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1 ABBREVIATIONS

AFP	Alpha-fetoprotein
ALBI	Albumin-bilirubin score
ALD	Alcoholic liver disease
ALT	Alanine aminotransferase
APC	Antigen-presenting cells
AUC	Area under the curve
B2M	β2 microglobulin
BCLC	Barcelona Clinic Liver Cancer
CCL5	CC-chemokine ligand 5
CTLA4	Cytotoxic T-Lymphocyte-Associated Protein 4
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ECOG	Eastern Cooperative Oncology Group
ED	Extrahepatic disease
EPCAM	Epithelial cell adhesion molecule
GSEA	Gene set enrichment analysis
HALS	Hand-assisted laparoscopic liver surgery
HBV	Hepatitis B
HCC	Hepatocellular carcinoma
HCV	Hepatitis C
HLA	Human leukocyte antigen
HPB	Hepato-Pancreatico-Biliary
iCC	Intrahepatic cholangiocarcinoma
ICI	Immune checkpoint inhibitor
IFN	Interferon
IGF2	Insulin-like growth factor 2
LAG3	Lymphocyte-activation gene 3
LLR	Laparoscopic liver resection
LT	Liver transplantation
MELD	Model for end-stage liver disease
MILL	Multiport laparoscopic liver surgery
MMR	Mismatch repair
MSI	Microsatellite instability
MVI	Macrovascular invasion
MWA	Microwave ablation
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NSCLC	Non-small cell lung cancer
OLR	Open liver resection
OR	Objective response
ORR	Objective response rate
OS	Overall survival
PD	Progressive disease
PD1	Programmed Cell Death Protein 1
PDFGR	Platelet-derived growth factor receptor
PD-L1	Programmed Cell Death 1 ligand 1
PFS	Progression-free survival

PKA	Proteinkinase A
RCT	Randomized controlled trial
RFA	Radiofrequency ablation
RNA	Ribonucleic acid
SD	Stable disease
TACE	Transarterial chemoembolization
TAM	Tumor-associated macrophages
TCR	T-cell receptor
TIL	Tumor-infiltrating lymphocytes
TKI	Tyrosine kinase inhibitor
TMB	Tumor mutational burden
VEGFR	Vascular endothelial growth factor receptor
WES	Whole-Exome sequencing

2 INTRODUCTION

Liver cancer represent a leading cause of cancer-related mortality globally and stands out among these extraordinarily lethal tumors due to its rising incidence[1]. Hepatocellular carcinoma (HCC), deriving from malignant transformation of hepatic parenchymal or progenitor cells, accounts for 80-90% of primary hepatic malignancies, whereas intrahepatic cholangiocarcinoma (iCC), originating in the cells lining the biliary duct system in the liver, represents ~10% of cases[2-5]. Predisposing risk factors for HCC include chronic viral hepatitis (hepatitis B and C), heavy alcohol consumption (alcoholic liver disease, ALD) and non-alcoholic fatty liver disease (NAFLD)[6, 7]. In the vast majority of patients, these underlying liver diseases cause cirrhosis, which is present in ~90% of patients with HCC[1, 8].

2.1 Treatment of HCC

As a unique feature of clinical management of HCC, treatment allocation is dictated by the globally recognized Barcelona Clinic Liver Cancer (BCLC) classification that takes into account tumor morphometrics, disease spread, hepatic function and general health (defined by ECOG performance status), instead of the TNM system which is used in most cancer types[9]. This classification defines very early (BCLC 0) and early-stage tumors (BCLC A) as well as intermediate (BCLC B), advanced (BCLC C) and terminal stages (BCLC D).

Across these disease stages several unmet needs exist in clinical practice that remain impediments towards improving outcomes. Despite considerable advances in the clinical management of HCC within the last 20 years, unfortunately, only patients at early disease stages (BCLC 0-A) are candidates for curative treatment options resection, local ablation and liver transplantation (LT) where median overall survival (OS) generally exceeds 5 years[8, 10-13]. However, in early-stage disease, recurrence rates remain high after resection or ablation with 50-70% of the population developing recurrence within 5 years[14, 15]. While the lack of effective neoadjuvant/adjuvant treatment options certainly remains a glaring issue in the field, recent studies have also indicated that complications after surgery may lead to early recurrence and thereby compromise oncologic outcomes[16, 17]. Thus, improving safety of curative treatment via resection and understanding vulnerable patient populations is critical for both short and long-term outcomes. In cases of more advanced tumors, characterized by the presence of macrovascular invasion (MVI), extrahepatic metastasis (ED) or poor

performance status (ECOG1-2), patients are generally only eligible for systemic treatment, but clinical practice is constrained by the near absence of precision oncology[8, 11]. Given the paucity of molecular studies that examine therapeutic vulnerabilities in patients with advanced stage disease to develop sound treatment rationales, current strategies rely on a one-fits-all approach despite severe heterogeneity of outcomes in patients treated with current state of the art treatment protocols[18, 19]. Developing precision oncology approaches is thus another priority of clinical and translational research in the field.

