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*REVIEW*

# Viral hepatitis and hepatocellular carcinoma: From molecular pathways to the role of clinical surveillance and antiviral treatment

Leonardo Stella, Francesco Santopaolo, Antonio Gasbarrini, Maurizio Pompili, Francesca Romana Ponziani

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## Abstract

Hepatocellular carcinoma (HCC) is a global health challenge. Due to the high prevalence in low-income countries, hepatitis B virus (HBV) and hepatitis C virus infections remain the main risk factors for HCC occurrence, despite the increasing frequencies of non-viral etiologies. In addition, hepatitis D virus coinfection increases the oncogenic risk in patients with HBV infection. The molecular processes underlying HCC development are complex and various, either independent from liver disease etiology or etiology-related. The reciprocal interlinkage among non-viral and viral risk factors, the damaged cellular microenvironment, the dysregulation of the immune system and the alteration of gut-liver-axis are known to participate in liver cancer induction and progression. Oncogenic mechanisms and pathways change throughout the natural history of viral hepatitis with the worsening of liver fibrosis. The high risk of cancer incidence in chronic viral hepatitis infected patients compared to other liver disease etiologies makes it necessary to implement a proper surveillance, both through clinical-biochemical scores and periodic ultrasound assessment. This review aims to outline viral and microenvironmental factors contributing to HCC occurrence in patients with chronic viral hepatitis and to point out the importance of surveillance programs recommended by international guidelines to promote early diagnosis of HCC.

**Key Words:** Hepatitis C virus; Hepatitis B virus; Hepatitis D virus; Hepatocellular carcinoma; Cirrhosis; Liver

**Core Tip:** Chronic hepatitis B virus (HBV), hepatitis C virus (HCV) and hepatitis D virus (HDV) infection represents a global health burden leading to liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC). Among these complications, HCC accounts for 3.5% of all deaths worldwide. Thus, the understanding of the role of chronic hepatitis virus infection in HCC development is necessary in order to clarify the mechanisms underlying oncogenesis and design future treatments for this cancer. This review outlines pathophysiological and molecular pathways that contribute to carcinogenesis in HBV, HCV and HDV chronic infection focusing on the impact of clinical surveillance and antiviral treatment on the risk of HCC development.

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## INTRODUCTION

Primary liver cancer is the sixth most common cancer worldwide accounting for 800000 new cases per year and one of the major causes of cancer-related death in men with 745000 deaths per year. It has been estimated that there will be an increased incidence after the year 2025 with more than 1 million cases per year[1]. Hepatocellular carcinoma (HCC) represents the most common histologic type among liver cancers (70%-90%)[2]. According to recent epidemiologic studies, the mortality rate from HCC is increasing in some of the European and North American countries. Indeed, HCC has been recognized as the cause of death in 54%-70% of patients with compensated cirrhosis of different etiologies[3]. Nevertheless, HCC is one of the most prevalent cancers in less developed world regions. About 60%-70% of HCCs have been linked with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, with a total incidence of 16/100000 cases globally (Table 1)[4], while the linkage with hepatitis D virus (HDV) is less clear[5].

HCC is associated with many risk factors (Table 2) with the highest odds ratio (OR) for viral ones. Cirrhosis precedes most cases of HCC and may exert a promoting effect *via* hepatocyte regeneration and chronic inflammation[4]. There are other environmental and genetic risk factors involved in the pathogenesis of HCC such as excessive alcohol consumption, aflatoxin intake, diabetes, obesity and hereditary hemochromatosis[6]. This review outlines the pathophysiology and molecular pathways that contribute to carcinogenesis in HBV, HCV and HBV/HDV chronic infection and investigates the impact of surveillance programs and antiviral treatments on the risk of HCC occurrence.

## HBV INFECTION AND HCC DEVELOPMENT

### *Epidemiology*

HBV infection is the most widespread chronic viral infection in the world. Currently, 2 billion people have been infected and more than 350 million are chronic carriers of the virus[7]. Worldwide HBV surface antigen (HBsAg) seroprevalence is 3.6% with the highest prevalence in Africa (8.83%) and in Western Pacific Asia (5.26%)[8]. In contrast, the prevalence of chronic HBV infection is low in most of European countries and it is estimated to be around 0.5%-0.7%[9]. Underdiagnosis and undertreatment are still unresolved issues and public awareness is largely suboptimal with less than 25% of infected Europeans aware of HBV transmission risk at the moment of their diagnosis[9]. Thus, chronic HBV infection is the tenth leading cause of global deaths accounting for 3.5% of all deaths worldwide (786000 deaths per year). Half of the total liver cancer mortality in 2010 has been attributed to HBV infection[10].

### *Molecular pathways and HCC development in chronic hepatitis B*

Chronic HBV infection is the most important cause of HCC worldwide. Prospective cohort studies show a 5-100-fold increase in the risk of developing HCC among patients with untreated chronic HBV infection[2]. A systematic review estimates a yearly HCC incidence rate of 0.2% in HBV inactive carriers, of 0.6% in those with chronic infection without cirrhosis and of 3.7% in those with compensated cirrhosis[11]. Meta-analyses show a 15-20 times greater relative risk for HCC occurrence in HBsAg positive individuals than in HBsAg negative individuals[2].

Most of HBV-infected individuals (70%-90%) develop HCC related to the onset of cirrhosis and secondary to chronic inflammation[2]. However, in up to one-third of patients, HCC occurs in the absence of cirrhosis[12]. Oncogenesis in HBV infected patients is a complex phenomenon involving

**Table 1 Hepatocellular carcinoma incidence and risk factors**

| Location | Chronic HBV infection | | Chronic HCV infection | | Alcoholic liver disease | | Non-alcoholic steatohepatitis | |
|---|---|---|---|---|---|---|---|---|
| | NC | CC | NC | CC | NC | CC | NC | CC |
| Europe | 0.12 | 2.2 | 0-1.8 | 3.7 | 0.1 | 1.8 | 0.5 | 1.1 |
| East Asia | 0.8 | 4.3 | F0/1 0.4; F2 1.5; F3 5.1 | 7.1 | 0.1 | 1.7 | a | a |

HBV: Hepatitis B virus; HCV: Hepatitis C virus; NC: Non cirrhotic; CC: Compensated cirrhosis; a: Lack of proper data in the selected area.

most of the cell cycle regulation molecular pathways (Table 3 and Figure 1). As a noncytopathic virus, the pathogenesis of HBV tissue damage is mainly mediated by cell-mediated immune response to HBV epitopes surface proteins. Persistent HBV replication triggers immune response against the virus leading to genetic damage and perpetrating oxidative stress. In addition to chronic inflammation, other viral mechanisms contribute to HBV carcinogenesis involving the HBs and HBx proteins, the insertional mutagenesis caused by HBV DNA integration into the host genome, which induces chromosomal instability and alters the expression of endogenous genes, the epigenetic modifications through DNA methylation and the regulation of microRNA (miRNA) expression[13].

There are other factors increasing HCC risk among HBV carriers that can be divided into: Viral factors (high rates of HBV replication, long term infection, coinfection with HCV/human immunodeficiency virus/HDV, high risk genotype) and host-related factors, such as genetic or demographic (older ages, male sex, Asian or African origin, family history of HCC), clinical (presence of cirrhosis) and environmental (diabetes, overweight, alcohol abuse, aflatoxin exposure)[14].

## VIRAL FACTORS

### *Role of HBV DNA level*

The risk of developing HCC strictly correlates with HBV viraemia. Multiple integrations have been detected in liver tissues and integrated HBV sequences have been observed in almost 90% of HBV-related HCCs[13]. The reveal-HBV study shows increased HCC-related mortality in subjects with baseline HBV DNA higher than $10^6$ copies/mL compared to those with baseline DNA lower than 300 copies/mL. A multivariate cyclooxygenase (COX) regression analysis identifies an increase in HBV DNA levels as the strongest independent predictor of death from HCC, after adjusting for age, sex, smoking, alcohol consumption and hepatitis B e antigen (HBeAg) serostatus[15]. Nevertheless, patients previously defined as "inactive carriers" (HBeAg negative, HBV DNA < 10000 copies/mL and normal liver function tests) have a 5-fold higher risk of developing HCC compared to HBsAg negative controls [16].

The integration of HBV DNA into the host genome represents a major pathogenetic pathway. The plasmid-like covalently closed circular DNA (cccDNA), derived from the conversion of HBV DNA soon after the entry into the hepatocyte, is recognized in the nucleus as a damaged product triggering DNA repair pathways such as the activation of cell cycle checkpoints and histone degradation, which enhances DNA recombination rates[17-19]. The integration of HBV DNA can also contribute to hepatocarcinogenesis by altering several cancer-relevant genes[20]. Viral promoter-driven human transcripts have also been identified adjacent to repetitive non-coding sequences, in particular long interspersed nuclear elements or short interspersed nuclear elements[20]. Evidence for fusion proteins have been collected for retinoic acid receptor-β and human cyclin A gene resulting in tumor-specific chimeric proteins endowed with pro-carcinogenic functions[21].

Despite this evidence, HBV integration is random and hardly ever leads to direct oncogene activation or tumor suppressor genes inactivation. However, it is widely accepted that integration contributes to hepatocytes genetic instability leading to clonal proliferation[22]; this can occur in HBV infected patients even before liver tissue damage is clinically evident[23].

### *Role of HBsAg*

A study shows that the presence of HBV-DNA integration increases more than 100 times the risk for HCC occurrence among HBsAg carriers compared with HBsAg negative individuals[13]. HBsAg seems to have effects on the mitochondrial function, as it reduces the lipid b-oxidative activity of enoyl-coenzyme A hydratase short chain 1. On one hand, this interaction induces cell apoptosis by decreasing the mitochondrial membrane potential and reduces the phosphorylation of a serine/threonine protein kinase (Akt). On the other hand, it leads to an increased reactive oxygen species (ROS) production and promotes UDP-glucose ceramide glycosyltransferase which contributes to the alteration of ceramide metabolism[24]. Furthermore, HBsAg can inhibit jumping translocation breakpoints leading to increased cell motility and decreased apoptosis[13].

**Table 2 Risk factors for hepatocellular carcinoma development[6,181-183]**

| Risk factors in hepatocellular carcinoma (other than liver cirrhosis) | | OR (95%CI) |
|---|---|---|
| Strong risk factors (OR > 10) | **Europe** | |
| | Untreated chronic HBV/HCV hepatitis | 191.0 |
| | Untreated chronic HCV hepatitis | 31.2 |
| | Untreated chronic HBV hepatitis | 18.8 |
| | **East Asia and Africa** | |
| | Untreated chronic HBV/HCV hepatitis | 75.6 |
| | Untreated chronic HBV hepatitis | 20.8 |
| | Untreated chronic HCV hepatitis | 11.5 |
| Moderate risk factors (OR = 2-10) | Aflatoxin B1 exposure | 5.9 |
| | Untreated chronic HDV infection | 3.9 |
| | Diabetes | 3.2 |
| | Asian race | 3.2 |
| | Male gender | 2.8 |
| | Alcohol intake | 2.3 |
| | Severe iron overload | 2.1 |
| Weak risk factors (OR < 2) | Obesity (BMI > 30 kg/m$^2$) | 1.9 |
| | Mild iron overload | 1.6 |
| | Current smoking | 1.6 |
| | HCV genotype 1b | 1.6 |
| | PNPLA3 rs738409 single nucleotide polymorphism | 1.4 |

HBV: Hepatitis B virus; HCV: Hepatitis C virus; HDV: Hepatitis D virus; OR: Odds ratio; CI: Confidence interval; BMI: Body mass index.

### *Role of HBeAg*

The association between HBeAg and HCC has been well established by epidemiological studies although the pathophysiological mechanisms of HBeAg-mediated oncogenesis remain unknown. A large study investigates the effect of HBV replication on HCC development risk among 11893 Taiwanese men followed up for a mean of 8.5 years: The incidence rate of HCC is 1169 per 100000 person-years among HBsAg/HBeAg positive men, 324 per 100000 person-years for HBsAg positive HBeAg negative men and 39 per 100000 person-years among HBsAg negative men[25]. HBeAg enhances cell proliferation by means of promotion by a G1/S transition. Data suggests that miR-106b upregulation mediated by HBeAg is involved in the pathogenesis of HBV-related HCC by downregulating retinoblastoma (RB) gene[26].

### *Role of HBV preS/S proteins, oxidative stress and interaction with endoplasmic reticulum*

The preS/S open reading frame encodes three envelope proteins (S or HBsAg, M, and L proteins)[27]. Various specific mutations in the preS/S gene may imbalance surface proteins' synthesis and their consequent retention within hepatocytes' endoplasmic reticulum (ER)[13,28]. This process occurs mainly in the presence of HBsAg or pre-S2 mutants: The viral proteins amassed in the ER can lead to oxidative stress, stimulate cell growth and survival signaling pathways and cause mutation through the generation of free radicals. The induced ER stress upregulates the cytoplasmic cyclin A pathway promoting chromosome instability through centrosome over-duplication[29]. Moreover, pre-S2 mutant protein in hepatocytes can directly interact with the c-Jun activation domain-binding protein 1 inducing hyperphosphorylation of the RB tumor-suppressor and its inactivation[13,30].

### *Role of HBx protein*

HBx is a 154 aminoacids long protein which is essential for the HBV viral life cycle. HBx is usually expressed at low levels during the first stages of infection but with the rise of HBV DNA integration frequency during infection, relative HBx expression can increase[31]. HBx is involved in multiple and complex molecular pathways linked to mitogen and apoptotic signaling cascades, resulting in one of the most relevant oncogenic proteins in HBV proteome. It does not interact directly with DNA, but rather it

**Table 3 Molecular pathways of hepatocellular carcinoma carcinogenesis in hepatitis B virus infected patients**

| HBx related pathways |
|---|
| **DNA repair impairment and DNA instability** |
| HBx - binds to DDB1 - instability of Scm5/6 - impairment in DNA replication and repair |
| HBx - interacts with TFIIH - impairment in DNA replication and repair |
| HBx - blocks BER pathway - impairment in DNA repair |
| HBx - binds to CRM1 and sequestering it in cytoplasm - aberrant centrosome duplication and chromatin's segregation - chromosome instability |
| **DNA replication increase** |
| HBx - upregulates RLF and CDT1 and downregulates geminin - DNA replication |
| HBx - binds to cccDNA - recruiting PCAF - histone H3 acetylation - inhibition of chromatin's methylation - DNA replication |
| **Cell cycle deregulation *via* signal pathways** |
| HBx - binds to p53 - impaired function of p53 - cell cycle dysregulation |
| HBx - induces AFP expression - activation of PTEN and PI3K/mTOR pathway - cell cycle deregulation |
| HBx - activates Notch1 and Notch4 receptor - cell cycle progression |
| HBx - upregulates NF-kB, AP-1, AP-2, c-EBP, RNA-polymerase, ATF - altered oncogenes expression and cell cycle deregulation |
| HBx - upregulates NF-kB - upregulation of EGR1 - upregulation of miR-3928v - downregulation of $VDAC_3$ - tumor suppressor inhibition |
| HBx - downregulates SFRP1 and SFRP5 - DNMT1 recruitment - inhibition of WNT/β-catenin pathway - epithelial mesenchymal transition |
| **Epigenetic modification impairment** |
| HBx - interacts with MBD2 and CBP - P3 and P4 promoters' activation through hypomethylation - recruitment of IGF2 - oncogenesis |
| HBx - stimulates deacetylation of IGFBP3 gene - upregulation of IGF1 - mitogenic and anti-apoptotic effects |
| HBx - upregulates DNMT1 - hypermethylation of RASSF1A - tumor suppressor inhibition |
| HBx - downregulates DNMT3a/DNMT3L and recruits HDAC1 - hypomethylation of oncogenes promoters including JAK/STAT3 - impairment in cell differentiation |
| HBx - downregulates CD82, MTA1, PCDH10 through hypermethylation - tumor progression |
| HBx - inhibits CDH1 through deacetylation - E-cadherin upregulation - metastasis promotion |
| **Apoptosis impairment** |
| HBx - upregulates Bcl2 and Mcl1, inhibits Bax - apoptosis inhibition |
| HBx - upregulates Foxo4 - increased resistance to ROS damage, avoiding cell death and apoptosis |
| HBx - upregulates NF-kB - increase of DR5 - TRAIL induced apoptosis |
| HBx - inhibits caspase-8 inhibitor A 20 - TRAIL induced apoptosis |
| **mi/lnc RNA related pathways** |
| HBx - impairs miRNA regulation and synthesis - cell cycle deregulation |
| HBx - impairs lncRNA regulation and synthesis - cell cycle deregulation |
| **Oxidative stress** |
| HBx - downregulates NQO1 - mitochondrial injury - ROS production |
| C-terminal truncated HBx - mitochondrial DNA damage - ROS production |
| **Neoangiogenesis** |
| HBx - upregulates VEGF, HIF1 and ANG2 - neoangiogenesis |
| **Unknown mechanism** |
| HBx - binds to HSP60 and HSP70 - unknown function but involved in HCC carcinogenesis |
| **Pre-S/S related pathways** |
| pre-S2 - retention of HBV proteins in ER - ROS increase - cell DNA damages |
| pre-S2 - retention of HBV proteins in ER - upregulation of CCNA - chromosome instability and centrosome overduplication |

| |
|---|
| pre-S2 - interacts with JAB1 - RB tumor suppressor inhibition |
| **HBsAg related pathways** |
| HBsAg - binds to ECHS1 - ROS increase - cell DNA damages |
| HBsAg - binds to JTB - decreased apoptosis and increased cell mobility |
| **HBeAg related pathways** |
| HBeAg - stimulates upregulation of miR-106b - RB tumor suppressor inhibition |
| **HBV DNA related pathways** |
| cccDNA - triggers DNA repair pathways - histone degradation and cell cycle checkpoints activation - enhanced DNA recombination rate |
| HBV DNA - genome integration - oncogene activation or tumor suppressor inhibition with evidence of fusion proteins |
| HBV DNA - genome integration - genetic instability - clonal proliferation |
| **Inflammatory pathways** |
| Increased cytokines production (TGF-β, IL-4, IL-10, IL-12, IL-13) - JAK/STAT3 activation - cell proliferation |
| CD4+ T follicular helper decrease - loss of growth inhibition and death control of cancer cells |
| CD8+ cell dysfunction - impaired growth inhibition and death control of cancer cells |
| Functional exhausted CD8+TIM-3+ T cells - increased viral replication - increased viral factors in HCC development |
| NK cells - increase in IL-4 and IL-13 - activation of HSCs - increased cytokines production - cell cycle deregulation |
| NK cells - miR-146a increase - reduced cytotoxicity and decreased IFN-γ production - reduction in immunosurveillance |
| Tregs - PD1 and CTLA4 overexpression - C |
| **Gut microbiota-related pathways** |
| HBV related dysbiosis - circulating LPS - TLR4 activation - cytokines production - JAK/STAT3 activation - cell proliferation |

DDB: Damage specific DNA binding protein; Scm5/6: Structural maintenance of chromosomes 5/6; TFIIH: Transcription factor II H; BER: Base excision repair; RLF: Rearranged L-Myc fusion gene protein; cccDNA: Covalently closed circular DNA; PCAF: P300/CBP-associated factor; AFP: Alpha fetoprotein; PTEN: Phosphatase and tensin homolog; PI3K: Phosphatidylinositol 3-Kinase; mTOR: Mechanistic target of rapamycin kinase; NF-kB: Nuclear factor-kappa B; AP-1: Activator protein 1; ATF: Activating transcription factor; EGR1: Early growth response protein 1; VDAC: Voltage dependent anion channel; SFRP: Secreted frizzled-related protein; DNMT: DNA methyltransferase; WNT: Wingless-related integration site; MBD: Methyl-CpG binding domain protein; CBP: CREB binding protein; IGF: Insulin like growth factor; IGFBP: Insulin like growth factor binding protein; MTA: Metastasis associated; Bcl2: B-cell lymphoma 2; Mcl1: Myeloid cell leukemia-1; PCDH: Protocadherins; miRNA: Micro-RNA; lncRNA: Long non coding RNA ; ROS: Reactive oxygen species; HIF: Hypoxia-inducible factor; ANG: Angiogenin; VEGF: Vascular endothelial growth factor; HSP: Heat shock protein; HCC: Hepatocellular carcinoma; ER: Endoplasmic reticulum; CCNA: Cytoplasmatic Cyclin A; RB: Retinoblastoma; ECHS: Enoyl-CoA hydratase short chain; JTB: Jumping translocation breakpoint; HBeAg: Hepatitis B e antigen; HBV: Hepatitis B Virus; TGF: Transforming growth factor; IL: Interleukin; NK: Natural killer; HSC: Hematopoietic stem cells; IFN: Interferon; PD1: Programmed cell death 1; CTLA4: Cytotoxic T-lymphocyte antigen 4; LPS: Lipopolysaccharides; TLR4: Toll-like receptor 4.

acts on cellular promoters, impairs DNA repair pathways, enhances DNA replication, alters epigenetic processing and inhibits apoptosis mechanisms. HBx effects are proven to contribute to HCC progression as well, by means of angiogenesis upregulation and stimulation of cell motility.

Some preclinical studies show that HBx interacts with cell cycle regulatory pathways activating a plethora of genes involved in mitogen signaling cascades and their downstream transcription factors, including, nuclear factor-κB (NF-κB), phosphatidylinositol 3-kinase (PI3K) and phosphatase and tensin homolog through upregulation of alpha-fetoprotein (AFP), Notch1 and Notch4, wingless-related integration site (WNT)/β-catenin signaling, activator protein 1 (AP-1), AP-2, CCAAT-enhancer-binding protein, RNA polymerase and nuclear factor of activated T cells. The activation of these pathways leads to altered expression of growth-control genes[32]. Other significant targets of HBx are the p53 family genes; The viral protein directly binds to p53 and impairs its function (p53-mediated apoptosis, p53-mediated transactivation properties and cell cycle regulation)[33].

*In vitro* studies have provided mechanistic insights towards the understanding of the role of HBx in HBV-mediated carcinogenesis showing an interesting effect on DNA replication. Indeed, HBx expressing cells exhibit increased DNA replication licensing factor, chromatin licensing and DNA replication factor 1, as well as reduced expression of geminin. The impairment in these molecules homeostatic balance leads to an increase in DNA replication[34]. Furthermore, HBx impairs DNA repair enzymes, interacting with transcription factor IIH, and deregulating all other proteins involved in the base excision repair pathway[32]. HBx is also involved in HBV transcription from cccDNA in the beginning and maintaining phase of viral replication. This leads to chromatin modulation and chromosomal instability because the HBx bond with cccDNA induces the recruitment of acetyltrans-

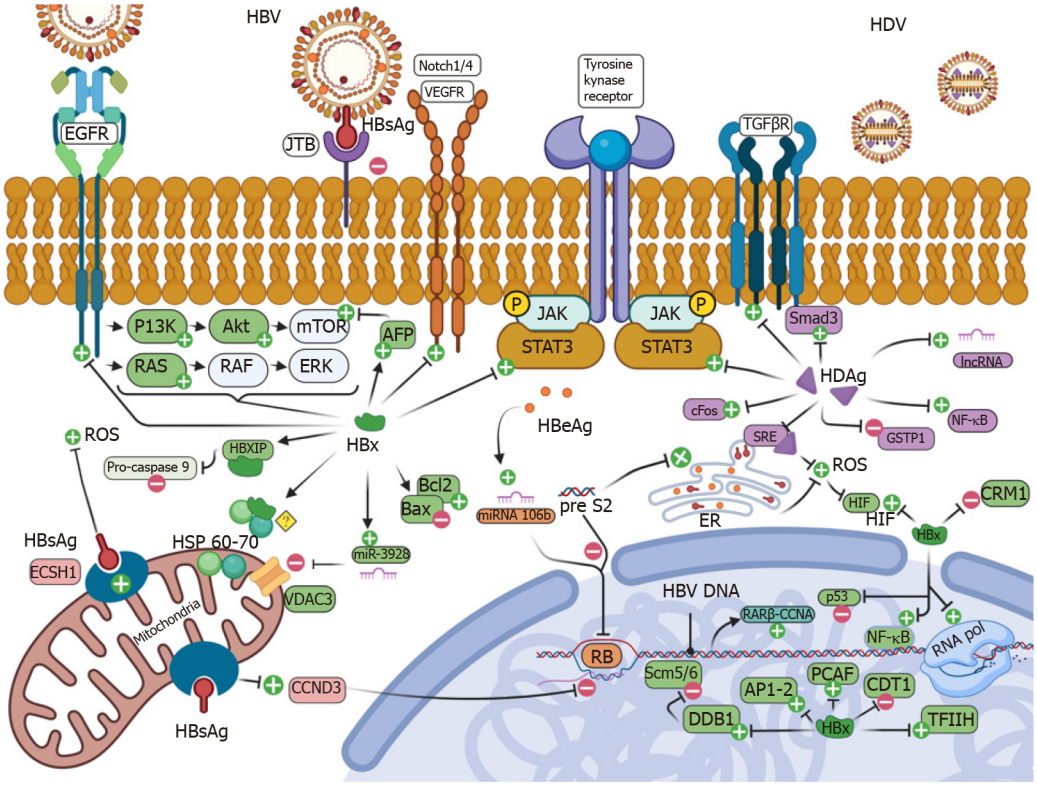


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**Figure 1 Molecular pathways in hepatitis B virus and hepatitis D virus carcinogenesis.** Created with BioRender.com. HBV: Hepatitis B virus; HDV: Hepatitis D virus; EGFR: Epidermal growth factor receptor; JTB: Jumping translocation breakpoint; HBsAg: Hepatitis B virus surface antigen; VEGFR: Vascular endothelial growth factor receptor; TGF: Transforming growth factor; PI3K: Phosphatidylinositol 3-kinase; Akt: AKT serine/threonine kinase; RAS: Rat sarcoma virus gene; RAF: Rapidly accelerated fibrosarcoma; mTOR: Mechanistic target of rapamycin kinase; JAK: Janus kinase; STAT3: Signal transducer and activator of transcription 3; Smad3: Mothers against decapentaplegic homolog 3; lncRNA: Long non coding RNA; NF-kB: Nuclear factor-kappa B; ROS: Reactive oxygen species; HSP: Heat shock protein; ECSH1: Enoyl-CoA hydratase short chain 1; VDAC: Voltage dependent anion channel; CCND3: Cytoplasmatic Cyclin D3; RB: Retinoblastoma; Bcl2: B-cell lymphoma 2; Bax: Bcl2 associated X; ER: Endoplasmic reticulum; HIF: Hypoxia-inducible factor; AP: Activator protein; DDB: Damage specific DNA binding protein; Scm5/6: Structural maintenance of chromosomes 5/6; PCAF: P300/CBP-associated factor; TFIIH: Transcription factor II H.

ferases P300/CREB-binding protein (CBP)-associated factor and acetylation of histone H3, inhibiting chromatin's methylation[35]. HBx seems to induce multipolar spindle formation, chromosome segregation defects and aberrant centrosome duplication, probably by sequestrating the nuclear transport receptor Chromosomal Maintenance 1 in the cytoplasm[36]. HBx binds to the HBx interacting protein regulating the centrosome duplication or to the UV-damaged DNA binding protein 1 that influences the stability of proteins such as the structural maintenance of chromosome proteins 5 and 6 complex which plays a role in nuclear DNA replication and repair[37,38].

In mitochondria, HBx binds heat shock proteins 60 and 70 (HSP60 and HSP70) and increased levels of the resulting complex have been found in HCC cells, even if the underlying carcinogenetic mechanism has not been understood yet[39]. HBx, through miR-3928v upregulation, downregulates voltage dependent anion channel 3 (VDAC3) expression in mitochondria leading to the loss of its tumor suppression activity[40].

The role of HBx on apoptosis has been controversial. On one hand, HBx upregulates B-cell lymphoma 2 (Bcl-2) and myeloid cell leukemia-1 that exert an anti-apoptotic role and downregulates Bcl-2-associated X protein, a cytosolic protein inducing apoptosis by its interaction with VDAC3 channels on the mitochondrial membrane. On the other hand, HBx stimulates apoptosis by increasing the cell surface concentration of death receptor 5 and inhibits caspase-8 inhibitor A20 that have central roles in tumor necrosis factor (TNF) α-related apoptosis-inducing ligand mediated apoptosis. Moreover, HBx increases resistance to apoptosis through the forkhead box protein 4 avoiding cell death primed by oxidative stress damage[32]. In addition, HBx controls hepatic angiogenesis, upregulating vascular endothelial growth factor (VEGF), hypoxia inducible factor 1 (HIF-1) and proangiogenic growth factor angiopoietin 2 (ANG2)[41].

### *HBx effects on DNA methylation and acetylation*

DNA methylation and acetylation are two of the most studied epigenetic modifications and usually occur in the early stage of HCC development. The genomic hypomethylation/hypoacetylation increases chromosome instability while localized hypermethylation/hyperacetylation decreases the expression of

**Table 4 Hepatocellular carcinoma surveillance in hepatitis B virus infected patients**

| HCC surveillance in HBV-infected patients | | |
|---|---|---|
| **Western medical societies** | | |
| EASL, 2017 | High-risk-patients: (1) HBV cirrhotic patients; (2) HBV and F3 fibrosis; and (3) HBsAg-positive patient on NA treatment with a PAGE-B of ≥ 18 at the onset of therapy. Medium risk-patients: HBsAg-positive patient on NA treatment with a PAGE-B of 10 - 17 at the onset of therapy | Screening with US examination with or without AFP every 6 mo for medium and high-risk patients. No specific HCC screening needed for low-risk patients |
| AASLD, 2018 | High-risk patients: (1) HBV cirrhotic patients; (2) Special population of HBsAg-positive adults: Asian or African men (> 40 yr) and Asian women (> 50 yr), first-degree family member with a history of HCC, HDV coinfected; and (3) HBsAg-positive children/adolescents with advanced F3 or cirrhosis and first-degree family member with HCC | Screening with US examination with or without AFP every 6 mo; if in areas where US is not readily available, screening with AFP every 6 mo |
| **Eastern medical societies** | | |
| JSH, 2014-2021 | Extremely-high-risk patients: HBV cirrhotic patients. High-risk patients: Special population of HBsAg positive patients: age ≥ 40, male, alcohol consumption, high HBV load, family history of HCC, HCV/HDV/HIV coinfection, F3 fibrosis, low platelet count associated with advanced fibrosis, genotype C, and core promoter mutation | Screening with US and tumor marker measurements (AFP, protein induced by vitamin K absence or antagonist-II and AFP-lectin fraction 3) every 3-4 mo in the super-high-risk population. A 6-12 mo dynamic CT scan or dynamic MRI should be performed. Screening every 6 mo in high-risk populations |
| APASL, 2016 | High-risk patients: All patients with HBV-related cirrhosis. HBsAg-positive without cirrhosis, based on the economic situation of each country and on the available risk scores | Surveillance by US and AFP should be performed every 6 mo and preferably every 3-4 mo in cirrhotic patients and those at high risk of HCC |
| KLCSG, 2014-18 | High-risk patients: HBV cirrhotic patients; chronic hepatitis B patients | Screening with US examination with or without AFP every 6 mo. If liver US cannot be performed properly, liver dynamic CT or dynamic contrast-enhanced MRI can be performed |

HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HDV: Hepatitis D virus; HIV: Human immunodeficiency virus; EASL: European Association for the Study of the Liver; AASLD: American Association for the Study of Liver Diseases; JSH: Japan Society of Hepatology; APASL: The Asian Pacific Association for the Study of the Liver; KLCSG: Korean Liver Cancer Study Group; US: Ultrasound; AFP: Alpha-fetoprotein; F3: Fibrosis; HBsAg: Hepatitis B virus surface antigen; CT: Computed tomography; MRI: Magnetic resonance imaging.

tumor suppressor genes[13]. DNA hypermethylation in the promoter region of specific oncosuppressor genes has been found in HBV-related HCC mainly due to HBx effect.

HBx upregulates DNA methyltransferases 1 (DNMT1) causing a hypermethylation of rat sarcoma (RAS) association domain family member 1 (RASSF1A), involved in cell cycle maintenance, altering its expression from the very early stages of HCC development[42]. On the contrary, the downregulation of DNA methyltransferases (DNMT) 3A and DNMT 3L and the recruitment of histone deacetylase 1 causes the hypomethylation of intragenic CpG islands resulting in dedifferentiation of liver cells[43]. The upregulation of methyl-CpG binding domain protein 2 and CBP triggers a molecular pathway leading to hypomethylation of promoters 3 (P3) and 4 (P4) of insulin-like growth factor 2 gene that stimulates cell duplication pathways as well as G1/S transition[44]. Hypomethylation has been found in the promoter region of Telomerase Reverse Transcriptase (TERT), increasing the proliferative potential of the cell, and in the promoter region of COX-2, leading to a proinflammatory condition[45]. Hypermethylation has also been found in a cluster of differentiation 82, a metastasis suppressor protein involved in the p53 pathway, metastasis-associated protein 1 and protocadherin 10. Deacetylation of Cadherin-1 promoter leads to E-cadherin upregulation and a progression to metastatic disease[32].

A growing number of studies identify and investigate the role of specific miRNA, small non-coding RNA molecules (up to 25 nucleotides in length) that regulate gene expression at transcriptional and post-transcriptional levels as mediators in the hepatocarcinogenic process. Recent publications, summarized by Sartorius *et al*[46] in a meta-analysis, have found miRNAs that are involved in HBV-related HCC leading to more aggressive cancer phenotypes by means of both tumor suppressor genes inactivation and oncogenes activation.

### *Role of occult HBV infection*

Studies with highly sensitive polymerase chain reaction assays show that HBV DNA may persist in the liver of people who have serologic recovery from acute HBV infection, as an occult HBV infection. A systematic review identifies a modest association between occult HBV infection and the risk of HCC occurrence [risk ratio (RR) = 2.83][47]. Another case-control study performed in Hong Kong shows an increased prevalence of occult HBV infection in cryptogenic HCC[48]. None of these studies is population based and most of them have a small sample size. Thus, the association between HBV occult infection and HCC lacks convincing evidence.

### *Role of HBV genotypes*

HCC is more common in patients with HBV genotype C, D and F than in those with genotype A[49].

The majority of retrospective and case-control studies indicate that patients with genotype C HBV have a more severe course of liver disease and a higher risk of HCC occurrence has been reported. A community-based Taiwanese cohort study shows that genotype C is associated with an increased risk of HCC in HBV carriers [hazard ratio (HR) = 2.35, 95% confidence interval (CI): 1.68-3.30, $P < 0.001$] as well [50]. The Eradicate-B study, by analyzing HBsAg positive patients without evidence of cirrhosis and a mean follow-up of 14.7 years, points out a higher annual incidence rate of HCC in genotype C than genotype B patients[51].

HBV genotype also influences the features of HCC. In genotype B patients, solitary HCC nodules are more frequent than in genotype C (94% *vs* 86%), but are more often associated with satellite nodules (22% *vs* 12%)[52]. Indeed, several reports show that HBV genotype type B is associated with a higher risk of HCC development in youth, whereas genotype C is associated with HCC development in the elderly[53]. Genotype D has been noticed to be prevalent in HCC patients younger than 40 years of age, as compared to other genotypes (63% *vs* 44%; $P = 0.06$). Furthermore, a study carried out among Alaska native HBV infected patients shows that the HCC is more prevalent in genotype F than other genotypes (OR= 7.73, 95%CI: 3.69-16.4, $P < 0.001$)[54]. Data about genotype E, endemic in Africa, are lacking and further studies are needed to outline the carcinogenic role of this genotype. Differently from HBV genotypes, the clinical significance of HBV subtypes is still unclear[55].

### *Role of HBV variants*

Several mutations in the X gene of the HBV genome are frequently found in HBV mutants of individuals with HCC. Some studies show that 3′-end X gene is commonly deleted in HCC cells leading to a C-terminal truncated HBx protein that may have a key role in the carcinogenetic process[56]. In a Taiwanese cohort, genotype C shows a higher prevalence of BCP A1762T/G1764A variant than genotype B. Patients with BCP A1762T/G1764A variant have a higher risk of developing HCC and a long-term follow up study reveals BCP A1762T/G1764A variant as an independent predictor for progression to HCC[57]. Mutations in enhancer II (C1653T) and elsewhere in the basal core promoter (T1753V) have also been found to be associated with HCC development[57].

## ENVIRONMENTAL FACTORS

### *Role of chronic inflammation*

Chronic tissue inflammation plays a central part in oncogenesis through various complex mechanisms. In the liver, continuous inflammation and regeneration increases the risk of hepatocarcinogenesis. Cytokines production and inflammation-mediated alteration of key signaling pathways, such as signal transducer and activator of transcription 3 (STAT3) and NF-κB pathways, play an important role as well [13,58]. The most common immune alterations found during HBV infection are: Cytokines production [such as tumor growth factor (TGF-β), interleukin (IL)-4, IL-10, IL-12, IL-13], decreased CD4+ and CD8+ cells function, Treg cells dysfunction and innate immune response suppression[59].

CD4+ Th1 and CD8+ cells play an important role in cell cycle regulation and death control of cancer cells; thus, T cells dysfunction can lead to a higher risk of HCC development[60]. The impairment of HBV-specific CD8+ T cell functions in patients with chronic hepatitis B is mainly shown by the high expression levels of inhibitory receptors such as cytotoxic T-lymphocyte antigen 4, programmed cell death protein (PD) 1 and T cell immunoglobulin and mucin-domain containing (TIM) 3. CD8+TIM-3+ T cells are functionally exhausted because of the continuous immune activation and are unable to effectively produce cytokines and exert anti-viral activity[60]. Additionally, non-specific CD8+ T cells with memory phenotypes secrete interferon (IFN)-γ, recruiting hepatic macrophages which promote HCC through the secretion of TNF-α, IL-6 and Monocyte Chemoattractant Protein 1[61]. The frequency of circulating CD4+ T follicular helper cells decreases and their function results are impaired during disease progression in patients with HBV-related HCC; CD4+ cells infiltrate seems to be significantly reduced in HCC tumor regions compared to non-tumor regions[62].

Natural killer (NK) cells from HCC patients show significant increase in the expression of miR-146a which downregulates NK cell's function and leads to a decreased IFN-γ and TNF-α production. Activation of non-virus-specific cells may result in widespread inflammation promoting HCC development. NK cells promote hepatic stellate cells (HSCs) activation in liver fibrogenesis through the production of the inflammatory cytokines IL-4 and IL-13[63]. HSCs enhance the recruitment of regulatory T cells (Tregs) in the liver increasing the fibrotic processes and therefore hepatocarcinogenesis. Indeed, Tregs show enhanced suppressive function by PD-1 over-expression leading to a more immunosuppressive and exhausted cellular microenvironment in HBV-related HCC compared to non-virus-related HCC[64]. Thus, HBV-induced immune imbalance is a major risk factor in the pathway of HCC development.

### *Role of the gut microbiota*

The link between the gut-liver axis and HCC has been studied in animal models as well as small cohorts of patients and is based on the theories that inflammation related to chronic liver disease causes

dysbiosis and intestinal barrier dysfunction. Translocation of bacterial products in portal and systemic circulation can trigger molecular pathways leading to inflammation, in particular lipopolysaccharide (LPS) from Gram-negative bacteria wall activates the Toll-like receptor (TLR) 4 causing an increase in inflammatory cytokines production. In turn, cytokines are linked to the activation of signaling pathways of cell proliferation, such as Janus kinase (JAK)/STAT3[65]. A well characterized pattern of inflammatory cytokines and chemokines has been associated with cirrhosis and HCC, characterized by increased plasmatic levels of IL-8, IL-13, C-C motif chemokine ligand (CCL) 3, CCL4 and CCL5[66]. This correlates alteration in gut microbiota with replication and immortalization of HCC cells. Indeed, several studies suggest that the gut microbiota might play a key role in the process. The inflammatory role of LPS is in fact mediated by TLR4; TLR4 knock-out mice show a much lower incidence of HCC [67]. Some other studies demonstrate that diethylnitrosamine (DEN)-exposed mice have an increased abundance of Gram-negative bacteria and therefore, of LPS (*Escherichia coli*, *Atopobium*, *Collinsella*, *Eggerthella* and *Coriobacterium)*[68]. High-dose probiotics administration in these animals is able not only to reverse dysbiosis but can reduce the number and size of cancer nodules as well.

There are limited studies comparing the gut microbiota of HBV-related HCC and non-HBV non-HCV-related HCC. A small Asian study enrolled 90 patients, divided in three groups: HBV-related HCC (B-HCC), non-HBV non-HCV-related HCC (NBNC-HCC) and healthy controls. The gut microbiota of B-HCC patients is more heterogeneous with higher bacterial alpha diversity indices, whereas, those of healthy controls and NBNC-HCC patients exhibits similar features. However, butyrate-producing bacteria (genus *Ruminococcus*, *Feacalibacterium*, *Clostridium*) present heterogeneity in B-HCC and NBNC-HCC in the study[69]. In addition, a set of *Bifidobacterium* species is found to mark predominant dysbiosis in HBV cirrhosis patients[70]. More studies are needed to clarify the possible oncogenic role of the gut microbiota related to HBV infection in the pathogenesis of HCC.

### HCC surveillance in patients with chronic hepatitis B

Eastern and western hepatological societies have the difficult task of identifying patient populations at high risk for HCC occurrence, in whom clinical and radiological surveillance is mandatory (risk above threshold, usually cumulative incidence > 1.5% yearly) and other populations where there is no certain benefit in carrying out the surveillance programs (risk unknown or below threshold)[71]. Based on these considerations, surveillance must include patients affected by cirrhosis of all causes and a subset of non-cirrhotic HBV carriers (Table 4). HCC screening and surveillance is recommended by the American Association for the Study of Liver Diseases (AASLD) guidelines in patients with cirrhosis of any cause and non-cirrhotic HBV carriers at higher risk (*i.e.,* Asian men older than 40-years-old, Asian women older than 50-years-old, patients with a family history of HCC and African or North American Blacks of any age)[72,73]. The European Association for the Study of the Liver (EASL) guidelines also include non-cirrhotic patients with high or medium risk calculated with the PAGE-B score[74,75]. The Japan Society of Hepatology (JSH) differentiates ''extremely-high-risk patients'' (hepatitis B cirrhosis) in whom surveillance is recommended every 3-4 mo and ''high-risk patients'', in whom surveillance is recommended every 6 mo and ''risk factors other than hepatitis virus infection or liver cirrhosis'' in whom the benefits of surveillance are uncertain[76,77].

### Risk scores

As individual HCC risk factors cannot adequately classify patients with chronic hepatitis B according to their HCC risk, several risk scores have been developed for those undergoing viral treatment with nucleotide analogues: CU-HCC, CAG-HCC, REACH-B, PAGE-B, modified PAGE-B (mPAGE-B)[78].

CU-HCC, GAG-HCC and REACH-B have been validated in a cohort of Asian patients with chronic hepatitis B, 22% of whom with cirrhosis, treated with entecavir (ETV): The 5-year HCC cumulative incidence rates were 12.9% in cirrhotic patients and 2.1% in non-cirrhotic ones while the accuracy of CU-HCC, GAG-HCC and REACH-B scores for HCC prediction at baseline was 0.8, 0.76 and 0.71, respectively[79]. Other studies confirmed an analogue predicting value of the REACH-B score in patients treated with tenofovir[80-82].

However, CU-HCC, GAG-HCC and REACH-B predictability is proven to be poor in Caucasian subjects with chronic hepatitis B. A large study including patients treated with ETV or tenofovir showed an accuracy of 0.66, 0.74 and 0.54, respectively[83]. Subsequently, Papatheodoridis *et al*[84] validated the PAGE-B score on 1815 Caucasians treated with ETV or tenofovir for at least 12 mo. The PAGE-B score had the advantage of including simple variables (platelets, age and gender) and was able to stratify the risk of HCC development within 5 years in: Low (0%), for PAGE-B score < 10; medium (4%), for PAGE-B score 10-17; or high (about 16%), for PAGE-B score > 17. Kim *et al*[85] developed the mPAGE-B score, adding serum albumin to the PAGE-B parameters, and validating the score on Asian populations. The mPAGE-B differentiated the 5-years HCC risk in low (< 8) or high (> 13). Therefore, the PAGE B score seems to perform with an adequate accuracy both in Caucasian and Asian chronic hepatitis B patients under the current oral antiviral drugs.

### Novel biomarkers

Recently, the usefulness of two novel biomarkers, hepatitis B core-related antigen (HBcrAg) and

**Table 5 Molecular pathways of hepatocellular carcinoma carcinogenesis in hepatitis D virus infected patients**

| Cell cycle deregulation *via* signal pathways |
|---|
| L-HDAg - Smad 3 activation - TGFβ upregulation - cells growth and dedifferentiation |
| L-HDAg - antagonizes c-Jun inhibitory effect over TGFβ - TGFβ upregulation - cells growth and epithelial-mesenchymal transition |
| L-HDAg - TNF-α stimulation - NF-κB activation - inflammation and proliferation |
| L-HDAg - activates STAT3 downstream protein - JAK/STAT pathway activation - cell growth |
| L-HDAg - stimulates c-Fos activation - cells growth and dedifferentiation |
| L-HDAg - downregulates GSTP1 - tumor oncosuppressor inhibition |
| **Oxidative stress** |
| L-HDAg - NF-κB and STAT3 activation - ROS production - DNA damage |
| L-HDAg - activates promoters of GRP78 and GRP94 - ROS production - DNA damage |
| L-HDAg - activates TGFβ1 - Nox4 activity - ROS production - DNA damage |
| S-HDAg and L-HDAg - increase in TRAF2 - inflammation and ROS production |
| S-HDAg and L-HDAg - bind to SRE - targeting proinflammatory genes - inflammation and ROS production |
| **Epigenetic mechanisms** |
| S-HDAg and L-HDAg - increased activity of histone acetyltransferases and CBP - histone H3 acetylation of clusterin promoter - increased clusterin expression - prolonged cell survival |
| S-HDAg - stimulates Histone H1e acetylation - clusterin promoter activation - prolonged cell survival |
| HDV - DNMT1 and 3b increased activity - tumor suppressor inhibition |
| S-HDAg and L-HDAg - hypermethylation of E2F1 promoter - cell cycle dysregulation |

HDAg: Hepatitis D virus antigen; Smad3: Small mother against decapentaplegic 3; TGF: Transforming growth factor; TNF: Tumor necrosis factor; NF-kB: Nuclear factor-kappa B; GSTP: Glutathione S-transferase Pi; ROS: Reactive oxygen species; GRP: Gastrin releasing peptide; HDV: Hepatitis D virus; STAT3: Signal transducer and activator of transcription 3; SRE: Stress response element; Nox4: NADPH oxidase; TRAF: Tumor necrosis factor-receptor associated factor; CBP: CREB-binding protein; DNMT1: DNA methyltransferases 1; JAK: Janus kinase.

Wisteria floribunda agglutinin-positive Mac-2 binding protein (M2BPGi), has been evaluated for the prediction of HCC occurrence. HBcrAg is a novel biomarker of HCC occurrence and consists of three products of the precore/core gene: HBeAg, hepatitis B core antigen and p22cr, a 22kDa protein present in DNA-negative Dane-like particles. It is used in Japan to support HCC screening in patients with chronic hepatitis B. Serum HBcr levels correlate with HBV replication, serum HBV DNA, intrahepatic cccDNA levels and transcriptional activity. In a large cohort study, HBcrAg is proven to be superior to HBV DNA in predicting HCC development in treatment-naïve patients with chronic hepatitis B. Tseng *et al*[51] showed that HBcrAg level is an independent risk factor of HCC in individuals with chronic hepatitis B and intermediate viral load. Another cohort study suggests a correlation between M2BPGi, a serum glycoprotein-based biomarker (glycobiomarker), fibrosis (FIB) stage and HCC. In patients with cirrhosis M2BPGi serum levels > 1.8 cut-off index are associated with a higher risk of HCC development ($P < 0.001$). Although the area under the receiver operating characteristic curves for M2BPGi and AFP are similar in cirrhotic patients of all etiologies (0.77 *vs* 0.72; $P = 0.15$), M2BPGi outperforms AFP in chronic hepatitis B patients (0.84 *vs* 0.75; $P = 0.02$)[86]. Furthermore, M2BPGi serum level before receiving NA and 48 wk after the beginning of treatment is predictive of HCC occurrence.

### *The impact of therapies for chronic hepatitis B infection on HCC occurrence*

Nowadays, only a few classes of drugs have been licensed and therefore approved for hepatitis B treatment: Nucleos(t)ide reverse transcriptase inhibitors (NRTIs) and IFN-α.

### *Lamivudine*

Lamivudine (LAM) has been the first nucleoside analogue used for the treatment of chronic HBV infection. In an Asian study, the cumulative incidence of HCC is 3.9% in the LAM arm *vs* 7.4% in the placebo arm ($P = 0.047$), yet the clinical benefits on disease progression and HCC occurrence are lost when patients developed resistance (11% *vs* 5% placebo)[87]. A meta-analysis of 5 studies including 1267 patients treated with LAM compared with 1022 untreated patients demonstrates that HCC incidence is reduced by 78% (2.5% *vs* 11.7%, RR = 0.22; $P < 0.001$)[87,88]. Therefore, LAM treatment seems to reduce but not to eliminate the risk of HCC.

### ETV and tenofovir

Second-generation NRTIs, ETV and tenofovir [tenofovir disoproxil fumarate (TDF) or tenofovir alafenamide), have a potent suppressive effect on the DNA synthesis mechanism during HBV replication, although they have poor effect on the intracellular concentration and activity of cccDNA, which persist for years in the infected liver due to the long half-life and the resistant molecular structure. In an Asian study, ETV treated patients have a 63% reduction in the incidence of HCC at 5 years compared to those not treated (3.7% *vs* 13.7%; $P < 0.001$)[89]. A Greek study followed 321 patients with chronic hepatitis B under ETV for 30 mo comparing them with 818 patients on LAM treatment; the cumulative HCC incidence in the ETV group is 4.8% *vs* 5.6% ($P = 0.096$)[49]. In another European multicenter cohort study after a median follow-up of 39 mo, HCC developed in 71 (4.3%) out of 1666 patients with chronic hepatitis B treated with ETV or TDF; the cumulative probability of HCC results 1.3% at the first year, 3.4% at the third year and 8.7% at the fifth year after the initiation of treatment [90]. ETV and tenofovir seem to improve liver FIB to prevent cirrhosis complications and to reduce, though not eliminate, the risk of HCC.

### IFN-α

Patients with a clinical response to conventional IFN-α therapy schemes show a lower incidence of cirrhosis decompensation and a decrease in HCC occurrence with a better overall survival compared with non-responders. A recent meta-analysis of 14 trials suggests that pegylated-IFN facilitates HBsAg clearance or seroconversion in chronic hepatitis B patients[15,91]. In another study evaluating long-term outcomes of IFN-α therapy in HBeAg seropositive patients by comparing 233 patients undergoing IFN-based treatment with 233 matched controls, the cumulative HCC incidence at the end of 15 years of follow-up is 2.7% *vs* 12.5% ($P = 0.01$)[92]. Finally, a meta-analysis on 2742 subjects pooled from 12 studies also shows that IFN-α treatment reduces the risk of HCC occurrence by 34% (RR = 0.66, 95%CI: 0.48-0.89)[93].

### HBV vaccines

HBV vaccination prevents children from becoming HBV carriers and reduces the incidence of HCC in vaccinated populations. In Taiwan, after a vaccination campaign, the average incidence of HCC in children (6 years to 14 years) fell from 0.70 per 100000 children in 1981-1986 to 0.57 in 1986-1990 and further to 0.36 in 1990-1994 ($P < 0.01$). Moreover, after the universal HBV immunization program in the 1990s, HCC incidence reduced from 0.293-0.117 per 100000 person-years[94]. Similar findings result from South Eastern Asian and Alaskan studies[95-97]. More than 70%-80% of HCC in all developing countries are linked to chronic hepatitis B infection so widespread vaccination is the most important step to prevent more than 500000 HCC cases per year worldwide[98].

## HDV INFECTION AND HCC DEVELOPMENT

### Epidemiology

Recent studies estimate that between 20 and 72 million people are infected with HDV[99]. Indeed, the precise global prevalence of HDV infection has remained unestablished due to the non-standardized screening programs in different nations and the inaccessibility to testing in most highly endemic areas [100,101]. In Western Europe, the prevalence of the infection has decreased in the last 20 years as a consequence of ameliorated socioeconomic conditions and national vaccination campaigns against HBV [102,103].

Chronic hepatitis D is considered the most severe form of chronic viral hepatitis leading to a rapid progression towards liver cirrhosis and a higher mortality rate compared to other types of viral hepatitis. More than 10% of chronically HDV infected patients develop liver cirrhosis within 5 years from infection and in 30 years, more than 80% of patients suffer from cirrhosis decompensation[104].

### Molecular pathways and HCC development in chronic hepatitis D infection

Recent cohort studies have found a risk as much as nine times higher in HDV-infected patients compared to HBV monoinfected ones despite the common belief that HDV doesn't represent a certain major risk factor for HCC development[105,106]. Furthermore, persistent HDV replication has been proven to be a risk factor for liver disease progression to liver cirrhosis and HCC[107]. A study by the European Concerted Action on Viral Hepatitis demonstrates an increase of HCC occurrence by a factor of 3.2 in anti-HDV positive cirrhotic patients compared to negative ones[105]. Other recent studies show an increased risk of HCC for HDV/HBV coinfected patients than in HBV monoinfected ones (adjusted HR = 9.30)[108]. One large study estimates a risk of HCC occurrence significantly higher among patients with chronic HDV infection (RR = 3.9) than among those with HBV monoinfection[109]. On the contrary, an English retrospective study reports that despite the increasing prevalence of HDV infected patients in South London, liver cancer occurrence is only slightly increased between HBV monoinfected and HBV/HDV coinfected patients (OR = 1.34)[110].

In conclusion, recent findings identify HDV infection as a risk factor for HCC even if a direct oncogenic mechanism is unlikely. So, it is uncertain whether HDV adds oncogenic effects beyond those carried by chronic inflammation, FIB and cirrhosis. The genomic signature of HDV-related HCC has been poorly understood but several studies have pointed out some potential oncogenic mechanisms involving: Interference of HDV antigen (HDAg) in the cell cycle through STAT3 and cyclophilin, alteration of protein synthesis, oxidative stress as a result of severe inflammation, aberrant silencing of tumor suppressor genes by DNA methyltransferases and a possible epigenetic control on HBV transcription[5]. These mechanisms are summarized in Table 5 and Figure 1.

## VIRAL FACTORS

### *Role of HDV replication levels*

Even if HDV replicative activity has been related to a poor prognosis, it is still uncertain whether levels of HDV DNA increase the risk of HCC development. Some studies find no correlation between viral load and HCC occurrence in HDV infected patients while some others show an increased risk of cirrhosis complications in patients with a higher HDV viraemia[104]. Further investigations are needed to point out the role of HDV replication as a risk factor in liver oncogenesis.

### *Role of HDAg*

HDAg expression alone does not appear to have oncogenic potential. Moreover, since high levels of antigen and viral RNA can lead to a cell cycle arrest in G1 phase, this mechanism can promote DNA damage and oncogenes mutation. L-HDAg plays a key role in HDV-related oncogenesis. It can stimulate inflammatory and cell growth pathways by enhancing TNF-α production and NF-kB upregulation triggering STAT3 downstream proteins in the JAK/STAT molecular signaling and recruiting small mothers against decapentaplegic (Smad) 3 protein, involved in TGF-β activation[104]. In a similar way, L-HDAg downregulates glutathione S-transferase pi 1 (GSTP1), a tumor suppressor, and antagonizes c-Jun, by neutralizing its inhibitory effect on TGF-β cascade[111]. HBx and L-HDAg can act synergistically on NF-kB and JAK/STAT regulation increasing each other's effect.

### *Oxidative stress*

The severe inflammation occurring in HDV infection can by itself produce free radicals through NF-kB and STAT3 pathways. In addition, L-HDAg-induced TGF-β1 can activate NADPH oxidase (Nox) 4, resulting in a further ROS levels increase[111]. Either L-HDAg and S-HDAg bind to ER stress response element and stimulate an increase in TNF receptor-associated factor 2 targeting proinflammatory genes in the generation of oxygen-derived reactive products[112]. Furthermore, HDAg can stimulate the promoters of immunoglobulin protein gastrin releasing peptide (GRP)78 and GRP94 targeting inflammatory genes and upregulating ROS production[111]. Even if the damage carried to the host genome by HDV through oxidative stress is indirect, it represents one of the most important oncogenic effects of the virus. Novel therapies such as bulevirtide can stop this process by inhibiting viral replication and consequently virus-related inflammation and oxidative stress.

### *Epigenetic regulation and DNA methylation/acetylation*

Both HBV and HDV-related HCC enhance oncogenes and inhibit tumor suppressor genes by epigenetic regulation mostly through DNMT1 and DNMT3b proteins involved in the maintenance of methylation patterns in the human genome. The molecular mechanism of DNMT1 deregulation in HDV infected patients has not been established yet, even if some studies identify STAT3 regulation over DNMTs protein as the culprit of DNA methylation impairment in HDV-related HCC[111].

Moreover, S and L-HDAg can increase E2F transcription factor 1 promoter leading to cell cycle deregulation (G2/M switch) and Nox4 activation[111]. HDAgs impair the DNA acetylation as well, mainly by the recruitment of histone acetyltransferases and P300/CBP[104]. Histone acetyltransferases increase clusterin expression and prolongs cellular survival through its antiapoptotic effect[43].

### *Potential role on HBV replication*

An epigenetic control of HDV over HBV transcription and regulation has been postulated. There are some evidences that long-lasting active proliferation of both viruses can lead to more severe disease until HCC development[5,113].

## ENVIRONMENTAL FACTORS

### *Potential role of inflammation*

The histological examination of liver tissue of chronically HDV-infected patients shows an increase in

HDAg specific T CD4+ lymphocytes that rises gradually along the course of the infection. Recent findings suggest that hepatitis D is an immune-mediated disease and chronic immune activation may promote hepatocarcinogenesis[5].

## PATHWAYS NEEDING FURTHER INVESTIGATION

### *Potential role of small and long non-coding RNAs*

There is increasing interest in the study of non-coding RNAs including small non-coding RNA and long non-coding RNAs (lncRNAs). According to a recent study Y3 lncRNA results significantly downregulated in HDV-related HCC suggesting a possible role in the pathogenesis of HDV-related HCC[114].

### *Impact of therapies for chronic hepatitis D infection on HCC occurrence*

Recent long-term studies point out that pegylated (PEG)-IFN treatment is independently associated with a better clinical outcome and a longer survival. A retrospective study shows a reduced rate of liver decompensation in PEG-IFNα treated patients without any difference in terms of HCC development. In addition, complete loss of HDV RNA reached with IFN-α therapy does not seem to reduce the HCC occurrence rate[5,115]. Whether PEG-IFN treatment can reduce the risk of HCC development in HDV infected patients is unknown and more studies are needed to clarify this point. Given the recent approval of bulevirtide, data on its effect on HCC occurrence is still lacking.

## HCV INFECTION AND HCC DEVELOPMENT

### *Epidemiology*

Global prevalence of HCV, based on detectability of anti-HCV antibodies, has been estimated to be 1.6% which corresponds to almost 115 million individuals[116]. The prevalence of viremic individuals is estimated at 1% or 71 million[117]. HCV seroprevalence varies across geographical regions and is highest in Central Asia, East Asia, North-Africa and the Middle East where more than 3.5% of the total population is affected[118]. Russia, Egypt, Nigeria, India, Pakistan and China account for more than 50% of the total viremic HCV infection[116,118] whereas Western countries contribute only a small percentage (seroprevalence < 1%)[119]. The number of HCV-related deaths due to liver cirrhosis decompensation or HCC has been increased from almost 900000 deaths in the 1990s to almost 1500000 deaths in the 2010s as the result of the high prevalence and the lack of therapies[120].

### *Molecular pathways and HCC development in chronic hepatitis C*

HCV infection has been identified as one of the major risk factors for HCC development worldwide. Prospective studies show a 15-30-fold increased risk of HCC occurrence among chronically HCV infected patients compared with HCV negative ones. HCV genetic material doesn't integrate into the host genome; therefore, HCV needs continuous replication to maintain chronic infection. Despite this, HCV exerts indirect and direct effects on hepatocarcinogenesis. On one hand, the indirect effect is related to FIB and cirrhosis. The probability of HCC development rises with the stage of FIB and most of HCV-related HCCs occur in patients with advanced FIB or cirrhosis (annual rate up to 8%, average 1%-4%)[121]. On the other hand, viral factors such as HCV core protein, non-structural proteins, structural proteins and HCV genotypes and subtypes have a direct effect on the risk of HCC development through the modulation of hepatocytes gene expression. The multifaceted HCV infection effects on cells molecular pathways and microenvironment are summarized in Table 6 and Figure 2.

## VIRAL FACTORS

### *Role of HCV core protein*

HCV core protein presents a broad intracellular distribution in host cells suggesting a role in the modulation of multiple metabolic and replicative processes. Host proto-oncogenes and tumor-suppressors are direct targets of the HCV core protein which interact with tumor-suppressor proteins such as p53, p73 and RB, downregulating their functions. Interaction between p73 and the HCV core protein leads to inhibition of cell growth arrest mechanisms[122].

