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Contents

Weekly Volume 27 Number 30 August 14, 2021

REVIEW

MINIREVIEWS

ORIGINAL ARTICLE

Basic Study

Clinical and Translational Research

Retrospective Study

Observational Study

Prospective Study

World Journal of Gastroenterology

Weekly Volume 27 Number 30 August 14, 2021

Contents

ABOUT COVER

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REVIEW

Diabetes and pancreatic cancer: Exploring the two-way traffic

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Abstract

Pancreatic cancer (PC) is often associated with a poor prognosis. Long-standing diabetes mellitus is considered as an important risk factor for its development. This risk can be modified by the use of certain antidiabetic medications. On the other hand, new-onset diabetes can signal towards an underlying PC in the elderly population. Recently, several attempts have been made to develop an effective clinical tool for PC screening using a combination of history of new-onset diabetes and several other clinical and biochemical markers. On the contrary, diabetes affects the survival after treatment for PC. We describe this intimate and complex two-way relationship of diabetes and PC in this review by exploring the underlying pathogenesis.

Key Words: Chronic pancreatitis; Diabetes; New onset diabetes; Pancreatic adenocarcinoma; Pancreatic cancer; Type 3c diabetes

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Core Tip: Type 2 diabetes mellitus can increase the risk of pancreatic cancer (PC) and certain antidiabetic medications can modify this risk. New onset diabetes in

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combination with other clinical and biochemical markers can serve as an effective screening tool for PC. On the contrary, the glycaemic status affects the treatment outcome of PC. More awareness among clinicians is required about the two-way relationship between diabetes mellitus and PC.

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INTRODUCTION

Pancreatic cancer (PC) is one of the few malignancies associated with a dismal prognosis. Its incidence is on the rise and is one of the leading causes of cancer-related death worldwide[1]. Similarly, type 2 diabetes mellitus (T2DM) accounts for a significant morbidity and mortality owing to a global increase in its incidence. Its prevalence is predicted to reach up to 700 million by 2045[2]. Longstanding diabetes has been regarded as a modest risk factor for PC. On the other hand, new-onset diabetes mellitus (NOD), especially after the 5th decade of life is often observed as a harbinger of an underlying PC. There is also a simultaneous increase in obesity worldwide, which plays a key role in development of both T2DM and PC[3]. Thus, this surge in diabetes and obesity prevalence may eventually increase the risk of PC in a significant number of population in near future[4].

Diabetes and PC have a multifaceted relationship. There are different types of diabetes as per the American Diabetes Association but two types of diabetes, namely T2DM and type 3c diabetes, merit attention in relation to PC[5]. T2DM is a chronic non-communicable disease characterised by hyperglycaemia resulting from the defective insulin secretion due to progressive beta cell dysfunction in the face of ongoing insulin resistance (IR)[5]. The diabetes associated with different exocrine pancreatic disorders is known as type 3c diabetes. The duration of DM has an important relationship with development of PC. However, the time duration cut-offs to define different types of diabetes are arbitrary and are varied. The time duration taken to define NOD in the context of PC is between 2-3 years[6]. On the contrary, when the diabetes is present for more than 2-3 years before the diagnosis of PC, it is considered as a long-standing T2DM. However, differentiating between this two entities is very difficult in a given subject of PC, since many patients of T2DM have a long asymptomatic undiagnosed period[5].

T2DM can have an impact on the outcome of different treatment modalities of the PC. Moreover, different drugs used for treating T2DM can affect the risk of PC as well. Metformin has gained particular attention in this context. In the appropriate clinical context, a recent worsening glycaemic profile requiring insulin might point towards the development of PC in the elderly diabetes subjects. The main obstacle in the diagnosis of PC in DM is to identify the candidates to be screened as routine evaluation for PC is not recommended in them. Ongoing research in identifying the screening population based on clinical characteristics and biomarkers and developing different models based on the combination of such parameters continues. In this background, we aim to review the current literature for unfolding the complex but intricate relationship between diabetes and PC.

SEARCH STRATEGY

The PubMed search was carried out for relevant articles by three authors (AR, JS, PM). The references of the pertinent articles were also searched for additional appropriate studies. The keywords and combinations included in the search were: 'diabetes'; 'new onset diabetes'; 'pancreatic cancer-related diabetes'; 'pancreatic cancer' and 'diabetes'; 'new onset diabetes' and 'pancreatic cancer'; 'long term diabetes' and 'pancreatic cancer'; 'pancreatic ductal adenocarcinoma' and 'diabetes'; 'metformin' and 'pancreatic cancer' and 'diabetes'; 'Type 3c diabetes' and 'pancreatic cancer'. The

search was restricted to only English literature and predominantly focused on the recent evidence. The appropriate articles to be included in this review were selected by SK, DN and RK.

RISK OF PC IN LONG-STANDING DIABETES MELLITUS

The evidence of association between NOD and the PC is consistent (see below); however, the evidence for risk of development of pancreatic ductal adenocarcinoma (PDAC) in long-standing diabetes is mixed. PDAC is the most common form of PC. Moreover, the risk is cumulative and is in continuum with the fasting blood glucose levels and the risk consistently increases from normal glucose tolerance to prediabetes to diabetes[7].

The increased risk of PC in long-standing T2DM has been suggested across different population of the world, including Asians[8-10]. A recent report involving a large population (n = 112818 females and 46207 males respectively) over 30 years of cumulative exposure showed an increased risk of PDAC with long-standing diabetes over time (age-adjusted hazard ratio [HR] 2.16 [95%CI: 1.78-2.60])[11]. Another recently published meta-analysis also suggested an increased PC related mortality with T2DM (relative risk [RR] 1.67; [95%CI: 1.30-2.14])[12].

The summary of the evidence suggests that the reported RR for developing PDAC in long-term diabetes is modest and varies between 1.4-2.1[8,13]. The risk may persist even after adjustment for obesity and smoking, two important and independent risk factors for PDAC[14]. Additionally, PDAC risk is significantly more in NOD and although the risk reduces subsequently, it may remain significant as the duration of the diabetes gets longer as per few meta-analysis[13,15]. However, a 2015 summary review of the available meta-analysis questioned the robustness of diabetes and PDAC association[16]. Importantly, other population based studies did not find any association between long-standing diabetes and the development of PDAC[17,18]. Thus, the elevated risk of PC in long-standing T2DM is confounded by the factor that may originate from a common soil of obesity and IR. Further, the role of different anti-diabetic medications as a risk modifier cannot be ignored while assessing the risk.

The Mendelian randomization (MR) studies looking into causal association between long-standing diabetes and PC have yielded conflicting results. While some studies showed causal association, others did not[19,20]. A pooled analysis performed on MR studies including 8374 PC patients by Yuan *et al*[21], found an odds ratio (OR) of 1.08 (95%CI: 1.02-1.14; P = 0.009) for this association. Although this evidence suggests a modest increase in the risk of PDAC in long-standing T2DM, more studies are required to confirm this association in future.

RISK OF PC IN TYPE 3C DIABETES

Chronic pancreatitis is defined as the chronic progressive inflammation and fibrosis of the pancreas caused by various aetiology and finally results in both endocrine and exocrine pancreatic dysfunction[22]. Diabetes is found in 35%-50% of subjects with CP in the observational studies[23-25] and the prevalence of DM increases with the increasing duration of CP and may reach up to 90%[25]. This type of diabetes is known as type 3c diabetes. Diabetes is more common in patients with pancreatic calcifications, pancreatic exocrine insufficiency and those who underwent surgery[23,24]. In a meta-analysis including fifteen studies (8970 patients), the incidence of DM was 30% and the prevalence increased after 5 years of CP diagnosis[26]. Diabetes in CP is often difficult to manage as a significant proportion of subjects require insulin therapy[23]. Importantly, CP itself is a risk factor for the development of PC. Kirkegård *et al*[27] had shown the risk of PC in CP varies with the duration of the disease and the effect estimates were 16.16, 7.90 and 3.53 at 2, 5 and 9 years after the diagnosis of CP, respectively. Another important entity is fibro-calculous pancreatic diabetes (FCPD), also known as tropical calcific pancreatitis, a relatively common cause of type 3c diabetes in certain tropical countries. FCPD also carries a very high risk for the development of PDAC[28].

Thus, it is important to look for CP in a given patient of diabetes and a closer follow-up with appropriate imaging is needed for diagnosis of PC in suspected cases. Since CP patients are often malnourished, progressive weight loss or anorexia despite adequate glycaemic control should alert the clinician for the possibility of PDAC.

RISK OF PC IN NEW-ONSET DIABETES

NOD has been considered as an important metabolic marker for the development of PDAC within the first 2-3 years of its diagnosis. NOD serves as a harbinger of PDAC in patients more than 45-50 years of age and hence calls for a careful follow up[29-31]. An earlier study demonstrated a 0.85% chance of development of PC within 3 years of diagnosis of diabetes in persons aged 50 years or more[32]. This study also showed that the risk was almost 8 times higher in patients with NOD. In a large cohort of 2.3 million Israeli population, a very high risk for developing PC was observed both in women and men (HR of 15.24 and 13.88 respectively) during the first year after the diagnosis of diabetes[33]. Two meta-analyses[13,14] also showed a 5-7 times elevated risk of PDAC in NOD, particularly within first year of diagnosis. Such an association was confirmed in different ethnicities like African Americans, Latinos[34] and Asians [35].

Agarwal *et al*[36] reported a very high prevalence of DM (68 %) in patients with PC compared to age matched other cancers subjects or non-cancer controls. Similarly, the number of NOD within the preceding 36 mo was markedly higher in PC than the other two groups (40% *vs* 3.3% *vs* 5.7%). About 50%-74% of the PC related diabetes is of recent onset (< 2-3 years duration)[6,37]. The prevalence of dysglycaemia in PDAC was more when standard oral glucose tolerance test (OGTT) was used instead of fasting glucose levels for diagnosis (78% *vs* 45%)[36,38]. The abnormalities in glucose metabolism are frequently missed in PDAC. The importance of making a preoperative diagnosis of glucose abnormality needs to be emphasized in this setting as it is shown to influence the surgical policy in up to 15% of patients[39].

It was also observed that a significant proportion of NOD in patients with PDAC resolved after pancreatic resection[37]. This indicates that PDAC by itself is causally related to the development of NOD, which is an early and specific biomarker for PDAC rather than a mere consequence. Besides the NOD, a deterioration of the existing glycaemic control in the form of elevated glycated hemoglobin **(**HbA1c) has also been associated with the development of PDAC[38].

MECHANISM OF DEVELOPMENT OF PC IN LONG-STANDING T2DM

The potential mechanism responsible for the development of PDAC in long-standing diabetes is poorly understood (Figure 1). The proposed theories are: (1) IR and the resulting direct effect of hyperinsulinemia[40]. A very recent study performed in a large prospective cohort (> 0.5 million subjects with a median follow-up of 8.4 years) has shown that higher IR as assessed by homeostatic model assessment- IR (HOMA-IR) is an important and independent risk factor for PC related mortality even in patients without diabetes[41]; (2) Cancer promoting role of the IGFs[42]; (3) The potential role of hyperglycaemia itself to alter several biochemical pathways involved in the carcinogenesis; (4) The synergistic effect of obesity and inflammation ('the common soil hypothesis'); and finally (5) Genetic predisposition to both these conditions. Experimental evidence is emerging to explain the molecular mechanism linking T2DM and PDAC. They include the roles of cellular senescence promoted by both T2DM and obesity[43], advanced glycation end products and its receptor[44], metabolic reprogramming by hyperglycaemia[45] and the interplay between non-alcoholic fatty pancreas development in the milieu of obesity and diabetes[46].

MECHANISM OF DEVELOPMENT OF NOD IN PC

PDAC by itself induces a potential 'diabetogenic' state. PC-associated NOD is grouped under Type 3c diabetes, which also includes diabetes caused by CP[47]. The mechanisms linking PC and NOD are shown in Figure 2. The hypothesis that NOD is the result of destruction of the endocrine pancreas by PDAC is not a plausible explanation because NOD can be present even before PC becomes radiologically detectable[48] and has also been shown to improve after surgery[37]. Hence, it is essential to search for systemic mediators of NOD in PC; until now, only a few of them have been substantiated.

Role of insulin resistance

Initial pioneering studies have shown that PC-associated NOD causes marked impairment in insulin action[49-51], more profound in patients who had diabetes[50].

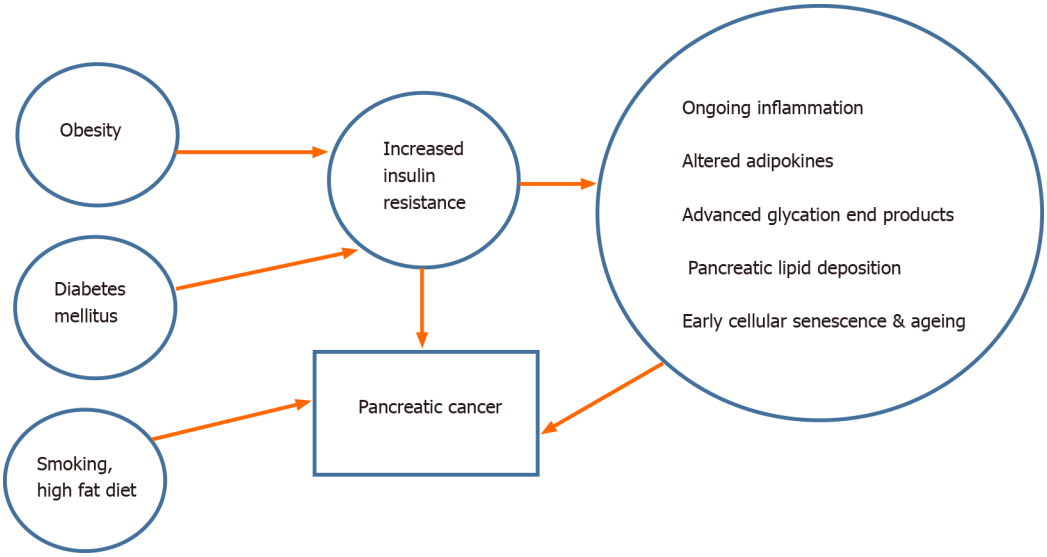

Figure 1 Schematic diagram depicting the interplay of various proposed factors leading to development of pancreatic cancer in long-standing diabetes mellitus.

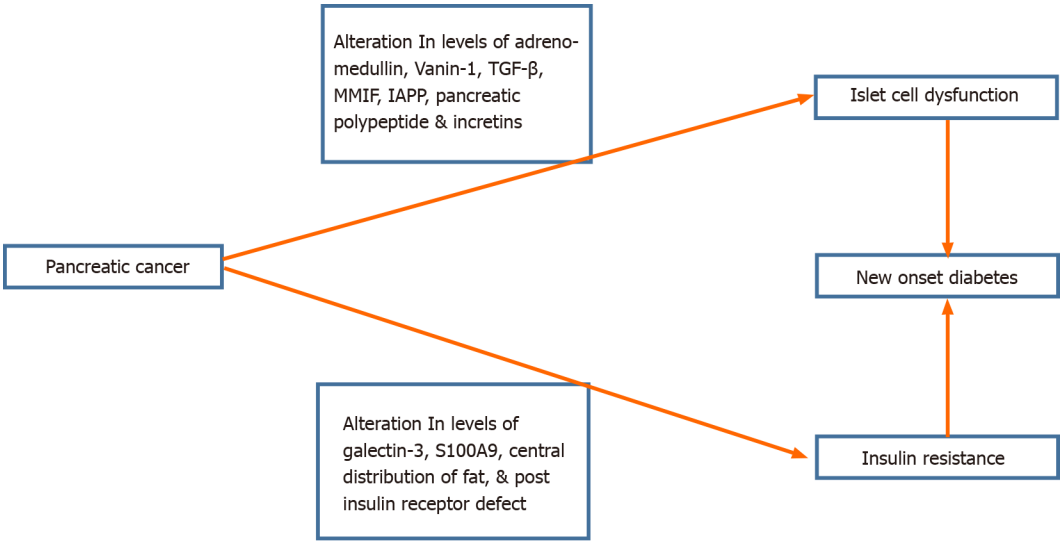

Figure 2 Schematic diagram for the possible mechanism for the pathogenesis of new-onset diabetes mellitus in pancreatic cancer. IAPP: Islet amyloid polypeptide; MMIF: Macrophage migratory inhibiting factor; TGF-β: Transforming growth factor-beta.

Insulin mediated glucose entry at the level of skeletal muscle was particularly found to be impaired significantly[52]. Interestingly, Permert *et al*[49] also demonstrated an improvement in whole body insulin sensitivity following surgery by using a hyperglycaemic clamp, which is considered to be the gold standard in evaluation of insulin sensitivity.

Thus, IR is an important determinant of the PDAC-related NOD, but the underlying mechanisms remain to be further studied. Currently available studies have suggested that IR may be related to the post-insulin receptor defect, particularly involving glycogen synthesis and storage pathways[53]. Recent experimental studies have found that PDAC-associated exosomes can inhibit the insulin receptor signalling pathway downstream of the receptor causing IR in skeletal muscle[54]. Another proteomic study revealed that galectin-3 and S100A9, which are overexpressed in PDAC-related NOD, can induce IR and can also serve as markers in distinguishing this entity from T2DM[55].

The role of islet amyloid polypeptide (IAPP) in the development of IR was initially suspected but later its clinical utility was not proven[56]. It was also suggested that PDAC-related NOD is due to the differential effect of ectopic fat as PDAC is characterized by subcutaneous fat loss and preservation of visceral fat[57]. However, a recent

study found that 30-18 mo before the diagnosis of PDAC, a significant proportion of patients had developed hyperglycaemia without any discernible change in the muscle or fat compartments[58,59].

Role of islet cell dysfunction

Pancreatic islet cell dysfunction is likely to be a crucial factor in the development of PDAC-related NOD. The morphology of the pancreatic islet in PC was recently characterized by Nagpal *et al*[60]. They demonstrated a significant reduction in islet density, beta and alpha cell area in PC compared to T2DM/control subjects. PC-related DM had lower IAPP deposition than T2DM. The lower IAPP deposit in PC related DM was also noted in an earlier study[61]. Future studies should explore the functional impact of such morphological changes.

Earlier studies have demonstrated a lower C-peptide response to glucagon stimulation suggestive of a beta cell secretory dysfunction in PDAC[62]. Beta cell function as assessed by HOMA-B was also found to be lower in PDAC patients with higher fasting glucose and diabetes[63]. In experimental studies, it was shown that beta cell in PDAC secrete increased amount of amylin preferentially while insulin secretion is diminished[64-66].

There is an experimental evidence for inhibition of insulin secretory function of the beta cell by adrenomedullin, which is released from PC-associated exosomes[67]. The role of adrenomedullin inhibiting beta cell insulin secretion in response to glucose was previously shown[68]. Moreover, adrenomedullin upregulation was noted in PC and its role in IR was also demonstrated[68]. Adrenomedullin was found to be a mediator for the increase in the exosome-induced lipolysis of the subcutaneous fat in PDAC [69]. The role of adrenomedullin as a screening biomarker is currently investigated in a prospective cohort study to identify patients with NOD and underlying PDAC[70].

Vanin-1 helps in hydrolysis of pantetheine and synthesis of vitamin B5 and cysteamine, which are required for lipid, energy and coenzyme A metabolism[71]. The role of vanin-1 is implicated in PDAC-induced paraneoplastic islet cell dysfunction, predominantly mediated by decreasing glutathione and elevating oxidative stress[72]. The same group earlier identified vanin-1 as a distinct marker of PDAC-related DM based on gene expression profile of the peripheral blood[73]. The role of transforming growth factor-beta (TGF-β) in the destruction of pancreatic beta cell had also been shown in animal studies[74,75] The macrophage migratory inhibiting factor was overexpressed in PDAC and was shown to decrease the beta cell secretory function [76].

A recent study has demonstrated that the markers of beta cell de-differentiations are consistently higher in non-diabetic PDAC patients suggesting the possible role of beta cell reprogramming in the early beta cell dysfunction even before the appearance of hyperglycaemia[77]. This dedifferentiation might be potentiated by the inflammatory milieu triggered by the PDAC.

Studies relating to alpha-cell function with PDAC-related DM are lacking. One study showed a higher glucagon/insulin ratio as a marker of NOD in PDAC[78]. Another small study revealed hyperglucagonemia in PDAC-related DM patients[79]. However, further studies are required to delineate the role of alpha cell dysfunction in PDAC-related diabetes.

Pancreatic polypeptide (PP) is released from the PP cells predominantly located in the head of the pancreas. PP cells have an important paracrine action including suppressive effect on glucagon secretion from alpha cell. Interestingly, one study had reported diminished PP response at 30 min following a mixed meal challenge in PC-related DM patients as compared to T2DM[80]. This was seen in tumours located in the ventral part of the pancreas. However, another study did not find any difference in fasting PP levels between PDAC-related DM, CP and T2DM[81]. Further studies should explore the role of PP in NOD and its use as an effective screening tool for PDAC.

Very few studies have evaluated the role of incretin hormones in the pathogenesis of PDAC-related DM. Interestingly, one study reported a lower gastric inhibitory polypeptide (GIP) and PP secretion in PDAC patients with diabetes as compared with T2DM patients, without any difference in glucagon-like peptide 1 (GLP-1) response [82]. Importantly, those with NOD or prediabetes with weight loss (> 2 kg) had significantly lower GIP. However, further studies are required to confirm this association. In-vitro studies had demonstrated that a lower GIP and GLP-1 response might be related to the inhibitory effect of the PDAC-exosomes on the proprotein convertase subtilisin/kexin type 1/3 enzyme which is responsible for cleaving the pro-glucagon molecule to generate the incretin peptides[83]. This study suggested the possibility of pancreatic exosome mediated dysfunction of the incretin hormones in

the gut.

EARLY DETECTION AND/OR SCREENING MODELS FOR PC IN DM

Clinical indicators

Till now, the recommendations regarding systemic screening of a person with diabetes to identify PDAC are not standardized. But, whom to screen and how to screen is not defined clearly by any guidelines till now to the best of our knowledge. So, it is necessary to develop a screening tool based on NOD and other risk factors. Since the yield of screening in such population is low, whether systematic screening is cost-effective and practically feasible remains an area of active debate.

The screening of patients with diabetes for PC is based on filtering of diabetes patients based on presence of associated clinical factors or level of biomarkers or a combination of such factors. NOD within 3 years of diagnosis increases the risk of PDAC 6-8 times more than the general population, but the prevalence of PDAC in such circumstances is low (0.8%-1%)[32].

It is a challenge to differentiate PC induced NOD from the more commonly encountered T2DM based on clinical and biochemical factors in clinical practice (Table 1). There are many overlaps between these two entities[84-86]. Munigala *et al* [87], identified age ≥ 65 years, heavy smoking, non-obese status at diagnosis, history of CP or gallstones as different risk factors of PC in a prospective cohort of NOD. One study reported a 40% higher risk of PC in patients with dyslipidaemia, although the association with specific lipid parameter was not mentioned[88].

Two other important factors that may provide a clinical clue for PC associated NOD are weight loss and worsening of hyperglycaemia. A continued weight loss in the presence of NOD was observed in a greater number of PC patients (59% *vs* 30%) than T2DM[89,90]. The amount of weight loss was also more in PC patients (8.3 ± 8.3 kg *vs* 0.8 ± 4.8 kg). Mueller *et al*[91] showed that weight loss of more than 10% had an adjusted OR of 3.58 (95%CI: 2.31-5.54) for development of PC. The presence of weight loss of more than 15% was not only associated with an increased odds of PC in NOD [91] but also in patients with long-standing diabetes[92]. Olson and colleagues showed that NOD and severe weight loss often occurred together before the diagnosis of PC [90]. Chen *et al*[11] observed that in a subject with NOD, when weight loss was unintentional or occurred in an individual with body mass index (BMI) less than 25 kg/m^2, then it substantially increased the risk of PC. Hence, weight change should be actively sought in elderly diabetes and warrant further investigation for PC.

Sah *et al*[58] reported worsening of hyperglycaemia, in the 18 to 6 mo before the diagnosis of PDAC. Similarly, rapid elevation of both blood glucose and HbA1c was observed by Huang *et al*[93] in the months preceding the detection of PC. The worsening of hyperglycaemia more often required the use of insulin treatment[90]. Thus, rapid deterioration of glycaemic control should alert a physician to screen for PDAC.

Another important feature is the loss of muscle mass, which is also known as sarcopenia. Sah *et al*[58] observed loss of subcutaneous adipose tissue even 6 mo before PDAC diagnosis. It was suggested that the preferential loss of subcutaneous adipose depot with relative preservation of the visceral adipose tissue might explain the IR and the worsening of glycaemic status[57]. However, a recent study did not find any difference in the prevalence of cachexia, skeletal muscle loss or weight loss between PC patients with or without DM[59]. Overall, sarcopenia suggests advanced disease and often portend poor survival, but its relationship with diabetes development need to be assessed in future studies[94].

Screening models

Interestingly, another upcoming approach is the development of a predictive model based on easily available clinical features in NOD. This model can identify NOD patients to be screened for PC and thus improve the detection rate while significantly decreasing the cost[30]. Sharma *et al*[95] came up with a model known as the Enriching New-Onset Diabetes for Pancreatic Cancer (END-PAC). This is a risk prediction model based on three different factors: change in weight, change in blood glucose, and age at the onset of DM. A score of 3 or more identified 78% of patients (n = 7/9) with 85% specificity. In the initial model, a score of more than 3 predicted a significantly increased risk of PDAC (4.4-fold) and a low END-PAC score of less than 0 had a very low risk of development of PDAC. This model was further assessed in a retrospective cohort of NOD patients (n = 13947) and 2% of high risk population (62 out of 3038)

Table 1 Factors that can help in differentiating pancreatic cancer associated new onset diabetes from type 2 diabetes mellitus

Clinical indicators	Biochemical markers
Age > 65 yr	Carbohydrate antigen 19-9
Heavy smoker	Galectin 3
Low body mass index	S100A9
History of chronic pancreatitis or gall stone disease	Insulin like growth factor-1
Recent worsening of hyperglycemia in an elderly patient	Osteoprotegerin
Weight loss associated with diabetes onset	Pancreatic polypeptide
Loss of subcutaneous fat and muscle mass in imaging studies like dual energy X-ray absorptiometry or magnetic resonance imaging	Thrombospondins- 1
	Vanin 1
	Matrix metalloproteinase-9
	MicroRNAs

were diagnosed with PDAC within 3 years yielding a sensitivity and specificity of 63% and 78% respectively at the score level of 3 or higher[96]. The positive predictive value (PPV) and negative predictive value were 2.0% and 99.7%, respectively. Another model was proposed by Boursi *et al*[97] based on The Health Improvement Network, which is a large primary care electronic research database from the United Kingdom. This prediction model included several easily available clinical parameters like age, BMI, change in BMI, presence or absence of smoking, use of proton pump inhibitors and other anti-diabetic medication including metformin. The laboratory parameters include levels of hemoglobin, creatinine, and alkaline phosphatase, HbA1c and cholesterol. The area under the curve (AUC) for the final model was 0.82 (95%CI: 0.75–0.89) and at a risk threshold of 1% for screening for PDAC, around 6% of patient with NOD would have to undergo systemic screening. The sensitivity, specificity and PPV at this level were 44.7%, 94.0% and 2.6%, respectively. Thus, though these model systems are encouraging and can narrow down on the screening population, they are limited by poor sensitivity and lower PPV[30] and further improvement is required before routine clinical use. Recently, a protocol of a multicentric, prospective observational study (NODES Trial) has been published, which intends to follow up new-onset (≤ 6 mo) diabetes patients over 60 years of age with both clinical and valid biomarkers [98]. The study also aims to evaluate for biomarkers that can distinguish patients with PDAC more precisely. Such studies will be invaluable in understanding and defining a screening protocol in NOD patients to identify PDAC as early as possible.

BIOMARKERS IN THE SCREENING OF PC IN DM

The role of different biomarkers in assisting the early diagnosis of PC among DM patients is crucial. A plethora of studies on different biomarkers have been published in the literature, though till now, none of them have reached the routine clinical use. The search for an easy-to-use clinically useful and cost-effective marker is still ongoing. Finding of suitable biomarkers is a difficult task in a relatively uncommon disease like PC and moreover, presence of diabetes can confound the measurements of different biomarkers in such setting[30]. A detailed discussion on this topic is out of the purview of this article, but a brief description on the latest biomarkers are discussed here. The proposed biomarkers are either measured in the blood or tissue fluids and they are the result of the 'multi-omics' studies involving proteomics, genomics and metabolomics.

Hormones involved in glucose homeostasis

The biomarkers, which draws our attention first is the biomarkers related to the glucose metabolism. Sharma *et al*[99] have shown that rising fasting plasma glucose itself predates the development of PDAC (36-60 mo before PDAC diagnosis) and is often related to the size of the tumour. Though fasting blood glucose levels increased concordantly with the volume of the tumour, no such relationship with the tumour gradation was reported. Another study reported a higher serum glucagon/insulin

ratio with a cut-off of 7.4 ng/mIU could differentiate PC induced NOD from T2DM with 77% sensitivity and 69% specificity[78]. A study demonstrated a higher glucose stimulated glucagon in PC patients with DM, suggesting glucagon as a potential biomarker[79].

Incretins involved in glucose homeostasis

The role of different gut polypeptides involved in the glucose homeostasis was also studied in PDAC patients. It was found that a significantly lower plasma concentrations of GIP and PP in patients with PC irrespective of the degree of glucose intolerance as compared to the T2DM and normal healthy controls[82]. A diminished PP response to a mixed meal was also observed earlier in PDAC associated diabetes in a small study[80]. However, another study could not find a difference in fasting PP levels between PDAC patients with or without diabetes and T2DM[81]. Thus the blunted PP response in PDAC can serve as an important tool for screening for PDAC, but the time-line is not well established and studies with a larger sample size may further consolidate PP as an important biomarker.

Carbohydrate antigen 19-9

A study by Choe *et al*[100] has shown that in asymptomatic NOD patients, a higher carbohydrate antigen 19-9 (CA19-9) levels above the upper normal limit had a 5.5 times risk of developing PC within 2 years of diagnosis. Another retrospective analysis showed similar results and found that the odds for development of PC in NOD patients with elevated CA19-9 was consistently higher, particularly in patients with elevated bilirubin levels[101]. Murakami *et al*[102] proposed that a cut-off of serum CA19-9 level of 75 U/mL can discriminate between patients with diabetes with or without PC. At this cut off, the sensitivity and specificity of CA19-9 for PC was 69.5% and 98.2%, respectively, while the AUC was 0.875 (95%CI: 0.826-0.924). A combination of elevated CA19-9 and carcinoembryonic antigen was also shown to detect PC among DM patients[103]. However, it is important to note that the utility of CA19-9 may be limited by the fact that it is affected by the levels of glycemia. CA19-9 levels must be interpreted in the context of ongoing glycaemic control and patients with diabetes per se may have elevated CA19-9[104].Thus there is a need to optimize the CA19-9 cut off level in DM patients for PC detection.

Thrombospondin -1

Another promising biomarker is thrombospondin-1 (TSP-1), a multimeric protein with anti-angiogenic properties. TSP-1 levels were found to be lower in PDAC patients, particularly those with diabetes as compared to non-diabetes and this lower levels were detected even 24 mo before the diagnosis of PDAC[105]. According to this study, TSP-1 levels in combination with CA19-9 yielded an AUC 0.86 in the detection of PDAC. Importantly, a lower TSP-1 levels were also noted in PDAC associated diabetes but not in the long-standing T2DM.

Vanin-1 and matrix metalloproteinase 9

Vanin-1, a protein involved in the oxidative stress pathway was found to be associated with paraneoplastic islet cell dysfunction (see earlier) and can serve as a potential biomarker in detecting PC among DM patients. Huang *et al*[73] have shown that the levels of *Vanin-1* genes were significantly upregulated in PDAC and an elevated levels of both vanin-1 and matrix metalloproteinase 9 (MMP9) in serum using quantitative real-time polymerase chain reaction could differentiate PDAC associated diabetes from T2DM. The AUC for the combination of both Vanin-1 and MMP9 was 0.950 with a sensitivity of 95% but the specificity of 76%. A combination of CA19-9 and MMP9 was also found to be helpful in discriminating PDAC-related diabetes from T2DM with an AUC of 0.886[106].

Galectin-3 and S100A9

Galectin-3 is a β-galactoside–binding lectin involved in the proliferation, migration and invasion of PC cells[107] whereas S100A9 protein is involved in the inflammation through toll-like receptor-4[108]. Liao *et al*[55] have shown that levels of both galectin-3 and S100A9 were higher in PDAC related DM than T2DM. They also found that the serum levels of both galectin-3 and S100A9 proteins can differentiate between PDAC related DM and T2DM with the AUC of 0.83 (95%CI: 0.74–0.92) and 0.77 (95%CI: 0.67–0.87) respectively.

MicroRNAs

Alteration in the profile of serum microRNAs (miRNAs) have been postulated as important biomarkers in recent times. One study reported that a panel of six miRNAs (miR-483-5p, miR-19a, miR-29a, miR-20a, miR-24, miR-25) could differentiate between PDAC related DM and T2DM[109]. The AUC for the differentiation of these two entities was 0.885 (95%CI: 0.784-0.986). However, the micro RNA profiles (miR-192, miR-196, miR-200, miR-21, miR-30 and miR-423) were found to be similar in PC patients with or without DM in a study by Skrha *et al*[110]. This list of miRNAs will be increasing in the future but the potential ones should come out from the prospective follow-up studies of the NOD patients.

Metabolomics

Studies have used extensive metabolomics approach either through liquid or gas chromatography and mass spectrometry or nuclear magnetic resonance imaging technique to identify various metabolites in order to identify specific biomarkers that can differentiate between PDAC-related DM and T2DM. One study revealed a distinct signature of 62 different serum metabolomes in PC related DM as compared to T2DM [111]. Out of them, two metabolites namely N-Succinyl-L-diaminopimelic-acid and PE (18: 2) had shown good sensitivity (93.3%) and specificity (93.1%) for PC in the logistic regression analysis. A recent nuclear magnetic resonance based study also identified a panel of eight metabolites with good accuracy (more than 80%) in the discrimination of PC and long-standing T2DM patients[112]. In future, possibly a panel of metabolites will help us to improve the precision medicine in identifying the cases requiring close follow-up for detection of PC among diabetes patients.

Other new biomarkers

There have been several other biomarkers proposed for the differentiation of the PDAC associated diabetes from T2DM. A very recent analysis of several immune related proteins including cytokines, chemokines and adhesion molecules revealed that a panel of different molecules (GM-CSF, IL-31, RANTES, RESISTIN, FASL, & ICAM1) were different between PDAC related DM and T2DM with an AUC of 0.96 (0.93–1.00)[113]. This study paved a new way in the screening of PC in diabetes patients. The other tools that can be used to screen PC are plasma free amino acid index[114], combination of either neutrophil-to-lymphocyte ratio or platelet-to-lymphocyte ratio and CA19-9[115], angiopoietin-like protein 2[116] among others with reported variable AUC.

In summary, although a great numbers of promising biomarkers have been studied to detect PC early in diabetes patients, a very few have reached routine clinical use as of now. As more translational research is emerging, the main requirement of a panel of clinically useful biomarkers for early detection of PC in DM will be fulfilled in near future.

DIABETES AND THE TREATMENT OUTCOMES OF PC

Diabetes has an important role to play as a prognostic marker in PC patients. Pancreatectomy is the initial management strategy in PDAC. Currently, the evidence that diabetes may portend an unfavourable impact on the overall outcome of PC, particularly after surgery is not concrete[117]. Whether the treatment of the diabetes modifies this risk is also not clear at present. Hank *et al*[118] showed that diabetes subjects had a poor median overall survival (18 *vs* 34 mo; $P < 0.001$). Moreover, diabetes was associated with higher 30-d mortality (3.2% *vs* 0.8%; $P = 0.019$). Importantly, a larger tumour size, a greater number of lymph node involvement and more peri-neural invasion were seen in diabetes patients with PC. A negative association of diabetes with overall survival was also noted in a meta-analysis[119]. However there are studies which do not agree on such association[120,121], and rather showed paradoxical reduction in the risk of death[122]. A 2013 review showed that diabetes patients had a higher risk of post-operative complications (45% *vs* 35%)[123]. Baseline HbA1c more that 6.5%-7.0% was also found to be associated with a shorter survival[124,125].

Long-standing DM vs new onset DM

Although studies have shown a poor outcome in all diabetes patients, the relative role of duration of diabetes on PC outcome needs further clarification. Very few studies

have shown the stratified analysis based on diabetes duration. Long-standing diabetes was found to have an association with diminished survival in prospective studies [126]. This was also confirmed in a meta-analysis[127] involving 18 studies (16181 patients). Several other studies[117,128,129] did not find any significant effect of long-standing diabetes on the survival in PC. Jeon *et al*[117] reported impact of long-term diabetes on decreased survival in those with resectable PDAC (HR, 1.42; 95%CI: 1.13–1.78) but not in advanced disease suggesting a role of staging in the outcome. It is important to note that the association of diabetes with prognosis became non-significant in most of the studies, after adjusting confounders like age, gender, BMI, smoking status and staging of the disease[128]. The evidence that diabetes patients can have a relatively larger pancreatic tumour size is well established[118,121,130,131].

On the other hand, the evidence is more consistent for a poorer outcome associated with NOD. A 2017 meta-analysis showed that only NOD was associated with shorter survival but not long-standing DM[119]. Similarly, other studies found that only NOD was a significantly independent predictor of decreased survival[129,132]. Importantly, Lee *et al*[133] have shown that NOD carries a higher risk of recurrence after pancreatic resection and may be a factor responsible for the poorer outcome. In contrast Jeon *et al* [117] did not find any impact of NOD on survival. Another point to consider is that whether post-surgery improvement of NOD has any impact on outcome. Though a study reported increased survival in patients where diabetes was resolved following surgery[134], future studies should substantiate this finding.

Impact of DM on the outcome after chemotherapy

Studies assessing the impact of diabetes in PC patients receiving chemotherapy have shown that, a prior diabetes status might be associated with a higher risk of death. Kleef *et al*[130] demonstrated a higher mortality rate in diabetes patients receiving adjuvant chemotherapy [HR 1.19 (95%CI: 1.01-1.40)]. Similarly, Hank *et al*[118] showed that median overall survival was lower in diabetes patients who received neo-adjuvant chemotherapy as compared to non-DM patients (18 mo *vs* 54 mo; $P < 0.001$). Another study showed diabetes to further add to the poorer outcome in metastatic disease treated with gemcitabine[135]. A meta-analysis looking at the impact of diabetes on the outcome following chemotherapy in PC (1034 with DM and 3207 without DM) demonstrated a lower survival and higher risk of death after chemotherapy in DM patients[136]. A high preoperative HbA1c was also found to be associated with non-completion of adjuvant chemotherapy and a higher risk of metastasis[137]. Diabetes also affects the survival in very advanced PC patients receiving palliative chemotherapy[138].

The mechanism behind the poorer outcome in PC with diabetes is not certain. Diabetes is associated with larger tumour size and hence a higher tumour stage. Hyperglycaemia has been shown to hasten the tumour development *via* sterol regulatory element binding protein 1 pathway[139]. There is also suggestion for an alteration in the tumour microenvironment in the presence of an elevated blood glucose level. Indeed, experimental studies have shown that hyperglycaemia increases the metastatic ability of the PC through aggravated hypoxia[140] or by increasing the perineural invasion[141]. The role of glycaemic variability is also suggested as a risk factor for promoting local invasion and metastasis *via* the retinoic acid receptor beta-runt related transcription factor 3-type VI collagen alpha 1 chain pathway[142].

EFFECT OF PC TREATMENT ON DIABETES

There is a complex relationship existing between patients undergoing surgery for PC and their glycaemic status. Glycaemic control is expected to worsen following pancreatectomy considering a significant loss of beta cells. However this is not often observed in clinical practice, particularly in patients with NOD after surgery. Studies[143-146] have either shown a significant improvement in their glycaemic control (75%) or resolution of NOD (20%-65%) after pancreatic surgery. It has also been reported that resolution of preoperative NOD after pancreatectomy may be a sign of a favourable outcome[134]. NOD has also been described in 15%-20% of patients[143,145,146] after surgery. One study reported deterioration of the glycaemic control in up to 40% post-operatively[147],when formal tests like OGTT and *i.v.* glucagon stimulation test were used. In the meta-analysis by Beger *et al*[148], cumulative incidence of NOD was found to be 15.5% after pancreatico-duodenectomy for malignant pancreatic tumours. Hence, it is necessary to assess the glycaemic status after pancreatico-duodenectomy even in those with pre-operative normoglycemia to achieve a better metabolic control after the

surgery.

ANTI-DIABETIC MEDICATIONS AND PC

Since diabetic patients will be receiving several medications for controlling hyperglycaemia, it is important to consider their effects in the context of PC. There are many excellent reviews[149] already available in this regard and we highlight salient points based on the recent available evidence.

Metformin

Metformin has garnered a lot of interest in recent times due to its anti-cancer effect and PC is not an exception. Metformin is the first line drug of choice for treating T2DM. A plethora of studies have looked into the three key aspects of metformin and their role in PC. They are: (1) Metformin as a risk modifier of PC development in T2DM[150-152]; (2) Effect of metformin on the overall survival following therapy[153]; and (3) Metformin as an adjuvant therapy in diagnosed PC[154].

Studies regarding metformin treatment as a risk modifier of PC have yielded mixed results. While some studies have shown risk reduction of PC in metformin users[155], other studies did not find such a beneficial impact of metformin in PC risk reduction [156,157] and even reported an increased association risk in metformin users[151]. Though earlier pooled analysis[150] had shown a decreased risk of PC, the studies included in those analysis were mostly retrospective and met with the significant lead time bias. Another complicating issue regarding the risk estimation is that the long duration of diabetes already present is itself a potential risk for PC and the NOD heralding the onset of PC often complicates the scenario further. Therefore, recent studies with better statistical designs are warranted to establish the role of metformin in PC prevention in a concrete manner.

Animal studies have shown that metformin decreases the PC cell proliferation[158, 159], its invasiveness[160] and thereby reduces the metastatic potential of PDAC[161]. Studies have also shown that metformin has a sensitization effect on chemotherapy, particularly gemcitabine[161,162]. The inhibition of TGF-β pathway is one of the several underlying mechanisms proposed to explain these effects[75,160]. Considering this finding, metformin might be expected to have beneficial result in PC. However, the clinical studies performed to assess the benefits of metformin have shown conflicting results. While few studies have suggested the survival benefit of metformin in PC patients[163-165], two phase 2 randomized controlled trials (RCTs)[166,167], observational studies[168,169] and other meta-analysis[170] refuted such finding.

The benefits of metformin observed in some earlier studies may be attributed to the immortal time bias that is inherent to meta-analysis studies[171]. Indeed, a meta-analysis taking into the account of immortal time bias did not show any additional survival benefit of metformin in DM patients with PC[170]. According to this meta-analysis, the effect size of reduction in the risk of survival was exaggerated by 18%. Again, the null effect of metformin on survival shown by the two RCTs was also flawed by the fact that metformin treatment was started late in the disease course and metastasis has already happened, leaving a small room to assess for the effect of metformin on survival outcome[166,167]. For this reason, it is important to focus on this area with well-designed RCTs in an earlier stage, including both DM and non-DM population. Further evidence is required to recommend treatment with metformin in PDAC patients with concurrent DM. Mild hyperglycaemia with obesity in early stage PC patients may be an ideal indication to start metformin. In summary, the risk reduction of PC and the overall survival following metformin therapy are not observed in recent well designed studies with improved statistical analysis taking into consideration of immortal time bias. Future phase 3 RCTs will be helpful in this context, mainly in selected PC candidates[172].

Insulin and insulin secretagogue

Insulin has a definite role in the pathogenesis of PDAC as hyperinsulinemia and IR are important risk factors for the development of PDAC (see above). Whether clinical use of insulin has any impact on PDAC development is a contentious issue. Long-term insulin use was not found to be a risk factor of PDAC development[173]. On the other hand, short-term insulin user (< 3-5 years) was found to have an elevated risk of PDAC (OR 5.60, 95%CI: 3.75-8.35)[173]. Perhaps this data reflects the other way around. It is likely that the worsening of hyperglycaemia or the severe hyperglycaemia requiring insulin injection might reflect the onset of PDAC or effect of PDAC on the

glycaemic control. Whereas few meta-analysis have suggested an elevated risk of PC in insulin users[174] the same evidence was not found in other studies[175] and also with newer insulin like glargine insulin[176]. In terms of survival benefit, insulin use had no impact on survival as shown in recent studies[120,177].

Insulin secretagogues like the sulfonylureas (SU) are also implicated as a risk modifier of PC. There are only few studies that have specifically looked into the link between SU and PC. However, studies including nation-wide cohorts[178], and meta-analyses[175] had pointed that SU use is associated an elevated risk of PC (OR varies between 1.5-1.7). However, with newer generation SU data is sparse and this association is further complicated by the effect of concurrent obesity and IR on the development of PC. Moreover, earlier analyses are met with significant methodological flaws and heterogeneity among studies[179].

Incretin based therapies: DPP-4 inhibitors and GLP-1 receptor agonist

Incretins are hormones secreted from the intestine and have a significant impact on the glycaemic control. GLP-1 analogues and inhibitors of dipeptidyl peptidase-4 (DPP-4) enzyme are established therapies for T2DM in clinical practice. Although there was a concern of acute pancreatitis and PC associated with their use from the initial preclinical[180] and adverse database review[181] studies, data regarding the risk for PC was inconsistent. Hence, both United States Food and Drug Administration and European Medicines Agency advised on continuous follow-up of patients started on these therapies for these two adverse events[182].

Earlier meta-analysis also did not find an increased risk of PC with DPP-4i treatment group[183]. Moreover, the recent meta-analysis involving 157 trials reporting PC (66897 patients in DPP-4 inhibitors and 61597 patients in control group) showed no associated risk with DPP-4 inhibitors use (OR: 0.84 [95%CI: 0.69-1.03], I^2 [for heterogeneity] = 0%). This association was found across different types of DPP-4i molecules and thus possibly reflects a class effect. Data from large population based studies also showed similar reassuring findings[184]. Due to several limitations of the trials like a shorter follow-up, reporting bias, small number of PC cases, it is important to keep a watch over this association in future. Moreover, one meta-analysis of the large cardiovascular outcome trials on DPP-4i showed an 75% increased risk of pancreatitis[185]. Such findings warrant longer duration follow-up studies and continued vigilance. A study[186] has shown that DPP-4i may be associated with increased risk of pancreatitis and PC in short-term without any relationship with exposure duration, thus implying that it might be the result of reverse causality rather than the DPP-4i exposure itself.

Similarly, more data are now available for different GLP-1 analogues. The larger cardiovascular outcome trials did not find any elevated risk of PC in GLP-1 analogue users[187,188]. Consequently, an updated pooled analyses from the cardiovascular outcome trials also did not show an excess risk of PC or pancreatitis with use of GLP-1 analogues[185]. However, it is noteworthy that such trials are not primarily meant to detect any increased malignancy risk. Thus, although the data is reassuring, a continued vigilance is warranted.

Other drugs (thiazolidinediones and sodium-glucose co-transporter type 2 inhibitors)

The other antidiabetic drugs are thiazolidinediones (TZD) and sodium-glucose co-transporter type 2 (SGLT-2) inhibitors. TZDs like pioglitazone and rosiglitazone primarily act through activation of the peroxisome proliferator-activated receptor-gamma pathway. This activation has direct and indirect implications in the PC biology. TZDs have shown to have inhibiting effect on several aspects of PC including cell proliferation and metastasis[189-191]. It also has the potential to modify the risk of PC through insulin sensitization, modification of the obesity and the inflammation [192]. However, these promising experimental findings of benefits of TZD have been replicated in clinical studies with mixed results.

While two meta-analyses did not find any association between TZD use and the risk of PC[175,193], one population based study had shown a protective role of TZDs against PC[178]. On the other hand, Lewis *et al*[194] demonstrated that TZD use might be associated with an increased risk of PC. As far as the prognostic role is considered, TZDs did not have any effect on survival[195,196].

SGLT2-inhibitors are the newest class of oral antidiabetic medication and have already made its place in the therapeutic algorithm of diabetes, owing to its cardiovascular benefits. Functional SGLT-2 are detectable in PC cells and hence, it was hypothesized that SGLT-2 inhibitors can inhibit tumour growth by blocking the entry

of the glucose within the cell[197]. An experimental study has shown canagliflozin, a SGLT-2 inhibitor to inhibit PC growth[198]. However, clinical studies are yet to confirm its effect on PDAC survival.

CONCLUSION

In this review, we have summarized the intricate relationship between DM and PC. Long-standing diabetes is considered as a risk factor for development of PC. On the other side, NOD in an elderly patient can be a manifestation of underlying PC. Though the exact mechanism remains to be eluded in future studies, the mechanism of the development of NOD in PC involves both IR and islet cell dysfunction. Diabetes has also been suggested to have an unfavourable effect on the overall survival of patients with PC.

Early detection of PC in a patient with DM is of utmost important and is a clinically challenging task. PC has a low prevalence in both general population and diabetes subjects. Thus, devising a strategy to screen diabetes population for PC is the need of the hour. There is an urgent need for a clinically useful and cost-effective screening tool to detect PC among patients with long-standing diabetes. The epiphenomenon of NOD can subserve as a potential clue along with recent onset worsening of glycaemic control and a continued weight loss. Apart from clinical pointers, many biomarkers have also been found to differentiate PC related DM from the commoner T2DM. Moreover, different clinical and biochemical parameters have been combined to develop different screening tools. Proper screening and early recognition of PC can improve the outcome of this devastating neoplasm.

Can we delay the occurrence or halt the progression of PC in a patient of DM? The strategies to improve IR like regular physical exercises, intermittent fasting, or low-fat diet can be explored in future. Moreover, other healthy behaviours like smoking cessation should be implemented in patients with long-standing DM. The role of glucose lowering medications like metformin in delaying the occurrence of PC needs to be explored further in longitudinal studies.

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REVIEW

Stress-activated kinases as therapeutic targets in pancreatic cancer

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Abstract

Pancreatic cancer is a dismal disease with high incidence and poor survival rates. With the aim to improve overall survival of pancreatic cancer patients, new therapeutic approaches are urgently needed. Protein kinases are key regulatory players in basically all stages of development, maintaining physiologic functions but also being involved in pathogenic processes. c-Jun N-terminal kinases (JNK) and p38 kinases, representatives of the mitogen-activated protein kinases, as well as the casein kinase 1 (CK1) family of protein kinases are important mediators of adequate response to cellular stress following inflammatory and metabolic stressors, DNA damage, and others. In their physiologic roles, they are responsible for the regulation of cell cycle progression, cell proliferation and differentiation, and apoptosis. Dysregulation of the underlying pathways consequently has been identified in various cancer types, including pancreatic cancer. Pharmacological targeting of those pathways has been the field of interest for several years. While success in earlier studies was limited due to lacking specificity and off-target effects, more recent improvements in small molecule inhibitor design against stress-activated protein kinases and their use in combination therapies have shown promising *in vitro* results. Consequently, targeting of JNK, p38, and CK1 protein kinase family members may actually be of particular interest in the field of precision medicine in patients with highly deregulated kinase pathways related to these kinases. However, further studies are warranted, especially involving *in vivo* investigation and clinical trials, in order to advance inhibition of stress-activated kinases to the field of translational medicine.

Key Words: Pancreatic cancer; Stress-activated protein kinases; Mitogen-activated protein kinases; c-Jun N-terminal kinases; Casein kinase 1; Small molecule inhibitor

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Core Tip: Since pancreatic cancer patients are generally confronted with poor prognosis, optimized therapeutic strategies are urgently needed. To establish new treatment options, efforts in drug development have increasingly focused on targeting protein kinases. In the cellular response to various stress signals, c-Jun N-terminal kinases (JNK) and p38 kinases as well as members of the casein kinase 1 (CK1) family are of special interest. Concentrating on pancreatic carcinoma in this review, we summarize the key roles of JNK, p38, and CK1 and provide an overview of recent achievements in the development of small molecule kinase inhibitors against these kinases.

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INTRODUCTION

Pancreatic cancer is a severe disease, with overall 5-year survival rates less than 10% and only very little improvement over the last decades[1]. It is currently the fourth most common cause of cancer-related deaths, and it is expected by 2030 that pancreatic cancer will have surpassed colon and breast/prostate cancer to move up to second rank of cancer-related deaths[2]. Contributing to the immense challenge of treating pancreatic cancer, dysregulation of multiple signaling pathways can frequently be detected. With the genetic hallmark mutation of *KRAS* in over 90% of all pancreatic cancer patients, the high relevance of kinase-driven pathways is underlined[3].

So far, classic chemotherapeutic agents have only shown moderate success in prolonging overall survival of patients suffering from pancreatic ductal adenocarcinoma (PDAC). However, more and more personalized therapy concepts are becoming promising options, especially the use of small molecule inhibitors specifically targeting newly identified drug targets, such as deregulated protein kinases. Of special interest are kinases activated in cellular stress situations, like mitogen-activated protein kinases (MAPKs) and members of the casein kinase 1 (CK1) family, which phosphorylate signal integration molecules like p53 and β-catenin finally resulting in activation of processes leading to cell cycle arrest or apoptosis.

MAPKs

MAPKs are key players in transducing extracellular stimuli into intracellular signaling cascades and therefore represent interesting drug targets. Multiple isoforms have been identified, which can be clustered into six groups of MAPKs. The most prominent of those are the extracellular-regulated kinases 1 and 2 (ERK1/2), the c-Jun N-terminal kinases 1, 2, and 3 (JNK1/2/3), and the p38 kinases α, β, γ, and δ[4,5]. As a response to various stimuli such as growth factors, cytokines, and environmental stress, MAPK-triggered phosphorylation of their target transcription factors (TFs) marks the endpoint of an intracellular kinase cascade. This cascade consists of ligands binding to their cell membrane receptors, recruitment of GTPase (*e.g.*, RAS) to the plasma membrane, and activation of MAPK kinase kinases (MKKKs or MAPKKKs, *e.g.*, RAF) as well as MAPK kinases (MKKs or MAPKKs, *e.g.*, MEK1/2)[5-7]. ERK1/2 belong to the best-studied kinases among MAPKs. Their relevance for pancreatic cancer has been well documented, especially as ERKs exert their functions downstream of mutant KRAS[8,9]. JNK and p38 can be grouped together as stress-activated protein kinases (SAPKs), as their pathways are regularly activated by environmental stressors, like nutrient deprivation, inflammatory cytokines, or ultraviolet irradiation[5,10].

CK1

A remarkable association with tumorigenesis and tumor progression has also been demonstrated for the CK1 family of protein kinases. Being among the first kinases described in history, involvement of CK1 isoforms in several essential signal transduction pathways has been reported within the last decades. As a key cascade in developmental processes, the (canonical) Wnt/β-catenin signaling pathway can also be involved in promoting cell proliferation through activation of oncogenes like c-myc

and cyclin D1[11,12]. All human CK1 isoforms were identified to fulfill negative as well as positive regulatory functions in canonical Wnt signaling, thereby either acting as tumor suppressors or contributing to Wnt-induced oncogenic processes[13-15]. CK1δ and ε might furthermore promote canonical instead of non-canonical Wnt signaling, consequently resulting in reduced JNK-mediated Wnt signaling and apoptosis[16,17]. In addition, apoptosis mediated by Fas can also be down-regulated by CK1δ- and ε-mediated stabilization of Bid[18]. Apart from signaling associated with proliferation, differentiation, and apoptosis, CK1 is also involved in further mechanisms of the cellular stress response, including functions in immune response and inflammation, regulation of microtubule dynamic processes, autophagy, and DNA damage-related signal transduction[19-21]. Especially well documented is the regulatory function of CK1 isoforms in p53-mediated signal transduction with CK1δ even forming an autoregulatory feedback loop with p53[19].

Cancer itself already forms a stressful environment on the tumor cells, induced by hypoxia and nutrient deprivation as well as metabolic and replication stress. Additionally, cancer cells face genotoxic stress exerted by chemo- and radiotherapies. In this regard, pancreatic cancer is no exception since tumors of the pancreas are known for their dense stroma with impaired vasculature and the association with metabolic stressors, like diabetic conditions. This review aims to elucidate the role of the stress-activated kinases: JNK and p38 but also CK1 in the pathogenesis of pancreatic cancer and their potential as therapeutic targets.

SAPKS: JNK AND P38

The first description of JNK as a 54 kDa protein kinase activated upon peritoneal cycloheximide injection into rats dates back to 1990[22]. So far, three different JNK isoforms have been identified in the human genome, including JNK1 (MAPK8), JNK2 (MAPK9), and JNK3 (MAPK10), whereas expression of JNK3 is mainly restricted to brain, heart, and testes[23]. Alternative splicing results in generation of at least 10 different JNK isoforms with molecular weights ranging from 46 kDa to 55 kDa[24]. Activation of JNKs is dependent on phosphorylation of threonine and tyrosine residues by upstream kinases. For JNK1, phosphorylation of Thr^{180} and Tyr^{182} within its kinase subdomain VIII has been demonstrated to be essential[25]. In the activation process of JNK1, the upstream kinases MKK4 and MKK7 both fulfill non-redundant functions, with MKK4 preferably phosphorylating Tyr residues while MKK7 favors Thr residues. Phosphorylation of Thr^{180} is sufficient for JNK1 activation, however, dual phosphorylation by both kinases is required for full JNK activation[26,27]. Further upstream of the signaling cascade, a large variety of at least 14 MKKKs can activate MKK4 and MKK7[23,28].

Merging the influence of multiple upstream kinases into fewer effector kinases enables the cells to respond to a variety of stimuli, like growth factors and cytokines, reactive oxygen species, physical interactions with other cells and the extracellular matrix, as well as cellular stressors[29]. The variety of different stimuli requires multiple cell membrane receptors engaging into the JNK pathway. These include G-protein coupled receptors, Wnt-receptors, transforming growth factor-β receptors, and tumor necrosis factor-α (TNF-α) receptors[30]. Signal transfer within the SAPK pathway is generally orchestrated by docking motifs for upstream kinases and downstream substrates, as well as scaffold proteins. Those scaffold proteins express docking sites for MKKKs, MKKs, and MAPKs and play an important role in the correct stimulus response through the kinase cascade[29,31].

Even more diverse than the upstream mediators are the possible JNK substrates. As all MAPKs, JNK is a proline-targeted serine/threonine kinase, thus preferably phosphorylating Ser-Pro as well as Thr-Pro motifs[4,32]. So far, the list of JNK substrates includes more than 100 targets, among them TFs like c-Jun, p53, c-myc, and β-catenin, microtubule-associated proteins, components of focal-adhesion-complex and cell-to-cell-adhesion, as well as apoptosis-regulating proteins like Bcl-2 and Bax [30]. Figure 1 shows an overview of MAPK-related cellular functions.

The first p38 MAPK was discovered in 1994, and today four isoforms (p38α, β, γ, and δ, corresponding to MAPK14, 11, 12 and 13) are known[33,34]. While p38α is ubiquitously expressed, the other isoforms show differential tissue distribution, with p38β being mainly expressed in the brain, p38γ in skeletal muscle, and p38δ in endocrine glands[35]. Dual phosphorylation of Thr^{180} and Tyr^{182} in a Thr-Gly-Tyr motif are required for full p38 kinase activation[36]. MKK3 and MKK6 specifically activate p38, but while MKK6 can activate all isoforms, MKK3 is unable to phosphorylate p38β

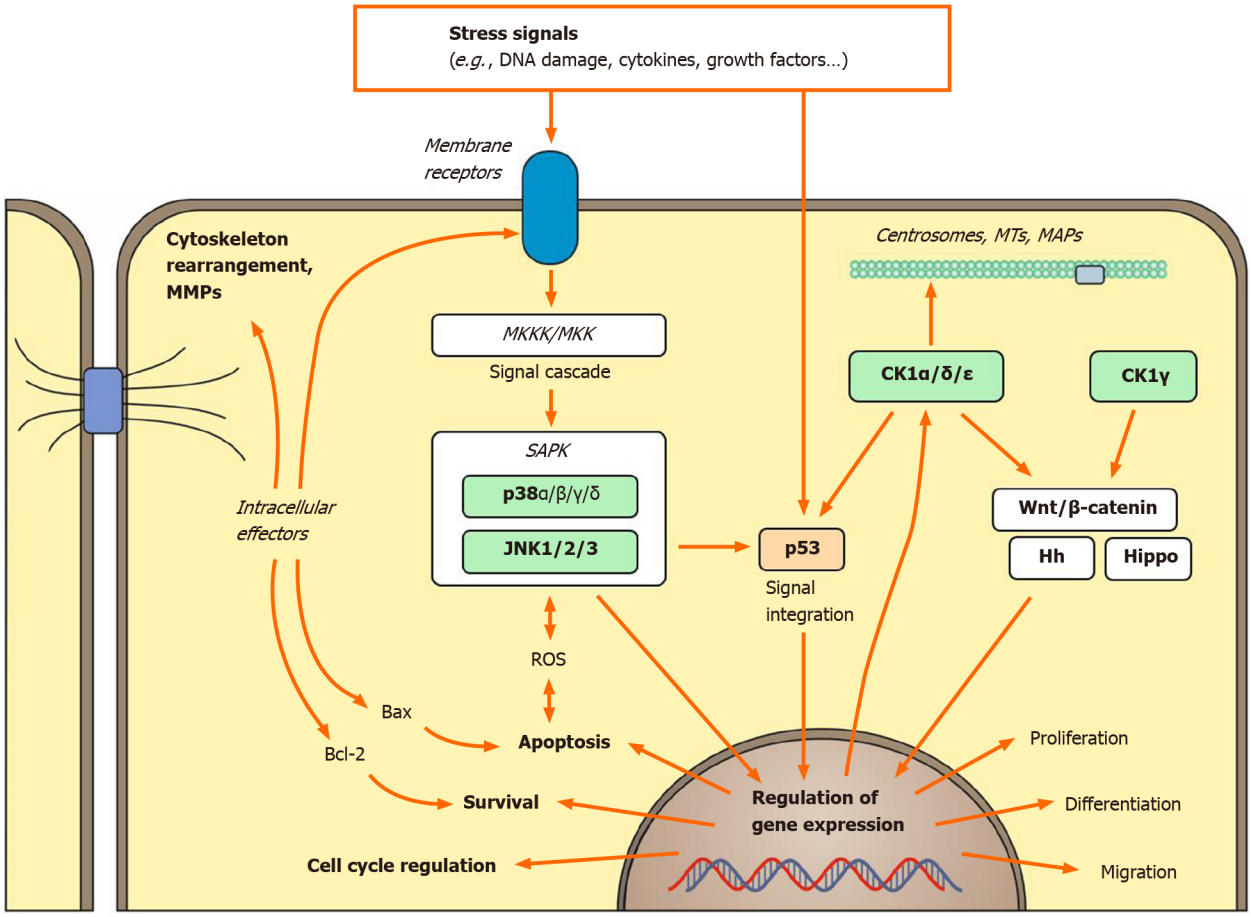

Figure 1 Signal integration mediated by stress-activated protein kinases. The stress-activated protein kinases (SAPKs) c-Jun N-terminal kinase (JNK) and p38 as well as the protein kinases of the casein kinase 1 (CK1) family are activated in response to endogenous and exogenous stress stimuli. Therefore, JNK and p38 are either activated directly or through upstream signaling cascades [mitogen-associated protein kinase kinase kinases (MKKKs), mitogen-associated protein kinase kinases (MKKs)] and subsequently exercise their functions through intracellular signal integrating effectors such as the transcription factors JNK, p53, c-myc, or β-catenin. In response to certain stress stimuli, the kinases of the CK1 family also take key functions in regulatory effects mediated *via* p53 or through various signal transduction pathways like the Wnt/β-catenin, Hedgehog (Hh), or Hippo pathway. Finally, cellular stress response including regulation of proliferation, differentiation, migration, cell cycle progression, survival, and apoptosis is initiated by modulation of gene expression. MAP: Microtubule-associated protein; MMP: matrix metalloproteinase; MT: Microtubule; ROS: Reactive oxygen species.

