on either evidence or expert consensus for the dietary management of GSD-IV. Improvement in clinical, anthropometric, and laboratory parameters was reported with a highprotein and low-carbohydrate diet[219,220]. Derks et al[201] recently reported improved clinical and biochemical outcomes after dietary interventions including a late evening meal, continuous nocturnal intragastric drip feeding, restriction of mono- and disaccharides, the addition of UCCS, and protein enrichment in patients with GSD-IV. Individual dietary plans should also aim to avoid hyperglycemia to minimize glycogen accumulation in the liver.

At present, there is no effective therapeutic approach other than liver transplantation for GSD-IV patients who are affected by progressive liver disease. However, anecdotal reports indicate that liver transplantation may not alter the extrahepatic progression of GSD-IV[217]. The presence of extrahepatic involvement, especially amylopectin storage in the myocardium, may lead to fatal complications following liver transplantation[221-223]. Careful assessment of cardiac function even in the absence of clinical decompensation or consideration of combined liver-heart transplantation is warranted for patients with GSD-IV[224]. Liver transplantation may provide beneficial effects not only for patients with liver disease but also for those affected by muscular involvement in GSD-IV [107,225,226]. This may be explained by systemic microchimerism (donor cells presenting in various tissues of the liver recipient) after liver allotransplantation and amelioration of pancellular enzyme deficiencies resulting in



a decrease in amylopectin in other organ systems^[12]. It has been suggested that the donor cells can transfer enzyme to the native enzyme-deficient cells[226].

In recent years, animal studies have been conducted to prevent glycogen and polyglucosan body accumulation in GSD-IV patients, and GYS inhibitor guaiacol and 144DG11 are promising in this regard [227,228]. The molecular target of 144DG11 is the lysosomal membrane protein lysosome-associated membrane protein 1 (LAMP1), which enhances autolysosomal degradation of glycogen and lysosomal acidification. In the adult polyglucosan body disease mouse model, 144DG11 reduced polyglucosan and glycogen in brain, liver, heart, and peripheral nerve^[228].

GSD-VI; Hers disease; liver glycogen phosphorylase deficiency

GSD-VI was first reported by Hers^[229] in three patients with hepatomegaly, mild hypoglycemia, an increased glycogen content and deficient activity of glycogen phosphorylase in the liver in 1959. GSD-VI is a rare autosomal recessive genetic disease caused by deficiency of hepatic glycogen phosphorylase. At least three human glycogen phosphorylases exist including muscle, liver, and brain isoforms[230]. In response to hypoglycemia, liver glycogen phosphorylase catalyzes the cleavage of glucosyl units from glycogen which results in the release of glucose-1-phosphate. The glucose-1-phosphate is subsequently converted to glucose-6-phosphate. The PYGL gene is currently the only known genetic locus associated with the development of GSD-VI and was mapped to chromosome 14q21-q22 in 1987[231]. Incidence of the disease is estimated to be 1:100000 and believed to be underestimated due to nonspecific and variable phenotypes, and a paucity of cases confirmed by genetic testing[232]. GSD-VI is more prevalent among the Mennonite community, with a prevalence of 1 in 1000, representing the only known population at higher risk for the disease [232].

GSD-VI is a disorder with broad clinical heterogeneity [232]. Infants with liver phosphorylase deficiency mainly present with hepatomegaly and growth retardation. The condition typically has a benign course, and symptoms tend to improve as the child grows[229]. Hepatomegaly usually normalizes by the second decade of life[233]. The child shows mild to moderate ketotic hypoglycemia related to prolonged fasting, illness, or stressful conditions[232]. As gluconeogenesis is intact in GSD-VI, hypoglycemia is usually mild. Despite gross hepatomegaly, the patient may be largely asymptomatic without hypoglycemia. However, there is a range of clinical severity in GSD-VI, with some patients experiencing severe and potentially life-threatening hypoglycemia. There is generally mild ketosis, growth retardation, abdominal distension due to marked hepatomegaly and mildly elevated levels of serum transaminases, triglycerides, and cholesterol. However, in patients with high residual enzyme activity, biochemical investigations may be normal [234,235]. Hypertriglyceridemia may persist despite treatment[108]. A few patients showing mild muscular hypotonia, muscle weakness or developmental impairment were observed, but otherwise, no neurological symptoms were reported in the literature [232]. Sleep difficulties and overnight irritability are common[236]. In contrast to GSD-I, serum levels of lactic acid and uric acid are generally within the normal range[15]. However, in a recent clinical study including 56 GSD-VI patients, hyperuricemia was reported as a complication in adolescent and adult patients with GSD-VI, which indicates the need for long-term monitoring of uric acid in older GSD-VI patients [237]. CK concentration is usually normal. In some patients, severe and recurrent hypoglycemia, pronounced hepatomegaly, and postprandial lactic acidosis have been reported [238]. Recently, children with GSD-VI have been reported to present with only ketotic hypoglycemia as the sole manifestation of the disease, without the characteristic hepatomegaly [239]. Mild cardiopathy has also been described for GSD-VI[233].

The clinical picture of GSD-VI virtually overlaps with phosphorylase kinase (PHK) deficiency (GSD-IX) and the differential diagnosis includes other forms of GSDs associated with hepatomegaly and hypoglycemia, especially GSD-I and GSD-III[236]. It is not possible to distinguish between GSD-VI and GSD-IX based on clinical or laboratory findings alone^[232].

Mutation analysis is the suggested method for the diagnosis of GSD-VI. A liver biopsy is not recommended to establish the diagnosis to avoid an invasive procedure. Excessive glycogen accumulation with structurally normal glycogen in the liver biopsy is consistent with GSD-VI. Fibrosis, mild steatosis, lobular inflammatory activity and periportal copper binding protein staining have also been reported in GSD-VI patients. Although it is possible to document glycogen phosphorylase deficiency in frozen liver biopsy tissue or blood cells including leukocytes and erythrocytes, normal *in vitro* residual enzyme activity may be seen and prevents establishment of a definitive diagnosis by an enzyme assay alone in some patients[234,235].

In GSD-VI, nutrition therapy aims to improve metabolic control and prevent primary manifestations such as hypoglycemia, ketosis, and hepatomegaly, as well as secondary complications including delayed puberty, short stature, and cirrhosis. Frequent meals, a high-protein diet providing 2-3 g protein/kg body weight/d, limitation but not prohibition of simple sugars such as sucrose, fructose, lactose, a late evening meal and use of UCCS are the main recommendations in GSD-VI patients[236]. The aim of the therapeutic approach is to achieve euglycemia and normoketosis by administration of the appropriate doses of cornstarch. The target level for blood glucose should be within 70-100 mg/dL, while the optimal range for blood ketones is 0.0-0.2 mmol/L[236]. An extended-release corn starch derived from waxy maize, marketed as Glycosade®, has been found to have a positive impact in delaying overnight hypoglycemia in children over 5 years of age and adults[87]. Some individuals with



GSD-VI may not require any treatment.

GSD-VI usually has a benign disease course. However, focal nodular hyperplasia, fibrosis, cirrhosis, and a degeneration to hepatocellular carcinoma have been reported in some patients[240-242]. Cirrhosis has been reported in patients as young as preschool age, even within the second year of life[242]. Based on these findings, aggressive treatment of GSD-VI has recently been suggested to maintain optimal metabolic control and prevent long-term complications[243]. Long-term monitoring of hepatic function is also recommended[236].

GSD-IX; PHK deficiency

Glucagon and epinephrine play a critical role in the regulation of glycogenolysis by activation of adenylate cyclase which leads to an increase in the cytosolic concentration of cyclic adenosine monophosphate (cAMP). The increased level of cAMP activates cAMP-dependent protein kinase which activates PHK. In the next step, PHK, a serine/threonine-specific protein kinase, functionally activates glycogen phosphorylase in the liver. PHK is a heterotetramer composed of 4 different subunits (α , β , γ , and δ). Each subunit is encoded by different genes that are located on different chromosomes and differentially expressed in a variety of tissues [244]. α and β subunits have regulatory functions, the γ subunit contains the catalytic site, and δ is a calmodulin protein [245]. PHK has a wide tissue distribution with multiple tissue-specific isoforms.

The α subunit has two isoforms, a muscle isoform, and a liver isoform, which are encoded by two different genes (PHKA1 and PHKA2, respectively) on the X chromosome [244]. The genetic loci of other subunits are mapped to autosomal chromosomes. The y subunit also has muscle and liver isoforms, each of which is encoded by a distinct gene (PHKG1 and PHKG2, respectively). There is only one gene encoding the β -subunit (*PHKB*). However, *PHKB* is expressed in both muscle and liver[246,247].

Liver PHK deficiency (liver GSD-IX) can be classified according to the involved gene, the X-linked form (GSD-IXa, X-linked glycogenosis) and autosomal recessive forms (GSD-IXb and GSD-IXc). GSD-IXa (PHKA2-related GSD-IX) is caused by pathogenic variants in the PHKA2 gene on X chromosome. GSD-IXb (PHKB-related GSD-IX) and GSD-IXc (PHKG2-related GSD-IX) are inherited in an autosomal recessive manner and caused by mutations in PHKB and PHKG2 genes, respectively (Table 1). GSD-IXa is further classified into subtypes XLG-I (formerly GSD-VIII) with no enzyme activity in liver or erythrocytes, and XLG-II with no enzyme activity in liver, but normal activity in erythrocytes[248,249].

GSD-IX is one of the most common forms of GSDs. Approximately 25% of all GSDs can be attributed to PHK deficiency [249]. The frequency of liver PHK deficiency was estimated to be 1:100000[15]. GSD-IXa, the most common subtype of liver PHK deficiency, accounts for 75% of all GSD-IX cases. On the X chromosome, there are two enzyme loci; one for the alpha subunit of muscle PHK, and one for the alpha subunit of liver PHK. In 1992, the liver PHK gene was located to Xp22.2-p22.1[244]. GSD-IXa is more common in males due to the X-linked inheritance pattern. Female carriers may become symptomatic due to X chromosome inactivation[250].

Hepatomegaly, growth retardation, delayed motor development, mild hypotonia, significantly elevated serum transaminase levels, hyperlipidemia, fasting hyperketosis, and hypoglycemia are the main symptoms and findings[251-254]. Rarely described clinical features include splenomegaly, liver cirrhosis, doll-like facies, osteoporosis, neurologic involvement, high serum lactate levels, metabolic acidosis, and renal tubular acidosis[233]. With increasing age, there is a gradual resolution of both clinical symptoms and laboratory abnormalities. Although puberty may be delayed, eventual attainment of normal height and complete sexual development is still possible [253]. Most adult patients are asymptomatic^[252]. Unusual presentations including asymptomatic hepatomegaly and isolated ketotic hypoglycemia without hepatomegaly have been reported in affected male children underscoring the importance of screening for GSD-IXa in male patients who are suspected of having GSD with atypical features [239,255]. More severe phenotypes including severe recurrent hypoglycemia and liver cirrhosis have also been reported[243,256,257]. Recent findings suggest that GSD-IXa is not a benign condition as is often reported in the literature and patients may have fibrosis even at the time of diagnosis^[258].

GSD-IXc is caused by autosomal recessive mutations in the *PHKG2* gene. There are two isoforms, encoded by different genes, for the gamma subunit: The muscle form (*PHKG1* gene) and the testis/liver form (*PHKG2* gene)[259]. The genetic locus of the liver form was located to 16p12.1-p11.2. The presence of *PHKG2* mutations has been linked to more severe clinical and biochemical abnormalities, such as an elevated risk for liver fibrosis and cirrhosis[260-262]. Liver cirrhosis can develop in infancy[263]. Cirrhosis related esophageal varices and splenomegaly, liver adenomas, renal tubulopathy and significant hypocalcemia were other reported clinical findings[236]. Patients with this condition commonly present with severe hypoglycemia requiring overnight feeding, show very low PHK activity in the liver, and exhibit highly elevated serum transaminase levels. A wide range of clinical symptoms can be observed, including hypoglycemia during fasting, hepatomegaly, elevated levels of transaminases, hepatic fibrosis, cirrhosis, muscle weakness, hypotonia, delayed motor development, growth retardation, and fatigue^[264].

The genetic cause of GSD-IXb is attributed to mutations in the PHKB gene, which is located on 16q12q13 and encodes the beta subunit of PHK[265]. The main features of the disease include marked hepatomegaly, increased glycogen content in both liver and muscle, and the development of hypoglycemic



symptoms after physical activity or several hours of fasting[265]. Patients with liver fibrosis, adenomalike mass, mild cardiopathy and interventricular septal hypertrophy were reported[233]. The muscle symptoms are generally mild or absent, affecting virtually only the liver. Distinction between GSD-IXb and individuals with pathogenic variants in PHKA2 or PHKG2 cannot be carried out based on clinical findings alone.

Genetic analysis is the preferred first-line diagnostic test in suspected patients. An approach using next-generation sequencing panels is advised due to the involvement of multiple genes. Liver biopsy can be a valuable diagnostic tool for confirming the diagnosis in cases where there are variants of unknown significance. Histopathological assessment of liver involvement is superior to biochemical parameters[266]. It is important to keep in mind that PHK enzyme activity can be normal in blood cells and even in liver tissue of affected patients. On the other hand, a reduction in PHK enzyme activity can also occur secondary to other metabolic defects such as pathogenic variants in GLUT2 in Fanconi-Bickel syndrome (FBS), PRKAG2 cardiomyopathy syndrome, or mitochondrial complex 1 deficiency [236].

In patients with GSD-IX, close monitoring of long-term liver and cardiac complications is recommended[233]. Aggressive structured dietary treatment with UCCS and relatively high protein intake was associated with considerable improvement in growth velocity, energy, biochemical abnormalities, hepatomegaly, and overall well-being of patients with GSD-IX. Radiographic features of fibrosis were also reported to be improved with early and aggressive dietary management^[243]. General nutritional recommendations for GSD-IX are similar to those for GSD-VI and have recently been published[236].

FBS (formerly GSD-XI)

The primary defect in FBS is deficiency of glucose transporter 2 (GLUT2), a monosaccharide carrier that is responsible for the transport of both glucose and galactose across the membranes in hepatocytes, pancreatic β -cells, enterocytes, and renal tubular cells. Utilization of both glucose and galactose is impaired in FBS[267]. Hepatorenal glycogen accumulation and proximal renal tubular dysfunction are the characteristic features of this rare disease [268,269]. FBS follows an autosomal recessive inheritance pattern. The responsible gene, GLUT2 gene (solute carrier family 2 member 2, SLC2A2), was localized to 3q26.1-q26.3 in 1988[270,271]. Since the first patient reported by Fanconi and Bickel in 1949, over 100 cases of FBS with various SLC2A2 mutations including missense, nonsense, frameshift/insertiondeletion, intronic, and compound heterozygous mutations have been reported[272].

Infants with FBS typically present between the ages of 3 to 10 mo. In addition to hepatorenal glycogen accumulation and proximal renal tubular dysfunction, FBS is characterized by fasting hypoglycemia, postprandial hyperglycemia and hypergalactosemia, rickets and marked growth retardation. Patients have entirely normal mental development. In older patients, dwarfism is the most notable finding. Puberty is significantly delayed, with other remarkable observations including a distended abdomen caused by hepatomegaly, deposition of fat on the abdomen and shoulders, and a moon-shaped face [273]. Some patients may not exhibit hepatomegaly during the early stages of the disease [269,274]. Hyperlipidemia and hypercholesterolemia are prominent and may cause acute pancreatitis. The development of generalized osteopenia occurs early and may result in fractures. Hypophosphatemic rickets and osteoporosis are characteristics of the disease that emerge later in life[275]. Tubular nephropathy is characterized by excessive glucosuria, moderate hyperphosphaturia along with persistent hypophosphatemia, hyperuricemia, hyperaminoaciduria, and intermittent albuminuria, collectively referred to as renal Fanconi syndrome[267,268]. Hypercalciuria is also evident. Due to increased renal losses, there is a frequent tendency towards hyponatremia and hypokalemia. Polyuria may develop due to high urinary osmotic load[276]. Progression to renal failure is not the case. Nephrocalcinosis was also reported in one third of the patients in a recent retrospective study^[277]. There may be mild metabolic hyperchloremic acidosis with normal anion gap due to renal loss of bicarbonate[267]. Cataracts, a frequently documented consequence of hypergalactosemia, are only present in a small number of patients[278].

Laboratory findings include fasting hypoglycemia and ketonuria, hyperglycemia and hypergalactosemia in the postabsorptive state, hypercholesterolemia, hyperlipidemia, moderately elevated alkaline phosphatase, mildly elevated transaminases, normal hepatic synthetic function, hypophosphatemia, hyperaminoaciduria, glucosuria, galactosuria, proteinuria, normal activity of enzymes involved in galactose and glycogen metabolism, normal fructose metabolism, and normal endocrinologic results[267]. FBS patients develop different patterns of dysglycemia, ranging from fasting hypoglycemia, postprandial hyperglycemia, glucose intolerance, to transient neonatal diabetes to gestational diabetes and frank diabetes mellitus[279]. The exact molecular mechanisms underlying the occurrence of dysglycemia in individuals with FBS are not yet fully understood. Sharari et al[272] recently suggested that SLC2A2 mutations cause dysglycemia either by a direct effect on GLUT2 expression and/or activity or, indirectly, by the dysregulated expression of microRNAs implicated in glucose homeostasis. Impaired renal glucose reabsorption, as well as the accumulation of glucose within the hepatocytes, which stimulates glycogen synthesis and inhibits gluconeogenesis and glycogenolysis, result in fasting ketotic hypoglycemia and hepatic glycogen deposition. Postprandial findings of hyperglycemia and hypergalactosemia are caused by impaired hepatic uptake and diminished insulin response[279]. Glycated hemoglobin A1c is usually within the normal range due to recurrent



hypoglycemia episodes [280]. Accumulation of glycogen and free glucose in renal tubular cells leads to general impairment in proximal renal tubular function. Histological evaluation of liver biopsy indicates an excessive buildup of glycogen along with steatosis. Due to the presence of galactose intolerance, newborn screening for galactosemia can sometimes identify patients with FBS[281]. The diagnosis is ultimately confirmed by genetic analysis of SLC2A2 gene.

The management of symptoms involves measures to stabilize glucose homeostasis and compensate for the renal loss of water and various solutes. Patients typically require replacement of water, electrolytes, and vitamin D, while also restricting galactose intake and adhering to a diabetes mellituslike diet. Frequent small meals with adequate caloric intake and administration of UCCS are important components of symptomatic treatment. In cases of renal tubular acidosis, it may be required to administer alkali to maintain acid-base balance. Catch-up growth was reported to be induced by UCCS [282]. Continuous nocturnal gastric drip feeding may be indicated in some cases with growth failure [283]. With these measures, the prognosis is good. However, a recent retrospective study reported poor outcome despite adequate metabolic management emphasizing the importance of early genetic diagnosis and facilitating prompt nutritional interventions[277].

GSDS INVOLVING MUSCLE

GSD-II; Pompe disease; acid alpha-glucosidase deficiency; acid maltase deficiency; alpha-1,4glucosidase deficiency

Pompe disease is a typical example of a lysosomal storage disease. The clinical manifestations of Pompe disease are variable, predominantly due to the varying amounts of residual acid alpha-glucosidase (GAA) activity linked with distinct mutations in the causative gene (GAA). GAA gene is mapped to chromosome 17q25.2-q25.3[284]. Enzyme deficiency results in intra-lysosomal storage of glycogen especially in skeletal and cardiac muscles. There is no genotype-phenotype correlation, but DD genotype in the angiotensin converting enzyme gene and XX genotype in the alpha actinin 3 gene are significantly associated with an earlier age of onset of the disease^[285].

There are mainly two types of GSD-II according to age of onset: Infantile-onset and late-onset Pompe disease. Patients with disease onset before the age of 12 mo without cardiomyopathy and all patients with disease onset after 12 mo of age are included in the late-onset form [286]. The combined frequency of infantile onset and late onset GSD-II varies between 1:14000 and 1:100000 depending on ethnicity and geographic region. In the infantile-onset form, cardiomyopathy and muscular hypotonia are the cardinal features and patients die around 1 year of age. The enzyme activity is less than 1% of normal controls, and the enzyme is deficient in all tissues. Patients also have feeding difficulties, macroglossia, failure to thrive, hearing impairment and respiratory distress due to muscle weakness. The liver is rarely enlarged unless there is heart failure. Hypoglycemia and acidosis do not occur[286]. In the lateonset form, involvement of skeletal muscles dominates the clinical picture, and cardiac involvement is generally clinically insignificant depending on the age of onset. Enzyme activity is partially deficient (2% to 40% of normal controls)[286]. Glycogen accumulation in vascular smooth muscle may cause the formation and subsequent rupture of an aneurysm[287]. Both severe infantile and asymptomatic adult forms of the disease were observed in two generations of the same family^[288]. Although women with GSD-II do not have an increased risk of pregnancy or delivery complications, pregnancy may worsen muscle weakness and respiratory complications [289]. As a rule, there is an inverse correlation between the age at disease onset and the severity of clinical manifestations with the level of residual enzyme activity^[286].

Laboratory testing reveals nonspecific elevations in CK, aldolase, aminotransferases, and lactate dehydrogenase. Elevated urinary tetrasaccharide is highly sensitive but not specific. To establish the final diagnosis, the measurement of enzyme activity in skin fibroblasts or muscle tissue or the demonstration of the responsible mutation is required [286].

Although it is not curative, ERT has changed the course of Pompe disease since its first use in 2001 [290]. Alglucosidase alfa, a lysosomal glycogen-specific recombinant enzyme, was approved by the European Medicines Agency (EMA) in 2006 in the European Union and by the Food and Drug Administration (FDA) in 2010 in the United States. The indication criteria were as follows: \geq 8 years and absence of cardiac hypertrophy (https://www.accessdata.fda.gov/drugsatfda_docs/Label/2010/125291Lbl. pdf; accessed on November 5, 2022). Based on data from later studies, treatment initiation was shifted to the neonatal period. The recommended dosage is 20 mg/kg body weight every two weeks by intravenous administration. A new formulation of GAA enzyme, avalglucosidase alfa, improves the delivery of the enzyme to target cells and has 15 times higher cellular uptake when compared with alglucosidase alfa. The FDA and EMA approved avalglucosidase in 2021 and in 2022, respectively, for the treatment of patients who are one year of age and older with late-onset Pompe disease[291]. Ongoing studies show that avalglucosidase is generally well tolerated in patients with infantile-onset Pompe disease[291]. The recommended dose is 5 to 20 mg/kg every 2 wk[291]. Criteria for starting and stopping ERT in adult patients with GSD-II are similar in different countries. While a confirmed diagnosis and being symptomatic are general criteria for starting ERT, patient wish, severe infusion



associated reactions, noncompliance with treatment, and lack of effect are criteria for stopping ERT [292]. Another way to increase the effectiveness of ERT is to use antibodies as an intracellular delivery vehicle. The 3E10 anti-nuclear antibody, that penetrates cells and localizes to the cell nucleus, has been used for this purpose. VAL-1221 is a fusion protein consisting of 3E10 antibody and GAA complex. The presence of 3E10 increases the delivery of GAA to both lysosomal and extra-lysosomal storage of glycogen within cells[293]. The earlier ERT is started, the better its effectiveness. Therefore, it is recommended that ERT is started before irreversible clinical symptoms begin. This concept has led to the development of screening programs for Pompe disease [294]. Recently, it has been shown that in utero alglucosidase alfa treatment, which was started at 24 wk 5 d of gestation and given 6 times at 2-wk intervals through the umbilical vein, was successful^[295].

Although antibodies against the enzyme may develop, a recent study showed that the development of antibodies did not affect the clinical course [296]. Whether additional treatments such as oral supplementation of L-alanine is beneficial is being investigated [297]. As an alternative to ERT, studies on gene therapy have also commenced[298].

Danon disease

Although Danon disease was previously classified as a variant of GSD-II with normal alpha-glucosidase activity, it is still controversial whether it is a real GSD. A lysosomal structural protein, LAMP2, is deficient in Danon disease. LAMP2 is involved in autophagosome maturation. Disruption of autophagy leads to accumulation of glycogen granules and autophagic vacuoles[299]. It is an X-linked (Xq24) dominant hereditary disease affecting both skeletal and cardiac muscles, and characterized by skeletal and cardiac myopathy, proximal muscle weakness and intellectual disability. Female patients have a milder disease predominantly involving cardiac muscle[300]. There is currently no treatment for Danon disease. There are ongoing studies evaluating the efficacy and safety of gene therapy[300].

AMP-activated protein kinase deficiency

Another glycogen storage cardiomyopathy results from PRKAG2 (the gene encoding gamma-2 noncatalytic subunit of adenosine monophosphate-activated protein kinase) mutations on chromosome 7q36.1. The disease is characterized by left ventricular hypertrophy due to altered glycogen metabolism and glycogen storage in cardiac muscle, similar to Danon disease 301-303]. It is inherited in an autosomal dominant pattern. PRKAG2 gene variants cause a syndrome characterized by cardiomyopathy, conduction disease, and ventricular pre-excitation[302]. It may cause atrial fibrillation/flutter or conduction abnormalities that may cause sudden cardiac death and severe heart failure typically in the third and fourth decade[301,302]. Mutations in the gamma-2 non-catalytic subunit of AMP-activated protein kinase may cause lethal congenital storage disease of the heart, and death in the first year of life [303]. It is important to differentiate the clinical picture related to *PRKAG2* mutations from Danon disease, as management and prognosis are different.

GSD-V; McArdle disease; myophospharylase deficiency; muscle glycogen phosphorylase deficiency

GSD-V is caused by mutations in *PYGM* gene which is the gene encoding the muscle isoform of glycogen phosphorylase. The PYGM gene is located on 11q13.1[304]. In a recently published European registry for patients with muscle glycogenosis, 95% of all patients had GSD-V[305]. The clinical manifestations generally occur during early adulthood with physical activity intolerance and muscle cramps characterized by muscle fatigue and pain, contracture, tachypnea, tachycardia, ptosis, and retinal dystrophy. Most of the patients are symptomatic at pediatric age (< 18 years)[306]. An improvement of exercise induced symptoms, named the "second wind phenomenon", characterized by the improvement of exercise tolerance after 8 to 10 min of aerobic exercise, is observed [306,307]. Exercise induced rhabdomyolysis can cause transient myoglobinuria, leading to acute renal failure. Hyperuricemia, gout development and thyroid dysfunction are not uncommon^[306]. Many patients are diagnosed with an incidental finding of abnormal serum CK levels[307].

Echaniz-Laguna et al[308] studied a family of 13 affected members with adult-onset muscle weakness, and reported a phenotype caused by a dominant myophosphorylase gene mutation (p.Asp639His). The first signs of the disease occurred after 40 years of age with proximal leg weakness, followed by proximal arm weakness. In contrast to McArdle disease, the patients did not have exercise intolerance, second wind phenomenon, markedly increased CK levels, or rhabdomyolysis. The authors concluded that specific *PYGM* mutations can cause either dominant or recessive GSDs[308].

GSD-VII; Tarui disease; muscle phosphofructokinase deficiency; GSD of muscle

The responsible gene is located on chromosome 12q13.3[309]. Exercise induced muscle cramps and myoglobinuria are the main characteristics of GSD-VII. Neurological examination does not reveal any abnormalities at rest. Muscle weakness and stiffness invariably occur in muscle groups that are subjected to intense or prolonged exertion. The ischemic exercise test is characterized by the absence of an increase in venous lactate level. Myoglobinuria may develop following exercise. Nausea and vomiting, icterus, elevated CK, hyperuricemia and reticulosis may also be observed [307]. In contrast to GSD-V, glucose intake prior to exercise worsens exercise capacity due to blocked use of both muscle



glycogen and blood glucose[307].

GSD-IXd: X-linked muscle PHK alpha-1 subunit deficiency

The gene is located on chromosome Xq13.1, and the disease is inherited recessively[310]. In most patients, clinical findings appear in adulthood and are characterized by muscle weakness and muscle cramps during exercise. Elevated serum CK level and myopathic findings on electromyography may guide the diagnosis[311].

GSD-X; muscle phosphoglycerate mutase deficiency

The last steps of glycogenolysis are abnormal. The disease is inherited in an autosomal recessive manner and characterized by exercise induced muscle cramps, myalgia, rhabdomyolysis and myoglobinuria. Serum CK level is increased between episodes[312].

GSD-XI; lactate dehydrogenase a deficiency

GSD-XI was first described by Kanno et al[313] in 1980 and characterized by easy fatigue, increase in serum CK, myoglobin, lactate, and pyruvate levels immediately after ischemic work. The gene locus is on chromosome 11p15.1[314].

GSD-XII; aldolase deficiency

GSD-XII is a very rare disease resulting from aldolase A deficiency and characterized by muscle glycogen accumulation, crisis of rhabdomyolysis induced by fever and/or exercise and hemolytic anemia with or without myopathy or cognitive dysfunction [315]. It is an autosomal recessive disorder, and the gene is located on chromosome 16p11.2[316].

GSD-XIII; muscle enolase 3 deficiency

GSD-XIII was first described by Comi et al [317] in 2001 in a 47-year-old man with severe deficiency of muscle enolase activity. The patient had recurrent exercise induced myalgia without cramps. Serum CK concentration was elevated while serum lactate level was normal following ischemic forearm exercise. The related gene is located on chromosome 17p13.2[317].

GSD-XV; glycogenin deficiency

Similar to Danon disease and PRKAG2 variants, glycogenin deficiency may cause left ventricular arrhythmogenic cardiomyopathy. Patients present with chest pain, progressive weakness, and vague presyncope spells[318].

CONCLUSION

There have been significant changes and improvements in the classification, diagnosis, and treatment of GSDs in recent years. We are now more aware that many GSDs, which were previously identified as childhood diseases, may present first in adulthood. Diagnosis can be challenging, especially for GSDs with milder phenotypes and those with only skeletal and/or cardiac muscle involvement. As early diagnosis and aggressive treatment is related to better prognosis, physicians should be aware of these conditions and include GSDs in the differential diagnosis of pediatric and adult patients with not only liver related manifestations but also skeletal and/or cardiac muscle, central nervous system, and multisystemic involvement.

FOOTNOTES

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REVIEW

Past, present, and future of long-term treatment for hepatitis B virus

Teresa Broquetas, José A Carrión

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Abstract

The estimated world prevalence of hepatitis B virus (HBV) infection is 316 million. HBV infection was identified in 1963 and nowadays is a major cause of cirrhosis and hepatocellular carcinoma (HCC) despite universal vaccination programs, and effective antiviral therapy. Long-term administration of nucleos(t)ide analogues (NA) has been the treatment of choice for chronic hepatitis B during the last decades. The NA has shown a good safety profile and high efficacy in controlling viral replication, improving histology, and decreasing the HCC incidence, decompensation, and mortality. However, the low probability of HBV surface antigen seroclearance made necessary an indefinite treatment. The knowledge, in recent years, about the different phases of the viral cycle, and the new insights into the role of the immune system have yielded an increase in new therapeutic approaches. Consequently, several clinical trials evaluating combinations of new drugs with different mechanisms of action are ongoing with promising results. This integrative literature review aims to assess the knowledge and major advances from the past of hepatitis B, the present of NA treatment and withdrawal, and the future perspectives with combined molecules to achieve a functional cure.

Key Words: Hepatitis B; Therapy; Antigen; Functional cure; Antiviral agents; Drug development

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Core Tip: Treatment for chronic hepatitis B has been used for decades, showing a good safety profile and high virological and clinical efficacy, decreasing hepatocellular carcinoma, clinical decompensation, and mortality. However, the low probability of hepatitis B virus surface antigen seroclearance with therapy made necessary indefinite treatment in a majority of patients. With the new insights about the immune system role in hepatitis B virus infection and the knowledge of the viral cycle phases, there has been in recent years increased activity in new therapeutic approaches. This review focuses on the past, present, and future of chronic hepatitis B therapy.

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INTRODUCTION

The hepatitis B virus (HBV) was identified in 1963 when the modern research history of viral hepatitis began. The Nobel Prize winner Baruch S. Blumberg (1925-2011) discovered, 60 years ago, an enigmatic serum protein named "Australia" antigen (AuAg)[1]. Some years later, the recognition of the HBV surface antigen (HBsAg) allowed for the first-time screening of blood donors. The HBsAg was the first marker assessed by a highly sensitive immune analysis, the HBV genome the first identified by deoxyribonucleic acid (DNA), the antibody against HBV core (anti-HBc) the first evaluated by the anti-µ capture technique, and the HBV vaccine the first produced by gene technology[2].

Infection by HBV may lead to acute or chronic hepatitis. Chronic hepatitis B (CHB) infection is defined as HBsAg serum detection for at least six months. The estimated world prevalence of CHB was 316 million in 2019, a major cause of cirrhosis and hepatocellular carcinoma (HCC)[3]. The treatment of choice during the last decades has been the long-term administration of nucleos(t)ide analogues (NA) with a high barrier to resistance. However, the low probability of HBsAg seroclearance made necessary an indefinite therapy.

This integrative literature review aims to review the major advances from the past of hepatitis B, the present of NA treatment and withdrawal, and the future perspectives for the new combined molecules to achieve a functional cure.

PAST

Virus detection: The "Australia" antigen and "Dane" particle

In the 1960s, virology was a young science, and techniques for the diagnosis of most viral diseases were complicated and suboptimal. The most often used method was the complement fixation reaction, which required four complex biological component mixtures from four different animal species. The first hint came from the American physician and geneticist Baruch S. Blumberg who used an immunological approach in 1967[1]. Blumberg's co-worker, Harvey J. Alter, discovered a new antigen in Australian aborigines named the AuAg.

Dane *et al*[4] were inspecting AuAg immune complexes under the electron microscope and identified virus-like particles of 42 nm in size. In 1971, Almeida *et al*[5] were able to free the core particles from the "Dane" particle and showed that patients formed, against this core antigen (HBcAg), antibodies (anti-HBc) suggesting that the Dane particle was the cause of hepatitis B, and the AuAg was the surface antigen of the HBV envelope (HBsAg)[5].

Many scientists, including Blumberg, recognized that the HBsAg did not allow to assess the severity of the disease. Magnius *et al*[6] discovered an additional marker when looking for HBsAg subtypes, the HB e antigen (HBeAg), which helped to distinguish highly infectious from less infectious HBV carriers. However, direct detection of the nucleic acid within HBV was not possible at that time since the HBV still could not be grown in cell cultures, and patient sera contained a few nanograms of Dane particles/ mL. It was in 1974 when Robinson *et al*[7] identified the polymerase activity, and the HBV-DNA. The nucleic acid of HBV was a small circular double-stranded DNA but not covalently closed such as the polyoma- or papillomaviruses. Therefore, the ability of the HBV-DNA polymerase to transcribe both RNA and DNA was similar to retroviruses[2,8].

After more than half a century, current international guidelines have recommended using these three viral markers, HBsAg, HBeAg, and HBV-DNA for characterizing HBV infection[9-11].

Hepadnaviral life cycle: The journey of Dane particle

HBV is a partially double-stranded virus that belongs to the *Hepadnaviridae* family[12]. The virus replicates in the host's hepatocytes. The first step is viral cell entry via the bile acid transporter sodium taurocholate cotransporting polypeptide (NTCP)[13,14]. The HBV is transported to the nucleus to release the relaxed circular DNA (rcDNA) genome^[15] and becomes a covalently closed circular DNA (cccDNA) that uses the host-cell DNA repair mechanism and can serve as a template for all viral transcripts that are translated into viral proteins. The HBV genome can be integrated into the host genome with the risk of hepatocyte transformation and carcinogenesis[16]. The pre-genomic RNA (pgRNA) is packaged and reversely transcribed to begin replication. The pgRNA is also enveloped and secreted. The HBV-integrated DNA encodes three HBsAg proteins: Large (L), middle (M), and small (S) HBs which are especially important in HBeAg-negative patients^[17]. The viral capsids will be enveloped with the HBsAg and released as infectious virions, non-infectious sub-viral spherical or filamentous particles[18], empty virions (HBsAg and core proteins), and RNA virions[19]. The role of subviral particles in the pathogenesis of chronic HBV infection is not fully understood, but they could act as an immune evasion mechanism by blocking the host's neutralizing antibodies (anti-HBs) and promoting the spread and persistence of the infection[20,21].

Pathogenesis and phases of HBV infection

The first step in HBV infection is a highly replicative phase before immune recognition begins, after weeks or months of delay. A vigorous cellular immune response suppresses viral replication and eliminates most of the HBV-infected hepatocytes resulting in acute hepatitis. During acute hepatitis B, HBsAg disappears within six months. If HBsAg persist longer than this period, is considered a CHB infection. CHB is a dynamic interaction between HBV and the host's immune system.

Infection of newborns or infants results in a persistent infection because of an ineffective immune response. After a long anergic phase, immune defense emerges and leads to the selection of escape mutants. The cellular immune responses against HBcAg appear and HBeAg loses its immunomodulatory function. Therefore, the HBeAg-positive chronic infection usually occurs in younger patients during the first years. Patients have a high viral load with normal liver function tests. The HBeAgnegative chronic infection is characterized by low levels of HBV-DNA with normal liver function and without significant fibrosis. Generally, treatment is not recommended in these two phases, but guidelines advocate treating older than 30-40 years HBeAg-positive patients[9-11]. Chronic hepatitis, defined by the elevation of transaminases and high viral load, induces liver fibrosis progression to cirrhosis and risk of developing HCC, being antiviral treatment recommended in these phases (Figure 1).

Antiviral treatments: Interferon and nucleos(t)ide analogues

In 1976, William Robinson and Thomas Merigan reported that interferon alpha suppressed HBV replication and cured some patients suffering from CHB[22]. However, further clinical studies showed that only a minority of the patients achieved the clearance of HBsAg while the majority showed a viral breakthrough under treatment or a relapse after the end of therapy (EoT). Conventional interferon alpha was approved in 1992 and one year later Wong et al^[23] demonstrated an HBeAg loss rate of 33%. Efficacy in HBeAg-negative patients was very limited and only 15% to 25% showed a sustained biochemical response after 12 mo of treatment^[24]. In 2005, pegylated interferon (Peg-IFN) replaced the standard form due to its improved pharmacokinetics and prolonged half-life. In HBeAg-positive patients, 12 mo of therapy achieved the sustained response (HBeAg loss with HBV-DNA < 2000 IU/mL, 6 mo after therapy) in 20% to 30% and the HBsAg loss in 3% to 7% [25]. The Peg-IFN therapy has important disadvantages, its high variability of response and its unfavorable safety profile with a significant number of patients ineligible or unwilling.

In 1995, Benhamou et al^[26] reported that HIV-co-infected patients with HBV who received the HIV drug named lamivudine (LAM) lost their HBV-DNA and improved their hepatitis (Figure 2). The same year Jules Dienstag showed the efficacy and safety of LAM in HBV-mono-infected patients[27]. LAM was well tolerated, but resistance soon developed in cases with high replication. After 5 years of therapy, 75% of the LAM-treated patients developed resistant HBV variants. The acyclic nucleotide analog adefovir (ADV) was approved in 2002 for LAM-resistant CHB patients. Unfortunately, its activity was relatively low and was rapidly replaced by the newer drug named tenofovir (TDF). This very similar drug was approved in 2001 for HIV and in 2008 for HBV[28]. The guanosine analog entecavir (ETV) was originally developed against the herpes simplex virus. In 1997, ETV showed its strong activity against HBV in the HepG2.2.15 cell line. In 2001, showed its efficacy in LAM-resistant CHB patients, and in 2005, it was approved over LAM[29]. In 2006, a synthetic thymidine nucleoside analogue named telbivudine (LdT) was approved but its activity was relatively low and was rapidly replaced. In 2015, the prodrug of TDF, tenofovir alafenamide (TAF), was approved for older patients with lower eGFR or lower bone mineral density[30,31].

Safety of long-term treatments

The safety profile of NA is very good even in HBV-infected patients with decompensated liver disease,





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Figure 1 Phases of hepatitis B virus infection. The persistence of hepatitis B virus surface antigen longer than six months is considered a marker for chronic hepatitis B infection. The HB e antigen (HBeAg)-positive chronic infection occurs in younger patients with high viral load and normal liver function tests. In contrast, HBeAg-negative chronic infection is characterized by low levels of hepatitis B virus-DNA with normal liver function and without fibrosis. Generally, treatment is not recommended in these two phases. Chronic hepatitis is defined by the presence of transaminases and high viral load that can induce liver fibrosis and risk of developing hepatocellular carcinoma, being antiviral treatment recommended[9-11]. HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; ALT: Alanine aminotransferase.



Figure 2 Hepatitis B virus antiviral treatment history. The figure shows the United States Food and Drug Administration approval dates for hepatitis B virus drugs. IFN: Interferon; Peg-IFN: Pegylated interferon; LAM: Lamivudine; ADV: Adefovir; TDF: Tenofovir; ETV: Entecavir; LdT: Telbivudine; TAF: Tenofovir alafenamide.

> liver transplants, extrahepatic manifestations, acute hepatitis, or severe exacerbation[32,33]. However, NA inhibits the HBV polymerase activity only during the time of administration. Long-term therapy with NA can produce a decline in bone mineral density and renal tubular dysfunction[34].

> TDF and ETV have renal metabolism and must be adjusted if patients have an estimated glomerular filtration rate (eGFR) of less than 50 mL/min per 1.73 m². A multicenter retrospective study including 6189 CHB patients treated with TDF (n = 2482) or ETV (n = 3707) showed that TDF was associated with a higher risk of worsening renal function (adjusted HR 1.26, 95% CI 1.11-1.43)[35]. A systematic review and meta-analysis showed that TDF and ETV can affect renal function and patients with cirrhosis under TDF had a higher risk of renal damage and hypophosphatemia[36]. The effects of TDF on bone mineral density are suspected to be related to an increased tubular phosphate metabolism, but with few clinical implications as they appear to be reversible after withdrawal[34]. Recently, it has been shown an improvement in bone density and renal function in patients previously treated with TDF after switching to TAF[37]. However, TAF is not approved in very low eGFR (below 15 mL/min per 1.73 m²).

> Even if side effects of NA are scarce, CHB patients usually start treatment at young ages, and with NA treatment, they may be treated for decades. Therefore, questions on NA treatment duration and withdrawal have been gaining interest in recent years.

PRESENT

Monitoring HBV infection: Quantification of HBsAg levels

Classically, three viral markers have been used to characterize HBV infection: (1) The qualitative HBsAg; (2) the HBeAg; and (3) the HBV-DNA. However, most patients under NA treatment have



undetectable HBV-DNA and normal alanine aminotransferase (ALT). Therefore, other biomarkers are needed to monitor antiviral activity. The quantified HBsAg is one of the most extensively studied biomarkers because HBsAg negativization, spontaneously or with treatment, has been related to a good prognosis[38]. It has been shown that the decline in HBsAg levels during NA therapy is very slow (around 0.1 Log IU/mL per year)[39-41] suggesting that it would take decades, even long life, to achieve the HBsAg loss [42,43]. Importantly, some patients have shown a faster and larger HBsAg decline during NA therapy achieving low HBsAg levels[41,43]. Importantly, low HBsAg levels after NA withdrawal (below 1000 in Caucasian and below 100 in Asian patients) can be predictors of HBsAg seroclearance[44-47].

Efficacy of long-term antiviral therapy

The NA can be classified as low (LAM, ADV, and LdT) or high (ETV, TDF, and TAF) barrier of resistance. Current international guidelines recommend the use of NA with a high barrier of resistance to prevent the progression of liver disease, decompensation of cirrhosis, the need for liver transplantation, the development of HCC and to improve survival[9-11].

The suppression of HBV-DNA to undetectable levels (virological response) is normally associated with a normalization of transaminases (biochemical response) and an improvement in survival. In the presence of cirrhosis, all patients with detectable HBV-DNA should be treated[9-11]. Indications for HBeAg-positive and HBeAg-negative CHB patients' treatment are generally based on the combination of three main criteria: Liver disease severity, HBV-DNA units, and ALT levels[9-11].

In HBeAg-positive CHB patients, long-term NA therapy can induce HBeAg loss and seroconversion, leading to a low replicative phase with partial immune control [9-11]. In these patients, antiviral treatment with ETV has demonstrated a 5 years cumulative probability with a virological response of 99%, HBeAg loss of 53%, but HBsAg loss of only 1.4% [48]. Similarly, TDF for 10 years has demonstrated an HBeAg loss of 52% but only a 4.9% of HBsAg loss[33].

In HBeAg-negative patients, the 5-year cumulative probability with ETV of virological response was 96%, although HBsAg loss was only achieved in 4.6% [32]. In the TDF registry study with patients treated for 10 years, 100% achieved a virological response, and 83% biochemical response, but only 3.4% succeeded in the HBsAg loss[33].

Similar rates of virological response have been described for TAF in HBeAg-positive[30] and HBeAgnegative patients[31].

This high efficacy in controlling viral replication causes histological and elastographic improvement. The long-term treatment with NA has shown a significant regression of liver fibrosis and even cirrhosis. A study with paired liver biopsies, after a median time of 6 years, in 57 patients receiving ETV[49] showed histological improvement (decrease \geq 2 points in the Knodell necroinflammatory score) in 96% of patients, and fibrosis improvement (decrease ≥ 1 point in the Ishak fibrosis score) in 88% of them. In a trial with TDF[50], including 348 patients with paired liver biopsies (at baseline and week 240), 87% showed histological improvement and 51% fibrosis regression (decrease ≥ 1 point in the Ishak fibrosis score). Among the 96 patients with cirrhosis at baseline, 74% achieved some degree of fibrosis regression.

A decrease in liver stiffness measurement (LSM) after long-term treatment with NA has been described[51]. After treatment initiation, the LSM decline is faster mainly related to the improvement in necroinflammatory activity. After 6 mo of therapy, the following LSM decrease is slower and could be associated with a true improvement of fibrosis[52,53].