Moreover, increased TERT gene activity, typical of cells undergoing malignant transformation, is observed in primary human hepatocytes transfected with HCV core protein. TERT expression seems to be one of the earliest neoplastic events in HCC conferring an immortalized phenotype to transformed hepatocytes[123]. The HCV core protein inhibits the expression of the cyclin-dependent kinase inhibitor p21/WAF that has a role in the regulation of the cell cycle and in the induction of apoptosis. In a similar way, HCV core protein activates the rapidly accelerated fibrosarcoma 1/mitogen-activated protein

**Table 6 Molecular pathways of hepatocellular carcinoma carcinogenesis in hepatitis C virus infected patients**

| HCV core protein-related pathways |
|---|
| **Signaling pathways** |
| HCV core protein - binds p53, p73 and RB - tumor suppressors inactivation |
| HCV core protein - increased TERT gene activity – oncogenesis |
| HCV core protein - induces expression of cyclin E/CDK2 - G1/S transition |
| HCV core protein - inhibits CKI1 - cell cycle deregulation |
| HCV core protein - induces RAF/MAPK pathway – oncogenesis |
| HCV core protein - inhibits E-cadherin expression and SFRP1 *via* histone modification - activation of WNT/β-catenin signaling - epithelial mesenchymal transition |
| HCV core protein - interacts with TBR1 - inhibit TGFβ signaling and prevent translocation of Smad - cell spreading, cell growth regulation |
| **Oxidative stress and mitochondrial impairment** |
| HCV core protein - impairs lipid β-oxidation - reduces mitochondrial electron transport chain - ROS production |
| HCV core protein - impairs mitophagy - mitochondrial damage - ROS production |
| HCV core protein -interacts with HSP60 - ROS production and inhibition of TNFα induced apoptosis |
| **Angiogenesis** |
| HCV core protein - stimulate an increasing in HIF1α and AP-1 - upregulation of VEGF expression - angiogenesis |
| HCV core protein - activates PI3K/Akt and JAK/STAT - AR activation - angiogenesis |
| HCV core protein - activates COX2, MMP-2 and MMP-9 – angiogenesis |
| **Inflammation** |
| HCV core protein - suppresses of NF-kB pathways - impaired immune response |
| HCV core protein - upregulates cytokines and deregulates HSCs activity - impaired immune response |
| **E2 protein-related pathways** |
| E2 protein - interacts with CD81 - impaired host immune system |
| E2 protein - activates MAPK/ERK pathway - promoting cell proliferation |
| E2 protein - inhibits PKR - inhibition of protein synthesis |
| **NS2 protein-related pathways** |
| NS2 - activates cyclinD/CDK4 - induces expression of cyclin E/CDK2 - G1/S transition |
| NS2 - binds p53 - tumor suppressors inactivation |
| **NS3-related pathways** |
| NS3 - inhibits p53 - tumor suppressor inactivation |
| NS3 - inhibits ATM - tumor suppressor inactivation |
| NS3 - suppresses of NF-kB pathways - impaired immune response |
| NS3 - blocks TLR3 and RIG-I - impaired immune response |
| **NS5A-related pathways: Signaling pathways** |
| NS5A - inhibits p53 - tumor suppression inactivation |
| NS5A - interacts with TGFBR1 - inhibit TGFβ signaling and prevent translocation of Smad 3/4 - cell spreading, cell growth regulation |
| NS5A - increases phosphorylation of GSK3β - activates β-catenin - upregulates c-Myc - cell growth |
| NS5A - activates Akt pathway – oncogenesis |
| NS5A - interacts with PI3K p85 subunit - upregulates cell survival cascade |
| NS5A - activates Twist 2 - epithelial mesenchymal transition |
| NS5A - activates RAS - enhance tumor cell invasiveness |
| NS5A - inhibits JAK/STAT pathway - blockage of IFN signaling |

| |
|---|
| NS5A - inhibits PKR - inhibition of protein synthesis |
| NS5A - activates TLR4 - amplified NANOG - Twist 1 induction - oncogenesis and epithelial mesenchymal transition |
| **NS5A-related pathways: Apoptosis** |
| NS5A - inhibits TNFα mediated apoptosis - cell immortalization |
| NS5A - inactivates caspase 3 - inhibition of apoptosis |
| NS5A - inhibits proteolytic cleavage of death substrates (PARPs pathway) - impaired DNA repair and apoptosis |
| **NS5A-related pathways: Oxidative stress** |
| NS5A - induces of WNT/β-catenin signaling - upregulation of c-Myc - ROS production |
| NS5A - increases calcium release from ER - mitochondrial calcium uptake - ROS production |
| **Epigenetic modifications** |
| HCV - alters histone mark H3K27ac - TNFα and IL2 pathways - cell growth deregulation and epithelial mesenchymal transition |
| HCV - upregulates DNMT1 and SMYD3 - increased methylation of CDKN2A, GSTP1, APC, SOCS1, RASSF1A - tumor suppressors inhibition |
| HCV - increases miR-141 - inhibition of DLC1 - tumor suppressor inhibition |
| **Inflammatory pathways** |
| HCV - activates CCL20-CCR6 - endothelial cell invasion and angiogenesis |
| Switch from Th1 to Th2 - increasing in IL4-5-8-10 - loss of death control on cancer cells |
| Switch from Th1 to Th2 - decreasing in IL1-2-12-15 - loss of death control on cancer cells |
| **Gut microbiota-related pathways** |
| HCV-related dysbiosis - circulating LPS - TLR4 activation - cytokines production - JAK/STAT3 activation - cell proliferation |

HCV: Hepatitis C virus; RB: Retinoblastoma; TERT: Telomerase reverse transcriptase; MAPK: Mitogen-activated protein kinase; SFRP: Secreted frizzled-related protein; WNT: Wingless-related integration site; TBR1: T-Box brain transcription factor 1; TGF: Transforming growth factor; Smad: Small mother against decapentaplegic; ROS: Reactive oxygen species; HSP60: Heat shock protein 60; TNF: Tumor necrosis factor; HIF: Hypoxia-inducible factor; AP-1: Activator protein 1; VEGF: Vascular-endothelial growth factor; PI3K: Phosphatidylinositol 3-kinase; MMP: Matrix metalloproteinase; NF-kB: Nuclear factor-kappa B; HSC: Hematopoietic stem cells; ATM: Ataxia telangiectasia mutated; TLR: Toll-like Receptor; RIG: Retinoic acid-inducible gene; TGFBR: Transforming growth factor beta receptor; GSK: Glycogen synthase kinase; RAS: Rat sarcoma virus gene; PKR: Protein kinase R; PARP: PolyADP-Ribose polymerase; IL: Interleukin; GSTP: Glutathione S-transferase Pi; DNMT1: DNA methyltransferase 1; SMYD: SET and MYND domain-containing proteins; APC: Adenomatous polyposis coli; SOCS1: Suppressor of cytokine signaling 1; RASSF1A: RAS Association Domain Family Protein 1A; DLC1: Deleted in liver cancer 1; LPS: Lipopolysaccharides; JAK: Janus kinase; STAT3: Signal transducer and activator of transcription 3; CCL: C-C motif chemokine ligand; PARPs: Poly(ADP-ribose) polymerases; CDK4: Cyclin-dependent kinase 4; COX2: Cyclooxygenase 2; RAF: Rapidly accelerated fibrosarcoma; ANG2: Angiopoietin 2; CCR: Chemokine receptor type.

kinase (MAPK) proto-oncogene pathway and nonspecific hepatitis-induced cell proliferation[124].

Furthermore, overexpression of the HCV core protein seems to be important for the regulation of epithelial mesenchymal transition (EMT) driving the activation of HSCs. The induction of EMT is mediated by the inhibition of E-cadherin expression and the induction of secreted frizzled-related protein 1 *via* DNA methylation and histone modifications. This pathway leads to activation of WNT/β-catenin signaling[123]. Finally, the HCV core protein can suppress NF-κB, impairing downstream immune response.

Indirect HCV core protein mediated damage is carried by oxidative stress through an impairment of lipid β-oxidation and alteration of intracellular lipid metabolism which is associated with a reduction of mitochondrial electron transport chain's function. This process induces further ROS production and regulates apoptosis through inhibition of the HSP60 leading to a TNF-α-mediated apoptosis. Recently, the HCV core protein has also been proven to impair mitophagy contributing to mitochondrial damage; the resulting oxidative stress has been identified as a key trigger of the hepatocarcinogenetic process [122]. Dysregulation in angiogenetic processes and upregulation of inflammatory pathways represent other targets of indirect damage mediated by the HCV core protein.

### *Role of other viral proteins*

HCV-encoded proteins have been directly involved in the oncogenic process through interaction with several signaling pathways mainly in experimental animal models. The HCV E2 glycoprotein interacts with CD81, a cell surface marker of NK cells. This process leads to the alteration of cytokines production and dysregulation of cytotoxic granules release which impairs the host immune system[14]. In addition, E2 protein leads to MAPK/extracellular signal-regulated kinases pathway activation promoting cell proliferation and maintaining cell survival[125]. The NS2 protein can activate cyclin D/cyclin-dependent kinase 4 (CDK4) inducing cyclin E expression and stimulating cell cycle progression from G1

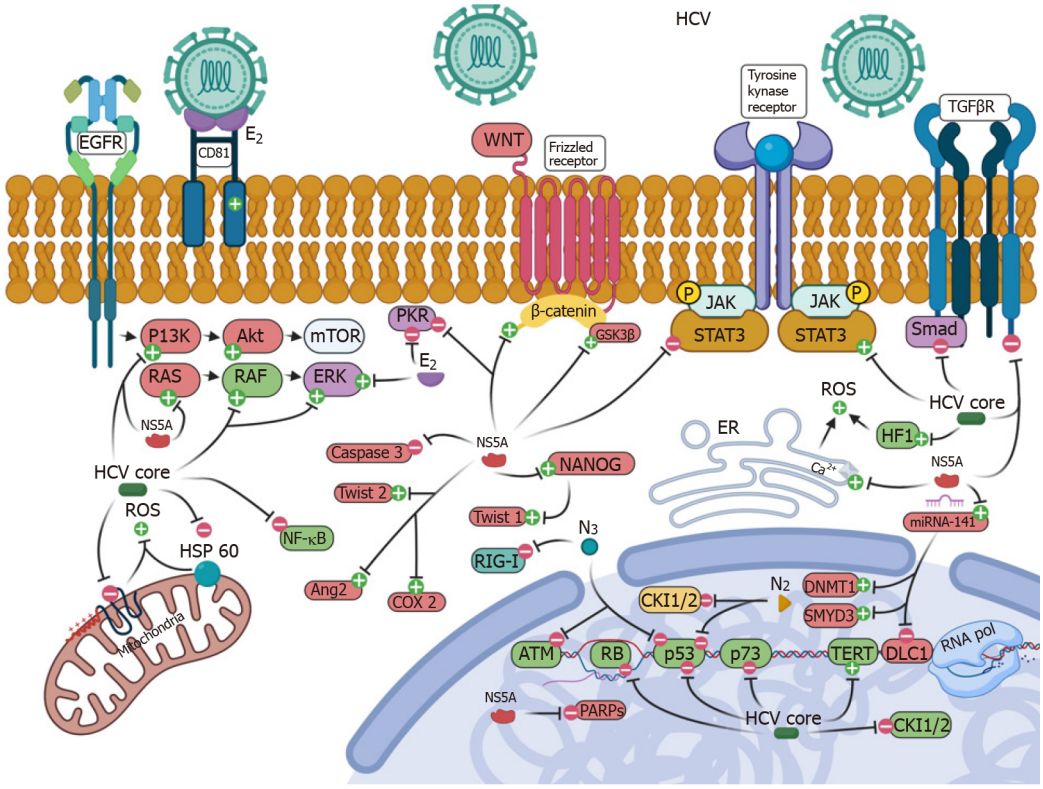


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**Figure 2 Molecular pathways in hepatitis C virus carcinogenesis.** Created with BioRender.com. HCV: Hepatitis C virus; EGFR: Epidermal growth factor receptor; WNT: Wingless-related integration site; TGF: Transforming growth factor; PI3K: Phosphatidylinositol 3-kinase; Akt: AKT serine/threonine kinase; RAS: Rat sarcoma virus gene; RAF: Rapidly accelerated fibrosarcoma; mTOR: Mechanistic target of rapamycin kinase; JAK: Janus kinase; STAT3: Signal transducer and activator of transcription 3; Smad3: Mothers against decapentaplegic homolog 3; NS5A: Non-structural protein 5A; NF-kB: Nuclear factor-kappa B; ROS: Reactive oxygen species; RIG1: Retinoic acid-inducible gene 1; ERK: Extracellular signal-regulated kinases; NANOG: Nanog homeobox; ATM: Ataxia telangiectasia mutated; RB: Retinoblastoma; ER: Endoplasmic reticulum; DNMT1: DNA methyltransferase 1; TERT: Telomerase reverse transcriptase; DLC: Deleted in liver cancer; CK1/2: Casein kinase 1/2; GSK: Glycogen synthase kinase.

phase to S phase. NS2 protein is involved in HCC carcinogenesis through tumor suppressor inactivation of p53 and consequent transcriptional activation[126]. The NS3 protein is involved in tumor cell transformation, by loss of contact inhibition and growth anchorage, evasion of innate immunity, interaction with mitochondrial signaling protein, tumor suppressors inactivation (p53 and ataxia telangiectasia mutated kinase or ATM) and alteration of immunoregulatory pathways such as TLR3, retinoic acid-inducible gene I (RIG-I) and NF-κB pathways[127].

Similarly, the NS5A protein plays a role in cell cycle dysregulation through signaling pathways, immune escape, impairment of cell apoptosis and ROS production. NS5A is involved in proliferative pathways by the inactivation of p53, blockade of IFN signaling through JAK-STAT cascade and inhibition of host protein synthesis through protein kinase R (PKR)[126]. In addition, NS5A protein, like HCV core protein, can inhibit TGF-β signaling through interaction with T-box brain transcription factor 1 or direct binding to TGF-β receptor 1 and through prevention of translocation of Smad proteins[128]. On the other hand, NS5A interacts with the PI3K p85 regulatory subunit that regulates apoptotic activity of PI3K and upregulates cell survival cascade, activating Akt and Twist family BHLH transcription factor 2 pathways favoring EMT, and interacts with RAS oncogene to enhance tumor cell invasiveness[129]. NS5A stimulates TLR4 activation and its signaling through homeobox protein NANOG and consequent Twist 1 activation leading to hepatocytes dedifferentiation[126].

NS5A affects apoptosis trough downregulatory effect on TNFα-mediated apoptosis. It deactivates caspase-3 and inhibits proteolytic cleavage of death substrates known as polyADP-ribose polymerases, impairing PD cascade[126]. Moreover, HCV seems to enhance ROS formation and aberrant cell-cycle arrest due to DNA damage through dysregulation of ER-mediated calcium uptake and induction of glycogen synthase kinase 3β phosphorylation with consequent beta-catenin-dependent upregulation of c-Myc *via* NS5A[122]. Non-structural viral proteins, together with HCV core protein, play a key role in HCV mediated oncogenesis. Their multifaceted interaction with basic hepatocytes signaling pathways favors deep dysregulation of cells homeostasis and accumulation of DNA damage until malignant transformation.

### Viral effect on angiogenesis

A higher vascular density has been reported in nodules of HCV-related HCC in relation to HCC nodules in patients affected by other liver diseases. Studies show upregulation of VEGF expression in patients with HCV-related HCC compared to the control group. HCV core protein can increase cellular VEGF expression through HIF-1α transcription factors and AP-1. Similarly, core protein can induce overexpression and stabilization of HIF-1α and ANG2 which upregulate VEGF expression[130]. Moreover, HCV core protein binds androgen receptor (AR), impairing transcriptional activity by activating several signaling pathways such as PI3K/Akt and JAK/STAT3. Since AR targets the VEGF gene in the liver tissue, HCV core protein upregulates VEGF expression through enhanced activity of the AR pathway[123].

Other mechanisms of HCV core-protein promoted angiogenesis and rely on upregulation of COX-2 and metalloproteinases 2 and 9 (MMP-2 and MMP-9)[126]. CCL20/C-C chemokine receptor type (CCR) 6 axis is directly involved in stimulating angiogenesis in the microenvironment of HCV-related HCC. *In vitro* experiments pointed out CCL20 as a direct promotor of angiogenesis inducing endothelial cells invasion and sprouting and migration of CCR6-positive leukocytes. A study demonstrates that HCV-induced CCL20 protein expression and secretion in hepatoma cells can be diminished and even abolished by means of antiviral treatment proving CCL20 expression to be dependent on HCV replication[131].

### Role of epigenetic modifications

Epigenetic modifications in patients' liver tissue can derive both from infected hepatocytes and from virus-induced inflammatory or fibrotic responses in the cells' microenvironment. HCV-induced histone modifications and fibrogenesis are interdependent during the course of liver disease; epigenetic changes orchestrate fibrogenesis through the activation of HSCs. Specific epigenetic modifications have been identified in patients' liver tissue and humanized mouse's liver tissue where no necrotic nor inflammatory response are present, therefore, it is probable that part of the changes observed can be a direct consequence of HCV-hepatocyte interaction.

HCV upregulates methyltransferase DNMT1 that hypomethylates tumor suppressor genes such as Cyclin Dependent Kinase Inhibitor 2A, GSTP1, RUNX family transcription factor 3, suppressor of cytokine signaling 1 and RASSF1A[126]. In a similar way, an upregulation of "suppressor of variegation, enhancer of Zeste, trithorax" and myeloid nervy deaf 1 domain-containing protein 3, also named SMYD3, leads to a specific hypermethylation of histone marks H3K4 and H4K5 in tumor suppressor genes, such as RASSF1A[126].

A recent study shows that chronic HCV infection induces changes in histone mark H3K27ac which are associated with HCC risk and seem to persist after HCV cure[132]. H3K27ac is involved in pathways related to TNF-α, inflammatory response, IL-2 and activator of transcription 5 signaling. Furthermore, lower levels of H3K27ac can alter pathways associated with metabolism and coagulative cascade, such as oxidative phosphorylation, impaired fatty acid regulation or adipogenesis. Several alterations in these pathways (*e.g.*, epigenetic alteration in TNF-α signaling, G2M-checkpoint, epithelial-mesenchymal transition, PI3K, Akt and mTOR) persist even after direct-acting antiviral agents (DAAs) therapy[132].

Recently, several studies outlined the role of miRNAs-mediated epigenetic alterations in HCV-related carcinogenetic processes. One study examines the expression profiles of miRNA in more than 50 HCV-related HCCs by quantitative real-time polymerase chain reaction. Five miRNAs are examined: MiR-122, miR-100, and miR-10a seem to be upregulated, while miR-198 and miR-145 are downregulated up to 5-fold in tumors than in normal liver tissue[133]. MiR-122 is highly expressed in the liver of HCV-infected individuals due to its essential role in the stability and replication of HCV RNA[134]. Another study develops an *in vitro* model of HCV infection in primary human hepatocytes to evaluate the role of miRNAs; miR-141, which targets the tumor-suppressor gene Deleted in Liver Cancer (DLC)-1, is upregulated in HCV genotypes 1a-, 1b-, and 2a-infected cells. The rise in miR-141 levels in HCV-infected cells correlates with an inhibitory effect on DLC-1 protein and a subsequent increase in HCC development risk[122].

### Role of HCV genotypes

HCV genotype 1b is associated with at least a doubled risk of HCC compared to other HCV genotypes [135,136]. HCV genotype 3 is considered a major risk factor for hepatic carcinogenesis. A multicenter Asian cohort study shows a 4.3-fold in the risk of HCC development in patients with chronic HCV genotype 3 infection compared to other genotypes[137]. Another Asian study shows a 5-year occurrence rate of HCC of 34% in genotype 3 and of 17% in non-genotype 3 groups, respectively ($P$ = 0.002); in a multivariate analysis, HCV genotype 3 infection is independently associated with a rise of HCC occurrence rates (HR = 3.54), even after adjustment for confounding factors[138]. A further study finds an approximately doubled risk of all-cause mortality and HCC in patients with HCV genotype 3 infection, related to individuals infected with other genotypes[139]. However, the exact role of HCV genotypes in hepatocarcinogenesis processes and in the worsening of liver disease still needs to be established.

## ENVIRONMENTAL FACTORS

### *Role of chronic inflammation and FIB*

Most of HCV-related HCCs occur in cirrhotic livers following decades of chronic inflammation underscoring the key role of the virus-induced inflammatory response in the hepatic carcinogenetic processes. Generally, the inflammatory response is beneficial to the host, but in HCV patients innate and adaptive immunity result ineffective. This cycle can be primed by HCV proteins but host immune response tends to self-maintenance.

HCV interferes with various defense mechanisms; TLR3 and RIG-I gene downstream pathogen-recognition signaling pathways are blocked by the HCV protease NS3/4A; HCV proteins NS5A and core proteins can block IFN signaling interfering with JAK–STAT pathway; HCV proteins E2 and NS5A inhibit PKR, an antiviral effector that impairs protein synthesis of infected cells; HCV core protein stimulates the release of cytokines that can lead to a direct decrease in plasmacytoid dendritic cells levels among HCV-infected patients[140,141].

The persistence of chronic inflammatory response leads to the release of free radicals such as ROS and nitric oxide (NO)[142]. NO contributes to viral persistence leading to antiapoptotic effects in hepatocytes and may induce viral mutations and promote suppression of Th1 cells[143]. Also, it directly influences liver cell survival by preventing apoptosis through the activation of the NF-κB pathway. ROS induce modifications in structure and function of cancer-related proteins and genes including those fundamental for cell-cycle control, apoptosis, and DNA repair[144]. There is evidence that HCV-mediated inflammation is responsible for the promotion of hepatic carcinogenesis through oxidative DNA damage[145].

In patients affected by HCC, Th1 dominance is progressively lost due to an increase of Th2 cells[146]. Some studies typify the microenvironment of HCC metastatic phenotype as composed by a Th2-like cytokine profile, consisting in higher levels of IL-4, IL-8, IL-10 and IL-5, and lower levels of Th1-like cytokines as IL-1α, IL-1β, IL-2, IL-12p35, IL-12p40, IL-15, TNF-α and IFN-γ[147]. Moreover, the peripheral blood and the tumor tissue of HCC patients show an increased number of Tregs (with a positive direct correlation with HCV RNA levels). IL-10 seems to be highly expressed in HCC patients and its presence correlates with disease progression[148].

In conclusion, inadequate immune response is a necessary factor for hepatic cells transformation but not a sufficient one. It builds a favorable microenvironment in which all factors (etiology, genetic, epigenetic, humoral and cellular inflammation, chronic liver injury) contribute to the development of FIB and liver cells proliferation. These oncogenic factors promote DNA abnormalities that ultimately transform the hepatocytes into malignant cells.

### *Role of the gut microbiota*

HCV-related cirrhosis is characterized by a disruption of the gut microbiota composition leading to an increased intestinal permeability and bacterial translocation. Compared to healthy individuals, bacterial alpha diversity seems to be lower in patients affected by chronic HCV infection with a reduction in bacteria of the order *Clostridiales* and an expansion in *Streptococcus species*. Gut dysbiosis begins to occur in the very early stages of the infection with the transient increase in *Bacteroidaceae* and *Enterobacteriaceae*. A decrease in the abundance of *Bifidobacteriaceae* and *Lactobacillaceae* with an increase of *Bacteroidaceae* and *Enterobacteriaceae* has been reported in the gut microbiota of patients with HCV-related HCC[149, 150]. Predicted metagenomics of microbial communities show an upregulation of urease gene, mainly encoded by *Streptococcus viridans* during chronic hepatic disease course, consistent with a significantly higher faecal pH than in healthy individuals[151]. A small study shows an increase in *Streptococcus salivarius* (*S. salivarius*) in oral and gut microbiota of patients with HCV-related HCC suggesting a possible role in oncogenesis. It has been noticed that *S. salivarius* impairs and deregulates the innate immune response of epithelial cells with a possible contribution in HCC progression[152].

DAAs treatment has proven to modulate the composition of the gut microbiota contributing to a decrease in inflammatory markers and liver stiffness but without effect on the gut barrier and its permeability. The eradication of the infection cannot resolve the intestinal dysfunction caused by liver cirrhosis but can improve the pre-existing dysbiosis[153]. Therefore, alterations of the gut microbiota may be focused as a predisposing factor for liver disease progression but further studies are necessary to establish its possible pathogenetic role in HCC development.

### *Role of host genetic factors*

The genetic background of the host has a growing role in hepatic carcinogenesis. A systematic review found 16 genes associated with HCV-related HCC[154]. The polymorphisms identified in patients with HCV-related HCC involve genes encoding for: TGF-β1, TNF-α, mitochondrial aldehyde dehydrogenase enzyme 2, catalase, EGF, glutathione S-transferase mu 1 protein, glutathione S-transferase theta 1, HLA-Bw4, HLA-B1814 and HLA-DR11, HSPA1B, leptin receptor, IL-1b, a regulator of the p53 tumor suppressor named mouse double minute 2 homolog, UDP glucuronosyltransferase 1 family-polypeptide A7, manganese-dependent superoxide dismutase mitochondrial protein, IFN lambda 3 and lambda 4 cytokine, patatin-like phospholipase domain–containing protein 3 (PNPLA3) and MHC class I

polypeptide-related sequence A (MICA/HCP5)[154-156]. Nowadays, the genetic risk score, designed using the combination of four high risk variants in the PNPLA3, TM6SF2, MBOAT7, and Glucokinase Regulator GCKR genes, estimates the inherited predisposition to accumulate liver fat (Genetic Risk Score) and is reported as an independent risk factor for *de novo* occurrence of HCC after DAAs treatment [157].

### HCC surveillance in patients with chronic hepatitis C

As already discussed, HCC surveillance in HCV-infected patients must include patients affected by cirrhosis of all causes and patients with chronic HCV infection with advanced liver FIB (Table 7). The AASLD and EASL practice guidelines recommend HCC screening and surveillance in patients with cirrhosis or with stage 3 FIB[73,75,158]. JSH still differentiates ''super-high-risk patients'' (hepatitis C cirrhosis) in whom surveillance is recommended every 3-4 mo and ''high-risk patients'', in whom surveillance is recommended every 6 mo[124].

### The impact of chronic hepatitis C treatment on HCC occurrence

The utmost importance of viral clearance in the reduction of HCC occurrence among chronically HCV-infected patients has been largely established. Since the first studies on IFN-based therapy, HCC incidence shows a significant difference between patients with sustained virological response (SVR) and non-SVR patients (5.1% and 21.8%)[159]. A large meta-analysis including 31528 HCV-infected patients shows that SVR is a key factor in reducing the incidence of HCC (1.5% in SVR patients *vs* 6.2% in non-SVR patients at 3-8 years of follow up)[139,160].

### PEG-IFNα

IFN-α was approved to treat HCV infection in the 1980s but its efficacy was limited (SVR rates 54%-56%). A meta-analysis of 8 long-term studies on 2649 patients with chronic hepatitis C demonstrates that HCC occurrence is significantly reduced in patients with SVR after IFN-based therapy compared to non-responder (4.2% *vs* 17.8%, HR = 0.23)[161]. The majority of the studies report that older ages, male gender, advanced liver FIB, fatty liver disease and a high post-treatment serum AFP level are the main risk factors for HCC development even after SVR[162].

### DAAs

The perspective of HCV therapy has changed over the years after the introduction of DAAs therapy, which has increased the SVR rate up to 90%, with excellent tolerance compared to IFN-based regimens. Even if the therapies demonstrated high efficacy, two retrospective studies, published in 2016 and conducted respectively in Spain and Italy, supposed the existence of a higher risk of HCC occurrence and recurrence following DAAs treatment course, raising the debate about the safety profile of DAAs and, in particular, the relation between SVR with and HCC occurrence and recurrence[163]. The Spanish study registered a high recurrence rate of HCC (27.6%) among 58 patients with previous history of HCC who received DAAs[164]. In the Italian study, HCC was detected in 7.6% of patients following DAAs treatment, 28.81% of patients with and 3.16% of patients without previous HCC[165]. Furthermore, an unpredictable increase in the amount of advanced stage HCC was noticed by the same authors.

Later debates arose owing some possible biases in these studies (the small size of the cohorts, lack of untreated controls and short follow-up periods). Subsequently, further data from large real-world retrospective cohort studies with extended follow-up periods after DAAs treatment have been released. On the heels of the studies listed so far, in 2017 another meta-analysis conducted by Waziry *et al*[166] reported lack of a statistically significant increase in HCC recurrence or occurrences in either patients having undergone DAAs or IFN treatment after achieving SVR. Subsequently in 2017, a systematic review from Ioannou *et al*[167] including 62354 patients treated with DAAs, IFN or a combination of both showed no significant difference in HCC occurrence or recurrence between the three treatment regimens. In fact, SVR was associated with a 71% decrease in HCC occurrence risk with no particular differences between DAAs and IFNs. Nahon *et al*[168] enrolled 1270 HCV-infected patients with compensated cirrhosis in France, demonstrating that there wasn't any statistically significant increase in the risk of HCC occurrence following DAAs treatment (HR = 0.89), after adjustment for age, diabetes and reduced liver function. In 2018, another American study from Singer *et al*[169] showed that DAAs therapy was associated with a lower risk of HCC as compared to untreated patients (HR = 0.84) and to IFN based treatment (HR = 0.69). During the same year, an Italian study found that SVR after DAAs treatment led to a lower incidence rate of HCC in patients with HCV-related cirrhosis over a follow up of 14 mo[170]. At the end of 2018, another Italian review from Guarino *et al*[171] outlined that the alarmist data on HCC recurrence after DAAs treatment and more aggressive pattern of disease have not been confirmed by subsequent studies.

Recently, a French prospective cohort study on 9895 HCV-infected patients with a long term follow-up (mean 33.4 mo) reported that after adjusting for several variables (including non-modifiable risk factor as age, sex, geographical origin, HCV genotype and modifiable ones as FIB score, alcohol consumption, diabetes, arterial hypertension, and model for end-stage liver disease score in patients with cirrhosis), DAAs exposure decreased all-cause mortality (adjusted HR = 0.48) and the risk of HCC

**Table 7 Hepatocellular carcinoma surveillance in hepatitis C virus infected patients**

| HCC surveillance in HCV infected patients | | |
|---|---|---|
| **Western medical societies** | | |
| EASL, 2018 | High-risk patients: HCV-related cirrhosis. Chronic hepatitis C and stage | Screening with US examination with or without AFP every 6 mo for high-risk patients (incidence > 1.5%/yr) |
| AASLD, 2018 | High-risk patients: HCV-related cirrhosis. Chronic hepatitis C and stage 3 fibrosis | Screening with US examination with or without AFP every 6 mo for high-risk group (incidence > 1.5%/yr) |
| **Eastern medical societies** | | |
| JSH, 2017-2021 | Extremely-high-risk patients: All patients with HCV-related cirrhosis. High-risk patients: Patients with chronic hepatitis C | Screening with US and tumor marker measurements (AFP, PIVKA-II and AFP-L3) every 3-4 mo in the super-high-risk population. A 6-12 mo dynamic CT scan, dynamic MRI should be performed or Sonazoid CEUS. Screening every 6 mo in high-risk populations |
| APASL, 2017 | High-risk patients: All patients with HCV-related cirrhosis. SVR patients with chronic hepatitis C with advanced liver fibrosis, independently of the histologic response to therapy. SVR patients with chronic hepatitis C with any histologic stage of HCV with comorbidities, such as alcohol abuse and DM | Surveillance by US and AFP should be performed every 6 mo and preferably every 3-4 mo in cirrhotic patients and those at high risk of HCC |
| KLCSG, 2014-2018 | High-risk patients: All patients with HCV-related cirrhosis. Patients with chronic hepatitis C and advanced fibrosis | Screening with US examination with or without AFP every 6 mo. If liver US cannot be performed properly, liver dynamic CT or dynamic contrast-enhanced MRI can be performed as an alternative |

HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; US: Ultrasound; AFP: Alpha-fetoprotein; SVR: Sustained virological response; PIVKA-II: Protein induced vitamin K absence or antagonist-II; CEUS: Contrast enhanced ultrasound; CT: Computed tomography; MRI: Magnetic resonance imaging; EASL: European Association for the Study of the Liver; AASLD: American Association for the Study of Liver Diseases; JSH: Japan Society of Hepatology; APASL: The Asian Pacific Association for the Study of the Liver; KLCSG: Korean Liver Cancer Study Group.

development (adjusted HR = 0.66)[172]. Another large American retrospective cohort study concluded that DAAs treatment and SVR resulted in a significant benefit in overall mortality[173].

Watanabe *et al*[174] recognized FIB-4 index ≥ 4.0 and albumin ≤ 3.8 g/dL before DAAs therapy and a FIB-4 index ≥ 4.0 and AFP ≥ 6.0 after the end of DAAs therapy as independent predictors for HCC occurrence. Another study confirmed a linkage between the risk of HCC and persistently high FIB-4/[aminotransferase/platelet ratio index (APRI)], either with or without cirrhosis. HCC development risk shows a significative reduction in cirrhotic patients who showed a decrease of FIB-4/APRI scores after DAAs therapy[175].

In 2020, an Egyptian study designed with three large independent cohorts, enrolled 4400 patients with chronic hepatitis C and advanced FIB, and identified predictors of HCC development after DAAs treatment developing and validating a simple risk score (GES risk score). GES identifies three groups of patients with a different risk of HCC occurrence along the follow-up by means of simple and readily available predictors: Age, male gender, low albumin, high AFP levels and presence of cirrhosis at baseline. The cumulative incidence of HCC for GES low-risk group at 2 years was 1.2% in the derivation cohort and 0.22% in validation cohorts, while GES high-risk group showed a 6 to 30 folds higher risk, with a cumulative incidence of 7.1% and 6.1% in the derivation and validation cohorts, respectively. The study enrolled mostly genotype 4-infected patients thus further studies are needed to assess the utility of this score in clinical practice[176].

These data underline that the rise in HCC incidence suggested by the first studies in patients with HCV-related cirrhosis treated with DAAs who achieve SVR compared to those treated with IFN-based therapy may be due to confounders as patients features (age, diabetes, reduced liver function) and/or lower screening frequency. Therefore, DAAs treatment should not be withheld for the potential risk of HCC occurrence or recurrence. According to long-term post-SVR observational studies, HCC development risk remains high in patients with cirrhosis who eliminate HCV, although it is significantly reduced compared to untreated patients or patients not achieving SVR (10-year cumulative incidence rate 21.8% *vs* 5.1%)[139].

## CLINICAL PRESENTATION OF HBV AND HCV-RELATED HCC: A COMPARISON

Several studies assessed the difference in clinical manifestation in HCC patients between different etiologies. Different HCC surveillance strategies need to be set according to the viral etiologies in relation to HCC age of presentation and clinical features. Two Italian studies noticed that HBV-infected patients are younger at HCC diagnosis, with a higher staging, increased probability of vascular invasion

and a worse prognosis[177]. An Asian study showed a peak incidence of HBV-related HCC in the 50-59 years old group whereas the peak annual incidence in HCV patients is over 70 years. In an HBV high endemic area, the outcome analysis of the study showed a reduced survival in HBV-related HCC compared to HCV-related HCC (1.34 years *vs* 2.17 years, adjusted for confounders). There was also a slight increase in frequency of large tumors (> 5 cm) and portal vein invasion at diagnosis in HBV-related HCC group, while multiple tumors occurred more frequently in HCV patients[178]. A cross sectional observational study from Pakistan, an HCV high endemic country, confirmed that HBV-related HCC patients were younger at presentation, but HCV-related HCC was larger, with higher AFP serum levels and more frequent vascular invasion at diagnosis[179]. Similar results were obtained in a large cohort including Asian and European patients[180-183]. These differences can be explained by the high prevalence of perinatal infections in high endemic areas and to the specific oncogenic potential of HBV. However, there was no difference in post-operative or post-treatment outcomes in the studies listed above between HBV and HCV-related HCC groups.

## CONCLUSION

Chronic HBV- and HCV-related hepatitis are still pandemic, with an increased spread in low and middle-income continents, such as Asia and Africa. HBV- and HCV-infected patients have a higher oncogenic risk compared to other liver disease aetiology, mostly because of the interactions between viral proteome and transcriptome and host cells molecular pathways involving inflammation and cells replication. New data about gut-liver axis and systemic inflammation are pointing out their key role as a cofactor in hepatocarcinogenesis. Therefore, viral hepatitis B and C still represent a major public health problem worldwide although health organizations have made substantial efforts to contain their spread in recent years through the global HBV vaccination campaign and the widespread accessibility of DAAs for HCV. The broad availability of generic antivirals would allow low- and middle-income countries to strengthen their contribution to the HCV eradication program. In the meantime, one of the most important challenges is to adapt the timing of HCC surveillance in these patients with the aim of ensuring early diagnosis and rapid management to improve prognosis. In the era of coronavirus disease 2019, well-organized and accessible HCC surveillance becomes even more important due to the limited healthcare resources allocated to secondary prevention and the reluctance of patients to access healthcare facilities. In the future, new biomarkers and risk stratification systems may help clinicians to identify patients for closer follow-up leading to a more personalized approach to HCC surveillance.

## FOOTNOTES

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MINIREVIEWS

# Updates in therapeutic drug monitoring in inflammatory bowel disease

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## Abstract

Biologics and immunomodulators (IMM) are generally considered the most effective therapies for the treatment of ulcerative colitis and Crohn's disease. However, despite the efficacy of these therapies, many patients either have a primary lack of response or a secondary loss of response to these medications. Therapeutic drug monitoring (TDM) is a systematic approach to managing such patients. In this review, we summarize the latest data on TDM, including reactive and proactive TDM, in patients with inflammatory bowel disease on biologics and/or IMM.

**Key Words:** Inflammatory bowel disease; Therapeutic drug monitoring; Crohn's disease; Ulcerative colitis



**Core Tip:** Inflammatory bowel disease (IBD), such as ulcerative colitis and Crohn's disease, are best treated with immunomodulators (IMM) or biologics. The rate of response clinically and endoscopically varies between the medications and within patient populations. Therapeutic drug monitoring (TDM) is a useful technique to assess drug and metabolite levels as well as anti-drug levels in patients on biologics or IMM in order to improve clinical outcome and prevent a multitude of complications. Here we discuss the role of TDM in patients with IBD with a focus on reactive *vs* proactive TDM.

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## INTRODUCTION

Since the approval of the first biologic for inflammatory bowel disease (IBD) in the 1990s, treatment for IBD has evolved tremendously. In addition to tumor necrosis factor (TNF) inhibitors, thiopurines, natalizumab, vedolizumab, ustekinumab, and tofacitinib have all been approved for the treatment of IBD. Previously, patients were treated based on symptoms, but we have now discovered that utilizing more objective parameters such as clinical and endoscopic remission reduces complications and leads to better outcomes[1].

Despite having effective treatments for ulcerative colitis (UC) and Crohn's disease (CD), one-third of patients (primary non-responders) will not respond to induction therapy after a biologic. Risk factors for primary non-response include long duration of disease, smoking, extensive small bowel disease, a normal C-reactive protein (CRP) at the start of therapy, and previous exposure to a biologic agent[2].

Secondary loss of response occurs when a patient initially had response to therapy but lost that benefit over time. This can occur in up to 50% of patients and can lead to the need for either dose intensification, or the use of an alternate agent. The formation of anti-drug antibodies (ADA) and inadequate drug exposure are the main factors contributing to secondary loss of response in patients on biologic therapies[1].

Therapeutic drug monitoring (TDM) is a way to optimize the dose of biologics and immunomodulators (IMM) to optimize treatment outcomes. The levels or metabolites, as well as the development of antibodies, are used to help guide drug dosing in order to enhance drug efficacy and reduce disease complications[3]. Current AGA guidelines published in 2017 recommend reactive TDM for patients with active IBD. Reactive TDM occurs when dosing of a therapy is changed following either primary non-response or secondary loss of response. Proactive TDM involves routine monitoring of drug levels and antibodies at set intervals with dose adjustments based on drug levels. Many studies have shown that there is a correlation between positive clinical outcomes and therapeutic ranges of serum drug concentrations for each agent available to treat IBD[4]. This review aims to discuss TDM for biologics and thiopurines in treatment of active IBD.

## TNF INHIBITORS

TNF inhibitors available for treating active IBD include infliximab, adalimumab, certolizumab, and golimumab. Studies have confirmed that there is a correlation between clinical response and drug concentrations of anti-TNF agents measured *via* serologic work-up.

Infliximab is a chimeric monoclonal anti-TNF agent approved for patients with active UC or CD. Studies have shown that higher infliximab concentrations lead to improved outcomes in patients with IBD. TAXIT, a prospective trial on patients with CD on infliximab, demonstrated a significant improvement in remission and lower rates of ADA with dose escalation[5]. The TAILORIX trial was a second prospective trial for patients with CD on infliximab that tried to assess whether increasing the dose of infliximab based upon a combination of symptoms, biomarkers, and serum drug concentrations leads to improved outcomes compared to dose intensification based purely upon symptoms. This trial did not reach its primary endpoint of sustained corticosteroid-free clinical remission from weeks 22 through 54[6]. However, a post-hoc analysis of the TAILORIX trial demonstrated that infliximab drug concentrations were higher in patients that achieved endoscopic remission by week 12 compared to patients who did not achieve remission, which supports TDM is beneficial for patients on infliximab[7]. Furthermore, the TAILORIX utilized an infliximab drug concentration of 3 μg/mL as a target, which is widely considered low based upon the results of several recent studies[8-11]. The low target infliximab level could have limited the efficacy analysis of TDM in the trial. Patients with UC on infliximab maintenance therapy were examined in a retrospective study that utilized TDM and endoscopic evaluation. This study was able to demonstrate that patients with endoscopic and histologic remission had significantly higher serum drug levels[12]. A cost-analysis performed on TDM for infliximab suggested that proactive TDM led to fewer flares than the reactive method, and that more patients remained on therapy with proactive TDM. Fewer flares paired with a reduction in the cost of infliximab over time suggests that proactive TDM may be more suitable for patients with IBD on biologic agents [13].

Adalimumab is a human monoclonal immunoglobulin G (IgG0 anti-TNF agent used for the treatment of active CD and UC. Numerous studies have demonstrated improved outcomes in patients with higher drug concentrations of adalimumab. Park *et al*[14] observed that higher serum drug levels of adalimumab were associated with more quiescent disease and normal CRP. The patients in this study also had higher rates of endoscopic and radiologic remission with higher serum concentrations of adalimumab. The POETIC study was a prospective study on patients with CD on adalimumab designed to evaluate the evolution of ADA over time, and its correlation with clinical outcomes. Many patients developed ADA as early as week 2, and the early development of antibodies correlated with primary non-response[15].

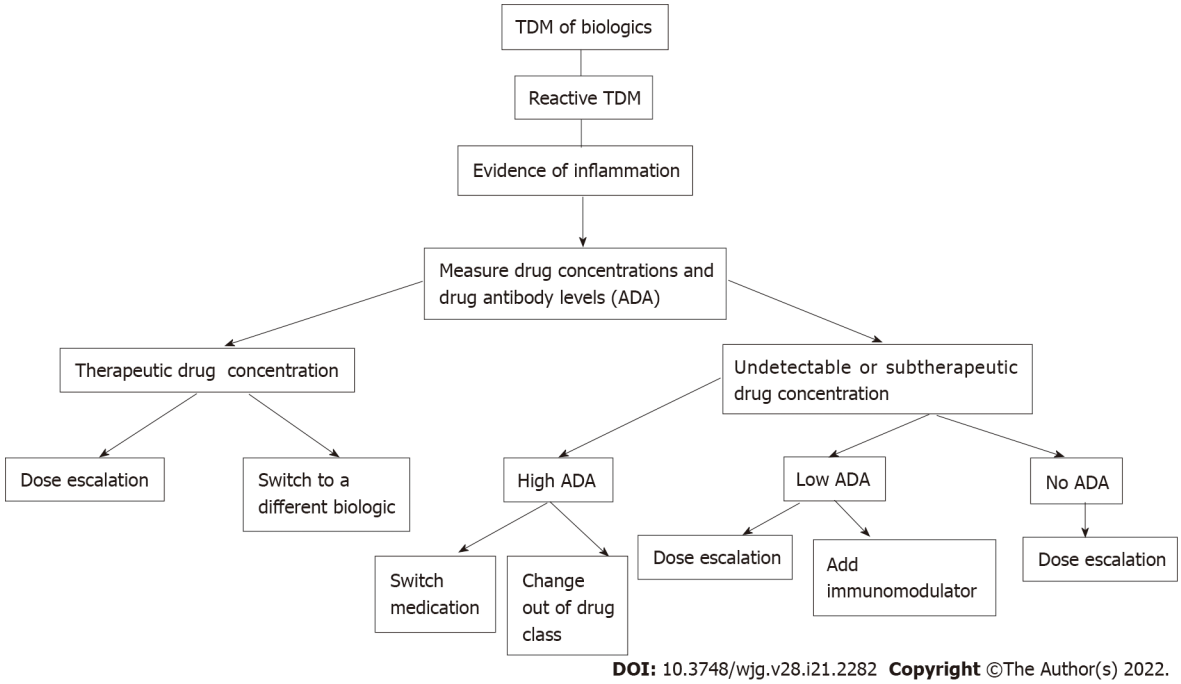


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**Figure 1 In-depth algorithm for reactive therapeutic drug monitoring.** TDM: Therapeutic drug monitoring; ADA: Anti-drug antibodies.

Golimumab is a human IgG1 kappa monoclonal anti-TNF therapy for patients with UC, which was approved based on the results from the PURSUIT trial. In the PURSUIT trial, serum golimumab concentrations and ADA were measured during induction as well as through maintenance therapy. An exposure-response relationship was noted; patients receiving the lowest dose of the drug had a higher incidence of ADA, as well as a higher fecal calprotectin and serum CRP[16]. Another study was able to demonstrate a positive correlation between golimumab concentrations and clinical and endoscopic outcomes[17].

Certolizumab pegol is a PEGylated Fab' fragment of a humanized monoclonal antibody that binds to TNF. It is unique in that it lacks the Fc component that other TNF inhibitors have, making it incapable of fixing complement or binding Fc receptors[18]. Current studies show that higher certolizumab plasma concentrations lead to increased remission as well as decreased levels of CRP[19]. Patients with higher concentrations of certolizumab as early as week 2 had clinical remission by week 6 of induction, as well as continued positive outcomes during maintenance therapy[20].

## VEDOLIZUMAB

Vedolizumab is a monoclonal antibody that binds $\alpha_4\beta_7$ integrin for the treatment of moderate-to-severe UC and CD. A post hoc analyses of the GEMINI data confirmed the relationship between higher vedolizumab exposure and clinical remission in patents with IBD. Both patients with UC and CD showed higher rates of remission at week 6 with higher drug concentrations, which correlated with clinical response and mucosal healing, thus confirming an exposure-efficacy relationship[21-23]. The LOVE-CD trial was a prospective trial in patients with active CD receiving vedolizumab that showed higher serum levels of vedolizumab correlated with higher rates of endoscopic and histologic remission at weeks 26 and 52[24]. One single-center, cross-sectional, retrospective study showed higher serum levels of vedolizumab correlated with lower CRP levels. However, this study failed to demonstrate a correlation between vedolizumab concentrations and mucosal healing[25]. Notably, the rate of ADA to vedolizumab seems to be relatively low[25,26].

## USTEKINUMAB

Ustekinumab is a human monoclonal antibody that binds to the p40 subunit of interleukin (IL)-12 and IL-23, thus preventing the interaction with the cell surface IL-12RB1 receptor. This prevents IL-12 and IL-23 mediated cell signaling[27]. The efficacy of ustekinumab for the treatment of moderate to severe CD was demonstrated in the UNITI-1 and UNITI-2 studies[28]. An analysis of data from phase 3 studies of patients with active CD on ustekinumab demonstrated a dose response. Drug serum concentrations

were positively associated with clinical remission and endoscopic improvement at week 44; there was also an inverse association with the CRP level. IMM were not found to have a significant effect on the serum concentration of ustekinumab[29,30]. The available data also suggest an extremely low rate of antibody formation in ustekinumab[28,29].

## THIOPURINES

IMM, including azathioprine (AZA) and 6-mercaptopurine (6-MP) have been used for the treatment of IBD for many years. AZA is converted to 6-MP using a non-enzymatic pathway. 6-MP is broken down in three different ways: Into 6-thiouric acid by xanthine oxidase, activated to 6-methyl-mercaptopurine (6-MMP) by thiopurine methyltransferase (TPMT), or to 6-thioguanine dehydrogenase (6-TGN) by three different enzymes. TPMT has variants that can lead to a reduction in activity, therefore patients should have their TPMT phenotype checked prior to use. Once a thiopurine has been added to a regimen, thiopurine metabolites should be assessed, as patients have better outcomes with higher 6-TGN levels and lower 6-MMP levels[31]. Thiopurines play a role in reducing the risk of antibody formation, particularly to TNF inhibitors A study on patients with multiple different autoimmune diseases showed a reduction in ADA with the use of supplemental immunosuppressants[32].

The SONIC study is a landmark prospective trial that showed a combination therapy of AZA and infliximab for CD was more effective than infliximab monotherapy in induction and maintenance of steroid-free clinical remission at 26 and 52 wk. Combination therapy also led to lower rates of antibodies to infliximab and higher serum drug concentrations[33].

## THE TRANSIENT NATURE OF SOME ADA

Although the formation of ADA can correlate to poor clinical outcomes, ADA levels may sometimes be transient[34,35]. Low levels of ADA may be overcome with higher serum drug concentrations and the addition of immunomodulators. However, if patients have sustained elevated levels of ADA, permanent loss of response is more likely to occur[36]. A small retrospective analysis of 5 patients investigated the addition of immunomodulators (thiopurines and methotrexate) to patients after the development of ADA to infliximab. All five patients had restoration of clinical response, and ADA levels gradually diminished over time[37]. It will be important for the future of IBD therapy to understand the role ADA formation plays in loss of response in patients on biologics, and the benefit of immunomodulators to recapturing response.

## REACTIVE TDM

Reactive TDM is the current standard of care when treating IBD patients who have a loss of response to biologic therapy[4]. This approach can identify the subset of patients that would benefit from dose escalation of their current agent *vs* transitioning to a different therapy. Once a patient has a flare of their symptoms, drug concentrations and ADA levels are measured, and further management is based upon these results (Figure 1). A retrospective study of patients with suspected loss of response was performed that determined that the measurement of trough levels of anti-TNF agents or ADAs during a suspected loss of response led to improved interventions. Patients with high ADA levels benefited more from switching agents than from dose escalation, whereas patients with no or low ADA levels did benefit from dose intensification. This study also demonstrated that patients with adequate levels of infliximab or adalimumab with inadequate response would benefit from an agent that is out of the anti-TNF class [38]. A retrospective analysis of patients receiving infliximab who underwent dose escalation was examined, and clinical decisions with or without the use of TDM were compared. Patients for whom decisions were based upon TDM had improved endoscopic outcomes, higher rates of clinical remission, fewer hospitalizations, and less steroid use[39]. Reactive TDM has the benefit of cost savings, as less drug can be utilized, as well as the ability to try to optimize drug levels and increase the chance of recapturing response in order to prevent treatment failure[7].

## PROACTIVE TDM

Proactive TDM differs from reactive TDM in that it aims to optimize drug concentrations by measuring serum drug concentrations and ADA levels at set intervals in order to prevent loss of response (Figure 2). Since the best therapeutic effect is obtained with the first biologic agent received, proponents of this approach consider it imperative to optimize the dose of the agent early in the treatment course

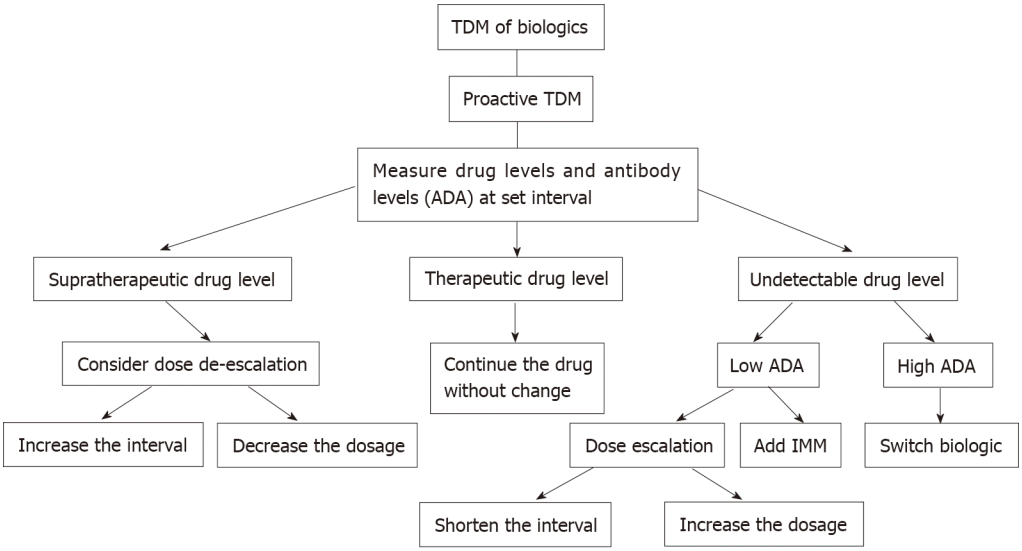


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**Figure 2 In-depth algorithm for proactive therapeutic drug monitoring.** TDM: Therapeutic drug monitoring; IMM: Immunomodulators; ADA: Anti-drug antibodies.

[40].

Fernandes *et al*[41] demonstrated in a prospective study on patients on infliximab therapy with CD and UC that proactive TDM had better outcomes than management without the use of TDM. Patients on infliximab underwent trough and ADA level measurements before the fourth infusion and at every 2 infusions, and dose-adjustment was made in order to keep a goal trough level between 3 and 7 μg/mL for CD and 5 and 10 μg/mL for UC. Compared to a retrospective cohort treated with infliximab without the use of TDM, the TDM group showed improved mucosal healing, fewer surgeries and hospitalizations, and less treatment discontinuation. A randomized, controlled trial of children with CD, the PAILOT trial, investigated proactive TDM *vs* reactive TDM. The primary endpoint was corticosteroid-free remission on adalimumab therapy. The results showed significantly higher rates of steroid-free remission in patients receiving proactive TDM than reactive TDM, as 31 children (82%) in the proactive group reached the primary endpoint, meanwhile 19 children (48%) reached the endpoint using reactive TDM[42]. Papamichael *et al*[43] performed a multicenter, retrospective study on patients receiving infliximab therapy for IBD. Proactive TDM had better clinical outcomes when compared to reactive TDM, including fewer surgeries, hospitalizations, lower ADA levels, longer time to treatment failure[44-46]. Much of the current data for TDM seems to demonstrate that proactive TDM leads to better clinical outcomes compared to reactive TDM. However, a universal analysis on cost-effectiveness is much more difficult given varying degrees of coverage on biologics and TDM assays[47,48].

## IMMUNOASSAY METHODS FOR THE DETECTION OF ANTIBODIES

Although it is well known that patients with inflammatory bowel disease are at risk of developing antibodies to biologics, more attention should be paid toward the optimal methodology used to detect these antibodies. The various immunoassay methods for detection of drug antibodies are suspected to yield varying results when assessing immunogenicity of biologics due to the presence of drug and the potential underestimation of ADA[49]. Drug interference limits the detection of ADA due to the formation of ADA-drug complexes in the assay. Drug tolerant assays were developed that can detect free ADA and ADA bound in a complex. This assay can dissociate the ADA from the drug to estimate the quantity of ADA more accurately in a sample. Drug-sensitive antibody detection methods such as the antibody binding test (ABT) and bridging enzyme-linked immunosorbent assay (ELISA) preceded the drug-tolerant assays[50].

A study by Ruwaard *et al*[49] compared the efficacy of three different immunoassays to detect ADA, including ABT, ELISA, and drug-tolerant assays in 86 patients on adalimumab. There was a significant difference in the ability to detect ADA between the assays, with drug-tolerant assays detecting ADA in 69% of patients, compared to 30% in the ABT, and 2% using the ELISA. This suggests that drug-tolerant assays should be the standard when detecting ADA in patients on adalimumab. A study by Wang *et al* [50] compared ELISA to a drug tolerant assay, the homogenous mobility shift assay (HMSA), in patients treated with infliximab. This study illustrated that the HMSA was significantly more sensitive in detecting ADA, especially in the presence of high serum drug concentrations. HMSA can overcome artifacts encountered using drug-sensitive assays, as it can dissociate the ADA from the drug. These

studies suggest that future studies should consider using drug-tolerant assays as their method of detecting ADA to standardize the methodology and prevent inconsistent results between different studies.

It is still well-known that ADA formation leads to lower drug concentrations and worse outcomes in patients with IBD on biologics. The data suggest that drug-tolerant assays are ideal for detection of ADA in patients on adalimumab and infliximab. Standardization in detection of ADA would improve the variability amongst studies, thus improving clinicians' ability to use and perform TDM. Unfortunately, the available data focus on TNF inhibitors, and the applicability to non-TNF inhibitor biologics is limited. Further studies with inclusion of all biologics could help lead to implementation of international standards and improve our understanding on the impact of ADA on clinical outcomes[51].

## CONCLUSION

TDM plays an important role in treatment outcomes for patients with IBD on biologic agents. TDM is useful for predicting loss of response and preventing treatment failure. Higher serum drug concentrations lead to improved outcomes, with fewer hospitalizations, surgeries, and treatment failures. Lower serum drug concentrations and the development of ADA lead to worse outcomes and loss of response. The addition of immunomodulators has not been standardized, but studies have shown that the addition of an immunomodulator to TNF inhibitors can lead to a reduction in the development of ADA as well as higher serum drug concentrations, thus eliminating the potential for failure of an agent.

Despite a multitude of studies, there are still limitations regarding the use of TDM in IBD patients. There is likely a fair amount of inter-individual variation regarding the appropriate serum concentration of various biologics, and the optimal target levels have therefore not been fully elucidated. Similarly, further research needs to be done regarding the significance of different levels of ADA. Different serum concentration and ADA assays can also complicate our interpretation of these values. Finally, analyses regarding cost-effectiveness of reactive *vs* proactive TDM in the setting of a variety of different health care settings are difficult to conduct.

## FOOTNOTES

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*MINIREVIEWS*

# Serological biomarkers for management of primary sclerosing cholangitis

David Tornai, Peter Laszlo Ven, Peter Laszlo Lakatos, Maria Papp

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## Abstract

Clinical manifestations and progression of primary sclerosing cholangitis (PSC) are heterogeneous, and its pathogenesis is poorly understood. The importance of gut-liver interactions in the pathogenesis has been clinically confirmed and highlighted in different theories. Recent advances regarding biomarkers of biliary-gut crosstalk may help to identify clinically relevant PSC subgroups assisting everyday clinical work-up (*e.g.*, diagnosis, disease stratification, or surveillance) and the exploration of potential therapeutic targets. Alkaline phosphatase produced by the biliary epithelium is consistently associated with prognosis. However, its level shows natural fluctuation limiting its use in individual patients. Inflammatory, cell activation, and tissue remodeling markers have been reported to predict clinical outcome. Elevated immunoglobulin (Ig) G4 level is associated with a shorter transplantation-free survival. IgG type atypical perinuclear anti-neutrophil cytoplasmic antibodies (P-ANCAs) are non-specific markers of various autoimmune liver diseases and may reflect an abnormal B-cell response to gut microbial antigens. IgG type atypical P-ANCA identifies PSC

patients with particular clinical and genetic (for human leukocyte antigens) characteristics. The presence of IgA type anti-F-actin antibody (AAA) may predict a progressive disease course, and it is associated with enhanced mucosal immune response to various microbial antigens and enterocyte damage. IgA type anti-glycoprotein 2 (GP2) antibodies identify patients with a severe disease phenotype and poor survival due to enhanced fibrogenesis or development of cholangiocarcinoma. Elevated soluble vascular adhesion protein-1 (sVAP-1) level is associated with adverse disease outcomes in PSC. High sVAP-1 levels correlate with mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expression in the liver that contributes to gut activated T-cell homing to the hepatobiliary tract. In the present paper, we review the evidence on these possible serological markers that could potentially help address the unmet clinical needs in PSC.

**Key Words:** Primary sclerosing cholangitis; Hepatobiliary; Serological biomarker; Immunoglobulin; Inflammatory; Tissue remodeling



**Core Tip:** Recent advances in biomarker research may help clinicians identify relevant subgroups of primary sclerosing cholangitis (PSC) and assist everyday clinical work-up. However, a diagnostic biomarker is still an unmet need. On the other hand, several biomarkers have been reported to predict outcome in PSC; however, most of them have not been validated by subsequent studies. The IgA type anti-glycoprotein 2 antibody is the first one to be supported by a satisfactory number of clinical studies and could be incorporated into clinical practice. These discoveries also reveal different aspects of PSC providing with potential therapeutic targets.

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## INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic and progressive inflammatory disease of the bile ducts, leading to the formation of intermitting bile duct strictures and dilatations, with periductal fibrosis that in most cases progresses to cirrhosis and decompensated disease stage. Moreover, PSC is associated with the development of cholangiocarcinoma (CCA) and colorectal cancer[1]. The etiology of PSC is poorly understood. However, the gut-liver interaction seems to be a driving factor in its development. It has been hypothesized that the interaction of the gut microbiome and certain genetically determined factors [such as certain human leukocyte antigens (HLA)] leads to the development of disrupted immunological pathways, as a result of an autoimmune reaction to yet unknown antigen(s). Most abnormalities discovered by genetic studies support a disruption of T cell functions. Gut-derived antigens significantly induce antigen presentation by major histocompatibility complexes (MHCs) on the surface of antigen-presenting cells to T cells, which, following clonal expansion, can be delivered to both the intestinal tract and the liver[2]. Abnormal T cell activation results in increased release of various cytokines [such as transforming growth factor-β (TGF-β)] involved in the development of fibrosis. Activation of B cells is indicated by the frequent appearance of bacterial translocation (BT) related serological antibodies [*e.g.*, anti-neutrophil cytoplasmic antibodies (ANCAs)].

The diagnosis of PSC is difficult, and no biomarker specific to the disease has been identified so far. In the presence of chronic cholestasis, the diagnosis is based on magnetic resonance cholangiography (MRCP) or, much less frequently today, endoscopic retrograde cholangiopancreatography (ERCP) with confirmation of biliary lesions and strictures. Careful exclusion of known causes of secondary sclerosing cholangitis (SSC) is also necessary. Immunoglobulin (Ig) G elevation and the presence of atypical perinuclear ANCA (P-ANCA) in serum are common but are not disease-specific serological alterations [3]. Routine liver biopsy is no longer routinely performed in the diagnosis of PSC and is only necessary in suspected cases of small duct PSC, when the cholangiogram is normal, or to confirm overlap syndrome with autoimmune hepatitis.

The disease course of PSC is variable. There are a few promising biomarkers to predict disease activity and progression, yet almost none has been validated in subsequent studies. A recently discovered serological biomarker [IgA type antibodies against pancreatic glycoprotein 2 (anti-GP2 IgA)] [4,5] is the first that has predictive value in PSC confirmed in multiple studies[6,7]. However, it has not

been integrated into clinical practice. Consequently, the question of accurate risk assessment, disease stratification, and follow-up strategy for patients with PSC is still unresolved[8].

Studying biomarkers characterizing the dialogue between the biliary tract and the intestine in PSC may not only identify clinically relevant subgroups for disease stratification, but can also unravel new pathogenesis-relevant correlations. This could in turn lead to the discovery of new therapeutic targets.

While biomarkers from other body fluids (*e.g.*, bile) can reveal important aspects of the pathogenesis, blood is more readily available for clinicians and can be obtained in a highly controlled fashion (in contrast to, *e.g.*, stool). This makes serological biomarkers the most appropriate for everyday clinical use, therefore these are the markers that we focus on in this review.

## ROUTINE LABORATORY TESTS AND COMPOSITE SCORES

### *Alkaline phosphatase*

Various clinical studies have consistently reported a correlation between alkaline phosphatase (ALP) levels and disease progression, but its individual application is difficult. In PSC, the fluctuation pattern of ALP is different from that observed in primary biliary cholangitis, and the degree of elevation is influenced by the development of various biliary complications (cholangitis, gallstones, or the appearance of a dominant stricture). Thus, it is not surprising that the ALP thresholds or values of change associated with disease outcomes in certain studies were not confirmed by other studies[9]. However, certain studies highlighted the importance of ALP fluctuation in CCA development free survival. The risk of developing CCA was significantly higher in patients with constantly high ALP levels without reduction[10-13]. Thus, further studies are needed to determine the significance of ALP level variation also in transplant free survival (due to progression of fibrosis) and treatment response.

### *Mayo risk score*

In the absence of individual predictive markers, researchers tried to develop predictive models incorporating multiple markers. These scoring systems, however, also contain clinical parameters besides serological markers. The first and still the most widely used clinical prognostic model is the Mayo risk score (MRS), which, as modified in 2000, no longer includes invasive or subjective parameters [14]. Abnormal value of the factors included in the MRS [serum bilirubin, albumin, and aspartate aminotransferase (AST) levels, and esophageal variceal bleeding], with the exception of age, represents advanced liver disease. Thus, the discriminatory potential of this scoring system is inadequate in the early stages of PSC. In addition, the predictable time period for disease progression is only around 4 years. The model was also not able to predict the adverse disease outcome associated to high-dose ursodeoxycholic acid treatment (28-30 mg/kg/d) either[15].

### *Amsterdam-Oxford model*

The Amsterdam-Oxford model (AOM) is another prognostic scoring system for PSC[16], which includes seven objective parameters (PSC subtype, age at diagnosis, platelet count, serum albumin, ALP, AST, and bilirubin levels). This scoring system was developed to overcome the limitations of MRS in the prediction of transplant-free survival. It can be used to estimate a longer-term prognosis of PSC (15 years) and can be recalculated at a later timepoint. However, the discriminative power of the model was only moderate (C-statistic: 0.68). In contrast, it has the advantage of being much more widely applicable (the reliability of the model was consistent in the two populations and at different timepoints) as it was developed using population-based data rather than data from transplant centers.

