CHAPTER THIRTEEN

# Immunotherapies for hepatocellular carcinoma and intrahepatic cholangiocarcinoma: Current and developing strategies

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## Abstract

Liver cancer including hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (iCCA) is the third leading cause of cancer-related deaths worldwide. HCC arises from hepatocyte or hepatic stem cells, while iCCA originates from biliary epithelial

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cells, and the respective biological context are very different. Despite screening programs, the diagnosis of liver cancer is in most cases made when curative treatments such as surgery or ablation are not possible. In 2020, after a decade of using only tyrosine kinase inhibitors (TKI), a combination of an immune-check point inhibitor (ICI) and a VEGF antagonist proved superior to a TKI as first line therapy of advanced HCC. In 2022, the addition of an ICI to standard chemotherapy demonstrated an improvement of patient survival in iCCA. Moreover, ICI offer an unprecedented rate of durable responses to HCC and iCCA patients. Nevertheless, still two thirds of patients do not respond to ICI-based combinations, and research efforts are focused on deciphering the mechanisms of immune evasion of these lethal cancers. Reliable predictive and prognostic biomarkers are still lacking, but the molecular phenotyping of the tumor microenvironment is currently providing potential candidates for patient stratification. In this review, we will summarize the current knowledge on the immune biology of the liver, the discovery of cell-intrinsic and immune cell-mediated mechanisms of immune evasion by means of high-resolution single cell data, the main targets of current immunotherapy approaches, and the recent milestones in immunotherapy of HCC and iCCA.

## Abbreviations

| | |
|---|---|
| **ACT** | adoptive cell therapy |
| **ALD** | alcohol-related liver disease |
| **AFP** | alpha fetoprotein |
| **CAR** | chimeric antigen receptor |
| **DAMP** | damage-associated molecular pattern |
| **DC** | dendritic cell |
| **GPC3** | glypican 3 |
| **HBV** | hepatitis B virus |
| **HCC** | hepatocellular carcinoma |
| **HCV** | hepatitis C virus |
| **iCCA** | intrahepatic cholangiocarcinoma |
| **ICB** | immune checkpoint blockade |
| **ICI** | immune checkpoint inhibitor |
| **IFN-γ** | interferon gamma |
| **KC** | Kuppfer cell |
| **MAFLD** | metabolic syndrome-associated fatty liver disease |
| **MDSC** | myeloid-derived suppressor cell |
| **MHC** | major histocompatibility complex |
| **NK** | natural killer cell |
| **NKT** | natural killer T cell |
| **OS** | overall survival |
| **ORR** | overall response rate |
| **PAMP** | pathogen-associated molecular pattern |
| **PFS** | progression-free survival |
| **TAA** | tumor-associated antigen |
| **TAM** | tumor-associated macrophage |
| **TGF-β** | transforming growth factor beta |
| **TKI** | tyrosine kinase inhibitor |
| **TME** | tumor micro-environment |

## 1. The immune biology of the liver

The liver is an organ with a blood supply that flows from the gut through the sinusoids, a unique fenestrated vasculature enabling optimal exchange of molecules for metabolic functions, together with generating an immune barrier to microbes and toxins. The liver has a notable capacity to remove gut-derived pathogens, and their derived Pathogen-Associated Molecular Patterns (PAMP), such as lipopolysaccharide (LPS), toxins and Damage-Associated Molecular Patterns (DAMP) from the portal circulation (Lumsden, Henderson, & Kutner, 1988). To cope with this injuring-prone environment the liver is specially armed with several types of innate and adaptive immune cells. But for the same reason, the liver possesses a key immunoregulatory role through its ability to maintain immunotolerance to non-pathological or constant inflammatory stimuli which prevents liver damage and induces systemic tolerance. To enable this immune regulatory function, the liver contains the largest number of resident macrophages in the body, the so called Kupffer cells (KCs). It also retains a high density of natural killer cells (NK cells), natural killer T cells (NKT cells), γδ T cells, and both liver-transiting and resident T lymphocytes (Jenne & Kubes, 2013).

Upon chronic liver damage, such as a viral infection (by hepatitis B virus [HBV] or hepatitis C virus [HCV]), abnormal continuous fat and carbohydrate metabolism disruption (for example in the Metabolic Syndrome-Associated Fatty Liver Disease or MAFLD), to chronic toxic injury (such as in Alcohol-related Liver Disease), or to less common causes of chronic injury, including protein misfolding in the hepatocyte (alpha 1 antitrypsin deficiency) and genetic iron or copper accumulation (in hemochromatosis or Wilson diseases, respectively) this immunological balance is initially deregulated towards an inflammatory environment, clinically detectable through the measurement of circulating hepatic enzymes, such as aminotransferases and/or glutamyl transferases. In these chronic diseases, the low-degree liver damage, if untreated, generates continual cell death and compensatory regeneration with hepatic stellate cell activation, which could lead to advanced liver fibrosis.

During the evolution of these chronic conditions, the relationship between the hepatocyte and the immune environment is maintained by a complex balance between proinflammatory cytokines (IL-2, IL-7, IL-12, IL-15 and IFN-γ) and anti-inflammatory cytokines (IL-10, IL-13 and

TGF-β). Due to the unique immunosuppressive environment and the regenerative potential of the liver, the global functional mass of hepatocytes is kept intact and patients with advanced fibrosis are mostly asymptomatic. In most patients, this chronic low-intensity damage evolves to cirrhosis, which becomes symptomatic after the first decompensation due to portal hypertension, associated with the development of ascites, esophageal variceal bleeding, or encephalopathy.

Primary liver cancers, particularly hepatocellular carcinoma (HCC), but also intrahepatic cholangiocarcinoma (iCCA), typically originate in this inflammatory and fibrotic environment. Inflammation and cell death leads to an intense replicative stress in both hepatocytes and hepatic progenitor cells. For oncogenesis to occur both a somatic mutation in a driver gene and a deficiency on immune surveillance are needed. Reversely, once the tumor clone niche is generated, HCC cells recruit tolerogenic cell types, further fostering evasion from immune mediated cell death.

In this review we summarize the evidence of immune cell dysregulation in HCC to understand the mechanisms of immune evasion and to gain a molecular insight on the mechanism of action of newly established and future immunotherapy-based treatments in HCC. We also detail the current status of immunotherapy for HCC and iCCA.

## 2. The immunosuppressive environment of hepatocellular carcinoma

Cohort-based histological and flow cytometry studies, bulk tumor RNA sequencing data, and recent single-cell RNA sequencing studies have illustrated the presence and clinical relevance of the immunosuppressive environment in human HCC samples, including regulatory T (Treg) cells, Kuppfer cells (KC) and monocyte-derived tumor-associated macrophages (TAMs), and myeloid-derived suppressor cells (MDSCs) (Zheng et al., 2017). The increase in the amount of these cell types correlates with cytotoxic T lymphocyte (CTL) impairment (Fu et al., 2007) and with the advanced stage (Shen et al., 2010). The following sections will summarize the pathogenic hijacking of these immune cell types by HCC.

### 2.1 The role of immune and other non-parenchymal cells

CTLs, through the secretion of Th1 type cytokines IFN-γ and IL2, have been associated with antigen-dependent anti-tumor responses. Nearly half of the lymphocytes in the liver express the T cell receptor (TCR), with

an enrichment of CD8+ T cells (Parker & Picut, 2005). While CD4+ T cells outnumber $CD8^+$ T cells by approximately two to one in the blood, in the liver this ratio is reversed. $CD8^+$ T cells are considered the primary mediators of cytotoxic anti-tumor effect, but recent findings have highlighted the importance of the specific phenotype of these cells for their cancer-related immune role. CD4+ and CD8+ tissue-resident memory T cells ($T_{RM}$ CD103+ Cells) originate from circulating T cells and, contrary to other circulating T cells, stably reside in an epithelial organ after the recognition of neoantigens (Pallett et al., 2020). In HCC, CD8+ $T_{RM}$ are important for tumor control (Amsen, van Gisbergen, Hombrink, & van Lier, 2018); however, circulating CD8+ T cells targeting neoantigens are only detected in ~15% of patients with HCC and are tolerized by Dendritic Cells (DC) migrating between the tumor and lymph nodes, and by TAMs (Zhang et al., 2019). On the other hand, exhausted CD8+ CXCR6+ T cells accumulate in NASH-derived HCC, promoting resistance to immunotherapy (Pfister et al., 2021). Another exhausted CD8+ T cell cluster expressing high levels of layilin (LAYN) was associated with reduced disease-free survival (Zheng et al., 2017). Two additional high-resolution single cell sequencing works showed that CD8+ *KLRB* + T Cells with innate-like low cytotoxic phenotype T cells are enriched in early HCC with poor prognosis (Sun et al., 2021), whereas CD8+ *XCL1* + *T cells* are associated with a better prognosis in viral HBV/HCV-related HCC (Song et al., 2020).

The role of CD4+ FOXP3+ T Regulatory Lymphocytes (Treg) is the antigen-specific inhibition of the antitumor immune response (Chen et al., 2003; Wei, Kryczek, & Zou, 2006), to avoid severe autoimmunity (Kim, Rasmussen, & Rudensky, 2007). In HCC, the abundance of Treg correlates with CTL impairment (Fu et al., 2007; Gao et al., 2007) and with advanced HCC stage (Shen et al., 2010). CD4+ Th17 T cells are involved in several autoimmune diseases and chronic inflammatory syndromes. Naive CD4+ T cells preferentially differentiate into the Th17 T cell subset in response to the combined signals of TGF-β and IL-6, commonly secreted in the TME of HCC. IL-23-induced Th17 (Kortylewski et al., 2009) promotes inflammation and angiogenesis, reducing active CD8+ T cell infiltration (Langowski et al., 2006) and antagonizing the tumor-suppressive role of IFN-γ producing CD4+ Th1 cells (Littman & Rudensky, 2010).