The most important outcome of antiviral therapy is to improve survival. Studies comparing untreated and ETV-treated patients demonstrated that antiviral therapy reduced the incidence of liver-related complications, HCC, and mortality [54,55]. Similarly, a multicenter study in TDF-treated patients with cirrhosis showed a reduced risk of developing, clinical decompensation, HCC or liver transplantation, and death compared to untreated patients[56]. However, patients treated with NA, are still in danger of developing HCC, and high-risk patients stratified by risk scores such as REACH-B or PAGE-B, should continue HCC surveillance^[57].

In recent years there has been a debate about the effect of the two main NA on HCC risk. Some data suggest that TDF is associated with significantly lower HCC risk compared to ETV in Asian patients [58]. A Chinese study including 29350 patients, showed a reduced HCC incidence in patients receiving TDF. However, there were differences in HCC risk factors between groups: TDF-treated patients were younger, and more frequently HBeAg-positive, females, without cirrhosis, and without diabetes. Thus, only 1% of patients with cirrhosis had received TDF[59]. Similarly, a recent meta-analysis of individual data including 42939 patients receiving TDF (n = 6979) or ETV (n = 35960) monotherapy, showed a lower risk of HCC with TDF, especially in patients older \geq than 50, males, HBeAg-positive, and nondiabetic subgroups [60]. In contrast, other studies have failed to demonstrate this association [61,62]. A large study including 1935 Caucasian patients from the PAGE-B cohort did not find differences in 5-year cumulative HCC incidence between ETV (5.4%) or TDF (6.0%) after a median follow-up of 7.5 years [62].

Benefits of functional cure

The "functional" cure defined as the HBsAg loss, with or without seroconversion of antibody against HBsAg (anti-HBs), is considered the optimal goal for antiviral treatments[10,38]. However, even if third-



generation NA are drugs with a high barrier to HBV resistance, and high efficacy, achieving in most patients a virological and biochemical response, the HBsAg seroclearance is anecdotic[63,64]. Table 1 summarizes the efficacy of available treatments to achieve the HBsAg loss.

In patients without advanced fibrosis, the HBsAg loss is associated with an improvement in survival, and minimal risk of developing cirrhosis, decompensation, or HCC[38]. A recent study of 1972 patients with HBsAg loss, showed an HCC annual incidence of 0.38 per 100 person-years (median follow-up of 5.6 years). The study did not show differences between patients who achieved the functional cure spontaneously or with NA[65]. A systematic review showed lower HCC incidence in patients with functional cure (1.86%) compared to those who did not achieve it (6.56%) (P < 0.001). Cirrhosis, age \geq 50, and male gender were the major risks for developing HCC after HBsAg seroclearance[66]. However, the 10-year HBsAg loss rate described in a large multicenter cohort (n = 4769) treated with ETV or TDF was only 2.1% with an annual incidence of 0.22%[64].

Rules to consider before treatment withdrawal

International guidelines of the main scientific societies recommend different treatment duration with NA. In non-cirrhotic HBeAg-positive CHB patients, NA treatment can be discontinued when HBeAg seroconversion is achieved and after one year of consolidation[9-11]. However, in non-cirrhotic HBeAg-negative patients, the NA treatment duration has been a matter of debate in recent years[39-43,67].

In 2008 the APASL guideline suggested the possibility of discontinuing NA therapy in HBeAgnegative patients after at least 2 years with undetectable HBV-DNA documented on three separate occasions 6 mo apart[68]. This approach in Asian patients was mostly driven by economics and reimbursement policies. Despite this, in 2012, Hadziyannis *et al*[69] reported in 33 Caucasian patients a high rate of HBsAg seroclearance after stopping ADV (39% at 5.5 years after withdrawal). In 2017, the first randomized controlled trial, the FINITE study, confirmed that the strategy of stopping therapy increased the HBsAg loss rate compared to continuing treatment (19% *vs* 0%)[70]. Based on these studies, in 2017 EASL guideline introduced the possibility of treatment withdrawal in non-cirrhotic HBeAg-negative CHB patients after 3 years of viral suppression[10]. In the last years, several studies have evaluated this strategy with different results (Table 1). Studies in Asian patients have shown a lower HBsAg loss rate compared to Caucasians. The different distribution of HBV-genotypes (especially genotype D), the inclusion or not of patients with advanced liver disease, and the different retreatment rules could explain these dissimilar results[45,71-73].

One of the important points to be considered before stopping treatment is the selection of the best candidates. The initial stopping rules proposed were based on the duration of treatment and virological suppression[9,10]. It has been suggested that NA withdrawal can be successful, only if the transcriptional activity of cccDNA has been silenced during treatment[74]. Additionally, it has been reported that long-term suppression of HBV-DNA can improve CD8 + T cell functions and restore the capacity of immune control by decreasing NK cell killing of HBV-specific T cells and increasing serum cytokines[44, 75-77]. This immune restoration can exert control of viral replication after treatment discontinuation, and a large proportion of patients remain as inactive carriers. Moreover, the recurrence of HBV replication after NA discontinuation can represent a trigger for the immune response[78] that can induce an accelerated HBsAg decline and may be beneficial to eradicate residual cccDNA-containing hepatocytes. Our group has recently observed that HBsAg decline was faster during the first year after NA cessation compared to the on-treatment decline[46].

HBsAg and hepatitis B core-related antigen (HBcrAg) levels have been evaluated as surrogate markers to select the patients with a better outcome after NA cessation. It has been shown that not only HBsAg levels at the EoT but also on-treatment HBsAg kinetics are good predictors of HBsAg loss[79, 80]. In a study performed by our group, we showed that on-treatment HBsAg kinetics had a high accuracy to predict the HBsAg loss one year after withdrawal[46]. Patients with an HBsAg decline > 1 Log IU/mL during treatment had a probability of 50% of achieving HBsAg loss one year after discontinuation. A large multicenter multiethnic study (n = 1552), the RETRACT-B study, showed different cut-offs of HBsAg at EoT in Asian and Caucasian patients. HBsAg levels at EoT < 100 IU/mL in Asian and < 1000 IU/mL in Caucasian patients were associated with a 4-year HBsAg loss probability of 33% and 41%, respectively[47]. Regarding levels of HBcrAg, the CREATE study, including 572 patients with NA discontinuation, showed lower HBcrAg levels at EoT in those with a virological response and HBsAg loss rates one year after withdrawal (65% and 12%, respectively). The same group confirmed in 1216 patients that non-Asian ethnicity, HBsAg levels < 100 IU/mL, and undetectable HBcrAg were associated with the highest HBsAg loss rate[81].

Another important point is to define standardized re-treatment criteria. Some authors recommend delaying retreatment, to allow patients with a "beneficial" flare to achieve a functional cure[82]. However, other authors suggested that re-treatment should be initiated in response to significant increases in serum HBV-DNA, regardless of other liver parameters as cases of fulminant hepatitis have been reported[83]. Table 1 summarizes the efficacy of NA withdrawal to achieve the HBsAg loss[84-93].

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Table 1 Efficacy of available antiviral treatments and nucleos(t)ide analogues withdrawal to achieve hepatitis B virus surface antigen loss						
Ref.	Treatment (duration)	HBeAg	Ethnicity	Follow-up	HBsAg loss (%)	
Current antiviral treatmen	ts					
Lau et al[84], 2005	Peg-IFNα (1 yr)	+	Mainly Asian	6 mo	2.9	
Marcellin et al[85], 2013	Peg-IFNα (1 yr)	-	Asian	60 mo	12.0	
Chang et al[48], 2010	ETV (5 yr)	+	Asian	-	1.4	
Ahn et al[<mark>32</mark>], 2016	ETV (5 yr)	-	Mixed	-	4.6	
Marcellin <i>et al</i> [33], 2019	TDF (10 yr)	+	Mixed	-	4.9	
Marcellin <i>et al</i> [33], 2019	TDF (10 yr)	-	Mixed	-	3.4	
Chan <i>et al</i> [30], 2016	TAF (3 yr)	+	Mixed	-	4.0	
Buti <i>et al</i> [<mark>31</mark>], 2016	TAF (3 yr)	-	Mixed	-	3.0	
Combined treatments						
Marcellin <i>et al</i> [86], 2016	Peg-IFN + TDF (1 yr)	+/-	Mixed	18 mo	9.1	
Bourlière <i>et al</i> [87], 2017	ETV, TDF, ADV, and LAM (1 yr)	-	Caucasian	12 mo	7.8	
Lim et al[88], 2022	NA add-on Peg-IFN (1 yr)	+/-	Asian	6 mo	12.1	
Mo et al[<mark>89</mark>], 2022	NAs (HBsAg < 1500 IU/mL, 1 yr)	-	Asian	48 mo	33.2	
Lim et al[88], 2022	NA switch to Peg-IFN (1 yr)	+/-	Asian	6 mo	9.7	
NA discontinuation						
Chen <i>et al</i> [90], 2018	2.7 yr	+	Asian	96 mo	19.6	
Song <i>et al</i> [91], 2021	2.9 yr	+	Asian	73 mo	9.5	
Kuo et al <mark>[92]</mark> , 2019	3.1 yr	+	Asian	24 mo	4.7 (ETV); 0 (TDF)	
Jeng et al[72], 2018	2.9 yr	-	Asian	36 mo	13	
Chen et al[90], 2018	2.9 yr	-	Asian	96 mo	33.1	
Kuo et al <mark>[92]</mark> , 2019	3.1 yr	-	Asian	36 mo	10 (ETV); 15 (TDF)	
Chen et al[93], 2020	3.2 yr	-	Asian	58 mo	20.8	
Song <i>et al</i> [91], 2021	2.7 yr	-	Asian	73 mo	14.6	
Hirode <i>et al</i> [47], 2022	3.0 yr	-	Mixed	17 mo	15.0	

Only studies with more than 100 patients have been included. HBsAg: Hepatitis B virus surface antigen; HBeAg: Hepatitis B e antigen; Peg-IFN: Pegylated interferon; LAM: Lamivudine; ADV: Adefovir; TDF: Tenofovir; ETV: Entecavir; TAF: Tenofovir alafenamide.

Risks of treatment withdrawal

The safety of treatment withdrawal is under discussion because on one hand hepatitis flare after NA cessation can induce hepatic decompensation but on the other hand early initiation of NA retreatment can inhibit the beneficial effect of flare-associated immune activation [47,72,94,95]. A study from Taiwan including 691 patients (44.6% with cirrhosis) showed a clinical relapse in 60.6% (7 developed a hepatic decompensation and 3 died)[72]. Similarly, the RETRACT-B study showed hepatic decompensation in only 1.22% (4.3% in patients with cirrhosis and 0.8% in those without cirrhosis; P < 0.01 [47]. In this study, 7 patients died (0.45%) by hepatic decompensation and 4 (0.25%) due to hepatitis B-associated flare.

Another important issue is the risk of developing HCC. The study of Jeng et al[72] included patients with (n = 308) and without cirrhosis (n = 383). The HCC incidence at 1, and 3 years after treatment discontinuation (0.15% and 1.00% for non-cirrhotic vs 1.30% and 4.00% for patients with cirrhosis) was similar to those during NA therapy (0.08% and 0.30% for non-cirrhotic vs 1.50% and 3.40% for patients with cirrhosis). Similarly, in the RETRACT-B study, the HCC incidence at 48 mo after NA withdrawal was 2.2% in patients with cirrhosis and 0.7% in those without cirrhosis[47]. Therefore, NA withdrawal does not seem to increase HCC rates. However, the tolerable level of circulating HBV DNA during longterm follow-up post-NA withdrawal has not been yet elucidated.

The evidence of increased HBsAg loss rates after NA cessation in some HBeAg-negative CHB patients must be balanced with safety. NA withdrawal should be performed only in centers with expertise in CHB management and in adherent patients willing stricter control after carefully discussing the pros and cons[74,96,97].

FUTURE

The need of expanding antiviral treatment criteria

Some recent studies have evaluated, with contradictory results, the use of NA in patients who do not meet strict criteria for treatment. On one hand, two retrospective studies in HBeAg-positive HBV-infected patients from Korea showed a lower risk of HCC and cirrhosis in those treated compared to untreated controls, suggesting a benefit of starting NA treatment at earlier phases[98,99]. Similarly, another Asian study including "minimally active" carriers showed a high risk of HCC (HR 9.9; 95%CI, 1.239-76.923; P = 0.031) when compared to inactive carriers[100]. But, the study included HBeAg-positive patients and those with a LSM > 9 kPa[101].

On the other hand, some European studies have not demonstrated significant benefits. A study from Italy, including patients without treatment with normal ALT and DNA < 20000 IU/mL, showed a benign course during a follow-up longer than 4 years[102]. Another study including Caucasian patients with HBeAg-negative chronic infection showed a high rate of HBsAg loss (15%) and low transition to CHB (6.3%) in patients with normal ALT and DNA < 2000 IU/mL or in the "grey zone" (HBV-DNA < 2000 IU/mL and ALT 40-80 U/L or, HBV-DNA 2000-20000 IU/mL and ALT < 40 U/L or ALT 40-80 U/L) after a median follow-up of 8.2 years[103]. However, a recent study from the United States has shown that patients without treatment indications, according to AASLD guidelines, had twice the risk of developing cirrhosis and/or HCC when compared to treated patients[104].

Simplifying indications for treatment initiation could be cost-effective and associated with survival improvement[105]. Therefore, it is plausible that antiviral treatment criteria will be expanded in the coming years to achieve the Global Hepatitis Elimination Goals, especially when new therapies with higher HBsAg loss rates will be available[106].

New HBV biomarkers to monitor antiviral therapy

The persistence of the cccDNA in the hepatocytes perpetuates HBV infection. So, detection and measurement of cccDNA levels would be the most precise tool to recognize HBV eradication. However, cccDNA can only be measured in liver biopsy samples by non-standardized or semi-automated techniques[107]. In recent years, there has been an effort to identify new biomarkers that could serve as cccDNA transcriptional activity surrogate markers.

The hepatitis B virus core-related antigen (HBcrAg) is an interesting biomarker composed of the hepatitis B core antigen (HBcAg), the HBeAg, and the 22-KDa pre-core protein (p22cr)[108]. These protein components are translated from cccDNA transcripts reflecting the cccDNA transcriptional activity. The HBcrAg is detected by automated chemiluminescence techniques using specific monoclonal antibodies with a limited sensitivity of 3.0 Log U/mL. The HBcrAg kinetics has been analyzed during NA therapy in a cohort of 222 CHB patients (132 HBeAg-negative and 90 HBeAg-positive) receiving ETV[109]. The decline of HBcrAg levels was 0.244 Log U/mL per year. HBcrAg levels were lower in HBeAg-negative patients compared to HBeAg-positive, but no differences were found between TDF or ETV[109,110]. On the other hand, the persistence of detectable HBcrAg at EoT has been related to severe aminotransferase flares after NA treatment cessation[111]. The main limitations of HBcrAg are its low sensitivity and its low levels after a long-treatment period in HBeAg-negative patients. Therefore, a great proportion of treated HBeAg-negative patients can show undetectable HBcrAg[112]. Recently, a new ultrasensitive assay has been described to improve the HBcrAg sensitivity, but further studies are needed to consider it in clinical practice[113].

The HBV-RNA is a newer marker under evaluation. The pgRNA is packaged in nucleocapsids and serves as the template for reverse transcription to HBV-DNA. In patients not receiving antiviral therapy, serum HBV-RNA levels are much lower than serum HBV-DNA levels. It can be detected in serum samples as pgRNA and spliced pgRNA variants produced by transcription of cccDNA as a marker of intrahepatic cccDNA transcriptional activity[114]. However, serum HBV-RNA is usually quantified by in-house assays and there are no commercial tests available for routine clinical practice. During NA therapy, reverse transcription of pgRNA to HBV-DNA is blocked, but cccDNA transcription to pgRNA persists, and HBV-RNA may be detected in the serum of patients with undetectable HBV-DNA. Therefore, the HBV-RNA could be useful to monitor NA-treated patients[115,116]. Recently, it has been shown that serum HBV-RNA reduced after treatment initiation from 1.46 Logs at week 48 to 1.77 Logs at week 96[117]. On one hand, serum pgRNA levels at wk 4 after NA initiation were correlated with low HBsAg levels ($\leq 100 \text{ IU/mL}$) and seroclearance during follow-up[118]. On the other hand, high levels of pgRNA have been associated with viral relapse after NA withdrawal[119].

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The recognition of HBsAg composition could be an interesting tool for following CHB patients because it changes across the stages of HBV infection. The main HBsAg component is S-HBs. The L-HBs and M-HBs proportion is lower in the inactive carrier stage compared to those in HBeAg-negative or HBeAg-positive CHB patients[120,121]. Interestingly, L-HBs and M-HBs proportions decrease prior to total HBsAg loss in patients receiving antiviral treatment[122].

Nowadays, the quantitative HBsAg is the most used and widely available biomarker as a consequence of its standardized and commercial tests. It includes the HBsAg produced not only from cccDNA but also by the integrated DNA that is the main source of the HBsAg in HBeAg-negative CHB patients, being more representative of the transcriptional activity. Moreover, quantitative HBsAg has shown a better predictive capacity compared to HBrcAg and HBV-RNA[123].

However, despite the potential utility of these new biomarkers for monitoring NA treatment, their usefulness should still be established for monitoring the new treatment strategies.

New drugs in development

After several years without major advances in novel therapies for CHB, currently, a multitude of new molecules are emerging, individually or in combination, as future treatment strategies. The last part of this review summarizes the main groups of evolving molecules evaluated in clinical trials (Table 2) and the combinations that are showing higher rates of sustained HBsAg loss (Table 3).

Several drugs are being evaluated in phase II clinical trials. These new molecules can be classified into two main groups according to their mechanism of action: (1) Molecules targeting different steps in the HBV life cycle; and (2) those acting as immunomodulators.

Molecules targeting HBV life cycle

Molecules targeting different steps in the HBV life cycle are summarized according to their treatment class, mechanism of action, and type in Table 2[124-143].

HBV entry inhibitors

HBV entry inhibitors protect naïve hepatocytes from infection. However, they do not directly target cccDNA. The recent identification of liver-specific bile acid transporter, the NTCP, has allowed exploring new therapeutics to block viral entry and reduce viral spread[144]. Bulevirtide consists of the preS1 domain of the large surface protein, which inhibits NTCP and prevents viral entry. In the phase 2 study, Bulevirtide monotherapy was evaluated in patients with CHB co-infected with the hepatitis D virus (HDV)[124]. HBV-DNA declined by > 1 Log in 32% of patients, although HBsAg was not affected. Its low effect on HBsAg levels made most studies focus on long-term treatment for HBV/HDV coinfected patients.

Capsid assembly modulators

The primary mechanism of action of nucleocapsid assembly modulators (CAMs) is to drive the nucleocapsid misassemble. These molecules interfere with capsid formation and disrupt the encapsidation of pgRNA. They have been classified into two main families (Class I and Class II). These agents have been shown to decrease serum HBV-DNA and RNA levels, although declines in HBsAg levels are negligible. Moreover, it has been shown HBV-RNA and HBcrAg rebounded after discontinuation of treatment [126]. Due to these limitations, to date, the role of CAMs in future treatments for CHB must be defined.

Post-transcriptional control inhibitors (small interfering RNA and antisense oligonucleotides)

RNA molecules, both pgRNA, and mRNAs encoding viral proteins, are essential for HBV replication. Small interfering RNA (SiRNA) is a specific post-transcriptional gene silencing mechanism that can inhibit the translation of viral proteins needed for cccDNA formation. SiRNA is 21-23 nucleotide duplexes processed from large double-stranded RNAs[145]. Chemically synthesized short RNA duplexes were shown to be capable of gene silencing *in vitro* without inducing an interferon response [146]. Different anti-HBV siRNA are being tested in clinical trials. Available clinical data are promising and show that siRNAs are safe and well-tolerated [127]. A study of siRNA therapy that targeted the common 3' ends of all HBV transcripts showed that while the treatment markedly reduced HBsAg levels in HBeAg-positive patients, it had minimal effects in HBeAg-negative patients[128].

Antisense nucleotides (ASOs) are single-stranded DNAs (8-10 nucleotides) that target HBV mRNA sequences, forming a DNA-RNA hybrid that is rapidly degraded by cytoplasmic ribonuclease-H[147]. Early results of ASOs, alone or in combination, have shown a profound HBsAg decline[130]. The B-Clear Study, a phase 2b clinical trial evaluating bepirovirsen 300 mg subcutaneously once weekly for 24 wk, showed undetectable HBsAg in 9% of patients who received bepirovirsen plus NA and in 10% who received bepirovirsen alone[131].

HBsAg release inhibitors: Nucleic acid polymers

HBsAg plays a major role in HBV infection and is the most abundant HBV antigen. Therefore, blocking its release could potentially prevent the release of new virions, decline the number of subviral particles and restore the HBV-specific immune response[148]. Nucleic acid polymers (NAPs) are amphipathic



Table 2 Mechanisms of action of the new drugs under development						
Treatment class	Mechanism of action	Туреѕ				
Drugs targeting HBV life cycle						
Entry inhibitors	Blockage of liver-specific bile acid transporter (NTCP)	Inhibitors of NTCP[124]; NMAb[125]				
Capsid assembly modulators	Interfere with capsid formation and disrupt the encapsidation of pgRNA	CAMs[126]				
Post-transcriptional control inhibitors	Post-transcriptional gene silencing by inhibition of the translation of viral proteins	SiRNA[127-129]; ASOs[130,131]				
HBsAg release inhibitors	Intracellular degradation of HBsAg via proteasomal and lysosomal degradation	NAPs[132,133]				
Immunomodulators						
Innate immune activator	Stimulation of innate immunity through TLRs and RIG-I	TLRs agonist[134-137]; RIG-I agonists[138, 139]				
Adaptive immune activator	Blocking the PD-1/PD-L1 pathway to reverse T-cell exhaustion; stimulation of host's immune response to generate CD4 and CD8 HBV-specific T cells	Checkpoint inhibitors[140,141]; therapeutic vaccines[142,143]				

HBV: Hepatitis B virus; NTCP: Sodium taurocholate co-transporting polypeptide; NMAb: Neutralizing monoclonal antibodies; CAMs: Nucleocapsid assembly modulators; SiRNA: Small interfering RNA; ASO: Antisense oligonucleotides; NAP: Nucleic acid polymers; TLRs: Toll-like receptors; RIG-I: Retinoic acid-inducible gene-1; PD-1: Programmed death receptor 1; PD-L1: Programmed cell death ligand 1; HBsAg: Hepatitis B virus surface antigen; HBeAg: Hepatitis B e antigen.

> phosphorothioate oligonucleotides. The antiviral effect of NAPs against HBV infection is the inhibition of HBsAg release, although the exact mechanism is unknown, resulting in rapid clearance of HBsAg [132]. In clinical studies, NAP monotherapy was accompanied by rapid HBsAg decline and seroconversion. Recently, NAP-based combination therapy with TDF and Peg-IFN achieved a functional cure in 35% of patients[133] Interestingly, in HBeAg-negative patients, HBsAg produced by integrated DNA was lost. However, more than 90% of patients experienced immune-mediated transaminase flares during therapy[132].

Immunomodulators

Immunomodulator molecules are summarized according to their treatment class, mechanism of action, and type in Table 2.

Immunomodulatory targets including therapeutic vaccines

The impaired immune response is one of the main barriers to eliminating HBV. T cell immune exhaustion due to the presence of high amounts of HBsAg and a defective response of the innate immune response has a role in HBV persistence. Thus, treatment strategies to restore HBV-specific immune responses with immunomodulatory therapies have been evaluated to achieve viral clearance.

Pegylated interferon: A "second life" for an old friend

Interferon was the first drug approved for CHB treatment. Despite the long use of this treatment, the exact mechanism of action is still unclear. It has been suggested that Peg-IFN has two main effects, acting on the transcriptional activity of cccDNA and also an immunomodulatory effect, enhancing both innate and adaptive immunity[149,150]. Due to its hypothetical immunomodulatory effects, the synergistic effects of combination with NA and Peg-IFN have been evaluated.

A recent meta-analysis found that NA and Peg-IFN combination improved HBsAg seroclearance rates (RR: 4.52, 95%CI: 1.95-10.47 for adding-on and RR: 12.15, 95%CI 3.99-37.01 for switching-to) compared to NA monotherapy. One of the strongest predictors of HBsAg seroclearance was the HBsAg level at the start of IFN therapy[151]. Our group also showed that the addition of Peg-IFN to NA produced a faster and larger decrease in HBsAg levels compared to NA monotherapy, especially in those patients with IL28CC polymorphism[112]. A better Peg-IFN response in patients with IL28CC polymorphism has been described especially in those infected with genotype D[152,153]. More recently, a Chinese real-world study (n = 3988), the Everest Project (NCT04035837), has reported that the addition of Peg-IFN alpha 2b to NA in HBeAg-negative patients achieved a 4-year HBsAg loss rate of 33% [89].

The main concerns on the use of Peg-IFN are side effects, poor tolerability, and major contraindication such as decompensated cirrhosis. These explain the preference to use NA in routine clinical practice. However, in selected patients with good tolerance, the adding-on or switching-to strategy may improve the HBsAg seroclearance. Indeed, some of the phase 2 trials evaluating new antivirals, have included concomitant treatment with Peg-IFN and have shown better outcomes.



Table 3 Efficacy of drug combinations to achieve sustained hepatitis B virus surface antigen loss

Drug class	Drug	Patients	Time therapy (wk)	Efficacy	Safety
NA +/- CAM	NA vs NA + JNJ-6379 (bersacapavir)	232	24-48	HBsAg decline 0.25 log IU/mL vs 0.41 log IU/mL	No major AE
SiRNA +/- NA	AB-729 vs NA + AB-729	43	8	HBsAg decline 2.03 log IU/mL monotherapy vs 2.16 log IU/mL combination	Injection site reactions; ALT flares
ASO + NA	ASO-GSK3228836 (bepirovirsen) ± NA	457	12-24	HBsAg < LoQ in 28%-29% and HBsAg loss in 9%-10% after 24 wk of EoT	Injection site reactions; few cases of grade 3-4 ALT flares
Inhibitor of NTCP + Peg-IFN	Bulevirtide + Peg-IFN in HDV-HBV co-infection	90	48	HBsAg loss 26.7% in one arm vs 0% in the other	Related to Peg-IFN; injection site reactions
NA + TLR agonists	NA + TLR7 agonist (vesatolimod, GS-9620)	162	24	No changes in HBsAg	Some grade 3 AE with higher doses (few treatment discontinuations)
NA + TLR agonists	NA + TLR8 agonist (selgantolimod)	48	24	HBsAg loss 5% at week 48	Mild and transient gastrointestinal AE
NA + checkpoint inhibitors	NA + PD-1 inhibitor (nivolumab)	12	1 dose (24 follow-up)	HBsAg reduction 0.48 log IU/mL (HBsAg loss in 5%)	No major AE
NA + checkpoint inhibitors	NA + PD-L1 inhibitor (ASC22, Menvafolimab)	48	24	HBsAg decline 0.38 log IU/mL (HBsAg loss in 19%)	Grade 1 and 2 ALT flares
NA + SiRNA +/- CAM	NA + JNJ-3989 (siRNA) + NA ± JNJ-6379 (CAM)	117	48	HBsAg decline 2.1 log IU/mL in double <i>vs</i> 1.8 log IU/mL in triple combination	No major AE
NAP + NA + Peg- IFN	REP2139 or REP 2165 + NA + Peg-IFN	40	48	HBsAg loss in 35% and HBsAg < 100 IU/mL in 75%	Related to Peg-IFN
SiRNA + NA +/- Peg-IFN	VIR 2218 (siRNA) + NA +/- Peg-IFN	80	24	HBsAg decline 2.03 log IU/mL in dual arm vs 2.55 log IU/mL in triple arm (HBsAg < 100 IU/mL in 95% and HBsAg < 10 IU/mL in 55%)	Related to Peg-IFN

CAM: Nucleocapsid assembly modulator; HBV: Hepatitis B virus; SiRNA: Small interfering RNA; NTCP: Sodium taurocholate co-transporting polypeptide; ASO: Antisense oligonucleotides; NAP: Nucleic acid polymers; TLRs: Toll-like receptors; RIG-I: Retinoic acid-inducible gene-1; PD-1: Programmed death receptor 1; PD-L1: Programmed cell death ligand 1; HBsAg: Hepatitis B virus surface antigen; HBeAg: Hepatitis B e antigen; ALT: Alanine aminotransferase; LoQ: Limit of quantification; EoT: End of therapy; AE: Adverse events.

Toll-like receptor and retinoic acid-inducible gene-1 agonists

HBV can be recognized by receptors such as toll-like receptors (TLRs)[154] and retinoic acid-inducible gene-1 (RIG-1)[155] TLRs are receptors that initiated intracellular pathways and induce antiviral molecules such as interferons, and cytokines. It has been reported that the activation of TLR-mediated pathways results in the suppression of HBV replication. An oral TLR 7 (TLR7) agonist, GS9620, has shown a decrease in serum HBV-DNA and HBsAg in chimpanzees[134]. However, a phase IIb trial in humans, showed a minimal decrease in HBsAg levels, despite the increase in cytokines and NK cell activity [135,136]. A more recent trial has shown that the combination of selgantolimod, a TLR8 agonist, with a NA resulted in a minimal reduction of HBsAg levels[137]. RIG-I constitutes an intracytoplasmic receptor that interacts with RNA viruses. Once activated, it leads to signal transduction through protein kinase complexes and activation of transcription factors that migrate to the nucleus and activate interferon-stimulable genes leading to the production of IFN[138]. Inarigivir activates the RIG-I pathway. Although early studies reported a reduction in HBV-DNA levels, there were cases of severe hepatotoxicity, with multiple cases of acute liver failure and one death, that lead to its discontinuation [139].

Checkpoint inhibitors

The immune system of HBV-infected patients shows high levels of programmed death receptor 1 (PD-1)-expressing CD8 T cells and high levels of programmed cell death ligand 1 (PD-L1) expression. This suggests that PD-L1 blockade could be a suitable strategy to treat chronic HBV infection. Furthermore, anti-PD-L1 blockade increases the number of IFN-y-producing T-cells and the amount of IFN-y produced per cell[156]. Checkpoint inhibitors against PD-1 and PD-L1 have been shown to be effective in several malignancies by reversing T-cell exhaustion and restoring immunity. However, the efficacy to achieve HBsAg clearance has been disappointing. In the CheckMate 040 trial, nivolumab increased

HBV-DNA 1 Log from baseline in 10% of HBsAg-positive patients [140]. PD-1 blockade showed limited antiviral activity, and no patient exhibited HBsAg seroconversion[141].

Monoclonal antibodies

HBV entry can be interrupted by small compounds such as neutralizing monoclonal antibodies (NMAb). In a phase 2 study, it has been evaluated the combination of NMAb VIR-3434 with small interfering RNA (SiRNA) VIR-2218 for up to 20 wk. Combination treatment was generally well tolerated with no serious adverse events. The majority of patients achieved low levels of HBsAg (< 10 IU/mL) but any patient lost the HBsAg during the study [125].

Therapeutic vaccines

These immunogenic molecules are developed to stimulate the host immune response generating CD4 and CD8 HBV-specific T cells to suppress viral replication and HBsAg loss. Early studies in humans showed a small decline in HBsAg[142]. However, several new therapeutic vaccines are in development probably to be used in combination with other drugs such as SiRNA[143].

Other immunomodulatory approaches

In recent years, a better understanding of the immune response to HBV has emerged with new therapeutic possibilities. T cell receptor (TCR)- redirected T cell and chimeric antigen receptor (CAR) T cell involve genetic engineering to confer specificity in front of HBV. CAR-T cells are transduced with an antibody-like receptor to recognize HBsAg on the surface of infected hepatocytes whereas TCRredirected T cells respond to HBV peptides[157]. However, the investigation in this field is still under development and is being tested in Phase I studies evaluating HBV-infected patients with HCC[158, 159].

Combinations of drugs in development

None of the new previously described molecules can achieve significant sustained HBsAg loss if used alone. Therefore, the most advisable strategy seems to be the combination of molecules with different mechanisms of action[160]. Table 3 summarizes the efficacy of the evolving combinations to achieve sustained HBsAg loss.

CONCLUSION

Long-term treatment for CHB has been used for decades, showing a good safety profile and high efficacy in controlling viral replication, improving histology, and decreasing the incidence of HCC, clinical decompensation, and mortality. However, the persistence of cccDNA and the low probability of HBsAg seroclearance with NA therapy made necessary indefinite treatment in a vast majority of patients. With the new insights into the role of the immune system in HBV infection persistence and the knowledge of the different viral cycle phases, there has been an increase in new therapeutic approaches in recent years. Moreover, several clinical trials evaluating new drugs with different mechanisms of action are ongoing with promising results. It is expected that in the coming years, there will be a paradigm shift in the treatment of CHB, as we have already seen in chronic hepatitis C, and functional cure in an important number of patients seems closer than ever. Meanwhile, an effort to improve diagnostic rates, and to assure access to treatment for all patients who need it must be urgently done.

FOOTNOTES

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REVIEW

Impact of gut microbiome in the development and treatment of pancreatic cancer: Newer insights

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Abstract

The gut microbiome plays an important role in the variation of pharmacologic response. This aspect is especially important in the era of precision medicine, where understanding how and to what extent the gut microbiome interacts with drugs and their actions will be key to individualizing therapy. The impact of the composition of the gut microbiome on the efficacy of newer cancer therapies such as immune checkpoint inhibitors and chimeric antigen receptor T-cell treatment has become an active area of research. Pancreatic adenocarcinoma (PAC) has a poor prognosis even in those with potentially resectable disease, and treatment options are very limited. Newer studies have concluded that there is a synergistic effect for immunotherapy in combination with cytotoxic drugs, in the treatment of PAC. A variety of commensal microbiota can affect the efficacy of conventional chemotherapy and immunotherapy by modulating the tumor microenvironment in the treatment of PAC. This review will provide newer insights on the impact that alterations made in the gut microbial system have in the development and treatment of PAC.

Key Words: Pancreatic cancer; Gut microbiome; Chemotherapy; Dysbiosis; Intratumoral microbiome; Gut flora

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Core Tip: Pancreatic cancer (PC) remains of on the most dismal in terms of prognosis. Treatment options are limited and even after complete surgical resection, the prognosis remains poor. The gut microbiome has been incriminated in the past for the development of certain cancers. Our review found that observation to be true as well for PC. Furthermore, we also found that it plays a role in efficacy and tolerance of certain regimens used to treat PC.

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INTRODUCTION

Pancreatic cancer (PC) usually refers to ductal pancreatic adenocarcinoma (PAC) (including its subtypes), which represents 85 to 90 percent of all pancreatic neoplasms. PC ranks fourth among cancer related mortality in the United States, only behind lung, colorectal, and prostate cancers in males, and lung, breast, and colorectal cancers in females. Although the incidence of PC has been relatively stable over time, the increasing use of imaging techniques such as endoscopic ultrasound and helical (spiral) abdominal computed tomography (CT) scans has revealed an increasing number of incidentally found cases of PC[1,2].

PC can run in some families, and approximately 10 percent of individuals with PC have a family history of the disease[3,4]. There are two broad categories of hereditary risk for PC which are inherited genetic predisposition syndromes associated with PC and familial PC (FPC), which is defined as a family with a pair of affected first-degree relatives who do not meet criteria for a known PC-associated genetic predisposition syndrome^[5].

The major gene causing most cases of hereditary PC remains unknown. Pathogenic germline variants (PGVs) in the breast cancer associated (BRCA) 1 and 2 genes are the most commonly associated mutations, occurring in 13 to 19 percent of FPC families[6]. Next generation sequencing helped uncover other genes causing hereditary pancreatic ductal adenocarcinoma: The partner and localizer of BRCA2 (PALB2) gene and the ataxia-telangiectasia mutated (ATM) gene [7,8]. PGVs are especially common in individuals with early onset PC (i.e., developing before age 50)[6]. Cigarette smoking contributes to the risk of PC in patients with hereditary pancreatitis and FPC and is associated with an earlier PC diagnosis by approximately 20 years[9].

In recent years, the role of gut microbiome in the development and treatment of several cancers, including PC, has been an area of active research. Porphyromonas gingivalis (P. gingivalis), Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans) and even Helicobacter Pylori (H. Pylori) are linked to an increased risk of PC[10,11]. Probiotics have been shown to be effective in reducing pancreaticoduodenectomy complications, by directly suppressing the growth of cancer cells. Postbiotics have been shown to have selective cytotoxicity against tumor cells. Prior literature revealed that fecal microbiota transplantation led to a reduction in tumor size for PC[12].

Carcinoembryonic antigen-related cell adhesion molecule 7 (CEACAM-7), also known as CGM2, is a glycoprotein expressed on the luminal surface of epithelial cells near the mouth of colonic crypts and on pancreatic ductal epithelial cells[13]. Most recently it has been shown that CEACAM7-directed chimeric antigen receptor (CAR) T cells can effectively mediate remission of late-stage patient-derived PAC xenograft tumors[13].

This review will provide a concise and up to date overview of the impact of commensal gut microbiota in the development and management of PAC. Furthermore, we will focus on the pathophysiology and pathogenesis by which the gut flora can gain oncogenetic attributes and to what extent their alteration can affect the treatment and outcome of PAC.

DIAGNOSIS OF PC

Recent advances in imaging techniques have elevated the diagnostic acumen for PC. Abdominal ultrasound (US) is a non-invasive approach, which can detect pancreatic masses with an accuracy of 50%-70% [14]. Although there are no tell-tale characteristic signs of different pancreatic masses, a hypoechoic mass, pancreatic and/or biliary duct dilation could point towards an ominous pathology [14, 15]. If a contrast enhanced US is available, the diagnostic accuracy could be significantly enhanced as hypovascularity of a mass point towards PAC whereas endocrine cell tumor is hyper vascularized and any pancreatitis associated mass is usually iso-vascularized [14,16].



CT with contrast is perhaps the most widely used-in detection and staging of PC. Hypovascularity, increased fibrous stroma, and decreased enhancement compared to surrounding tissue points towards PAC[17]. In hypoattenuating lesions and in instances where CT is equivocal, multi-detector row CT can be helpful as it provides three dimensional images and various phases of contrast enhancementparenchymal, portal venous, and arterial-leading to earlier detection and accurate staging of the cancer [18-20]. Enhanced magnetic resonance imaging (MRI), due to better soft tissue visualization, has been shown to be equal or superior to CT imaging for blood vessel invasion and local extent, however, it is poor in detecting the involvement of portal venous system or duodenum [21-23]. The most accurate and sensitive method for detection of even the smallest tumors with or without vascular invasion is EUSsuperior to MRI, CT, or US. It is also an excellent modality for diagnosis when combined with biopsy and has incredibly high sensitivity to detect metastasis to the lymph nodes as well as vascular invasion [24-27]. The biggest challenge in diagnosis is differentiating between chronic pancreatitis and PAC, this is when EUS with biopsy comes in handy.

STAGING OF PC

The staging of PC at the time of diagnosis is pivotal for prognosis and treatment planning as the aggressive or palliative care approach could be applied based on the stage. The role of CT imaging with contrast is pivotal in determining the stage, however, sometimes, sophisticated modalities such as enhanced MRI, EUS, or fluorodeoxyglucose-positron emission tomography could be needed. The tumor size, location in the pancreas, surrounding structures involvement-with or without vascular involvement, and spread to surrounding lymph nodes or metastasis are the components involved in staging for PC.

The T (tumor), N (Node), and M (Metastasis) is the widely accepted staging systems for PC as per The American Joint Committee for Cancer^[28]. The T stage is classified based on the tumor size within the pancreas and/or involvement of vascular structures. The N and M stage is classified based on the involvement of regional lymph nodes and sites of metastasis, if any. Subsequently, based on the imaging, cancer is characterized as resectable, borderline resectable, locally advanced, or metastatic disease. Stages I and II do not involve any major blood vessels, stage III is a localized tumor but with involvement of a major blood vessel, whereas Stage IV is metastatic disease^[28].

The National Comprehensive Cancer Network stages PC primarily based on tumor extent. This is primarily in the absence of metastatic disease and resection options are localized advanced/ unresectable, borderline resectable, and resectable disease. Locally advanced/unresectable disease is predominantly when the tumor involves major vascular structures such as aorta, superior mesenteric or portal vein (unreconstructable), or > 180 degrees of tumor contact with the Superior mesenteric artery or celiac artery. Resectable disease or borderline resectable is defined as no involvement of any vascular structures mentioned above or ≤ 180 degrees of involvement[29].

PC-MODALITIES OF TREATMENT

Depending on the staging of PC, there are various modalities which are employed for the treatment. Surgical resection is always desirable, however, due to the relatively silent clinical course of PC, only $1/5^{\text{th}}$ of patients have resectable tumors at the time of diagnosis[30,31]. The most utilized surgical procedures are total pancreatectomy, distal pancreatectomy, and Whipple's procedure depending on the staging of the cancer^[32]. Previously, in patients presenting with jaundice, preoperative biliary stenting was considered if there was a tumor on the head of the pancreas causing biliary obstruction, however, recent studies have shown that this modality is associated with increased time to surgery, increased rates of infection, and complications; preoperative biliary stenting is as a result, no longer recommended for head of the pancreas tumors which have not metastasized and can be easily resected [33,34]. However, preoperative stenting can be considered in patients who are undergoing neoadjuvant chemotherapy, if surgery is postponed by logistical constraints, or have severe jaundice [33,34].

In patients who present with PC s of 'borderline resectability', neoadjuvant therapy prior to surgical resection is a consideration. However, data is conflicting. While there is some evidence on increased survival amongst borderline resectable tumors with neoadjuvant gemcitabine-based chemotherapy, there are also studies which suggest an increased postoperative stay and increased surgical challenges in locally resectable tumor patients who received neoadjuvant chemotherapy [35-37]. Also, it is important to note that histological diagnosis is mandatory prior to starting the chemotherapy, which may further delay the time to surgery.

The overall prognosis of PC is abysmal, even post complete surgical resection. As a result, 5-Fluorouracil (with Leucovorin) or Gemcitabine adjuvant chemotherapy is frequently employed postsurgical resection. Which agent is better though, does remain a topic of discussion. Studies are equivocal with some showing no difference between the two whereas others favor gemcitabine[38]. In patients with metastatic disease, the armamentarium consists of psychosocial support, chemotherapy, treating a



variety of other comorbid conditions, and targeted therapy. As far as chemotherapy is concerned in such a setting, Gemcitabine has been shown to be superior, by far and remains the first line standard of care[39]. Arguably, Conroy et al[40] have proven that FOLFIRINOX can super side Gemcitabine, as patients on FOLFIRINOX demonstrated not only a better response rate, but also improved one year, progression free, and overall survival^[40]. In patients who are non-tolerant to first line gemcitabine, second line treatment consisting of oxaliplatin with fluoropyrimidines have demonstrated some clinical benefit[41,42]. Furthermore, if FOLFIRINOX was used as the first line, gemcitabine-based therapy should be tried as second line and has some clinical evidence of being beneficial [40].

Newer modalities of treatment include but are not limited to the use of epidermal growth factor receptor (EGFR) inhibitors. Medications like Cetuximab and Erlotinib which target the EGFR have been developed recently for targeted therapy and have been shown to be effective in many clinical trials. A combination of gemcitabine with Erlotinib is shown to increase overall survival rates and decrease the progression of PC[43]. PC cells are notorious to adapt in order to decrease the drug delivery to them by production of desmoplastic stroma and lead to resistance to chemotherapeutic agents[44]. Several therapies have recently been developed to decrease this stromal tissue and improve drug penetration despite the desmoplastic stroma, including nab-paclitaxel[45].

Radiation therapy has a somewhat beneficial role alongside surgery and chemotherapy. Neoadjuvant radiation therapy for PC has been described in prior literature. Pisters et al[46] demonstrated that minimal toxicity and a very small recurrence rate can be obtained with preoperative fractionation chemoradiation based on 5-Fluorouracil, Whipple's procedure, and intraoperative radiation[46]. In another study, utilizing a similar strategy for treatment but replacing 5-Fluorouracil with paclitaxelbased chemotherapy, the results were similar, however, the toxicity levels were higher [47].

To improve the patient's overall prognosis, radiation therapy is frequently being utilized for management of PC alongside chemotherapy. In the United States, adjuvant radiation therapy is a common norm after the Gastrointestinal Tumor Study Group's prospective study in 1985. Patients with resectable PC were enrolled in this trial and were found to have a significantly longer and medial survival rate when treated with adjuvant chemoradiation[48]. Owing to this trial, adjuvant chemoradiation, the most commonly used adjuvant treatment for patients with resectable PC, is being practiced to date in the United States.

Novel techniques like stereotactic body radiotherapy have also been developed in recent years for targeted delivery of radiation. However, it has only been shown to slow local progression of the disease but has no effect on overall survival rates as the majority of mortality in PC patients is secondary to systemic and distant metastasis^[49-51]. As PC is genetically a heterogeneous malignancy, there have also been baby steps in personalized chemotherapeutic regimen based on the patient's genome to significantly increase the rates of chemotherapeutic efficacy by decreasing the resistance and making the response to chemotherapy consistent across all individuals. However, further research is needed on this novel therapeutic approach.

GUT MICROBIOTA AND PC

Mechanisms via which microbes regulate pancreatic oncogenesis

The gut microbiome, which refers to microbes naturally present in the human mucosal surfaces, has shown, when altered, to lead to oncogenesis and to some extent affect the response to therapy of several cancers, among which PAC[52]. The exact mechanisms by which oral and intestinal microbiota reach the pancreas remains unknown, but the proposed mechanisms involve the translocation via biliary/ pancreatic ducts or through the blood circulation[52]. A summary can be found in Table 1.