These different unmet needs intersect, particularly in early-stage disease, where, increasingly, clinical trials evaluate systemic treatment in a perioperative setting (neo-/adjuvant) with the goal of reducing recurrence rates. Given that follow-up is long in this population these studies are time- and cost-intensive. It is therefore common to test only those drugs that have already proven efficacy to decelerate tumor progression in advanced stage patients. Across cancer types this strategy has yielded several positive findings in adjuvancy, although not in HCC, where sorafenib proved futile in reducing recurrence rates in the STORM trial[20, 21]. One of the main explanations for this disappointing result is that the modest benefit provided by sorafenib and other TKIs may be too little in a patient population with an expected OS beyond 5 years compared to advanced stage where the natural course of the disease is expected to be ~8 months[20]. In recent years, however, treatment of advanced stage disease, the domain of systemic treatment where precision approaches are developed, has advanced considerably and several new compounds have been proven efficacious in improving OS and progression-free survival (PFS). Specifically, the introduction of immunotherapy has heralded in a new era, where novel frontline treatments have emerged, and ongoing trials heavily revolve around the use of checkpoint inhibitors[22]. These drugs can provide outstanding outcomes with immense improvements in survival, but only to a subset of the population [23-25]. Defining determinants of response and resistance to these new treatments for advanced HCC could maximize efficient drug deployment and introduce precision medicine in this space. Furthermore, this may inform trial design in early-stage HCC to tackle high recurrence rates. Developing biomarker-driven precision approaches is hence a priority in the field. Gradually, these new therapies are tested perioperatively within trials for earlier disease stages. In this regard, uneventful postoperative courses are required, as complications can markedly postpone systemic treatment and even render it ineffective with patients missing the

therapeutic window. This underscores the need to improve perioperative safety and develop precision oncology for systemic treatment to convey meaningful advances for patients with HCC.

The treatment landscape of HCC is shifting with indication for curative treatment options being extended and systemic treatment options extending towards intermediate and early-stage disease[26-28]. This work focuses on the key requirements to safeguard the further development of these trends: (I) to ensure high-quality safety outcomes after curative-intent surgical treatment and (II) to enable precision medicine for systemic treatment with checkpoint inhibitors by providing a granular picture of mechanisms of response and resistance as well as immune-based molecular tumor subclasses and developing clinically informative predictive biomarkers.

2.1.1 Surgical treatment of HCC

Resection and LT are the backbone of curative therapies for HCC and are, in principle, applied to treat early-stage disease[12, 29]. Percutaneous treatment via radiofrequency ablation (RFA) or microwave ablation (MWA) can achieve non-inferior outcomes to surgical resection in nodules <3cm, leaving larger tumors to be addressed via surgical intervention[30, 31]. Given that LT entails both treatment of the malignant lesion as well as the underlying liver disease, it is regarded as the state-of the art approach for patients with HCC and portal hypertension or compromised hepatic function, whereas surgical resection is the mainstay option for non-cirrhotic patients as well as those with compensated cirrhosis. Both treatment modalities provide a median OS beyond 5 years, although LT is able to markedly reduce recurrence rates within 5 years to 10-15% compared to 50-70% after resection[13, 14, 29, 32]. However, deployment of LT, where scarcity of donor organs has to be considered, is contingent on strict inclusion and exclusion criteria. To be eligible for LT, tumor burden is required to be within the Milan criteria (single nodule < or = 5 cm or two or three nodules < or = 3 cm)[29], although numerous extended criteria have provided evidence that this threshold can be pushed considerably while maintaining acceptable oncologic outcomes[33-37]. Overall, the decision between surgical resection and LT has to factor in patients' characteristics such as liver function, performance status, severity of hepatic dysfunction, presence of portal hypertension as well as tumor characteristics (size and number of nodules, vascular involvement and alpha-feto protein levels) and local regulations that guide organ allocation. In recent years, as regional shortages of donor organs became