B lymphocytes have dual tumor-promoting and antitumoral roles in HCC. Peritumoral CXCR3+ B cells are associated with early HCC recurrence (Liu et al., 2015), while peritumoral $PD1^{high}$ and $FcgRII^{low/-}$ B cells

suppress antigen-specific antitumor immunity (Ouyang et al., 2016; Xiao et al., 2016). Immunosuppressive IgA-producing PD1 + plasma cells, inhibit antigen-specific CTL activity and tumor regression (Shalapour et al., 2017). Interestingly, together with T lymphocytes, B lymphocytes can form Tertiary Lymphoid Structures (TLS) that resemble lymphoid organs within inflamed tissues. Although in some cancers TLS have been associated to better prognosis, such as in early stage colorectal and non-small cell lung cancer (Di Caro et al., 2014; Dieu-Nosjean et al., 2008). In inflamed livers TLS promote the formation of microniches for stem cell proliferation and cancer progression and are associated with HCC late recurrence (Finkin et al., 2015). In contrast, intratumoral TLS were associated with lower early recurrence of HCC after surgical resection (Calderaro et al., 2019). Whether this apparent contradiction has to do with the degree of maturation of tumoral TLS, from primary follicles type (FL-I) to secondary follicle type (FL-II), needs to be investigated. On the other hand, plasmacytic B cells can produce antitumor specific antibodies and durable immunity, thus enhancing the response to immunotherapy (DeFalco et al., 2018).

Gamma delta (γδ) T cells are unconventional T cells, defined by expression of T-cell receptors (TCRs) composed of γ and δ chains instead of the conventional ab-containing TCRs. γδ T cells show tissue-specific subsets all of which share the same TCR. The liver contains one of the largest populations of γδ T cells in the body, which comprise 15–25% of the T cells in the liver (Abo, Kawamura, & Watanabe, 2000; Nemeth, Baird, & O'Farrelly, 2009). γδ T cells infiltrate HCCs and other cancers. Recently, the blockade of the immune inhibitory ligand butyrophilin (BTN), belonging to the B7-family of ligands, enabled the coordinated antigen-specific antitumor activity of γδ and ab T and tumor regression in ovarian cancer, constituting a new attractive therapeutic target (Payne et al., 2020). Other T cell subtypes related to HCC promotion are IL-22 producing T cells (Th22), (Chen et al., 2016), IL-21-producing $CXCR5^{-}$ $PD1^{-}$ $CD69^{high}$ Follicular Helper T Cells ($T_{FH}$) (Chen et al., 2016) and IL-9 and IL-10 producing T cells (Th9) (Tan, Wang, & Zhao, 2017).

Hepatic Innate Lymphoid Cells (ILC) such as NK cells are lymphoid cells lacking specific antigen receptors which can respond to an array of cell-surface ligands expressed by infected, damaged or transformed cells, and can acquire cytotoxic-like properties and secrete IFN-γ, perforin and granzyme (Notas, Kisseleva, & Brenner, 2009). One-third to one-half of the lymphocytes in the liver are NK cells, more than a three-fold enrichment over that observed in blood (Crispe, 2009; Doherty & O'Farrelly, 2000).

NK are strongly activated when the class I MHC is downregulated in tumor cells, a common mechanism of immune evasion. NK can also induce MHC expression by hepatocytes and hepatic stellate cells (Crispe, 2009). Several studies have shown that the dysfunction or decrease in the abundance of NK cells are associated with higher rate of HCC progression and poor survival (Juengpanich et al., 2019). Despite its immune effector phenotype, their antitumor activity in HCC might be limited by TGF-β-mediated impairment of oxidative phosphorylation (Zecca et al., 2020). Subsets of HCC-infiltrating CD11b–CD27–NK cells have a decreased cytolytic activity and a defective IFN-γ production (Zhang et al., 2017). Macrophages are responsible for NK dysfunction in HCC (Wu et al., 2013). High expression of the inhibitory receptor NKG2A and its ligand HLA-E in HCC cells lead to an exhausted NK CD56dim phenotype and are associated with poor prognosis (Sun et al., 2017). Conversely, NKG2D is an important activating receptor, expressed also in γδ and some T lymphocytes, which promotes cytotoxicity of NK cells against HCC through the interaction with its ligands MHC-I-related chain molecules A and B (MICA/B) in the membrane of cancer cells (Jinushi et al., 2003).

Invariant NK T cells (iNKT) are a distinct population of T cells that express both an invariant αβ TCR, that responds to glycolipid antigens presented in the context of the MHC-I–like molecule CD1d, and several NK-type cell surface molecules. iNKT cells are the only liver-resident lymphocytes to actively patrol the liver vasculature in search of pathogens (Bricard et al., 2009). They can express CD4, CD8 or be double negative. In HCC, the tumor is enriched in CD4+ iNKTs harboring a Treg-phenotype, reduced cytolytic activity, and are capable of antigen-specific CD8+ T cell expansion (Bricard et al., 2009).

KC are CD68+ liver-resident macrophages located in the vasculature, adherent to LSECs and directly exposed to the contents of blood. KCs express several types of scavenger, Toll-like, complement and antibody receptors, which enable KC to phagocyte pathogens and associated molecules. In HCC, KCs seem to have an antitumor role. KC depletion during partial hepatectomy enhances HCC recurrence in mice, where KC-secreted TNF-α plays a key role in tumor rejection (Hastir et al., 2020). Nevertheless, although KCs can activate T cells, continual exposure to PAMPs from the gut, appears to dampen the ability of KCs to activate the adaptive immune response (Huang et al., 2013).

In association with HCC evolution, TAMs originated from blood monocytes become recruited into the tumor microenvironment. In early-stage

HCC, type 1 macrophages (M1-TAM) are recruited to the liver where through pro-inflammatory CXCL19 and CXCL10 and can attract and promote the differentiation of CD4 Th1 and Th17 T cells, as well as NK cells (Biswas & Mantovani, 2010). In this stage, IRF5 induces the expression of IL12, and represses IL10 in TAM (Krausgruber et al., 2011), which should promote immune surveillance. In advanced HCC, type 2 macrophages (M2-TAM) secrete an array of immunosuppressive cytokines such as IL-10, IL-23, IL13, TGF-β, IL-8, CCL17, CCL22 or CCL24, which promote CD4 Th2 differentiation and recruitment (Movahedi et al., 2010). Th2 T Cells, in turn, will promote an M2 phenotype in TAMs. TAMs both increase the expression of HLA-DR and immune checkpoint molecules with inhibitory capacity, such as Programmed Death 1 ligand 1 (PD-L1), which could lead to CTL extinction (Kuang et al., 2009). M2-TAM derived-IL10 promotes the expansion of IL-17-expressing CD4 ($T_H17$) and CD8+ (Tc17 cells) T cells (Kuang et al., 2010). Th17 and Tc17-secreted IL-17 further induces PD-L1 expression in peritumoral TAMs, which in turn, suppresses CTL function (Zhao et al., 2011). M2-TAMs also support tissue remodeling and angiogenesis through the secretion of VEGFA, VEGFC and EGF (Biswas & Mantovani, 2010; Murdoch, Muthana, Coffelt, & Lewis, 2008). Angiopoietin receptor TIE2-expressing TAMs are increased in the blood and tumor tissue of HCC patients treated with surgical resection or radiofrequency ablation and are associated with higher micro-vessel density (Matsubara et al., 2013). High TAM density is directly correlated with tumor size and advanced stage. Peritumoral macrophage colony-stimulating factors (M-CSF) and macrophage density predicted the patients' death and disease recurrence in resectable HCC (Zhu et al., 2008). In contrast, other studies have found that TAM-associated PD-L1 expression predicts better survival, in contrast to intratumoral PD-L1 that was associated with poor survival (Liu et al., 2018). This latter finding stresses the need of spatial highly multiplexed histological analyses to allow for a better understanding of the immune tumor microenvironment.

MDSCs represent a heterogeneous population of immature myeloid cells not yet committed into macrophages, dendritic cells, or granulocytes (Bronte et al., 2016). Marked by CD11b surface expression, they may be either CD14+, Monocyte-type (M-MDSC) or CD15+ Polymorphonuclear-type (PMN-MDSC). In mouse models, HCC-derived GM-CSF and CXCL1 seem responsible for MDSC recruitment (Kapanadze et al., 2013). The tumor microenvironment inhibits the natural maturation of MDSCs. Instead, MDSC

expand and activate as an immature myeloid cell population. MDSCs induce the depletion of essential amino acids L-Arginine (through the enzyme arginase 1 or ARG1) and tryptophan (through the increased expression of indoleamine oxidase1 or IDO1), thus restricting CTL and NK activation and proliferation (Hornyak et al., 2018; Rodriguez, Quiceno, & Ochoa, 2007; Wang et al., 2012). MDSCs also exhibit an increased expression of Nitric Oxide Synthase-2 (NOS2) and secretion of Nitric Oxide (NO), MMP9 and VEGF, which inhibit inflammatory responses and promote blood vessel formation (Shojaei, Zhong, Wu, Yu, & Ferrara, 2008). Finally, MDSCs secrete TGF-β, IL-10, and other immunosuppressive cytokines to promote Treg recruitment and expansion (Condamine & Gabrilovich, 2011; Gabrilovich & Nagaraj, 2009) and to induce tolerogenic Dendritic cell expansion (Li, Harden, Anderson, & Egilmez, 2016).