P. gingivalis, which is a bacterium mainly found in the mouth and associated with periodontal diseases, has shown the ability to disseminate and affect immune response. P. gingivalis infection has shown an involvement of toll-like receptors (TLRs) including TLR4, involved in protective immunity. TLR signaling, especially TLR4, has been shown to play an important role in human pancreatic tumors [53]. Furthermore, periodontal diseases, such as the ones caused by *P. gingivalis* can lead to an increased production of nitrosamines[54]. Nitrosamines can be metabolized by Cytochrome P450 and produce electrophiles that can effectively interact with the DNA and lead to the formation of DNA adducts that have a carcinogenic potential if not repaired [55]. Porphyromonas Peptidyl Arginine Deaminase (PPAD) is a protein produced by *P. gingivalis* that has been associated with cancer development by the way of P53 activity and KRAS (Kirsten-ras) mutation[52]. P53, which is a tumor suppressor gene, if mutated can lead impairment of cell cycle arrest and decrease of apoptosis increasing the risk of malignancy. KRAS, which is an oncogene with hydrolyzing effect on guanosine triphosphate, can lead uncontrolled and inappropriate cell proliferation, thus increasing the risk of malignancy [52]. P53 is a transcription factor that can activate the transcription of numerous genes, including the Cyclin-dependent kinase (CDK) inhibitor p21.

P53 is rapidly degraded and therefore not detectable within the cell. Mutation of the P53 gene results in a protein that fails to bind DNA effectively. Therefore, expression of the CDK inhibitor P21 gene is decreased, and P21 protein production is decreased. P21 protein is not available to stop the entry of the



Table 1 Gut microbiota associated with Pancreatic oncogenesis						
Bacteria	Primary site	Potential mechanism	Subsequent effect	Ref.		
Porphyromonas gingivalis	Mouth	TLR signaling disruption; Nitrosamines production; PPAD production	Loss of protective immunity; DNA adducts formation; P53 overactivity (loss of apoptosis); KRAS mutation (cellular overproliferation)	[52- 55]		
Aggregatibacter actinomycetemcomitans	Mouth	Nitrosamine production; DNA double-strand breaks; CagE production	DNA adducts formation; genome instabilities; dysregulation of DNA methylation expression	[52, 58,59]		
Fusobacterium nucleatum	Mouth	FadA production; Fap2 production	activation of the Wnt/β-catenin pathway; suppression of the cytotoxic effects of NK cells and lymphocytes	[59, 61-63]		
Helicobacter Pylori	Stomach	cagA, cag PAI and vacA production	Disruption of host intracellular signaling pathways	[<mark>64</mark>]		
Bacteroides fragilis, Bacteroides vulgatus, Listeria monocytogenes, Clostridium, Lactobacillus, Bifidobacterium	Large bowel	Transformation of primary bile acids to secondary bile acids	Reduction of susceptibility to apoptosis, induction of inflammatory mediators, and perturbation of membranes and cellular movement	[76]		
Escherichia coli	Large bowel	Polyamines production	Polyamines upregulation	[81- 83]		

TLR: Toll-like receptor: PPAD: Porphyromonas Peptidyl Arginine Deaminase: CagE: Cytotoxin-associated gene E: NK: Natural killer: PAI: Pathogenicity island; cagA: Cytotoxin-associated gene A.

> cell into S phase, again resulting in unregulated cell cycle progression, potentially leading to carcinogenesis[56]. The KRAS gene, an oncogene, is one of the most frequently mutated genes in PC. This gene is the human homolog of a transforming gene isolated from the Kirsten rat sarcoma virus, hence the name KRAS. Mutations in this gene, the vast majority of which are at codon 12, are activating, leading to abnormal activation of the protein product of the gene^[57].

> A. actinomycetemcomitans is also an oral microbiome that has been incriminated in PAC^[52]. Similar to P. gingivalis, it can lead to periodontal infections and lead to increased nitrosamine production[54]. A. actinomycetemcomitan can also induce DNA double-strand breaks in host cells, independently of apoptosis, and cause the risk of genome instabilities and subsequently increase the risk of carcinogenesis[58]. Furthermore, the bacteria can produce the cytotoxin-associated gene E (CagE). CagE may have helicase activity, and its role in regulating DNA methylation expression is considered as possible mechanisms of tumorigenesis. CagE gene has been widely expressed in various cancer cell lines and cancer tissues including PC[59]. Fusobacterium nucleatum (F. nucleatum), which is another oral microbiome produces Fusobacterium adhesin A (FadA), that showed capacity of binding to host cells and is also the most characteristic virulence factor of F. nucleatum[59]. The host receptors for FadA are members of the cadherin family, mainly E-cadherin and vascular endothelial (VE) cadherin (CDH5) [60]. FadA binds to E-cadherin of epithelial cells, leading to phosphorylation and internalization of Ecadherin on the membrane; afterwards, canonical Wnt pathway is activated, accompanied by decreased phosphorylation of β -catenin, which accumulates in the cytoplasm and translocate to the nucleus[59]. Increase in Wnt signaling activity and subsequent activation of the Wnt/ β -catenin pathway, has shown to be essential in the initiation of PC[61,62]. Furthermore, FadA binds VE-cadherin on VE cells, increasing endothelial penetrability[59]. Therefore, FadA not only directly invades host cells but also allows dissemination of itself and other bacteria into blood by increasing endothelial permeability[59]. F. nucleatum can produce a protein called familial adenomatous polyposis 2, which binds and interacts to human inhibitory receptor T cell immunoreceptor on natural killer (NK) cells and lymphocytes. Thus, suppressing the cytotoxic effects of NK cells and lymphocytes, leading to protection of tumors from the immune system and fostering a flourishing inflammatory context^[63]. By a mechanism similar to P. gingivalis, F. nucleatum can be involved with the TLRs and lead to carcinogenesis as discussed previously [53].

> H. Pylori is notorious for its association with gastric cancer and yields various virulence factors that may disrupt host intracellular signaling pathways and lower the threshold for neoplastic transformation. Of all virulence factors, cytotoxin-associated gene A and its pathogenicity island (cag PAI) and vacuolating cytotoxin A are the major pathogenic factors[64]. Whether H. Pylori infection is associated with PAC remains controversial with conflicting data in the literature. A study by Kumar et al[65] showed a very low incidence of *H. Pylori* among patients with PAC, whereas studies by Hirabayashi et *al*[66], and Nilsson *et al*[67] found an association.

> Several members of the gut microbial community (especially of the large bowel), including Bacteroides fragilis, Bacteroides vulgatus, Listeria monocytogenes, Clostridium, Lactobacillus, Bifidobacterium, and Escherichia, are involved in the transformation of primary bile acids to secondary bile acids, either by



deconjugation, oxidation, dehydroxylation, or epimerization[68-70]. Bile acids have multiple nuclear receptors, including farnesoid-X-receptor (FXR), liver-X receptor, CAR, vitamin D receptor (VDR), pregnane X receptor (PXR), and a non-nuclear receptor Takeda G Protein-Coupled Receptor 5/Gprotein-coupled bile acid receptor (TGR5), that may impact carcinogenesis [71,72]. Secondary bile acids can behave as both pro- and anti-carcinogens, depending on the cancer concerned and the concentration of the bile acid present [71-73]. Furthermore, bile acids can modulate the composition of the microbiome and facilitate bacterial translocation into tissues, which is a key step in the carcinogenesis of PAC[74]. Bile acid levels have been shown to be elevated in PAC^[75]. Bile acids can also affect risk factors for PAC such as pancreatitis and bile acid efflux disorders, type II diabetes, obesity, and hyperlipidemia; and they can reduce susceptibility to apoptosis, induce inflammatory mediators, and may perturb membranes and cellular movement^[76]. A secondary bile acid, deoxycholic acid can bind to TGR5 and activate EGFR, mitogen-activated protein kinase, and signal transducer and activator of transcription 3 signaling in PAC cells, inducing cell cycle progression[77]. Other bile acid receptors such as VDR, FXR and PXR are also found to be highly expressed in PAC tissues compared to normal tissues [78-80].

Polyamines can be produced, accumulated, or used by the following gut bacteria Escherichia coli (E. coli), Enterococcus faecalis (E. faecalis), Staphylococcus aureus, Haemophilus influenzae, Neisseria flava, Pseudomonas aeruginosa, Campylobacter jejuni, Yersinia pestis, Vibrio cholerae, Bacteroides dorei, Bacteroides thetaiotaomicron, Bacteroides fragilis, Bacillus subtilis, and Proteus mirabilis[81,82]. A mouse study revealed that bacterial polyamine biosynthetic capacity was upregulated and aggravated by tumor progression in PAC and there was a correlated elevated serum level of polyamines[83].

As evidence by the work of Riquelme et al [84], Fecal Microbiotal Transplant from human subjects to mice, yielded from PC long term murine survivors, showed a significant reduction in tumor growth, however, that effect was lost with the use of antibiotics altering the fecal microbiota[84,85]. Furthermore, it was found that long term survivor mice that did not receive antibiotics were rich in CD8+ T-cell, enhancing the tumor immune cell infiltration. On the other hand, mice that were treated with antibiotics, thus altering the fecal microbiota, showed an increased number of CD4+FOXP3+ T-regs and myeloid derived suppressor cells which are well known to lower the immune system, thus promoting tumor growth[84,85].

NK cells are a group of cells that play an important role by mediating tumor initiation and progression. NK cells are often found in the circulation, preventing tumor cells from metastasizing[86, 87]. When a patient is NK cells depleted, tumor escape and growth may ensue[86]. NK cells having the ability to inhibit CD8+ T cell responses during chronic infections, it has been hypothesized that NK cells can facilitate solid tumors infiltration, among which PC[86].

Hepatotropic viruses such as hepatitis B virus (HBV) and hepatitis C virus (HCV) have been incriminated in pancreatic oncogenesis. HBV and HCV have the ability to delay host immune system clearance of the virus by integrating the DNA, modifying tissue viscoelasticity, and modulating the PI3K/AKT signaling pathway, which promotes metabolism, proliferation, cell survival, growth and angiogenesis in response to extracellular signals, via the HBV X protein, thus leading to oncogenesis[87].

It has been shown that fungal microbiota including Candida, Saccharomyces, Aspergillus or Malassezia spp. are involved in pancreatic oncogenesis. One proposed mechanism is that ligation of mannosebinding lectin, which binds to glycans of the fungal wall may lead to activation of the complement cascade and oncogenic progression[88].

Short-chain fatty acids (SCFA) which are metabolites from the gut microbiota and cathelicidin-related antimicrobial peptides secreted by normal pancreatic β -cells protect against tissue inflammation and control pancreatic bacterial overgrowth [89,90]. It has been shown that patients with PC have an abundance of a higher abundance of lipopolysaccharide-producing bacteria, and a reduction in beneficial microbes, such as butyrate-producing bacteria[91]. Butyrate, which is a SCFA produced by certain bacteria of gut possesses anti-inflammatory and anti-neoplastic properties in regard to PC by the means of "pro-differentiation, anti-proliferation, anti-invasion, pro-apoptosis" and chemo-sensitization effects[91]. Another SCFA from the gastrointestinal (GI) microbiota, acetate, induces insulin secretion via the microbiome-brain β -cell axis controlling pancreatic bacterial overgrowth [92,93]. Tryptophan metabolism can serve as an immunomodulatory factor by overexpression of indoleamine2,3dioxygenase1 which inhibits the maturation of CD11c and dendritic cells, and T-cell proliferation and by high expression of Kyn which leads to induction and activation of the aryl hydrocarbon receptor, leading to upregulation of programmed cell death protein 1 expression; enhancing the efficacy of antitumor adoptive T-cell therapy and reducing the rate of migration and invasion in both tumorbearing mice and patients with PC[94-96].

IMPACT OF GUT MICROBIOME ALTERATION ON THE TREATMENT OF PC

PAC is only resectable in approximately 15% to 20% of cases at the time of diagnosis, however, surgical resection offers the only chance of cure. PAC carries a dismal prognosis even after surgical resection with negative margins given its high rate of recurrence. Therefore, systemic chemotherapy, radiation therapy, and combined approaches (chemoradiotherapy) have been used both prior to and following



surgical resection in an effort to improve cure rates[97]. More recently, immunotherapy and CAR T-cell therapy have gained favor for use in the treatment of PAC[13]. The gut microbiome has been shown to interact with those treatment modalities and affect their efficacy[13,42]. Furthermore, the gut microbiome has also shown some cytotoxic effect in PAC[42].

E. coli and Staphylococcus aureus strains have the potential to produce Cytolysin A (ClyA), which is a pore-forming cytotoxin that possesses anticancer properties [98]. ClyA exerts its cytotoxicity, by creating multimeric pores and imposing cell death in the eukaryotic membrane by the caspase-dependent pathway[99]. E. coli, A. actinomycetemcomitans, Campylobacter and Helicobacter are known to produce Cytolethal distending toxin (CDT)[100]. CDT is known to have genotoxic attributes by DNase activity which creates DNA double stranded breaks, leading to cell cycle arrest and cytotoxicity^[49]. Streptococcus pyogenes secretes streptolysin O which is implicated in cytolysis and apoptosis[101].

Prebiotics are defined as nutrients that are degraded by gut microbiota and may affect not only the intestinal microenvironment but also distant organs. In a mice study by Trivieri et al[102] using xenograft mice model confronted with PC gene expression dataset (GSE16515) and investigating the impact of high levels of prebiotic resistant starch diet (RSD) on miRNA expression profiles in tumor tissues, RSD was associated with dysregulation of 19 miRNAs genes expression in comparison to control. subsequent analysis revealed that part of genes participating in the regulation of processes such as the development of carcinoma, inflammatory response, abdominal cancer, metabolic disease, growth, invasion, and metastasis were downregulated in a group of mice fed with RSD in comparison to control. Furthermore, genes participating in the synthesis of carbohydrates, glucose metabolism disorder, and cell death of cancer cell lines were significantly upregulated in mice fed with RSD. Thus, the authors concluded that there is prolonged overall survival and beneficial value of RSD in PAC[103].

Lactobacillus casei is a probiotic that can produce Ferrichrome, which has the potential to suppress the growth of refractory PC cells by inhibiting cancer cells progression and dysregulating cell cycle by activating P53[102,103]. Next-generation probiotics such as Akkermansia muciniphila (A. muciniphila), are identified using next-generation sequencing and bioinformatics tools. A. muciniphila has been shown to inhibit the proliferative activity of INS-1 (rat pancreatic islet cell tumor cells) in a mouse model[104].

FOLFIRINOX, which is a commonly used regimen in PAC is composed of leucovorin calcium (folinic acid), fluorouracil, irinotecan hydrochloride, and oxaliplatin. Oxaliplatin has an immunomodulatory effect as well, potentiating tumoricidal T-cell immunity. In a mice model, a group with a defective TLR signaling pathway, demonstrated no response to oxaliplatin treatment[105]. Agonistic TLR molecules from microbial membranes were reported to help stimulate the immune system and increase reactive oxygen species production, thus enhancing the tumoricidal activity of oxaliplatin[105]. Irinotecan is characterized by common GI side effects limiting the dose and effectiveness of treatment. Those side effects can be modulated by enzymatic activity of the gut microbiome, with some bacteria improving the side effects profile, while others may worsen the side effects. The β-glucuronidase enzyme produced by intestinal bacteria cleaves the active irinotecan metabolite SN-38G into a toxic form that damages the colonic mucosa and causes GI side effects. The literature revealed that antibiotics or modification of gut microbiomes significantly alleviated the GI toxicity in cancer patients[106]. Furthermore, reduced risk of developing irinotecan toxicity has been shown with the use of indigestible fibers, using appropriate probiotics and adequate butyrate intake[107].

Several animal studies showed that mice housed in germ-free conditions and animals treated with broad-spectrum antibiotics showed reduced effects of immunotherapy by a combination of TLR-9 antagonist and anti-interleukin-10 antibody. Furthermore, the ineffectiveness of cancer immunotherapy directed against the major negative regulator of T cell activation cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) was observed when applied to antibiotic-treated animals or germ-free mice[108]. Monoclonal antibodies that neutralize CTLA-4 have been shown to rely on the intestinal microbiota, in particular, Bacteroidales and Burkholderiales [108]. A recent study used a gut microbe-derived metabolite trimethylamine N-oxide (TMAO) that showed enhanced anti-tumor immunity to PAC. TMAO was delivered either intraperitoneally or via a dietary choline supplement to orthotopic PAC bearing mice, and lead to reduced tumor growth and associated with an immunostimulatory tumorassociated macrophage phenotype and activated effector T cell response in the tumor microenvironment. The combination of TMAO and immune checkpoint inhibitors (ICI) such as programmed cell death 1, in a mouse model of PAC, proved to be superior in reducing tumor burden and improving survival compared to either therapy alone [109].

CAR T-cell therapy has shown tremendous results in hematologic malignancies. Only recently, it was tried on non-hematologic malignancies with promising preliminary data. CAR T-cell therapy of solid tumors faces a major issue in that commonly targeted tumor antigens are expressed at low levels in normal tissues, leading to on-target off-tumor toxicity. CEACAM7, which has low to undetectable expression in all normal tissues and with strong surface expression on a subset of primary human PAC tumors was identified as a potential target antigen for CAR T-cell therapy of PAC. CAR T-cells targeting CEACAM7 were generated in a study by Raj et al [13] and showed significant antitumor activity against patient-derived PAC tumor cultures both in vitro and in vivo[13]. A brief summary can be found in Table 2

Oncolytic adenoviruses have been engineered to replicate in cancer cells and controlling tumor progression. Oncolytic adenovirus AdNuPARmE1A with miR-222 binding sites, are made to withdraw



Table 2 Impact of gut microbiota in the treatment of Pancreatic cancer						
Bacteria	Primary site	Potential mechanism	Subsequent effect	Ref.		
Escherichia coli	Large bowel	Cytolysin A production; Cytolethal distending toxin production	Imposing cell death in the eukaryotic membrane by the caspase-dependent pathway; DNA double stranded breaks, leading to cell cycle arrest and cytotoxicity	[49, 86,87]		
Staphylococcus aureus	Variable	Cytolysin A production	Imposing cell death in the eukaryotic membrane by the caspase-dependent pathway	[<mark>86</mark>]		
Streptococcus pyogenes	Variable	Streptolysin O production	Increase in apoptosis and cytolysis	[<mark>88</mark>]		
Aggregatibacter actinomycetem- comitans, Campylobacter and Helico- bacter	Mouth, stomach, large bowel	Cytolethal distending toxin production	DNA double stranded breaks, leading to cell cycle arrest and cytotoxicity	[87]		
Lactobacillus casei	Mouth and small intestine	P53 activation	Upregulation of apoptosis	[<mark>90</mark>]		
Bacteroidales, Burkholderiales	Large bowel	CTLA-4 upregulation	Enhancing activity of monoclonal antibody against CTLA-4	[95]		

CTLA-4: Cytotoxic T-lymphocyte-associated protein 4.

the miRNA from the cellular environment. AdNuPAR-E-miR222-S mediated-decrease of miR-222 expression in pancreatic cancer cells was shown to strongly improve the viral yield and enhance the adenoviral cytotoxic effects[110].

INTRATUMORAL MICROBIOME IN PANCREATIC CA

Intratumoral microbiome is derived from 3 basic mechanisms; (1) Sloughing of the mucosal barrier; (2) Adjacent normal tissues; and (3) Hematogenous spread[111]. Interestingly, Nejman et al[112] demonstrated that amongst the tumors that they studied, every tumor was associated with a completely different microbiome composition[112]. The pancreas is traditionally thought to be a 'bacteria-free' organ. However, bacterial DNA belonging to the Proteobacteria phylum was very abundantly found in pancreatic cancer[112]. Another study which confirmed increased amounts of bacterial DNA in pancreatic cancer vs normal pancreatic tissue was the study by Geller et al[113]. Bacterial ribosomal DNA was detected via FISH technique in 76% of patients with PAC vs 15% of patients with normal pancreatic tissue[88]. Similarly, by using the FISH technique, Aykut et al[88] demonstrated that Pseudomonadota, Bacillota, and Bacteroides were the most abundant bacteria found intratumorally in pancreatic cancer patients[88]. Interestingly, the fungal mycobiome in pancreatic tissue samples obtained from patients with PAC was also found to be very distinct from healthy individuals with a high prevalence of Malassezia^[88].

MICROBIOME IMBALANCE AND PANCREATIC CA

Dysbiosis or imbalance in the microbiome has been shown to impact the inflammatory cascade in a nonphysiological way and in turn, contribute to the development of cancer[114]. The known risk factors for pancreatic carcinoma are smoking, advancing age, type 2 diabetes mellitus, chronic pancreatitis, and obesity. Interestingly, many of these risk factors have been recently found to be associated with an imbalance in the microbiome, which may increase the risk of PAC[115-118]. A meta-analysis by Maisonnuevve demonstrated a positive correlation between periodontal disease and PAC[119]. This may be related to an imbalance in the oral microbiome. Oral microbiomes have been shown to be associated with carcinogenesis via inducing systemic inflammation, and the most important being Porphyromonas Gingivalis[120-122]. A case control study demonstrated that the risk of PAC was 2-fold higher in patients with a higher level of antibodies against a specific strain of *P. Gingivalis*, whereas higher levels of antibodies against commensal oral microbiome were actually protective against PAC, with an almost 50% lower risk of the cancer in patients who had these antibodies[123]. In-vivo studies have shown that P. gingivalis enhances the proliferation of pancreatic tumor cells, regardless of the concentration of TLR-4. Furthermore, the concentration and proliferation of *P. gingivalis* is greatly increased in PAC tissue secondary to hypoxia, which is very prevalent in the cancer microenvironment[124]. Furthermore, bacteria that cause periodontitis are also found to cause K-ras and p53 mutations, and those have in turn been associated with poor prognosis in patients with pancreatic cancer[125]. They also demonstrated that the number of cases of pancreatic cancer were higher in patients who had GI infections from H.



Pylori, Enterobacter, and Enterococcus species[125].

This prior literature leads us into sensibly concluding that possibly, an imbalance in the oral microbiome is associated with an increased risk of PAC, however, reverse causation is an important factor that needs to be excluded before exploring this aspect further. One study evaluated this and found that 2 oral bacteria-P. gingivalis and Aggregatibacter actinomycetemcomitans are associated with an increased risk whereas Leptotrichia genus of Fusobacterium species was associated with a reduced risk of PAC. Interestingly, even after excluding patients who developed the cancer within 2 years from the date of sample collection, the risks remained elevated [10]. This significantly reduces the likelihood of reverse causation. Another significant study going in favor of a causality between E. faecalis, and pancreatic cancer is the one by Maekawa et al[126], wherein the level of antibodies against E. faecalis capsular polysaccharide were found to be increased in the serum of pancreatic cancer patients[126]. However, larger cohort studies are needed on the subject to conclusively establish causation.

CONCLUSION

Despite advances in medicine and the discovery of newer anticancer therapies, the prognosis of pancreatic cancer remains dismal. By the way of this review, we found that a prebiotic resistant starch diet has been associated with better overall survival in PAC. We also found that periodontal diseases increase the risk of developing PAC. This is especially important as periodontal diseases should be avoided and promptly treated in patients with a family history of PAC, other risk factors for PAC, and those with known/suspected genetic mutations susceptible for the development of PAC. Furthermore, we found that the use of concomitant antibiotics can positively or negatively affect treatment of PAC. Some gut microbiomes can enhance the effect of therapy and improve tolerance to therapy as well. Thus, neutropenic diet can be avoided in select patients meeting the requirements. Newer therapeutics such as ICI and CAR T-cell therapies can play a major role in the outcome of PAC, however, most promising studies are done in animal models. We hope that in the near future, there will be more clinical trials in human subjects replicating the promising results from animal studies which will possibly offer newer ways to handle this very deadly malignancy.

FOOTNOTES

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MINIREVIEWS

Insulin resistance and adipose tissue interactions as the cornerstone of metabolic (dysfunction)-associated fatty liver disease pathogenesis

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Abstract

The relationship between metabolic derangements and fatty liver development are undeniable, since more than 75% of patients with type 2 diabetes mellitus present with fatty liver. There is also significant epidemiological association between insulin resistance (IR) and metabolic (dysfunction)-associated fatty liver disease (MAFLD). For little more than 2 years, the nomenclature of fatty liver of non-alcoholic origin has been intended to change to MAFLD by multiple groups. While a myriad of reasons for which MAFLD is thought to be of metabolic origin could be exposed, the bottom line relies on the role of IR as an initiator and perpetuator of this disease. There is a reciprocal role in MAFLD development and IR as well as serum glucose concentrations, where increased circulating glucose and insulin result in increased de novo lipogenesis by sterol regulatory elementbinding protein-1c induced lipogenic enzyme stimulation; therefore, increased endogenous production of triglycerides. The same effect is achieved through impaired suppression of adipose tissue (AT) lipolysis in insulin-resistant states, increasing fatty acid influx into the liver. The complementary reciprocal situation occurs when liver steatosis alters hepatokine secretion, modifying fatty acid metabolism as well as IR in a variety of tissues, including skeletal muscle, AT, and the liver. The aim of this review is to discuss the importance of IR and AT interactions in metabolic altered states as perhaps the most important factor in MAFLD pathogenesis.

Key Words: Metabolic (dysfunction)-associated fatty liver disease; Insulin resistance; Adipose tissue; Fatty liver; Metabolic syndrome; Adipokine

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Core Tip: In this review, we outline the main arguments that support the importance of insulin resistance (IR) in fatty liver pathogenesis, stressing its role in metabolic dysfunction. IR and other genetic and molecular mechanisms play a pivotal role not only in metabolic dysfunction-associated fatty liver disease development but also in some of its complications and comorbidities, such as chronic kidney disease.

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INTRODUCTION

The term non-alcoholic fatty liver disease (NAFLD) was initially used by Klatskin and colleagues in 1979[1], while Ludwig coined the term non-alcoholic steatohepatitis after witnessing similar clinical features in patients with liver steatosis^[2]. Even though this nomenclature has been used for almost four decades, in 2020 a group of experts suggested a change of nomenclature from NAFLD to metabolic (dysfunction)-associated fatty liver disease (MAFLD) due to the large multifactorial basis for the disease and the fact that non-alcoholic does not accurately describe the disease pathogenesis[3-5]. By contrast, metabolic dysfunction accounts for a great deal of the pathogenesis of fatty liver whenever large alcohol consumption amounts are not present. MAFLD diagnosis is based on the presence of hepatic steatosis in addition to one of the following: obesity or overweight, type 2 diabetes mellitus (T2DM), or metabolic dysregulation. Metabolic dysfunction (in this context) accounts for the following conditions: increased waist circumference, systemic hypertension, dyslipidemia, prediabetes and insulin resistance (IR) (measured through the homeostatic model assessment [HOMA] > 2.5).

The development of MAFLD is linked to dyslipidemia, obesity, and IR. These are also the main features of metabolic syndrome (MetS). MetS constitutes a cluster of metabolic abnormalities which stem from IR and chronic low grade inflammation and on the long run increase the risk for cardiovascular disease (CVD) and T2DM[6]. The criteria for diagnosing MetS have changed since they were first established by the World Health Organization in 1988[7], evolving since the knowledge of the pathogenesis and its implications expanded. The criteria include the presence of visceral adiposity, IR, atherogenic dyslipidemia, and endothelial dysfunction, among others[8].

On a very important note, the change of nomenclature to MAFLD is very well justified by the sole fact that about 90% of NAFLD patients have one or more MetS component[9]. Furthermore, Marchesini et al^[10] showed that the presence of MetS among patients with hepatic steatosis carry a significantly increased risk of developing steatohepatitis and fibrosis, with odds ratios of 3.2 and 3.5, respectively [10]. Also, MetS is a useful index for the prediction of the severity of obesity-related fatty liver[11].

One way in which we can argue in favor of IR's importance in MAFLD pathogenesis is by analyzing the causes of the established items in the metabolic risk abnormalities checklist. Another way is by refuting the counterarguments for the use of this new term. In this review, we will cover both issues.

One of the most valid points involves the fact that given MAFLD is a heterogeneous and complex disease, considering a single postulation to explain is pathogenesis is absurd. We agree that MAFLD is a multifactorial disease, and similar to diabetes, systemic hypertension and many more involve genetic and environmental mechanisms. In fact, there is progressively more data proving the influence of different environmental factors on fatty liver genesis, including air pollution and cigarette smoke. However, in MAFLD as a multifactorial disease, there is one specific overarching concept that accounts for most of the cases and pathogenesis of MAFLD, which is metabolic dysfunction.

To give an educated opinion on the subject, we must first understand what IR is, how it manifests on different endocrine organs, how it influences triglyceride (TG) metabolism in the liver, and its role in metabolic dysfunction.

IR: The basis

Insulin is the main anabolic hormone in the body, primordial for glucose homeostasis as well as other functions in tissue growth and development. Glucose homeostasis is maintained by regulating gluconeogenesis and glycogenolysis in the liver, as well as by inducing insulin-mediated glucose uptake in skeletal and cardiac muscle, as well as in adipose tissue (AT)[12]. IR refers to the impaired response of target tissues to insulin stimulation. This abnormality can be a result of altered number of receptors, or malfunction of the existing ones. In reality, there is not a single cause for IR development; instead


multiple factors together lead to this metabolic abnormality. These include the mentioned receptor abnormalities, as well as defects in the insulin signaling cascade, negative regulation of the cascade by inhibitors, or the presence of a proinflammatory internal milieu. There has even been a proposal regarding the induction of IR by fatty acids, which because of their abnormal metabolism lead to lipid accumulation in the muscle and liver. In any given case, whenever there is a decreased sensitivity of a hormonal stimulus, the result is a positive feedback loop that increases the concentration of the effectlacking hormone (in this case, insulin). Increased insulin serum concentrations have a variety of effects due to its trophic effects on various tissues. Insulin serum concentrations are the basis for determining the presence of IR in an individual, principally through the use of the HOMA.

During the past decade, the "glucocentric" view of IR has shifted to the "lipocentric" view, regarding its pathogenesis and associated mechanisms. We can appreciate how much the focus of IR effect on glucose metabolism prevailed before the year 2000 in any scientific literature, even if we don't look in depth. For instance, IR had been included within a concept denominated the "IR syndrome", which considered the presence of dyslipidemia, hypertension and impaired glucose tolerance as factors leading to increased cardiovascular risk, however, that's the farthest lipids' involvement got[13]. Chronic hyperglycemia leads to glucotoxicity, directly inducing IR and the IR degree is one of the strongest predictors for T2DM onset in populations at risk[14,15]. While all of this is a fact, the role of fatty acid metabolism had been overlooked since in 1965 Randle and colleagues first suggested increased serum free fatty acids (FFAs) as one of the primary causes for decreased glucose oxidation and IR development^[16]. The last decade has had an increased body of research supporting this fact, centering the role of lipids, along with that of glucose, in the development of fatty liver.

IR is present in a variety of metabolic disorders, such as MetS and T2DM. IR in the liver presents as increased gluconeogenesis and decreased hepatic glycogenesis, resulting in increased glucose production and release[17].

Adipose tissue IR-Which came first: The resistance or the fat?

AT has long been known to be an endocrine organ, both by releasing hormones such as leptin and adiponectin (adipokines), and by regulating proinflammatory mediator secretion and metabolic processes. AT IR refers to the impaired suppression of lipolysis in the presence of high insulin serum levels (Figure 1). One of the key hormones involved in AT-IR is adiponectin, which contributes to the development of obesity-related IR and CVD[18]. While in this case adiponectin levels are lower, other adipokines such as leptin are increased [19], the former one acting as a protective factor for hepatic steatosis development[20].

Whenever IR ensues, there is a disinhibition of lipolysis in the AT, which results in higher breakdown of stored triglycerides in AT and higher release of FFA into the blood. The circulating FFAs lead to activation of the proinflammatory nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KB) pathway in the liver, ultimately resulting in lipotoxicity. Lipotoxicity, however, is not the end result of a single pathway, but rather the combination of the role of FFAs, TGs, biliary acids (BAs), free cholesterol, ceramides, and lysophosphatidyl cholines[21].

When it comes to metabolic health, AT is the major determinant given the ability of subcutaneous AT (SAT) to store excess fat (through adipocyte de novo lipogenesis [DNL] instead of allowing it to deposit in the non-fatty tissues, otherwise known as "ectopic" deposition of fat. Whenever this ability becomes impaired, aberrant adipocyte tissue develops, where the adipocytes undergo hypertrophy along with decreased number of glucose transporter-4 (GLUT-4) receptors. Under physiologic conditions, adipocytes carry out recruitment of adipogenic precursor cells, along with adequate angiogenesis. Dysregulation of the signaling pathway between the wingless-related integration site (WNT) and the bone morphogenetic protein 4 (BMP-4) results in alterations in the recruitment, proliferation and differentiation of the precursors. BMP-4 has also been found to contribute to "browning" of white AT in mice, where brown AT (BAT) tallies to the oxidative phenotype of lipid-storing adipocytes[22]. The alteration in AT expansion means that not only do pathologic adipocytes have decreased number of GLUT-4 receptors but they also have altered blood supply, leading to hypoxia and consequently to activation of hypoxia-inducible factor 1 alpha (HIF-1α). By having a reduced number of GLUT-4 receptors, glucose influx is impaired, which in turn limits DNL. The induction of HIF-1 α factor in this pathological AT state increases systemic inflammatory conditions. Transcriptome analysis of AT macrophages in obese mice revealed rewiring of the metabolic pathways within these macrophages with increased glycolysis and oxidative phosphorylation, rendering them as inflammatory macrophages [23]. Macrophage HIF-1 α is involved in the formation of crown-like structures, which lead to maintenance of inflammatory processes and inhibition of angiogenesis in preadipocytes, leading to a vicious circle of added hypoxia and expansion of the aberrant AT[24] (Figure 2). Pathologic expansion of AT leads to systemic IR, as mentioned throughout this review.

Briefly expanding upon the importance of BAT, a 2011 paper proved how cold-induced browning of AT in rats controlled TG rich lipoprotein metabolism by boosting their turnover and channeling lipids into AT browning[25]. There has been a number of studies proving that the presence of BAT in adulthood is independently associated with lower probability of developing liver steatosis[26,27], for which multiple mechanisms have been uncovered. For instance, the uncoupling protein 1 (UCP-1) expressed specifically in BAT reverses obesity and also antagonizes liver inflammation and pathology





Figure 1 Effect of insulin resistance on adipose tissue lipolysis. Under normal circumstances, insulin inhibits lipolysis in adipose tissue by inducing the hormone sensitive lipase, thus decreasing the release of excessive free fatty acids (FFAs) into the serum. However, in an insulin resistance state, inhibition of lipolysis is blocked, increasing serum FFAs, which eventually increase the influx of lipids into the liver. All the figures were created using BioRender. AT: Adipose tissue; IR: Insulin resistance.



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Figure 2 Adipose tissue dysfunction in metabolic dysfunction-associated fatty liver disease. Adipocyte precursor cells undergo initial proliferation through the wingless-related integration site (WNT) signaling pathway and thereafter commitment to the adipogenic lineage by bone morphogenetic protein 4 (BMP-4) stimulus, until its conversion to preadipocytes and later on to mature adipocytes. White adipose tissue can undergo beiging or browning under the influence of two main stimuli: BMP-4 and irisin. Browning of adipose tissue (AT) implies higher catabolic and oxidation rates, In the case of WNT, BMP-4, and WNT-1 inducible signaling pathway protein 2 pathway dysregulation, there is hypertrophy of AT. Physiologically, hyperplasia through the proliferation process mentioned is the appropriate mechanism for AT expansion. Pathologically, however, hypertrophy of AT leads to decreased levels of intracellular glucose transporter 4 and limited angiogenesis. Limited angiogenesis stimulates the hypoxia inducible factor 1 alpha (HIF-1a), stimulating further AT hypertrophy, creating a vicious cycle in the expansion of pathological AT. WAT: White adipose tissue; WISP-2: WNT-1 inducible signaling pathway protein 2; GLUT-4: Glucose transporter 4.

> [28]. Interestingly, uric acid transporters have been seen to influence fatty liver[19]; a study carried out by Tanaka et al^[29] found that the use of dotinurad (urate reabsorption inhibitor) showed amelioration of IR in rats by reducing liver steatosis and promoting rebrowning of AT[29].

> Novel discovery of a group of lipids known as the fatty acid esters of hydroxy fatty acids (FAHFAs) released from the AT when appropriate levels of GLUT-4 and adipogenesis are present shed light to another pathway through which AT, and specially SAT, regulates systemic IR and inflammation[30]. FAHFAs induce metabolic health by stimulating insulin-dependent glucose transport in various tissues, as well as glucagon-like peptide 1 and insulin release from the gut enteroendocrine cells and the

pancreatic β-cells, respectively. Important anti-inflammatory effects have also been shown by studying docosahexaenoic acid (DHA)-derived FAHFAs' effects in cultured human hepatoma-derived cells, finding potent activation of nuclear factor erythroid 2-related factor 2 with tenable antioxidant function [31].

The answer to the question "what came first: the resistance or the fat?" could go both ways, i.e. liver fat build-up could be attributed to some degree to IR and hyperinsulinemia or directly from excessive FFA availability, which consequently brings about IR. Amount of AT, hepatic steatosis, and low-grade subacute inflammation are all correlated with the development of IR and MetS[32]. For this reason, the interaction between fat and IR is not causal, but rather reciprocal.

Skeletal muscle IR

Skeletal and cardiac muscle play important roles in glucose metabolism. Studies in humans have shown that it is the principal insulin-stimulated glucose uptake site (about 75% of postprandial serum glucose), whereas AT presents with relatively lower uptake[33]. Similar to hepatic IR, one of the ways skeletal muscle IR develops is by increased FFA supply, which cannot be processed by the tissue.

The association between skeletal muscle dysfunction and the progression of MAFLD has been widely recognized. There are several muscular conditions that are directly related to fatty liver, such as myosteatosis and sarcopenic obesity. We briefly touched upon the fact that AT releases adipokines key to metabolic regulatory systems. However, skeletal muscle also has the capacity to release hormonally active molecules termed myokines, which exert their function in an autocrine, paracrine, or endocrine fashion[34]. There are hundreds of myokines and the specific function of most has not been fully elucidated; however, many have shown effects in a multisystemic manner, including cognition, bone composition, AT "browning," as well as lipid and glucose metabolism. Myostatin, myonectin, irisin and a series of interleukins are among the most important myokines. Myostatin has a negative effect on MAFLD progression, given that it enhances liver inflammation and fibrogenesis by hepatic stellate cell stimulation[35]. Irisin, on the other hand, has the opposite effect by stimulating white AT browning and UCP-1 expression reducing adipose IR. This is key, given that one of the hallmarks of treatment for MAFLD is physical exercise. Irisin, among others, is an exercise-inducible myokine; this represents one of the few pathways through which moderate or rigorous exercise can reduce progression in MAFLD by targeting IR in the liver and AT (Figure 2). An additional feature of irisin is FFA oxidation, which is a method for lipid removal from ectopic tissue; this will later be explained in the intrahepatic triglyceride content section. Lastly, myonectin plays a role in FFA oxidation in the AT and the liver, as well as thermogenesis. Therefore, we already see how different myokines can have both beneficial or detrimental effects on metabolic health depending on the collective organ characteristics. A specific phenotype based on lifestyle characteristics defines the myokines that will be released, e.g., irisin, and therefore the IR in other tissues that affects the development of liver steatosis.

Lean MAFLD

The absence of overt metabolic dysfunction in the lean population with MAFLD does not exclude the presence of metabolic abnormalities at cellular level. An important measurement of our hypothesis would be measuring the presence or absence of overt IR (e.g., as per international criteria, mainly the HOMA) in a follow-up period of the population.

IR causes fatty liver from the inside out and not the other way around. In other words, cellular level alterations can, on their own, cause increased TG accumulation in the liver, even if obesity, acanthosis nigricans or even HOMA are not present or are within out of normal range in the latter case.

Individuals with normal body mass index (BMI) also develop MAFLD, and many studies (mainly in non-Caucasian populations) have shown a lack of IR in patients with MAFLD. A study conducted by Ahmed *et al*^[27] studied the presence of NAFLD in patients with different BMI (non-obese, overweight, and obese) and evaluated whether these NAFLD individuals presented with IR. The results showed that a significant number of individuals without IR had NAFLD; however, there was no analysis of whether the NAFLD individuals without IR had non-obese BMI. This small aspect could be quite significant, given that we claim that the two main contributors to MAFLD development are IR and AT dysfunction. Furthermore, regarding lean MAFLD, it is known that IR unrelated to obesity can occur in various hyperglycemic states.

It is important to establish that IR drives DNL in the liver. Fatty liver diagnosis is defined based on the total amount of intrahepatic TG (IHTG). A recent study showed that hepatic DNL is an important regulator of IHTG content, concluded after correcting for the potential confounding contribution of AT in DNL. It was also noted that increases in serum glucose and insulin stimulate hepatic DNL. Glucose and insulin promote DNL by inducing the carbohydrate response element binding protein, as well as the sterol regulatory element binding protein 1c (SREBP 1c) and the acetyl CoA carboxylase, respectively[36]. Increased serum insulin is a compensatory mechanism during IR when there is appropriate endocrine pancreas activity, by having increased insulin release, DNL is stimulated further.

Thus, one of the most important arguments in favor of IR as the base of pathogenesis is the fact that it directly stimulates DNL. The question here would be: can DNL alone be contribution enough to increase IHTG up to MAFLD levels? The answer is no, even though DNL contributes about 26% to total IHTG content, while most of it originates from increased influx of FFA and their esterification in the



liver, accounting for 59% of total lipid content[37]. A smaller percentage, 15%, is attributable to diet TG consumption, as shown by Donnelly *et al*[37].

Mechanisms of IHTG accumulation

Increased lipid content in the liver originates from an imbalance between FFA uptake by the liver, DNL, lipid oxidation, and hepatic very low density lipoprotein (VLDL) export rate[38]. TG synthesis and lysis are the main ways in which the liver regulates the storage of FFA in serum when levels are high, whereas in the case of energy expenditure, it releases VLDL particles containing FFA to the muscle and fat tissue[39]. There was a previous misconception on the role of hepatic TG storage as a cause of lipotoxicity; it is now known that TG storage and secretion of VLDL particles are protective mechanisms against FFA-induced lipotoxicity. An elegant study by Listenberg et al[40] proved that through unsaturated fatty acid supplementation in CHO an 25RA cells, a protective effect against lipotoxicity through TG synthesis induction was achieved[40].

TG storage in the liver is carried out through the conversion of FFAs into glycerol-3-phosphate. TGs, along with cholesterol esters are neutral lipid particles which can be stably stored in the liver or can be released as VLDL particles[41]. There are a number of mechanisms involved in FFA oxidation and lipid metabolism. As we briefly touched upon, irisin is one of hundreds of exercise-induced myokines secreted by skeletal muscle, which plays an important role in the AT-muscle-liver axis. It also regulates the adenosine monophosphate-activated protein kinase signaling pathway, thus increasing FFA oxidation in myocytes[42].

Another mechanism that helps keep the balance between the IHTG is fatty acid β -oxidation in the mitochondria or peroxisomes. This process leads to the production of adenosine triphosphate or in the case of excess FFAs, the production of ketone bodies [43]. Alterations in β -oxidation contribute to the development of hepatic steatosis. For instance, downregulation in peroxisome proliferator-activated receptor alpha (PPAR- α), which serves as an FFA sensor, leads to decreased fatty acid catabolism and intrahepatic lipid accumulation. These alterations also determine the severity to which steatosis will develop depending on the nutritional status of an individual^[44]. Stimulation of PPAR- α in mice enhances the expression of cytochrome P450 4A and enhances lipid turnover in the liver, decreasing the risk of developing dietary steatohepatitis[45]. Furthermore, PPAR-α activation increases peroxisomal fatty acid β -oxidation by inducing acyl-coenzyme A oxidase (Acox1), the rate-limiting enzyme in the oxidation of very long-chain fatty acids[46,47]. Acox1 is also associated with spontaneous liver damage in humans, as well as spontaneous steatosis, steatohepatitis, and hepatocellular carcinoma development in mice [46]. Despite what we discussed, alterations in PPAR- α are not the only ones leading to hepatic steatosis. Instead, a number of altered function in nuclear receptors such as the pregnane and xenobiotic receptors, the liver X receptor, and the farnesoid X receptor (FXR) contribute to the pathogenesis of MAFLD[48].

What about the comorbidities in MAFLD? Let's not forget CKD: The multiple effects of gene mutations

We have already discussed two primordial concepts in the understanding of MAFLD: AT dysfunction and IR. Even though these attributes explain a great deal of the pathogenic mechanisms involved in MAFLD, saying that overseeing genetic alterations involved is a mistake would hardly be an overstatement.

As it has been already reviewed in multiple studies and around a number of countries, there is a significant amount of genetic mutations that highly predispose populations to MAFLD. Even though there are multiple genetic mutations, the most common involves the gene patatin-like phospholipase domain-containing protein 3 (PNPLA3), which encodes for a protein called adiponutrin that exerts lipolytic action on TGs and reduces DNL within the liver. The PNPLA3 gene is present in many tissues in the body; however, it is most highly expressed in the liver and the kidney.

MAFLD is known for its large range of associated comorbidities; it is not only the hepatic manifestation of MetS but is a rather multisystemic disease on its own. A recent meta-analysis evaluated the risk of having NAFLD (previous nomenclature) and the risk of developing chronic kidney disease (CKD). In total, the data from more than a million patients were analyzed, and the study concluded that pre-existing NAFLD is associated with about a 1.45-fold increased risk of incident CKD stage \geq 3[49]. The same team carried out a meta-analysis in 2017 showing 40% increased risk of CKD in patients with NAFLD[50]. Another group of researchers reached the same conclusions, showing that the presence and severity of NAFLD are associated with an increased risk and severity of CKD[51]. However, another group concluded that the association was not because of a causal relationship between MAFLD and CKD but was due to shared risk factors between them, namely diabetes, age, hypertension, and hyperuricemia[52]. This recent cross sectional study from The National Health and Nutrition Examination Survey 2017-2018 showed that MAFLD and CKD were not independently related after propensity score matching[52].