[37,38]. MKK4 can also contribute to p38 activation[39,40]. Multiple MKKKs contribute to the activation of MKK3 and MKK6, among which some are shared with the JNK pathway. By engaging specific MKKKs in response to defined stimuli, cells are enabled to elicit the correct stress response[35]. In T cells, a kinase cascade-independent pathway of p38α activation has also been described[41]. Similar to JNK, there are many p38-specific substrates, ranging from TFs to other protein kinases and apoptosis-regulating proteins[35].

Relevant SAPK-related pathways in cancer

The involvement of JNK and p38 in the pathogenesis of cancer has been studied extensively. Their role has been debated controversially since both can exhibit pro- as well as anti-tumorigenic functions[42,43]. For both kinases, the cellular effects provoked by JNK and p38 depend on the type, strength, and duration of the stimulus [44,45]. However, the influence of SAPKs on cancer development and progression is apparent as major cancer characteristics like cell proliferation, migration, and apoptosis are influenced by p38 and JNK. Pathways associated with these characteristics will now be discussed in general and specifically in pancreatic cancer.

A primary example of differential regulation in SAPK-related pathways is the interaction of JNKs and c-Jun. In non-stimulated cells, JNK2 activity leads to degradation of c-Jun, while after stimulation, JNK1 phosphorylates and stabilizes c-Jun. Consequently, knockdown of JNK1 decreased fibroblast proliferation through reduced activity of Activator protein-1 (AP-1), a TF of which phospho-c-Jun is a vital component[46,47]. Phosphorylation of c-Jun has also been identified as a critical step in RAS-induced tumorigenesis. Oncogenic RAS uses the same phosphorylation sites as

JNK on c-Jun and promotes transformation of rat embryonic fibroblasts, while *c-Jun*^{null} cells are resistant to RAS-induced transformation[48,49]. However, the role of JNK in this process is under dispute. In *TP53*$^{-/-}$ mouse embryonic fibroblasts, dual knockout of JNK1 and 2 reduced *KRAS*^{G12D}-induced transformation and suppressed *in vivo* growth. Furthermore, *KRAS*^{G12D}-induced lung tumor formation in mice was similarly reduced by JNK knockout[50]. This effect was in part attributed to JNK2, as only JNK2- but not JNK1-deprived mouse embryonic fibroblasts resisted RAS-induced transformation, although increased levels of AP-1 and phospho-c-Jun were observed[51]. However, not all transforming effects of RAS seem to be controlled by JNK, and in contrast to the before-mentioned studies, loss of JNK in RAS mutant cells may also contribute to enhanced tumorigenesis through apoptosis regulation[52]. A possible explanation for these findings is JNK-controlled cell cycle progression, as fibroblasts with knockdown of JNK2 show faster G1/S progression, while their JNK1-deprived counterparts show an opposing phenotype[46].

Besides proliferation, pro- and anti-apoptotic signals are also mediated by JNK in dependency of the stimulus. JNK regulates the expression of Bcl-2 family members and thereby influences apoptosis mediated *via* the mitochondrial pathway[44]. In TNF-mediated apoptosis, at early time-points, JNK activation triggers pro-survival pathways, while functioning JNK signaling is required for TNF-mediated apoptosis under persistent stimulation[53]. In murine cancer models, JNK1 was shown to promote chemically induced liver cancer, a finding that was also confirmed in human hepatocellular carcinoma[54,55]. On the other hand, knockdown of JNK1 rendered mice more susceptible to chemically induced skin tumors, while knockdown of JNK2 exerted an opposite effect[56].

The influence of p38 on oncogenesis is generally thought to be tumor suppressive, but protumorigenic functions like promotion of invasiveness has also been reported [44]. p38α controls proliferation through regulation of cell cycle progression at the G1/S and G2/M phases[57]. p38 can inhibit G1/S progression, *e.g.*, through downregulation of cyclin D1[40,58] or by phosphorylation of p53 and retinoblastoma protein [59-61]. Alternatively, p38 can also promote cell cycle progression through the induction of cyclin A or interference with the retinoblastoma protein pathway[62,63]. p38-mediated phosphorylation of p53 also activates the G2/M checkpoint in response to DNA double-strand breaks. Ironically, this also offers a survival benefit for tumor cells and increases therapy resistance, as DNA damaging drugs become less effective with functional p38 signaling[64-66]. Other tumor-promoting roles include formation of a pro-invasive phenotype through induction of matrix metalloproteinases and tumor cell dormancy, enabling metastatic relapse[42].

p38-related implication for involvement of the SAPK pathway in pancreatic cancer

The influence of the SAPK pathway on pancreatic cancer has been studied on patient samples as well as genetically engineered cell lines and mouse models, while patient cohorts are especially helpful to study population risk factors. Handra-Luca *et al*[67] offered an immunohistochemical analysis of the MAPK pathway in 99 surgically resected pancreatic cancer specimens. While high immunoreactivity for ERK1/2 was consistently associated with a worse prognosis, high expression levels of p38 could be associated with shorter recurrence-free survival in patients without adjuvant treatment. Strong staining for MKK4 was associated with increased proliferation[67]. Contrarily hereto, phosphorylated p38 with activated downstream TFs was identified as a favorable biomarker after surgical resection and associated with reduced number of lymph node metastases. Labeling of phospho-p38 showed no changes through the different cancer stages. Furthermore, pharmacologically inhibiting p38 *in vitro* and *in vivo* resulted in enhanced JNK signaling and enhanced cell growth[68]. Phospho-p38 was reported to increase during tumor progression, which is consistent with reports claiming that p38 has tumor suppressive functions during early carcinogenesis but switches towards a tumor promoting phenotype later on[69]. In our own previous work, we were able to dissect the isoform-specific functions of p38 in pancreatic cancer by genetically targeting p38 isoforms α and β. We confirmed an *in vivo* tumor suppressive phenotype of p38α but also showed a pro-invasive function. Additionally, we showed a tumor suppressive role of p38β, opposing p38α[70]. Interestingly, oncogenic KRAS induced activation of p38 and phenotypically increased invasion[71, 72].

JNK-related implication for involvement of the SAPK pathway in pancreatic cancer

Increased phospho-JNK staining was observed in pancreatic cancer tissues compared to normal controls[73] and increased phospho-JNK1 staining was determined as an independent predictor of peritoneal spread[74]. Furthermore, serum auto-antibodies

against JNK2 were identified as potential biomarker in pancreatic cancer patients[75]. By isoform-specific knockdown of JNK1 and 2 in MiaPaCa-2 and Panc-1 cells, a tumor promoting role could be attributed to JNK1, while JNK2 seems to exert suppressive functions in pancreatic cancer[76]. Upstream kinases of JNK have also been studied in pancreatic cancer, but their distinct role in tumor formation and progression remains elusive. MEKK1, as a representative of JNK-activating MKKKs, was shown to contribute to pancreatic cancer cell survival. However, unlike in other cancer cell lines, JNK signaling was not affected by knockout of MEKK1 in the PDAC cell line Panc-1 [77]. MKK4, a direct upstream kinase of JNK, was expressed in the majority of resected specimens, while expression levels were reduced in matched metastatic samples. As further hints for a potential tumor suppressive role, patients with loss of MKK4 were associated with shorter survival, and pancreatic cancer cell lines frequently showed loss-of-heterozygosity for MKK4[78,79]. However, ectopic expression of MKK4 stimulated proliferation and migration of ASPC-1 and BxPC3 cells[80].

Both kinases also act in pancreatic cancer outside neoplastic cells. *Ptf1a*$^{Cre/+}$; *Kras*$^{G12D/+}$; *JNK1*$^{-/-}$ mice showed significantly smaller tumors than their *JNK1*$^{+/-}$ counterparts. Tumors induced by transplantation of murine PDAC cells were larger in wild-type mice than in *JNK1*$^{-/-}$ mice lacking JNK signaling in stromal and immune cells. Interestingly, mice heterozygous for JNK1 showed less infiltrating $CD8^+$ T cells, possibly due to JNK-mediated downregulation of chemokine secretion of tumor-associated fibroblasts[81]. On the other hand, alternative activation of p38 through the T cell receptor in $CD4^+$ T cells resulted in more aggressive disease through secretion of pro-inflammatory cytokines like interleukin-17 and TNF-α[82].

SAPK inhibitors and their role for the treatment of pancreatic cancer

Due to the involvement of SAPKs within a variety of cellular processes and diseases, multiple researchers and the pharmaceutical industry have focused on identifying pharmacological inhibitors.

Generally, JNK small molecule inhibitors can be grouped into adenosine triphosphate (ATP)-competitive and non-ATP-competitive inhibitors. ATP-competitive inhibitors represent the majority of compounds, and most of them act as pan-JNK inhibitors, as the ATP-binding pocket is highly conserved among all three isoforms [83]. Non-ATP-competitive inhibitors target the interaction of JNK with upstream and downstream targets as well as scaffolding proteins. p38 inhibitors can also be grouped by their way of action. Similar to JNK inhibitors, many p38 inhibitors act as ATP-competitive inhibitors, either binding to the active (type 1 inhibitors) or inactive conformation of p38 (type 2 inhibitors)[84]. So far, p38 as well as JNK have been targeted in therapeutic intention for multiple pathologic conditions, like neurodegenerative diseases (*e.g.*, Alzheimer's and Parkinson's disease) and inflammatory diseases (*e.g.*, rheumatoid arthritis and inflammatory bowel disease)[85-87]. Consequently, pharmacological inhibition of both kinases has also been tested in order to explore new therapeutic strategies for the treatment of neoplastic diseases. Table 1 summarizes selective SAPK inhibitors studied in the context of cancer.

Strikingly, most studies report an antitumor effect of pharmacological SAPK inhibition. However, it needs to be noted that all inhibitors were pan-JNK inhibitors and p38α or pan-p38 inhibitors, respectively. As mentioned above, we previously reported on marked isoform-specific differences. By using genetic pathway disruption, isoform-specific tumor suppressive functions of JNK2 and p38β were detected and consequently, targeting of these isoforms might increase the risk of failure in clinical studies[76]. This effect could not be observed in our study, as pharmacological inhibition of JNK also reduced cell growth in various cell lines. Up to now, clinical studies using p38 inhibitors failed or only showed moderate success. Only one JNK inhibitor (CC-401) has been clinically evaluated for the treatment of cancer (NCT00126893), but this study has been discontinued[88,89].

In an early study, Ding *et al*[90] showed that the p38 inhibitor SB203580 [half maximal inhibitory concentration (IC_{50}) = 34 nmol/L] increases the number of Panc-1 cells in S phase as well as their proliferation. Decreased p38 activity was confirmed by detection of reduced levels of phospho-activating transcription factor. However, the same study also revealed increased phosphorylation levels of ERK1/2 and JNK[90]. Increased activation of ERK1/2, possibly as a compensation mechanism or off-target effect under SB203580 treatment, has also been shown in other reports[91,92]. Therefore, it remains unclear if the observed increased proliferation is a consequence of the loss of p38-dependent tumor suppressive actions or rather of increased ERK1/2 signaling. More recently, off-target effects of SB203580 and the closely related compound SB202190 were also described by Shanware and colleagues[93], reporting on cellular effects interestingly caused by off-target inhibition of CK1. Similar to the

Table 1 Small molecule inhibitors of stress-activated protein kinases tested for treatment effects on pancreatic carcinoma cells *in vitro* and *in vivo*

Inhibitor	IC_{50} (μmol/L)	Observed effects in cell culture and *in vivo* data	Ref.
JNK inhibitor II(SP600125)	0.040 (JNK1); 0.040 (JNK2); 0.090 (JNK3)	Antitumor effects in cancer cell lines of thyroid, stomach, lung, colon, pancreas, and brain	[104, 185-189]
JNK inhibitor XVI(JNK-IN-8)	0.005 (JNK1); 0.019 (JNK2); 0.980 (JNK3)	Covalent binding to JNK inactivates kinase function; Sensitizes pancreatic cancer cells and triple negative breast cancer cells to 5-FU/FOLFOX and triple negative breast cancer cells to lapatinib treatment	[190-192]
Bentamapimod(AS602801)	0.080 (JNK1); 0.090 (JNK2); 0.230 (JNK3)	Cytotoxic effects observed on cancer stem cells derived from pancreatic cancer, non-small cell lung cancer, ovarian cancer, and glioblastoma	[103, 193]
SB203580	0.034 (p38)	Synergistic effects observed in combination with cisplatin *in vitro* and *in vivo*; Inhibition of gemcitabine-induced apoptosis in combination therapy (tested on PK-1 and PCI-43 PDAC cell lines); $IC_{50(p38)}$ = 0.08-0.20 μmol/L *in vivo*)	[194-198]
SB202190	0.050 (p38α); 0.100 (p38β2); 0.600 (CK1)	Inhibition of gemcitabine-induced apoptosis in combination therapy (tested on PK-1 and PCI-43 PDAC cell lines); Inhibits resistance of colon cancer cell lines towards irinotecan	[93,197, 199,200]
SB239063	0.044 (p38α and β)	Dose-dependent growth inhibition observed in three pancreatic cancer cell lines	[68,201]

CK1: Casein kinase 1; EC_{50}: Half maximal effective concentration; 5-FU: 5-fluorouracil; IC_{50}: Half maximal inhibitory concentration; JNK1/2/3: c-Jun N-terminal kinases 1, 2, and 3.

above mentioned studies, Zhong *et al*[68] reported on the growth enhancing effects not only for SB203580 but also for SB202190 and SB239063 on three different pancreatic cancer cell lines. Interestingly, while environmental stressors (hypoxia and reduced serum levels) led to reduced proliferation in PDAC cell lines, p38 inhibition abolished these effects. Again, increased phospho-JNK levels after p38 inhibition were reported, and JNK inhibition through SP600125 abolished the effects of p38 inhibition *in vitro*. The pan-JNK inhibitor SP600125 ($IC_{50(JNK1)}$ = 40 nmol/L; $IC_{50(JNK2)}$ = 40 nmol/L; $IC_{50(JNK3)}$ = 90 nmol/L) also reduced the *in vivo* growth of cell lines with high phospho-p38 Levels [68]. The crosstalk of p38 and JNK has been described in various contexts previously. Although there is evidence for a synergistic role of both SAPKs in activation of downstream targets[94], an opposing function of both has also been well documented [95-97]. Possible regulation mechanisms of JNK through p38 include upstream MKKKs (MLK3, TAK1) as well as nuclear factor-κB[44]. Consequently, targeting single MAPKs is highly challenging. An alternative approach to using inhibitors could be selectively activating pathways of interest. The small molecule triptonide was shown to selectively activate the MEKK4-MKK4-p38 pathway without significantly altering phosphorylation levels of JNK and ERK1/2. This resulted in dose-dependent growth reduction of six pancreatic cancer cell lines as well as *in vivo* xenografts by inducing G2/M arrest and reduced expression of cyclin-dependent kinase 3[98].

In contrast to those studies showing an overall growth restraining effect of p38 in pancreatic cancer, Yang *et al*[99] performed a screen of p38α expression in various cancer samples of The Cancer Genome Atlas database and identified an overexpression of p38α in PDAC samples. The same study also reported enhanced phospho-p38 labeling in PDAC tissues compared to adjacent normal tissue and mostly attributed phospho-p38 labeling to cancer cells. When treating Pan02 cells with SB203580 or the p38α- and β-specific inhibitor LY2228820, Yang *et al*[99] reported growth-restricting effects. However, it needs to be noted that the used inhibitor concentrations were relatively high and potential off-target effects cannot be excluded. Finally, in order to address the issue of lacking sensitivity, possible binding pockets in p38, enabling the design of more selective inhibitor compounds in future, were identified by *in silico* modeling[99].

Previous studies indirectly suggested a growth-promoting effect of JNKs on pancreatic cancer. Takahashi *et al*[73] observed growth inhibition *in vitro* and *in vivo* after treatment with SP600125. This was associated with G1 arrest and downregulation of cyclin D1 *in vitro*. In genetically-engineered mouse models (*Ptf1a*$^{cre/+}$, *LSL-Kras*$^{G12D/+}$, and *Tgfbr2*$^{flox/flox}$) SP600125 reduced neoangiogenesis and expression levels of CD44 in PDAC cells[73]. Together with CD133, CD44 is considered as a potential marker for cancer stem cells (CSCs) or CSC-like cells (CSCLCs)[100,101]. Increased levels of phospho-JNK was also shown in CSCLCs of pancreatic cancer and other human malignancies[102]. Inhibition of JNK by the pan-JNK inhibitors SP600125 or AS602801 as well as genetic targeting of JNK1 and 2 *via* small interfering RNA-mediated knockdown reduced levels of $CD133^+$ cells in an isolated subpopulation of pancreatic

cancer-derived CSCLCs and abolished their self-renewal capacity *in vitro* and *in vivo* [102,103]. Besides growth suppression and interference with CSCs, induction of cellular differentiation can be another mechanism of JNK inhibition-mediated tumor suppression[104].

In clinical practice, SAPK inhibition will rather be used for combination therapy approaches instead of single-agent therapy. Therefore, the interference of SAPK inhibitors with standard of care chemotherapeutics is highly relevant. In addition to single treatment of CSCLCs, the group of Suzuki *et al*[105] also investigated the effects of JNK inhibition in combination with gemcitabine and 5-fluorouracil (5-FU). While CSCLCs expectedly were more resistant to these agents, pretreatment with SP600125 had a synergistic effect in combination with gemcitabine and 5-FU in a reactive oxygen species-based way of action[105]. The observed synergistic effect can furthermore be explained by JNK-mediated effects on multidrug resistance. Multidrug resistance is not only a hallmark of CSCs but of cancer cells in general, and multidrug transporters like P-glycoprotein reduce intracellular drug levels[106]. In this context, high JNK levels were shown to decrease P-glycoprotein levels in pancreatic and gastric cancer, thereby increasing intracellular drug concentrations as well as drug sensitivity[107].

The interplay of the p38 pathway and gemcitabine treatment has been well studied in pancreatic cancer cells. Apoptosis mediated through gemcitabine was consistently associated with p38 activation as well as caspase-dependent cleavage of poly (ADP-ribose) polymerase and heat shock protein 27 phosphorylation. Inhibition of p38 by SB203580 reversed these effects. Similarly, inhibition of MAPK-activated protein kinase 2, a downstream target of p38, abolished gemcitabine-mediated apoptosis in pancreatic cancer. However, combination of p38 inhibitors with mitomycin C showed synergistic effects[108].

PROTEIN KINASE CK1 FAMILY

In 1954 for the first time, an enzyme was isolated from liver tissue, which was able to phosphorylate the milk protein casein[109]. Fifteen years later, two distinct protein kinases with the ability to phosphorylate casein (at least *in vitro*) were described and termed casein kinase 1 (CK1) and casein kinase 2 (CK2), meanwhile renamed protein kinases CK1 and CK2[110]. Despite their common nomenclature and the ability to phosphorylate casein, protein kinases CK1 and CK2 are highly different with respect to their classification and cellular functions. While CK2 belongs to the CMGC [containing cyclin-dependent kinase, MAPK, glycogen synthase kinase 3 (GSK3), and cdc2-like kinase families] group of the human kinome, CK1 forms an independent family of protein kinases[111].

In the human genome, six CK1 isoforms are encoded (α, γ1, γ2, γ3, δ, and ε), and several splice variants can originate from post-transcriptional processing. While all CK1 isoforms are highly conserved within the kinase domain, the sequences of the N- and C-terminal noncatalytic domains can be quite variable[20,112]. Although the protein kinases of the CK1 family are generally considered to be constitutively active, several regulatory mechanisms have been described. Expression and/or activity levels of CK1 isoforms can be enhanced by insulin or cellular stress executed by viral transformation, topoisomerase inhibitor treatment, or γ-irradiation. Regulation of enzymatic activity is also possible on the protein level, *e.g.*, by modulation of subcellular localization, interaction with other proteins, or (auto-)phosphorylation in particular targeting the C-terminal regulatory domain but also the kinase domain[20,112].

Most CK1 isoforms are localized in the cytosol. Only the CK1αL variant possessing a second nuclear localization signal in the L-exon can be localized to the nucleus[113]. Due to C-terminal palmitoylation, CK1γ can be associated with the plasma membrane [114-116]. By modulation of subcellular localization, CK1 isoforms can be brought in proximity with different substrate pools. Substrate recognition motifs for CK1 can generally be found on most cellular proteins and, to date, more than 150 substrates being phosphorylated by CK1 isoforms at least *in vitro* have been reported[20]. Thereby, CK1 shows strong preferences for acidic or phospho-primed substrates presenting the canonical consensus sequence (phospho-Ser/phospho-Thr-X-X-(X)-Ser/Thr). In addition, several alternative noncanonical motifs targeted by CK1 have been described[117,118].

The broad range of substrates phosphorylated by CK1 gives a hint of the numerous cellular processes potentially regulated by CK1 family members. These processes involve cell proliferation and differentiation, DNA processing and repair, as well as

cytoskeleton maintenance just to name few of them. In particular, essential signal transduction pathways involving CK1-mediated regulation include Wnt and Hedgehog (Hh) signaling as well as regulation of circadian rhythm[20]. Consequently, deregulation or dysfunction of CK1 isoforms involved in regulation of these signaling pathways can result in deregulated signal transduction and subsequent development of pathological states.

Relevant CK1-related pathways in cancer

CK1 isoforms have been implicated in several signaling pathways such as the canonical and noncanonical Wnt as well as Hh and Hippo signaling pathways, which play an important role in tissue development, growth, and homeostasis[119-122]. Aberrant signaling as well as mutations of key regulator proteins of these pathways can lead to various cancer entities[123-127]. The connection between CK1 and cancer has been strengthened through the discovery of their targets such as β-catenin, p53, and mouse double minute homologue 2 and 4, which hold important roles as key regulators in signaling pathways and are generally thought to be involved in cancer development (Figure 1)[128,129]. Considering the reported CK1-mediated phosphorylation of numerous substrates essential for signal transduction, it is not surprising that CK1-specific mutations resulting in altered expression levels or enzyme activity are likely to be accompanied by dramatic changes affecting CK1-regulated signal transduction pathways.

As one of the best characterized CK1-regulated processes, the Wnt signaling pathway has an important regulatory role in cell proliferation, differentiation, and cell polarity[120,130-133]. Altered expression levels of key regulators within the pathway are associated to oncogenesis, both through increased expression of positive regulators and decreased expression of negative regulators[134-137]. Several studies showed that all CK1 isoforms are implicated in the Wnt signaling pathway and either exert positive or negative regulatory functions, respectively[14]. Acting as positive regulators of the canonical Wnt signaling pathway, CK1γ, δ, and ε were found to initiate the transcription of proto-oncogenes like cyclin D1 and c-myc resulting in increased cell proliferation and cell survival[13,138,139]. For instance, mutations within the C-terminal region of CK1δ were shown to alter its physiological role, increase the oncogenic potential, and promote colonic adenoma development[140]. Additionally, CK1 isoforms exhibit oncogenic characteristics associated to the inhibition of apoptotic processes. This assumption is supported by the findings that CK1δ and CK1ε contribute to the switching mechanism between the canonical and the non-canonical Wnt/Rac1/JNK pathway, where they may favor the canonical Wnt pathway to the detriment of JNK-mediated apoptosis[17,141]. In many Wnt-driven cancers, CK1α protein expression is suppressed, leading to an activation of proliferative processes *via* the Wnt pathway. In addition, the absence of CK1α leads to a critical involvement of p53 in controlling invasiveness, which was shown in a model for colon cancer[15].

The importance of CK1 isoforms within various signaling pathways is strengthened by reports linking CK1 to phosphorylation of components in Hh signaling pathway. Although the activity of the Hh signaling pathway is reduced in adulthood, it is critical for embryonic development, organogenesis, and maintenance of healthy adult cells[119]. In the adult organism, Hh signaling contributes to the regulation of epithelial maintenance and tissue regeneration; consequently mutations and dysregulation of components of this signaling pathway promote tumorigenesis and cancer development[142-145]. As seen in Wnt signaling, CK1 isoforms appear to have contrasting effects on Hh signaling. Acting as a negative regulator, CK1 promotes proteolysis of GLI TF and prevents target gene transcription[146-148]. In order to fulfill its positive function, CK1α and G-protein coupled receptor kinase 2 phosphorylate the positive Hh regulator Smoothened homologue precursor, thereby inducing its active conformation[149].

The major functions of the Hippo pathway have been defined to correct organ maturation through restriction of organ size by regulating cell proliferation and apoptosis[150]. As such, dysregulated Hippo signaling can trigger tumorigenesis and cancer. CK1 isoforms have been proposed to regulate Hippo signaling through phosphorylation of a phosphodegron signal in Yes-associated protein after receiving priming phosphorylation by large tumor suppressors 1 and 2. As a result, the phospho-degron signal mediates recruitment of βTrCP ubiquitin ligase causing Yes-associated protein degradation and inhibition of cell growth and differentiation[150]. Additionally, a more recent publication proposed an interaction between the Wnt and the Hippo pathways mediated through CK1ε. In this context, the Hippo upstream kinase MST1 inhibits the Wnt signaling pathway by directly binding CK1ε and thereby suppressing phosphorylation of Disheveled[151].

Implication for CK1 involvement in pancreatic cancer

In a recent study analyzing messenger RNA-based gene expression data of the International Cancer Genome Consortium Pancreatic Cancer Australia cohort, high expression levels of CK1δ detected in patients with pancreatic cancer were correlated with poor survival. Increased expression of CK1δ could be found in patients with metastatic pancreatic carcinoma, and CK1δ expression was furthermore strongly correlated with the tumor grade[152]. This observation is in line with previous studies reporting upregulation of CK1 isoforms in PDAC in general[153,154] and describing increased expression of CK1δ and CK1ε in a patient cohort with higher-graded PDAC [155]. Cell line-specific elevated expression levels of CK1δ and/or CK1ε were also detected in various tumor cell lines[152,155]. Independent of the detected protein levels, CK1δ- and CK1ε-specific kinase activities in extracts obtained from various pancreatic cancer cell lines (MiaPaCa-2, BxPC3, PancTu-1, and Colo357) significantly differed from each other by up to six orders of magnitude[156].

In general, due to the involvement of CK1 isoforms in various pathways related to tumorigenesis, altered expression and/or activity levels of CK1 isoforms can be associated with increased oncogenic potential. Using the breast cancer cell line MCF7, a regulatory function of CK1ε has been identified in the Akt pathway[157]. This is of particular interest because Akt is frequently upregulated in PDAC[158,159]. In detail, CK1ε is able to inhibit protein phosphatase 2B, consequently resulting in increased Akt phosphorylation levels and enhanced Akt kinase activity. Inhibition of CK1ε in MCF7 cells by the small molecule inhibitor IC261 has been demonstrated to reduce Akt phosphorylation as well as Akt-mediated phosphorylation of GSK3β[157]. Quite similar findings could be made using PDAC cells. Also in this case, phosphorylation of Akt was reduced in response to treatment with IC261[160]. However, these results were only based on observations made in preliminary experiments, and effects were obtained by using extremely high concentrations of the rather unspecific early-stage inhibitor IC261.

Apart from altered expression and/or activity levels, mutations in the coding sequence for CK1 isoforms can also be associated with increased oncogenic functions of the resulting CK1 mutant proteins. Several mutations in *CSNK1D*, the gene coding for human protein kinase CK1δ, identified in different types of cancer (*e.g.*, colorectal carcinoma, lung squamous cell carcinoma, bladder urothelial carcinoma, and pancreatic carcinoma) were analyzed for their enzyme kinetic parameters and their sensitivity towards the treatment with several CK1-specific small molecule inhibitors. Among the tested mutants, hyperactive (*e.g.*, R127L and R127Q) as well as nearly inactive (*e.g.*, E247K and L252P) variants could be characterized. Especially, the hyperactive CK1δ mutant R127Q showed enhanced sensitivity towards the treatment with various CK1-specific inhibitors. The two tested CK1δ mutants exclusively detected in PDAC (Q399* and H414Y) only showed slightly reduced kinase activity when compared to wild-type CK1δ[161]. The online analysis tool cBioPortal for Cancer Genomics lists even more mutations detected in PDAC and affecting CK1 isoforms, among them frameshift deletions as well as nonsense and missense mutations[162-165]. However, these mutations have so far not been investigated for their oncogenic potential.

Unfortunately, no detailed information on the role of CK1 isoforms in formation of metastasis from primary tumors located in the pancreas has been available so far. In general, the zinc-finger TF Snail is phosphorylated by CK1ε and GSK3β in a hierarchical manner. Snail can promote epithelial-mesenchymal transition by repressing expression of E-cadherin but is degraded by the proteasome upon phosphorylation by CK1ε and GSK3β. Pharmacological inhibition (using the inhibitor IC261) or RNA interference-mediated downregulation of CK1δ inhibits phosphorylation of Snail and promotes cell migration[166]. In addition, reduced proliferation and invasion could be linked with CK1ε-mediated inhibition of Wnt/β-catenin signaling and downregulation of Wnt3a, β-catenin, proliferating cell nuclear antigen, and matrix metalloproteinase 9 in colorectal cancer cells[167].

Inhibitors of CK1 and their role for the treatment of pancreatic cancer

Results obtained from numerous studies conducted within the last 10-15 years characterized the protein kinases of the CK1 family as well-established drug targets. While early-stage small molecule inhibitors (*e.g.*, IC261[168]) only demonstrated low target selectivity, several recent-stage CK1-specific inhibitors with enhanced selectivity and improved potency in the nanomolar range are available to date (Table 2)[20,112,169]. So far, none of these inhibitors advanced to the stage of clinical trials, and the use of these compounds was limited to biochemical and cell culture-based testing or animal

Table 2 Casein kinase 1-specific small molecule inhibitors tested for treatment effects on pancreatic carcinoma cells *in vitro* and *in vivo*

Inhibitor	IC_{50} CK1 (μmol/L)	Observed effects in cell culture and *in vivo* data	Ref.
IC261	1.000 ± 0.30 (CK1δ)	Reduced growth of ASPC-1, BxPC3, Capan-1, Colo357, MiaPaCa-2, Panc-1, Panc89, and PancTu-1 at 1.25 μmol/L concentration of IC261; Subcutaneous xenograft model using PancTu-2: reduced tumor size with IC261 or gemcitabine (no synergism with gemcitabine), downregulation of anti-apoptotic genes/upregulation of cell cycle- and cell death-associated regulators; Notable off target effects (affecting the cytoskeleton and ion channels)	[155, 168, 182-184, 202]
compound 11b	0.004 ± 0.001 (CK1δ); 0.025 ± 0.004 (CK1ε); 0.010 (p38α)	Cytotoxic effects observed on Colo357 (EC_{50} = 3.5 ± 0.3 μmol/L) and Panc89 (1.5 ± 0.4 μmol/L)	[174]
compound 3c	1.600 (CK1δ/ε)	In a panel of cell lines only effective against Panc-1 (EC_{50} = 9.3 ± 0.0 μmol/L); Cytotoxic effects observed on A549 (lung carcinoma) and Hek293 (normal cells) significantly higher EC_{50} values	[175]
compound 2	0.070 ± 0.01 (CK1δkd); 0.520 ± 0.05 (CK1ε)	Cytotoxic effects observed on BxPC3 (EC_{50} = 0.11 ± 0.01 μmol/L), Colo357 (0.13 ± 0.02 μmol/L), MiaPaCa (0.26 ± 0.02 μmol/L), PancTu-1 (0.70 ± 0.02 μmol/L), and Panc-1 (0.35 ± 0.08 μmol/L); Cell line-specific effects observed in screening against a panel of 82 tumor cell lines	[178]
IWP-4	1.020 ± 0.13 (CK1δ); 7.070 ± 2.01 (CK1ε)	Cytotoxic effects observed on A818-6 (EC_{50} = 0.93 ± 0.07 μmol/L), MiaPaCa-2 (0.23 ± 0.01 μmol/L), Panc-1 (0.23 ± 0.02 μmol/L), Panc89 (0.58 ± 0.12 μmol/L), and Capan (0.23 ± 0.01 μmol/L); Inhibition of Wnt signaling (Wnt3A overexpression, autocrine/paracrine) with IC_{50} = 0.71 ± 0.38 μmol/L; Inhibition of Wnt signaling (Wnt3A-conditioned medium, autocrine/paracrine) with EC_{50} = 1.47 ± 0.55 μmol/L	
SR-3029	0.044 (CK1δ); 0.260 (CK1ε)	Cytotoxic effects observed on Panc-1 (EC_{50} = 0.023 μmol/L), MiaPaCa2 (0.370 μmol/L), and BxPC3 (0.131 μmol/L); Mouse pharmacokinetic studies with promising results for animal model use of SR-3029; Orthotopic xenograft model using Panc-1, reduced tumor size using SR-3029 and/or gemcitabine (synergism with gemcitabine due to upregulation of dCK)	[152, 180]

CK1: Casein kinase 1; EC_{50}: Half maximal effective concentration; IC_{50}: Half maximal inhibitory concentration.

models.

Quite recently, we characterized optimized 4,5-diarylimidazoles as highly effective ATP-competitive inhibitors of CK1δ. Substituted isoxazoles were originally designed as inhibitors of p38α MAPK, but they share the same pharmacophore moiety that is necessary to inhibit CK1δ[93,170-172]. Substituting the isoxazole scaffold with an imidazole scaffold resulted in the generation of highly potent dual-specific inhibitors of p38α MAPK and CK1δ[173]. By further optimizing these imidazole-based compounds, CK1 isoform-specific inhibitors with IC_{50} values in the low nanomolar range like compounds 11b [$IC_{50(CK1\delta)}$ = 4 nmol/L], 12a (19 nmol/L), and 16b (8 nmol/L) could be developed, which represent the most potent CK1δ-specific inhibitors described so far. Because IC_{50} values determined for the highly related isoform CK1ε are increased by six to 12 orders of magnitude (with 25, 227, and 81 nmol/L for 11b, 12a, and 16b, respectively), these compounds can also be considered to be selective for CK1δ. Compound 11b even demonstrated superior selectivity towards CK1δ among a panel of more than 321 protein kinases. However, full selectivity with respect to side-effects on p38α MAPK could still not be achieved for this set of compounds, but IC_{50} values determined for p38α are three-fold higher compared to CK1δ. Finally, 11b demonstrated significant effects on pancreatic cancer cell lines, with half maximal effective concentration (EC_{50}) values in the low micromolar range [$EC_{50(Colo357)}$ = 3.5 μmol/L, $EC_{50(Panc89)}$ = 1.5 μmol/L)[174].

Apart from isoxazole- and imidazole-derived molecules, the quinazoline-based inhibitors (N-(1H-pyrazol-3-yl)quinazolin-4-amines) 3c and 3d have been shown to inhibit CK1δ and ε ($IC_{50(CK1\delta/\varepsilon)}$ = 1.6 and 1.4 μmol/L, respectively). In a panel of human cancer cell lines, compound 3c even demonstrated selective cytotoxicity against the PDAC cell line Panc-1, with an EC_{50} value of 9.3 μmol/L [for all others no EC_{50} value could be determined (> 100 μmol/L), except for A549 with 29.7 and HEK293 with 71.1 μmol/L]. Compound 3d also demonstrated effects on Panc-1 cells but only with an extremely high EC_{50} value of 69.4 μmol/L. However, the mechanism of selectivity of the tested quinazoline-based inhibitor remains to be determined[175].

Within the last decade, several benzimidazole-based inhibitors have demonstrated significant inhibition of CK1δ variants and superior isoform selectivity over CK1δ. The series of compounds described by Leban *et al*[176] originates from piperidinyl-thiazoles originally designed to inhibit nuclear factor-κB[176]. Following modification, these compounds also demonstrated significant inhibition of CK1 family members. Most significant inhibition of CK1δ kinase domain (CK1δkd) with superior isoform selectivity over CK1ε could be determined for compound 5 ($IC_{50(CK1\delta kd)}$ = 29 nmol/L, $IC_{50(CK1\varepsilon)}$ = 199 nmol/L). Compound 5 also induced apoptosis in various tumor cell lines

with cell line-specific effects and only moderate levels of apoptosis in Colo357 pancreatic cancer cells (tested at 4 μmol/L concentration)[177]. As reported by Richter and colleagues[178], the highly related but structurally slightly different compound 1 showed three-fold stronger inhibition of CK1δkd (IC_{50} = 10 nmol/L). By further improving the physicochemical properties of this difluoro-dioxolo-benzoimidazole derivative, inhibitor potency *in vitro* could be maintained for modified compound 2 ($IC_{50(CK1\delta kd)}$ = 0.07 μmol/L, $IC_{50(CK1\varepsilon)}$ = 0.52 μmol/L) while significantly increasing the effects observed on a panel of cancer cell lines. In comparison to compound 1, the effects on cell viability were significantly increased for cell lines treated with compound 2, among them the pancreatic cancer cell lines BxPC3, Colo357, MiaPaCa, PancTu-1, and Panc-1 (see Table 2 for EC_{50} data)[178].

Being structurally related to benzimidazole-based inhibitors, compounds derived from inhibitors of Wnt production (IWP) have recently been described as CK1-specific inhibitors. IWP-2 and IWP-4 as well as the further optimized compound 19 displayed rather potent inhibition of CK1δkd *in vitro* ($IC_{50(CK1\delta)}$ = 0.32, 1.02, and 0.09 μmol/L for IWP-2, IWP-4, and compound 19) and also demonstrated significant effects on the proliferation of pancreatic cancer cell lines as determined for IWP-4-treated A818-6 (EC_{50} = 0.93 μmol/L), MiaPaCa (0.23 μmol/L), Panc-1 (0.23 μmol/L), and Panc89 (0.58 μmol/L) cells[179].

As a benzimidazole-based inhibitor containing a purine scaffold compound SR-3029 has been described as highly potent and selective inhibitor of CK1δ ($IC_{50(CK1\delta)}$ = 44 nmol/L, $IC_{50(CK1\varepsilon)}$ = 260 nmol/L)[180]. SR-3029 shows improved cellular activity on the human melanoma cell line A375 (EC_{50} = 86 nmol/L) and the triple-negative breast cancer cell line MDA-MB-231[181]. These results suggested favorable cell penetration for SR-3029, and mouse pharmacokinetic properties indicated that SR-3029 actually was sufficient for use in xenograft studies[180].

Recently, SR-3029 has been tested for its effects on the proliferation of PDAC cell lines Panc-1, MiaPaCa-2, and BxPC3, thereby obtaining EC_{50} values in the submicromolar range (23, 370, and 131 nmol/L, respectively). Furthermore, synergistic effects have been detected for the treatment of MiaPaCa-2 and Panc-1 cells with a combination of SR-3029 and gemcitabine, the standard of care used in treatment of locally advanced and metastatic PDAC. Same effects could be observed after silencing of CK1δ by small interfering RNA. The mechanism of synergy could be explained by upregulation of deoxycytidine kinase subsequent to inhibition of CK1δ by SR-3029, resulting in enhanced metabolism and anti-proliferative effects of gemcitabine. Anti-proliferative effects of SR-3029 and synergy with gemcitabine could also be observed *in vivo* by using an orthotopic xenotransplantation mouse model. Tumors obtained from injection of Panc-1 cells into the pancreas were significantly smaller after treatment with SR-3029 or gemcitabine, and tumor size was even more reduced after combination therapy[152].

In a previous xenotransplantation study, the early-stage CK1-specific inhibitor IC261 had already demonstrated therapeutic potential. Tumor cell growth of a panel of established pancreatic cancer cell lines (ASPC-1, BxPC3, Capan-1, Colo357, MiaPaCa-2, Panc-1, Panc89, and PancTu-1) was significantly reduced by treatment with 1.25 μmol/L IC261 *in vitro*, and the size of tumors obtained after subcutaneous injection of PancTu-2 cells was significantly smaller after treatment with IC261. In the tumor tissue, downregulation of several anti-apoptotic genes (*e.g.*, Bcl-2 family members) and upregulation of cell cycle- and cell death-associated regulators (*e.g.*, p21, ataxia-telangiectasia mutated kinase, checkpoint kinase 1) could be observed following treatment with IC261 or gemcitabine[155]. However, and in contrast to the above mentioned recent study by Vena and colleagues[152], IC261 failed to sensitize gemcitabine-resistant PancTu-1 cells to treatment with gemcitabine, and no synergistic or additive action in combination with gemcitabine could be demonstrated for IC261. This failure can be due to the unspecific effects meanwhile described for IC261. Apart from its specific action on CK1 family members, IC261 is able to bind tubulin with an affinity similar to the spindle poison colchicine. IC261 can therefore be considered as a microtubule polymerization inhibitor by directly exerting its effects on microtubules independent of CK1 blockage[182,183]. Moreover, within the concentration range necessary to block CK1 kinase activity, IC261 is also able to block voltage-gated sodium channels, and consequently, well-characterized recent-stage CK1-specific inhibitor compounds like SR-3029 should be used for targeting CK1 isoforms instead of using the unspecific early-stage inhibitor IC261[184].

CONCLUSION

In recent years, there has been a lot of evidence for the involvement of stress-activated kinases like JNK and p38 but also CK1 in the pathogenesis of pancreatic cancer. Furthermore, remarkable progress has been made in designing specific small molecule inhibitors to effectively target these kinases *in vitro* and *in vivo* and to reduce off-target effects. Interestingly, due to similarities in protein structure, some inhibitor compounds even demonstrate dual inhibition of p38 and CK1 isoforms. However, further mechanisms and benefits from dual kinase inhibition have not been studied in detail. Furthermore, conclusive results from using specific inhibitors in clinical trials remain to be obtained, and knowledge on the interplay of these inhibitors with standard of care chemotherapeutics needs to be acquired in future studies.

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REVIEW

Malnutrition and liver disease in a developing country

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Abstract

Malnutrition is a highly prevalent and under recognized condition in developing countries of South Asia. The presence of malnutrition causes a severe impact on patients with liver cirrhosis. The etiology of cirrhosis differs in the South Asian region compared to the West, with hepatitis B and C still being the leading causes and the prevalence of nonalcoholic fatty liver disease increasing over time. Comorbid malnutrition worsens outcomes for cirrhosis patients. Urgent attention to address malnutrition is needed to improve patient outcomes. The etiology and pathophysiology of malnutrition in liver diseases is multifactorial, as reduction in liver function affects both macronutrients and micronutrients. A need for nutritional status assessment for liver disease patients exists in all parts of the world. There are many widely studied tools in use to perform a thorough nutritional assessment, of which some tools are low cost and do not require extensive training. These tools can be studied and evaluated for use in the resource limited setting of a country like Pakistan. Treatment guidelines for proper nutrition maintenance in chronic liver disease exist for all parts of the world, but the knowledge and practice of nutritional counseling in Pakistan is poor, both amongst patients and physicians. Emphasis on assessment for nutritional status at the initial visit with recording of vital signs is needed. Simultaneously, treating physicians need to be made aware of the misconceptions surrounding nutritional restrictions in cirrhosis so that patient education is done correctly based on proper scientific evidence.

Key Words: Liver cirrhosis; Malnutrition; Nutrition assessment; Liver diseases; Nutritional and metabolic diseases; Micronutrients

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Core Tip: Malnutrition in liver cirrhosis is a serious problem in South Asia where the etiology differs from the Western population. As malnutrition is generally highly prevalent in the region, it causes an impact on patients with liver cirrhosis. Urgent attention to address malnutrition is needed to improve patient outcomes. Emphasis on assessment for nutritional status at the initial visit with recording of vital signs is also needed. Simultaneously, treating physicians need to be made aware of the misconceptions surrounding nutritional restrictions in cirrhosis so that patient education is done correctly based on proper scientific evidence.

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INTRODUCTION

Malnutrition (undernutrition) is defined as an insufficient intake or assimilation of nutrients essential for development and prevention of disease[1]. Malnutrition presents as a common complication of end-stage liver failure (cirrhosis), which is characterized by mass systemic complications such as refractory ascites, spontaneous bacterial peritonitis, hepatorenal syndrome and variceal hemorrhage[2]. Due to the deterioration of health that cirrhosis causes, approximately a million people die from cirrhosis globally, making it a significant disease burden to be addressed.

Several studies have evaluated nutritional status in patients with liver cirrhosis of different etiologies and varying degrees of liver insufficiency[3,4] leading to a consensus that malnutrition is recognizable in all forms of cirrhosis[5] with the prevalence of malnutrition in cirrhosis having been estimated to range from 65%-100%[6,7]. Thus, malnutrition becomes an important prognostic indicator of clinical outcomes, which include survival rate, the length of hospital stay, post-transplantation morbidity and quality of life in patients with cirrhosis[8], particularly in patients with severe cirrhosis and alcoholic cirrhosis where malnutrition is more likely to be present[9]. While malnutrition remains potentially modifiable, it has been observed starting from the initial stages of liver disease that results in an almost direct relationship between the liver disease severity and the extent of malnutrition, with malnutrition becoming easier to detect in severe cirrhosis[10].

The causes of malnutrition in liver disease are complex and multifactorial due to the liver forming a fundamental part of the body's metabolism. It results from impaired dietary intake due to the malfunctioning liver, chronic inflammation and subsequently the altered macronutrient and micronutrient metabolism. Low patient activity is also attributed to it[7,11]. Additionally, anorexia, gastroparesis, nausea, increased leptin levels, encephalopathy and gastritis are found to be contributors of malnutrition. Ascites, frequent paracentesis, some drugs such as diuretics and lactulose, a sodium-restricted diet and alcohol consumption (decreases appetite) can also result in metabolic malfunctioning and thus contribute to a reduction in dietary intake, which leads to malnutrition in liver disease[12,13]. The deficiency of biliary salts due to liver malfunction and decreased assimilation of nutrients, abnormal motility of the digestive tract and an increased bacterial growth may subsequently lead to an impaired uptake and metabolism of nutrients, resulting in malnutrition[14]. Portal hypertension leads to an increased permeability of the intestinal mucosa, thereby causing an increased loss of proteins that may be similarly noted in cases of bleeds caused by varices or ulcers[15]. A poor nutritional status then poses significant consequences for postoperative complications among transplant candidates as well because this is an important prognosticator for mortality and morbidity among chronic liver disease (CLD) patients[9]. Subsequently, malnutrition itself becomes an independent predictor of mortality in patients with CLD.

The resultant malnutrition comprises of both macro and micronutrient deficiencies. The macronutrients (being proteins, carbohydrates and fats) have their metabolism affected[16], while protein deficiency makes patients more susceptible to hepatocellular disease, in particular. On the micronutrient level, patients with a history of cholestatic liver disease are more liable to have an insufficient calorie intake with a

higher risk of vitamin deficiencies. Detecting micro or macronutrient deficiencies at an earlier stage is crucial so that nutritional supplementation can be initiated in a timely manner, which has been proven to decrease the risk of infection and in-hospital mortality while enhancing liver function. Therefore, it has been recommended that all patients with CLD should be screened to have an early identification of those at risk of complications so that relevant interventions can be started[17].

In this article, we will be reviewing the prevalence, underlying mechanism and factors associated with malnutrition and the impact of malnutrition in cirrhosis in the setting of a developing country. This will pave the way for increased awareness regarding malnutrition in cirrhosis and its consequences to subsequently treat the patients accordingly for better outcomes.

MALNUTRITION IN SOUTH ASIA

While about 2 billion people in the world are suffering from various forms of malnutrition[18], the prevalence of malnutrition due to cirrhosis is estimated to range from 65%-100% globally[6,7]. In South Asia particularly, malnutrition is rampant with a high prevalence of low adult body mass index (BMI) (23.4% in men and 24.0% in women in 2014)[19]. In this region, Pakistan in particular has 45.3 million people (28 percent of the population) being victims of food insecurity according to the National Nutrition Survey Report 2011[20]. The global nutrition report 2020 has studied the progress of attaining 10 global nutrition targets of 2025 in 194 countries (which include anemia, low birth weight, exclusive breastfeeding, childhood stunting, childhood wasting, childhood overweight, adult obesity and adult diabetes, salt intake and raised blood pressure). Progress is not assessed at the country level for salt intake and blood pressure. Data collected for 8 of the 10 targets shows that Pakistan is on track for only 2 targets out of 8. In Pakistan, more than half of children grow up stunted or wasted. Pakistan is recognizing this issue as important, as is evident by it being 1 of the 25 countries to have revisited their nutrition budget, which has resulted in allocating increased funds towards nutrition thrice since 2015. Of this, the highest increase is towards nutrition sensitive allocation of social protection. The report highlights the importance for subnational level of financing for nutrition in areas like Balochistan in Pakistan and Rajhasthan in India. Pakistan and India comprise two of the three countries with the highest number of stunted children with 10.7 million stunted children in Pakistan and 25.5 million in India. This data reflects the severity of malnutrition in the region and can be extrapolated to the adult population of the South Asian countries[21].

LIVER DISEASES IN SOUTH ASIA

While most of the burden of liver cirrhosis in western countries is nonalcoholic fatty liver disease, most of it for South Asian countries is related to hepatitis B and C. However, increasing prevalence of nonalcoholic fatty liver disease is being seen in the South Asian population as well[22]. A survey conducted in 2015 showed the incidence of cirrhosis in South Asia to be 21.39% overall with 6.98% being hepatitis B virus related, 4.87% hepatitis C virus related, 4.84% alcohol related and 4.69% due to other etiologies[23]. The incidence of cirrhosis is estimated to be 23.6 per 100000 in Southeast Asia in 2017[24,25]. A study comparing liver resection patients between the East and the West of the world showed that patients from the East had worse Child Pugh scores and had a more advanced stage of cancer than the West[26]. A multicenter study from India revealed that at least a third of the patients presenting with CLD present at a very advanced stage of decompensated cirrhosis[27].

ETIOLOGY AND PATHOPHYSIOLOGY

The flowcharts in Figures 1 and 2 describe the pathophysiology of malnutrition as a consequence of cirrhosis and the multiple pathways that lead to it[28].

Metabolic changes occurring in CLD

Multiple changes in nutrient metabolism are observed in cirrhosis, which subsequently contributes to malnutrition. Around 14% to 40% of patients with cirrhosis

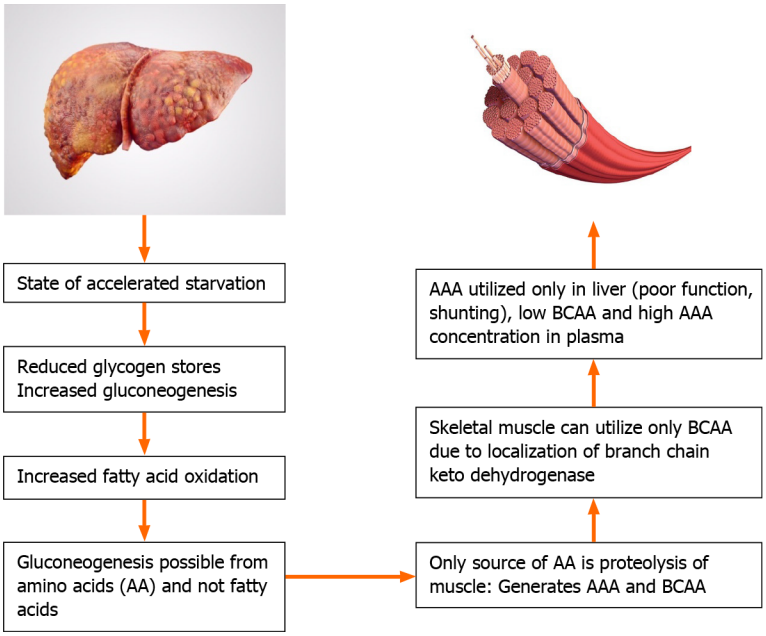

Figure 1 Pathophysiology of malnutrition as a consequence of cirrhosis and the multiple pathways that lead to it. AAA: Aromatic amino acids; BCAA: Branched chain amino acids.

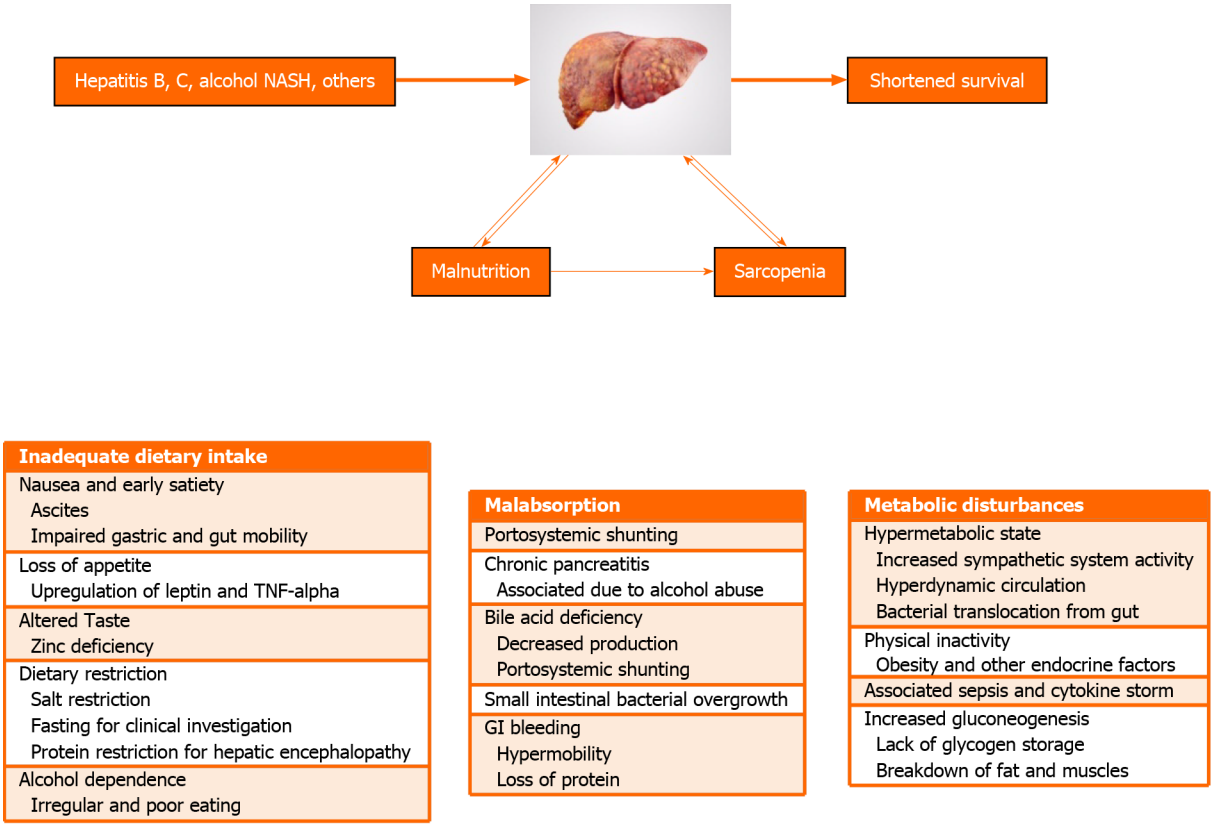

Figure 2 Pathophysiology of malnutrition as a consequence of cirrhosis and the multiple pathways that lead to it. GI: Gastrointestinal; NASH: Nonalcoholic steatohepatitis; TNF: Tumor necrosis factor.

have been diagnosed with type 2 diabetes mellitus due to glucose intolerance or end stream resistance to insulin seen in an estimated 70% of cirrhotic patients[29]. It has also been observed that the levels of hepatic and muscle glycogen are decreased, leading to a reduction in the availability of glucose as an energy substrate, with the body switching to the consumption of fats and proteins as alternative energy sources [30].

Regarding the rest energy expenditure (REE), a measure of resting metabolic rate and defined as the energy expended to sustain homeostasis at rest, conflicting results have been reported. Up to 34% of patients have been claimed to have a REE out of range by being about 120% higher, while some results show most of the patients with a REE within range[9,31,32]. Further measuring the calorie consumption, a cross-sectional study of 473 patients with cirrhosis found that 34% had hypermetabolism, measured by indirect calorimetry. The increase in REE was associated with lean body mass and an increase in beta-adrenergic activity but not with the severity or type of liver disease. Further studies have demonstrated that hypermetabolism can persist at least 1 year after liver transplant and correlates with a reduction in survival, also indicating prognosis[33]. Ascites can also contribute to an increase in REE due to which its removal results in a significant reduction in the REE when measured by indirect calorimetry. The mechanism underlying this observation is unclear[34].

The muscle plays a significant role in metabolism particularly of amino acids (AA). It also constitutes an important site for glutamine synthesis and gluconeogenesis. It should be noted that gluconeogenesis plays a direct role in the destruction of muscle tissue, with the body deriving protein substrates from the muscle for metabolism. This destruction of muscle subsequently causes a decrease in the synthesis of proteins and in its break down, also causing these patients to become protein deficient. Therefore, the most common complication of cirrhosis is a presentation of sarcopenia, observable in almost 60% of patients[35,36]. The ratio of branched chain AA (BCAA) to aromatic AA declines in CLD. A similar effect is seen in sepsis and major trauma. It is due to this change that malnourished individuals often develop hepatic encephalopathy (HE). This has established sarcopenia as an important risk factor for HE[36,37]. Further contributing to the increase in the consumption of AA by the muscles is the factor of any overnight fast that promotes an increase in ketogenesis and gluconeogenesis, even with lipids representing 75% of the calories expended during such a period. Healthy subjects after 3 d of fasting may also have a similar effect, but it occurs much slower when compared with cirrhotic patients[38].

There is an overall loss of protein from reduced synthesis of urea and hepatic proteins, reduced intestinal protein absorption and increased urinary nitrogen excretion[39]. The deficiency of AA and thus proteins gives rise to specifically a protein-calorie malnutrition (PCM). Each stage of CLD is affected by PCM with PCM being present in 65%-90% of patients with advanced disease. PCM also detrimentally causes systemic complications. There is an increased risk of HE and hepatorenal syndrome, while esophageal varices may often be seen as well. There is also a reduced regeneration capacity of the liver with a declining trend of liver function and an increased risk of surgical morbidity as well as mortality, all due to the protein and AA deficiency causing subsequent metabolic dysfunction[40,41].

Peripheral insulin resistance and β-cell dysfunction due to abnormal cell signaling pathways in cirrhotic patients leads to pancreatic dysfunction, giving rise to hepatogenous diabetes, steatorrhea and overall pancreatic disease[42,43]. The improper carbohydrate metabolism preferentially causes lipids to be oxidized instead for energy. This increases lipolysis and fatty acid oxidation, while an increase in the production of ketones may also occur. Therefore, reduced levels of plasma triglycerides, phospholipids, cholesterol, apoproteins and polyunsaturated fatty acids occurs in cirrhotic patients over time. These decreasing levels can be correlated with the severity of the liver disease, becoming an independent predictor of mortality in alcoholic cirrhosis. The respiratory quotient is also lowered when compared to non-diseased patients, also pointing towards poorer patient outcomes[44,45].