Dendritic cells (DC) are responsible for capturing cancer antigens and presenting them to naïve T cells in the lymph nodes. Potent proinflammatory CD11c + CD141 + myeloid DCs, present in healthy livers, can stimulate strong T cell responses via IFN-γ and IL-17. Chronically inflamed livers are depleted of these DCs (Kelly et al., 2014). Conversely, a specific subtype of DC expressing CD14 (CD14 + DCs), which is increased in blood of HCC patients, was found to intratumorally express high levels CTLA4 and PD1 and was able to suppress T cell responses through IL10 and IDO (Han et al., 2014).

In early resected HCC, the abundance of intratumoral CD66b + neutrophils was associated with poor prognosis and the ratio neutrophil-to-CD8 + T cell was a good predictor of outcome, a finding confirmed in other malignancies (Ilie et al., 2012; Li et al., 2011). Upon damage, neutrophils are usually capable of platelet recruitment as a rapid initiating mechanism of hemostasis. The aggregation of platelets on adherent neutrophils induces the release of neutrophil extracellular traps (NETs), a potent web-like structure consisting of DNA, histones, and antimicrobial molecules. IL8 produced by cancer cells, MDSCs and fibroblasts, seems to be determinant for neutrophil recruitment to the tumor, the extrusion of NETs and the resistance to immunotherapy (Teijeira et al., 2021). NETs were related to inflammation and HCC development in mouse models (van der Windt et al., 2018).

Cancer Associated Fibroblasts (CAFs), probably derived from HSCs, are the major source of collagen in the HCC stroma. They differ from normal fibroblasts in their ability to secrete high levels of stromal cell-derived factor 1 (SDF-1) and CXCL12 and promote tumor growth and angiogenesis

(Orimo & Weinberg, 2006). Several mechanisms have been linked to the important pro-tumoral role of CAFs in liver cancer (Affo, Yu, & Schwabe, 2017). CAFs can reduce the immune surveillance increasing Treg survival, inhibiting CTL infiltration (Zhao et al., 2011), recruiting MDSCs (Zhao et al., 2014) or promoting the shift of recruited monocytes to a M2-TAM immunosuppressive phenotype (Ji et al., 2015). CAFs are also important producers of TGFb, a potent inhibitor of antitumor immunity that strongly promotes the tolerogenic fate of TAMs, DCs, neutrophils, NK, NKT, Tregs and CD4+ and CD8+ effector T cells (Flavell, Sanjabi, Wrzesinski, & Licona-Limon, 2010).

Liver sinusoidal endothelial cells (LSECs), which conform the highly specialized fenestrated vascular web of the liver, are not mere structural components for inflow transport from arterial and portal circulation, but actively participate in pathogen detection, capture and antigen presentation (Limmer et al., 2000). LSECs comprise ~50% of the nonparenchymal cells in the liver, making these cells more than twice as abundant as either liver-resident macrophages or lymphocytes in the liver (Racanelli & Rehermann, 2006). LSECs express a wide variety of pattern recognition receptors (PRRs), such as Toll-like receptors TLR3, TLR4, TLR7 and TLR9 (Knolle & Limmer, 2003; Wu et al., 2010) and constitutively express major histocompatibility complex (MHC) I and MHC II, costimulatory molecules (CD80 and CD86), and lymphocyte adhesion molecules (Knolle & Limmer, 2003). Recognition of antigens by T cells results in an upregulation of PDL1 in the LSEC (Diehl et al., 2008) and the expansion of antigen-specific CD8+ T cells which do not acquire an effector phenotype, thus being a key element for the induction of central tolerance (Berg et al., 2006; Jenne & Kubes, 2013; Limmer et al., 2000). Constant low-level activation of LSECs by some MAMPs (for example, lipopolysaccharide) and KC-derived IL-10 downregulate major histocompatibility complex and costimulatory molecules and thereby facilitate T cell tolerance (Knolle et al., 1999). In HCC, LSEC can foster an immunosuppressive environment through the secretion of IL10 and the recruitment of Treg, and the secretion of CXCL1, and CXCL2 promoting the recruitment of MDSCs (Wilkinson, Qurashi, & Shetty, 2020). Conversely, LSEC can mediate antitumor surveillance. In mice with modulated commensal bacteria, microbiome-mediated primary-to-secondary bile acid conversion elicited an increased CXCL16 expression in LSEC, leading to a higher cytotoxic NKT activation and tumor growth suppression (Ma et al., 2018).

## 2.2 The role of tumor cells

Tumor cell-specific genomic alterations can differently shape the tumor microenvironment. Tumor-associated Antigens (TAA) are cancer-specific epitopes originated from newly derepressed oncofetal or cancer-testis antigens, viral peptides (in case of chronic HBV or HCV infection) or neoantigens caused by coding non-synonymous mutations that are expressed, presented through MHC-I and immunogenic. Naturally occurring TAA-specific CD8+ T-cell responses are present as part of the normal T-cell repertoire in patients with HCC and these responses correlate with patient survival (Flecken et al., 2014). The most common derepressed antigens in HCC are the cancer/testis antigen melanoma-associated antigen 3 (MAGEA3), the oncofetal antigen α-fetoprotein (AFP) and glypican 3 (GPC3). Neoantigens can derive from both passenger or driver mutations, and depending on the dominance of the subclone, they could lead to different degrees of exposure, immune tolerance, and T cell exhaustion (Hou, Zhang, Sun, & Karin, 2020). Therefore, it is not clear that the most common driver mutations in HCC (those affecting TERT promoter, TP53, ARID1A, ARID2, CTNNB1, AXIN1 and PIK3CA genes), are good candidates for inducing robust antitumor responses. The tumor mutational burden (TMB), expressed as mutations per megabase (mut/Mb), reflects the amount of non-synonymous mutations in a particular tumor, and is supposed to correlate with the neoantigen load (Rizvi et al., 2015). In highly mutagenic tumors such as melanoma or non-small cell lung cancer, or in microsatellite instability high (MSI-H) colon cancer, high TMB has been linked to response to immunotherapy, suggesting that it is possible to activate exhausted antigen-specific T cell responses in these patients, as well as fostering the development of personalized vaccines or cell therapy directed towards specific tumor neoantigens. Nevertheless, neoantigen editing during tumor evolution, copy-number loss of previously clonal neoantigens, loss of heterozygosity (LOH) in human leukocyte antigens (HLA) or the promoter hypermethylation of neoantigen-bearing coding genes, are just some of the mechanisms of immune evasion enacted by cancer cells under antigen-specific immune pressure (Rosenthal et al., 2019). In patients with HCC, with a reported median TMB of less than 3 mut/Mb, higher TMBs have not been associated with higher levels of immune infiltration (Sia et al., 2017) or response to immunotherapy (Spahn et al., 2020). Importantly, the occurrence of Copy Number Alterations (CNA) and large chromosomal aberrations are associated with LOH in antigen-presentation genes and

with immune exclusion in cancer. In line with this, HCC with high levels of chromosomal aberrations displayed immune desertification (Bassaganyas et al., 2020).

Tumor cell-specific signaling cascades can also affect the immune microenvironment of HCC. Around one third of human HCC bear gain-of-function mutations on β-catenin (encoded by CTNNB1 gene) as putative driver gene (Sanchez-Vega et al., 2018). These tumors are usually well differentiated, present a microtrabecular pattern, lack inflammatory infiltrates and are histologically defined by intense nuclear β-catenin staining and strong glutamine synthetase expression (Calderaro et al., 2017). In metastatic melanoma, constitutive activation of β-catenin pathway, by either defined mutations or increased expression in key WNT/CTNNB1 pathway elements results in less infiltration by lymphocytes (Spranger, Bao, & Gajewski, 2015). One of the postulated mechanisms would be the ATF3-mediated repression of the DC-attractant chemokine CCL4 by cancer cells, hampering the recruitment of conventional type 1 DC, known to be a critical factor for T cell and NK infiltration (Spranger et al., 2015). In mice, HCC bearing MYC overexpression and CTNNB1 constitutive activation, endogenously generated in the liver by tail vein hydrodynamic injection, exhibited downregulation of CCL5, recruited less DC1, and were insensitive to immune checkpoint blockade (Ruiz de Galarreta et al., 2019). In human HCC, a downregulation of the NKG2D ligands MIC-A and MIC-B was seen in CTNNB1 activated tumors, which could explain NK recruitment. Interestingly though, this phenotype was related to less aggressiveness, indicating that immune evasion does not necessarily mean worse survival (Cadoux et al., 2021). Glutamine synthesis by cancer cells has been associated with immune depletion and the blockade by glutamine antagonist JHU083 led to enhanced efficacy of immunotherapy and durable responses in syngeneic colon cancer mouse models (Leone et al., 2019). Since glutamine synthesis in human CTNNB1-activating HCC is constitutively active, one could infer that glutamine blockade could be useful to increase immune infiltration in these tumors. However, not all human HCC bearing CTNNB1 mutations are immune excluded. In a recent refinement of transcriptomic classification of HCC, which leverages on bulk RNA sequencing deconvolution to infer immune infiltration, one third of CTNNB1-signature bearing tumors belonged to the so-called inflammatory class, with high expression of IFN-γ signaling, infiltration of CD8+ T cells and M1-TAM, and increased immune cell-attractant chemokines, including

CCL5 (Llovet et al., 2021). Genomic level information from phase III clinical trials with immunotherapy is now needed to firmly determine if and how CTNNB1-activating human HCCs respond differently to immune checkpoint inhibition.