We propose an explanation for this, which might account for the high prevalence of CKD in people with MAFLD, and also shed light on the reason why these two diseases, even when highly correlated, are not independently related.



As previously mentioned, the PNPLA3 gene is most highly expressed in two tissues: the liver and kidney. Mutations in the PNPLA3 gene, especially the PNPLA3 I148M variant, leads to CKD in a similar pattern as how the mutation predisposes to MAFLD. Montovani and his team followed this line of thought and by studying 157 patients with T2DM, found that the presence of this mutation (especially in the podocytes on the renal cortex) was associated with lower glomerular filtration rates (GFR) and higher risk of CKD, 63.6% vs 24.2% risk in people without homozygous mutation [53]. It is important to establish that the association between the PNPLA3 I148M variant and the risk of lower GFR and CKD development is independent of liver disease severity as well as other factors [53]. This association has also been found among children with MAFLD, where the PNPLA3 G/G genotype leads to decreased kidney function and increased 24-h proteinuria^[54].

By having established this, we can understand the multifactorial nature of MAFLD and its comorbidities, as in this case, CKD's. Whether or not MAFLD predisposes to kidney malfunction could be studied in a group of patients who develop both entities but lack mutations in the PNPLA3 gene.

While PNPLA3 gene mutations might be a common factor in the predisposition for both CKD and MAFLD, there are a number of nuclear transcription factors that contribute to the pathogenesis of both diseases. These factors include the peroxisome proliferator-activated receptor (PPAR) family, FXR, and SREBP2, which modify their respective molecular pathways and influence the progression of both CKD and hepatic steatosis [55]. For instance, the downregulation of PPAR- α , PPAR- δ , and PPAR- γ causes a myriad of cellular alterations in the nephron, including increased podocyte apoptosis leading to altered glomerular barrier integrity, increased mesangial cell hypertrophy and enhanced matrix deposition, as well as NF-kB activation with consequent proinflammatory cytokine secretion in the glomerular endothelium[56]. These same factors under physiologic circumstances suppress fibrogenesis by inhibiting the transforming growth factor β in stellate cells, lower the M1/M2 Kupffer cell phenotype ratio (thus decreasing inflammatory stimulus in the liver) and increases catalase activity in hepatocytes, among other functions. It is clear how downregulation of the PPAR family of factors hinders these protective mechanisms in the liver and promotes the development of fatty liver, as well as CKD. The same situation of multiorgan damage comes about with decreased expression of FXR and upregulation of SREBP-2, given that FXR inhibits SREBP-1c-mediated DNL in hepatocytes while decreasing reactive oxygen species formation in mesangial cells and increasing endothelial nitric oxide synthase in the glomerular epithelium[55]. Finally, SREBP-2 upregulation leads to increased cholesterol synthesis and decreased excretion in both liver and renal cells [57,58]. With this brief compilation of the molecular pathway similarities between CKD and fatty liver development, it would be of no surprise to find in the near future novel discoveries on further overlapping mechanisms and genetic predisposition for both diseases.

CONCLUSION

In conclusion, although MAFLD pathogenesis is multifactorial and complex, we consider IR to be the basis for the development of the disease, the abnormal metabolic profile in patients, and disease complications. Further research is required to fully understand and test this hypothesis along with others that may develop. Understanding the basis of the disease and the many variables that play a role in its development will lead to appropriate targeted therapies for MAFLD.

FOOTNOTES

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MINIREVIEWS

Recent advances and current challenges in endoscopic resection with the full-thickness resection device

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Abstract

Endoscopic full-thickness resection (EFTR) has emerged as a viable technique in the management of mucosal and subepithelial lesions of the gastrointestinal tract (GIT) not amenable to conventional therapeutic approaches. While various devices and techniques have been described for EFTR, a single, combined fullthickness resection and closure device (full-thickness resection device, FTRD system, Ovesco Endoscopy AG, Tuebingen, Germany) has become commercially available in recent years. Initially, the FTRD system was limited to use in the colorectum only. Recently, a modified version of the FTRD has been released for EFTR in the upper GIT as well. This review provides a broad summary of the FTRD, highlighting recent advances and current challenges.

Key Words: Endoscopic full-thickness resection; Full-thickness resection device; Colorectal neoplasm; Subepithelial lesions; Scarred non-lifting polyps

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Core Tip: Endoscopic full-thickness resection (EFTR) is an emerging technique for tissue resection of lesions in the gastrointestinal tract (GIT) not amenable to conventional resection approaches. The novel full-thickness resection device (FTRD) is a combined full-thickness resection and closure device that allows for EFTR of lesions in the GIT. EFTR with FTRD is feasible, safe, and efficacious and should be considered as a viable option for resection of select lesions in the lower and upper GIT.



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INTRODUCTION

Traditionally, flexible endoscopic removal of gastrointestinal neoplasms has been accomplished by standard polypectomy, endoscopic mucosal resection (EMR), or endoscopic submucosal dissection (ESD). More recently, endoscopic full-thickness resection (EFTR) has emerged as a complementary resection technique for the removal of colorectal lesions, particularly for lesions that are scarred or nonlifting, early cancers with deeper invasion, and subepithelial lesions, where resection via standard endoscopic techniques is difficult or not possible[1,2]. EFTR also has the potential added benefit of decreasing procedural time and providing a minimally-invasive organ-sparing alternative approach, obviating need for surgery. As its name implies, EFTR refers to the technique whereby a lesion is resected endoscopically in its entirety including all layers of the gastrointestinal wall[1]. The two main techniques for EFTR include the "resect and close" method, where the full-thickness resection is performed first exposing the peritoneal cavity, followed by complete closure of the defect, and "close and resect" method, where approximation of the wall layers deep to the lesion is performed first followed by full-thickness resection without exposure and contamination of the peritoneum. Various strategies and devices have been developed to accomplish EFTR, including but not limited to needleknives, ESD knives, nylon loop accompanied by standard through-the-scope clips, omental patches, endoscopic plicating and suturing devices, and endoscopic staplers[3-9]. These methods will not be covered in this review.

Recently, a single, combined full-thickness resection and closure device referred to as the fullthickness resection device (FTRD system, Ovesco Endoscopy AG, Tuebingen, Germany) has become available and is quickly being adopted across centers worldwide to accomplish EFTR. Its appeal over other strategies includes the ability to perform resection and closure with a single device integrating an over-the-scope clip (OTSC) and electrosurgical snare. It can offer a safe and nonsurgical alternative for removal of lesions that may be challenging to resect with conventional endoscopic methods and might otherwise require surgery. Main indications for the FTRD include non-lifting or scarred polyps, partially resected or residual polyps with submucosal fibrosis, early cancers, subepithelial lesions, and diagnostic EFTR for pathologic assessment of the gut wall. It is approved for use in the colorectum, and recently, received additional approval from the U.S. Food and Drug Administration for use in the upper gastrointestinal tract (GIT)[10].

This review article will provide a summary of the major studies on FTRD, highlighting recent advances and current challenges, in the hopes that practicing endoscopists will be better equipped to understand the role and limitations of, and potentially utilize, this novel and important resection technique.

DEVICE

The FTRD system, a combined full-thickness resection and closure device, was first made available in Europe in 2014[11]. It has since been approved for use in the United States for colorectal EFTR in 2017 and gastroduodenal EFTR in 2020[12].

The FTRD system is based on the principle of "close and resect" for EFTR. It uses the well-established OTSC system (Ovesco Endoscopy AG, Tuebingen, Germany), which allows for full-thickness tissue apposition deeper to the lesion first, followed by full-thickness resection above the clip, thereby performing EFTR without exposure of the peritoneal cavity^[13]. The FTRD system (Figure 1) consists of a clear, plastic distal attachment cap that has a mounted ready-for-use 14 mm nickel titanium alloy (Nitinol) clip, integrated 14 mm monofilament snare and thread, thread retriever, endoscope sleeve with fixation tapes, and a hand wheel. Separately, an FTRD Marking Probe (Figure 2A) and FTRD Grasper (Figure 2B) are provided as part of the FTRD set. These individual instruments can be advanced through the working channel for marking and mobilization of the target lesion, respectively. The FTRD system is suitable for use for endoscopes having a diameter of 11.5-13.2 mm and a working channel diameter of 3.2 mm. The cap diameter is 21 mm and the cap depth is 23 mm.

The initial FTRD system was fashioned for use in the colorectum and is now marketed as the colonic FTRD©. With recent advances demonstrating the role of the FTRD in the upper GIT, an additional FTRD system called the gastroduodenal FTRD© has now become available. The gastroduodenal FTRD system is smaller in caliber and additionally comes with an insertion balloon and guide wire to help facilitate passage of the FTRD through the upper esophageal sphincter and pylorus (Figure 2C). A





Figure 1 The colonic full-thickness resection device system. A: Hand wheel; B: Thread Retriever; C: Thread; D: Distal attachment cap with affixed overthe-scope-clip; E: Endoscope sleeve; F: Integrated snare; G: Snare lock. Images provided by Ovesco Endoscopy.



Figure 2 Full-thickness resection device separately included items. A: Marking probe; B: Grasper; C: Guidewire and balloon (diagram) - for gastroduodenal full-thickness resection device (FTRD)[®]; D: Modified anchor — for gastroduodenal FTRD[®]; E: prOVE cap. Images courtesy of Ovesco Endoscopy.

separate modified Anchor (Figure 2D) is also available to allow for better tissue mobilization (*i.e.*, for subepithelial lesions). The gastroduodenal FTRD is suitable for endoscopes having a diameter of 10.5-12.0 mm and a working channel diameter of 3.7 mm. The cap diameter is 19.5 mm and the cap depth is 23 mm.

Recently, a third FTRD system called the diagnostic FTRD© was made available for use in the colorectum for diagnostic resection, or full-thickness biopsy. This system is nearly identical to the colonic FTRD system but is smaller in size allowing for full-thickness resection of lesser tissue volume when desired for diagnostic purposes. The diagnostic FTRD system can also be applied to a pediatric colonoscope allowing for increased flexibility and mobility. The diagnostic FTRD system is suitable for endoscopes having a diameter of 10.5-12.0 mm and a working channel diameter of 3.2 mm. The cap diameter is 19.5 mm and the cap depth is 23 mm.

TECHNIQUE

Once a target lesion in the GIT is identified, its margins are delineated with the FTRD Marking Probe ensuring a margin of normal surrounding tissue (Figure 3A). The endoscope is then withdrawn from the patient. It is often cumbersome to advance the endoscope (with the attached stiff FTRD cap) to the lesion (especially lesions located in the right colon), and sometimes difficult to grasp and bring a scarred lesion into the cap. Hence many endoscopists will first attach a test cap – the FTRD prOVE cap (a "blank" cap similar to the FTRD cap but without the mounted clip or snare; see Figure 2E) – to the endoscope to perform a "test run" without using the FTRD, which may otherwise be wasted if unable to be advanced to the lesion. Once it is confirmed that the endoscope with the attached prOVE cap can be advanced to the lesion and the lesion able to be brought inside the cap, the endoscope is withdrawn and fitted with the "real" FTRD system. The hand wheel (Figure 1A) is first inserted into the working channel of the endoscope. The thread retriever (Figure 1B) is then inserted into the working channel and used to retrieve the thread (Figure 1C) allowing for attachment of the plastic attachment cap (Figure 1D) onto the distal end of the endoscope. The distal attachment cap has a preloaded clip attached to the thread that runs inside the working channel of the endoscope and attaches to the hand wheel, as well as



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Figure 3 Technique for accomplishing endoscopic full-thickness resection using the full-thickness resection device. A: Borders of target lesion marked using full-thickness resection device (FTRD) Marking Probe; B: FTRD Grasper positioned over target lesion; C: FTRD Grasper opened over target lesion; D: Target lesion grasped and pulled into cap; E: Over-the-scope-clip deployed; F: Specimen resected with electrosurgical snare. Images provided by Ovesco Endoscopy.

a preloaded snare that runs outside of the endoscope. The accompanying endoscope sleeve (Figure 1E) and adhesion tapes help affix the snare (Figure 1F) to the outside of the endoscope and prevent inadvertent twisting of the snare around the endoscope or entrapment of tissue between the snare and the endoscope. Turning of the hand wheel allows for adjusting tension on the thread, and eventually, deployment of the clip. A snare lock (Figure 1G) helps prevent inadvertent snare opening and can be removed to "unlock" the snare once ready for use. The endoscope with the attached FTRD system is then re-inserted into the GIT to the target lesion. Next, with the tip of the cap appropriately positioned over the lesion (Figure 3B), the FTRD Grasper is used to grasp (Figure 3C) and mobilize the lesion completely within the cap (Figure 3D). All markings made by the FTRD Marking Probe should be visible inside the cap. Suction should not be used to draw the lesion into the cap as this may bring in extraluminal structures (e.g., small bowel) into the cap causing significant injury to adjacent organs and structures. Finally, the clip is deployed (Figure 3E) by turning the hand wheel, and the lesion immediately resected with the snare using an electrosurgical current (Figure 3F) while being firmly grasped with the Grasper. It should be noted that once the lesion is brought into the cap, these steps should be performed quickly and sequentially in this specific order to prevent perforation and/or the lesion slipping out of the cap. The resected specimen is retrieved by withdrawing the endoscope with the lesion held by the Grasper. The endoscope without the attached FTRD is then reinserted to inspect the resection site for complete resection and confirm the absence of adverse events (AEs) (Figure 4).

PROOF OF CONCEPT AND PRECLINICAL DATA

Earlier studies established efficacy and safety of the OTSC system for endoscopic closure of gastrointestinal perforations and surgical anastomotic leaks and fistulas [14,15]. Following these data, several observational experiences and case series were described using an OTSC-assisted method for resection of gastrointestinal lesions, whereby the OTSC system was used to deploy the clip deep to a lesion prior to snare resection [16-18]. Based on these experiences, the basis for the FTRD system – a combined device containing an OTSC system plus a preloaded snare – was developed.

An early preclinical study using the FTRD in an animal (porcine) model demonstrated safety and efficacy[11]. The FTRD was used for resection of the colon wall in 11 pigs, and 7 and 28-d follow up study sessions were performed to evaluate the clip application site. Clips were still present in all but one case at 28 d and adherent stool and fibrin were found on the clips. There were mild inflammatory reactions seen histologically at day 7, but no acute inflammation was noted on day 28. The underlying tissue at the clip bed showed no signs of dehiscence or ischemia. Manometric pressure tests demonstrated no leak. Histology assessments revealed successful full-thickness resection (mucosa to serosa) in all cases.



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Figure 4 Endoscopic images of full-thickness resection device. A: Full-thickness resection device (FTRD) Marking Probe used to mark the target lesion (non-lifting polyp); B: FTRD Grasper used to grasp the lesion and pull into the cap followed by successful clip deployment; C: Resection site after FTRD; D: Resected specimen. Images courtesy of Dr. Wagh MS.

CURRENT APPLICATIONS AND CLINICAL DATA

Study search and inclusion criteria

A summary of the major studies investigating the FTRD are included below. The keywords "endoscopic full thickness resection", "full thickness resection device," and "FTRD" were used for the initial query on PubMed. Studies published within a 10-year period from January 1, 2011-December 31, 2021, were considered for inclusion. To be considered for inclusion in this review, studies had to be original research studies using the Ovesco Endoscopy FTRD (other devices described for EFTR were not included) and be designed as prospective or retrospective studies. Observational studies, case reports, preclinical animal studies and proof of concept studies were not included. Studies that did not report the type of lesion, technical success rates, R0 resection rates, full-thickness resection rates, and rates of AEs were also not included.

Colorectal FTRD

The first clinical experience using the FTRD was published in 2015[19]. In this retrospective, two-center European study, 24 patients who underwent resection of colorectal lesions with the FTRD were identified. Indications included recurrent or incompletely resected adenomas with a non-lifting sign (n)= 11), appendiceal adenomas (n = 5), untreated adenomas with a non-lifting sign (n = 2), incomplete resection of T1 carcinoma (n = 2), submucosal tumors (n = 2), adenoma within diverticulum (n = 1), flat adenoma in a coagulopathic patient (n = 1), and diagnostic resection in a patient with suspected Hirschsprung's disease (n = 1). Technical success (complete *en bloc* resection) was achieved in 20/24 (83.3%) patients and R0 resection (histologically complete resection with tumor-free margins) in 18/24 (75.0%) patients. Two patients developed post-polypectomy syndrome and required antibiotics, but there were no other reported AEs including no perforations or major bleeding events.

This study has been followed by numerous retrospective and prospective studies demonstrating safety and efficacy of FTRD in the colon and rectum (see Table 1 for summary of major clinical studies with current applications of FTRD for EFTR)[12,20-27]. In the largest, prospective multicenter study to date, 181 patients were recruited to receive EFTR with the FTRD system[25]. Indications for EFTR included "difficult" adenomas (n = 143), early carcinomas (n = 15), and subepithelial tumors (n = 23). "Difficult" adenomas included adenomas with a non-lifting sign, adenomas at the appendiceal orifice, and adenomas at a diverticulum. Technical success was achieved in 89.5% of cases and R0 resection in 76.9% of cases. AEs were noted in 18/181 (9.9%) patients and included bleeding (4/18), postpolypectomy syndrome (3/18), perforation (6/18), acute appendicitis (3/18), abdominal pain without clear etiology on laboratory and radiographic work-up (1/18), and enterocolonic fistula formation (1/18)18). All four patients with bleeding were treated conservatively and did not require transfusion of blood



Table 1 Summary of major clinical studies with current applications of full-thickness resection device for endoscopic full-thickness resection

	Ref.	Study type	Total FTRD cases, n	Site (s)	Type of lesion	Technical success, %	R0 resection, %	Full- thickness resection, %	Adverse events, %
1	Schmidt <i>et al</i> [19]	Retrospective	24	Colon; Rectum	Non-lifting polyps ($n = 13$); Appendiceal polyps ($n = 5$); Diagnostic ($n = 3$); Subepithelial tumor ($n = 2$); Polyp involving diverticulum ($n = 1$)	83.3	75.0	87.5	8.3
2	Schmidt <i>et al</i> [25]	Prospective	181	Colon; Rectum	Difficult adenomas (79.0%); Cancer (8.3%); Subepithelial tumor (12.7%)	89.5	76.9	81.0	9.9
3	Meier <i>et al</i> [<mark>12</mark>]	Retrospective	1178	Colon; Rectum	Difficult adenomas (67.1%); Cancer (18.4%); Subepithelial tumor (6.8%); Diagnostic (1.3%)	88.2	80.0	89.9	12.1
4	Boger <i>et al</i> [23]	Retrospective	68	Colon; Rectum	Non-lifting polyps ($n = 29$); Cancer ($n = 13$); Subepithelial tumor ($n = 9$); Polyps in appendix or diverticulum ($n =$ 17)	88.2	76.8	NA	5.9
5	Hajifathalian et al <mark>[37]</mark>	Retrospective	56	Stomach; Duodenum	Mesenchymal neoplasm including GIST ($n = 26$); Diagnostic ($n = 10$), NET ($n =$ 9); Adenomas ($n = 6$); Adenocarcinoma ($n = 5$)	93.0	68.0	NA	21.0
6	Meier <i>et al</i> [<mark>39</mark>]	Prospective	29	Stomach	Subepithelial tumor ($n = 29$)	89.7	76.0	65.5	31.0
7	Ichkhanian et al[<mark>20]</mark>	Retrospective	66	Colon/appendiceal orifice	Polyps involving appendiceal orifice	89.0	93.0	91.0	17.0
8	Zwager <i>et al</i> [28]	Prospective	367	Colon; Rectum	Difficult polyps ($n = 133$); T1 cancer ($n = 71$); Incomplete resection ($n = 150$); Subepithelial tumors ($n = 13$)	83.9	82.4	83.2	9.3
9	Zwager <i>et al</i> [29]	Retrospective	330	Colon; Rectum	Primary resection of T1 cancer ($n = 132$); Secondary resection of T1 cancer ($n = 198$)	87.0	85.6	NA	2.2

FTRD: Full-thickness resection device; GIST: Gastrointestinal stromal tumors; NA: Not available; NET: Neuroendocrine tumor.

products. Emergency surgery was required in 3/181 (2.2%) patients.

The results of a recent large retrospective analysis using the German colonic FTRD registry (n = 1178) reported similar findings, corroborating the previously reported findings of smaller studies[12]. Indications included difficult adenomas, early carcinomas, subepithelial tumors, and diagnostic resection. The technical success rate was 88.2% and R0 resection rate 80.0. The AE rate was 12.1% with 3.1% being major AEs. A similar but smaller retrospective study from the UK FTRD registry (n = 68) reported similar outcomes²³ with technical success rate 88.2%, R0 resection rate 76.8%, and AE rate 5.9%. This has since been further replicated in a larger Dutch FTRD registry (n = 367) with technical success rate 83.9%, R0 resection rate 82.4%, full-thickness resection rate 83.2%, and AE rate 9.3% [28].

Colorectal FTRD for early cancer, lesions at the appendix, and subepithelial lesions

Studies have reported slight variations (albeit with comparable success) in outcomes for FTRD depending on the indication for EFTR. Regarding FTRD use for early cancer, a recent study of 156 patients with a histologically proven diagnosis of adenocarcinoma showed FTRD to have a technical success rate of 92.3% and R0 resection rate of 71.8%. Serious AEs were reported in 3.9% of patients [24]. In a subgroup analysis from the previously mentioned retrospective study using the German colonic FTRD registry by Meier et al[12], 217 cases of FTRD in early cancers were identified. Technical success was reported to be 84.6% and R0 resection in 82.8% of cases. In a separate analysis by Schmidt *et al*[25], 29 cases of FTRD for early carcinomas were identified (14 harbored unsuspected cancer and 15 were known cancers). R0 resection was achieved in 72.4% of cases, but curative resection could only be achieved in 44.8% of cases. Similarly, a study from the Dutch FTRD registry showed overall technical



success, R0 resection, and AE rates of 87.0%, 85.6%, and 2.2%. However, the curative resection rate was only 60.3% with a curative resection rate of 23.7% for primary resection of T1 cancers^[29]. Hence, given these low curative resection rates, there is some uncertainty regarding the role of FTRD as primary use for early cancers.

For lesions at the appendiceal orifice, a recent study of 66 patients demonstrated a technical success rate of 89% and R0 resection rate of 79% [20] using FTRD. Of note, 17.2% of patients developed appendicitis and 10.3% of patients (60% of those who developed appendicitis) required surgical appendectomy. Factors associated with risk of appendicitis included male sex (odds ratio: 1.2, P = 0.03) and failure to achieve histologic full-thickness resection (odds ratio: 1.5, P = 0.04)[20]. The high rates of appendicitis for this indication have raised concerns regarding the use of FTRD for appendiceal lesions. Further highly powered studies are needed to clarify the safety of FTRD for these lesions.

There is a lack of highly powered studies investigating FTRD for subepithelial tumors. A recent study of 40 patients with rectal neuroendocrine tumors (NETs) did report a technical success rate of 100%, R0 resection rate of 100%, and no AEs[21]. While these outcomes should be interpreted cautiously in the setting of a small sample size, the reported success is nonetheless encouraging and in line with the success reported in other FTRD studies[12,22,23,25]. In a subgroup analysis using the German colonic FTRD registry by Meier et al[12], 80 cases of FTRD for subepithelial tumors were identified. The technical success rate was 97.3% and R0 resection rate was 97%. R0 resection was significantly higher for subepithelial tumors compared with that for other lesions in the German FTRD registry (77.2% for difficult adenomas, 82.8% for early cancers). Data regarding AE rates within the subgroup analysis were not available. In a separate subgroup analysis from Schmidt et al[25], 23 cases of FTRD for indication of subepithelial lesions were identified, with R0 resection rate of 87%. Overall, FTRD for subepithelial lesions seems to be very effective, with greater technical success and R0 resection than FTRD for other lesions.

Subgroup analyses have also been performed in studies to determine whether differences in outcomes may be related to other factors such as lesion location, lesion size, and prior treatment. Findings have varied[12,25], though by and large, there does not appear to be convincing evidence of any significant differences in technical success and other outcomes based on lesion location, size, or prior treatment^[12]. Further studies may help better clarify what factors independently predict these outcomes of success.

Similar technical success and R0 resection rates have been reported for EMR and ESD[30,31]. ESD has been associated with greater R0 resection rates than EMR, but is also associated with greater rates of AEs like perforation and major bleeding. Direct comparisons of technical success and R0 resection between EMR/ESD and FTRD are difficult to make, as the majority of indications for FTRD currently are for lesions deemed difficult or challenging to resect via EMR or ESD. Few studies have reported on the efficacy of salvage ESD (ESD performed for residual or locally recurrent non-lifting lesions), which would likely be a better comparator group for FTRD, but these few small studies do appear to suggest comparable rates of success[32,33]. Similar AE rates have been reported for ESD and FRTD[31]. However, procedural time for ESD is considerably longer than FTRD[31].

Overall, EFTR with the FTRD system in the colorectum appears to be safe and efficacious. Common indications for FTRD in the lower GIT include select lesions difficult to resect by more conventional approaches (*i.e.*, non-lifting adenomas, adenomas at appendiceal orifice, adenomas at diverticulum), early cancers, subepithelial lesions, and diagnostic resection. Very large lesions > 3 cm may not be appropriate for FTRD.

Gastroduodenal FTRD

Recently, a newer and smaller caliber FTRD, the gastroduodenal FTRD©, has become available for use in the upper GIT.

Prior to the development of the newer gastroduodenal FTRD system, experiences using the traditional FTRD system (now known as the colonic FTRD[©]) in the upper GIT were reported [34-36]. These were primarily case reports and small case series dating as far back as 2014 (when the FTRD first became available in Europe) reporting safe and successful use of the FTRD in the stomach and duodenum in patients with malignant or large, nonmalignant subepithelial lesions who were deemed unsuitable for surgery [34-36]. Several retrospective analyses have followed demonstrating safety and efficacy of FTRD in the UGIT[37,38]. The largest of these was an international multicenter trial of 56 patients across 13 centers[37]. Common indications included gastrointestinal stromal tumors (GIST), adenomas, hamartomas, and adenocarcinomas. Results showed a technical success rate of 77% and R0 resection rate of 68%. The AE rate was 21%, though none were classified as severe AEs. Common AEs included intraprocedural and delayed bleeding. No perforations were reported.

Recently, a prospective multicenter pilot study (RESET trial) investigated the new gastroduodenal FTRD in the upper GIT[39]. 29 patients underwent EFTR with the gastroduodenal FTRD in this trial for gastric subepithelial tumors, which included leiomyomas, lipomas, schwannomas, ectopic pancreas, GISTs, and NETs. The technical success rate was 89% and the R0 resection rate was 76%. Minor bleeding occurred in 31% of cases and was able to be managed intraprocedurally in all cases. No transfusion of blood products was required nor was any further bleeding observed. No other AEs were reported. The success rates and AE rates reported here are similar to those reported with FTRD in the lower GIT.



To our knowledge, there is only one single report on the use of the FTRD in the esophagus[37]. However, this was reported as part of a larger multicenter study, and it is not evident whether this case was technically successful or associated with any AEs.

Similar to FTRD in the lower GIT, various subgroup analyses have been performed to ascertain whether outcomes of FTRD in the upper GIT are associated with other variables. Thus far, variables such as indication, lesion size, lesion location, prior treatment, participating center, or number of FTRD cases performed by the endoscopist have not been clearly associated with technical success and other success outcomes[37,39]. Further studies are needed to help delineate the impact of these individual factors on gastroduodenal FTRD outcomes.

Overall, early data suggests that FTRD in the upper GIT appears to be feasible, safe, and efficacious. Common indications include gastric and duodenal subepithelial lesions, adenomas, and early cancer. However, given the paucity of data of FTRD in the upper GIT compared to the lower GIT, further studies are needed to further clarify the indications, especially for the indication of early cancer, as well as clarify overall safety and efficacy.

EMERGING APPLICATIONS

Hybrid FTRD

In cases with lesions > 3 cm or where *en bloc* EFTR appears challenging for other reasons, various hybrid techniques have been proposed. These have been referred to by varying names including Hybrid FTRD, EMR + FTRD, and ESD + FTRD. In general, these hybrid techniques refer to using either piecemeal EMR or ESD followed by FTRD of scarred areas or residual abnormal appearing tissue. Hybrid techniques have shown some promise in small case series [40-44]. However, further evaluation in larger scale studies is needed to draw conclusions on their routine use.

TECHNICAL CHALLENGES

Several limitations of the FTRD have been reported. One technical challenge is the difficulty in resecting larger lesions. With a cap diameter of 21 mm and cap depth of 23 mm for the standard colonic FTRD (and smaller for the gastroduodenal FTRD and diagnostic FTRD), it is often difficult to adequately grasp large lesions (> 3 cm) to accomplish en bloc resection. In addition, these lesions are often scarred and fixed to the colon wall, and therefore may be difficult to grasp and bring into the cap even if they are smaller than 3 cm. The length and stiffness of the plastic cap can also interfere with visualization and flexibility at the scope tip, thereby making it quite cumbersome to advance the colonoscope with the FTRD across a tortuous sigmoid and especially to the right colon. As described earlier, a proprietary test cap called the FTRD prOVE Cap[©] is available to help circumvent some of these issues and to determine whether a target lesion can be reached and might be suitable for resection with FTRD. The long plastic sheath that wraps around the scope shaft can increase friction of the device and also make advancement of the scope through tight or tortuous anatomic locations difficult. Some endoscopists leave a guidewire in the colon after the diagnostic colonoscopy is performed. The colonoscope with the attached FTRD is then advanced alongside the guidewire, following the guidewire to the lesion, overcoming the limited visualization and maneuverability with the FTRD cap. Device failure (failure of the clip to fully cinch down or complete malfunction of the clip or snare's opening and closing functions) and premature clip deployment leading to immediate perforation or incomplete resection have been reported[12]. Risk factors for incomplete or partial resection include tissue fibrosis, right-sided colonic lesions, and size > 3 cm[25]. It should be emphasized that clip placement must be immediately followed by prompt snare resection, often requiring good communication and teamwork between the endoscopist and assistants handling the snare and grasper. It is important to have the endoscopist fire the clip and have two separate assistants operate the grasper and snare. Delay in snare resection after clip placement, slippage of the lesion from the grasper, or inadvertently performing snare resection before firing the clip can result in incomplete resection or immediate perforation. As with other endoscopic devices and techniques, EFTR with the FTRD requires adequate training and endoscopic skill. Completion of mandatory training is required prior to the purchase and use of FTRD.

FUTURE DIRECTIONS

Currently, there is a lack of direct comparison between FTRD and other standard resection approaches such as EMR and ESD. Direct comparisons between FTRD and more standard approaches are needed to further clarity the role of FTRD, whether it is merely adjunctive to standard approaches or may be a suitable primary or "standard" approach itself. Cost-effectiveness studies may help further clarify this role as well. Recently, laparoscopic and endoscopic cooperative surgery (LECS) has been described as a



less invasive approach to resecting lesions in the GIT, such as GIST[45]. Prospective studies comparing FTRD with LECS and other similar novel minimally-invasive surgical techniques will help clarify the advantage of FTRD over these novel surgical approaches. Studies investigating longer term outcomes are also needed, especially regarding AEs and the ability to perform adequate endoscopic examination of the full-thickness resection site during follow-up and surveillance. This may be challenging if the FTRD clip is still in place and not fallen off. There are still concerns regarding AEs with EFTR with FTRD, especially for resection of lesions at the appendiceal orifice and with regards to delayed perforation. Long term data are needed on specific outcomes such as the development of an appendiceal mucocele or delayed appendicitis after using FTRD in this location. It is also tempting to consider whether there might be safe applications for the FTRD in the esophagus, particularly in the treatment of early carcinomas or subepithelial lesions. Future studies may help clarify this and other expanded roles of FTRD.

CONCLUSION

EFTR can be successfully and safely accomplished with the FTRD system. The role of FTRD has expanded in recent years and the FTRD is currently available for use in the lower and upper GIT. EFTR with FTRD is a crucial addition to the endoscopic armamentarium for resection of gastrointestinal lesions and reducing need for more invasive surgery. Common indications for its use include resection of lesions difficult to resect with conventional techniques like EMR and ESD, such as scarred or nonlifting polyps, early cancers, subepithelial lesions, and diagnostic resection for evaluation of various neuromuscular or entero-infiltrative conditions (e.g., Hirschsprung disease, gastrointestinal amyloidosis). EFTR with FTRD has a high technical success and R0 resection rate, and AE rates are acceptable, especially when used as a less invasive option for lesions not amenable for standard endoscopic resection. Common AEs include bleeding and perforation, and further long-term data on unique complications such as post-polypectomy syndrome, delayed perforation, acute appendicitis, and enterocolonic fistula are needed. Several technical challenges exist and endoscopists should be adequately trained on the FTRD system prior to use in clinical practice. FTRD, used in its proper context, has the ability to be a major advancement in endoscopic resection for populations felt to be too high risk for traditional resection techniques and surgery (e.g., elderly patients, patients at high risk of bleeding, high risk for anesthesia).

FOOTNOTES

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MINIREVIEWS

Dynamic contrast enhanced ultrasound in gastrointestinal diseases: A current trend or an indispensable tool?

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Abstract

Contrast enhanced ultrasound (CEUS) has been widely implemented in clinical practice because of the enormous quantity of information it provides, along with its low cost, reproducibility, minimal invasiveness, and safety of the secondgeneration ultrasound contrast agents. To overcome the limitation of CEUS given by the subjective evaluation of the contrast enhancement behaviour, quantitative analysis of contrast kinetics with generation of time-intensity curves has been introduced in recent years. The quantification of perfusion parameters [named as dynamic-CEUS (D-CEUS)] has several applications in gastrointestinal neoplastic and inflammatory disorders. However, the limited availability of large studies and the heterogeneity of the technologies employed have precluded the standardisation of D-CEUS, which potentially represents a valuable tool for clinical practice in management of gastrointestinal diseases. In this article, we reviewed the evidence exploring the application of D-CEUS in gastrointestinal diseases, with a special focus on liver, pancreas, and inflammatory bowel diseases.

Key Words: Quantitative perfusion analysis; Gastrointestinal diseases; Time-intensity curve; Multiparametric ultrasound

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Core Tip: Contrast-enhanced ultrasound (CEUS) has been widely implemented in clinical practice in recent years. Despite its several advantages, the qualitative evaluation of this exam and the lack of objectivity could lead to variability between different operators and ultrasound equipments. Dynamic-CEUS (D-CEUS) with the measurement of perfusion parameters is aimed at overcoming this important limitation. The purpose of this review is to explore the usefulness of D-CEUS in gastroenterological diseases.

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INTRODUCTION

Contrast enhanced ultrasound (CEUS) has been widely implemented in clinical practice as a result of the enormous quantity of information it provides, along with its low cost, reproducibility, minimal invasiveness, and safety of the second-generation ultrasound contrast agents (UCAs)[1-4]. Despite its numerous advantages, one of the most significant limitations of CEUS is the subjective evaluation of contrast enhancement related behaviour of the explored tissues [5]. In recent years, dynamic-CEUS (D-CEUS) has been explored to overcome this limitation.

D-CEUS represents the quantitative analysis of UCA-kinetics in a specific region of interest (ROI)[6]. This technique allows two types of analysis in the examined tissue: Disruption-replenishment analysis and wash-in/wash out analysis^[7]. The first analysis consists in the evaluation of microbubbles replacement after destroying them with high mechanical index. Requiring the continuous intravenous infusion over five to twenty minutes of UCA, the disruption-replenishment analysis are infrequently used due of their complex methodology[8]. Consequently, the second form of analysis is more frequently employed in clinical practice. It consists of measuring the average intensity of a ROI following a bolus injection of UCA and generating a time-intensity curve (TIC). Hence, multiple parameters are derived from the TIC to quantitatively characterize the different stages of the wash-in and wash-out phases. The fundamental parameters derived from TIC are summarized in Table 1[9,10] and a schematic representation of TIC is shown in Figure 1. Generally, these parameters are obtained from different softwares and might consequently have varying nomenclature but can be divided into two categories: Amplitude parameters and time parameters. These criteria reflect various vascularization features: Amplitude parameters are mainly related to blood volume in the ROI, while blood flow is mostly correlated with time parameters[11]. Tracking microbubbles circulation provides the spatial representation of blood flow patterns and the derivation of parametric values of tissue perfusion since microbubbles strictly remain within the vasculature compartment[12].

Examining the pros and cons, D-CEUS is a widely accessible, radiation-free, non-nephrotoxic and cost-effective technique that allows objective enhancement quantification, image comparison, real-time evaluation of the microcirculation perfusion by a strictly intravascular blood pool agent. This is crucial after the introduction of updated response evaluation criteria in solid tumor (RECIST) criteria based on tumour perfusion as D-CEUS potentially enables the monitoring of changes in vascularization even shortly after tumor treatment[13,14]. According to current European Federation for Ultrasound in Medicine and Biology recommendations, D-CEUS is useful for quantifying tumor enhancement objectively, to characterize focal lesions and evaluate the therapeutic response[7]. In contrast, D-CEUS should ideally be uniform regardless of the ultrasound equipment, data collecting, and analysis software, as different approaches and technical issues may influence the results' validity. Lastly, the technical limitations of the method must be addressed, particularly in the abdomen, where intestinal, respiratory, and probe motion artefacts could make this exam challenging, as well as the patient's ability to accomplish the instructions based on his mental and physical state[15].

The first D-CEUS examination was performed on oncological renal illness more than two decades ago [16]. Since then, this technique has spread to several medical specialties, especially in the gastroenterological setting and not only for oncological diseases. In this review we summarize the evidence exploring the application of D-CEUS in gastrointestinal diseases.

LIVER DISEASES

In liver diseases, D-CEUS has been explored primarily for its usefulness in characterizing focal liver lesions (FLLs). Several applications of D-CEUS in predicting biological behaviour, differential diagnosis, and prognosis have been explored. These studies are summarized in Table 2, and Figure 2 illustrates the



Table 1 Dynamic contrast-enhanced ultrasound parameters in time-intensity curve								
Abbreviation	Parameter	Definition	Unit					
АТ	Arrival time	Time from administration of UCA to the beginning of the curve	s					
AUC or WiWoAUC	Area under the curve or wash-in and wash-out area under the curve		AIU					
FT	Fall time	Time from PE to point where tangent of descending curve across x-axis	S					
IMAX or MI	Maximum intensity	Maximum intensity of the curve	AIU					
MTT	Mean transit time	Mean time taken by contrast to pass through the ROI	s					
PE	Peak enhancement	Maximum intensity of the curve	AIU					
PI	Peak intensity	Maximum intensity of the curve	AIU					
Pw	Slope coefficient of wash-in	Coefficient of the enhancement wash-in slope	AIU × s					
RT	Rise time	Time from PE to point where tangent of ascending curve across x-axis	S					
TPI or TTP or TP	Time to peak	Time from the beginning of the curve to peak	s					
WiAUC	Wash-in area under the curve	AUC from the beginning of the curve to PE	AIU × s					
WoAUC	Wash-out area under the curve	AUC from the PE to the end of the curve	AIU × s					
WiR	Wash-in rate	Tangent at the ascending part of the curve	AIU × s					
WoR	Wash-out rate	Tangent at the descending part of the curve	AIU × s					

AIU: Absolute intensity unit; ROI: Region of interest; UCA: Ultrasound contrast agent.



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Figure 1 Time-intensity curve and derived parameters. AIU: Absolute intensity unit; AT: Arrival time; FT: Fall time; MTT: Mean transit time; PE: Peak enhancement; s: Second; TTP: Time to peak; WiAUC: Wash-in area under the curve; WiR: Wash-in rate; WiWoAUC: Wash-in and wash-out area under the curve; WoAUC: Wash-out area under the curve; RT: Rise time.

use of D-CEUS to characterize liver lesions.

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Table 2 Dynamic contrast-enhanced ultrasound and liver diseases

Ref.	Study design/number of patients	Object of D- CEUS	Population/groups	Machine/UCA/software	Significant results (<i>P</i> < 0.05)
Wildner <i>et</i> <i>al</i> [<mark>31</mark>], 2022	Prospective/17	Melanoma liver metastasis	Patients with melanoma liver metastasis treated with sorafenib/Responders' vs non- responders	Sequoia 512/SonoVue/Qontrast	Increase of MTT and TTP is associated with response to treatment and prognosis
Gu et al[<mark>30</mark>], 2022	Retrospective/97	НСС	Patients with HCC underwent thermal ablation	Acuson Sequoia, Phillip EpiQ7/SonoVue/VueBox	WiAUC, WoAUC, and WiWoAUC ratios between HCC and surrounding parenchyma before ablation were predictors of survival
Huang <i>et al</i> [<mark>18]</mark> , 2022	Prospective/120	НСС	Patients with HCC underwent biopsy/Low-Ki-67 <i>vs</i> high-Ki-67	Logiq E9/Sonazoid/NovoUltrasound Kit	PE difference between HCC and distal liver parenchyma was different in the Kupffer phase
Li et al <mark>[22]</mark> , 2022	Retrospective/31	НСС	Patients with HCC underwent surgery/MVI-positive <i>vs</i> MVI- negative	Phillip EpiQ7/Sonazoid/built-in auto contrast software	None of the D-CEUS parameters was related to MVI
Zocco et al [28], 2013	Prospective/46	Liver parenchyma	Cirrhotic patient underwent HVPG/clinically significant portal hypertension <i>vs</i> severe portal hypertension	iU22/SonoVue/QLAB	Negative correlation between PI, Pw and HVPG. Positive correlation with MTT. AUROC of 1.00 for PI < 23.3 AIU to predict clinically significant portal hypertension
Dong <i>et al</i> [<mark>21]</mark> , 2021	Retrospective/16	НСС	Patients with HCC underwent surgery/MVI-positive <i>vs</i> MVI-negative	Acuson Oxana, Logiq E9, Siemens Acuson Sequoia/SonoVue, Lumason/VueBox	WiAUC and WoAUC were higher in MVI positive group
Schwarz <i>et</i> <i>al</i> [<mark>24</mark>], 2021	Retrospective/139	Focal liver lesion	Patients with diagnosed focal liver lesion/malignant versus benign	Acuson Sequoia, S2000 or S3000 and Phillip EpiQ7/SonoVue/VueBox	RT and late phase ratio were different between malignant and benign liver lesion
Xuan <i>et al</i> [<mark>19]</mark> , 2021	Retrospective/128	НСС	Patients with HCC underwent biopsy or surgery/highly-differ- entiated <i>vs</i> moderate-differen- tiated <i>vs</i> poorly-differentiated	-/SonoVue/-	RT and MTT increased from poorly- to moderate- to highly-differentiated. Enhancement rates decreased from poorly- to moderate- to highly-differ- entiated
Amadori <i>et</i> <i>al</i> [32], 2018	Prospective/37	CRC Liver metastasis	Patients underwent chemotherapy/chemotherapy <i>vs</i> chemotherapy plus bevacizumab	iU22 vision 2008/SonoVue/QLAB	Reduction of PI and AUC and increase of TPI correlated with higher PFS in chemotherapy plus bevacizumab group
Wildner <i>et</i> al[25], 2019	Prospective/148	Focal liver lesion	Patients with focal liver lesion and subsequent final diagnosis/HCC, CCC, PCA, CRC, BC, MM, FNH	Sequoia 512/SonoVue/VueBox	Higher PE and WiWoAUC in HCC than CRC. Lower Relative intensity signal for PCA and CRC compared to HCC at 30 and 120 s after PE
Mogensen <i>et al</i> [<mark>33</mark>], 2017	Prospective/12	CRC liver metastasis	Patients underwent chemotherapy/chemotherapy vs chemotherapy plus bevacizumab	Logiq E9/SonoVue/VueBox	Early changes of PE correlate with tumor shrinkage at CT scan
Zocco et al [28], 2013	Prospective/28	НСС	Patients treated with sorafenib/Responders' <i>vs</i> non- responders	iU22/SonoVue/QLAB	PI, Pw and AUC 10% decrease correlate with response to therapy. AUC 10% decrease and increased/unchanged TPI and MTT are associated with longer survival. Decrease of Pw is associated with PFS
Zhan <i>et al</i> [20], 2019	Prospective/35	НСС	Patients with HCC underwent microwaves ablation	Acuson Sequia/Sonovue/SonoLiver	Positive correlation between MVD, VEGF and IMAX; negative correlation

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					between MVD and TTP. TTP was an independent predictor of OS
Wildner <i>et</i> al[<mark>26</mark>], 2014	Prospective/43	HCC, ICC	Patient with proven HCC and ICC	Acuson Sequoia 512/SonoVue/VueBox	FT and MTT were lower in ICC than HCC. Relative signal intensity was lower in ICC than HCC in all time point after PE
Frampas <i>et</i> al[69], 2013	Prospective/19	HCC	Patients with HCC treated with sorafenib or sunitinib/RECIST progressor <i>vs</i> non-progressor	Aplio XV/SonoVue/Vascular Recognition Imaging" mode	AUC decrease ≥ 40% correlated with RECIST non-progression
Lassau <i>et al</i> [<mark>29</mark>], 2011	Prospective/42	НСС	Patients with HCC treated with Bevacizumab	Aplio scanner/SonoVue/Contrast Harmonic Imaging-Quantification software	AUC, WiAUC, WoAUC and TPI decrease correlate with tumor response. TPI decrese correlate with PFS. AUC and WoAC decrease correlate with OS

AIU: Arbitrary intensity unit; AUC: Area under the curve; AUROC: Area under the receiver operating characteristic; BC: Breast cancer; CCC: Cholangiocarcinoma; CRC: Colorectal cancer; CT: Computed tomography; D-CEUS: Dynamic contrast-enhanced ultrasound; FNH: Focal nodular hyperplasia; HCC: Hepatocellular carcinoma; HVPG: Hepatic vein portal pressure; MM: Malignant melanoma; MTT: Mean transit time; MVD: Microvascular density; MVI: Microvascular invasion; OS: Overall survival; PCA: Pancreatic adenocarcinoma; PE: Peak enhancement; PFS: Progression free survival; PI: Peak intensity; Pw: Slope coefficient of wash-in; RECIST: Response evaluation criteria in solid tumors; RT: Rise time; TPI: Time to peak intensity; TTP: Time to peak; UCA: Ultrasound contrast agent; WiAUC: Wash-in area under the curve; WoAUC: Wash-out area under the curve; WiWoAUC: Wash-in and wash-out area under the curve.