### *UK-PSC scores*

The UK-PSC scores were also developed to predict transplant-free survival[17]. Two models were created for 2-year and for 10-year prediction, derived from a cohort of 1001 PSC patients and validated in two independent cohorts adding up to 451 patients. The incorporated variables were serum bilirubin, ALP, albumin, platelets, presence of extrahepatic biliary disease, and variceal hemorrhage. "Both short- and long-term UK-PSC risk scores had better performance than MRS and AST-to-platelet ratio index when predicting outcomes [C-statistics for 2-year survival (short-term) were 0.81 *vs* 0.75 and 0.81 *vs* 0.63, respectively; for 10-year survival (long-term) were 0.80 *vs* 0.79 and 0.80 *vs* 0.59, respectively]."

### *PSC risk estimate tool*

PSC risk estimate tool (PREsTo) was developed to predict the advent of hepatic decompensation (ascites, variceal hemorrhage, or encephalopathy). The model was derived from data of a multicenter North American cohort and validated in an international multicenter cohort including 509 and 278 subjects, respectively. Individuals with advanced PSC or CCA at baseline were excluded. PREsTo incorporates nine variables: Bilirubin, albumin, serum ALP times the upper limit of normal, platelets, AST, hemoglobin, sodium, patient age, and number of years since the diagnosis of PSC. In predicting hepatic decompensation, PREsTo performed better than model of end stage of liver disease (MELD)

score and MRS (C-statistics: 0.90 *vs* 0.72 and 0.90 *vs* 0.85, respectively)[18]. The equation is not provided, and only an online calculator is available.

### Enhanced liver fibrosis test

The enhanced liver fibrosis (ELF) test (Siemens ADVIA Centaur) is based on purely serological measurements summarizing three direct components of fibrogenesis (hyaluronic acid, tissue inhibitor of metalloproteinase 1, and type III procollagen amino-terminal propeptide). It has been reported to be a strong predictor of prognosis (mortality and liver transplantation) in PSC independently of MRS. Validation of this result was successful in multiple studies (C-statistic: 0.79-0.81)[19,20]. In a recent study, the ELF score slightly increased in PSC patients over a 5-year follow-up period and showed low intrapersonal variability supporting the consistency of this score[21].

## IMMUNOGLOBULINS

### IgG4

About 10% of patients have elevated serum IgG4 levels without IgG4-associated disease. Recently, IgG4 determination is recommended at least once in all patients with PSC[22]. Elevated serum IgG4 levels (> 140 mg/dL) in PSC patients were associated with higher values of liver function tests, higher MRS, and shorter time to liver transplantation, which clearly indicates a more severe disease course in this subgroup of patients[23]. It is unknown whether the addition of a systemic glucocorticoid to treatment in the high IgG4 subtype of PSC may be beneficial in curbing disease progression[24].

### ANCAs

Various other serum autoantibodies have been described in PSC patients; however, they are considered unspecific, since they may be present also in other diseases. The most frequently observed antibodies in PSC are ANCAs, but antinuclear antibodies and anti-smooth muscle antibodies were also reported to be frequently associated with PSC[25].

The occurrence of IgG isotype atypical P-ANCAs in PSC are common (up to 80% of cases). The putative role of intestinal bacteria in ANCA formation (homology between human beta-tubulin isotype 5 and bacterial FtsZ protein, molecular mimicry) has been described[26]. Early, small scale, case-control clinical studies[27] found no correlation between ANCA formation and clinical or genetic features of PSC. Hov *et al*[28], however, in a large comprehensive case-control study, demonstrated that risk genes with a strong association to PSC formation (HLA-B*08 and DRB1*03) were also associated with ANCA formation. Interestingly, association between the presence of PSC related HLA alleles (B*08, C*07, and DRB1*03) and increased risk of acute rejection syndrome in liver transplant recipients has also been reported (while DRB1*04 was found to be a protecting factor)[29].

The antibody against serine proteinase-3 (PR3) is a type of cytoplasmic ANCA (C-ANCAs). PR3-ANCA is typically observed in small vessel vasculitis[30] but recently has been reported in ulcerative colitis (UC)[31], which prompted researchers to investigate this antibody in PSC as well. Interestingly, in the setting of PSC, PR3-ANCA seems to be associated with worse liver biochemistry rather than with a co-diagnosis of UC[32]. In a recent study by Wunsch *et al*[7], besides worse liver function tests and MELD score, PR3-ANCA has been shown to be associated with CCA development [hazard ratio (HR) = 9.8, 95% confidence interval (CI) = 1.3-74.5; $P$ = 0.03] and risk of shorter transplant free survival (HR = 1.8; 95%CI = 1.3-2.8; $P$ = 0.01). However, the predictive capacity of PR3-ANCA was not confirmed in the validation cohort where the frequency of PR3-ANCA was found to be surprisingly low (5% in contrast to 54% in the discovery cohort) and CCA development was also less prevalent[7].

### IgA type autoantibodies

IgA is the most important immunoglobulin isotype involved in mucosal immunity[33]. Since the gut-liver interaction plays a central role in the pathogenesis of PSC, the formation of IgA type autoreactive antibodies is a characteristic feature of the disease. Under physiological conditions, monomeric IgA in serum inhibits immunological processes (so-called immunosuppressive effect). However, in pathological conditions, immune complexes formed by aggregation of monomeric IgA molecules can activate immunological processes by binding to myeloid cells[34]. IgA, produced by plasma cells lining the gut and biliary epithelium, is a highly abundant immunoglobulin in bile and plays a central role in the defense against intestinal pathogens. Biliary epithelial cells (that are the cellular targets of injury in PSC) and intestinal epithelial cells transport the IgA molecules into the biliary and intestinal lumens, respectively (→secretory IgA)[35-37]. However, in the past, most studies on serological antibodies in autoimmune liver diseases, like in the case of ANCA, have focused mainly on IgG isotype antibodies, and the IgA isotype is only recently gaining attention[38].

The work of Berglin *et al*[39] is an example, which highlighted the described importance of IgA isotype antibody related pathogenetic pathways in PSC. They demonstrated that in the presence of IgA isotype autoantibodies against biliary epithelial cells, disease progression was faster than in their

absence. No similar correlation was observed for IgG isotype antibodies.

In 2015, our group reported the association between PSC and the presence of anti- GP2 IgA antibodies in inflammatory bowel disease (IBD) patients[40]. Subsequently, anti-GP2 IgA positivity was demonstrated to be able to predict a progressive disease course in PSC by our and another group[4,5]. These results were confirmed later by further studies[6,7]. In total, 1111 PSC patients have been evaluated for the presence of anti-GP2 IgA and the prevalence was found between 30.8% and 52.2%[8]. Anti-GP2 IgA was consistently reported to be associated with a more severe PSC phenotype with a 2- to 5-fold higher risk to develop end stage liver disease with a need for liver transplantation (Figure 1). Consequently, anti-GP2 IgA may soon be integrated into clinical practice for risk assessment in PSC. Moreover, anti-GP2 IgA can also identify a subset of PSC patients with existing biliary tract cancer or with an increased risk of developing it during the disease course. This association between anti-GP2 IgA and CCA was reported by Jendrek *et al*[4] and confirmed by Wunsch *et al*[7] both evaluating two cohorts each, providing strong evidence that incorporation of anti-GP2 IgA into CCA surveillance protocols could be beneficial (Figure 1).

IgA autoantibody against F-actin has been also reported to be associated with enhanced mucosal immune response to various microbial antigens and enterocyte damage in PSC, and to identify PSC patients with progressive disease (HR = 4.54; 95%CI = 1.14-18.18; $P$ = 0.032)[41]. Cytoskeletal F-actin initiates an extracellular damage-associated molecular pattern signaling pathway through DNGR-1/CLEC9A and Syk-SFK that results in antigen cross-presentation to $CD8^+$ T cells[42-45]. $CD8^+$ T cells, after activation in the gut, can be recruited to the liver and induce immune-mediated cholangitis in mice [46].

## OTHER SEROLOGICAL BIOMARKERS

### *Interleukin-8*

As a result of increased lipopolysaccharide (LPS) exposure, biliary epithelial cells produce interleukin-8 (IL-8), which has a proliferation-promoting effect and enhances fibrogenesis related gene expression. In the study by Vesterhus *et al*[47], using modern antibody array technology, elevated serum IL-8 showed the strongest association with poor transplant-free survival among pro-inflammatory markers. However, the predictive value of MRS and ELF test for clinical outcomes was found to be higher than that of IL-8.

### *Macrophage activation markers*

Bossen *et al*[48] found that macrophage activation markers, soluble CD163 (sCD163) and mannose receptor (sMR), were increased according to disease severity in two independent PSC cohorts of 138 and 159 patients. In both cohorts, sCD163 and sMR levels rose in parallel with increasing liver enzymes, MRS, and ELF test. Patients with high baseline levels of sCD163 (> 3.86 mg/L) had decreased transplant-free survival during the 8-year follow-up in both cohorts (35.2% *vs* 83.0% in the combined cohort). sMR showed similar association only in one of the cohorts with more severe disease features. In Cox regression, sCD163 also performed better in the more severe cohort (HR = 3.15 and 2.89) but it lost significance in the multivariate model against ELF test and AOM score.

### *BT markers*

In a study investigating serum markers of BT, serum levels of zonulin, intestinal fatty acid binding protein, soluble CD14 (sCD14), LPS, and LPS-binding protein (LBP) were measured in 166 PSC patients and 100 healthy controls[49]. LBP and sCD14 levels were higher, whilst zonulin levels were lower in PSC patients compared to controls. This latter difference disappeared with the exclusion of PSC patients with elevated prothrombin time (indicating advanced liver disease). In patients with CCA, sCD14 levels were higher compared to patients without it. High sCD14 and LBP values (> 1638 ng/mL and > 13942 ng/mL, respectively) were associated with reduced transplantation-free survival independently of MRS (HR = 2.26; 95%CI = 1.15-4.43; $P$ = 0.018 and HR = 2.00; 95%CI = 1.17-3.43; $P$ = 0.011, respectively). Further studies are needed to validate these findings.

### *Extracellular remodeling markers*

In a cohort of 138 large-duct PSC patients (74% with IBD) using 52 UC patients as controls, Nielsen *et al* [50] investigated the predictive potential of four extracellular remodeling markers related to collagen formation (PRO-C3 and PRO-C5) and collagen degradation (C3M and C4M). All markers were elevated in PSC compared to UC patients, with PRO-C3 showing the most robust difference. High serum levels of three markers (with the exception of C3M) were associated with a shorter transplant-free survival during a 4-year follow-up period. In the univariate Cox regression model (using tertiles of the markers), PRO-C3 had the highest HR (HR = 3.02; 95%CI = 1.96-4.67, $P$ < 0.001) but it failed to predict survival in the multivariate model including ELF test, while PRO-C5 remained an independent predictor (multivariate HR = 1.92; 95%CI = 1.28-2.88; $P$ = 0.002). The combination of PRO-C3 and PRO-C5 resulted in a significantly improved odds ratio (OR = 47.3; $P$ < 0.001) compared to the individual markers and

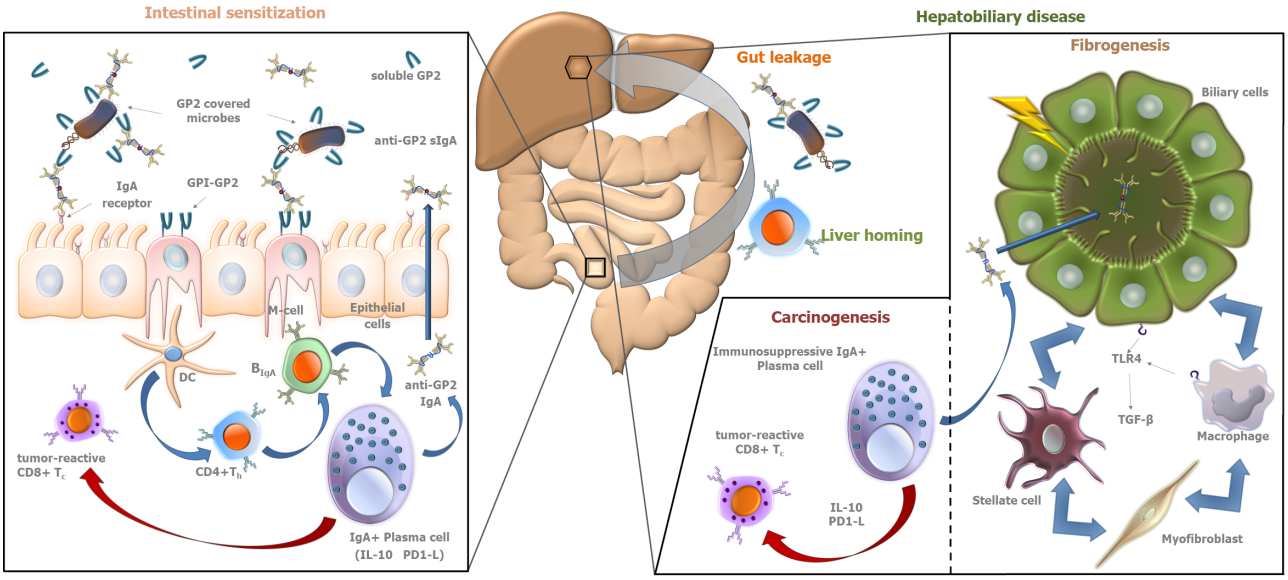


**Figure 1 Autoimmunity-driven putative role of pancreatic glycoprotein 2 in fibro- and tumorigenesis in primary sclerosing cholangitis.** Along with digestive enzymes, glycoprotein 2 (GP2) is secreted from pancreatic acinar cells into the intestinal lumen. In addition, GP2 is also expressed on the luminal surface of microfold cells [M-cells in Peyer's patches anchored by glycosylphosphatidylinositol (GPI)]. Both forms of the molecule interact with FimH+ bacteria and opsonize them. The anchored form may be involved in the transcytosis of bound ligands (FimH+ microbes) through M cells, which pass them to antigen-presenting cells like dendritic cells (DC) located in the mucosa-associated immune system. Microbe-bound GP2 epitopes are presented to CD4-positive T helper cells ($CD4^+ T_h$) along with bacterial antigens that lead to loss of tolerance to GP2. After clonal expansion, these sensitized cells can "home" to both the gut and the liver where they trigger (blue arrows) the differentiation of $IgA^+$ B cells into $IgA^+$ plasma cells. The produced anti-GP2 IgA is actively transported by epithelial cells to the intestinal and biliary lumens as secretory IgA (sIgA), where it binds to GP2. Epithelial cells express IgA receptors on their luminal surface that are involved in active retrograde transport of sIgA molecules (typically coupled by antigens). This process may contribute to bacterial overload of the gut mucosa, elevating the levels of bacterial components in the circulation. In the hepato-biliary tract, bacterial components trigger the Toll-like receptor 4 (TLR4)-transforming growth factor-β (TGF-β) pathway, facilitating fibrosis and cirrhosis. Meanwhile, as a response to the continuous inflammation, a line of $IgA^+$ plasma cells develops an immunosuppressor phenotype expressing interleukin-10 (IL-10) and programmed cell-death 1 Ligand (PD1-L). These molecules inhibit (red arrow) tumor-suppressing cytotoxic $CD8^+$ T cells ($CD8^+$ Tc), contributing to tumor development in the hepatobiliary and intestinal tract. Citation: Tornai D, Papp M. Editorial: serologic antibodies in primary sclerosing cholangitis a tell-tale sign of compromised gut-liver immunity? *Aliment Pharmacol Ther* 2021; 53: 350-351[8]. Copyright ©The Authors 2021. Published by John Wiley and Sons. (Supplementary material).

ELF test. However, in this latter test, the authors compared the outcomes associated with the first (lowest) tertile of the markers to those associated with the third (highest) tertile. Further studies are needed to confirm the importance of these findings as well.

### *MicroRNA-122*

MicroRNA-122 (miR-122) is the most abundant microRNA in the liver, orchestrating molecular pathways in hepatocytes and regulating liver functions including lipid and cholesterol homeostasis. MiR-122 deficiency leads to inflammation, cholestasis, and ultimately fibrosis of the liver[51]. Friedrich *et al*[52] investigated the predictive potential of miR-122 in 114 PSC patients with a prospective follow-up of 10 years. They divided the population to patients with low and high miR-122 levels based on the median value of the marker (CT-value of 28.5). Low miR-122 levels were associated with a higher risk of death or need for liver transplantation (HR = 1.27; 95%CI = 1.04-1.39; $P$ = 0.009) even in the multivariate Cox regression model including MRS, presence of dominant stricture, and IBD (HR = 1.19; 95%CI = 1.00-1.43; $P$ = 0.045).

### *Soluble vascular adhesion protein 1*

Expression of vascular adhesion protein 1 (VAP-1) and amine oxidase enzyme activity in the liver endothelium are increased in PSC. The former results in an elevation of its soluble form (sVAP-1) in patients' sera. Elevated levels of sVAP-1 (> 529 ng/mL) in PSC are associated with an adverse disease outcome, independent of the presence of cirrhosis (HR = 3.85; 95%CI = 1.57 to 9.34; $P$ = 0.003)[53].

Increased VAP-1 activity results in the expression of mucosal addressin cell adhesion molecule (MAdCAM-1) in liver tissue, which is otherwise expressed only in the intestinal endothelium. MAdCAM-1 binds to α4β7 integrin receptors on effector T lymphocytes, which is required for lymphocyte homing. Thus, abnormally expressed MAdCAM-1 in the liver results in the entry of gut activated effector T lymphocytes into liver tissue[53]. The increased expression and intrahepatic activity of VAP-1 may be attributed to the altered intestinal flora and the increased amine influx into the portal tract due to the inflamed, permeable bowel. The pathological amine substrate of VAP-1 is cysteamine [53], which causes colitis and colorectal carcinoma in mice. The *Escherichia* genus, which is able to

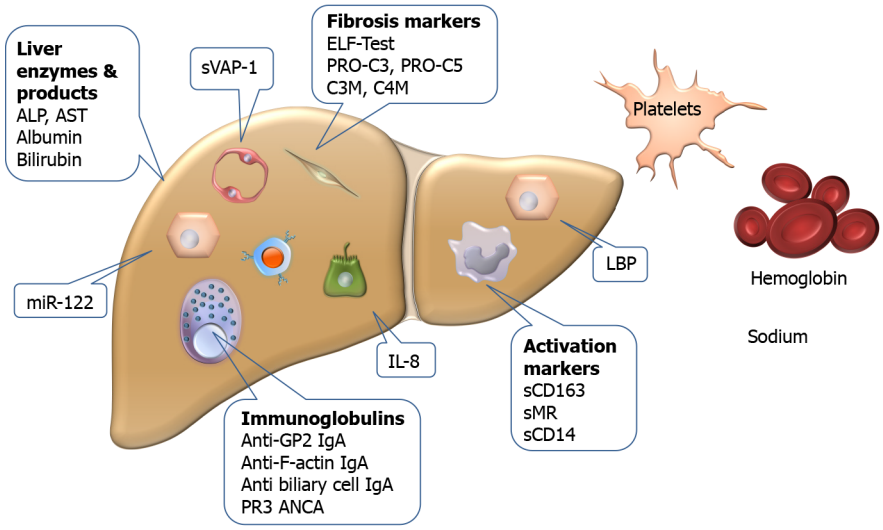


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**Figure 2 Biomarkers with potential to predict the clinical course of primary sclerosing cholangitis.** The markers have various cellular origins, and some (LPS-binding protein, interleukin-8, and enzymes) have multiple sources that limit their specificity. ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; GP2: Pancreatic glycoprotein 2; IL: Interleukin; LBP: Lipopolysaccharide binding protein; miR: Micro-ribonucleic acid; sMR: Soluble mannose receptor; sVAP: Soluble vascular adhesion protein.

produce cysteamine *in vitro*, is upregulated in the mucosa-associated microbiota population in PSC. However, the intestinal epithelium itself is capable of cysteamine production *via* the ectoenzyme vanin-1. The observation that cysteamine enhances colonic inflammation and carcinogenesis is consistent with the increased colon carcinoma formation observed in PSC. The aldehyde derivative formed during the metabolism of cysteamine causes abnormal collagen cross-binding, which could theoretically contribute to increased fibrogenesis. Increased VAP-1 activity in liver and colonic tissue is most likely an attempt to counteract the increased amine load resulting from colitis.

The newly discovered VAP-1 pathway is one explanation of how colitis and increased amine production lead to damage of liver tissue. VAP-1 may be a potential therapeutic target in the future due to its effect in promoting α4β7/MAdCAM-1 interaction. A VAP-1 antagonist may also be able to regulate the migration of effector T lymphocytes from the inflamed intestine to the liver and thereby inhibit fibrogenesis[53].

The biomarkers used individually or as parts of score systems are summarized in Figure 2.

## SEROLOGICAL BIOMARKERS FOR DETECTING CHOLANGIOCARCINOMA IN PSC

Carbohydrate antigen 19-9 (CA19-9) is the most commonly used serological marker for screening CCA, but its application is limited in PSC by the lack of comprehensive studies comparing PSC patients with and without CCA. Most studies were too small and ultimately provided inconsistent cut-off values[54]. A recent development in this field is the discovery of the role of fucosyltransferase (FUT) 2 and 3 in influencing the serum levels of CA19-9 in patients with PSC. These enzymes catalyze the final steps of CA19-9 biosynthesis. The study identified FUT genotypes with low, intermediate, and high CA19-9 synthesis capacity displaying distinct CA19-9 serum levels[55]. According to these data, CA19-9 is not an appropriate marker for CCA screening in patients with a low CA19-9 biosynthesis genotype since they do not synthetize CA19-9. This group can be identified by a Lewis-negative blood group which is also determined by these enzymes. In contrast, the high CA19-9 biosynthesis genotype might be a reason for false positive results[55]. In addition, bacterial cholangitis can be another explanation for increased CA19-9 level in PSC patients without CCA[56].

Finally, a study demonstrated a significant influence of FUT2 genotype also on CEA serum levels. The most prominent effect was observed in the subgroup incapable of CA19-9 biosynthesis[57]. Studies with other serological biomarkers, like angiopoetin-2[58] or cytokeratin fraction 21-1[59], have not reached satisfactory results in terms of identifying individuals with CCA among PSC patients.

## CONCLUSION

Several promising discoveries have been made on biomarkers for PSC. The first validated prognostic biomarker (anti-GP2 IgA) is finally ready to be incorporated into clinical practice, and the investigations

highlighted important pathophysiological mechanisms for future research that might open new avenues for medical therapy. However, we still lack specific serological markers that could support the diagnosis of PSC. Additionally, a serological marker with satisfactory discriminative power to aid the recognition of CCA in PSC patients is another unmet need.

## FOOTNOTES

**Author contributions:** Tornai D and Ven PL performed the literature review; Tornai D wrote the manuscript; Papp M and Lakatos PL supervised the work and provided expert insights; all authors have read and approved the final manuscript.

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ORIGINAL ARTICLE

Basic Study

# Impact of radiotherapy on the immune landscape in oesophageal adenocarcinoma

Noel E Donlon, Maria Davern, Fiona O'Connell, Andrew Sheppard, Aisling Heeran, Anshul Bhardwaj, Christine Butler, Ravi Narayanasamy, Claire Donohoe, James J Phelan, Niamh Lynam-Lennon, Margaret R Dunne, Stephen Maher, Jacintha O'Sullivan, John V Reynolds, Joanne Lysaght

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## Abstract

### *BACKGROUND*

In the contemporary era of cancer immunotherapy, an abundance of clinical and translational studies have reported radiotherapy (RT) and immunotherapies as a viable option for immunomodulation of many cancer subtypes, with many related clinical trials ongoing. In locally advanced disease, chemotherapy or chemoradiotherapy followed by surgical excision of the tumour remain the principal treatment strategy in oesophageal adenocarcinoma (OAC), however, the use of the host immune system to improve anti-tumour immunity is rapidly garnering increased support in the curative setting.

### *AIM*

To immunophenotype OAC patients' immune checkpoint (IC) expression with and without radiation and evaluate the effects of checkpoint blockade on cell viability.

### *METHODS*

In the contemporary era of cancer immunotherapy, an abundance of studies have demonstrated that combination RT and IC inhibitors (ICIs) are effective in the immunomodulation of many cancer subtypes, with many related clinical trials ongoing. Although surgical excision and elimination of tumour cells by chemotherapy or chemoradiotherapy remains the gold standard approach in OAC, the propagation of anti-tumour immune responses is rapidly garnering increased support in the curative setting. The aim of this body of work was to immunophenotype OAC patients' IC expression with and without radiation and

to establish the impact of checkpoint blockade on cell viability. This study was a hybrid combination of *in vitro* and *ex vivo* models. Quantification of serum immune proteins was performed by enzyme-linked immunosorbent assay. Flow cytometry staining was performed to evaluate IC expression for *in vitro* OAC cell lines and *ex vivo* OAC biopsies. Cell viability in the presence of radiation with and without IC blockade was assessed by a cell counting kit-8 assay.

***RESULTS***

We identified that conventional dosing and hypofractionated approaches resulted in increased IC expression (PD-1, PD-L1, TIM3, TIGIT) *in vitro* and *ex vivo* in OAC. There were two distinct subcohorts with one demonstrating significant upregulation of ICs and the contrary in the other cohort. Increasing IC expression post RT was associated with a more aggressive tumour phenotype and adverse features of tumour biology. The use of anti-PD-1 and anti-PD-L1 immunotherapies in combination with radiation resulted in a significant and synergistic reduction in viability of both radiosensitive and radioresistant OAC cells *in vitro.* Interleukin-21 (IL-21) and IL-31 significantly increased, with a concomitant reduction in IL-23 as a consequence of 4 Gray radiation. Similarly, radiation induced an anti-angiogenic tumour milieu with reduced expression of vascular endothelial growth factor-A, basic fibroblast growth factor, Flt-1 and placental growth factor.

***CONCLUSION***

The findings of the current study demonstrate synergistic potential for the use of ICIs and ionising radiation to potentiate established anti-tumour responses in the neoadjuvant setting and is of particular interest in those with advanced disease, adverse features of tumour biology and poor treatment responses to conventional therapies.

**Key Words:** Oesophageal Cancer; Radiotherapy; Immunotherapy; Immunology; Surgery; Oncology



**Core Tip:** This body of work evaluates the impact of radiotherapy on the immune profile in oesophageal adenocarcinoma with an added caveat of immunotherapy effects on tumour cell killing.

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## INTRODUCTION

Oesophageal adenocarcinoma (OAC) is rapidly increasing in incidence in the western world, and five year survival rates rarely exceed 40%[1]. Multimodal therapy alongside surgical resection has become standard of care for locally advanced cancer of the oesophagus or the oesophagogastric junction[2]. One option is the CROSS regimen, which includes preoperative administration of carboplatin and paclitaxel with concomitant radiotherapy (RT)[3]. In Europe, radiation is delivered in 23 fractions of 1.8 Gray (Gy), giving a total dose of 41.4 Gy but this varies worldwide, with North American centres delivering up to 50.0-51.4 Gy[4], while Asian regimens can feature cumulative doses of 60 Gy[5].

Hypofractionated RT is where radiation is delivered in fewer fractions of 2.4 Gy to 5.0 Gy, but often the same cumulative dose[6]. This has the potential to reduce costs, increase patient comfort, and could be more effective compared to conventional treatment[7]. Randomised trials in breast and prostate cancer have found that both high- (≥ 5 Gy per fraction) and moderately (2.4-3.4 Gy per fraction) hypofractionated RT is non-inferior to traditional regimens[7-10]. As RT is a mainstay of treatment, and oesophageal malignancies are associated with considerable morbidity, there is interest in evaluating whether this paradigm can be applied in the upper gastrointestinal context.

Disappointingly, a pathologic complete response to treatment is observed in less than 30% of patients with oesophageal cancer undergoing chemoradiotherapy[11], and it is this small subgroup that benefits most in terms of survival[12]. More effective strategies are therefore required. One emerging approach is combining chemoradiotherapy with immune checkpoint blockade (ICB). The most widely used ICB

involves blocking the interaction of PD-1 expressed on T cells and it's ligand, PD-L1 expressed on tumour cells[13], and seeks to re-invigorate anti-tumour cytotoxic T cells[14]. Phase III trials of single agent ICB have delivered mixed results in chemorefractory advanced oesophagogastric cancer[15], but some recent encouraging results have been reported in earlier stage disease[16,17].

Radiation can sensitise tumours to immunotherapy through three main mechanisms[18]. First, radiation can increase neoantigen expression and induce immunogenic cell death, whereby release of damage associated molecular patterns (DAMPs) results in more efficient tumour antigen presentation and immune stimulation[19]. Second, radiation induced DNA damage can activate the GMP-AMP stimulator of interferon genes (cGAS-STING) cytosolic DNA sensor, resulting in type I interferon production[20,21]. Finally, RT can result in remodeling of the tumour microenvironment (TME), promoting infiltration of immune cells[22]. The latter effect is particularly affected by radiation dosage and some limited preclinical evidence suggests that hypofractionated RT can have more immunostimulatory effects than conventional fractionation[18]. However, the majority of studies in the literature to date have focused on more inherently immunogenic tumour models like melanoma or non-small cell lung cancer, or common malignancies like breast or colon cancer. There are a number of clinical studies evaluating hypofractionation in the context of squamous cell cancer of the oesophagus, however, data is lacking for OAC. In addition, there are no translational studies characterising immune response in OAC in the context of immunotherapy and thus was the premise for this study.

This study assessed the effects of hypofractionated RT on IC expression in oesophageal cancer cells *in vitro* and *ex vivo* and correlated this with clinical outcomes. We also assessed the synergistic effects of ICB and radiation on OAC cell lines. Through this, we aimed to enhance our understanding of the interplay between immunotherapy, radiation and the TME in oesophageal cancer, with the goal of identifying the most effective radiation dosing strategy to combine with immunotherapy.

## MATERIALS AND METHODS

### Ethics statement

We secured ethical approval for this study from the Tallaght/St James's Hospital Ethics Committee. All patients provided formal written consent for all sample and variable data collection. During the course of all steps of sample and data collection good clinical practice was maintained and ethical standards upheld. We also pseudonymised patient data to protect privacy.

### Specimen collection

We secured tissue from those patients who consented to participate from 2018-2021. Tumour biopsies were obtained from patients with OAC prior to treatment at the National Centre for Oesophageal and Gastric Cancer at St James's Hospital, Dublin. A total of 17 biopsies were used for analysis with all patient samples being treatment naïve prior to having neoadjuvant therapies to ensure clinical relevance of the study population. A total of 12 men and 5 women with a mean age of 64.23 years (SD11.5) in the study. All patients had locally advanced disease and were $T3N_{any.}$

### Generation of tumour conditioned media

Tumour treatment naïve tissue samples were added to L-15 (Leibovitz) Lonza™ BioWhittaker™ X-vivo media for in a 12 well plate and subsequently cultured for a period of 24 h at 37 °C, 5% $CO_2$. After the 24 h period expired, the tissue conditioned media was collected for storage at -80 °C.

### Quantification of serum immune proteins

Tumour conditioned media (TCM) was collected based on a standard operating procedure designed as per MSD United States instructions (Meso Scale Diagnostics, United States). To assess markers of angiogenesis, vascular injury, pro-inflammatory, cytokines, chemokine as well as soluble checkpoints from TCM, a 54-plex enzyme-linked immunosorbent assay (ELISA) kit was used (Meso Scale Diagnostics, United States). The ELISA was utilized to determine the level of secretions of the following markers: C-reactive protein (CRP), Eotaxin, Eotaxin-3, FGF (basic), Flt-1, GM-CSF, ICAM-1, IFN-γ, interleukin-10 (IL-10), IL-12/IL-23p40, IL-12p70, IL-13, IL-15, IL-16, IL-17A, IL-17A/F, IL-7, IL-8, MCP-1, MCP-4, MDC, MIP-1α, MIP-1β, MIP-3α, PlGF, SAA, TARC, Tie-2, TNF-α, TNF-β, TSLP, VCAM-1, vascular endothelial growth factor (VEGF)-A, VEGF-C and VEGF-D and ICs TIM-3, TIGIT, PD-1, PD-L1, CD276 and CD80 from TCM. These assays were processed according to a standard operating procedure following consultation of the manufacturer's guidelines. The derived data with respect to all markers were normalised to protein content as determined using a Pierce bicinchoninic acid assay.

### Neoplastic tissue sample digestion

In preparation for flow cytometry, the tissue samples were digested to enable phenotyping of the cancer cells. The tissue was resected using a surgical blade and added to collagenase solution (2 mg/mL of collagenase type IV (Sigma) in Hanks Balanced Salt Solution (GE healthcare) supplemented with 4%

(v/v) foetal bovine serum) at 37 °C and 1500 rpm on an orbital shaker. The cancer cells were stained with flow cytometry antibodies for subsequent analysis.

### Cell culture of OAC cell lines

Human OAC cell lines OE33 were purchased from The European Collection of Authenticated Cell Cultures (ECACC), established from a poorly differentiated stage IIA adenocarcinoma of the lower oesophagus of a 73-year old female patient. An in-house isogenic radioresistant model was generated [23].

### Cell viability cholecystokinin octapeptide assay

A cell counting kit-8 (CCK-8) viability assay was used to determine the impact of ionising radiation on the viability of OE33P and passage matched OE33R cells. The impact of anti-PD-1, and anti PD-L1 therapies in isolation, and dual ICB with and without radiation, at both hypofractionation and bolus dosing of clinically relevant doses on the viability of OE33P and R cells was also assessed using a CCK-8 assay. OAC cells ($5 \times 10^3$) were adhered in a 96 well plate at 37 °C, 5% CO2 overnight. Cells were treated with bolus dosing or three consecutive fractionated doses of radiation with an interval of 24 h using the X-Strahl RS225 irradiator. In addition to this, the cancer cells were treated with and without radiation in the absence or presence of pembrolizumab (10 μg/mL), atezolizumab (10 μg/mL), nivolumab (10 μg/mL) or combination atezolizumab (10 μg/mL) and nivolumab (10 μg/mL), or dual atezolizumab (10 μg/mL) and pembrolizumab (10 μg/mL). All of the data were analysed from three independent experiments (Supplementary material).

### Flow cytometry staining for in vitro OAC cell lines and ex vivo OAC biopsies

OE33 cells were trypsinised and stained with zombie aqua viability (Biolegend, United States) dye. Antibodies used for OAC cell lines included: PD-L1-FITC, PD-L2-PE, TIGIT-PE/Cy7, PD-1-APC/Cy7 (Biolegend, United States), OE33P and OE33R cells were fixed with 1% paraformaldehyde solution and acquired using BD FACs CANTO II (BD Biosciences) using Diva software and analysed using FlowJo v10 software (TreeStar Inc.). Tumour tissue biopsies were stained with zombie aqua viability dye (Biolegend, United States) as per manufacturer's recommendations.

### Statistical analysis

GraphPad Prism 9 was utilized to analyze the results. In order to determine statistical differences between treatments in cell lines, a paired parametric statistical *t*-test was utilized. In order to determine the differences between the OE33P and OE33R cell lines an unpaired parametric *t*-test was performed. To evaluate any differences between paired treatments of patient samples, Wilcoxon signed rank test was performed. Statistical significance was pre-determined as $P \leq 0.05$.

## RESULTS

### IC expression by an isogenic model of radioresistance following bolus and hypofractionated RT dosing

In order to ascertain if different expression levels of IC proteins were detectable on a radiosensitive (OE33P) and a radioresistant (OE33R) OAC cell line at baseline and following variable fractions of radiation, cells were stained with antibodies for a range of IC proteins and assessed by flow cytometry 24 h after the last dose. The administration of fractionated dosing resulted in significantly higher expression of PD-1, PD-L1, PD-L2 and TIGIT ($P < 0.05$) in both parental and resistant cell lines when compared to bolus dosing (Figure 1). There was a significantly higher expression of checkpoints and their ligands in the parental cell line compared to the passage matched radioresistant cell line. There was also a significantly higher expression of PD-1 and its ligands PD-L1 and PD-L2 with bolus dosing 10 Gy and 20 Gy in OE33P cell lines compared to the radioresistant passage matched cell line ($P < 0.05$). Globally there was a higher expression of PD-1, PD-L1 and PD-L2 on the parental cell line with fractionated dosing regimens of 3X1 Gy, 3X2 Gy and 3X8 Gy compared to the radioresistant cell line ($P < 0.05$). In the case of TIGIT, there was a significantly higher upregulation in the OE33P cell line compared to the radioresistant cell line following fractionated dosing of 3X4 Gy and 3X8 Gy ($P < 0.05$) (Figure 1).

### Cell viability in the context of radiation and IC blockade

IC blockade alone reduced the viability of both OE33P and OE33R cell lines, for both anti-PD-1 and anti-PD-L1 therapies. Multimodal use of both anti-PD-1 and anti-PD-L1 therapies with ionising radiation resulted in a synergistic reduction in viability in both cell lines (Figure 2).

In the OE33P cell line, 2 Gy radiation alone reduced viability to 78.49% (± 2.05, $P < 0.01$) and 4 Gy to 35.48% (± 2.08, $P < 0.01$) compared with unirradiated cells and there was a significant reduction in viability when comparing 2 Gy to 4 Gy ($P < 0.05$). In the OE33R cell line, 4 Gy reduced viability to

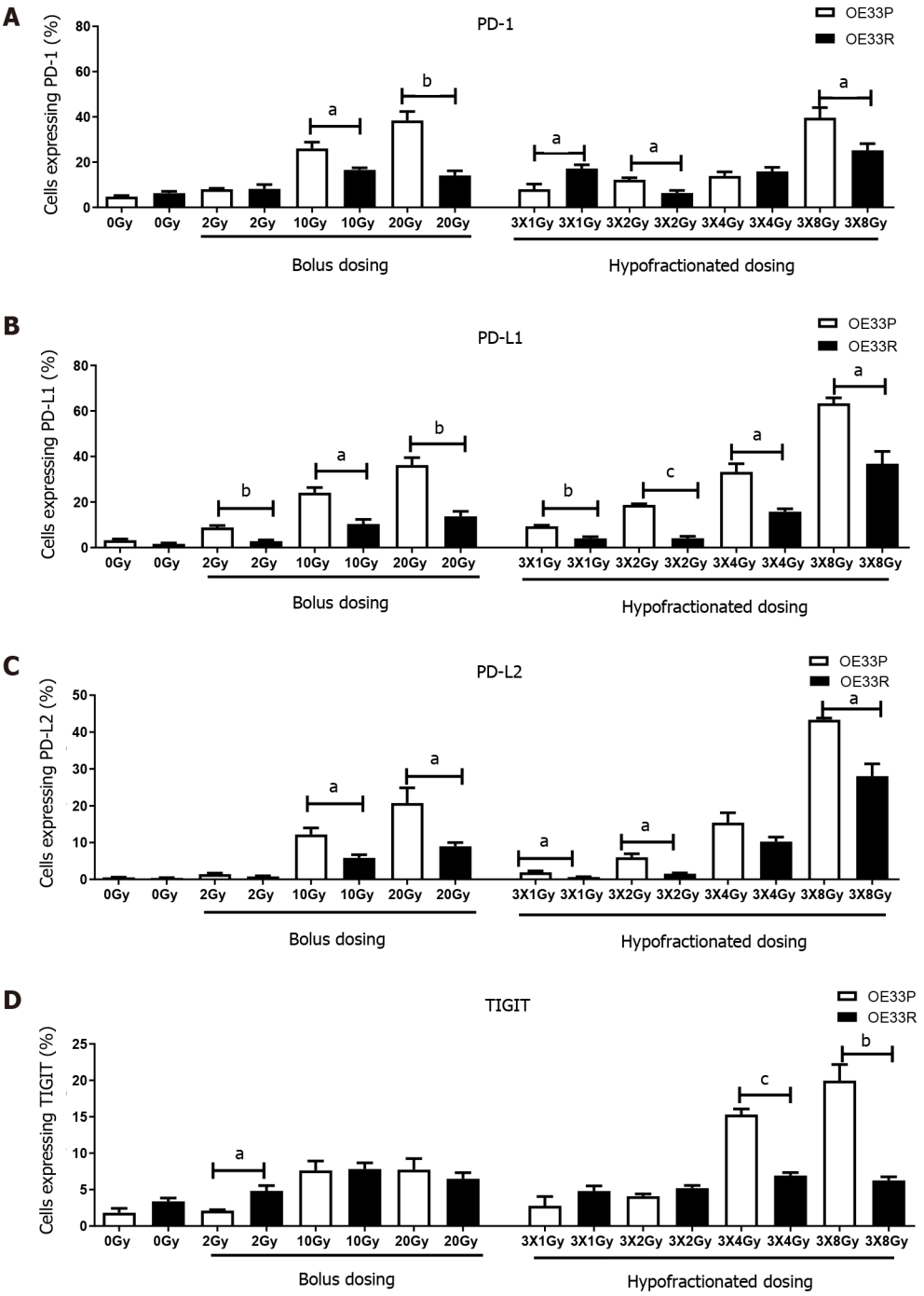


**Figure 1 OE33P and R cell lines were screened for the surface expression of immune checkpoints by flow cytometry.** Inhibitory immune checkpoints are expressed at a higher level on parental cell lines than the passage matched radioresistant cell line ($n$ = 3). A: PD-1; B: PD-L1; C: PD-L2; D: TIGIT. Graph shows % expression (± SE). [a]$P$ < 0.05; [b]$P$ < 0.01; [c]$P$ < 0.001 by unpaired parametric $t$-test.

63.33% (± 2.67, $P$ < 0.05). Both 2 Gy and 4 Gy radiation resulted in a significantly greater reduction in viability in the OE33P cell line compared to the OE33R ($P$ < 0.05) (Figure 2).

Compared with untreated cells, when the OE33P cells were treated with Atezolizumab alone, viability was reduced to 91.3% (± 0.30, $P$ < 0.01) and with the addition of 2 Gy radiation viability reduced to 66.57% (± 2.40, $P$ < 0.01) and to 48.92% (± 5.76, $P$ < 0.01) with 4 Gy radiation. Compared with untreated OE33R cells, viability of OE33R cells treated with Atezolizumab alone was reduced to 88% (± 2.65,$P$ < 0.05), with the addition of 2 Gy radiation viability was reduced to 75.67% (± 2.33, $P$ < 0.01) and 38% (± 3.06, $P$ < 0.01) with 4 Gy radiation (Figure 2B).

In the OE33P cells, Pembrolizumab treatment alone non-significantly reduced viability to 91.54% (± 2.67) compared with the untreated cells, however with the addition of 2 Gy radiation viability reduced to 65.36% (± 2.81, $P$ < 0.01) and 48.18% (± 3.20, $P$ < 0.01) with 4 Gy radiation when compared to untreated OE33P cells. When the OE33R cells were treated with Pembrolizumab, viability was non-significantly reduced to 92% (± 2.52), but the addition of 2 Gy radiation reduced viability to 75.33% (±

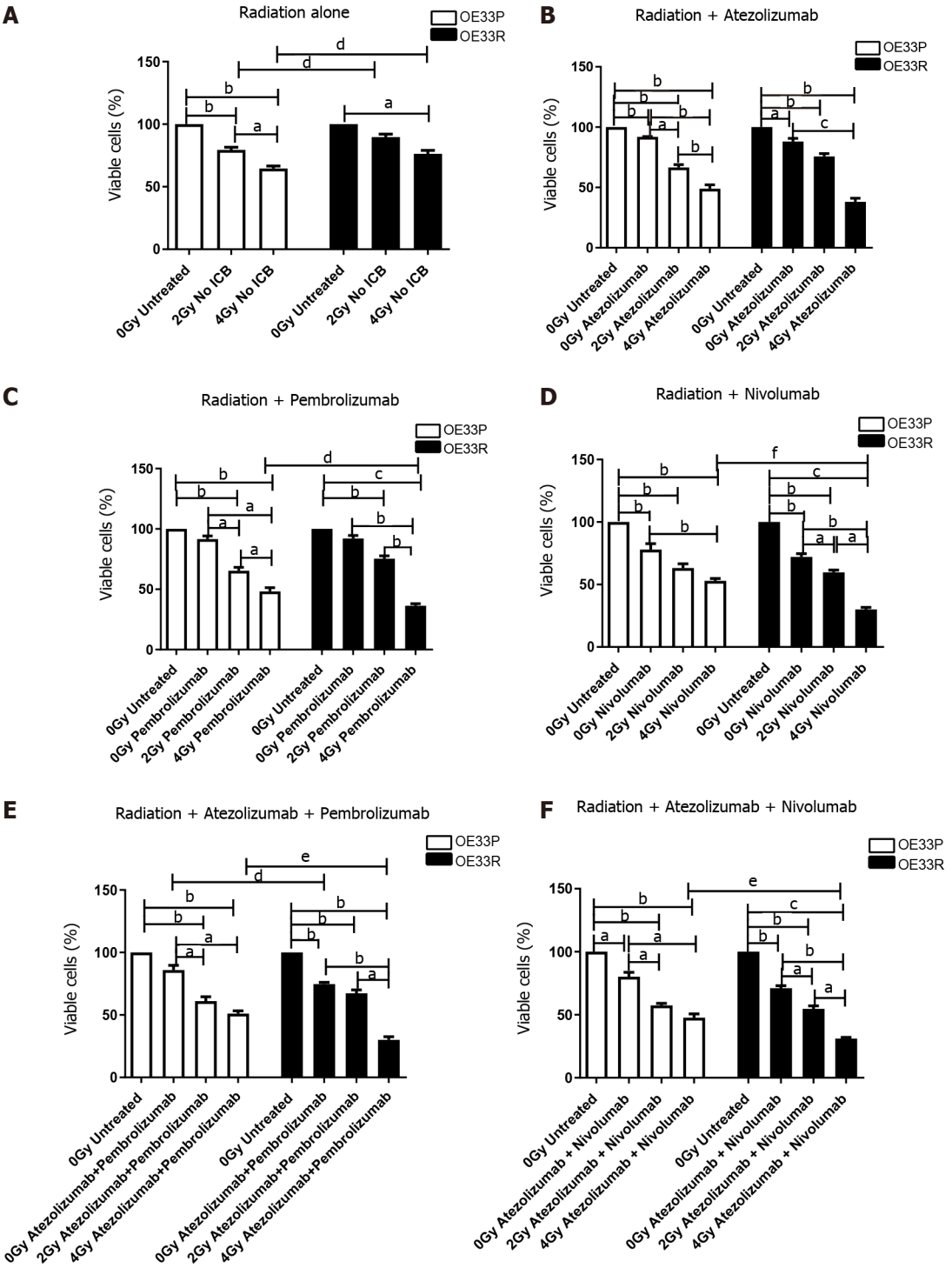


**Figure 2 Viability (± SE) of OE33P and OE33R cells were assessed using a cell counting kit-8 assay with or without radiation (*n* = 3).** Ionising radiation with immune checkpoint blockade results in a greater reduction in cell viability when compared to either modality alone. Graph shows % expression (± SE). A: Treatment with radiation dosing only; B: Treatment with radiation and single agent immunotherapy Atezolizumab; C: Treatment with radiation and single agent immunotherapy Pembrolizumab; D: Treatment with radiation and single agent immunotherapy Nivolumab; E: Treatment with radiation and dual immunotherapy agents Atezolizumab & Pembrolizumab; F: Treatment with radiation and dual immunotherapy agents Atezolizumab & Nivolumab. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ paired *t*-test; $^{d}P < 0.05$, $^{e}P < 0.01$, $^{f}P < 0.001$ unpaired *t*-test.

2.33, $P < 0.01$) and 36.33% (± 1.67, $P < 0.001$) with 4 Gy radiation. Four Gy radiation with Pembrolizumab resulted in a significantly greater reduction in viability in the radioresistant OE33R cell line compared to the radiosensitive OE33P cell line ($P < 0.05$) (Figure 2C).

In the OE33P cells Nivolumab reduced viability to 77.94% (± 4.79, $P < 0.05$) and with the addition of 2 Gy radiation viability reduced to 63.21% (± 3.41, $P < 0.01$) and 52.98% (± 1.82, $P < 0.01$) with 4 Gy radiation compared with untreated OE33P cells. When the OE33R cells were treated with Nivolumab, viability was reduced to 72% (± 2.62, $P < 0.01$) and with the addition of 2 Gy radiation viability reduced to 59.67% (± 1.86, $P < 0.01$) and 30% (± 1.73, $P < 0.001$) with 4 Gy radiation compared with untreated

OE33R cells. Treatment with 4 Gy radiation and Nivolumab resulted in a significantly greater reduction in viability in the OE33R cell line compared to the radiosensitive cell line ($P < 0.001$) (Figure 2D).

In the OE33P cells, combination Atezolizumab and Pembrolizumab non-significantly reduced viability to 85.94% (± 3.79) but the addition of 2 Gy radiation significantly reduced viability to 61.10% (± 3.44, $P < 0.01$) and 51.07% (± 2.27, $P < 0.01$) with 4 Gy radiation compared with untreated OE33P cells. When the OE33R cells were treated with combination Atezolizumab and Pembrolizumab, viability was significantly reduced to 74.67% (± 1.33, $P < 0.01$), and with the addition of 2 Gy radiation viability was reduced to 67.33% (± 2.73, $P < 0.01$) and 30% (± 2.52, $P < 0.01$) with 4 Gy radiation. Four Gy radiation with combination Atezolizumab and Pembrolizumab resulted in a significantly greater reduction in viability in the OE33R cell line compared to the radiosensitive cell line ($P < 0.01$) (Figure 2E).

In the OE33P cells combination Atezolizumab and Nivolumab reduced viability to 80.18% (± 3.48, $P < 0.05$) and with the addition of 2 Gy radiation reduced viability further to 57.48% (± 1.64, $P < 0.01$) and 47.63% (± 3.11, $P < 0.01$) with 4 Gy radiation, compared with untreated OE33P cells. When the OE33R cells were treated with combination Atezolizumab and Nivolumab, viability was reduced to 71% (± 2.08, $P < 0.01$) and with the addition of 2 Gy radiation viability reduced to 54.67% (± 2.40, $P < 0.01$) and 31% (± 1.03, $P < 0.001$) with 4 Gy radiation compared with untreated OE33R cells. Treatment with 4 Gy radiation and combination Atezolizumab and Nivolumab resulted in a significantly greater reduction in viability in the OE33R cell line compared to the OE33P cell line ($P < 0.01$) (Figure 2F).

### Profiling IC expression in fresh patient tissue samples

The *in vitro* data revealed an increase in IC expression on OAC cells post irradiation. To determine if this held true in *ex vivo* OAC tumour tissue, we profiled IC expression post irradiation with 2 Gy and 4 Gy. Subcohorts of patients demonstrated an upregulation and others a downregulation in checkpoint expression upon exposure to conventional radiation doses and hypofractionation for $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$ tumour-infiltrating T cells. There was a significant increase in the frequency of $CD3^+PD\text{-}1^+$ and $CD3^+CD8^+PD\text{-}1^+$ T cells in tumour tissue when irradiated with 2 Gy (26.76 ± 8.80 *vs* 16.62 ± 5.40 at 0 Gy, and 14.8 ± 4.1 *vs* 7.70 ± 2.01 at 0 Gy, respectively, $P < 0.05$). There was a significant increase in the frequency of $CD3^+CD4^+$ $PD\text{-}L1^+$ cells with 4 Gy radiation (19.4 ± 2.9 *vs* 6.27 ± 1.02 at 0 Gy, $P < 0.05$). There was an significant increase in $CD3^+TIGIT^+$ and $CD3^+CD4^+TIGIT^+$ expression with both 2 Gy (55.6 ± 8.6 *vs* 40.12 ± 5.40, and 61.29 ± 8.20 *vs* 52.17 ± 7.70, respectively, $P < 0.05$) and 4 Gy radiation dosing regimens (48.06 ± 3.10 *vs* 40.12 ± 5.40, and 65.16 ± 6.90 *vs* 52.17 ± 7.90, respectively, $P < 0.05$) when compared with unirradiated cells, and an increase in $CD3^+CD8^+$ $TIGIT^+$ expression following 4 Gy irradiation (49.55 ± 4.90 *vs* 31.07 ± 7.70, $P < 0.05$). Of interest, this population was significantly associated with advanced disease at initial presentation, poorer treatment responses, and adverse features of tumour biology, notably, lymphovascular invasion and perineural invasion.

In the cohort of patients which displayed a reduction in IC protein expression following radiation, there was a significant decrease in expression of PD-1 by $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ cells in tumour tissue when irradiated with 2 Gy *vs* 0 Gy (18.44 ± 5.90 *vs* 26.48 ± 7.50, $P < 0.05$; 10.33 ± 3.40 *vs* 14.46 ± 3.90, $P < 0.01$; 12.96 ± 5.10 *vs* 17.77 ± 8.20, $P < 0.05$; respectively) and PD-1 expression by $CD3^+$ and $CD3^+CD4^+$ when irradiated with 4 Gy *vs* 0 Gy (21.06 ± 6.90 *vs* 26.48 ± 7.50, and 10.04 ± 4.20 *vs* 14.46 ± 3.90, respectively, $P < 0.05$). There was also a significant decrease $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ cells expressing PD-L1 (15.6 ± 4.2 *vs* 20.6 ± 5.5, $P < 0.01$; 18.73 ± 11.50 *vs* 25.1 ± 13.1, $P < 0.05$; and 4.13 ± 0.90 *vs* 11.17 ± 2.80, $P < 0.05$; respectively) , TIGIT (48.61 ± 5.60 *vs* 60.13 ± 6.20, 59.88 ± 4.50 *vs* 69.57 ± 4.10, and 21.67 ± 6.40 *vs* 30.76 ± 4.50, respectively, $P < 0.05$) and TIM-3 (3.24 ± 0.90 *vs* 6.86 ± 2.50, 4.07 ± 1.30 *vs* 10.91 ± 3.30, and 3.37 ± 119.00 *vs* 9.13 ± 3.10, respectively, $P < 0.01$) with 2 Gy radiation. Similar findings were identified with 4 Gy irradiation compared to basal expression by $CD3^+CD8^+$ for PD-L1 (3.51 ± 0.60 *vs* 11.17 ± 2.80, $P < 0.05$) and TIM-3 (2.93 ± 0.70 *vs* 9.13 ± 3.10, $P < 0.05$) (Figure 3).

### Clinical correlations

In order to understand potential clinical implications of these cohorts with increased and decreased IC expression post radiation, clinicopathological correlations were made based on patient tumour stage, adverse features of tumour biology, radiation and IC positivity (Table 1). There was a positive correlation basally with $PD\text{-}1^+CD3^+$ cells and lymphovascular invasion ($P = 0.04$). In terms of tumour staging, clinically there was a positive association of increasing tumour stage and $PD\text{-}L1^+CD3^+$ ($P = 0.02$) basally, $PD\text{-}L1^+CD3^+$, $TIM\text{-}3^+CD3^+$ and $TIM\text{-}3^+CD4^+$ at 2 Gy, and $TIM3^+CD8^+$ at 4 Gy ($P < 0.05$). There was a negative association between $PD\text{-}1^+CD8^+$ at 2 Gy ($P = 0.01$). In terms of clinical nodal status, there was a positive association with nodal positivity and $PD\text{-}L1^+CD4^+$ basally ($P < 0.001$) and $PD\text{-}1^+CD4^+$, $TIM\text{-}3^+CD4^+$, $TIM\text{-}3^+CD8^+$ at 4 Gy ($P < 0.05$). Pathologically, advancing tumour stage was negatively associated with $TIGIT^+CD3^+$ at 2 and 4 Gy ($P < 0.01$). Pathological nodal positivity was associated with $PD\text{-}L1^+CD4^+$ basally at 0 Gy, and $TIGIT^+CD3^+$ at 4 Gy ($P < 0.05$). It was negatively associated with $PD\text{-}L1^+CD8^+$ cells and $TIGIT^+CD3^+$ cells at 2 Gy ($P < 0.05$).

### Release of angiogenic markers, cytokines, co-stimulatory molecules and soluble checkpoints post irradiation

Given the complex interplay in the tumour microenvironment between immunosuppressive factors and

**Table 1 Clinicopathological characteristics of the study population illustrating the correlation for the percentage of $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ cells expressing immune checkpoints present in oesophageal adenocarcinoma tumour tissue**

| Clinical factor | IC expression | Radiation dose | Spearman *r* | *P* value (two-tailed) |
|---|---|---|---|---|
| Lymphovascular invasion | PD-1 $CD3^+$ | 0 Gy | 0.6396022 | 0.046435 |
| Clinical T stage | PD-L1 $CD3^+$ | 0 Gy | 0.6411189 | 0.024659 |
| | PD-1 $CD8^+$ | 2 Gy | -0.7000000 | 0.016471 |
| | PD-L1 $CD3^+$ | 2 Gy | 0.7768986 | 0.004908 |
| | TIM-3 $CD3^+$ | 2 Gy | 0.7171372 | 0.012993 |
| | TIM-3 $CD4^+$ | 2 Gy | 0.7171372 | 0.012993 |
| | TIM-3 $CD8^+$ | 4 Gy | 0.6963106 | 0.025293 |
| Clinical N stage | PD-L1 $CD4^+$ | 0 Gy | 0.8568931 | 0.000370 |
| | PD-1 $CD4^+$ | 4 Gy | 0.7311262 | 0.016282 |
| | TIM-3 $CD4^+$ | 4 Gy | 0.6614951 | 0.037241 |
| | TIM-3 $CD8^+$ | 4 Gy | 0.6614951 | 0.037241 |
| Pathological T stage | TIGIT $CD3^+$ | 2 Gy | -0.7395740 | 0.014492 |
| | TIGIT $CD3^+$ | 4 Gy | -0.8964215 | 0.006267 |
| Pathological N stage | PD-L1 $CD4^+$ | 0 Gy | 0.6510135 | 0.041473 |
| | PD-L1 $CD8^+$ | 2 Gy | -0.6443043 | 0.044345 |
| | TIGIT $CD3^+$ | 2 Gy | -0.7471188 | 0.013014 |
| | TIGIT $CD4^+$ | 4 Gy | 0.8981774 | 0.006011 |

Positive values indicate positive correlation, negative values indicate negative correlation. Spearman correlation. Only significant data shown. Spearman *r* = 0.40-0.59 moderate, 0.60-0.79 strong and 0.80-1.00 very strong. IC: Immune checkpoint.

anti-tumour immunity, we investigated the expression of cytokines, ICs, co-stimulatory molecules, markers of angiogenesis and vascular injury with and without radiation. The administration of 4 Gy radiation was effective in significantly reducing angiogenic markers over that of untreated 0 Gy tissue; basic fibroblast growth factor (bFGF, 57.7 ± 24.5 *vs* 197.5 ± 76.2, $P < 0.05$), Flt-1 (113.5 ± 47.7 *vs* 364.7 ± 145.8, $P < 0.05$), placental growth factor (PIGF, 18.7 ± 12.1 *vs* 32.8 ± 17.8, $P < 0.05$). Whereas, a significant reduction was observed in VEGF-A following 4 Gy radiation over that of 2 Gy (522.8 ± 144.2 *vs* 583.7 ± 86.2, $P < 0.05$) (Figure 4).

There was a significant increase in the level of IL-21 (1.98 ± 0.30 *vs* 1.3 ± 0.3, $P < 0.01$) and IL-31 (0.24 ± 0.04 *vs* 0.18 ± 0.02, $P < 0.01$) with the administration of 4 Gy radiation compared to 0 Gy with a significant decrease in IL-23 (3.53 ± 1.40 *vs* 5.24 ± 1.90, $P < 0.05$) following 4 Gy radiation compared with 2 Gy. CRP, a marker of vascular injury increased significantly with 2 Gy radiation dosing compared to untreated 0 Gy tissue (7568 ± 5750 *vs* 5425 ± 2925, $P < 0.05$) but was reduced with 4 Gy compared to non-irradiated tissue (Figure 5).

In terms of IC receptor and ligand expression, there was a significant reduction in levels of soluble PD-1 (6.44 ± 2.40 *vs* 15.72 ± 6.20, $P < 0.05$), PD-L1 (3.76 ± 0.70 *vs* 8.12 ± 1.70, $P < 0.05$), TIM-3 (24.11 ± 6.20 *vs* 76.02 ± 23.50, $P < 0.05$), TIGIT (4.26 ± 1.50 *vs* 39.0 ± 3.6, $P < 0.05$) and CD276 (58.81 ± 12.80 *vs* 164.30 ± 61.02, $P < 0.05$) in the TCM following 4 Gy radiation compared with 0 Gy. In addition, 4 Gy radiation also induced a significant decrease in the release of the soluble co-stimulatory molecules CD28 (82.18 ± 27.70 *vs* 163.2 ± 56.3, $P < 0.05$), glucocorticoid-induced TNF receptor (GITR, 5.27 ± 4.10 *vs* 5.7 ± 3.8, $P < 0.05$) and OX-40 (6.7 ± 2.1 *vs* 11.9 ± 2.9, $P < 0.05$) compared to untreated tissue 0 Gy (Figure 6).

## DISCUSSION

The visceral appeal of modulating the host immune system is one of simplicity in an effort to harness a profound anti-tumour response and is a principle that has existed since the development of the field of cellular immunology as an entity. Quintessentially, skewing the hosts innate immune system to boost anti-tumour immunity consists of two processes compliant to exploitation: These being the stimulant as well as the reaction. The most logical way to perturb the tumour and its microenvironment is through promoting tumouricidal effects, through systemic chemotherapy or the radiation therapy delivered.