Overexpression of MYC, occurring in a majority of human HCC, led to increased PD-L1 translation transforming KRAS-activated liver cancer from hot to cold tumors in a mouse model. This translational enhancement of PD-L1 was reversed by Eukaryotic Translation Initiation Factor 4E (eIF4E) inhibition (Xu et al., 2019). Around one-third of HCC bear *TP53-null* mutations. These tumors produce high AFP levels and are poorly differentiated and enriched in the macrotrabecullar massive histological subtype, presenting a poor prognosis in the pre-ICB era (Calderaro et al., 2017). Interestingly, in non-small cell lung cancer, TP53 mutations were independently associated with overall survival and longer responses when treated with ICI (Assoun et al., 2019). ARID1A is a core member of the polymorphic BRG/BRM-associated factor chromatin remodeling complex. Mutations in ARID1A gene include around 8–10% of human HCC (Wheeler & Network, 2017). Although ARID1A mutations lead to genomic instability and the generation of unrepaired DNA mismatches and higher TMB-driven immunogenicity, ARID1A aberrations can also limit chromatin accessibility to IFN-responsive genes, impairing IFN gene expression, and response to immunotherapy (Li et al., 2020). TP53-null and ARID1A HCCs are two examples demonstrating that immune exclusion in animal models or histological studies, does not equal resistance to immunotherapy.

Tumor-specific secretome defined by these genomic and functional features can also shape the immune microenvironment. Oncofetal AFP highly expressed by TP53-null HCC can modify the immune infiltrate towards a more tolerogenic one (Pardee, Shi, & Butterfield, 2014; Ritter et al., 2004). HCC bearing VEGF amplification (Horwitz et al., 2014), or IDO overexpression (Li et al., 2018) show increased secretion of VEGFA or Kinurenines, respectively, thus strongly shaping the immune environment. In early stage, resected HCCs, the expression of IDO1 in tumor cells has been associated with higher CD8 + T cell infiltration and patient survival (Li et al., 2018), but in HCC cell lines and animal models, IDO was found to be increased in immune checkpoint-resistant tumors and its inhibition led to higher anti-CTLA4-induced responses (Brown et al., 2018). Equally important, genomic rewiring of HCC cells by driver genes make them

increasingly avid for myeloid derived cytokines or growth factors, as exemplified by the TGF-β receptor pathway activation (Chen, Gingold, & Su, 2019), or cMET activation (Wang et al., 2020) cause them to be exquisitely dependent on TGF-β1 and HGF, respectively.

Tumor-specific immune ligandome, defined by the density of ligands related to immune recognition and killing include: the expression and membrane localization of MHC-I, upregulated in HCC cell lines (Huang, Cai, & Wei, 2002), the immune costimulatory molecules CD80 (B7-1), CD86 (B7-2) shown to be downregulated in HCC (Tatsumi et al., 1997), the increased expression of the costimulatory ligand B7-H3 (Sun et al., 2012; Wang et al., 2014), and the increased expression of inhibitory ligands PD-L1 (Huang et al., 2021), or Galectin 9 (Zhou et al., 2018). Whether specific driver gene mutations are associated with a change in expression of these ligands in human HCC is still to be explored.

The complexity of the immune tumor microenvironment speaks against simplistic analysis and conclusions regarding the potential of immunotherapy in liver cancer. Fig. 1 summarizes the most important actors in this play. In the future, a more detailed genomic, transcriptomic, and proteomic characterization of HCC patients treated with immunotherapy in phase III clinical trials will lead to a better understanding of how these driver mutations define the response or resistance, knowing that the genomic profile of a tumor is an evolving landscape affected by immune pressure, Darwinian subclone selection, and the appearance of new immune evasion mechanisms.

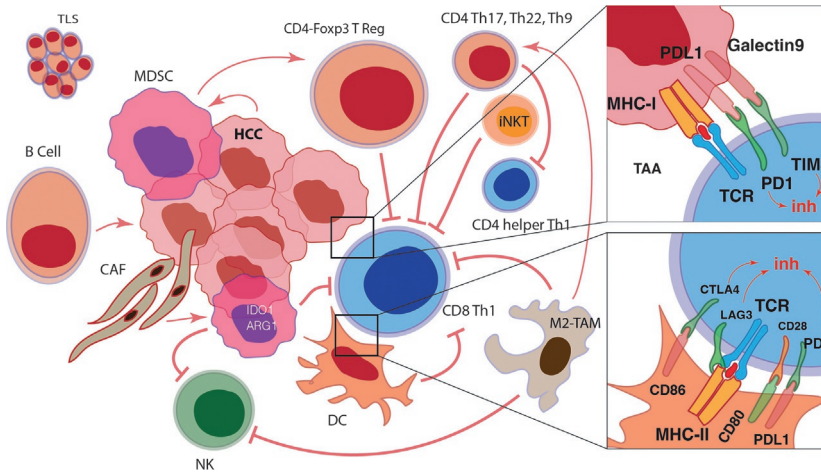


**Fig. 1** Mechanisms of immune evasion.

## 2.3 Etiology of liver cancer and mechanisms of immune evasion

Recent studies have suggested that there may be differences in response to immune checkpoint blockade in patients with diverse cancer etiology. According to some of these studies, patients and animal models of non-alcoholic steatohepatitis (NASH), would not benefit as much from immunotherapies. A hepatocyte-specific Endoplasmic Stress-prone mouse model of NASH-related HCC was used in the preclinical evidence of this phenomenon. Using high resolution single cell mass spectrometry and RNA sequencing, the authors found that livers of NASH mice and humans were enriched in exhausted CD8+ PD1+ T cells and were not responsive to immune checkpoint inhibition, which instead, caused more damage and fibrosis in these already damaged livers. In this model, the depletion of CD8 T cells led to less tumor burden (Pfister et al., 2021). Other mechanisms that could shape NASH livers differently include the activation of dysfunctional immune cells, NKT cells (Wolf et al., 2014), T helper 17 ($T_H17$) cells (Gomes et al., 2016), and $IgA^+$ plasma cells (Shalapour et al., 2017) that could impact tumor immune surveillance and favor hepatocarcinogenesis. In the genetically modified mouse model of NASH, the depletion of CD4+ T, induced by fatty acid-dependent oxidative damage, contributes to hepatocarcinogenesis (Ma et al., 2016). In Alcohol-related Liver Disease (ALD), an increased gut permeability leads to translocation of bacterial pathogen-associated molecular patterns (PAMPs) which suppresses KC activation, increases the abundance of M2-TAMs, promotes the recruitment of MDSC infiltration and suppresses CD8+ T cell activation, a mechanism that could dampen HCC immunogenicity.

A meta-analysis using data from the main phase III clinical trials with the anti-PD1 antibodies nivolumab and pembrolizumab and the anti-PDL1 atezolizumab suggested no benefit over sorafenib in patients with non-viral etiology (Pfister et al., 2021). However, trials included in this study employed different therapies and different patient populations, and the meta-analysis showed a high heterogeneity. In fact, recently communicated data from a phase III clinical trial exploring first line anti-PDL1 durvalumab combined with anti-CTLA4 tremelimumab do not support this hypothesis and showed similar benefit in HBV-infected and uninfected patients (Abou-Alfa et al., 2022).

Hepatitis B Virus (HBV) is a DNA virus with hepatic tropism, transmitted through blood contact (transfusions, IV drugs, sexual intercourse, maternal delivery), that causes acute infection and complete surface and core

antigen-directed T-cell-mediated clearance in a minority of infected individuals (Knolle & Thimme, 2014). In most patients, CD8+ T cell exhaustion and recruitment of tolerogenic cell types such as granulocytic MDSC, $T_{reg}$ or regulatory B cells ($B_{reg}$) in the infected livers, mediate disease chronicity (Das et al., 2008; Pallett et al., 2015). The cycle of damage and regeneration, the integration of HBV DNA, and the expression of viral oncoproteins such as HBx, are related to oncogenesis that can arise in non-cirrhotic livers. HBV infection cannot be cured. Antivirals such as lamivudine or entecavir are therapeutic options for patients with high replication rates and HBV-mediated damage or in the preventive setting in immunosuppressed patients. Highly suppressive PD-$1^{hi}$ $T_{reg}$ cells are selectively enriched in HBV-related versus non-viral HCCs and are associated with a poor prognosis, while CD8+ $T_{RM}$ cells are related to good outcomes in patients with HBV-related HCC (Lim et al., 2019). Thus, the liver immune milieu of an HCC-bearing chronically HBV-infected patient trends towards a tolerogenic state and this could hamper the success of immunotherapy.