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Figure 2 Dynamic contrast-enhanced ultrasound and time-intensity curves of the liver. A: Hypoechoic mass (hepatocellular carcinoma) of IV liver segment visualized in B-mode ultrasound; B and C: Contrast-enhanced ultrasound with corresponding time-intensity curve of the liver mass.

Biological behaviour

The vascular structure of a tumour lesion is closely associated with microscopic features such as the degree of differentiation, the proliferation index and growth rate, the presence of necrosis, the angiogenesis and the vascular invasion[17]. Therefore, the analysis of vascularization parameters could be different according to each of the aforementioned characteristics.

Huang et al[18] investigated the correlation between perfusion parameters and Ki-67 in hepatocellular carcinoma (HCC) patients. Prospective analysis of one hundred twenty patients showed that the peak enhancement (PE) difference between HCC and distal liver parenchyma in the Kupffer phase was significantly higher in low Ki-67 (< 10%) group compared to high Ki-67 (≥ 10%) group, probably due to lower concentration of Kupffer cells in poorly differentiated neoplasms[18]. Supporting this, differences in D-CEUS parameters were found among HCC differentiation classes. Specifically, rise time (RT) and time to peak (TTP) demonstrated a significant positive correlation with differentiation degree, whereas



enhancement rate was significantly higher in lesions with less differentiation^[19].

Regarding the pure vascular features of tumors, Zhan et al^[20] demonstrated that histological determined microvessel density and vascular endothelial grow factor stain positively correlated with maximum intensity (MI), while microvessel density negative correlated with TTP[20]. This suggested that D-CEUS parameters could provide a reliable characterization of the microvascular scaffold of lesions. In another study Dong *et al*[21] perform a retrospective analysis of D-CEUS characteristics of HCC to investigate the capability to predict microvascular invasion in a cohort of 16 patients who underwent subsequent surgery. They found that wash-in area under the curve (WiAUC) and wash-out area under the curve (WoAUC) were significantly higher in microvascular invasion positive group (P <0.05), especially when the ROI was positioned in the marginal area of the lesion. This phenomenon is likely to be attributed to the formation of arteriovenous fistulas during vascular invasion, which leads to an increase in blood flow [21]. In contrast, using different UCA, Li et al [22] found no correlation between quantitative parameters and microvascular invasion in thirty-one resected HCCs[22]. One of the most significant differences between second-generation UCAs is their resistance to US wave pressure, which could explain this apparent contradiction in results[23].

This evidence suggests that D-CEUS may serve as a biomarker of the biological behaviour and microscopic characteristics of HCC, detecting the abnormal vascularization characteristics that developed as the disease progressed.

Differential diagnosis

Quantitative analysis of perfusion parameters demonstrated a promising potential to distinguish between benign and malignant FLLs. In a retrospective study including one hundred and thirty-nine FLLs of which forty-four benign and ninety-five malignant, benign lesions showed a significantly higher late-phase ratio (ratio between signal intensities of lesion and surrounding tissue in late phase, LPR) compared to malignant counterpart, showing an area under the curve (AUC) of 0.9, with maximal sensitivity (100%) but low specificity (56.8%). Interestingly, the difference in LPR remains significant also comparing hypoechoic haemangiomas to malignant lesions, suggesting the ability to distinguish a real wash-out from other phenomena. Although RT demonstrated a lower AUC (0.58) for distinguishing benign from malignant lesions, it demonstrated outstanding accuracy (AUC: 0.91) when applied to the distinction between haemangioma and malignancy. Furthermore, considering only benign lesions, haemangioma and adenoma displayed longer mean RT values than other benign lesions[24]. This suggests that quantitative analysis could increase the diagnostic accuracy between benign liver lesions and in challenging situations, such as benign lesions with moderate hyperenhancement (e.g., thrombosed haemangioma) or modest hypoenhancement in late phase (e.g., certain subtypes of hepatic adenoma).

D-CEUS might be helpful to differentiate hypervascular tumours like HCC from other malignant liver lesions that are predominantly necrotic and hypovascular. Wildner et al[25], analysing D-CEUS parameters in HCC and different secondary liver lesions showed that PE normalized for parenchyma signal and wash-in-wash-out area under the curve (WiWoAUC) were significantly higher in HCC compared to colorectal cancer (CRC) metastasis and relative signal intensity at 30 and 120 s after PE was significantly lower for pancreatic adenocarcinoma and CRC liver metastasis compared to HCC[25]. These results clearly suit to the hypervascular nature of HCC in contrast to the more necrotic and weakly centrally vascularized secondary liver lesions of other primitive cancers. While arterial phase parameters are significantly different between HCC and CRC metastatic liver masses, their applicability to differentiate HCC from other primary intrahepatic malignancies is unfitted. Previously, the same authors had investigated the differences between HCC and intrahepatic cholangiocellular carcinoma (ICC) showing no significant differences in arterial phase parameters while ICC group showed lower values of mean transit time (MTT) and fall time in portal and venous phase. Furthermore, relative signal intensity was significantly lower in ICC compared to HCC in all time points after PE at 40 s, 80 s, 100 s and 120 s^[26]. Actually, the main difference between HCC and ICC at CEUS is the early and marked wash-out in portal phase for ICC[4]. Objective quantification of the wash-out phenomenon using D-CEUS could improve the diagnostic accuracy of differentiating lesions with similar portal and late phase hypoenhancement.

Prognosis prediction

One of the most promising applications of D-CEUS is the assessment of liver tumour response to treatment, particularly in HCC where chemotherapy regimens are mostly based on vascular-targeting agents[27]. To this purpose, Zocco et al[28] investigated the role of D-CEUS to early detect vascular changes in HCC patients treated with sorafenib and to predict response to therapy and prognosis. The results showed that a decrease in AUC, peak intensity (PI), and slope of wash-in (Pw) between T0 (baseline) and T1 (after fifteen days of therapy) was significantly associated with response to therapy assessed with RECIST criteria after two months of treatment. Furthermore, 10% decrease in AUC was significantly associated with longer survival as increased/unchanged of time to PI (Tp) and MTT, while a Pw reduction was significantly associated with progression-free survival (PFS)[28]. Similar results were obtained by Lassau *et al*^[29] considering patients with advanced HCC treated with bevacizumab. D-CEUS was performed before treatment and at days 3, 7, 14, and 60 after treatment; and every 2 mo



thereafter. Interestingly, the results showed that very early changes in D-CEUS characteristics correlated with response to therapy and prognosis. Particularly, the decreases in AUC, AUC during wash in, AUC during washout, and time to PI (TPI) at day 3 were significantly associated with RECIST response at 2 mo. Furthermore, PI, AUC and AUC during washout changes at day 3 were correlated with PFS and overall survival (OS)[29]. These results suggest that effects of antiangiogenetic treatments can be early assessed quantifying the perfusion parameters and could allow for a tempestive intervention when relative prognosis is unfavourable.

D-CEUS could also have a role in predicting the prognosis of HCC patients who received locoregional treatments. In HCC patients undergoing microwave ablation, TTP evaluated before the procedure was confirmed as an independent predictor of OS[20]. Similarly, the WiAUC, the WoAUC and the WiWoAUC ratio between HCC lesion and surrounding liver parenchyma evaluated before thermal ablation were significantly associated with survival [30]. As a consequence, quantitative perfusion evaluation might provide additional information useful to plan treatment procedures.

Considering non-HCC malignant liver lesions, different studies evaluated D-CEUS modifications to predict early response to therapies. In patients with liver metastasis of melanoma treated with sorafenib, TTP and MTT increased significantly in responders group at 15 and 56 d assessment[31]. Furthermore, CRC-metastatic patients treated with chemotherapy plus bevacizumab showed changes in derived PI, TPI and AUC at day 15 that were significantly correlated with PFS, however these modifications were not related with tumor response or survival[32]. In contrast, Mogensen et al[33] observed a significant association between PE early variation and computed tomography (CT) dimensional tumour decrease in patients treated with chemotherapy plus bevacizumab[33]. Variability in these results could be explained by limitations of 2-dimensional imaging techniques used in all the discussed studies. In the future, 3-dimensional D-CEUS might provide a more accurate evaluation of entire tumor features[34].

Non-oncological hepatic application of D-CEUS

Despite most studies primarily focused on neoplastic liver disease, the application of quantitative analysis of perfusion parameters has also been explored in chronic liver disease. The TICs of the liver parenchyma can provide information's of intrahepatic blood flow and, indirectly, of portal vein pressure, either that could be altered by liver fibrosis.

In the past, different studies evaluated transit time between vessels to estimate the intrahepatic blood flow and to assess liver fibrosis stage. It was showed that hepatic vein transit time decreased as severity of histologically proved chronic hepatopathy increased, thus allowing diagnosis of severe fibrosis with an accuracy of 79% [35,36]. In addition, hepatic vein arrival time and intrahepatic transit time were correlated with hepatic venous pressure gradient (HVPG), with an area under the receiver operating characteristic (AUROC) of 0.97 for HVPG > 10 mmHg and 0.94 for HVPG > 12 mmHg, respectively[37, 38].

Recently, perfusion parameters analysis of liver parenchyma showed a decrease of amplitudeparameters and an increase of time-dependent parameters according to grade of portal pressure assessed with HVPG. Interestingly, PI resulted significantly negative correlated with portal hypertension and showed high accuracy (100% for both specificity and sensitivity) to predict clinical significant portal hypertension using a cut-off of 23.3 dB in patients with liver cirrhosis[39].

PANCREATIC DISEASES

Existing evidence about the usefulness of D-CEUS for pancreatic diseases is very limited and is resumed in Table 3. Characterization and differential diagnosis of benign and malignant pancreatic lesions are the focus of the available research. Figure 3 depicts an example of D-CEUS in pancreatic disease.

One of the first studies regarding the perfusion analysis of pancreatic cancer was conducted by D'Onofrio et al[40]. Prospectively, ten patients with suspected pancreatic ductal adenocarcinoma (PDAC) (as confirmed by histology) underwent CEUS with subsequent quantitative perfusion analysis. The results showed a significant difference in PE and ascending curve between PDAC and normal pancreatic parenchyma, providing an objective quantification of enhancement for the assessment of pancreatic lesion[40].

Chronic pancreatitis (CP), localized autoimmune pancreatitis (AIP), and paraduodenal pancreatitis can present CT and magnetic resonance imaging abnormalities identical to PDAC and, vice versa, potentially resectable malignant lesions can be misdiagnosed due to their similarities with benign masses[41]. In such instances, D-CEUS may reveal the pathophysiological differences between highly vascularized inflammatory lesions and essentially necrotic malignant masses, enabling a correct differentiation between benign lesions and cancer. To compare CP to PDAC, Kersting et al[42] performed D-CEUS in sixty undetermined pancreatic lesions that were histologically characterized as PDAC (fourtyfive) or inflammatory lesion in CP (fifteen). The grouped analysis of TICs showed that TTP and arrival time were significantly prolonged in PDAC compared to CP. On the contrary, no differences were detected in MI and AUC between the two pathological conditions^[42]. Regarding AIP, D-CEUS with quantitative analysis has the potential to make pre-operative differential diagnosis between focal-type



Table 3 Dynamic contrast-enhanced ultrasound and pancreatic diseases

Ref.	Study design/number of patients	Object of D- CEUS	Population/groups	Machine/UCA/Software	Significant results (<i>P</i> < 0.05)
Yang <i>et al</i> [47], 2023	Retrospective/42	pNET	Patients with histopatholo- gically proved pNET/G1, G2, G3, pNEC	Acuson Sequoia, Acuson Oxana2/SonoVue/VueBox	rPE, rMTT and rAUC were higher in pNETs G1/G2 than G3/pNECs
Zhang <i>et al</i> [4 5], 2020	Prospective/11	LAPC	Patient with LAPC underwent chemoradio- therapy	Acuson Oxana2/SonoVue/SonoLiver	MI decreased after chemora- diotherapy
Vitali <i>et al</i> [<mark>43</mark>], 2015	Prospective/20	PC, focal AIP	Patients with diagnosis of AIP <i>vs</i> histologically proved PC	Acuson Sequoia 512, S200/SonoVue/VueBox	The difference in PE (dPE) between lesion and surrounding parenchyma in AIP was lower compared to dPE in PC
D'Onofrio <i>et</i> al[40], 2014	Prospective/10	Suspected PDAC	Patients with suspected and then histologically proved PDAC	Acuson S2000/SonoVue/VueBox	PE and ascending curve values were different between lesion and adjacent parenchyma
Kersting <i>et al</i> [42], 2009	Prospective/60	Undefined pancreatic lesion	Patients with undefined pancreatic lesion underwent biopsy/PDAC <i>vs</i> CP	Sonoline Elegra/SonoVue/Axius ACQ	TTP and AT were longer in PDAC compared to focal masses in CP

AIP: Autoimmune pancreatitis; AT: Arrival time; CP: Chronic pancreatitis; D-CEUS: Dynamic contrast-enhanced ultrasound; LAPC: Local advanced pancreatic cancer; MI: Maximum intensity; PC: Pancreatic cancer; PDAC: Pancreatic ductal adenocarcinoma; PE: Peak enhancement; pNET: Pancreatic neuroendocrine tumor; pNEC: Pancreatic neuroendocrine carcinoma; rAUC: Relative area under the curve; rMTT: Relative mean transit time; rPE: Relative peak enhancement; TTP: Time to peak; UCA: Ultrasound contrast agent.



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Figure 3 Dynamic contrast-enhanced ultrasound and time-intensity curves of the pancreas. A: Hypoechoic mass (adenocarcinoma) of pancreatic head in B-mode ultrasound; B and C: Contrast-enhanced ultrasound with corresponding time-intensity curve of the pancreatic lesion.

> AIP and PDAC non-invasively. Vitali et al[43] compared D-CEUS parameters of three patients with focal AIP with seventeen PDAC patients. Specifically, the difference between PE of PDAC and circumjacent normal parenchyma was significantly lower as compared to AIP. Significant was also the difference between wash in index perfusion (WiPI = WiAUC/RT) of PC and AIP[43]. In another study, TICs of AIP lesions showed delayed and higher enhancement compared to PDAC. Among all CEUS perfusion parameters, ratio of PE, WiAUC, wash-in rate (WiR), WiPI, WoAUC, WiWoAUC, and wash-out rate (WoR) between pancreatic lesion and surrounding normal pancreatic tissue were significantly higher in AIP lesions than PDAC lesions[44]. In cases of diffuse AIP, quantitative perfusion analysis is not suggested since there is no healthy parenchyma for comparison, which is necessary to enhance the



comparability of the results regardless of exam- or patient-related factors[42].

Similarly to perfusion analysis for malignant liver lesions, D-CEUS could be considered to evaluate the response to therapies in PDAC. Recently, Zhang et al[45] investigated the role of D-CEUS to monitor the response to chemoradiotherapy in eleven patients with local advanced pancreatic cancer. They performed D-CEUS at baseline and after four weeks of therapy and analyzed the variation of TICs and related parameters. The rising and falling slope rates of TICs diminished after four weeks, and the percentage of MI decreased significantly compared to the surrounding normal parenchyma^[45]. Since MI is related to tumour microvascular density, its reduction is coupled with a decrease in lesion blood perfusion, and the quantification of this consequence might reflect the objective efficacy of chemotherapy.

Lastly, D-CEUS could provide information in other types of pancreatic tumors. It has been proven that pancreatic neuroendocrine tumors (pNETs) with different histopathological grades have differences in tumor microvascular perfusion [46], therefore Yang et al [47] analyzed the correlation between perfusion analysis and histopathological grades of pNETs. In forty-two patients, the TICs shape of grade 1 (G1)/grade (G2) lesions were significantly different compared to TICs of grade 3 (G3)/ pancreatic neuroendocrine carcinomas (pNECs). Significant differences were revealed at relative RT, relative MTT and relative AUC which were higher in G1/G2 than G3/pNECs. ROC analysis showed that relative AUC had the higher accuracy to distinguish the two groups^[47]. The D-CEUS analysis and quantitative parameters have the potential value to non-invasively predict the biological behaviour of pNETs.

INFLAMMATORY BOWEL DISEASES

Table 4 summarizes the evidence regarding the role of D-CEUS in inflammatory bowel diseases, and Figure 4 depicts a TIC derived from intestinal wall examination. These studies are mainly focused on Crohn's disease (CD).

D-CEUS and inflammatory/fibrotic disease

TICs represent the perfusion of a tissue, and we would expect a difference in perfusion parameters not only when comparing fibrosis to inflammation, but also when considering various degrees of inflammation.

Girlich et al[48] described for the first time the difference in perfusion parameters between CD and healthy gut. As expected, the differences were substantially with significantly higher PE and regional blood volume, and longer TTP of thickness bowel wall of CD patients[48]. In another study, the same authors investigated the correlation between perfusion parameters and histopathological characteristics of the gut wall in surgically treated CD patients, confirming that TTP was negatively correlated with the histological inflammatory score[49]. Similarly, a higher PE, regional blood flow and regional blood volume, and shorter TTP were significantly correlated with high vascular density defined as the presence of more than two hundred sixty five vessels per field on histological examination[50]. On the contrary, Ripollés et al[51] showed a non-significant association of TTP with inflammatory or fibrosis histological score, however they used different histological scores and had longer time between CEUS and surgery $(34.5 \pm 17.3 vs 4.7 \pm 4.7 d)$ [51].

To assess the differences between fibrotic or inflammatory CD, Nylund *et al*[52] compared sixteen patients with fibrotic disease undergoing surgery to seventeen patients with medically treated active inflammatory disease. In inflammatory disease, the blood volume and blood flow were significantly higher compared to fibrotic disease, while MTT was not significantly different between the two groups. Interestingly, blood volume/bowel wall thickness ratio showed a high accuracy to predict surgery[52]. Similarly, Quaia et al[53] found a significant difference in blood volume related parameters (PE, AUC, WiAUC, WoAUC, WiR and, WiPI) between fibrotic strictures and inflammatory strictures among patients with CD, with the latter having higher values. However, TTP showed no significant differences between the two groups[53]. These findings imply that D-CEUS can detect the microvascular differences between fibrotic and inflammatory tissue and allows non-invasive differentiation of CD phenotypes.

Regarding ulcerative colitis (UC), only one prospective study investigated the relationship between perfusion parameters and histopathological findings. In this study, TTP/Peak(%) showed a strong negative correlation with histopathological inflammatory activity score[54].

D-CEUS and clinical outcomes

Given the negative correlation with inflammation, Peak > 40.5% and TTP < 35 seconds had a high positive predictive value (94%) and a high negative predictive value (92.3%) for active disease, respectively^[50].

It has also been shown that the evaluation of PE and AUC is a useful tool to assessing the severity of CD when the ultrasound global assessment and colorDoppler imaging criteria are indeterminate. Particularly, PE > 23 dB showed a sensitivity and specificity of 90% and 89.5%, respectively, to distinguish moderate from severe disease [55]. Using this cut-off to identify patients with severe disease, Wilkens et



Table 4 Dynamic contrast-enhanced ultrasound and inflammatory bowel diseases

Ref.	Study design/number of patients	Object of D- CEUS	Population/groups	Machine/UCA/Software	Significant results (<i>P</i> < 0.05)
Laterza <i>et al</i> [60], 2021	Prospective/44	CD	Patients with CD treated with anti- TNFa/responders <i>vs</i> non-responders	iU22/SonoVue/QLAB	Correlation between decrease in PI, AUC, Pw, and MTT and response to therapy
Goertz <i>et al</i> [57], 2018	Prospective/18	CD and UC	Patients with CD or UC treated with vedolizumab/responders <i>vs</i> non-responders	Acuson S2000/SonoVue/VueBox	WiR was lower in responders' group after 14 wk
Wilkens <i>et al</i> [<mark>56</mark>], 2018	Retrospective/104	CD	Patients with severe CD underwent CEUS/normal vs atypical intensity decline on CEUS	iU22/Definity/QLAB	AUC, wash-out time, and intensity at 60s and 120s were higher in atypical decline group and this correlated with bad outcomes
Quaia et al[<mark>53]</mark> , 2017	Prospective/65	CD	Patients with CD with terminal ileal loop stricture histologically charac- terized/inflammatory <i>vs</i> fibrostenotic disease	iU22/SonoVue/VueBox	PE, WiR, WiPI, AUC, WiAUC and WoAUC were higher in inflammatory group compared to fibrostenotic gruoup. TTP was not different between the two groups
Medellin- Kowalewski <i>et</i> al[55], 2016	Retrospective/127	CD	Patients with CD underwent US and CEUS	iU22/Definity/QLAB	PE correlate with wall thickness
Quaia et al <mark>[61]</mark> , 2016	Prospective/50	CD	Patient with CD underwent medical treatment/responders <i>vs</i> non- responders	iU22/SonoVue/VueBox	Changes in PE, WiR, WoR, WiPI, AUC, WiAUC, and WoAUC from baseline to six weeks after therapy differed between responders and non- responses
Socaciu <i>et al</i> [<mark>58]</mark> , 2015	Prospective/38	CD and UC	Patients with CD or CU candidate for medical treatment	Logiq 7/SonoVue/SonoLiver	Logarithm of AUC correlated with endoscopic improvement in both diseases
Saevik <i>et al</i> [59], 2014	Prospective/14	CD	Patients with CD started medical treatment/remission <i>vs</i> treatment failure	Logiq E9/SonoVue/VueBox	PE, WiR, WoR, and WiAUC were different between two groups at 1 mo of treatment
Romanini <i>et al</i> [50], 2014	Prospective/33	CD	Patients with CD undergoing colonoscopy and biopsy	Sequoia 512/SonoVue/Qontrast	Correlation between high vascular density and Peak% and regional blood flow
Ripollés <i>et al</i> [<mark>51</mark>], 2013	Prospective/25	CD	Patients with CD undergoing elective bowel resection/inflammatory vs fibrostenotic disease	Aplio 80/SonoVue/Software in Aplio 80 system	The percentage of increase in contrast enhancement of the bowel wall in inflammatory lesions was greater than fibrotic lesions
Nylund <i>et al</i> [<mark>52]</mark> , 2013	Prospective/33	CD	Patient with CD underwent surgery or medical treatment/inflammatory <i>vs</i> fibrostenotic disease	Logiq E9/SonoVue/Custom software	Blood flow and blood volume were higher in the medical group compared to surgery group
Girlich <i>et al</i> [<mark>54]</mark> , 2012	Prospective/11	UC	Patients with UC undergoing endoscopy	Logiq E9/SonoVue/Qontrast	Negative correlation between TTP/Peak% and histopatho- logical score
Girlich <i>et al</i> [<mark>49]</mark> , 2011	Prospective/20	CD		Logiq 9/SonoVue/Qontrast	Negative correlation between TTP and histopathological score. Positive correlation with single items of the score
Girlich <i>et al</i> [48], 2009	Prospective/20	CD	Patients with active CD vs healthy volunteers	Logiq 9/SonoVue/Qontrast	Higher PE and regional blood volume and shorter TTP in CD

AUC: Area under the curve; CD: Crohn's disease; CEUS: Contrast-enhanced ultrasound; D-CEUS: Dynamic contrast-enhanced ultrasound; MTT: Mean transit time; PE: Peak enhancement; PI: Peak intensity; Pw: Slope coefficient of wash-in; TNF-a: Tumor necrosis factor-a; TTP: Time to peak; UC: Ulcerative colitis; UCA: Ultrasound contrast agent; US: Ultrasound; WiAUC: Wash-in area under the curve; WiPi: Wash-in ratio index; WiR: Wash-in rate; WoR: Wash-out rate.

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Figure 4 Dynamic contrast-enhanced ultrasound and time-intensity curves of the bowel wall. A: B-mode ultrasound features of ileal bowel wall thickening in Crohn's disease; B and C: Contrast-enhanced ultrasound with corresponding time-intensity curve of the bowel wall thickening.

> al[56] investigated the clinical outcomes in twenty patients with severe disease in whom they observed an atypical, prolonged intestinal washout due to the stuck bubble phenomenon (AUC > 20000 AIU) compared to patient with severe disease and AUC < 20000 AIU (control group). They found a significative higher rate of surgery and a trend toward more combination therapies in study group. Interestingly, they ascribed the stuck bubbles phenomena to the attachment of microbubbles to active leukocytes on the endothelium; if confirmed, this might be utilized as a tool for targeted therapy [56].

> Assessment of perfusion parameters is important for identifying disease status, as well as monitoring and predicting the efficacy of therapies. Indeed, D-CEUS measurements changed significantly between clinical- and endoscopic-assessed responders and non-responders with CD and UC following treatments[57,58]. In a prospective study, Saevik et al[59] included fourteen patients with acute CD who started treatment with steroids or tumor necrosis factor- α (TNF- α) inhibitors. CEUS was performed before starting therapy and at one, three and twelve months. At one month, the differences between patients who achieved clinical remission and those who had treatment failure during the follow-up period were significant. Particularly, PE, WiAUC, WiR and WoR was significantly lower in effective treatment group[59]. The reduction in perfusion parameters could be related to a decrease in inflammation and, thus, a treatment response. Recently, it was demonstrated that the reduction in PI, AUC, Pw, and MTT was higher in patients responding to anti-TNF- α therapy after two weeks than in patients who relapsed within six months of treatment initiation, who displayed not only a lower early reduction in perfusion parameters but also an increase in PI after twelve weeks[60]. Changes in quantitative perfusion analysis parameters between baseline and six weeks after therapy initiation distinguished responders from non-responders defined by clinical and endoscopic evaluation at twelve weeks[61]. This highlights the potential for D-CEUS to detect therapy-induced modifications in the pathologic bowel wall and support clinicians in disease management.

OTHER GASTROINTESTINAL DISEASES

The investigation of perfusion parameters with D-CEUS could be an informative tool in the diagnosis and prognosis of other gastrointestinal diseases, such as gastric cancer[62]. In a prospective study including forty-three patients with advanced gastric cancer, Joo et al[63] showed a good feasibility of CEUS (88.4%) and a significant difference in PI and AUC according to differentiation status of the tumor. Localization in the upper stomach and an ulcerated phenotype were the limiting variables for D-CEUS feasibility[63]. Regarding the CRC, the difference in AUC was significantly related with tumor



necrosis and T stage[64], suggesting a possible role of D-CEUS in predicting tumor microenvironment characteristics and behavior. Furthermore, dynamic contrast enhanced endoscopic ultrasound (D-CE-EUS) showed a significant correlation between RT and vessel density in patients with left side colonic tumors[65], indicating that perfusion analysis might be useful to predict outcomes of antiangiogenic treatment as Lassau *et al*[66] has showed in a multicentric study including over one thousand D-CEUS evaluations in more than five hundred patients with solid tumor treated with antiangiogenic therapy [66]. In particular, D-CEUS blood volume related parameters showed significant early changes in gastrointestinal stromal tumours treated with masitinib, predicting positron emission tomography-CT outcome^[67].

Concerning non-neoplastic bowel diseases, different studies described the CEUS aspects of various inflammatory bowel diseases, including abscesses, acute appendicitis, diverticulitis, vascular bowel disease, such as intestinal ischemia, and graft vs host disease[68]. To the best of our knowledge, no specific study employing D-CEUS in the aforementioned conditions has been published yet; however, it would be desirable to investigate the potential usefulness of quantitative perfusion analysis in predicting pathological features and prognosis also in this field.

CONCLUSION

The quantification of perfusion parameters in CEUS has several applications in gastrointestinal neoplastic and inflammatory disorders. Everything that is visible with ultrasound can be measured, and this has allowed D-CEUS to be employed within the pancreas and digestive system in addition to the liver evaluation. The objective assessment of tissue perfusion is crucial for the evaluation of all disorders in which the vascular component plays a key pathophysiological role, such as malignant tumours and inflammatory bowel disease. However, the limited availability of large studies and the heterogeneity of the technologies employed have precluded the standardization of this approach which potentially represents a valuable tool for clinical practice in management of gastrointestinal diseases.

FOOTNOTES

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MINIREVIEWS

Fractal nature of human gastrointestinal system: Exploring a new era

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Abstract

The morphological complexity of cells and tissues, whether normal or pathological, is characterized by two primary attributes: Irregularity and self-similarity across different scales. When an object exhibits self-similarity, its shape remains unchanged as the scales of measurement vary because any part of it resembles the whole. On the other hand, the size and geometric characteristics of an irregular object vary as the resolution increases, revealing more intricate details. Despite numerous attempts, a reliable and accurate method for quantifying the morphological features of gastrointestinal organs, tissues, cells, their dynamic changes, and pathological disorders has not yet been established. However, fractal geometry, which studies shapes and patterns that exhibit self-similarity, holds promise in providing a quantitative measure of the irregularly shaped morphologies and their underlying self-similar temporal behaviors. In this context, we explore the fractal nature of the gastrointestinal system and the potential of fractal geometry as a robust descriptor of its complex forms and functions. Additionally, we examine the practical applications of fractal geometry in clinical gastroenterology and hepatology practice.

Key Words: Fractals; Geometry; Gastroenterology; Esophagus; Stomach; Colon; Pancreas;


Liver; Time-series; Shape; Behavior

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Core Tip: Utilizing fractal analysis to estimate the fractal dimension is a potent tool for investigating the geometrical properties of the gastrointestinal system. This approach has the potential to improve diagnostic and prognostic rates, leading to better patient care and a deeper understanding of the underlying pathophysiological mechanisms.

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INTRODUCTION

"In One Dimension, did not a moving Point produce a Line with two terminal points? In Two Dimensions, did not a moving Line produce a Square with four terminal points? In Three Dimensions, did not a moving Square produce-did not this eye of mine behold it-that blessed Being, a Cube, with eight terminal points? And in Four Dimensions shall not a moving Cube-alas, for Analogy, and alas for the Progress of Truth, if it be not so-shall not, I say, the motion of a divine Cube result in a still more divine Organization with sixteen terminal points? Behold the infallible confirmation of the Series, 2, 4, 8, and 16 is not this a Geometrical Progression? Is not this-if I might quote my Lord's own words-strictly according to Analogy?" From Flatland: A romance of many dimensions, Edwin A. Abbott.

The complexity of nature, characterized by its various degrees of irregularity and diverse physical properties[1], is a challenging topic. The Scottish biologist and mathematician D'Arcy Wentworth Thompson (1860-1948) proposed that the form of an anatomical entity can be identified through its dimensions and contour. However, Euclidean-based morphometric analyses, which involve measurements like area, perimeter, and form factor, fall short in accurately describing natural, including anatomical, entities due to their irregular shapes that cannot be quantified by means of the classical Euclidean geometry, which is only able to describe regular geometrical objects. Mandelbrot[2] (1924-2010) introduced the notion of fractal geometry[3] and coined the term "fractal" to describe spatial or temporal irregular patterns[4,5]. Fractal geometry has been suggested as an instrument to define the geometry of various natural entities, such as mountains, coastlines, clouds, trees, molecular structures, and an array of forms within anatomical systems along with their underlying dynamic processes [6]. The gastrointestinal or digestive system is a complex network of organs including the mouth, pharynx, esophagus, stomach, small and large intestines, rectum, and anus, as well as the salivary glands, liver, gallbladder, and pancreas. The morphological intricacy of the gastrointestinal system, both in its natural state and under pathological conditions, is mainly characterized by its irregularity and self-similarity across varying scales^[7]. Classical examples of irregularly-shaped histological entities that defy Euclidean geometric principles include gastric pits, which are indentations in the stomach signifying entrances to tubular gastric glands, and villi, which are finger-like structures that significantly increase the surface area of the small intestine^[8]. The human stomach is known to have millions of these pits across the surface of its lining epithelium. The adult small intestine, with an average inner surface area of around 30 m²[9,10], necessitates such a large surface for effective metabolism and nutrient absorption. This is achieved by forming unique irregularly-shaped structures like villi and microvilli. The functional efficiency of epithelia is enhanced by their fractal organization, which expands the surface area for processes like absorption or sensory reception, thereby enabling more efficient interaction with the external environment[11]. This level of surface area and functional efficiency would be unattainable if the colonic mucosa were to be represented by a two-dimensional Euclidean plane. However, the fractallike characteristics observed in epithelial tissues, which line organ surfaces and body structures, are not universal but rather specific to certain instances. The liver development, commonly described as a fractal-like phenomenon, involves intricate processes and interactions among various cell types. During embryonic development, a bud-like structure known as the hepatic diverticulum, which emerges from the endodermal layer of the early gut tube, forms the liver. The hepatic diverticulum then undergoes branching morphogenesis, leading to a complex network of interconnected structures that enable liver tissue expansion and development of bile ducts, blood vessels, and functional units like lobes or lobules. Similarly, pancreas development is a regulated process involving growth and branching. While the developing liver and pancreas' branching patterns may superficially resemble fractal structures, it's



crucial to acknowledge that true fractals display self-similarity across multiple scales, a trait not precisely seen in liver development. Fractal geometry has been used to describe pathological entities within the gastrointestinal system as well. The irregularity in the shape of the interface between epithelial and connective tissues is a recognized characteristic of malignant and pre-malignant epithelial lesions. However, efforts to objectively assess this have been sparse. Landini and Rippin[12] have applied fractal geometry as an instrument for evaluating this irregular epithelial-connective tissue interface[13]. Intestinal polyps, characterized by abnormal tissue overgrowth within the intestinal lining, can display varying shapes and structures based on factors like the type of polyp (i.e., adenomatous polyps, hyperplastic polyps), their location, and size. Some polyps, such as villous adenomas, appear to exhibit a self-similar form, hinting at a possible fractal structure within a specific magnification range. The fractal dimension has been leveraged to quantify these polyps, proving to be a more effective discriminator between diagnostic groups than Euclidean measures like area or perimeter length[14]. Several studies suggest that fractal estimation could offer a novel method for examining the nonlinear dynamics of the gastrointestinal system. This approach sheds light on the complexity and unevenness of colonic pressure activity during different periods, providing potential applications for understanding gastrointestinal system functions. As fractal analysis becomes increasingly prevalent in biomedical research, it serves to quantify the irregularities of anatomical entities, their generation patterns, and their physiological and pathological processes. In this context, we delve into the potential of fractal geometry as a tool for understanding the gastrointestinal system and its possible applications in clinical practice.

FRACTAL GEOMETRY: BASIC PRINCIPLES

Fractals, whether anatomical or natural, are characterized by four primary attributes: (1) Irregularity in shape; (2) self-similarity in structure; (3) non-integer or fractal dimension; and (4) scaling. Scaling indicates that the measured features of a fractal depend on the scale at which they are observed[15]. The most significant characteristic of fractal objects is their ability to display similar patterns at different scales, resulting in the component parts having a form that is similar to the whole in all dimensions. Complex curves such as coastline shapes serve as typical examples of this property, where each segment can be regarded as a smaller version of the complete structure in a statistical sense (Figure 1). The term "self-similarity" refers to a property of fractals that can be either geometric or statistical. Geometric selfsimilarity occurs when every smaller piece of an object is an exact replica of the whole object. For instance, the "snowflake" and the "curve", both described by Swedish mathematician Niels Fabian Helge von Koch (1870-1924) in 1904, are examples of geometrically self-similar figures[16]. Another geometrically self-similar figure is the "Sierpinski triangle", originally described by Polish mathematician Waclaw Sierpinski (1882-1969) in 1915. In contrast, statistical self-similarity pertains to biological objects, including natural objects (Figure 2) and anatomical forms (Figure 3). While small components of anatomical systems are not typically exact replicas of the entire system, they still exhibit self-similarity. For instance, if we examine a segment of a tree branch or vascular vessel, it may not look identical to the entire tree or vessel, but it still displays self-similarity. Many anatomical structures have been identified as statistical self-similar entities, including the circulatory system, the biliary tree of the liver, the dendritic structure of neuronal cells, the ductal system of glands, the cell membrane, and the extracellular matrix (ECM) in cases of chronic liver disease[17].

The concept of "dimension" is a fundamental aspect of fractals and represents a characteristic value of the object. There are two definitions of dimension: the first is the "topological dimension", introduced by Austrian mathematician Karl Menger (1902-1985), which assigns an integer number to each point in three-dimensional Euclidean space (E3). For example, a "point" is assigned a dimension of 0, a "straight line" a dimension of 1, a "plane surface" a dimension of 2, and a "three-dimensional figure" a dimension of 3. The second definition was introduced by Felix Hausdorff (1868-1942) and Abram Besicovitch (1891-1970), who assigned a "real number" between the topological dimension and 3 to every natural object in E3. Mandelbrot designated Menger's dimension with the symbol D_y and Hausdorff and Besicovitch's dimension with the symbol D. For all Euclidean figures, D_y and D are equivalent (D_y = D). However, this equality does not apply to all fractal natural objects, as the inequality D > D_y is true. Many methods have been proposed to estimate the fractal dimension, with the "box-counting method" being the most widely used in biomedical sciences to estimate the space-filling properties of anatomical objects in two-dimensional and three-dimensional space.

It applies the following formula: $D = \lim \epsilon \rightarrow 0 [\log N(\epsilon) / \log(1/\epsilon)]$, where D is the box-counting fractal dimension of the object, ϵ is the side length of the box, and $N(\epsilon)$ is the smallest number of boxes of side ϵ required to cover the outline or the surface of the object completely. Because the zero limit cannot be applied to natural objects, the dimension was estimated by the formula D = d, where d is the slope of the graph of Log $N(\epsilon)$ against Log $(1/\epsilon)$. The linear segments of these graphs were identified using the least-squares method of regression, and the gradients of these segments are assessed using an iterative resistant-line method[15,18-25].

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Figure 1 Examples of geometrical self-similar objects. A: "Curve" from Niels Fabian Helge von Koch; B: "Snowflake" from Niels Fabian Helge von Koch; C: "Sierpinski triangle"; D: Mandelbrot set; E: Julia set named after the studies on the geometrical complexity by the French mathematicians Gaston Julia e Pierre Fatou.



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Figure 2 Examples of natural statistical self-similar structures resembling mathematical fractals. A: Marine invertebrates corals; B: Tree branches; C: Water breaking waves; D: Sand dunes.

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Figure 3 Some self-similar tissues in the gastrointestinal system and their corresponding idealized mathematical fractals. A: Intestine villi; B: Liver; C: Pancreatic Langerhans islet; D: Mandelbrot set; E: Diffusion-limited aggregation model; F: Julia fractal islet.

The fractal esophagus

Wax et al[26] conducted a quantitative analysis of nuclear morphometry in epithelial cells using an animal model of esophageal carcinogenesis in 2003. They observed changes in the size and texture of cell nuclei caused by neoplastic transformation and chemopreventive action [26]. However, the fractal dimension did not enhance the diagnostic ability of the technique for classifying neoplasia^[26]. Instead, the increase in fractal dimension observed in dysplastic tissues corresponded with changes in the texture of cell nuclei observed in histological images, where clumped and grainy cell nuclei are typical of dysplasia. The authors concluded that the increased fractal dimension resulted from alterations in the organization of chromatin and proteins within the nucleus, as demonstrated by the changes in the visual appearance of cell nuclei in histological images[26]. A framework for creating a two-dimensional, globally interconnected map to simulate epithelium dynamics has been proposed by Hosseini and Hashemi Golpayegani^[27]. The aim of this model was to facilitate early-stage dysplasia detection using microscopic imagery from endoscopic biopsies. Their findings suggested a potential direct correlation between the system's structural complexity and the unpredictability of its dynamics[27]. Textural analysis of positron emission tomography (PET) and other imaging techniques is gaining interest in measuring intra-tumoral heterogeneity.

Studies have demonstrated that measuring tumor heterogeneity through textural analysis in 18Ffluorodeoxy glucose PET can provide predictive and prognostic information. In a study by Doumou et al[28], sixty-four patients with adenocarcinoma of the lower esophagus underwent 18F-FDG PET/CT imaging for clinical staging before surgery. Using in-house software implemented under MATLAB (The MathWorks Inc.) and calculating 57 textural features, including fractal indexes, they discovered that altering the "bin width" produced poor agreement for most second- and high-order features, with less impact on fractal parameters. The authors concluded that fractal analysis features were relatively resistant to changes in smoothing, segmentation, and bin width. In 2005, Gardner et al[29] noted that in normal subjects, patients with gastroesophageal reflux disease (GERD), and individuals with GERD treated with a proton pump inhibitor, both esophageal and gastric pH reflect an underlying stochastic process that generates a fractal pattern over time. The authors hypothesized that the fractal pattern of esophageal pH and gastric pH, along with the statistical association of sequential pH values for up to 2 h, encodes information regarding gastric acidity. They proposed that depending on the value of gastric acidity, gastroesophageal reflux causes the esophagus to signal the stomach to alter gastric acidity by influencing gastric secretion of acid or bicarbonate^[29]. The authors subsequently confirmed their hypothesis by investigating 26 healthy adults with no history of gastrointestinal disease or symptoms and 27 adult subjects with a history of GERD who experienced heartburn at least four times per week for at least 6 mo[30] Their findings suggest that the esophagus can signal the stomach to alter gastric acidity, depending on the value of gastric pH, and that this ability is impaired in subjects with GERD.



Thus, the self-similar, fractal pattern encodes information about gastric acidity that the esophagus can decode and, when necessary, signal the stomach to reduce gastric pH.

The fractal stomach

In 1994, Demling and Ell[31] conducted a study titled "Fractal stomach ulcer. A new measurement parameter for benign and malignant changes for the future", proposing that fractal geometry could be beneficially applied for the spatial and temporal analysis of the stomach. Abdominal sound analysis is known to aid in the diagnosis of gastrointestinal diseases, and bowel sounds originating from the stomach and intestine are highly variable over time. They are described as loose successions or clusters of sudden bursts. Realistic recordings of abdominal sounds are contaminated with noise and artifacts that must be differentiated from the bowel sounds. More recently, Kölle et al[32] suggested a method of intrinsic mode function-fractal dimension filtering based on a multivariate empirical mode decomposition approach. The intrinsic mode function-fractal dimension filtering method has been applied to a realistic, contaminated dataset, with close to 100% of manually labeled bowel sounds correctly identified[32]. In a novel approach, West et al[33] analyzed fluctuations in human myoelectric gastric activity noninvasively measured from the surface of the abdomen. They estimated the "scaling index" in 17 healthy individuals and found it to be statistically different from a set of surrogate data. The authors also demonstrated that the dynamical pattern of the gastric rate variability time series was significantly reduced in systemic sclerosis patients compared to healthy individuals, indicating a decrease in complexity of the underlying gastrointestinal control system. In terms of clinical relevance, employing fractal image analysis to model the conduction patterns of slow waves (SWs) through detailed networks of interstitial cells of Cajal can enhance our comprehension of the principles driving gastrointestinal myoelectric activity, as well as the diseases linked to its malfunction[34]. Interstitial cells of cajal (ICC) are specialized pacemaker cells in the gastrointestinal system responsible for generating and propagating SWs of depolarization throughout the muscularis propria. These SWs regulate the motility of the gastrointestinal system tract, which is essential for digestion, nutrient absorption, and waste elimination. By using a combined approach based on image analysis and fractal geometry of detailed ICC networks, researchers have demonstrated that modeling SW conduction patterns can aid in improving our understanding of the mechanisms underlying gastrointestinal system myoelectric activity and the diseases associated with its dysfunction[35,36].

Watanabe et al[37] applied fractal analysis to contrast-enhanced computed tomography (CT) images to estimate structural abnormality in gastric tumors and evaluated its biomarker value for predicting survival in surgically treated gastric cancer patients. The authors found that high fractal dimension values of tumors were significantly associated with high T stage and high pathological stage. Moreover, in Kaplan-Meier analysis, patients with higher fractal dimension tumors had significantly worse disease-specific survival, indicating that contrast-enhanced CT fractal analysis could be a useful biomarker for gastric cancer patients, reflecting clinic-pathologic features and survival.

In a study by Liliac *et al*[38], the pattern of E-cadherin at the cell membrane level was analyzed using fractal dimension analysis on fluorescence immunohistochemistry samples labeled with E-cadherin in gastric well/moderate and solid gastric adenocarcinoma from patients without any associated chemotherapeutic treatment or radiotherapy. The images were binarized using a fixed threshold of the E-cadherin fluorescence channel, and the fractal dimension of the binarized image outlines was calculated to assess the ruggedness of the cellular membranes. The study demonstrated that the morphological analysis of a simple marker for the cell membrane can identify and distinguish tumor cells.

The fractal colon

Cross *et al*^[14] suggested that colorectal polyps have a self-similar structure that may contain fractal elements, and that the fractal dimension could be a useful morphometric discriminator. The authors estimated the fractal dimensions of images from hematoxylin and eosin-stained sections of 359 colorectal polyps, including 214 tubulovillous adenomas, 41 tubular adenomas, 29 villous adenomas, 68 metaplastic polyps, and 7 inflammatory polyps, and found that colorectal polyps exhibit a fractal structure within a defined range of magnification[14]. They concluded that fractal dimension can provide a new method of estimating polyp shape and serve as a useful morphometric discriminator between diagnostic categories[14].