Deficiency of micronutrients

Micronutrient deficiency is common in cirrhosis, most observed in alcoholic disease. The most common deficiency seen is that of group B vitamins. Deficient levels of micronutrients are explained mainly by a decreased oral intake, malabsorption and declining hepatic reserves[46]. Fat-soluble vitamins such as Vitamin A, D, E and K have been seen to be deficient in alcoholic disease, along with steatorrhea, cholestasis and bile salt deficiency, negatively impacting patient outcomes[12,47]. Thiamine deficiency is also often found particularly in alcoholic disease as well as other forms of cirrhosis. Intake of alcohol causes a decline in the intestinal absorption and metabolization of thiamine. This increases the risk of developing Wernicke encephalopathy and Korsakoff dementia due to a decline in neurotransmitter synthesis, nucleic acid synthesis and synthesis of steroids and fatty acids[48]. Decreasing liver reserves cause a vitamin B12 and folic acid deficiency. While the levels of vitamin B12 in the serum may be increased, decreased tissue levels are observed. Anemia, glossitis and neurological symptoms may subsequently occur[12,49].

Retinol deficiency also occurs in cirrhosis and is related with decreased absorption and impaired hepatic mobilization due to hepatic stellate cell injury. This may result in dermatitis, night blindness, dyslipidemia or photophobia and increase the risk of hepatocellular carcinoma and other neoplastic disorders[50-52]. Vitamin A supplementation should be administered carefully because its excess causes hepatotoxicity [53]. Vitamin K deficiency results from declining hepatic reserves, increasing the risk of bleeding; while vitamin D deficiency can be caused from a reduced ingestion and absorption. Absorption is impaired in underexposure to ultraviolet light as well as in cholestatic disease or portal hypertension enteropathy[54]. Patients with abnormal liver functioning have deficient vitamin D levels as hydroxylation is required to synthesis calcidiol. A significant part of the liver loses function before calcidiol production is seen to be decreased. It is due to this that patient presentation with biochemical or histological evidence of osteomalacia is rare, unless a vitamin D deficiency is present concomitantly[55,56]. Therefore, decreasing vitamin D levels directly increase the risk of patient mortality where CLD is also present. It also contributes to failure of treatment of liver diseases especially in patients being treated for hepatitis C [57].

Other deficiencies present in patients with alcoholic and nonalcoholic liver disease include that of zinc and selenium. Their supplementation has improved CLD outcomes[58]. Zinc deficiency is caused by a reduction in intake and systemic assimilation. Treatment with diuretics is also a contributor. The levels of ammonia rise in the circulation, which causes dysregulation of hepatocyte functioning and an increased risk of HE. Anorexia can also be caused with this deficiency, along with the dysfunction of the immune system[38,59]. Magnesium deficiency is also noted with alcohol consumption causing not just folate deficiency but also contributing to decreasing magnesium levels. Magnesium deficiency was observed in 30% to 80% of alcoholics. Dysfunctional magnesium transport and homeostasis in vital organs such as the brain, skeletal muscle, heart and liver can result due to alcohol intake. Often magnesuria can also occur[60]. The subsequent magnesium deficiency can cause dysgeusia and contribute to minimal HE, which further decreases the patients' intakes and worsens outcomes. Clinical improvement is therefore noted with magnesium supplementation [54]. Table 1 summarizes the effects of each micronutrient deficiency caused by liver disease.

NUTRITIONAL ASSESSMENT IN CLD

A nutritional assessment determines both the macro and micronutrient state of a patient. It is necessary to identify nutritional risks that affect morbidity and mortality and helps develop a targeted therapy to benefit the patient.

Malnutrition is more evident in decompensated compared to compensated cirrhosis. The 2019 European Association for the Study of the Liver guidelines recommend a screening of all advanced CLD patients for malnutrition (undernutrition), as it is a recognized complication of liver cirrhosis. Criteria for patients at high risk of malnutrition are: (1) a BMI < 18.5 kg/m^2; and (2) advanced decompensated cirrhosis (Child-Pugh C patients)[57].

Thus, an assessment and diagnosis of malnutrition should be done thoroughly starting with a detailed history and physical examination. A thorough history can help identify poor intake, eating habits, loss of appetite, weight changes and any episodes of diarrhea and malabsorption as a potential source of malnutrition in a patient. Calculation of the patient's BMI and determination of the Child Pugh Score for severity of disease is also important with the history, as a direct correlation exists between severity of disease and malnutrition. A physical examination also provides insight about any macro and micronutrient deficiencies. Specific physical findings, such as pallor in iron deficiency anemia, are very important to observe and record[9, 61].

Skeletal muscle loss (sarcopenia) has been established as the most common complication of cirrhosis[59]. It is associated with severe outcomes in patients with liver cirrhosis and is considered useful for the global assessment of patients with CLD, therefore a physical examination for it is important[62]. A multitude of different standardized nutritional status assessment tools also exist that can be used for examination.

Subjective global assessment scale

This includes components of history and physical exam and does not rely on any lab

Table 1 Effects of each micronutrient deficiency caused by liver disease

Micronutrient	Effect caused by deficiency in liver disease
Vitamin A	Dermatitis, night blindness, dyslipidemia, photophobia, increased risk of neoplasia
Vitamin B1	Wernicke encephalopathy and Korsakoff dementia
Vitamin B9	Dementia
Vitamin B12	Anemia, glossitis and neurological symptoms (numbness, muscle weakness and ataxia)
Vitamin D	Rare osteomalacia, increased risk of mortality
Vitamin E	Reduced antioxidation
Vitamin K	Increased risk of bleeding
Zinc	Hepatocyte dysfunction, increased risk of hepatic encephalopathy
Magnesium	Dysgeusia, increased risk of hepatic encephalopathy

parameters[63]. Weight changes, dietary intake changes, presence of gastrointestinal symptoms, functional capacity and the metabolic demand associated with the disease state are the historical components, while the physical examination components include the presence of edema, ascites, muscle wasting and subcutaneous fat loss. The scale classifies patients from well-nourished to severely malnourished[64]. However, this scale underestimates the presence of sarcopenia and is not a good outcomes predictor[65].

Royal free hospital subjective global assessment

This is a modified version of the subjective global assessment and includes some objective variables as well like anthropometry and gender of the patient[66].

Assessment of sarcopenia

Computed tomography scan: Computed tomography scan can be utilized to calculate the cross-sectional area of muscles at the L3 vertebral level. The psoas, paraspinal and abdominal wall muscles are used to calculate their skeletal muscle area. The skeletal muscle index is calculated by dividing the skeletal muscle area by the patient height. A value of the skeletal muscle index less than 50 for men and less than 39 for women is considered to meet the criteria for sarcopenia. Computed tomography scan is an expensive tool and not routinely performed but if done for screening of hepatocellular carcinoma, evaluation for liver transplant or vascular shunt can be utilized for assessment of sarcopenia and calculation of the skeletal muscle index[67,68].

Anthropometry: Anthropometry as a screening tool for nutritional assessment can prove to be important in resource limited settings. The easiest and least technically challenging is the measure of the BMI using anthropometric measurements. However, the use of BMI as a nutritional assessment tool in patients with advanced liver disease is limited as patients are often in a volume overload state, which affects their body weight[9]. A corrected BMI can be calculated instead after paracentesis of ascitic fluid or by using dry body weight[65,69]. Mid arm muscle circumference is an important alternative measurement and is not reflective of the overall body volume status. Mid arm muscle circumference shows good correlation with body cell mass measurement, which is a proven marker for PCM in CLD patients. Mid arm muscle circumference is related to mortality risk in CLD patients and hence holds prognostic value as well[70]. Skin fold thickness measurement can also be used as a nutritional status assessment tool. Thickness can be measured at the triceps, biceps, subscapular and supra-iliac areas and is representative of fat reserve. It recognizes malnutrition earlier than the BMI[61].

Dual-energy X-ray absorptiometry scan: Dual-energy X-ray absorptiometry is a radiological modality that is used to measure bone mass, fat mass and fat free mass, which is representative of lean muscle mass[71]. Its use is limited in cirrhotic patients as fat free mass can also include water mass.

Bioelectrical impedance analysis

Bioelectrical impedance analysis is a low cost, easy to administer screening tool. Alternating current is applied using two electrodes, and the impedance recorded. This

can be performed at the bedside. Bioelectrical impedance analysis is used to estimate total body water, fat mass and hence fat free mass. However, bioelectrical impedance analysis was shown to underestimate malnutrition status as compared to the PCM score in a study from Pakistan. PCM score is thus calculated using anthropometric measurements and some lab parameters[40].

Liver specific tools

A few specific nutrition screening tools have been developed for liver disease patients and these include the Royal Free Hospital-Nutrition Prioritizing Tool and the Liver Disease Undernutrition Screening Tool. The Royal Free Hospital-Nutrition Prioritizing Tool score includes components involving weight loss, volume overload, BMI and reduced oral intake. Patients are then classified as being at low, moderate or high risk for malnutrition[72]. The Liver Disease Undernutrition Screening Tool includes components of oral intake, weight loss, loss of subcutaneous fat or muscle mass, volume overload and functional status[73,74]. These tools have not been studied in detail in the South Asian Population.

Dietary assessment

A dietary interview with the patient provides important insight to the patient's nutritional status. It recognizes barriers to nutritional intake and any deficiencies that need to be addressed. A thorough evaluation includes number of meals eaten, time between each meal, protein and fluid intake and total calories consumed as well as any nausea, vomiting, diarrhea or constipation. A thorough evaluation takes time and requires trained staff. Additionally, patient factors such as poor recall and lack of cooperation also play a role. A 3 d food diary is the best method for a dietary assessment, but it requires patient cooperation as well as patients being capable of writing and maintaining, which may be difficult in the mostly uneducated population of South Asia. Therefore, repeated short-term 24 h recalls are recommended and may prove to be important in this population[75,76]. Patients should at least be asked if their food intake has changed and by how much over what period of time.

In a study evaluating reasons for food aversion in a Pakistani population, it was concluded that factors such as advice from family doctor, close friends and relatives and alternative medicine practices influenced dietary restrictions in patients with advanced liver disease[77]. Lack of awareness about proper dietary intake was prevalent in both educated and uneducated populations with CLD in this population[78]. Table 2 summarizes the methods of nutritional assessment.

TREATMENT

General caloric and protein intake

A multidisciplinary approach for the treatment of malnutrition in liver cirrhosis is important for improving patient survival and long-term outcomes[79]. Nutritional counseling should be included in the patient management. According to the 2020 European Society for Clinical Nutrition and Metabolism guidelines, patients of CLD and a sedentary lifestyle should consume a daily calorie intake of 1.3 × REE (Resting Energy Expenditure)[80], where indirect calorimetry has been used to calculate the REE.

Patients with cirrhosis are recommended to consume 3 to 5 meals per day ensuring there are short starvation periods in between. A late evening carbohydrate snack has been shown to improve protein metabolism in cirrhosis by affecting nitrogen balance [81-83]. Patients should have an energy intake of 30-35 kcal/kg per day and a protein intake of 1.2-1.5 g/kg per day, whereas non-malnourished patients are recommended to intake 1.2g/kg per day of protein, and malnourished and sarcopenic patients should consume 1.5 g/kg per day of protein. However, a higher caloric intake is recommended for patients with acute decompensation or conditions with increased energy expenditure. REE should be calculated whenever possible[80]. In patients with HE, there is no recommendation for protein restriction[84-86].

Use of BCAA

In patients who are protein intolerant (develop HE with ingestion of mixed proteins), consumption of BCAA (at the rate of 0.25 g/kg per day) or vegetable derived proteins is recommended[80,87].

Table 2 Nutritional assessment methods for chronic liver disease

Nutritional assessment methods	Description
Subjective global assessment (SGA)	Uses components of history and physical exam
Royal free hospital subjective global assessment (RFH-SGA)	Modified version of SGA, includes anthropometry and gender
Sarcopenia assessment	
Computed tomography scan	Used to calculate skeletal muscle area and the skeletal muscle index at the L3 vertebral level
Anthropometry	Mid arm muscle circumference, skin fold thickness, BMI calculation
Dual-energy X-ray absorptiometry	A radiological modality that is used to measure bone mass, fat mass and fat free mass
Bioelectrical impedance analysis	Alternating current is used to estimate total body water, fat mass and fat free mass
Liver specific tools	
Royal Free Hospital-Nutrition Prioritizing Tool	Includes weight loss, volume overload, BMI, and reduced oral intake, classifies patients according to risk of malnutrition
Liver Disease Undernutrition Screening Tool (LDUST)	The LDUST includes components of oral intake, weight loss, loss of subcutaneous fat or muscle mass, volume overload, and functional status
Dietary assessment	Includes dietary review, 3 d food diary, 24 h diet recall

BMI: Body mass index; SGA: Subjective global assessment.

Prescription of long-term BCAA supplement is recommended for patients with advance cirrhosis. Use of BCAA enriched formula has been shown to improve survival in patients with alcoholic steatohepatitis and cirrhosis with an improved mental state in protein intolerant cirrhotic patients with HE[6,88-91]. However, the cost of BCAA supplements needs to be considered when prescribing them to patients.

Micronutrients

In cirrhotic patients, any micronutrient deficiency should be corrected. Micronutrient deficiencies may include both water soluble (especially thiamine) and lipid soluble deficiencies[92,93]. While there are no proven therapeutic effects directly by correcting micronutrient deficiencies, supplementation of zinc and vitamin A may improve dysgeusia and hence nutritional intake and affect the nutritional status indirectly[93,94]. While zinc deficiency has been associated with HE, supplementation has not shown any significant therapeutic effect on the encephalopathy[95-99]. Practically, a liberal supplementation is recommended in the first 2 wk of therapy, as a diagnosis of a deficiency would be costly and take time[80].

If a low sodium diet is prescribed in the scenario of ascites, the increased risk of lower intake should be considered due to the non-palatability of such a diet[100,101]. Ensuring adequate nutrition intake is important with sodium restriction.

Enteral and parenteral nutrition

If patients are unable to tolerate diet orally or if the nutritional target is unmet with oral nutrition, enteral nutrition (EN) is recommended. An adequate nutrient intake is the primary goal in cirrhotic patients. EN has been proven to be beneficial in survival and liver function[84,89]. However, with EN a risk of overfeeding exists, as the energy intake can exceed the recommended intake.

If the patient is unable to maintain oral and EN, parenteral nutrition (PN) should be used. When administering parenteral nutrition, patients are more prone to infection and sepsis through intravenous lines[25].

CONCLUSION

Malnutrition in liver cirrhosis is a serious problem in South Asia where the etiology differs from the Western population. As malnutrition is generally highly prevalent in the region, it causes an impact on patients with liver cirrhosis. Urgent attention to address malnutrition is needed to improve patient outcomes. Emphasis on assessment for nutritional status at the initial visit with recording of vital signs is needed.

Simultaneously, treating physicians need to be made aware of the misconceptions surrounding nutritional restrictions in cirrhosis so that patient education is done correctly based on proper scientific evidence.

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REVIEW

Brain-gut-liver interactions across the spectrum of insulin resistance in metabolic fatty liver disease

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Abstract

Metabolic associated fatty liver disease (MAFLD), formerly named "nonalcoholic fatty liver disease" occurs in about one-third of the general population of developed countries worldwide and behaves as a major morbidity and mortality risk factor for major causes of death, such as cardio-vascular, digestive, metabolic, neoplastic and neuro-degenerative diseases. However, progression of MAFLD and its associated systemic complications occur almost invariably in patients who experience the additional burden of intrahepatic and/or systemic inflammation, which acts as disease accelerator. Our review is focused on the new knowledge about the brain-gut-liver axis in the context of metabolic dysregulations associated with fatty liver, where insulin resistance has been assumed to play an important role. Special emphasis has been given to digital imaging studies and in particular to positron emission tomography, as it represents a unique opportunity for the noninvasive in vivo study of tissue metabolism. An exhaustive revision of targeted animal models is also provided in order to clarify what the available preclinical evidence suggests for the causal interactions between fatty liver, dysregulated endogenous glucose production and insulin resistance.

Key Words: Metabolic associated fatty liver disease; Nonalcoholic fatty liver disease; Endogenous glucose production; Insulin resistance; Steatohepatitis; Inflammation

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Core Tip: From studies using tissue-targeted animal models, it emerges that neither insulin resistance per se induces hepatic steatosis, nor steatosis induces whole-body insulin resistance. However, it is evident that reducing inflammation has several beneficial effects both at the hepatic and whole-body level. In fact, either hepatic or systemic inflammation act as major throttle of progressive liver and systemic diseases.

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INTRODUCTION

In recent years, nonalcoholic fatty liver disease (NAFLD) has become the leading cause of chronic liver disease worldwide, and the endpoint complication of nonalcoholic steatohepatitis (NASH), a major indication for liver transplantation[1]. The magnitude of the problem is highlighted by a recent model that estimated a 178% increase in deaths caused by liver disease related to NASH by 2030[2]. Among noncirrhotic NAFLD patients, the leading cause of death is cardiovascular disease[3]. Fatty liver associates with the metabolic syndrome and predisposes to all diseases (cardiovascular, digestive, metabolic, neoplastic and neurodegenerative) that are major causes of death in developed countries. As metabolic dysfunctions play a major role in the pathogenesis of fatty liver, a panel of experts has recently proposed to change the term "NAFLD," and its definition, to metabolic associated fatty liver disease (MAFLD)[4,5] in an attempt to identify clinical criteria to give a "positive" diagnosis of the disease. Furthermore, the definition of "nonalcoholic" was misleading because it is virtually impossible to exclude the endogenous production of alcohol by an intestinal autobrewery[6]. According to the new consensus, MAFLD means the evidence of hepatic steatosis accompanied either by type 2 diabetes (T2D) or overweight/obese or in normal weight/lean subjects by at least two metabolic risk abnormalities.

There are many complex physio-pathologic connections within the brain, gut, and liver (BGL) axis (Figure 1). While MAFLD per se contributes to an increased risk of neurodegeneration[7,8], one well known alteration of this axis is linked to hepatic encephalopathy (HE) a debilitating neuropsychiatric condition often associated with acute liver failure and/or cirrhosis[9]. However, the pathophysiologic mechanisms and treatment options involved in HE are much different from those involved with insulin resistance in MAFLD. Consistently, a recent study demonstrated that the nonabsorbable antibiotic rifaximin, a standard of care for HE had no effect on improving insulin resistance, adipose tissue inflammation, or plasma lipopolysaccharide (LPS) levels following an oral lipid test in obese subjects[10].

Our review is focused on the early pathophysiology of BGL in the context of insulin resistance and specifically addresses two pillars of hepatic insulin resistance, namely dysregulated endogenous glucose production and MAFLD. Of note, highly selected patients with MAFLD without any features of the metabolic syndrome, have altered endogenous glucose production[11]. Specific emphasis will be given to novel findings from imaging studies, since imaging provides noninvasive in vivo "snapshots" of the tissues of interest. We and others have highlighted that there is an imperative need of noninvasive techniques, including imaging to identify effective biomarkers and early prognostic patterns of MAFLD[12,13]. Finally, we discuss new insights that can be gained from targeted animal models in which interventions such as the knockout (KO) of the insulin receptor or the GLUT4 glucose transporter in different tissues, or the primary upregulation of lipid synthesis help to elucidate the effect of insulin resistance on hepatic steatosis and *vice versa*.

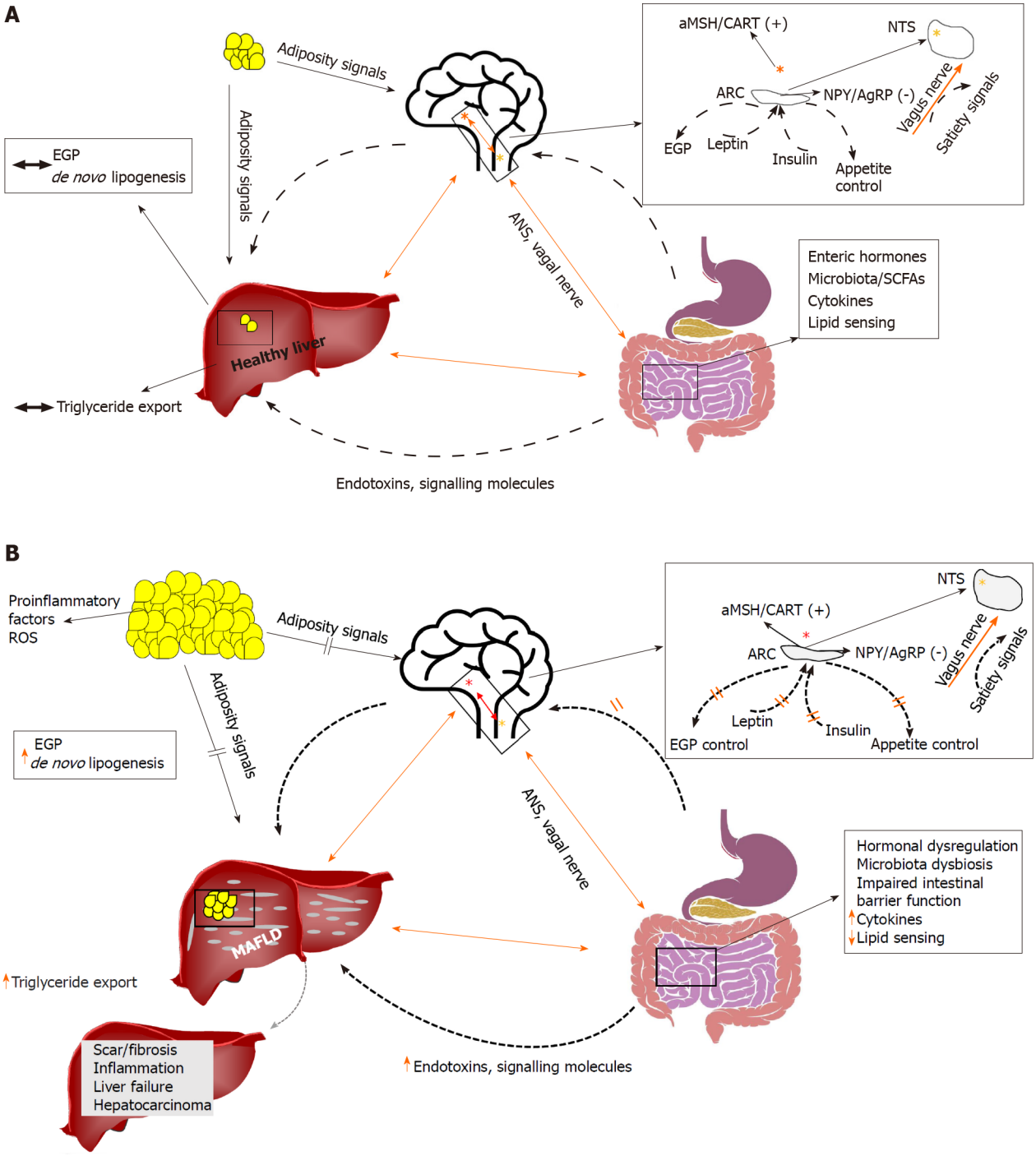


Figure 1 A summary of some interactions (A) of the brain, liver, and gut in health, and (B) in the context of insulin resistance. Several lines of research have shown that the brain may directly control endogenous glucose production. Recent evidence suggests that the brain may also control the rate of lipid turnover in the liver, thus promoting or defending from metabolic associated fatty liver disease (MAFLD). The liver is anatomically in close relationship to the gut, which represents the first line of defense against gut-derived endotoxins and signaling molecules (*e.g.*, short-chain fatty acids). Altered gut microbiota and/or a leaky gut may contribute directly to establishment of MAFLD. The gut also produces substantial amounts of hormones that, through endocrine signals, act on the brain and the liver. The autonomic nervous system and the vagus nerve constitute the basis of the brain, gut and liver axis interconnections. Orange line: liver–brain–gut neural arc; dotted line: other ways of communication (*e.g.*, hormonal, adipocytokines). Red star denotes the hypothalamic nuclei; yellow star denotes the nucleus tractus solitaries. αMSH: α-melanocyte-stimulating hormone; AgRP: Agouti-related protein; ANS: Autonomous nervous system; ARC: Arcuate nucleus; CART: Cocaine- and amphetamine-regulated transcript; EGP: Endogenous glucose production; MAFLD: Metabolic associated fatty liver disease; NPY: Neuropeptide Y; NTS: Nucleus tractus solitarius; ROS: Reactive oxygen species; SCF: Short-chain fatty acid.

MAFLD-INSULIN RESISTANCE-INFLAMMATION: A VICIOUS CIRCLE

Whereas the association between MAFLD and insulin resistance is well established, there is debate on their cause-effect relationships. Thus, it is not clear whether systemic insulin resistance induces the accumulation of lipids in the liver[11] or if hepatic

steatosis is a major determinant of systemic insulin resistance[14]. In any case, once hepatic steatosis is established, other typical characteristics are observed such as insulin resistance, insufficient suppression of endogenous glucose production (EGP), increased insulin secretion, decreased whole-body glucose disposal, increased lipolysis with consequent enhanced lipid oxidation[11], decreased insulin clearance[15], and chronic oxidative stress[16]. Both insulin resistance and MAFLD are characterized by elevated circulating inflammatory markers[17,18]. The interplay between insulin resistance, ectopic fat accumulation in the liver, and inflammation is characterized by mutual positive regulation, *i.e.* a vicious circle[19]. On one hand, ectopic fat accumulation in the liver leads to lipotoxicity, low-grade inflammation and insulin resistance in the liver[19]. On the other hand, insulin resistance enhances lipotoxicity through unsuppressed lipolysis[19]. Finally, proinflammatory markers such as tumor necrosis factor (TNF)-α and interleukin (IL)-6, which are typically increased in conditions of insulin resistance may further aggravate both insulin resistance and MAFLD[19].

In MAFLD patients, intrahepatic inflammation is the most important prognostic determinant of liver disease progression and systemic inflammatory markers correlate with hepatic inflammation[20]. A plausible hypothesis holds that in the context of MAFLD, inflammation (hepatic and/or systemic) acts as major disease accelerator[13].

BRAIN-LIVER AND BLG AXIS

Control of EGP

Preclinical studies have shown that insulin acting directly on the brain may affect EGP. More specifically, Obici *et al*[21] have shown that an intracerebroventricular (ICV) injection of insulin suppresses EGP in rats. Human evidence is slowly accumulating, and recent clinical studies have confirmed the presence of a "brain-liver axis". More specifically, intranasal insulin (INI) administration during the euglycemic hyperinsulinemic clamp was shown to suppress EGP in lean, but not in overweight, individuals[22]. Under the same euglycemic hyper-insulinemic conditions, brain imaging with ^{18}F-fluorodeoxyglucose (FDG) positron emission tomography (PET) has shown that brain glucose uptake (BGU) correlates positively with EGP in morbidly obese individuals, but not in healthy lean individuals[23]. On the contrary, when INI was given during fasting conditions EGP was not affected, and similarly no correlation was found between BGU and EGP in the post-absorptive state. Taken together, the data suggest that under conditions of high systemic insulin levels like those typically seen in the postprandial state, the brain may directly control (*i.e.* suppress) EGP, but the control is lost with increased adiposity.

Other lines of research also suggest that the brain may control EGP. Intracerebroventricular administration of brain-derived neurotrophic factor (BDNF) lowers blood glucose levels and suppresses hepatic glucose production (HGP) and hyperglucagonemia[24]. Of note, leptin activates BDNF-expressing hypothalamic neurons, which in turn stimulate BDNF synthesis[25]. Also, Bercik *et al*[26] have shown that the intestinal microbiota affects both the central levels of BDNF and behavior in mice.

Lipid delivery into the upper intestine has also been shown to suppress EGP through central action. More specifically, lipid delivery leads to long chain fatty acyl-CoA production that suppresses EGP, and the effect is abolished either after coadministration of the anesthetic tetracaine, by gut vagal deafferentation, or by hepatic vagotomy[27]. Wang and colleagues further demonstrated that in rats with insulin resistance induced by a high-fat diet (HFD), upper intestine lipid delivery failed to suppress EGP, suggesting a potential mechanism for dysregulated EGP in the context of insulin resistance. Along the same line, cholecystokinin (CCK) has also been reported to trigger gut-brain-liver axis control of HGP, and that HFD impaired CCK-induced afferent vagal signals to suppress HGP[28].

Enteric hormones (enteroendocrine system)

The gastrointestinal tract is a major producer of hormones, among which GLP-1, ghrelin, cholecystokinin are particularly involved in the BGL axis. GLP-1, an incretin hormone secreted from the L cells of the intestine in response to a meal, exhibits high fasting levels in subjects with insulin resistance and in whom GLP-1 does not increase sufficiently in response to a meal[29]. GLP-1-receptor mRNA has been found both in the hepatic portal region and in neurons[30]. Preclinical studies have suggested that GLP-1 may act on the liver through the nerve endings of the intestinal wall. Insulin clamp studies in GLP-1 receptor knockout mice showed a defective suppression of EGP[31], and the intraportal GLP-1 injection in rats increased the firing rate of the

hepatic afferent of the vagus nerve[32]. In healthy individuals under conditions of pancreatic clamping (*i.e.* with stable insulin and glucagon concentrations), GLP-1 inhibits EGP, and the effect is mediated either through direct GLP-1 action on the liver or through neuron-mediated inhibition[33]. GLP-1 also contributes to suppression of appetite[34]. Thus, GLP-1 may play an important role in the pathophysiology of MAFLD; and in preliminary clinical studies, exenatide, a GLP-1 receptor agonist, was shown to decrease hepatic fat content and liver enzymes[35], and to improve liver histology[36]. Similarly, liraglutide was shown to improve NASH[37].

Ghrelin is a hormone that is mainly derived from the stomach and duodenum, and its main function is to control food intake by inducing appetite. Ghrelin has recently been shown to participate in the BGL axis, as under conditions of pancreatic clamping, intraduodenal ghrelin infusion resulted in increased HGP through neural-mediated action, as administration of ghrelin, while inhibiting the vagal afferent neurotransmission, abolished the ghrelin-induced increase of HGP[38]. Similarly, vagotomy or use of N-Methyl-D-aspartate blockers abolished the ghrelin effects on HGP. Ghrelin has also been shown to block the action of cholecystokinin. The ghrelin nutrient-sensitive effects on the gut may thus be attributed to its inhibition of cholecystokinin.

CCK is released from intestinal endocrine cells during feeding, and it binds to CCK-A receptors on gut vagal fibers that project the signal to the brainstem, causing termination of the meal[39]. Insulin and CCK have complimentary actions in inducing satiety, as ICV administration of insulin enhances the satiety effect of CCK[40], whereas fasting decreases it[41]. Similar complementary effects with CCK have been proposed for leptin, as ob/ob mice and Zucker rats have been shown to be relatively insensitive to the satiety effects of CCK[42].

Adipocytokine signaling

Leptin is an adipokine, or adipocytokine with structural similarities with the cytokines of the type I cytokine family. Circulating leptin levels are directly related to expanded fat mass, but obesity is characterized by leptin resistance, and leptin resistance consists at least partially in a decreased capacity for leptin transport into the brain[43]. Even though secreted by adipose tissue, the main site of action of leptin is the central nervous system (CNS), and particularly in the hypothalamic nuclei. Leptin and insulin central actions are largely interconnected; both act on the arcuate nucleus to suppress the expression of the orexigenic peptides neuropeptide Y and agouti-related protein. Their action on other neurons is different and more complex, as leptin stimulates while insulin inhibits proopiomelanocortin neurons[44]. Apart from controlling EGP, appetite (and thus body weight), leptin is also implicated in MAFLD as it has been demonstrated that leptin deficient ob/ob mice have marked steatosis[45]. A recent preclinical study has shown that CNS-leptin signaling promotes hepatic triglyceride export and decreases de novo lipogenesis[46]; the authors propose intranasal leptin administration as potential new treatment of MAFLD.

Vagus nerve and the enteric nervous system

As already highlighted, the tenth cranial, or vagus nerve, plays a pivotal role in the BGL axis communications, which are summarized in Figure 1. The enteric nervous system, also named the "second brain" or "brain in the gut" is considered as one of the autonomic nervous system divisions and consists of approximately 500 million neurons which produce a variety of neurotransmitters including acetylcholine, adrenaline, VIP and serotonin (5-HT). It has been shown that 5-HT promotes lipid accumulation in hepatocytes in vitro[47]. In line with that, short-term treatment with tryptophan inhibitors prevented the formation of 5-HT, which is a metabolite of tryptophan, and inhibited the development of hepatic steatosis in mice fed with a high carbohydrate diet without increasing the energy expenditure in adipose tissues[48]. In addition, the same study showed that inhibition of gut-derived serotonin ameliorated hepatic steatosis. Taken together, the data suggest that gut-derived serotonin is a regulator of hepatic lipid metabolism through a gut-liver axis. On the other hand, the gut microbiota regulates both the 5-HT synthesis and its release from the enteroendocrine cells, and 5-HT plays its role on the CNS as one of the most important central neurotransmitters in the regulation of mood, sleep, and pain[49]. Consistently, modification of central 5-HT levels was shown by Pagoto *et al*[50] to affect food preferences. Following acute tryptophan depletion (transient decrease of both peripheral and central 5-HT levels), overweight individuals increased their sweet calorie intake and preferred sweet foods. Of significant note is that most tryptophan was converted to kynurenine rather than to 5-HT, and under conditions of inflammation, the rate-limiting enzyme for this transformation could be upregulated. As kynurenine and 5-HT compete to cross the blood-brain barrier through the same

transporter, it follows that inflammation-associated changes in kynurenine levels could impact on central 5-HT concentrations[51,52]. Thus, this is another pathway through which inflammation could decrease central 5-HT levels to prompt affected patients to increase their intake of sweets.

Gut microbiota

An altered gut microbiota composition is associated with obesity[53], and in obese humans specific microbiota compositions may be associated with impaired glucose control[54]. It is well established that a diet rich in fibers is healthy, with improvement of insulin sensitivity and glucose tolerance. The beneficial effects of increased fiber consumption are hypothesized to be mediated by the production of the short-chain fatty acids (SCFAs) acetate, propionate, and butyrate after fermentation of the fibers by the gut microbiota. SCFAs do not act just as substrate for colonocytes and enterocytes [55], but also as signaling molecules. For instance, SCFAs can stimulate the secretion of GLP-1 and peptide YY, and decrease the secretion of ghrelin[56-58]. Propionate and butyrate have been shown to activate intestinal gluconeogenesis (IGN), and interestingly, increased IGN was shown to have beneficial effects on glucose homeostasis even if the resulting increase in EGP is a key feature of T2D[59]. In the case of propionate, the effect occurs *via* a gut-brain neural circuit[60], as it was shown that after denervation of the periportal nervous system, propionate feeding no longer affected IGN. Even though butyrate feeding (which has a direct effect on IGN shown by gene expression *via* a cAMP-dependent mechanism) could still enhance IGN, both the beneficial effects of IGN and portal glucose sensing were lost[60].

Decreased levels of the SCFA butyrate have also been associated with tight-junction abnormalities and increased intestinal permeability[61], which have been implicated in MAFLD pathogenesis and progression. Other microbiota-derived molecules might play also an important role. Deficiency of the macronutrient choline, which is implicated in the prevention of liver steatosis by promoting the assembly and excretion of very-low-density lipoprotein[62], has been observed in NAFLD patients and was associated with abundance of specific bacteria (Erysipelotrichia taxa), which are able to metabolize choline to trimethylamine (TMA) and its oxidized form TMAO, with the net effect of reducing choline bioavailability and increasing that of steatogenic TMAO[63].

Amino acid metabolism is also important. The branched-chain amino acids (BCAAs) valine, isoleucine and leucine, contribute to insulin resistance and hepatic steatosis, and can be synthesized and metabolized by specific gut bacteria. An intervention study in rats showed that the dietary administration of BCAA reduced the accumulation of liver fat through the modification of gut microbiota[64]. The effect occurred through the gut-brain axis, accompanied by microbiota-mediated production of the SCFA acetate, which activated the parasympathetic nervous system[65]. Other amino acids, such as tryptophan, phenylalanine, and tyrosine can be metabolized by gut bacteria that produce derivates with effects on metabolism and inflammation. For example, the essential amino acid tryptophan is the precursor of serotonin and can be converted into its indole intermediate, which in turn can reduce hepatic lipogenesis and inflammation[66].

Furthermore, the bacterial-derived endotoxin lipopolysaccharide (LPS) might contribute to local and systemic inflammation by the activation of the toll-like receptor 4 pathway. Increased abundance of endotoxin-producing bacterial strains has been found in the gut of obese patients compared with controls[67], suggesting its potential implication in the development of MAFLD and its progression to NASH, with the involvement of CNS dysfunction and inflammation.

Dietary patterns are able to modulate brain lipid composition and function[68] as well as hepatic lipid content[69]. It has been shown that unhealthy diets rich in saturated or monounsaturated fatty acids have unfavorable effects on gut microbiota composition[70], promoting an increase of LPS-producing bacteria and reduction of SCFAs leading to a systemic proinflammatory state occurring through the BGL axis [71].

Finally, gut microbiota-dependent regulation of neurotransmitters interacts with vagal afferent pathways to affect liver metabolism through the gut-brain axis. Gut bacteria can modify serotonin release[72], which has a brain-dependent effect on gastrointestinal motility and secretion, and energy expenditure. It promotes liver steatosis by local endocrine mechanisms[73], and modulates the production of several other molecules, such as gamma-aminobutyric acid, acetylcholine, histamine, norepinephrine, dopamine, and endocannabinoids that affect glucose and lipid metabolism and inflammation, as deeply reviewed elsewhere[74].

A useful experimental model for studying the effects of specific microbiota on host metabolism is provided by germ-free mice, which are resistant to high-fat diet-induced obesity[75] and protected from MAFLD. In a seminal study Le Roy *et al*[76] showed that germ-free mice that received fecal transplantation from C57BL/6J mice with HFD-induced hyperglycemia and increased plasma concentrations of proinflammatory cytokines, developed hepatic steatosis. On the contrary, germ-free mice that received stool from C57BL/6J mice that were non-responders to HFD and without hyperglycemia and increased proinflammatory cytokines, did not develop hepatic steatosis. Moreover, the gut microbiota controlling the balance between proinflammatory and anti-inflammatory signals may contribute to the progression to NASH. Consistently, short-term treatment of patients with steatosis and NASH with rifaximin led to an improvement of liver enzymes levels[77]. The effect was thought to have been caused by a change of the gut microbiota composition leading to a direct reduction of leaky gut, and consequent improvement of hepatic inflammation, rather than an effect on insulin sensitivity. It was recently demonstrated by Finlin *et al*[10] that rifaximin did not improve insulin resistance.

Bile acids

Bile acids (BAs) are synthesized in the liver from cholesterol, stored in the gallbladder, and secreted after gallbladder emptying into the intestinal lumen upon food ingestion. As BAs move along the intestinal lumen, they contribute to the absorption of lipids and lipophilic vitamins. The majority of BAs (~95%) are re-absorbed by enterocytes and then transferred back to the liver where they are reused (*i.e.* enterohepatic circulation). BA are transformed to secondary BAs by the gut microbiota[78] and only in a small amount reach the systemic circulation and increases of plasma BA levels were reported after meals, suggesting that BAs could be a postprandial systemic signal [79].

In the last decades, important new insights have been gained, proposing BAs as important determinants of glucose homeostasis. Early studies showed that KO of the BA farnesoid X receptor (FXR) induced insulin resistance, whereas administration of BA agonists enhanced insulin sensitivity[80]. BA receptors are also present in the CNS, and it is now believed that the BA signal reaches the brain through three different pathways, one direct and two indirect[81]. The direct pathway consists in activating central FXR and Takeda G protein-coupled receptor (TGR5R) signaling after crossing the blood-brain barrier. Indirect activation of intestinal FXR and TGR5R results in the release of FGF19 and GLP-1, both of which can signal to the CNS. The pathways have been extensively reviewed by Mertens *et al*[81]. Even though major pathways of communication of the BGL axis through BAs were identified, their importance in pathophysiology warrants further investigation.

Nutrient intake: Role of fructose

In Western societies, increased consumption of fructose began in the 1970s after the introduction of high-fructose corn syrup as a sweetener in soft drinks. Since then, the prevalence of obesity and T2D have substantially increased and a link between high fructose consumption and MAFLD has been established[82]. Fructose is a 5-carbon carbohydrate with peculiar characteristics, as upon entry in the cell it is phosphorylated to fructose-1-phosphate by phosphofructokinase, decreasing the cell's ATP levels because of the rapid depletion of phosphate. AMP degradation increases uric acid levels, which has a proinflammatory affect in the intracellular compartment. In the liver, fructose can be transformed into free fatty acids that can either be secreted into the circulation as triglycerides or stored as intrahepatic lipids, contributing to MAFLD. How does fructose affect the liver through the BGL axis? Studies in mice have shown that fructose consumption causes a strong binge-eating response that is attributed to release of orexin from the lateral hypothalamus[83]. Chronic fructose intake leads to leptin resistance and weight gain[84]. Similar findings were confirmed in humans, who after fructose assumption, experienced increased hunger and desire for sweet foods than after glucose administration[85]. Furthermore, Spruss *et al*[86] showed that the long-term intake of fructose was associated with a marked reduction of the protein in the tight junctions of the duodenum that led to an increase in translocation of bacterial endotoxin and activation of toll-receptor-4-dependent signaling cascades in the liver. Interestingly, metformin a drug that reduces insulin resistance, was shown to protect from fructose-induced steatosis[86].

RESULTS OBTAINED FROM TISSUE-TARGETED ANIMAL MODELS

Based on the frequent coexistence of fatty liver, steatohepatitis, obesity, T2D, dysbiosis, insulin resistance, and low-grade inflammation, there is general acceptance of their possible causal interactions. However, their exact nature and implication in different subgroups of patients remains to be elucidated. In order to identify more specific causal relationships, we reviewed studies in tissue-targeted animal models in which the known primary event was either insulin resistance or hepatic steatosis or gut microbiota depletion or the induction or reduction of inflammation (Figures 2 and 3; Figure 2 is given more extensively in Supplementary Table 1). All the studies indicate that unless extreme lipodystrophy occurs (FIRKO-90%), severe insulin resistance in the whole body or skeletal muscle and/or adipose tissue does not cause hepatic steatosis and liver insulin resistance or inflammation[87-89]. GLUT4-null mice do not have hepatic steatosis or glucose intolerance and have normal EGP-related enzyme expression in their liver[90-92]. In the absence of insulin receptors or GLUT4 in both muscle and adipose tissue, glucose tolerance is normal or is less affected than expected from the degree of insulin resistance[93-95]. The key compensatory organs appeared to be adipose tissue (with upregulation in glucose uptake, increase in small adipocyte number), and the liver (with upregulated glucose uptake, balanced increase in lipid synthesis *vs* export). A chronic lack of insulin action in only the liver leads to unsuppressed EGP, resulting in severe glucose intolerance from early life onward, low insulin clearance, glycogen depletion, low expression of lipogenesis pathways, and resistance to high-fat diet-induced steatosis[96-99]. The effects of a short-term lack of insulin action are controversial[98,100]. With a normal diet in the chronic model, moderate liver steatosis occurs in older animals, with an elevation in liver enzymes, focal dysplasia, no fibrosis, and low circulating triglycerides, which may depend on blunted triglyceride export because of chronic brain exposure to hyperinsulinemia. In fact, acute *vs* chronic central insulin infusions have shown a transition from stimulation to suppression of hepatic triglyceride export[101], modulating liver fat content. Lack of insulin action in the brain also causes a moderate degree of hyperphagia in females (either with or without overweight), insulin resistance without hyperglycemia, or glucose intolerance and high or normal triglyceride levels[101-103]. Instead, the selective KO of brain GLUT4 results in normal peripheral insulin sensitivity, but unsuppressed EGP leading to glucose intolerance[104]. Although the intestine is assumed to contribute little to EGP, it was noted that the absence of insulin action in enterocytes ameliorated glucose tolerance, *via* reduced intestinal glucose absorption and downregulation of intestinal EGP enzyme expression[105,106]. From these studies, EGP (dys)regulation resulting from the action of insulin on the liver, brain, and gut seems to be the most prominent determinant of glucose (in)tolerance.

On the other hand, the primary induction of liver steatosis does not cause whole-body insulin resistance, glucose intolerance, or hepatic inflammation, but provokes an increase in hepatic ceramide and diacylglycerol content, and the enrichment of liver triglycerides with polyunsaturated fatty acids, which may increase susceptibility to inflammatory damage[107-111]. The exposure to toxins (including LPS) caused liver and lipid inflammation and reduced fasting glucose, insulin and triglyceride levels [108-111]. Consistently, older studies have shown that an injection of LPS leads to a major increase in fasting glucose consumption by the whole body, with a several-fold elevation in the liver and spleen glucose uptake lasting 48 h, possibly because of the content of macrophages[112]. More recent studies on chronic LPS infusion have revealed that, while inducing overweight and inflammation in the liver and in adipose tissue and muscle in chow and HFD fed mice, LPS caused steatosis and extra-hepatic insulin resistance only in HFD mice or hepatic insulin resistance (EGP) only in chow-fed mice, with a small impact on glucose tolerance[113]. This is in agreement with observations in germ-free mice lacking LPS showing lower liver fat, better glucose tolerance, higher insulin sensitivity, and normal circulating triglyceride and free fatty acid levels compared with colonized mice[114-116]. However, the selective inoculation of bacteria producing or not producing LPS in germ-free mice showed a direct effect only on adipose tissue inflammation and without hepatic or systemic impact[116]. All the evidence suggests that both the metabolic and hepatic effects of LPS require other microbial or dietary components and support a role for liver inflammation, but not steatosis per se, in the regulation of peripheral metabolism. In line with this, anti-inflammatory drugs have been shown to ameliorate liver function, steatosis, inflammation, and insulin resistance, with glucose and lipid lowering effects observed only in diabetic animals[117-121].

Primary defect	Species	Targeted model	Liver Steatosis	Liver	EGP during clamp	Whole body IR-IS	Fasting TG-FFA	Fastingglucose	Glucose intolerance	Body weight	Adiposity	Reference
Peripheral insulin resistance	Mouse	MIRKO										Bruning Mol Cell 1998, Kim J Clin Invest 2000, Ealey Am J Physiol 2008
	Mouse	FIRKO 50%										Bluher DevCell 2002, Boucher Diabetes 2016, Softic Diabetes 2016
		FIRKO 90%										
	Mouse	GLUT4-KO-M (muscle), GLUT4-KO-M+AT (muscle + adipose tissue)										Carvalho AJP 2005, Zisman Nat Med 2000, Kim JCI 2001, Kotani JCI 2004
	Mouse	GLUT4-null										Lin and Accili J Biol Chem 2011, Ranalletta Diabetes 2005 and AJP 2007, Katz Nature 1995
Insulin resistance in liver, brain, intestine	Mouse	LIRKO										Michael Mol Cell 2000, Fisher & Kahn JCI 2003, Buettner JCI 2005, Coen J Biol Chem 2007, Biddinger Cell Metab 2008, Haas Cell Metab 2012
	Mouse	LGSKO										Irimia J Biol Chem 2010, 2017
	Mouse	NIRKO (or ins. Infusions, or WB ins. Receptor deletion or sparing brain)										Bruning Science 2000, Diggs-Andrews Diabetes 2010, Scherer Diabetes 2016
	Mouse	Brain specific GLUT4 KO + chow or HFD										Reno Diabetes 2017
	Mouse	Intestinal-IRKO										Andres Am J Physiol 2014, Ussar Diabetes 2017
Hepatic steatosis ± inflammation	Mouse	Hepatic DGAT2 or MTTP overxpression										Monetti Cell Metab 2007, Minehira J Lip Res 2008 Raabe JCI 1999, Bjorkegren J Biol Chem 2002, Jornayvaz PNAS 2011
	Mouse	MTTP overexpr + LPS or ConA or PEA										Bjorkegren J Biol Chem 2002
Microbiota ± inflammation	Mouse	Gut microbiota KO (Germ-free)										Rabot FASEB J 2010, Bäckhed PNAS 2007, Caesar Gut 2012
	Mouse, rat	LPS, effects of microbiota in chow diet (in HFD normal EGP, peripheral IR, liver steatosis)										Meszaros J Biol Chem 1987, Cani Diabetes 2007, Caesar Gut 2012
Inflammation-targeting	Mouse, rat	NSAID (cox-inhib, aspirin, indomethacin, IL1Ra) effect in high-risk models (diet, BDL, TAA, LDLR-KO, GK)										Madrigal-Perez Int J Clin Exp Med 2015, Paik Gut 2009, Tian Plos One 2014, Murali J Lip Res 2012, Ehses PNAS 2009

Figure 2 Tissue-targeted animal models evaluating the independent effects of tissue-specific insulin receptor knockout, or GLUT4 knockout, or the induction of steatosis or inflammation on metabolic outcomes, including liver steatosis, endogenous glucose production, glucose tolerance, and body weight. Negative effects are shown in red and abnormalities reported as mild-moderate or not consistent in all studies in light red. Beneficial effects (dark) or no effect (light) are shown in green. Orange indicates opposite (high *vs* low) findings between studies. Light blue refers to a decrease that cannot be unequivocally interpreted as being beneficial or adverse to health. BDL: Bile duct ligation; ConA: Concanavalin A; DGAT2: Diacylglycerol O-acyltransferase 2; FFA: Free fatty acids; FIRKO: Fat-specific insulin receptor knockout; GK: Goto-Kakizaki; HFD: High-fat diet; IL-Ra: Interleukin-1 receptor antagonist; IR: Insulin resistance; IRKO: Insulin receptor knockout; KO: Knockout; LDLR-KO: Low-density lipoprotein cholesterol receptor knockout; LIRKO: Liver-

specific insulin receptor knockout; LGSKO: Liver glycogen synthase knockout; LPS: Lipopolysaccharide; MIRKO: Muscle-specific insulin receptor knockout; MTTP: Microsomal triglyceride transfer protein; NIRKO: Brain-specific deletion of the insulin receptor; PEA: P. aeruginosa exotoxin A; TAA: Thioacetamide; TG: Triglycerides

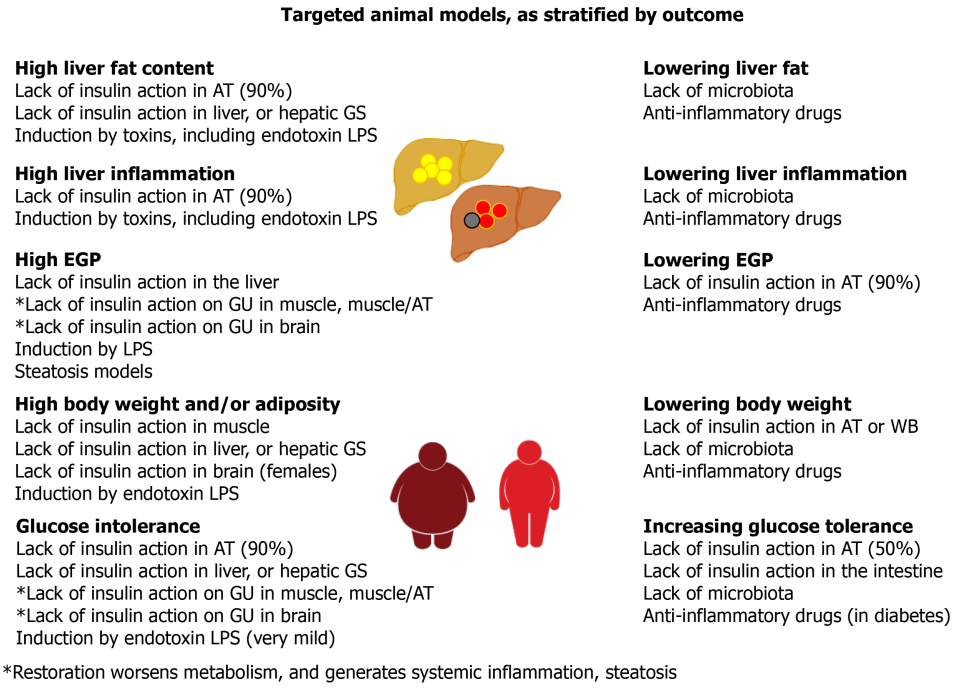


Figure 3 Summary of the outcomes yielded by targeted animal models. AT: Adipose tissue; LPS: Lipopolysaccharide; GS: Glycogen synthase; GU: Glucose uptake; WB: Whole body.

DISEASE MONITORING BY DIGITAL IMAGING: FOCUS ON PET AND MECHANISTIC UNDERSTANDING

Liver biopsy is the current gold standard in both the diagnosis and follow-up of liver disease. The presence of ballooning degeneration of hepatocytes being the hallmark of steatohepatitis, but biopsy is invasive and unsuitable for frequent monitoring[122]. Liver function tests are useful, but not diagnostic or predictive of NASH and/or fibrosis in individual patient. Imaging tools can capture and measure liver steatosis. Among them, magnetic resonance imaging (MRI)-magnetic resonance spectroscopy (MRS) has the highest sensitivity, but it is complex and not always accessible. Recently mono- and multiparametric scores obtained by the AI-processing of common ultrasound images have been proposed for repeated follow-up of liver fat and are validated against spectroscopic magnetic resonance technology[123]. Vibration-controlled transient elastography and magnetic resonance elastography provide useful measures of the combined inflammation-fibrosis index, but a reliable distinction between them remains to be achieved in the diagnostic field[12].

MRI can measure the proton density fat fraction (PDFF) and has been shown to be an objective, accurate, and reproducible quantitative indicator of hepatic fat content across the entire liver. MRI-PDFF has been validated against liver histology, and shown to be more sensitive in detecting changes in hepatic fat content and treatment response in clinical trials[124-126].

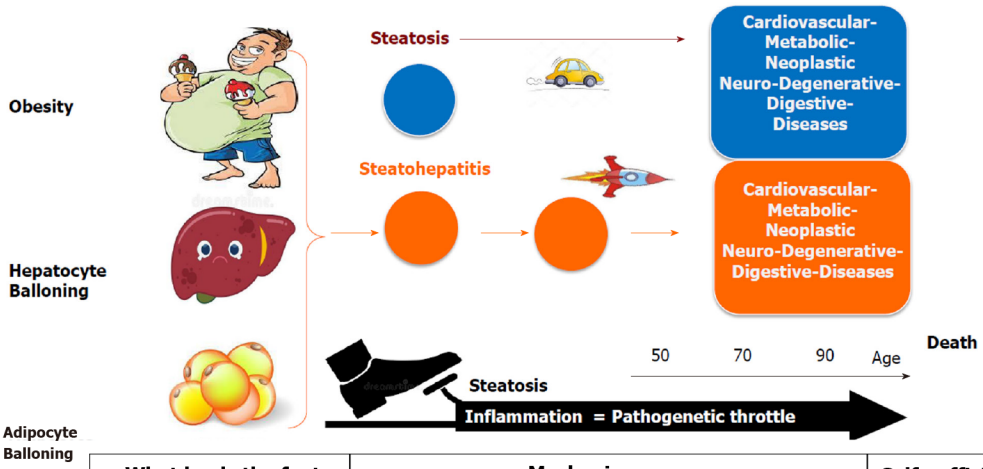
Finally, multiparametric MRI makes it possible to establish scores for assessment and quantification of liver fibrosis and inflammation, with accurate prediction of clinical outcomes in patients with chronic liver disease of mixed etiologies and/or steatosis[127]. The animal studies discussed above showed that LPS-induced liver inflammation is characterized by very high hepatic glucose uptake, possibly because of macrophages. The notion that activated macrophages and lymphocytes have high glucose-avidity has supported the use studies with PET imaging of the glucose analogue (^{18}F)-FDG in inflammatory conditions, such as osteomyelitis, sarcoidosis, vasculitis, or vulnerable atherosclerosis plaques[128,129]. Some studies, although lacking liver biopsies, have explored the relationship between computed tomography-

determined steatosis and fasting (^{18}F)-FDG-PET imaging[130-132], yielding controversial results. Two recent reports that used biopsy-proven liver diagnosis and compartmental modeling of (^{18}F)-FDG in the liver, found an inverse relationship between hepatic inflammation grades and liver blood flow, *i.e.* the K_1 rate constant representing the flow-dependent delivery of (^{18}F)-FDG to the liver[133,134]. Other rate constants (*e.g.*, fractional extraction) did not correlate with histology grades. Unfortunately, these human studies addressed relative indices and not the absolute rate of hepatic glucose uptake (HGU), which is given by the product of (^{18}F)-FDG fractional uptake × circulating glucose levels, and they did not quantify EGP.

We have previously validated a method to simultaneously estimate EGP [by (^{18}F)-FDG plasma clearance] together with HGU (by imaging) during (^{18}F)-FDG-PET, addressing their relationship with liver steatosis (by MRI-MRS) in type 2 diabetic or morbidly obese patients. The studies indicate that hepatic insulin resistance and steatosis are, to some extent, proportional and improve after weight loss by bariatric surgery in morbidly obese individuals[135]. However, very-low-calorie diets in less severe obesity had effects on glucose tolerance, EGP, and liver fat, but not on HGU [136], whereas glucose lowering by SGTL2 inhibitors in diabetic patients had a significant effect on glucose control and liver fat, but not on EGP or HGU[137]. Taken together, the studies suggest that liver fat is not a cause of hepatic dysmetabolism, but rather a consequence of glucose intolerance. It is also important to keep in mind that the euglycemic insulin clamp that was used in the studies, did not reflect the daily metabolic physiology of patients, in which glucose and insulin levels increase and decrease together after meals or under fasting conditions. HGU and EGP are dependent on the changing insulin and glucose levels, and chronic hyperglycemia and hyperinsulinemia are commonly present. For example, PET imaging studies in minipigs underscore the relevance of circulating glucose by showing that hyperglycemic- compared with euglycemic-hyperinsulinemia enhanced HGU, hepatic triglyceride content and triglyceride release in proportion to glycemia[138]. The euglycemic clamp thus provides relevant information on the sole action of insulin on tissue metabolism, being insufficient to characterize the more complex relationship between glucose and lipid metabolism occurring in the liver in real life.

By using a fatty acid PET tracer, we demonstrated that overweight was characterized by an elevation in fasting hepatic fatty acid oxidation, with normal rates of triglyceride incorporation[139]. Liver steatosis occurred in obese subjects, in whom weight loss was able to reduce hepatic fatty acid uptake and liver steatosis in a proportional manner, and EGP[140]. Notably, a chronic, *i.e.* 1-wk treatment with acipimox, suppressing fatty acid levels and liver fatty acid uptake provoked a significant improvement in systemic and liver insulin sensitivity and decreased circulating triglycerides and liver enzymes, but did not change liver fat content, as measured with MRS in healthy individuals[140]. Thus, liver and systemic insulin sensitivity were improved, together with liver function and independent of hepatic triglyceride accumulation. Again, in spite of cross-sectional correlations and consensual changes after weight loss, intervention studies disconnect liver steatosis per se from other adverse metabolic consequences, at least in healthy subjects.

In spite of the new light on pathophysiology shed by the above studies, two major needs remain unmet. Firstly, none of the above PET imaging studies included sufficient histologic information to address the progression of liver steatosis into steatohepatitis and/or fibrosis, thus the specific factors of disease progression are not yet identified. However, sufficient knowledge exists to design targeted studies for a more effective demonstration of the potential of PET-CT as diagnostic tools. Secondly, the relevance of other organs in compensating or aggravating liver disease needs a better understanding in order to address appropriate treatment strategies and targets, and intervention-time windows. We have just started to examine the brain-liver-gut axis in humans by PET imaging. Our studies during euglycemic clamp revealed a positive relationship between BGU and EGP and the predictive value of BGU of glucose homeostasis in diabetic subjects following bariatric surgery[23]. We also detected a high fasting-uptake of fatty acids in the brain in obese and morbidly obese individuals[141]. A greater elevation of BGU was also observed in reward-related but not in behavior-controlling regions in response to sensory stimulation by chocolate stimuli in overweight women with high food-addiction scores, compared with women with lower scores, independent of peripheral substrate and hormone levels, which were shown to be similar. Only in the former group was BGU reduced after a low-calorie diet, independent of similar peripheral changes[142]. Thus, high or unbalanced BGU is associated with a variety of high-risk behavioral and metabolic aspects. More important, the study underscores that the same phenotype can result from different mechanisms, and that mechanistic or intervention studies pooling patients based on a



What loads the foot	Mechanisms	Self-sufficient
Extreme deficiency in adipose tissue substrate storage	Build up of reactive oxygen species in the steatotic liver, lipid peroxidation, macrophage infiltration, later dysplasia	Yes
Hepatic triglyceride composition	Unsaturated fatty acids render the steatotic liver more susceptible to (endo/exo) toxin-mediated oxidative damage, lipid peroxidation	No
Unestablished gut microbiota factors	Germ-free (GF) conditions trigger protective responses	Yes
Lipopolysaccharide (LPS)	Hepatic proinflammatory factor, but not self-sufficient (proinflammatory effect lost in GF conditions)	No

Figure 4 Threesome ballooning hallmark of progressive fatty liver disease. Liver steatosis is associated with cardiovascular, digestive, metabolic, neoplastic, and neurodegenerative diseases; but it is the concomitant presence of inflammation that markedly accelerates the progression of these diseases. We illustrate this concept, representing steatosis as a "pedal" on which a series of aggravating factors may press as a "foot" that pushes on the "throttle", namely inflammation.

similar phenotype (*e.g.*, obesity or T2D) may be misleading both on detection of cause and on the evaluation of treatment efficacy. Evaluating the BGL axis by PET imaging with double-tracer oral glucose loading, we showed that the administration of exenatide (a GLP-1R agonist) in subjects with impaired glucose tolerance decreased EGP and HGU. A decrease in the intestinal absorption of oral glucose resulted in lower insulin levels, with an increased proportion of orally ingested glucose that was retained by the liver and increased BGU in most brain regions[143]. That underlines the importance of integrating intestinal metabolism and absorptive effects under real life circumstances in the study of the BGL axis. The quantification of intestinal glucose uptake by PET imaging has been recently validated, showing that intestinal insulin resistance in the jejunum was improved by bariatric surgery in obese subjects and in the large and small intestine by metformin and mildly improved in the small intestine of diabetic patients by rosiglitazone. Intestinal fatty acid uptake was elevated and further increased in obese subjects after bariatric surgery. Interestingly, parallel animal model observations showed that the human body could release glucose and fatty acids from the circulation into the gut lumen[144], which suggests that the gut can be a way to actively eliminate excess substrate and that the body feeds substrates to the gut microbiota, potentially modulating its composition and function. EGP was either decreased, unchanged or increased in the studies, again indicating a possible confounding effect of morbid obesity or a disconnect between insulin action in gut and liver. These studies, primarily planned to address insulin sensitivity, have set the stage for the design of gut-targeted and BGL-targeted imaging approaches under metabolic conditions that are relevant to this interaction, including the study of microbiomics.