Hepatitis C Virus (HCV) is another blood-borne transmitted single-stranded RNA which acute infection is cleared in around 20% of infected individuals. This clearance is related to vigorous induction of CD4 and CD8 antigen specific responses and the subsequent narrowing of quasi-species diversity (Farci et al., 2000). In most patients, viral high replication and hypermutation overwhelms the adaptive response and the disease becomes chronic, causing a low-degree inflammatory environment that in the long term promotes neoplastic transformation of injured hepatocytes (Hedegaard et al., 2017). Viral factors inhibit the key sensors of virus infection RIG-I and MDA5 (Cao et al., 2015). High efficacy of direct acting antivirals (DAA) has dramatically impacted the field of hepatology, changing the prevalence of viral etiology in cirrhotic patients and in patients with viral-related HCC. In patients cured from HCV infection, nevertheless, circulating TCF1+CD127+PD1+ HCV-specific exhausted CD8+ T-cell subsets have been found, indicating that once the antigen stimulation has ceased, the immune system is still shaped by the chronic insult (Wieland et al., 2017). In cured patients, the inflammation resolves shortly after the end of treatment (Huang et al., 2021) but it is not clear to what extent the degree of fibrosis regression is, especially in obese (McPhail et al., 2021). Whether the immunotherapy of HCC in HCV-infected versus HCV-cured individuals could have different outcomes is yet unknown, but a probable scenario is the progressive reduction of HCC cases due to this etiology.

The growing debate on the clinical relevance of the etiology in HCC patients has highlighted the need and increased the interest in reviewing the diverse immune context of HCV and HBV infections, NASH and ALD and the study of whether this could account for differences in tumor biology, immune infiltration, and most importantly impact in their response to immunotherapies. Certainly, a difficulty to perform this type of analysis arises from the lack of a good definition of non-viral etiologies of liver cancer in clinical trials and in many observational studies where the immune environment of HCC has been characterized. The animal models of viral-induced HCC are inexistent or poorly relevant to human disease, those of toxic-induced HCC are abnormally mutagenic and those diet-induced need additional toxic or genetical insults to be technically feasible. Another even more important obstacle is the overlap of NASH and ALD and with HCV or HBV infection in real life of humans which should prompt a better reporting of metabolic profile and alcohol consumption in every patient with HCC. But even at the biological level it may be impossible to separate these mechanisms. For example, HCV infection induces liver steatosis and contributes to the development of metabolic dysfunction, and it is precisely the combination of HCV and obesity that increases the risk of developing HCC (Leslie, Geh, Elsharkawy, Mann, & Vacca, 2022). It has been reported that DAA treatment can result in weight gain in a proportion of HCC patients and these metabolic changes could potentially lead to malignant transformation (Leslie et al., 2022).

## 3. Immunotherapy of HCC

In the previous section, the main features of immune biology of HCC have been delineated. For more than a decade, the multi-tyrosine kinase inhibitor (TKI) sorafenib has been the standard of care for patients with advanced HCC (Llovet et al., 2008). In recent years an array of new TKI such as lenvatinib in first line, and regorafenib, cabozantinib and ramucirumab in second line after sorafenib have been approved. The success of a new way of understanding and treating cancer, through the unleashing of immune evasion, exemplified by the unprecedented responses in advanced melanoma patients, promoted the study of these drugs in other cancers. The immunotherapy aims at reinvigorating exhausted T cell responses, by the activation of costimulatory or the blocking of coinhibitory membrane receptors or their ligands, globally called immune checkpoints. In Fig. 2, the array of immune checkpoints interfacing the tumoral cell,

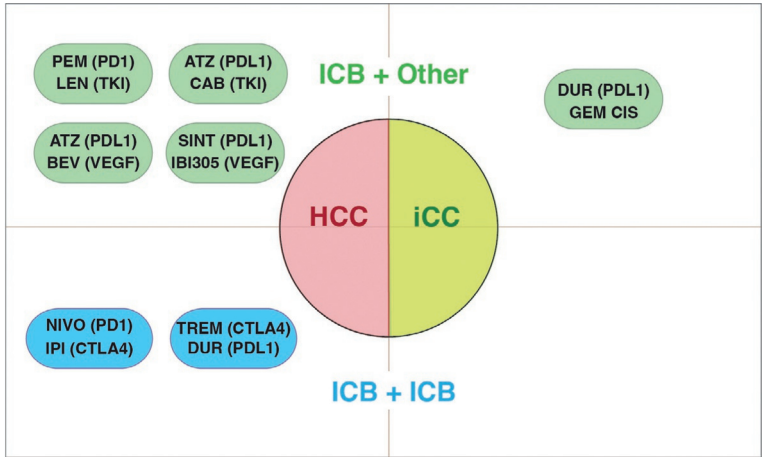


**Fig. 2** Immunotherapies with reported or ongoing phase III clinical trials as first line systemic therapy in liver cancer.

the antigen-presenting cell, and the lymphocyte is depicted. Antibodies against the immune checkpoints CTLA4, PD-1 and PD-L1 have reached the clinical stage of development and are responsible for the first successes in liver cancer. Others, such as LAG3, TIM3 or VISTA are currently being tested in phase II trials.

## 3.1 Combination therapies

In 2022, combination immunotherapy should be considered the preferred option for an HCC patient in need of systemic therapy, including those with vascular invasion or extrahepatic spread, with too much liver burden to be adequately treated by intra-arterial therapies or with liver disease progressing to intra-arterial therapies (Reig et al., 2021). Table 1 summarizes the results of the main phase II and III trials using immune checkpoint inhibitors (ICI) that have been conducted in HCC patients.

The combination of the PD-L1 inhibitor atezolizumab and a relatively high dose (15 mg/kg) of the VEGF-inhibitor bevacizumab resulted in prolonged OS and PFS compared to sorafenib in the IMBRAVE-150 trial with a hazard ratio (HR) of 0.58 (95%CI 0.42–0.79) for overall survival (OS) and 0.59 (95%CI 0.47–0.76) for progression-free survival (PFS) (Finn, Qin, et al., 2020). With more prolonged follow-up, median OS was 19.2 months in patients treated atezolizumab plus bevacizumab (vs. 13.4 with sorafenib) and median PFS was 6.9 months (vs. 4.3 with sorafenib), while ORR was

**Table 1** Phase III trials of ICIs in advanced HCC.

| Agent (dose) | N | MVI (%) | EHD (%) | AFP > 400 ng/mL (%) | ORR (CR) (%) | mPFS (months) | mOS (95% CI) (months) | OS HR (95% CI) | Reference |
|---|---|---|---|---|---|---|---|---|---|
| *KEYNOTE-240 (second line after sorafenib)* | | | | | | | | | |
| Pembrolizumab (200 mg q3w) | 278 | 13 | 70 | 46 | 18 (2) | 3 | 13.9 (11.6–16) | 0.71 (0.61–0.96) | Finn et al. (2020), Merle et al. (2021) |
| Placebo | 135 | 12 | 69 | 43 | 4 (0) | 2.8 | 10.6 (8.3–13.5) | | |
| *CheckMate 459 (first line)* | | | | | | | | | |
| Nivolumab (240 mg q2w) | 371 | 75[#] | 75[#] | 33 | 15 (4) | 3.7 | 16.4 (13.9–18.4) | 0.85 (0.72–1.02) | Yau et al. (2022) |
| Sorafenib (800 mg qd) | 372 | 70[#] | 70[#] | 38 | 7 (1) | 3.8 | 14.7 (11.9–17.2) | | |
| *IMbrave150 (first line)* | | | | | | | | | |
| Atezolizumab (1200 mg q3w + Bevacizumab (15 mg/kg q3w) | 336 | 38 | 63 | 38 | 30 (8) | 6.8 | 19.2 | 0.66 (0.52–0.85) | Finn et al. (2021), Finn et al. (2020) |
| Sorafenib (800 mg qd) | 165 | 43 | 56 | 37 | 12 (0) | 4.3 | 13.4 (10.4-NE) | | |
| *HIMALAYA (first line)* | | | | | | | | | |
| STRIDE Tremelimumab (300 mg 1 dose) + Durvalumab (1500 mg q4w) | 393 | 26 | 53 | 37 | 20 (3) | 3.8 | 16.4 (14.2–19.6) | 0.78 (0.65–0.92) | Abou-Alfa et al. (2022) |
| Durvalumab (1500 mg Q4W) | 389 | 24 | 54 | 35 | 17 (2) | 3.7 | 16.6 (14.1–19.1) | 0.86 (0.73–1.03) | |
| Sorafenib (800 mg qd) | 389 | 27 | 52 | 32 | 5 (0) | 4.1 | 13.8 (12.3–16.1) | | |

*Continued*

**Table 1** Phase III trials of ICIs in advanced HCC.—cont'd

| Agent (dose) | N | MVI (%) | EHD (%) | AFP > 400 ng/mL (%) | ORR (CR) (%) | mPFS (months) | mOS (95% CI) (months) | OS HR (95% CI) | Reference |
|---|---|---|---|---|---|---|---|---|---|
| *ORIENT-32 (first line)* | | | | | | | | | |
| Sintilimab (200 mg q3w) + Bevacizumab biosimilar (15 mg/kg q3w) | 380 | 28 | 73 | 43 | 24 (1) | 4.6 | NR | 0.57 (0.43–0.75) | Ren et al. (2021) |
| Sorafenib (800 mg qd) | 191 | 26 | 75 | 42 | 8 (0) | 2.8 | 10.8 (8.5-NR) | | |
| *COSMIC-312 (first line)* | | | | | | | | | |
| Cabozantinib (40 mg qd) + Atezolizumab (1200 mg q3w) | 432 | 31 | 54 | 38 | 11 (0) | 6.8 | 15.4 (13.7–17.7) | 0.90 (0.69–1.18) | Kelley et al. (2022) |
| Sorafenib (800 mg qd) | 217 | 28 | 56 | 30 | 4 (0) | 4.2 | 15.5 (12.1-NE) | | |
| Cabozantinib (60 mg qd) | 188 | 36 | 54 | 35 | 6 (0) | 4.3 | N/A | | |

N, number of patients; MVI, macrovascular invasion; EHD, extrahepatic disease; AFP, alpha-fetoprotein; mPFS, median progression free survival; mOS, median overall survival; HR, hazard ratio; NR, not reached; #, MVI and EHD were merged.