apparent, increasing efforts have been made to push the envelope in terms of patient selection for surgical resection, thereby challenging the existing guidelines that have advocated to select ideal patients for resection in order to ensure best outcomes[38-40]. This orthodoxy is under increasing scrutiny as emerging studies have revealed that resection of tumors in suboptimal candidates may still offer superior outcomes compared to the loco-regional treatments that these patients would otherwise default to[41, 42]. Adherence to selection criteria for resection, which have been formulated by Western guidelines (single tumor, Child Pugh A liver function with bilirubin <1mg/dl in the absence of clinically significant portal hypertension, preserved performance status (ECOG 0)) limits perioperative mortality to below 3%[8, 11]. Notably, the predictive models that are applied to stratify patients for resection such as the model for end-stage liver disease score (MELD), the albumin-bilirubin score (ALBI) and the Child-Pugh classification relate to the risk of post-hepatectomy liver failure as the driver of poor outcomes[43, 44]. Extending the applicability of surgical resection to patients with limited hepatic dysfunction is thus key to improve outcomes for the overall patient population. A pivotal development in this regard has been the reduction of the operative trauma through minimally-invasive procedures, which lower postoperative morbidity in this susceptible patient population[45].

2.1.2 Laparoscopic liver resection

While minimally-invasive procedures have been considered standard practice for many years in several domains of abdominal surgery, its application in the hepato-biliary field has been considerably decelerated. Despite early evidence suggesting laparoscopic liver resection (LLR) to be safe and feasible in select cases, the challenging anatomical and physiologic properties of the liver have been regarded as obstacles towards establishing LLR[46]. Initially prevailing concerns were directed mostly at intraoperative safety, where particularly parenchymal transection and hilar dissection entail higher risk of intraoperative hemorrhage compared to other fields where laparoscopy is well established such as colorectal cancer. The presence of liver cirrhosis adds a layer of complexity with more brittle parenchyma, compromised haemostasis and higher risk for postoperative complications such as liver failure. Moreover, the appropriateness of LLR has been drawn into question by reservations regarding oncologic outcomes, specifically the ability to achieve adequate resection margins and avoid tumor cell dissemination[47].

LLR indeed poses several technical difficulties such as atraumatic liver retraction and accurate dissection of the parenchyma to expose vascular and biliary structures to avoid inadvertent harm to the liver remnant. Within the last decade, however, a growing body of evidence has highlighted the ability of LLR to convey safe outcomes after resection of primary liver tumors HCC as well as iCC[48-51]. Indeed, HCC has been consistently reported as the most frequent indication for LLR despite the higher prevalence of colorectal liver metastasis in the overall population[47]. Over the past years, several reports have emerged that have validated LLR to achieve at least non-inferior oncologic outcomes compared to liver resection (OLR) with 5-year OS rates of 50-70% in HCC[49, 52, 53]. Importantly, LLR has been shown to also markedly ameliorate the operative trauma, leading to reduced postoperative morbidity including less hepatic decompensation[54]. While definitive proof of pathophysiologic underpinnings is amiss, it is hypothesized that the reduction of the operative trauma with less pain and preservation of the abdominal wall vessels play a key role in lowering morbidity. Favourable safety outcomes after LLR in HCC were, interestingly, pronounced in patients with liver cirrhosis, suggesting that in those patients with compensated cirrhosis the benefits of LLR might outweigh perceived hazards[55]. Collectively, these results have led to LLR being established as standard practice for several types of liver resections. At high-volume centers LLR, indeed, represents the default approach to treat hepatic lesions, even when major resection is required, meaning the removal of at least three of the eight liver segments.

It needs to be acknowledged, however, that the anatomical properties of the liver, the different resection types and liver function impact the complexity of a resection. Over the past five years multiple scores have been reported and, in some instances, applied in clinical practice to ascertain the difficulty of a resection type. The Iwate criteria, which have been independently validated, have emerged as the most commonly used score and take into account the technical approach, hepatic function, tumor size, proximity to major vessels and, crucially, the location of the lesion[56, 57]. Nodules within the posterosuperior segments (segments IVa, VII, VIII) have in the Iwate criteria and others been consistently described as the most challenging indications for LLR[56, 58].

Despite the encouraging progress of LLR in practice, clinical problems persist and relate heavily to patient selection. The extension of the indication requires a refined grasp of predictors of postoperative morbidity with capacity as a preoperative

predictive marker to improve patient selection and avoid disproportional risk. Particularly in patients where tumor location already entails challenging technical constraints, understanding whether compromised hepatic function puts these patients at further risk is important to deploy the correct therapeutic modality.