CONCLUSION

We reviewed the most recent knowledge of the complex interplay among the organs of the BGL axis in the pathophysiology of insulin resistance and MAFLD, presenting the best established interconnections between brain, gut and liver in the context of insulin resistance and hepatic steatosis. From studies using tissue-targeted animal models it

emerges that insulin resistance per se does not induce hepatic steatosis, nor does steatosis induce whole-body insulin resistance. However, it is evident that reducing inflammation has several beneficial effects both at the hepatic and whole-body level. In fact, inflammation (either hepatic or systemic) acts as major throttle of progressive liver and systemic diseases. This paradigm is illustrated in Figure 4 together with the important diagnostic and prognostic role of the three hallmark characteristics of progressive fatty liver disease, namely visceral obesity (abdominal ballooning) and/or ballooning degeneration of hepatocytes and adipocytes (thus predominant adipocyte hypertrophy rather than hyperplasia).

There is currently no approved treatment for MAFLD, which is a multifaceted syndrome caused by pathogenetic mechanisms that, in animal studies, consistently appear to be diverse. Understanding and being able to identify and measure different factors that trigger and/or accelerate the pathogenesis of steatosis and its progression is a key issue for successful risk-stratification, prevention, and drug development.

With several drugs being potentially beneficial in the treatment of MAFLD, future clinical investigations should address carefully the most appropriate stratification of MAFLD patients to study their specific effects on liver and systemic inflammation, liver fat, and insulin resistance. On the other hand, the approach of both system biology and medicine has to be applied to address the unmet needs in the understanding of the pathophysiology of insulin resistance in different subsets of patients with MAFLD. The pooling of MAFLD patients just on the basis of their common phenotypic characteristic in clinical studies and trials can be highly misleading for both mechanistic understanding and for new drug development as the same phenotype can result from different and time/stage-evolving mechanisms, each requiring a very targeted approach (*i.e.* personalized, timely, and adequate for the disease-stage). Studies targeting the BGL axis might unveil new underlying mechanisms and fill the existing knowledge gaps in the causal links between insulin resistance and MAFLD pathophysiology, paving the way for the development of innovative diagnostic and therapeutic approaches.

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REVIEW

Gut microbiome in acute pancreatitis: A review based on current literature

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Abstract

The gut microbiome is a complex microbial community, recognized for its potential role in physiology, health, and disease. The available evidence supports the role of gut dysbiosis in pancreatic disorders, including acute pancreatitis (AP). In AP, the presence of gut barrier damage resulting in increased mucosal permeability may lead to translocation of intestinal bacteria, necrosis of pancreatic and peripancreatic tissue, and infection, often accompanied by multiple organ dysfunction syndrome. Preserving gut microbial homeostasis may reduce the systemic effects of AP. A growing body of evidence suggests the possible involvement of the gut microbiome in various pancreatic diseases, including AP. This review discusses the possible role of the gut microbiome in AP. It highlights AP treatment and supplementation with prebiotics, synbiotics, and probiotics to maintain gastrointestinal microbial balance and effectively reduce hospitalization, morbidity and mortality in an early phase. It also addresses novel therapeutic areas in the gut microbiome, personalized treatment, and provides a roadmap of

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human microbial contributions to AP that have potential clinical benefit.

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Core Tip: We live in a world of microbes. There is a distinct microbiome sighted in every niche of our body. This review is based on current knowledge to define an overview of how the gut microbiota has accelerated the frontiers of understanding recently and empowered its importance in influencing human physiology through its potential role in various diseases. It further explores the possible application of microbiota-targeted therapeutics in routine clinical practice, meaning manipulating gut microbiota into the current therapeutics to minimize the potential risk of various diseases, including acute pancreatitis.

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INTRODUCTION

Recent research has confirmed the importance of the human gut microbiota in maintaining health and its involvement in disease. The human gastrointestinal (GI) tract harbors a diverse microbial population of more than 10^{14} microorganisms, comprising more bacterial cells than human body cells[1] and more than 3.3 million unique genes[2]. The predominant commensal bacteria in the human GI tract are members of the phyla *Firmicutes* and *Bacteroidetes*, constituting 80%–90% of the total gut microbiota[3]. Other phyla include *Proteobacteria, Actinomyces, Actinobacteria, Fusobacteria,* and *Verrucomicrobia*[4]. The composition of the gut microbiome plays a key role in modulating human immune responses to invasive pathogens, and it also prevents the pathogens from crossing the intestinal barrier[5].

Recent improvement of advanced sequencing methods, such as next-generation sequencing and metagenomics, have added to our understanding of the involvement of gut microbiome in human health and disease[6] and the potential therapeutic value of interventions that target the composition of the gut microbiome. Such interventions would manipulate the host-microbiome community by eliminating harmful taxa or reconstituting missing beneficial taxa[6,7]. Recent evidence shows that, under ideal conditions, symbiotic relationships among the microbial species in the host GI tract can function to prevent opportunistic and nosocomial infections that have become more frequent because of the widespread use of antibiotics to treat various diseases[8-11].

Research performed over the past two decades has revealed the significance of gut dysbiosis in the pathogenesis of many diseases[12], including inflammatory bowel disease[13], irritable bowel syndrome[14], colon cancer[15], Alzheimer's disease[16], coronary heart disease[17], obesity[18], and diabetes mellitus[19]. Changes in the diversity, proportions, and dominant species of the gut microbiome are probably associated with intestinal barrier dysfunction that influences onset and clinical course of multiple diseases, including pancreatic disorders[20]. In a healthy individual, no gut microbes are present in the pancreas, but changes of the gut microbiota may be involved in the pathogenesis of pancreatic disease, including acute pancreatitis (AP)[21]. AP is a common disorder of the digestive system with high morbidity and mortality worldwide. Managing AP is challenging and has a heavy financial burden on the patient and society[22,23]. Therefore, it is important to understand the primary causes and mechanism of the pathogenesis and progression of AP to facilitate early diagnosis and treatment, avoid a course leading to severe disease, and reduce AP-associated fatality[23]. Ongoing studies of AP in humans have found that premature activation of trypsinogen, dysfunctional calcium signaling, impaired endoplasmic reticulum stress-related autophagy, unfolded protein response, and mitochondrial

dysfunction all promote AP. However, the cause of multiorgan dysfunction in AP is poorly understood[24]. The potential role of the intestine in promoting systemic inflammation and organ dysfunction is of interest.

In AP, hypovolemia, reflex splanchnic vasoconstriction, intestinal ischemia, and reperfusion injury due to fluid resuscitation may result in bacterial translocation [25, 26]. Systemic inflammatory response syndrome accompanied by intestinal bacterial translocation is associated with high AP mortality[26]. A change in gut permeability/motility that causes bacterial translocation and leads to the activation of gut-associated lymphoid tissues may result in systemic complications in AP[27]. This review summarizes relevant human and animal studies that provide insights into the potential role of the gut microbiome in AP pathogenesis. It also summarizes treatment perspectives that target the gut microbiome.

PATHOPHYSIOLOGY OF AP

Previous studies of microbiome involvement in AP described a complex cascade of events with significant involvement of pancreatic acinar cells, but the mechanisms involved in the initiation of AP are still poorly understood[28]. Development of a well-defined clinical management protocol is challenging. Most investigations of AP pathophysiology have documented injury or disruption of the pancreatic acini that triggered the activation of pancreatic enzymes (trypsin, chymotrypsin, and elastase) in pancreatic tissue. Activated proteases (trypsin and elastase) and lipase breakdown tissue and cell membranes, leading to edema, vascular damage, hemorrhage, and necrosis[28].

During the initial phase of pancreatic injury, acinar cells release proinflammatory cytokines, like tumor necrosis factor (commonly referred to as TNF)-α, interleukin (IL)-1 and IL-6, and anti-inflammatory mediators, such as IL-10 and IL-1 receptor antagonist[29]. These mediators recruit neutrophils and macrophages to enter the pancreatic parenchyma, to propagate both local and systemic responses. Reactive oxygen metabolites, prostaglandins, platelet-activating factor, and leukotriene may also be involved[29].

Recent epidemiological studies have shown that a local inflammatory response aggravates pancreatitis by increasing permeability, which damages the microcirculation and results in local hemorrhage and pancreatic necrosis in cases of severe AP. Some of the inflammatory mediators released by neutrophils aggravate pancreatic injury by activating pancreatic enzymes (see Figure 1)[26,30]. Figure 1 is a schematic description of AP pathogenesis. The acinar cells of the pancreas cause trypsin activation followed by impairment of cell membrane trafficking and activation of the zymogen cascade mediated by trypsin. Attraction and activation of leukocytes occur with the release of pro- and anti-inflammatory cytokines and chemokines. Overt, sustained activation of proinflammatory mediators leads to systemic inflammatory response syndrome (SIRS) and may progress to multiorgan failure, infection, pancreatic necrosis, and sepsis as late complications of AP.

CLASSIFICATION OF AP

Approximately 15%–25% of patients diagnosed with AP may progress to severe AP. The 2012 revised Atlanta classification and definitions by international consensus include three degrees of AP severity (*i.e.* mild, moderately severe, and severe)[31]. The three degrees primarily manifest as transient organ failure, persistent organ dysfunction, and local or systemic AP[31]. The determinant-based classification of AP is highly dependent on clinical data, and also on the available feedback from patients (to a lesser extent)[32]. Table 1 summarizes both the Atlanta and determinant-based classifications.

VARIOUS SCORING SYSTEMS USED IN THE DIAGNOSIS OF AP

The initial diagnosis of AP made on arrival at a clinic or hospital relies on known medical history, comprehensive physical examination, and increased serum amylase or lipase, with or without additional imaging evaluation. Currently, there are no specific laboratory tests with consistent accuracy and reliability for predicting AP

Table 1 Revised Atlanta classification and determinant-based classification of acute pancreatitis

Revised Atlanta classification of disease severity	
Mild AP	No organ failure
	No local or systemic complications
Moderately severe AP	Organ failure that resolved within 48 h (transient organ failure) and /or
	Local or systemic complications without persistent organ failure
Severe AP	Persistent organ failure > 48 h
	Single organ failure
	Multiple organ failure
A modified Marshal score defines a persistent organ failure	
Determinant-based classification of disease severity	
RACAP	**DBCAPS**
Mild AP	Mild AP
Absence of organ failure	Absence of organ failure
Absence of local complications	Absence of (peri-) pancreatic necrosis
Moderately severe AP	Moderate AP
Local complications and/or	Sterile (peri-) pancreatic necrosis and/or
Transient organ failure	Transient organ failure
Severe AP	Severe AP
Persistent organ failure	Persistent organ failure or
	Infected (peri-) pancreatic necrosis
	Critical AP persistent organ failure
	Infected (peri-) pancreatic necrosis

AP: Acute pancreatitis; DBCAPS: Determinant-based classification of acute pancreatitis severity; RACA: Revised Atlanta classification. Persistent organ failure is defined by a modified Marshal score or a sepsis-related organ failure assessment score.

severity. However, several scoring systems are available and are routinely used by hospital physicians to predict AP severity and prognosis (Table 2)[33-41].

BIOMARKERS AND PREDICTORS TO CONFIRM THE SEVERITY OF AP

In addition to the various clinical scoring systems, several biomarkers have also been applied as predictors of AP severity and are shown in Table 3[42-47].

microRNAs as biomarkers in AP diagnosis

Recently, circular microRNAs have been studied as potential diagnostic biomarkers in AP because of their specific properties, such as stability in biological fluids, simple identification, and sequence conservation among different species (Table 4)[48-60].

GUT MICROBIOME AND MICROBIOME IN AP

The human GI tract is home to a diverse and complex microbial community of bacteria, viruses, and fungi that help to maintain health and are involved in the pathogenesis of various diseases. The gut contains at least 1000 bacterial species and 100-fold more genes than have been identified in the human genome[4,61]. The microbiome is considered a hidden "metabolic organ", and it has a significant impact on well-being because of its influence on our metabolism, physiology, nutrition, and immune function[62]. It has been shown that the gut microbiome co-evolves with us;

Table 2 Prediction scoring systems used in acute pancreatitis diagnosis

No.	Multifactorial scoring system	Timeline	Threshold	Area under the curve	Ref.
1	Ranson score	48 h	≥ 3	0.81–0.88	[33-35]
2	Glasgow score	48 h	2	0.73–0.784	[36,37]
3	Acute Physiology and Chronic Health Evaluation-II score (APACHE-II)	24 h	7	0.80–0.895	[33,38,39]
4	Acute Physiology and Chronic Health Evaluation II score-Obesity (APACHE:-O)	24 h	7	0.893	[40]
5	Bedside Index of Severity score (BISAP)	24 h	≥ 3	0.79–0.875	[33-35,41]
6	Pancreatitis Activity Scoring System (PASS)	24 h	> 160	0.71	[36]
7	Systemic inflammatory response syndrome (SIRS)	24 h	≥ 2	0.73	[34,39]

Table 3 Blood biomarkers predicting disease severity in acute pancreatitis

No.	Blood biomarkers	Timeline	Threshold	Area under the curve	Ref.
1	Interleukin 8	Preoperative	196 pg/mL	0.778	[42]
2	Interleukin 6	24 h	50 pg/mL	0.9	[39]
3	Hepcidin	24 h	234.4 ng/mL	0.82	[43]
4	Red blood cell distribution width	24 h	13.35%	0.787	[44]
5	Procalcitonin	24 h	1.77 ng/mL	0.797	[45]
6	Blood urea nitrogen	24 h	5.945 mg/dL	0.677	[44]
7	Oleic acid chlorohydrin	24 h	32.40 nM	1	[46]
8	C-reactive protein	24 h	150 mg/L	0.61	[47]
9	C-reactive protein	48 h	150 mg/L	0.73–0.91	[33,39,47]

hence, any changes to the microbial community can have significant consequences, both beneficial and harmful[63]. Disruption of the gut microbiota, or dysbiosis, has been associated with diverse systematic conditions, such as obesity[64,65], malnutrition[66], diabetes[67], and chronic inflammatory diseases, such as inflammatory bowel disease, ulcerative colitis, and Crohn's disease[68].

Researchers have continued to study the microbiome at an accelerated pace over the past two decades, revealing the myriad ways these microorganisms affect our day-to-day lives. The microbiome in our gut is now understood to be a significant contributor to the development of chronic disease. Gut microbiota is now known to play a critical role in human health and disease. With the advances in microbiome research over time, more and more data has become available showing the gut microbes' overall composition and functional potential. Additionally, the number of diseases associated with changes in our gut microbial community has increased simultaneously[4,6]. The human gastrointestinal tract is a habitat crowded with microorganisms contributing to the host's immunity and pathogenesis of several diseases, including acute pancreatitis [69]. Human gastrointestinal microflora is divided into three different types by the way they present themselves in the body and perform multiple functions: *e.g.*, There are three major categories of bacteria: Physiological bacteria, that hold over 90% and are nourishing and immune-modulating; opportunistic bacteria, which are pathogenic in situations of lower immune resistance or antibiotic abuse; and pathogenic bacteria, which have lower numbers and invade difficultly[70]. Progression in AP has been more complicated by gastrointestinal motility dysfunction, which is probably related to the neuroendocrine system, hypoxia-ischemia, ischemia-reperfusion injury (IRI), inflammatory mediators, and cajal cells[71].

Since many discoveries have postulated that commensal intestinal microbiomes play a crucial role in humans' health, immune system, and homeostasis recently, there has been a surge of interest in this area of study. The overall function of the intestine in the entire mechanism of AP pathogenesis (such as in acute and critical illnesses) is essential to understand, but often it is overlooked. This pathogenesis mechanism involves several factors contributing to the loss of gut barrier function, allowing bacteria and endotoxins to translocate into the bloodstream, which is critical for

Table 4 MicroRNAs used as biomarkers in the diagnosis of acute pancreatitis

No.	miRNAs	Patients	Sample	Expression change	Reference gene	Ref.
1	miR-216a	AP	Plasma	Up	None	[49]
2	miR-551b-5p	AP	Plasma	Up	miR-16	[50]
3	miR-216a-5p, miR-375, and miR-551b-5p	AP	Serum	Up	miR-103a-3p	[51]
4	miR-7, miR-9, miR-122, and miR-141	AP	Serum	Up	Exogenous reference genes	[52]
5	miR-216a	AP	Plasma	Up	U6	[53]
6	miR-551-5p	AP	PBMC-	Up	U6	[54]
7	miR-155	AP	Serum	Up	U6	[55]
8	miR-29a	AP	Plasma	Up	U6	[56]
9	miR-24-3p, miR-222-3p, miR-361-5p, and miR-1246	HTG-AP	Serum	Up	U6	[57]
10	miR-1260b, miR-762, miR-22-3p, miR-23b, and miR-23a	AP-associated ALI	Serum	Up	U6	[58]
11	miR-92b, miR-10a, and miR-7	AP	Plasma	Down	miR-16	[50]
12	miR-155	AP	Serum	Down	Not mentioned	[59]
13	miR-181a-5p	HTG-AP	Serum	Down	U6	[57]
14	miR-550a, miR-324-5p, miR-484, miR-331-3p, miR-140-3p, miR-342-3p, and miR-150	AP-associated ALI	Serum	Down	U6	[58]
15	miR-127	AP-associated ALI	Plasma	Down	miR-16	[60]

ALI: Acute lung injury; AP: Acute pancreatitis; HTG-AP: Hypertriglyceridemic-acute pancreatitis; mi: Micro.

generating the second inflammatory hit of AP[72]. The data of Johnson *et al*[73] suggests gut bacteria translocation (*via* hematogenous, lymphatic, and reflux) is involved in AP infection progression, which indicates the presence of a possible correlation between gut microbiota and AP infection progression. An abnormality of the gastrointestinal microbiota (dysbiosis) is associated with the systematic inflammatory response syndrome (SIRS) and a broad range of diseases[74].

When the mucosal barrier of the intestine is damaged, intestinal bacteria may migrate into the blood or to other tissues and organs, further accelerating AP[75]. In recent years, several studies have been conducted investigating changes in intestinal flora associated with AP severity. AP progression involves the abnormal release of trypsin and destruction of pancreatic tissue due to abnormal cells. Several recent studies examined the changes in intestinal flora during AP development concerning disease severity. It is observed that abnormal trypsin secretion has occurred due to AP progression and that pancreatic structure destruction leads to an abnormal pancreatic secretion, resulting in the intestinal flora and homeostasis changes[76,77].

Numerous studies have now demonstrated the function of normal gut microbes to promote healthy gut mucosa. Gut mucosal ischemia and reperfusion during AP progression can compromise the integrity of the gut barrier, causing bacterial reabsorption from the gut to other parts of the body and causing local and systemic infections[78]. Some further research findings have also revealed that intestinal mucosal barrier injury is a significant complication in many AP patients. The intestinal mucosal barrier can be destroyed by affecting intestinal inflammation and the immune response[75]. Many studies are supporting now to demonstrate that normal gut microbes play a primary role in maintaining gut mucosal integrity. However, gut mucosal ischemia and reperfusion during AP progression can damage the overall integrity of the gut barrier and lead to gut bacterial translocation to other locations, causing local and systemic infections[78]. Thus, a significant complication of an AP patient's condition involves intestinal mucosal barrier damage. This is caused by intestinal inflammation and immune response defects. Other research has also found injuries of the intestinal mucosal barrier to patients with AP[75].

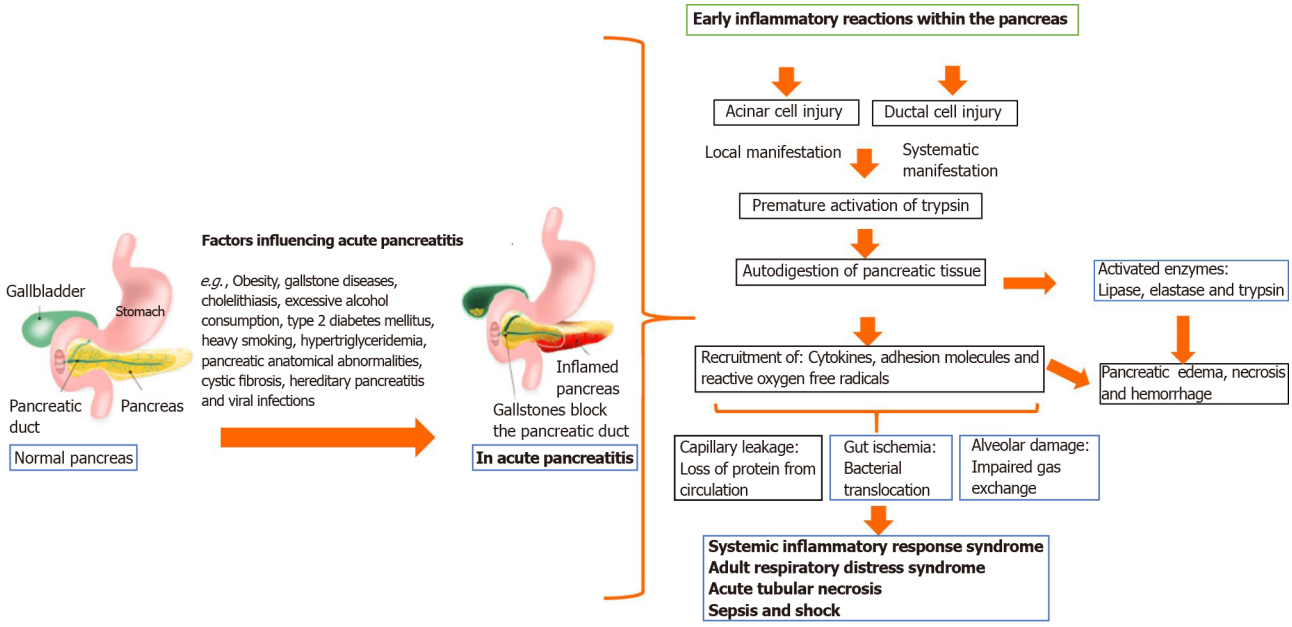

Figure 1 Pathophysiology of acute pancreatitis. Damage in the pancreas' acinar cell causes trypsin activation following cell membrane trafficking impairment, with subsequent activation of the zymogen cascade by trypsin. Attraction and activation of leukocytes occur with the release of many proinflammatory and anti-inflammatory cytokines, as well as chemokines. An overt and sustained activation of proinflammatory mediators leads to systemic inflammatory response syndrome (SIRS), which may further proceed to multiorgan failure, pancreatic necrosis and sepsis with late complications of acute pancreatitis.

In brief, the pancreas-gut communication has been described as being in AP, with bacterial translocation as a possible consequence and the homeostatic host response noted. Translocation of bacteria from the lower gastrointestinal tract occurs *via* the portal circulation - the oral course and/or the mesenteric lymph nodes. The acinar cells of the pancreas secrete pancreatic antimicrobial peptides (AMPs). AMPs have homeostatic bidirectional communication with the gastrointestinal tract[79]. The lower level of the microbiome in the gastrointestinal tract may increase pancreatic antimicrobial peptide production by short-chain fatty acid metabolites. Consequently, it induces a pancreatic immunoregulatory environment which decreases proinflammatory immune cells. Conversely, decreased antimicrobial peptide production facilitates the overgrowth of the gastrointestinal microbiota leading to the induction of proinflammatory immune cells. Thus, it subsequently alters the gut microbiome and the intestinal immune system[79].

POTENTIAL ROLE OF THE GUT MICROBIOME IN AP

Metagenomics and next-generation sequencing have facilitated the investigation of the involvement of the gut microbiome in human physiology and various diseases. The findings have allowed for consideration of the gut microbiome as a hidden organ[80]. The close interaction of the gut microbiome with host physiology can account for the harmful effects of disruptions in the former caused by various internal or external events that initiate inflammatory conditions and some types of cancer. Therefore, it is essential that specific microbial signals maintain the host immune response and other physiological functions that protect against pathogens[81].

Injury of the microcirculation and hypovolemia that occur during AP can lead to gut mucosal ischemia and reperfusion injury that result in loss of gut barrier function. Subsequent translocation of gut bacteria can result in local pancreatic and systemic infections[82]. Leading causes of AP mortality include pancreatic infection and peripancreatic necrosis[78]. The initial onset of cerulean-driven AP depends on NOD1 activation in acinar cells by commensal microbiota that have translocated from the gut. Following activation, NOD1 induces the expression of inflammatory mediators[83]. The role of the gut in neutrophil priming and release of proinflammatory cytokines is important for the initiation and propagation of inflammation and sepsis[84]. The loss of gut barrier function has been implicated in the pathogenesis of AP-related infections. Ahuja *et al*[76] reported that secretion of antimicrobials from pancreatic acinar cells regulated gut microbiota composition and innate immunity[76]. Blocking

acinar cell exocytosis in mice has been found to lead to gut dysbiosis, inflammation, systemic bacterial translocation, and ultimately, death. Additional evidence has revealed additional examples of crosstalk between pancreas acinar cells and the gut microbiome[76,77].

An ongoing investigation of the microbiota and AP has shown that microcirculatory disturbances associated with the loss of fluid into the "third space," lead to hypovolemia, ischemia, and reperfusion injury in AP patients. The gut is affected by AP, but it is not a passive victim because it plays an active role in the worsening of the illness[85]. In addition to bacterial translocation, the translocation of inflammatory compounds produced in the intestinal wall and the gut's toxic products might also be responsible for initiating SIRS and distant organ injury in AP patients[86]. The contribution of the gut microbiome for protection against pathogens in AP patients has not been clearly elucidated. An increase of such pathogenic bacteria as *Enterobacteriaceae* and *Firmicutes*, and a decrease in beneficial bacteria like *Bacteroidetes* and *Lactobacillus* has been observed in AP patients[87]. Furthermore, increased serum IL-6 has been found to be positively related to increased *Enterobacteriaceae* and *Enterococcus* and inversely related to *Bifidobacterium* and *Clostridium* cluster number. Tan *et al*[87] reported that the extent of gut microbiota modification predicted pancreatitis severity and the occurrence of systemic complications. Gerritsen *et al*[88] found that "AP-associated microbiota" replaced the normal intestinal flora in a study performed in a mouse model. In AP, changes in the populations of specific commensal bacteria have been associated with reduced levels of the inflammatory cytokines IL-1b, TNF-a, CXCL1, and IL-18, and inversely correlated with pancreatitis severity and the occurrence of systemic infectious complications. The evidence highlights the restoration of the physiological gut microbiota composition as a valuable strategy to treat AP[89].

The 16S rRNA gene is highly conserved in bacteria, and it is highly species-specific. Consequently, 16S rRNA gene sequencing is widely used to study the gut microbiota in various disease states[90]. Zhu *et al*[69] reported that the relative abundance of commensal microbiota in AP patients differed from that in a healthy individual. Members of the *Bacteroidetes* phylum decreased significantly, but *Proteobacteria* were over-represented in AP. AP patients also had a relative overabundance of *Escherichia/Shigella* compared to healthy control[69]. Increased abundance of two common opportunistic pathogens, including *Enterococcus* and an unknown genus in the *Enterobacteriaceae* family, were also observed in AP patients. Linear discrimination and effect size analysis revealed significant increases in *Acinetobacter, Stenotrophomonas,* and *Geobacillus* with decreased *Bacteroides, Alloprevotella, Blautia* and *Gemella* in patients with severe AP, as compared to those with mild and moderately severe AP[69]. Table 5[21,69,75,91] and Table 6[69,75,87,90,92] summarize the significant changes in the microbiome composition of healthy controls and in patients with mild, moderately severe, and severe AP.

Changes in the gut microbiota in AP include overexpression of opportunistic pathogens, such as *Escherichia/Shigella*, and reduced abundance of beneficial genera, such as *Bifidobacterium*[69]. It has been hypothesized that a reduction of beneficial bacteria might facilitate microbial translocation across a damaged gut barrier, thereby promoting the progression of AP. Studies performed by Li *et al*[75] and other investigators[93,94] found that dysbiosis that included the depletion of short-chain fatty acid-producing bacteria was associated with an impaired gut barrier and worsening of AP. Changes in the gut microbiome may thus serve as a diagnostic tool in AP. Restoring gut microbiota homeostasis and stabilizing the gut barrier might have therapeutic value in AP patients, as shown in Figure 2[21,30,69,75,87,93,94]. The overall literature suggests that there is an association between the gut microbiome and the severity of AP[95]. Additionally, in experimental acute pancreatitis, changes to the gut microbiome, *e.g.*, administration of *Clostridium butyricum* can suppress AP[27] pointing to the therapeutic potential of this approach is promising.

ANTIBIOTICS, PREBIOTICS, SYNBIOTICS, AND PROBIOTICS FOR THE TREATMENT OF AP

Antibiotic therapy in AP

In the past decade, substantial advancements have been made not only in understanding the pathophysiology of AP but also in treatment strategies and multidisciplinary management. Necrotizing pancreatitis occurs in about 30% of patients and has a poor prognosis and high mortality. About 80% of AP deaths are caused by infections

Table 5 Altered microbiome composition in acute pancreatitis *vs* healthy controls

No.	Techniques used for microbiome profiling	Healthy control	Acute pancreatitis	Ref.
1	qPCR (Fecal samples)	*Firmicutes*↑	*Firmicutes*↓	[84]
		Bacteroidetes↓	*Bacteroidetes*↑	
		Proteobacteria↓	*Proteobacteria*↑	
		Actinobacteria↑	*Actinobacteria*↓	
		Tenericutes↓	*Tenericutes*↑	
2	16S rRNA gene sequencing (Fecal samples)	*Proteobacteria*↓	*Bacteroidetes*↓	[83]
			Proteobacteria↑	
			Escherichia/Shigella↑	
			Enterococcus↑	
			Enterobacteriaceae↑	
			Prevotella↓	
			Faecalibacterium↓	
			Bifidobacterium↓	
3	16S rRNA gene sequencing (Fecal samples)	NA	*Bacteroidetes*↑	[85]
			Proteobacteria↑	
			Firmicutes↓	
			Actinobacteria↓	
4	16S rRNA gene sequencing (Fecal samples)	NA	*Enterobacteriaceae*↑	[21]
			Enterococcus↑	
			Bifidobacteria↓	

NA: Not available. ↑: Higher level; ↓: Lower level.

secondary to AP that might be attributable to gut translocation of intestinal bacteria [82,96]. Current guidelines recommend against routine antibiotic prophylaxis in AP [97]. Recent studies did not find benefits of antibiotic prophylaxis in reducing AP mortality, infections not involving the pancreas, or surgical interventions. The data on infections accompanying pancreatic necrosis in adults are conflicting[98,99].

The use of antibiotics, starting with carbapenems, quinolones, and metronidazole, has been advised in patients with AP and concomitant cholangitis symptoms, infected necrosis, or necrotizing pancreatitis accompanying a deteriorating clinical status[97]. Delaying surgical interventions decreases morbidity and mortality[100]. In some instances of AP, where infection is clinically suspected or confirmed, the use of antibiotics is recommended to avoid development of antimicrobial resistance. The predictive value of fine-needle aspiration for sampling and determination of bacterial sensitivities in diagnosing peri-pancreatic infection is comparable to that of clinical signs and imaging. The routine use of fine-needle aspiration is not recommended[101].

Early enteral nutrition is recommended in AP because it protects mucosal nutrition, the gut mucosal barrier, and gut-pancreas homeostasis[102]. In a previous randomized trial, decontamination of the gut with norfloxacin, colistin, amphotericin and standard AP therapy did not reduce mortality[103]. At present, selective gut decontamination cannot be recommended for AP patients.

Despite constant improvement in targeted therapeutics, a third of adult AP patients develop moderately severe AP and/or severe AP with SIRS, organ failure, and an increased risk of infection[104]. The gut mucosal barrier reduces the risk of infected pancreatic necrosis and thus helps to decrease mortality risk[85,105]. Microbiome-targeted therapies, such as genetic engineering of modified strains to outcompete pathogens, selective nutrient or prebiotic supplementation, or engineered bacteriophages, could steer the altered microbiome toward a healthy phenotype or change the course of critical illness[81]. Probiotics offer substantial health benefits and support the homeostasis of gut flora[81]. The most widely used probiotic bacteria in clinical trials are *Lactobacillus* and *Bifidobacterium*, which are easy to isolate from human feces or the

Table 6 Altered microbiome composition observed in acute pancreatitis of increasing severity

No.	Techniques used for microbiome profiling	MAP	MSAP	SAP	Ref.
1	qPCR (Fecal samples), performed only on MAP and SAP patients	*Enterococcus*↑ *Enterobacteriaceae*↑ *Bifidobacterium*↓	NA	*Enterococcus*↑ *Enterobacteriaceae*↑ *Bifidobacterium*↓	[79]
2	16S rRNA gene sequencing (Fecal samples)	*Finegoldia*↑	NA	*Acinetobacter*↑ *Stentrophomonas*↑ *Geobacillus*↑ *Bacteroides*↓ *Alloprevotella*↓ *Blautia*↓ *Gemella*↓	[83]
3	16S rRNA sequencing (Fecal sample)	*Enterobacteriaceae*↑ *Enterococcus*↑ *Bifidobacterium*↓	NA	*Enterobacteriaceae*↑ *Enterococcus*↑ *Bifidobacterium*↓ *Blautia*↓	[85]
4	16S rRNA gene sequencing (Rectal swab)	*Bacteroides*↑ *Escherichia/Shigella*↑ *Enterococcus*↑ *Finegoldia*↑ *Blautia*↓	*Bacteroides*↑ *Escherichia/Shigella*↑ *Enterococcus*↑ *Anaerococcus*↑ *Eubacterium hallii*↓	*Bacteroides*↑ *Escherichia/Shigella*↑ *Enterococcus*↑ *Eubacterium hallii*↓ *Acinetobacter*↓ *Stenotrophomonas*↓ *Bacteroides*↓ *Blautia*↓	[82]
5	Shotgun metagenomics (Fecal sample)	*Thermoprotei*↑ *Crenarchaeota*↑ *Streptococcus*↑ *Anaerostipes hadrus*↓	*Sulfolobus*↑ *Methanobrevibacter ruminantium*↑ *Methanosarcina - Thermophila*↑ *Anaerostipes hadrus*↓ *Escherichia coli*↑	*Sulfolobus*↑ *Methanomicrobiales - archaeon 53_19*↑ *Enterococcus*↑ *Blautia*↓	[86]

MAP: Mild acute pancreatitis; MSAP: Moderately severe acute pancreatitis; NA: Not available; SAP: Severe acute pancreatitis; ↑: Higher level; ↓: Lower level.

intestinal mucosa. Prebiotics are nondigestible foods required to propagate probiotics, and they stimulate the growth and activity of the healthy gut flora. Synbiotics are nutritional supplements that include both probiotics and prebiotics[106].

A randomized controlled trial by Pan *et al*[107] evaluated the ability of synbiotics to restore intestinal barrier damage and reduce the infection rate in early AP. A group of 45 patients were given either live or heat-inactivated *Lactobacillus plantarum* 299 with an oat fiber supplement as early enteral nutrition. Supplementation plus the symbiotic significantly reduced both pancreatic necrosis and surgical interventions[107]. A subsequent clinical trial included 62 severe AP patients treated with early enteral nutrition with four different prebiotics (inulin, beta-glucan, resistant starch, and pectin) together with four *Lactobacillus* probiotic preparations. The treatment resulted in a reduced incidence of SIRS and organ failure, supporting the use of early enteral symbiotic nutrition in severe AP[108]. Olah *et al*[109] randomized 45 AP patients to receive either a freeze-dried preparation containing 10^9 live *L. plantarum* 299 in each dose together with oat fiber or a heat-inactivated *Lactobacillus* controlled by nasojejunal tube for one week. Infected pancreatic necrosis and abscesses were significantly lower

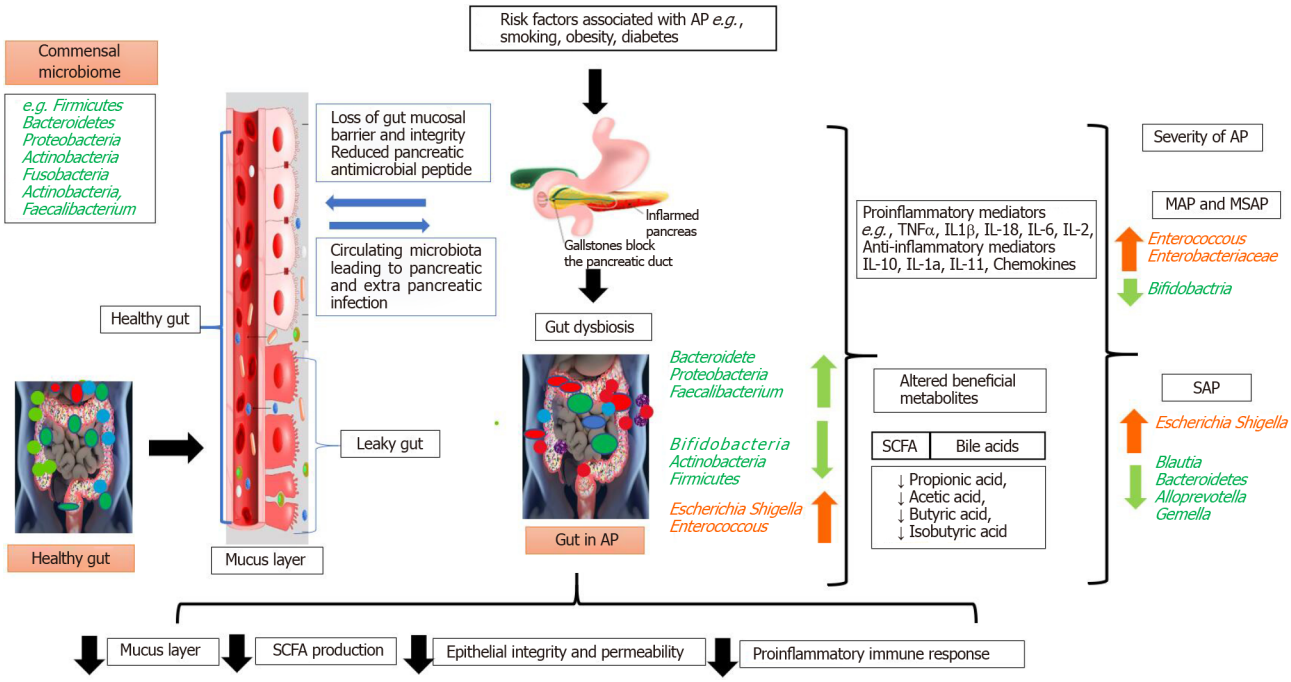

Figure 2 Role of the gut microbiota in inflammation of the pancreas and acute pancreatitis. Breakdown of the relationship between physiologic and pathogenic bacteria, the immune system, and the intestinal epithelial barrier leads to gut dysbiosis. Inflammation and gut dysbiosis causes the translocation of microbes to the pancreas. The translocation of bacteria results in pancreatic inflammation due to toxin diffusion and complications like fibrosis, digestive and absorption disorders, diabetes, and other metabolic disorders. AP: Acute pancreatitis; MAP: Mild acute pancreatitis; MSAP: Moderate severe acute pancreatitis; TNF: Tumor necrosis factor; IL: Interleukin; SAP: Severe acute pancreatitis; SCFA: Short-chain fatty acids.

in the treatment group than in the control group.

Other experimental pancreatitis studies in rat models confirmed the efficacy of *L. plantarum* spp. in reducing microbial translocation and as a possible alternative to antimicrobials[110]. Several studies have shown that probiotics containing *Faecalibacterium* and *Bifidobacterium* species had beneficial effects, including stabilizing the gut barrier, increasing anti-inflammatory responses, and attenuating bacterial translocation[111,112]. Some studies have not found any significant benefit or adverse effect of probiotics in severe AP. Still, it is essential to note the considerable patient and probiotic regimen heterogeneity in the published clinical trials of probiotics. The PROPATRIA Probiotics[113] in Pancreatitis Trial randomized 298 patients with predicted severe AP to either a multispecies probiotic mixture containing two different *Bifidobacterium*, three *Lactobacillus*, and one *Lactococcus* species or a placebo. The infectious complications in the two groups were similar, but the probiotic group had higher mortality (16% *vs* 6%) and incidence of bowel ischemia (6% *vs* 0%) compared with the placebo group. The high load of the probiotic mixture used in the study was thought to have been responsible for the increased mortality[114]. The findings highlight the challenges of supplementing the gut microbiome with beneficial microbial species in the setting of AP. Nevertheless, eight years later, the PROPATRIA trial was reevaluated by Bongaerts *et al*[115]. The team of researchers analyzed and addressed all shortcomings identified in the trial. PROPATRIA researchers contend that a lethal combination of predominantly proteolytic pancreatic enzymes and probiotic therapy was responsible for the high mortality rate, and that elevated levels of lactic acid produced by bacterial fermentation of carbohydrates significantly contributed to the high death rate. Additionally, one of them was the latency time in the first administration of probiotics; indeed, some patients were treated 24 h after onset of symptoms. Furthermore, there were errors in randomization; in fact, the onset of multi-organ failure was already present during admission in more patients in the first group than in the placebo group (41 patients *vs* 23 patients). Finally, last but not least, the team of researchers suggested that in future studies, when considering substituting probiotics in AP, it is necessary to assess the appropriate, effective doses of probiotics. However, caution should be mandatory to prevent bacterial overgrowth while conducting clinical trials in AP patients.

Previous studies have shown that *L. plantarum* decreased the occurrence of infective necrosis in AP patients[110] and that *Saccharomyces boulardi* spp. administered concomitantly with antibiotics such as ciprofloxacin decreased histopathologic scores in acute

necrotizing pancreatitis[111]. Animal studies have provided strong evidence in support of probiotic benefits in animal models of AP. A mixture of *Lactobacillus acidophilus, Streptococcus thermophilus,* and *Bifidobacterium lactis* given by oral gavage reduced pancreatitis, bacterial translocation to extra-intestinal sites, and mortality in male albino rats because of reduced duodenal bacterial overgrowth[112].

Injury of the GI barrier is a key event in the development of AP. Few studies have reported prevention of disruption of the intestinal barrier with modulation of gut microbiome balance. In one such study conducted in a mouse model of AP, *Clostridium butyricum*, a producer of small-chain fatty acids, which have immunomodulatory properties, reduced infiltration of neutrophils and dendritic cells in the pancreas and inhibited inflammatory responses mediated by NLRP3 and TLR4 signaling pathways in the pancreas and colon[27]. In summary, probiotics help to maintain gut homeostasis. Research should improve the designs of future studies, for example, by detecting a peculiar strain of microorganisms (*i.e.*, their type), standardizing the dose and duration of treatment, or standardizing the state of disease progression when considering to use in current therapy scenarios.

CONCLUSION

The gut microbiome plays a significant role in health and diseases. The resident microbiota in the human GI tract influences host metabolism, physiology, and immune system development. Disruption of this bacterial community results in GI disease. Ongoing medical and clinical research has produced a substantial body of evidence of a clear correlation between changes in the commensal microbiota and the occurrence of pancreatic disease. Application of biochemical, microbiological, and molecular biological methods have provided a description of the constituents of the gut microbiome in health and disease, their niches, and their physiological roles. Additional study is needed to explain whether microbial dysbiosis is a cause or an effect of diverse pathologies. The microbiome profile and changes in dysbiosis may influence an increase in AP severity during its clinical course.

Damage of the intestinal mucosal barrier allows migration of intestinal microbes to the blood or other tissues and organs, which enhances or aggravates AP. Changes in the resident species and abundance of the intestinal flora during AP are closely related to damage of the intestinal mucosal barrier system. Regulating the intestinal flora to repair the intestinal mucosal barrier and restore its function may be useful in AP treatment. Changes in the gut microbiota composition in AP include over-representation of opportunistic pathogens such as *Escherichia/Shigella* species and a significant decrease in the beneficial *Bifidobacterium* genus. Early dysbiosis of the gut microbiota, especially the depletion of small-chain fatty acid-producing bacteria, is probably associated with impairment of the gut barrier and increased AP severity.

The mechanisms of gut dysbiosis and the etiology of AP are not yet fully understood. The relationship of GI microbial symbiosis and AP are avenues for further research. The concomitant use of probiotics and antibiotics together with conventional treatment, such as surgery, radiotherapy, chemotherapy and targeted therapies, are further areas for research. In summary, the clinical significance of GI homeostasis during AP is emerging step by step. Thus, restoring the homeostasis of gut microbiota and stabilizing the gut barrier could be a promising therapeutic target in preventing AP progression. Challenges and specific problems that stand in the way to developing a research platform for understanding AP and its interaction with the microbiome need to be overcome. This review has explored the role of the gut microbiome in AP and the targeted use of probiotics, prebiotics, and synbiotics to maintain or restore GI microbial balance.

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MINIREVIEWS

Role of exercise in preventing and restoring gut dysbiosis in patients with inflammatory bowel diseases: A review

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Abstract

Inflammatory bowel diseases (IBD) include a spectrum of chronic inflammatory disorders of the gastrointestinal tract whose pathogenesis is yet to be elucidated. The intestinal microbiome has been studied as a causal component, with certain microbiotic alterations having been observed in subtypes of IBD. Physical exercise is a modulator of the intestinal microbiome, causing shifts in its composition that are partially corrective of those observed in IBD; furthermore, physical exercise may be beneficial in patients with certain IBD subtypes. This review studies the effects of physical exercise on the human gut microbiome while investigating pathophysiologic mechanisms that could explain physical activity's clinical effects on patients with IBD.

Key Words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Pouchitis; Microbiome; Exercise

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Core Tip: Inflammatory bowel diseases (IBD) are a spectrum of diseases that are characterized by their complex pathogenesis. The intestinal microbiome is thought to be a part of their pathogenesis, with certain alterations having been associated with

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IBD subtypes. Physical exercise is a modulator of the intestinal microbiome that has, furthermore, been associated with positive clinical outcomes in certain patients with IBD. Herein we discuss certain types of physical exercise, their effect on the intestinal microbiome, and its clinical effects on patients with IBD, as well as investigating underlying pathophysiologic mechanisms that could mediate the observed associations.

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INTRODUCTION

Inflammatory bowel disease (IBD) comprises a spectrum of chronic inflammatory diseases that primarily but not exclusively affect the gastrointestinal tract, including ulcerative colitis (UC), Crohn's disease (CD), and other related conditions[1]. Their pathogenesis is classically thought to include interactions between genetic, immune-mediated, and environmental factors[2]. Studies on the molecular epidemiology of IBD have shown that the gut microbiome composition is a biomarker of prognostic importance for CD and UC[3]. Other molecular phenotypes include *NOD2*, *MHC*, and *MST1* genotypes, which are correlated to disease location and activity, microRNA miR-215 levels, and DNA methylation, which are correlated to disease activity, *FOXP3* haplotype, which is of prognostic importance, and oncostatin M and IL-1β levels, which predict response to anti-tumor necrosis factor therapy[3,4]; furthermore, serum C-reactive protein levels correlate to disease activity but are generally nonspecific[4]. Increased serum miR-595 and miR-1246 levels are associated with active IBD[4]. Serum interleukin (IL)-2 and IL-6l and positivity for anti-bacterial flagellin, anti-outer membrane porin C, anti-A4-Fla2, and anti-Fla-X antibodies predict recurrence of disease[4]. The heterogeneity of IBD phenotypes underlines the need for new markers that can subclassify, diagnose, inform prognosis, and guide IBD treatment; but owing to the low sensitivity or specificity of known molecular markers, current knowledge is far from adequate to support their use in everyday clinical practice, a limitation that includes the microbiome as biomarker[3,4].

Early studies on animal models have shown that immune cells could not cause inflammation in the absence of intestinal bacteria, therefore suggesting a putative role for the intestinal microbiome in the induction and/or maintenance of local inflammation and disease[5]. This was further supported by the observation that intestinal inflammation in IBD was greatest in parts of the bowel richer in bacteria[2]. Further studies have demonstrated that certain patterns of microbiotic alterations, including increases or reductions in the plethora of bacterial, fungal, and viral species, were likely linked to the risk for IBD[2].

Physical exercise is a possible modulator of intestinal microbiome composition, altering the functional activity of the gut ecosystem. Exercise is associated with increased biodiversity and a beneficial metabolic function, while exhaustive exercise training might be associated with dysbiosis of the gut microbiota, promoting negative metabolic effects and inflammation[6,7]. Thus, physical activity/exercise has been studied as a significant modifier of the intestinal microbiome in animal[8,9] and human[6,7,10] studies. Specifically, more active individuals' microbiomes tend to harbor a higher abundance in *Akkermansia muciniphila*, a health-promoting species, as well as decreased Bacteroidetes bacteria and increased bacterial diversity[11,12]. Specific types of physical exercise have not only been associated with microbiotic signatures, but also with a reduction in endotoxemia and serum inflammatory markers [11,13,14]. These alterations persist as statistically significant, even when normalizing for age, weight, body composition, and nutritional habits as confounding factors[15]. Nevertheless, it should be noted that researchers have also reported a lack of correlation between a certain type of exercise and changes in the microbiome[16,17]; moreover, some contradictions as to the observed patterns of microbiotic alterations associated with exercise are evident[18]. This ambiguity is, however, expected to be partly clarified when common definitions and detailed description of exercise charac-

teristics [*i.e.* frequency, intensity, type (mode), time (duration), and volume/dose (duration x intensity)] and methods of fecal sampling are utilized among the same study groups.

Given the established pathobiological role for the microbiome in IBD and taking into account the more recent data assessing patterns of microbiotic alterations associated with exercise, this review aims to summarize and clarify the important findings linking dysbiosis in IBD to physical activity, with a focus on preventative medicine and therapeutics.

METHODLOGY

Our literature search utilized the PubMed literature database. Search keywords included "inflammatory bowel disease", "IBD", "ulcerative colitis", "Crohn's disease", "indeterminate colitis", "microbiome", "microbiota", "physical activity", "exercise", as well as combinations of the aforementioned with the AND/OR operators. Articles were first filtered by title, followed by abstract screening, and the remaining were finally selected based on their full text.

EXERCISE AND ITS INFLUENCE ON THE MICROBIOME

In healthy mice, *Allobaculum* and *Clostridiales* are more abundant in exercised mice than in controls[19]. In diabetic mice, total intestinal bacteria and Enterobacteriaceae are lower in the exercise groups than in diabetic controls[9]. In high-fat diet obese mice, exercise increases Bacteroidetes as well as increases the Bacteroidetes/Firmicutes ratio in the cecum and colon[20]. In an early obesity and non-alcoholic fatty liver disease model, where male rats fed a control or a high-fat diet, a combined aerobic and resistance exercise training resulted in increased *Parabacteroides*, *Bacteroides*, and *Flavobacterium* genera, while *Blautia*, *Dysgonomonas*, and *Porphyromonas* exhibited an opposite pattern[8] (Table 1).

Several factors that modify the human gut microbiome have been identified; those include country of residence, specific genotypes (such as those affecting the ABO antigens), delivery by caesarian section, diet, cigarette smoking, breastfeeding, gastroenteritis, increased hygiene, use of antibiotics, obesity, immune response, as well as physical exercise[2,13,21,22]. Human studies have shown that sedentary individuals have a predominance of *Bacteroides* and *Parabacteroides* in their gut microbiome, while participants with a higher level of activity, as gauged by accelerometers, have a predominance of *Coprococcus*, *Blautia*, and *Eubacterium*[23]. Women with an active lifestyle have a higher proportion of *Faecalibacterium prausnitzii*, *Roseburia hominis,* and *Akkermansia muciniphila* bacteria in their gut than sedentary women[24]. Aerobic brisk walking increases *Bacteroides* species in healthy elderly women[25]. Endurance training has been observed to reduce *Streptococcus*, *Proteobacteria*, *Porphyromonadaceae*, *Odoribacter*, *Desulfovibrionaceae*, and *Enterobacteriaceae* and to increase *Verrucomicrobiaceae*, *Bifidobacteriaceae*, *Dorea*, *Anaerofilum*, and *Akkermansia* bacteria in overweight women[26]. In obese children, a strength and endurance combined training program led to an increase in *Blautia*, *Dialister,* and *Roseburia* species, accompanied by a reduction in inflammasome activation[14]. In elderly men, endurance exercise has been observed to reduce *Clostridium difficile* and increase intestinal populations[27] Male elite rugby players have been found to have a more diverse microbiome than body mass index (BMI)-matched controls, with the athletes having higher proportions of *Akkermansiaceae* and *Akkermansia* than high-BMI controls and lower proportions of *Lactobacillaceae*, *Bacteroides,* and *Lactobacillus* than low-BMI controls[10]. In swimmers, a reduction in training volume is accompanied by a significant reduction in *Coprococcus* and *Faecalibacterium* populations[28]. In marathon runners, *Lentisphaerae* and *Acidobacteria* increase in intestinal population after running[29]. Ultra-endurance exercise has been observed to increase butyrate-producing bacteria, such as *Subdoligranulum* and *Roseburia hominis*, which are thought to reduce intestinal inflammation by producing butyrate[30]. In martial arts athletes, *Parabacteroides, Phascolarctobacterium, Bilophila, and Oscillibacter* are higher in higher-level athletes than in lower-level ones, with *Allisonella*, *Citrobacter,* and *Megasphaera* found at lower levels[31]. Other researchers have reported no change in gut bacterial diversity or composition after short-term high-intensity interval training in lean and overweight men[16]. Exercise has also been found to induce microbial transformations in the context of damaging intestinal conditions, such as a high-fat diet and toxic substances[32] (Table 1). These changes

Table 1 Effect of exercise on the gut microbiome

Ref.	Study subject	Sample type	Exercise protocol	Exercise *vs* controls	Notes
Lambert *et al* [9]	Mouse, type 2 diabetic, (C57BL/KsJ-leprdb/leprdb)	Cecal matter	Treadmill, 5 d/wk, 66 min/d, 2.87 m/min	↑ Clostridium leptum ↑ Lactobacillus ↓ Total bacteria ↓ Bacteroides ↓ Bifidobacterium ↓ Methanobrevibacter ↓ Prevotella	↑ Bifidobacterium with exercise in non-diabetic mice
Campbell *et al*[19]	Mouse C57BL/6NTac, male	Fecal matter from the distal colon	Free running wheel	↑ Allobaculum ↑ Clostridiales ↑ Faecalibacterium prausnitzi	Normal-diet, not observed in a high-fat diet, except for Faecalibacterium prausnitzi
Denou *et al* [20]	Mouse, C57 BL/6, high-fat diet, obese	Feces from anal area, then full intestinal sampling	Treadmill, 6 wk total, 3 d/wk, 1 h/d, 17 m/min at 5% grade for 2 min + 2 min rest, increase by 1 m/min every week	↑ Bacteroidetes/Firmicutes ratio in the cecum ↑ Bacteroidetes/Firmicutes ratio in the rectum	-
Carbajo-Pescador *et al*[8]	Juvenile male Wistar rats on early obesity and non-alcoholic fatty liver disease onset	Fecal matter	Treadmill, 11 wk total, 60 min/d combined aerobic and resistance training (10-min running; eight 2-min progressive incline run from 10°-25° at 20-25 cm/s /1 min rest; 30 min aerobic exercise)	↑ Parabacteroides ↑ Bacteroides ↑ Flavobacterium genera ↓ Blautia ↓ Dysgonomonas ↓ Porphyromonas	
Clarke *et al* [10]	Human, rugby player, male	Fecal matter, self-collected	Rugby training, capacity determined by EPIC-Norfolk questionnaire	↑ Akkermansia (than high-BMI controls) ↓ Bacteroides (than low-BMI controls) ↓ Lactobacillus (than low-BMI controls)	-
Bressa *et al* [24]	Human, female, premenopausal, BMI 20-25 kg/m^2	Fecal matter, self-collected	No forced exercise, physical activity level gauged by accelerometers	↑ Akkermansia muciniphila ↑ Faecalibacterium prausnitzii ↑ Roseburia hominis	-
Munukka *et al*[26]	Human, female, sedentary, BMI > 27.5 kg/m^2	Fecal matter, self-collected	Ergometer, Weeks 1-2: at 60 rpm, low intensity, 3 d/wk, 40 min/d Weeks 3-4: 3 d/wk, 50 min/d, every other session 3 10-min intervals of moderate-intensity cycling, the rest low intensity Weeks 5-6: 3 d/wk, 60 min/d, four 10-min moderate intensity intervals, the rest low intensity	↑ Akkermansia ↑ Anaerofilum ↑ Bifidobacteriaceae ↑ Dorea ↑ Verrucomicrobiaceae ↓ Desulfovibrionaceae ↓ Enterobacteriaceae ↓ Odoribacter ↓ Porphyromonadaceae ↓ Proteobacteria ↓ Streptococcus	-
Taniguchi *et*	Human, male, age > 60	Fecal matter,	Cycling, Weeks 1-2: 3 d/wk, 30	↓ Clostridium difficile	-

al[27]	yr, healthy	self-collected	min/d, 60% of VO_2peak (week 1), 70% of VO_2peak (week 2) Weeks 3-5: 3 d/wk, 45 min/d, 70% of VO_2max (week 3), 75% of VO_2max (weeks 4-5)	↑ Oscillospora	
Zhao *et al*[29]	Human, marathon runners	Fecal matter, self-collected	The 2016 Chongqing half marathon, before and after the race	↑ Acidobacteria ↑ Lentisphaerae	Post- *vs* pre-running
Castellanos *et al*[23]	Human	Fecal matter, self-collected	No forced exercise, physical activity level gauged by accelerometers	↑ Blautia ↑ Coprococcus ↑ Eubacterium ↓ Bacteroides ↓ Parabacteroides	-
Keohane *et al* [30]	Human, male, athlete	Fecal matter, self-collected	Rowing race, 33 d 22 h, 151.8 km per day	↑ Roseburia hominis ↑ Subdoligranulum	Post-ultra-endurance exercise
Liang *et al* [31]	Human, martial arts athlete	Fecal matter, self-collected	Martial arts, athletes, divided into higher- and lower-level based on General Administration of Sport of China criteria	↑ Bilophila ↑ Oscillibacter ↑ Parabacteroides ↑ Phascolarctobacterium ↓ Allisonella ↓ Citrobacter ↓ Megasphaera	Higher- *vs* lower-level athletes
Morita *et al* [25]	Human, female, age > 65 yr, sedentary	Fecal matter, self-collected	Trunk strengthening training, 12 wk, 1 h/wk: 5-10 min of warm-up + 45 min of targeted resistance training of trunk muscles + 5 – 10 cool-down and at-home exercise daily	↑ Bacteroides	After 12 wk of aerobic training
Hampton-Marcell *et al* [28]	Human, age 18-24 yr, swimmers	Cotton swab sample	Self-reporting of daily swimming distance and duration during daily practice	↑ Coprococcus ↑ Faecalibacterium	Before *vs* after reduction of training volume
Quiroga *et al* [14]	Human, age 7-12 yr, obese	Fecal matter, self-collected	Strength and endurance training 12 wk, 2 d/wk: Warm-up on an ergometer for 7 min, low-medium load, 60 rpm Third minute onwards, a sprint of 30s at 3’30”, 4’30”, 5’30”, and 6’30” Strength exercises for five muscle groups, initially 3 sets of 12 repetitions at 30% 1RM, up to 3 sets of 8 repetitions at 70% 1RM Cool-down at an elliptical cardiovascular device, 7 min, 50 rpm, 4 min low-medium load + 3 min high load	↑ Blautia ↑ Dialister ↑ Roseburia	After a 12-wk strength and endurance training program
Rettedal *et al* [16]	Human, male, age 20-45 yr	Fecal matter, self-collected	9 sessions of high-intensity interval training on non-consecutive days over 3 wk: 60 s cycling at VO2peak 75 s rest 8 intervals initially, up to 12 intervals by the end of the protocol	No significant changes in composition	Before and after high-intensity interval training

BMI: Body mass index.

could in part be attributed to increased gut motility during exercise, which promotes shedding of loosely bound bacteria and the growth of health-promoting species[33].

ROLE OF THE MICROBIOME IN IBD

Patients with CD have a diminished diversity of the fecal intestinal microbiome, with Lachnospiraceae, Bacteroidetes, and the species *Clostridium leptum* being decreased, with Proteobacteria, Actinobacteria, and the genus *Prevotella* being increased[13]. Patients with inflammatory bowel disease also have increased *Fusobacterium*, *Pasturellaceae*, *Ruminococcus gnavus*, *Veillonellaceae*, *Candida albicans*, *Candida tropicalis*, *Clavispora lusitaniae*, *Cyberlindnera jadinii*, *Kluyveromyces marxianus,* and *Caudivirales* in their gut microbiota, as well as decreased *Bacteroides*, *Bifidobacterium*, *Clostridium* XIVa, *Clostridium* IV, *Faecalibacterium prausnitzii*, *Roseburia*, *Suturella*, and *Saccharomyces cerevisiae* [2]. Therefore, it can be argued that intestinal dysbiosis may be a component of IBD pathogenesis[34].