30% by RECIST 1.1, including 8% complete responses (Cheng et al., 2021). This combination also showed a favorable safety profile and delayed deterioration of health-related quality of life (HRQoL) (11.2 vs. 3.6 months; HR 0.63, 95% CI 0.46–0.85) (Toh et al., 2022). A similar combo of the anti-PD1 sintilimab with a bevacizumab biosimilar (IBI305) was also superior to sorafenib in the ORIENT-32 phase III trial with very comparable HRs of 0.56 (95% CI 0.46–0.70) for OS and 0.56 (95% CI 0.46–0.70) for PFS (Ren et al., 2021a). None of these two trials compared single agent atezolizumab or bevacizumab versus sorafenib. However, in a phase 1b trial which randomized patients to atezolizumab plus bevacizumab or atezolizumab alone, PFS was better with the combination than with single agent atezolizumab (5.6 vs. 3.4 months) despite a similar response rate (20% vs. 17%) (Lee et al., 2020). In an exploratory biomarker analysis of this study using archival or fresh biopsies, TMB was not associated with response to atezolizumab plus bevacizumab or with more prolonged PFS but pre-existing immunity was, as illustrated by a high PD-L1 expression and a simple T effector signature including granzyme B, PRF1, CXCL9 genes (Zhu et al., 2020). High VEGFR2 expression, Treg and myeloid inflammation were also associated with longer PFS in patients treated with the combination compared atezolizumab monotherapy (Table 2).

Very recently, the combination of the PD-L1 inhibitor durvalumab with a single high (300 mg) priming dose of the CTLA4 inhibitor tremelimumab (STRIDE regime) resulted in prolonged OS compared to sorafenib in the HIMALAYA trial with a HR of 0.78 (95%CI 0.65–0.92) (Abou-Alfa et al., 2022). Median OS was 16.4 months in patients treated with durvalumab plus tremelimumab vs.13.8 with sorafenib, while ORR

**Table 2** Phase III trials of ICIs in advanced BTC.

| Agent (dose) | N | ORR (CR) (%) | mPFS (months) | mOS (95% CI) (months) | OS HR (95% CI) | Reference |
|---|---|---|---|---|---|---|
| *TOPAZ-1 (first line)* | | | | | | |
| GemCis (gemcitabine 1000 mg/m2 + Cisplatin 25 mg/m2 D1, D8 q3w | 344 | 18.7 (0.6) | 5.7 | 11.5 (10.1–11.5 | 0.80 (0.66–0.97) | Oh et al. (2020) |
| GemCis + Durvalumab (1500 mg q3w) | 341 | 26.7 (2.1) | 4.2 | 12.8 (11.1–14.0) | | |

N, number of patients; mPFS, median progression free survival; mOS, median overall survival; HR, hazard ratio.

was 20.1% by RECIST 1.1 (vs. 5.1%), including 3% complete responses. As it happened in several trials testing single agent ICIs, there was no improvement in PFS. This combination also showed a favorable safety profile with 26% of patients experiencing treatment-related adverse events (TRAE) grade 3 or 4 (vs. 37% with sorafenib) and 8% having TRAE leading to treatment discontinuation (vs. 11% with sorafenib).

There is no question that HCC is sensitive to the CTLA4 blockade. Indeed, the first evidence of the potential benefit of ICIs in HCC was seen in a small trial in patients with concurrent HCC and HCV infection treated with tremelimumab (Sangro et al., 2013) where an ORR of 17% was observed. Interestingly, a reduction in HCV viral load was also detected in some patients indicating the activation of exhausted CD8 HCV-specific responses. In another small trial testing tremelimumab plus subtotal tumor ablation with TACE or RFA, an even higher ORR of 26% was observed. In this study, tremelimumab alone increased the number of activated CD4+ and CD8+ cells in the peripheral compartment and tumor infiltration by CD3+ and CD8+ cells were increased in all patients with paired biopsies before and after tremelimumab and was higher in responders than in non-responders.

When used in combination with PD-(L)1 inhibitors, the dose of CTLA4 is important, as it happens in other tumors. A cohort of the CheckMate-040 trial randomized sorafenib-experienced patients to 3 arms with different doses of the CTLA4 inhibitor ipilimumab and the PD-1 inhibitor nivolumab (Yau et al., 2020). In two of them, lower doses of ipilimumab and higher doses of nivolumab (1 and 3 mg/kg, respectively) were used. Outcomes were better using a higher dose of ipilimumab (3 mg/kg) and a lower dose of nivolumab (1 mg/kg), with an ORR of 32%, median OS of 22.9 months and a remarkable 42% survival rate at 3 years (El-Khoueiry et al., 2021). The HIMALAYA trial has shown that a single high dose of tremelimumab at treatment initiation is able to improve the efficacy of durvalumab to a point where the combination outperforms sorafenib as first-line systemic therapy. Indeed, in a phase 2 study such a priming dose of tremelimumab resulted in the most intense early peripheral burst of proliferating CD8+ cells, a finding associated with response (Kelley et al., 2021). As expected, higher doses of ipilimumab increased the rate of TRAEs and we will have to wait until the results of the CheckMate-9DW trial are presented to learn if this combination prolongs OS compared to sorafenib and shows an acceptable safety profile in the first-line setting.

The COSMIC-312 phase III trial has compared the combination of atezolizumab and cabozantinib vs. sorafenib. In the final analysis (Kelley et al., 2022), the combination resulted in prolonged PFS (median 6.8 vs. 4.2 months, HR 0.63, 95 CI 0.44–0.91, $P = 0.0012$) but the same benefit was not shown for OS in the interim analysis (median 15.4 vs. 15.5 months, HR 0.90, 95 CI 0.69–1.18, $P = 0.438$). Inhibition of VEGF signaling targeting the ligand or the receptors may enhance T cell stimulation by PD-(L)1 inhibitors through different mechanisms, including normalization of the tumor vasculature and promotion of a more favorable TME by reducing suppressor cells like MDSC and M2-polarized TAMs, and increasing CD8+ T cells (Rahma & Hodi, 2019; Shigeta et al., 2020). In COSMIC-312, the combination of cabozantinib and atezolizumab resulted in a low ORR (11%) compared to the other combinations (15–30%) but we should wait for the final OS analysis and full report of this trial before raising any hypothesis about this somewhat unexpected result.

In animal models, cabozantinib had little effect on tumor infiltration by macrophages and T cells and did not modify PD-L1 expression by tumor cells while the combination with an anti-PD-L1 antibody did not improve the therapeutic efficacy of cabozantinib monotherapy (Shang et al., 2021). The immune effects of cabozantinib have also been studied in a small trial that investigated preoperative cabozantinib and nivolumab in resectable HCC patients (Ho et al., 2021). Cabozantinib alone induced an increase of effector and memory CD4+ and CD8+ T cells in the peripheral compartment. Within the TME (with transcriptional profile analyzed in only four paired biopsy samples), cabozantinib reduced the expression of some immunosuppressive genes like IDO1, TGF-β2, CXCL1 or CX3CR1, but also increased the expression of others like IL8/CXCL8. When nivolumab was added to cabozantinib and patients with major or complete pathological responses were compared to non-responders, the former had higher infiltration by CD4+ and CD8+ T cells and CD20+ B cells, often forming TLS, and an enrichment of IFN-γ producing effector memory CD4+ and CD8+ T cells as well as granzyme B+ effector CD8+ T cells. Besides, responders had more abundant myeloid cells including immunosuppressive M2-macrophage clusters.

Nevertheless, TKIs inhibit a wide spectra of tyrosine kinases and their effect in the TME should not be considered comparable to one another. In a phase 2 trial, the combination of pembrolizumab and lenvatinib doubled (36%) the ORR observed with single agent pembrolizumab and lead to a

promising 20-month median OS, at a price of more intense toxicity (TRAE grade 3 or higher in 67% of patients (Finn et al., 2020). The impact of this combination on overall survival as first-line therapy compared to sorafenib is now under investigation in the LEAP-02 trial (Llovet et al., 2019).

## 3.2 Single agents

Altogether, current scientific evidence shows that, if locally available, patients with advanced HCC should receive atezolizumab plus bevacizumab or durvalumab plus tremelimumab. Contraindications to ICIs include active autoimmune diseases targeting relevant organs and solid organ recipients in whom the risk of provoking a severe flare or rejection, respectively, is high and worrying (Pinter, Scheiner, & Peck-Radosavljevic, 2021). The safety of ICIs has not been studied and therefore ICIs cannot be recommended in patients with HBV infection that is not effectively treated with antiviral agents or in those with HBV and HDV while the use in patients with HIV infection or HBV and HCV coinfection should be considered individually (Sangro, Bruix, Chan, Galle, & Rimassa, 2022).