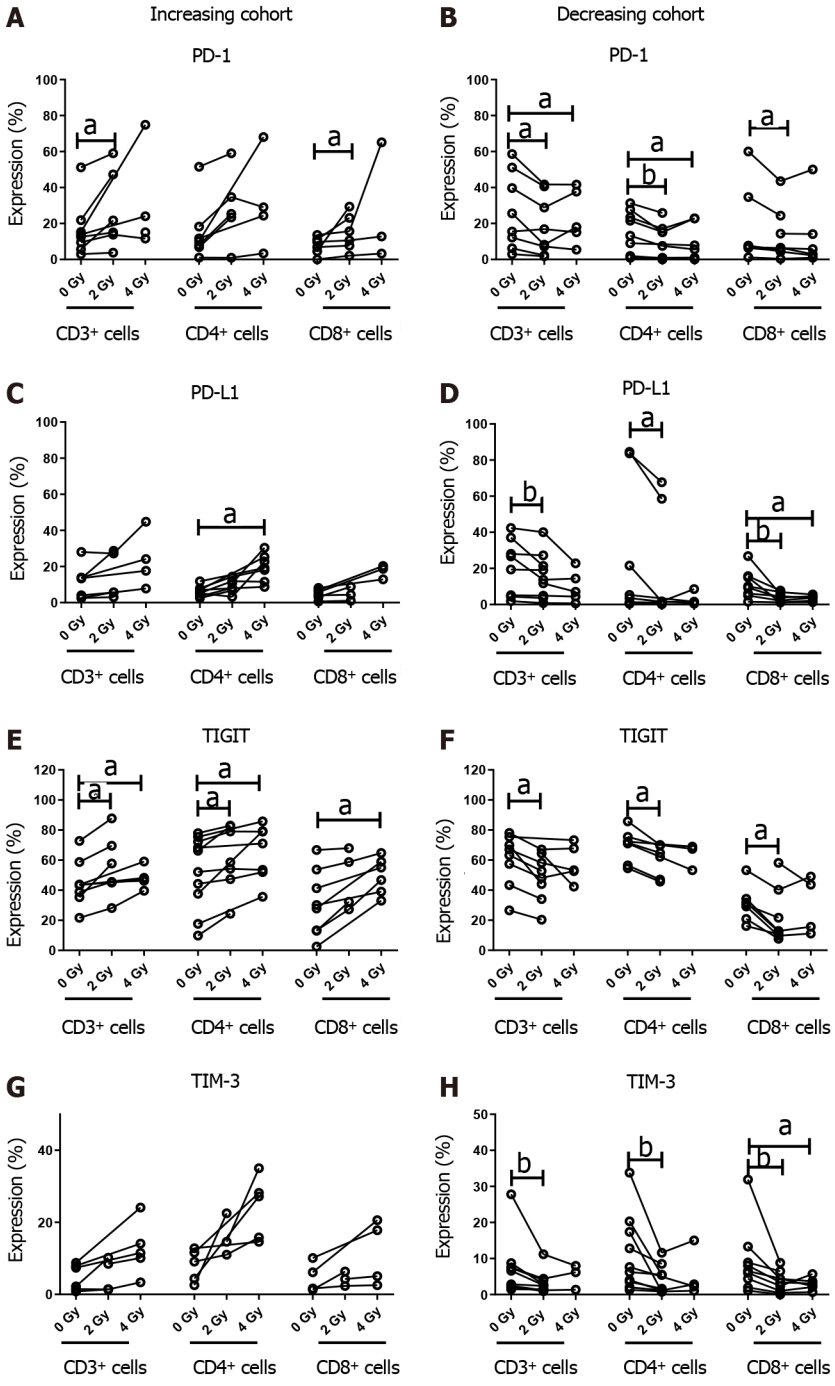


**Figure 3 Oesophageal adenocarcinoma patients were screened for the surface expression of immune checkpoints *ex vivo* by flow cytometric analysis.** Subcohorts where ionising radiation induced upregulation and downregulation of immune checkpoints (ICs). Inhibitory ICs are expressed at a higher level with conventional and hypofractionated dosing regimens in one cohort ($n$ = 8). Inhibitory ICs are expressed at a lower level with conventional and hypofractionated dosing regimens in a separate cohort ($n$ = 9). A and B: Increasing and decreasing cohort of PD-1; C and D: Increasing and decreasing cohort of PD-L1; E and F: Increasing and decreasing cohort of TIGIT; G and H: Increasing and decreasing cohort of TIM-3. [a]$P < 0.05$; [b]$P < 0.01$ by Wilcoxon signed rank test.

Increasing and propagating the anti-tumour responses thereby facilitating immune activation with optimal kinetics may achieve a synergistic anti-tumour response, producing a more profoundly durable effect on the immune system than chemo (radio) therapy alone. In this context, the landmark Checkmate-577 trial has demonstrated significantly improved disease free survival in the adjuvant setting of resectable gastroesophageal cancer[16]. The findings of increased IC expression *in vitro* and *ex vivo* through the use of radiation in the current body of work provides promising translational therapeutic rationale for their use in the multimodal paradigm. RT propagates the priming and effector phases of the anti-tumour immune response rendering it an appropriate combination with IC inhibitors (ICIs)[24]. However, inherently radioresistant tumours may pose a particular therapeutic dilemma, as they may not have a similar synergism with ICB as radiosensitive tumours. Ionising radiation is currently under investigation in metastatic oesophageal cancer with pembrolizumab (NCT02642809)

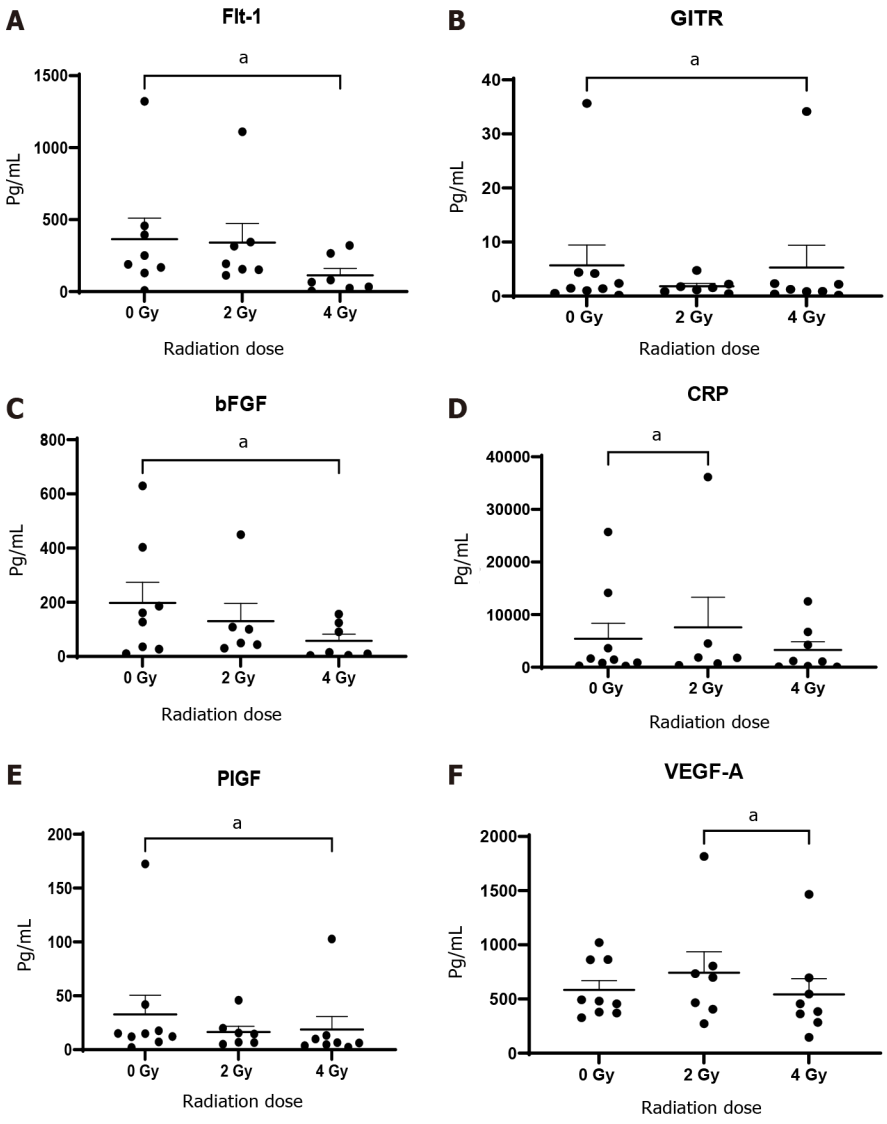


**Figure 4 Conditioned media generated using oesophageal adenocarcinoma patient tumour was screened for markers by multiplex immunosorbent assay kit.** Angiogenic markers Flt-1, basic fibroblast growth factor (bFGF), placental growth factor (PlGF) and vascular endothelial growth factor (VEGF)-A and vascular injury marker C-reactive protein (CRP) decrease significantly with 4 Gy radiation ($n$ = 9). A: Flt-1; B: GITH; C: bFGF; D: CRP; E: PlGF; F: VEGF-A. $^{a}P < 0.05$ by Wilcoxon signed rank test.

and is currently under investigation in the curative setting with neoadjuvant trimodal therapy of Pembrolizumab and chemoradiotherapy in oesophageal squamous cell carcinoma (NCT03792347), with a similar trial investigating durvalumab and chemoradiotherapy in squamous cell carcinoma (SCC) and OAC (NCT02735239). The SKY-SKRAPER-07 trial is currently evaluating anti-PD-L1 Atezolizumab with anti-TIGIT therapy following chemoradiotherapy in advanced oesophageal cancer (NCT04543617). Of note, in a study by Zhao *et al*[25], they reported that with PD-1 positivity correlated with TIM-3 expression, and CD8$^{+}$ tumour infiltrating lymphocyte density as a risk factor for recurrence free and overall survival (OS) in oesophageal SCC. The increasing expression in OAC cells and a cohort of patients following RT in this study represents promising therapeutic targets in OAC.

The clinically important observation in this study that half of the patients assessed displayed a reduction in IC expression post RT is an interesting caveat, one which suggests very different susceptibility to ICB in combination with RT and therefore, the stratification of patients into potential responders and non-responders should be addressed. In the same vein, the activation of cGAS-STING signaling, which has been recognized to potentiate systemic anti-tumour immunity and subsequent tumour rejection by dual RT and checkpoint blockade administration is promising even in those with checkpoint downregulation. A study by Vanpouille-Box *et al*[26], highlighting the importance of the cGAS-STING pathway in response to combination RT and immunotherapy, reported the knockdown of cGAS in murine cancer cells abrogated the priming of CD8$^{+}$ T cells in tumour-draining lymph nodes and spleen, and prevented the infiltration of abscopal tumours by CD8$^{+}$ T cells. Importantly, the synergistic and significant reduction in viability of radioresistant OAC cancer cells which we observed in this study following the dual administration of ICIs and ionising radiation is very promising.

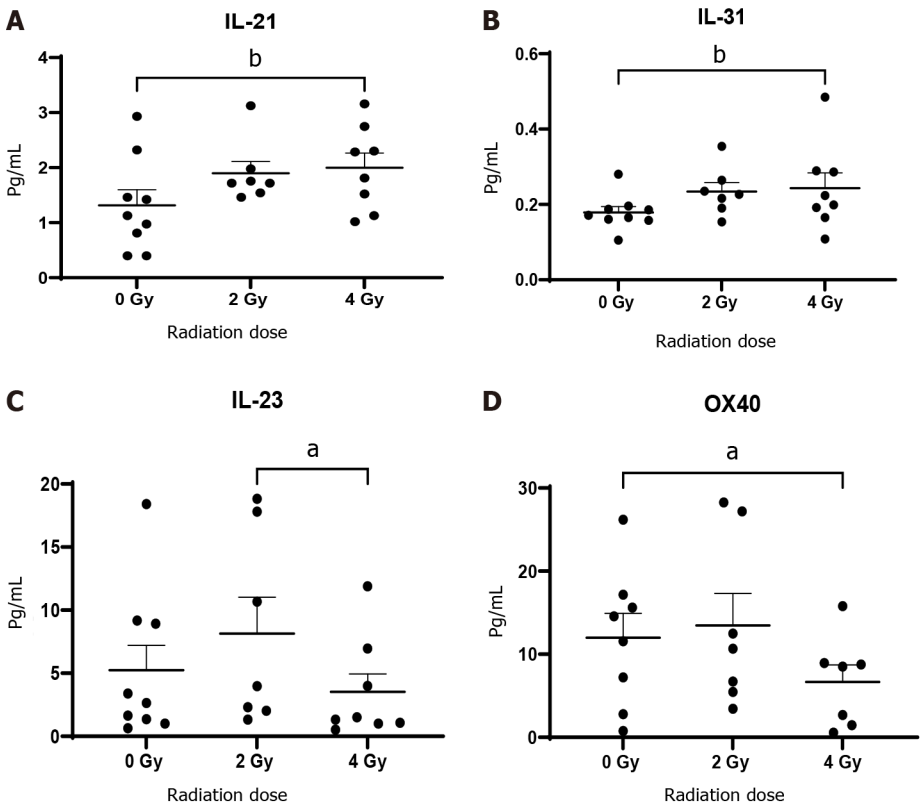


**Figure 5 Oesophageal adenocarcinoma patients tumour conditioned media were screened by multiplex immunosorbent assay kit (*n* = 9).** The cytokines interleukin (IL)-21 and IL-31 increase with ionising radiation while IL-23 and OX-40 decrease. A: IL-21; B: IL-31; C: IL-23; D: OX-40. [a]*P* < 0.05; [b]*P* < 0.01. Wilcoxon signed rank test to compare expression between basal levels and dosing regimens.

With respect to the use of hypofractionation in the curative setting for oesophageal cancer, there is an increasing volume of evidence demonstrating the safety and efficacy of this approach[27]. There are studies demonstrating a survival benefit of this approach particularly in the context of metastatic nodal disease. In one such study, hypofractionated radiotherapy (HFR) administered with taxane based chemotherapy in the management of post-surgery tracheoesophageal groove lymph node (TGLN) metastasis demonstrated improved OS in the HFR group compared with that of the conventional dosing treatment arm [24.1 mo (95%CI, 16.2-32.1 mo) *vs* 11.9 mo (95%CI, 9.2-14.4 mo), $P$ = 0.024)[28]. Importantly, the study did not find a significant difference in pulmonary complications such as radiation pneumonitis (grades 3-4, 16.0% *vs* 7.1%; $P$ = 0.314)[28].

Radiation induced lymphopenia is a frequent complication of multimodal cancer therapy and poorer outcomes are directly linked to the severity of lymphopenia[29]. Furthermore, it has been demonstrated that circulating lymphocyte count during neoadjuvant chemoradiation (CRT) in oesophageal cancer patients can predict pathological complete response (pCR) rates and low absolute circulating lymphocytes are associated with poorer outcomes[30]. The widespread adoption of immunotherapy has garnered new support and focus on the preservation of a pool of lymphocytes that are functional in enhancing immune function in the circulation and a study in pancreatic adenocarcinoma demonstrated hypofractionated CRT of 10 Gy in 3 doses over one week resulted in a decrease in the loss of T cells systemically compared to 28 daily doses of 1.8 Gy equating to 50.4 Gy[31,32].

The literature to date is concentrated primarily on evaluation of adverse events and of RT or immunotherapy in isolation. With improvements in targeted radiation delivery modalities, and technological advances, hypofractionated RT is now utilised without evidence of increased toxicities in a number of malignancies[33,34]. The Hypofractionated RT used in this instance was safe, well tolerated and provided robust survival results in those who could not receive chemoradiotherapy[35]. Furthermore, there is data in lung that conventional radiation dosing and immunotherapy is safe and feasible with no increases in adverse events[36-38].

The ATTRACTION 3 trial demonstrated a 50% reduction in serious adverse events in those treated with nivolumab *vs* conventional chemotherapy in Esophageal Squamous cell carcinoma[39]. In a study evaluating and immunotherapy in renal cell cancer, melanoma and lung, fatigue and pneumonitis were the most common adverse event. They found that toxicity did not correlate with hypofractionation or tumour type. Hypofractionated RT of pulmonary lesions was found to induce a complete response more consistently than in other sites. This study found that combining body Hypofractionated RT with immunotherapy is safe and viable, however, level I evidence is needed[40].

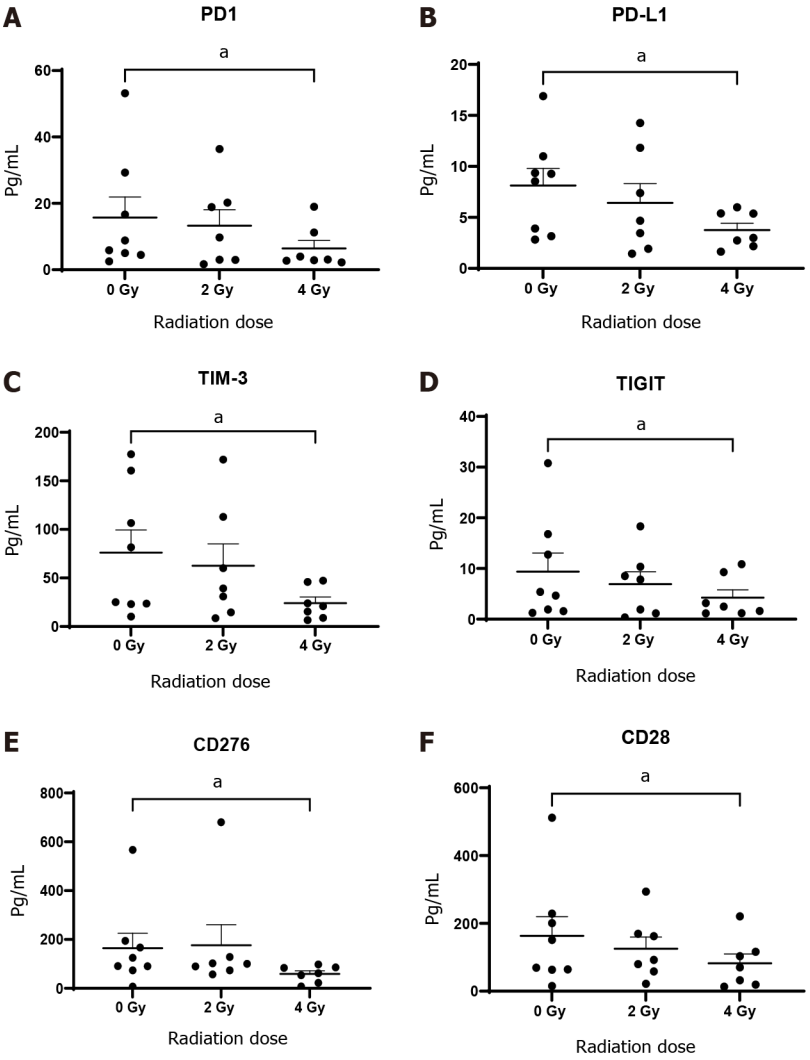


**Figure 6 Oesophageal adenocarcinoma patient's tumour conditioned media were screened by multiplex immunosorbent assay kit.** The inhibitory checkpoints PD-1 and its ligand PD-L1, TIGT, TIM3, immunosuppressive molecule and checkpoint CD276 (B7-H3) and costimulatory molecule CD28 significantly decrease with fractionated radiotherapy ($n$ = 8). A: PD-1; B: PD-L1; C: TIM-3; D: TIGIT; E: CD276; F: CD28. $^{a}P < 0.05$ by Wilcoxon signed rank test.

VEGF is a mitogen essential for angiogenesis and Ramucirumab is approved for use in advanced gastroesophageal cancer patients. The use of anti-VEGF agents have shown promise in promoting improved survival when used in combination with chemoradiotherapy in colon cancer, however treatment resistance is a common problem[41]. This can be due compensatory mechanisms resulting in resistance, namely hypoxia- induced increases of other angiogenic promoters such as PIGF[42]. VEGF, bFGF and PIGF are crucial angiogenic promoters linked with tumourigenesis and Flt-1, is involved in tumour growth and metastatic dissemination, most likely *via* stimulation of macrophage-lineage cells [43]. PIGF/Flt-1 signaling can contribute to colorectal cancer progression through increasing the phosphorylation of p38 mitogen-activated protein kinase (MAPK), thereby upregulating MMP9 expression; resulting in increasing cellular migration/invasion. Therefore inhibition of PIGF/Flt-1 signalling will have therapeutic potential in lower gastrointestinal cancers[44]. In the current study radiation therapy was demonstrated to reduce the expression of these promoters of angiogenesis, which is crucial in the mitigating the risk of metastatic disease for upper gastrointestinal cancers.

The subset of cytokines expressed post radiation and immunotherapy treatment play a key role in determining the subsequent immune response elicited. In this study the OAC tumour tissue released significantly more anti-tumour IL-21 and IL-31 in response to radiation. IL-21 is produced by numerous T helper cells, such as Th1 and Th17 cells, activated Natural Killer T cells[45]. It promotes B cell differentiation into plasma cells, regulates immunoglobulin production, reshaping the tumour microenvironment and influencing the proliferation and/or effector function of both $CD4^+$ and $CD8^+$ T cells, while limiting the differentiation of Tregs[46]. IL-21 has distinct anti-tumour properties as a consequence of its ability to increase the availability of $CD8^+$ T cells through the induction of an early differentiation phenotype and Natural Killer cells[33]. IL-31 has immunoregulatory properties, with a study demonstrating that mice infused with IL-31 had tumour growth disruption and a decreased metastatic burden, supporting the use of IL-31 to offset the risk for metastatic disease development[47]. Similarly,

in a breast cancer murine model, the tumouricidal effects of T cells are increased, and myeloid derived suppressor cells and tumour-associated macrophages are reduced in tumours with high expression of IL-31, with an immunophenotype supporting antitumour immunity[48]. While both IL-21 and IL-31 were significantly increased in the TCM, the expression of IL-23, which has been documented to promote tumour metastases was decreased. IL-23 has metastases promoting properties *via* suppressing the anti-tumour properties of T cells and the anti-metastatic function of NK cells[49]. In addition to this, IL-23 was found to be overexpressed in many human cancers including colorectal and gastric cancer, and was found to be a negative prognostic indicator[50]. Of note CRP, an acute phase protein and marker of vascular injury was found to increase with 2 Gy radiation in our study. There have been epidemiologic studies to suggest that elevated CRP levels in circulation are linked with poorer outcomes in those with solid cancers, whereas elevated levels in apparently healthy subjects, is a potential independent risk factor for future risk of developing cancer of any type including lung, colorectal and gastric cancers due to chronic low inflammatory states, which is of particular relevance in OAC[51]. Therefore, the exact role of CRP in response to RT and immunotherapy requires further study.

Co-stimulatory and IC molecules can have both immunostimulatory and immunosuppressive effects. In this study a range of soluble ICIs and ligands were significantly downregulated following 4 Gy radiation treatment of OAC tumour explants. The role of soluble receptors however, and its effects on immune function remain yet to be elucidated and therefore its potential use as an oncological treatment remain unclear. Through this body of work, we observed a significant down regulation of PD-1, PD-L1, TIM-3 and TIGIT and this was paralleled by a concomitant increase in OAC cell line surface expression and a cohort of OAC tumour explants, which may go some way to explain the decrease in the soluble forms of these IC proteins post irradiation. B7-H3 (also known as CD276) is an IC molecule, with many cancers exhibiting aberrant overexpression and such upregulation is associated with aggressiveness and a poor clinical prognosis[52]. Furthermore, there are studies demonstrating a vital role for B7-H3 in promoting tumourigenesis and metastatic dissemination, proliferation, invasion and migration[52-54]. CD276 promotes tumour proliferation and invasion[55]. In addition, soluble CD276 was found to stimulate the invasion and metastatic dissemination of pancreatic adenocarcinoma cells *via* the Toll like Receptor 4/nuclear factor kappa-light-chain-enhancer of activated B cells pathway (50). Overall, additional studies are required in gastroesophageal cancers to determine the true function of soluble IC proteins and how they pertain to treatment response and immune regulation.

CD28 which is a co-stimulatory molecule is essential in the augmentation of T cell activation and metabolism, driving tumour-infiltrating T cell glycolysis. It is antagonized by CTLA-4 and PD-1[56]. In the current study, soluble CD28 is reduced with radiation, which may be immunosuppressive. Soluble CD80-Fc has been found to maintain IFN-γ release by PD-1$^+$ specific activated T cells even with PD-L1$^+$ tumour cells[57]. Soluble GITR, which was reduced in this study, represents a potential immunotherapeutic target and is found to be expressed at high levels on Tregs[58,59]. A number of phase 1 trials have identified anti-GITR antibodies to have safe pharmacological profiles, with phase II trials ongoing evaluating its combination with RT and anti-PD-1 therapy (NCT04225039). New promising approaches are focusing on the activation of co-stimulatory pathways to enhance antitumour immune responses. GITR activation can result in the inhibition of T-cell (Treg) function and promote effector T-cell function [58], and may also provide theoretical basis for the clinical application of combinations with monoclonal antibody therapy such as bFGF in molecular targeted therapies[60].

Lastly, OX40 has been demonstrated to have a crucial part to play in maintaining the immune responses in the immediate term and ongoing responses through enhancing T cell expansion, differentiation, and survival[61]. OX40 activation can have a significant impact T cell receptor (TCR) signaling through the PI3-K/PKB pathway, influencing T cell division, survival and cytokine production. This can directly increase calcium influx, and lead to IL-2, IL-4, IL-5, and IFN-γ secretion[62]. OX40 triggering regressed Treg cells, thus this allows Dendritic cells to reach the lymph nodes draining the tumour and in doing so prime the tumour-specific CD8 lymphocytes response [63]. However, in the current study radiation induces a downregulation of OX40 in TCM which may indeed be an immunosuppressive consequence of radiation therapy and one which requires further investigation. Again, more robust studies will be helpful to determine the functions of soluble co-stimulatory molecules as they may have alternate functions compared with their cell membrane bound counterparts in the tumour microenvironment.

## CONCLUSION

The introduction of ICIs has resulted in enhanced survival in melanoma and non-small cell lung cancer treatment and has evolved to involve the spectrum of solid gastrointestinal malignancies with positive results of the landmark Checkmate 577 trial in the adjuvant setting most notable to date. However, there remains many issues to be interrogated including an appropriate RT regimen in conjunction with immunotherapies. There is considerable translational, preclinical and clinical data in favour of fractionated RT, and timing of RT delivery and target delineation, there remains disparity and no universal approach applicable to the clinical setting. In the current study, IC blockade in combination

with radiation synergistically reduces viability in radioresistant cells and Nivolumab appears most efficacious. There remains a need to delineate the effects of RT on host anti-tumour immunity. Additionally, lymphopenia induced by RT delivery may negate the effects of immunotherapy on offsetting T cell exhaustion, thus protocols that can minimize lymphopenia need careful design for maximal therapeutic potential. Finally, more concentrated and robust studies to determine and validate potential biomarkers to predict those who will be suitable for these treatment modalities are urgently required with profiling next-generation sequencing of tumour mutation burden based profiling, immune signatures, gene profiling signatures and the repertoire of T-cell receptors potential avenues to elucidate this.

## ARTICLE HIGHLIGHTS

### *Research background*

Oesophageal cancer is represents a difficult treatment dilemma with poor 5 year overall survival due to presentation at advanced stages due to its indolent nature as well as poor treatment responses to conventional therapies.

### *Research motivation*

The advent of immunotherapy represents a shift in the multimodal treatment paradigm for esophageal cancer and has had mixed results in many solid tumours to date. The Checkmate 577 trial is a landmark study and is sure to revolutionize immune checkpoint blockade as the treatment modality of choice in the adjuvant setting.

### *Research objectives*

To determine the impact of radiotherapy (RT) on immune checkpoint expression, and to determine the prevailing immune milieu in terms of markers of angiogenesis, cytokines and metastatic markers.

### *Research methods*

This hybrid *in vitro* and *ex vivo* study is a mixture of flow cytometry, enzyme-linked immunosorbent assay kit work and cell viability by a cell counting kit-8 assay.

### *Research results*

Radiation results in a decrease in angiogenic and metastatic markers with an increase in anti-tumour cytokines. There were two distinct subpopulations, with one cohort of patients demonstrating increased checkpoint expression as a consequence of radiation and a separate cohort demonstrating the opposite effects. The cohort with increased checkpoint expression had poorer treatment responses and were associated with adverse tumour biology.

### *Research conclusions*

Oesophageal cancer represents an immune active tumour and is a viable target in both the neoadjuvant and adjuvant setting and should be combined with RT to exert maximum synergistic effects.

### *Research perspectives*

This seminal study is the first of its kind and is a truly clinical and translational evaluation of the immune landscape of esophageal cancer.

## FOOTNOTES

**Author contributions:** Donlon NE and Davern M contributed equally to this work; Donlon NE and Davern M contributed to experimental design and execution, and manuscript drafting and revision; O'Connell F and Sheppard A contributed to experiments; Heeran A, Bhardwaj A, and Butler C contributed to sample acquisition; Narayanasamy R, Donohoe C, Phelan JJ, Lynam-Lennon N, Dunne MR, and Maher S contributed to concept design; Phelan JJ contributed to statistical analysis; O'Sullivan J, Reynolds JV, and Lysaght J contributed to paper revision and supervision of the project.

**Institutional review board statement:** The study was reviewed and approved by the Tallaght/St James's Hospital Ethics Committee.

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**Data sharing statement:** Data generated in this study will be available upon specific request from the corresponding author.

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ORIGINAL ARTICLE

Case Control Study

# Intermittent hypoxia is involved in gut microbial dysbiosis in type 2 diabetes mellitus and obstructive sleep apnea-hypopnea syndrome

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## Abstract

***BACKGROUND***

Obstructive sleep apnea (OSA)-hypopnea syndrome (OSAHS) has been recognized as a comorbidity of type 2 diabetes mellitus (T2DM); more than half of T2DM patients suffer from OSAHS. Intermittent hypoxia (IH) plays an important role in metabolic diseases, such as obesity and OSAHS, through various mechanisms, including altering the gut microecological composition and function. Therefore, it is important to study the role of gut microbiota in T2DM patients with OSAHS, which has a high incidence and is prone to several complications.

***AIM***

To assess whether IH is involved in altering the fecal microbiome in T2DM patients with OSAHS.

***METHODS***

Seventy-eight participants were enrolled from Henan Province People's Hospital and divided into healthy control (HC, $n$ = 26), T2DM ($n$ = 25), and T2DM + OSA ($n$ = 27) groups based on their conditions. The fecal bacterial DNA of the research participants was extracted and subjected to 16S ribosomal RNA sequencing. The clinical indices, such as insulin resistance index, homocysteine (HCY) concentration, and the concentrations of inflammatory factors in the peripheral blood, were assessed and recorded.

***RESULTS***

Group T2DM + OSA had the highest apnea-hypopnea index (AHI) (2.3 *vs* 3.7 *vs* 13.7), oxygen desaturation index (0.65 *vs* 2.2 *vs* 9.1), HCY concentration (9.6

μmol/L *vs* 10.3 μmol/L *vs* 13.81 μmol/L) and C-reactive protein (CRP) concentrations (0.3 mg/L *vs* 1.43 mg/L *vs* 2.11 mg/L), and lowest mean oxygen saturation (97.05% *vs* 96.6% *vs* 94.7%) among the three groups. Twelve and fifteen key differences in amplicon sequence variants were identified when comparing group T2DM + OSA with groups T2DM and HC, respectively. We found progressively decreased levels of *Faecalibacterium*, *Eubacterium,* and *Lachnospiraceae,* and an increase in the level of *Actinomyces,* which strongly correlated with the HCY, CRP, fasting plasma glucose, and hemoglobin A1c concentrations, AHI, mean oxygen saturation, and insulin resistance index in group T2DM + OSA ($P < 0.05$).

***CONCLUSION***

For T2DM patients with OSAHS, IH may be involved in selective alterations of the gut microbiota, which may affect the pathophysiological development of T2DM and DM-related complications.

**Key Words:** Gut microbiota; Obstructive sleep apnea-hypopnea syndrome; Type 2 diabetes mellitus; Intermittent hypoxia; Obstructive sleep apnea



**Core Tip:** Clinically, type 2 diabetes mellitus (T2DM) patients have a significantly higher prevalence of obstructive sleep apnea-hypopnea syndrome (OSAHS) than non-T2DM patients and are more prone to diabetes-related complications and metabolic syndrome, including obesity and hypertension. In recent years, the imbalance of gut microbiota has been found to be associated with various metabolic disorders. This study revealed that intermittent hypoxia was associated with changes in the gut microbiota in patients with T2DM complicated by OSAHS. These changes may be involved in the progression of metabolic disorders through increased proinflammatory factors and impaired intestinal barrier function.

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## INTRODUCTION

Obstructive sleep apnea (OSA)-hypopnea syndrome (OSAHS) is characterized by recurrent partial or complete pharyngeal obstruction during sleep[1], resulting in intermittent hypoxia (IH); cyclical adverse changes in heart rate, blood pressure (BP), and sympathetic activity; and disruption of sleep architecture [2]. Epidemiological studies have shown that OSAHS is a globally prevalent chronic sleep disorder, especially in adults with type 2 diabetes mellitus (T2DM), with rates varying from 23% to 87%[3,4]. Moderate to severe OSAHS has been found to be associated with an increase in the incidence of T2DM, and the incidence of OSAHS in patients with T2DM was > 50% higher than that in those without diabetes, independent of traditional risk factors, such as obesity and other confounders. IH, a hallmark of OSAHS, plays an important role not only in the pathogenesis of OSAHS but also in reducing glycemic control and insulin resistance in patients with T2DM and diabetes-related complications[5-7], probably by activating several systematic inflammatory mediators and enhancing the oxidative stress cascade and hypothalamic-pituitary-adrenal function, among others[8].

Commensal bacteria, termed microbiota, cover every surface of our body exposed to the external environment, with 70% of these residing in the gastrointestinal tract[9]. The microbiome is a vast and complex polymicrobial ecosystem that coexists with the human organism and has been identified as playing important roles in the development of host immunological phenotypes. Gut microbiota dysbiosis has been linked to a series of metabolic disorders, such as diabetes and hypertension[10]. In a study of IH mimicking OSAHS, in an *Ldlr*$^{-/-}$ mice fed a high-fat diet, the imbalance of gut microbiota was found to be associated with adverse cardiovascular events and metabolic disorders[11,12]. Similarly, sleep fragmentation induced by OSAHS alters feeding behavior and promotes obesity and metabolic abnormalities, while the host gut microbiome changes, leading to increased intestinal permeability and chronic inflammation of adipose tissues[11,12]. For T2DM patients with OSAHS, only a few studies have focused on the relationship between severe metabolic disorders caused by IH and gut microbial dysbiosis, independent of conventional risk factors, such as obesity and hypertension. Hence, our study aimed to investigate the gut microbiota changes in T2DM patients with OSAHS.

Clinical indices, such as inflammatory factors and homocysteine (HCY), were compared for the groups as well.

## MATERIALS AND METHODS

### Participants

In total, 78 participants, including 25 hospitalized patients with T2DM (Group T2DM), 27 hospitalized patients with T2DM complicated by OSAHS (Group T2DM + OSA), and 26 healthy controls (HC) who were examined with a type IV portable monitor (PM, Sleep Fairy-A7, China) overnight (from 10 p.m. to 7 a.m.), were recruited from July 2019 to July 2020. Most of the sleep recording took place in a quiet and comfortable hospital ward at Henan Provincial People's Hospital; the remainder was performed by participants sleeping at home after being systematically trained in using the PM. The patient-reported periods before sleep onset and after awakening in the morning were excluded before manual scoring [13]. We collected fasting blood and fecal samples the next morning. General questionnaires were used to collect information on demographic characteristics and health status.

### OSAHS assessment

Pulse oximetry was used to assess oxyhemoglobin saturation, and respiratory effort was measured with a pneumatic sensor attached to an effort belt. Nasal airflow was recorded using a nasal cannula connected to a pressure transducer. The final data were automatically generated by the software system. The scoring rules were based on the 2007 American Academy of Sleep Medicine manual. The apnea-hypopnea index (AHI) was calculated as the total number of episodes of apnea (continuous cessation of airflow for at least 10 s) and hypopnea (reduction in airflow for ≥ 10 s with oxygen desaturation of ≥ 4%) divided by the total duration of sleep events, and OSAHS was defined as an AHI of 5 events/h.

### Inclusion and exclusion criteria

The inclusion criteria were as follows: (1) Age of 18-70 years; (2) Treatment-naïve type 2 diabetes based on the 1999 World Health Organization Criteria[14]; (3) 6.5% ≤ glycated hemoglobin A1c (HbA1c) ≤ 11%; (4) No antibiotic use within the 12 wk before enrollment and no probiotics and/or prebiotics; and (5) No glucose-lowering drugs other than insulin during the 12 wk before enrollment in patients with diabetes.

The exclusion criteria were as follows: (1) Body mass index (BMI) of > 28 kg/m$^2$; (2) Diagnoses of chronic respiratory disease, central system sleep apnea syndrome, severe heart failure, BP of ≥ 140/90 mmHg, and severe organic diseases such as cancer, myocardial infarction, and stroke; (3) Diagnoses of other types of diabetes, for example, type 1 diabetes; (4) Diagnoses of inflammatory bowel disease, coagulation disorders, connective tissue diseases, or gastrointestinal surgery, except for appendicitis and hernia surgery; (5) Participation in other programs during the previous 3 mo; (6) Alcoholism (drinking more than five times in 1 wk: > 100 g of spirits, 250 g of rice wine, or 5 bottles of beer); (7) Pregnancy; and (8) Taking drugs that may affect respiratory function, for example, anxiolytics, hypnotics, or mood stabilizers.

### Sample collection

Biological samples and anthropometric data were obtained without medical treatment. Blood samples were collected after overnight fasting and centrifuged using centrifuge (Multifuge X3R, Thermo Fisher Scientific, United States) at 3000 rpm for 20 min after standing at 24 °C for 30 min to obtain serum. Fresh fecal samples, morning urine, and serum samples were immediately frozen on dry ice after collection and stored at -80 °C until further analysis.

### Clinical indices

Fasting plasma glucose (FPG) concentrations were measured with the Automatic Biochemical Analyzer (TBA-120 FR, Toshiba, Japan). Fasting insulin concentrations were measured using immunochemiluminometric assays (ADVIA Centaur, Siemens A.G., German). Plasma HbAlc concentrations were measured by high-performance liquid chromatography (Bio-Rad D-10, Bio-Rad Laboratories Co., Ltd., Germany). Routine blood tests were performed using the Swelab Alfa Cell analyzer (Boule Diagnostics AB, Sweden). The Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was calculated using the following formula: FPG*FIN/22.5, where FIN is fasting insulin.

### Inflammatory factors and HCY

C-reactive protein (CRP) and HCY concentrations were measured with the Automatic Biochemical Analyzer. The determination of the plasma concentrations of the lipopolysaccharide-binding protein (LBP), interleukin (IL)-6, IL-17, and transforming growth factor (TGF)-β1 was performed using human ELISA kits according to the manufacturer's instructions. The ELISA kits for the assessment of the concentrations of human LBP, tumor necrosis factor (TNF)-α, IL-17, and TGF-β1 were all from Cusabio

Biotech, Wuhan, China. The kits for high sensitivity IL-6 were purchased from Multi-Science, Hangzhou, China.

### 16S rRNA gene amplification sequencing

The DNA of the participants was extracted using the E.Z.N.A.® Stool DNA Kit (Omega Bio-tek, Inc., Norcross, GA, Unite States). The isolated DNA was used as the template for polymerase chain reaction (PCR) amplification of the V3-V4 region of 16S rRNA genes. The forward primer (341F) was 5'-CCTACGGGNGGCWGCAG-3', and the reverse primer (805R) was 5'-GACTACHVGGGTATC-TAATCC-3.' PCR was performed with an EasyCycler 96 PCR system (Analytik Jena Corp., AG), and the products from different samples were indexed and mixed at equal ratios for sequencing by Shanghai Mobio Biomedical Technology Co. Ltd. using the Miseq platform (Illumina Inc., San Diego, CA, Unite States) according to the manufacturer's instructions.

### Sequencing data analysis

Amplicon sequence variants (ASVs) were identified with the DADA2 algorithm. The representative sequences for each ASV were annotated using the SILVA reference database (SSU138). The alpha diversity metrics (Shannon–Wiener diversity index and Simpson diversity index) were assessed by using Mothur v1.42.1. The non-parametric Mann–Whitney U test was used to test for significant differences between the two groups. The comparison of multiple groups was performed using a nonparametric Kruskal–Wallis test. Principal coordinates analysis (PCoA) based on Bray–Curtis and unweighted UniFrac distance was conducted using the R program (version 3.6.0, http://www.R-project.org/) to display microbiome space between samples. The key ASVs associated with T2DM and OSA were identified by random forest models, and those significantly associated with BMI selected by MaAsLin2 (https://github.com/biobakery/Maaslin2) were removed. A heatmap plot was drawn to indicate the distributions of the rest of the key ASVs. The linear discriminant analysis effect size (LEfSe) method (lefse 1.1, https://github.com/SegataLab/Lefse) was used to detect taxa with differential abundance. PICRUSt2 v2.4.1 (https://github.com/picrust/picrust2/wiki) was used to predict functional abundance based on 16S rRNA gene sequences.

### Statistical analyses

Continuous variables are expressed as means ± SD or medians with interquartile ranges for normal distribution data or non-normal distribution data, respectively. Categorical variables are expressed as percentages. All the statistical analyses, including one-way analysis of variance, Kruskal–Wallis test, Mann-Whitney U test, and least significant difference *t*-test, were performed using SPSS version 26.0, with a two-sided $P$ value of $< 0.05$ denoting statistical significance. Significant differences were adjusted by Bonferroni correction. The correlation coefficients for the concentrations of the gut microbiota, inflammatory factors, and HCY were evaluated using Spearman correlation.

## RESULTS

### Clinical parameters and inflammatory levels among three groups

Applying the strict inclusion and exclusion criteria described above, a total of 78 fecal samples were collected for analysis after PM assessment. We characterized the clinical indices and gut microbiomes of patients in groups HC, T2DM, and T2DM + OSA (Figure 1). There were no significant differences in sex and age among the three groups ($P > 0.05$). Compared with group T2DM, there were no significant differences in the insulin resistance index (HOMA-IR), BMI, waist-to-hip ratio, neck circumference, FPG, HbA1c, systolic BP, and diastolic BP in group T2DM + OSA; however, they were significantly increased compared with those in group HC ($P < 0.05$).

The HCY level was the highest in group T2DM + OSA compared with the other two groups. Our PM data revealed that the AHI and oxygen desaturation index (ODI) increased significantly, accompanied by a decrease in the mean oxygen saturation ($SpO_2$); group T2DM + OSA had lower $SpO_2$ than group HC. Further details on the clinical parameters of the participants with diverse severities are shown in Table 1.

The concentration of CRP increased gradually from the HC group to the T2DM group and even more in the T2DM + OSA group. In addition, the levels of TNF-α, IL-17, IL-6, and LBP increased and the levels of TGF-β1 decreased in group T2DM + OSA compared with those in group HC, while the level of TNF-α increased compared with that in group T2DM ($P < 0.05$) (Figure 2 and Supplementary Table 1).

### IH is related to differences in the gut microbiota of T2DM + OSA patients

The resulting rarefaction curves showed that the microbial richness of the sampled guts was near saturation at the applied sequencing depth (Supplementary Figure 1), which was sufficient to identify most of the bacterial community members for each microbiome. The alpha diversity of the gut microbiota expressed by the Shannon estimator, ACE estimator, and Simpson index (Figure 3A-C and

**Table 1 Participant characteristics and clinical parameters**

| | HC (*n* = 26) | T2DM (*n* = 25) | T2DM + OSA (*n* =27) | *P* value |
|---|---|---|---|---|
| Sex, male/female | 17/9 | 17/8 | 21/6 | 0.584 |
| Age, yr | 45.58 ± 8.81 | 45.92 ± 13.89 | 47.59 ± 5.18 | 0.728 |
| BMI, kg/m$^2$ | 24.63 ± 2.61 | 25.84 ± 3.45 | 27.03 ± 2.11[b] | 0.009 |
| WHR, cm | 0.85 ± 0.06 | 0.95 ± 0.07 | 0.98 ± 0.05[b] | < 0.001 |
| NC, cm | 32.88 ± 3.70 | 36.25 ± 4.75 | 38.37 ± 2.31[b] | < 0.001 |
| FPG, mmol/L | 5.30 (4.70, 5.43) | 6.90 (6.30, 8.05) | 8.00 (6.40, 9.25)[b] | < 0.001 |
| HOMA-IR | 1.50 (1.09, 2.03) | 2.10 (1.02, 3.53) | 2.38 (1.37, 3.22)[a] | 0.099 |
| HbA1c, % | 5.30 (5.10, 5.60) | 9.70 (7.30, 10.80) | 8.70 (7.70, 9.70)[b] | < 0.001 |
| SBP, mmHg | 117.50 (112.00, 123.25) | 130.00 (117.00, 143.00) | 131.00 (128.00, 144.00)[b] | < 0.001 |
| DBP, mmHg | 71.96 ± 5.44 | 81.13 ± 11.33 | 82.26 ± 8.58[b] | < 0.001 |
| HCY, μmol/L | 9.60 (8.30, 12.53) | 10.30 (8.05, 12.03) | 13.81 (10.73, 20.54)[b,c] | < 0.001 |
| AHI | 2.30 (1.48, 3.05) | 3.70 (2.00, 4.15) | 13.70 (9.80, 20.10)[b,c] | < 0.001 |
| Mean $SpO_2$, % | 97.05 (96.50, 97.53) | 96.60 (96.05, 96.95) | 94.70 (93.80, 95.20)[b,c] | < 0.001 |
| Lowest $SpO_2$, % | 85.50 (82.00, 90.25) | 85.00 (76.00, 87.50) | 81.00 (73.00, 84.00)[b] | 0.002 |
| ODI | 0.65 (0.40, 1.23) | 2.20 (1.10, 5.45) | 9.10 (5.90, 15.30)[b,c] | < 0.001 |

[a]*P* < 0.05 *vs* HC.
[b]*P* < 0.01 *vs* HC.
[c]*P* < 0.01 *vs* T2DM.
*P* value: Comparison of three groups. Data are presented as the mean ± SD or median with interquartile range. The *P* value was based on Kruskal–Wallis test or one-way ANOVA. Mann–Whitney U test or least significant difference *t*-test was used to compare two groups. Significant differences were adjusted by Bonferroni correction. WHR: Waist-hip ratio; NC: Neck circumstance; FPG: Fasting plasma glucose; HOMA-IR: Insulin resistance index; HbA1c: Hemoglobin A1c; SBP: Systolic pressure; DBP: Diastolic pressure; HCY: Homocysteine; AHI: Apnea–hypopnea indices; ODI: Oxygen desaturation index; HC: Healthy control; T2DM: Type 2 diabetes mellitus; OSA: Obstructive sleep apnea.

Supplementary Table 2) showed that there were no significant differences in groups HC, T2DM, and T2DM + OSA ($P > 0.05$). The overall structures of the gut microbiota in the three groups showed a minimal difference as revealed by the PCoA plot (Figure 3D); however, the difference was not significant (Adonis, $P > 0.05$).

We further analyzed the taxonomic composition and alterations of the gut microbiome. The composition and abundance of the bacterial community in each sample at the phylum and genus levels are shown in Supplementary Figure 2. At the phylum level, *Firmicutes* and *Proteobacteria* were the dominant bacteria in groups HC, T2DM, and T2DM + OSA (Figure 4A). At the genus level, we found no significant differences in the relative abundance of *Escherichia−Shigella,* which had the highest abundance among the three groups ($P > 0.05$) (Figure 4B). The relative abundance of the following genera significantly differed among groups: *Faecalibacterium* ($P = 0.0434$), *Streptococcus* ($P = 0.0393$), *Haemophilus* ($P = 0.0286$), *Phascolarctobacterium* ($P = 0.0242$), and *Oscillibacter* ($P = 0.0274$) (Figure 4C). Among the above genera, the levels of *Phascolarctobacterium* decreased and the levels of *Oscillibacter* increased in group T2DM + OSA compared with group T2DM, and the levels of *Faecalibacterium* significantly decreased in group T2DM + OSA compared with group HC. A gradually decreasing trend of *Faecalibacterium* abundance from group HC to groups T2DM and T2DM + OSA was observed (Figure 4C). Through LEfSe, we also found a significant decrease in the level of *Faecalibacterium* in group T2DM + OSA (Figure 4D).

We found 12 ASVs associated with gut microbial dysbiosis in group T2DM + OSA compared with group T2DM, including ASV632 (*Streptococcus*), ASV450 (*Clostridiaceae_Clostridium_sensu_stricto*), ASV352 (*Faecalibacterium*), ASV511 (*Roseburia*), ASV307 (*[Eubacterium]_hallii_group*), ASV1000 (*[Eubacterium]_eligens_group*), ASV995 (*Blautia*), ASV584 (*Eggerthella*), ASV535 (*Erysipelotrichaceae*_UCG-003), ASV87 (*Phascolarctobacterium*), ASV1112 (*Prevotella*), and ASV548 (*Oscillibacter*). On comparing groups HC and T2DM + OSA, 15 ASVs were different: ASV450 (*Clostridiaceae_Clostridium_sensu_stricto*), ASV511 (*Roseburia*), ASV1000 (*[Eubacterium]_eligens_group*), ASV266 (*Lachnospiraceae_unclassified*), ASV763 (*Lachnospiraceae_unclassified*), ASV367 (*Faecalibacterium*), ASV779 (*Actinomyces*), ASV986 (*Haemophilus*), ASV352 (*Faecalibacterium*), ASV436 (*Faecalibacterium*), ASV265 (*Streptococcus*), ASV535 (*Erysipelotrichaceae_UCG-003*), ASV68 (*Blautia*), ASV347 (*Saccharimonadales*), and ASV1022 (*Acinetobacter*) (Figure 5A). Therein, the relative abundance of ASV1022 (*Acinetobacter*), ASV367 (*Faecalibacterium*),

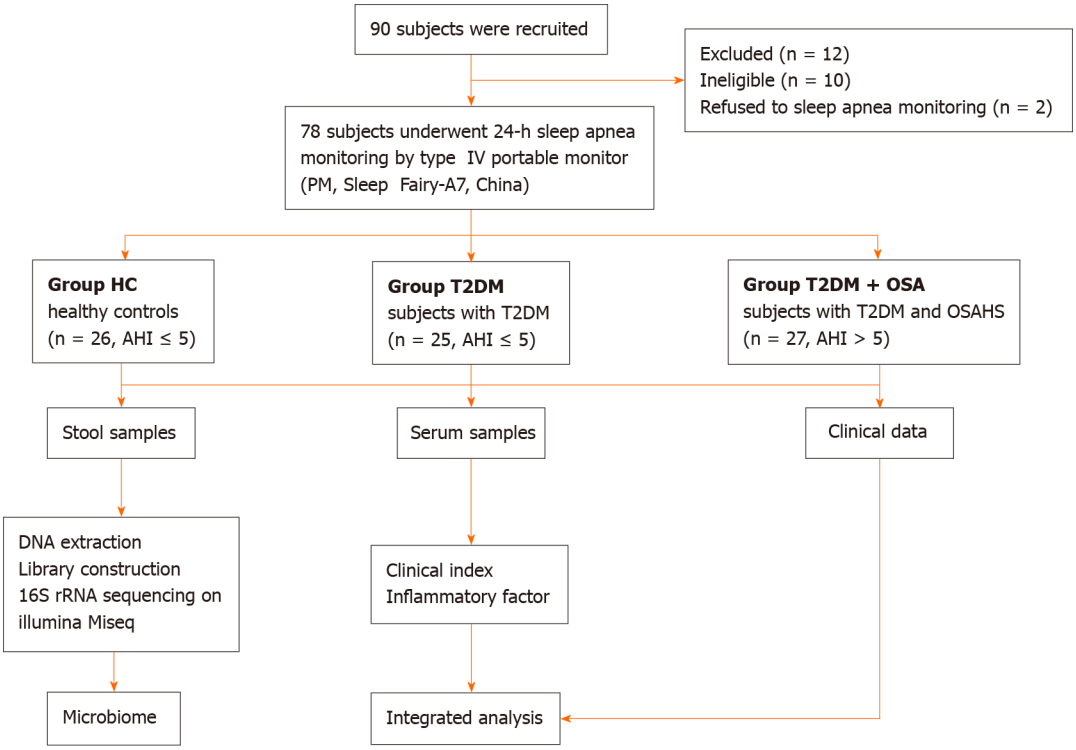


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**Figure 1 Flowchart of the study.**

ASV436 (*Faecalibacterium*), ASV763 (*Lachnospiraceae_unclassified*), and ASV266 (*Lachnospiraceae_unclassified*) were progressively decreased, and the abundance of ASV779 (*Actinomyces*) increased from group HC to group T2DM and to group T2DM + OSA (Figure 5B).

### *IH-related gut microbiota dysbiosis was associated with abnormal metabolic and inflammatory indicators of T2DM + OSA patients*

We further studied the correlations among metabolic indicators, inflammatory factors, and the above six key ASVs with ascending or decreasing trends. Spearman correlation analysis revealed that the decreased abundance of ASVs showed a significant negative correlation with indicators related to IH or respiratory disorders, such as the ODI and AHI; glucose metabolism indicators, such as HbA1c, FPG, and HOMA-IR; cardiovascular disease-related metabolic indicators, such as HCY; and inflammatory factors, such as CRP, TNF-α, and LBP. The ASV436 (*Faecalibacterium*) was negatively correlated with the AHI, ODI, FPG, HbA1c, CRP concentrations, and HOMA-IR, while the ASV763 (*Lachnospiraceae_unclassified*) was negatively correlated with the AHI and FPG, HbA1c, CRP, and LBP concentrations ($P < 0.05$). Furthermore, the ASV436 (*Faecalibacterium*), ASV1000 (*[Eubacterium]_eligens_group*), and ASV367 (*Faecalibacterium*) were positively correlated with the mean $SpO_2$, another IH-related indicator. Among the three groups, the gradually increasing abundance of ASV779 (*Actinomyces*) showed an opposite relationship; it was significantly positively correlated with the AHI, ODI, and TNF-α concentration and negatively correlated with the mean $SpO_2$ ($P < 0.05$) (Figure 6 and Supplementary Table 3).

## DISCUSSION

T2DM has been linked to gut dysbiosis and chronic inflammation in several clinical and animal experiments[15-17], which may be a consequence of the loss of or deficiency in a beneficial function, such as short-chain fatty acid (SCFA)-producing bacteria production from carbohydrate fermentation, in the gut ecosystem[16]. Diseases previously partially attributed to lifestyle, such as obesity and OSAHS, are now considered microbiota-related as well[12,18]. Although much epidemiological and clinical evidence has suggested that OSAHS is an independent risk factor for the development of T2DM[19], the underlying pathogenesis of altered glucose metabolism in T2DM patients with OSAHS remains to be elucidated. Meanwhile, a longitudinal cohort study over a period of 6 years found that the insulin resistance index (HOMA-IR) was a predictor of incident "witnessed apnea", independent of obesity [20]. This showed that dysglycemia and insulin resistance may lead to the development of OSAHS. Together with the findings of the above-mentioned studies, this strongly suggests that the relationship

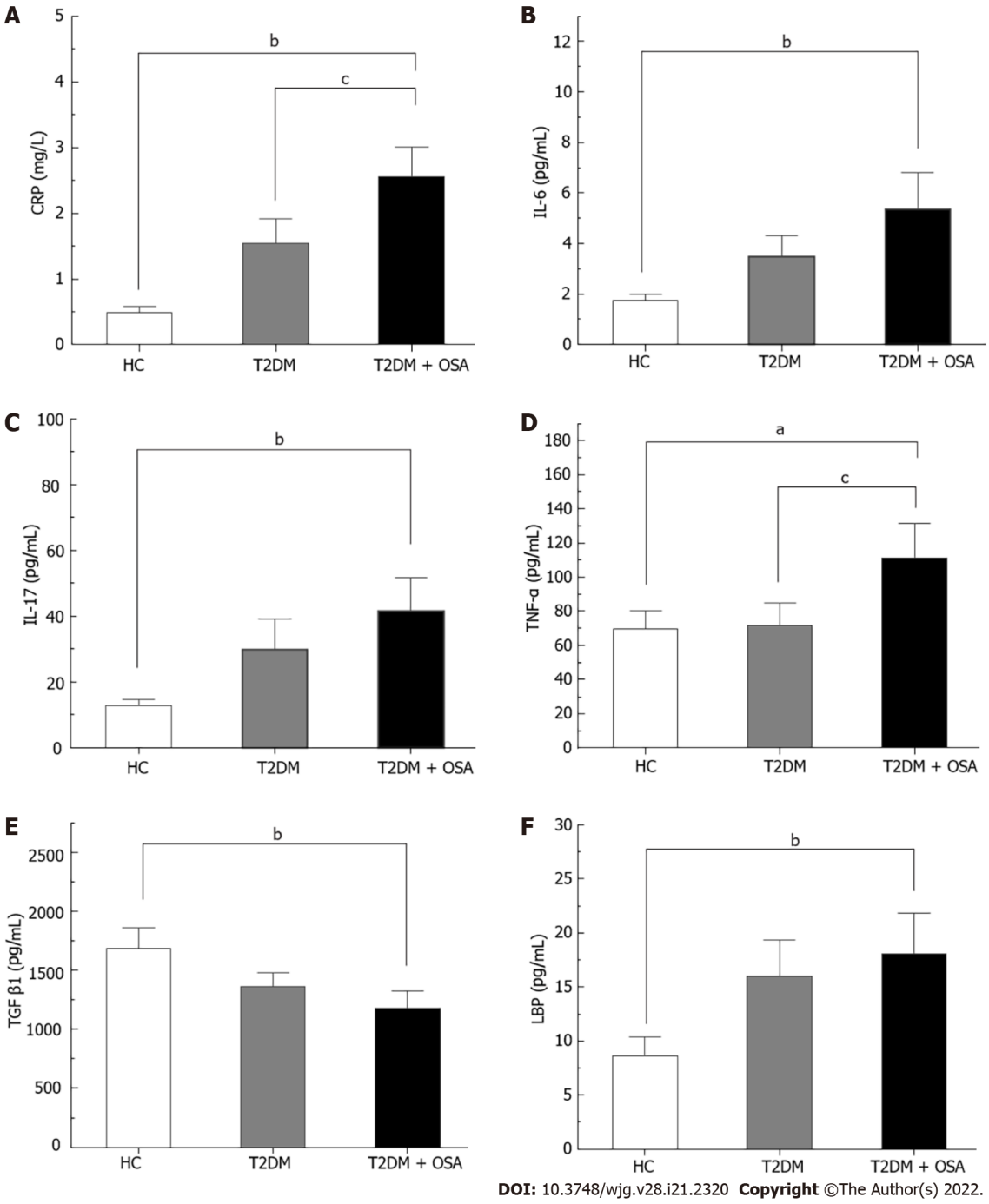


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**Figure 2 The concentrations of inflammatory factors in groups healthy control, type 2 diabetes mellitus, and type 2 diabetes mellitus plus obstructive sleep apnea.** A: CRP; B: IL-6; C: IL-17; D: TNF-α; E: TGF-β1; F: LBP. Bar charts show the means ± SE of the mean. [a]*P* < 0.05 *vs* healthy control (HC); [b]*P* < 0.01 *vs* HC; [c]*P* < 0.05 *vs* type 2 diabetes mellitus. The *P* value was based on Kruskal–Wallis test or one-way ANOVA. A significant difference was shown by the least significant difference *t*-test or Mann–Whitney U test. Significant differences were adjusted by Bonferroni correction. CRP: C-reaction protein; IL-6: Interleukin-6; IL-17: Interleukin-17; TNF-α: Tumor necrosis factor-α; TGF-β1: Transforming growth factor beta 1; LBP: Lipopolysaccharide-binding protein; HC: Healthy control; T2DM: Type 2 diabetes mellitus; OSA: Obstructive sleep apnea.

between T2DM and OSAHS may be bidirectional[2]. Therefore, it is necessary to investigate whether the imbalance of the intestinal microbiota plays a key role in the pathophysiology underlying metabolic dysfunction of patients with T2DM complicated by OSAHS.

In our study, the sequencing analysis of the 16S rRNA gene-tags applied to fecal samples from T2DM patients complicated by OSAHS showed differences in the relative abundances of the predominant taxa of the genera levels. We found that the concentrations of various IH-related gut bacteria, including SCFA-producing bacteria such as *Faecalibacterium* and *Lachnospiraceae,* were significantly correlated with the concentrations of FPG HbA1c and HOMA-IR, as well as the concentration of HCY, a risk predictor of hypertension and arteriosclerosis[21,22]. IH can result in hypoxia/re-oxygenation cycling events within the gut microbiome and, as a result, the biological diversity of gut microorganisms may be

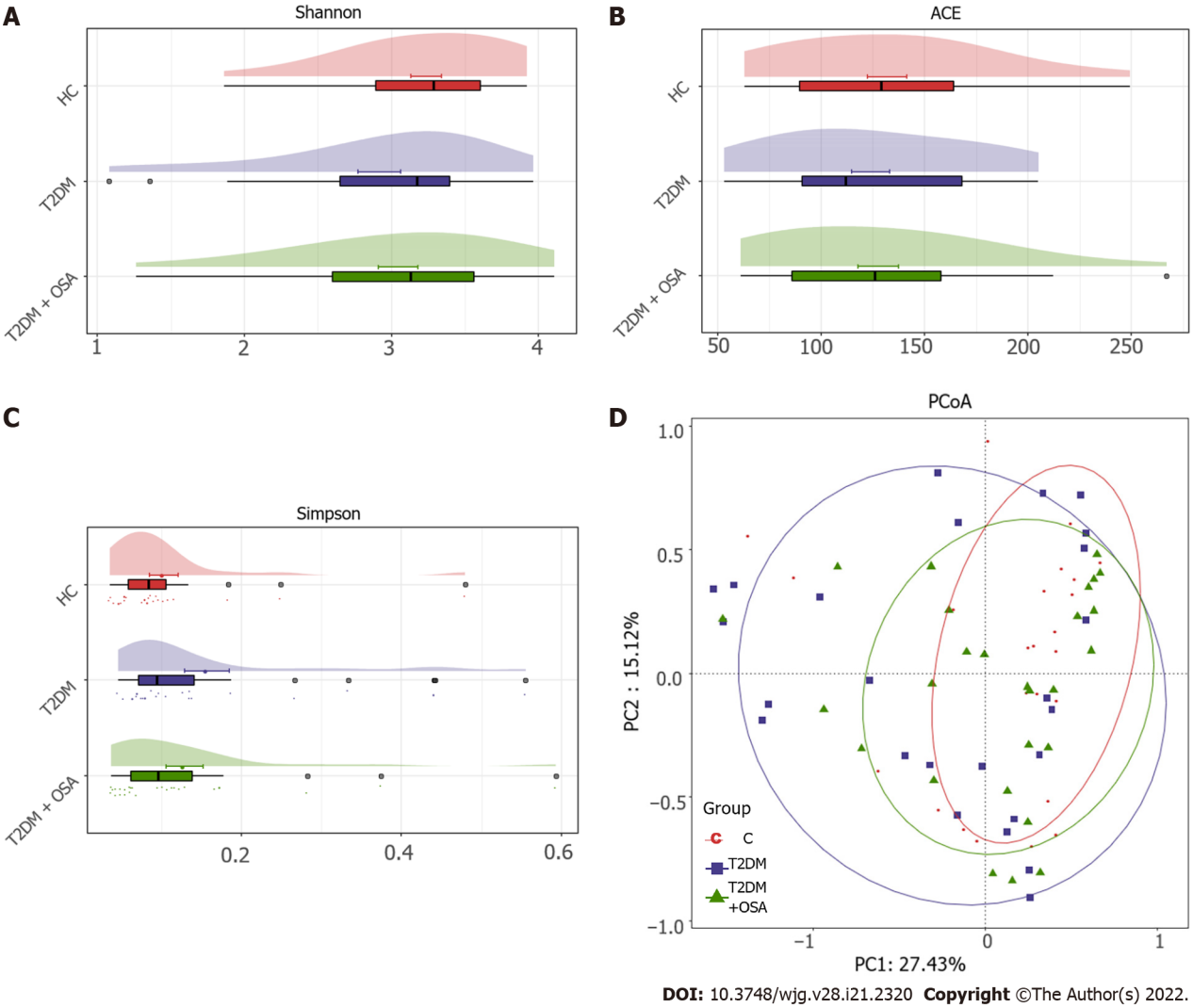


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**Figure 3 The gut microbial diversity and structures of the gut microbiota in groups healthy control, type 2 diabetes mellitus, and type 2 diabetes mellitus plus obstructive sleep apnea.** A: Shannon index; B: ACE estimator; C: Simpson index; D: Principal coordinates analysis plot of the gut microbiota among groups healthy control, type 2 diabetes mellitus, and type 2 diabetes mellitus plus obstructive sleep apnea. HC: Healthy control; T2DM: Type 2 diabetes mellitus; OSA: Obstructive sleep apnea.

modified[16]. Although the intestinal epithelium is significantly resistant to hypoxia, regulating the absorption and barrier function of the intestinal epithelium is sensitive to the oxygen level in the intestine[23]. Hypoxia/re-oxygenation can directly impair cellular function *via* an increase in permeability and bacterial translocation and a decrease in tight junction integrity[16]. In addition, studies have shown that after prolonged normoxic recovery after IH exposure, the gut microbiota and circulating endotoxemia remain negatively affected[8]. Our results do not show significant differences in α-diversity and β−diversity. However, the gradual decrease in the relative abundance of SCFA-producing bacteria (such as the ASVs of *Faecalibacterium*, *Eubacterium*, and *Lachnospiraceae*) associated with abnormal indicators of oxygen metabolism, as well as elevated levels of inflammatory indicators (including CRP, IL-17, and TNF-α), which are critically involved in the development of insulin resistance and pathogenesis of T2DM, was observed in T2DM + OSA patients[24]. Emerging evidence shows that SCFAs can modulate glycemic control, exhibit anti-inflammatory and antitumorigenic activity, and decrease oxidative stress[25-28]. Short chain fatty acids contribute to mucin synthesis, decrease bacterial translocation, maintain gut integrity, and mitigate inflammation in the intestine[29, 30]. Thus, we speculated that SCFAs may be regarded as potential targets for recognizing metabolic comorbidities in patients with T2DM complicated by OSAHS.