The involvement of the microbiome in the pathogenesis of IBD is further supported by the effectiveness of antibiotic therapy in the treatment of certain IBD phenotypes, such as perianal CD and pouchitis, and in the prevention of postoperative relapse in patients with CD[2].

Fecal microbiota transplantation involves the transfer of feces from a donor to the GI tract of a recipient, as an attempt to enrich the recipient's gut microbiota and correct any dysbiosis. Fecal microbiota transplantation is currently systematically used in the treatment of *Clostridium difficile* colitis[35]. Some researchers have also investigated its implementation in IBD therapeutics, with some promising findings having been reported, although no concrete conclusions can yet be drawn about its efficacy and safety, plausibly owing to the dissimilarities between the associated studies[35].

ANIMAL CLINICAL DATA

Gut microbiota transplant from exercise-trained mice leads to a reduction in inflammatory markers in the distal colons of sedentary mice, combined with an attenuated colitis histology score[36]. In mouse models of colitis, voluntary treadmill exercise has been found to reduce inflammation while forced exercise exacerbates tissue damage and leads to increased mortality[37]. The same researchers then attributed part of this effect to an increase in *Tenericutes* bacteria in the large intestine in the forced-exercise group, since the family *Mollicutes*, a member of the phylum *Tenericutes*, has been linked to UC in humans[38]. In another study, exercise was shown to ameliorate the symptoms of chemically induced colitis and to alter significantly gut microbiota, with decreased populations of *Bacteroides vulgatus* and increased numbers of *Akkermansia muciniphila*[39]. In mice born without a normal mucus layer in their intestines due to a genetic knockout of mucin-2, exercise neither significantly alters the gut microbiome nor reduces the severity of chronic colitis, in contrast to wild-type mice where both effects have been observed[40].

HUMAN CLINICAL DATA

Based on current literature, it is not clear whether dysbiosis is a cause or a result of IBD[13]; however, some conclusions could be drawn from certain scientific findings: Disease activity is mostly focused on bowel segments where the fecal stream is slower and bacterial populations are higher[2]. Mutations in genes affecting the functions of intestinal Paneth cells (*e.g.*, *NOD2*), which defend the small intestine against bacteria, are risk factors for IBD[2]; moreover, exposure to antibiotics in early life is linked to IBD later in life[13]. Antibiotics are, however, effective in certain conditions involving IBD, such as in inflammation of the ileoanal pouch after colectomy indicated by IBD-related complications[41]. Surgical fecal diversion is beneficial in the treatment of CD, as bowel segments excluded from the fecal stream tend to show remission[2]; furthermore, probiotics and fecal transfer are a therapeutic option for inducing and maintaining IBD remission[13]. These findings suggest that there exists a causal component to the microbiotic patterns associated with IBD, despite the inconsistencies between observed microbiotic changes as reported by different studies[13].

Regarding the probable protective effect of exercise in IBD, it is thought to stem from anti-inflammatory actions[6]. These include the secretion of myokines by skeletal muscles, such as myostatin, irisin, IL-15, brain-derived neurotrophic factor, myonectin, decorin, and secreted protein acidic and rich in cysteine, mediators with autocrine, paracrine, and endocrine anti-inflammatory actions[42]; moreover, in obese humans, exercise has been found to alter the gut microbiome and reduce endotoxemia, as

measured by the levels of the endogenous protein lipopolysaccharide binding protein [11], thus suggesting another probable anti-inflammatory effect for physical activity. Exercise training has also been found to reduce the levels of NLR Family Pyrin Domain Containing 3 and caspase 1, proteins that participate in the inflammasome activation pathway, in obese children[14].

Several mechanisms have been proposed by which exercise may influence gut microbiota. These include crosstalk between muscles and the gut microbiota through the 5′ adenosine monophosphate-activated protein kinase and fasting-induced adipose factor pathways as well a reduction of fecal bile acids, an increase in production of short-chain fatty acids, an increase in gut luminal immunoglobulin A, a reduction in luminal transit time, and the activation of the stress hypothalamic-pituitary-adrenal axis, effects found to be produced by exercise[43-46].

The functions of a healthy microbiome include metabolic functions, such as the production of anti-inflammatory short-chain fatty acids, vitamin K, and biotin, protective effects, such as induction of secretions that attack pathogenic bacteria, triggering of mucosal proliferation through the Toll-like receptor pathway, inhibition of adhesion of pathogenic bacteria, and trafficking of neutrophils, as well as trophic functions, *i.e.* protecting the intestinal mucosa from immune-mediated damage[46-48]. Part of the pathogenesis of IBD is thought to include a loss of these effects, probably caused by a damaging shift to the microbial composition in the gut[1]. These include reductions in Bacteroidetes, *Clostridium leptum*, *Prevotella*, *Bifidobacterium*, and *Roseburia* as well as increases in Proteobacteria, as mentioned above. All of these alterations are restored by exercise interventions in animal and human studies (Table 1), suggesting a plausible mechanism for the beneficial effect of exercise in IBD.

Current exercise guidelines from the American Heart Association for adults aged 18 years to 65 years of age recommend moderate-intensity aerobic exercise for at least 30 min, 5 d a week or vigorous-intensity exercise physical activity for 20 min, 3 d a week [49]. No specific guidelines exist for patients with IBD, however, evidence suggests that mild-to-moderate exercise harbors multiple benefits for patients with at least mild IBD, and excessive exercise could pose hazards for patients' health[41]; therefore, physicians should be cautious when prescribing exercise for patients with IBD, being on the lookout for exercise addiction[41].

Clinical data studying the association of IBD with exercise suggest that sedentary occupations confer a higher risk for IBD than more physically demanding occupations, as found in a retrospective study of German employees[50]; moreover, both CD and UC have been associated with low physical activity during childhood[51]. Exercise has been reported to decrease the risk of relapse in patients with IBD in remission[52]. Mild-to-moderate exercise is beneficial in patients with at least mild IBD[41]. A recent study reported that prolonged moderate-intensity walking did not appear to increase blood cytokine levels in patients with IBD more than it did in healthy controls, and fecal calprotectin levels were found to be comparable between patients who walked and patients who did not walk, suggesting that exercise does not cause exacerbation of IBD[53].

CONCLUSION

The findings of the present review imply that there exists a promising field of research regarding exercise-induced changes of the microbiome in IBD. What needs to be elucidated is whether the microbiome is a passive "bystander" in the systemic effects induced by physical activity, *i.e.* observing and reacting to activity-related systemic metabolic and endocrine signals by altering its composition, or whether it is a necessary physiological intermediate in the restoration of immune tolerance and normal gastrointestinal function in the context of IBD. Further research should also focus on disease determinants, such as age, sex, type, localization, histology, refractory phenotype, disease activity, molecular markers, and performance status, which could affect the disease's response to certain types of physical exercise. Besides, considering that there is not only one optimal microbiota composition for the IBD patients, more studies are also needed to reveal how microorganisms interact with each other and with their host to identify different healthy microbiota schemes and an optimal, potentially personalized, dose of exercise for these patients. Lastly, an interesting field might exist for the microbiome as an index predictive or indicative of exercise-induced amelioration of IBD clinical symptoms, as part of current research on the molecular epidemiology of IBD.

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MINIREVIEWS

Gastrointestinal mucosal immunity and COVID-19

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Abstract

As the gastrointestinal tract may also be a crucial entry or interaction site of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the role of the gut mucosal immune system as a first-line physical and immunological defense is critical. Furthermore, gastrointestinal involvement and symptoms in coronavirus disease 2019 (COVID-19) patients have been linked to worse clinical outcomes. This review discusses recent data on the interactions between the virus and the immune cells and molecules in the mucosa during the infection. By carrying out appropriate investigations, the mucosal immune system role in SARS-CoV-2 infection in therapy and prevention can be established. In line with this, COVID-19 vaccines that stimulate mucosal immunity against the virus may have more advantages than the others.

Key Words: Mucosa; Gut mucosa; Mucosa-associated lymphoid tissue; SARS-CoV-2; COVID-19; Secretory immunoglobulin A; Gut microbiota

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Core Tip: The gastrointestinal tract is a frequent route of infection with severe acute

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respiratory syndrome coronavirus 2. Given the complex interactions between the virus and the mucosal immune system after exposure, additional research is needed to elucidate the immune mechanisms and processes in the gut mucosa. The hallmark of all immune responses is the recruitment of various immune cells, such as neutrophils, dendritic cells, macrophages, and T cells in the gut mucosa. However, the mucosal inflammatory response could change intercellular space between enterocytes, leading to an increase in intestinal permeability that allows various bacterial antigens and toxins to enter the bloodstream, further complicating the disease state of coronavirus disease 2019 patients.

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INTRODUCTION

It is well-established that nasopharynx-associated lymphoid tissue (NALT) and mucosa-associated lymphoid tissue (MALT) are first-line defenses. Therefore, airborne infections start by penetrating the upper airway mucosa, where a higher viral load is found, compared with the throat. NALT is involved in the induction of the immune response towards the microorganisms by promoting the differentiation and activation of immune cells such as Th1- and Th2 cells, dendritic cells, macrophages, resident microfold M cells, innate lymphoid cells, immunoglobulin (Ig)A-switched B cells, as well as immune mediators and molecules (*i.e.* beta-defensins, galectins, collectins, and cytokines)[1]. The same goes for the gut mucosal immune system.

It is thus not surprising that NALT exerts "gate control" on many infections that penetrate the mucosa, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)[2]. SARS-CoV-2 acts cytopathically at the mucosal level by inducing injury and death of the infected cells. That can be accomplished by pyroptosis as a consequence of activation of host-cell released damage-associated (DAMPs) and viral pathogen-associated molecular patterns (PAMPs) and innate immunity along with the secretion of many cytokines [interleukin (IL)-6, interferon (IFN)-gamma, MCP1, and IP-10)][1]. On the other hand, pattern-recognition receptors and their soluble forms are mainly involved in SARS-CoV-2 infection[3,4].

Amongst the immune molecules, the collectins have a significant role in recognizing glycoside structures of the virus. For example, deficiency of mannose-binding lectin (MBL) has been associated with increased susceptibility to viral infections, including SARS-CoV[5]. Additionally, MBL can inhibit S protein by binding to it and inhibiting structural S rearrangements needed for optimal infection, thus leading to reduced virulence of SARS-CoV-2. Furthermore, as the expression of MBL declines with aging, older adults may be more prone to infection because of lack of an effective innate response[5].

Additionally, the presence of natural, pre-existing IgM and IgA antibodies produced in the absence of any antigen, provides the first-line defense[6]. Furthermore, some of the natural antibody subsets can recognize ABO blood-group antigens, which are expressed on many epithelial cells, including the lung[7]. As enveloped viruses like SARS-CoV-2 are highly glycosylated, it is thought that when virions reproduce in the alveolar epithelial cells in people with group A or B blood, those antigens may be expressed on their envelope. Thus, one may suggest that natural antibodies against A and B antigens may be protective[8]. Studies have so far revealed that anti-A antibodies can inhibit S protein binding to angiotensin-converting enzyme 2 (ACE2) receptors when the host cells express A antigen. Individuals with group O blood have a reduced risk of infection compared with those who have non-O blood groups, and those with group A blood are prone to severe coronavirus disease 2019 (COVID-19)[8].

EPIDEMIOLOGICAL SIGNIFICANCE OF MUCOSAL PENETRATION AND REPLICATION FOR THE SPREAD OF SARS-COV-2

COVID-19 is an infectious disease in which the primary mode of transmission of its causative agent, SARS-CoV-2, is by transfer of saliva microdroplets between people in close contact. The microdroplets are produced while coughing, sneezing, or talking. Infection by contact with contaminated surfaces followed by touching the face is less common. Most microdroplets fall to the ground or surfaces and are not effective over long distances. The first 3 d after the onset of symptoms is when the patient is most contagious. Transmission may occur before symptoms appear. Asymptomatic people may thus be contagious, too[9].

COVID-19 is an airborne viral disease. It was shown that SARS-CoV-2 penetration into the upper airways is the first step of the infection, as higher viral loads were found in nasal swabs than throat swabs[10,11]. The same distribution as in symptomatic patients was observed in asymptomatic patients, implicating the nasal epithelium as a portal for initial infection and transmission[12]. The nose is a critical component of mucosal immunity, providing protection in the upper airway. It is involved both in host protection and immune homeostasis between the commensal microbiota and invading pathogens. The mucosal immune system is the first line of physical and immunological defense against invading pathogens[13]. Current evidence indicates that SARS-CoV-2 enters the human body mainly through the ACE2 + transmembrane protease/serine subfamily member 2 (TMPRSS2) + nasal epithelial cells. The initial host response to this pathogen begins in the NALT system[1].

VIRAL FACTORS OF SARS-COV-2 AND IMPACT ON THE MUCOSA

Coronaviruses are enveloped, positive-sense single-stranded RNA viruses that are members of families *Coronaviridae*, order Nidovirales. There are four known genera, *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*. SARS-CoV-2 belongs to the family *Coronaviridae* and genus *Betacoronavirus*[14,15]. Diseases caused by coronaviruses comprise symptoms that range from mild respiratory illness like the common cold to severe infections causing death. These viruses can infect humans, mammals, and avian species, including farm and companion animals (pets). Hence they represent not only a challenge for public health but also are of veterinary and economic concern. From the beginning of the 21st century, the SARS epidemic in 2002-2003, the Middle East respiratory syndrome (MERS) in 2012, and the emergence of the new SARS-CoV-2, are examples of human infections caused by coronaviruses[16,17].

Coronaviruses are spherical, enveloped RNA viruses containing an impressively large (25 kb to 32 kb), nonsegmented, single-stranded, positive-sense RNA genome, which is the same sense as the messenger RNA (mRNA) found in cells. The genome codes four main structural proteins, nonstructural proteins (NSPs), and accessory proteins. The structural proteins, which include the spike (S), nucleocapsid (N), membrane (M), and envelope (E) proteins, play essential roles in the arrangement of the virus particles and other aspects of the viral life cycle[18]. Among the structural proteins, the most important is S protein, which is required for viral entry as it binds to the target cell receptors and initiates fusion with the cell membrane[19].

SARS-CoV-2 spike (S) protein is a large glycosylated transmembrane, homotrimeric protein. Each monomer has a molecular mass of about 150-200 kDa[18]. Each subunit of the protein consists of two functionally distinct domains, S1 and S2. S1 forms the bulb portion of the spike protein on the virion surface. S2 attaches the S proteins to the viral membrane. The receptor-binding domain is located on S1; S2 is necessary for membrane fusion to allow the viral cell entry[20]. Transmembrane ACE2 is the functional host receptor for SARS-CoV-2[21]. ACE2 is widely expressed in the ciliated, goblet, and surfactant-producing type-2 alveolar cells of the lungs, intestinal, cardiac, and vascular endothelia, the kidney, and the liver[22].

Other receptors, such as DC-SIGN, L-SIGN, Neuropilin-1, furin, and cathepsin B and L can serve as portals of virus entry into the cell. Taken together, the findings provide a possible explanation for the occurrence of COVID-19 complications in organs expressing those receptors. The binding of S protein to the host ACE2 receptor alone is not enough for the viral fusion. The spike protein needs to be cleaved by cell surface serine proteases at specific sites (S1/S2 boundary and S2`), releasing the S1 domain, which subsequently activates the S2 domain, leading to fusion of the viral cellular membranes[23]. Host-cell surface serine proteases shown to cleave the S proteins include, but are not limited to, TMPRSS2, furin, and trypsin. Both ACE2 and

TMPRSS2 (also furin) are highly expressed in the gastrointestinal (GI) tract, particularly intestinal epithelial cells[24]. The primary entry site of SARS-CoV-2 is host lung cells. Nevertheless, the GI may also be a crucial entry or interaction site. SARS-CoV-2 viral particles are preferentially released apically and not at the basement of the airway cells. Thus, the released SARS virus may be removed by mucociliary clearance with access to the GI *via* luminal exposure. Moreover, the early appearance of gastrointestinal symptoms such as nausea, vomiting, abdominal pain, and diarrhea in almost 30% of COVID-19 patients supports this hypothesis.

Viral factors of SARS-CoV-2 and host innate immune response

After the fusion with the cell membrane, the viral genome is released into the host-cell cytoplasm, and the highly controlled process of viral RNA replication and transcription occurs[23]. The virus interacts with cellular compartments and proteins to make its RNAs and proteins. It has been shown that some viral proteins influence critical host-cell processes such as apoptosis, necrosis, innate immunity, and others. One of the structural proteins, nucleocapsid (N) protein, despite its central role in binding to the viral RNA genome, was shown to inhibit type I IFN production and signaling. Recent studies provide evidence that NSP1 (participates in host-cell mRNA degradation and translation inhibition[25]. Hence, the translation of vital cellular proteins, including type I IFN, is shut down, allowing viral RNA to be translated effectively[26]. That may be the reason why NSP1 is cleaved and activated immediately after the production of polypeptide pp1a.

PLpro and Mpro (3CLpro) viral proteases are necessary for the proteolytic cleavage of the polyproteins (pp1a and pp1ab)[20]. Furthermore, it has been shown that the proteases play a role in inhibition of type I IFN signaling. PLpro is responsible for only a few cleavage events in pp1a, but it also can act as a deubiquitinase and deISGylating (removal of IFN-stimulated gene 15 from proteins), which are enzyme activities that lead to evasion and the initial steps of the antiviral response[27,28].

During viral RNA replication and transcription, various PAMPs are produced in the form of double-stranded RNA intermediates. It has been suggested that some of the viral proteins (*i.e.* E, N, NSPs) are involved in the formation of convoluted membranes and double-membrane vesicles to create a protective microenvironment for genomic RNA replication and transcription of subgenomic mRNAs[23]. Furthermore, the PAMPs are recognized by endosomal Toll-like receptors (TLR 3, 7, 8) or cytoplasmic RNA PRRs, such as retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5). The proper function of TLRs, RIG-I, and MDA-5 is crucial for host-cell survival, as the receptors provide a first-line defense against infections[29-31]. The viral genome attachment to the receptors, especially the TLRs, gives rise to innate immune response signaling pathways. In leukocytes (*e.g.*, dendritic cells, macrophages, natural killer cells, T cells of the adaptive immune system, and B cells), interaction with viral RNA triggers innate immune responses and development of acquired antigen-specific immunity[31]. The innate immune system senses the foreign viral materials that are possibly pathogenic, which initiates downstream signaling to the nucleus, and in turn promotes the expression of types I and III IFNs and other proinflammatory cytokines. Once activated, the IFNs induce a cascade of cellular and molecular events that lead to the suppression of viral replication and reduction in the number of infected cells. Many viral proteins influence the IFN signaling pathway, thus providing a favorable environment for virus development. PLpro, NSP1, ORF3b (a viral accessory protein), and N inhibit two proteins (IRF3 and IRF7) required for INF transcription, thus inhibiting the first steps in the innate immune response against the virus[29-31]. ORF3a and ORF3b are viral proteins that induce caspase-independent necrotic cell death and initiate an inflammatory cascade through activation of the NLRP3 inflammasome[32].

It should be noted that necroptosis and pyroptosis are highly inflammatory mechanisms of cell death that lead to increased secretion of proinflammatory cytokines and chemokines, thus contributing to further tissue damage. Several studies have found that expression of N, E, M, ORF3a, ORF3b, ORF7a, ORF8a, or ORF9b proteins in various cell lines triggered apoptosis through cytochrome C release and caspase-dependent pathways. Apoptosis is a form of noninflammatory cell death that often serves as a host response during viral infection. At the moment, it is not yet clearly elucidated what the exact role of SARS-CoV-2 induced cell death is. It may be an exit strategy to increase viral spread, a form of immune evasion, or just an indirect effect of viral replication on the host cell cycle[23].

Proteome analysis of SARS-CoV-2 has shown that viral proteins interact with more than 300 host-cell proteins, leading to cellular mRNA degradation, inhibition of translation, inhibition of IFN production and signaling, induction of apoptosis and

necrosis, and other activities. The hallmark of all these functions is an evasion of host innate immune responses that could facilitate viral spreading to nearby cells. The changes in infected enterocytes could result in the recruitment of neutrophils, dendritic cells, macrophages, and T cells in the gut mucosa. The mucosal inflammatory response could change the intercellular space between enterocytes, leading to an increase in intestinal permeability that provides an opportunity for bacterial antigens and toxins to enter the bloodstream and further complicated the disease state of COVID-19 patients.

SARS-COV-2 AND MUCOSAL IMMUNITY OF THE GI

An effective and powerful adaptive immune response follows the early antiviral innate response in the mucosa. Expansion of CD4+ T-helper cells, CD8+ cytotoxic T cells, and plasma cells simultaneously with the ongoing innate immune response is critical for virus elimination[33]. However, many factors can alter the immune response and control of the viral replication cycle. External factors such as smoking, pollutants, temperature, humidity, and internal factors such as age and genetics negatively affect the effective response to SARS-CoV-2. Additionally, defects in the immune response towards the virus, such as reduced MBL levels and natural antibodies, anti-IFN autoantibodies, and impaired cytotoxic CD8+ cells, may lead to severe infection because of a lack of effective control of viral replication and the spread of propagation from the upper to the lower airway. Simultaneously, a vast release of proinflammatory cytokines and the recruitment of neutrophils, macrophages, and other cell types contribute to uncontrolled systemic inflammation and cytokine storm [33].

We have to mention the thick layer of mucus on the respiratory, gastrointestinal, and reproductive mucosal surfaces in most mammals that contributes to the first-line defense against various infections. Many studies have reported the essential role that mucins play in infectious diseases, including COVID-19[34,35]. In addition, glycans are complex molecules glycans that play a critical role in communication between cells, including adhesion. The extracellular N-terminal domain and intracellular C-terminal domain undergo biochemical changes during bacterial, viral, and parasitic infections by directly influencing both proinflammatory and anti-inflammatory responses[36]. Mucins sense ligands of pathogenic origin and pass the information downstream by activating immunomodulatory pathways. Currently, 22 genes for membrane-bound and secretory mucins have been documented in humans. Recent data suggest that they can be an entry and/or exit for SARS-CoV-2[36]. Furthermore, mucin levels in bronchoalveolar lavage fluid were shown to correlate with cytokine levels, predicting the magnitude of inflammation (*i.e.* cytokine storm), the hallmark of severe COVID-19 and acute respiratory disease syndrome (ARDS). Prognosis and the response to therapy were also influenced by mucin levels[34].

As the virus can enter through the mouth mucosa or the conjunctival surface of the eye, an excellent immune system response would begin with primed and activated immune cells and molecules, including secretory IgA, and SIgA and then spread through the entire mucosa. Moreover, bronchus-associated lymphoid tissue might contribute to the greater resistance to COVID-19 in children, adolescents, and young people compared with older adults[37]. Particular attention should be paid to SIgA, which plays an effective role in protection against various pathogens by neutralization, inhibition of adherence, and agglutination. As SIgA does not activate the classical complement cascade pathway, it is more anti- than proinflammatory[38]. Furthermore, IgA can inhibit IgM or IgG antibody-activated complement. It has been shown that the mucosal immune response with involvement of SIgA begins around 6-10 d after SARS-CoV-2 infection, with the expression of α4β7 integrin mucosal homing receptors and terminal differentiation of B cells into pIgA-secreting plasma cells in NALT[39]. Serum and salivary IgA antibodies against the spike protein of SARS-Cov-2 have also been reported[40]. Moreover, the salivary IgA has been shown to persist for at least 3 mon. Indeed, IgA antibodies against SARS-CoV-2 were found to be higher in the nasal mucosal fluids, tears, and saliva of infected subjects[41,42], IgA-switched plasmablasts that bear the mucosal chemokine receptor CCR10 were increased in the peripheral blood of SARS-CoV-2-infected subjects[42]. Thus, now we have more data on the IgA antibody production in response to SARS-CoV-2 infection.

In addition to MALT, mucosal-associated invariant T cells were also described. They comprise innate-like T cells (*e.g.*, invariant natural killer T, innate lymphoid cells, and γδ T cells) involved in pulmonary mucosal antiviral immunity and tissue protection

and repair after resolving the infection[43]. Pearson *et al*[44] focused on local mucosal responses during viral infection, particularly with SARS-CoV-2 in both lungs and gut. They found that IL-33 and IL-8 were increased in fecal samples of COVID-19 patients due to intestinal involvement[44,45]. Simultaneously, cytokines such as IL-1b, tumor necrosis factor alpha, and IL-6 were found to decrease. IL-7, a critical cytokine for T cell development and survival was also increased during gastrointestinal infection [46]. In addition to the other T cells in the gut mucosa during COVID-19 infection, and enhanced effector function of Th17 cells has also been seen[47]. By secreting many cytokines, they contribute largely to the acute lung injury observed in severe COVID-19 cases. However, their role in mucosal SARS-CoV-2 infection needs to be elucidated.

Considering the route of infection and the relative independence of mucosal and systemic immune responses, one can suggest that appropriate investigations can establish the role of mucosal immune system in SARS-CoV-2 infection for therapy and prevention. In line with this, intranasal COVID-19 vaccines are an additional hope to promote mucosal immunity against the virus, an apparent advantage of other nasal vaccines (*i.e.* influenza)[48]. Moreover, the advantages of such vaccines including generation of both mucosal (SIgA) and circulating (IgG and IgA) antibodies and SARS-specific effector and memory T cell responses have not be seen in conventional vaccines[49].

It is speculated that anti-SIgA antibodies can neutralize and eliminate SARS-CoV-2 in the mucosa without inflammatory consequences. Furthermore, testing for IgA antibodies in nasal and saliva samples might indicate the presence of mucosal immune responses against SARS-CoV-2. Additionally, serum IgA is distinct from locally secreted IgA^+ dimers in plasma cells in the lamina propria of mucosal tissues[50]. The interaction between the virus and the mucosal immune system is shown in Figure 1.

GASTROINTESTINAL INVOLVEMENT DURING SARS-COV-2 INFECTION

Along with its other functions, ACE2 participates in the uptake of amino acids in intestinal epithelial cells, expression of antimicrobial peptides, and gut microbiome ecology[21,51]. As stated above, ACE2 is expressed in almost all human organs, but in varying degrees. Active replication of the SARS-CoV-2 virus has been detected in small-intestine enterocytes isolated from fecal specimens[52]. Other studies showed that the SARS-CoV-2 spike glycoprotein had a 10- to 20-fold higher binding affinity to ACE2 compared with SARS-CoV[53]. ACE2 is highly expressed in the GI[11], and in addition to the small intestine, ACE2 is also highly expressed in the pancreas[54]. Recent studies of single-cell mRNA expression found enriched expression of ACE2 and TMPRSS2 in enterocytes and mucus-producing cells[11,55].

The physiological activities of ACE2 include the absorption of nutrients from digested food. It also maintains osmotic and electrolyte balance across the GI lining epithelium by regulating sodium-dependent amino acid and glucose transporters in the enterocyte brush border[51]. Infectious diarrhea and malabsorption disorders that result from SARS-CoV-2 infection can be explained from a pathophysiological standpoint by the dysregulation of intestinal ion transporters[56]. Studies also suggest dysregulation of these transporters leads to inflammation and GI symptoms[57]. A similar mechanism of enhanced ACE2 expression is known to occur in irritable bowel disease patients who present symptoms similar to those of patients with SARS-CoV-2 [58]. GI cells are potential sites for virus replication of SARS-CoV-2 because of the enriched expression of ACE2 receptors in the mucosal glands and enterocytes[53]. A study using a recombinant strain of SARS-CoV-2 confirmed in situ that the virus could potentially infect and replicate in human intestinal tissue[52]. Once the virus enters the GI cells, it can replicate there, and viral toxin-mediated cell injury can cause gastroenteritis-like symptoms, including diarrhea, nausea, vomiting, and abdominal pain[59].

Infection caused by SARS-CoV-2 is often associated with typical respiratory response and prevalent gastrointestinal symptoms. ACE2 receptors in the GI play a vital role in the genesis of gastrointestinal symptoms. The mechanism underlying the gastrointestinal symptoms may involve damage to the intestinal mucosal barrier and promote the production of inflammatory factors[60]. Studies show that the incidence of gastrointestinal symptoms in SARS-CoV-2 and MERS-CoV infection is more than 20%[61]. Gastrointestinal symptoms may include vomiting, diarrhea, or abdominal pain in the disease's early phases[62]. The cause of the symptoms is an alteration of intestinal permeability and enterocyte dysfunction[63]. One of the first COVID-19 studies included 204 patients from Wuhan, China, infected with the virus with typical respiratory symptoms, many of whom also showed gastrointestinal symptoms, most

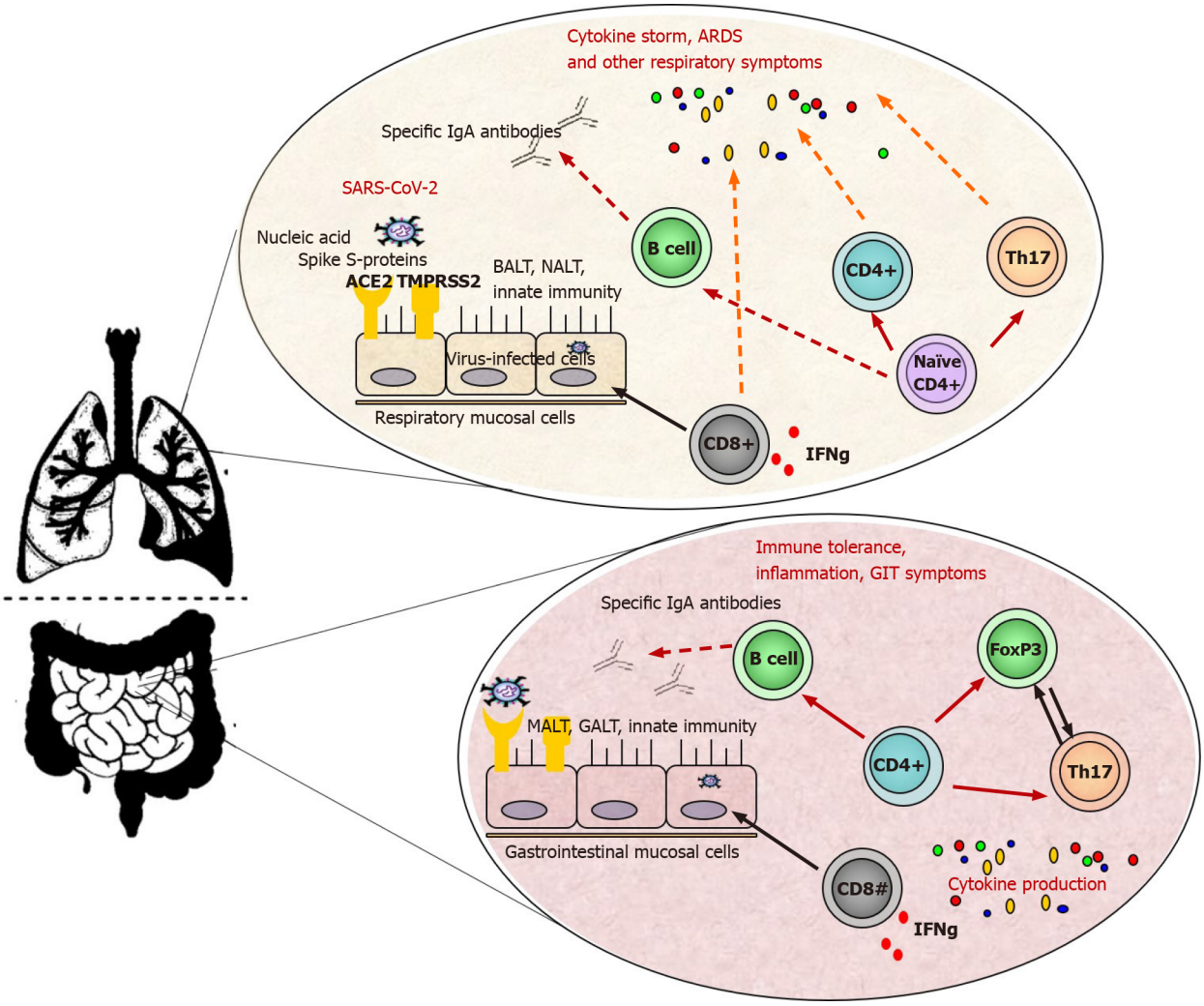

Figure 1 Nasal-, bronchial- and mucosa-associated lymphoid tissue are the first line of defense. Airborne infections usually penetrate the upper airway mucosa, where a higher viral load is found. Nasopharynx-associated lymphoid tissue is involved in the induction of the immune response against the microorganisms by promoting the differentiation and activation of immune cells such as Th1- and Th2 cells, dendritic cells, macrophages, resident microfold M cells, innate lymphoid cells, immunoglobulin (Ig)A-switched B cells, as well as immune mediators and molecules (*i.e.* beta-defensins, galectins, collectins, cytokines). Similar immune processes are also observed in the gut mucosa. However, the expansion of CD4+ T-helper cells, CD8+ cytotoxic T cells, and plasma cells simultaneously with the ongoing innate immune response is critical for virus elimination. Additionally, specific secretory IgA (SIgA) plays an effective role in protection against severe acute respiratory syndrome coronavirus 2 by neutralization, inhibition of adherence, and agglutination. Additionally, SIgA does not activate the classical complement cascade pathway, and thus greater anti- than proinflammatory activity. Innate immune system and some innate receptors (PAMPS, DAMPS) are not shown for simplification of the figure. ACE2: Angiotensin-converting enzyme 2; ARDS: Acute respiratory distress syndrome; BALT: Bronchial-associated lymphoid tissue; GALT: Gut-associated lymphoid tissues; GIT: Gastrointestinal tract. IFN: Interferon; Ig: Immunoglobulin; MALT: Mucosa-associated lymphoid tissue; NALT: Nasopharynx-associated lymphoid tissue; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; TMPRSS2: Transmembrane protease/serine subfamily member 2.

commonly diarrhea. Patients with digestive symptoms have a worse clinical outcome and a longer hospital stay than patients who do not suffer from these symptoms[64].

Although the underlying pathophysiology of gastrointestinal involvement of infection with SARS-CoV-2 is not fully understood, some loss of intestinal barrier integrity and gut microbes is observed. A disruption of intestinal barrier integrity activates innate and adaptive immune cells, which in turn release proinflammatory cytokines into the circulatory system, leading to systemic inflammation[65]. One piece of evidence that SARS-CoV-2 causes an inflammatory response in the gut is elevated levels of fecal calprotectin in patients infected with the virus[66]. Researchers suggest that measuring calprotectin concentrations may play a role in tracking patients infected with SARS-CoV-2. In patients with diarrhea as a symptom, the calprotectin concentrations are elevated, and higher serum IL-6 levels have been reported. It is possible that the disruption of the gut microbiota may be caused by the entry of inflammatory cells, including neutrophils and lymphocytes, into the intestinal mucosa [67]. Studies show that 34% of COVID-19 patients have digestive symptoms, with anorexia and diarrhea being the most common symptoms in adults, while vomiting is

more common in children[68]. Patients with severe COVID-19 have a higher incidence of gastrointestinal symptoms, such as diarrhea and abdominal pain, compared with patients with a mild form of the virus[69]. Nausea and/or vomiting, diarrhea, and loss of appetite are the digestive system's three most common symptoms. Their overall prevalence is around 15% in SARS-CoV-2 infections according to a recent systematic study and meta-analysis involving 6686 patients with GI manifestations. The same study also reported a loss of appetite, ranging from 1% to 79%[69]. The analysis showed that the most common symptom was anorexia (26.8%), but the mechanism remains unclear. The presumption is that widely spread taste and olfactory dysfunctions played a role[70]. Liver injury has also been reported in some patients, with an incidence of 39.6% to 43.4%. The most commonly found elevations are of alanine aminotransferase and aspartate aminotransferase as well as hypoalbuminemia [71].

GUT MICROBIOTA AND COVID-19

The gut microbiota consists of 10^{14} resident microorganisms, including bacteria, viruses, archaea, and fungi[72]. Principally, the gut bacteria in healthy people is dominated by four phyla Actinobacteria, Proteobacteria, Firmicutes, and Bacteroidetes [73]. The gut microbiota has a validated health role through its protective, trophic, and metabolic actions[73]. Loss of healthy commensal bacteria and overgrowth of pathogenic microbes is described as dysbiosis and critical illness. Dysbiosis is related to increased susceptibility to sepsis, multiorgan failure, and nosocomial infections[74]. The development of gut microbiota alternations in COVID-19 depends on SARS-CoV-2 occurrence, the pharmacotherapy of COVID-19, and the disease-associated GI symptoms[75,76].

In a recent study, Xu *et al*[76] described a decrease of beneficial genera, such as *Lactobacillus* and *Bifidobacterium*, in some patients with COVID-19. In another study, Zuo *et al*[77] investigated gut microbiota in 15 SARS-CoV-2 patients by taking fecal samples 2-3 times during their hospital stay. They found reduced commensal bacteria (*Faecalibacterium prausnitzii*, *Eubacterium ventriosum*, *Roseburia*, and *Rachnospiraceae* taxa) and an increased amount of opportunistic pathogens (*Actinomyces viscosus*, *Clostridium hathewayi*, and *Bacteroides nordii*). Furthermore, the abundance of *Clostridium ramosum*, *Coprobacillus*, and *Clostridium hathewayi* was associated with COVID-19 severity[78]. Another exciting study noted that COVID-19 patients had a significantly reduced microbial diversity, a higher abundance of opportunistic bacteria (Streptococcus, Rothia, Veillonella, and Actinomyces), and an increased abundance of beneficial microbes. Additionally, it showed that the microbial signature in the patients was different from those with influenza A and in healthy controls[78]. In summary, the gut microbiota in SARS-CoV-2 infected patients is modified by the reduction of commensal microbes, loss of bacterial diversity, and increased opportunistic pathogens.

The pharmacological therapies used to treat COVID-19 contribute to gut microbiota alterations. A variety of drugs used to treat COVID-19. Among them are chloroquine phosphate, lopinavir, ritonavir, and remdesivir. In cases with pneumonia, broad-spectrum antibiotics are also administered[79]. Antibiotics are well-known modifiers of gut microbiota, and even if short-term use can reduce microbial diversity and cause dysbiosis[80]. Angelakis *et al*[81] demonstrated that gut microbiota alterations were associated with long-term doxycycline and hydroxychloroquine use, leading to significantly decreased amounts of Bacteroidetes, Firmicutes, and *Lactobacillus*. Such changes may also occur in COVID-19 patients, causing gut dysbiosis. Therefore, they may cause the development of gut dysbiosis-related diseases even after improvement of COVID-19 infection. Consequently, it is suggested to screen stool samples taken from recovered patients at least 35 d after the clearance of the virus from the respiratory tract. Before 35 d, SARS-CoV-2 may still be detected in feces[82]. It is also advised to screen the composition and the activity of gut microbiota to describe its balance.

CONCLUSION

As the virus can enter through mouth mucosa, the expectation is that the triggered immune response that occurs somewhere in the mucosa will spread throughout the entire mucosa. This is especially valid for the GI, which may also be a crucial entry or

interaction site of SARS-CoV-2 infection, leading to complex immune activation, digestive symptoms, altered microbiome, development of complications, and eventually to severe COVID-19 and fatal outcome. However, the role of the gut mucosal immune system as the first line of physical and immunological defense is critical. By carrying out appropriate investigations, the mucosal immune system's role in SARS-CoV-2 infection for therapy and prevention can be established. In line with that, COVID-19 vaccines that stimulate mucosal immunity against the virus may have more advantages than other types of vaccines.

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Basic Study

Estrogen augmented visceral pain and colonic neuron modulation in a double-hit model of prenatal and adult stress

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Abstract

BACKGROUND

Chronic stress during pregnancy may increase visceral hyperalgesia of offspring in a sex-dependent way. Combining adult stress in offspring will increase this sensitivity. Based on the evidence implicating estrogen in exacerbating visceral hypersensitivity in female rodents in preclinical models, we predicted that chronic prenatal stress (CPS) + chronic adult stress (CAS) will maximize visceral hyperalgesia; and that estrogen plays an important role in colonic hyperalgesia.

AIM

The aim was to illuminate the role of estrogen in colonic hyperalgesia and its underlying mechanisms.

METHODS

We established a CPS plus CAS rodent model in which the balloon was used to distend the colorectum. The single-fiber recording *in vivo* and patch clamp experiments *in vitro* were used to monitor the colonic neuron's activity. The reverse transcription-polymerase chain reaction, western blot, and immunofluorescence were used to study the effects of CPS and CAS on colon primary afferent sensitivity. We used ovariectomy and letrozole to reduce estrogen levels of female rats respectively in order to assess the role of estrogen in female-specific enhanced primary afferent sensitization.

RESULTS

Spontaneous activity and single fiber activity were significantly greater in females than in males. The enhanced sensitization in female rats mainly came from low-threshold neurons. CPS significantly increased single-unit afferent fiber activity in

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L6-S2 dorsal roots in response. Activity was further enhanced by CAS. In addition, the excitability of colon-projecting dorsal root ganglion (DRG) neurons increased in CPS + CAS rats and was associated with a decrease in transient A-type K^+ currents. Compared with ovariectomy, treatment with the aromatase inhibitor letrozole significantly reduced estrogen levels in female rats, confirming the gender difference. Moreover, mice treated with letrozole had decreased colonic DRG neuron excitability. The intrathecal infusion of estrogen increased brain-derived neurotrophic factor (BDNF) protein levels and contributed to the response to visceral pain. Western blotting showed that nerve growth factor protein was upregulated in CPS + CAS mice.

CONCLUSION

This study adds to the evidence that estrogen-dependent sensitization of primary afferent colon neurons is involved in the development of chronic stress-induced visceral hypersensitivity in female rats.

Key Words: Chronic prenatal stress; Estrogen; Visceral pain; Neuronal sensitization; Excitability; Letrozole

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Core Tip: We investigated whether estrogen re-enhanced visceral hyperalgesia in chronic prenatal stress plus chronic adult stress models. After using physical ovariectomy or chemical inhibition with letrozole to reduce estrogen levels, we found that visceral hyperalgesia, colonic afferent neuronal excitability, nerve growth factor and brain-derived neurotrophic factor, and estrogen were all increased. The findings indicate that chronic stress-induced visceral hypersensitivity was estrogen dependent and that the hypersensitivity was mediated by estrogen-dependent sensitization of primary afferent colon neurons. The preclinical models provide key scientific evidence in support of developing gender-based visceral pain management.

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INTRODUCTION

Visceral pain of colonic origin is the most prominent symptom in irritable bowel syndrome (IBS) patients[1]. Female IBS patients report more severe pain that occurs more frequently and with longer episodes than in male patients[1,2]. The ratio of female to male IBS is about 2:1 among patients seen in medical clinics[3]. Moreover, females have a higher prevalence of IBS co-morbidities such as anxiety and depression [4,5] and are more vulnerable to stress-induced exacerbation of IBS symptoms compared with males[3,6,7].

Clinical studies show that early life adverse experiences are risk factors for the development of IBS symptoms, including visceral pain and ongoing chronic stress, especially abdominal pain[8-10]. These factors contribute to the development of visceral hypersensitivity, a key component of the IBS symptom complex and one that may be responsible for symptoms of pain[11,12]. Our previous research found that the female offspring of mothers subjected to chronic prenatal stress (CPS) had a markedly greater visceral sensitivity than their male littermates following challenge by another chronic adult stress (CAS) protocol. A critical molecular event in the development of this female-enhanced visceral hypersensitivity is upregulation of brain-derived neurotrophic factor (BDNF) expression in the lumbar-sacral spinal cord of female CPS + CAS rats[13]. However, the neurophysiological changes underlying the enhanced female-specific visceral hypersensitivity and the role of hormone in the development of stress-induced visceral hypersensitivity are not well understood.

Visceral hypersensitivity in IBS involves abnormal changes in neurophysiology throughout the brain-gut axis. In IBS, there is evidence for sensitization of primary afferents to jejunal distention and electrical stimulation[14], and there is evidence for increased sensitivity of lumbar splanchnic afferents[15,16]. In animal models of either early life adverse events or adult stress-induced visceral hypersensitivity[17], there is evidence of colon primary afferent sensitization. However, the studies were performed in male rodents. Therefore, in this study, we established a CPS and CAS rodent model to analyze the impact on female colon afferent neuron function and the role of estrogen. Our hypothesis was that female CPS offspring subjected to chronic stress as adults would exhibit greater colonic dorsal root ganglion (DRG) neuron sensitization compared with their male littermates, and that the enhanced visceral sensitization and primary afferent sensitization in females was estrogen dependent.

MATERIALS AND METHODS

Animals

The Institutional Animal Care and Use Committee of the University of Texas Medical Branch at Galveston, TX approved all animal procedures. Experiments were performed on pregnant Sprague Dawley rats and their 8-wk-old to 16-wk-old male and female offspring. Rats were housed individual cages with access to food and water in a room with controlled conditions (22 ± 2 °C, relative humidity of 50% ± 5%), and a 12 h light/12 h dark cycle.

CPS and CAS models

Pregnant dams were subjected to a CPS protocol that consisted of a random sequence of twice-daily applications of one of three stress sessions, a 1-h water-avoidance, 45-min cold-restraint, or a 20-min forced swim starting on day 6 and continuing until delivery on day 21. Male and female offspring of the stressed dams were designated CPS rats. Control dams received sham stress and their offspring were designated control rats. As adults at 8-16 wk of age, control and prenatally stressed offspring were challenged by the same CAS protocol for 9 d. Ovariectomy (OVX) or sham surgery was performed on female prenatal-stress offspring on day 56. Daily letrozole treatment was initiated on day 49, 2 wk prior to initiation of adult stress. Treatment was continued through the stress protocol. A schematic diagram of the study procedures is shown in Figure 1A.

Rat treatment

Before OVX or letrozole treatment, vaginal smears were used to identify the estrus cycle phase. OVX or sham surgery was performed on female prenatal-stress offspring on day 56. The aromatase inhibitor letrozole [4,4′-(1H-1,2,4-triazol-l-yl-methylene)-bis-benzonitrile], (Novartis) 1.0 mg/kg was orally administered in the experimental group and vehicle (hydroxypropyl cellulose 0.3% in water) was given in the control group once daily for 14 d. Direct transcutaneous intrathecal injections of estrogen and letrozole were performed as described by Mestre *et al*[18].

In vivo single fiber recording of L6-S2 DRG rootlets

Multiunit afferent discharges were recorded from the distal ends of L6-S2 dorsal rootlets decentralized close to their entry into the spinal cord. A bundle of multiunit fibers was distinguished into 2-6 single units off-line using wave mark template matching in Spike 2 software that differentiates spikes by shape and amplitude. Colonic afferent fibers were identified by their response to graded colorectal distention (CRD). A balloon was used to distend the colorectum. Isoflurane, 2.5%, followed by 50 mg/kg intraperitoneal sodium pentobarbital induced general anesthesia that was maintained by infusing a mixture of pentobarbital sodium + pancuronium bromide + saline by intravenous infusion through the tail vein. The adequacy of anesthesia was confirmed by the absence of corneal and pupillary reflexes and stability of the end-tidal CO_2 level. A tracheotomy tube connected to a ventilator system provided a mixture of room air and oxygen. Expired CO_2 was monitored and maintained at 3.5%. Body temperature was monitored and maintained at 37 °C by a servo-controlled heating blanket. A laminectomy from T12 to S2 exposed the spinal cord. The head was stabilized in a stereotaxic frame.

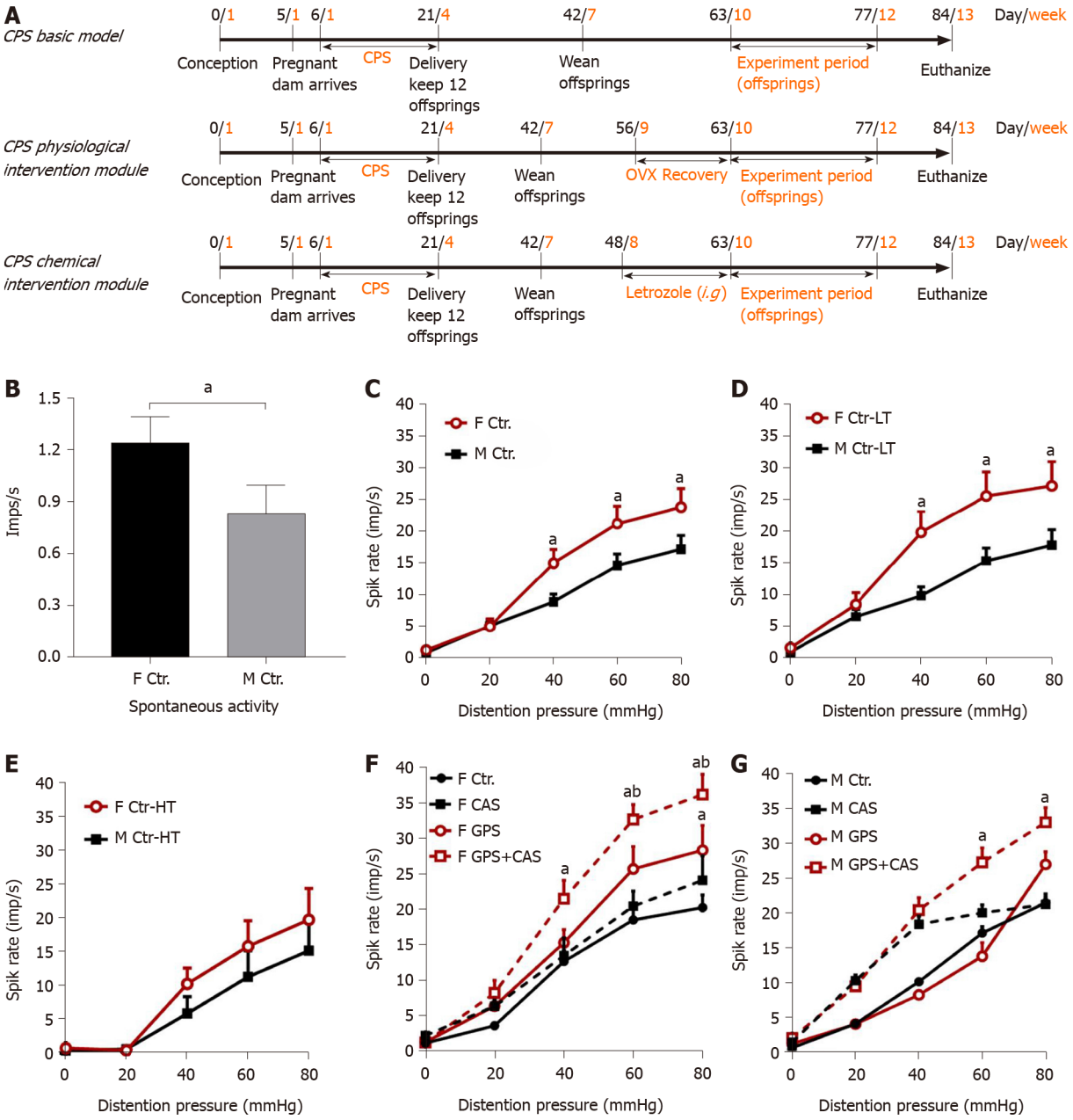


Figure 1 Primary afferent responses to colorectal distention. A: Chronic prenatal stress (CPS) plus chronic adult stress (CAS) model. Pregnant dams were subjected to prenatal stress from on day 11 of gestation. Ovariectomy (OVX) or sham surgery was performed on female prenatal-stress offspring on day 56. Daily Letrozole was initiated on day 49, 2 wk prior to initiation of adult stress. Treatment was continued through the stress protocol; B: Spontaneous activity (SA) of single afferent units in male and female control rats (n = 70 fibers in 6 rats in each group, t-test, $^{a}P < 0.05$); C: Average response to graded colorectal distention (CRD) of 56 afferent fibers in 6 male and 70 afferent fibers in 6 female control rats; two-way analysis of variance (ANOVA; $^{a}P < 0.05$ *vs* the same pressure male group); D: Responses of low-threshold (LT) fibers to CRD in 42 fibers in 6 male rats and 40 fibers in 6 female control rats (ANOVA, $^{a}P < 0.05$ *vs* the same pressure male group); E: Responses of high-threshold (HT) afferent fibers to CRD in 14 fibers in 6 male and 29 fibers in 6 female control rats; F: Effects of CAS on afferent fiber responses to CRD from 59 fibers in 6 control and 99 fibers in 6 CPS female rats; (two-way ANOVA, $^{a}P < 0.05$ *vs* the same pressure control group, $^{b}P < 0.05$ *vs* the same pressure CPS group); G: Effects of CAS on afferent fiber responses to CRD in control and CPS male rats (n = 6 rats, 57 fibers for control and 95 fibers for CPS female group; two-way ANOVA, $^{a}P < 0.05$ *vs* the same pressure-control group).

In vitro patch clamp recordings in colonic DRG neurons

Retrograde fluorescence label injections: Labeling of colon-projecting DRG neurons was performed as previously described[13]. Under general 2% isoflurane anesthesia, the lipid soluble fluorescent dye, 1,1′-dioleyl-3,3,3′,3′-tetramethylindocarbocyanine methane-sulfonate (9-DiI, Invitrogen, Carlsbad, CA) was injected (50 mg/mL) into the muscularis externa on the exposed distal colon in 8 to 10 sites (2 μL each site). To prevent leakage, the needle was kept in place for 1 min following each injection.

Dissociation and culture of DRG neurons: Rats were deeply anesthetized with isoflurane followed by decapitation. Lumbosacral (L6–S2) DRGs were collected in ice

cold and oxygenated dissecting solution, containing (in mM) 130 NaCl, 5 KCl, 2 KH_2PO_4, 1.5 $CaCl_2$, 6 $MgSO_4$, 10 glucose, and 10 HEPES, pH 7.2 (305 mOsm). After removal of the connective tissue, the ganglia were transferred to a 5 mL dissecting solution containing collagenase D (1.8 mg/mL; Roche) and trypsin (1.0 mg/mL; Sigma, St Louis, MO), and incubated for 1.5 h at 34.5 °C. DRGs were then taken from the enzyme solution, washed, and put in 0.5-2 mL of the dissecting solution containing DNase (0.5 mg/mL; Sigma). Cells were subsequently dissociated by gentle trituration 10 to 15 times with fire-polished glass pipettes and placed on acid-cleaned glass coverslips. The dissociated DRG neurons were kept in 1 mL DMEM (with 10% FBS) in an incubator (95% O_2/5% CO_2) at 37 °C overnight.

Whole-cell patch clamp recordings from dissociated DRG neurons: Before each experiment, a glass coverslip with DRG neurons was transferred to a recording chamber perfused (1.5 mL/min) with external solution containing (10 mM): 130 NaCl, 5 KCl, 2 KH_2PO_4, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose, pH adjusted to pH 7.4 with NaOH (300 mOsm) at room temperature. Recording pipettes, pulled from borosilicate glass tubing, with resistance of 1-5 MΩ, were filled with solution containing (in mM): 100 $KMeSO_3$, 40 KCl, and 10 HEPES, pH 7.25 adjusted with KOH (290 mOsm). DiI-labeled neurons were identified by fluorescence microscopy. Whole-cell currents and voltage were recorded from DiI-labeled neurons using a Dagan 3911 patch clamp amplifier. Data were acquired and analyzed by pCLAMP 9.2 (Molecular Devices, Sunnyvale, CA). The currents were filtered at 2–5 kHz and sampled at 50 or 100 s *per* point. While still under voltage clamp, the Clampex Membrane Test program (Molecular Devices) was used to determine membrane capacitance (Cm) and membrane resistance (Rm), during a 10 ms, 5 mV depolarizing pulse form a holding potential of −60 mV. The configuration was then switched to current clamp (0 pA) to determine other electrophysiological properties. After stabilizing for 2–3 min, the resting membrane potential was measured. The minimum acceptable resting membrane potential was −40 mV. Spontaneous activity (SA) was then recorded over two 30 s periods separated by 60 s without recording, as described by Bedi *et al*[19].

Transient A-type K^+ current (I_A) recording method in patch studies: To record voltage-gated K^+ current (Kv), Na^+ in control external solution was replaced with equimolar choline and the Ca^{2+} concentration was reduced to 0.03 mM to suppress Ca^{2+} currents and to prevent Ca^{2+} channels becoming Na^+ conducting. The reduced external Ca^{2+} would also be expected to suppress Ca^{2+}-activated K^+ currents. The current traces of Kv in DRG neurons were measured at different holding potentials. The membrane potential was held at −100 mV and voltage steps were from −40 to +30 mV to record the total Kv. The membrane potential was held at −50 mV to record the sustained Kv. The I_A currents were calculated by subtracting the sustained current from the total current. The current density (in pA/pF) was calculated by dividing the current amplitude by cell membrane capacitance.

Real time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using RNeasy Mini Kits (QIAGEN, Valencia, CA). One microgram of total RNA was reverse-transcribed using the SuperScript™ III First-Strand Synthesis System. PCR assays were performed on a StepOnePlus thermal cycler with 18 s as the normalizer using Applied Biosystems primer/probe set Rn02531967_s1 directed against the translated exon IX. Fold-change relative to control was calculated using the ΔΔCt method (Applied Biosystems).

Western blot

Samples were lysed in RIPA buffer containing protease inhibitor cocktail and phenylmethanesulfonyl fluoride. Lysates were incubated for 30 min on ice and then centrifuged at 10 000 × *g* for 10 min at 4 °C. The protein concentration in the supernatant was determined using bicinchoninic acid (BCA) assay kits with bovine serum albumin as a standard. Equal amounts of protein (30 μg per lane) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes (Bio-Rad, United States). The membranes were blocked in Li-Cor blocking buffer for 1 h at room temperature and then incubated with primary antibodies. BDNF antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) was used at 1:200 dilution; nerve growth factor (NGF) antibody (Abcam, MA) was used at 1:1000 dilution; β-actin antibody (Sigma Aldrich, St Louis, MO) was used at 1:5000 dilution. The secondary antibodies were donkey anti-rabbit Alexa Fluor 680 (Invitrogen) and goat anti-mouse IRDye 800 (Rockland). Images were

acquired and band intensities measured using a Li-Cor Odyssey system (Li-Cor, Lincoln, NE).

Immunofluorescence

Frozen sections of colon tissue from control, CAS, CPS and CPS + CAS female rats were mounted on glass slides, and rehydrated in phosphate buffered saline at room temperature. The slides were treated for antigen retrieval and blocked with 10% normal goat serum diluted in 0.3% phosphate buffered saline-Triton for 1 h, and then incubated with NGF primary antibody in antibody diluent (Renoir Red, Biocare Medical, Concord, CA) at 4 °C overnight. The slides were exposed to fluorescent dye-conjugated secondary antibody for 2 h at room temperature, counterstained with 4',6-diamidino-2-phenylindole and coverslipped. Images were taken in fluorescence mode on an Olympus laser scanning confocal microscope and the average signal intensity was calculated by the bundled software.

Serum estradiol and norepinephrine levels

Serum estradiol, adrenocorticotropic hormone (ACTH), and norepinephrine levels were measured using specific enzyme-linked immunosorbent assay kits for each analyte (CSB-E05110r, CSB-E06875r, CSB-E07022, Cusabio Bioteck CO., United States) following the manufacturer's instructions.

Data analysis

Single fiber responses (impulses/second) to CRD were calculated by subtracting SA from the mean 30 s maximal activity during distension. Fibers were considered responsive if CRD increased their activity to 30% greater than the baseline value. Mechanosensitive single units were classified as high threshold (> 20 mmHg) or low threshold (≤ 20 mmHg) on the basis of their response threshold and profile during CRD. Single fiber activity data were analyzed by analysis of variance with repeated measures; CRD intensity was the repeated factor and the experimental group was the between-group factor. If significant main effects were present, the individual means were compared using the Fisher post-hoc test. All authors had access to the study data and reviewed and approved the final manuscript.

RESULTS

Effects of CPS plus CAS on primary afferent responses to CRD in male and female rats

The basal activity of a spinal afferent fiber was defined as the average number of action potentials per second (impulses/sec) in the 60 s period before the onset of a distention stimulus. In male controls, 66% of the afferent fibers under study displayed SA and SA was significantly higher in female controls than in male controls (0.71 ± 0.21 *vs* 1.24 ± 0.20 imp/sec; Figure 1B). The average single fiber activity in response to CRD was significantly higher in female control rats compared with male controls (Figure 1C). We found that the enhanced sensitization in female rats mainly came from the low-threshold fibers (Figure 1D and E).

To assess the effects of CPS + CAS on colon afferent fiber activities, we compared average single colon afferent fiber activities projecting from dorsal roots S1-L6 in response to CRD in male and female control, CPS, control + CAS and CPS + CAS rats recorded approximately 24 h after the last stressor. In females, CPS significantly increased single-unit afferent activity in response to CRD *vs* control female rats (Figure 1F). CAS alone enhanced single-unit activity compared with control. The increase in average afferent responses after CAS in prenatally stressed female rats (44.0%) was significantly greater than the increase in female control rats (39.3%). In males, CPS had no significant effect on primary afferent responses (Figure 1G). When we compared males to females within each experimental group, we found that the average single fiber activity was significantly higher in female compared with male CPS + CAS rats (Figure 1F, G). The increased activity may contribute to the enhanced female visceral hypersensitivity previously reported in this model. Average single-fiber activities were significantly greater in control and CPS and CAS females than in their corresponding male experimental groups (Figure 1F and G). Both CAS and CPS + CAS rats had significantly increased primary afferent responses compared with control and CPS rats. Thus, our CPS and CAS protocols sensitized colon-projecting primary afferent fibers, with the greatest effects produced by the combination of CPS +

CAS in both males and females.