The results from doublets have certainly curtailed the interest in single agent ICIs. However, there may still be a place for them in selected patients or in health environments where combinations are not available. PD-1 inhibition with nivolumab or pembrolizumab can induce deep, durable responses associated with prolonged survival in around 15% of patients naïve or sorafenib-experienced (El-Khoueiry et al., 2017; Zhu et al., 2018). After sorafenib, a benefit of pembrolizumab over placebo was not proven in the KEYNOTE-224 trial, because of not reaching the predetermined cutoff significance of $P<0.01$ (HR 0.78; 95% CI, 0.61 to 0.99; $P=0.024$), although OS was numerically better with pembrolizumab (median OS 13.9 vs. 10.6 months) (Merle et al., 2021). More recently, an Asian trial with a similar design has shown a significant prolongation of OS vs placebo (HR 0.7; 95% CI 0.63–0.99; $P=0.018$; median 14.6 vs. 13 months) (Qin et al., 2022). In naïve patients, a significant improvement in OS was not observed when nivolumab and sorafenib were compared in the CHECKMATE-459 trial (HR 0.85; 95% CI 0.72–1.02; $P=0.075$), although again OS was numerically better after nivolumab (median OS 16.4 months vs. 14.7 months) and substantially delayed deterioration of HRQoL (Yau et al., 2022). In the HIMALAYA trial, a secondary endpoint was the comparison of OS of durvalumab monotherapy vs. sorafenib with a non-inferiority margin of 1.08 (Abou-Alfa et al., 2022). Non-inferiority was

met with a HR of 0.86 (95% CI 0.73–1.03) while median OS was 16.6 vs. 13.8 months for durvalumab and sorafenib, respectively. With a higher ORR (17% vs. 5%) and a better tolerability (TRAE of any grade and grade 3 or 4 occurring in 52% vs. 85% and 13 vs. 37% of patients, respectively), durvalumab seems to be a better option for first-line systemic therapy.

One potential obstacle for efficacy in atezolizumab-containing regimes is the occurrence of anti-drug antibodies (ADA). The prevalence of ADA following treatment with ICB seems to be higher for atezolizumab (54%) than for nivolumab (10%) or pembrolizumab (1.5%) (Enrico, Paci, Chaput, Karamouza, & Besse, 2020). Whether the presence of ADA hampers the efficacy of the drug and impacts on patient's survival is currently under investigation.

Immune checkpoint ligands such as LAG3, TIM3 or VISTA, and others are being investigated as targets in HCC, mainly in multi-cancer phase II trials with no solid data at this moment. Many other new strategies investigated for other cancers have boosted the design of multicancer trials where some HCC patients can be enrolled.

## 3.3 Biomarkers of response or resistance

Only around one third of the patients treated with current ICI combinations show objective remissions and 20 to 40% have progressive disease as the best response. It is thus crucial to identify upfront those patients that will or will not respond to the ICB. PD(L)1 immunohistochemistry (IHC) has been intensely investigated. In 2015, in a trial testing the efficacy of the anti-PD1 antibody pembrolizumab in patients with non-small cell lung cancer, the PD-L1 positive staining with linearly correlated with overall response rates, which impacted on biomarker-based indication of pembrolizumab in patients with NSCLC (Kang et al., 2017). This was revalidated in several other trials with pembrolizumab. Currently, Head and Neck Squamous Cell, Breast, Cancer and Urothelial treatments with the anti-PD1 pembrolizumab, nivolumab, durvalumab and the anti-PD-L1 atezolizumab are PD-L1 IHC biomarker-based. Unlike in the above-mentioned cancer types, the predictive role of PD-L1 in HCC is not entirely clear. In KEYNOTE-224 trial testing pembrolizumab in the second line the CPS but not the TPS was associated with response (Zhu et al., 2018). In the CHECKMATE-459 trial, the expression of PD-L1 in more than 1% of the area was associated with longer median OS in nivolumab-treated patients (Yau et al., 2022). In the CHECKMATE-040 trial, PD1 and

PDL1 staining were associated with improved OS but only PD1 staining was associated with ORR (Sangro et al., 2020). Median OS was 28.1 (95% CI 18.2–n. a.) vs. 16.6 months (95% CI 14.2–20.2) for patients with tumor PD-L1 $\geq$ 1% vs. <1% ($P = 0.03$). Although this could seem promising, complete or partial tumor responses were observed in both PD-L1–positive and PD-L1–negative patients treated with nivolumab monotherapy (Sangro et al., 2020). In the adjuvant setting, the frequency of tumor CD3+ PD1+ expression in post-treatment was higher in responders. No differences were seen in pretreatment tissues. In this study CD8 and CD68 staining pre- or post-treatment were not associated with outcomes (Agdashian et al., 2019). Regarding flow cytometry analyses, baseline frequency of CD4+ PD1+ cells in Peripheral Blood Mononuclear Cells was higher in patients treated with radiofrequency ablation or chemoembolization who responded to tremelimumab adjuvancy (Agdashian et al., 2019). Despite the efforts to find a good predictive marker in HCC, PD-L1 or PD1 IHC show limited positive or negative predictive values and thus have not been implemented for patient stratification in clinical trials or in treatment decisions in clinical practice.

The Tumor Mutational Burden (TMB), a calculation that reflects the number of mutations per megabase of sequenced tumor DNA, has been shown to be associated with both prognosis and predictive power in clinical trials using ICB (Havel, Chowell, & Chan, 2019). Despite this, the fact that HCC has low median TMBs could be the cause of the lack of correlation of Whole Exome Sequencing (WES)-calculated TMB with survival in first line atezolizumab and bevacizumab clinical trial (Zhu et al., 2020). Copy number alterations (CNA), another proxy of high mutational load in cancer cells, which may be related to immune exclusion in HCC (Bassaganyas et al., 2020) has not yet been tested as a predictive biomarker in clinical trials.

Gene expression profiling has been studied mainly in RNA sequencing (RNA-seq) studies of fresh-frozen liver biopsies or surgical pieces. From the first RNA-seq based transcriptomic classifications of HCC, there has always been the evidence of different degrees of inflammatory gene expression signatures in HCC (Rebouissou & Nault, 2020), with certain association of β-Catenin mutation with low immune signature, consistent with findings in melanoma and other tumors. The most recent study in this field identified a subgroup, referred to as an "immune class," that was shown to include 2 distinct clusters with an exhausted immune response, which may help determine why some apparently "inflamed" tumors are poor responders to ICIs

(Sia et al., 2017). A recent study in samples from a first line trial testing nivolumab in patients with advanced HCC, examined these published signatures and its relation to survival and or response to ICB (Sangro et al., 2020). There was a significant or trend association of *T-cell exhaustion (CD274 (PD-L1), CD276, CD8A, LAG3, PDCD1LG2, TIGIT)*, Gajewski 13-gene inflammatory (*CCL2*, *CCL3*, *CCL4*, *CD8A*, *CXCL10*, *CXCL9*, *GZMK*, *HLA-DMA*, *HLA-DMB*, *HLA-DOA*, *HLA-DOB*, *ICOS*, *1RF1*), 6-gene interferon gamma (*CXCL10*, *CXCL9*, *HLA-DRA*, *IDO1*, *IFNG*, *STAT1*), Ribas 10-gene interferon gamma (*CCR5*, *CXCL10*, *CXCL11*, *CXCL9*, *GZMA*, *HLA-DRA*, *IDO1*, *IFNG*, *PRF1*, *STAT1*) and Interferon gamma biology (*CCL5*, *CD27*, *CXCL9*, *CXCR6*, *IDO1*, *STAT1*) signatures with response to nivolumab (Sangro et al., 2020). This indicates that the baseline inflammation of the tumor will mark the efficacy of ICB.

Some easier-to-perform laboratory parameters could point to good ICB responders. In nivolumab-treated advanced HCC patients, complete and partial responses to nivolumab were seen in patients with low Neutrophil to Lymphocyte and Platelet to Lymphocyte Ratios (NLR, PLR) (Sangro et al., 2020). In the explorative setting, plasma analysis using ELISA or higher multiplexed techniques have enabled biomarker discovery studies. In a small phase I trial exploring pembrolizumab in the second line, high levels of TGF-β ($>200$ pg/mL) were associated with poor outcomes. Other proinflammatory and antiinflammatory cytokines (IL1b, IL6, IL8, IL12, IL18, IFN-γ, IL10, CXCL9, CCL4, CCL5) and soluble checkpoint ligands (PDL1 and PDL2) were not associated with prognosis nor were related to objective responses (Feun et al., 2019).

## 3.4 Beyond immune checkpoint inhibitors

The success of ICIs has set the proof of principle that tumor immune rejection is achievable and can be exploited therapeutically. This has opened the door to testing other forms of immunotherapy in liver cancer. A detailed analysis of these other strategies is beyond the scope of this manuscript, but we will summarize the key strategies that have initiated clinical development.

Adoptive cell therapies (ACT) consist of the use of self-patient lymphocytes which are sensitized and/or expanded in vitro and reinfused to the patient. Several cell types such as lymphokine-activated killer (LAK), cytokine-induced killer (CIK), NK, Tumor Infiltrating Lymphocytes

(TIL) and redirected circulating T cells have been explored. In the case of peripheral blood lymphocytes, two main strategies have been explored, namely the use of transgenic tumor-antigen specific TCR and chimeric antigen receptors (CAR). In ACT, patients are usually treated with a preconditioning cytotoxic regimen to induce lymphodepletion and support in vivo expansion of transferred cells. In a phase III trial in patients with curative HCC resection, the infusion of CIK improved both PFS and OS when compared with patients with no adjuvant therapy (Lee et al., 2015). Feasibility of TIL treatment in HCC was shown in a pilot phase I trial (Jiang et al., 2015). Immunotherapy using ex vivo-expanded allogeneic NK cells in HCC treated with resection was recently shown to be safe in a phase I trial (Kim et al., 2021). Other ongoing trials with NK cells in HCC have been detailed elsewhere (Mantovani, Oliviero, Varchetta, Mele, & Mondelli, 2020). These strategies are nevertheless not regularly used in clinical practice in part due to the lack of in-house cell therapy facilities and the regulatory nuances of ACT.