2.2 Systemic treatment of advanced stage HCC

Advanced stage tumors are characterized by the presence of either MVI or ED independent of the size or number of nodules. Alternatively, patients with reduced general health as accounted for by limited performance status (ECOG1-2) are also defined to be at advanced stage, where outcome is dismal and the natural history of disease progression assumes a median OS of ~8months without any treatment[9]. While intraarterial therapies including radioembolization with Yttrium-90 and transarterial chemotherapy have been tested in large, randomized trials, level one evidence for treatment of advanced HCC exists only for systemic therapy[1, 59, 60].

2.2.1 Tyrosine-kinase inhibitors and antiangiogenics

Long known for its resistance towards conventional chemotherapy, a globally recognized systemic treatment for HCC was amiss until 2008, when the tyrosine-kinase inhibitor sorafenib was shown to convey a modest but significant increase in media OS (10.7 vs 7.9 months) compared to placebo in the seminal SHARP trial[20]. Sorafenib targets several receptors involved in angiogenesis, a key molecular feature of HCC, including VEGFR1-3, PDGFR and c-KIT[61]. These findings were confirmed in the simultaneously conducted Asia-Pacific (AP) trial that attained a similar magnitude of benefit from sorafenib although median OS was lower owing to the enrolment of patients with more aggressive tumors[62]. Importantly, the SHARP trial still functions as the benchmark for clinical trial design in advanced HCC, that established selection criteria as well as stratification factors, including the etiology of the underlying liver disease, tumor characteristics as well as liver function[60].

Disappointingly, within the following decade clinical trials that were thought to build upon the expanding molecular insight into HCC represented setbacks and failed to improve survival. These phase III investigations testing among others TKIs brivanib, sunitinib, linifanib and erlotinib did not meet their respective endpoints of prolonging OS[63-66]. Since 2017, the field has advanced incrementally, and new treatments have emerged that significantly prolong OS after progression or intolerance to sorafenib. First, the TKI regorafenib, that differs on a molecular level from sorafenib only by a

single fluoro substituent was approved after the positive RESORCE trial[67]. Subsequently, TKI cabozantinib and anti-VEGF monoclonal antibody ramucirumab augmented the therapeutic armamentarium after the CELESTIAL and REACH-2 trials, respectively[68, 69]. Of note, ramucirumab first failed to meet the endpoint of improving OS in the REACH trial that followed classical enrolment criteria for systemic treatment trials in HCC[70]. In the subanalysis however, a significant benefit was observed in patients with high AFP, indicative of an aggressive phenotype. This was confirmed in the subsequent REACH-2 trial that only enrolled patients with AFP≥400ng/ml. To date, this represents the only instance of biomarker-driven precision oncology in clinical care of patients with advanced stage HCC. Finally, the REFLECT trial, an open label RCT established the TKI lenvatinib as an alternative to sorafenib in frontline treatment[71]. The trial was powered for both superiority and non-inferiority in terms of OS but only met the latter endpoint.

Overall, the introduction of TKIs have led to an improvement in outcomes of patients with HCC, although the benefit was limited in magnitude and antitumoral efficacy was confined to advanced stage, whereas TKI treatment in an adjuvant setting failed to reduce recurrence rates [21]. With the onset of immunotherapy in clinical oncology, however, studies assessing their applicability in HCC are dominating the trial landscape.

2.2.2 Immune-checkpoint inhibitors

The immune system plays a critical role in impeding cancer progression. Herein, T cells are exposed to a tumoral neoantigen, a structurally and potentially functionally altered protein that derives from a mutation in a given tumor cell. To exert antitumoral immunity, however, T cells require a 2nd signal, often referred to as co-stimulation which is elicited through the binding of CD28 with CD80/CD86 on antigen-presenting (APC) or tumor cells[72, 73]. This process primes the effector T cell, enabling it to conduct immune surveillance and thus contain cancer growth. This protection of the host against cancer is contingent on several steps: (i) in the cellular machinery, including but not limited to transcription, RNA-editing and HLA-binding affinity of the neoantigen and (ii) sustained antitumoral cytotoxicity exerted by the effector immune cell. These contingencies highlight vulnerabilities through which immunosurveillance can be rendered ineffective: first, this antitumoral immunity collaterally places evolutionary pressure on malignant cells to undergo phenotypical changes. Second, upon chronic

antigen exposure, immune effector cells are more likely to express inhibitory receptors, termed immune checkpoints, such as PD-1 and CTLA4[74]. While PD-1 binds PD-L1 on tumoral cells or APCs to abrogate T cell activation, CTLA4 binds CD80/CD86 at a much higher affinity than its natural ligand CD28, thereby inhibiting the costimulatory signal[75]. This process renders T cells exhausted and virtually incapacitated for immunosurveillance[76]. Harnessing and boosting antitumor T cell function is thus a very effective therapeutic strategy in clinical oncology. The first generation of checkpoint inhibitors has been developed on this premise with drugs targeting PD-1, PD-L1 and CTLA4[75].