It is clear that the invasive front of colorectal cancers (CRCs) can vary in complexity, ranging from smooth to highly complex when the front breaks up into small cell clusters or single cancer cells[39]. Currently, the degree of complexity is estimated visually and/or semi-quantitatively. Franzén et al[39] compared the visual estimation of CRC invasive front irregularity to different quantitative image analytical techniques and developed a "complexity index" for the invasive margin. They analyzed cytokeratin 8-stained tissue sections from 29 CRCs and found that the fractal dimension and tumor cell clusters provided the best correlation to visual grading using discriminant analysis. Moreover, the fractal dimension separated tumors up to a certain level of complexity, while tumor cell cluster counting distinguished cells above and beyond the fractal dimension when the tumor front fragmented into small cell clusters. These results suggest that the fractal dimension could be a valid index for objectively quantifying the complexity of tumor invasive fronts. In another study, Hahn-Strömberg et al[40]



correlated the complexity of colon carcinoma invasive fronts with cell adhesion protein expression and polymorphisms in their genes. They constructed a "complexity index" using computer-assisted morphometry to estimate fractal dimension and tumor cell clusters, followed by tree analysis, for 32 colon carcinomas. Hahn-Strömberg et al[40] found that adhesion protein distribution was significantly altered in most carcinomas, but no correlation was observed between the complexity of the invasive border and protein distribution or genetic alterations. These findings indicate that the complexity of CRC invasion is not solely dependent on genetic derangements in adhesion protein genes or protein distribution, but also on aberrations in the function of other proteins related to adhesive proteins.

In their research, Stefănescu *et al*[41] utilized fractal analysis and neural network modeling of colon mucosa images generated by confocal laser endomicroscopy to develop an automatic diagnosis algorithm for CRCs. They examined 1035 images of normal mucosa and tumor regions, processed using a computer-aided imaging system to achieve an automatic diagnosis. The researchers found that nonmalignant colon mucosa has polyhedral crypt structures while malignant colon mucosa has irregular and interrupted crypts. They estimated seven parameters for each image, with only contrast, homogeneity and feature number showing significant differences between natural and cancer images. Their findings suggest that computed aided diagnosis through fractal analysis of glandular structures can be used in combination with traditional histological and minimally invasive imaging methods [41]. Another study by Shaleve et al^[42] used the fractal dimension to predict clinically significant postendoscopic bleeding (CSPEB) by analyzing blood vessel morphology within post-colonic endoscopic mucosal resection (EMR) mucosal defects. They introduced the fractal dimension estimate as a measure of the complexity of blood vessel contour, and on multivariate analysis, found that fractal dimension and four other independent variables correlated with CSPEB. This suggests that the morphometric characteristics of blood vessels in post-EMR defects can be used to predict delayed bleeding following colonic EMR. Ren et al[43] measured curvature, fractal dimension, and volumetric feature maps to assist radiologists in reducing interpretation time and improving detection results in computed tomographic colonography.

In their study, Mah et al[34] investigated the use of fractal dimension to distinguish between healthy and cancerous colon images and to examine the relationship between fractal dimension and traditional texture analysis features. They found a highly significant difference between the groups using fractal analysis. Interestingly, they also reported that micro-architectural analysis of the histologically examined colon mucosa provided unprecedented sensitivity for early detection of CRC[34]. They observed significant alterations in spectral slope, fractal dimension, and principal component 3 in mouse mucosa at the earliest time points, and these alterations increased over time, indicating the micro-architectural underpinnings of subsequent tumorigenesis.

Additionally, these indexes spatially correlated with future adenoma development. The concept of "deformability" has been introduced as a hallmark of the CRC process leading to metastatic spread.

Furthermore, Streba et al[44] used fractal analysis to estimate elements obtained from images taken from pathological and immunohistochemical investigations of colonic biopsy fragments in patients who underwent surgery for previously diagnosed CRC. They introduced the concept of "deformability" as a hallmark of the CRC process leading to metastatic spread. The authors performed a fractal analysis using an in-house tool.

Streba et al [44] found that the fractal dimensions were significantly different between adenocarcinomas and other types of colonic cancers, but no significant differences were found between most types of CRCs. They also reported a significant statistical difference when comparing well-differentiated tumors with all other stages. The authors concluded that fractal analysis is a novel and interesting tool for determining the pathologic diagnosis of CRCs, and may further improve diagnostic and prognostic rates, thus enhancing patient care.

Several initial studies have suggested that the novel agent polyethylene glycol (PEG) may have the necessary chemopreventive properties for widespread clinical use[45]. Despite reports attesting to its remarkable efficacy, the mechanisms of action of PEG remain largely unexplored. To determine the stage of carcinogenesis that PEG targets, Roy et al[45] assessed fractal dimension, one of the earliest markers of neoplastic transformation of the colon, in fresh colonic tissue using four-dimensional elastic light-scattering fingerprinting.

Studies have shown that PEG can suppress morphologic variables and restore micro-architectural organization, as indicated by the fractal dimension estimate. Changes in fractal dimension have been observed prior to the development of aberrant crypt foci in azoxymethane-treated rats and adenomas in mouse models, making fractal dimension a sensitive early marker in cancer prevention. Short-term treatment with PEG has been shown to completely normalize fractal dimension, indicating that PEG can reverse the earliest changes of colon carcinogenesis.

Strauss et al[46] assessed the impact of quantitative parameters on the differentiation of primary CRCs from normal colon tissue. They applied compartment and non-compartment modeling to dynamic PET data in 22 patients with CRCs prior to surgery, five of whom also had liver metastases at the time of the PET study. The non-compartment modeling was used to calculate the fractal dimension of the time-activity data. The study found that using quantitative data had the advantage of not being primarily dependent on the individual assessment and experience of the physician visually evaluating the FDG PET data. Additionally, the analysis of dynamic PET data of the corresponding primary tumor



could predict the presence of metastatic lesions.

Strauss *et al*[46] found that the fractal dimension, standardized uptake value, influx, and k3 were the most important single parameters for lesion differentiation[46]. The study also demonstrated that the highest accuracy for lesion differentiation was achieved with the fractal dimension[46]. The use of quantitative data has the advantage that the detection of CRC is not solely dependent on the individual assessment and experience of the physician visually evaluating the FDG-PET data. Quantitative FDG-PET studies provide accurate data for differentiating primary CRCs from natural tissue, and most of these studies conclude that the presence of metastatic lesions can be predicted by analyzing the dynamic PET data of the corresponding primary tumor[46,47].

Studies have found that 18F-FDG kinetics are primarily dependent on the expression of genes associated with glucose transporters and hexokinases, but may also be modulated by other genes. Strauss *et al*[47] investigated the dependency of 18F-FDG kinetics on angiogenesis-related gene expression. They evaluated a series of 25 patients with primary CRCs using PET and 18F-FDG within 2 d before surgery.

During surgery, tissue specimens were obtained from the tumor and adjacent non-tumoral colon, and gene expression was assessed using gene arrays. Strauss *et al*[47] identified 23 angiogenesis-related genes with a tumor-to-normal ratio exceeding 1.50, and found a significant correlation between k1 and vascular endothelial growth factor, as well as between fractal dimension and angiopoietin-2. They also noted a negative correlation between k3 and VEGF-B, and a positive correlation for angiopoietin-like 4 gene. In a recent study, Goh *et al*[48] evaluated the feasibility of using fractal analysis to assess the spatial pattern of CRC perfusion at dynamic contrast-enhanced CT (perfusion CT).

Twenty CRC patients who underwent a 65-second perfusion CT study. A perfusion parametric map was generated using validated commercial software. The tumor was identified by an experienced radiologist, segmented *via* thresholding, and fractal analysis was applied using in-house software. Fractal dimension, abundance, and lacunarity were assessed for the entire outlined tumor, as well as for selected representative areas within the tumor of low and high perfusion.

The study by Goh *et al*[48] concluded that fractal values were higher in cancer than in normal colon, and that fractal values were lower in areas of high perfusion compared to low perfusion areas. Lacunarity curves were shifted to the right for cancer compared to normal colon. The results suggest that CRCs mapped by perfusion CT demonstrate fractal properties, and thus fractal analysis is feasible, potentially providing a quantitative measure of the spatial pattern of tumor perfusion.

Lętowska-Andrzejewicz *et al*[49] have investigated the effects of 5-fluorouracil, interferon, and dexamethasone, on the healing of colon anastomosis by assessing morphometric and fractal parameters of the colonic wall. Lętowska-Andrzejewicz *et al*[49] performed an experimental anastomosis of the ascending colon in 60 male Wistar rats and subsequently randomly assigned them to four groups. On the second to sixth post-operative days, the rats were administered 5-fluorouracil, interferon- α , dexamethasone, or 0.9% NaCl solution as a control. The healing of the anastomosis was assessed through macroscopic, histomorphometric, and microbiological evaluations. The histomorphometric parameter changes were most evident on the seventh and fourteenth post-operative days in all treatment groups. Connective tissue fractal dimension significantly decreased in those animals treated with interferon and dexamethasone. All three pharmaceutical agents impaired healing of the anastomosis and promoted infection in the anastomosis and skin wound sites. Dexamethasone was considered the most detrimental in this study, as it induced both morphometric and macroscopic alterations.

The inability of cytotoxic anticancer therapies to effectively treat tumors may lead to changes in their morphology and increased tissue invasion. This effect is more prominent in cancer cells with strong metastatic capabilities. Researchers, Pasqualato *et al*[50], have established a clear correlation between alterations in cell shape and the development of a more aggressive phenotype in colon cancer cells that are resistant to 5-FU treatment, specifically HCT-8FUres.

The observed changes in cell shape were strongly associated with an increase in the speed of cellular movement. Pasqualato *et al*[50] developed a method to quantitatively measure the shape of both wild-type and chemoresistant HCT-8 cells during a wound healing assay. They assessed various shape descriptors such as the area/perimeter ratio, circularity, roundness, fractal dimension, and solidity to characterize the biological behavior of these two cell lines. Solidity was identified as the most effective parameter for distinguishing between the chemoresistant and wild-type cells. Moreover, this parameter was found to capture the differences in cell shape at each time point of the migration process. Furthermore, a negative correlation was observed between solidity and motility speed.

The fractal pancreas

Studies that attempt to quantitatively analyze the growth and development of pancreatic islets in both normal and pathological conditions have encountered significant challenges in terms of methodology and conceptualization. To address these challenges, Hastings *et al*[51] conducted a study that utilized the "geometry of random fractals" to investigate the regeneration of islets in guinea pigs that had been treated with alloxan.

The authors of the aforementioned study have since demonstrated that the power-law distribution of pancreatic islets is consistent across various mammalian species and is maintained throughout ontogenetic development in guinea pigs[52]. This suggests that islet formation adheres to an iterative or fractal pattern that is common among mammals. The islets of Langerhans, which are responsible for regulating blood glucose levels, are known to be unevenly distributed throughout the pancreas. In fact, it has been reported that there exists a universal power-law that governs the fractal spatial distribution of islets in two-dimensional pancreatic sections^[52].

In a study conducted by Jo et al [53], the three-dimensional spatial distribution of islets in intact mouse pancreata was examined using optical projection tomography. The results showed a power law distribution with a fractal dimension of 2.1. The authors also analyzed two-dimensional pancreatic sections from human autopsies and found that the distribution of human islets followed a universal power law with a fractal dimension of 1.5 in adult pancreata. This value is consistent with previous findings in smaller mammalian pancreas sections. Finally, the authors developed a growth model to explain the development of islet distribution and suggested that the fractal nature of the spatial islet distribution may be due to self-avoidance in the branching process of vascularization in the pancreas[53].

Pancreatic cancer is a highly aggressive disease with a poor prognosis, despite surgical intervention. To improve patient outcomes and avoid unnecessary laparotomies, it is crucial to identify reliable factors that can predict the resectability of pancreatic tumors. Vasilescu et al[54] conducted a study to determine if nuclear morphometry and fractal dimension of pancreatic nuclear features could serve as preoperative indicators for assessing pancreatic resectability. The study involved sixty-one patients diagnosed with pancreatic cancer, who were divided into two groups: those with resectable cancer and those with non-resectable pancreatic cancer. The authors evaluated several morphometric parameters, including nuclear area, length of minor and major axes, and nuclear shape and chromatin distribution of pancreatic tumor cells, which were estimated using fractal dimension analysis. The morphometric analysis conducted by Vasilescu et al [54] revealed significant differences in the nuclear area between the resectable and non-resectable groups. The non-resectable group was also found to have a higher value of fractal dimension in both nuclear outlines and chromatin distribution. Based on these findings, the authors suggested that objective measurements should be utilized to enhance risk assessment and treatment decision-making for pancreatic cancer. Additionally, they proposed that the fractal dimension of nuclear shape and chromatin distribution could be a valuable tool for conventional pathological analysis. In another study, fractal analysis was used to demonstrate that ghrelin administration increased structural complexity and tissue disorder in rat exocrine pancreas[55].

The study involved 40 male Wistar rats, where pancreas tissue sections were stained with hematoxylin and eosin and visualized through light microscopy. The average values of tissue fractal dimension, lacunarity, and co-occurrence matrix texture parameters were determined for each animal using computer-aided image analysis. The authors found that regardless of age, administration of ghrelin increased the fractal dimension and textural entropy of the exocrine pancreas, while decreasing its lacunarity. This is the first study to examine the effects of ghrelin on the morphological properties of pancreatic tissue and the first to utilize fractal and textural analysis methods to quantify the architecture of exocrine pancreas tissue.

According to Metze^[56], fractal characteristics of chromatin have been identified through light or electron microscopy in the past 20 years. These features can be easily estimated from digitized microscopic images and are useful for cancer diagnosis and prognosis. Studies have shown that an increase in the fractal dimension of stained nuclei occurs during carcinogenesis and tumor progression in various cancers, including pancreatic cancer. Researchers have also reported potential connections between changes in chromatin organization during carcinogenesis and tumor progression and an increase in the fractal dimension of stained chromatin.

The fractal dimension of chromatin in routine histological or cytological preparations has been found to increase during various stages of tumor development, including from pre-neoplastic stages to cancer, and from initial cancer stages to advanced stages. Additionally, higher fractal dimensions are associated with poor prognosis, while lower fractal dimensions are observed in patients with a better prognosis. Al-Mrabeh et al [57] conducted a study to determine whether the low volume and irregular border of the pancreas in patients with type-2 diabetes could be normalized following the reversal of diabetes.

The study conducted by Al-Mrabeh et al [57] utilized three-dimensional volume-rendering and fractal dimension analysis of MRI-acquired images, along with three-point Dixon imaging to measure fat content. The results indicated no change in pancreas volume six months after diabetes reversal compared to baseline. However, the reversal of diabetes was associated with an increase in the irregularity of pancreas borders between baseline and eight weeks, followed by a decrease at six months. In contrast, no changes in fractal dimension were observed in the non-reversed group. The authors concluded that combining three-dimensional volume segmentation of the pancreas with fractal dimension analysis could provide a promising mathematical definition of the pancreas border morphology.

Fractal dimension analysis revealed a significant increase in pancreas complexity after acute weight loss in the responder group, followed by a significant decrease resulting in a smoother pancreas border by the end of the study. Conversely, no changes in the pancreas border were observed in the nonresponder group. Although the effect of insulin on the endocrine pancreas has been extensively studied,



there have been few quantitative morphometric investigations of the exocrine pancreas. Recently, Pajevic *et al*[58] conducted a study to investigate the effect of acute and chronic insulin administration on the morphology of rat pancreas acini. The results showed that acute insulin treatment, regardless of the applied doses, increased the fractal dimension of the pancreas acini while decreasing their lacunarity.

The study showed that chronic low-dose insulin treatment decreased the fractal dimension and increased the lacunarity of pancreas acini, whereas high-dose insulin had the opposite effect. These findings suggest that fractal analysis could be used to detect fine architectural changes in acini, making it a potential alternative or addition to routine stereology.

In recent years, endoscopic ultrasound (EUS) elastography has been demonstrated as a useful technique for characterizing solid pancreatic lesions (SPLs). Carrara *et al*[59] introduced a combined analysis using EUS elastography (strain ratio) and fractal analysis to characterize the morphology of SPLs. A total of 102 SPLs from 100 patients were analyzed, and the results showed that both the parenchymal strain ratio (pSR) and wall strain ratio (wSR) were significantly higher in malignant SPLs than benign ones during elastography.

Fractal analysis revealed a significant difference in mean surface fractal dimensions between malignant lesions and neuroendocrine tumors, suggesting that combining EUS elastography with pSR and fractal-based analysis is a promising method for quantitatively characterizing SPLs[60].

It is widely accepted that the desmoplastic reaction is a hallmark of pancreatic cancer, although its impact on tumor behavior remains debated. Grizzi *et al*[61] recently developed a computer-aided method for quantifying the amount of pancreatic collagenic ECM, its spatial distribution pattern, and its degradation process.

The study conducted by Grizzi *et al*[61] demonstrated a progressive increase in pancreatic collagenic ECM from normal to inflammatory and pancreatic ductal adenocarcinoma. The two-dimensional fractal dimension revealed a significant difference in the spatial complexity of collagenic ECM between normal and inflammatory and pancreatic ductal adenocarcinoma. Moreover, a significant difference was observed when comparing the number of cycles required to degrade pancreatic collagenic ECM in normal *vs* inflammatory and pancreatic ductal adenocarcinoma. The mean velocity of collagenic ECM degradation was also found to be faster in inflammatory and pancreatic ductal adenocarcinoma than in normal. These findings highlight the potential of computer-aided methods and fractal analysis in quantifying the pancreatic ECM and its role in pancreatic diseases.

The study has demonstrated that inflammatory and pancreatic ductal adenocarcinomas are characterized by an increased amount of pancreatic collagenic ECM, as well as changes in its spatial complexity and degradation. These findings define new features of the pancreatic collagenic ECM and provide a basis for further investigations into the clinical behavior of pancreatic ductal adenocarcinoma and the development of therapeutic strategies. Overall, the study highlights the potential of computeraided methods and fractal analysis in quantifying the pancreatic ECM and its role in pancreatic diseases, ultimately contributing to improved diagnostic and therapeutic approaches for patients with pancreatic cancer.

Recently, researchers investigated the relationship between ductal morphometry and ramification patterns in the submandibular gland and pancreas to confirm their shared fractal dimension[62]. The study found that while the length of the intraglandular submandibular duct and the main pancreatic duct were correlated, other morphometric features of the ducts were not. This suggests a more intricate relationship between the two digestive glands beyond a simple shared fractal dimension.

Fractal analysis offers maps of the FD, which allows for a more dependable and size-independent measurement utilizing gross pathology or multi-parametric MRI as reference standards[63]. This approach quantifies perfusion chaos, the underlying pathophysiological principle, and can differentiate the more chaotic tumor rim from the tumor core and nearby non-tumorous pancreatic tissue.

Abdominal CT biomarkers, both inside and outside the pancreas, have the potential to diagnose type 2 diabetes mellitus. Tallam *et al*[64] conducted a study on a large clinical dataset using fully automated deep learning to investigate abdominal CT biomarkers for type 2 diabetes mellitus. Their findings revealed that the most accurate predictors of type 2 diabetes mellitus include intrapancreatic fat percentage, pancreatic fractal dimension, plaque severity between the L1 and L4 vertebra levels, average liver CT attenuation, and BMI.

The fractal liver

In 1989, Dioguardi[65-67] (1921-2019) proposed the liver as a composite system comprised of a set of operative microunits that receive a continuous flow of matter, energy, and information. The development of mathematical models for non-Euclidean geometry has led to the creation of models for highly complex natural phenomena[68]. In developmental biology, branching morphogenesis has been explained through self-similar iterative branching rules that have helped to elucidate branch patterns observed in various tissue types. However, in solid viscera, the issue of geometry is more complex, as there is no readily available marker for geometry in parenchymal tissue. The mosaic pattern provides such a marker for studying solid viscera.

The patches observed in mosaic liver have been shown to exhibit fractal properties, indicating that the pattern may have arisen from a self-similar process in which small areas are representative of, although not necessarily identical to, the whole object. This observation provides a new analytical approach to the study of biological structure in organogenesis[68]. It is well-established that partial hepatectomy induces compensatory, non-neoplastic growth and regeneration in mammalian liver[69]. Previous experimental evidence has suggested that compensatory liver growth occurs uniformly, without focal centers of proliferation. Given this information, fractal analysis was used to test various hypothesized patterns of regenerative growth in the liver. The results of this analysis indicate that the mosaic pattern does not change significantly during the regenerative process. The area and perimeter of patches (the area occupied by or perimeter around cells of like lineage) increase during compensatory liver growth in chimeric rats without altering the geometric complexity of patch boundaries (the boundaries around cells of like lineage). These tissue findings are consistent with previously reported computer models of growth in which repetitive application of simple decisions assuming uniform growth created complex mosaic patterns.

These findings support the idea that an iterating, self-similar cell division program is sufficient for the regeneration of liver tissue following partial hepatectomy. Such programs are important because they suggest a way in which complex patterns, or morphogenesis, can be efficiently created from a small amount of stored information. In geometrical terms, liver fibrosis is an example of self-similar natural fractal structure[70].

To evaluate the usefulness of a reliable and reproducible mathematical scoring system based on fractal geometry for quantifying the irregular pattern in fibrosis commonly seen in liver biopsy specimens from chronic liver diseases, Dioguardi et al[70] investigated 26 liver biopsy sections obtained from patients with chronic hepatitis C virus-related liver disease. The degree of fibrosis in each section was estimated using a quantitative scoring system based on the computer-assisted evaluation of both the fractal and spectral dimensions of deposited collagen. The findings indicated that the fractional dimension of its irregular shape defines fibrosis as a natural fractal structure.

The study by Dioguardi et al[70] proposed a method for quantifying irregular patterns in liver tissue using fractal geometry. The method is based on a quantitative scoring system that evaluates the fractal and spectral dimensions of collagen deposited in liver biopsy sections obtained from patients with chronic hepatitis C virus-related liver disease. The results showed that the fractional dimension of the irregular shape of fibrosis defines it as a natural fractal structure, and a single numerical score can be used to quantify the complex distribution of collagenous components. The method is reproducible, rapid, and inexpensive, and eliminates subjectivity and external factors that can influence staging and classification. The method uses a rectified meter implemented in a computer-assisted planar image analysis system to give metric measures of irregular outlines and surfaces, which can be used to produce an index that quantifies the typical wrinkledness of biologic objects[71]. A computer-aided morphometric method employing fractal geometry and Delaunay triangulation was introduced to quantify the necro-inflammatory phase in liver biopsy specimens. The method was applied to twomicrometer thick sections from 78 chronic hepatitis C virus-infected patients, immunohistochemically treated to identify inflammatory cells[62]. An automatic image analysis system was used to define the inflammatory cell network based on Delaunay triangulation, and the cells were categorized geometrically into clusters or irregular distributions within the tissue. This automatic method is fast and objective, producing accurate results represented by scalar numbers and allowing the organ's condition to be represented by Hurst's exponent with an error of no more than 12%. The availability of precise measurements and a reasonably representative assessment of the organ's overall state raises questions about revising the indications for hepatic biopsy, taking into account its invasiveness and subjective interpretation. A computer-aided method utilizing fractal principles was applied to digitized histological biopsy sections from 209 patients with varying degrees of chronic hepatitis C virus-related fibrosis or cirrhosis^[72]. This model offers the first metric evaluations of fibrosis's geometric properties and quantitative liver tissue architectural changes [72]. To evaluate the sampling variability of computeraided, fractal-corrected fibrosis measurements in liver biopsies, Grizzi et al[73] examined samples from different liver parts removed from 12 patients with clinically and histologically proven cirrhosis who underwent orthotopic liver transplantation. A computer-aided image analysis system automatically measured fibrosis surface, perimeter, fractal surface and dimensions, wrinkledness, and Hurst coefficient in Sirius red-stained sections. High inter-sample variability was found in fibrosis surface and wrinkledness measurements, while Hurst's exponent variability was low. The study suggests that Hurst's exponent is a valuable histological estimate of fibrosis in the entire organ for clinical use, emphasizing the crucial role biopsy sections play in qualitatively diagnosing chronic hepatitis and quantitatively estimating architectural changes in liver tissue.

A technique for analyzing liver biopsy samples was proposed, which involves the use of the "Dioguardi Histological Metriser" machine to automatically measure various parameters related to liver health, including residual hepatocyte mass, inflammation, fibrosis, and loss of liver tissue tectonics [74]. This technique was validated by analyzing digitized images of liver biopsy sections from 398 patients and comparing the results to a semi-quantitative scoring system. The method provides measurements for the extent of hepatocyte mass, including abnormal lipid accumulation; the size of the inflammation basin and the number of leukocytes within it; the degree of collagen islets, which are classified into three



magnitudes; and the tectonic index, which quantifies the degree of liver tissue organization disorders.

The findings of the proposed technique represent the first standardized method for measuring the geometric properties of liver tissue, including parenchyma, inflammation, fibrosis, and tectonics of biopsy sections. Other authors have also reported on the accuracy and feasibility of using fractal analysis to measure liver fibrosis. A study involving 77 rats, including 10 sham, 46 with fibrosis due to bile duct ligation, and 21 with fibrosis resulting from CCl₄ intoxication, was conducted to investigate various measurements, such as the fractal dimension of Kolmogorov, histologic lesions, the area of fibrosis by image analysis, liver hydroxyproline content, messenger RNA fibronectin, serum hyaluronate level, and portal pressure^[75]. The results showed that the fractal dimension of Kolmogorov was correlated with other markers of fibrosis, including the area of fibrosis, hydroxyproline content, serum hyaluronate level, and portal pressure.

The study found that the fractal dimension of Kolmogorov was significantly different between the two fibrosis models, unlike the area of fibrosis, and this relationship was independent of other histologic lesions. These results suggest that fractal analysis is an appropriate technique for analyzing liver fibrosis and has excellent reproducibility. In fact, this method is the only quantitative morphometric approach that can distinguish between different fibrosis models and is sensitive enough to detect pharmacologically-induced changes in liver fibrosis. Meanwhile, Fibroscan is a widely used technique for assessing liver fibrosis in chronic hepatitis C by measuring liver stiffness. However, it remains unclear how liver steatosis, which is a common feature of chronic hepatitis C and other chronic liver diseases, affects the accuracy of liver stiffness evaluation (LSE).

A quantitative morphometric analysis was conducted on 650 patients with chronic hepatitis C who underwent liver biopsy and LSE. The liver specimens were evaluated using both optical analysis (Metavir F and A, steatosis grading) and computerized morphometry to determine the area and fractal dimension of liver fibrosis and steatosis. The precise evaluation of liver histology using computerized morphometry revealed that liver stiffness measured by Fibroscan was associated with liver fibrosis, activity, and steatosis. In cases of high levels of steatosis, Fibroscan measurements may result in misinterpretation of liver fibrosis. Meanwhile, Nielsen et al[76] used a polygonization-based method to estimate the fractal dimension and other scalar lacunarity features from digitized transmission electron micrographs of mouse liver cell nuclei.

The aim of Nielsen *et al*^[76] was to determine whether a small set of fractal features could differentiate between natural liver samples, hyperplastic nodules, and hepatocellular carcinomas (HCC). They found that several single fractal features estimated from the periphery of the cell nuclei could distinguish between samples from hyperplastic nodules and HCC and those from normal liver tissue. Recent studies suggest that the cellular environment's mechanical influences can have significant effects on gene activity, cell differentiation, and proliferation, in addition to chemical factors[77]. These mechanisms involve a tissue matrix system that includes the ECM, nuclear matrix, and cytoskeleton. As hepatocytes during fetal development are a useful model for studying such variations, Vassy et al[77] investigated hepatocyte differentiation from fetal to adult livers using computerized quantitative image analysis of cytokeratin 8 immunofluorescent localization.

Vassy et al[77] investigated a set of line features, including the number and length of lines, orientation of lines, and the fractal dimension of the filament network, to analyze hepatocyte differentiation from fetal to adult livers. The study found highly significant differences in the features studied throughout liver development, including an increase in the total amount of cytokeratin filaments, demonstrating the feasibility of objective and quantitative analysis of the differences in the pattern of the cytokeratin filament network. Meanwhile, the organization of the hepatic microvascular network has been widely studied in recent years, particularly in cirrhosis. A quantitative mathematical approach based on fractal and Fourier analyses performed on photomicrographs has been used to recognize the distinctive vascular patterns in cirrhotic livers compared to natural livers^[78]. The study found that the natural hepatic sinusoidal network had higher complexity than the cirrhotic network, with more significant morphological changes observed in rats with bile duct-ligation cirrhosis than those with CCl₄-induced cirrhosis. These findings could provide insight into the pathophysiological alterations of the liver and may have diagnostic value in future clinical research. Additionally, aging-related modifications have been shown to cause marked changes in both the structure of the liver sinusoidal endothelial cell and liver perfusion^[79]. Fractal and Fourier analyses and micro-CT showed that age did not affect sinusoidal dimensions, sinusoidal density, or dispersion number, but there were changes in the geometry and complexity of the sinusoidal network as determined by fractal dimension and degree of anisotropy.

Small age-related changes in the architecture of the liver sinusoidal network can influence hepatic function and may reflect broader aging changes in the microcirculation. Fractal analysis has been applied to HCC as well[80-83]. The fractal dimension has been investigated as a quantifier of non-Euclidean two-dimensional vascular geometry in a series of paired specimens of primary HCC and surrounding non-tumoral tissue. Blood vessels had a higher fractal dimension in primary tumors compared to liver metastasis, and this approach allowed for differentiation between primary liver tumors with and without neurodifferentiation. Fractal geometry has also been used to investigate alphafetoprotein behavior in patients with HCC waiting for liver transplant[84]. Furthermore, it has been shown that the chromatin of mouse hepatocytes exhibits age-related reduction of fractal dimension, suggesting that this index can be a potential indicator of age-associated changes in chromatin structure



[83].

The study of fractal architecture in various organs and structures has expanded the possibilities for determining their functioning and structural arrangement, leading to the development of new approaches to diagnostics and therapeutic interventions. Recently, Azmaiparashvili et al[85] investigated the spatial geometry of the biliary tree and assessed the significance of both its fractal and Euclidean characteristics for developing approaches that provide a comprehensive description of intrahepatic biliary tract architecture. This configuration of the biliary bed is essential for ensuring the smooth implementation of the physiological function of the liver and its adaptation to different pathologies accompanied by biliary hypertension. Furthermore, the findings could be considered in the diagnosis and assessment of ductular reaction, biliary regeneration, and/or carcinogenesis.

There is a growing interest among researchers in employing fractal geometry-based algorithms for modeling, monitoring, and treating hepatitis B virus infections, as comprehending the intricate relationships between pathogens, immune systems, and overall liver function is of vital importance[86].

Recently, fractal geometry has been employed to model portal hypertension[87]. Specifically, the model encompasses the entire process of blood flow, starting from the sinusoids and culminating in the portal vein. It can be adjusted to account for the diagnosis of portal hypertension caused by thrombosis and liver cirrhosis. Moreover, this approach offers a novel way to detect portal vein pressure noninvasively from a biomechanical perspective. Furthermore, the combination of qualitative MRI features with fractal analysis has enabled the identification of subtypes of hepatocellular adenomas HCCs in patients with non-cirrhotic livers [88]. This approach also enhances the differentiation of lesions with a high-risk of malignant transformation from those with a low-risk [88]. Liver and tumor segmentation from CT scans plays a critical role in hepatic surgical planning. As manual segmentation is both timeconsuming and labor-intensive, a fully automated approach is highly sought after. Chen *et al* [89] introduced an automatic two-step liver and tumor segmentation method. Full-field optical coherence tomography (FF-OCT) has been recognized for its label-free subcellular imaging capabilities. Recently, Zhu et al[90] demonstrated the promise of their classifier using FF-OCT images for label-free, quantified tumor detection. This suggests that a fractal dimension-based classifier could potentially assist clinicians in identifying tumor boundaries for surgical resection in the future.

CONCLUSION

Irregularity and self-similarity under scale changes are the main attributes of the morphologic complexity of cells and tissues, either normal or pathologic. Fractal analysis is used in biomedical studies for the exact characterization of the complexity of analyzed structures. Fractal analysis has been demonstrated as a suitable method for quantifying heterogeneity from histological, endoscopic, radiological, and nuclear medicine images under a variety of conditions and in different organs. The application of nontraditional mathematics models to GI tract oncology may represent a comprehensive way to interpret the physical and biological nature of tumors opening new windows in the field of knowledge. Further research is required to exploit physiologically proven fractal behavior in the clinical setting. It is encouraging to see mathematicians, biologists, and clinicians collaborating towards a common quantitative understanding of anatomical complexity. This interdisciplinary approach has the potential to clarify concepts, interpret new and old experimental data, identify alternative experiments, and categorize acquired knowledge based on similarities and shared behaviors of different types of anatomical entities.

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FOOTNOTES

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Abstract

BACKGROUND

Cholangiocarcinoma (CCA) is a devastating malignancy and has a very poor prognosis if tumors spread outside the liver. Understanding the molecular mechanisms underlying the CCA progression will likely yield therapeutic approaches toward treating this deadly disease.

AIM

To determine the molecular pathogenesis in CCA progression.

METHODS

In silico analysis, in vitro cell culture, CCA transgenic animals, histological, and molecular assays were adopted to determine the molecular pathogenesis.

RESULTS

The transcriptomic data of human CCA samples were retrieved from The Cancer Genome Atlas (TGCA, CHOL), European Bioinformatics Institute (EBI, GAD-00001001076), and Gene Expression Omnibus (GEO, GSE107943) databases. Using Gene set enrichment analysis, the cell cycle and Notch related pathways were demonstrated to be significantly activated in CCA in TCGA and GEO datasets. We, through differentially expressed genes, found several cell cycle and notch associated genes were significantly up-regulated in cancer tissues when compared



with the non-cancerous control samples. The associated genes, *via* quantitative real-time PCR and western blotting assays, were further examined in normal human cholangiocytes, CCA cell lines, mouse normal bile ducts, and mouse CCA tumors established by specifically depleting P53 and expressing Kras^{G12D} mutation in the liver. Consistently, we validated that the cell cycle and Notch pathways are up-regulated in CCA cell lines and mouse CCA tumors. Interestingly, targeting cell cycle and notch pathways using small molecules also exhibited significant beneficial effects in controlling tumor malignancy. More importantly, we demonstrated that several cell cycle and Notch associated genes are significantly associated with poor overall survival and disease-free survival using the Log-Rank test.

CONCLUSION

In summary, our study comprehensively analyzed the gene expression pattern of CCA samples using publicly available datasets and identified the cell cycle and Notch pathways are potential therapeutic targets in this deadly disease.

Key Words: Bile duct cancer; Notch; Cell cycle; Liver cancer; Therapeutic target

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Core Tip: Molecular profiling of cholangiocarcinoma (CCA) has been conducted using various cohorts. However, the identified targets vary among different cohorts. In the current study, we combined different cohorts of CCA RNA sequencing datasets and refined the potential therapeutic targets in human CCA malignancy. We validated the findings using human CCA cell lines, the Kras^{G12D} and P53 mutation transgenic mouse model, and human CCA clinical data, thus supporting the potential of targeting the identified pathways in clinical trials.

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INTRODUCTION

Cholangiocarcinoma (CCA) is a devastating malignancy with "silent" clinical manifestations, genetically heterogeneous, anatomically distinct, but limited treatment options[1]. CCA can be classified as three subtypes depending on the origin of anatomical site, including intrahepatic (iCCA), perihilar (pCCA) and distal (dCCA) CCA[2]. If CCAs are diagnosed at the early stage, surgical resection may be a treatment option. However, CCA tumors are generally diagnosed at advanced stages due to lack of symptoms. Therefore, it is urgent to develop early detection markers and effective therapies for CCA patients. Understanding the molecular pathogenesis of CCAs may help identify early detection markers and potential therapies for this deadly disease.

Advances in whole-exome and transcriptome sequencing have paved the way for precision medicine in patients with advanced-stage or unresectable lesions[3-5]. Several genetic mutations have been identified in CCA patients[6]. Among them, IDH1 mutations have been well studied and the IDH1 mutation specific inhibitor has been developed for targeting malignant tumors with this mutation. In the ClarIDHy clinical trial, it was found that Ivosidenib-IDH1 mutation inhibitor could improve CCA patient overall survival (OS) and progression free survival[7,8], which eventually led to the FDA approval of Ivosidenib use in CCA patients with IDH1 mutation. Nevertheless, the 5-year survival of these patients treated with Ivosidenib is not significantly improved[7,8]. Besides, there is no effective therapy for those CCA patients with wild-type IDH1, despite that cisplatin and gemcitabine could extend the median OS for 3.6 mo but is not curative. Therefore, there is still an unmet need for identifying effective therapies in CCA patients.

In the current study, we utilized the transcriptomic profiling of CCA to explore the oncogenic driver events, analyzed the relevant genes expression, and identified the feasibility of target therapies with respect to those tumorigenic lesions.

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MATERIALS AND METHODS

Human data

A total of 2 archival tissue blocks from patients with eCCA were retrieved from the First Affiliated Hospital of Zhengzhou University (Zhengzhou, Henan Province, China) and the specimens were independently diagnosed by two hepatobiliary pathologists. This study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of the same institution (Approval No. 2022-KY-1514-002). Informed consents were obtained from all patients.

Cell Lines and reagents

Human CCA cell lines, including H1 (OCUCh-LM1-H1)[9], HuCCT1[10], SSP-25[11], and ETK1[12] cell lines were kindly provided by Dr. Jack Wands. TFK-1 and RBE were purchased from RIKEN Cell Bank (Tsukuba, Japan). The non-malignant human bile duct cells hBD was purchased from Celprogen Inc (Torrance, CA, United States, Catalog Number: 36755-12). CCA cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS; Gibco™, Thermo Scientific, Waltham, MA, United States; Cat# 10099141C), 100 µg/mL streptomycin, and 100 U/mL penicillin (HyClone™, Logan, UT, United States). The hBD cell line was grown in DMEM containing 10% FBS, 100 µg/mL strep. All cell lines were authenticated by STR and were negative for contamination of mycoplasma recently. Arcyriaflavin A, Flavopiridol, Roscovitine, and γ-secretase inhibitor, DAPT (N-[N-(3, 5-difluorophenacetyl)-lalanyl]-s-phenylglycinet-butyl ester) were all purchased from Cayman Chemical (Ann Arbor, MI, United States).

Data collection

RNA-Seq, DNA methylation, and clinicopathological data of 36 patients with cholangiocarcinoma (liver/normal bile duct = 9, iCCA = 30, pCCA = 4, dCCA = 2) were retrieved from The Cancer Genome Atlas (TCGA) database. The Genomic Data Commons mRNA quantifications were calculated by STAR [13], and then annotated by the reference gene model GENCODE v36. iCCA and the related paracancerous normal livers from 30 patients, and their survival information were employed (GSE107943) [14]. Fastq files of RNA-Seq from 162 samples of CCA (iCCA = 122, pCCA = 14, dCCA = 26) and the associated survival status of patients were fetched from the European Genome-phenome Archive[15].

Survival analysis

The Kaplan-Meier analysis was performed to evaluate the OS and disease-free survival (DFS) of patients from GEO and EBI cohorts. The optimal cutoff point for two groups was estimated based on maximally selected rank statistics[16], and then significance of survival analysis was calculated using log-rank test between the two groups.

Gene set enrichment analyses

Gene set enrichment analysis (GSEA) was conducted using R package "Pi" [17]. Hallmark (H) and KEGG subset of Canonical Pathways (CP) gene sets were selected for GSEA[18].

Tumor-infiltrating immune cell analysis

Single-sample GSEA was performed to analyze the tumor-infiltrating lymphocytes (TILs) based on the 28 immune cell types[19]. Correlations between the indicated genes expression and each infiltrating immune cells levels were calculated based on Spearman correlation (ρ).

Genetically engineered mouse

All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Rhode Island Hospital, and all experiments were conducted in accordance with the guidelines of this IACUC. The specifical Kras $^{\mbox{\tiny G12D}}$ (LSL-Kras $^{\mbox{\tiny G12D}}$), floxed-p53 (p53L/L), and albumin-Cre mutants strains were purchased from the Jackson Laboratory (Bar Harbor, ME, United States). They were intercrossed to achieve the mutant mice as indicated in the results section. The specific primers and genotyping protocol were performed following the instruction suggested by the Jackson Laboratory. Mice were bred in a pathogen-free environment and provided with free access to food and sterilized water Ad Libitum. Animals within experimental cohorts were monitored until the appearance of illness, including diminished activity, abdominal bloating, and cachexia, followed by full necropsy and the subsequent histopathological analysis.

MTT cell growth assay

Three CCA cell lines, including HuCCT1, H1, RBE, were seeded at 2 × 10³ cells/well into 96-well plate in six replicates. And then the above-mentioned cells were treated with small molecular inhibitors targeting cell cycle (Arcyriaflavin a, Flavopiridol, and Roscovitine) and Notch (DAPT) pathways 24 h post-seeding, respectively. The treatments were changed as indicated at days 1, 3, 5, and 7. Cell proliferation was measured using MTT at the following day (day 0 and day 1) and every 48 h after (up to day



7). Absorbance at OD570 nm and 650 nm were read and then normalized to vehicle control (0.1% DMSO or the corresponding medium). The relative cell proliferation rates were calculated as OD570-OD650.

RNA extraction and real-time guantitative PCR

Total RNA was extracted from CCA cell lines using TRIzol™ (Invitrogen, Thermo Scientific; Cat# 15596026) according to the manufacturer's protocol. 1 µg of total RNA was used to reverse transcription using iScript™ Reverse Transcription Supermix (Bio-Rad, Hercules, CA, United States, Cat#1708840). The qRT-PCR was conducted using SYBR® Green Realtime PCR Master Mix (Bio-Rad, Cat#1725270) by QuantStudio[™] 5 Real-Time PCR System (Applied Biosystems). The relative mRNA expression was calculated based on 2-AACt protocol. All primers were listed in Supplementary Table 1.

Western blotting

Whole-cell protein extractions was acquired using RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, Cat# 89901), supplemented with protease inhibitor (Thermo Fisher Scientific, Cat#78430). The concentration of protein was quantified using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Cat# J63283.QA) before mixed with protein loading buffer (3×). Then the protein samples were separated by 10% SDS-PAGE, and the detailed procedures conducted as previously described[20].

Antibodies including α-Tubulin, GAPDH, Cyclin D1, P21, PCNA, E2F1, CDK2, CDK4, CDK6, RB1, JAG1, JAG2, Caspase 8, BAX, XIAP, BCL-2L12, ATR, ATM, Rad50, Mre11, PIK3C3, LC3A/B, and ULK1 were purchased from Cell Signaling Technology (Danvers, MA, United States) and Santa Cruz Biotechnology (Dallas, TX, United States).

Statistical analysis

Statistical analyses were performed using STATA software 16.0 (StataCorp, College Station, TX, United States) and R software (version 4.2.0). Statistical analysis was performed using unpaired Student's t and Mann-Whitney U test with respect to Gaussian distribution or not, respectively, unless otherwise indicated. Kruskal-wallis test was performed to compare the expression of genes examined by PCR, and the post-hoc tests within two groups were adjusted by Holm's correction. Spearman correlation coefficient (ρ) is used to evaluate the correlations between DNA methylation levels of CpG islands and the associated genes expression. Wilcoxon signed-rank test was utilized to compare the expression level of cell cycle and Notch related genes between iCCA and its corresponding normal tissues. Two-way repeated measures Anova was utilized to compare optical densities of CCA cell lines treated with the serial gradient concentration of drugs, which measured more than once. Then simple effect at day 7 was calculated when there were interaction effects between time and dosages. P values were shown as: ^{a}P < 0.05, ${}^{b}P < 0.01$, and ${}^{c}P < 0.001$.

RESULTS

CCA oncogenesis is highly associated with elevated cell cycle and Notch associated pathways

To determine what molecular events are up-regulated in cholangiocarcinogenesis, we downloaded the transcriptomic data of CHOL from the TCGA database. We performed the GSEA and found that cell cycle associated pathways were preferentially activated in tumors compared with the normal samples, such as genes involved in G2M checkpoint, E2F Targets, and Mitotic spindle (Figure 1A, upper). Besides, the pathways related with Notch were also induced in tumors, e.g., genes implicated in epithelial mesenchymal transition (EMT), Hedgehog, and Apical Junction (Figure 1A, lower). We retrieved the leading edge gene sets from those pathways, which drive the enrichment score and dominate the tumorigenicity of those pathways. Consistently, the genes from those leading edges are distinctly up-regulated in tumors compared with the normal samples, in line with the oncogenic properties (Figure 1B and C). Together, through the analysis of GSEA and differentially expressed genes, we found cell cycle and Notch pathways are up-regulated in CCA patients compared to nonmalignant patient controls.

Cell cycle and Notch associated pathways were found upregulated in CCA cell lines compared to normal biliary epithelial cells

To facilitate the application of identified genes in preclinical studies, we must validate if these findings are reproducible in established normal biliary epithelial cells and CCA cells. In this case, we would be able to evaluate whether these pathways can be targeted using the *in vitro* cell culture system. Thus, we examined several leading-edge genes associated with these pathways in normal biliary epithelial cells and several CCA cell lines. The results suggest that several cell cycles associated genes were upregulated in CCA cancer lines, including PCNA, E2F1, CDK2, CDK4, and CDK6 (Figure 2A). Besides, JAG1 and JAG2 which serve as the Notch ligand also substantially increased in CCA cell lines when compared with normal biliary epithelial cells (Figure 2B). We also examined several genes involved in autophagy, cell death, and DNA damage pathways since we wanted to illustrate if cell cycle associated







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Figure 1 Cholangiocarcinogenesis driven by cell cycle and Notch associated pathways. A: Rank-based gene set enrichment analysis of genes preferentially activated in tumors identified the tumorigenic pathways, cell cycle and Notch from Hallmark gene sets, in CHOL transcriptome from TCGA database. Upper: Cell cycle associated pathways, including G2M checkpoint, E2F Targets, and Mitotic spindle gene sets; Lower: Notch associated pathways, including epithelial mesenchymal transition, Hedgehog, and apical junction gene sets. The mRNA expression levels from the leading edges of cell cycle (B) and Notch (C) associated pathways were analyzed in human normal bile ducts (n = 9) and CCA samples (n = 35) using TCGA database, suggesting up-regulation of cell cycle and Notch associated genes in CCA samples. TPM: Transcripts per kilobase million; Red: Cholangiocarcinoma (iCCA/eCCA), Blue: Normal bile ducts and/or paracancerous liver; EMT: Rpithelial mesenchymal transition. °P < 0.001 when compared with the normal control.