CAR T cell has shown to be of great success in hematological malignancies (Larson & Maus, 2021), but there are specific challenges, specially the lack of good tumor-specific surface antigens. In HCC, the most explored cancer antigens to be targeted with ACT have been GPC3 and AFP. GPC3 is expressed in the surface of the HCC cell, so GPC3-targeting CAR-T cells has been shown to be efficacious in animal models and are being tested in phase I clinical trials (NCT04121273, NCT02715362, NCT03130712). AFP, instead, is found intracellularly and secreted, so AFP-specific TCR transgenic T lymphocytes are being tested. AFP-specific TCR T cells have obtained some objective tumor remissions reported in an ongoing clinical trial of in HCC (Sangro et al., 2022, NCT03132792). Other strategies such as bispecific GPC3-S-Fabs designed to attract NK cells to GPC3-positive cells, bispecific T-cell Engagers (BiTE) binding GPC3 and CD3, are still in their preclinical phases (Huang et al., 2021). Therapeutic vaccines aim at either de novo priming of T cells against antigens expressed by tumor cells that are unable to spontaneously trigger a response, or enhancing already existing responses, or it can also widen the repertoire and breadth or tumor specific responses (Tagliamonte et al., 2018). In HCC, many trials based on tumor lysates have failed to produce consistent results (Prieto, Melero, & Sangro, 2015). HCC peptide vaccines targeting telomerase, GPC3 or AFP have elicited specific responses in some cases, but none has provided clinically meaningful results (Greten et al., 2010; Sawada et al., 2012). Vaccines targeting true tumor-specific neoantigens identified by a

multi-omic approach could be a step forward. An off-the-shelf vaccine including 5 HLA-A*24 and 7 HLA-A*02 as well as 4 HLA-DR restricted peptides identified and selected from human HCC, in combination with a novel RNA-based adjuvant was able to induce immune responses but lacked clinical activity as a single therapy (Buonaguro et al., 2020).

## 4. Immunotherapy of intrahepatic cholangiocarcinoma

Biliary tract cancer (BTC) includes cholangiocarcinomas and gallbladder cancer. Cholangiocarcinoma (CCA) is a molecularly and clinically heterogeneous entity. The immune environment of cholangiocarcinoma is poorly understood. CCA is an epithelial tumor that arises from the bile ducts and 90% of them are adenocarcinomas (Valle, Kelley, Nervi, Oh, & Zhu, 2021). Anatomically, they are divided into intra- and extrahepatic cholangiocarcinomas. Intrahepatic CCA (iCCA) arises within the hepatic parenchyma whereas extrahepatic (eCCA) arises in the extrahepatic portion of the bile duct. From a clinical perspective they can be divided into resectable versus unresectable disease. Non-resectable disease includes localized or locally advanced disease non amenable to surgical treatment upfront or overt metastatic disease. Due to the low incidence of these entities, randomized clinical trials for non-resectable disease usually include any type of BTC, both iCCA or eCCA. This explains why treatment guidelines for non-resectable or metastatic BTC usually do not differentiate between them, although from a clinical, biological, and molecular perspective they are different entities. There is an obvious need to better understand the best treatment for these entities and guidelines, such as NCCN, describes primary treatment options for patients with unresectable or metastatic BTC as enrolment on a clinical trial, administering systemic treatment or providing best supportive care (Benson et al., 2021).

Standard of care for first line advanced or metastatic ICCA is gemcitabine plus cisplatin based on the ABC-02 trial that showed a median OS of 11.7 months and a mPFS of 8.0 month in the combination arm compared to 8.1 months OS and 5.0 months PFS in the gemcitabine alone arm (Valle et al., 2010). Other accepted combinations based on different phase II trials are gemcitabine plus oxaliplatin (Andre et al., 2008; Jang et al., 2010; Kim et al., 2009), gemcitabine plus capecitabine (Knox et al., 2005; Koeberle et al., 2008; Riechelmann, Townsley, Chin, Pond, & Knox, 2007) gemcitabine plus nab-paclitaxel (Shroff et al., 2019), capecitabine plus oxaliplatin (CAPOX) (Nehls et al., 2008), or the triad including folinic acid,

fluorouracil, and oxaliplatin (FOLFOX) (Nehls et al., 2002). In recent years, the interest in better understanding the differences across BTC subtypes has led to the identification of patient populations defined by genomic alterations with improved survival when those alterations are amenable to a therapeutic intervention, enabling the indication of targeted therapies. Some examples include FGFR2 translocations (13–14% of iCCA) targeted with pegatinib or other FGFR kinase inhibitors, IDH1 mutations (10–23% of iCCA) with ivosidenib or similar IDH1 inhibitors, BRAF V600E (3% of iCCA) with BRAF or BRAF plus MEK inhibitors (Goeppert et al., 2014) and NTRK fusions (0.75% of BTC) recently successfully treated (Demols et al., 2020).

## 4.1 Single agents

Small subgroups of iCC have shown to be sensitive to ICIs. iCCA having mismatch repair deficiency with high microsatellite instability (dMMR/MSI-H) which accounts for around 10% of patients (Ando et al., 2022; Silva et al., 2016), or bearing high TMB defined as 10 or more mutations per megabase, which accounts for 5% of patients (Cao et al., 2020) have responded to monotherapy with the anti-PD1 pembrolizumab. As a result, pembrolizumab has been approved by the US FDA for this subset of patients based on the KEYNOTE-158 phase II trial that tested the efficacy of this drug in non-colorectal MSI-H/dMMR cancers (Marabelle et al., 2020). In iCCA patients ($n = 22$) pembrolizumab showed an ORR of 40.9% (95% CI, 20.7%–63.6%) with a median PFS of 4.2 months and a median OS of 24.3 months. iCCA TMB-high patients had 29% of ORR compared to 6% in the non-TMB-high population in the same trial (Marabelle et al., 2020). Similar ORR has been reported with the investigational PD1 antibody dostarlimab in MSI-H/dMMR patients, although only 2 patients with BTC were included in the non-endometrial cohort ($n = 106$) (Berton et al., 2021).

In the non-selected BTC population, the role of ICI monotherapy is disappointing. The largest published data is the joint analysis of the KEYNOTE-158 ($n = 104$, all comers) and KEYNOTE-028 ($n = 24$, tumor PD-L1 expression). The ORR after pembrolizumab in iCCA patients ranged from 6% to 13% with no difference by baseline PD-L1 expression (Piha-Paul et al., 2020). Similar data were seen with Nivolumab in a phase II trial that reported an ORR of 11% (Kim et al., 2020). These were early trials and had several limitations like the use of different PD-L1 expression

detection methodologies and the lack of definition by anatomical origin of BTCs (iCCA vs eCCA vs gallbladder cancer). As a positive note, it is interesting to reflect that in the three described trials there were long responders among the few BTC patients that responded. ICI combinations without chemotherapy have not been successful and although data is scarce (Klein et al., 2020; Sahai et al., 2020; Yoon et al., 2021), it does not seem to be the way moving forward at this point, at least with unselected BTC cohorts. Several additional trials are ongoing evaluating anti-PD-1/PD-L1 antibodies as monotherapy or in combination (Hilmi, Vienot, Rousseau, & Neuzillet, 2019; Vogel, Bathon, & Saborowski, 2021).

## 4.2 Combination therapies

Based on the positive results in other tumor types and the rationale that chemotherapy could reshape the tumor microenvironment and the immune system (Salas-Benito et al., 2021) several trials combining the standard of care with ICIs have been running. Some early data of chemo-immunotherapy combination has already been partially presented in several international meetings (Oh et al., 2020; Sahai et al., 2020) and several trials are ongoing (Manne, Woods, Tsung, & Mittra, 2021; Vogel et al., 2021). Very recently, the TOPAZ-1 trial, testing the anti-DP-L1 antibody durvalumab in combination with gemcitabine plus cisplatin, has become the first phase III positive randomized clinical trial in BTC that may be able to shift the role of immunotherapy in this cancer (Oh et al., 2022). OS was significantly improved with a HR of 0.80 (95% CI 0.66–0.97) and median OS was 12.8 months in the durvalumab plus chemotherapy arm vs 11.5 months in the chemotherapy only group. Based on an initially planned stratification, iCCA showed a larger benefit compared to eCC/GBC. In addition, Asian race and Asia as a region showed higher benefit compared to non-Asian race or the rest of the world. This should be analyzed further as there is data pointing towards genetic differences in iCCA between Eastern and Western patients that may have impacted the overall results of the trial (Cao et al., 2020). Other trials with a similar design using Pembrolizumbab (NCT04003636) or Atezolizumab (NCT04677504) as ICI backbone are also ongoing. Other Immunotherapy approaches in BTC. Based on positive signals shown in HCC, trials with immunotherapy plus TKI are ongoing and some have shared early data with promising responses (Lwin et al., 2020) although it is still unclear the role these combinations may play in the future.

## 5. Concluding remarks

In only a decade we have witnessed the dawn of immunotherapy of liver cancer leading to a stage where it has become or will soon become part of the standard of care for patients with HCC and iCCA (Fig. 2). While this represents an enormous advance for those patients who will obtain a high-impact benefit, the success comes with important challenges. Financial issues is one of them and not the least important. In coming years, much translational and clinical research is needed to optimize treatment efficacy and patient selection, but the outcome is worth the effort.

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