Increase in excitability of colon-projecting lumbosacral DRG neurons in female CPS + CAS rats

To elucidate the electrophysiological basis of enhanced stress-induced primary afferent activity in female rats, we performed patch clamp studies on acutely dissociated retrograde-labeled colon-projecting neurons from the L6-S2 DRGs in control, prenatal stress, adult stress only, and CPS + CAS female rats isolated 24 h after the last adult stressor (Figure 2A). Input resistance (Figure 2B) and rheobase (Figure 2C) were significantly decreased in neurons from CPS + CAS rats compared with the other three groups. The number of action potentials elicited at either 2 × or 3 × the rheobase were significantly greater in adult stress and CPS + CAS neurons compared with control and to CPS neurons (Figure 2D, E). CAS significantly increased action potential overshoot with or without CPS (Figure 2F), but it did not significantly alter other electrophysiological characteristics, such as number of spontaneous spikes, membrane capacitance (pF), resting membrane potential, cell diameter, time constant, and DRG neuron action potential amplitude and duration (Table 1).

The percentage of neurons with SA in was significantly greater in CPS + CAS rats than in control or CPS only rats (Figure 2G). Under voltage clamp conditions (Figure 2H), neurons from female CPS + CAS, CAS, CPS and control groups had I_A and sustained outward rectifier K^+ currents (I_K). Compared with the other three groups, DRG neurons from CPS + CAS rats had significantly reduced average I_A ($P < 0.05$). The average I_K density was decreased but the change was not significant.

Effects of CPS and/or CAS on plasma estrogen concentration

We did a vaginal smear test to identify the estrus cycle phases by identifying the vaginal cytological cell types. Estrogen concentration was significantly higher in the CPS proestrus/estrus phase compared with control diestrus, control proestrus/estrus, and CPS diestrus proestrus ($P < 0.05$; Figure 3A). Comparison of the plasma estrogen concentrations in control, CAS, CPS, CPS + CAS showed that CPS significantly increased plasma estrogen levels compared with the control rats and that CAS increased plasma estrogen level compared with the control and CPS rats (Figure 3B).

To determine whether estrogen contributed to stress-induced visceral hypersensitivity in prenatal stressed females, we reduced plasma estrogen levels by either OVX or letrozole treatment. OVX significantly lowered serum estradiol levels before and after CAS (Figure 3C). Treatment was continued throughout CAS. After treatment with letrozole, serum estradiol levels were significantly reduced (Figure 3D). To study the effects of gender and stress on norepinephrine and ACTH levels, we measured plasma norepinephrine levels in female rats from all four experimental groups. CAS alone significantly increased plasma norepinephrine levels compared with both the controls and with CPS alone (Figure 3E) Plasma norepinephrine levels were significantly increased in CPS + CAS rats compared with CAS alone as well as with controls and CPS. Plasma ACTH levels were significantly increased in CPS + CAS rats compared with controls. (Figure 3F).

Effects of Letrozole treatment on colon DRG neuron excitability

We performed patch clamp experiments on acutely isolated retrograde-labeled DRG neurons from CPS + CAS females with or without letrozole treatment 24 h after the last adult stressor. Letrozole treatment significantly increased rheobase (Figure 4A), and significantly reduced input resistance (Figure 4B). Action potential overshoot (Figure 4C) and the number of action potentials elicited by a current injection at either 2 × or 3 × rheobase were significantly reduced by letrozole treatment (Figure 4D). Other electrophysiological properties were not significantly altered (Table 2). We also recorded electromyographic activity to determine whether the reduction in visceral sensitivity in female CPS + CAS rats caused by OVX or systemic letrozole treatment reduced visceromotor responses. The findings demonstrated a significant decrease in excitability of colon-projecting L6-S2 neurons.

Spinal cord BDNF levels regulated by estrogen

To investigate the effect of estrogen on BDNF expression, we measured BDNF mRNA and protein levels in the lumbar-sacral spinal cords of OVX and Sham CPS + CAS female rats. Systemic estradiol administration to naïve cycling females produced significant increases in plasma estrogen (Figure 5A), lumbar-sacral spinal cord BDNF mRNA (Figure 5B), and protein (Figure 5C). We also measured BDNF mRNA and protein levels in the lumbar-sacral spinal cords of OVX and Sham CPS + CAS female

Table 1 Electrophysiological characteristics of colon related DRG neuron

Classification	Ctr., *n* = 36	CAS, *n* = 34	CPS, *n* = 29	CAS + CPS, *n* = 45
Spontaneous spike number	7.4 ± 4.0	16.9 ± 6.6	11.7 ± 4.8	44.8 ± 14.6[c]
Membrane capacitance (pF)	72.1 ± 4.9	95.4 ± 5.8	85.1 ± 6.2	93.8 ± 8.9[a]
Action potential threshold (mV)	-27.1 ± 2.5	-29.2 ± 1.8	-34.6 ± 1.6	-38.6 ± 1.4[c]
Resting membrane potential (mV)	-60.1 ± 1.7	-50.1 ± 1.3	-53.8 ± 1.5	-56.4 ± 1.3
Cell diameter (μm)	32 ± 0.9	29 ± 0.6	31 ± 0.8	31 ± 0.6
Time constant (μm)	545.5 ± 51.1	737.7 ± 70.4	595.9 ± 54.1	535.3 ± 42.5
Action potential amplitude (mV)	79.0 ± 4.7	80.2 ± 3.9	77.4 ± 4.2	85.6 ± 3.7
Duration (ms)	8.38 ± 0.97	11.2 ± 0.95	12.3 ± 1.83	8.89 ± 0.60

Values means ± standard error.
[a]$P < 0.05$;
[c]$P < 0.001$ *vs* control group. CAS: Chronic adult stress; CPS: Chronic prenatal stress.

Table 2 Electrophysiological characteristics of colon related DRG neuron after Letrozole treatment

Classification	Veh. + CAS + CPS, *n* = 60	Let + CAS + CPS, *n* = 27
Spontaneous spike number	34.6 ± 11.3	8.04 ± 4.62[c]
Membrane capacitance (pF)	86.8 ± 7.1	83.7 ± 6.7
Action potential threshold (mV)	-34.6 ± 1.6	-27.6 ± 2.1[a]
Resting membrane potential (mV)	-54.9 ± 1.3	-54.6 ± 2.2
Cell diameter (μm)	31 ± 0.5	27 ± 1.6
Time constant (um)	502.7 ± 34.5	454.0 ± 36.6
Action potential amplitude (mV)	90.9 ± 3.7	89.8 ± 7.2
Duration (ms)	8.67 ± 0.63	10.70 ± 2.22

Values are means ± standard error.
[a]$P < 0.05$;
[c]$P < 0.001$ *vs* control group. CAS: Chronic adult stress; CPS: Chronic prenatal stress.

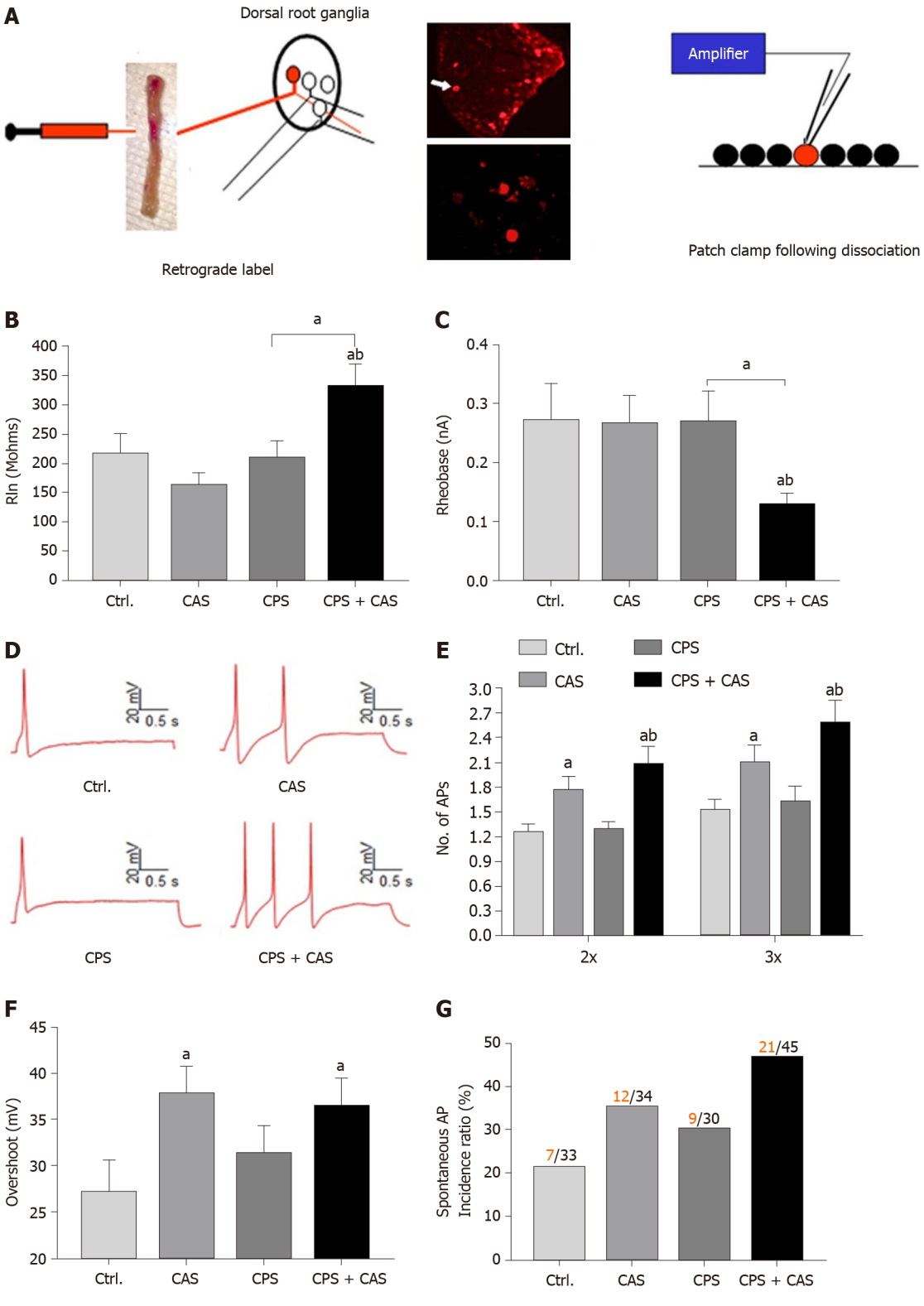
rats. BDNF mRNA and protein expression were significantly suppressed by OVX compared with sham rats. Another experiment showed that intrathecal infusion of estrogen into naïve female rats significantly increased BDNF protein levels, which proved that estrogen reversed the experimental results and contributed to the response to visceral pain[13].

Peripheral NGF level increased in CPS + CAS female rats

We examined NGF expression in the colons of females from all four experimental groups by immunohistochemistry (Figure 6A). Morphometric analysis showed that CAS and CPS + CAS significantly increased NGF levels in the colon wall, with the increase in CPS + CAS significantly greater that of CAS alone (Figure 6B). Western blotting showed that NGF protein was significantly upregulated in CPS + CAS rats compared with controls (Figure 6C).

DISCUSSION

Enhanced CPS-induced visceral hypersensitivity in female rats was associated with an increase in the responses of lumbosacral nerve fibers to CRD in both male and female offspring. These findings are further supported by data showing increased excitability of colon-projecting DRG neurons from females in patch clamp studies. The magnitude of the sensitization was the greatest in female CPS + CAS rats, suggesting that it made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Dorsal root ganglia
Retrograde label
Amplifier
Patch clamp following dissociation
B
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20 mV
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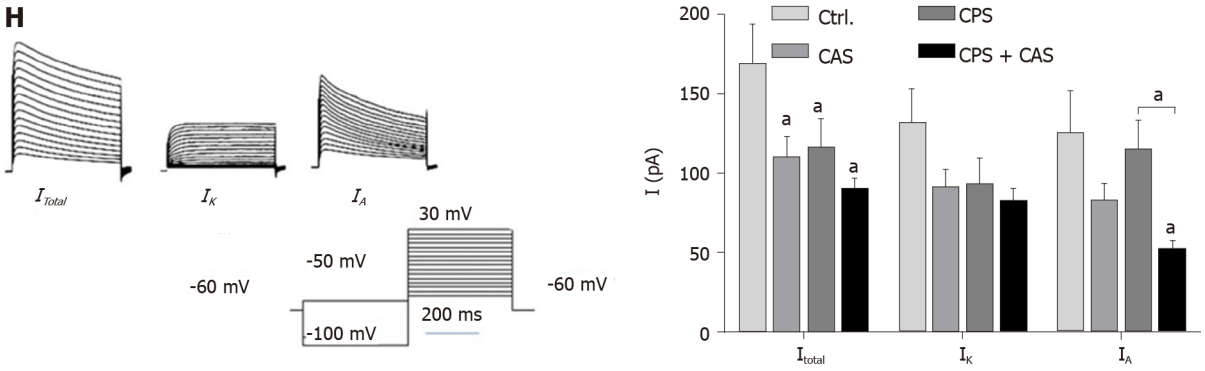

Figure 2 Patch clamp recording in colonic dorsal root ganglion neurons from female rats. A: Patch clamp process of cell labeling. Under isoflurane anesthesia, the lipid soluble fluorescent dye 9-DiI was injected into muscularis externa of the exposed distal colon (left figure). Lumbosacral (L6–S2) dorsal root ganglions (upper photograph) were isolated and DiI-labeled neurons were identified by fluorescence microscopy (lower photograph). Electrophysiological properties of each neuron were measured using whole-cell current and voltage clamp protocols (right figure); B: Rheobase from all four experimental groups (n = 5 rats, 45 cells in each group, one-way ANOVA, $^aP < 0.05$ *vs* control or $^bP < 0.05$ *vs* CAS); C: Representative action potentials (APs) elicited by current injection at 2 × the rheobase in neurons from control, chronic adult stress (CAS), chronic prenatal stress (CPS) and CPS + CAS female rats; D: Membrane input resistance from all four groups (n = 5 rats, 45 cells in each group, one-way ANOVA, $^aP < 0.05$ *vs* control; $^bP < 0.05$ *vs* CPS); E: Number of APs elicited by current injection at either 2 × and 3 × the rheobase in all four experimental groups (two-way ANOVA, $^aP < 0.05$ *vs* control; $^bP < 0.05$ *vs* CPS); F: AP overshoot recorded from all four experimental groups ($^aP < 0.05$ *vs* control); G: The proportion of neurons from each experimental group exhibiting spontaneous APs. Red numbers represent spontaneous AP firing cells; black numbers represent total cells; H: Representative total, I_K and I_A current tracings and average values of potassium currents: I_{total}, I_K and I_A are shown in female CPS + CAS, CAS, CPS (n = 15 neurons, from 5 rats in each group), and control groups (n = 12 neurons from 5 rats); two-way ANOVA, $^aP < 0.05$ *vs* each control group.

a major contribution to the observed enhanced female visceral hypersensitivity in our model.

Chronic stress is known to increase the excitability of colon-projecting DRG neurons in rats and mice. In adult male Sprague Dawley rats, colon DRG neuron sensitization was shown to be driven by increases in NGF expression in the colon muscularis externa[13]. In our model, we also observed a significant increase in colon NGF, but its potential role in primary afferent sensitization and visceral hypersensitivity was not investigated.. Other studies in male mice showed that stress, in the form of water avoidance, significantly increased the excitability of colon-projecting DRG neurons and that the combined activity of the stress mediators corticosterone and norepinephrine increased DRG neuron excitability in vitro[20,21]. We previously found significant increases in the serum levels of norepinephrine in CPS + CAS females. However, daily systemic treatment with adrenergic antagonists during the adult stress protocol failed to reduce visceral hypersensitivity in female neonatal + adult stress rats [13] suggesting that norepinephrine did not play a major role in the acquisition of enhanced female visceral hypersensitivity or primary afferent sensitization in our model.

When we tested on lumbar-sacral afferent fibers and dissociated neurons in patch clamp studies, we found significant decreases in transient potassium I_A currents in neurons isolated from CPS + CAS females compared with the other three experimental groups. Declines in A-type Kv currents in DRG neurons have been associated with persistent pain in multiple chronic pain models[22]. Whether the decline was caused by changes in channel properties or expression was not investigated in this study. However, another study demonstrated that estrogen significantly shifted the activation curve of I_A currents in the hyperpolarizing direction and that estrogen inhibited Kv (+) channels in mouse DRG neurons through a membrane ER-activated nongenomic pathway[23].

Our results showed that the excitability of colon-projecting neurons in CPS + CAS females was significantly reduced by systemic letrozole treatment, suggesting that estrogen contributed to the sensitization process. Previous studies show that estrogen receptors expressed on primary afferent neurons contributed to enhanced sensitivity in various pain models[24-26]. One study found no decline in the responses of colon-projecting nerve fibers to CRD following OVX and found no detectable estrogen receptor alpha immunoreactivity in colon-projecting DRG neurons[27]. The reasons for the differing results are not clear, but local production of estrogen in DRG neurons could be sufficient to sustain sensitization.

NGF and its receptors play important roles in the mechanism of visceral pain and hyperalgesia in women. For example, endometriosis is estrogen dependent and is commonly diagnosed. The main symptoms are various types of pelvic pain that have a

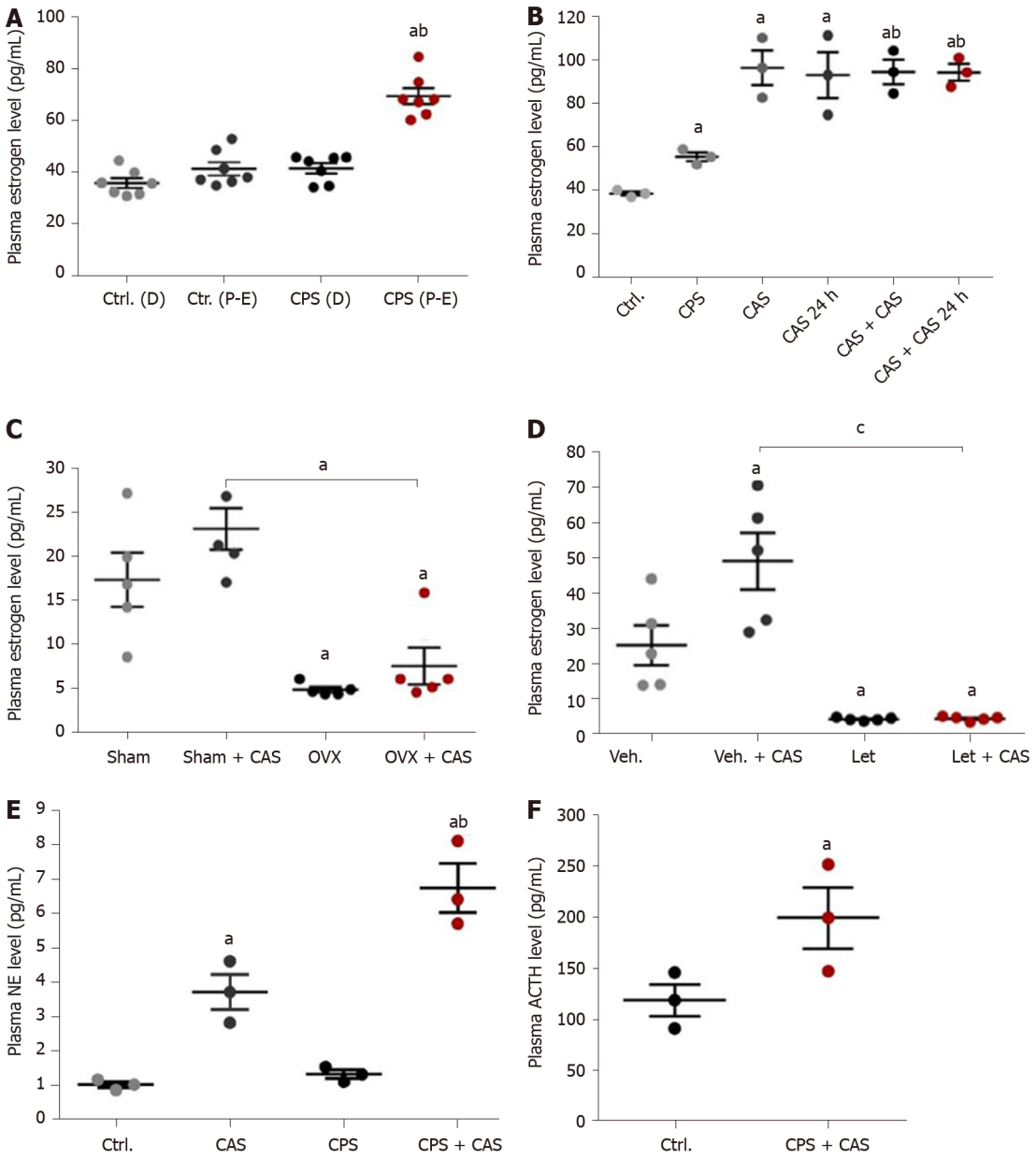

Figure 3 Effects of chronic prenatal stress, chronic adult stress, ovariectomy, and letrozole treatment on plasma estrogen levels in female rats. A: Plasma estrogen level in control and chronic prenatal stress (CPS) rats by estrus cycle phase (n = 8 rats, one-way ANOVA, $^{a}P < 0.05$ *vs* control proestrus/estrus (P-E) phase; $^{b}P < 0.05$ *vs* CPS diestrus (D) phase); B: Plasma estrogen levels increased in CPS rats and following chronic adult stress (CAS) 24 h after the last adult stressor (n = 8 rats, one-way ANOVA, $^{a}P < 0.05$ *vs* control; $^{b}P < 0.05$ *vs* CPS); C: Ovariectomy (OVX) significantly reduced CPS female rat plasma estrogen levels before and after CAS (n = 5 rats, one-way ANOVA, $^{a}P < 0.05$ *vs* sham group); D: Letrozole treatment significantly reduced CPS female rat plasma estrogen levels before or after CAS (n = 5 rats, one-way ANOVA, $^{a}P < 0.05$ *vs* vehicle group; $^{c}P < 0.0001$); E: Plasma norepinephrine levels from control, CAS, CPS and CPS + CAS group female rats (n = 5 rats, one-way ANOVA, $^{a}P < 0.05$ *vs* control; $^{b}P < 0.05$ *vs* CPS); F: Plasma adrenocorticotropic hormone (ACTH) levels from control and CPS + CAS group female rats (n = 5 rats, t-test, $^{a}P < 0.05$ *vs* control).

serious effect on physical and mental health, but the mechanisms of abdominal pain are still unclear. Studies have shown NGF to be an inflammatory mediator and modulator of pain in adulthood[28].

CONCLUSION

In this study, we examined the sex differences and effects of estrogen on the acquisition of enhanced visceral hypersensitivity in the offspring of rats in a model of prenatal and adult stress as shown in Figure 7. Our study shows that estrogen acted in the spinal cord and the primary afferent neurons to enhance visceral nociception. Acute blockade of the endogenous synthesis of estrogens in rat spinal cord

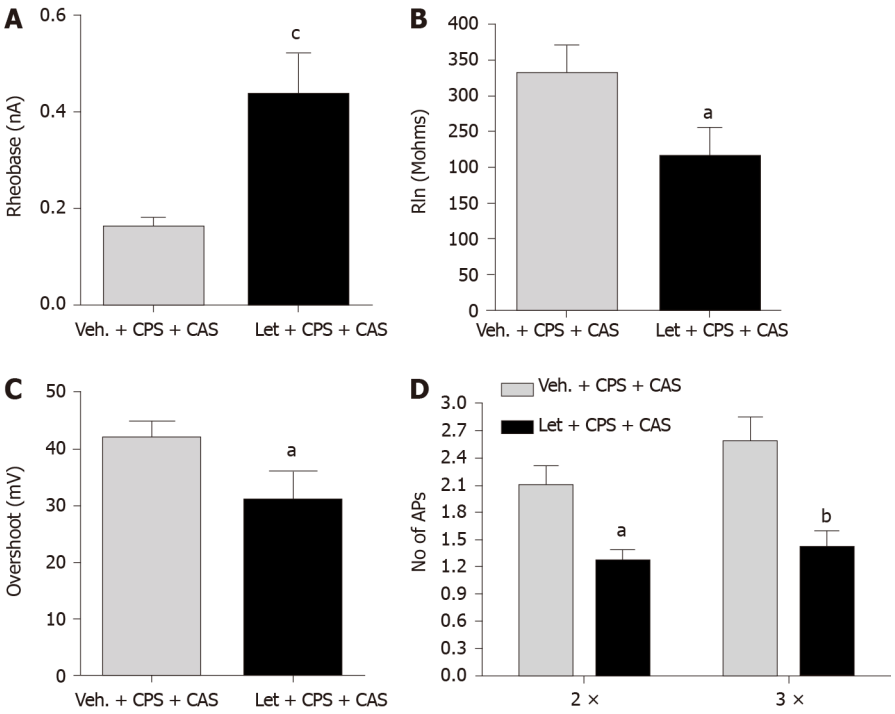

Figure 4 Effects of Letrozole treatment on colon dorsal root ganglion neuron excitability. A: Rheobase (n = 45 cells in 6 rats in each group, t-test, $^{c}P < 0.001$ *vs* Veh. + chronic adult stress [CAS] + chronic prenatal stress [CPS]); B: Membrane input resistance (Rin) (t-test, $^{a}P < 0.05$); C: Action potential (AP) overshoot (t-test, $^{a}P < 0.05$); D: Number of APs elicited by current injection at 2 × and 3 × rheobase (two-way ANOVA, $^{a}P < 0.05$; $^{b}P < 0.01$).

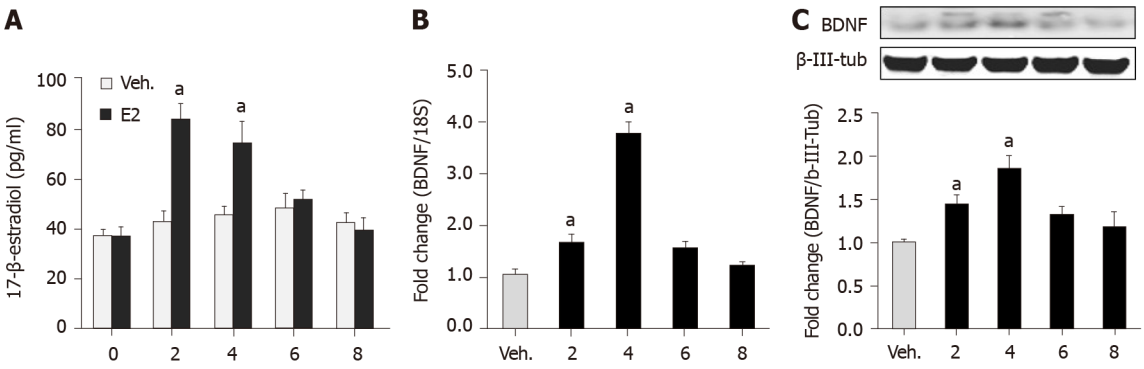

Figure 5 Brain-derived neurotrophic factor expression in lumbar-sacral spinal cord is regulated by estrogen. A: Plasma estrogen levels in cycling females that received a bolus estradiol (E2) infusion on day 1; B: Lumbar-sacral spinal cord brain-derived neurotrophic factor (BDNF) mRNA following bolus estrogen infusion; C: Lumbar-sacral spinal cord BDNF protein following bolus estrogen infusion. (n = 8 rats in each group, two-way ANOVA, $^{a}P < 0.05$ *vs* vehicle group).

significantly reduced visceral hypersensitivity, suggesting that locally produced estrogen in the central nervous system can regulate nociceptive neurons to modulate visceral hypersensitivity. The chronic stress-estrogen-BDNF axis sensitized visceral hypersensitivity in the offspring of females subjected to CPS. The development of chronic stress-induced visceral hypersensitivity in female rats was estrogen dependent. A key component of this hypersensitivity was estrogen-dependent sensitization of primary afferent colon neurons. Our findings provide key scientific evidence in a preclinical model in support of developing gender-based treatment for abdominal pain in IBS.

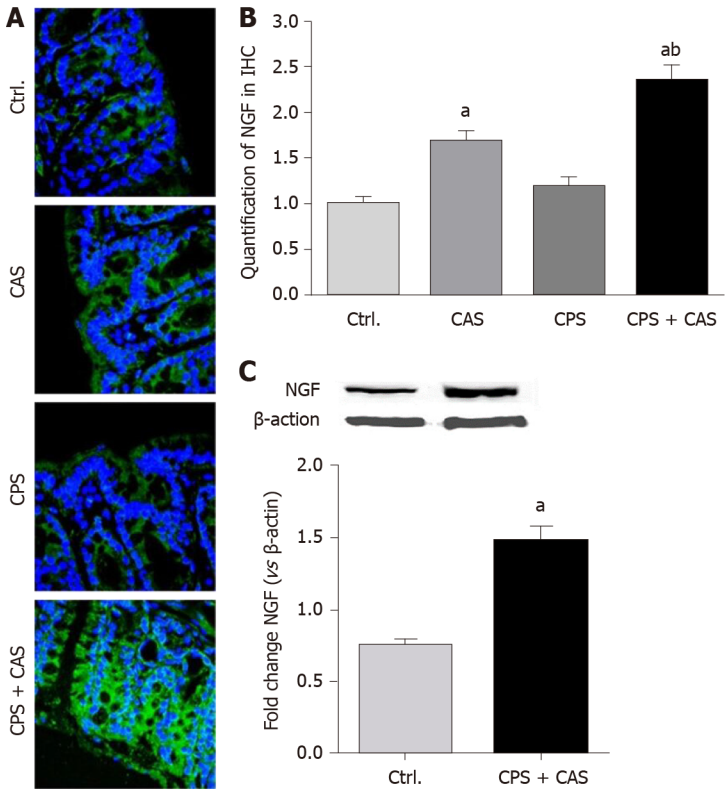

Figure 6 Nerve growth factor expression level in the colon wall. A: Immunohistochemical staining of nerve growth factor (NGF; green) was detected with nuclear counterstaining staining (blue) in controls, chronic adult stress (CAS), chronic prenatal stress (CPS) and CPS + CAS group female rat colon walls. × 400 magnification representative pictures were shown; B: Quantification of NGF levels from colon wall in immunohistochemistry (IHC) (n = 4 rats in each group, one-way ANOVA, $^{a}P < 0.05$ *vs* control group; $^{b}P < 0.05$ *vs* CPS group); C: Western blots of NGF protein from control and CPS + CAS female rats colon wall tissue (n = 6 rats in each group, t-test, $^{a}P < 0.05$ *vs* control group).

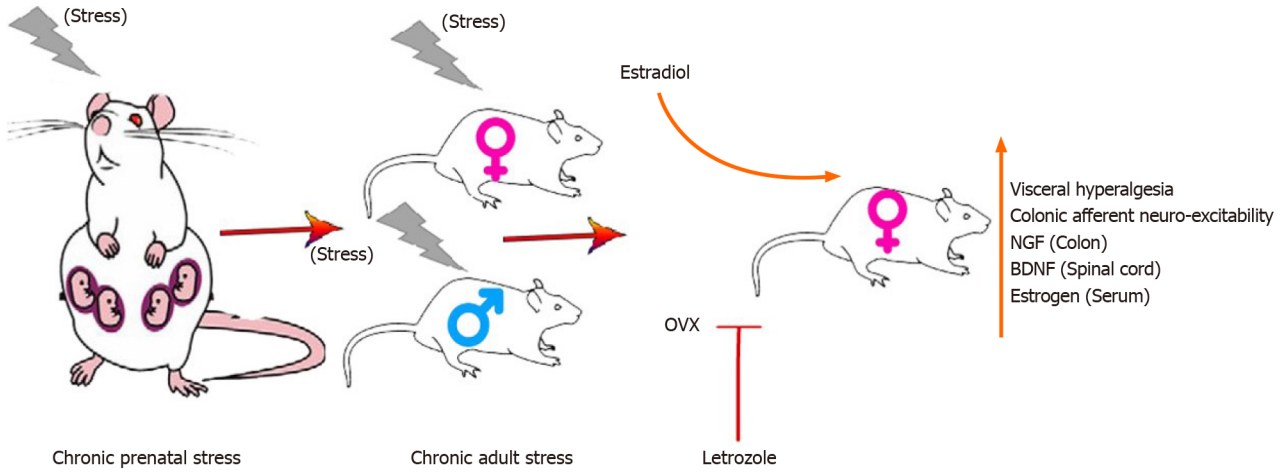

Figure 7 Summary diagram of estrogen re-enhanced visceral hyperalgesia investigated in chronic prenatal stress plus chronic adult stress models. BDNF: Brain-derived neurotrophic factor; NGF: Nerve growth factor.

ARTICLE HIGHLIGHTS

Research background

Chronic stress during pregnancy may increase visceral hyperalgesia in the offspring. Combining adult stress in offspring will increase this sensitivity. Therefore, based on the evidence implicating estrogen exacerbates visceral hypersensitivity in female rodents in preclinical models, we predicted that chronic prenatal stress (CPS) + chronic adult stress (CAS) would maximize visceral hyperalgesia and that estrogen has an important role in colonic hyperalgesia.

Research motivation

The mechanisms of visceral hypersensitivity are not well defined. Understanding the neurophysiological mechanisms driving visceral hypersensitivity will spur the development of female pain-specific therapies.

Research objectives

The objective was to identify the enhancement of visceral hypersensitivity in a CPS + CAS model and explain the role of estrogen in that process.

Research methods

A CPS + CAS rodent model was established. Single fiber recording *in vivo* and patch clamp experiments *in vitro* were used to monitor the activity of colonic neurons. Reverse transcription-polymerase chain reaction (RT-PCR), western blots, and immunofluorescence were used to study the effects of CPS and CAS on colon primary afferent sensitivity. We used ovariectomy (OVX) and letrozole to reduce estrogen levels in female rats in order to assess the role of estrogen in female-specific enhanced primary afferent sensitization.

A CPS + CAS rodent model was established. Single fiber recording *in vivo* and patch clamp experiments were used to monitor the colonic neuron activity in vitro. RT-PCR, western blots, and immunofluorescence were used to study the effects of CPS and CAS on colon primary afferent sensitivity. We used OVX and letrozole to reduce estrogen levels of female rats in order to assess the role of estrogen in female-specific enhanced primary afferent sensitization.

Research results

Spontaneous activity and single fiber activity were significantly greater in females than in males. The enhanced sensitization in female rats mainly came from the low-threshold neurons. CPS significantly increased single-unit afferent fiber activity in the L6-S2 dorsal roots in response. Activity was further enhanced by CAS. In addition, the increased excitability of colon-projecting DRG neurons in CPS + CAS rats was associated with a decrease in transient A-type K+ currents. Compared with OVX, letrozole treatment further reduced the estrogen levels of female rats, which confirmed the gender difference. Moreover, rats treated with letrozole had decreased colonic DRG neuron excitability. The intrathecal infusion of estrogen increased BDNF protein levels and contributed to the response to visceral pain. Western blots showed that nerve growth factor protein was upregulated in CPS + CAS rats.

Research conclusions

This study adds to the evidence of the development of chronic stress-induced visceral hypersensitivity in females, and that it involves estrogen-dependent sensitization of primary afferent colon neurons.

Research perspectives

This study demonstrated that CAS + CPS induced visceral hypersensitivity and that estrogen played a role in the process. Understanding the molecular and neurophysiological mechanisms driving this response will spur the development of female pain-specific therapies.

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ORIGINAL ARTICLE

Clinical and Translational Research

Association of gene and protein expression and genetic polymorphism of CC chemokine ligand 4 in colorectal cancer

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Author contributions: Dimberg J and Wågsäter D designed and coordinated the study; Shamoun L and Balboa-Ramilo A performed the experiments, acquired and analyzed data; Shamoun L, Balboa-Ramilo A, Landerholm K, Andersson RE, Dimberg J, and Wågsäter D interpreted the data; Landerholm K and Andersson RE recruited the patients; Shamoun L, Dimberg J, and Wågsäter D wrote the manuscript; All authors made critical revisions of the manuscript and approved the final version of the article.

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Abstract

BACKGROUND

Leukocytes, such as T cells and macrophages, play an important role in tumorigenesis. CC chemokine ligand (CCL) 4, which is produced by lymphocytes and macrophages, has been found to be expressed in the mucosa of the gastrointestinal tract and is a potent chemoattractant for various leukocytes.

AIM

To examine CCL4 expression and its genetic polymorphism rs10491121 in patients with colorectal cancer (CRC) and evaluate their prognostic significance.

METHODS

Luminex technology was used to determine CCL4 Levels in CRC tissue (n = 98), compared with paired normal tissue, and in plasma from patients with CRC (n = 103), compared with healthy controls (n = 97). Included patients had undergone surgical resection for primary colorectal adenocarcinomas between 1996 and 2019 at the Department of Surgery, Ryhov County Hospital, Jönköping, Sweden. Reverse transcription quantitative PCR was used to investigate the CCL4 gene expression in CRC tissue (n = 101). Paired normal tissue and TaqMan single

nucleotide polymorphism assays were used for the CCL4 rs10491121 polymorphism in 610 CRC patients and 409 healthy controls.

RESULTS

The CCL4 protein and messenger RNA expression levels were higher in CRC tissue than in normal paired tissue (90%, $P < 0.001$ and 45%, $P < 0.05$, respectively). CRC tissue from patients with localized disease had 2.8-fold higher protein expression levels than that from patients with disseminated disease. Low CCL4 protein expression levels in CRC tissue were associated with a 30% lower cancer-specific survival rate in patients ($P < 0.01$). The level of plasma CCL4 was 11% higher in CRC patients than in healthy controls ($P < 0.05$) and was positively correlated ($r = 0.56$, $P < 0.01$) with the CCL4 protein level in CRC tissue. The analysis of CCL4 gene polymorphism rs10491121 showed a difference ($P < 0.05$) between localized disease and disseminated disease in the right colon, with a dominance of allele A in localized disease. Moreover, the rate of the A allele was higher among CRC patients with mucinous cancer than among those with non-mucinous cancer.

CONCLUSION

The present study indicates that the CRC tissue levels of CCL4 and CCL4 gene polymorphism rs10491121, particularly in the right colon, are associated with clinical outcome in CRC patients.

Key Words: CC chemokine ligand 4; Gene polymorphism; Gene and protein expression; Chemokine; Survival rate; Colorectal cancer

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Core Tip: Our data suggest that the CC chemokine ligand (CCL) 4 rs10491121 polymorphism is associated with colorectal cancer (CRC), particularly mucinous CRC and CRC in the right colon. Moreover, we observed that CCL4 protein expression was upregulated in CRC tissues compared with in normal tissues, was correlated with disease stage and that low expression was related to a 30% lower cancer-specific survival rate.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies and one of the leading causes of cancer death[1]. In most cases, the disease occurs sporadically through a transition from dysplastic adenomatous polyps to carcinoma. Genetic alterations and various genetic pathways cause this transition by affecting CRC induction and progression[2,3]. Details of the relationship between inflammation and CRC induction, progression, and metastasis, as well as the importance of tumor suppressor genes in inhibiting carcinogenesis, have been established by us and others [4-6]. Inflammatory factors expressed in CRC cells or in the tumor microenvironment appear to play an important role in local immune regulation and may either promote or suppress CRC pathogenesis. Communication between tumor cells and their microenvironment through secretion of factors is thought to be crucial for tumor growth. Particularly, the interaction between tumor cells and infiltrating leukocytes such as macrophages is vital for CRC progression and prognosis[4,7-9]. There is an ongoing search for molecular biomarkers to facilitate early diagnosis, determine prognosis, and guide the selection of personalized therapy for CRC patients[6,10]. The patients are identified based on pathological and clinical parameters, with CRC

consent was obtained from each of the participants.

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

Conflict-of-interest statement: The authors declare no conflicts of interest.

Data sharing statement: No additional data are available.

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staging primarily based on the tumor-node-metastasis (TNM) system, as described in the American Joint Committee on Cancer classification[11].

The connection between inflammatory factors, such as cytokines and chemokines (chemotactic cytokines), and CRC is well-established[4,12]. Chemokines and their receptors have many roles in immune regulation[13] and some chemokines promote and regulate neoplastic progression, metastasis, angiogenesis, and immune cell infiltration[14-16].

The CC chemokine ligand (CCL) 4, also called macrophage inflammatory protein-1 beta, is a potent chemoattractant for monocytes/macrophages, dendritic cells, and T cells, *via* its cognate receptor CCR5. It is an especially effective chemoattractant for Th1 cells, which have an antitumor role[17-19]. CCL4 is mainly produced by lymphocytes and macrophages[17,18], but is also expressed in the intestinal mucosa and upregulated in gastric cancer and CRC tissue[20-22]. Genetic variations, such as single nucleotide polymorphisms (SNPs), in inflammatory genes are suggested to play a role in CRC risk and the survival of CRC patients[23,24]. The human CCL4 gene is located on chromosome 17 and recent studies have shown a relationship between the nonsynonymous SNP CCL4 rs10491121 (G > A) and oral[25], breast[26], and hepatocellular cancer[27]. To our knowledge, little is known about this SNP and CCL4 expression in patients with CRC. Therefore, the aim of the present study was to examine expression of CCL4 and its genetic polymorphism rs10491121 in patients with CRC and to evaluate their prognostic significance by identifying associations with various clinicopathological parameters and long-term survival.

MATERIALS AND METHODS

Patients and controls

Blood samples were obtained from 610 patients (344 males and 266 females) with a median age of 71 years (range: 25–94 years) who underwent surgical resection for primary colorectal adenocarcinomas between 1996 and 2019 at the Department of Surgery, Ryhov County Hospital, Jönköping, Sweden. Follow-up for the estimation of cancer-specific survival ended on the date of death or on February 23, 2021.

The study included 274 patients with rectal cancer and 336 patients with colon cancer, grouped based on the site of the primary cancer. In accordance with Liang *et al* [28], the colon cancer patients were divided into those with cancer localized in the right colon (cecum, ascending colon, hepatic flexure, transverse colon) (n = 194) and those with cancer localized in the left colon (splenic flexure, descending colon, sigmoid colon) (n = 142). The tumors were classified in accordance with the American Joint Committee on Cancer classification: Stage I in 105 cases, stage II in 228 cases, stage III in 196 cases, and stage IV in 81 cases. The degree of differentiation was categorized as high/medium (478 cases) or poor (132 cases) and the tumors were characterized as non-mucinous (n = 528) or mucinous (n = 82).

Healthy blood donors (n = 347) at Ryhov County Hospital, with no known CRC history and from the same geographical region as the CRC patients, were selected as the control population. This cohort comprised 216 males and 193 females, with a median age of 58 years (range: 33–68). Blood samples were collected at the start of surgery for the patients and at the time of blood donation for the controls. All blood samples were centrifuged to separate plasma and blood cells and then stored frozen, at -70°C, until analysis.

The investigation was approved by the Regional Ethical Review Board in Linköping, Sweden, and informed consent was obtained from each participant.

Genotyping of CCL4 gene polymorphism

DNA was isolated from each blood sample using QiaAmp DNA Blood Kit (Qiagen, Hilden, Germany). The TaqMan SNP genotype assays were used for analysis of the *CCL4* rs10491121 (ID C-11626804-10) genotypes (Applied Biosystems, Foster City, CA, USA). Ten nanograms of DNA were mixed with TaqMan Genotyping Master Mix (Applied Biosystems) and analyzed with the 7500 Fast Real-Time PCR System (Applied Biosystems). Amplification was performed using an initial cycle at 50°C for 2 min, followed by one cycle at 95°C for 10 min, and, lastly, 40 cycles at 95°C for 15 s and at 60°C for 1 min. The manual calling option in the allelic discrimination application ABI PRISM 7500 SDS software version 1.3.1 (Applied Biosystems) was used to assign the genotypes.

Cell lines

Two established human colon cancer cell lines, Caco-2 and HT-29, were purchased from American Type Culture Collection (Rockville, MD, USA). The cell lines were grown in accordance with the supplier's instructions and the growth media were Essential Medium (Caco-2) and Mc Coy 5a (HT-29). The cell lines were stored frozen, at -78°C, until analysis.

Tissue samples and lysates

This study utilized tumor and paired normal tissue samples from 98 of the CRC patients, of whom 52 were males and 46 females, with a median age of 68 years (range: 29–90). The tumors were located in the colon in 53 patients and in the rectum in 45 patients and were classified as stage I in 18 cases, stage II in 33 cases, stage III in 22 cases, and stage IV in 25 cases. Colorectal cancer tissue and adjacent normal colorectal mucosa (within about 5 cm from the tumor) were excised from each patient and immediately frozen at -78°C until analysis. Frozen tumor, paired normal tissue, and cell lines were thawed and homogenized in ice-cold radioimmunoprecipitation assay lysis buffer (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, United States) containing a protease inhibitor cocktail, in accordance with the manufacturer's instructions. The lysate was placed on ice for 30 min and then centrifuged at 18000 *g* for 10 min. The protein content of the supernatant fluid was determined for each sample using the Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, United States).

Plasma samples

Of the CRC patients and controls, 103 and 97, respectively, were available for CCL4 protein analysis in plasma. Blood samples were drawn at the start of surgery for the patients and at the time of blood donation for the controls. The CRC patient group comprised 52 males and 51 females with a median age of 68 years (range: 29–90). Forty-six tumors were in the colon and 57 tumors in the rectum; they were classified as stage I in 18 cases, stage II in 34 cases, stage III in 25 cases, and stage IV in 26 cases. Controls for plasma samples included 51 males and 46 females with a median age of 63 years (range: 55–68).

Determination of CCL4 protein levels in tissue, cell lines, and plasma

The levels of CCL4 in plasma, colorectal tissue, and cell line lysates were measured using a Luminex bead-based technology (Bio-Rad Laboratories, Inc.) and commercially available Luminex assay for CCL4 (Bio-Rad Laboratories). The tissue and cell line levels of CCL4 protein were expressed as pg/mg of protein and the plasma CCL4 protein concentration from patients and controls as pg/mL.

Immunohistochemistry

Formalin-fixed and paraffin-embedded CRC tissues from patients with CRC were obtained from the Department of Pathology of the County Hospital Ryhov, Jönköping, Sweden. Among the eight patients, six had tumors localized in the colon and two in the rectum; these were classified as stage I in two cases, stage II in two cases, stage III in two cases, and stage IV in two cases. The staining was performed on 3.5 μm sections of paraffin-embedded tissue samples to detect the CCL4 protein expression. In brief, antigen retrieval was finished by cooking at 110°C for 52 min in Diva Decloaker, 10X (Biocare Medical, Concord, CA, United States). The slides were treated with hydrogen peroxide for 5 min to prevent the occurrence of endogenous peroxidase, which might alter the interpretation of the color. Primary rabbit polyclonal CCL4 antibody against human CCL4 (Abcam, Tokyo, Japan) was used at a dilution of 1:300. The antibody was applied to the tissue sections, which were then incubated for 30 min at room temperature. The MACH 4 Universal HRP-Polymer Detection kit (Biocare Medical) was used, and the reaction was visualized using Betazoid DAB Chromogen Kit (Biocare Medical). Human kidney tissue was used as a positive control for CCL4 expression and rabbit IgG, polyclonal isotype control (Abcam) was used as a negative control and included along with each patient tissue section.

RNA extraction, complementary DNA synthesis, and reverse transcription quantitative PCR

One hundred and one of the CRC patients were available for the analysis of CCL4 messenger RNA (mRNA) expression, of whom 52 were males and 49 females, with a median age of 72 years (range: 43–89). The tumors were located in the colon in 56 patients and in the rectum in 45 patients and were classified as stage I in 16 cases, stage

II in 43 cases, stage III in 38 cases, and stage IV in four cases. Eighty-two available paired normal tissue samples were analyzed.

All CRC tissue samples and paired normal tissue samples were stored in RNA protect tissue reagent (Qiagen) to maintain good RNA quality. RNA was purified using the RNeasy Mini kit (Qiagen), in accordance with the manufacturer's instructions, and eluted in nuclease-free water. The concentration and purity of the RNA and RNA integrity were determined using an Agilent 2100 Bioanalyzer with the Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, United States), in accordance with the manufacturer's instructions. RNA (297 ng) was reverse transcribed in a total volume of 20 μL using Super Script III Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher Scientific, Carlsbad, CA, United States), in accordance with the manufacturer's instructions. The resulting complementary DNA and the remaining RNA were stored at -80°C.

Complementary DNA was amplified through reverse transcription quantitative PCR with TaqMan™ Fast Universal PCR Master Mix (Cat.no 4366072, Applied Biosystems) and TaqMan Gene Expression Probes and Primers (RPLP0 Cat.no HS99999902 m1 and CCL4 Cat.no HS99999148 m1, Applied Biosystems) on a QuantStudio5 Real Time PCR system (Applied Biosystems), with each sample run in duplicate. Results were normalized to expression levels of human RPLP0. Relative quantification of gene expression was performed using the standard curve method and expressed in arbitrary units.

Statistical analysis

The differences in the frequencies of the CCL4 gene polymorphism between CRC patients and controls and between clinical characteristics within the CRC subgroups were analyzed using the chi-squared test. The Hardy-Weinberg equilibrium was assessed for the genotypes. Survival analysis was performed through Kaplan-Meier analysis with log-rank test. The Wilcoxon's signed-rank test and the Mann-Whitney *U*-test were used for the analysis of the related and independent parameters and the Kruskal-Wallis test was used for comparing three or more classes. Correlations between parameters were analyzed using Spearman's rank correlation test. Statistical analyses were performed using Stata Statistical Software Release 15 (Stata Corp., College Station, TX, United States) and SPSS software for Windows, version 14.0 (SPSS Inc., Chicago, IL, United States). Results were considered statistically significant at $P < 0.05$. The statistical methods of this study were reviewed by Roland E Andersson from Region Jönköping County, Sweden.

RESULTS

CRC risk, clinicopathological features, and survival in relation to CCL4 gene polymorphism

No statistically significant differences in the genotypic or allelic frequencies were observed between the patients and the healthy control group for CCL4 rs10491121 (Table 1). Stratification analysis of associations between this SNP and the tumor location showed no statistically significant differences between colon and rectum, or between right and left colon (data not shown). However, we noted a difference between localized (stages I + II) and disseminated (stages III + IV) cancer in the right colon, but not in the left colon, regarding the genotypic ($P = 0.003$) and allelic ($P = 0.013$) distributions (Table 2). The presence of allele A in the right colon was more common in stages I + II (40.5%) than in stages III + IV (28.2%) (Table 2) with an odds ratio (OR) = 1.73 (95% confidence interval = 1.12–2.68, $P = 0.013$). Analysis of the association between the genotypic or allelic distributions in non-mucinous or mucinous CRC showed statistically significant differences (Table 3). The rate of the A allele was higher (45.1%) among patients with mucinous cancer than among those with non-mucinous cancer (34.6%) with an OR = 1.56 (95% confidence interval = 1.12–2.17, $P = 0.009$).

Based on data from our cohort with up to 25 years follow-up, the Kaplan-Meier analysis revealed no differences in cancer-specific survival overall. No significant association was found between the genotypes or alleles when patients were stratified by gender, age, or degree of differentiation (data not shown). Neither the patient group nor the control group showed significant deviation from the Hardy-Weinberg equilibrium (data not shown).

Table 1 Genotypic and allelic distributions of the CC chemokine ligand 4 gene polymorphism (rs10491121) in *n* (%), among 610 patients with colorectal cancer and 409 healthy controls

Genotype/allele	Patients	Controls
G/G	(250 (41.0)	176 (43.0)
G/A	281 (46.1)	181 (44.3)
A/A	79 (12.9)	52 (12.7)
G	781 (64.0)	533 (65.2)
A	439 (36.0)	285 (34.8)

CCL4: CC chemokine ligand 4.

Table 2 Genotypic and allelic distributions of the CC chemokine ligand 4 gene polymorphism (rs10491121) in *n* (%), regarding tumour location and disease stage among patients with colon cancer

Variable	Cases	Genotype			Allele	
Right colon		G/G	G/A	A/A	G	A
Stages I + II	116	38 (32.8)	62 (53.4)	16 (13.8)	138 (59.5)	94 (40.5)
Stages III + IV	78	44 (56.4)[b]	24 (30.8)	10 (12.8)	112 (71.8)[a]	44 (28.2)
Left colon						
Stages I + II	67	23 (34.3)	34 (50.8)	10 (14.9)	80 (59.7)	54 (40.3)
Stages III + IV	75	27 (36.0)	37 (49.3)	11 (14.7)	91 (60.7)	59 (39.3)

[a]$P < 0.05$.
[b]$P < 0.01$. CCL4: CC chemokine ligand 4.

Table 3 Genotypic and allelic distributions of the CCL4 gene polymorphism (rs10491121) in *n* (%), regarding non-mucinous and mucinous cancer among patients (*n* = 610) with colorectal cancer

Genotype/allele	Non-mucinous	Mucinous
G/G	222 (42.0)	28 (34.1)
G/A	247 (46.8)	34 (41.5)
A/A	59 (11.2)	20 (24.4)[b]
G	691 (65.4)	90 (54.9)
A	365 (34.6)	74 (45.1)[b]

[b]$P < 0.01$.
CCL4: CC chemokine ligand 4.

Protein expression in CRC tissue and plasma in relation to clinical features and survival

The level of CCL4 protein was 90% higher in CRC tissue compared with in normal paired tissue ($P < 0.001$; Table 4). Moreover, the level of plasma CCL4 was 11% higher in patients than in controls ($P < 0.050$; Table 4) and showed a positive correlation ($r = 0.56$, $P < 0.01$) with levels in the CRC tissue.

The CRC tissue showed a gradual decrease in CCL4 protein level in relation to disease stage, and localized disease (stages I + II) showed a 2.8-fold higher CCL4 protein level than disseminated disease (stages III + IV, $P = 0.001$; Table 5). There was no difference in tissue levels of CCL4 protein for any of the other reported clinical features such as age, gender, location, differentiation grade, or mucinous state. Moreover, plasma levels of CCL4 were not associated with any of the investigated clinical features (data not shown). In the colon cancer cell lines, we noted that the

Table 4 Tissue levels and plasma levels of CC chemokine ligand 4 in colorectal cancer patients

Variable	Cases, *n*	CCL4 protein
Tissue		
Colorectal cancer tissue	98	137 [5–3413] pg/mg[b]
Paired normal tissue	98	72 [5–2205] pg/mg
Plasma		
Patient	103	63 [8–3428] pg/mL[a]
Control	97	57 [2–178] pg/mL

Data are shown as median [range].
[a]$P < 0.05$.
[b]$P < 0.001$. CCL4: CC chemokine ligand 4.

Table 5 Disease stage in relation to cancer tissue levels of CC chemokine ligand 4 in colorectal cancer patients

Variable	Cases, *n*	CCL4 protein in pg/mg
Stage		
I	18	262 [12–3326]
II	33	188 [5–2797]
III	22	125 [24–3413]
IV	25	71 [10–1188][b]
I + II	51	235 [5–3326]
III + IV	47	84 [10–3413][b]

Data are shown as median [range].
[b]$P < 0.001$. CCL4: CC chemokine ligand 4.

average protein level of CCL4 was 7.8 pg/mg in HT-29 and 2.9 pg/mg in Caco-2.

When CRC tissue levels of the CCL4 protein were used to group patients into tertiles (with low and medium/high levels), we found that the patients with low tissue levels of the CCL4 had a 30% lower cancer-specific survival rate ($P = 0.005$, log-rank test, Figure 1).

CCL4 is expressed in epithelial cells

Immunohistochemical evaluation of CCL4 expression in CRC and normal tissue showed positive and negative to weak staining, respectively (Figure 2A-D). Cytoplasmic staining was evident predominantly in the epithelial cells. The staining of slides with isotype antibody control and with secondary antibody revealed only negative staining (Figure 2E and F).

CCL4 mRNA expression in CRC and paired normal tissue

The CCL4 mRNA expression was 45% higher in CRC tissue (median 61 AU, range: 2–494) compared with normal paired tissue (median 42 AU, range: 4-160, $P = 0.044$). When the relationship between the CCL4 mRNA expression and the mucinous state of the CRC tissue was analyzed, the expression level was found to be higher in mucinous CRC tissue (median 78 AU, range: 11–243) than in non-mucinous CRC tissue (median 56 AU, range: 2–494, $P = 0.043$). CCL4 mRNA expression levels were not associated with other reported clinical features (data not shown).

DISCUSSION

Previous studies have shown that CCL4 gene polymorphism rs10491121 (G > A) is associated with susceptibility to several cancer diseases[25-27], but little is known

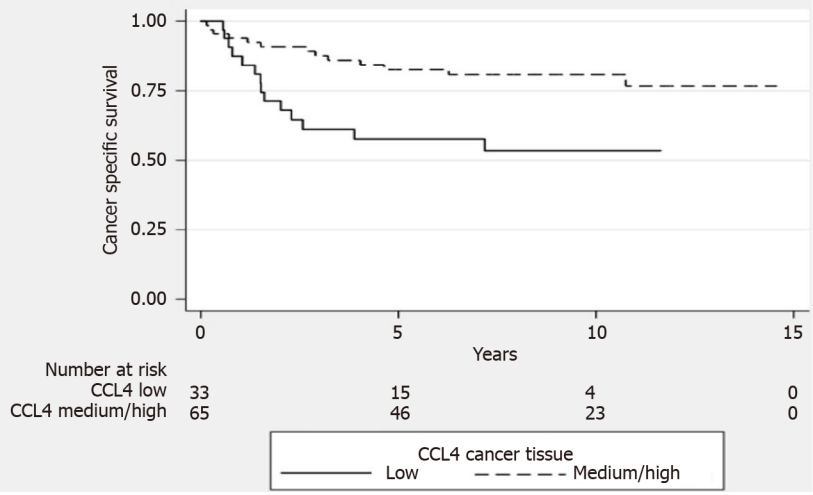

Figure 1 Kaplan-Meier curves between expression levels of CC chemokine ligand 4 protein grouped into tertiles (low and medium/high) and cancer-specific survival rates in colorectal cancer patients. CCL4: CC chemokine ligand 4.

about this SNP in relation to CRC. The present study found no statistically significant differences between CRC patients and controls in genotype distribution or allelic frequencies of this SNP. Further, no associations were identified with clinical characteristics other than location, stage, and mucinous state. The genotypes of CCL4 gene polymorphism rs10491121 may influence gene expression, protein levels, and protein function of CCL4. However, to the best of our knowledge, there is limited data to describe the functional activity of this SNP. Regarding cancer, it has previously been shown that patients carrying the AG or GG genotype are at lower risk of developing distant metastases in breast cancer[26]. In our study, stratification analysis showed a difference in the frequency of this SNP between localized disease and disseminated disease in the right colon, but not in the left colon. Specifically, in the right colon, a dominance of the allele A was seen in localized disease as compared with in disseminated disease. Rectal, left, and right colon cancer differ with respect to histology, clinical outcomes, and expression of distinguishable genes, which has led to the hypothesis that they constitute different disease entities[28-30]. In our study, it is possible that the genotype distribution of CCL4 gene polymorphism rs10491121, together with other gene or gene-environment interactions, contributed to development towards localized or disseminated disease in the right colon.

Mucinous cancer is a subtype of CRC and is more common in proximal colon cancer than in distal colon and rectal cancer. There are conflicting results in the literature regarding the prognosis and survival rates for mucinous CRC[31]. Factors involved in the development of mucinous CRC are not yet known, but mucinous cancer has a different molecular signature than non-mucinous cancer[31]. This study demonstrated that the *CCL4* gene polymorphism rs10491121 was associated with mucinous CRC, with a higher rate of the A allele than non-mucinous cancer. Moreover, we noted that higher CCL4 mRNA expression levels were associated with mucinous cancer. As far as we know, this is the first time data relating to this investigated genetic variant of CCL4 and CCL4 gene expression in mucinous CRC have been published. Further studies are needed to verify our findings and to investigate the potential function of this SNP on CCL4 expression and for prediction of the mucinous state.