There are several potential mechanisms by which IH mediates its effect on metabolic dysfunction. It induces macrophages to polarize toward the pro-inflammatory subtype of M1, leading to the production of more pro-inflammatory mediators in visceral adipose tissue, such as TNF-α, IL-6, and IL-8, resulting in subsequent impairment of insulin signaling pathways and insulin resistance[7]. Here, we found that the concentration of *Lachnospiraceae* was negatively correlated with that of LBP, which is one of the reference indicators of intestinal barrier disruption, suggesting that the induction of inflammatory

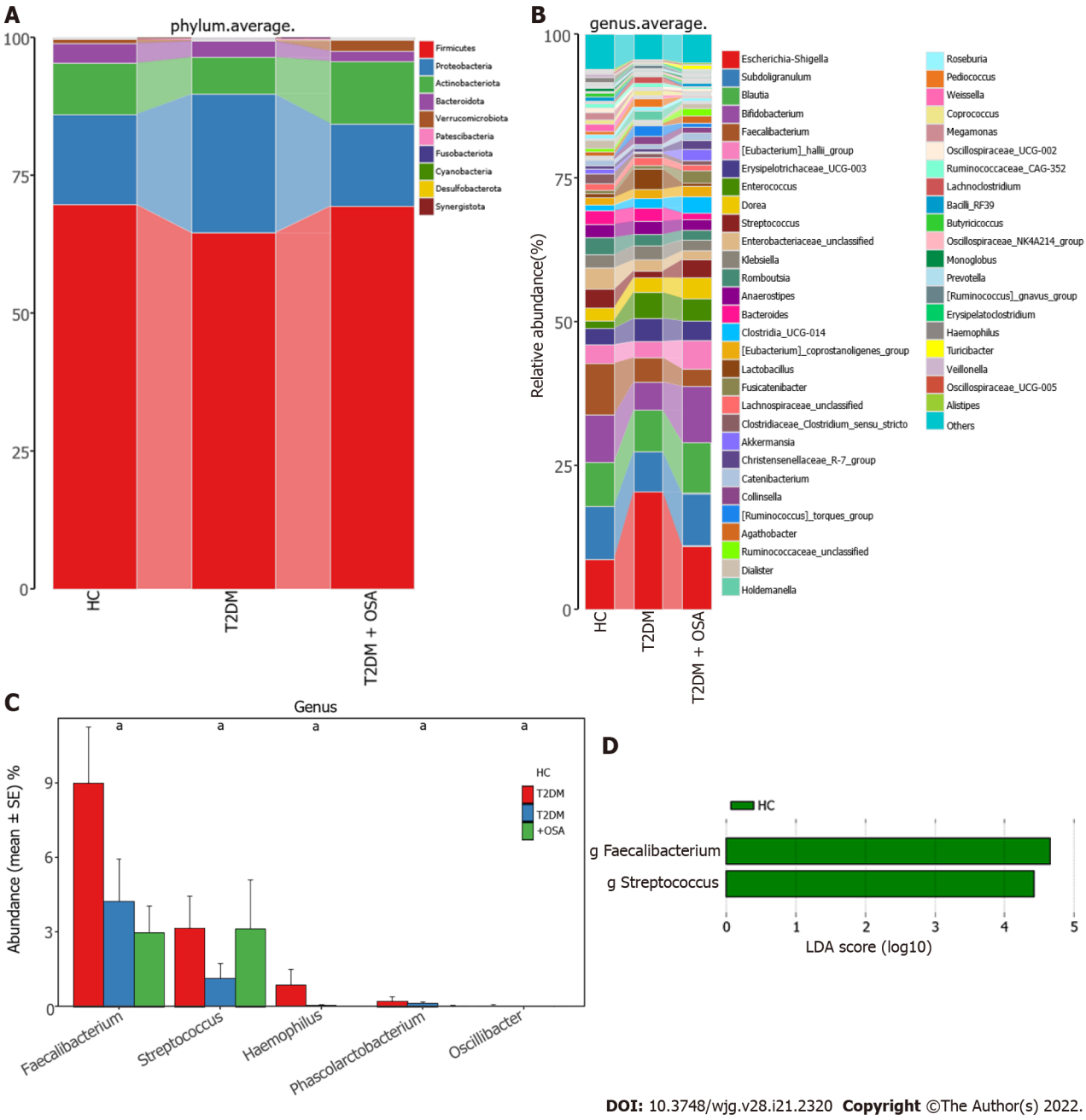


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**Figure 4 Average compositions and relative abundance of the bacterial community among three groups at different levels.** A: Average relative abundance histogram of dominant species composition stratified by group at the phylum levels; B: Average relative abundance histogram of dominant species composition stratified by group at the genus levels; C: Histograms for genera with significant differences. Bar chart showing the mean ± SE of each group; D: Linear discriminant analysis analysis column diagram. LDA: Linear discriminant analysis. $^{a}P < 0.05$. Significant difference shown by the Kruskal–Wallis rank-sum test.

processes may be due to the leakage of microbial metabolites into the circulation induced by IH. We found certain changes in the gut microbiota among the three groups over time; however, there were no significant differences between patients with T2DM with and without OSAHS. Nonetheless, the concentrations of inflammatory indicators, such as CRP, TNF-α, and IL-17, were significantly increased in T2DM patients with OSAHS, which indicates that the changes in gut microbiota may have been delayed relative to the chronic inflammatory changes in T2DM patients with OSAHS. On the other hand, some patients with relatively mild disease were included to prevent confounding by factors, including hyperglycemia and obesity, which affect the composition of intestinal microbiota[31]; as a result, the intestinal flora appeared not to have changed remarkably.

Various respiratory diseases have been associated with dysbiosis not only in the airway microbiota but also in the intestinal microbiota[32,33]. This evidence reinforced the existence of a gut-lung axis and the close relationship between intestinal and respiratory compartments; changes at one of the two sites could impact the other[34]. As a minor constituent of the airway microbiota, *Actinomyces* is related to anaerobic enzymes through GLUT1-dependent glucose elevation and MCT4-dependent lactate transport[35,36]. In our study, we observed that the increase in the relative abundance of *Actinomyces*

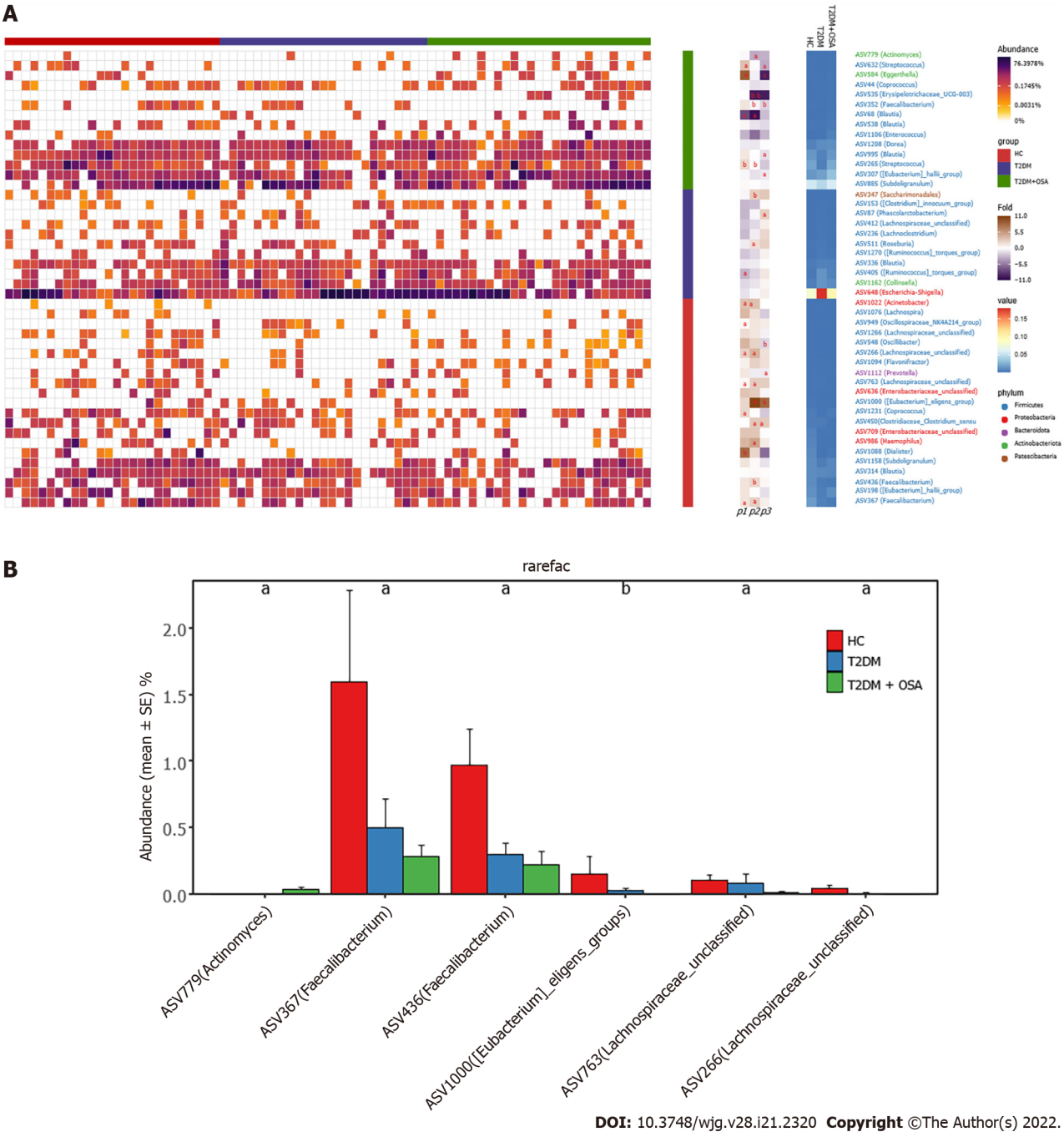


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**Figure 5 Amplicon sequence variants contributing to the changes in the gut microbiota in groups healthy control, type 2 diabetes mellitus, and type 2 diabetes mellitus plus obstructive sleep apnea.** A: The heatmap shows the relative abundance of the 46 amplicon sequence variants (ASVs) significantly different in the three groups. The fold ratio (log2-transformed) indicated the relative abundance of the 46 ASVs between the two different groups [*P*1: Type 2 diabetes mellitus (T2DM) + obstructive sleep apnea (OSA) *vs* T2DM; *P*2: T2DM + OSA *vs* healthy control (HC); *P*3: HC *vs* T2DM]. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. Significant difference shown by the Mann–Whitney U test; B: With the increase in disease components, six key ASVs showed increasing or decreasing trends. $^{a}P < 0.05$, $^{b}P < 0.01$.

was positively correlated with OSAHS severity indices and the concentration of TNF-α. We did not test for lung microbes; however, we speculate that they may be related to the gut-lung axis. Further experimental approaches to exploring causal links may be needed.

There are some limitations to our study. First, the sample size was relatively small. Second, the causal relationship between T2DM complicated by OSAHS and the gut microbiota was unclear. Large-scale clinical trials and gnotobiotic mice model validation may be required in the future.

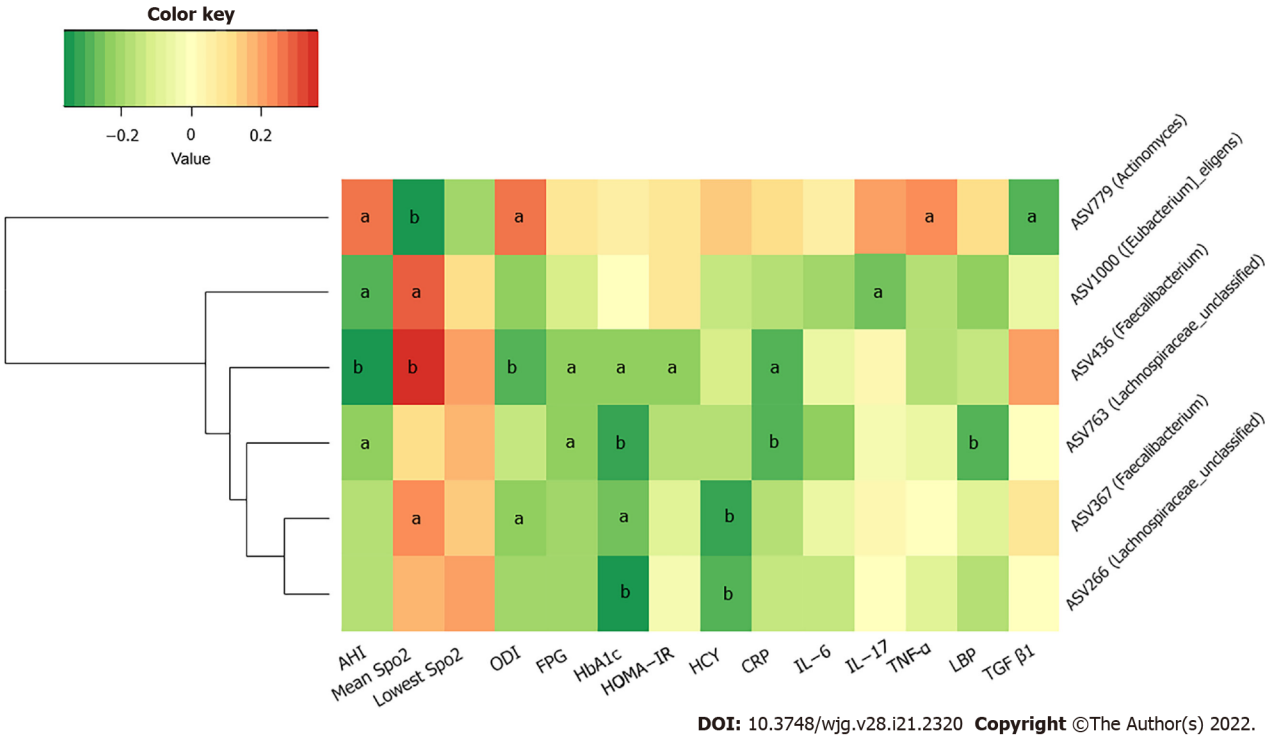


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**Figure 6 Heatmap of correlation among key amplicon sequence variants of gut microbiota and metabolic and inflammatory indicators.** The color of the cells represents the Spearman's correlation coefficient between each amplicon sequence variant and clinical parameter. [a]*P* < 0.05; [b]*P* < 0.01.

## CONCLUSION

T2DM patients with OSAHS may have a higher prevalence of gut microbial dysbiosis. IH may be involved in selective alterations of gut microbiota, which may be related to increased gut permeability and concurrent systemic inflammatory changes in patients with T2DM complicated by OSAHS. These findings provide foundations for further studies on the mechanisms and interventional approaches aimed at restoration of the gut microbiota to prevent or to palliate the adverse effects of T2DM patients with OSAHS.

## ARTICLE HIGHLIGHTS

### *Research background*

Obstructive sleep apnea (OSA)-hypopnea syndrome (OSAHS), as a chronic and treatable sleep disorder, has a high prevalence in type 2 diabetes mellitus (T2DM) patients. As a landmark feature of OSAHS, intermittent hypoxia (IH) plays an important role in the occurrence and development of related complications in T2DM patients. However, the pathological mechanisms are varied and unknown. Therefore, it is important to study the role of gut microbiota, a meaningful new target, in T2DM patients with OSAHS.

### *Research motivation*

In recent years, it has been found that gut microbiota imbalance is related to metabolic diseases. However, most studies have not discussed the relationship between gut microbiota changes and T2DM patients with OSAHS.

### *Research objectives*

In this study we focused on IH that might be involved in altering the gut dysbiosis in T2DM patients with OSAHS. Meanwhile, we further assessed the changes of clinical indicators and inflammatory factors related to dysbiosis, aiming to provide new targets and perspectives for the pathogenesis and prevention strategies of T2DM patients complicated with OSAHS.

### *Research methods*

A case-control study was conducted to select subjects who were divided into T2DM + OSA group, T2DM group, and healthy control group. They were examined with a type IV portable monitor

overnight. The clinical indexes, respiratory parameters, inflammatory indexes, and gut microbial community of the three groups were measured.

### Research results

Among the three groups, T2DM + OSA group showed the most severe changes in sleep apnea parameters and increased systemic inflammatory factors. We found the decreased levels of short-chain fatty acid-related *Faecalibacterium, Eubacterium,* and *Lachnospiraceae* and the increased levels of *Actinomyces* at the amplicon sequence variant level. The changes in these gut microbiotas were closely related to clinical indicators as well.

### Research conclusions

IH may be involved in the selective changes of intestinal microbiota, which may be related to the increased intestinal permeability and systemic inflammation response in T2DM patients with OSAHS.

### Research perspectives

This study shows that IH may change the state of gut microbiota and systemic inflammation, which participate in the occurrence and development of T2MD complicated with OSAHS. In the future, large-scale clinical randomized controlled prospective trials and animal trials may be needed to further explore the corresponding causality.

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## FOOTNOTES

**Author contributions:** Tang SS designed the research and conducted microbiome analysis; Liu YL conducted the research except the microbiome analysis; Tang SS and Liang CH analyzed the microbiome data and wrote the paper; all other data were analyzed by Shi XY and Deng XR with oversight by Wei W; Zhang LJ and Wang LM were responsible for the data acquisition and input and proofreading of the manuscript; Tang SS and Yuan HJ had primary responsibility for the final content; all authors have read and approved the final manuscript.

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**Informed consent statement:** All study participants provided informed written consent before the study enrollment.

**Conflict-of-interest statement:** All authors declare no conflicts of interest.

**Data sharing statement:** Raw sequencing data and associated metadata were deposited in the NCBI short-read archive with BioProject accession number PRJNA788301.

**STROBE statement:** The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.



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ORIGINAL ARTICLE

**Retrospective Study**

# Serotonin type 3 receptor subunit gene polymorphisms associated with psychosomatic symptoms in irritable bowel syndrome: A multicenter retrospective study

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## Abstract

***BACKGROUND***

Single-nucleotide polymorphisms (SNPs) of the serotonin type 3 receptor subunit (*HTR3*) genes have been associated with psychosomatic symptoms, but it is not clear whether these associations exist in irritable bowel syndrome (IBS).

***AIM***

To assess the association of *HTR3* polymorphisms with depressive, anxiety, and somatization symptoms in individuals with IBS.

***METHODS***

In this retrospective study, 623 participants with IBS were recruited from five specialty centers in Germany, Sweden, the United States, the United Kingdom, and Ireland. Depressive, anxiety, and

somatization symptoms and sociodemographic characteristics were collected. Four functional SNPs — *HTR3A* c.-42C>T, *HTR3B* c.386A>C, *HTR3C* c.489C>A, and *HTR3E* c.*76G>A — were genotyped and analyzed using the dominant and recessive models. We also performed separate analyses for sex and IBS subtypes. SNP scores were calculated as the number of minor alleles of the SNPs above. The impact of *HTR3C* c.489C>A was tested by radioligand-binding and calcium influx assays.

***RESULTS***

Depressive and anxiety symptoms significantly worsened with increasing numbers of minor *HTR3C* c.489C>A alleles in the dominant model ($F_{depressive}$ = 7.475, $P_{depressive}$ = 0.006; $F_{anxiety}$ = 6.535, $P_{anxiety}$ = 0.011). A higher SNP score (range 0-6) was linked to a worsened depressive symptoms score ($F$ = 7.710, *P-linear trend* = 0.006) in IBS. The potential relevance of the *HTR3C* SNP was corroborated, showing changes in the expression level of 5-HT$_3$AC variant receptors.

***CONCLUSION***

We have provided the first evidence that *HTR3C* c.489C>A is involved in depressive and anxiety symptoms in individuals with IBS. The SNP score indicated that an increasing number of minor alleles is linked to the worsening of depressive symptoms in IBS.

**Key Words:** Irritable bowel syndrome; 5-HT$_3$ receptor subunit gene polymorphisms; Single-nucleotide polymorphism score; Depression; Anxiety; Somatization



**Core Tip:** Bringing together high quality data as well as methodological expertise, our results show that: In the dominant model, *HTR3C* c.489C>A was correlated with depressive and anxiety symptoms in irritable bowel syndrome (IBS); a higher number of minor alleles, which was computed by combining the individual SNP status of *HTR3A* c.-42C>T, *HTR3B* c.386A>C, *HTR3C* c.489C>A, and *HTR3E* c.*76G>A, was linked to more severe depressive symptoms in IBS; and the potential relevance of the *HTR3C* SNP was corroborated in functional assays showing changes in the expression level of 5-HT$_3$AC variant receptors. These results will contribute towards standardization and harmonization of genetic research strategies in IBS.

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## INTRODUCTION

Irritable bowel syndrome (IBS) is a chronic functional gastrointestinal (GI) disorder characterized by abdominal pain and altered bowel habits[1-4]. The pathophysiology of IBS has not entirely been resolved, but is understood to be biopsychosocial and affected by an impaired function of the central and enteric nervous systems and their crosstalk *via* the brain-gut axis[5,6]. IBS patients often present with increased comorbid depressive and anxiety symptoms[7-11], highlighting the complex relationship between visceral sensitivity and subjective psychological perceptions[12,13]. Nevertheless, about 50% of IBS patients report GI symptoms but show no comorbid affective symptoms[14].

There is evidence that disturbances of the serotonergic system are important in GI disorders such as IBS and in mental disorders, both of which interact *via* the brain-gut axis[15,16]. The serotonin type 3 receptors (5-HT$_3$R) modulate key functions in the GI tract[17,18]. In line with such functions, 5-HT$_3$R antagonists are beneficial in the treatment of diarrhea-predominant IBS (IBS-D)[19-22]. 5-HT$_3$Rs are also involved in emotional processing, mood regulation, and visceral perception and have been associated with depressive and anxiety symptoms that represent comorbid phenotypes in IBS[23]. Single-nucleotide polymorphisms (SNPs) in the 5-HT$_3$R subunit genes (*HTR3*), namely *HTR3A* c.-42C>T (rs1062613), *HTR3B* c.386A>C (rs1176744), *HTR3C* c.489C>A (rs6766410), and *HTR3E* c.*76G>A

(rs56109847), are associated with IBS according to studies investigating the effects of sex or IBS subtypes [12,24-30]. However, whether *HTR3* polymorphisms are associated with IBS and comorbid depressive and anxiety symptoms has not been determined because existing studies have missing phenotypic data on comorbidities and small sample sizes. These studies had case-control designs and investigated associations between these polymorphisms in individuals with IBS phenotypes or mental behavioral conditions and controls rather than combining genetic data with specific psychosocial characteristics of IBS patients.

This multicenter observational study focused on a large IBS patient cohort comprising 768 participants from centers in Germany, Sweden, the United States, the United Kingdom, and Ireland with the aim of meeting three objectives: (1) To explore the associations between functional *HTR3* polymorphisms and psychosomatic burden (*i.e.*, depressive, anxiety, and somatization symptoms) within an IBS population; (2) To investigate the impact of the *HTR3* SNP score (computed as the number of minor alleles) on psychosomatic burden, based on our hypothesis that the observed number of minor alleles was associated with specific mental characteristics in IBS patients; and (3) To perform a functional analysis of variant 5-HT$_3$AC receptors.

## MATERIALS AND METHODS

### Subjects

The study population was pooled from five different tertiary care expert centers. German participants were recruited from the Specialty Clinic for Functional GI Disorders at the Department of General Internal Medicine and Psychosomatics of Heidelberg University Hospital[31] and from our clinical partners in the IBS-Net in Hamburg, Krefeld, Berlin, Vilsbiburg, and Munich (www.ibs.uni-hd.de). Swedish participants were recruited at the specialized unit for patients with functional GI disorders at Sahlgrenska University Hospital in Gothenburg. United States participants were recruited at Washington University, Barnes-Jewish Hospital in St. Louis, Missouri. United Kingdom participants were recruited at the Nottingham Digestive Diseases Center and participants from Ireland from a specialty clinic at Cork University Hospital. Participant recruitment is shown in Figure 1.

Written informed consent was obtained from all participants and the experiments were in accordance with the principles of the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. All studies were approved by the following local Ethics Committees: Heidelberg, Germany: Ethical Committee, Medical Faculty of the Heidelberg University Hospital (S067/2010); Cork, Ireland: Clinical Research Ethics Committee (APC024); Gothenburg, Sweden: Regional Ethical Review Board in Gothenburg (S489-02 and 731-09); Nottingham, United Kingdom: registered at clinical trial clinicaltrials.gov (identifier NCT00745004) and approved by Nottingham Research Ethics Committee 2 (REC reference number 08/H0408/134)[21]; and St-Louis, United States: Washington University St. Louis, Human Research Protection Office (IRB ID #: 201103220).

### Inclusion/exclusion criteria

Only patients diagnosed with IBS according to the ROME III criteria were included in the analysis. All participants were of Caucasian ancestry and had comparable population stratification. Patients under 18 years of age or without SNP test results were excluded.

### Genotyping

Genomic DNA was isolated from IBS patient blood samples using ethylenediaminetetraacetic acid according to standard protocols[32]. Four polymorphic *HTR3* loci, namely *HTR3A* c.-42C>T (rs1062613), *HTR3B* c.386A>C (rs1176744), *HTR3C* c.489C>A (rs6766410), and *HTR3E* c.*76G>A (rs56109847) were selected as target SNPs for this study. The corresponding primers were designed and synthesized using AssayDesigner 3.1 software. Genotyping was performed at the Department of Human Molecular Genetics at Heidelberg University Hospital using the KASPar® SNP Genotyping System (KBiosciences, Ltd, Hoddesdon, United Kingdom). To analyze *HTR3* SNPs, the fluorescence plate reader of the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, California, United States) was used as recommended. About 10% of the samples were repeat tested to ensure genotyping accuracy.

### Data collection

In addition to sociodemographic characteristics and IBS diagnosis, we also collected data on depressive, anxiety, and somatization symptoms[33] and genetic markers of the serotonergic system.

**IBS diagnosis:** The diagnostic classification of IBS was based on the Scoring Algorithm for Rome III Diagnostic Questionnaire for Adult Functional GI Disorders (SA for Rome III-DQ)[34,35] in all five centers. Percentages of the different IBS subtypes, *i.e.*, constipation-predominant IBS, IBS-D, IBS with mixed bowel habits, and unclassified IBS, were also calculated.

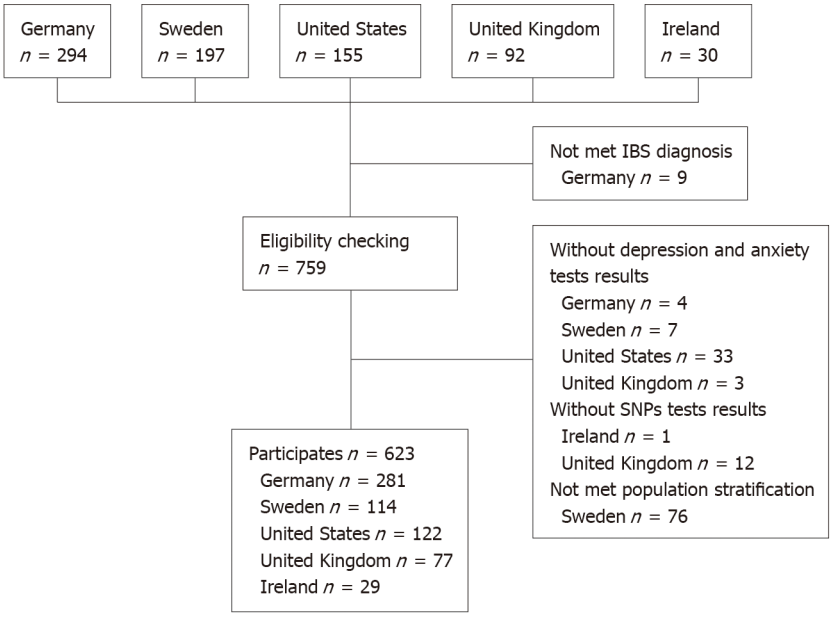


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**Figure 1 Recruitment strategy.** IBS: Irritable bowel syndrome.

**Depressive symptoms:** The nine-item depression module from the Patient Health Questionnaire (PHQ-9)[36,37] was used to measure depressive symptoms in the German cohort. The Hospital Anxiety and Depression Scale depression subscale[38] was used to identify depressive symptoms in participants from Sweden, the United Kingdom, and Ireland and the Beck Depression Inventory[39] was used to measure the severity of depressive symptoms in United States participants.

**Anxiety symptoms:** In the German cohort, symptoms of generalized anxiety were assessed using the brief measurement for generalized anxiety disorder (GAD-7)[40]. In the cohorts from Sweden, the United Kingdom, and Ireland, the HADS anxiety subscale was used to identify anxiety symptoms. The Beck Anxiety Inventory[41] was used to assess anxiety in the United States cohort.

**Somatization symptoms:** In the cohorts from Germany, Sweden, and the United States, the 15-item somatization module from the PHQ-15[42] was used to identify somatization symptoms.

**Genetic markers of the serotonergic system:** The four functional SNPs *HTR3A* c.-42C>T (rs1062613), *HTR3B* c.386A>C (rs1176744), *HTR3C* c.489C>A (rs6766410), and *HTR3E* c.*76G>A (rs56109847) were selected for validation based on previous reports as outlined above[25].

### *Ligand binding and calcium influx assays*

These procedures are described in the Supplementary material.

### *Statistical analysis*

All statistical procedures were carried out using IBM SPSS Statistics 22.0 for Windows. Variables with a skewed distribution were log-transformed prior to further analysis. If different measurements had been collected in the five centers, *z* values were calculated to enable pooling. The Hardy-Weinberg equilibrium (HWE) of genotype frequency distribution was tested using SHEsis[43]. Genome-wide SNP data were generated by the Bellygenes team of D'Amato M using the Illumina Global Screening array and platform[44,45]. We used the multidimensional scaling approach to correct for population stratification in PLINK[46]. Following the guidance provided at https://github.com/MareesAT/GWA_tutorial/, our data were anchored by data of the 1000 Genomes project (http://www.1000genomes.org/). The 10 main components were used as covariates in the association tests to correct for population stratification[46] and exclude outliers. Polymorphisms were analyzed separately using the dominant and the recessive models. Also, stratified analyses based on sex and IBS subtypes were carried out. ANOVA was used to analyze group differences and to check for linear trends in depressive, anxiety, and somatization symptoms. For the independent variable, a SNP score was computed based on the number of minor alleles (*i.e.*, continuous from 0 to 8 for the four SNPs). Based on the first human *HTR3* locus-specific variant database (www.htr3.uni-hd.de)[25], scoring criteria were as follows: major allele homozygous variant gene = 0; heterozygous variant gene = 1; and minor allele homozygous variant gene = 2 (for details see Table 1). Statistical comparisons were made between the two groups using the $\chi^2$ test or Fisher's exact test for frequencies and *t* tests or Mann-Whitney *U* tests for metric

**Table 1 Strategy for computing the single-nucleotide polymorphism score**

| | Major allele homozygous genotype | Heterozygous variant genotype | Minor allele homozygous genotype |
|---|---|---|---|
| *HTR3A* c.-42C>T (rs1062613) | CC | CT | TT |
| *HTR3B* c.386A>C (rs1176744) | AA | AC | CC |
| *HTR3C* c.489C>A (rs6766410) | CC | CA | AA |
| *HTR3E* c.*76G>A (rs56109847) | GG | GA | AA |

variables. Normal distribution and variance homogeneity were checked as conditions. Statistical tests were two-sided based on an alpha error of 0.05%. All analyses were explorative and not confirmatory. False discovery rates (FDRs) were calculated based on overall *P* values using the Benjamini-Hochberg method[47]. Significant values that were no longer significant after FDR multiple testing correction were named "nominally significant".

## RESULTS

### Sociodemographic and symptomatic characteristics

In total, 623 participants from five independent expert centers were included in this study (45.1% from Germany, 18.3% from Sweden, 19.6% from the United States, 12.4% from the United Kingdom, and 4.7% from Ireland). We excluded 76 Swedish participants who did not meet the population stratification criteria (Supplementary Figure 1). Participants from the United Kingdom and Ireland were excluded from the main analysis because the sample size was small. Table 2 presents the sociodemographic characteristics, IBS subtypes, and psychosomatic symptoms of the included participants. Participants had a mean ± SD age of 41.7 ± 16.1 years and 69.5% were female. Overall, IBS patients showed minimal to mild levels of depressive and anxiety symptoms, moderate levels of IBS symptoms, and moderate levels of somatization symptoms.

### HTR3 SNP genotypes and allele frequencies

Genotype and allele frequencies of the functional *HTR3A* c.-42C>T, *HTR3B* c.386A>, *HTR3C* c.489C>A, and *HTR3E* c.*76G>A polymorphisms were calculated. No significant differences in genotype frequency were observed between sexes or IBS subtypes. For *HTR3A* c.-42C>T, the frequency of the minor *T* allele was 21.5%; for *HTR3B* c.386A>C, the frequency of the minor *C* allele was 29.6%; for *HTR3C* c.489C>A, the frequency of the minor *A* allele was 41.6%; and for *HTR3E* c.*76G>A, the frequency of minor *A* allele was 6.1%. The genotypic distribution of the four polymorphic loci of rs1062613, rs1176744, rs6766410, and rs56109847 were in accordance with HWE (all $P > 0.05$). The results are shown in Supplementary Tables 1-3.

### HTR3 SNP analysis using the dominant and the recessive model

*HTR3* SNPs were separately analyzed using the dominant model and the recessive model stratified for sex and IBS subtypes. Depressive and anxiety symptoms worsened significantly with increasing numbers of minor alleles of *HTR3C* c.489C>A in the dominant model ($F_{depressive} = 7.475$, $P_{depressive} = 0.006$; $F_{anxiety} = 6.535$, $P_{anxiety} = 0.011$). This seemed to be driven by female sex ($F_{depressive} = 7.040$, $P_{depressive} = 0.008$; $F_{anxiety} = 7.550$, $P_{anxiety} = 0.006$) and IBS-D ($F_{depressive} = 5.670$, $P_{depressive} = 0.018$; $F_{anxiety} = 13.444$, $P_{anxiety} < 0.001$). The same trend was also found for *HTR3A* c.-42C>T in male participants with depressive symptoms in the dominant model ($F_{depressive} = 4.149$, $P_{depressive} = 0.043$). For the recessive model, depressive and somatization symptoms worsened with increasing numbers of minor alleles of *HTR3C c.489C>A* ($F_{depressive} = 6.190$, $P_{depressive} = 0.014$) and *HTR3B c.386A>C* ($F_{depressive} = 6.482$, $P_{depressive} = 0.011$), respectively in IBS-D participants. *F* values from the ANOVA are shown in Table 3. As mentioned above, the analyses of participants from the United Kingdom and Ireland are presented separately in the Supplementary Table 4.

### Effect of SNP score on psychosomatic symptoms

SNP scores ranged from 0 to 6; 37.1% had one or zero minor alleles of the analyzed *HTR3* SNPs and 30.8% had three or more minor alleles of the analyzed *HTR3* SNPs. No significant differences in SNP scores were observed between sexes ($F = 3.550$, $P = 0.060$) or IBS subtypes ($F = 1.485$, $P = 0.227$). ANOVAs were conducted and linear trends were checked to analyze the effect of SNP scores on depressive, anxiety, and somatization symptoms. Overall, an increasing number of minor alleles was linked to worsening depressive symptoms ($F = 7.710$, *P-linear trend* = 0.006). However, material trends did not reveal a link between more minor alleles and worsening anxiety or somatization symptoms. By stratifying analyses for sex, an increasing number of minor alleles was linked to worsened depressive

**Table 2 Sociodemographic and symptomatic characteristics of the study participants**

| | Total | Centers | | | | |
|---|---|---|---|---|---|---|
| | | Germany | Sweden | United States | United Kingdom | Ireland |
| *n* | 100.0 (623) | 45.1 (281) | 18.3 (114) | 19.6 (122) | 12.4 (77) | 4.7 (29) |
| Age | 41.7 ± 16.1 (18, 91) | 40.7 ± 16.4 (18, 88) | 33.7 ± 11.8 (18, 60) | 54.9 ± 14.6 (25, 91) | 40.8 ± 11.6 (18, 70) | 30.3 ± 8.6 (19, 51) |
| Sex | | | | | | |
| Female | 69.5 (433) | 63.3 (178) | 69.3 (79) | 77.9 (95) | 72.7 (56) | 86.2 (25) |
| IBS subtypes | | | | | | |
| IBS-C | 14.1 (87) | 8.9 (25) | 12.3 (14) | 36.7 (44) | 0 | 13.8 (4) |
| IBS-D | 41.7 (258) | 43.4 (122) | 19.6 (22) | 28.3 (34) | 100.0 (77) | 10.3 (3) |
| IBS-M | 42.5 (263) | 45.6 (128) | 67.0 (75) | 31.7 (38) | 0 | 75.9 (22) |
| IBS-U[1] | 1.8 (11) | 2.1 (6) | 0.9 (1) | 3.3 (4) | 0 | 0 |
| Depressive symptoms | - | 9.4 ± 5.9[2] | 5.2 ± 3.5[4] | 13.2 ± 9.6[5] | 5.9 ± 4.0[4] | 4.3 ± 3.2[4] |
| Anxiety symptoms | - | 7.7 ± 4.8[3] | 8.3 ± 4.5[4] | 14.6 ± 9.7[6] | 9.6 ± 4.6[4] | 7.8 ± 4.5[4] |
| Symptom severity | - | 280.3 ± 90.7[7] | 297.3 ± 100.5[7] | 43.2 ± 16.2[8] | - | - |
| Somatization symptoms[9] | 14.8±6.3 | 16.2 ± 7.3 | 13.3 ± 4.7 | 13.8 ± 5.0 | - | - |

Sample size, sex, IBS subtypes, and severity of depressive and anxiety symptoms are presented as % (*n*). Age is presented as mean ± SD (range). Scores of depressive and anxiety symptoms are presented as mean ± SD.

[1]Was not included in the later analysis because of the small sample size.

[2]Evaluated by the nine-item depression module from the Patient Health Questionnaire.

[3]Evaluated by the brief measurement for generalized anxiety disorder.

[4]Evaluated by Hospital Anxiety and Depression Scale.

[5]Evaluated by Beck Depression Inventory.

[6]Evaluated by Beck Anxiety Inventory.

[7]Evaluated by irritable bowel syndrome (IBS) symptom severity scale.

[8]Evaluated by the gastrointestinal symptom rating scale for IBS.

[9]Evaluated by the somatization module for the Patient Health Questionnaire.

IBS: Irritable bowel syndrome; IBS-C: Constipation-predominant IBS; IBS-D: Diarrhea-predominant IBS; IBS-M: IBS with mixed bowel habits; IBS-U: Unclassified IBS.

symptoms in female participants ($F$ = 5.770, *P-linear trend* = 0.017). There was no significant association between SNP score and depressive, anxiety, or somatization symptoms when looking at IBS subtypes separately (Table 4). As mentioned above, the analyses of participants from the United Kingdom and Ireland are presented separately in the Supplementary Table 5.

### *Functional analysis of variant 5-HT$_3$AC receptors*

The *HTR3C* SNP encodes the amino acid exchange p.Asn163Lys (p.N163K), and recombinantly expressed 5-HT$_3$AC receptors harboring variant 5-HT$_3$C subunits that mimic the homozygous minor allele and the heterozygous state presented with increased cell surface expression and enhanced 5-HT maximum response. Radioligand-binding assays revealed higher $B_{max}$ values of 117.1% ± 4.38% and 111.9% ± 1.79%. Calcium influx assays showed increased 5-HT-induced maximum effects of 137.2% ± 9.0% and 151.9% ± 17.3% for the minor allele 5-HT$_3$AC 163K or combined 5-HT$_3$AC 163N/5-HT$_3$AC 163K receptors compared with the major allele representing 5-HT$_3$AC 163N receptors, respectively. The affinity of the specific 5-HT$_3$ receptor antagonist [$^3$H]GR65630, as reflected by the Kd values, and the potency of 5-HT, as reflected by the $EC_{50}$ values, did not differ between the receptor variants (Figure 2).

## DISCUSSION

### *Main findings*

The 5-HT$_3$ receptors modulate essential functions in the GI tract such as GI motility as well as mood and emotions[19], and *HTR3* SNPs have been associated with depression, anxiety, and IBS[25]. In this study, we showed that: (1) In the dominant model, *HTR3C c.489C>A* was correlated with depressive and anxiety symptoms in IBS; (2) A higher number of minor alleles (*i.e.*, a higher SNP score, which was computed by combining the individual SNP status of *HTR3A c.-42C>T, HTR3B c.386A>C, HTR3C*

**Table 3 Differences between single-nucleotide polymorphisms in depressive and anxiety symptoms, separately analyzed with ANOVA using the dominant model and the recessive model**

| Models | Mental symptoms | SNPs | Total | Sex | | IBS subtypes | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Male | Female | IBS-C | IBS-D | IBS-M |
| Dominant model | Depressive symptoms | *HTR3A* c.-42C>T (rs1062613) | 2.541 | **4.149** ↑ | 0286 | 0.998 | 0.376 | 0.694 |
| | | *HTR3B* c.386A>C (rs1176744) | 0.067 | 0.005 | 0.198 | 0.282 | 0.124 | 0.232 |
| | | *HTR3C* c.489C>A (rs6766410) | **7.475** ↑ | 0.672 | **7.040** ↑ | 0.261 | **5.670** ↑ | 2.328 |
| | | *HTR3E* c.*76G>A (rs62625044) | 0.054 | 0.013 | 0.180 | 0.208 | 0.461 | 0.010 |
| | Anxiety symptoms | *HTR3A* c.-42C>T (rs1062613) | 0.511 | 0.210 | 0.280 | 0.604 | 0.672 | 0.162 |
| | | *HTR3B* c.386A>C (rs1176744) | 0.926 | 0.406 | 0.801 | 0.468 | 1.369 | 0.770 |
| | | *HTR3C* c.489C>A (rs6766410) | **6.535** ↑ | 0.158 | **7.550** ↑ | 0.186 | **13.444** ↑ | 0.045 |
| | | *HTR3E* c.*76G>A (rs62625044) | 1.055 | 0.429 | 0.853 | 0.000 | 0.495 | 1.109 |
| | Somatization symptoms | *HTR3A* c.-42C>T (rs1062613) | 0.016 | 0.141 | 0.106 | 0.086 | 0.017 | 0.203 |
| | | *HTR3B* c.386A>C (rs1176744) | 0.217 | 0.332 | 0.326 | 0.407 | 0.415 | 2.125 |
| | | *HTR3C* c.489C>A (rs6766410) | 0.784 | 0.035 | 1.085 | 0.269 | 0.000 | 1.962 |
| | | *HTR3E* c.*76G>A (rs62625044) | 0.002 | 1.554 | 0.263 | 0.128 | 0.729 | 0.354 |
| Recessive model | Depressive symptoms | *HTR3A* c.-42C>T (rs1062613) | 0.002 | 0.421 | 0.132 | 0.093 | 1.320 | 1.064 |
| | | *HTR3B* c.386A>C (rs1176744) | 1.118 | 2.027 | 0.107 | 1.741 | 0.197 | 0.744 |
| | | *HTR3C* c.489C>A (rs6766410) | 0.047 | 0.821 | 0.401 | 2.306 | **6.190** ↑ | 0.676 |
| | | *HTR3E* c.*76G>A (rs62625044) | 0.677 | - | 0.574 | 0.541 | - | - |
| | Anxiety symptoms | *HTR3A* c.-42C>T (rs1062613) | 0.479 | 0.027 | 0.549 | 0.057 | 0.315 | 0.350 |
| | | *HTR3B* c.386A>C (rs1176744) | 0.872 | 0.081 | 1.691 | 0.476 | 0.361 | 1.923 |
| | | *HTR3C* c.489C>A (rs6766410) | 0.028 | 2.562 | 0.344 | 0.570 | 3.509 | 2.093 |
| | | *HTR3E* c.*76G>A (rs62625044) | 0.216 | - | 0.179 | 0.242 | - | - |
| | Somatization symptoms | *HTR3A* c.-42C>T (rs1062613) | 0.217 | 0.018 | 0.257 | 0.138 | 0.092 | 0.431 |
| | | *HTR3B* c.386A>C (rs1176744) | **6.482** ↑ | 2.245 | 3.575 | 0.142 | **14.033** ↑ | 0.341 |
| | | *HTR3C* c.489C>A (rs6766410) | 0.468 | 1.813 | 0.128 | **4.715** ↓ | 0.002 | 0.113 |
| | | *HTR3E* c.*76G>A (rs62625044) | 0.001 | - | 0.011 | 0.000 | - | - |

*F* values are shown in the table. Arrows represent the direction of the associations. ↑ dependent variable increases from major to minor alleles; ↓ dependent variable decreases from major to minor alleles. The underlined values were significant. Values in bold were "nominally significant" (*i.e.*, lacking

significance after false discovery rate multiple testing correction). IBS: Irritable bowel syndrome; IBS-C: Constipation-predominant IBS; IBS-D: Diarrhea-predominant IBS; IBS-M: IBS with mixed bowel habits; SNPs: Single-nucleotide polymorphisms.

**Table 4 Association of depressive, anxiety, and somatization symptoms with the single-nucleotide polymorphism score of the four tested polymorphisms**

| | Total | Sex | | IBS subtypes | | |
|---|---|---|---|---|---|---|
| | | Male | Female | IBS-C | IBS-D | IBS-M |
| Depressive symptoms | | | | | | |
| *F* value | **7.710** | 1.336 | **5.770** | 1.551 | 2.159 | 0.196 |
| *P-linear trend* value | **0.006** | 0.250 | **0.017** | 0.184 | 0.144 | 0.659 |
| Anxiety symptoms | | | | | | |
| *F* value | 2.150 | 0.028 | 2.514 | 1.372 | 2.400 | 1.412 |
| *P-linear trend* value | 0.143 | 0.866 | 0.114 | 0.245 | 0.123 | 0.236 |
| Somatization symptoms | | | | | | |
| *F* value | 1.15 | 0.354 | 1.028 | 0.716 | 2.718 | 0.628 |
| *P-linear trend* value | 0.283 | 0.553 | 0.311 | 0.614 | 0.102 | 0.429 |

Values in bold were "nominally significant" (*i.e.*, lacking significance after false discovery rate multiple testing correction). IBS: Irritable bowel syndrome; IBS-C: Constipation-predominant IBS; IBS-D: Diarrhea-predominant IBS; IBS-M: IBS with mixed bowel habits.

*c.489C>A*, and *HTR3E c.*76G>A*) was linked to more severe depressive symptoms in IBS; and (3) The potential relevance of the *HTR3C* SNP was corroborated in functional assays showing changes in the expression level of 5-$HT_3$AC variant receptors. These findings are discussed in more detail below.

### Sample characteristics

Participants with IBS were more frequently female than male, in line with previous findings[48,49] that IBS is more prevalent in young and middle-aged females. Most participants with IBS were only mildly affected by depression and anxiety symptoms. Of note, German participants only visited the Specialty Clinic for Functional GI Disorders at Heidelberg University Hospital after a long history of dealing with IBS[31]; therefore, these participants reported more severe depressive symptoms. However, causal relationships between IBS symptoms, depression, and anxiety are still controversial.

The investigated SNPs rs1062613, rs1176744, rs6766410, and rs56109847 were in accordance with HWE. There was no population stratification, and the sample was representative and excluded genotyping errors. Although participants were recruited from various centers in different countries, there were no obvious selective differences.

### Connections between HTR3 SNPs and mental symptoms

***HTR3A* c.-42C>T (rs1062613):** Depressive symptoms were "nominally significantly" more severe with increasing numbers of minor *HTR3A* c.-42C>T alleles in male participants according to the dominant model. This SNP has been associated with bipolar affective disorder[50], IBS symptom severity, amygdaloid activity[29,51], early life trauma[52], altered emotional networks in the human brain, and the onset of depression[53]. However, these studies only included single sex participants and did not include subgroup analyses.

***HTR3B* c.386A>C (rs1176744):** Somatization symptoms worsened significantly with increasing numbers of minor *HTR3B c.386A>C* alleles in the dominant model. The *HTR3B* variant p.Tyr129Ser (rs1176744) has been associated with bipolar affective disorder in males and with major depression in females as well as with pain catastrophizing, a coping style characterized by excessively negative thoughts and emotions related to pain[30,54-56]. This discrepancy may be due to an enhancement or weakening of this association by polymorphic interactions in the serotonin pathways[56].

***HTR3C* c.489C>A (rs6766410):** Depressive and anxiety symptoms worsened significantly with increasing numbers of minor *HTR3C* c.489C>A alleles in the dominant model. This effect seemed to be driven by female sex and IBS-D. *HTR3C* c.489C>A was previously associated with IBS-D in female patients[57], but the proportion of male patients was small in this study, which may limit the applicability of these findings. In the recessive model, depressive and anxiety symptoms "nominally

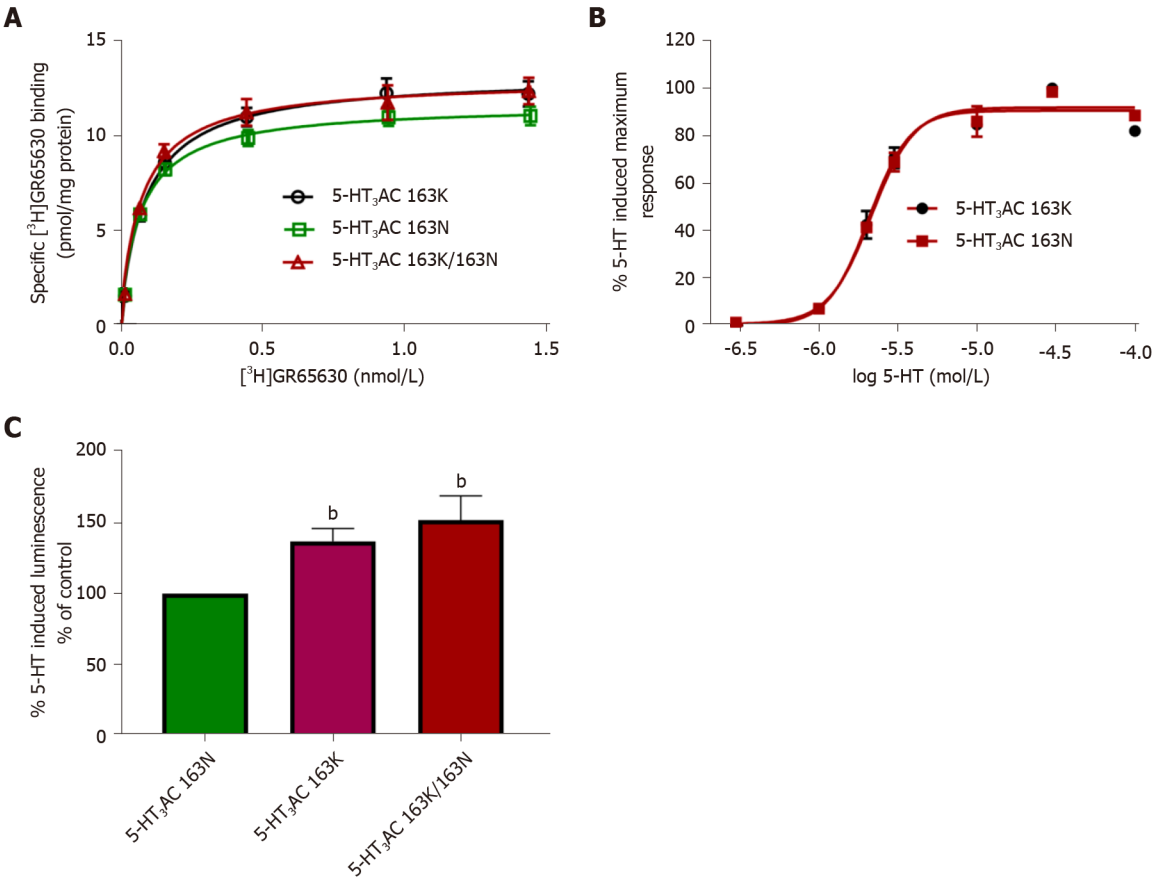


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**Figure 2 Comparative analysis of 5-HT$_3$AC 163N (major allele homozygous), 5-HT$_3$AC 163K (minor allele homozygous), and 5-HT$_3$AC 163K/163N (heterozygous) receptors.** A: Radioligand-binding studies Saturation experiments were performed in membranes of HEK293 cells transiently expressing different 5-HT$_3$ subunit combinations in triplicate with six increasing concentrations of [3H]GR65630 (0.02-1.5 nmol/L); Kd values were 0.07 ± 0.01 for 5-HT$_3$AC 163N; 0.08 ± 0.01 for 5-HT$_3$AC 163K, and 0.07 ± 0.01 for 5-HT$_3$AC 163K/163N. *n* = 6 experiments; B: Concentration response curves were assessed in a calcium influx assay (aequorin assay) in HEK293 cells transiently transfected with different 5-HT$_3$ subunit combinations in quadruplicate with seven increasing concentrations of 5-HT. pEC50 values were as follows: 68 ± 0.03 for 5-HT$_3$AC 163N 5 and 5.66 ± 0.02 for 5-HT$_3$AC 163K; C: Respective maximal calcium influx values ($E_{max}$) evoked by 5-HT (10 μmol/L). Data are expressed as percentages of the $E_{max}$ of the heteromeric 5-HT$_3$AC 163N receptor (% of control), *n* = 8 experiments. Bars represent mean ± SE. $^bP < 0.01$ from ANOVA followed by Dunnett's post-test or unpaired Student's test for comparison of only two groups.

significantly" worsened with increasing numbers of minor alleles of *HTR3C* c.489C>A in Irish participants. However, these results should be interpreted with caution because the Irish sample size was low. As far as we are aware, *HTR3C* c.489C>A has not been analyzed in individuals with affective disorders before.

***HTR3E* c.*76G>A (rs56109847):** *HTR3E* is restrictedly and robustly expressed in the GI tract[58,59], suggesting that it plays a special role in 5-HT$_3$ receptor function in the gut. In this study, we did not find a relationship between functional polymorphisms of *HTR3E* and depressive and anxiety symptoms in IBS patients. This may be attributed to a floor effect because depressive and anxiety symptoms were minimal to mild in our sample[60].

### *The SNP score and its impact on depressive symptoms*

A single gene variant is not sufficient to explain all symptoms shaping the clinical phenotype of a complex disorder like IBS[61]. By computing SNP scores based on the number of minor alleles of rs1062613, rs1176744, rs6766410, and rs56109847, our study revealed that an increasing number of minor alleles is linked to increasing severity of depressive symptoms. However, there was no obvious association between an increasing number of minor alleles and the severity of anxiety or somatization symptoms. Stratification for sex revealed a correlation between increasing numbers of minor alleles and worsening depressive symptoms in female participants.

### *Functional properties of variant 5-HT$_3$AC receptors*

*HTR3* genes encode different 5-HT$_3$ subunits to make up heteromeric receptors. The 5-HT$_3$A subunits play a major role in these receptors because they can form functional receptors on their own. The other

subunits can only form functional receptors with 5-HT$_3$A and seem to modulate the function and properties of the receptors[62]. How these native receptors might contribute to the pathogenesis of IBS, particularly regarding co-expression patterns of *HTR3*, has not been established yet. The *HTR3A* and *HTR3E* variants reside within untranslated regions and the respective SNPs correlate with increased expression levels, whereas the *HTR3B* variant changes the channel properties[25]. To gain insight into the pathophysiological relevance of the associated *HTR3C* variant c.489C>A (rs6766410), we characterized the pharmacological and functional properties of those 5-HT$_3$AC receptors that altered the 5-HT-mediated maximum response and expression of variant 5-HT$_3$AC receptors. However, how structural modifications in these receptors affect their function *in vivo* and how they modulate the serotonergic system to influence mood, emotional processing, and the manifestation of IBS and comorbid conditions remains to be determined.

### Limitations and strengths

Our study has some limitations. First, different instruments were used by different centers to assess phenotypic features. To correct for this, scale scores were converted into *z* standard scores. However, given that the participants reported no severe psychosomatic symptoms, the discovery chances might be limited. Second, there is no sufficient evidence to show the relationship between risk alleles and respective major/minor alleles as patients and healthy controls were not compared in this study. Similarly, the relative strength of the cumulative effect represented by the SNP score was also affected to a certain extent. Third, our participants were all Caucasian, so the results may not apply to other ethnic groups.

Despite these limitations, our study has some strengths. First, this was a multicenter study so had a large sample size. Large, well-characterized samples like ours are necessary to identify molecular causes of IBS and comorbid conditions[12]. Second, this study investigated the association between polymorphisms in *HTR3* genes and comorbid psychosomatic symptoms for the first time. We conducted population stratification tests to ensure that the included populations were comparable. We also performed stratified analyses of sex and IBS subtypes and a more stringent multiple testing correction by FDR. Third, SNP scores have higher power and are better suited to testing multiple instead of single variants. This is useful because the pathogenesis of IBS is complex with multiple factors contributing to the manifestation of various subtypes. Also, individual genes may only play a minor role[12].

### Clinical implications and further research

IBS is a complex condition. The continuous improvement of the allelic variation database for *HTR3*[25] and deep phenotyping combined with gene information (also in other datasets) may help to identify disease subgroups accurately and consistently, thereby facilitating future treatment[33,63]. This will be an important step towards standardization and unification of IBS genetic research strategies.

## CONCLUSION

Our results provide the first evidence that the accumulation of *HTR3* SNPs (reflected by the SNP score computed by *HTR3A* c.-42C>T, *HTR3B* c.386A>C, *HTR3C* c.489C>A, and *HTR3E* c.*76G>A) may play a role in the pathophysiology of depressive and anxiety symptoms in IBS. This study has revealed that depressive and anxiety symptoms significantly worsened from the major to the minor allele of *HTR3C* c.489C>A in the dominant model and an increasing number of minor alleles are linked to more severe depressive symptoms in IBS.

## ARTICLE HIGHLIGHTS

### Research background

Over the past decades, genetic evidence on the key players within the serotonergic system including the serotonin type 3 (5-HT$_3$) receptor subunit genes (*HTR3*) accumulated showing association with irritable bowel syndrome (IBS) as well as mental illnesses. However, it has never been explored whether associations of the single-nucleotide polymorphisms (SNPs) of *HTR3* genes to depressive and anxiety symptoms can be replicated within IBS.

### Research motivation

In order to address this knowledge gap, This multicenter observational study focused on a large IBS patient cohort comprising 768 participants from centers in Germany, Sweden, the United States, the United Kingdom, and Ireland.

### Research objectives

The objectives are: (1) To explore the associations between functional *HTR3* polymorphisms and psychosomatic burden within an IBS population; (2) To investigate the impact of the *HTR3* SNP score on psychosomatic burden, based on our hypothesis that the observed number of minor alleles was associated with specific mental characteristics in IBS patients; and (3) To perform a functional analysis of variant 5-HT$_3$AC receptors.

### Research methods

In this retrospective study, 623 participants with IBS were recruited from five specialty centers in Germany, Sweden, the United States, the United Kingdom, and Ireland. Depressive, anxiety, and somatization symptoms and sociodemographic characteristics were collected. Four functional SNPs — *HTR3A* c.-42C>T, *HTR3B* c.386A>C, *HTR3C* c.489C>A, and *HTR3E* c.*76G>A — were genotyped and analyzed using the dominant and recessive models. We also performed separate analyses for sex and IBS subtypes. SNP scores were calculated as the number of minor alleles of the SNPs above. The impact of *HTR3C* c.489C>A was tested by radioligand-binding and calcium influx assays.

### Research results

Bringing together high quality data as well as methodological expertise, our results show that: (1) In the dominant model, *HTR3C c.489C>A* was correlated with depressive and anxiety symptoms in IBS; (2) A higher number of minor alleles (*i.e.*, the higher the SNP score, which was computed by combining the individual SNP status of *HTR3A c.-42C>T, HTR3B c.386A>C, HTR3C c.489C>A*, and *HTR3E c.*76G>A*) was linked to more severe depressive symptoms in IBS; and (3) The potential relevance of the *HTR3C* SNP was corroborated in functional assays showing changes in the expression level of 5-HT$_3$AC variant receptors.

### Research conclusions

Our results provide the first evidence that the accumulation of *HTR3* SNPs (reflected by the SNP score computed by *HTR3A* c.-42C>T, *HTR3B* c.386A>C, *HTR3C* c.489C>A, and *HTR3E* c.*76G>A) may play a role in the pathophysiology of depressive and anxiety symptoms in IBS.

### Research perspectives

We are confident that these results are of interest to your readership, as they contribute substantially to update current knowledge regarding the role of accumulation of *HTR3* SNPs in depressive and anxiety symptoms in IBS patients. In turn, our data will contribute towards standardization and harmonization of genetic research strategies in IBS.

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## FOOTNOTES

**Author contributions:** Mönnikes H, Keller J, Walstab J and Wahl V performed the experiments; Dong Y, Berens S, Schaefert R, Clevers E and Walstab J carried out data analyses; Berens S, Engel F, Gauss A, Herzog W, Spiller R, Clarke G, Dinan TG, Quigley EM, Sayuk G, Simrén M, Tesarz J, Sayuk G and IBS-Net Germany characterized and enrolled the patients; Dong Y, Berens S, and Schaefert R drafted the manuscript; all authors contributed to discussing and editing of the manuscript; Niesler B, Schaefert R and van Oudenhove L designed and supervised the study and revised and finalized the manuscript; Berens S and Dong Y equally contributed to the paper; Schaefert R and Niesler B shared last authorship.

**Institutional review board statement:** The study was reviewed and approved by the Ethical Committee, Medical Faculty of the Heidelberg University Hospital (approval No. S067/2010).

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

**Conflict-of-interest statement:** APC Microbiome Ireland has conducted studies in collaboration with several

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ORIGINAL ARTICLE

Retrospective Study

# Differentiating malignant and benign focal liver lesions in children using CEUS LI-RADS combined with serum alpha-fetoprotein

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## Abstract

### *BACKGROUND*

Contrast-enhanced ultrasound (CEUS) can be used to diagnose focal liver lesions (FLLs) in children. The America College of Radiology developed the CEUS liver imaging reporting and data system (LI-RADS) for standardizing CEUS diagnosis of FLLs in adult patients. Until now, no similar consensus or guidelines have existed for pediatric patients to improve imaging interpretation as adults.

### *AIM*

To evaluate the performance of CEUS LI-RADS combined with alpha-fetoprotein (AFP) in differentiating benign and malignant FLLs in pediatric patients.

### *METHODS*

Between January 2011 and January 2021, patients ≤ 18 years old who underwent CEUS for FLLs were retrospectively evaluated. The following criteria for diagnosing malignancy were proposed: Criterion I considered LR-4, LR-5, or LR-M lesions as malignancies; criterion II regarded LR-4, LR-5 or LR-M lesions with simultaneously elevated AFP (≥ 20 ng/mL) as malignancies; criterion III took LR-4 Lesions with elevated AFP or LR-5 or LR-M lesions as malignancies. The sensitivity, specificity, accuracy and area under the receiver operating characteristic curve (AUC) were calculated to determine the diagnostic value of the aforementioned criteria.