> pathways play a dominant role or cell death pathways dominate the CCA tumorigenesis. Interestingly, we found several up-regulated genes that are associated with DNA damage and cell death pathways, but not autophagy (Figure 2C-E). Moreover, the negative correlations between the methylation status (β value) and its expression of those genes suggested that the aberrant expression might be attributed to the hyper- or hypomethylation (Supplementary Figure 1).

Up-regulated cell cycle and Notch associated gene expression levels were correlated with protein expression profiles in CCA cell lines

To determine if the identified genes have increased protein expression levels or promote other protein expression levels of the same pathway in CCA cell lines compared with the control biliary epithelial cells, we decided to examine protein expression of several major representative genes in each pathway (Figure 3, Supplementary Figure 2). As shown, several cell cycles associated genes were up-regulated in CCA cell lines when compared with the control biliary epithelial cells, including CDK2, CDK4, CDK6, and E2F1. Unexpectedly, several cyclins which include cyclin D1 and cyclin E1 were found decreased (Figure 3A). When we examined Notch ligands which include JAG1 and JAG2, we found JAG1 and JAG2 were all significantly increased in CCA cell lines compared to the biliary epithelial cells (Figure 3B). We further examined autophagy associated proteins and found no significant difference in autophagy between normal and malignant cells based on the autophagy definition (Figure 3C)[21]. In line with the mRNA change data, several apoptosis and DNA damage associated genes that were identified elevated in CCA cell lines were found increased as well (Figure 3D and E). Together, the mRNA and protein profile data demonstrated that it is feasible to identify potential target genes through mining public datasets and validating through *in vitro* human CCA cell lines.

Validation of the identified genes in a triple transgenic CCA mouse model

It has been well characterized that KRAS and P53 are two of the most mutant genes in human CCA tumor samples^[22]. Thus, a previous study has established the CCA transgenic mouse model by





Figure 2 Cell cycle and Notch associated pathways are increased in cholangiocarcinoma cell lines in vitro. The mRNA expression levels of leading-edge genes from (A) Notch, (B) cell cycle, (C) autophagy, (D) cell death, and (E) DNA damage were examined in cholangiocarcinoma (CCA) cell lines compared to normal biliary epithelial cells (hBD), indicating up-regulation of these genes in CCA tumor samples. CCA tumor cell lines including HuCCT1, ETK-1, H1, RBE, TFK-1 and SSP25. Data are relative to hBD cell line and normalized to 18S rRNA.

conditionally activating KrasG12D and depleting P53 (either P53 homozygous or P53 heterozygous knockout) in mouse liver using the Albumin promoter driven cre recombinase[23]. We also established the CCA transgenic mouse (Figure 4A) as evidenced by the genotyping data showing positive cre, Kras^{G12D}, and heterozygous floxed P53 genes (Figure 4B). The gross images of representative mouse

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Figure 3 The cell cycle and Notch pathway associated protein expression levels are up-regulated in cholangiocarcinoma cell lines compared to the normal human bile duct cells. The protein expression levels of leading-edge genes from (A) Notch, (B) cell cycle, (C) autophagy, (D) cell death, and (E) DNA damage were determined in cholangiocarcinoma (CCA) cell lines compared to hBD. Several cell cycle and Notch associated genes, including CDK4, CDK6, E2F1, JAG1, and JAG2, were up-regulated in CCA cell lines. CCA tumor cell lines including HuCCT1, ETK-1, H1, RBE, TFK-1 and SSP25. α-Tubulin served as the loading control.

tumor were showed (Figure 4C). When comparing with human non-malignant bile duct and CCA tumor samples, the mouse CCA tumors present almost identical CCA histology to human CCA tumor samples (Figure 4C and D). To further determine whether mouse CCA and human CCA tumors have similar molecular profiles, we examined the genes that we identified using public human CCA dataset and validated using normal biliary epithelial cells and human CCA cell lines. Intriguingly, mouse and human CCA both have highly elevated genes associated with cell cycles and Notch, including CDK2, CDK4, CDK6, JAG1, and JAG2 (Figure 4E and Supplementary Figure 3). In line with CCA cell line data, several DNA damage genes, including RAD50 and MRE11, were found increased in mouse CCA tumor samples as well (Figure 4E). Given all these facts, our data demonstrated that the CCA tumors derived from the CCA transgenic mouse model have very high similarity to human CCA tissues and cell lines. Besides, the results of CCA transgenic mouse tumor data further demonstrated that refining the public human RNA sequencing data would help identify dysregulated pathways which may be druggable.

Targeting cell cycle and Notch pathways inhibited cell growth of human CCA cell lines

As we have found that cell cycle and Notch associated pathways are highly elevated in human CCA tumor samples and validated these findings in CCA cell lines, we proposed to determine whether targeting these pathways using small molecular inhibitors could achieve therapeutic potential in CCA cell lines, including H1, HuCCT1 (Kras and P53 mutations), and RBE (IDH1 mutation). It was found that the D1-dependent CDK4 inhibitor Arcyriaflavin a significantly suppressed cell growth in HuCCT1 and RBE CCA cell lines, but to a less extent in H1 (Figure 5A). The pan-CDK inhibitor Flavopiridol and the selected CDK inhibitor Roscovitine, which targets multiple CDKs, both displayed substantial growth inhibition in H1, HuCCT1, and RBE CCA cell lines (Figure 5B and C). It was noted that RBE CCA cells with IDH1 mutation are very vulnerable upon the treatments of CDK inhibitors (Figure 5A-C). Regarding the treatment using a Notch inhibitor, a γ -secretase inhibitor-DAPT was used to challenge these CCA cell lines. Although the Notch inhibitor could exert anti-tumor effects in CCA cell lines, the suppressive effects are not as strong as CDK inhibitors. Furthermore, the close relationships between the levels of TILs and those genes expressed indicated that cell cycle related pathways are associated with immune check point therapies (Supplementary Figure 4). Taken together, our results suggested that targeting cell cycle and/or Notch pathways could be considered as alternative modalities for patients with CCA.

Validation of identified cell cycle and Notch related pathways in other datasets

It is essential to verify whether our findings are applicable to other datasets. Thus, we utilized another dataset (GSE107943) to validate the oncogenic traits of cell cycle and Notch related pathways in CCA. Consistently, the leading edge of GSEA showed pathways pertaining to cell cycle (Figure 6A, upper) and Notch (Figure 6A, lower) were significantly activated in tumors compared with the normal





Figure 4 Validation of the identified cell cycle and Notch associated genes in the triple transgenic cholangiocarcinoma mouse model. A: The cartoon diagram of the genetically engineered mouse modeling (GEM) strategies. Triple transgenic mice bearing Albumin-Cre transgene, LSL-Kras^{G12D}, and floxed-p53 were conditionally activated KrasG12D and heterozygous knocked out P53 in the liver. B: The genotyping data presenting positive Cre (red triangle),

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KrasG12D (blue triangle), and homozygous floxed P53 genes (green triangle). C: The gross image of representative tumor (upper right, scale bar = 5 mm), and the corresponding histopathology (lower right, scale bar = 100 µm) with inset showing glandular appearance (arrow, scale bar = 20 µm). D: Histology of human bile duct (upper, scale bar = 100 µm) with inset showing biliary epithelium (arrow, scale bar = 20 µm), and cholangiocarcinoma (lower, scale bar = 100 µm) with inset showing mitotic figure (arrowhead, scale bar = 20 µm) and glandular appearance (arrow, scale bar = 20 µm). E: The protein expression levels of leading-edge genes from cell cycle and Notch associated pathways, and genes involved in autophagy, cell death and DNA damage in CCA tumor tissues from the GEM (50 wk) compared to the normal bile ducts of control mice (50 wk). GAPDH was used as the loading control.

> samples. Besides, the analysis of differentially expressed genes also revealed that upregulation of cell cycle related genes, including CDK2, CDK4, CCNE1, and E2F1, were observed in human CCA tumor samples compared with the adjacent normal liver tissues. Similarly, several Notch associated genes, including NOTCH1 and JAG2, were found up-regulated in CCA tumor samples as well (Figure 6C). Together, with the validation data derived from another dataset, it is therefore suggested that our findings are applicable to other clinical human CCA tumor samples.

Cell cycle and Notch associated genes were correlated with poor prognosis in CCA patients

The cell cycle and Notch pathways were refined from the analysis of GSEA. These genes were found upregulated in CCA tumor samples as well when compared with adjacent normal liver tissues. As we have found that targeting these pathways significantly suppressed CCA cell growth in vitro, we speculated that high expression of these genes may correlate with poor prognosis. To determine the correlation of these genes and CCA patient prognosis, we conducted the Kaplan-Meier survival analysis using the cell cycle and Notch pathways associated genes in CCA patients derived from two datasets, including GSE107943 and EBI (EGAD00001001076). It was found that high expression of cell cycle genes, including CDK2, CDK6, CCNE1, and E2F1, were negatively correlated with OS of CCA patients derived from the EBI database (EGAD00001001076) (Figure 7A). Similarly, high expression of Notch related genes, including NOTCH1 and JAG2, were associated with poor prognosis in these patients (Figure 7B). These findings were further validated using the GSE107943 database. Consistently, high CDK2, CDK4, CCNE1, and E2F1 were correlated with poor OS of CCA patients (Figure 7C). High NOTCH1 expression was associated with poor OS and DFS in these patients as well (Figure 7D). Collectively, these data demonstrated that refining the signaling pathways associated with tumorigenesis of human CCA using bioinformatics approach could identify potential therapeutic targets for patients with this deadly disease (Figure 8).

DISCUSSION

The current study demonstrated that cell cycle and notch associated pathways are elevated in human CCA patients compared with adjacent normal tissues. These findings were verified using human biliary epithelial cells and CCA cell lines. Consistently, cell cycle and Notch associated genes were found increased in CCA cell lines compared with non-malignant human biliary epithelial cells. As it has not been investigated if the cell cycle and Notch associated pathways are up-regulated in the CCA transgenic mice, our studies therefore disclose the similar but novel findings in the transgenic CCA mouse model. Cell cycle and Notch associated genes were up-regulated in mouse CCA tumors when compared with age-matched mouse normal bile ducts. Besides, we demonstrated that targeting cell cycle and Notch pathways could inhibit cell growth in human CCA cell lines. Furthermore, we validated the GSEA findings using another dataset. Most importantly, we demonstrated that high expression of several cell cycle and Notch associated genes are highly associated with poor prognosis in human CCA patients in two databases.

Refining RNA sequencing results have been well studied in liver malignancy, including hepatocellular carcinoma and CCA[3,4,24,25]. By combining whole genomic sequencing and RNA sequencing data, it was previously suggested that intrahepatic CCA could be classified as inflammation and proliferation groups which have different clinical outcomes, specifically worse prognosis in the proliferation group[26]. Recently, it was also suggested that extrahepatic CCA could be classified as immune, mesenchymal, metabolic, and proliferation groups. Further analysis revealed specific potential therapeutic targets correspondent to different groups, *i.e.*, PD1/PD-L1 inhibitors were suggested to be potential therapies for the immune group of extrahepatic CCA patients^[4]. Nevertheless, these potential therapeutic approaches have not been evaluated in preclinical models due to the possible reasons that no preclinical models could represent the individual group specifically. However, the recent study investigated the microenvironment-based classification of intrahepatic CCA and identified the inflammatory stroma class of intrahepatic CCA with T cell exhaustion and KRas mutation. Thus, they evaluated if the combination of KRas inhibitor and PD1 antibody could inhibit the malignant progression of CCA tumors established by using tail vein hydrodynamic injection of SB13 transposase, CRISPR/Cas9-sg-p19, pCaggs-KrasG12D. The model has high similarity to the inflammatory stroma intrahepatic CCA group. Their findings demonstrated that Kras inhibitor and PD1 antibody could





Figure 5 Targeting cell cycle and Notch pathways inhibited cell growth of human cholangiocarcinoma cell lines. Growth curves of three cholangiocarcinoma cell lines with different genetic background, *e.g.*, HuCCT1 (KRAS and P53 mutations), RBE (IDH1 mutation), and H1, treated with cell cycle and Notch inhibitors, respectively. For targeting cell cycle pathways, (A) D1-dependent CDK4 inhibitor Arcyriaflavin a, (B) pan-CDK inhibitor Flavopiridol, (C) selected CDK inhibitor Roscovitine were utilized, and (D) the γ -secretase inhibitor (DAPT) was used against Notch pathway. Absorbance at OD570 nm and 650 nm analyzed by MTT, and then normalized to vehicle control (0.1% DMSO or the corresponding medium; *n* = 6 wells/dose point). The relative cell proliferation rates were calculated as OD570 - OD650. ^aP < 0.05, ^bP < 0.01, and ^cP < 0.001 when compared with the vehicle control at day 7.

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Figure 6 The generalizable oncogenic traits of cell cycle and Notch associated pathways in cholangiocarcinoma. GSEA identified cell cycle and Notch associated pathways significantly activated in tumors compared to the corresponding adjacent normal livers from GEO database (GSE107943) (A). Upper: Cell cycle associated pathways, including G2M checkpoint, E2F Targets, and Mitotic spindle gene sets; lower: Notch associated pathways, including epithelial mesenchymal transition, Hedgehog, and apical junction gene sets. The mRNA expression levels from the leading edges of cell cycle (B) and Notch (C) associated pathways were analyzed in CCA samples compared to the corresponding adjacent normal livers (pairs = 27). RPKM: Reads per kilobase million; Red: Intrahepatic cholangiocarcinoma; Blue: Para-cancerous normal liver; $^{\circ}P < 0.001$ when compared with the relevant normal control.

substantially inhibit tumor malignant progression[3]. Taken together, it is very likely to identify potential therapeutic targets by refining publicly available RNA sequencing datasets.

By using this approach, we found that Notch and cell cycle associated pathways are potential therapeutic targets in CCA tumors. It has been previously demonstrated that specific overexpression of intracellular domain of Notch 1 could lead to CCA development possibly through cyclin E1[27]. The Notch1 Ligand, JAG1 could activate Notch1 signaling cascade to initiate cyclin D1 transcriptional expression[28]. Both JAG1 and Notch1 contain the epidermal growth factor like domain[29] which could be hydroxylated by aspartate beta-hydroxylase[30-32]. Indeed, previous studies have demonstrated that aspartate beta-hydroxylase could promote CCA progression through the Notch1-mediated cyclin D1 pathway[33]. Cell cycle is tightly controlled by the tumor suppressor retinoblastoma protein (RB1)[34]. Although RB1 mutation is not a common event in CCA patients^[35], dysregulated cell cycle regulating genes are often found in these patients. Dysregulated cell cycle control would lead to cancer cell proliferation. As discussed, intrahepatic CCA patients could be classified as proliferative and inflammatory groups^[26]. Previous studies also demonstrated the therapeutic potential of targeting CCA tumors by using the specific inhibitor targeting cyclin dependent kinases 4 and 6[36,37] which are important mediator controlling RB1 phosphorylation. Phosphorylation of RB1 prevents its binding from the E2F1 transcriptional factor, which finely modulates cell cycle regulating genes[38]. Thus, phosphorylation of RB1 would lead to inactivation of RB1 tumor suppressor function, in turn promoting cell cycle progression. It was previously reported that aspartate beta-hydroxylase could promote RB1 phosphorylation to disrupt cell cycle regulation in CCA tumors[39]. Interestingly, aspartate betahydroxylase was reported to be highly expressed in CCA but barely detectable in normal bile duct tissues^[39], suggesting the linkage of identified Notch1 and cell cycle associated pathways to aspartate beta-hydroxylase.

The current study has several limitations. (1) We did not evaluate if targeting Notch1 and cell cycle pathways could inhibit CCA progression using preclinical models. Actually, the previous studies have demonstrated that Notch1 inhibitor could be a potential therapeutic approach[27] and that targeting cell cycle with CDK4/6 inhibitors could suppress CCA progression[36,37]. Although the current study was





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Figure 7 Cell cycle and Notch associated genes correlate with dismal survival in patients with cholangiocarcinoma. Kaplan-Meier analysis of cholangiocarcinoma patients in EBI-EGAD00001001076 (A and B) and GSE107943 (C and D) database. The optimal cutoff of genes from the leading edges of cell cycle (A and C) and Notch (B and D) associated pathways was calculated by maximally selected rank statistics. And then the difference between two groups was analyzed by log-rank test. Red: Higher expression of the indicated genes, Blue: Lower expression of indicated genes; DFS: Disease-free survival, OS: Overall survival.



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Figure 8 The summary of dysregulated cell cycle and Notch pathways in cholangiocarcinoma progression. The cholangiocarcinogenesis driven by aberrant cell cycle and Notch pathways, which may be targeted by small molecular inhibitors.

initiated from a different angle by using transcriptomic analysis, it ended up with similar findings that Notch1 and cell cycle pathways could be potential targets in CCA patients; (2) We did not separate intrahepatic and extrahepatic CCA patients. We pooled the available data by combining intrahepatic and extrahepatic transcriptomic datasets due to the limited datasets publicly available; and (3) our studies did not investigate if elevation of cell cycle and Notch associated pathways may be used as early detection markers. Future studies will be needed to further determine potential therapeutic approaches specifically for intrahepatic and extrahepatic CCA patients, respectively.

CONCLUSION

In conclusion, our data, through mRNA and protein profiling, identified very high similarity between CCA tumors derived from the CCA transgenic mice and human CCA tumors, thus providing a potential preclinical CCA model for investigating the CCA tumorigenesis. Besides, our data suggested that cell cycle and Notch associated pathways could be potential therapeutic targets in CCA patients.

ARTICLE HIGHLIGHTS

Research background

The molecular pathogenesis of cholangiocarcinoma (CCA) remains largely unknown. Investigating the molecular mechanisms underlying CCA progression will potentially yield results toward identifying therapeutic targets.

Research motivation

Several mRNA sequencing (Seq) datasets in CCA samples are publicly available, but it remains unclear if the data can be validated in CCA cell lines, the transgenic CCA mouse model, and human CCA samples.

Research objectives

To summarize the mRNA Seq results and validate the findings in CCA cell lines, the transgenic CCA mouse model, and human CCA samples.

Research methods

Bioinformatic analysis, cell culture studies, transgenic mouse model, human CCA samples, and molecular strategies were used to determine molecular pathogenesis of CCA.

Research results

Through bioinformatic analysis, we found that cell cycle and Notch associated pathways are upregulated in human CCA samples, compared to the non-tumor controls. We validated the findings in human CCA cell lines and the transgenic CCA mouse model.

Research conclusions

Our data, through mRNA and protein profiling, identified very high similarity between CCA tumors derived from the CCA transgenic mice and human CCA tumors, thus providing a potential preclinical CCA model for investigating CCA tumorigenesis. Besides, our data suggested that cell cycle and Notch associated pathways could be potential therapeutic targets in CCA patients.

Research perspectives

Systemic chemotherapy, gemcitabine and cisplatin, is recommended for CCA patients. However, the 5year survival of CCA patients has not been significantly improved, suggesting an urgent need to develop novel therapeutic approaches.

FOOTNOTES

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ORIGINAL ARTICLE

Retrospective Study Non-invasive model for predicting high-risk esophageal varices based on liver and spleen stiffness

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Abstract

BACKGROUND

Acute bleeding due to esophageal varices (EVs) is a life-threatening complication in patients with cirrhosis. The diagnosis of EVs is mainly through upper gastrointestinal endoscopy, but the discomfort, contraindications and complications of gastrointestinal endoscopic screening reduce patient compliance. According to the bleeding risk of EVs, the Baveno VI consensus divides varices into high bleeding risk EVs (HEVs) and low bleeding risk EVs (LEVs). We sought to identify a non-invasive prediction model based on spleen stiffness measurement (SSM) and liver stiffness measurement (LSM) as an alternative to EVs screening.

AIM

To develop a safe, simple and non-invasive model to predict HEVs in patients with viral cirrhosis and identify patients who can be exempted from upper gastrointestinal endoscopy.

METHODS

Data from 200 patients with viral cirrhosis were included in this study, with 140 patients as the modelling group and 60 patients as the external validation group, and the EVs types of patients were determined by upper gastrointestinal endoscopy and the Baveno VI consensus. Those patients were divided into the HEVs group (66 patients) and the LEVs group (74 patients). The effect of each parameter on HEVs was analyzed by univariate and multivariate analyses, and a non-invasive prediction model was established. Finally, the discrimination ability,



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calibration ability and clinical efficacy of the new model were verified in the modelling group and the external validation group.

RESULTS

Univariate and multivariate analyses showed that SSM and LSM were associated with the occurrence of HEVs in patients with viral cirrhosis. On this basis, logistic regression analysis was used to construct a prediction model: $Ln [P/(1-P)] = -8.184 - 0.228 \times SSM + 0.642 \times LSM$. The area under the curve of the new model was 0.965. When the cut-off value was 0.27, the sensitivity, specificity, positive predictive value and negative predictive value of the model for predicting HEVs were 100.00%, 82.43%, 83.52%, and 100%, respectively. Compared with the four prediction models of liver stiffness-spleen diameter to platelet ratio score, variceal risk index, aspartate aminotransferase to alanine aminotransferase ratio, and Baveno VI, the established model can better predict HEVs in patients with viral cirrhosis.

CONCLUSION

Based on the SSM and LSM measured by transient elastography, we established a non-invasive prediction model for HEVs. The new model is reliable in predicting HEVs and can be used as an alternative to routine upper gastrointestinal endoscopy screening, which is helpful for clinical decision making.

Key Words: Cirrhosis; High-risk esophageal varices; Non-invasive prediction model; Spleen stiffness measurement; Liver stiffness measurement; Upper gastrointestinal endoscopy

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Core Tip: The non-invasive prediction model for predicting high risk esophageal varices (HEVs) in patients with viral cirrhosis was successfully established based on the spleen stiffness measurement and liver stiffness measurement. It is a novel model that has not been reported. The model was shown to be better than previous prediction models. The new model is reliable in predicting HEVs and can be used as an alternative to routine upper gastrointestinal endoscopy screening, which is helpful for clinical decision making.

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INTRODUCTION

Liver cirrhosis is the end stage of chronic liver disease. When hepatic venous pressure gradient (HVPG) \geq 10 mmHg is defined as clinically significant portal hypertension (CSPH), the patients who meet this criteria may suffer from complications such as esophageal variceal bleeding, ascites, hepatic encephalopathy, and jaundice due to portal hypertension and liver insufficiency[1,2]. Rupture of esophageal varices (EVs) is a common and life-threatening complication in patients with liver cirrhosis. The incidence of EVs bleeding is approximately 5%-15% per year, the re-bleeding rate within 6 wk after EVs rupture bleeding is 30% to 40%, and the mortality rate is 15%-25% [3-5]. The severity of liver cirrhosis, the size of EVs and the presence or absence of the red sign (RS) are related indicators of EVs bleeding [6]. For routine assessment of the above indicators, the Baveno VI consensus recommends that patients with cirrhosis need to undergo regular screening upper gastrointestinal endoscopy so that appropriate preventive treatment can be administered to prevent variceal bleeding events[7]. To date, HPVG and upper gastrointestinal endoscopy are considered the gold standards for the assessment of PH and EVs, respectively[6]. However, the prevalence of varicose veins requiring treatment (VNT), as defined by the Baveno VI guidelines, is very low in Compensated advanced chronic liver disease (cACLD) patients who are detected at an early stage[8]. HPVG and EGD are invasive procedures and expensive, and the patient compliance associated with them is poor[9,10]. Therefore, in clinical practice, it is necessary to develop a safe, non-invasive and patient-acceptable prediction model that can not only prevent frequent HPVG or gastrointestinal endoscopy examinations but also better predict HEVs in patients with viral cirrhosis. With the development of transient elastography (TE), studies have shown that liver stiffness



(LS) and spleen stiffness (SS) detected by TE are associated with liver fibrosis, significant portal hypertension, and EVs. SS is increased in patients with viral hepatitis, and SS is positively correlated with HVPG, which has good predictive performance for CSPH and EVs in patients with cACLD[11-13].

At present, in addition to ultrasound, CT, MRI, and other imaging methods, there are also several common prediction models[14,15]. The LS-spleen diameter to platelet (PLT) ratio score (LSPS), variceal risk index (VRI), aspartate aminotransferase (AST) to alanine aminotransferase (ALT) ratio (AAR) and Baveno VI model have all achieved good clinical effects in predicting HEVs[16-19]. In addition, the Baveno VI consensus states that LS measurement (LSM) combined with PLT helps to exclude HEVs[8, 19,20]. To initially predict HEVs in patients with viral cirrhosis, the aim of this study is to establish a non-invasive prediction model that can predict HEVs based on SS measurement (SSM) and LSM and to evaluate the accuracy of the new model in identifying HEVs in patients with viral cirrhosis who can be exempted from upper gastrointestinal endoscopy.

MATERIALS AND METHODS

Patients and study design

The study was authorized by the Ethics Committee of the Second Affiliated Hospital of Xi'an Jiaotong University (Xi'an, Shaanxi Province, China). As a retrospective study; therefore, the Ethics Committee waived the informed consent. This study retrospectively analyzed the data of patients with viral cirrhosis who were admitted to the Second Affiliated Hospital of Xi'an Jiaotong University and underwent upper abdominal computed tomography (CT) examination from March 2020 to November 2022. The inclusion criteria were: (1) Age > 18 years old; (2) patients with hepatitis B and hepatitis C cirrhosis; (3) patients who underwent endoscopy, upper abdominal CT, and laboratory examinations with complete results; and (4) the interval between two examinations was not more than 3 mo. Exclusion criteria: (1) Other liver injury factors, such as alcoholic liver disease, autoimmunity, metabolic liver disease, occult liver cirrhosis, etc; (2) suspicious liver tumor; (3) history of liver resection, liver transplantation or splenectomy; (4) frequent use of proton pump inhibitors; (5) other diseases that may impact the haemodynamics of the splenic vein or portal vein, such as cavernous degeneration, thrombosis, embolism; (6) cirrhotic patients with moderate or massive ascites; (7) previous treatment of portal hypertension, such as splenectomy, transjugular intrahepatic portosystemic shunt, endoscopic therapy, and nonselective β -blocker therapy; (8) diseases may affect the liver or spleen size, such as cysts, leukaemia, thrombocytopenic purpura, haemolytic anaemia, multiple myeloma, etc; (9) patients with a history of esophageal bleeding undergoing endoscopic or surgical treatment; (10) severe malnutrition or weight loss; (11) unreliable LSM: Quartile range/median > 0.3, success rate < 60%, or the number of effective measurements < 10; or (12) other conditions affecting LSM, such as body mass index > 35 kg/m^2 .

A total of 140 patients who met the inclusion criteria were selected as the modelling group. According to the results of upper gastrointestinal endoscopy and the Baveno VI criteria, the patients were divided into the HEVs group and the LEVs group; 66 patients were HEVs patients, and 74 patients were LEVs patients. In addition, 60 patients who met the inclusion criteria were used as the validation group. The patient's data were collected when the model was established, and the data collection procedure and the model application did not interfere with each other.

Definition of EVs

Patients with liver cirrhosis were graded and scored using the Child-Pugh scoring system[21]. According to Baveno VI criteria, HEVs were defined as EVs with a diameter \geq 5 mm, EVs with a diameter \leq 5 mm and a positive RS, EVs in patients with Child grade C, and EVs that did not meet these criteria were LEVs with a low risk of bleeding[22,23].

CT scan-based liver and spleen volume measurement

The CT examinations were conducted by a multislice spiral CT scanner (GE 128-slice spiral CT scanner; Linux Medical System, United States) with a 5 mm reconstructed layer thickness, and the time interval was 5 s.

Actual liver volume measured by CT (CTLV), actual spleen volume measured by CT (CTSV), portal vein diameter (PVD) and spleen long diameter (SLD) were simultaneously measured by experienced radiologists who did not know the basic information of the patients. CTLV and CTSV were obtained by manually tracing the surface area of the liver and spleen at each level and multiplying by the layer thickness. The entire measurement process requires active avoidance of large blood vessels, gallbladder, and fissure. The SLD was defined as the length of the superior pole to the inferior line of the spleen at the plane of maximum surface area. The PVD needs to be measured at the midpoint between the portal vein bifurcation site and the vein confluence site[24].

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Laboratory examination

For each patient, data on age, sex, height, weight, medical history, medication use, the presence or absence of ascites, and Child-Pugh score were collected. The white blood cell count, red blood cell count, PLT, ALT, AST, total bilirubin (TBil), alkaline phosphatase (ALP), glutamine transferase (GGT), albumin (ALB), total cholesterol (TCHO), prothrombin time (PT), international prothrombin ratio (INR), and prothrombin activity (PTA) of all patients included in the study were collected. The blood test was tested by an XN-9000 analyser (Xisen Meikang Medical Electronics Co. Ltd., Shanghai, China), and the coagulation function was tested by a Sysmex CO-CS-1500 system (SYSMEX Co., Ltd, Kobe, Japan) and the liver function test was performed using a Cobas 8000 analyser (Roche Diagnostics, Mannheim, Germany).

Liver and SS measurements by TE

LSM and SSM were measured in all patients using FibroScan (Echosens, Paris, France) and FibroTouch (Hai's Medical Technology Center, Beijing, China). The LSM was assessed by a trained and experienced operator after at least 4 h of fasting, and the SSM was performed on the same day as the LSM assessment. All measurements were obtained by experienced operators who had performed at least 300 tests in patients with chronic liver disease. The TE results of the patients were collected retrospectively, and the obtained results were expressed in kilopascals (kPa). The interquartile range (IQR) was defined as the intrinsic variation index between the 25^{th} and 75^{th} percentiles of the LS results containing 50% of the valid measurements. Therefore, LSM and SSM values were considered to be reliable when at least 10 valid measurements were obtained and the results were reliable, with an overall success rate of more than 60% and IQR/median $\leq 0.3[25,26]$.

Upper gastrointestinal endoscopy

Upper gastrointestinal endoscopy was performed by an endoscopic operator who was experienced in the assessment of patients with cirrhosis (with a minimum of 500 endoscopic procedures). Endoscopic examinations were performed to determine whether the patients had EVs, and if so, the EVs were graded according to the location (L), shape and size (F), colour (C), and presence or absence of RS of the lesion.

Non-invasive score of EVs

The non-invasive prediction models we choose to compare were as shown blow: LSPS = [LSM (KPa) × SLD (cm)]/PLT (× 10⁹/L)[16]; VRI = -4.364 + 0.538 × SLD-0.049 × PLT-0.044 × LSM + 0.001 × (LSM × PLT)[17]; AAR = AST/ALT[18] and the Baveno VI criteria proposed by the consensus conference. The Baveno VI criteria were defined as LSM < 20 kPa and platelet count > 150×10^{9} /L. The extended Baveno VI criteria were defined as LSM < 20 kPa and platelet count > 110×10^{9} /L[8,27]. The results of upper gastrointestinal endoscopy were used as the gold standard. The receiver operating characteristic (ROC) curves of LSPS, VRI, AAR and Baveno VI were drawn, and the area under the ROC curve (AUC), sensitivity, specificity and Youden index were calculated to evaluate the performance of the new model and the previous four models in identifying HEVs. The point with the largest sum of sensitivity and specificity was selected as the best cut-off value for the diagnosis of HEVs.

EVs saluation of new prediction models

The discrimination ability of the new model was assessed by the ROC curves in the modelling group and the external validation group. The Z test was used to evaluate differences in ROC curves. If there was no significant difference in the ROC between the two groups and AUC > 0.7, the model was considered to have good discrimination ability. The calibration ability of the prediction model was evaluated by the Hosmer-Lemeshow test and the two sets of calibration scatter plots. Decision curve analysis (DCA) was performed to evaluate the clinical efficacy of the new model.

Statistical analysis

SPSS 26.0 and R software (IBM SPSS, Chicago, IL, United States) were used for statistical analysis. Data are presented as the mean \pm SD. The chi-square test was used to compare the measurement data between the HEVs group and the LEVs group. The Mann-Whitney *U* test was used for univariate analysis of continuous variable measurement data, and WALD backwards regression analysis was used for multivariate analysis. SPSS 26.0 software was used to draw the ROC curve and calculate the AUC to evaluate the diagnostic performance of the model. The maximum corresponding point of the Youden index was selected as the best cut-off value, and a positive prediction result was defined as equal to or greater than the best cut-off value. The best discrimination probability threshold, sensitivity, specificity and predictive value were calculated, and the diagnostic accuracy was compared. The higher the Youden index (1% or 100%), the more effective the correlation. Hosmer-Lemeshow test results, calibration charts and DCA were obtained using R software. All statistical tests were two-sided, with an alpha value of 0.05 and a statistical significance threshold of *P* < 0.05.

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RESULTS

Baseline characteristics of the patients

Tables 1 and 2 List the baseline characteristics of the modelling group and the external validation group, respectively. In the LEVs group, the mean age was 50.88 years ± 11.6 years, 43 (58.1%) were male, and 61 (82.4%) had hepatitis B. The age of the HEVs patients was 55.36 years ± 11.1 years old, 37 (56.1%) were male, and 53 (80.3%) had hepatitis B. In the modelling group, there was a significant difference in age between the HEVs group and the LEVs group (P < 0.05) but no significant difference in sex or hepatitis type (P > 0.05), and the two groups were comparable. In the external validation group, there was no significant difference in sex, age or hepatitis type (P > 0.05).

Univariate analysis of HEVs

T tests and non-parametric rank sum tests were used for the univariate analysis. The summarized results are shown in Table 3. There were significant differences in SSM, PLT, LSM, ALT, AST, GGT, SLD, PT, INR, PTA, PVD, CTLV, and CTSV between the HEVs group and the LEVs group (P < 0.05). There were no significant differences in ALP, TBil, and TCHO between the two groups (P > 0.05).

Multivariate analysis of HEVs

The parameters shown in Table 3 with statistically significant differences between the HEVs and LEVs groups were analyzed by multivariate analysis using backwards WALD regression analysis. As shown in Table 4, the SSM and LSM between the HEVs group and LEVs group were significantly different (P <0.05). There were no significant differences in PLT, ALT, AST, GGT, SLD, PT, INR, PTA, PVD, CTLV, and CTSV (*P* > 0.05).

Establishment of a non-invasive prediction model

Based on the results of the multivariate analysis, the parameters with no statistically significant difference between the two groups were excluded, and the parameters with statistically significant differences, including SSM and LSM, were used to establish a non-invasive prediction model. The logistic regression analysis showed that SSM and LSM were independent factors affecting the occurrence of HEVs and were statistically significant (P < 0.05). As shown in Table 5, the model was as follows: $Ln [P/(1-P)] = -8.184 - 0.228 \times SSM + 0.642 \times LSM$. HEVs in patients with viral cirrhosis were negatively correlated with SSM and positively correlated with LSM.

Comparison of non-invasive prediction models

The new model was compared with other models reported to predict EVs in patients with liver cirrhosis, namely, the LSPS, VRI, AAR, and Baveno VI models. The sensitivity, specificity and AUC of the new model based on the SSM and LSM and LSPS, VRI, AAR and Baveno VI models were calculated. The cut-off value of the model was defined as the maximum value of the sum of the specificity and sensitivity. Patients were considered to have HEVs when the P value calculated by the established formula was greater than the cut-off value. As shown in Figure 1A and Table 6, the AUC of this model was 0.965, while the AUCs of LSPS, VRI, AAR and Baveno VI were 0.835, 0.744, 0.641, and 0.675, respectively. The AUC > 0.7 indicated the good discrimination power of the model. The higher the AUC is, the better the discriminative power of the model, so the new model has good discriminative power.

Diagnostic accuracy of non-invasive tests for predicting HEVs

The accuracy, positive predictive value and negative predictive value of LSPS, VRI, ARR, Baveno VI, and the new model of 140 patients in the modelling group were calculated according to the calculation formula. As shown in Table 7, the non-invasive prediction model shown in this study had an accuracy of 89.30% and a positive predictive value of 83.52%. The accuracy and positive predictive values suggest the likelihood that the new model can correctly diagnose HEVs, with higher values indicating a more correct diagnosis.

Discriminating ability edicting HEVs

Evaluation of the ability of the non-invasive model was performed by drawing the ROC curve of the established new model in the external validation group and using the Z test to compare the AUC curve between the modelling group and the external validation group and to evaluate the discriminative power of the new model. The AUC of the new model for predicting HEVs in the modelling group was 0.965, which was higher than that of the LSPS, VRI, AAR, and Baveno VI models. The AUC of the new model in the external validation group was 1. The Z test result was 0.896, and the P value was 0.37, indicating that there was no significant difference between the modelling group and the external validation group. The ROC curve of the external validation group is shown in Figure 1B.

EVs saluation of the calibration ability of the new model

The Hosmer-Lemeshow test was used to calculate the χ^2 value of the modelling group and the external



Table 1 Comparison of general characteristics in the modelling group					
Parameter	Patients with LEVs, <i>n</i> = 74	Patients with HEVs, <i>n</i> = 66	<i>T</i> value/ χ ² value	<i>P</i> value	
Age in yr	50.88 ± 11.60	55.36 ± 11.10	-2.333	0.021	
Male (%)	43 (58.1%)	37 (56.1%)	0.060	0.807	
Etiology, HBV/HCV	61/13	53/13	0.105	0.746	

P < 0.05 is considered statistically significant. HEVs: High-risk esophageal varices; LEVs: Low-risk esophageal varices; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

Table 2 Comparison of general characteristics in the external validation group						
Parameter	Patients with LEVs, <i>n</i> = 28	Patients with HEVs, <i>n</i> = 32	<i>T</i> value/χ² value	P value		
Age in yr	52.54 ± 13.70	54.97 ± 10.40	-0.780	0.438		
Male (%)	15 (53.6%)	14 (43.8%)	0.577	0.448		
Etiology, HBV/HCV	25/3	30/2	0.024	0.876		

P < 0.05 is considered statistically significant. HEVs: High-risk esophageal varices; LEVs: Low-risk esophageal varices; HBV: Hepatitis B virus; HCV; Hepatitis C virus.

Table 3 Univariate analysis of parameters of patients with high-risk esophageal varices and low-risk esophageal varices					
Parameter	Patients with LEVs, <i>n</i> = 74	Patients with HEVs, <i>n</i> = 66	tlZ	<i>P</i> value	
SSM, KPa	22.70 ± 6.00	19.06 ± 4.90	3.880	< 0.001	
PLT, $\times 10^9/L$	108.55 ± 68.10	62.53 ± 29.00	5.096	< 0.001	
LSM, KPa	14.90 ± 5.10	24.83 ± 4.30	-12.354	< 0.001	
ALT, IU/L	37.50 (26.00, 49.50)	23.00 (16.00, 35.00)	-4.278	< 0.001	
AST, IU/L	43.00 (31.75, 69.00)	34.50 (27.75, 45.25)	-2.796	0.005	
ALP, IU/L	108.00 (81.25, 137.25)	93.50 (78.00, 128.75)	-1.012	0.311	
GGT, IU/L	60.50 (28.00, 114.00)	33.00 (19.75, 58.75)	-3.609	< 0.001	
SLD, mm	13.58 ± 3.10	15.10 ± 3.30	-2.806	0.006	
TBIL, μmol/L	22.63 (16.01, 34.96)	27.80 (18.05, 39.65)	-0.960	0.337	
ALB, g/dL	37.16 ± 8.30	36.02 ± 5.80	0.935	0.351	
TCHO, mmol/L	3.57 ± 1.40	3.18 ± 0.90	1.981	0.050	
PT, s	12.45 ± 2.20	13.34 ± 2.50	-2.284	0.024	
INR	1.13 ± 0.20	1.21 ± 0.20	-2.175	0.031	
РТА, %	82.15 ± 20.80	75.09 ± 16.10	2.228	0.028	
PVD, mm	12.20 ± 1.90	13.64 ± 2.30	-4.024	< 0.001	
CTLV, cm ³	1031.88 ± 361.20	920.85 ± 241.50	2.111	0.037	
CTSV, cm ³	558.11 ± 338.70	808.25 ± 409.90	-3.951	< 0.001	

P < 0.05 is considered statistically significant. HEVs: High-risk esophageal varices; LEVs: Low-risk esophageal varices; SSM: Spleen stiffness measurement; LSM: Liver stiffness measurement; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: Glutamine transferase; SLD: Spleen long diameter; TBIL: Total bilirubin; ALB: Albumin; TCHO: Total cholesterol; PT: Prothrombin time; INR: International prothrombin ratio; PTA: Prothrombin activity; PVD: Portal vein diameter; CTLV: Actual liver volume measured by computed tomography; CTSV: Actual spleen volume measured by computed tomography.

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Table 3 Univariate

Table 4 Multivariate analysis of parameters of patients with high-risk esophageal varices and low-risk esophageal varices					
Parameter	Patients with LEVs, <i>n</i> = 74	Patients with HEVs, <i>n</i> = 66	t/Z	<i>P</i> value	
SSM, KPa	22.70 ± 6.00	19.06 ± 4.90	3.880	0.009	
PLT, $\times 10^9/L$	108.55 ± 68.10	62.53 ± 29.00	5.096	0.606	
LSM, KPa	14.90 ± 5.10	24.83 ± 4.30	-12.354	< 0.001	
ALT, IU/L	37.50 (26.00, 49.50)	23.00 (16.00, 35.00)	-4.278	0.669	
AST, IU/L	43.00 (31.75, 69.00)	34.50 (27.75, 45.25)	-2.796	0.125	
	60.50 (28.00, 114.00)	33.00 (19.75, 58.75)	-3.609	0.790	
SLD, mm	13.58 ± 3.10	15.10 ± 3.30	-2.806	0.952	
PT, s	12.45 ± 2.20	13.34 ± 2.50	-2.284	0.883	
INR	1.13 ± 0.20	1.21 ± 0.20	-2.175	0.777	
РТА, %	82.15 ± 20.80	75.09 ± 16.10	2.228	0.920	
PVD, mm	12.20 ± 1.90	13.64 ± 2.30	-4.024	0.220	
CTLV, cm ³	1031.88 ± 361.10	920.85 ± 241.50	2.111	0.892	
CTSV, cm ³	558.11 ± 338.70	808.25 ± 409.90	-3.951	0.713	

P < 0.05 is considered statistically significant. HEVs: High-risk esophageal varices; LEVs: Low-risk esophageal varices; SSM: Spleen stiffness measurement; LSM: Liver stiffness measurement; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; SLD: Spleen long diameter; PT: Prothrombin time; INR: International prothrombin ratio; PTA: Prothrombin activity; PVD: Portal vein diameter; CTLV: Actual liver volume measured by computed tomography; CTSV: Actual spleen volume measured by computed tomography.

Table 5 Parameters used to establish the non-invasive prediction model						
Parameter	В	S.E.	Wald	Р	Exp (B)	95%Cl of exp (B)
SSM	-0.228	0.074	9.647	0.002	0.796	0.689-0.919
LSM	0.642	0.123	27.245	< 0.001	1.900	1.493-2.418
Constant	-8.184	2.300	12.659	< 0.001	0.000	-

SSM: Spleen stiffness measurement; LSM: Liver stiffness measurement; CI: Confidence interval.

Table 6 Comparison of various parameters of each model						
	Area	SE	Р	95%Cl of exp (B)		
LSPS	0.835	0.033	< 0.001	0.771-0.900		
VRI	0.744	0.041	< 0.001	0.663-0.824		
AAR	0.641	0.046	0.004	0.550-0.732		
Baveno VI	0.675	0.045	< 0.001	0.586-0.764		
The new model	0.965	0.015	< 0.001	0.936-0.995		

LSPS: Liver stiffness-spleen diameter to platelet ratio score; VRI: Variceal risk index; AAR: Aspartate aminotransferase/alanine aminotransferase ratio; BavenoVI: Baveno VI criteria were defined as liver stiffness measurement < 20 kPa and platelet count > 150 × 10⁹ / L; CI: Confidence interval.

> validation group to evaluate the calibration ability of the new model. The results showed that χ^2 was -10.39 in the modelling group and 0.03 in the external validation group. The P values were 0.999 and 1.000, respectively. Both the groups' P value were greater than 0.05, indicating that the model could predict HEVs accurately. The calibration scatter plots of the two groups were shown in Figure 2. As seen from the figure, all scatter points fluctuated around the baseline without significant deviation because the P values of both groups were greater than 0.05 and the difference between the groups was not statistically significant. The results showed that the HEVs patients who were predicted to have viral



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Table 7 Comparison of various parameters of each model						
	Sensitivity	Specificity	Accuracy, %	Positive predictive value, %	Negative predictive value, %	Cutoff value
LSPS	89.39	62.16	74.30	67.78	86.81	3.12
VRI	74.24	67.57	69.30	67.09	74.66	0.03
AAR	75.76	52.70	57.90	58.78	70.95	1.27
Baveno VI ¹	98.48	36.49	65.70	57.99	96.42	-
The new model	100.00	82.43	89.30	83.52	100.00	0.27

¹Baveno VI criteria were defined as liver stiffness measurement < 20 kPa and platelet count > 150×10^9 /L.

LSPS: Liver stiffness-spleen diameter to platelet ratio score; VRI: Variceal risk index; AAR: Aspartate aminotransferase/alanine aminotransferase ratio.



Figure 1 Area under the curve of various models in predicting high-risk esophageal varices of patients. A: Modelling group; B: External validation group. The area under the curve of the new model in predicting high-risk esophageal varices of patients was 0.965 in the modelling group, which was higher than that of liver stiffness-spleen diameter to platelet ratio score, variceal risk index, aspartate aminotransferase/alanine aminotransferase ratio and Baveno VI; and it was 1 in the external validation group. LSPS: Liver stiffness-spleen diameter to platelet ratio score; VRI: Variceal risk index; AAR: Aspartate aminotransferase/alanine aminotransferase ratio.