Higher CCL4 mRNA expression levels in tumor tissue than in normal tissue in colon (P = 0.07) was suggested in a previous study including 20 patients[21]. In the present study, with 101 CRC patients, we confirmed that the CCL4 mRNA expression levels were higher (P = 0.044) in CRC tissue than in normal tissue. Two studies focused on CCL4 protein expression found higher levels in CRC tissue compared with in adjacent normal tissue[20,32]. These studies included only 10[20] and 25 patients[32], respectively, and found no association between CCL4 protein levels and cancer stage. In our study, CCL4 protein expression levels were examined in a large series of 98 patients with CRC. We found that CCL4 protein levels were higher in CRC tissue than in normal paired tissue. We also found that localized disease was associated with higher CCL4 protein levels than disseminated disease. The interaction between tumor cells and infiltrating leukocytes such as macrophages is a powerful relationship that affects CRC progression and prognosis[4,7-9]. CCL4 protein can be produced by lymphocytes and macrophages[17,18] and, in this study, immunostaining revealed

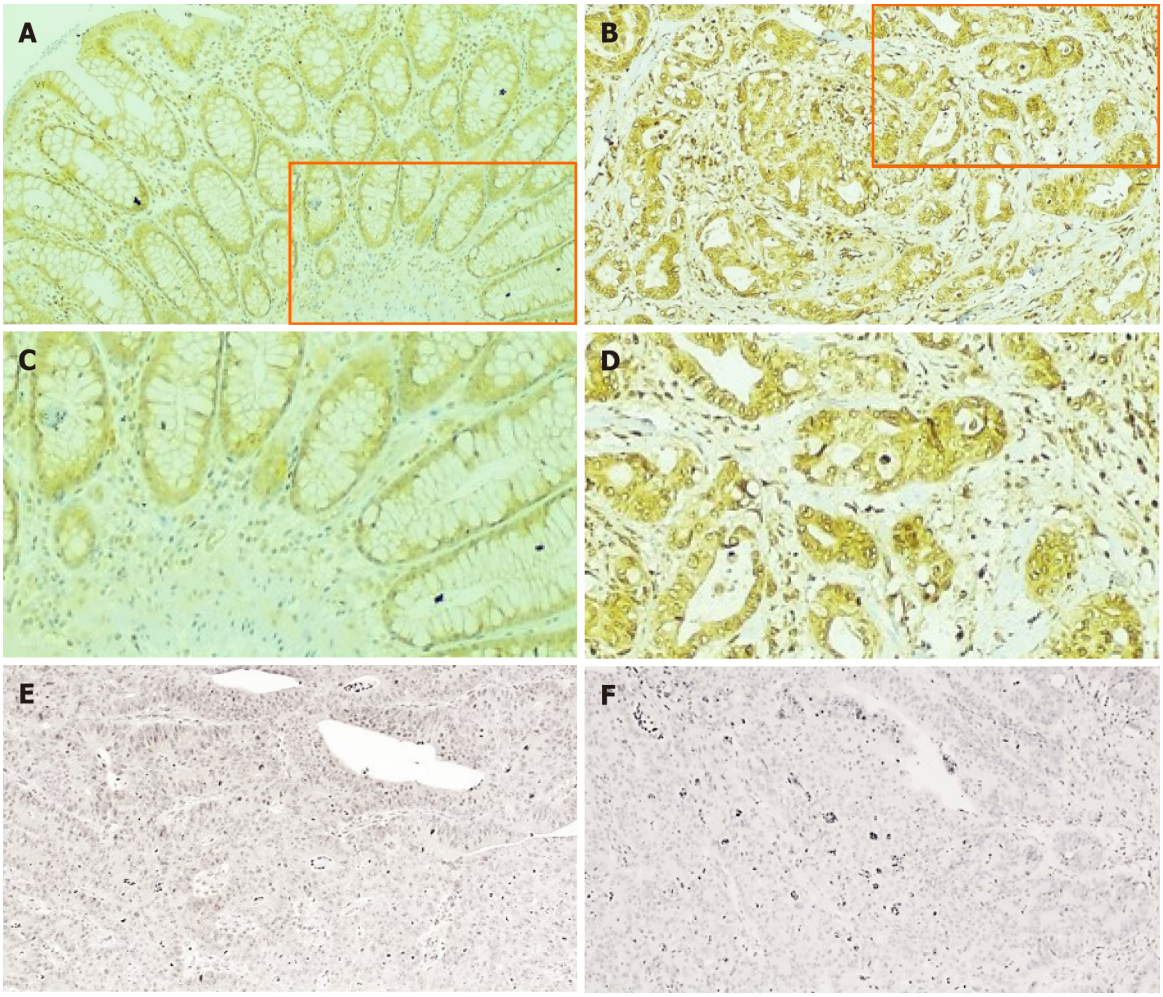

Figure 2 Representative immunohistochemical staining of CC chemokine ligand 4. A: Normal tissue (× 100 magnification); B: Colon cancer tissue (× 100 magnification); C: Normal tissue (× 400 magnification) of highlighted area; D: Colon cancer tissue (× 400 magnification) of highlighted area; E: Isotype control antibody in colon cancer tissue (× 100 magnification); F: Primary antibody omitted in colon cancer tissue (× 100 magnification).

expression of CCL4 in CRC tissue predominantly in epithelial cells. We also detected CCL4 protein in the colon cancer cell lines HT-29 and Caco-2. The lack of association between CCL4 mRNA and CCL4 protein levels in the present study could be explained by differences in transcription rate, mRNA stability, or translation efficiency within cell types such as lymphocytes, macrophages, and epithelial cancer cells in the colon and rectum.

When we grouped CRC tissue levels of the CCL4 protein as low or medium/high, we found that patients with low CCL4 protein levels in CRC tissue had lower cancer-specific survival rates. Thus, based on our data, higher CCL4 protein levels in CRC tissue are associated with less advanced disease stage and better prognosis. The properties of the tumor microenvironment and the interactions between tumor cells and infiltrating leukocytes, such as macrophages and T cells, affect CRC progression and prognosis[4,7-9]. Tumor-associated macrophages (TAMs) are commonly found in CRC tissue[7,8]. Macrophages are an important component of the tumor microenvironment and coordinate various aspects of immunity. TAMs promote tumor growth by facilitating angiogenesis, immunosuppression, and inflammation, and can also influence tumor relapse after anticancer therapies. Depending on activation status, macrophages can exert dual influences on tumorigenesis, either by antagonizing the cytotoxic activity in immune cells or by enhancing antitumor responses[7,8]. The classic view is that TAMs have one of two phenotypes, M1 or M2, which have antitumoral or protumoral activities, respectively[7,8].

The chemokine CCL4 is a potent chemoattractant for monocytes/macrophages, dendritic cells, and T cells, *via* its cognate receptor CCR5, and it is an especially effective chemoattractant for Th1 cells, which have an antitumor role[17-19]. TAMs adapt their phenotype in response to environmental stimuli and the presence of interferon gamma and tumor necrosis factor alpha (TNF-α) promotes polarization into

phenotype M1[7,8]. In a previous study, we showed that TNF-α protein level was significantly higher in CRC tissue than in normal tissue[5]. It could be speculated that a higher level of CCL4 increases the prevalence of Th1 and TAMs, with a shift towards M1 macrophages that is stronger at lower stages than at higher stages, thus negatively affecting tumor development. However, the impact of macrophages in tumor progression remains to be fully elucidated, in part due to the contrasting roles they play depending on their polarization[7,8]. Further studies with immunohistochemical approaches will be conducted to clarify the number of TAMs, their phenotypic patterns, and the presence of T lymphocytes in our analyzed CRC tissue. From a molecular standpoint, the underlying mechanisms responsible for the differing levels of CCL4 at different disease stages remain to be clarified.

This study demonstrated that levels of plasma CCL4 were higher in patients compared with controls and were positively correlated with levels in CRC tissue. Plasma CCL4 concentration could possibly reflect levels in CRC tissues. However, we failed to detect a stage-dependent alteration of plasma CCL4 Level, which is consistent with data from another study[33]. Due to the general immunological imbalance indicated by the systemic cytokine pattern in CRC patients[33,34], we hypothesize that the production of CCL4 by lymphocytes and monocytes may be altered. Thus, the relationship between concentration of CCL4 in plasma and cancer stage may be masked.

Some limitations of our study should be mentioned. It is important to note that this study was of an exploratory nature. The patients and controls were selected from one hospital and may not represent the general populations. Additional studies with larger and more diverse populations of patients and controls are needed to validate our findings.

CONCLUSION

Our data suggested that the CCL4 rs10491121 polymorphism was associated with CRC, particularly mucinous CRC and CRC in the colon. Moreover, we observed that CCL4 protein expression was upregulated in CRC tissues compared with in normal tissue, correlated with disease stage, and that low expression was related to a lower cancer-specific survival rate. Detailed functional analysis is required to reveal the mechanisms underlying our observed associations that different levels of CCL4 in CRC tissue reflect different stages of disease. Moreover, studies with immunohistochemical approaches will be conducted to clarify the contribution of TAMs and their phenotypic patterns and the presence of T lymphocytes in our cohort of CRC tissue.

ARTICLE HIGHLIGHTS

Research background

Colorectal cancer (CRC) is one of the most common malignancies and one of the leading causes of cancer death. Inflammatory factors expressed in CRC cells or in the tumor microenvironment play an important role in local immune regulation. Among these factors, chemokines like CC chemokine ligand (CCL) 4 play an important role in facilitating recruitment of leukocytes.

Research motivation

There is an ongoing search for molecular biomarkers to facilitate early diagnosis, and to determine the prognosis of CRC patients.

Research objectives

The aim of the present study was to examine expression of CCL4 and its genetic polymorphism rs10491121 in patients with CRC and to evaluate their association to clinicopathological parameters and prognostic impact.

Research methods

Blood, tumor and paired normal tissue from patients with CRC and blood samples from healthy controls were subjected to an analysis of CCL4 protein using Luminex technology. Reverse transcription quantitative PCR was used to investigate the CCL4 gene expression in CRC tissue and paired normal tissue. For studies on the CCL4

rs10491121 polymorphism in CRC patients and healthy controls, TaqMan genotype assays based on polymerase chain reaction were used.

Research results

The CCL4 protein and messenger RNA expression levels were higher in CRC tissue than in normal paired tissue and the level of CCL4 protein in CRC tissue from patients with localized disease was higher than that in CRC tissue from patients with disseminated disease. Low levels of CCL4 protein in CRC tissue were associated with a 30% lower cancer-specific survival rate in patients. The level of CCL4 in plasma was higher in CRC patients than in healthy controls and was positively correlated with the CCL4 protein level in CRC tissue. There was a difference in polymorphism rs10491121 between localized disease and disseminated disease in the right colon, with a dominance of allele A in localized disease.

Research conclusions

The results from this study indicate that CCL4 expression and gene polymorphism are good markers of prognosis. However, the findings need to be reproduced in larger cohorts.

Research perspectives

The chemokine CCL4 deserves further attention as a clinical prognostic biomarker in CRC. Detailed functional analysis is required to reveal the mechanisms underlying the observed associations between different levels of CCL4 in CRC tissue and different stages of disease.

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ORIGINAL ARTICLE

Retrospective Study

Clinical efficacy of antiviral therapy in patients with hepatitis B-related cirrhosis after transjugular intrahepatic portosystemic shunt

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Abstract

BACKGROUND

As a country with a high burden of hepatitis B, China has about 86 million cases of hepatitis B virus infection, ranking the first in the world. Currently, there are about 390000 deaths due to hepatitis B-related complications such as liver cirrhosis and liver cancer every year. Consequently, how to control portal hypertension, improve liver functional reserve, and reduce the incidence of hepatic failure and liver cancer in such patients is the focus of current clinical attention. Previous clinical study in our center suggested that at 24 mo after transjugular intrahepatic portosystemic shunt (TIPS), the liver functional reserve of patients with hepatitis B cirrhosis was better than that of patients with alcohol-induced and immune cirrhosis, which may be related to the effective etiological treatment.

AIM

To investigate the clinical efficacy of three first-line antiviral drugs recommended by the guidelines of prevention and treatment for chronic hepatitis B in China (2019) in the treatment of patients with hepatitis B-related cirrhosis who had received a TIPS.

METHODS

The clinical data of 137 patients with hepatitis B-related cirrhosis with portal hypertension after receiving TIPS at our centre between March 2016 and December 2020 were analysed retrospectively. According to different anti-viral drugs, the patients were divided into entecavir (ETV) (n = 70), tenofovir alafenamide fumarate (TAF) (n = 32), and tenofovir disoproxil fumarate (TDF) (n = 35) groups. The cumulative incidence of hepatic encephalopathy and hepatocellular carcinoma, survival, and changes in hepatic reserve function and

glomerular filtration rate in patients treated with different antiviral drugs within 24 mo after surgery were investigated.

RESULTS

At 24 mo after surgery, the Child–Pugh score in the TAF group (6.97 ± 0.86) was lower than that in the TDF (7.49 ± 0.82; t = -2.52, P = 0.014) and ETV groups (7.64 ± 1.17; t = -2.92, P = 0.004). The model for end-stage liver disease score in the TAF group at 24 mo after surgery was 9.72 ± 1.5, which was lower than that in the TDF (10.74 ± 2.33; t = -2.09, P = 0.040) and ETV groups (10.97 ± 2.17; t = -2.93, P = 0.004). At 24 mo after surgery, the estimated glomerular filtration rate (eGFR) in the TAF group (104.41 ± 12.54) was higher than that in the TDF (93.54 ± 8.97) and ETV groups (89.96 ± 9.86) (F = 21.57, P < 0.001).

CONCLUSION

At 24 mo after surgery, compared with TDF and ETV, TAF has significant advantages in the improvement of liver functional reserve and eGFR.

Key Words: Transjugular intrahepatic portosystemic shunt; Hypertension; Antiviral; Hepatitis B-related cirrhosis; Liver reserve function

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Core Tip: As a country with a high burden of hepatitis B, China has about 86 million cases of hepatitis B virus (HBV) infection, ranking the first in the world. Hepatitis B cirrhosis complicated with portal hypertension is characterized by persistent HBV replication and aggravated liver inflammation and fibrosis. Considering the fact that there is currently no report on the clinical efficacy of antiviral therapy for patients with hepatitis B cirrhosis after transjugular intrahepatic portosystemic shunt, we believe that this study has appreciated clinical reference value for the selection of anti-HBV drugs in such patients.

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INTRODUCTION

Patients with chronic hepatitis B (CHB) cirrhosis complicated with severe portal hypertension-related complications rarely accept liver transplantation because of limited liver source and high medical costs. As a result, how to improve the liver functional reserve, reduce the incidence of liver cancer, and enhance the survival rate of such patients has become the focus of clinical research. Transjugular intrahepatic portosystemic shunt (TIPS) is an effective method to treat the complications secondary to liver cirrhosis complicated with portal hypertension. While portal hypertension-related complications in patients with CHB cirrhosis are effectively controlled, early initiation of antiviral therapy can significantly reduce long-term liver-related complications, such as hepatocellular carcinoma (HCC) and hepatic failure, and improve the survival rate. At present, tenofovir disoproxil fumarate (TDF), entecavir (ETV), and tenofovir alafenamide fumarate (TAF) are recommended as the first-line treatment options for CHB patients by major international and domestic guidelines for the diagnosis, treatment, and management of CHB. Moreover, previous clinical study[1] in our center suggested that the use of an 8-mm stent during TIPS could not only effectively shunt, but also relieve portal pressure. Twenty-four months after surgery, the liver functional reserve of patients with hepatitis B cirrhosis was better than that of patients with alcohol-induced and immune cirrhosis, which may be related to the effective etiological treatment. Based on the above studies, the clinical data of patients with hepatitis B cirrhosis after TIPS treated with different antiviral drugs were

data access agreement. Proposals should be directed to jpqqing@163.com.

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retrospectively analyzed in this study. However, TAF entered the Chinese market since November 2018. Current data on the efficacy and safety of TAF in Chinese people are rare, especially in patients with CHB cirrhosis complicated with portal hypertension whose portal vein pressure is relieved after TIPS. Therefore, we evaluated the clinical efficacy of the three antiviral drugs through this retrospective study, with an aim to provide reference for antiviral treatment of patients with CHB cirrhosis after TIPS.

MATERIALS AND METHODS

Subjects

The clinical data of 137 patients with CHB cirrhosis complicated with portal hypertension treated by TIPS with a Viator stent at our center from March 2016 to December 2020 were collected. The inclusion criteria included: (1) Adult patients with CHB cirrhosis complicated with portal hypertension treated by TIPS for the first time; (2) patients receiving TIPS with a shunt channel dilated by an 8-mm balloon and an 8-mm TIPS-specific stent (Viator stent) implanted; and (3) patients undergoing monotherapy (ETV/TDF/TAF) for hepatitis B virus (HBV) for more than one year, with complete virological response maintained 3, 6, and 12 mo after surgery, as evidenced by a low HBV DNA (the lower limit of detection of HBV DNA was 100 IU/mL). The exclusion criteria were as follows: (1) Patients with liver cancer confirmed by imaging examination before surgery, and a history of splenectomy or partial splenic artery embolization; (2) patients with stent dysfunction; (3) patients with preoperative Child-Pugh score for hepatic function > 13 and underlying nephropathy [estimated glomerular filtration rate (eGFR) < 90 mL/min]; (4) patients with cavernous transformation of the portal vein or portal thrombosis; (5) patients with preoperative hepatic encephalopathy (HE), hepatorenal syndrome, and hepatopulmonary syndrome; (6) patients with a history of major liver surgery such as hepatectomy, surgical shunt, and liver transplantation; (7) patients with severe coagulation disorders; (8) patients with severe right heart failure and pulmonary hypertension; (9) patients with uncontrolled intrahepatic infection [alanine aminotransferase (ALT) was more than 10 times higher than the upper limit of normal] and systemic infection; (10) pregnant and lactating patients or patients with pregnancy plan; and (11) patients with liver cirrhosis treated with warfarin and other anticoagulants after surgery. All the patients met the indications of the United States TIPS guidelines[2] and the American Association of the Study of Liver Diseases (AASLD) guidelines for the prevention and treatment of CHB[3]. This study was approved by the hospital ethics committee. The patients and their families were fully informed and signed an informed consent form.

Equipment and materials

Digital subtraction angiography (DSA) was carried out using the AXIOM-Artist DSA system (Siemens, Germany) and Mark V high-pressure syringes. Surgical materials included a RUPS-100 puncture kit, a straight-needle multi-side hole catheter, an Opta Pro balloon catheter, embolization coils, a Cobra catheter (Cook, United States), a stiffening exchange guide wire, and a Viatorr stent (Gore and Associates, United States). Additionally, TDF (300 mg/tablet, Beite Pharmaceutical Co., Ltd.), ETV tablets (0.5 mg/tablet, Sino-American Shanghai Squibb Pharmaceutical Co., Ltd.), and TAF tablets (25 mg/tablet, Gilead, United States) were used in this study.

Research methodology

Preoperative preparation: Preoperatively, routine examinations including routine blood test, liver and kidney function test, HBV-DNA quantitation, and prothrombin time test were performed. All the patients underwent enhanced computed tomography (CT) of the liver and three-dimensional (3D) reconstruction of the hepatic vein-portal vein. The anatomic relationship between the hepatic vein and the portal vein was analyzed to guide the puncture of the portal vein during surgery.

TIPS approach: All the patients received TIPS *via* the right jugular vein. Under the guidance of a guide wire, the puncture system passed through the superior vena cava and the right atrium to the right hepatic vein or the hepatic segment of the inferior vena cava. According to the images obtained from preoperative enhanced CT of the liver and 3D reconstruction of the hepatic vein-portal vein, the puncture of the portal vein branch was guided. After the safety of the puncture was evaluated, portal vein

pressure was measured and the collateral circulation vessels causing esophagogastric varices were embolized. Then, a stent was inserted after balloon dilation puncturing the channel, followed by another portal vein angiography and measurement of portal vein pressure. The detailed operations have been reported in a previous study[4]. All operations were successfully completed by the same group of professionals using an 8-mm balloon and an 8-mm Viatorr stent, without severe operation-related complications.

Postoperative follow-up: The data on HBV-DNA quantitation, liver and kidney function, and coagulation were collected before surgery and 1, 3, 6, 12, and 24 mo after surgery. Ascites and HE were understood by ultrasonography and follow-up records. Child-Pugh grade and the model for end-stage liver disease (MELD) were obtained, respectively. The follow-up endpoints of follow-up was death at 24 mo after surgery, no response to follow-up for consecutive 2 times or more, liver transplantation, or study deadline (December, 2020). The evaluation methods of follow-up were referred to the references[5-7].

Statistical analysis

All measurement data are expressed as the mean ± SD or percentages. The portal pressure gradient before and after treatment was analyzed using the paired *t*-test or chi-square test. The incidence of HE and liver cancer and survival rate were calculated with the Kaplan-Meier curve and compared by the log-rank test. All the data were statistically analyzed using SPSS 22.0. $P < 0.05$ was considered significantly significant.

RESULTS

Patient characteristics

A total of 137 patients with liver cirrhosis were enrolled, including 75 males and 62 females, with an average age of 54.0 ± 9.1 years. According to different antiviral drugs, the patients were divided into an ETV group ($n = 70$), a TAF group ($n = 32$), and a TDF group ($n = 35$). Gender, age, preoperative and postoperative portal pressure gradient, preoperative and postoperative Child-Pugh grade, MELD score, the proportion of hepatitis B e antigen (HBeAg)-positive patients, median HBV DNA level, median time to TAF/TDF/ETV therapy, and kidney function index (eGFR or sCr) showed no statistically significant differences among the three groups (Table 1).

TIPS operations

TIPS *via* the right jugular vein was performed successfully in all the 137 patients, without severe intraoperative complications such as abdominal hemorrhage and bile duct hemorrhage. The collateral circulation causing esophagogastric varices was embolized and the stent was implanted successfully. The success rate of surgery was 100%, and the short-term hemostasis rate was 100%. The portal pressure gradient (mmHg) decreased from 25.71 ± 5.32 preoperatively to 9.95 ± 2.75 postoperatively ($t = 37.32$, $P < 0.001$).

Survival, HE, and liver cancer in three groups

The median follow-up time was 24 (20, 24) mo. The survival rates at 12 and 24 mo after surgery was 93.3% and 89.6% in the TAF group, 93.9% and 86.9% in the TDF group, and 91.2% and 84.3% in the ETV group, respectively (log rank $\chi^2 = 0.517$, $P = 0.772$) (Figure 1A). During the follow-up, a total of 17 patients died, with a median time to death of 12 (7, 20) mo, including three cases in the TAF group, four in the TDF group, and ten in the ETV group. The causes of death included primary liver cancer in 11 (2/2/7) patients, sepsis/infection in 4 (1/1/2), and hepatorenal syndrome in 2 (0/1/1).

One, three, six, twelve, and twenty-four months after surgery, the incidence of HE was 6.2%, 12.5%, 12.5%, 15.6%, and 19.5% in the TAF group, 2.9%, 14.3%, 14.3%, 17.6%, and 25.7% in the TDF group, and 5.7%, 13.1%, 16.1%, 20.9%, and 28.3% in the ETV group (log rank $\chi^2 = 0.712$, $P = 0.700$) (Figure 1B). During the follow-up, HE occurred in 32 patients, with a median time to occurrence of 3 mo. There were six cases of HE in the TAF group (HE grade I/II/III-IV, 4/2/0), eight in the TDF group (HE grade I/II/III-IV, 6/1/1), and 18 in the ETV group (HE grade I/II/III-IV, 14/2/2).

Table 1 Basic clinical data

Clinical characteristic	TAF (*n* = 32)	TDF (*n* = 35)	ETV (*n* = 70)	Statistic	*P* value	Total (*n* = 137)
Gender, *n* (%)				$X^2 = 1.033$	0.597	
Male	20	18	37			75
Female	12	17	33			52
Age (yr, mean ± SD)	53.8 ± 8.5	54.4 ± 7.3	54.5 ± 10.3	$t = 0.234$	0.815	54.3 ± 9.2
Child-Pugh classification				$X^2 = 1.623$	0.805	
A	9	14	21			44
B	18	17	37			72
C	5	4	12			21
Child-Pugh score	6.94 ± 1.29	7.46 ± 2.14	7.26 + 1.64	F = 1.119	0.330	7.31 ± 1.75
MELD score	10.37 ± 2.94	9.69 ± 2.96	10.41 ± 2.93	F = 0.775	0.463	10.22 ± 2.93
Prothrombin time (s)	14.40 ± 1.86	15.14 ± 2.81	14.95 ± 2.73	F = 0.930	0.354	14.74 ± 2.59
Albumin (g/L)	35.27 ± 5.65	34.14 ± 5.01	34.43 ± 4.33	F = 0.496	0.610	34.55 + 4.82
eGFR (mL/min/1.73 m^2)	91.22 ± 10.67	90.37 ± 14.24	90.19 ± 12.05	F = 0.078	0.925	90.47 ± 12.27
Cr (μmol/L)	75.04 ± 36.60	78.07 ± 39.72	71.56 ± 23.70	F = 0.940	0.349	74.04 ± 31.51
Total bilirubin (μmol/L)	36.83 ± 14.74	38.62 ± 17.21	34.10 ± 15.69	F = 0.868	0.387	35.89 ± 15.89
PPG before TIPS (mmHg)	25.41 ± 4.82	25.71 ± 5.49	25.84 ± 5.49	F = 0.073	0.929	25.71 ± 5.31
PPG after TIPS (mmHg)	9.52 ± 2.28	9.97 ± 3.04	10.13 ± 2.81	F = 0.530	0.590	9.95 ± 2.75
Deaths in 24 mo, *n* (%)	3 (9.4)	4 (11.4)	10 (14.3)	$X^2 = 0.517$	0.772	17 (13.7)
HCC in 24 mo, *n* (%)	2 (6.3)	2 (5.7)	7 (10)	$X^2 = 0.712$	0.700	11 (9.3)
HE in 24 mo, *n* (%)	6 (18.8)	8 (22.9)	18 (25.7)	$X^2 = 0.770$	0.681	32 (25.4)

MELD: Model for end-stage liver disease; TIPS: Transjugular intrahepatic portosystemic shunt; PPG: Portal pressure gradient; HE: Hepatic encephalopathy; HCC: Hepatocellular carcinoma; eGFR: Estimated glomerular filtration rate; TAF: Tenofovir alafenamide fumarate; TDF: Tenofovir disoproxil fumarate; ETV: Entecavir.

Twelve and twenty-four months after surgery, the incidence of liver cancer was 3.4% and 7.3% in the TAF group, 3.6% and 7.3% in the TDF group, and 6.1% and 11.3% in the ETV group, respectively (log rank $\chi^2 = 0.77$, $P = 0.681$) (Figure 1C). During the follow-up, liver cancer occurred in 11 patients, with a median time to occurrence of 11 (8, 14) mo (TAF *vs* TDF *vs* ETV, 2/2/7).

Child-Pugh score and constituent ratio before and after TIPS in three groups

Child-Pugh score showed no significant differences in the three groups before and 12 mo after surgery ($P > 0.05$). Twenty-four months after surgery, Child-Pugh score in the TAF group was 6.97 ± 0.86, which was lower than those in the TDF group (7.49 ± 0.82; $t = -2.52$, $P = 0.014$) and ETV group (7.64 ± 1.17; $t = -2.92$, $P = 0.004$) (Figure 2).

Twenty-four months after surgery, the constituent ratio of Child-Pugh stage A/B/C (60.8/34.8/4.4) in the TAF group was improved compared with that (28.1/56.3/15.6) before surgery ($\chi^2 = 6.47$, $P = 0.039$), but the constituent ratio of Child-Pugh stage A/B/C in the TDF/ETV group presented no difference from that before surgery ($P > 0.05$) (Table 2).

MELD score before and after surgery in three groups

No statistically significant differences were found in MELD score before and 12 mo after surgery in the three groups ($P > 0.05$). Twenty-four months after surgery, MELD score in the TAF group was 9.72 ± 1.5, which was lower than those in the TDF group (10.74 ± 2.33; $t = -2.09$, $P = 0.040$) and ETV group (10.97 ± 2.17; $t = -2.93$, $P = 0.004$) (Figure 3).

eGFR change before and after surgery in three groups

In the three groups, eGFR change showed no significant differences 3 mo after surgery

Table 2 Child-Pugh constituent ratio

	Baseline	1 mo	3 mo	6 mo	12 mo	24 mo
TAF, *n* (%)	9/18/5 (32)	4/23/5 (32)	3/23/6 (32)	6/22/4 (32)	7/18/2 (27)	14/8/1 (23)
Child-Pugh A/B/C (%)	28.1/56.3/15.6	12.5/71.9/12.7	12.7/74.6/15.6	18.8/68.8/12.5	25.9/66.7/7.4	60.9/34.8/4.3
TDF(*n*)	14/17/4 (35)	4/25/6 (35)	4/23/8 (35)	7/21/5 (33)	6/20/3 (29)	7/11/3 (21)
Child-Pugh A/B/C (%)	40.0/48.6/11.4	11.4/71.4/12.0	11.4/65.8/22.8	21.2/63.6/15.2	20.7/68.9/10.4	33.3/52.4/14.3
ETV, *n* (%)	21/37/12 (70)	9/47/13 (69)	6/47/16 (69)	11/44/11 (66)	11/42/6 (59)	11/24/5 (40)
Child-Pugh A/B/C (%)	30.3/52.9/17.1	13.1/68.1/18.8	8.7/68.1/23.2	16.7/66.6/16.7	18.6/71.2/10.2	27.5/60.0/12.5

TAF: Tenofovir alafenamide fumarate; TDF: Tenofovir disoproxil fumarate; ETV: Entecavir.

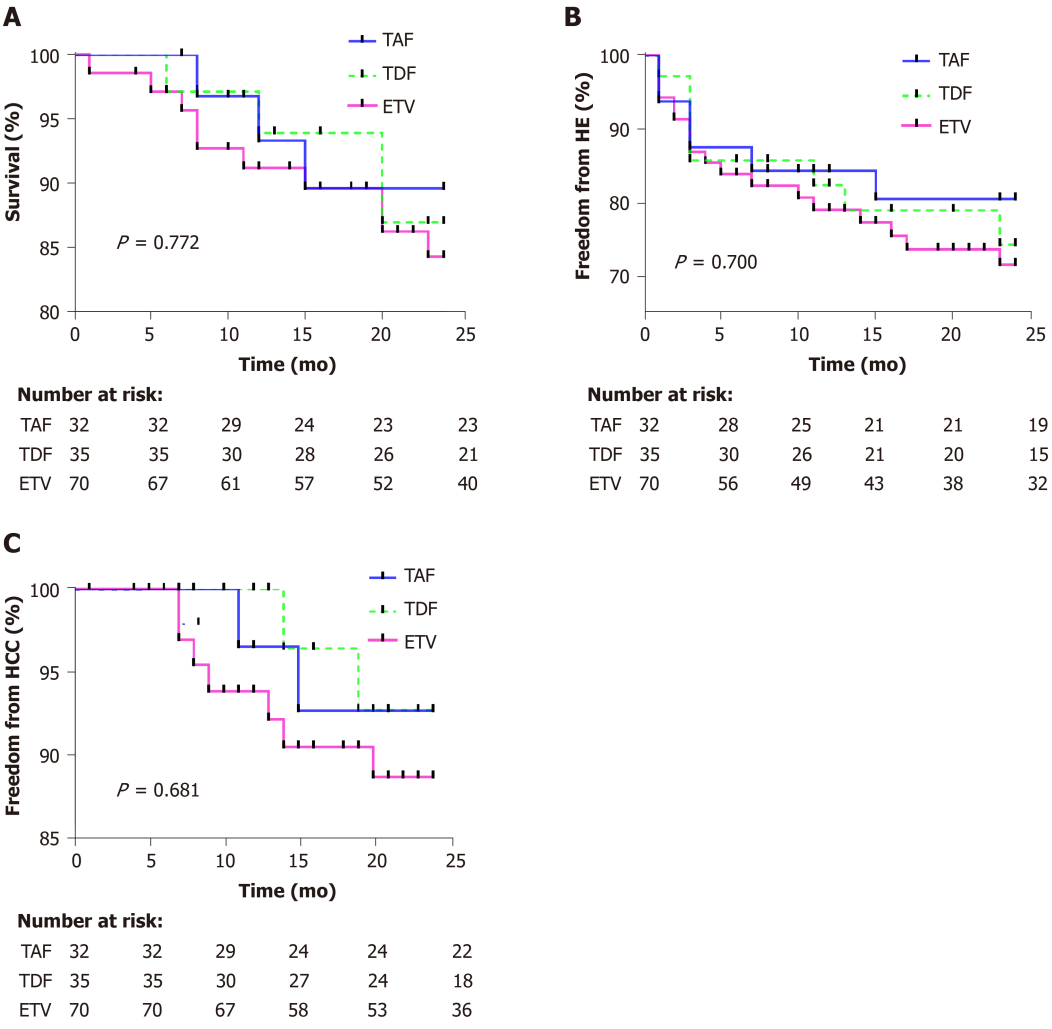

Figure 1 Overall survival, hepatic encephalopathy free survival, and hepatocellular carcinoma free survival rate in tenofovir alafenamide fumarate, tenofovir disoproxil fumarate, and entecavir groups. A: Survival; B: Freedom from hepatic encephalopathy; C: Freedom from hepatocellular carcinoma. HCC: Hepatocellular carcinoma; HE: Hepatic encephalopathy; TAF: Tenofovir alafenamide fumarate; TDF: Tenofovir disoproxil fumarate; ETV: Entecavir.

($P > 0.05$). Six months after surgery, eGFR level in the TAF group was 99.72 ± 11.52, which was higher than that in the ETV group (91.24 ± 12.60; $t = 3.24$, $P = 0.002$), but not significantly different from that in the TDF group (94.97 ± 12.52; $t = 1.61$, $P = 0.112$). Twelve months after surgery, eGFR level in the TAF group was 103.44 ± 13.02, which was higher than those in the TDF group (94.11 ± 12.34; $t = 3.08$, $P = 0.004$) and ETV group (90.03 ± 11.04; $t = 5.37$, $P < 0.001$). Twenty-four months after surgery, eGFR level in the TAF group was 104.41 ± 12.54, which was higher than those (93.54 ± 8.97) in the TDF (93.54 ± 8.97) and ETV (89.96 ± 9.86) groups ($F = 21.57$, $P < 0.001$) (Figure 4).

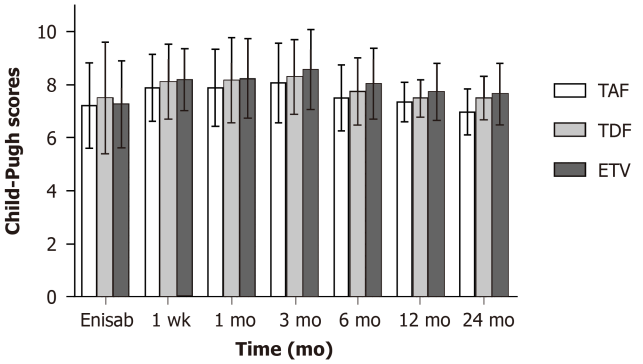

Figure 2 Preoperative and postoperative Child-Pugh scores and ratios in the three patient groups. TAF: Tenofovir alafenamide fumarate; TDF: Tenofovir disoproxil fumarate; ETV: Entecavir.

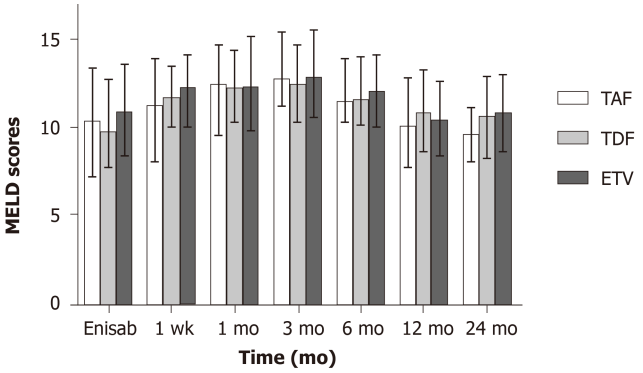

Figure 3 Changes in preoperative and postoperative model for end-stage liver disease scores in the three patient groups. MELD: Model for end-stage liver disease; TAF: Tenofovir alafenamide fumarate; TDF: Tenofovir disoproxil fumarate; ETV: Entecavir.

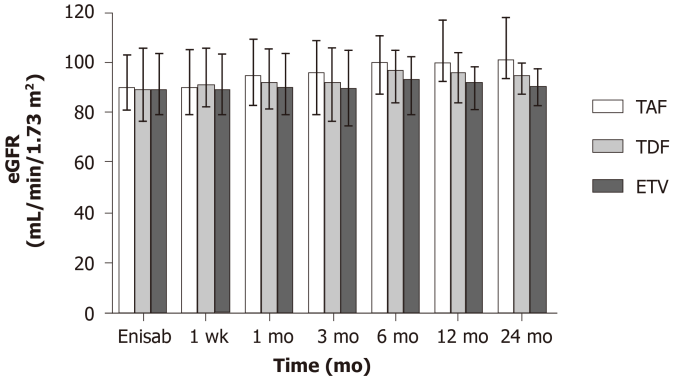

Figure 4 Changes in preoperative and postoperative estimated glomerular filtration rates in the three patient groups. eGFR: Estimated glomerular filtration rate; TAF: Tenofovir alafenamide fumarate; TDF: Tenofovir disoproxil fumarate; ETV: Entecavir.

DISCUSSION

As a country with a high burden of hepatitis B, China has about 86 million cases of HBV infection, ranking the first in the world. Currently, there are about 30 million CHB patients, and 390000 deaths due to hepatitis B-related complications such as liver cirrhosis and liver cancer every year[8,9]. Hepatitis B cirrhosis complicated with portal hypertension is characterized by persistent HBV replication and aggravated liver inflammation and fibrosis. Without timely control, it may progress to severe post-cirrhosis complications such as esophagogastric variceal bleeding, HE, intractable hydrothorax and ascites, and even hepatic failure and liver cancer, leading to death. Consequently, how to control portal hypertension, improve liver functional reserve, and reduce the incidence of hepatic failure and liver cancer in such patients is the

focus of current clinical attention. TIPS is an effective minimally invasive interventional method for the treatment of portal hypertension-related complications. It is worthy of further clinical exploration on the management of patients with hepatitis B cirrhosis using individualized antiviral therapy after surgery. Considering the fact that there is no report on the clinical efficacy of antiviral therapy for patients with hepatitis B cirrhosis after TIPS, we believe that this study has appreciated clinical reference value for the selection of anti-HBV drugs in such patients.

CHB is one of the main causes of HCC, cirrhosis-related complications, and liver-related death all over the world[10]. Chronic HBV infection is mainly treated by maximizing the long-term inhibition of HBV replication, relieving the inflammation and necrosis of hepatocytes and fibrous hyperplasia of the liver, delaying and reducing the occurrence of hepatic failure, decompensated cirrhosis, HCC, and other complications, improving the quality of life of the patients, and prolonging their survival time[11-13]. Antiviral therapy is a long-term or even lifelong process. The efficacy and safety of drugs are important factors that clinicians and patients need to consider. Previous studies have shown that in CHB patients with initial treatment, the incidence of drug resistance 5 years after ETV therapy is about 1.2%[14,15], while the clinical drug resistance of TDF and TAF has not been definitely reported[16,17]. Moreover, these three drugs are the first choice of oral antiviral drugs recommended by international and domestic guidelines, characterized by high barrier to resistance, strong effect, and high safety. In this study, all the included patients were those with hepatitis B cirrhosis after TIPS, and treated with the three antiviral drugs. During follow-up, the percentage of complete virological response of HBV DNA at 3, 6, and 12 mo after surgery was 100%, without breakthrough in virology. We believe that after continuous antiviral therapy, complete virological response can be achieved, inflammatory activity of the liver can be reduced or even become static, liver fibrosis can be reversed, and the regeneration ability of residual hepatocytes can be enhanced, which can further improve the liver functional reserve of the patients. In this study, TAF showed a better effect in improving the liver functional reserve of patients with hepatitis B cirrhosis at 24 mo after TIPS than TDF and ETV, the potential mechanism of which may be attributed to the stronger antiviral effect and ALT normalization ability of TAF. Previous global phase III clinical studies 108/110 on TAF[18,19] showed that compared with TDF, CHB patients treated with TAF could achieve a higher proportion of ALT normalization at both the 48th and 96th weeks. Moreover, an increasing number of real-world studies[20-22] demonstrated that about 30% of CHB patients receiving long-term ETV therapy failed to achieve complete virological response (the lower limit of detection of HBV DNA was 20 IU/mL), while most patients could achieve complete virological response after switching to TAF therapy. All these studies suggest that TAF has more prominent advantages in antiviral ability and/or ALT normalization. In our center, the lower limit of detection of HBV DNA was 100 IU/mL, which could not be used to accurately identify the CHB population with a low level of HBV DNA replication. However, the above findings may still have certain reference value for antiviral therapy in patients with hepatitis B cirrhosis complicated with portal hypertension after TIPS.

In fact, for patients with cirrhosis, especially for those with decompensated cirrhosis, liver transplantation is the only way to remove the lesion and radically cure cirrhosis. However, transplantation is rarely conducted in Asia. Especially in China, fewer patients can receive liver transplantation because of the limitation in funds and liver sources. For patients with hepatitis B cirrhosis, effective etiological treatment combined with control of portal hypertension and improvement of liver functional reserve is the key to long-term survival of patients with decompensated hepatitis B cirrhosis who cannot accept liver transplantation. TIPS is the only minimally invasive method for relieving portal hypertension. Previous clinical study[1] in our center suggested that the use of an 8-mm stent during TIPS could not only effectively shunt, but also relieve portal pressure. Postoperatively, the liver functional reserve was affected by shunt in a short time, but the special stent with an inner diameter of 8 mm had limited shunt. The liver functional reserve could be restored to the preoperative level 12 mo after surgery. Twenty-four months after surgery, the liver functional reserve of patients with hepatitis B cirrhosis was better than that of patients with alcohol-induced and immune cirrhosis, which may be related to the effective etiological treatment. Based on the above studies, the clinical data of patients with hepatitis B cirrhosis after TIPS treated with different antiviral drugs were retrospectively analyzed in this study. Before this study, many studies[23-27] in the past 2 years have explored the effects of different antiviral drugs on the long-term occurrence of HCC in patients with CHB, most of which are retrospective cohort studies, and some were systematic reviews and meta-analyses, with non-unified

conclusions. However, it can be concluded that nucleotide drugs (represented by TFV) are equal or superior to nucleoside analogues (represented by ETV) in the long-term risk of HCC, especially in some patients with high-risk factors for HCC, such as compensated cirrhosis or decompensated cirrhosis. In this study, it was found that during the follow-up, the incidence of HE and liver cancer as well as survival rate had no statistical significance among the three groups. We believe that it was related to the small number of patients enrolled in this study and short follow-up time. Because TAF has not been clinically applied in China for a long time, the long-term clinical outcomes of the three different antiviral drugs in the treatment of patients with hepatitis B cirrhosis after TIPS need to be further clarified by expanding the sample size and prolonging the observation time.

According to the Chinese guidelines for TIPS operation in 2019[28], TIPS can effectively improve glomerular filtration rate, increase renal blood flow, and reduce serum creatinine and aldosterone levels. The eGFR of all the patients included in this study was improved at varying degrees 6 mo after surgery. We believe that the short-term changes in renal function are related to the changes in systemic hemodynamics after TIPS, and effective control of portal pressure can improve renal blood supply and glomerular filtration rate. The safety of TAF for the bone and kidney has been proved to be higher than that of TDF in previous global phase III clinical studies 108/110[18, 19]. Additionally, the 2018 AASLD HBV guidelines[11] suggest no advantages or disadvantages between ETV and TDF for the risk of long-term renal and skeletal complications. Compared with TDF, TAF is associated with a lower proportion of patients with skeletal and renal abnormalities. Moreover, in the 2017 EASL guidelines [12], TAF or ETV is superior to TDF in CHB patients > 60 years old with bone diseases and renal changes. With the same baseline, eGFR of the TAF group was better than the other two groups 12-24 mo after surgery in this study. We believe that in the limited follow-up time, there were differences among the three drugs in the treatment of patients with hepatitis B cirrhosis after TIPS. TAF may have a smaller effect on the renal function of patients with decompensated hepatitis B cirrhosis than the other two drugs, which further indicates that TAF may have a smaller effect on the renal function of patients with CHB cirrhosis. Through clinical practice, we believe that patients with hepatitis B cirrhosis after TIPS can benefit from long-term treatment by active antiviral therapy and choosing TAF, which has a smaller effect on renal function and can potently inhibit viruses.

This study has several limitations. First, this is a retrospective cohort study. Although most indexes in different treatment groups were consistent at the baseline in this study, there were still potential unpredictable biases. Second, in this study, HBeAg status, HBsAg titer level, and ALT that may affect the long-term prognosis were not recorded and analyzed. Finally, the time from the approval of TAF in China to its clinical application is still short, and the number of patients receiving TAF therapy is relatively small. Therefore, the sample size of this study is small, and we need to further expand the sample size, and use propensity score matching statistical method to avoid excessive biases and baseline mismatch as much as possible. This study is continuing to determine whether the beneficial effect of TAF will last for a long time, and we will also conduct a longer-term clinical observation on the antiviral therapy in patients with hepatitis B cirrhosis after TIPS.

In our center, the operation and clinical management were performed by the same group of doctors. Through clinical practice, we believe that improving the long-term survival rate of patients with hepatitis B cirrhosis after TIPS involves a complex situation, which is determined by a large number of non-hemodynamic factors. Age, degree of renal failure, chronic inflammation, urease-producing intestinal bacteria, bacterial translocation and malnutrition/atrophy are other very important factors in the regulation of the treatment. Early initiation of antiviral therapy and optimization of antiviral therapy are also important factors. In conclusion, compared with TDF and ETV, TAF has significant advantages in the improvement of liver functional reserve and eGFR. The difference in the long-term effect of TAF on HCC occurrence needs further observation and clarification.

CONCLUSION

At 24 mo after surgery, compared with TDF and ETV, TAF has significant advantages in the improvement of liver functional reserve and eGFR. The difference in the long-term effect of TAF on HCC occurrence needs further observation and clarification.

ARTICLE HIGHLIGHTS

Research background

Hepatitis B cirrhosis complicated with portal hypertension is characterized by persistent hepatitis B virus (HBV) replication and aggravated liver inflammation and fibrosis. Without timely control, it may progress to severe post-cirrhosis complications such as esophagogastric variceal bleeding, hepatic encephalopathy (HE), intractable hydrothorax and ascites, and even hepatic failure and liver cancer, leading to death. Consequently, how to control portal hypertension, improve liver functional reserve, and reduce the incidences of hepatic failure and liver cancer in such patients is the focus of current clinical attention. Previous clinical study in our center suggested that the use of an 8-mm stent during transjugular intrahepatic portosystemic shunt (TIPS) could not only effectively shunt, but also relieve portal pressure. Twenty-four months after surgery, the liver functional reserve of patients with hepatitis B cirrhosis was better than that of patients with alcohol-induced and immune cirrhosis. Based on the above studies, the clinical data of patients with hepatitis B cirrhosis after TIPS treated with different antiviral drugs were retrospectively analyzed in this study.

Research motivation

TIPS is an effective minimally invasive interventional method for the treatment of portal hypertension-related complications. It is worthy of further clinical exploration on the management of patients with hepatitis B cirrhosis using individualized antiviral therapy after surgery. Considering the fact that there is no report on the clinical efficacy of antiviral therapy for patients with hepatitis B cirrhosis after TIPS, we believe that this study has appreciated clinical reference value for the selection of anti-HBV drugs in such patients.

Research objectives

To explore the clinical efficacy of the three antiviral drugs through this retrospective study, so as to provide reference for antiviral treatment of patients with chronic hepatitis B (CHB) cirrhosis after TIPS.

Research methods

The clinical data of 137 patients with hepatitis B-related cirrhosis with portal hypertension after receiving TIPS at our center between March 2016 and December 2020 were analysed retrospectively. According to different anti-viral drugs, the patients were divided into entecavir (ETV) (n = 70), tenofovir alafenamide fumarate (TAF) (n = 32), and tenofovir disoproxil fumarate (TDF) (n = 35) groups. The cumulative incidence of HE and hepatocellular carcinoma (HCC), survival, and changes in hepatic reserve function and glomerular filtration rate in patients treated with different antiviral drugs within 24 mo after surgery were investigated.

Research results

At 24 mo after surgery, compared with TDF and ETV, TAF had significant advantages in the improvement of liver functional reserve and estimated glomerular filtration rate (eGFR).

Research conclusions

Through clinical practice, compared with TDF and ETV, TAF has significant advantages in the improvement of liver functional reserve and eGFR. The difference in the long-term effect of TAF on HCC occurrence needs further observation and clarification. The author's team believes that improving the long-term survival rate of patients with hepatitis B cirrhosis after TIPS involves a complex situation, which is determined by a large number of non-hemodynamic factors. Early initiation of antiviral therapy and optimization of antiviral therapy are important factors. Age, degree of renal failure, chronic inflammation, urease-producing intestinal bacteria, bacterial translocation, and malnutrition/atrophy are other very important factors in the regulation of the treatment.

Research perspectives

The difference in the long-term effect of TAF on HCC occurrence needs further observation and clarification.

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ORIGINAL ARTICLE

Observational Study

Assessing disease activity using the pediatric Crohn's disease activity index: Can we use subjective or objective parameters alone?

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Abstract

BACKGROUND

The pediatric Crohn's disease activity index (PCDAI) is used as a standard tool to assess disease activity in clinical trials for pediatric Crohn's disease.

AIM

To examine which items on the PCDAI drive assessment of disease activity, and how subgroups of subjective and objective items reflect change in disease state over time.

METHODS

Selective raw data from three prospectively collected datasets were combined, including 703 children with full PCDAI data at baseline, at 3-mo (Q1, n = 670), and 1-year (Q4, n = 474). Change in individual PCDAI scores from baseline to Q1 and to Q4 were examined using the non-weighted PCDAI.

RESULTS

Abdominal pain, well-being, weight, and stooling had the highest change scores over time. Objective indicators including albumin, abdominal exam, and height velocity followed. Change scores for well-being and abdominal exam did not explain significant variance at Q1 but were significant predictors at Q4 ($P < 0.001$ and $P < 0.05$). Subjective and objective subgroups of items predicted less variance

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(18% and 22%) on total PCDAI scores at Q1 and Q4 compared to the full PCDAI, or a composite scale (both 32%) containing significant predictors.

CONCLUSION

Although subjective items on the PCDAI change the most over time, the full PCDAI or a smaller composite of items including a combination of subjective and objective components classifies disease activity better than a subgroup of either subjective or objective items alone. Reliance on subjective or objective items as stand-alone proxies for disease activity measurement could result in misclassification of disease state.

Key Words: Crohn's disease; Pediatric Crohn's disease activity index; Patient reported outcome measurement; Disease activity; Clinical trials; Pediatric

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Core Tip: The pediatric Crohn's disease activity index (PCDAI) is commonly used to assess disease activity in clinical trials. The PCDAI is a multi-item index incorporating subjective (*e.g.*, patient well-being) and objective (*e.g.*, laboratory tests) items. In response to a call from the Food and Drug Administration our team reexamined functioning of this index. Although subjective items on the PCDAI changed the most over time, the full PCDAI or a smaller composite of items that includes both subjective and objective components better classifies disease activity. Use of subjective or objective items on their own may result in misclassification of disease state.

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INTRODUCTION

The pediatric Crohn's disease activity index (PCDAI) has been the standard tool to assess clinical disease activity and response to treatment in clinical trials of pediatric Crohn's disease (CD) for the past two decades[1-4]. The PCDAI was developed by a group of clinicians and focuses on: (1) Subjective reporting of the degree of abdominal pain, stool pattern, and general well-being; (2) Extra-intestinal manifestations, such as fever, arthritis, rash, and uveitis; (3) Physical examination findings including abdominal pain, perirectal disease, extraintestinal manifestations, weight and height; and (4) Laboratory data, including hematocrit, erythrocyte sedimentation rate (ESR), and serum albumin[1] . This index has high inter-rater reliability, and good construct validity with physician global assessments of disease activity[1]. Validity has been further demonstrated in several studies assessing its psychometric properties[5-9].

Over time, concerns over both feasibility and short-term responsiveness to change in clinical status have arisen, due to the inclusion of perianal examination, as well as height velocity, which are not expected to change in a short-term (typically 8-12-wk period for induction of remission) clinical trial. These feasibility concerns are problematic in use of the PCDAI as the completion rate in routine practice of the PCDAI is approximately half (48% *vs* 98%)[10] that of the pediatric ulcerative colitis activity index (PUCAI), which does not include laboratory values, physical examination, or growth[11]. In response, other versions of the PCDAI have been developed, which have demonstrated increased feasibility: the abbreviated PCDAI[10,12,13], the Short PCDAI[10], and the modified PCDAI[14], but they have had lower face, construct and discriminant validity than the original PCDAI[5]. Subsequently, a mathematically weighted version (wPCDAI) that removed redundant and low feasibility items was established[5]. Despite multiple versions, there remain concerns around construct validity due to the mismatch between clinical symptoms relied upon in the wPCDAI and objective markers of inflammation as visualized endoscopically, or

Grade A (Excellent): A, A
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

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reflected *via* C-reactive protein or fecal calprotectin[15-17].

Traditionally, endpoints chosen for clinical trials have been based on multi-item indices[18,19], such as the PCDAI, which can incorporate symptoms, signs, laboratory tests, and endoscopic measures[20]. The PCDAI is still in use as an endpoint in many pediatric CD trials despite the feasibility concerns of these multi-item indices. As such, the United States Food and Drug Administration (FDA) sponsored the Gastroenterology Regulatory Endpoints and Advancement of Therapeutics (GREAT) workshops to reassess their use[20]. The FDA is seeking to redefine endpoints used in clinical trials, based on the need for objective measures of disease state. Additionally, recent regulatory guidance mandates that symptoms should be scored directly from the patient without a proxy[21]. This guidance recommends the development and use of Patient Reported Outcomes (PROs)[22-25] to measure clinical trial end-points, alongside objective markers of disease (such as endoscopic tools)[26] and health related quality of life[27,28].

The PCDAI is a composite of subjective items derived from a clinical observer, as well as objective items (laboratory studies), and as such it does not meet criteria set forth by the FDA for a PRO[22]. Although recent work has repurposed pieces of the PCDAI (ESR, stooling) with fecal calprotectin and C-reactive protein to develop a non-invasive index of mucosal healing in pediatric CD[29], there is no validated measure of subjective disease state in this population. A PRO for pediatric CD disease activity is still in the early stages of development[30] and is years away from implementation and use as an endpoint in clinical trials. Meanwhile, adult inflammatory bowel disease (IBD) trials are currently moving away from multi-item indices as endpoints and moving towards FDA guidance involving use of a PRO to measure functional components of disease alongside an objective marker of inflammation[31]. As such, as part of the GREAT workshops, the academic and pharmaceutical communities were asked to investigate and redefine the drivers of remission on the PCDAI given the urgent need for a PRO in pediatric CD.

In response to the FDA call we have set out to evaluate whether subgroups of items on the PCDAI could serve as an indicator of objective disease activity, or subjective disease activity until a PRO is ready for use. To date, post-hoc analyses of the PCDAI have not evaluated subcomponents that may serve as interim proxies of subjective or objective components, nor have they evaluated how individual items change over time. In the current study we evaluated the functioning of items on the PCDAI to determine how well subgroups of subjective and objective PCDAI items can capture disease activity.

MATERIALS AND METHODS

Ethics

Appropriate ethics approvals were attained across all participating sites at the original time of data collection. No additional ethics approvals were required for the purpose of this study.

Participants

Three prospectively collected datasets were merged and used for analysis, as follows.

The Registry[8]: The pediatric inflammatory bowel disease (IBD) collaborative research group registry. Data from Canada and the United States were collected on children (< 16 years old) newly diagnosed with IBD across 22 sites between 2002 and 2014. For children with CD, PCDAI, as well as clinical and demographic characteristics, physician global assessment of disease activity, and therapies were recorded at time of diagnosis or at study enrolment, which sometimes differed by up to a few weeks (hereinafter referred to as baseline), 30 d after diagnosis, and then quarterly. Children were not selected for inclusion based on disease severity. In the current study, data from baseline, first quarter (Q1), and at 1-year post diagnosis (Q4) were extracted for 414 children with PCDAI scores available.

The REACH study[4]: Clinical trial of infliximab use in pediatric CD. This randomized controlled trial of infliximab use included 112 children 6-17 years old who had moderate to severe CD (PCDAI > 30) at baseline. This trial involved a 10-wk induction phase, followed by a 1-year maintenance phase. Data at baseline, 10 wk (considered Q1), and 1-year (Q4) were extracted for the current study.

The IMAgINE trial[32]: Clinical trial of adalimumab use in pediatric CD. In this randomized controlled trial, 192 patients between 6 and 17 years old, with moderate to severe CD (PCDAI > 30) received open-label adalimumab at baseline and at 2 wk

before being randomized in blinded fashion at week 4 to high or low dose alternate weekly adalimumab maintenance for an additional 48 wk. Patients were stratified prior to randomization by week 4 responder status and prior exposure to infliximab. Data for the current study were extracted at baseline, 12 wk (Q1) and 52 wk post-treatment initiation (Q4).

Measures

The original version of the 11-item PCDAI (*i.e.*, non-weighted) was used in this study [1], as this was in keeping with how data were collected, scored, and analyzed in each of the original studies. The PCDAI was used to assess disease activity, with the acknowledgement that there is no current gold standard of disease activity in pediatric CD assessment. Severity for each item is assigned a score of 0 (normal), 5 (mild abnormality), or 10 (severe abnormality) except for hematocrit and ESR which are scored as 0, 2.5, or 5. A minimum total index score is zero, and a maximum score is 100. Remission was defined as a total PCDAI score ≤ 10, congruent with the REACH and IMAgINE trials[4,32].

Data acquisition

Relevant raw data were provided from each of the studies and were merged for group analysis. Basic demographic characteristics (age, gender, disease duration), and PCDAI scores at the above-mentioned time points were included. Physician Global Assessment (REACH, Registry), Subject and Parent Global Assessment (REACH), as well as IMPACT, a 35-item health related quality of life self-report measure for paediatric IBD patients[33] (REACH, Registry) were also provided, but were not included in the current analysis. Only data relevant to the proposed analyses were provided, thus, for further information on disease location, or types of treatment, please see the original manuscripts for the REACH[4] and IMAgINE[32] trials.

Inclusion criteria

Only participants with complete PCDAI data at baseline (i.e. had a score for each item on the questionnaire) were included. Thus, sample sizes for clinical trial datasets differ from the original reports, which used different missing data rules (*e.g.*, last value carried forward) as appropriate to the methodology of each respective trial. Sample sizes differ when examining change in PCDAI scores over time, as not all participants had complete data at each respective timepoint. Data were determined to be missing at random (*i.e.* did not attend scheduled follow-up appointment, some clinical or laboratory data parameters not collected due to established PCDAI feasibility issues).

Statistical analysis

Differences in demographic characteristics and baseline scores for participants with and without complete PCDAI data at each timepoint were compared using Independent samples Mann-Whitney U tests. PCDAI change scores were calculated by subtracting the score at baseline from the score at each follow-up time point (either Q1 or Q4, as appropriate). Repeated measures general linear models were run, which examined the mean change from baseline to Q1 and from baseline to Q4 for individual PCDAI items. Fisher's least significant difference tests for pairwise comparisons were run within the general linear model to examine the rank order of change scores across items. Multivariate linear regressions were run to examine the relative contribution of individual PCDAI change scores (entered in weighted units) from baseline to total disease activity at Q1 and Q4. Unstandardized beta values are reported. Subsequent models were run to examine the relative performance of subjective and objective item change scores that significantly contributed to the overall score at Q1 and Q4 follow-up, as well as models looking at only those items that significantly contributed to the overall score at each follow-up time point.

Serial receiver operating characteristic curves (ROC) were run to determine the optimal total score cut-point that differentiates between remitters and non-remitters for the two subscales containing subjective items and objective items. The best cut-off score maximized sensitivity and specificity, corresponding to the upper left shoulder of the ROC curve. The cut-points were then used to examine how well subjective and objective scales alone would similarly classify patients as achieving remission (remitters) *vs* non-remitters compared to the full PCDAI scale score.

All analyses were carried out using SPSS V24.0 (Chicago, IL, United States).

RESULTS

A total of 703 children, with full PCDAI data at baseline, were included in the current study (Table 1). Patients enrolled in the Registry were all at diagnosis, of varying disease severity, and treated per physician dictate, not by protocol. Patients enrolled in the REACH and IMAGINE studies had moderate to severe disease activity at study entry, had a mean disease duration of approximately two years since diagnosis, and were treated by the clinical trial protocol. Basic demographic information, including age, disease duration, and mean PCDAI scores, are presented separately for each dataset as the Registry sample was significantly different than each of the trials with respect to age at baseline (younger), age at diagnosis (younger), and baseline PCDAI (lower) (all $P < 0.001$). The data were then merged and analyzed together for all subsequent analyses, as the goal with merging the data was to have a diverse and representative sample. Sample sizes differ for each follow-up time point as some participants had incomplete PCDAI data at those follow-ups. There were some differences between those who had full PCDAI data at Q1 ($n = 670$), as participants who did not have full Q1 data ($n = 39$) were somewhat older (13.5 ± 2.3 *vs* 12.5 ± 2.8 years, $P = 0.059$), but were significantly younger at diagnosis (10.2 ± 2.9 *vs* 11.5 ± 2.8 years, $P = 0.004$), and had higher baseline PCDAI scores (42.1 ± 9.6 *vs* 34.9 ± 13.7, $P < 0.001$). Participants who did not have full PCDAI data at Q4 ($n = 235$) were significantly younger (12.0 ± 2.7 *vs* 12.8 ± 2.8, $P < 0.001$) and had lower baseline PCDAI scores (32.3 ± 14.7 *vs* 36.89 ± 12.7, $P < 0.001$) compared to those with full PCDAI data at Q4 ($n = 474$). There were no differences in gender distribution between those who did and did not have complete data at each time point (both $P > 0.53$).

The mean absolute change of individual PCDAI items was examined over time for individuals who had complete PCDAI data from baseline to Q1 ($n = 670$), and from baseline to Q4 ($n = 474$) (Figure 1). Of note is that accurate comparisons of mean change cannot be made between hematocrit or ESR and the other PCDAI items as they are scored on 5-point *vs* the 10-point scale of the other PCDAI items. The mean change of individual items was similar at both Q1 and Q4. Abdominal pain, well-being, weight, and stooling had a larger degree of change from baseline to Q1 and Q4 in comparison to the other items ($P < 0.05$). Other objective indicators of disease activity followed, with albumin, abdominal exam, height velocity, and ESR being within the next four most highly ranked items showing similar degrees of absolute change over time to both follow-up time points.