### *RESULTS*

The study included 63 nodules in 60 patients (mean age, 11.0 ± 5.2 years; 26 male). There were no statistically significant differences between the specificity, accuracy, or AUC of criterion II and criterion III (95.1% *vs* 80.5%, 84.1% *vs* 87.3%, and 0.794 *vs* 0.902; all $P > 0.017$). Notably, criterion III showed a higher diagnostic

sensitivity than criterion II (100% *vs* 63.6%; $P < 0.017$). However, both the specificity and accuracy of criterion I was inferior to those of criterion II and criterion III (all $P < 0.017$). For pediatric patients more than 5 years old, the performance of the three criteria was overall similar when patients were subcategorized by age when compared to all patients in aggregate.

*CONCLUSION*
CEUS LI-RADS combined with AFP may be a powerful diagnostic tool in pediatric patients. LR-4 with elevated AFP, LR-5 or LR-M lesions is highly suggestive of malignant tumors.

**Key Words:** Pediatric; Contrast-enhanced ultrasound; Liver imaging reporting and data system; Diagnosis; Focal liver lesions; Alpha-fetoprotein



**Core Tip:** Contrast-enhanced ultrasound liver imaging reporting and data system (CEUS LI-RADS) is used for the diagnosis of focal liver lesions (FLLs) in adult patients at high risk of hepatocellular carcinoma. CEUS has recently been approved to be used in characterization of FLLs in children. Our study investigated the diagnostic value of CEUS LI-RADS in association with serum alpha-fetoprotein (AFP) in differentiating malignant from benign FLLs in pediatric patients. Our study demonstrated that CEUS LI-RADS combined with AFP may be a powerful diagnostic tool for pediatric patients. LR-4 with elevated AFP, LR-5 or LR-M lesions are highly indicative of malignant tumors.

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## INTRODUCTION

Pediatric patients have different treatment strategies regarding benign and malignant focal liver lesions (FLLs)[1]. Hepatoblastoma (HB) constitutes the most common malignant tumor, accounting for 67% of all pediatric malignant FLLs, followed by hepatocellular carcinoma (HCC), at 28%[2-4]. Thanks to the development of surgical techniques and chemotherapy, the overall 5-year survival rates of HB exceed 80% with timely treatment, and those of nonmetastatic HCC patients who can be treated surgically are 70%-80%[5]. In comparison, the survival rate of children with inoperable hepatic malignancies was less than 20%[6].

Although computed tomography (CT) and magnetic resonance imaging (MRI) are usually recommended for the differential diagnosis of pediatric FLLs[7], both have some limitations. CT increases children's radiation exposure, while MRI requires a long imaging time and high cost[8-10]. Furthermore, children are exposed to the risk of contrast-induced nephrotoxicity and potential use of sedation[11]. Serum alpha-fetoprotein (AFP) is the most widely used tumor biomarker for the screening of HCC and HB in high-risk pediatric populations[12]. However, AFP levels remained in the normal range in 30%-40% of HCC patients and 10% of HB patients. Moreover, the positive predictive value of AFP is poor, making the value of AFP alone as a diagnostic tool very limited[13,14]. Therefore, developing a potent diagnostic method for differentiating benign from malignant FLLs in children is urgently needed.

The American College of Radiology developed the Liver Imaging Reporting and Data System (LI-RADS) to standardize the diagnosis of HCC and assist in the diagnosis of other hepatic malignant tumors[7]. In addition, CEUS can overcome the shortcomings of the aforementioned imaging modalities [15]. Moreover, CEUS has been approved for use in the diagnosis of FLL in the pediatric population [16]. Therefore, this study aimed to evaluate the diagnostic performance of the combination of CEUS LI-RADS and AFP in differentiating benign and malignant FLLs in a pediatric population.

## MATERIALS AND METHODS

This retrospective study was approved by the institutional review board of our hospital, and informed consent was waived.

### Patient selection

From January 2011 to January 2021, hepatic CEUS examinations performed in a tertiary academic medical center were retrospectively collected.

The inclusion criteria were: (1) Age ≤ 18 years at the time of examination; (2) visible liver nodules at baseline US; and (3) sufficient images of the arterial phase, portal phase, and parenchymal phase.

The exclusion criteria were: (1) Lesions previously treated; (2) known or strongly suspected active extrahepatic primary malignancy; (3) poor image quality; and (4) no accepted reference standard (see more detail in a later section).

### Ultrasound examination

Conventional and contrast-enhanced US examinations were performed using a Philips IU22 system (Philips Medical Solutions; Mountain View, CA, United States) with a C5-1 convex or an L9-3 Linear probe. After routine ultrasound examinations, all pediatric patients underwent CEUS examinations using the pulse inversion harmonic imaging technique with a mechanical index less than 0.1. A bolus injection of 1.2 mL of ultrasound contrast agent (SonoVue; Bracco, Milan, Italy) was administered through a vascular catheter needle placed in the anterior cubital vein. The imaging timer was started immediately upon completion of the contrast agent injection. A 5 mL flush of 0.9% sodium chloride solution followed the ultrasound contrast agent injection. The target area was continuously scanned in the first 60 s, followed by intermittent scans and records until the examiner confidently observed washout or faded liver parenchymal enhancement, typically 5 min or longer. CEUS imaging was digitally stored for further evaluation.

### Reference standards

Pathological diagnosis from surgical resection or percutaneous biopsy was taken as the reference standard. In addition, lesions without pathological diagnosis were considered benign if their size increased less than 50% at the 12-mo imaging follow-up. Meanwhile, serum AFP ≥ 20 ng/mL was regarded as elevated[13].

### Diagnostic criteria for differentiating benign and malignant fills

In a previous study, lesions with categories of LR-1, LR-2 or LR-3 were considered benign, while LR-4, LR-5 or LR-M was defined as malignancy[16]. Moreover, the meta–analysis conducted by Christian *et al* [17], including 17 studies (2760 patients, 3556 lesions), showed that 80% of LR-4, 97% of LR-5, and 93% of LR-M lesions were malignant. Therefore, we proposed the following criteria for the diagnosis of malignancy in the pediatric population: Criterion I considered LR-4, LR-5, or LR-M lesions as malignancies; criterion II regarded LR-4, LR-5 or LR-M lesions with simultaneously elevated AFP (≥ 20 ng/mL) as malignancies; and criterion III took LR-4 lesions with elevated AFP or LR-5 or LR-M lesions as malignancies.

### Imaging analysis

Two certified radiologists (Qiu TT and LI JW, with more than 3 years and 5 years of experience in hepatic CEUS, respectively) who were blinded to the reference standard and other clinical data reviewed the CEUS examinations of all cases independently and assigned a category according to the CEUS LI-RADS (2017 version). When there was an inconformity, arbitration from a blinded expert radiologist (Lu Q, with 17 years of experience) was performed. Briefly, the main diagnostic criteria of CEUS LI-RADS are nodule size, enhancement degree and pattern in the arterial phase, timing and degree of washout. Moreover, the ancillary features for category adjustment are nodule-in-nodule architecture and mosaic architecture.

### Statistical analysis

Qualitative data are presented as the numbers and percentages. Quantitative data are presented as a combination of the mean values and standard deviations. The comparison of numeric variables was performed using $t$ tests. Differences in categorical variables were analyzed using $\chi^2$ tests or Fisher's exact tests. The unit of analysis is each FLL rather than each patient. The accuracy, sensitivity, specificity, area under the curve (AUC) and 95% confidence intervals (CI) were calculated to evaluate the diagnostic power of CEUS LI-RADS in association with AFP in distinguishing benign and malignant FLLs. The performance of the diagnostic criteria was further assessed by the fourfold table and compared by using the McNemar test. A $P$ value less than 0.05 was considered statistically significant. The $P$ values were corrected for multiple comparisons through the Bonferroni method (Bonferroni-adjusted $P$ values < 0.017). Given that HCC more commonly occurs in children over 5 years old among pediatric patients[18], subgroup analysis was also conducted. Based on the value of $\kappa$, the strength of agreement is defined as follows: $\kappa$ < 0.20 suggests poor agreement, 0.21-0.40 suggests fair agreement, 0.41-0.60 suggests moderate agreement, 0.61-0.80 suggests good agreement, and 0.80-1.00 suggests almost perfect agreement. Statistical analyses were performed using statistical software (MedCalc10.4.7.0; MedCalc Software, Ostend, Belgium).

## RESULTS

### *Patients and liver nodule characteristics*

According to the inclusion and exclusion criteria, 63 lesions from 60 patients were enrolled in this study (Figure 1), among which 3 patients had 2 FLLs. The main clinical characteristics of the patients, including age, sex, serum AFP, tumor size, and high-risk factors for HCC, are shown in Table 1. The average size of the 63 lesions was 68 ± 39 mm, ranging from 11 to 163 mm. Males accounted for 43.3% of the included patients, and AFP levels exceeding 20 ng/mL were present in 14 patients. The AFP level of malignant lesions (Figure 2) was higher than that of benign lesions [63.6% (14/22) *vs* 4.9% (2/41), $P < 0.0001$]. In our study, 14 patients had high-risk factors for HCC, including 8 chronic hepatitis B and 6 cirrhosis.

Histopathological results and follow-up results of the lesions are summarized in Table 2. Histopathological results of 52 (82.5%) lesions were obtained by surgical resections or US-guided core needle biopsies. The 11 (17.5%) lesions were regarded as benign through the one-year follow-up.

The distribution of FLLs in CEUS LI-RADS categories and lesions with elevated AFP levels are displayed in Table 3. In this study, 2 benign lesions were classified as LR-M, including one granulomatous inflammation and one abscess. Furthermore, 4 benign lesions in LR-5 included one adenomatoid hyperplasia, one abscess, and 2 focal nodular hyperplasia (FNH). Among the lesions defined as LR-4, there were only two lesions with elevated AFP. Postoperative pathology confirmed them as a regenerative nodule and an infantile hemangioendothelioma (Figure 3). The CEUS characteristics of various FLLs are presented in Table 4.

### *Interobserver agreement in CEUS LI-RADS classification*

The rating of liver nodules according to CEUS LI-RADS of the two readers indicated good agreement, with a $\kappa$ value of 0.76 (95%CI: 0.62-0.90).

### *The diagnostic performance of CEUS LI-RADS combined with AFP*

Table 5 summarizes the diagnostic performances of different diagnostic criteria in differentiating benign and malignant FLLs in children. Table 6 shows a comparison of different criteria on indicators of diagnostic performance. Notably, there was no statistically significant difference between the specificity, accuracy, or AUC of criterion II and criterion III (95.1% *vs* 80.5%, 84.1% *vs* 87.3%, and 0.794 *vs* 0.902; all $P > 0.017$). Notably, criterion III showed a higher diagnostic sensitivity than criterion II (100% *vs* 63.6%; $P < 0.017$). However, both the specificity and accuracy of criterion I was inferior to those of criterion II and criterion III (all $P$ values < 0.017).

### *Diagnostic performance of CEUS LI-RADS combined with AFP in pediatric patients > 5 years of age*

In total, 53 FLLs were included in this subgroup analysis. The diagnostic performance of CEUS LI-RADS in association with AFP for predicting overall hepatic malignancy and HCC among patients older than 5 years is shown in Supplementary Table 1. Moreover, a comparison of indicators for diagnostic power among the three criteria is shown in Supplementary Table 2. The performance of the three criteria was similar overall when patients were subcategorized by age when compared to all patients in aggregate. In short, there was no statistically significant difference between the specificity, accuracy, or AUC of criterion II and criterion III (97.2% *vs* 86.1%, 83.3% *vs* 87.0%, and 0.780 *vs* 0.931; all $P > 0.017$). Notably, criterion III showed a higher diagnostic sensitivity than criterion II (100% *vs*58.8%; $P < 0.017$). However, both the specificity and accuracy of criterion I was inferior to those of criterion II and criterion III (all $P < 0.017$). Interestingly, if LR-5 lesions with elevated AFP were regarded as HCC in this subgroup, the sensitivity, specificity, accuracy, and AUC of diagnosing HCC were 80.0% (95%CI: 44.4%-97.5%), 95.4% (95%CI: 84.2%-99.4%), 94.4% (95%CI: 84.6%-98.8%) and 0.877 (95%CI: 0.757-0.951), respectively.

## DISCUSSION

Proper differentiation between benign and malignant FLLs is essential in the treatment of pediatric liver disease. We found that CEUS LI-RADS in association with AFP presented an effective way to differentiate benign tumors from malignancies in pediatric patients. The sensitivity and specificity of criterion III (LR-4 with elevated AFP or LR-5 or LR-M lesions) reached 100.0% and 80.5%, respectively.

The specificity (29.3%) of diagnostic criterion I (LR-4, LR-5, or LR-M lesions) was significantly reduced compared to criteria II and III. This may be because there were a considerable number of benign lesions in LR-4. Notably, differentiation between benign and malignant FLLs in pediatric patients by CEUS LI-RADS alone had an accuracy of 54.0% and specificity of 29.3%, suggesting that CEUS LI-RADS alone is not suitable for this scenario. CEUS LI-RADS was mainly used as a diagnostic tool for HCC in adults at high risk. This study explored the possibility of expanding the application of this diagnostic algorithm in pediatric patients. However, only a few pediatric patients have high-risk

**Table 1 The clinical characteristics of enrolled 60 patients**

| Characteristics | All Patients (*n* = 60) | Patients with malignant lesions (*n* = 20) | Patients with benign lesions (*n* = 40) | *P* value[2] |
|---|---|---|---|---|
| Age, yr; mean ± SD, (range) | 11.0 ± 5.2 (0-18) | 9.7 ± 5.4 (0-18) | 11.7 ± 5.1 (0-18) | 0.98 |
| Gender, *n* (%) | | | | 0.54 |
| Male | 26 (43.3) | 10 (50.0) | 16 (40.0) | |
| Female | 34 (56.7) | 10 (50.0) | 24 (60.0) | |
| AFP level (ng/mL), *n* (%) | | | | < 0.05 |
| AFP > 20 | 14 (23.3) | 12 (60.0) | 2 (5.0) | |
| AFP < 20 | 46 (76.7) | 8 (40.0) | 38 (95.0) | |
| High-risk factors[1] | | | | 0.24 |
| High risk for HCC[1] | 14 (23.3) | 7 (35.0) | 7 (17.5) | |
| No high risk for HCC[1] | 46 (76.7) | 13 (75.0) | 33 (82.5) | |

[1]High risk for hepatocellular carcinoma (HCC) in focal liver lesions in contrast-enhanced ultrasound liver imaging reporting and data system included cirrhosis, chronic hepatitis B viral infection, and current or prior HCC.
[2]*P* values showed whether there were significant differences in age, gender, alpha-fetoprotein level or high-risk factors between the benign and malignant groups.
Note-data are numbers of patients with percentages in parentheses. AFP: Alpha-fetoprotein; HCC: Hepatocellular carcinoma.

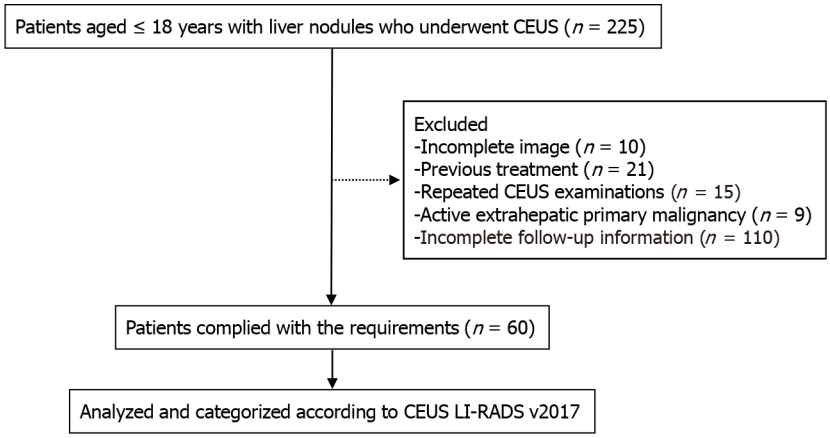


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**Figure 1 Flow diagram for the study population.** CEUS: Contrast-enhanced ultrasound; LI-RADS: Liver imaging reporting and data system.

factors for HCC, and the disease spectrum of FLLs between adults and children is different. HB and HCC account for a majority of pediatric hepatic malignancies, while hemangioma and FNH account for a majority of pediatric hepatic benign lesions. Because a significant difference in AFP was found between benign and malignant FLLs[19], CEUS LI-RADS combined with serum AFP is proposed for better characterization of FLLs in pediatric patients.

Compared with criterion III, the sensitivity (63.6%) of criterion II decreased significantly. A possible explanation was that 2 HCC patients and 6 patients with other hepatic malignancies presented normal serum AFP values (< 20 ng/mL), resulting in false negatives of the aforementioned lesions according to criterion II.

In this study, 13 FNHs were assigned to LR-4, and 2 FNHs were assigned to LR-5. A retrospective study by Kong *et al*[20] found that 42.9% of FNHs displayed global homogeneous hyperenhancement, and 42.9% of FNHs showed centrifugal enhancement in the arterial phase. Centrifugal arterial enhancement was often present in FNH < 3 cm. This is probably because the blood supply of larger lesions is more abundant[21]. Moreover, atypical FNHs could demonstrate washout in the portal and late phases[22]. Due to the above reasons, FNHs could be classified as LR-4 or LR-5 Lesions. However, AFP in patients with FNH is generally within the normal range[23]. Therefore, the combination of CEUS LI-RADS and AFP may potentially avoid diagnosing FNH as a malignancy.

**Table 2 Number of included fills with each diagnosis, stratified by reference standard**

| Diagnosis | All fills (*n* = 63) | FIls from Patients > 5 yr (*n* = 53) |
|---|---|---|
| Pathologic analysis | 2 | 42 |
| Malignant liver lesions | 22 | 17 |
| HCC | 10 | 10 |
| HB | 6 | 2 |
| Undifferentiated sarcoma | 2 | 1 |
| Non-Hodgkin's lymphoma | 1 | 1 |
| Neuroendocrine carcinoma | 1 | 1 |
| Desmoplastic small round cell tumor | 1 | 1 |
| Perivascular epithelioid cell tumor | 1 | 1 |
| Benign liver lesions | 30 | 25 |
| FNH | 14 | 12 |
| RN/DN | 3 | 3 |
| Area of granulomatous inflammation | 3 | 3 |
| Adenomatoid hyperplasia | 3 | 3 |
| Infantile hemangioendothelioma | 2 | 0 |
| Liver abscess | 1 | 0 |
| Other benign tumors | 3 | 3 |
| Follow-up < 50% size increase in 12 mo | 11 | 11 |
| Hemangioma | 3 | 3 |
| FNH | 3 | 1 |
| RN/DN | 2 | 2 |
| Other benign tumors | 3 | 3 |

FLLs: Focal liver lesions; HCC: Hepatocellular carcinoma; HB: Hepatoblastoma; FNH: Focal nodular hyperplasia; DN: Dysplastic nodule; RN: Regenerative nodule.

**Table 3 All focal liver lesions in contrast-enhanced ultrasound liver imaging reporting and data system categorization and distribution of elevated alpha-fetoprotein**

| CEUS LI-RADS | No. of nodules (*n* = 63) | No. of malignant lesions (*n* = 22) | No. of benign lesions (*n* = 41) | AFP > 20 ng/mL (*n* = 16) |
|---|---|---|---|---|
| LR-1 | 4 | 0 | 4 | 0 |
| LR-2 | 0 | 0 | 0 | 0 |
| LR-3 | 8 | 0 | 8 | 0 |
| LR-4 | 23 | 0 | 23 | 2 |
| LR-5 | 22 | 18 | 4 | 13 |
| LR-M | 6 | 4 | 2 | 1 |

FLLs: Focal liver lesions; CEUS LI-RADS: Focal liver lesions in contrast-enhanced ultrasound liver imaging reporting and data system; AFP: Alpha-fetoprotein.

We also performed subgroup analysis by the age of 5 to explore whether those patients could use CEUS LI-RADS combined with AFP to identify malignant FLLs or even HCC. For differentiating malignant from benign FLLs, the results of subgroup analysis were similar to the overall analysis. LR-5 in adult patients had a high diagnostic specificity for HCC. In this study, LR-5 Lesions with elevated AFP for diagnosing HCC presented high specificity (95.4%) in pediatric patients over 5 years old.

**Table 4 Imaging characteristics of different types of focal liver lesions**

| Image features | Malignant lesions | | | Benign lesions | | |
|---|---|---|---|---|---|---|
| | HCC (*n* = 10) | HB (*n* = 6) | Other malignant lesions (*n* = 6) | FNH (*n* = 17) | RN/DN (*n* = 5) | Other benign tumors (*n* = 18) |
| Gray-scale echogenicity | | | | | | |
| Hyperechoic | 3 | 4 | 5 | 4 | 2 | 9 |
| Hypoechoic | 7 | 2 | 1 | 13 | 3 | 9 |
| Arterial phase, hyperenhancement | | | | | | |
| Homogeneous | 4 | 2 | | 9 | 1 | 4 |
| Inhomogenous | 6 | 4 | 5 | 8 | | 5 |
| Rim | | | 1 | | | 2 |
| Peripheral nodular | | | | | | 3 |
| Isoenhancement | | | | | 2 | 2 |
| Hypoenhancement | | | | | 2 | 2 |
| Late phase | | | | | | |
| Hyperenhancement | | | | 10 | | 5 |
| Isoenhancement | | | | 5 | 5 | 8 |
| Hypoenhancement | 10 | 6 | 6 | 2 | | 5 |
| Washout | | | | | | |
| < 60 s | | 1 | 3 | | | 1 |
| Marked, ≤ 120 s | | | 1 | | | |

Data are numbers of nodules. FLLs: Focal liver lesions; HCC: Hepatocellular carcinoma; HB: Hepatoblastoma; FNH: Focal nodular hyperplasia; DN: Dysplastic nodule; RN: Regenerative nodule.

**Table 5 Performance of various diagnostic criteria for differentiating benign and malignant focal liver lesions**

| Diagnostic criteria | Sensitivity (%) | Specificity (%) | Accuracy (%) | AUC |
|---|---|---|---|---|
| Criterion I | 100.0 (84.6-100.0) | 29.3 (16.1-45.5) | 54.0 (40.9-66.6) | 0.646 (0.516-0.763) |
| Criterion II | 63.6 (40.7-82.8) | 95.1 (83.5-99.4) | 84.1 (72.7-92.1) | 0.794 (0.673-0.885) |
| Criterion III | 100.0 (84.6-100.0) | 80.5 (65.1-91.2) | 87.3 (76.5-94.4) | 0.902 (0.801-0.963) |

Criterion I considered LR-4, LR-5, or LR-M lesions as malignancies; criterion II regarded LR-4, LR-5 or LR-M lesions with simultaneously elevated alpha-fetoprotein (AFP, ≥ 20 ng/mL) as malignancies; criterion III took LR-4 lesions with elevated AFP or LR-5 or LR-M lesions as malignancies. AUC: Area under the curve.

Consequently, we speculate that CEUS LI-RADS combined with AFP has the potential to diagnose HCC in children older than 5 years. Nevertheless, the number (*n* = 10) of pediatric HCC patients included in this study was too small. Further study with a larger sample is needed to validate this hypothesis.

In this study, a 19-hour-old newborn patient with infantile hemangioendothelioma presented a significant increase in AFP levels (AFP > 1210 ng/mL). Regarding the features of CEUS, the patient showed inhomogeneous hyperenhancement in the arterial phase and isoenhancement in the portal and delayed phases, and there were areas of nonenhancement within the lesion. The aforementioned feature indicated that the lesion was likely a benign lesion. However, because the lesion was diagnosed as malignant by contrast-enhanced CT, the patient underwent surgical resection of the hepatic mass. Postoperative pathology confirmed that the lesion was an infantile hemangioendothelioma. Within 60 ± 24 h after birth, the serum AFP of newborns can range from 9700 to 11190 ng/mL and drop rapidly to a level close to the normal level of adults within one year[24]. Therefore, we should be meticulous with elevated AFP in differentiating FLLs of newborns. In addition, infantile hemangioendothelioma is a common benign tumor in newborns, most of which do not require surgical treatment[25]. Therefore, the diagnosis of benign and malignant FLLs in newborns should be made with caution, and the diagnostic

**Table 6 Comparison of different criteria on indicators of diagnostic performance**

| *P* value | Sensitivity | Specificity | Accuracy | AUC |
|---|---|---|---|---|
| Criterion I *vs* criterion II | < 0.017 | < 0.0001 | < 0.017 | > 0.017 |
| Criterion I *vs* criterion III | - | < 0.0001 | < 0.0001 | < 0.0001 |
| Criterion II *vs* criterion III | < 0.017 | > 0.017 | > 0.05 | > 0.05 |

Criterion I considered LR-4, LR-5, or LR-M lesions as malignancies; criterion II regarded LR-4, LR-5 or LR-M lesions with simultaneously elevated alpha-fetoprotein (AFP, ≥ 20 ng/mL) as malignancies; criterion III took LR-4 lesions with elevated AFP or LR-5 or LR-M lesions as malignancies. AUC: Area under the curve.

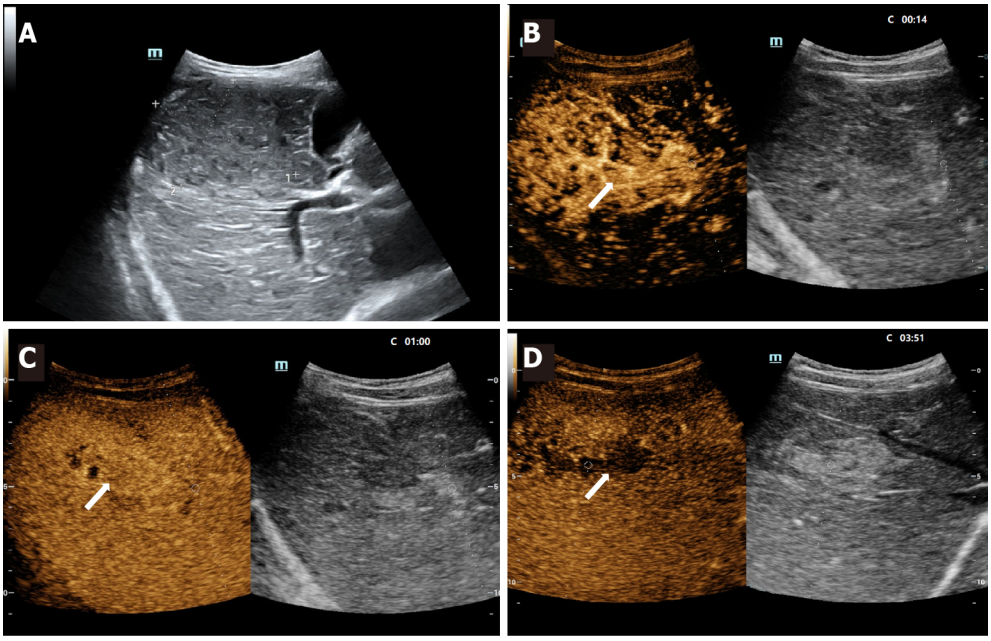


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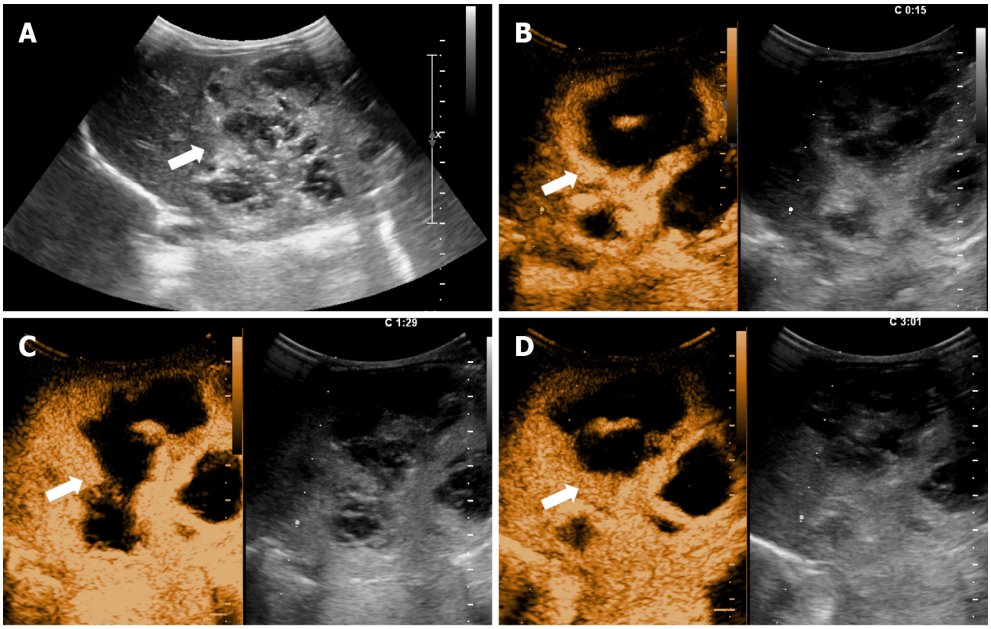
**Figure 2 LR-5 nodule in a 10-year-old boy.** A: A hypoechoic nodule (arrow) measuring 7.3 cm in the right lobe of the liver was shown at conventional gray-scale US; B: The lesion was inhomogeneously hyperenhanced (arrow) in the arterial phase (14 s) at contrast-enhanced US; C: The lesion was seen iso-enhanced in the portal phase (60 s); D: Mild washout in the late phase (231 s) was shown. There were small areas of nonenhancement within the lesion during the whole process. The patient had a chronic hepatitis B viral infection. The serum AFP level was greater than 1210 ng/mL. This lesion was assigned to LR-5 and was confirmed as hepatocellular carcinoma by histopathologic analysis.

method needs to be further explored.

This study had several limitations. First, this was a retrospective study with a relatively small sample size, which may inevitably lead to selection bias. Second, CEUS LI-RADS was mainly used for patients at risk of HCC, while only 14 patients in this study met the prerequisites for risk factors. Moreover, the risk factors for HCC in children do not exactly correspond to those in adults. Lastly, there were a considerable number of benign lesions confirmed by histopathology results, which might have led to the selection of benign lesions with atypical imaging findings. Thus, the specificity of the diagnostic criteria may have been underestimated.

## CONCLUSION

We propose a novel method that might be a powerful diagnostic tool to differentiate malignant from benign FLLs in pediatric patients. LR-4 with elevated AFP, LR-5 or LR-M lesions could effectively differentiate benign and malignant tumors in pediatric patients.




**DOI:** 10.3748/wjg.v28.i21.2350 

**Figure 3 LR-4 nodule in a 19-hour-old newborn.** A: An inhomogeneous hyperechoic nodule measuring 7.2-cm (arrow) in the left lobe of the liver was shown at conventional gray-scale US; B: The lesion was inhomogeneously hyperenhanced (arrow) with large area of unenhancement in the arterial phase (15 s) at contrast-enhanced US; C: The enhanced area of the lesion was seen slightly hyperenhanced (arrow) in the portal phase (89 s); D: The enhanced area of the lesion was seen iso-enhanced (arrow) in the late phase (181 s). There were patchy areas of nonenhancement within the lesion during the whole process. The serum AFP level was greater than 1210 ng/mL. Infantile hemangioendothelioma was confirmed at histopathologic analysis.

## ARTICLE HIGHLIGHTS

### *Research background*

Contrast-enhanced ultrasound (CEUS) has recently been approved to be used in characterization of focal liver lesions (FLLs) in children. The America College of Radiology developed the CEUS liver imaging reporting and data system (LI-RADS) for standardizing CEUS diagnosis of FLLs in adult patients. However, it is not suitable for pediatric patients.

### *Research motivation*

To explore a method for differentiating benign and malignant FLLs in pediatric patients.

### *Research objectives*

To evaluate the performance of CEUS LI-RADS combined with alpha-fetoprotein (AFP) in differentiating benign and malignant FLLs in pediatric patients.

### *Research methods*

The following criteria for diagnosing malignancy were proposed: Criterion I considered LR-4, LR-5, or LR-M lesions as malignancies; criterion II regarded LR-4, LR-5 or LR-M lesions with simultaneously elevated AFP (≥ 20 ng/mL) as malignancies; criterion III took LR-4 Lesions with elevated AFP or LR-5 or LR-M lesions as malignancies. The sensitivity, specificity, accuracy and area under the receiver operating characteristic curve (AUC) were calculated to determine the diagnostic value of the aforementioned criteria.

### *Research results*

There were no statistically significant differences between the specificity, accuracy, or AUC of criterion II and criterion III. Notably, criterion III showed a higher diagnostic sensitivity than criterion II. However, both the specificity and accuracy of criterion I was inferior to those of criterion II and criterion III. For pediatric patients more than 5 years old, the performance of the three criteria was overall similar when patients were subcategorized by age when compared to all patients in aggregate.

### *Research conclusions*

We propose a novel method that might be a powerful diagnostic tool to differentiate malignant from benign FLLs in pediatric patients. LR-4 with elevated AFP, LR-5 or LR-M lesions could effectively differentiate benign and malignant tumors in pediatric patients.

### *Research perspectives*

CEUS LI-RADS combined with AFP might be a powerful diagnostic tool to differentiate malignant from benign FLLs in pediatric patients.

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## FOOTNOTES

**Author contributions:** Jiang ZP and Lu Q designed the research; Jiang ZP, Li JW, Qiu TT, Luo Y and Lu Q performed the research; Jiang ZP, Zeng KY, Huang JY, Yang J and Yang R collected the data; Jiang ZP and Lu Q drafted the manuscript and review the literatures; Luo Y and Lu Q revised the manuscript; all authors contributed to approval of final version of submitted manuscript and agree to ensure that any questions related to the work are appropriately resolved.

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ORIGINAL ARTICLE

Observational Study

# Socioeconomics and attributable etiology of primary liver cancer, 1990-2019

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## Abstract

### *BACKGROUND*

Primary liver cancer (PLC) is a major contributor to cancer-related deaths. Data on global and country-specific levels and trends of PLC are essential for understanding the effects of this disease and helping policymakers to allocate resources.

### *AIM*

To investigate the association between the burden of PLC and socioeconomic development status.

### *METHODS*

Cancer mortality and incidence rates were obtained from the Global Burden of Disease (GBD) 2019, and the data were stratified by country and territory, sex, and the Socio-demographic Index (SDI) level. The association between the attributable etiology of PLC and socioeconomic development status, represented using the SDI, was described. The attributable etiology of PLC included hepatitis B, hepatitis C, alcohol use, and nonalcoholic steatohepatitis. The association between the attributable etiology of PLC and SDI was further stratified by sex and geographical location. A confidence analysis was also performed based on bootstrap draw.

*RESULTS*

The age-standardized incidence rate of PLC was 6.5 [95% confidence intervals (CI): 5.9-7.2] per 100000 person-years, which decreased by -27.5% (-37.0 to -16.6) from 1990 to 2019. Several countries located in East Asia, South Asia, West Africa, and North Africa shouldered the heaviest burden of PLC in 2019. In terms of incidence rates, the first leading underlying cause of PLC identified was hepatitis B, followed by hepatitis C, alcohol use, and nonalcoholic steatohepatitis. Regarding stratification using the SDI, the incidence rate of PLC was the highest for high and middle SDI locations. Further, the leading attributable etiologies of PLC were hepatitis B for the middle and high middle SDI locations while hepatitis C and nonalcoholic steatohepatitis for the high SDI locations.

*CONCLUSION*

The pronounced association between socioeconomic development status and PLC burden indicates socioeconomic development status affects attributable etiologies for PLC. GBD 2019 data are valuable for policymakers implementing PLC cost-effective interventions.

**Key Words:** Epidemiology; Public health; Socioeconomics; Primary liver cancer; Hepatitis; Alcohol



**Core Tip:** Primary liver cancer (PLC) is a common cancer with high morbidity and mortality rates. PLC usually occurs as a preventable disease. An association was identified between socioeconomic development status and PLC burden. The leading attributable etiologies of PLC were hepatitis B for the middle and high middle Socio-demographic Index (SDI) locations, and hepatitis C and nonalcoholic steatohepatitis for the high SDI locations. Our findings are valuable to implement tailored prevention strategies for PLC.

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## INTRODUCTION

Primary liver cancer (PLC) was the third leading cause of cancer deaths in 2020 following lung and colorectal cancer[1]. In terms of cancer-related mortality, PLC was the third leading cause in China[2] and the fifth leading cause in the United States[3]. The burden of PLC varies significantly in terms of sex and geographic region due to different risk-factor exposure. The major risk factors include chronic viral infections [hepatitis B virus (HBV), hepatitis C virus (HCV)], alcohol use, and nonalcoholic steatohepatitis (NASH), and they have been widely studied in recent years[4]. PLC is caused by chronic hepatitis B (CHB) (60%) in Africa and East Asia, whereas chronic hepatitis C (CHC) appears to be the major risk factor in the Western world[5]. Thus, it is expected that the appropriate handling of risk factors can significantly contribute to the overall reduction of PLC-related deaths in the near future.

The Global Burden of Disease (GBD) database has been constructed to improve health systems and eliminate disparities; this database comprises a comprehensive catalog of censuses, vital statistics, surveys, and other health-related data. Policymakers can benefit from the GBD database as it enables them to understand the true nature of the health challenges of a country and the shifting challenges over time. In recent years, the prevention of PLC has been eclipsed by substantial improvements in PLC treatment. Given the marked lag between risk factor exposure and the development of PLC, even the well-proven prevention approaches would take decades to reduce of the PLC burden. Although several prior studies have focused on the global prevalence of PLC[4,6], few studies focus on the tailored prevention of PLC. In this study, we focused on identifying the effect of socioeconomic development status on the attributable etiologies of PLC from a global perspective. We hope our findings will be helpful contributions for developing specialized prevention strategies for PLC. Considering the heavy burden of PLC, characterizing this association will help health workers to design tailored prevention strategies and policymakers to allocate research and clinical resources for implementing cost-effective interventions for PLC.

## MATERIALS AND METHODS

### Data sources

For this study, the incidence and death rates of PLC were acquired from the GBD 2019 (http://ghdx.healthdata.org/gbd-2019) that covered 204 countries and territories[7]. The incidence and death rates were age-standardized according to the GBD 2019 world population recorded per 100000 person-years. The International Classification of Diseases, Tenth Revision (ICD-10) was adopted. The ICD-10 codes for PLC are C22-C22.4, C22.7-C22.8, and Z85.05 (Supplementary material, page 17). Mortality and non-fatal estimates have been described in detail in previous studies[8,9]. Additional information is provided in the Supplementary material.

### Confidence analysis

We assumed that the incidence or death rates in each year followed a log-normal distribution and that the rates in different years were independent of each other. Based on these assumptions, in each bootstrap draw, we measured the increase rates and 95%CIs based on the 25$^{th}$ and 975$^{th}$ ranked values across all 1000 draws. The 2.5% and 97.5% quantiles from the 1000 draws of the posterior distribution were used to generate 95%CIs.

### Socio-demographic Index

The Socio-demographic Index (SDI) incorporates the mean education level for individuals aged 15 years and older, the total fertility rate in women under the age of 25 years, and lag-distributed income per person. The method of generating the SDI is described in the report by the GBD 2016 Mortality Collaborators[10]. Further, the SDI was used to evaluate the effect of the development levels of a country or region on the burden of PLC based on data obtained from the GBD 2019 (Supplementary material, pages 1-15). The values of the SDI range from 0 to 1, which correspond to the development level of a country or region from the worst to the best. The SDI was categorized based on the references bound as low SDI, low middle SDI, middle SDI, high middle SDI, and high SDI, as shown in the Supplementary material, page 16.

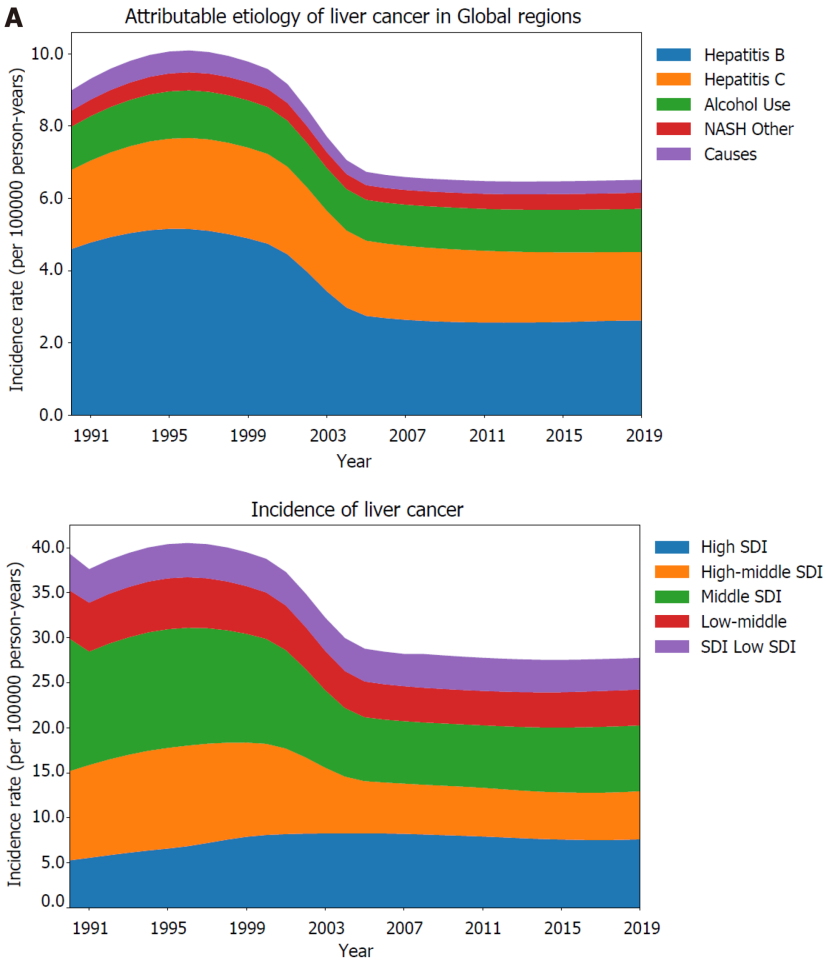
### Ethic statement

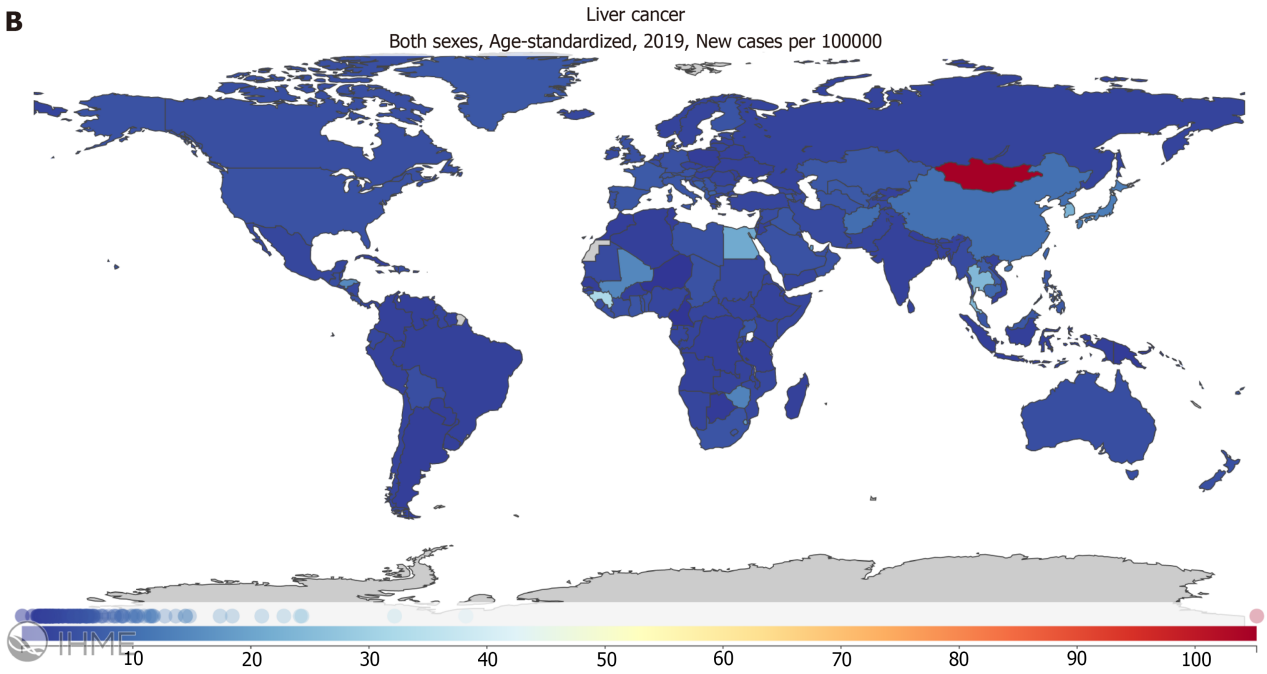
The study was reviewed and approved by the Ethics Committee of First Affiliated Hospital of Fujian Medical University (MTCA, ECFAH of FMU[2015]084-1).

## RESULTS

### Burden of liver cancer

Liver cancer is one the most common cancers. In 2019, the global age-standardized incidence rate of PLC was 6.5 (95%CI: 5.9-7.2) per 100000 person-years (Supplementary material, page 25). Fortunately, the incidence rate of PLC has declined significantly by -27.5% (-37.0 to -16.6) from 1990 to 2019 (Figure 1A; Supplementary material, page 18). The main contributor for this drop was the decreasing burden of PLC caused by hepatitis B and the declining burden of PLC in the middle SDI locations. Between 1990 and 2019, the global incidence rate of PLC peaked in 1995-1996, and then, it decreased gradually. However, the incidence rate of PLC has not declined further since 2010 (Figure 1A; Supplementary material, pages 23-25). Before 2004, the incidence rate of PLC for middle SDI locations surpassed that for high SDI locations whereas high SDI locations exceeded middle SDI locations in terms of the burden of PLC after 2004 (Figure 1A; Supplementary Figure 1B; Supplementary material, pages 29-32). In terms of the incidence rates, the leading underlying cause of PLC was HBV, followed by HCV, alcohol use, and NASH (Figure 1A). Hepatitis B manifested the most drastic decline between 1990 and 2019 as the underlying causes of PLC [57.0% (45.3-71.4)] (Figure 1A; Supplementary material, pages 19-20). Stratified using the SDI, the age-standardized incidence rate of PLC was found to be the highest for high and middle SDI locations compared to those for high middle, low middle, and low SDI locations (Figure 1A; Supplementary Figure 1B; Supplementary material, pages 29-32). Further, a declining pattern was observed for the age-standardized incidence rate of PLC in the high middle [53.8% (45.1-64.5)] and middle SDI locations [49.7% (41.1-59.9)] compared with the increasing trend in the high SDI locations [144.5% (130.3-159.6)] (Figure 1A; Supplementary Figure 1B; Supplementary material, page 18). Between 1990 and 2019, PLC caused by hepatitis B and hepatitis C showed a decreasing trend in the death rate (Figure 1C; Supplementary material, pages 21-22). Stratified using the SDI, the high middle, middle, and low middle SDI locations showed decreasing trends in the age-standardized death rate of PLC. In contrast, the high SDI location showed an increasing trend in the age-standardized death rate of PLC (Figure 1C; Supplementary material, page 18). Several countries located in East Asia, South Asia, West Africa, and North Africa shouldered the heaviest burden of the PLC incidence and death rates. For the age-standardized incidence rate of PLC, Mongolia demonstrated the highest burden [105.2 (82.6-131.5)] per 100000 person-years), followed by Gambia and Guinea (Figure 1B; Supplementary material,


A
Attributable etiology of liver cancer in Global regions
Incidence rate (per 100000 person-years)
Year
Hepatitis B
Hepatitis C
Alcohol Use
NASH Other
Causes
Incidence of liver cancer
Incidence rate (per 100000 person-years)
Year
High SDI
High-middle SDI
Middle SDI
Low-middle
SDI Low SDI


B
Liver cancer
Both sexes, Age-standardized, 2019, New cases per 100000
10
20
30
40
50
60
70
80
90
100

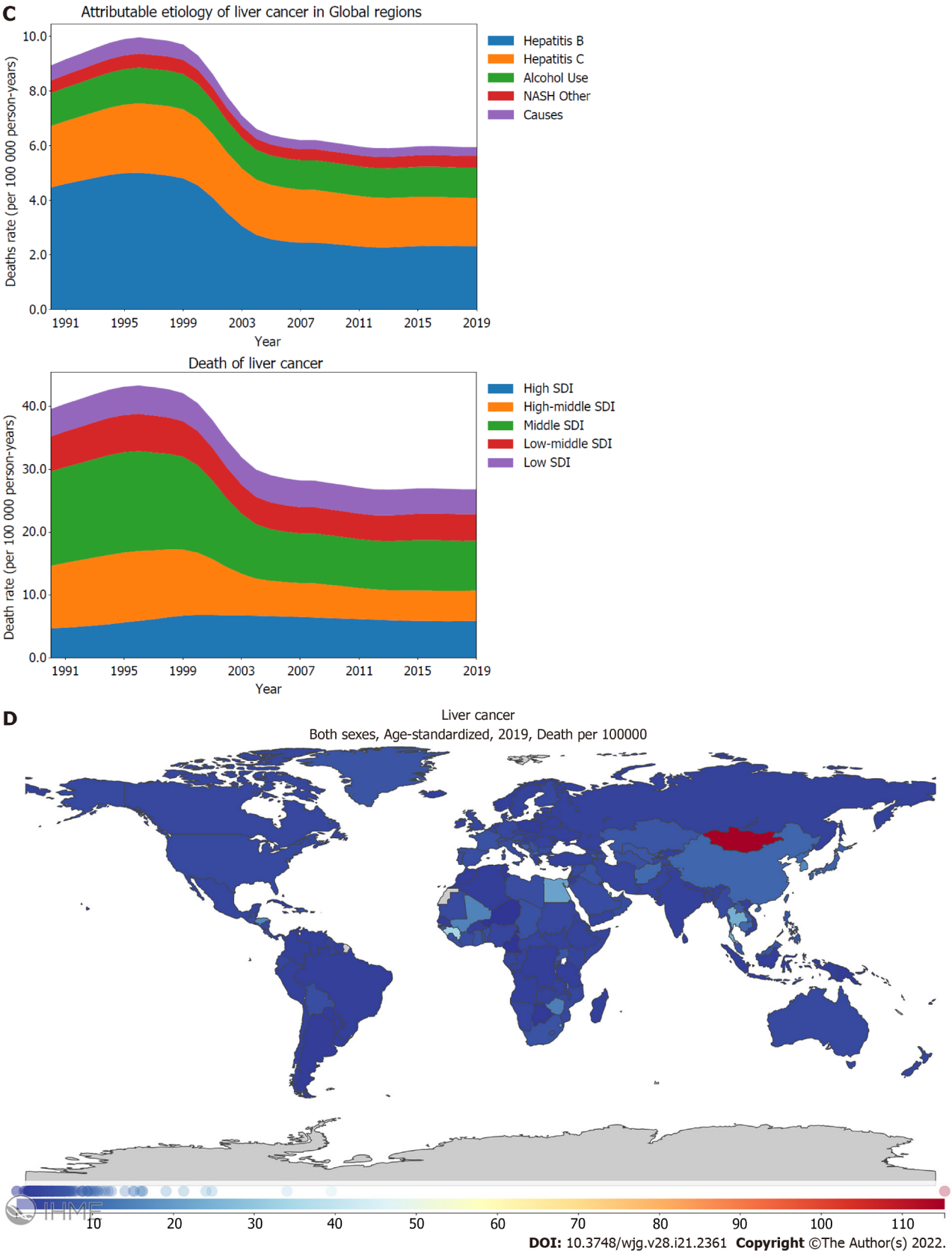



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**Figure 1 Burden of liver cancer for 204 countries and territories.** A and C: Age-standardized incidence (A) and death (C) rates per 100000 population for liver cancer from 1990 through 2019, stratified by the attributable etiology of liver cancer or the Socio-demographic Index; B and D: Age-standardized incidence (B) and death (D) rate of liver cancer per 100000 person-years by country and territory, in 2019. The maps in (B) and (D) are generated using the Global Burden of Disease 2019 tool. SDI: Socio-demographic Index; NASH: Nonalcoholic steatohepatitis.

pages 33-35). Countries that possessed the highest burden PLC incidence rate also had the highest burden PLC death rate (Figure 1D; Supplementary material, pages 46-48).

### Burden of liver cancer caused by hepatitis B

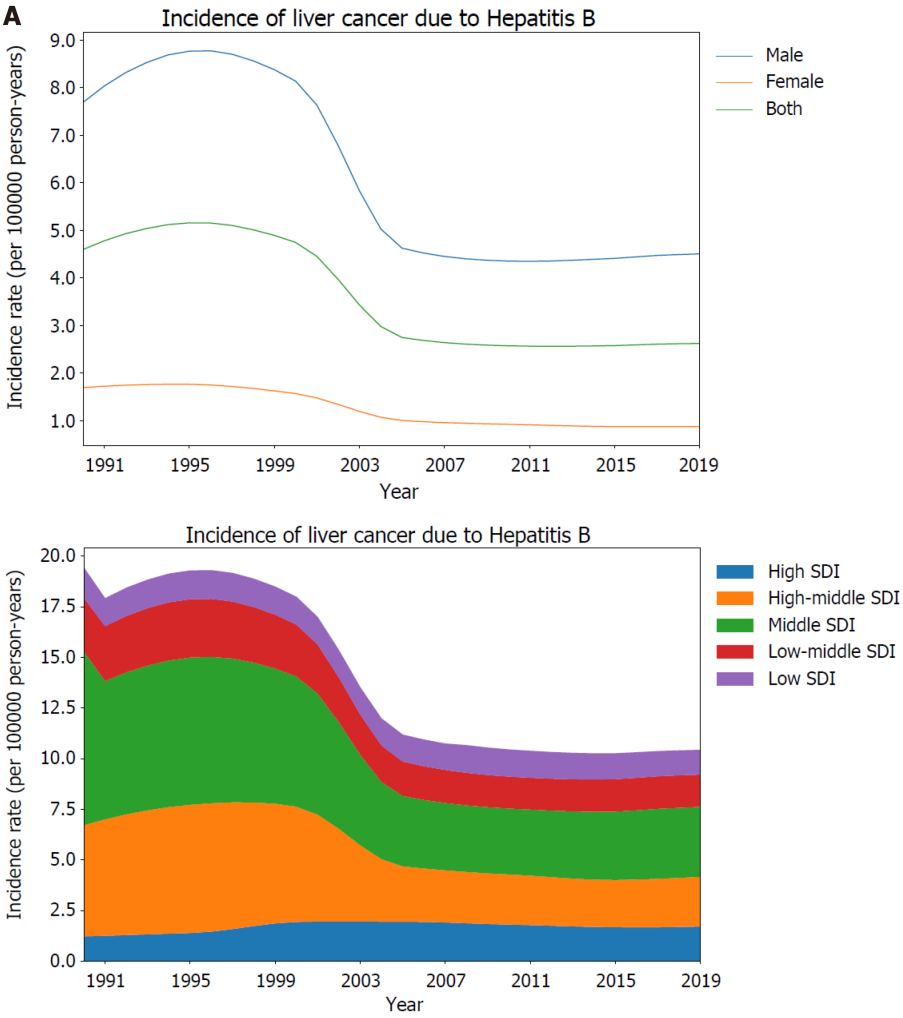
The global age-standardized incidence rate of PLC caused by hepatitis B reached its peak in 1995-1996, and then decreased gradually. However, the burden of the incidence rate has remained stable and has not declined further since 2005. By stratification using sex, the age-standardized incidence rate of PLC caused by hepatitis B was found to be four times higher in males than that in females (Figure 2A; Supplementary material, pages 49-50). Moreover, the age-standardized incidence rate of PLC caused by hepatitis B was found to be higher for middle and high middle SDI locations than for high, low middle, and low SDI locations (Figure 2A; Supplementary Figure 2A; Supplementary material, pages 51-57). Between 1990 and 2019, the decreasing trend in the age-standardized incidence rate of PLC caused by hepatitis B differed significantly based on SDI regions, with the highest declines in the middle [40.3% (31.1-51.8)] and high middle SDI locations [44.8% (34.2-58.8)]. In contrast, high SDI locations showed an increasing trend [139.3% (112.1-173.3)] (Figure 2A; Supplementary Figure 2A; Supplementary material, pages 19-20). In 2019, the incidence rate of PLC caused by hepatitis B differed dramatically between countries or regions. In particular, the highest age-standardized incidence rate was recorded in Mongolia with 27.3 (18.0-39.1) per 100000 person-years, followed by Gambia and Guinea (Figure 2B; Supplementary material, pages 58-61). Similar to the age-standardized incidence rate of PLC caused by hepatitis B, the burden of the PLC death rate caused by hepatitis B was higher for males than that for females (Figure 2C; Supplementary material, pages 62-63). Between 1990 and 2019, the age-standardized death rate of PLC caused by hepatitis B decreased significantly in the high middle [39.0% (30.2-50.6)] and middle SDI locations [44.7% (34.7-57.4)]. However, the high SDI locations showed an increasing trend [113.4% (90.6-141.6)] (Figure 2C; Supplementary Figure 2B; Supplementary material, pages 21-22). In 2019, Mongolia had the highest age-standardized death rate with 28.2 (18.9-40.8) per 100000 person-years, followed by Gambia and Guinea (Figure 2D; Supplementary material, pages 67-70).

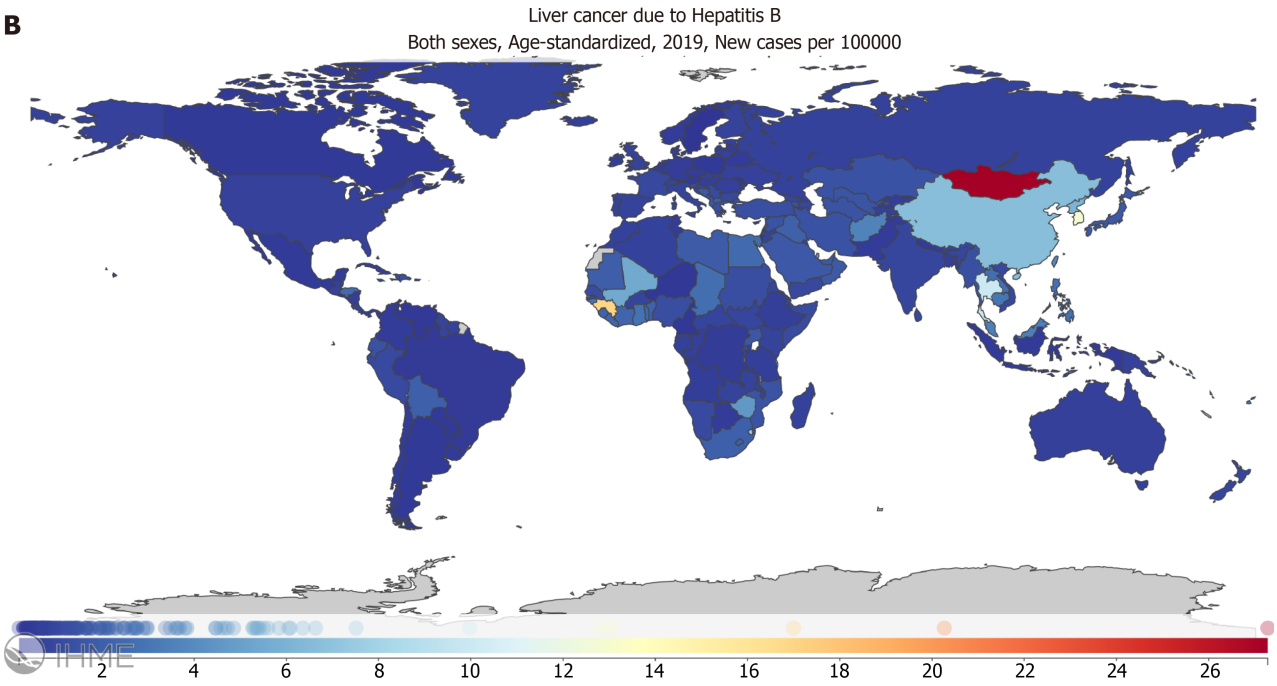
### Burden of liver cancer caused by hepatitis C

Hepatitis C is the second leading cause of PLC. By stratification using sex, the age-standardized incidence rate and mortality rate of PLC caused by hepatitis C in males was found to be higher than those in females (Figure 3A and C; Supplementary material, pages 71-72 and 80-81). Further, the age-standardized incidence rate of PLC caused by hepatitis C was higher for high and middle SDI locations than for high middle, low middle, and low SDI locations (Figure 3A; Supplementary Figure 3A; Supplementary material, pages 73-75). From 1990 through 2019, the age-standardized incidence rate of PLC caused by hepatitis C differed significantly between the SDI regions, with the middle [59.5% (46.5-76.3)] and high middle SDI locations [63.3% (51.3-78.0)] exhibiting declining trends whereas the high SDI location [133.4% (112.5-158.2)] showed increasing trends (Figure 3A; Supplementary Figure 3A; Supplementary material, pages 19-20). In 2019, the incidence rate of PLC caused by hepatitis C manifested a substantial variance between countries or regions. The highest age-standardized incidence rate was recorded in Mongolia with 35.0 (24.7-46.8) per 100000 person-years, followed by Egypt and Japan (Figure 3B; Supplementary material, pages 76-79). Between 1990 and 2019, the age-standardized death rate of PLC caused by hepatitis C decreased significantly in high middle [57.9% (47.5-71.1)] and middle SDI locations [58.7% (46.2-74.4)]. However, the high SDI locations showed an increasing trend [119.5% (101.8-139.8)] (Figure 3C; Supplementary Figure 3B; Supplementary material, pages 21-22). In 2019, Mongolia had the highest age-standardized death rate with 40.3 (28.6-53.3) per 100000 person-years, followed by Egypt (Figure 3D; Supplementary material, pages 85-88).