Figure 2 Calibration scatter plot of data of patients. A: Modelling group; B: External validation group. In predicting patients in the modelling group and external validation group, the scattered points fluctuated around the reference line without significant deviations.

cirrhosis by using the new model were in good agreement with the actual HEVs patients.

EVs salvation of the clinical efficacy of the new model

DCA was plotted against the probability of actual HEVs occurrence by predicting the probability of the modelling group and the external validation group by the new model. The DCA of the two groups is shown in Figure 3. In the DSA curve, the two dashed lines represent the two extreme cases, and the black line indicates that the new model predicts there was no HEVs, and a net clinical benefit of zero. The other grey line with a negative slope indicates that the new model predicts HEVs in all patients with viral cirrhosis, and the net clinical benefit is a back-slope with a negative slope[28]. The red line is the new model's DCA. As the DCA curve shown, the red line was higher than the black and grey lines, indicating that when the new model was applied to the modelling group and the external validation group, both groups of patients could benefit, so the new model had certain clinical efficacy.



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Figure 3 Adjusted decision curve analysis of data of patients. A: Modelling group; B: External validation group. The black line indicates that in extreme cases, the new model predicted that there were no high-risk esophageal varices in all patients with viral cirrhosis, and the clinical net benefit was 0. The gray curve indicates that in extreme cases, the new model predicts there are high-risk esophageal varices in all patients with viral cirrhosis, the clinical net benefit is the negative slope. The red line indicates that the new model has a clinical net benefit. The red line is higher than the black and gray lines, indicating that patients in the modelling group can benefit from the new model.

DISCUSSION

Long-term chronic viral hepatitis can lead to liver cirrhosis and is associated with high morbidity and mortality; therefore, it is a public health concern that deserves attention[29-31]. EVs rupture and bleeding are common causes of death in patients with liver cirrhosis. Clinical guidelines recommend the use of upper gastrointestinal endoscopy in screening and periodic reexamination of patients with cirrhosis regardless of the disease cause[9]. According to the results of upper gastrointestinal endoscopy and the Baveno VI criteria, EVs were divided by the low bleeding risk EVs (LEVs) and the high bleeding risk EVs (HEVs). For patients with HEVs, early precation measures can significantly reduce the esophageal variceal bleeding risk[22,32]. However, the invasiveness of upper gastrointestinal endoscopy, the high price, and the risk of anaesthesia make the compliance of patients to upper gastrointestinal endoscopy very low [9,10]. Considering that many patients do not have EVs in the early stage of liver cirrhosis, there is a need for non-invasive, simple, and safe means to identify liver cirrhosis patients with HEVs. In addition to upper gastrointestinal endoscopy, a variety of imaging methods, such as ultrasound, CT, and MRI, can be used to predict HEVs. However, these three methods cannot visually observe EVs, and the accuracy of identifying HEVs is poor[33]. A number of non-invasive models have been developed to predict HEVs, and several studies have shown that the LSPS, VRI, AAR, and Baveno VI models have achieved good results in predicting HEVs. Measurement of LS and SS by TE (using FibroScan) is a fast, non-invasive, easy to perform and reproducible procedure for predicting the presence of clinically significant EVs and PH, so LS and SS were measured by TE in this study [34]. In this study, to ensure the homogeneity of aetiology, we included patients with cirrhosis and HBV/ HCV infection. Our study showed that LSM and SSM were two independent variables associated with the presence of HEVs. The results of our study are similar to those of several studies. LSM is associated with PH, and LSM combined with other indicators can predict HEVs[35,36]. Splenomegaly is common in patients with chronic viral cirrhosis, and splenic blood flow enters the portal vein system through the splenic vein. Therefore, SSM can simultaneously reflect static resistance fibrosis of the liver (LSM can also reflect) and dynamically capture PH-related visceral hypoperfusion, changes in spleen results, changes in blood flow, and PH-induced splenic fibrosis[37].

In this study, we constructed a non-invasive prediction model including LSM and SSM. The AUC of the new model was 0.965, the accuracy was 89.30%, which was better than that of the LSPS, VRI, AAR, and Baveno VI models, and the new model showed good diagnostic performance. When the optimal cut-off value was 0.27, the sensitivity and negative predictive value (NPV) of the new model in the modelling group were both 100%, and due to the Baveno VI criteria and other models, the new model could best identify non-HEVs patients so that these patients could be spared from undergoing upper gastrointestinal endoscopy. Morishita et al[38] used a similar approach in 135 patients with HCV-related cirrhosis to predict the presence of HEVs in patients with viral cirrhosis using a single indicator of LSM, and their results showed that the AUC of LSM for predicting the presence of HEVs was 0.868. The sensitivity, specificity, positive predictive value and negative predictive value were 81%, 82%, 69%, and 89%, respectively[38]. Similarly, in another study, Stefanescu et al[5] used a single-indicator SSM to assess the presence of HEVs, selecting an SSM@50 Hz with a 95% sensitivity for the best cut-off value [5]. Moreover, many studies have shown that when the HVPG value is \geq 12 mmHg, LSM alone cannot reliably diagnose or exclude the risk grade of varices because of the poor correlation of extrahepatic factors. However, SSM can evaluate the severity of PH, the presence of EVs and the risk of bleeding but cannot predict the grade of EVs[39]. Therefore, this study developed and validated whether a noninvasive prediction model based on the combination of SSM and LSM indicators can be used as a useful tool to assess the severity of EVs and the risk of upper gastrointestinal bleeding (UGIB). These results of our study suggest that in the majority of patients with viral cirrhosis evaluated, the new model can



accurately exclude patients with HEVs, thereby allowing these patients to avoid endoscopy or prophylactic therapy. Furthermore, the higher NPV and sensitivity, regardless of the cirrhosis severity, suggest that use of the new model may be more cost-effective, as endoscopic screening of patients with both compensated and decompensated cirrhosis proved to be cost-effective[40]. In addition, a second independent dataset was used to externally validate the clinical utility of the new model, and the results showed that the new model had high discrimination power. In addition, DCA was cited to illustrate the clinical benefit of the new model, and both the modelling group and the external validation group could benefit from the new model. In addition, the included indicators in the new model can be obtained by TE and B-ultrasound, which are non-invasive, inexpensive, do not require radiation, are highly feasible in clinical practice, and are easy to popularize in clinical work.

Generally speaking, we have successfully developed a non-invasive prediction model using LSM and SSM indicators to predict the presence of HEVs in patients with viral cirrhosis, which has not been reported in the literature. Compared with other models LSPS, VRI, AAR, and Baveno VI, the new model has a good diagnostic performance, a high discrimination ability, calibration ability and a clinical application value. In addition, we enrolled patients with viral cirrhosis, which provided good consistency while minimizing bias in the results. However, this retrospective study has limitations. First, the sample size of this study is relatively small, and more data need to be collected for evaluation. Second, the patients in this study were all Chinese, and it is unclear whether this model can be applied in other ethnic groups. Additionally, because the patients in this study were all patients with viral cirrhosis and because changes in liver and spleen volume can vary with cirrhosis from different causes, it is unclear whether the new model can be applied to cirrhosis from other causes.

In conclusion, the new model based on SSM and LSM indicators, $\ln [P/(1-P)] = -8.184 - 0.228 \times SSM + 0.228 \times SM +$ $0.642 \times LSM$, can effectively rule out the presence of HEVs in patients with viral cirrhosis, and this model needs to be further verified in prospective trials. This model helps physicians recognize the presence of HEVs in patients with viral cirrhosis, make more informed decisions, and provide appropriate preventive treatment.

CONCLUSION

The new model can effectively rule out the presence of HEVs in patients with viral cirrhosis and can be used as an alternative to routine upper gastrointestinal endoscopy screening.

ARTICLE HIGHLIGHTS

Research background

Acute bleeding due to esophageal varices (EVs) is a life-threatening complication in patients with cirrhosis. The diagnosis of EVs is mainly through upper gastrointestinal endoscopy, but the discomfort, contraindications and complications of gastrointestinal endoscopic screening reduce patient compliance.

Research motivation

To develop a safe, simple and non-invasive model to predict high risk EVs (HEVs) in patients with viral cirrhosis and identify patients who can be exempted from upper gastrointestinal endoscopy.

Research objectives

To establish a non-invasive prediction model based on spleen stiffness measurement (SSM) and live stiffness measurement (LSM) as an alternative to EVs screening.

Research methods

Two hundred Chinese adults, from March 2020 to November 2022, were included at the Second Affiliated Hospital of Xi'an Jiaotong University. Required data were collected by the medical records, and the EVs types of patients were determined by upper gastrointestinal endoscopy and the Baveno VI consensus. The effect of each parameter on HEVs was analyzed by univariate and multivariate analyses, and a non-invasive prediction model was established, and then the effect of each parameter on HEVs was analyzed by univariate and multivariate analyses, and a non-invasive prediction model was established.

Research results

After univariate and multivariate analyses, SSM and LSM were used to established a prediction model. The new non-invasive model was better than other four models to predict HEVs in patients with viral cirrhosis.



Research conclusions

The new model is reliable in predicting HEVs and can be used as an alternative to routine upper gastrointestinal endoscopy screening, which is helpful for clinical decision making.

Research perspectives

In the future, we will try to apply the new model to predict HEVs in patients with viral cirrhosis.

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FOOTNOTES

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Observational Study

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ORIGINAL ARTICLE

Real-world effectiveness and safety of direct-acting antivirals in hepatitis C virus patients with mental disorders

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Abstract

BACKGROUND

It is estimated that 58 million people worldwide are infected with the hepatitis C virus (HCV). Patients with severe psychiatric disorders could not be treated with previously available interferon-based therapies due to their unfavorable side effect profile. This has changed with the introduction of direct-acting antivirals (DAA), although their real-life tolerance and effectiveness in patients with different psychiatric disorders remain to be demonstrated.

AIM

To evaluate the effectiveness and safety of DAA in patients with various mental illnesses.

METHODS

This was a retrospective observational study encompassing 14272 patients treated with DAA for chronic hepatitis C in 22 Polish hepatology centers, including 942 individuals diagnosed with a mental disorder (anxiety disorder, bipolar affective disorder, depression, anxiety-depressive disorder, personality disorder, schizophrenia, sleep disorder, substance abuse disorder, and mental illness without a specific diagnosis). The safety and effectiveness of DAA in this group were compared to those in a group without psychiatric illness (n = 13330). Antiviral therapy was considered successful if serum ribonucleic acid (RNA) of HCV was undetectable 12 wk after its completion [sustained virologic response (SVR)]. Safety data, including the incidence of adverse events (AEs), serious AEs (SAEs), and deaths, and the frequency of treatment modification and discontinuation, were collected during therapy and up to 12 wk after treatment completion. The entire study population was included in the intent-to-treat (ITT) analysis. Per-protocol (PP) analysis concerned patients who underwent HCV RNA evaluation 12 wk after completing treatment.

RESULTS

Among patients with mental illness, there was a significantly higher percentage of men, treatmentnaive patients, obese, human immunodeficiency virus and hepatitis B virus-coinfected, patients with cirrhosis, and those infected with genotype 3 (GT3) while infection with GT1b was more frequent in the population without psychiatric disorders. The cure rate calculated PP was not significantly different in the two groups analyzed, with a SVR of 96.9% and 97.7%, respectively. Although patients with bipolar disorder achieved a significantly lower SVR, the multivariate analysis excluded it as an independent predictor of treatment non-response. Male sex, GT3 infection, cirrhosis, and failure of previous therapy were identified as independent negative predictors. The percentage of patients who completed the planned therapy did not differ between groups with and without mental disorders. In six patients, symptoms of mental illness (depression, schizophrenia) worsened, of which two discontinued treatments for this reason. New episodes of sleep disorders occurred significantly more often in patients with mental disorders. Patients with mental illness were more frequently lost to follow-up (4.2% vs 2.5%).

CONCLUSION

DAA treatment is safe and effective in HCV-infected patients with mental disorders. No specific



psychiatric diagnosis lowered the chance of successful antiviral treatment.

Key Words: Hepatitis C; Mental disorders; Direct-acting antivirals

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Core Tip: In the population of patients with mental illness, the prevalence of hepatitis C virus infection is many times higher than in the general population. In the era of interferon-based therapy, these patients had minimal access to antiviral treatment due to contraindications to interferon with the risk of exacerbation of psychiatric diseases. Their situation has improved with the introduction of direct-acting antivirals, but data from real-world studies are scarce. This retrospective analysis carried out in clinical practice confirmed that the good safety profile and treatment effectiveness are not inferior to those obtained in a population without mental disorders.

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INTRODUCTION

Hepatitis C virus (HCV) infection, currently affecting approximately 58 million individuals worldwide, still poses a severe global issue[1]. It remains a major cause of chronic liver disease worldwide, liable to progress to cirrhosis and hepatocellular carcinoma, and can lead to various extrahepatic manifestations [2]. The prevalence of HCV infection is significantly increased (3-fold to 20-fold) in individuals with mental disorders compared to the general population. This includes patients with depression, bipolar disorder, schizophrenia, and personality disorders. In patients with substance abuse, particularly with intravenous drug use, the reported percentage of HCV infection is as high as 60%-70% [3,4]. In addition, preexisting mental illness may complicate HCV treatment by adversely affecting treatment adherence[5-7]. Moreover, HCV infection itself can further exacerbate or accelerate psychiatric symptoms [6,8].

In the past, treatment of patients with severe psychiatric disorders infected with HCV was highly challenging due to neuropsychiatric side effects (e.g., anxiety, affective, cognitive, and psychotic symptoms) of previously used interferon (IFN)-based therapies[9]. Their situation has improved with the introduction of direct-acting antivirals (DAA), which are well tolerated and easier to dose[10]. Whether they are also effective in different mental disorders requires further studies. Observations from the IFN-era period indicate that these disorders were not necessarily associated with a worse sustained viral response (SVR) rate[11-13]. However, the observations encompassing the period when DAA were introduced are scarce, include small study groups, only particular diseases (e.g., depression), or treat them collectively as mental disorders [14-17]. The results of one multicenter study evaluating a single DAA regimen among psychiatric patients by type of mental disorder have recently been published, but evidence is still needed from large populations of real-world experience that discriminate between individual psychiatric diseases treated with different DAA options and compare the results to the group without mental disorders while controlling for confounding variables[18].

To this end, the present study aimed to evaluate the effectiveness and safety of DAA in patients with various mental illnesses: Anxiety, anxiety-depressive disorder, bipolar disorder, depression, personality disorder, schizophrenia, sleep disorder, substance abuse, and mental illness without a specific diagnosis. In order to achieve this goal, a retrospective, real-world analysis of the Polish population of HCV-infected patients treated with DAA between 2015 and 2022 was conducted.

MATERIALS AND METHODS

Data collection

The data of 14272 patients treated with DAA for chronic hepatitis C (CHC) from 1 July 2015 to 30 December 2022, included in the EpiTer-2 observational study database, were analyzed. This study was conducted under the auspices of the Polish Association of Epidemiologists and Infectiologists and



included data from 22 Polish hepatology centers on treating CHC in everyday practice. The choice of CHC therapy was at the physician's discretion and followed the existing recommendations of the Polish Group of Experts for HCV and the recommendations of the National Health Fund. The data were collected retrospectively and included age, sex, body mass index, the severity of liver disease, HCV genotype (GT), human immunodeficiency virus (HIV) and/or hepatitis B virus (HBV) coinfection, previous and current CHC treatment, presence of hepatocellular carcinoma, HCV viral load, alanine transaminase activity, albumin, hemoglobin, creatinine, and platelets level.

Assessment of mental disorders

The patients were divided into two groups: 942 patients with concomitant mental illness (group A) and 13330 without psychiatric disorders (group B). Mentally ill patients were under the care of psychiatrists, who diagnosed their mental illness, prescribed and monitored psychiatric treatment.

Assessment of liver disease severity

The severity of liver disease was assessed using non-invasive methods of fibrosis assessment: Transient elastography or shear-wave elastography with an Aixplorer device (SuperSonic Imagine, Aix-en-Provence, France) or transient FibroScan. Based on the METAVIR score, according to the guidelines of the European Association for the Study of the Liver, the cutoff value of 13 kPa was used to predict individuals considered to be cirrhotic[19]. These patients were evaluated for the presence of decompensation of liver function (in the past and at the beginning of antiviral therapy) and were assessed on the Child-Pugh (CP) scale. Patients scored as B or C on the CP scale were considered to be decompensated. Data were also collected on the past diagnosis of HCC and liver transplantation.

Effectiveness assessment

Antiviral therapy was considered successful if ribonucleic acid of the virus (HCV RNA) in serum was undetectable 12 wk after its completion, meaning that the patient achieved a sustained virologic response (SVR). Patients with detectable viral load at this time point were considered virologic failures, while those lost to follow-up without HCV RNA evaluation 12 wk after the end of therapy were recognized as non-virological failures and not included in the PP analysis.

Safety assessment

An analysis of potential drug-drug interactions was carried out before the start of DAA therapy using an online tool on the University of Liverpool website[20]. The results of this analysis influenced the choice of DAA regimen or modification of psychiatric therapy. Safety data, including the incidence of adverse events (AEs), serious AEs (SEAs), and deaths, as well as the frequency of treatment modification and discontinuation, were collected during therapy and up to 12 wk after treatment completion.

Ethics

The data were originally collected not for scientific purposes but to evaluate the effectiveness and safety of real-world treatment with registered drugs. Patients were not exposed to any experimental interventions. According to local law (Law of 6 September 2001, Pharmaceutical Law, Article 37al), non-interventional studies do not require ethics committee approval. Due to the retrospective design of the analysis, the requirement for patient consent was not necessary. Patient data were collected and analyzed in accordance with applicable data protection rules.

Statistical analysis

Statistical analyses were performed using Statistica v. 13 (StatSoft, Tulsa, OK, United States) and MedCalc v. 15.8 (MedCalc Software Ltd, Ostend, Belgium). The entire study population was included in the ITT analysis. The PP analysis concerned patients who underwent HCV RNA evaluation 12 wk after completing treatment. Differences in frequencies of events between groups with and without mental disorders were assessed with χ^2 Pearson's test or Fisher exact test (when the number of observations was < 10). The differences in data expressed on the interval scale were evaluated with a non-parametric Mann-Whitney *U* test because they did not meet the Gaussian assumption (Shapiro-Wilk's test, *P* < 0.05). Multiple logistic regression was used to predict the odds of no response to HCV treatment for mental disorders (for which a lower frequency of SVR was observed) while controlling for known factors affecting the chance of SVR in the IFN and IFN-free era: male sex, obesity, GT3, cirrhosis, history of HCV treatment failure, HIV coinfection, HBV coinfection, and three antiviral combinations: asunaprevir (ASV) and daclatasvir (DCV), sofosbuvir (SOF) and velpatasvir (VEL), and SOF/VEL with ribavirin (RBV)[21-24]. A *P* value below 0.05 was deemed statistically significant.

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RESULTS

Characteristics of the studied groups

The demographic characteristics of the groups are summarized in Table 1. Group A included 503 patients who had depression (55.6%), 121 with schizophrenia (12.8%), 36 with mental illness without a specific diagnosis (3.8%), 26 with anxiety-depressive disorders (2.8%), 24 with bipolar affective disorder (2.6%), 21 with anxiety disorders (2.2%), 16 with personality disorders (1.7%), and 6 with sleep disorders (0.6%). Substance abuse disorder was diagnosed in 159 cases (16.9%). Moreover, in 30 patients (3.2%), substance abuse and other mental illness coexisted: Depression in 21 patients (2.2%), personality disorders in 4 (0.4%), schizophrenia in 2 (0.2%), anxiety disorders in 2 (0.2%), and an anxiety-depressive disorder in 1 patient (0.1%).

The mean age of patients in both groups was comparable. Group A included a higher percentage of men, obese individuals, patients with more advanced liver fibrosis, and a higher percentage of CP class B and C patients. Similar to group B, the GT1b prevailed, although its frequency was lower, while the higher percentage of HCV-infections with GTs 3 and 4 were noted. Coinfections with HIV and/or HBV were also more common in the group of patients with mental disorders (Table 2).

This group was also characterized by a higher baseline HCV viral load. Most patients in group A (80.6%) were treatment-naïve, while 16.7% (n = 157) of the cases had previously been treated with IFN. Of these, 34 patients discontinued IFN-based therapy due to AEs. During current therapy, 12/34 (35%) had mild AEs, the most common of which was headache (n = 3), and one patient in this group experienced increased insomnia. All patients in this group completed therapy as planned, 31 achieved SVR, and 1 patient was lost to follow-up. Patients with mental illnesses were more often treated with pangenotypic therapy for CHC (Table 3).

Treatment effectiveness

There were no statistically significant differences in the achievement of SVR in groups A and B in both the ITT (92.8% vs 95.3%, respectively) and PP (96.9% vs 97.7%) analyses (Figure 1A). A significantly lower percentage of SVR was observed in patients with bipolar disorder (Figure 1B). In multivariate analyses, a lower chance of SVR was associated with male sex, GT3, cirrhosis, history of previous HCVtreatment failure, and two DAA regimens: ASV + DCV and SOF + VEL + RBV (Table 4).

Treatment safety

Patients with mental illness more often belonged to the group lost to follow-up (4.2% vs 2.5%). In six patients symptoms of mental illness (depression - 5, schizophrenia - 1) worsened during antiviral therapy, of which two permanently discontinued treatment for this reason. New episodes of sleep disorders occurred significantly more often in group A during DAA therapy. In group B, one patient experienced an episode of schizophrenia, and six were diagnosed with depression. In both groups, a similar percentage of patients completed the planned treatment. There were no statistically significant differences in discontinued and modified treatments (Table 5).

Modification of therapy concerned only a change in the dose of RBV. Possible drug interactions were analyzed prior to antiviral therapy. Patients in group A were treated with neuroleptics, anticonvulsants, antidepressants, sedatives, anxiolytics, and methadone. Only carbamazepine could have clinically relevant interactions. This drug was used in 3 patients in group A and in 12 patients in group B. All patients with mental diseases using carbamazepine completed the treatment as planned without AEs, and all achieved SVR.

DISCUSSION

By documenting the high rate of SVR in patients with mental diseases, our study proves that this patient population does not differ in their response to DAA treatment from those with CHC not affected by psychiatric conditions supporting the findings of a recently published multicenter analysis [18]. Thus, there is no rationale for considering these patients as a difficult-to-treat population in terms of virologic response to therapy, as they were previously recognized in the era of IFN-based regimens. Antiviral therapy for mentally ill patients has been a challenge due to contraindications to the use of IFN and the side effects of this cytokine [9,25]. Neuropsychiatric disorders of various caliber during IFN therapy have been widely reported [26-28]. Most were mild in severity and transient in nature, but severe psychosis and suicide attempts have also been described as a reason for discontinuing therapy. Such side effects of therapy have been described even in patients with no prior mental disorders^[29]. Due to the risk of exacerbating psychiatric conditions, many patients with a diagnosed mental illness were not eligible for treatment. The mental state of those included in therapy had to be closely monitored, requiring cooperation with the patient's attending psychiatrist[30]. This was the only way to keep the patient in therapy and in the event of exacerbation of mental disorders, to react swiftly and to modify or discontinue antiviral treatment, if necessary for safety reasons[5,31]. At the same time, this was a population of patients in whom successful antiviral therapy offered a chance for mental improvement



Table 1 Baseline characteristics of chronic hepatitis C patients with (group A) and without (group B) psychiatric disorders, %, n					
Parameter	All patients (<i>n</i> = 14272)	Group A (<i>n</i> = 942)	Group B (<i>n</i> = 13330)	<i>P</i> value	
Age, mean (min-max yr)	51.2 (18-92)	50.8 (19-90)	51.2 (18-92)	0.599 (<i>U</i> = 6216478)	
Men/women	49.3/50.7 (7035/7237)	54.9/45.1, 507/435	49.0/51.0, 6528/6802	0.004 ($\chi^2 = 8.3$)	
BMI, mean (SD)	26.2 (4.5)	26.9 (5.1)	26.1 (4.5)	0.0001 (U = 5811710)	
BMI ≥ 30	17.4 (2486)	23.9 (225)	17.0 (2261)	$< 0.0001 \ (\chi^2 = 29.4)$	
Comorbidities					
Hypertension	32.0 (4567)	34.4 (324)	31.8 (4243)	0.103 ($\chi^2 = 2.6$)	
Diabetes	11.3 (1614)	13.0 (122)	11.2 (1492)	0.100 ($\chi^2 = 0.5$)	
Renal disease	3.8 (543)	3.4 (32)	3.8 (511)	0.498 ($\chi^2 = 0.5$)	
Autoimmune disease	2.1 (304)	2.0 (19)	2.1 (285)	0.803 ($\chi^2 = 0.06$)	
Non-HCC tumor	2.0 (279)	2.1 (20)	1.9 (259)	0.700 ($\chi^2 = 0.15$)	
ALT (IU/L), median (IQR)	58.0 (37.0-97.0)	59.0 (36.0-96.0)	58.0 (37.0-97.0)	0.581 (<i>U</i> = 6204221)	
Albumin (g/dL), median (IQR)	4.1 (3.8-4.4)	4.1 (3.8-4.4)	4.1 (3.8-4.4)	0.633 (<i>U</i> = 6222478)	
Bilirubin (mg/dL), median (IQR)	0.6 (0.5-0.9)	0.6 (0.5-0.9)	0.6 (0.5-0.9)	0.060 (<i>U</i> = 6050856)	
Hemoglobin (g/dL), median (IQR)	14.5 (13.4-15.5)	14.4 (13.4-15.4)	14.5 (13.4-15.6)	0.307 (<i>U</i> = 6120464)	
Platelets (× 1000/µL), median (IQR)	197.0 (146.0-244.0)	190.0 (136.0-240.0)	197.0 (146.0-244.0)	0.01 (<i>U</i> = 597757)	
Creatinine (mg/dL), median (IQR)	0.8 (0.7-0.9)	0.8 (0.7-0.9)	0.8 (0.7-0.9)	0.798 (<i>U</i> = 6101372)	
HCV RNA, × 10 ⁶ IU/mL, median (IQR)	0.97 (0.32-2.53)	1.17 (0.38-3.0)	0.96 (0.31-2.48)	0.0005 (<i>U</i> = 5856849)	

The reported P value relates to Pearson's chi-square test (or Fisher's exact test if the number of observations in a category was less than 10) or the Mann-Whitney U test. BMI: Body mass index; ALT: Alanine transaminase; HCC: Hepatocellular carcinoma; IQR: Interquartile range; HCV: Hepatitis C virus; RNA: Ribonucleic acid.

> [32]. In addition to causing liver disease, HCV can potentially induce extrahepatic manifestations, *i.e.*, disorders of other organs and systems that are pathogenetically associated with infection[33]. One of them is the central nervous system, whose involvement results in neurological and psychiatric disorders [6,7,34]. The mechanism of the virus's impact on the nervous system is complex and includes both a direct cytopathic effect related to the infection of nerve cells, as well as the impact of the immune response triggered by the HCV and the effects of psychogenic stressors[8,34,35].

> The frequent co-occurrence of CHC and psychiatric disorders was confirmed by numerous studies analyzing this issue in Real World Evidence (RWE) populations[36,37]. The estimated frequency of active mental illness in patients with CHC assessed in the large cohort of American Veterans was as high as one-third, while in our analysis, the percentage of patients was 6.6% [38]. Among them, depression, addiction, and schizophrenia were the most common. On the other hand, the pooled prevalence of HCV infection among patients with severe psychiatric diseases evaluated in a metaanalysis that included papers from 1989-2020 was 8%, which is several times higher compared to the general population[38,39]. However, the fact that patients with mental illnesses are disproportionately affected by HCV is also influenced by other factors beyond the pathogenetic relationship. One that seems to be historical is exposure to infection through frequent contact with healthcare facilities, and it cannot be overlooked that hospitalizations for mental illness are very long. Another factor contributing to the higher prevalence of HCV infections in this population, the importance of which persists to the present day, is that persons diagnosed with mental disorders are much more likely to abuse substances, including injection drugs and needle sharing used for intravenous drug administration is the most common route of HCV transmission[17]. This was also documented in our analysis. Moreover, the population of HCV-infected patients with mental diseases, which was underserved in the era of IFN, has gained its opportunity in the time of DAA therapy. The much shorter course of treatment and the lack of contraindications to the drugs has enabled patients with psychiatric disorders to access therapy on the same principles as for other HCV-infected individuals and has provided a basis for predicting HCV microelimination in this population[18]. In the current analysis, we found no statistically significant differences in the course of treatment in mentally ill patients compared to others. The percentage of those who had their therapy modified or discontinued was comparable; the modification



Table 2 Characteristics of liver disease in patients with chronic hepatitis C with (group A) and without (group B) psychiatric disorders, %, <i>n</i>					
Parameters	All patients (<i>n</i> = 14272)	Group A (<i>n</i> = 942)	Group B (<i>n</i> = 13330)	<i>P</i> value	
Genotype					
1a	4.4 (633)	4.1 (39)	4.5 (594)	0.649 ($\chi^2 = 0.2$)	
1b	77.3 (11029)	67.8 (639)	77.9 (10390)	$< 0.0001 \ (\chi^2 = 51.2)$	
1	2.1 (303)	2.4 (23)	2.1 (280)	0.483 ($\chi^2 = 0.5$)	
2	0.2 (29)	0.2 (2)	0.2 (27)	$> 0.948 \ (\chi^2 = 0.004)$	
3	11.1 (1580)	17.5 (165)	10.6 (1415)	$< 0.0001 (\chi^2 = 42.6)$	
4	4.9 (695)	8.0 (74)	4.7 (621)	$< 0.0001 \ (\chi^2 = 19.4)$	
5	0.007 (1)	0.0 (0)	0.007 (1)	1.0	
6	0.01 (2)	(0.0) 0	0.01 (2)	1.012	
Liver fibrosis					
F0	2.5 (361)	1.4 (13)	2.6 (348)	0.02 ($\chi^2 = 5.4$)	
F1	40.8 (5831)	34.8 (328)	41.3 (5503)	$< 0.0001 \ (\chi^2 = 15.2)$	
F2	19.2 (2743)	21.0 (198)	19.1 (2545)	0.147 ($\chi^2 = 2.1$)	
F3	13.5 (1925)	13.2 (124)	13.5 (1801)	0.763 ($\chi^2 = 0.09$)	
F4	23.9 (3412)	29.6 (279)	23.5 (3133)	$< 0.0001 \ (\chi^2 = 18.1)$	
Child-Pugh					
Child-Pugh-B	2.8 (398)	4.4 (41)	2.7 (357)	0.003 ($\chi^2 = 9.1$)	
Child-Pugh-C	0.2 (23)	0.4 (4)	0.1 (19)	0.061	
HCC history	1.3 (191)	1.2 (11)	1.3 (180)	0.637 ($\chi^2 = 0.2$)	
Coinfection					
HIV	5.9 (848)	9.1 (86)	5.7 (762)	$< 0.0001 \ (\chi^2 = 18.4)$	
HBV	13.5 (1933)	17.5 (165)	13.3 (1768)	0.0002 ($\chi^2 = 13.6$)	
HIV/HBV	1.6 (225)	3.4 (32)	1.4 (193)	$< 0.0001 (\chi^2 = 21.6)$	

The reported P value relates to Pearson's chi-square test or Fisher's exact test if the number of observations in the category was less than 10. HCC: Hepatocellular carcinoma; HIV: Human immunodeficiency virus; HBV: Hepatitis B virus.

> in both groups concerned only RBV dose reduction according to the label. The safety profile was also comparable with the percentage of patients experiencing SEAs and deaths. Similar conclusions on the good tolerability of DAA can be drawn from the observations of other researchers in RWE cohorts[40, 41]. When analyzing psychiatric complications occurring during therapy, we found a significantly higher percentage of sleep disorders in patients with psychiatric diseases than in those without mental conditions. Other researchers evaluating the quality of sleep in patients with mental disorders did not find any significant differences during DAA therapy compared to the baseline, demonstrating that DAA regimens are free of mental side effects regardless of current and/or past psychiatric history[14,15].

> In our analysis, however, in six patients, the underlying disease (five cases of depression and one of schizophrenia) worsened during antiviral therapy, which in 2 cases was the reason for discontinuing treatment. However, we cannot exclude the potential influence of other factors. It should be noted that the cited analyses were prospective, while ours is a retrospective study with limitations in data collection. Interestingly, another prospective study reported some degree of neuropsychiatric impairment associated with IFN-free treatment in 10 cirrhotic patients, suggesting their susceptibility to mild DAA neurotoxicity[42].

> Despite the significant change in access to treatment, there are still some challenges for a group of patients with mental illness. These are related to potential drug interactions. For this reason, this is carefully reviewed before therapy, and if there are contraindications to combining medications, psychiatric drugs are modified to allow the patient to receive antiviral treatment. This is not always possible; in our study, three patients were on carbamazepine, which could not be discontinued as it was the only drug that stabilized their mental state. Although this drug is not recommended in combination with DAA because it can reduce their levels below therapeutic levels, all patients completed the

Table 3 Treatment characteristics in chronic hepatitis C patients with (group A) and without (group B) psychiatric disorders, %, n					
Parameter	All patients (<i>n</i> = 14272)	Group A (<i>n</i> = 942)	Group B (<i>n</i> = 13330)	<i>P</i> value	
History of antiviral treatment					
Treatment-naïve	78.7 (11238)	80.6 (760)	78.6 (10478)	0.132 ($\chi^2 = 2.2$)	
Nonresponder to IFN-based regimens	19.6 (2791)	16.7 (157)	19.8 (2634)	$0.02 (\chi^2 = 5.3)$	
Nonresponder to IFN-free regimens	1.7 (247)	2.7 (25)	1.6 (222)	0.02 ($\chi^2 = 5.1$)	
Current treatment regimen					
Pangenotypic regimens	35.2 (5028)	43.0 (405)	34.7 (4619)	$< 0.0001 (\chi^2 = 26.0)$	
Genotype-specific regimens	64.8 (9248)	57.0 (537)	65.3 (8711)		
Current-RBV-containing regimens	16.2 (2308)	16.5 (155)	16.1 (2153)	0.807 ($\chi^2 = 0.05$)	
Genotype-specific treatment regimens					
ASV + DCV	0.8 (118)	1.3 (12)	0.8 (106)	0.117 ($\chi^2 = 2.5$)	
SOF/LDV ± RBV	19.6 (2795)	21.8 (205)	19.4 (2590)	0.081 ($\chi^2 = 3.1$)	
$OBV/PTV/r \pm DSV \pm RBV$	25.4 (3626)	14.1 (133)	26.2 (3493)	$< 0.0001 (\chi^2 = 67.8)$	
GZR/EBR ± RBV	16.8 (2400)	16.3 (154)	16.8 (2246)	0.691 ($\chi^2 = 0.1$)	
Other SOF ± SMV ± DCV ± RBV, SMV ± DCV ± RBV	2.2 (305)	3.2 (33)	2.1 (272)	$0.02 (\chi^2 = 5.7)$	
Pangenotypic regimens					
GLE/PIB	20.6 (2940)	20.6 (194)	20.6 (2746)	0.997 ($\chi^2 = 0.0$)	
GLE/PIB + SOF + RBV	0.05 (6)	0.0 (0)	0.05 (6)	1.0	
SOF/VEL ± RBV	14.4 (2058)	22.2 (209)	13.9 (1849)	$< 0.0001 (\chi^2 = 49.3)$	
SOF/VEL/VOX	0.2 (24)	0.2 (2)	0.2 (22)	0.670	

The reported P value relates to Pearson's chi-square test or Fisher's exact test if the number of observations in the category was less than 10. CHC: Chronic hepatitis C; IFN: Interferon; RBV: Ribavirin; ASV: Asunaprevir; DCV: Daclatasvir; LDV: Ledipasvir; SOF: Sofosbuvir; OBV: Ombitasvir; PTV: Paritaprevir; r: Ritonavir; DSV: Dasabuvir; GZR: Grazoprevir; EB: Elbasvir; SMV: Simeprevir; GLE: Glecaprevir; PIB: Pibrentasvir; VEL: Velpatasvir; VOX: Voxilaprevir.

> treatment as planned without AEs and achieved SVR. It should be emphasized that a possible modification in psychiatric treatment in case of expected drug interactions, as well as monitoring the mental state of the patient, requires the cooperation of a hepatologist and a psychiatrist. However, the scope of supervision is significantly smaller than in the case of IFN-based therapy[17,43]. The issue we highlighted in our analysis was the significantly higher percentage of patients lost to follow-up without SVR evaluation in the population of patients with mental illness compared to those without mental disorders, 4.2% vs 2.5%, respectively. The problem with adherence, concerning not only the use of drugs which was not evaluated in our study but, as in this case, keeping the dates of follow-up visits, is also raised by other authors[18]. However, some analyses did not report the difference in adherence between mentally ill patients (including those with substance abuse disorder) and the general HCV-infected population[3,40,44].

> In patients with available SVR, we did not observe significant differences in the effectiveness of DAA therapy depending on the diagnosis of mental illness, which is consistent with reports from other RWE cohorts[40,45]. A 96.9% cure rate was achieved in the mentally affected group compared to 97.7% in the patients without psychiatric disorders, despite a higher proportion of men, those with liver cirrhosis, and a higher prevalence of GT3 infection in this population, which are considered negative predictors of SVR in HCV-infected patients treated with DAA[21]. Significantly lower SVR rates have been documented in patients with bipolar disorder, which is consistent with the results of the analysis by Wedemeyer *et al*[18]. However, this multicenter study did not control for potential confounders, while in our research, the multivariate analysis clearly excluded bipolar disorder as an independent predictor of non-response to treatment. The identified independent negative predictors included the presence of liver cirrhosis, GT3 infection, history of prior therapy failure, and male gender, which is in line with available data concerning the general HCV-infected population[21,46]. However, apart from the abovementioned factors, administration of the ASV + DCV regimen or SOF/(VEL + RBV) combination was also associated with a lower chance of SVR. The ASV + DCV option was registered only for patients with GT1b infection and used until 2018. Due to its cumulative efficacy of 85%, evaluated in a metaanalysis of 9 clinical trials, it was considered suboptimal^[23]. The SOF/(VEL + RBV) regimen is

Table 4 Logistic multiple regression results on the association between lack of sustained viral response, bipolar disorder (for which lower frequency of sustained viral response was observed in the present study), and known risk factors of non-response to antiviral treatment in the studied cohort (*n* = 14272)

Parameter	OR	95%CI	<i>P</i> value
Bipolar disorder	3.52	0.76-16.34	0.109
Male sex	1.93	1.51-2.45	< 0.0001
Obesity (BMI > 30 kg/m^2)	1.08	0.82-1.43	0.579
GT3	3.26	2.50-4.27	< 0.0001
Cirrhosis	2.55	2.01-3.22	< 0.0001
HIV-coinfection	1.37	0.88-2.13	0.165
HBV-coinfection	0.85	0.61-1.18	0.323
History of HCV-treatment failure	1.46	1.14-1.87	0.003
ASV+DCV	4.97	2.60-9.55	< 0.0001
SOF/VEL	0.86	0.61-1.20	0.371
SOF/VEL+RBV	2.37	1.34-4.17	0.003

BMI: Body mass index; GT: Genotype; HIV: Human immunodeficiency virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; ASV: Asunaprevir; DCV: Daclatasvir; SOF: Sofosbuvir; VEL: Velpatasvir; RBV: Ribavirin; OR: Odds ratio; CI: Confidence interval.

Table 5 Safety of direct-acting antiviral treatment in chronic hepatitis C patients with (group A) and without (group B) p	sychiatric
disorders, %, n	

Parameter	All patients (<i>n</i> = 14272)	Group A (<i>n</i> = 942)	Group B (<i>n</i> = 13330)	P value
Treatment course				
According to schedule	97.5 (13848)	96.8 (907)	97.6 (12941)	$0.164 (\chi^2 = 1.9)$
Therapy discontinuation	1.1 (157)	1.4 (13)	1.1 (144)	$0.394 (\chi^2 = 0.7)$
Therapy modification	1.4 (194)	1.8 (17)	1.3 (177)	0.222 ($\chi^2 = 1.5$)
No data	0.5 (73)	0.5 (5)	0.5 (68)	0.813
Death	0.5 (71)	0.4 (4)	0.5 (67)	1.0
SAE	1.0 (145)	1.6 (15)	0.97 (130)	$0.068 (\chi^2 = 3.3)$
Psychiatric complications				
New incidence of depression	0.1 (8)	0.2 (2)	0.05 (6)	0.093
New incidence of schizophrenia	0.0 (1)	0.0 (0)	0.0 (1)	1.0
New incidence of sleep disorders	1.8 (262)	3.3 (31)	1.7 (231)	0.001 ($\chi^2 = 10.4$)
New incidence of anxiety	0.0 (1)	0.1 (1)	0.0 (0)	1.0
Drinking binge	0.0 (1)	0.1 (1)	0.0 (0)	1.0
Exacerbation of psychiatric disease		0.6 (6)		
Depression		0.5 (5)		
Schizophrenia		0.1 (1)		

The reported *P* value relates to Pearson's chi-square test or Fisher's exact test if the number of observations in the category was less than 10. SAEs: Serious adverse events.

registered in patients with decompensated cirrhosis, and its efficacy in a randomized clinical trial was 83%[47]. In our analysis, 23/26 (88.5%) patients with mental disorders responded to this type of treatment. There are no papers evaluating this regimen exclusively in patients with mental illness, so we cannot relate our results to another study.

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Figure 1 The sustained virological response rate in the group with mental disorders compared to the control group in intention-to-treat and per protocol analyses and calculated per protocol analysis in the population with mental disorders. A: The sustained virological response rate (SVR) in the group with mental disorders (n = 942) compared to the control group (n = 13330) in intention-to-treat and per protocol analyses; B: SVR calculated per protocol analysis in the population with mental disorders considering the type of the psychiatric illness in comparison to the control group. All statistical comparisons were performed with Pearson's chi-square or Fisher's exact test. ITT: Intention-to-treat; PP: Per protocol.

Our study has some limitations which we wish to stress. This is a retrospective study with all its consequences, such as incomplete data, including information on side effects, data entry errors, and possible bias. The database includes information from baseline to 12 wk after the end of therapy, and there are no data on the number of patients who had their psychiatric treatment modified before treatment, as well as the nature of these modifications. Additionally, no data were collected on the adjustment of psychiatric treatment during DAA therapy. Another limitation is the lack of systematic monitoring of the patient's mental state, such as with appropriate questionnaires. The main strength of the current study is the large group of patients from different centers in our country treated in routine clinical practice, which affects the quality of the results and the possibility of generalizing conclusions. To the best of our knowledge, this is the first study to address the problem of treating CHC with various DAA regimens in patients with mental disorders, highlighting the differences in specific psychiatric illnesses.

CONCLUSION

This study provides real-world evidence for the effectiveness and safety of DAA treatment of HCV infection in patients with preexisting psychiatric disorders. As shown, most of these patients achieve SVR. No specific psychiatric diagnosis lowered the chance of successful DAA treatment. Negative predictors of virologic response do not differ for these patients compared to the general HCV-infected population and include cirrhosis, GT3 infection, male gender, and failure of previous therapy.

ARTICLE HIGHLIGHTS

Research background

In the era of interferon (IFN)-based therapies, patients with psychiatric disorders had limited access to therapy due to their unfavorable safety profile. This changed with the introduction of direct-acting antiviral (DAA) drugs.

Research motivation

We aimed to evaluate the tolerability and effectiveness of IFN-free therapies in patients with various psychiatric disorders and to identify predictors of virologic response in this patient population.

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Research objectives

Data of 14272 patients treated with IFN-free regimens for chronic hepatitis C included in the EpiTer-2 database were analyzed. The patients were divided into two groups: 942 patients with mental disorders and 13330 without psychiatric diseases.

Research methods

The effectiveness and safety of antiviral treatment were compared between both populations. Effectiveness was assessed by the percentage of patients with sustained virologic response (SVR). Multiple logistic regression was used to predict the odds of no SVR in patients with mental illness. Safety data were collected through the treatment period and 12 wk of follow-up.

Research results

The cure rate was not significantly different in the two groups analyzed, with an SVR of 96.9% in patients with psychiatric disorders and 97.7% without psychiatric disorders. Patients with bipolar disorder achieved a lower SVR (90.5%), but multivariate analysis excluded it as an independent predictor of treatment non-response. Male gender, genotype 3 infection, cirrhosis and failure of previous therapy were identified as independent negative predictors. The percentage of patients who completed the planned therapy did not differ between the groups with and without mental disorders. In six patients, symptoms of mental illness (depression, schizophrenia) worsened, of which two discontinued treatment for this reason. New episodes of sleep disorders occurred significantly more often in patients with mental disorders. Patients with mental illness were more frequently lost to follow-up (4.2% vs 2.5%).

Research conclusions

DAA treatment is safe and effective in hepatitis C virus (HCV)-infected patients with mental disorders. No specific psychiatric diagnosis lowered the chance of successful antiviral treatment.

Research perspectives

The data obtained provide real-world evidence of the effectiveness and safety of DAA for HCV infection in patients with mental disorders, indicating that there are no differences compared with the general HCV-infected population.

FOOTNOTES

Author contributions: Dybowska D, Zarębska-Michaluk D, Rzymski P, Pawłowska M and Flisiak R conceived the study design; Dybowska D and Dybowski M cured data and prepared data for analysis; Dybowska D and Rzymski P analyzed and interpreted the data; Rzymski P performed the statistical analysis; Rzymski P prepared tables and figures; Dybowska D, Zarebska-Michaluk, Rzymski P, Pawłowska M, and Flisiak R drafted the manuscript; Dobrowolska K prepared the manuscript for submission; Berak H, Lorenc B, Sitko M, Dybowski M, Mazur W, Tudrujek-Zdunek M, Janocha-Litwin J, Janczewska E, Klapaczyński J, Parfieniuk-Kowerda A, Piekarska A, Sobala-Szczygieł Barbara, Dobrowolska K, and Pawłowska M acquired the data and approved the final version of the manuscript.

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