Two linear regression analyses were run to examine the contribution of individual PCDAI change scores to disease activity at Q1 and Q4 (Table 2). At Q1, abdominal pain, stooling, ESR, albumin, weight, height velocity, and perirectal examination were all significant predictors of disease activity (all $P < 0.05$). Three additional models were run: (1) Subjective; (2) Objective; or (3) A composite scale of PCDAI items that were significant predictors in the full model. All the models were significant (all $P < 0.001$), however, the proportion of variance accounted for by the PCDAI questions included was highest for the total and composite scales (both $R^2 = 0.32$), where less variance was accounted for on the models including only the subjective ($R^2 = 0.18$) and objective ($R^2 = 0.21$) items. Similar results were found at Q4, except that the PCDAI change scores from baseline to Q4 were also significant for well-being ($P < 0.001$), abdominal exam ($P < 0.05$), and extraintestinal manifestations ($P < 0.05$). The three additional models also all accounted for a significant amount of variance (all $P < 0.001$), with the subjective scale at this time point also including well-being, and the objective scale included abdominal exam and extraintestinal manifestations, as previously discussed. Similar to Q1, more variance was accounted for by the full and composite models (both $R^2 = 0.45$), than for the subjective ($R^2 = 0.22$) or objective ($R^2 = 0.36$) models.

ROC curves were run to examine how well these sub-scales of subjective and objective items would function on their own, as a proxy to measure disease activity. Items included on the subjective and objective subscales were made based on which item change scores significantly predicted disease activity at the first follow-up (Q1) as this is a similar timeframe to many clinical trials and corresponds with the degree of clinical change observed during a feasible assessment time frame. Cut-points to define remission were defined at Q1 ($n = 670$), using a composite of the significant subjective items (abdominal pain and stooling) (Figure 2A) and objective items (ESR, albumin, weight, height velocity, and perirectal examination) (Figure 2B). A cut-point of ≤ 2.5 (out of a maximum score of 20) best defined remission for the subjective composite, based on an area under the curve of 0.84 (95%CI: 0.80-0.87), with a sensitivity of 74% and a specificity of 83%. For the objective composite, a cut-point of ≤ 3.75 (out of 45) defined remission, with an area under the curve of 0.85 (95%CI: 0.82-0.88), sensitivity of 71% and specificity of 78%.

Table 1 Descriptive statistics

	Registry (*n* = 414[1])		REACH (*n* = 101[1])		IMAgINE (*n* = 188[1])	
	Range	Median (IQR)	Range	Median (IQR)	Range	Median (IQR)
Age at baseline	1.5-16.1	12.2 (3.6)	6.0-17.8	13.8 (3.2)	6-17.0	14.0 (4.0)
Age at diagnosis	1.5-15.9	12.2 (3.6)	4.8-16.6	11.8 (4.1)	2.4-16.4	10.6 (3.2)
Gender (female), *n* (%)	180 (43.5)		40 (39.6)		82 (43.6)	
	Range	mean ± SD	Range	mean ± SD	Range	mean ± SD
PCDAI BL	0-82.5	31.39 ± 15.34	25-62.5	41.14 ± 8.18	7.5-62.5	40.97 ± 7.48
PCDAI Q1	0-52.5	10.25 ± 9.75	0-40	9.77 ± 8.55	0-57.5	16.78 ± 12.64
PCDAI Q4	0-50.0	8.58 ± 9.21	0-70	12.07 ± 14.10	0-70	19.56 ± 16.34

[1]The baseline characteristics describe participants with full pediatric Crohn's disease activity index (PCDAI) data (*i.e.* had individual PCDAI scores collected) at baseline. Q1 PCDAI scores describe participants with full baseline and Q1 PCDAI data available (*n* = 414, 97, and 159 participants for the Registry, REACH, and IMAgINE datasets respectively). Q4 PCDAI scores describe participants with full baseline and Q1 PCDAI data available (*n* = 216, 75, and 183 participants for the Registry, REACH, and IMAgINE datasets respectively). PCDAI: Pediatric Crohn's disease activity index.

Remission status at Q1 and Q4 was examined using subscales of subjective and objective items with the cut-points identified above to examine the comparability of using subscales compared to the full PCDAI (Table 3). This table demonstrates the proportion of patients that would be classified the same or different as the original PCDAI scale by using subscale scores only, thus either in remission ("remitters") or having active disease ("non-remitters"). At Q1, of those classified as in remission using the full PCDAI, only 73.8% would be classified as in remission using the subjective subscale, and thus 26.2% of patients would be classified incorrectly. Using the objective scale, 71.5% of patients deemed remitters using the full PCDAI would be classified as being in remission. For patients not in remission using the full PCDAI, 16.7% would be differently classified using the subjective scale, and 21.2% using the objective scale. At Q4 73.7% of those in remission according to the full PCDAI scale would be in remission based on the subjective subscale, and 74.5% would be in remission according to the objective subscale. For those with active disease at Q4, 77.5% would be similarly defined as having active disease using the subjective subscale, and 72.0% would have active disease using the objective subscale.

DISCUSSION

In response to the FDA call for reevaluation of subcomponents of the PCDAI, and through work generated through the GREAT-III workshops, we have evaluated items on the PCDAI to determine the suitability of these subjective components in assessing disease activity on their own–while we await the development of a PRO for pediatric CD that fulfills FDA guidance. The results of this study indicate that relying on these items, as they are currently measured (*i.e.*, clinician report, nominal variable scoring), will not suffice as a substitution for a robust PRO outcome measure. However, the results do indicate that subjective items such as abdominal pain and stooling in the short-term, and well-being in a longer-term measurement are adequate indicators of disease activity and response to treatment.

We examined both absolute change in scores over time, in addition to looking at what change scores explained unique variance in disease activity at a 3-mo and 1-year follow-up. Items that changed the most over this period were similar. Subjective items including abdominal pain, well-being, and stooling were consistently among the top four items that changed the most over these periods. The rank order of the remaining items varied across these two time points, although objective markers of disease activity including weight, albumin, and abdominal exam were amongst the next group of items that changed the most over time. Results were similar when looking at the unique contributions of the change in item scores to the follow-up total PCDAI score at each time point. However, well-being was not a unique predictor at the 3-mo follow-up, nor was abdominal exam. At the 1-year follow-up however, almost all the change scores from baseline to 1-year added unique variance to the total score, except for hematocrit.

Table 2 Linear regression analysis examining the contribution of pediatric Crohn's disease activity index change scores to Q1 and Q4 pediatric Crohn's disease activity index disease activity

	PCDAIΔ Q1 to BL (n = 670)			
	Full scale	Subjective	Objective	Composite
	β values	β values	β values	β values
1. Abdominal pain	0.57[b]	0.80[b]		0.65[b]
2. Stooling	0.42[b]	0.53[b]		0.45[b]
3. Well-being	0.13			
4. HCT	-0.09			
5. ESR	0.94[b]		1.27[b]	0.96[b]
6. Albumin	0.46[b]		0.41[b]	0.45[b]
7. Weight	0.30[b]		0.52[b]	0.32[b]
8. Height Velocity	0.50[b]		0.56[b]	0.48[b]
9. Abdominal exam	0.08			
10. Perirectal exam	0.47[b]		0.45[a]	0.48[b]
11. Extraintestinal	0.13			
R^2	0.32	0.18	0.21	0.32
	PCDAIΔ Q4 to BL (n = 474)			
1. Abdominal Pain	0.45[b]	0.65[b]		0.45[b]
2. Stooling	0.42[a]	0.52[b]		0.42[a]
3. Well-being	0.58[b]	0.87[b]		0.58[b]
4. HCT	-0.01			
5. ESR	1.32[b]		1.56[b]	1.32[b]
6. Albumin	0.81[b]		0.84[b]	0.81[b]
7. Weight	0.49[b]		0.63[b]	0.49[b]
8. Height velocity	0.45[b]		0.48[b]	0.45[b]
9. Abdominal exam	0.35[a]		0.83[b]	0.35[a]
10. Perirectal exam	0.78[b]		0.90[b]	0.78[b]
11. Extraintestinal	0.42[a]		0.67[a]	0.42[b]
R^2	0.45	0.22	0.36	0.45

[a]$P < 0.05$.

[b]$P \leq 0.001$. PCDAI: Pediatric Crohn's disease activity index; HCT: Hematopoietic cell transplantation; ESR: Erythrocyte sedimentation rate.

Dominant PCDAI items (*i.e.*, items that contributed significant, unique variance) were similar to previous work examining functioning of the PCDAI and the contribution of individual items to change in disease activity[9]. At the 3-mo follow-up, well-being, hematocrit, abdominal exam, and extraintestinal manifestations did not explain unique variance in the total PCDAI score. This is similar to previous work demonstrating the limited responsiveness of specific items on the PCDAI, where abdominal exam, height, and hematocrit were removed from the currently recommended weighted PCDAI as they were found to be redundant in predicting change in disease activity as measured by physician global assessment[5]. At the 1-year follow-up, change scores for all items contributed significant unique variance to the total PCDAI score except for hematocrit. The strength in the current work is the examination of change scores over a 1-year period, highlighting the composite nature of the PCDAI, and that the change in items such as height velocity, abdominal exam, and extraintestinal manifestations take a longer time to demonstrate statistically unique or meaningful change but are nonetheless important components of overall well-being and functioning in patients with CD.

Table 3 Correct and incorrect identification of remitters and non-remitters

Full PCDAI-remission status	Subjective-remission status		Objective-remission status	
	Remitters	Non-remitters	Remitters	Non-remitters
	n (%)	*n* (%)	*n* (%)	*n* (%)
Remitters at Q1 (*n* = 397)	292 (73.8)	105 (26.2)	284 (71.5)	113 (28.5)
Non-remitters at Q1 (*n* = 273)	46 (16.7)	227 (83.3)	58 (21.2)	215 (78.8)
Remitters at Q4 (*n* = 274)	202 (73.7)	72 (26.3)	204 (74.5)	70 (25.3)
Non-remitters at Q4 (*n* = 200)	45 (22.5)	155 (77.5)	56 (28.0)	144 (72.0)

Subjective subscale is composed of abdominal pain and stooling; Objective subscale is composed of erythrocyte sedimentation rate, albumin, weight, height velocity, and perirectal examination. Shaded cells = number of patients that would be incorrectly (or differently) classified as having active or inactive disease based on the subjective components alone (*e.g.*, At the Q1 time point, 397 patients were classified as being in remission, but based on the subjective only scoring, 292 (73.8%) of those patients would be classified as being in remission, while the other 26% would be classified as having active disease). PCDAI: Pediatric Crohn's disease activity index.

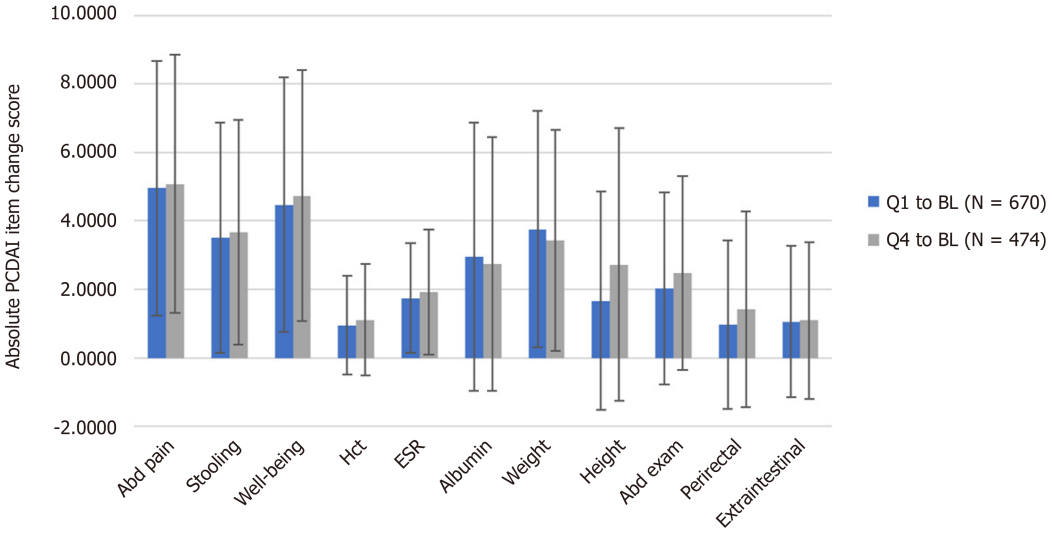

Figure 1 Mean change over time (from baseline to Q1 and Baseline to Q4). Hematocrit and erythrocyte sedimentation rate are scored on a 0-5-point scale, whereas all other items are scored on a 0-10 point scale.

Further examination of the subjective and objective subcomponents highlighted that change in both subcomponents over time does contribute significantly to disease activity. However, change in either subjective or objective groups of items on their own did not perform as well as the full-scale, or to a subgroup of questions (items that were significant unique predictors of total scores) that served as a composite measure of disease activity. If these subcomponents were used to classify patients into remission or active disease, a large portion of patients would be classified differently compared to using the full-scale. The outcomes at the 3-mo and 1-year assessments were similar, where approximately 25% of patients in remission with the full scale would be classified as having active disease with either the subjective or objective scales.

For patients with active disease, the percentage of patients misclassified ranged from about 16% to 22% with the subjective scale, whereas the range was 21% to 28% misclassified with the objective scale at both follow-up time points. Although the composite of items showed similar performance to the full PCDAI, the goal of this work was not to recommend or endorse a composite PCDAI measure as this work has already been completed[5,10], and our findings produced similar results as previously described. Rather, the focus of the current work around these smaller subgroups of items reflects the limited ability of either subjective or objective components alone, as measured on the PCDAI, to fully characterize disease activity.

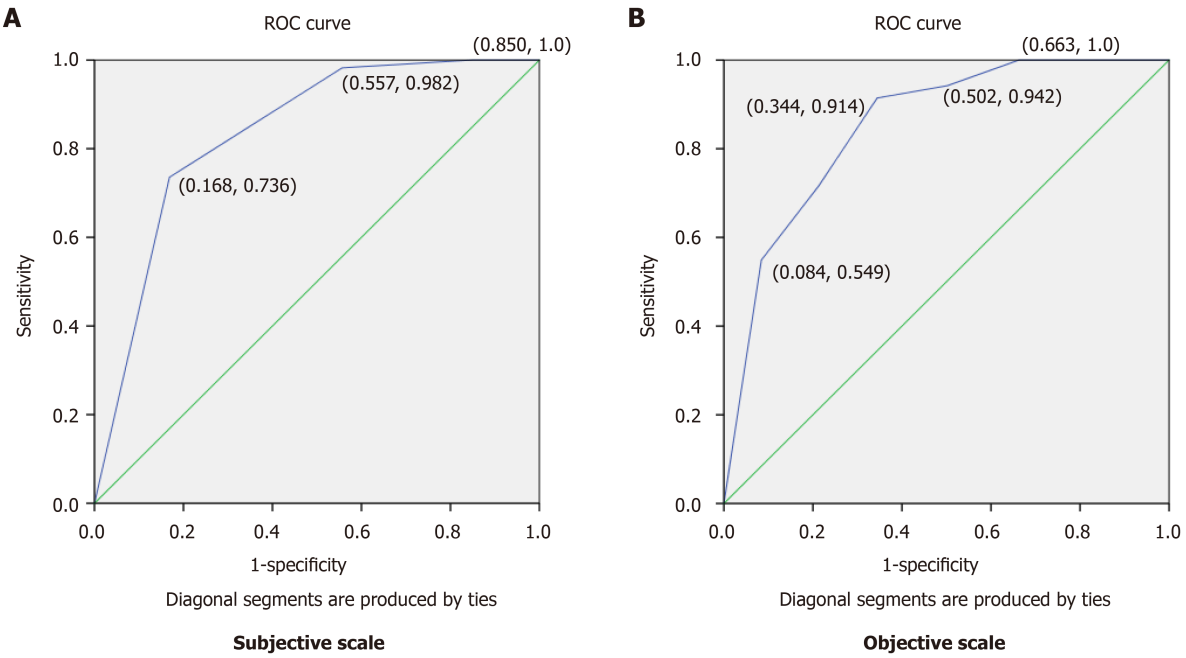

Figure 2 Receiver operator characteristic curve to identify the equivalent remission cut point of the subjective and objective scales. A: Subjective scale; B: Objective scale. ROC: Receiver operator characteristic.

Our study has several limitations, where the main limitation in the current work is the lack of "gold standard" measurement of disease activity beyond physician global assessment. The examination of the change in subjective and objective items on the PCDAI was compared over time but in relation to measurement of disease activity with the same measure at follow-up time points. Other work in trying to assess the psychometric properties of existing measures of disease activity in pediatric CD has found limited correlation with objective measurement of mucosal inflammation with the Simple Endoscopic Score for CD[15,16]. Additionally, work to identify biomarkers that accurately identify disease activity in pediatric CD is ongoing[34], but currently suggests that fecal calprotectin and C-reactive protein are reliable markers with utility in management of a patient's condition[35]. These indices also demonstrate limited correlation with C-reactive protein, and a poor correlation with fecal calprotectin[15,16]. Although there were some differences (younger at diagnosis, higher disease activity at baseline) between participants who had complete follow-up data at 1 year and those who did not, these differences were not considered problematic in the context of the current study. The objective of this study was to examine performance of the PCDAI rather than response to treatment, thus differences between those with and without complete data were not thought to be influential factors that would affect outcomes. In the absence of a universal gold standard measure, the current study is a first step in further understanding the functioning of subjective and objective assessment of disease activity as measured on the primary outcome measure used in clinical trials-the PCDAI.

Without a pediatric CD PRO ready for use, it is tempting to consider using measures already in existence to serve as an interim substitution. However, our study found that looking at subjective items alone in a large sample of patients with a range of disease activity is likely to misclassify many of these patients by relying on subjective items alone. Although subjective items on the PCDAI change the most over time, these items do not capture disease activity as well as a composite of PCDAI items containing both subjective and objective components, or in comparison to the full PCDAI scale. It is not surprising that a composite scale might outperform individual items given the protean nature of CD. This may be particularly relevant when looking over longer periods of time when changes in growth parameters may be more evident.

CONCLUSION

Our analysis highlights the limitations of relying only on subjective or objective items as currently measured on the PCDAI as stand-alone proxies for disease activity

measurement in pediatric patients with CD. Measuring improvements in health outcomes should be informed by evidence-based information produced by patients [36]. Where improvement in disease state may not reflect improvements in a patients well-being[37], our data reiterate that complementary subjective (*i.e.*, PROs)[28] and objective markers of disease[26] should be used to evaluate the outcomes of treatment.

ARTICLE HIGHLIGHTS

Research background

The pediatric Crohn's disease activity index (PCDAI) is a standard tool to assess disease activity in clinical trials for pediatric Crohn's disease. Over time, concerns over both feasibility and short-term responsiveness to change in clinical status have arisen. Based on feasibility concerns, and new guidance recommending that symptoms are scored directly from a patient, the PCDAI was reexamined.

Research motivation

In response to a call from the Food and Drug Administration our team reexamined functioning of this index.

Research objectives

The objective of this study was to examine which items on the PCDAI drive assessment of disease activity, and how subgroups of subjective and objective items reflect change in disease state over time.

Research methods

We retrospectively examined data from three completed studies – one registry study and two clinical trials involving pediatric patients with Crohn's disease. Data was collected at baseline, at 3-mo (Q1) and 1-year (Q4). Change in individual PCDAI scores from baseline to Q1 and to Q4 were examined using the non-weighted PCDAI.

Research results

Abdominal pain, well-being, weight, and stooling had the highest change scores over time. Objective markers of disease activity including weight, albumin, and abdominal exam were amongst the next group of items that changed the most over time. Subjective and objective subgroups of items predicted less variance on total PCDAI scores at Q1 and Q4 compared to the full PCDAI, or a composite scale containing significant predictors.

Research conclusions

Although subjective items on the PCDAI change the most over time, the full PCDAI or a smaller composite of items including a combination of subjective and objective components classifies disease activity better than a subgroup of either subjective or objective items alone. However, the results do indicate that subjective items such as abdominal pain and stooling in the short-term, and well-being in a longer-term measurement are adequate indicators of disease activity and response to treatment. Reliance on subjective or objective items as stand-alone proxies for disease activity measurement could result in misclassification of disease state.

Research perspectives

Subjective or objective items alone, as currently measured on the PCDAI, do not serve as a substitute for a robust patient-reported outcome measure. Complementary subjective (*i.e.*, patient reported outcome measures) and objective markers of disease should be used to evaluate the outcomes of treatment.

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ORIGINAL ARTICLE

Prospective Study

Naïve hepatitis B e antigen-negative chronic hepatitis B patients are at risk of carotid atherosclerosis: A prospective study

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Abstract

BACKGROUND

There is an increased risk of atherosclerosis in patients with chronic hepatitis C or human immunodeficiency virus, but there is scarce data on hepatitis B virus infection. The hypothesis of this study is that hepatitis B virus infection increases the risk of carotid plaques and subclinical atherosclerosis in naïve hepatitis B e antigen (HBeAg) negative subjects.

AIM

To assess the rate of carotid plaques and subclinical atherosclerosis in naïve HBeAg negative subjects in comparison with a cohort of healthy controls.

METHODS

Prospective case-control collaborative study conducted in two tertiary hospitals. Four hundred and two subjects prospectively recruited at the outpatient clinic were included from May 2016 to April 2017: 201 naïve HBeAg-negative hepatitis

B virus-infected [49 chronic hepatitis B (CHB) and 152 inactive carriers(ICs)] and 201 healthy controls. Anthropomorphic and metabolic measures, liver stiffness and carotid Doppler ultrasound were performed. Subclinical atherosclerosis was established on an intima-media thickness increase of ≥1.2 mm and/or the presence of carotid plaques. Normally distributed quantitative variables were compared with the Student *t* test and those with a non-normal distribution with the Mann-Whitney *U* test. Categorical variables were compared between groups using the χ^2 or Fisher exact test.

RESULTS

Carotid plaques were found more often in CHB (32.7%) than ICs (17.1%) or controls (18.4%) ($P = 0.048$). Subclinical atherosclerosis was also increased in CHB (40.8%) *vs*ICs (19.1%) or controls (19.4%) ($P = 0.003$). No differences in the risk of atherosclerosis were observed between controls and ICs. The factors independently associated with the presence of carotid plaques were age [odds ratio(OR) 1.43, $P < 0.001$] and CHB (OR 1.18, $P = 0.004$) and for subclinical atherosclerosis, age (OR 1.45, $P < 0.001$), CHB (OR 1.23, $P < 0.001$) and diabetes (OR 1.13, $P = 0.028$). In the subset of young subjects (< 50 years), carotid plaques (12.5% *vs* 1.1%, $P = 0.027$) and subclinical atherosclerosis (12.5% *vs* 2.2%, $P = 0.058$) were more frequent among CHB than ICs.

CONCLUSION

Untreated HBeAg-negative CHB is an independent risk factor for carotid plaques and subclinical atherosclerosis, while ICs present a similar risk to controls.

Key Words: Hepatitis B virus; Carotid plaques; Subclinical atherosclerosis; Cardiovascular risk; Endothelial dysfunction; Intima-media thickness

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Core Tip: This prospective case-control collaborative study aimed to assess whether chronic infection by hepatitis B was associated with risk of carotid plaques and subclinical atherosclerosis. Overall, 402 subjects were recruited, 201 naïve hepatitis B e antigen-negative hepatitis B virus-infected and 201 healthy controls Patients with hepatitis B e antigen-negative chronic hepatitis B presented a higher rate of carotid plaques than non-infected controls,but no differences were observed between controls and hepatitis B inactive carriers. These results suggest that hepatitis B infection may have a role as a cardiovascular risk factor in patients with chronic hepatitis B.

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INTRODUCTION

More than 257 million people worldwide are infected with hepatitis B virus (HBV)[1], and more than 780000 die each year due to the infection[2]. Chronic HBV infection is a dynamic condition that passes through several phases, being the hepatitis B e antigen (HBeAg)-negative form the most common in Western countries[1]. Currently, patients are classified as HBeAg-negative chronic hepatitis B (CHB) when they have increased HBV DNA and alanine aminotransferase (ALT) levels and liver fibrosis and/or necroinflammation or as HBeAg-negative chronic infection/inactive carriers (ICs)when they have low HBV DNA and normal ALT levels and associated with absent or mild liver damage[3].

were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. It was approved by the Ethics Committee of both hospitals (PR(AG)245/2015).

Informed consent statement: Informed verbal consent was obtained from all individual participants included in the study and recorded at the medical records.

Conflict-of-interest statement: Riveiro-Barciela M has received research grants from Gilead and served as speaker for Gilead and Grifols. Esteban R has received research grants from Gilead and has served as advisors for Gilead, Bristol-Myers Squibb and Novartis. Buti M has received research grants from Gilead and has served as advisors for Gilead, Bristol-Myers Squibb and Novartis. The rest of authors have no personal or financial conflicts of interest.

Data sharing statement: Technical appendix, and dataset available from the corresponding author at [mbuti@vhebron.net]. Participants gave informed verbal consent for data sharing.

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An increased risk of cardiovascular events has been associated with some viral infections like hepatitis C virus (HCV)[4] or human immunodeficiency virus (HIV)[5] as well as autoimmune diseases[6]. The cause of atherosclerosis in these patients is not fully explained by conventional risk factors, and endothelial dysfunction has been suggested as the underlying mechanism causing the early atherosclerotic process. This endothelial dysfunction is mainly associated with the persistent inflammatory state linked to these diseases (HCV, HIV and autoimmune diseases). In fact, eradication of HCV infection has shown a positive impact on carotid atherosclerosis[7]. Both the presence of carotid plaques or measurement of the intima-media thickness (IMT) are accepted and validated surrogate markers for early diagnosis of subclinical atherosclerosis leading to increased cardiovascular risk[8].

Chronic HBV infection has been associated with a propensity to mount proinflammatory immune reactions[9,10], including higher oxidative stress[11], that may predispose to a higher subclinical atherosclerosis.

The aim of this study was to assess whether the stage of HBeAg-negative chronic HBV infection impacts the presence of both carotid plaques and subclinical atherosclerosis. Another aim was to evaluate if the risk of both carotid plaques and subclinical atherosclerosis in HBeAg-negative patients differ to those of healthy controls.

MATERIALS AND METHODS

Patients

Two hundred and one patients with chronic HBV infection and naïve to antiviral therapy were prospectively recruited at the outpatient clinics of two tertiary hospitals (Di.Bi.M.I.S., University of Palermo, Italy and Vall d'Hebron Hospital, Spain) from May 2016 to April 2017. Inclusion criteria were hepatitis B surface antigen (HBsAg) positive for more than 6 mo, HBeAg-negative and no prior exposure to antiviral therapy. Exclusion criteria were previous cardiovascular events (acute myocardial infarction or ischemic stroke), liver transplantation, HCV, hepatitis D or HIV coinfection, history of hepatocellular carcinoma or evidence of liver disease of mixed etiology (autoimmune hepatitis, Wilson's disease, hemochromatosis, α1-antitriypsin deficiency). In addition, 201 healthy individuals matched for sex, age and body mass index were recruited as controls at the outpatient clinics from the same centers. In particular, no patient had a history of previous cardiovascular events, evidence of HBV infection (HBsAg and anti-HBc negative), HCV or HIV, or history of rheumatic or oncological disease. Importance of selection of naïve patients was crucial in view of the effect of antiviral therapy in both liver immunity and carotid plaques in subjects with HCV treated with direct-acting antivirals[7].

Naïve patients with HBV infection were classified into CHB and IC according to the recommendations of European Association for the Study of the Liver[3]: HBeAg-negative CHB was established on HBV DNA > 2000 IU/mL plus fluctuating or persistently elevated ALT levels and/or histological evidence of at least moderate fibrosis and/or necroinflammation; HBeAg-negative chronic infection or IC state was established on persistently normal ALT levels plus HBV DNA < 2000 IU/mL or HBV DNA 2000-20000 IU/mL plus evidence of mild or absent hepatic necroinflammation and fibrosis. Diagnosis of liver cirrhosis was established by liver biopsy (Ishak score 5 or 6) or transient elastography values > 13.1 kPa[12]. This study was conducted in accordance with the Declaration of Helsinki guidelines and the principles of Good Practice and was approved by the Ethics Committee of both hospitals (PR(AG) 245/2015).

Baseline clinical and laboratory assessment

Data on demographics (sex, age and race), toxic exposure (alcohol, tobacco), cardiovascular risk factors (on-treatment arterial hypertension, diabetes and dyslipidemia) and anthropomorphic characteristics (height, weight, and waist circumference) were prospectively collected at the time of enrollment. A blood test was performed including hematology and a standard biochemical panel as well as insulin level, glycated hemoglobin, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, C-reactive protein, HBV serology (quantitative HBsAg, anti-HBc, HBeAg and anti-HBe) and HBV virology. HBV DNA was determined using the COBAS 6800 HBV test (Roche Diagnostics, Mannheim, Germany), with a lower limit of quantification of 20 IU/mL and lower limit of detection of 10 IU/mL. Antibodies against HCV, hepatitis D virus and HIV were also tested.

Central obesity was defined as a waist circumference greater than 102 cm in men and 88 cm in women. Insulin resistance was determined with the homeostasis model assessment[13]. The nonalcoholic fatty liver disease (NAFLD) score was also calculated, and values > 0.675 were considered suggestive of advanced NAFLD-related fibrosis[14]. Liver elastography (Fibroscan® 502 Touch, Echosens, Paris, France), including the control attenuation parameter (CAP) as a marker to quantify hepatic steatosis, was carried out in all patients. CAP was chosen as marker of liver steatosis because it has been pointed out as more accurate than other scores such as Hepatic Steatosis Index in patients with chronic infection by HBV[15].

Carotid artery evaluation

Carotid Doppler ultrasound study (Vivid I, General Electric, GE Healthcare, Horten, Norway, equipped with a 3.5-10 MHz linear transducer) was performed to determine the IMT. B-mode ultrasound with a semi-automatic edge-detection algorithm was used to measure the medium and maximum IMT on the far wall of both the right and left side of the common carotid artery at 1 cm before the bifurcation, measuring at least 250 mm of a straight arterial segment. The presence of an atheroma plaque was established based on the Manheim criteria, as a focal structure that encroached into the arterial lumen by at least 0.5 mm or 50% of the surrounding IMT value or demonstrated a thickness > 1.5 mm as measured from the media-adventitia interface to the intima-lumen interface[16]. The presence of plaques was investigated in the common carotid artery and internal and external carotid arteries. Subclinical atherosclerosis was established on an increased IMT (≥ 1.2 mm) and/or detection of a carotid plaque[16]. To avoid interobserver variability, ultrasound measurements were performed by operators specifically trained in carotid ultrasound cardiovascular risk assessment. Moreover, measurement of the IMT at the common carotid artery presented high reproducibility and interobserver agreement in previous multicenter studies[17].

Statistical analysis

Normally distributed quantitative variables were compared with the Student *t* test and those with a non-normal distribution with the Mann-Whitney *U* test. Quantitative variables were expressed as the median and interquartile range or mean and standard deviation depending on the group size. Categorical variables were compared between groups using the χ^2 or Fisher exact test, as appropriate.Variables with a *P* value < 0.10 in the univariate model were analyzed in a multivariate logistic regression model. Quantitative variables were also introduced as categorical (median or mean of the overall cohort) in order to increase the potency of the models. In the case of homeostasis model assessment, values from included patients were contrasted with the normal from general population[18]. Odds ratios (ORs) and 95% confidence intervals were calculated for the independent predictive factors of carotid plaques and subclinical atherosclerosis. Only patients with available data for all the variables considered in the analysis were included in the multivariate logistic regression models.

Because enrollment of patients with CHB was difficult due to the limitation to naïve subjects, the number of CHB and ICs differed. For this reason, a propensity score analysis matched by sex, age and main cardiovascular risk factors was carried out by using the package of R[19]. *P* values < 0.05 were considered statistically significant. All statistical analyses were performed using IBM SPSS, version 26.0 (SPSS Inc, Armonk, NY, United States).

RESULTS

Baseline characteristics of patients

In total, 402 individuals were enrolled: 201 chronic HBV-infected and 201 healthy controls. Overall, 218 (54.2%) were males, and the more common cardiovascular risk factors were active or former smokers (33.3%), alcohol intake (25.8%) and dyslipidemia (19.9%). Both alcohol intake and central obesity were more common in the control group. Table 1 shows the baseline characteristics of the included cohorts of patients.

In the HBV-infected group, 152 (75.6%) were ICs and 49 (24.4%) CHB. In the latter, 12 (24.4%) patients had liver cirrhosis. Baseline characteristics according to the classification of HBV infection were summarized in Table 1. Most patients were Caucasian (68.2%), and the median age was 47 years. Demographical features did not differ between the two groups. Dyslipidemia was more common in ICs than in patients with CHB, whereas the prevalence of the remaining cardiovascular risk factors was similar. ALT, HBV DNA and HBsAg values as well as liver stiffness were significantly higher

Table 1 Baseline characteristics of included subjects and comparison between infected and non-infected subjects and among patients infected by hepatitis B virus according to the phase of the infection (chronic hepatitis B *vs* inactive carriers)

	Controls	Chronic hepatitis B	Inactive carriers		
	n = 201	*n* = 49	*n* = 152	*P* value[1]	*P* value[2]
Age, yr	48.1 ± 10.2	48.4 ± 12.0	46.5 ± 13.4	0.29	0.28
Male sex (%)	103 (51.2)	31 (64.6)	84 (54.9)	0.13	0.16
Race (%)				< 0.001	0.48
Caucasian	186 (92.5)	35 (72.9)	102 (66.7)		
Asian	2 (1.0)	6 (12.5)	11 (7.2)		
African	0 (0)	6 (12.5)	28 (18.3)		
Hispanic	13 (6.5)	1 (2.1)	12 (7.8)		
Cardiovascular risk factors (%)					
Tobacco exposure	74 (36.8)	15 (31.3)	45 (29.6)	0.09	0.48
Alcohol intake[3]	70 (34.8)	11 (22.9)	23 (15.4)	< 0.001	0.17
Hypertension	40 (19.9)	11 (22.9)	27 (17.8)	0.46	0.28
Diabetes	4 (2.0)	4 (8.3)	6 (3.9)	0.08	0.2
Dyslipidemia	46 (22.9)	3 (6.3)	31 (20.4)	0.08	0.02
Central obesity	52 (25.9)	10 (20.8)	27 (17.9)	0.04	0.4
BMI, kg/m^2	25.3 ± 3.6	26.0 ± 3.9	25.2 ± 4.0	0.76	0.22
Liver cirrhosis (%)	0 (0)	12 (24.4)	0 (0)	< 0.001	< 0.001
ALT, IU/mL	22.6 ± 12.7	59.7 ± 48.6	25.6 ± 16.7	< 0.001	< 0.001
GGT, IU/mL	30.4 ± 30.9	60.7 ± 87.9	31.5 ± 63.3	0.24	< 0.001
LDL, mg/dL[4]	131.9 ± 38.3	116.9 ± 30.7	118.1 ± 32.6	0.002	0.82
Triglycerides, mg/dL	108.0 ± 56.7	96.4 ± 46.3	106.8 ± 59.1	0.54	0.26
C-reactive protein, mg/dL[5]	0.29 ± 0.42	0.84 ± 1.90	1.00 ± 9.00	0.42	0.88
HOMA index[5]	2.05 ± 1.84	4.20 ± 3.50	3.40 ± 3.90	< 0.001	0.18
HBsAg, logIU/mL	-	3.6 ± 0.8	2.9 ± 1.2	-	0.001
HBV DNA, logIU/mL	-	4.4 ± 1.8	2.4 ± 1.1	-	< 0.001
Transient elastography, kPa	4.5 ± 1.4	11.3 ± 10.9	5.5 ± 2.4	< 0.001	< 0.001
CAP, dB/m	246.5 ± 54.5	227.4 ± 55.0	227.2 ± 56.2	0.001	0.98

Data are expressed as the median (interquartile range) or as the *n* (%).
[1]Comparison between hepatitis B virus-infected and non-infected controls.
[2]Comparison between patients with chronic hepatitis B and inactive carriers.
[3]Significant alcohol intake was defined as > 30 g per day for men and > 20 g per day for women.
[4]These data were available in 132 non-infected subjects.
[5]This data was available in 83 non-infected subjects. ALT: Alanine transaminase; CAP: Control attenuation parameter; GGT: Gamma glutamyltransferase; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; BMI: Body mass index; LDL: Low-density lipoprotein; HOMA: Homeostasis model assessment.

in patients with CHB.

Carotid plaques and subclinical atherosclerosis in HBV-infected group in comparison with the control group

No differences were observed between the HBV-infected group and the control group in terms of gender and age, although some cardiovascular risk factors such as central obesity and dyslipidemia were more common among non-HBV infected individuals (Table 1). In fact, although increased values of liver stiffness were observed in patients with HBV infection, CAP levels were higher in subjects within the control group.

Table 2 Factors associated with the presence of carotid plaques and subclinical atherosclerosis

	Subclinical atherosclerosis			Carotid plaques		
	Univariate analysis	Multivariate analysis		Univariate analysis	Multivariate analysis	
		OR (95%CI)	*P* value		OR (95%CI)	*P* value
Age, years	< 0.001			< 0.001		
Age > 50 years	< 0.001	1.45 (1.24-1.48)	< 0.001	< 0.001	1.43 (1.21-1.44)	< 0.001
Male sex	0.004		0.336	0.003		0.212
Central obesity	0.008		0.073	0.007		0.141
Tobacco exposure	0.003		0.081	0.002		0.187
Alcohol intake[1]	0.109		-	0.073		0.929
Arterial hypertension	< 0.001		0.949	< 0.001		0.690
Diabetes mellitus	0.004	1.13 (1.03-1.59)	0.028	0.01		0.082
Dyslipidemia	0.001			< 0.001		0.095
Chronic hepatitis B	0.001	1.23 (1.11-1.41)	< 0.001	0.016	1.18 (1.06-1.34)	0.004
Transient elastography, kPa	0.01		0.090	0.073		0.438
Transient elastography > 5.7 kPa	0.008			0.048		
CAP, dB/m	< 0.001		0.989	< 0.001		0.577
CAP > 238 dB/m	< 0.001			< 0.001		
AST, IU/mL	0.115		-	0.152		-
AST > 27 IU/mL	0.102			0.131		
GGT, IU/mL	< 0.001		0.067	0.001		0.947
GGT > 36 IU/mL	< 0.001			0.001		
Triglycerides, mg/dL	0.011		0.059	0.018		0.957
Triglycerides > 106 mg/dL	0.009			0.009		
LDL, mg/dL	0.651		-	0.180		-
HOMA index	0.038		0.073	0.278		0.105
HOMA index > 1.2	0.150			0.489		
HOMA index > 3	0.002			0.018		

Data are given as mean ± SD or as *n* (%).

[1]Significant alcohol intake was defined as > 30 g per day for male and > 20 g per day for female. At the multivariate logistic regression model only patients with available data for all the variables were included. The cut-off for inclusion was a *P* value < 0.10 in the univariate model. AST: Aspartate transaminase; CAP: Control attenuation parameter; GGT: Gamma glutamyltransferase; CI: Confidence interval; OR: Odds ratio; HOMA: Homeostasis model assessment; LDL: Low-density lipoprotein.

Overall, patients with HBV infection presented higher rates of both carotid plaques (20.9% *vs* 18.4%) and subclinical atherosclerosis (24.4% *vs* 19.4%), though these differences did not reach statistical significance ($P = 0.31$ and $P = 0.14$, respectively). When the three groups were analyzed separately, taking into account the state of HBV infection, we learnt that patients with CHB had higher rates of carotid plaques (32.7%) and subclinical atherosclerosis (40.8%) than controls (18.4% and 19.4%, respectively), as shown in Figure 1. However, the rates were similar when only ICs and controls were compared (carotid plaques: 17.1% *vs* 18.4%, $P = 0.446$; subclinical atherosclerosis: 19.1% *vs* 19.4%, $P = 0.525$). Although the typical cardiovascular risk factors were linked with both carotid plaques and subclinical atherosclerosis on the univariate analysis, on the multivariate (Table 2) the only factors independently associated with the presence of subclinical atherosclerosis were older age (OR 1.45, $P < 0.001$), diagnosis of CHB (OR 1.23, $P < 0.001$) and diabetes (OR 1.13, $P = 0.028$). Similar results were observed

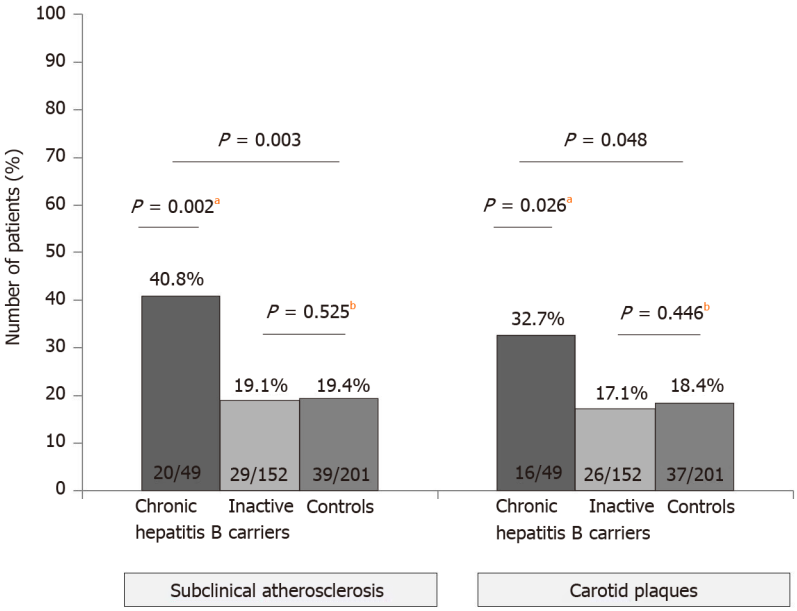

Figure 1 Rate of carotid plaques and subclinical atherosclerosis (defined as intima-media thickness ≥ 1.2 mm and/or presence of atheroma plaques) in the overall cohort. [a]*P* < 0.05; [b]*P* < 0.01.

regarding the carotid plaques, with age over 50 years (OR 1.43, *P* < 0.001) and CHB (OR 1.18, *P* = 0.004) as independent risk factors.

Though this is a prospective study, due to the different number of HBV-infected subjects included in each group, a propensity score analysis including all patients with CHB (*n* = 49) and a cohort with the same number of IC and controls, balanced by age, sex and main cardiovascular risk factors, was carried out. The multivariate analysis of this propensity score revealed similar results as shown the analysis performed with the overall cohort, with older age (OR 1.30, *P* = 0.01) and CHB state (OR 1.26, *P* = 0.03) as independent risk factors associated with the presence of carotid plaques (Table 3).

Carotid plaques and subclinical atherosclerosis in CHB and HBV ICs

Overall, 49 (24.4%) patients had subclinical atherosclerosis, including 42 (20.9%) with carotid plaques, 19 (9.5%) with increased IMT (≥ 1.2 mm) and 12 (6%) with both findings. The prevalence of both subclinical atherosclerosis (*P* = 0.003) and carotid plaques (*P* = 0.019) was higher in patients with CHB than ICs (Figure 2). Liver cirrhosis was associated with an increased risk of subclinical atherosclerosis (42.0% *vs* 23.0%) although the difference did not reach statistical significance (*P* = 0.13). The impact of CHB on the presence of subclinical atherosclerosis remained when patients were stratified by age (Figure 2). In those ≤ 50 years, the prevalence of subclinical atherosclerosis was 12.5% in CHB patients and only 2.2% in ICs (*P* = 0.058). In patients aged over 50 years, those with CHB also had a higher prevalence of subclinical atherosclerosis (68.0% *vs* 45.8%, *P* = 0.051). Age was strongly associated with the presence of subclinical atherosclerosis (*P* < 0.001). On multivariate analysis, factors independently associated with the presence of subclinical atherosclerosis were older age (OR 1.11, *P* < 0.001), increased values of gamma-glutamyltransferase (OR 5.9, *P* = 0.007) and CHB (OR 3.35, *P* = 0.017). When age was introduced as a categorical variable (threshold of 50 years), both CHB and age remained as predictive factors of subclinical atherosclerosis (Table 4).

In terms of carotid plaques, impact of CHB was especially important in patients aged ≤ 50 years (Figure 2). On the multivariate analysis, only age (age > 50 years, OR 1.45, 95%confidence interval 1.30-1.62, *P* < 0.001) and increased gamma-glutamyltransferase levels (gamma-glutamyltransferase > 36 IU/mL, OR 1.19, 95%confidence interval 1.04-1.37, *P* = 0.012) independently impacted the presence of carotid plaques.

Four patients with CHB and liver cirrhosis presented a NAFLD score > 0.675, suggesting significant fibrosis. Two of them had subclinical atherosclerosis, but none had a history of diabetes, and their body mass index was < 25 kg/m^2 and CAP < 250 dB/m. Otherwise, they presented an HBV DNA > 2000 IU/mL, suggesting that fibrosis was likely related to CHB.

Table 3 Factors associated with the presence of carotid plaques in a propensity score matched by age, sex and main cardiovascular risk factors

	Groups of study			Univariate analysis	Multivariate analysis	
	Chronic hepatitis B, *n* = 49	Inactive carriers, *n* = 49	Controls, *n* = 49	*P* value	OR (95%CI)	*P* value
Age, yr	48.4 ± 12.0	48.7 ± 13.0	47.1 ± 11.3	0.78		
Age > 50 yr (%)	25 (51.0)	24 (49.0)	23 (46.9)	0.92	1.30 (1.12-1.50)	0.01
Male sex (%)	31 (64.6)	31 (63.3)	31 (63.3)	1		0.72
Caucasian race (%)	35 (72.9)	32 (65.3)	38 (77.6)	0.41		-
Cardiovascular risk factors (%)						
Tobacco exposure	15 (31.3)	13 (26.5)	16 (32.7)	0.80		0.71
Hypertension	11 (22.9)	10 (20.4)	11 (22.4)	0.96		0.40
Diabetes	4 (8.3)	5 (10.2)	3 (6.1)	0.76		0.57
Dyslipidemia	3 (6.3)	3 (6.1)	5 (10.2)	0.68		0.54
Central obesity	10 (20.8)	10 (20.4)	10 (20.4)	1		0.06
BMI, kg/m^2	26.0 ± 3.9	26.3 ± 4.9	26.4 ± 3.8	0.90		0.16
ALT, IU/mL	48.6 ± 9.4	26.9 ± 9.4	25.2 ± 9.6	< 0.001		0.08
GGT, IU/mL	60.7 ± 87.9	42.7 ± 106.0	37.7 ± 50.9	0.45		0.07
LDL, mg/dL[1]	116.9 ± 30.7	120.3 ± 32.6	112.7 ± 35.3	0.72		0.87
Triglycerides, mg/dL	96.4 ± 46.3	99.0 ± 57.5	115.3 ± 77.1	0.30		0.41
HOMA index[2]	4.2 ± 3.5	4.7 ± 5.8	2.6 ± 2.6	0.14		0.40
Transient elastography, kPa	11.3 ± 10.9	5.7 ± 3.0	5.0 ± 7.9	< 0.001		0.80
CAP, dB/m	227.4 ± 55.0	232.1 ± 48.6	251.5 ± 62.8	0.095		0.49
Chronic hepatitis B state (%)	49 (100)	0 (0)	0 (0)	< 0.001	1.26 (1.09-1.47)	0.03

[1]Only available for 34 controls.

[2]Only available for 25 non-infected controls. ALT: Alanine transaminase; CAP: Control attenuation parameter; GGT: Gamma glutamyltransferase; BMI: Body mass index; LDL: Low-density lipoprotein; CI: Confidence interval; OR: Odds ratio; HOMA: Homeostasis model assessment.

DISCUSSION

The results of this prospective collaborative study including well-characterized HBeAg negative chronic HBV infection show that CHB is independently associated with the presence of both carotid plaques and subclinical atherosclerosis. These results suggest that HBV infection may have a role as a cardiovascular risk factor in naïve patients with CHB.

There are few studies assessing the potential effect of HBV infection on development of carotid atherosclerosis, and they are all cross-sectional with a limited number of HBsAg-positive populations (Supplementary Table 1). In two of these studies, an association was observed between HBsAg positivity and early atherosclerosis[11,20]. The severity of liver disease was not determined in any of these studies, and therefore no data on the possible impact of CHB was reported. In our cohort, similar to HCV and HIV, patients with HBV infection had greater risk of subclinical atherosclerosis and carotid plaques than controls. In this line, a study focusing on early atherosclerosis in liver disease (NAFLD, HCV and HBV) found that all three conditions were strongly associated with early atherosclerosis (OR 1.96, 1.61 and 1.40 respectively), regardless of the patients' classical risk factors, including insulin resistance and metabolic syndrome [20].

Table 4 Baseline characteristics and analyses of factors associated with the presence of subclinical atherosclerosis of patients with hepatitis B virus infection

	Subclinical atherosclerosis	No subclinical atherosclerosis	Univariate analysis	Multivariate analysis		Adjusted multivariate analysis	
	n = 49	*n* = 152	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Age, yr	57 (53.5-62.0)	42 (33.0-52.0)	< 0.001	1.10 (1.06-1.16)	< 0.001	1.19 (1.12-1.25)	< 0.001
Age > 50 yr (%)	45 (91.8)	46 (30.3)	< 0.001	21.9 (6.7-71.8)	< 0.001		
Male sex (%)	31 (63.3)	84 (55.3)	0.207	-	-		
BMI, kg/m^2	70.0 (64-77)	69.5 (62-80)	0.261	-	-		
Central obesity (%)	13 (26.5)	24 (16.0)	0.079	-	0.298		0.356
Tobacco exposure (%)	22 (44.9)	38 (25.2)	0.008	-	0.208		0.920
Alcohol intake[1] (%)	14 (28.5)	20 (13.5)	0.016	-	0.876		0.092
Arterial hypertension (%)	18 (36.7)	20 (13.2)	0.001	-	0.789		0.419
Diabetes mellitus (%)	5 (10.2)	5 (3.3)	0.067	-	0.994		0.457
Dyslipidemia (%)	16 (32.6)	18 (11.9)	0.001	-	0.876		0.786
Chronic hepatitis B (%)	20 (40.8)	28 (18.4)	0.002	3.35 (1.20-9.10)	0.017	1.89 (1.75-2.04)	< 0.001
Liver cirrhosis (%)	5 (10.2)	7 (4.6)	0.138	-	-		
ALT, IU/mL	29 (22.0-43.5)	24 (17.0-34.0)	0.310	-	--		
ALT > ULN (%)	10 (20.4)	22 (14.5)	0.220	-	-		
GGT, IU/mL	31 (18.0-62.5)	20 (16.0-28.0)	< 0.001	-	0.120		
GGT > ULN (%)	16 (32.7)	8 (5.3)	< 0.001	5.90 (1.60-21.40)	0.007	1.27 (1.19-1.36)	< 0.001
HbA1c, %	5.5 (5.3-5.8)	5.4 (5.1-5.6)	0.004	-	0.78		0.551
HbA1c ≥ 6% (%)	7 (14.6)	6 (4.1)	0.018	-	-		
HOMA index	3.3 (2.2-6.0)	2.4 (1.7-3.6)	0.038	-			0.054
HOMA index > 3 (%)	22 (45.8)	51 (34.2)	0.102	-	-		
HBsAg, logIU/mL	3.2 (2.5-3.6)	3.3 (2.4-4.0)	0.321	-	-		
HBV DNA, logIU/mL	3.1(2.4-3.8)	2.9 (2.3-3.5)	0.533	-	-		
Transient elastography, kPa	6.2(4.2-10.3)	5.2 (4.2-6.9)	0.059	--	0.327	1.01 (1.00-1.01)	< 0.001
CAP, dB/m	246 (210.0-289.0)	213 (185.0-261.5)	0.004	--	0.220	1.000 (1.000-1.001)	0.006
CAP > 227 dB/m (%)	29 (67.4)	51 (38.3)	0.001	--	0.172		

Data are given as mean ± SD or as *n* (%).

[1]Significant alcohol intake was defined as > 30 g per day for male and > 20 g per day for female.At the multivariate logistic regression model only patients with available data for all the variables were included (*n* = 250). The cut-off for inclusion was a *P* value < 0.10 in the univariate model. ALT: Alanine transaminase; BMI: Body mass index; HBV: Hepatitis B virus; CI: Confidence interval; CAP: control attenuation parameter; HbA1c: Hemoglobin A1c; GGT: Gamma glutamyltransferase; OR: Odds ratio; ULN: Upper limit of normality; HOMA: Homeostasis model assessment; HBsAg: Hepatitis B surface antigen.

The suggested mechanisms to explain HBV-related atherosclerosis is direct vascular damage by the virus and particularly accelerated oxidative damage and the pro-inflammatory state of chronic HBsAg carriers[21]. Knowledge about the immune response in HBV-infected patients has increased considerably in recent years[9]. The production of proinflammatory cytokines (*e.g.*, interleukin1b, tumor necrosis factoralpha) steadily increases during early life until it reaches the state of chronic low-

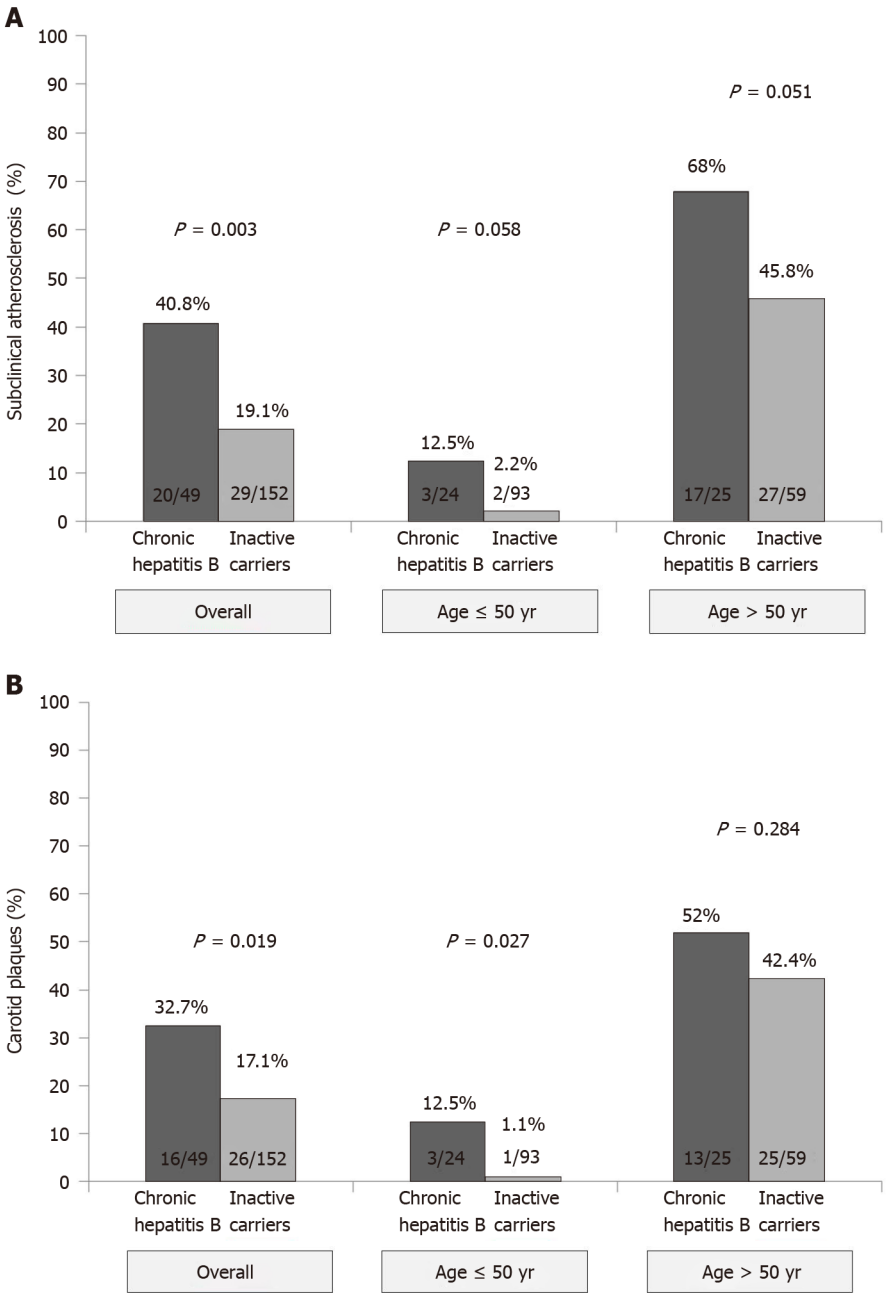

Figure 2 Rate and impact of age and hepatitis B e antigen negative phase of infection (chronic hepatitis *vs* inactive carriers) in subclinical atherosclerosis and carotid plaques in the cohort of patients chronically infected by hepatitis B virus. A: Subclinical atherosclerosis; B: Carotid plaques.

grade systemic inflammation that occurs in elderly persons[22]. HBeAg-negative CHB [3] has been linked with a propensity to mount proinflammatory immune reactions [9]. In this population, liver inflammation is triggered by HBV-specific CD8 T cells, and it is associated with increased levels of chemokines and natural killer cell activation[23]. This proinflammatory state is independent of ALT levels and even HBV DNA levels, which usually fluctuate in this stage of the disease[24]. However, it has been clearly associated with progression of liver disease[9].

In our study, neither ALT levels nor HBV DNA were associated with an increased prevalence of subclinical atherosclerosis. This fact may be explained by the inclusion of patients with CHB with normal ALT but increased values of HBV DNA and liver damage at liver biopsy. On the other hand, some of the patients with liver cirrhosis presented relatively low HBV DNA levels. Older age and CHB status were independent factors associated with increased carotid plaques and subclinical atherosclerosis, in line with the proinflammatory state induced by older age and progression of liver damage.

Serum paraoxonase-1 and arylesterase activities, plasma free sulfhydryl groups and total antioxidant capacity, all factors associated with increased susceptibility to atherogenesis[24,25], are lower in HBV patients than in non-infected controls[26]. This finding can also contribute to the development of atherosclerosis in patients with HBV infection. Moreover, the association between fibrosis progression and exacerbated immune responses in patients with CHB is well established[9,10,27], so this dysfunctional immunological response might also bring an increase in cardiovascular risk.

Accordingly, HCV infection has been linked with increased prevalence of carotid plaques in those patients with evidence of advanced liver fibrosis[4]. In that study, Petta *et al*[4] showed that 73 of 174 HCV patients (42%) had carotid plaques, with older age and liver fibrosis as independent factors associated with carotid atherosclerosis, results in line with our findings because age and CHB were the two variables independently linked with increased risk of both carotid plaques and subclinical atherosclerosis. The role of liver damage is especially relevant in view of the lack of statistical differences when HBV ICs were compared with controls, suggesting that HBV infection may predispose to cardiovascular risk only when it is associated with a proinflammatory state, as described in patients with CHB[9,27].

This study has some limitations. First, the fact that only naïve patients were included turned out in a relatively low number of patients with HBeAg negative CHB and inferior to the cohort of HBV ICs. However, these patients were well characterized, and all met the European Association for the Study of the Liver criteria for CHB, including 24% with liver cirrhosis. Second, there were some differences among the groups. In order to minimize this potential bias, a propensity score was carried out, confirming the role of CHB status as cardiovascular risk factor. Moreover, data presented herein derived from a prospective, collaborative cohort of well-characterized patients, including different ethnicity and therefore HBV genotypes.

Interestingly, since reversion of liver fibrosis in patients with CHB is possible due to nucleos(t)ide analog therapy[28], it would be appealing to assess the potential impact of oral antiviral therapy on early atherosclerosis related to HBV infection, especially to view the effect of antiviral treatment for HCV in the overall cardiovascular risk and specifically in the carotid plaques[7].

CONCLUSION

In conclusion, in this prospective, case-control collaborative study, presence of subclinical atherosclerosis and carotid plaques were more frequent in patients with HBV infection than controls. The presence of liver damage was an independent factor associated with subclinical atherosclerosis and carotid plaques, regardless of the classical cardiovascular factors.

ARTICLE HIGHLIGHTS

Research background

There is an increased risk of atherosclerosis in patients with chronic hepatitis C and also in individuals with human immunodeficiency virus infection.

Research motivation

There is scarce data on the potential role of hepatitis B virus infection as a cardiovascular risk factor.

Research objectives

To assess whether the stage of hepatitis B e antigen (HBeAg)-negative chronic hepatitis B virus infection impacts the presence of both carotid plaques and subclinical atherosclerosis and to evaluate if the risk of both carotid plaques and subclinical atherosclerosis in HBeAg-negative patients differ to those of healthy controls.

Research methods

Prospective case-control study with 402 subjects prospectively recruited at the outpatient clinic. Anthropomorphic and metabolic measures, liver stiffness and carotid Doppler ultrasound were performed.

Research results

Patients with HBeAg-negative chronic hepatitis B presented a higher rate of carotid plaques than healthy controls (32.7% *vs* 18.4%, P = 0.002), but no differences were observed between controls and hepatitis B inactive carriers. HBeAg-negative chronic hepatitis B was an independent risk factor for carotid plaques as well as age, dyslipidemia and central obesity.

Research conclusions

These results suggest that hepatitis B infection may have a role as a cardiovascular risk factor in patients with chronic hepatitis B.

Research perspectives

Further studies should assess the potential impact of oral antiviral therapy on early atherosclerosis related to hepatitis B virus infection.

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