### Burden of liver cancer caused by alcohol use

For PLC caused by alcohol use, the age-standardized incidence rate in males was four times higher than that in females (Figure 4A; Supplementary material, pages 89-90). Similar to PLC caused by hepatitis C, the age-standardized incidence rate of PLC caused by alcohol use was found to be higher for high SDI locations than other SDI locations when stratified using the SDI (Figure 4A; Supplementary Figure 4A; Supplementary material, pages 91-93). From 1990 through 2019, there was a notable difference in the trends for age-standardized incidence rates of PLC caused by alcohol use between SDI regions; high middle SDI locations [72.7% (54.3-96.4)] showed a significant decline. In contrast, high SDI locations showed a significant increase [163.5% (126.4-209.8)] (Figure 4A; Supplementary Figure 4A; Supplementary material, pages 19-20). In 2019, the highest incidence rate of PLC caused by alcohol use was recorded in Mongolia with 31.8 (21.3-44.7) per 100000 person-years, followed by Gambia and Thailand (Figure 4B; Supplementary material, pages 94-97). Males showed a higher burden of death rate of PLC caused by alcohol use than females, which corresponds with the higher incidence rate of PLC caused by alcohol use in males (Figure 4C; Supplementary material, pages 98-99). Between 1990 and 2019, the age-standardized death rate of PLC caused by alcohol use decreased significantly in high middle SDI locations [67.6% (50.9-88.9)]. However, high SDI locations showed an increasing trend [141.2% (111.0-179.2)] (Figure 4C; Supplementary Figure 4B; Supplementary material, pages 21-22). In 2019, Mongolia had the highest age-standardized death rate with 34.2 (23.1-47.8) per 100000 person-years, followed by


A
Incidence of liver cancer due to Hepatitis B
Incidence rate (per 100000 person-years)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
1991
1995
1999
2003
2007
2011
2015
2019
Year
Male
Female
Both
Incidence of liver cancer due to Hepatitis B
Incidence rate (per 100000 person-years)
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
Year
High SDI
High-middle SDI
Middle SDI
Low-middle SDI
Low SDI


B
Liver cancer due to Hepatitis B
Both sexes, Age-standardized, 2019, New cases per 100000
IHME
2
4
6
8
10
12
14
16
18
20
22
24
26

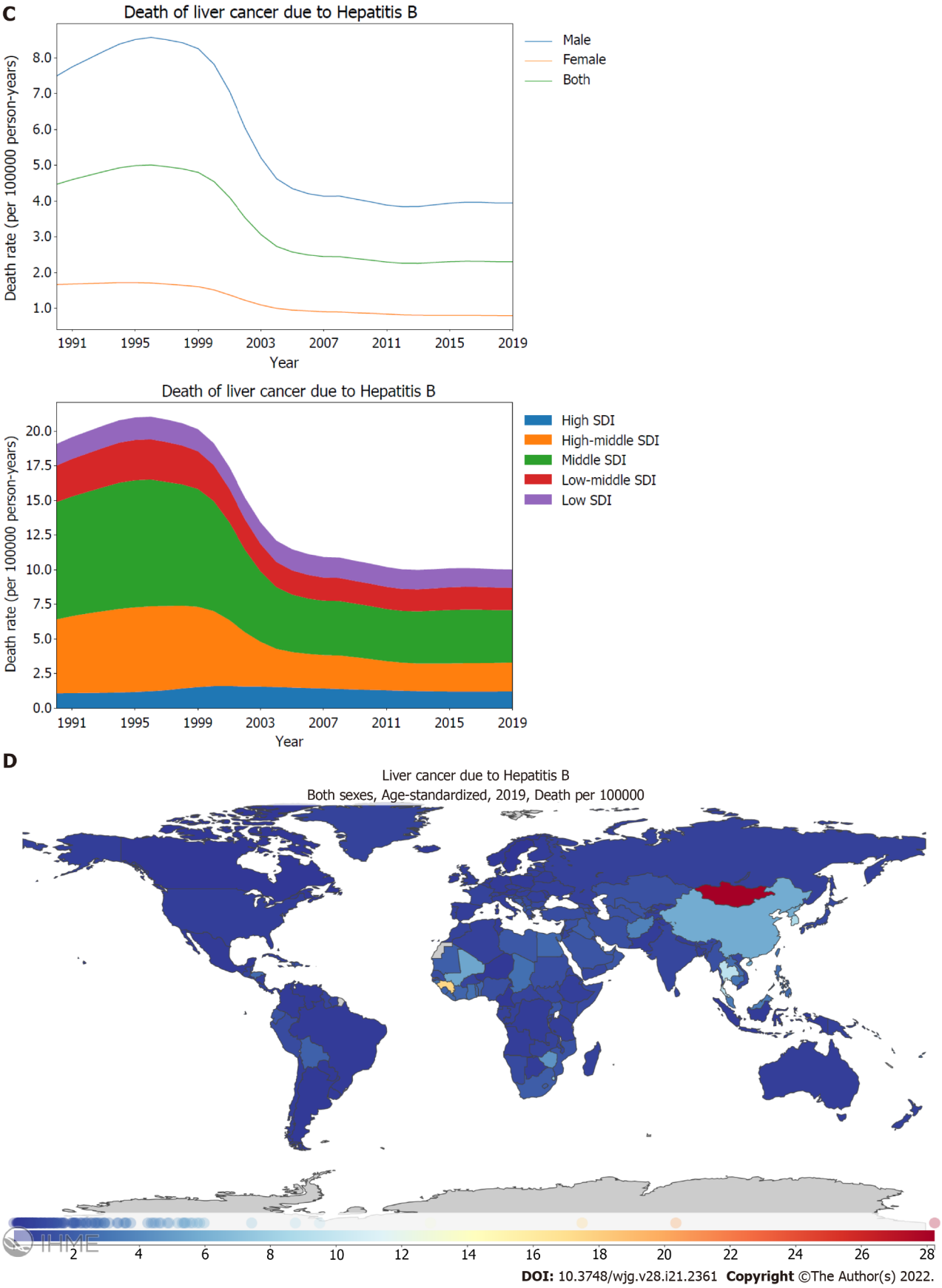
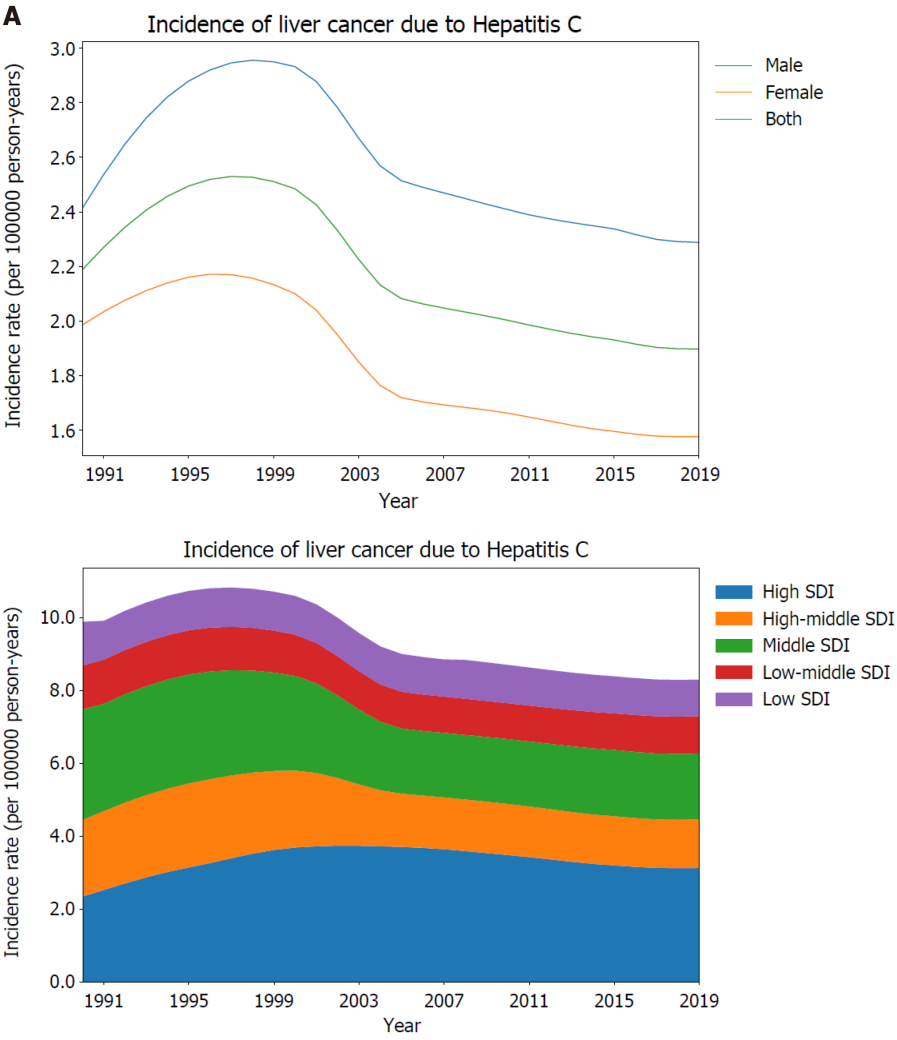
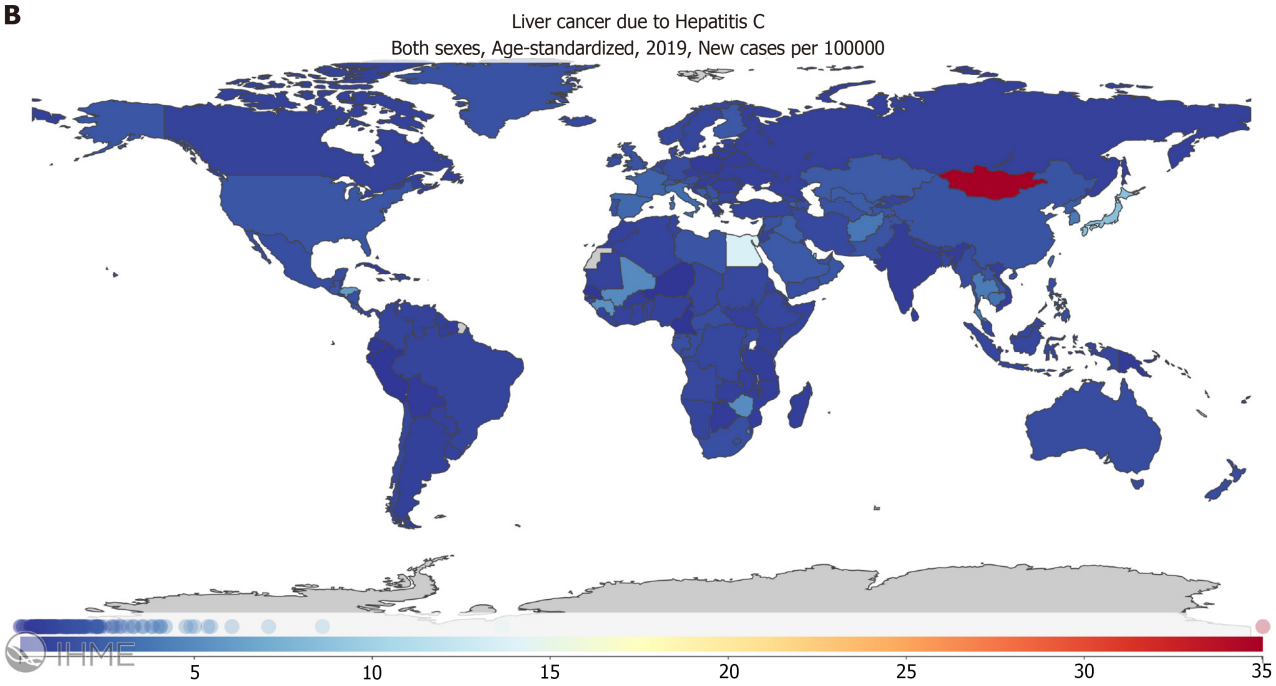



**Figure 2 Burden of liver cancer caused by hepatitis B for 204 countries and territories.** A and C: Age-standardized incidence (A) and death (C) rates per 100000 population of liver cancer caused by hepatitis B from 1990 through 2019, stratified by sex or the Socio-demographic Index; B and D: Age-standardized incidence (B) and death (D) rate of liver cancer caused by hepatitis B per 100000 person-years by country and territory, in 2019. The maps in (B) and (D) are generated using the Global Burden of Disease 2019 tool. SDI: Socio-demographic Index.


A
Incidence of liver cancer due to Hepatitis C
Incidence rate (per 100000 person-years)
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1991
1995
1999
2003
2007
2011
2015
2019
Year
Male
Female
Both
Incidence of liver cancer due to Hepatitis C
Incidence rate (per 100000 person-years)
10.0
8.0
6.0
4.0
2.0
0.0
Year
High SDI
High-middle SDI
Middle SDI
Low-middle SDI
Low SDI


B
Liver cancer due to Hepatitis C
Both sexes, Age-standardized, 2019, New cases per 100000
IHME
5
10
15
20
25
30
35

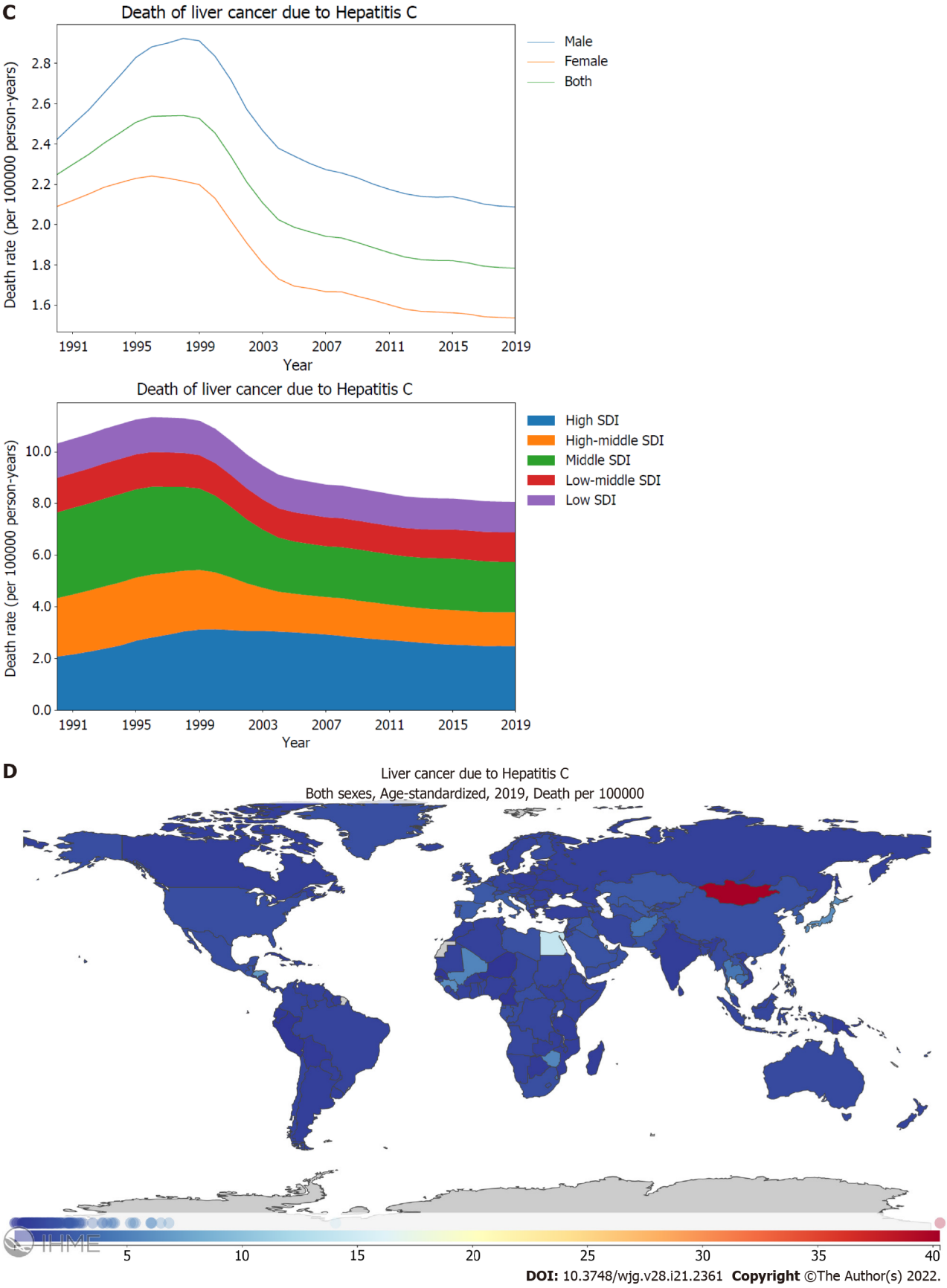



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**Figure 3 Burden of liver cancer caused by hepatitis C for 204 countries and territories.** A and C: Age-standardized incidence (A) and death (C) rate per 100000 population of liver cancer caused by hepatitis C from 1990 through 2019, stratified by sex or the Socio-demographic Index; B and D: Age-standardized incidence (B) and death (D) rate of liver cancer caused by hepatitis C per 100000 person-years by country and territory, in 2019. The maps in (B) and (D) are generated using the Global Burden of Disease 2019 tool. SDI: Socio-demographic Index.

Gambia and Thailand (Figure 4D; Supplementary material, pages 103-106).

### Burden of liver cancer caused by NASH

By stratification using sex, the age-standardized incidence and mortality rates of PLC attributed to NASH in males was found to be higher than in females (Figure 5A and C; Supplementary material, pages 107-108 and 116-117). Similar to the geographical variance observed in PLC caused by alcohol use, the highest age-standardized incidence and death rates of PLC attributed to NASH were reported in the high and middle SDI locations (Figure 5A and C). Between 1990 and 2019, a remarkable difference was observed in the trends of age-standardized incidence rates of PLC attributed to NASH between SDI regions, with the high middle SDI locations [72.9% (55.1-96.0)] showing a declining trend and the high SDI locations showing an increasing trend [182.9% (135.4-248.6)] (Figure 5A; Supplementary Figure 5A; Supplementary material, pages 19-20). The changing pattern for the age-standardized death rate across SDI locations was comparable to that observed in the incidence rate of the same period. In 2019, Mongolia [7.6 (4.9-11.4)] depicted the highest age-standardized incidence rate, followed by Gambia and Qatar (Figure 5B; Supplementary material, pages 112-115). Similar to the order of age-standardized incidence rate, Mongolia [8.7 (5.6-12.9)], Gambia, and Guinea had the highest age-standardized death rate (Figure 5D; Supplementary material, pages 121-124).

### Burden of liver cancer attributed to other causes

In terms of sex variance, the age-standardized incidence and mortality rates of PLC attributed to other causes were found to be higher in males (Figure 6A and C). When stratified using the SDI, higher incidence and mortality rates of PLC attributed to other causes were observed for high and middle SDI locations than for low middle and low SDI locations (Figure 6A and C). Between 1990 and 2019, there were remarkable geographical differences in the changing trend of age-standardized incidence rates of PLC attributed to other causes across the SDI regions; the high middle, middle, and low middle SDI locations showed a declining trend, whereas the high SDI locations showed an increasing trend [144.8% (112.8-186.3)] (Figure 6A; Supplementary Figure 6A; Supplementary material, pages 19-20). The geographical differences observed in the age-standardized death rate of PLC attributed to other causes across the SDI regions were comparable to the incidence rate (Figure 6C; Supplementary Figure 6B; Supplementary material, pages 21-22). The highest incidence and death rates of PLC attributed to other causes were observed for Mongolia, Gambia, and Guinea (Figure 6B and D; Supplementary material, pages 130-133 and 139-142).

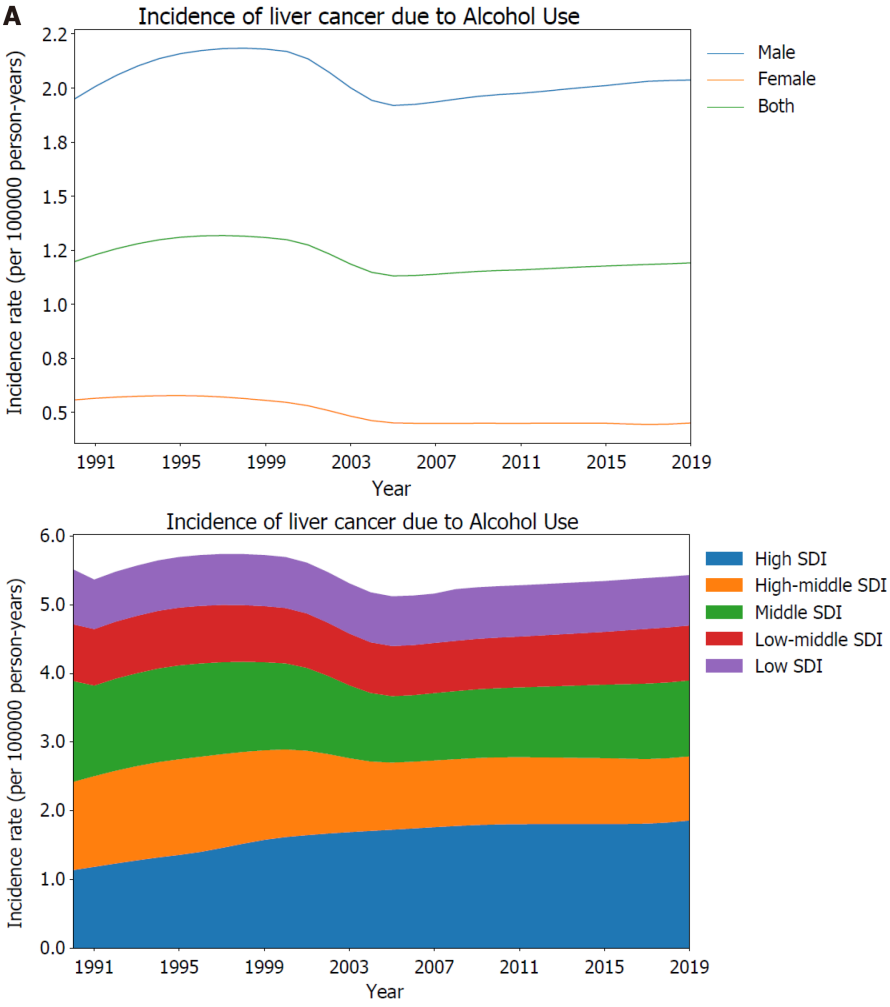
## DISCUSSION

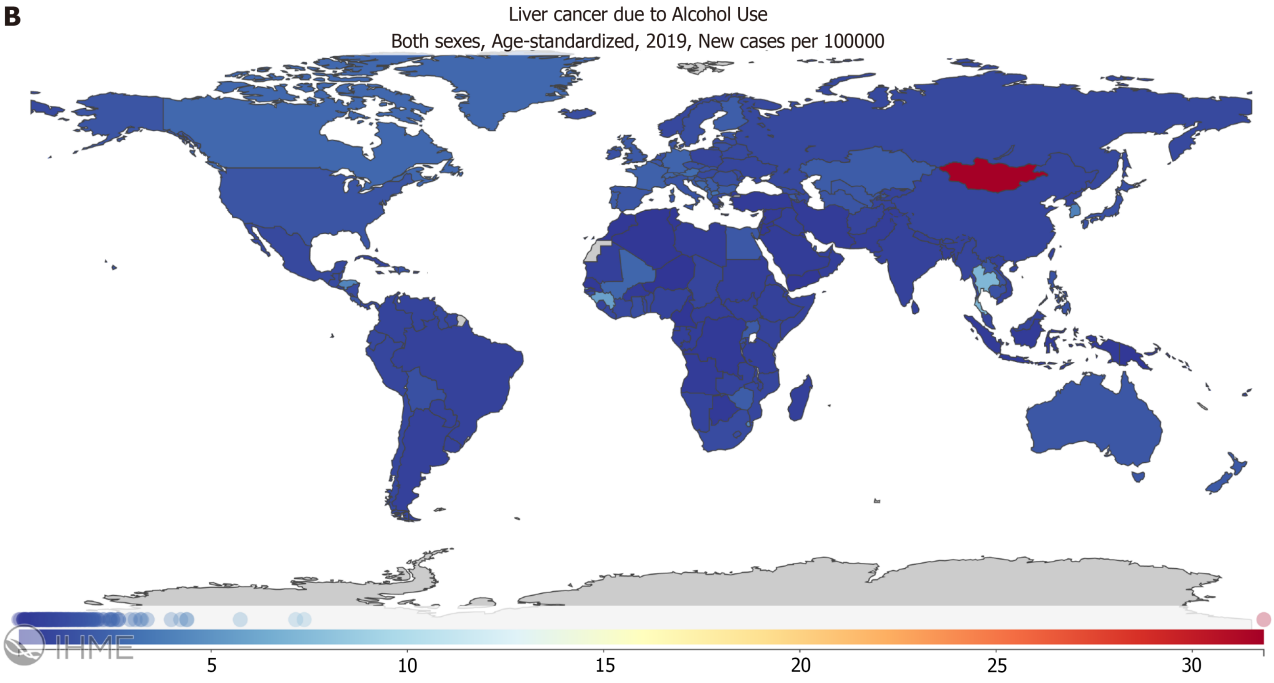
### Main findings

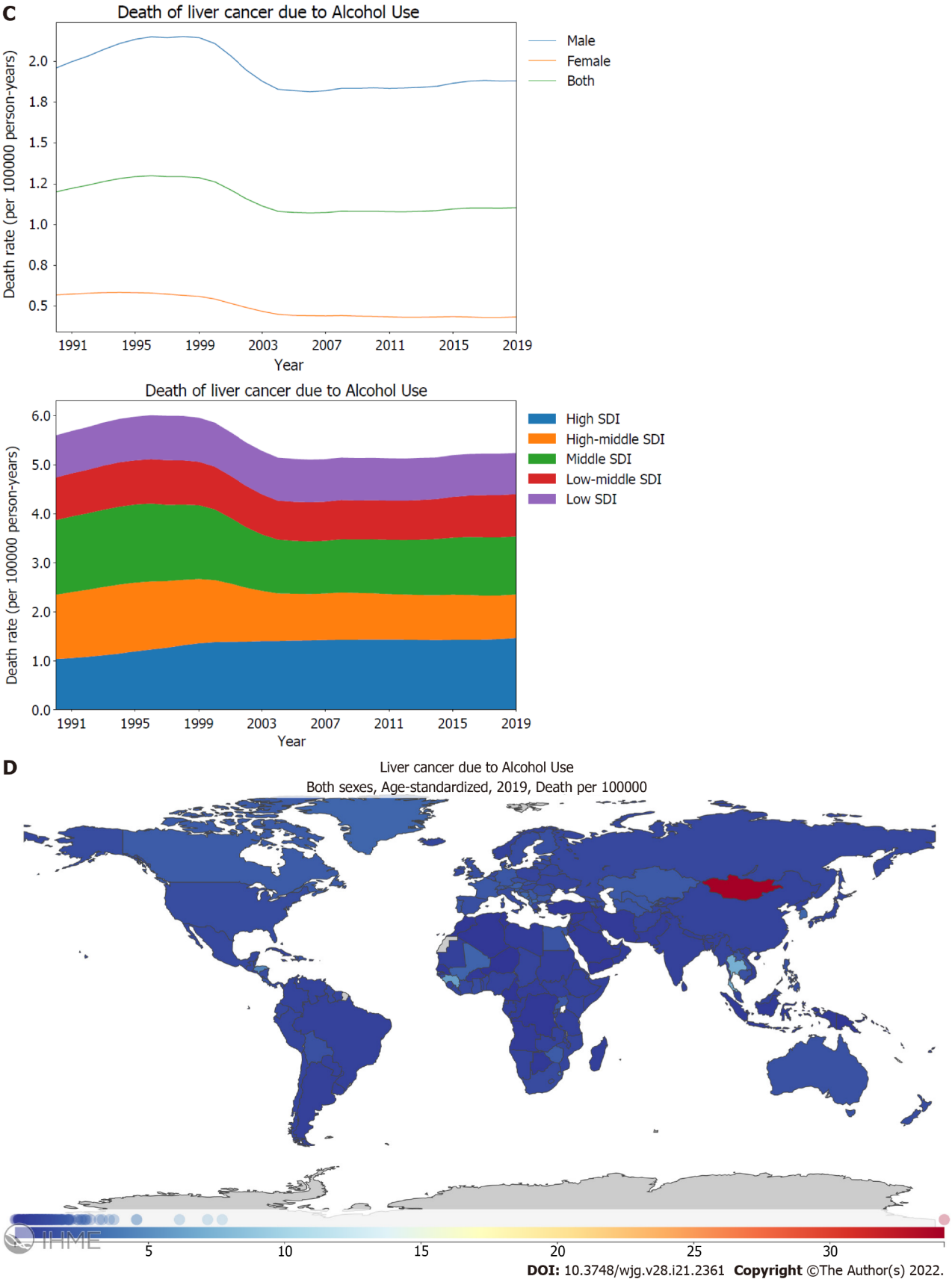
Based on the data from the GBD 2019, we explored the global burden of PLC and focused on the relationship between socioeconomics and the attributable etiologies of PLC. Our main findings are listed below: (1) Global incidence and mortality rates of PLC declined between 1990 and 2019. The decreasing burden of PLC caused by hepatitis B and the declining PLC burden in middle SDI locations was considered the main driver for this favorable trend; (2) PLC had higher prevalence in males; (3) The highest attributable etiology of PLC was hepatitis B, followed by hepatitis C, and alcohol use; (4) The leading attributable etiology of PLC in the middle SDI locations was hepatitis B; and hepatitis C and alcohol use in the high SDI locations; (5) Before 2004, the middle SDI locations surpassed high SDI locations in terms of PLC burden. However, the high SDI locations exceeded the middle SDI locations in terms of PLC burden after 2004; (6) Between 1990 and 2019, the incidence rate of PLC decreased for the high middle SDI locations; it increased for the high SDI locations, according to the stratified causes of PLC including hepatitis B, hepatitis C, alcohol use, and NASH; and (7) In 2019, several countries located in East Asia, South Asia, West Africa, and North Africa shouldered the heaviest burden for incidence and death rates of PLC.

### Liver cancer

The risk factors for liver cancer include HBV, HCV, alcohol consumption, metabolic syndrome, diabetes [11]. Although there are substantial variations between countries in the underlying etiologies; globally, HBV accounts for 33%; alcohol, 30%; HCV, 21%, and other causes, 16% of liver cancer deaths[4]. Similar to these findings, we found that the leading attributable etiology of PLC was hepatitis B, followed by hepatitis C, alcohol use, NASH, and other causes, based on the GBD 2019. CHB and CHC cause sustained or repeated inflammatory damage, followed by liver fibrosis and cirrhosis. After liver cirrhosis is established, the risk of hepatocellular carcinoma (HCC) increases substantially. Further, liver cirrhosis caused by NASH substantially increases the risk for HCC[12]. A superimposed condition can enhance the possibility of PLC. For example, alcohol use can contribute to the occurrence of PLC in the setting of CHC.

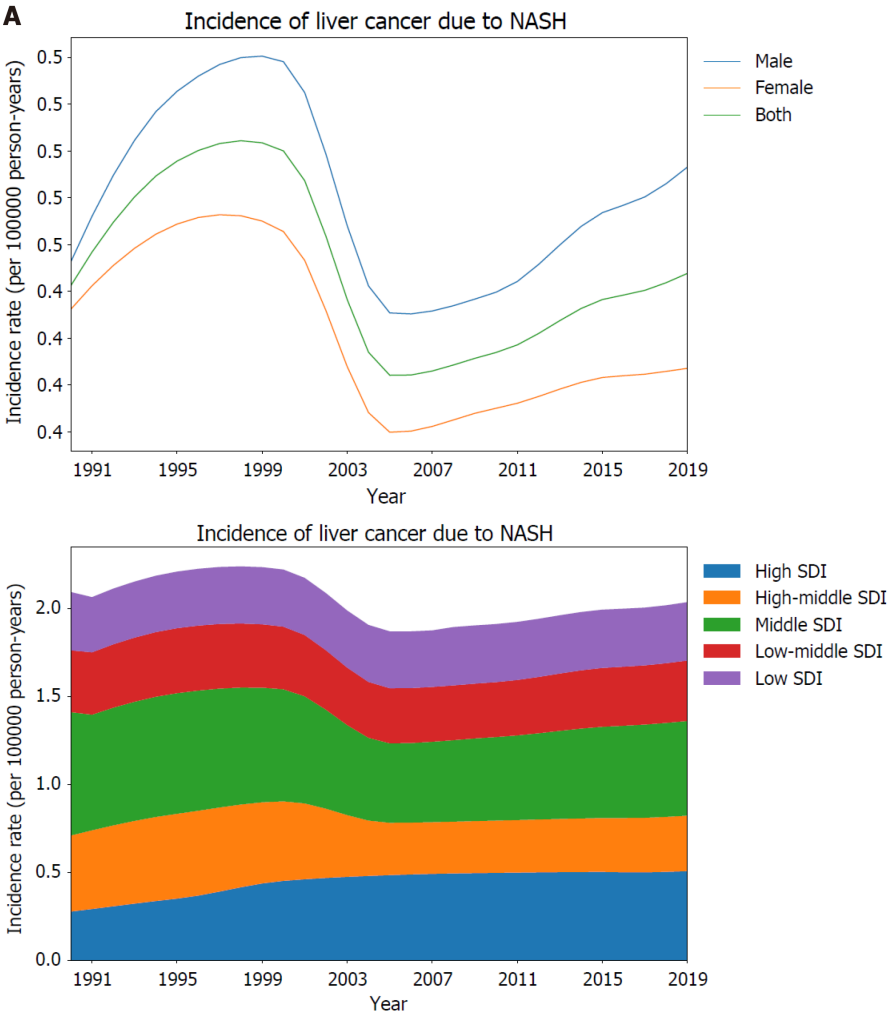

A
Incidence of liver cancer due to Alcohol Use
Incidence rate (per 100000 person-years)
Year
Male
Female
Both
Incidence of liver cancer due to Alcohol Use
Incidence rate (per 100000 person-years)
Year
High SDI
High-middle SDI
Middle SDI
Low-middle SDI
Low SDI

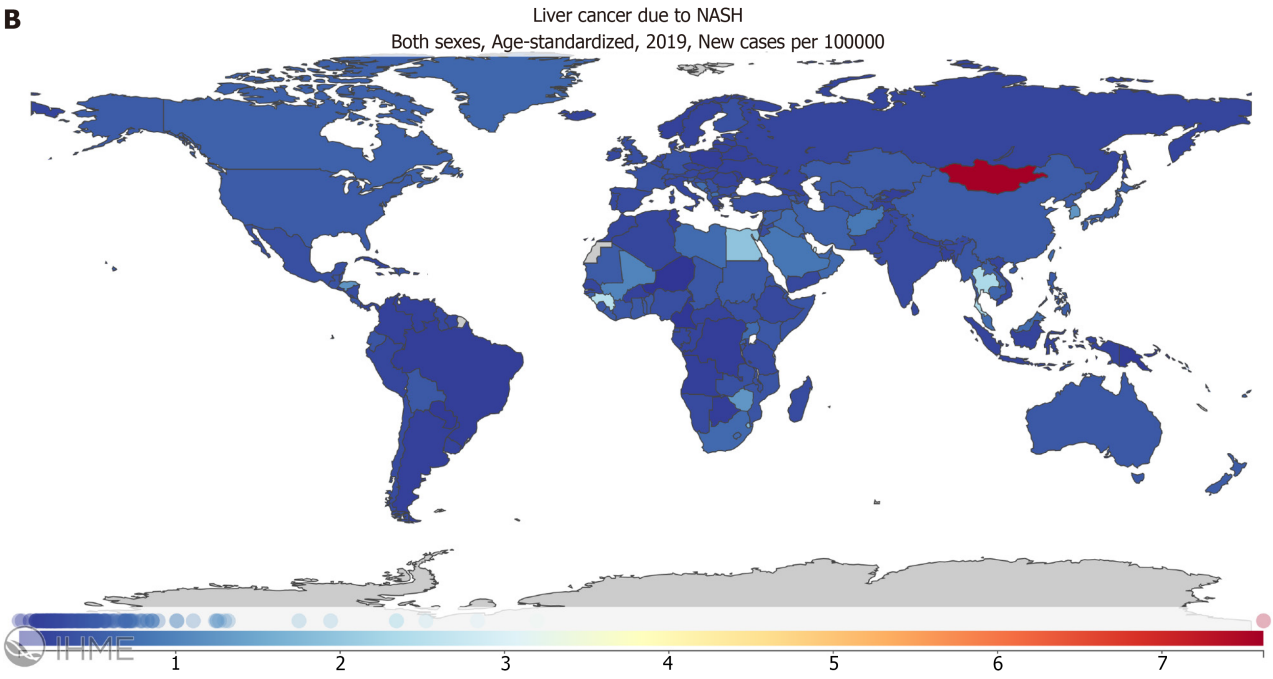

B
Liver cancer due to Alcohol Use
Both sexes, Age-standardized, 2019, New cases per 100000
IHME
5
10
15
20
25
30

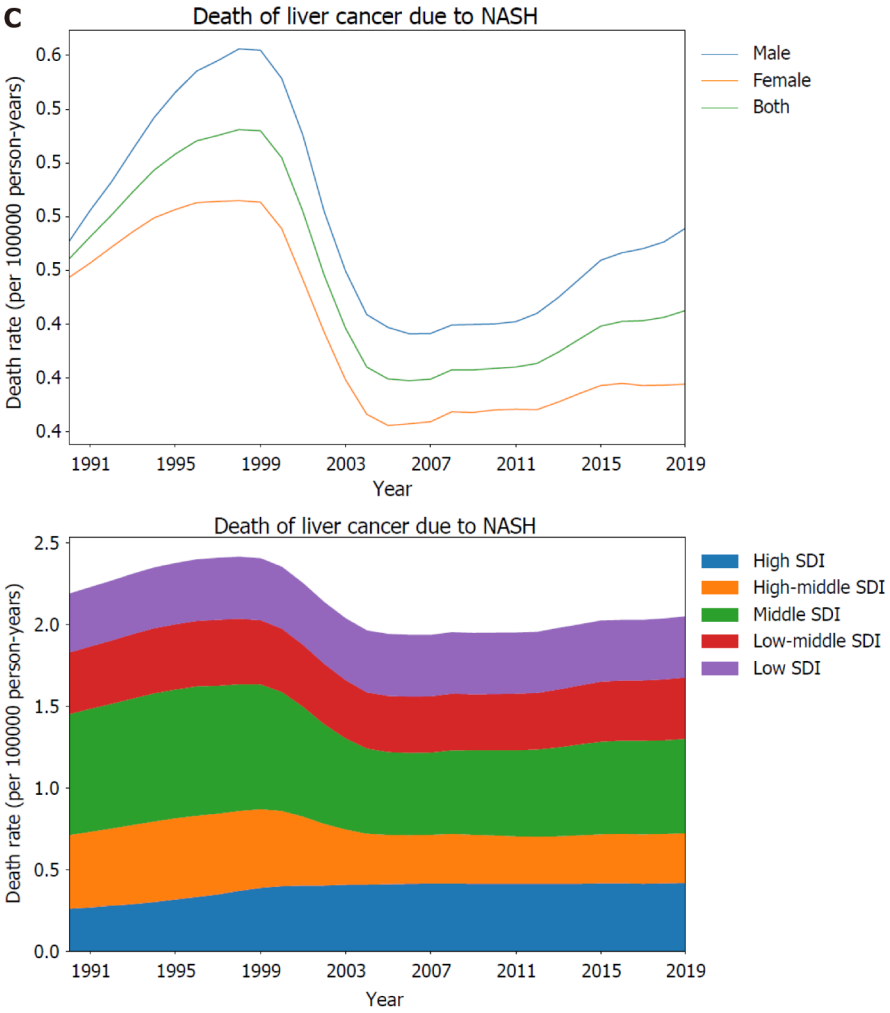


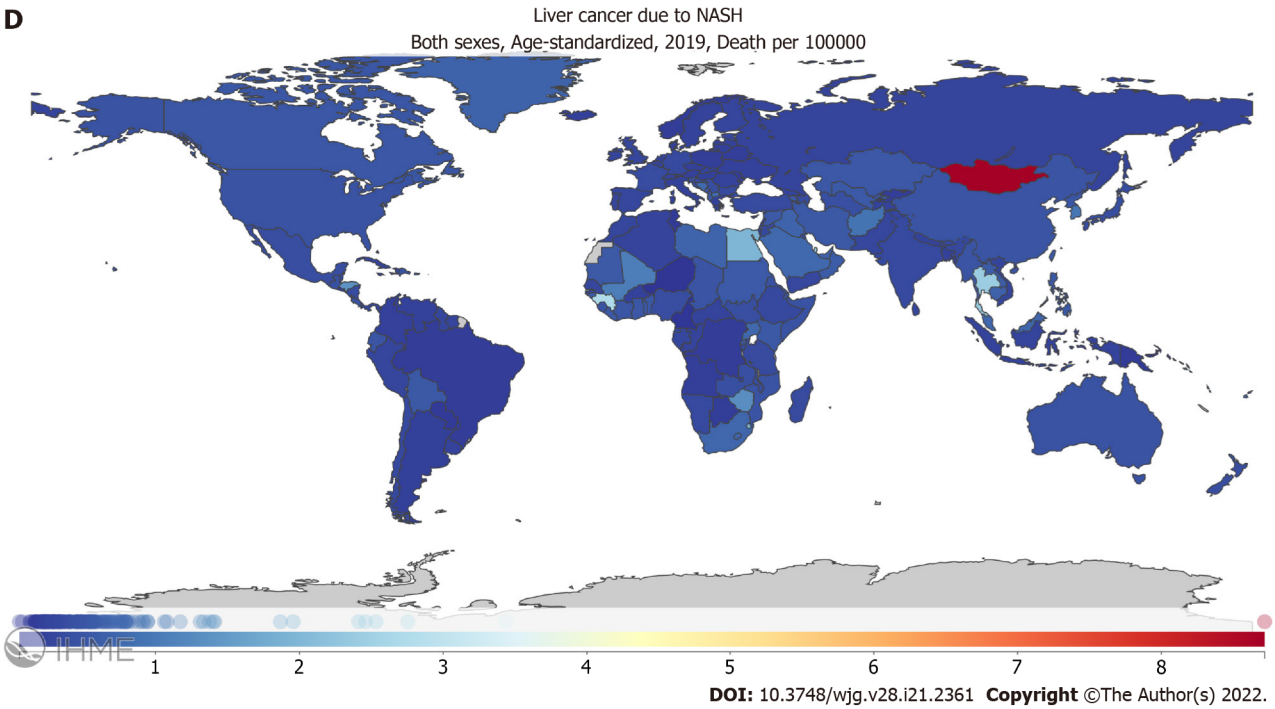

**DOI:** 10.3748/wjg.v28.i21.2361 

**Figure 4 Burden of liver cancer attributed to alcohol use for 204 countries and territories.** A and C: Age-standardized incidence (A) and death (C) rate per 100000 population of liver cancer attributed to alcohol use from 1990 through 2019, stratified by sex or the Socio-demographic Index; B and D: Age-standardized incidence (B) and death (D) rate of liver cancer attributed to alcohol use per 100000 person-years by country and territory, in 2019. The maps in (B) and (D) are generated using the Global Burden of Disease 2019 tool. SDI: Socio-demographic Index.


A
Incidence of liver cancer due to NASH
Incidence rate (per 100000 person-years)
0.5
0.5
0.5
0.5
0.5
0.4
0.4
0.4
0.4
1991
1995
1999
2003
2007
2011
2015
2019
Year
Male
Female
Both
Incidence of liver cancer due to NASH
Incidence rate (per 100000 person-years)
2.0
1.5
1.0
0.5
0.0
1991
1995
1999
2003
2007
2011
2015
2019
Year
High SDI
High-middle SDI
Middle SDI
Low-middle SDI
Low SDI


B
Liver cancer due to NASH
Both sexes, Age-standardized, 2019, New cases per 100000
IHME
1
2
3
4
5
6
7




**DOI:** 10.3748/wjg.v28.i21.2361 

**Figure 5 Burden of liver cancer attributed to nonalcoholic steatohepatitis for 204 countries and territories.** A and C: Age-standardized incidence (A) and death (C) rate per 100000 population of liver cancer attributed to nonalcoholic steatohepatitis (NASH) from 1990 through 2019, stratified by sex or the Socio-demographic Index; B and D: Age-standardized incidence (B) and death (D) rate of liver cancer attributed to NASH per 100000 person-years by country and territory, in 2019. The maps in (B) and (D) are generated using the Global Burden of Disease 2019 tool. SDI: Socio-demographic Index.

We observed an impressive association between socioeconomic status and the attributable etiologies of PLC. For high middle and middle SDI regions, hepatitis B was the main etiology of PLC whereas hepatitis C was the main etiology of PLC for high SDI regions; this was in accordance with another similar study[6]. In addition to the heavier burden of PLC caused by hepatitis C, the high SDI locations had a higher prevalence of PLC attributed to alcohol use. Given that the prevalence of drinking is greatest for high SDI locations and the least in low middle SDI locations[13], this finding was expected. Although viral hepatitis including CHB and CHC remains the most common cause of liver deaths, nonalcoholic fatty liver disease (NAFLD) is a rapidly growing contributor to liver mortality and morbidity. A similar phenomenon has been observed in China. In 2016, NAFLD cases requiring inpatient care in China outnumbered their counterparts for chronic viral hepatitis[14]. In the United States, the attributable population factors for HCC were greatest for metabolic disorders[15]. Interestingly, the global age-standardized incidence rate of PLC due to hepatitis B reached its peak in 1995-1996, then decreased gradually, as was shown in the GBD 2019. Wide HBV vaccine coverage may have been the potential cause of this beneficial phenomenon. A genetically engineered hepatitis B vaccine was available in 1986. In China, vaccination against HBV began in 1985 using a plasma-derived hepatitis B vaccine. In 1992, a genetically engineered hepatitis B vaccine was licensed in China and managed nationally. The integration of the HBV vaccination into the Expanded Program on Immunization in China has reduced chronic HBV infection by 90% among children < 15 years of age [16]. One of our studies found that the global incidence of acute hepatitis B has decreased gradually since 1990[17]. Usually, a declining trend for HBV incidence precedes a decreasing trend of PLC incidence due to hepatitis B by 10 to 20 years. Similarly, wide HBV vaccine coverage may have contributed to the declining PLC burden in high middle SDI locations since hepatitis B was the most important attributable etiology of PLC in these regions.

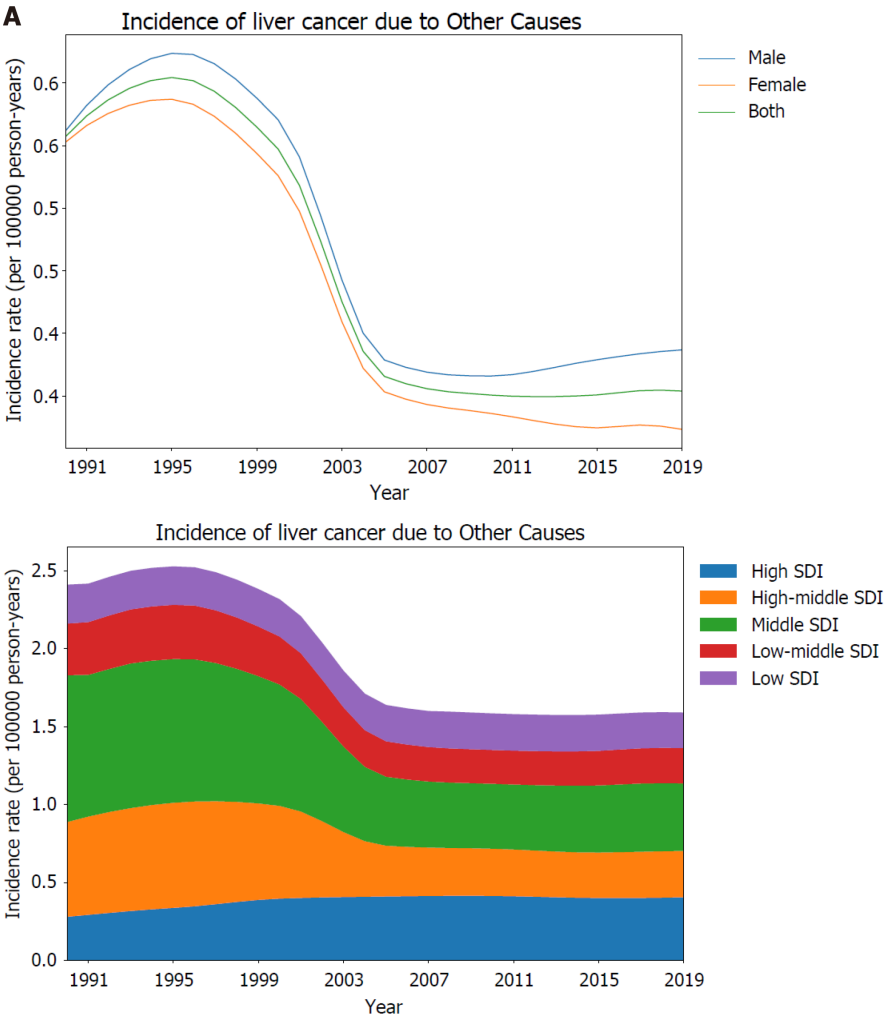
According to the GBD 2019 data, PLC is more prevalent in males. In fact, this is in line with several other observations[4,11]. MyD88-dependent IL-6 production, Foxa1, and Foxa2 play a role in the gender disparity in PLC[18,19]. Furthermore, according to the GBD 2019, there were 534000 (487000-589000) incident cases, and 485000 (444000-526000) deaths attributed to liver cancer globally in 2019; these were significantly lower than those reported in the GBD 2017 and GLOBOCAN 2020[1,6]. In the GBD 2019, the mapping of ICD-10 C22.9 was changed to a garbage code because this would have included both primary and secondary or metastatic cancers (see also https://www.thelancet.com/pb-assets/Lancet/gbd/summaries/diseases/Liver-cancer.pdf). In clinical practice, liver metastasis originating from colorectal cancer or stomach cancer is rather common. Therefore, fewer deaths were mapped for liver cancer in the GBD 2019 than in the GBD 2017. That is, the data of PLC from the databases of the GBD 2016 and GBD 2017 may have unintentionally included cases of metastatic liver cancer.

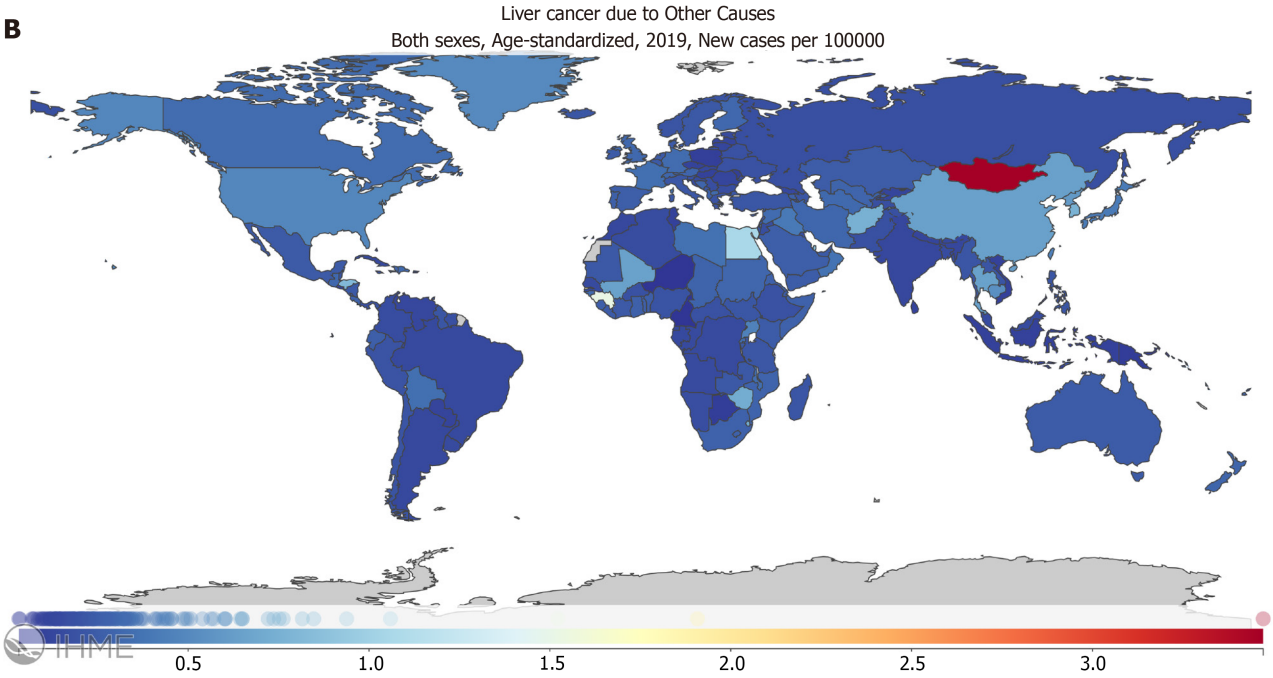
### Prospects

In recent years, incidence and mortality rates of PLC have declined in middle SDI locations, such as China and other Eastern and Southeastern Asian countries[20-22]. In line with these findings, the PLC burden has been declining in the high middle and middle SDI locations from 1990 to 2019 according to the GBD 2019; this decline has benefited from the decreasing trend of viral hepatitis, such as CHB. However, the incidence and mortality rates of PLC increased in high SDI locations during the same period, which is in line with several other studies[4,23,24]. After 2004, the PLC burden in high SDI locations surpassed that in middle SDI locations. Several factors contributed to this reversal. First, hepatitis B was the leading attributable etiology of PLC in middle SDI locations. However, vaccination coverage for hepatitis B contributed to the declining trend of PLC in the middle SDI locations. Second, the increasing trend of PLC burden in high SDI locations was attributed to the increasing prevalence of alcohol use and metabolic risk factors for HCC, including metabolic syndrome, obesity, type II diabetes, and NASH[11,13]. As shown in Figures 4A, 4C, 5A and 5C, the gradually increasing burdens of alcohol use and NASH aggravated the burden of PLC in high SDI locations. The epidemiology of HCC has been shifting away from a disease predominated by viral hepatitis to NASH. A similar phenomenon was observed in the United States[25]. Thus, maintaining adequate surveillance of alcohol abuse and NASH is vital to develop strategies against the burden of PLC caused by these conditions.

### Prevention

Although PLC causes a heavy burden of cancer incidence and mortality, it (to be precise, HCC) can be prevented by avoiding the risk factors. Compared with the cohort without vaccination, universal HBV vaccination reduced the relative prevalence of HBsAg to 0.24 (0.16-0.35)[26]. Similarly, escalating vaccination policy in China has significantly reduced the prevalence of HBsAg in the recent three decades[16]. Given the heavy burden of PLC caused by hepatitis B in middle and high middle SDI locations, universal HBV vaccination in these areas is considered a practical and principal strategy to minimize the liver cancer burden. Data have indicated that universal HBV vaccination has contributed to a dramatic decline in the PLC burden in several countries and regions[27,28]. For CHB or HBV-related liver cirrhosis, effective antiviral treatment should be provided based on the relative guidelines [29]. Treatment with > 5 years of oral antiviral therapy effectively decreases the HCC incidence regardless of whether patients have baseline cirrhosis[30]. The early diagnosis of CHB and CHC before liver cirrhosis is important considering that liver cirrhosis substantially contributes to the risk of PLC. In


A
Incidence of liver cancer due to Other Causes
Incidence rate (per 100000 person-years)
0.6
0.6
0.5
0.5
0.4
0.4
1991
1995
1999
2003
2007
2011
2015
2019
Year
Male
Female
Both
Incidence of liver cancer due to Other Causes
Incidence rate (per 100000 person-years)
2.5
2.0
1.5
1.0
0.5
0.0
1991
1995
1999
2003
2007
2011
2015
2019
Year
High SDI
High-middle SDI
Middle SDI
Low-middle SDI
Low SDI


B
Liver cancer due to Other Causes
Both sexes, Age-standardized, 2019, New cases per 100000
IHME
0.5
1.0
1.5
2.0
2.5
3.0

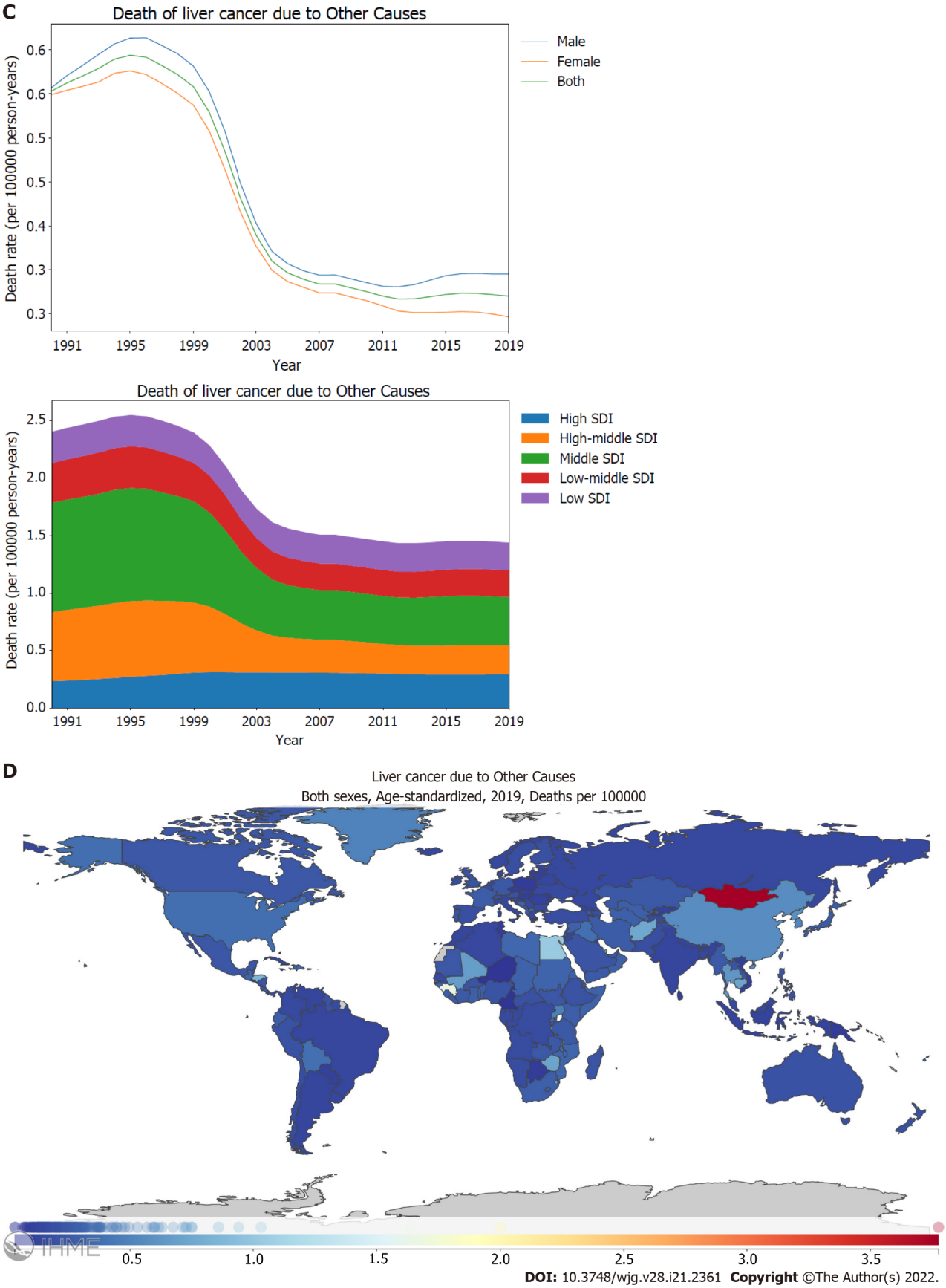



**Figure 6 Burden of liver cancer attributed to other causes for 204 countries and territories.** A and C: Age-standardized incidence (A) and death (C) rate per 100000 population of liver cancer attributed to other cause from 1990 through 2019, stratified by sex or the Socio-demographic Index; B and D: Age-standardized incidence (B) and death (D) rate of liver cancer attributed to other causes per 100000 person-years by country and territory, in 2019. The maps in (B) and (D) are generated using the Global Burden of Disease 2019 tool. SDI: Socio-demographic Index.

areas where conditions permit, performing non-invasive examinations, such as liver stiffness measure, for individuals with high risk may be potentially beneficial. Unfortunately, there is no effective vaccine for HCV available now; however, DAA have made the eradication of HCV a reality. The achievement of an HCV cure before HCC diagnosis is associated with improved survival[31].

Globally, alcohol use was the seventh leading risk factor for deaths in 2016[13]. As shown in the GBD 2019, alcohol use is a major cause of PLC, especially in high SDI locations. This highlights the need for developing strategies to decrease alcohol use. NAFLD is the third-most common cause of cancer-related deaths worldwide and the seventh most common cause in the United States[32]. Considering the increasing trend of PLC due to NASH, especially in high SDI and middle SDI locations, the control or even reversal of NASH is of critical importance, and this can be attained with lifestyle changes comprising diet, exercise, and weight loss.

### Limitations

There are several limitations to this study: (1) There is a possibility of the underestimation of the PLC burden in low middle and low SDI locations because of inadequate cancer screening. However, underestimation of the PLC burden is an inevitable problem, especially in low middle and low SDI locations owing to inadequate cancer screening and lack of registration. Similar limitations have been reported in cervical cancer screenings in low- and middle-income countries[33]. Additionally, in one of our previous studies, the underestimation of acute hepatitis in low-income countries was evident[17]; (2) Insufficient disclosure of geographical variances in large countries such as China and the United States. The GBD reports cancer burden by country or region; however, a large country has significant geographical variances in cancer burden for the urban or rural regions; (3) The lack of finer data for complex cancer, as PLC can be further divided into HCC and cholangiocarcinoma. These subgroups of cancer tend to have different etiologies and exhibit different features in terms of incidence and mortality rates; and (4) The inclusion of undefined etiologies in "other causes" can be leading causes in certain locations.

Despite these limitations, the GBD 2019 data are valuable for policymakers to implement cost-effective interventions, address modifiable risk factors, and prevent PLC efficiently.

## CONCLUSION

The pronounced association between socioeconomic development status and PLC burden indicates socioeconomic development status affects attributable etiologies for PLC. GBD 2019 data are valuable for policymakers implementing PLC cost-effective interventions.

## ARTICLE HIGHLIGHTS

### Research background

Primary liver cancer (PLC) is a common cancer with high morbidity and mortality rates. PLC usually occurs as a preventable disease. Data on global and country-specific levels and trends of PLC are essential for understanding the effects of this disease and helping policymakers to allocate resources.

### Research motivation

The association between socioeconomic development status and attributable etiologies for PLC is still unclear.

### Research objectives

To investigate the association between the burden of PLC and socioeconomic development status.

### Research methods

Cancer mortality and incidence rates of PLC were obtained from the Global Burden of Disease (GBD) 2019, and the data were stratified by the Socio-demographic Index (SDI) level. The association between the attributable etiology of PLC and SDI was described.

### Research results

Several countries located in East Asia, South Asia, West Africa, and North Africa shouldered the heaviest burden of PLC in 2019. In terms of incidence rates, the first leading underlying cause of PLC identified was hepatitis B. The incidence rate of PLC was the highest for high and middle SDI locations. The leading attributable etiologies of PLC were hepatitis B for the middle and high middle SDI locations and hepatitis C and nonalcoholic steatohepatitis for the high SDI locations.

### *Research conclusions*

Socioeconomic development status significantly affects attributable etiologies for PLC.

### *Research perspectives*

Our findings are valuable to implement tailored prevention strategies for PLC.

## FOOTNOTES

**Author contributions:** Pan JS had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis; Pan JS was responsible for its conception and design; Xing QQ, Li JM, Dong X, Zeng DY, Chen ZJ, and Lin XY were responsible for the acquisition, analysis, or interpretation of data; Pan JS drafted the manuscript; Li JM and Dong X made critical revision of the manuscript for important intellectual content; Li JM and Pan JS conducted the data analysis; Xing QQ, Li JM, and Dong X contributed equally to the study.

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**Institutional review board statement:** The study was reviewed and approved by the Ethics Committee of First Affiliated Hospital of Fujian Medical University (MTCA, ECFAH of FMU[2015]084-1).

**Informed consent statement:** Not required.

**Conflict-of-interest statement:** All authors report no conflicts interests.

**Data sharing statement:** All data are available in the Supplementary material.

**STROBE statement:** The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.



**Country/Territory of origin:** China

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**S-Editor:** Yan JP
**L-Editor:** A
**P-Editor:** Guo X

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SYSTEMATIC REVIEWS

# Endoscopic ultrasound-guided injectable therapy for pancreatic cancer: A systematic review

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## Abstract

### *BACKGROUND*

Given the low survival rate in pancreatic cancer, new therapeutic techniques have been explored, especially for unresectable or borderline resectable disease. Endoscopic ultrasound (EUS) provides real-time imaging and minimally invasive access for local and targeted injection of anti-tumor agents directly into the pancreatic tumor. Limited studies have been reported using this technique for the treatment of pancreatic ductal adenocarcinoma (PDAC).

### *AIM*

To evaluate the progress made with EUS-guided injectable therapies in the treatment of PDAC.

### *METHODS*

All original articles published in English until July 15, 2021, were retrieved *via* a library-assisted literature search from Ovid Evidence-Based Medicine Reviews and Scopus databases. Reference lists were reviewed to identify additional relevant articles. Prospective clinical studies evaluating the use of EUS-guided injectable therapies in PDAC were included. Studies primarily directed at non-EUS injectable therapies and other malignancies were excluded. Retrieved manuscripts were reviewed descriptively with on critical appraisal of published studies based on their methods and outcome measures such as safety, feasibility, and effectiveness in terms of tumor response and survival. Heterogeneity in data outcomes and therapeutic techniques limited the ability to perform comparative statistical analysis.

### *RESULTS*

A total of thirteen articles (503 patients) were found eligible for inclusion. The EUS-injectable therapies used were heterogeneous among the studies consisting of immunotherapy ($n$ = 5) in 59 patients, chemotherapy ($n$ = 1) in 36 patients, and viral and other biological therapies ($n$ = 7) in 408 patients. Eleven of the studies reviewed were single armed while two were double armed with one randomized

trial and one non-randomized comparative study. Overall, the included studies demonstrated EUS-guided injectable therapies to be safe and feasible with different agents as monotherapy or in conjunction with other modalities. Promising results were also observed regarding their efficacy and survival parameters in patients with PDAC.

*CONCLUSION*
EUS-guided injectable therapies, including immunotherapy, chemotherapy, and viral or other biological therapies have shown minimal adverse events and potential efficacy in the treatment of PDAC. Comparative studies, including controlled trials, are required to confirm these results in order to offer novel EUS-based treatment options for patients with PDAC.

**Key Words:** Pancreatic ductal adenocarcinoma; Endoscopic ultrasound-guided fine-needle injection; Local injectable therapy; Immunotherapy; Chemotherapy; Oncolytic viral therapy



**Core Tip:** Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal malignancy. Resistance to systemic therapies may be attributable to the dense stromal matrix in the pancreatic tumor mass. Endoscopic ultrasound-guided fine-needle injection (EUS-FNI) is a novel technique to deliver various anti-tumor agents locally in real-time and may overcome this limitation. This review examines the EUS-FNI therapies used to treat PDAC.

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## INTRODUCTION

Pancreatic cancer is associated with a 5-year survival rate of approximately 10% at diagnosis and is the seventh leading cause of cancer-related deaths worldwide[1]. Pancreatic ductal adenocarcinoma (PDAC) accounts for more than 90% of cases of pancreatic cancer. The very low survival rate is partly due to the lack of early diagnosis and limited response to systemic therapies[2]. Only 15%-20% of patients present with surgically resectable disease and less than 10% undergo complete resection, which is the only curative intervention[3,4]. The majority of patients who present with unresectable locally advanced pancreatic cancer (LAPC) or metastatic disease are managed with systemic chemotherapy and/or radiotherapy with a very limited prognosis. The introduction of newer chemotherapy combinations and regimens have shown some promising results but still the overall survival (OS) remains dismal[5,6]. One of the many reasons for failure of systemic chemotherapy has been hypothesized as poor delivery of these agents due to abundant stromal matrix and deficient vasculature[7]. This justifies the rationale to explore the use of direct intratumoral injection for targeted delivery of an anti-tumor agent into the tumor mass while minimizing systemic complications. Percutaneous injection of direct intratumoral agents under ultrasound or computed tomography (CT) guidance has been demonstrated to be safe and feasible in phase I trials but this is technically cumbersome and difficult for administering multiple doses[8,9]. Endoscopic ultrasound (EUS) provides the opportunity for real-time visualization of the pancreatic mass and allows minimally invasive access for injectable therapies. This systematic review focuses on the methodology and outcomes of previously published clinical studies on EUS-guided fine needle injection (EUS-FNI) of anti-tumor agents in patients with PDAC.

## MATERIALS AND METHODS

### *Literature search*

An expert librarian conducted searches of the Ovid Evidence-Based Medicine Reviews (Embase, MEDLINE, Cochrane Central Register of Controlled Trials, Cochrane Database of Systematic Reviews) and Scopus databases to identify studies published between database inception until July 15, 2021, using the search strategy in Supplementary Table 1. The search was limited to full reports and articles published in English. The titles and abstracts were screened by two independent reviewers (JK and VC) and were assessed for eligibility based on the evaluation of the full manuscript. Disagreements between

the two reviewers were resolved by discussion. Additional studies were identified from searching through references and were screened similarly.

### Inclusion criteria

The review is restricted to published prospective studies reporting the effects of injectable interventions primarily using the EUS-FNI technique in patients with PDAC, irrespective of the stage. Therapeutic interventions may include any form of immunotherapy, chemotherapy, or biological agents. Studies that utilized co-interventions and other modes of therapy delivery along with EUS-FNI were included. Studies were eligible if they assessed at least one of the outcomes of interest: Safety, feasibility, and efficacy in terms of tumor response and/or survival.

### Exclusion criteria

Benchtop and animal models were excluded as were studies using non-EUS directed therapies. Studies investigating other pancreatic tumors and multiple gastrointestinal cancers where data for the PDAC group was not separately reported were also excluded. Studies were not considered eligible for inclusion if they did not focus on the treatment of PDAC and rather explored the effects of the interventions on palliation and symptom control.

### Data extraction

Data was extracted on studies' characteristics of interest- participants, study design, interventions (*e.g.*, therapeutic agent, dosage, and EUS-FNI technique), prior therapies and co-interventions, outcome measures, and results. Relevant data from the included articles were recorded in itemized tables using Microsoft Excel for Microsoft 365 (MSO 16.0.13801.21002) 64 bit.

### Outcomes

Outcome parameters of toxicity and clinical efficacy (tumor response and/or survival parameters) were reported as defined by the individual studies. Grade 3-4 AEs included those with severe or life-threatening toxicity.

### Statistical analysis

Heterogeneity in data outcomes and therapeutic techniques limited the ability to perform comparative statistical analysis.

### Quality assessment

The risk of bias in the included studies was assessed by two independent reviewers (JK, VJ) using the NIH Study Quality Assessment tools for controlled intervention studies and the before-after (pre-post) studies with no control group[10]. These guidelines help to rate the studies as good, fair, or poor based on a set of quality criteria questions. The tools were adapted keeping in mind the nature of the study being reviewed by identifying and reporting some questions as non-applicable as deemed by the reviewers. The results were compared, and any differences were resolved by discussion.

## RESULTS

The literature search yielded 101 publications. Title and abstract screening further yielded 30 potentially eligible publications. A full review of manuscripts identified 9 eligible reports along with 4 eligible reports found after backward reference searching leading to a total of 13 full-text articles with 503 patients that were included in the systematic review. The baseline characteristics of these studies are included in Table 1. All were single arm studies except 2, one of which was a randomized controlled trial (RCT) while the other was a non-randomized study. The EUS-injectable therapy administered was heterogeneous among the studies and consisted of immunotherapy ($n$ = 5) in 59 patients, chemotherapy ($n$ = 1) in 36 patients, and viral and other biological therapies ($n$ = 7) in 408 patients.

The quality assessment process identified 11 studies as good and 2 as fair, the details of which are attached in Supplementary Tables 2 and 3.

### EUS-guided fine needle injection

Linear array echoendoscopes have facilitated the simultaneous visualization of a target lesion and advancement of a needle from the distal tip of the echoendoscope under precise control to aspirate, inject, or gain access to the organ[11]. This has expanded the role of EUS into the realm of therapeutic interventions with a wide range of applications. EUS-FNI has demonstrated safety and feasibility in applications such as celiac plexus block/neurolysis for the management of pancreas-related pain or pancreatic cyst ablation[12,13]. More recently, its use has been explored for the injection of anti-tumor agents in patients with pancreatic cancer as an attractive method of delivery of such agents considering its minimal invasiveness and low rate of adverse events (AEs)[14].

**Table 1 Characteristics of published clinical studies using endoscopic ultrasound-guided fine-needle injection for pancreatic ductal adenocarcinoma**

| Ref. | Disease | Country | No. of subjects, No. of groups | Study type | EUS-FNI injectable agent | Type of therapy | Aes | Tumor response | Median survival |
|---|---|---|---|---|---|---|---|---|---|
| Chang *et al* [17], 2000 | Unresectable PDAC | United States | 8, single arm | Phase I | Allogeneic mixed lymphocyte culture | Immunotherapy | DLT-0 | Partial remission 25%, minor response 12.5% | 13.2 mo (OS) |
| Irisawa *et al* [21], 2007 | Unresectable PDAC refractory to gemcitabine | Japan | 7, single arm | Pilot clinical study | Dcs | Immunotherapy | Aes-0 | Mixed response 28.6%, stable disease 28.6% | 9.9 mo (OS) |
| Hirooka *et al* [22], 2009 | LAPC | Japan | 5, single arm | Phase I | OK-432-pulsed dcs | Immunotherapy | Grade 3 or 4 aes-0 | Effective response 60% (partial remission 20%, stable disease 40%) | 15.9 mo (OS) |
| Endo *et al*[24], 2012 | Resectable PDAC | Japan | 24, two arms | Phase I | Idcs and OK-432 | Immunotherapy | Grade 3 aes-1 | NA | No difference |
| Hirooka *et al* [25], 2017 | LAPC | Japan | 15, single arm | Phase I/II | Zoledronate-pulsed dcs | Immunotherapy | DLT-0 (grade 3 aes-4) | Stable disease 46.7% | 11.5 mo (OS) |
| Levy *et al*[27], 2017 | Unresectable PDAC | United States | 36, single arm | Prospective non-randomized | Gemcitabine | Chemotherapy | Aes-0 | Partial response 25%, stable disease 57% | 10.4 mo (OS) |
| Hecht *et al*[31], 2003 | Unresectable PDAC without liver metastasis | United States | 21, single arm | Phase I/II | ONYX-015 | Viral therapy | Aes-8 (four related to the virus and four to the injection technique) | Partial response 10%, stable disease 38% | 7.5 mo (OS) |
| Hecht *et al*[9], 2012 | LAPC | United States | 50, single arm | Phase I/II | Tnferade Biologic | Viral therapy | DLT-3 | Complete response 2%, partial response 6%, minor response 8%, stable disease 24% | 297 d (OS) |
| Herman *et al* [33], 2013 | LAPC | United States | 304, two arms | Randomized phase III | Tnferade Biologic | Viral therapy | No difference in grade 3 to 4 aes | No difference | 10.0 mo (OS) for both arms |
| Hirooka *et al* [35], 2018 | LAPC | Japan | 12, single arm | Phase I | HF-10 | Viral therapy | DLT-0, Serious aes-2, Grade 3 aes-5 | Effective response 78% | 5.5 mo (OS) |
| Lee *et al*[36], 2020 | LAPC | South Korea | 9, single arm | Phase I | Ad5-DS | Viral therapy | DLT-0 | Overall response 11%, disease control rate 100% | 11.4 mo (PFS) |
| Nishimura *et al* [40], 2018 | Unresectable PDAC | Japan | 6, single arm | Prospective non-randomized | STNM01 | RNA oligonuc-leotide | Aes-0 | NA | 5.8 mo (OS) |
| Hanna *et al* [42], 2012 | Unresectable PDAC | United States, Israel | 6, single arm | Phase I/IIA | BC-819 | DNA plasmid | DLT-1 | Overall response 33.3% and 66.7% in the two dose cohorts respectively | 100% and 66.7% (six-month survival) in the two dose cohorts |

AEs: Adverse events; NA: Not available; PDAC: Pancreatic ductal adenocarcinoma; LAPC: Locally advanced pancreatic cancer; iDC: Immature dendritic cell; DLT: Dose-limiting toxicity; OS: Overall survival; PFS: Progression-free survival.

### *Immunotherapy*

Cancer immunotherapy aims to harness the inherent ability of the host immune system to mount an effective anti-tumor response against cancer cells through multiple strategies. Therapeutic cancer vaccines stimulate the activation of cytotoxic T lymphocytes (CTLs) against unique immunogenic tumor antigens by enhancing the delivery of these antigens[15].Targeting immune checkpoint inhibitor molecules aids in disrupting the immune suppressive mechanisms developed by cancer cells to evade immunosurveillance while the adoptive transfer of engineered lymphocytes expressing tumor epitopes or chimeric receptors aims to mediate anti-tumor response[16]. After mixed results with studies employing systemic immunotherapy in PDAC, direct administration with EUS-FNI has been used.

**Allogeneic mixed lymphocyte culture:** The first reported clinical study that used the novel delivery technique of EUS-FNI as local injectable therapy for PDAC was reported in 2000[17]. It was also the first attempt at administering biological response modifier or cellular-based immune therapy for the treatment of PDAC. The authors used allogeneic mixed lymphocyte culture prepared by coincubation of peripheral blood mononuclear cells from the patient and an allogeneic blood donor to generate a mixed lymphocyte reaction (MLR). It was based on the hypothesis that the MLR results in a high concentration of cytokines within the tumor which upregulates host anti-tumor effector mechanisms to aid in tumor regression. Patients with unresectable PDAC underwent a single session EUS-FNI procedure using 3, 6, and 9 billion cells in a dose-escalation manner. The median OS was documented to be 13.2 mo although there were only 2 partial responses and 1 minor response on either CT or EUS. Dose-limiting toxicity (DLT) was not reached and there were no procedure-related AEs were reported. Low-grade fever was the most common AE but was not associated with leukocytosis and was treated with acetaminophen. There were three grade 3 gastrointestinal toxicities and three grade 3 elevations in bilirubin which were transient and resolved after replacing the preexisting biliary stents in the patients. The encouraging results led to a multicenter RCT comparing EUS-guided injection of the allogeneic mixed lymphocyte culture to conventional IV gemcitabine therapy, but it was not completed as interim results suggested better survival and tumor response in the gemcitabine arm.

**Dendritic cells as cancer vaccine:** Dendritic cells (DCs) act as potent antigen-presenting cells (APCs) to generate anti-cancer immunity through stimulation of host primary T cell response. Immature or unloaded DCs (iDCs) acquire specific tumor-derived antigens, process them in situ, and migrate to lymphoid organs for presentation to CTLs[18]. Although vaccine strategies for delivering immunogenic tumor antigens themselves as synthetic peptides have been developed for different tumors, these require identification of specific antigens and evidence of their immunogenicity in the tumor microenvironment (TME). Despite autologous tumor cell lysates being used to provide specific tumor antigens in the clinical studies involving cancers with poorly defined antigens, there is evidence to support APCs as potent vaccines to more effectively cross-prime the CTLs with apoptotic tumor cells and apoptotic bodies[19,20]. Considering the difficulty in attaining sufficient quantities of tumor cells for *ex vivo* loading of DCs, it is reasonable to inject iDCs that can get exposed to tumor antigens *in vivo* after administration of apoptosis-inducing therapy like radiotherapy or chemotherapy.

The first report of the use of iDC injection for pancreatic cancer was a pilot clinical study in patients with unsuccessfully treated (gemcitabine resistant) unresectable PDAC[21]. Seven patients underwent cycles of EUS-FNI of unpulsed iDCs in the dose of 10 million cells or more at 2 to 3 sites within the pancreatic mass. iDCs were injected on days 1, 8, and 15 with cycles repeated every 28 d. Five patients received prior radiation therapy to induce apoptosis and facilitate tumor antigen cross-presentation. No procedural AEs were noted, and all DC injections were tolerated without clinical toxicity. Two patients demonstrated a mixed response and two others had stable disease for more than 6 mo. Median patient survival was 9.9 mo despite resistance to gemcitabine.

Hirooka *et al*[22] explored DC-based vaccination as first-line therapy for unresectable LAPC and combined it with gemcitabine based on its known apoptosis-inducing effects. It was postulated to release tumor antigens slowly over time for processing and presentation by DCs. The study used DCs pulsed with OK-432, penicillin killed and lyophilized preparation of a low-virulence strain of Streptococcus pyogenes, which acts as an immunopotentiating agent reported to stimulate DC maturation and T cell activation[23,24]. Conventional lymphokine-activated killer cells stimulated with anti-CD3 monoclonal antibody (CD3-LAKs) were also administrated systemically to induce additional anti-cancer activity. The results showed the combined therapy to be safe and synergistically effective with a median survival of 15.9 mo. Effective radiological tumor response was evidenced in three patients with 1 partial response and 2 long stable diseases for more than 6 mo. Interestingly, the patient with partial remission and the longest survival of 25.4 mo also exhibited significant immunological response with respect to the number of interferon gamma producing cells in peripheral blood lymphocytes (PBLs) and tumor-antigen specific CTL activity.

Based on this study, the authors reported a recent clinical study assessing the safety and efficacy of comprehensive immunotherapy combined with IV gemcitabine as first-line therapy for patients with LAPC[25]. Twelve cycles of EUS-FNI were performed using zoledronate-pulsed DCs rather than the previously used OK-432 pulsed DCs along with systemic administration of adoptive activated T lymphocytes (αβT) and gemcitabine every 14 d. DLT was reported. Grade 3 toxicity was recorded in

four patients, including the two patients that were attributed to gemcitabine. Seven of the 15 patients showed a stable disease tumor response with most showing long-term clinical responses. Patients receiving this therapy were noted to have a higher quality of life assessments as well as the immunological response which was evaluated by the ratio of the number of $CD8^+$ T cells to that of regulatory T cells ($CD8^+$/Treg ratio) was found to be significantly higher in patients with stable disease. The median OS and progression-free-survival (PFS) of 15 patients were 12.0 mo and 5.5 mo, respectively. Patients with pre-treatment neutrophil/lymphocyte ratio (NLR) lower than 5.0 demonstrated significantly longer survival. In an analysis limited to patients with an NLR lower than 5.0, the patients whose $CD8^+$/Treg ratio increased more than twofold survived longer. This suggests that using precise biomarkers such as NLR and $CD8^+$/Treg ratio can make comprehensive immunotherapy more beneficial for subgroups of patients with PDAC.

Another study compared 9 patients who received EUS-FNI of iDCs and OK-432 prior to the pancreatectomy surgery to 15 patients who did not receive this therapy[24]. The intervention group patients also received intra-operative radiotherapy to the retroperitoneal space. There were no severe toxicities following the pre-operative iDC injection except for one transient grade 3 fever. The incidence of postoperative complications was similar in both DC and non-DC groups. Although there was no statistically significant difference in OS times of both groups, the authors reported that 2 patients from the DC group, one of which was stage IV with distant lymph node metastasis, survived more than 5 years without requiring adjuvant therapy. Immunohistochemical examination of the surgically dissected lymph nodes revealed significantly higher $CD83^+$ cells in the regional lymph nodes and higher accumulated $Foxp3^+$ cells in both regional and distant lymph nodes in the DC group. This study not only demonstrated the safety and feasibility of preoperative EUS-FNI but also illustrated the potential of inducing an effective immune response against PDAC.

### Chemotherapy

Although systemic chemotherapy forms the basis of standard of care (SOC) treatment for unresectable PDAC primarily LAPC, only one clinical study has so far reported the use of a chemotherapeutic agent being injected locally *via* the EUS-FNI technique.

### Gemcitabine

Gemcitabine has been standard therapy for surgically unresectable PDAC since 1997 with an established record of use, safety, and relative benefit when administered intravenously[26]. Given its safety profile, it was selected to be administered in a prospective study conducted at our institution[27]. Patients with locally advanced ($n$ = 20) and metastatic ($n$ = 13) PDAC in whom surgical resection was not performed were included to undergo a single session of EUS-FNI using gemcitabine in the concentration of 38 mg/mL injected *via* a 22-gauge needle. The needle tip was placed 0.5-1.0 cm from the distal tumor edge with injection as the needle retracted proximally to inject approximately 50% of the dose uniformly along the perimeter of the tumor at sites of local infiltration (*e.g.*, blood vessels) and 50% within the remainder of the tumor. Multiple needle passes were performed (median 3, range 1-4) until the injectate was not limited within the tumor but instead began to infiltrate along needle tract or peritumoral sites, leading to varied injection volumes. The median volume of injectate per patient was 2.5 mL (range, 0.7-7.0 mL) corresponding to an intratumoral gemcitabine dose of 95 mg (range, 27-266 mg). Patients underwent subsequent conventional multimodality therapy: Chemoradiotherapy ($n$ = 22), chemotherapy alone ($n$ = 10), no therapy ($n$ = 1), or indeterminate therapy ($n$ = 3). There were no AEs attributable to the EUS-FNI procedure. OS at 6 mo, 12 mo, and 5 years were 78%, 44%, and 3%, respectively. The median OS was 10.4 mo [95% confidence interval (CI): 2.7-68.0]. From the 20 patients with stage III unresectable disease, 4 (20.0%) were downstaged and underwent an R0 resection. Patients who had a more complete therapy based on a visual score showed the greatest increase in median survival ($P$ < 0.0001) with a consistent trend of increasing survival as completeness increased. Although completeness of therapy corresponded to prolonged survival, the significance of this finding is potentially limited by the subjective nature of its assessment.

### Oncolytic viral therapy

Oncolytic viruses (OVs) are increasingly being explored as a therapeutic option because of their ability to be engineered for tumor selectivity and express genes of interest within the tumor cells to cause cytotoxic effects and cell death[28,29]. Unlike gene therapy, OVs are replication-competent and propagate within tumor cells, generating infectious progeny that further spreads to surrounding cells after tumor cell lysis. Therefore, in theory, OVs have the potential for efficient oncolysis in solid tumor masses[30].

**Onyx-015:** ONYX-015 is the first replication selective virus used in clinical trials. It is a chimeric human group C adenovirus with a deletion in the E1B-55kD gene inhibiting p53 function which is already lost in most cancer cells making them susceptible to this agent[31]. In a clinical study of 21 patients with unresectable PDAC, eight sessions of EUS-FNI with ONYX-015 were administered over 8 wk along with systemic gemcitabine therapy. The viral agent was administered in the dosage of $2 \times 10^{10}$ particles/session ($n$ = 3) and $2 \times 10^{11}$ particles/session ($n$ = 3) in phase I, and $2 \times 10^{11}$ particles/session ($n$ = 15) in

phase II of the same study which was the MTD (maximum tolerated dose). EUS-FNI was performed using a transgastric or transduodenal approach with a 22-gauge needle in a fanning pattern during the withdrawal of the needle. Two cases of duodenal perforation that were observed were attributed to the stiff tip of the echoendoscope and thus the protocol was modified only allowing for transgastric FNI. Subsequently, no luminal perforations were noted. Additionally, two cases of sepsis were noted, which may have been related to the injection technique. Thus, the protocol was again modified such that the needles were not fully retracted into the lumen during repositioning and repassage of the needle. Furthermore, the study authors instituted prophylactic administration of oral ciprofloxacin. ONYX-015 itself was well tolerated. Asymptomatic grade 3 and 4 increases in amylase and lipase were detected in 10% of patients, but no clinical pancreatitis was observed. After the combination therapy, objective partial regression of > 50% was seen in 2 out of 21 patients (10%) treated. Two patients demonstrated minor radiographic response to treatment, 6 had stable disease, and the remaining 11 had progressive disease or had to go off study because of treatment toxicity. This study established that EUS guided transgastric injection of ONYX-015 adenovirus into PDAC was both feasible and safe and that such an approach may be extended to other novel biological agents.

**TNFerade:** TNFerade™ Biologic (also called "AdGVEGR.TNF.11D" or "TNFerade"), is a replication-deficient adenoviral vector for selective delivery of tumor necrosis factor-α (TNF-α) into tumor cells. It consists of Egr-1 promoter gene located upstream to the cDNA which is radiation inducible, hence providing spatial and temporal control of the cytotoxicity by TNF-α[9]. TNF- α may also act as a radiosensitizer and enhance the effect of subsequent radiation therapy[32]. In a multicenter study investigating the safety and feasibility of intratumoral gene therapy with TNFerade Biologic along with standard chemoradiotherapy as first-line treatment for LAPC, subjects were administered either EUS-FNI or percutaneous injection under ultrasound or CT guidance[9]. Twenty-seven patients underwent EUS-FNI and 23 received a percutaneous injection of TNFerade once a week for 5 wk in a dose-escalation manner. A total volume of 2 mL was administered per session of EUS-FNI and injected as four 0.5 mL injections into different areas of the tumor. The maximum tolerated dose was calculated as $4 \times 10^{11}$ PU after the appearance of DLT of pancreatitis and cholangitis in 3 patients at the highest dose. Overall grade 3 and 4 toxicities included GI bleeding, deep vein thrombosis (DVT), pulmonary emboli, pancreatitis, and cholangitis which had an unclear attributability to the treatment which was therefore concluded to be well tolerated. There occurred 1 complete response (2%), 3 partial responses (6%), 4 minor response (8%) and 12 had stable disease (24%). Seven patients subsequently had an operative resection, 6 of which had clear margins, and 3 had a survival of more than 24 mo. Furthermore, the overall outcome was not influenced by the mode of delivery of TNFerade Biologic (either by EUS or percutaneous injection).

Based on these results, a multicenter RCT was conducted in patients with LAPC who were assigned to receive either TNFerade along with SOC chemoradiotherapy or SOC alone[33]. TNFerade intratumoral injection was delivered in the dose of $4 \times 10^{11}$ PU using a CT or ultrasound-guided percutaneous transabdominal approach (PTA) or EUS-guided transgastric or transduodenal approach before the first fraction of radiotherapy each week for 5 wk. The mode of delivery was based on the discretion of the individual study sites. SOC consisted of continuous infusion 5-fluorouracil and radiotherapy, followed by gemcitabine or gemcitabine plus erlotinib maintenance therapy. The trial was discontinued based on futility after planned interim analysis. Median OS of TNFerade plus SOC *vs* SOC alone (10.0 mo *vs* 10.0 mo; HR: 0.90; 95%CI: 0.66-1.22; $P = 0.26$) and median PFS (6.8 mo *vs* 7.0 mo, respectively; HR: 0.96; 95%CI: 0.69-1.32; $P = 0.51$) were similar in both groups. Multivariate analyses showed that the EUS-FNI approach rather than the percutaneous transabdominal approach was a risk factor for lower PFS (HR: 2.08; 95%CI: 1.06-4.06; $P = 0.032$). Higher rates of definite or probable grade 1 and 2 fever and chills were observed in TNFerade plus SOC *vs* SOC arm alone. Significantly more grade 2 to 4 toxicities were present in the TNFerade plus SOC arm, but this was not dose-limiting suggesting that conditional expression of TNF-α through Egr-1 promoter limits systemic toxicity. TNFerade administration in this study did not prove effective in prolonging survival in patients with LAPC.

**HF-10:** HF-10 is a spontaneously mutated oncolytic herpes simplex virus-1 reported to have high tumor selectivity and reduced neuro invasiveness[34]. A phase 1 trial published in 2018 evaluated the safety and anti-tumor effectiveness of a triple combination therapy consisting of EUS-guided intratumoral injection of HF-10 along with systemic gemcitabine and erlotinib therapy for unresectable LAPC[35]. Patients underwent twice-weekly HF10 injections to a total of four injections unless DLT appeared. Three cohorts were designed in a dose escalation of $1 \times 10^6$, $3 \times 10^6$, and $1 \times 10^7$ pfu/d. Five patients developed grade III myelosuppression due to chemotherapy and two had serious AEs (perforation of duodenum and grade IV hepatic dysfunction) which were concluded to be unrelated to HF-10. Out of the nine subjects who completed the treatment, the tumor response was three partial responses and four stable diseases. Although the median PFS was relatively short as 6.3 mo, the median OS was 15.5 mo and two patients achieved long-term survival over 3 years. Infiltration of $CD4^+$or $CD8^+$cells was well documented in surgical specimens of two patients who ultimately downstaged and underwent surgery, highlighting the idea that oncolytic viruses might not only aid in tumor destruction but may also trigger host anti-tumor response.

**Ad5-DS:** Ad5-DS is a second-generation, replication-competent oncolytic adenovirus containing double suicide genes that convert prodrugs, 5-fluorocytosine, and valganciclovir, to active cytotoxic metabolites. In a recent Phase I study, nine patients with newly diagnosed LAPC received EUS-guided injection of Ad5-DS with concomitant oral 5- fluorocytosine and valganciclovir along with standard-dose intravenous gemcitabine[36]. The dose cohorts were $1 \times 10^{11}$, $3 \times 10^{11}$, and $1 \times 10^{12}$ viral particles (viral particles)/mL. The therapy was reported to be well tolerated no DLT occurred. Tumor response from nine patients who underwent this therapy showed that one patient had a partial response while the other eight had stable disease at 12 wk. The overall response rate was 11%, and the disease control rate was 100%. Disease progression was noted in two patients at 6.5 mo (median PFS of 11.4 mo). Adenoviral DNA was detected in the peripheral blood of 4 patients at 8 wk. Although the trends in tumor size and carbohydrate antigen 19-9 levels seemed more favorable in patients who received higher doses of Ad5-DS, no dose-response relationship was established statistically[37].

### *Other gene transfer therapies*

**STNM01:** Carbohydrate sulfotransferase 15 (CHST15) is a specific enzyme that has been shown to initiate pancreatic cell mobilization and invasion through its product chondroitin sulphate-E which cleaves CD44 and releases the sCD44 variant into the extracellular space. STNM01 is a synthetic double-stranded RNA oligonucleotide that selectively represses CHST15 expression[38,39]. Six patients with unresectable pancreatic cancer were administered STNM01 *via* a single EUS-FNI procedure with 16 mL (250 nM) injectate using a conventional 22-gauge needle in an open-labeled trial[40]. The agent was injected into 16 different sites within the tumor (1 mL each). Additional STNM01 injections were delivered after 4 wk of observation for AEs and were continued until disease progression occurred. All patients tolerated the procedure well and no AEs were observed. Median tumor size changed from 31 mm to 29 mm along with a significant decrease in median serum soluble CD44 variant, 6 which may reflect CHST15 inhibition and decreased cleavage of CD44, although this finding is limited by the lack of a reference range for sCD44v6 in healthy individuals. Histological evidence of high baseline expression of CHST15 positive cancer cells was noted which showed a large reduction in 2 patients after 4 wk of treatment. Interestingly, these patients also demonstrated tumor necrosis and longest OS (15.5 mo and 18 mo, respectively) indicating that STNM01 acts on CHST15 positive cells to reverse invasion and induce local tumor necrosis although this needs further confirmatory data in future studies.

**BC-819:** BC-819 is a double-stranded DNA plasmid that carries the cytotoxic gene for diphtheria toxin. Its expression is controlled by the presence of the H19 promoter sequence, which is overexpressed in some tumors like PDAC, leading to selective tumor cell destruction[41]. In a clinical study involving nine patients with unresectable locally advanced PDAC (positive for H19 expression), 2 wk of twice-weekly intratumoral injection of BC-819 under either CT ($n$ = 3) or EUS ($n$ = 6) guidance was administered[42]. The mode of delivery was determined by the principal investigator depending on tumor size, location, and ease of injection. Injection volumes of 1 mL (4 mg of BC-819) and 2 mL (8 mg of BC-819) were delivered in a dose-escalation manner in the two cohorts using a 21- to a 22-gauge needle in a clockwise alternating injection site scheme for maximum distribution. The treatment was safe and well-tolerated. Asymptomatic elevation of lipase in one patient was considered as DLT but MTD was not reached. Partial response was observed in 3 of the 6 patients treated with the higher dose (8 mg) at three-month follow-up. Resectability assessment showed that two individuals who received chemotherapy or chemoradiation therapy after experimental treatment were down staged to resectable PDAC at three months with one patient subsequently undergoing surgery with negative margins. This indicates that BC-819 may provide additional therapeutic benefits for advanced PDAC along with systemic chemotherapy.

## DISCUSSION

Pancreatic cancer remains a highly lethal malignancy. One of the challenges hypothesized with systemic administration of therapeutic agents is their lack of penetration into the pancreatic tumor bed owing to surrounding desmoplasia. Direct injection therapies are an attractive option as they can lead to greater intratumoral concentration of the drug or biologic agent while minimizing systemic side effects. EUS-FNI has emerged as an attractive delivery option as this modality can visualize the tumor and surrounding structures in real-time. This allows for precise intratumoral delivery of biological agents while minimizing the risk for AEs such as avoiding vascular or surrounding structures. Multiple candidate agents for local therapy have been identified.

Successful local delivery of chemotherapeutics is a logical option given their proven safety profile with systemic therapy. Some of these agents have been delivered into normal pancreatic tissue in animal studies using EUS-FNI with no significant AEs[43,44] Results of the clinical study using EUS-guided gemcitabine injections are encouraging although additional data is required to confirm these findings with regulated delivery of standard multimodality therapy and controlled trials assessing the effect of multiple sessions and escalating doses towards significant clinical advantage. In theory, immuno-

therapy is an attractive option, but various challenges with PDAC include the immune-suppressive TME including stromal cellular and molecular components, and other multiple immunological barriers making PDAC a "cold" tumor. Direct delivery of these iDCs into the tumor mass makes tumor antigen loading theoretically more effective for inciting T-cell response mechanisms and has provided a renaissance in the exploration of immunotherapy for PDAC. Although the mentioned limited clinical reports have established safety, feasibility, and some immunological response in their studies, there appears to be a need for increased understanding of the complex immunotherapeutic pathways in PDAC for determining the most efficacious DC activating agent and most suited combination therapy for improving outcomes. Further studies are required to confirm survival benefits, explore synergism with immune checkpoint inhibitors, and select the most appropriate patient population to benefit from these immunotherapies based on precise biomarkers like neutrophil to lymphocyte ratio (NLR) and $CD8^+$/Treg ratio[25] Novel molecular markers may also help in identifying patients with predominantly locoregional complications from PDAC that would benefit from localized therapies.

Oncolytic viral therapy is among the most promising agents for local delivery in pancreatic tumors. Although different strains of adenovirus, herpes virus, measles virus, and other viruses have shown positive results in cancer cell lines and preclinical models, limited clinical studies have performed intratumoral injection of these agents using EUS-FNI[45,46]. As PDAC mass consists of islands of neoplastic cells interspersed with dense stroma which can hinder the spread of injectable agents, OVs can overcome this problem to some extent through their replicative potential and hence increased dissemination within the tumor. Other future areas of research can include viruses targeted towards extracellular matrix disruption and combination with anti-stromal agents to allow better penetration of viral therapy.

EUS-FNI technique can further enhance tumor penetration regardless of tumor cell distribution and composition of the surrounding stroma. Injection into multiple sites in the pancreatic mass using EUS-FNI may assist with even distribution of the agent throughout the tumor. Furthermore, EUS can be used to assess response, and allow for subsequent FNI therapy. EUS, however, is associated with the need for sedation, which may add additional cost and risks associated with sedation. Further studies are needed to compare and firmly establish the most effective EUS-FNI delivery technique including the use of multiple injection sites within the tumor, multiple passes, or use of newer designed needle devices for enhanced dispersion of the agents within the desmoplastic pancreatic stroma[47].

EUS-FNI is an emerging modality for enhanced local intratumoral drug delivery[48]. Current data demonstrate that EUS-guided injectable therapies are safe for the treatment of PDAC. Larger studies, including RCTs should consider using EUS-FNI and these data are needed to establish efficacy and survival data, identify the most suitable anti-tumor agents, including combination therapy, and determine the best patient populations that may benefit from local drug delivery. Regenerative therapies, including the use of immunotherapy, DCs, and oncolytic viruses offer new hope in the management of PDAC. These advances towards novel EUS-FNI therapies should more actively involve endoscopists as part of the multidisciplinary treatment team as we hope to improve survival of our patients with PDAC.

## CONCLUSION

EUS-guided injectable therapies, including immunotherapy, chemotherapy, and viral or other biological therapies have shown minimal AEs and potential efficacy in the treatment of PDAC. Comparative studies, including controlled trials, are required to confirm improved survival and establish the most effective therapeutic options. Further research is needed to offer novel EUS-based therapies as a promising treatment for patients with PDAC in the future.

## ARTICLE HIGHLIGHTS

### *Research background*

Many new treatment options for pancreatic cancer are being explored owing to its poor prognosis. Advent of therapeutic Endoscopic ultrasound (EUS) guided therapies in recent years paved the way to explore the local delivery of injectable agents. In the last 22 years, very few studies have explored the use of EUS-guided fine-needle injection (EUS-FNI) to treat pancreatic ductal adenocarcinoma (PDAC). These are mostly phase I/II clinical studies using different agents and varied methodologies with mixed results.

### *Research motivation*

EUS-FNI has the theoretical advantage of targeted delivery of anti-tumor agents under real-time visualization and minimal invasiveness. It can also overcome the limitations of systemic therapy mainly the low penetration of these agents into the desmoplastic tumor mass of PDAC. Limited literature and

heterogeneity in methodologies and outcomes necessitated a systematic review of the present literature to understand and guide future research in this promising field.

### Research objectives

To evaluate the current status of research in the novel area of EUS-guided injectable treatment for PDAC. This has helped to understand the progress made so far and draw meaningful conclusions based on the limitations and gaps found in the literature. This has also enabled the development of focused future directives for research on this topic which can potentially advance the treatment of PDAC.

### Research methods

A systematic and comprehensive review of clinical studies which used EUS-guided injectable therapy for the treatment of PDAC was done. Expert librarian assisted in the electronic search of various databases. Screening of papers for eligibility was done by two study members independently. Data were collected in a standardized manner with regard to the methodologies and outcomes of these studies. A critical appraisal of the present literature on this topic was performed.

### Research results

Our study demonstrates that immunotherapy, chemotherapy, oncolytic viral, and other biological therapies have been used *via* EUS-guided injection technique in different ways to study the safety and efficacy of such treatment in PDAC patients. The review of the present literature indicates that these therapies are well tolerated and feasible overall. Mixed results are demonstrated in terms of clinical efficacy.

### Research conclusions

This study concludes that EUS-FNI based treatment may be administered to patients with advanced PDAC without significant toxicity. Clinical efficacy with respect to the standard of care (SOC) is not yet established. Further research should be undertaken to find out the most effective therapeutic agent, dose, and techniques that may be employed to the appropriate population of PDAC patients who would benefit the most from these.

### Research perspectives

The direction of future research should be to design controlled studies and phase III trials using the data from present literature to establish efficacy in terms of tumor response and survival with respect to the SOC. Anti-tumor agents may be administered at higher doses and multiple EUS-FNI sessions to maintain the appropriate concentration in the tumor bed. Studies using appropriate combination therapies (using chemotherapy and/or radiotherapy) and different EUS-FNI techniques, for example, multiple needle passes should be encouraged as they may help in overcoming hostile tumor microenvironment of pancreatic cancer.

## FOOTNOTES

**Author contributions:** Kaur J and Chandrasekhara V conceived and designed the study and critically reviewed the manuscript; Kaur J and Chandrasekhara V conducted the literature search, screened for eligibility, and drafted the manuscript; Kaur J and Jaruvongvanich V collected, analyzed, and interpreted the data; all authors reviewed the literature and revised the manuscript, read and approved the final manuscript.

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LETTER TO THE EDITOR

# Peripancreatic paraganglioma: Lesson from a round table

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## Abstract

We described the case of a peripancreatic paraganglioma (PGL) misdiagnosed as pancreatic lesion. Surgical exploration revealed an unremarkable pancreas and a large well-defined cystic mass originating at the mesocolon root. Radical enucleation of the mass was performed, preserving the pancreatic tail. Histologically, a diagnosis of PGL was rendered. Interestingly, two previously unreported mutations, one affecting the *KDR* gene in exon 7 and another on the *JAK3* gene in exon 4 were detected. Both mutations are known to be pathogenetic. Imaging and cytologic findings were blindly reviewed by an expert panel of clinicians, radiologists, and pathologists to identify possible causes of the misdiagnosis. The major issue was lack of evidence of a cleavage plane from the pancreas at imaging, which prompted radiologists to establish an intraparenchymal origin. The blinded revision shifted the diagnosis towards an extrapancreatic lesion, as the pancreatic parenchyma showed no structural alterations and no dislocation of the Wirsung duct. *Ex post*, the identified biases were the emergency setting of the radiologic examination and the very thin mesocolon sheet, which hindered clear definition of the lesion borders. Original endoscopic ultrasonography diagnosis was confirmed, emphasizing the intrinsic limit of this

technique in detecting large masses. Finally, pathologic review favored a diagnosis of PGL due to the morphological features and immonohistochemical profile. Eighteen months after tumor excision, the patient is asymptomatic with no disease relapse evident by either radiology or laboratory tests. Our report strongly highlights the difficulties in rendering an accurate pre-operative diagnosis of PGL.

**Key Words:** Peripancreatic paraganglioma; Pancreatic neuroendocrine tumor; Solid pseudopapillary neoplasm; S100; Succinate dehydrogenase subunit B gene and expression; Fine needle biopsy



**Core Tip:** Our report strongly supports that paraganglioma should be included in the differential diagnosis of peripancreatic/pancreatic masses, highlighting the difficulties in establishing the accurate preoperative diagnosis, even after a second-round evaluation. In fact, due to the deep localization and the lack of specific clinical manifestations and imaging data, early diagnosis often relies solely on a level of suspicion, thus making it more probable to have a missed diagnosis or misdiagnosis. Moreover, the limited time spent with a patient in an emergency setting might impair the accuracy of the diagnosis, lowering quality and outcomes of healthcare delivery. A multidisciplinary team approach, involving skilled radiologists, endoscopists, pathologists, and surgeons, is of foremost importance for proper diagnosis and management, preventing undue surgical resections.

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## TO THE EDITOR

We read with interest the paper authored by Lanke *et al*[1] on a peripancreatic paraganglioma (PPGL) successfully diagnosed pre-operatively by endoscopic ultrasonography (EUS)-fine needle aspiration. After a careful review of the literature, the report highlights the enduring difficulties in achieving an accurate diagnosis of this rare entity prior to surgery. Among the 47 pancreatic/peripancreatic PGL reported so far, only 8 (17%) were correctly identified pre-operatively; the majority ($n$ = 29, 62%) were misdiagnosed as pancreatic neuroendocine tumors or pancreatic neoplasms/cysts. In almost all cases (80%), the treatment of choice was surgery, demonstrating the uncertainty of classification for such lesion and its unpredictable behavior[1].

In November 2020, a 23-year-old woman complaining of left hypocondrial pain, diarrhea, and sweating lasting for 4 d, was referred to the Emergency Unit of Ospedale Unico della Versilia. The physical examination was unremarkable, but the abdominal US showed a large cystic, liquid-filled mass close to the pancreatic tail. Whole-body computed tomography (CT) scan confirmed the presence of a 95-mm cystic lesion with thickened, contrast-enhanced walls and a marked inhomogeneous boundary with the pancreatic tail. The mass seemed to be adherent to the left renal artery, left kidney hilum, and homolateral ureter (Figures 1A and 1B). Imaging work-up was completed with abdominal nuclear magnetic resonance (NMR) revealing T1 signal hyperintensity (Figure 1C) and positron emission tomography disclosing abnormal hyperactivity of the mass (standard uptake value 4.7%) (Figure 1D). The suspicion of a solid-pseudopapillary neoplasm (SPN) *vs* a cystic pancreatic tumor was posed. Blood tests, including vasoactive intestinal peptide, C-peptide, pancreatic polypeptide, serotonin, carcinoembryonic antigen (CEA), Ca-125, and Ca-19.9, were within normal range; whereas chromogranin A (CgA) serum levels exceeded 1800 ng/mL (reference range: 0-108 ng/mL). EUS-fine needle biopsy (FNB) was scheduled. EUS examination showed a 10-cm lesion of the pancreatic tail. The lesion was hypoechoic with well-defined borders and a cystic component (Figures 1E and 1F). Two FNB passes were made using a 20 Gauge needle with Rapid On-Site Evaluation to ensure adequate sampling[2]. Intracystic fluid was aspirated and analyzed with the following results: Glucose, 45 mg/dL; pancreatic amylase, 5 U/L; CEA, 1.0 ng/mL; Ca-19.9, 9 U/mL. Cell block examination disclosed nests of small to medium-sized epithelioid cells exhibiting moderate amount of amphophilic granular cytoplasm and oval to round nuclei with smooth contours and dense chromatin (Figure 2A). Occasionally, small nucleoli (Figure 2A dots), sparse mitotic figures (Figure 2A arrows), and intra-cytoplasmic hyaline globules (Figure 2A encircled) were noted, as well as vague rosette-like formations and branching hyalinized

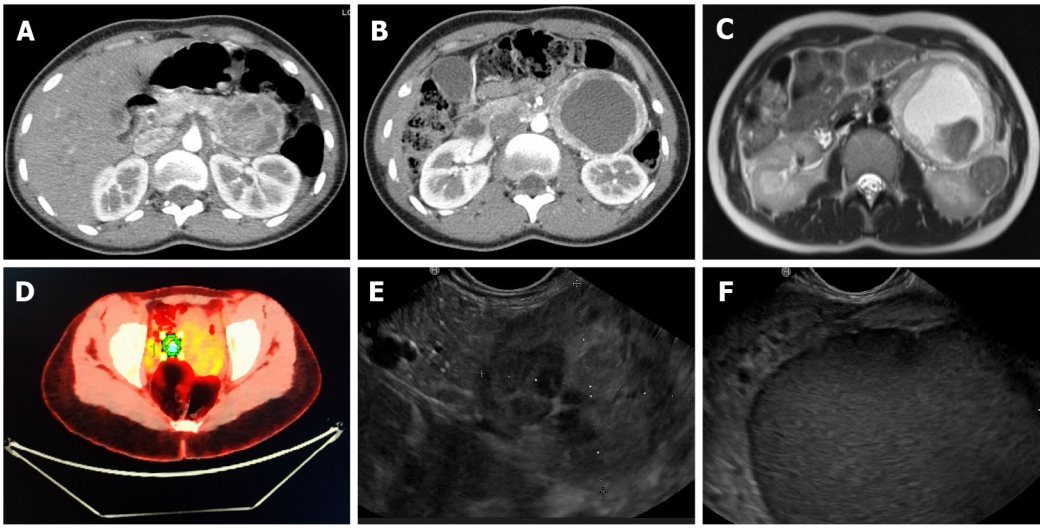


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**Figure 1 Radiologic findings.** A and B: Computed tomography-scan showed a 95-mm cystic lesion with no cleavage plane from the pancreas; C: Nuclear magnetic resonance evidenced that the lesion was hyper-intense in T1; D: Positron emission tomography demonstrated a 4.7% standard uptake value; E and F: Endoscopic ultrasonography identified a hypoechoic mass close to the pancreatic tail.

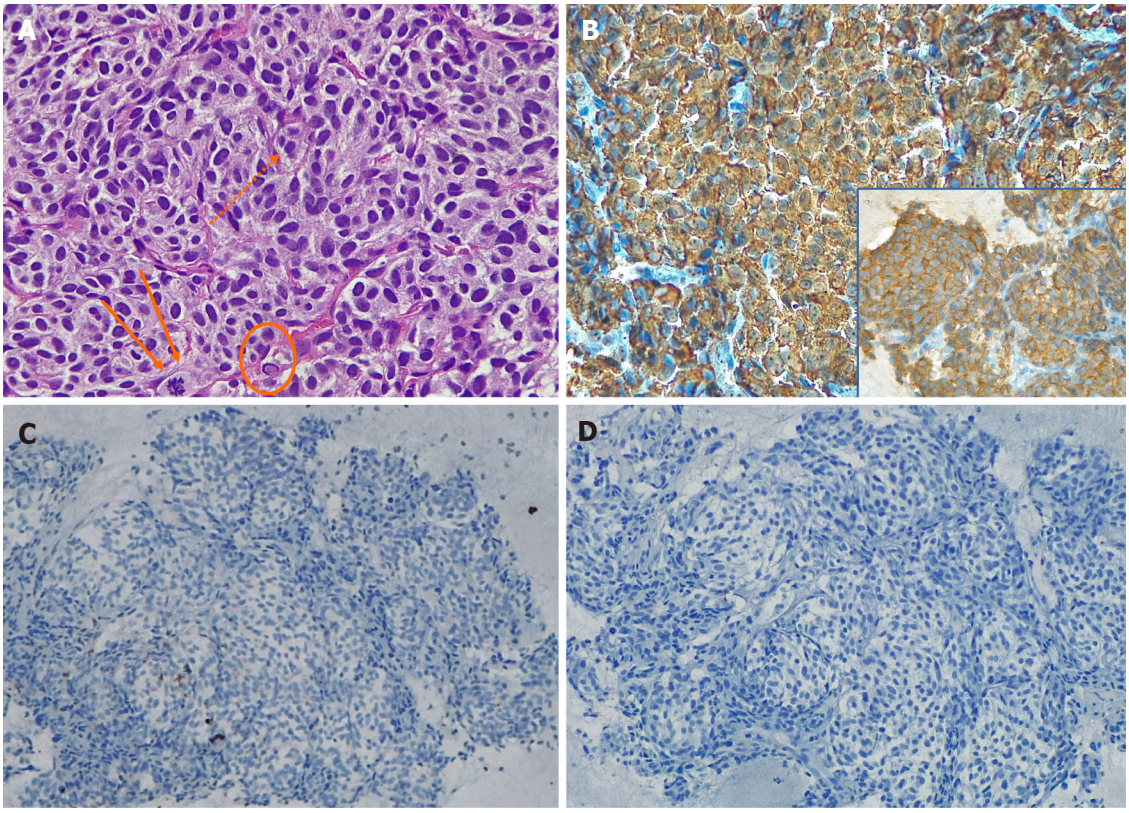


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**Figure 2 Fine needle biopsy findings.** A: Proliferation of small to medium-sized cells arranged in a nest pattern was evident, the cells occasionally showed small nucleoli (dots), mitotic figures (arrows), and intra-cytoplasmic hyaline globules (encircled), hematoxylin and eosin original magnification (O.M) × 40; B: Chromogranin A (CgA) positivity of neoplastic cells; inset: Synaptophysin positivity of neoplastic cells, CgA stain, O.M. × 20; B inset, synaptophysin stain, O.M. × 20; C: AE1/AE3 cytokeratins expression in scattered cells, cytokeratin AE1/AE3 stain, O.M. × 20; D: S100 negativity, S100 stain, O.M. × 20).

fibrovascular cores lined by neoplastic cells. Tumor cells diffusely expressed CgA (Figure 2B), synaptophysin (Figure 2B, inset), and GATA-3. Scattered cells were dot-like positive for AE1/AE3 cytokeratins (Figure 2C). Epithelial membrane antigen (EMA), PAX-8, and S100 protein (Figure 2D) were negative. A provisional diagnosis of neoplasm with neuroendocrine differentiation (either PGL or PanNET) *vs* SNP was made.

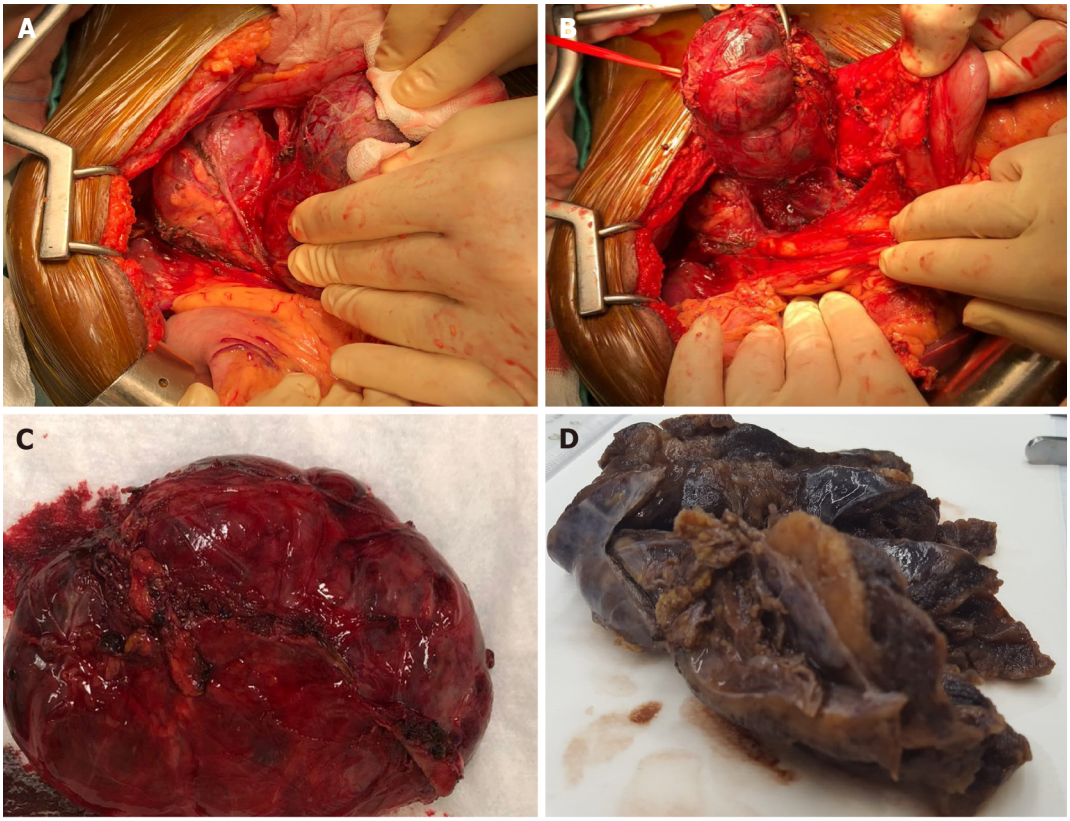


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**Figure 3 Macroscopic findings.** A and B: At surgical exploration a large well-defined cystic mass located underneath the mesocolon plane was found; C: Radical enucleation of the lesion; D: Grossly, the cystic lesion showed a thick fibrous wall with a solid component and a yellowish, lobulated appearance on cut surface.

Surgical exploration revealed an unremarkable pancreas and a large well-defined cystic mass underneath the mesocolon plane, originating at the mesocolon root (Figures 3A and 3B). The lesion extended to the inferior margin of the pancreas and was strictly adherent but did not involve the organ parenchyma. As the capsule could be easily detached from the mesocolon sheets and vessels, radical enucleation was performed, preserving the pancreatic tail (Figure 3C).

The surgical specimen consisted of a 12-cm hemorrhagic cystic lesion, surrounded by a thick fibrous wall, with a solid component showing a yellowish, lobulated appearance on cut surface (Figure 3D). Microscopically, the tumor was composed of polygonal cells with abundant finely granular eosinophilic to amphophilic cytoplasm, mostly arranged in nests and anastomosing cords (Figure 4A). Areas with a diffuse growth pattern were also noted. Nuclei were regular, round to oval, with granular fine chromatin, small nucleoli, and occasional pseudoinclusions. The stroma was richly vascular. In the more cellular areas, pleomorphic cells with prominent nucleoli and occasional mitotic figures (2/high power field) were observed. There was evidence of focal capsular disruption. Cells were confirmed to be positive for CgA (Figure 4B), synaptophysin, GATA-3 (Figure 4C), and substantially negative for AE1/AE3 cytokeratins, EMA, and PAX-8. S100 and GFAP stained scanty cells encircling cell nests (Figure 4D). A final pathologic diagnosis of PGL, Grading System for Adrenal Phaeochromocytoma and Paraganglioma score 6 (intermediate risk), was rendered[3,4].

Given the young age of the patient and the rarity of the tumor, succinate dehydrogenase subunit B (SDHB) protein expression was evaluated, and next generation sequencing (NGS) analysis, using the commercial kit "Miriapod NGS-IL" (Diatech) on MiSeq Illumina Platform, was carried out to identify mutations affecting genes associated with syndromic and/or familial conditions (*i.e.*, *SDHB*, *VHL*, *NF1*, *RET*). *SDHB* expression was preserved (Figure 4D, inset) and none of the above-mentioned genes were found to be mutated, thus ruling out a hereditary condition[5]. Interestingly, we detected two previously unreported mutations, one affecting the *KDR* gene in exon 7 (c.889G>A missense variant)[6], and another on the *JAK3* gene (c.394C>A missense variant) in exon 4[7,8] (Figures 4E and 4F). Both mutations are known to be pathogenetic in olfactory neuroblastoma[6], some forms of leukemia, and solid tumors, such as neuroendocrine tumors[7,8].

Imaging and cytologic findings were blindly reviewed by an expert panel of clinicians, radiologists, and pathologists to identify possible causes of misdiagnosis. The major issue was lack of evidence of a cleavage plane from the pancreas at US, CT, and NMR, which prompted radiologists to establish an intra-parenchymal origin. The blinded revision shifted the diagnosis towards an extra-pancreatic lesion

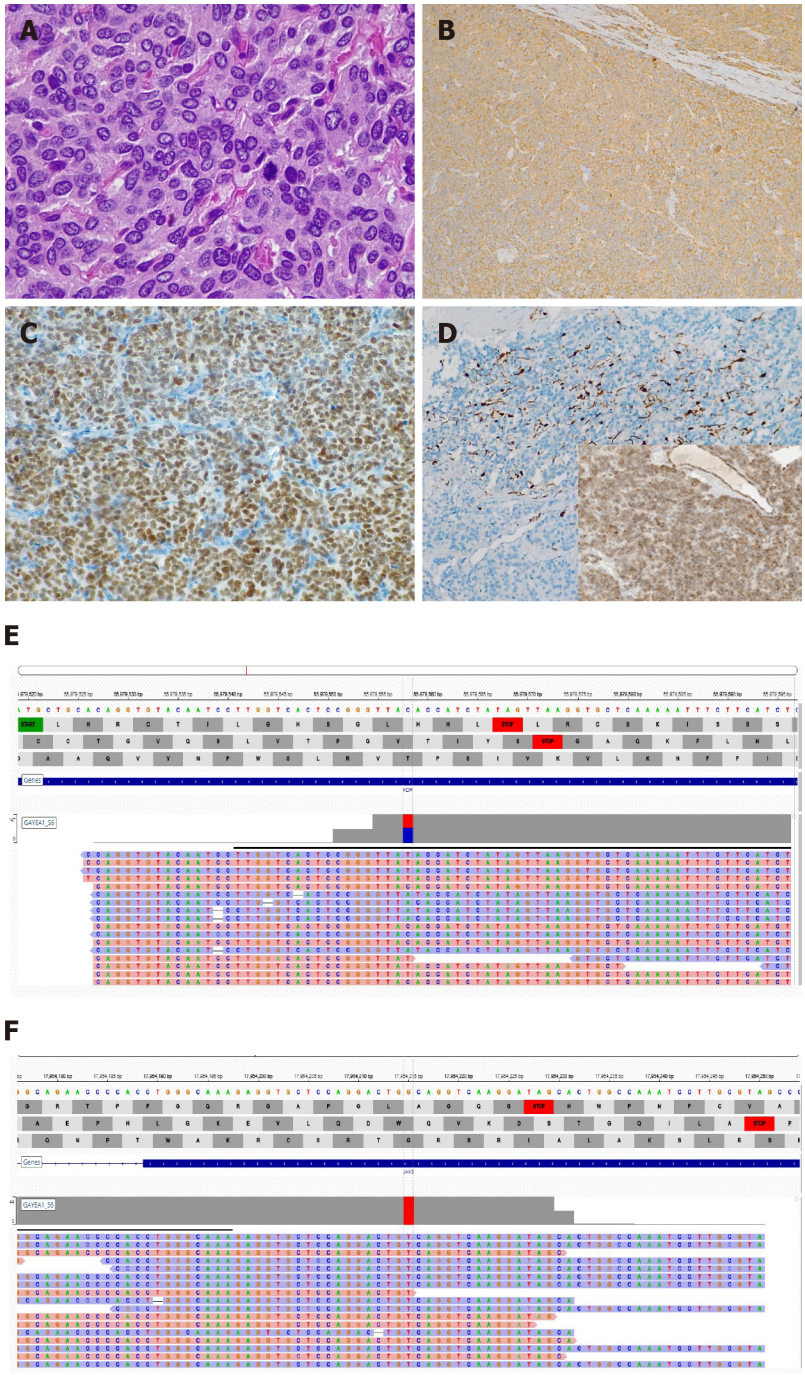


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**Figure 4 Histologic and molecular findings.** A: The lesion was composed of nests and cords of polygonal cells with abundant granular cytoplasm, and occasional mitotic figures in the more cellular areas, hematoxylin and eosin original magnification (O.M) × 60; B: Chromogranin A (CgA) positivity in neoplastic cells; inset: Synaptophysin positivity in neoplastic cells, CgA stain, O.M × 10; C: GATA-3 positivity in neoplastic cells, GATA-3 stain, O.M. × 10; D: S100-positive cells were scattered; inset: Succinate dehydrogenase subunit B (SDHB) expression was preserved, S100 stain, O.M. × 10; inset, SDHB stain, O.M. × 10; E: *KDR* mutation; F: *JAK3* mutation.

as the pancreatic parenchyma showed no structural alterations and no dislocation of the Wirsung duct. *Ex post*, the identified biases were the emergency regimen of radiologic examination, which impaired a precise assessment, and the very thin mesocolon sheet, probably on account of the patient's leanness, which hindered clear definition of the lesion borders, also masked by the extensive hemorrhagic component. Original EUS diagnosis was confirmed, emphasizing the intrinsic limit of the bi-dimensional image of trans-abdominal US in detecting a large mass in the same examination field. Finally, pathologic review of the FNB findings favored a diagnosis of PGL due to substantial negativity for cytokeratins and PAX-8, usually positive in PanNet[9,10], and absence of folded nuclei with longitudinal grooves, cholesterol crystals, and foamy macrophages, usually found in SPN[9]. Furthermore, diffuse cytoplasmic CgA reactivity is typically not seen in SPN[9]. The detection of S100-positive

sustentacular cells and zellballen pattern, considering the hallmarks of PGL, has limited value in the diagnosis of PGL, since they may not be present in small samples or in sympathetic PGLs, as in our case [2,11]. Eighteen months after tumor excision, the patient is asymptomatic with no disease relapse evident with CT or NMR imaging; CgA levels are within the reference range.

In conclusion, our report strongly supports the suggestion by Lanke *et al*[1] that PGL should be included in the differential diagnosis of peripancreatic/pancreatic masses, and highlights the difficulties in establishing an accurate preoperative diagnosis even after a second round evaluation. Indeed, due to the deep localization and lack of specific clinical manifestations and imaging data, early diagnosis of PGL often relies solely on the level of suspicion, thus easily resulting in misdiagnosis or missed diagnosis[12,13]. A multidisciplinary team approach, involving skilled radiologists, endoscopists, pathologists, and surgeons, is of foremost importance for proper PGL diagnosis and management in order to prevent undue surgical resections[14]. In the present case, the first radiologic mistake affected all the diagnostic work-up, even after second round evaluation, and only the long-lasting experience of the surgeon avoided over-treatment (*i.e.*, distal splenopancreasectomy, as reported in most of the cases described so far)[1]. Since there are no definite criteria for malignancy, a close follow-up after surgery is mandatory[14,15]. Pathologists play a key role in providing clues that may disclose genetic profile and predict malignant potential of the tumor[1,3,4,14,15].

## FOOTNOTES

**Author contributions:** Ambrosio MR, Amorosi A, and Arganini M contributed to the conception and design; Fratini G and Sbrozzi-Vanni A acquired the data; Cavazzana A, Giusti A, and Nesi G analyzed and interpreted of the data; Ambrosio MR, Fratini G, and Petrelli F drafted the article; Amorosi A, Arganini M, Manta R, Nesi G, and Vignali C contributed to the critical revision of the article for important intellectual content; and all authors approved the final manuscript to be published.

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