Collectively, these immune checkpoint inhibitors (ICI) have revolutionized clinical care as they have been demonstrated to effectively prolong OS and PFS alone or in combination with other drugs in several solid cancer types, including HCC[18, 77, 78]. As a major caveat of ICI treatment, outcome patterns among patients are highly heterogeneous. Treatment with classical chemotherapy or TKIs has generally been able to elicit a modest benefit for the broad patient population. ICIs on the other hand, convey an outstanding survival benefit for a subset of the population[72]. Therefore, the proportion of patients exhibiting radiological objective response (OR) dictates the applicability of ICI in each cancer type. In advanced HCC, most patients undergoing single-agent ICI treatment exhibit either stable (SD) or progressive disease (PD) whereas only 15-20% of patients respond[24, 79]. The relatively small size of this subset has contributed to the failure of phase III trials of nivolumab (anti-PD1)[25] and pembrolizumab (anti-PD1)[24] in front- and second line, respectively. However, combining ICI therapy with anti-angiogenics has been demonstrated to be an effective tool in expanding the immune sensitive population, culminating in the positive findings from the IMbrave150 trial that established anti-PD-L1 atezolizumab with bevacizumab (anti-VEGF) as standard of care in frontline, where an OR rate of 33% was observed[20]. More recently, positive findings have been reported for the combination of tremelimumab (anti-CTLA4) and durvalumab (anti-PD-L1), a dual ICI regimen that will represent a first-line alternative providing median OS of 18.7 months and boosting the OR rate to 24%[19]. Likewise, the combination of camrelizumab (anti-PD1) and rivoceranib (VEGFR2 inhibitor) was reported to achieve a median OS of 22.1 months with an OR rate of 25% at ESMO2022[80]. These positive findings have spawned intense efforts to leverage the improved drug efficacy of ICI-based regimens to reduce recurrence rates at earlier disease stages. In this regard, early evidence testing

cemiplimab (anti-PD1) or nivolumab combined with ipilimumab (anti-CLTA4) in neoadjuvancy has provided promising results[28, 81]. Furthermore, a recent trial from Johns Hopkins has highlighted the ability of ICI-based therapy (nivolumab + TKI cabozantinib) to convert locally advanced disease into resectable tumors,enabling subsequent curative treatment options[82]. Overall, the emergence of ICI and combination treatments in clinical care of HCC have been shown to statistically prolong survival for the entire population. Nevertheless, a considerable proportion of patients retain little to no clinical benefit and a comprehensive understanding of the mechanisms guiding response and resistance to ICI is still lacking.

2.3 Biomarkers of response and resistance to ICI across cancer types

While ICIs did not enter the clinical space in HCC until 2017, experience in other solid cancer types, most notably melanoma and non-small cell lung cancer (NCLC) dates further back[78, 83]. Specifically, clinical and translational data from melanoma, the first cancer type for which an ICI was approved (ipilimumab in 2011), have given rise to general concepts about response and resistance to ICIs[84-86]. The foundation that has powered these scientific discoveries was mostly the advent of next-generation sequencing technology to interrogate the genomic and transcriptomic landscape of tumors via whole-exome sequencing (WES), RNA-sequencing (RNAseq)/Whole genome microarray, respectively[87]. These platforms have enabled researchers to overcome typical constraints such as limited availability of tissue and isolated nucleic acids via simultaneous readout of the vast majority of coding genes on a mutational and transcriptomic level. Fortunately, the development of ICI and the widespread availability of next-generation sequencing platforms have coincided, hence fueling biomarker discovery in this research domain.

In solid cancer types with more extensive experience with ICI, translational studies have shown that biomarkers of response and resistance to ICI can originate in several layers of tumor as well as host biology, that are discussed in this and subsequent sections. Since effector T-cell activation is contingent on priming of the T-cell by a specific antigen either directly by the tumor cell or through a dendritic cell, an intact antigen presentation machinery is paramount to induce sustained antitumoral immunity. When this process is impeded within either the host or the tumoral cell through mutations of genes that code for key proteins in this process, patients might be not amenable to ICI treatment. For example, mutations in B2M, a component of the

MHCI complex renders treatment with anti-CTLA4 and anti-PD1 less efficacious[88, 89]. Moreover, the host's microbiome has been previously shown to drive outcomes after ICI in melanoma patients, where distinct microbiome compositions were linked to treatment response. Accordingly, fecal transplant was able to salvage outcomes in a murine model of primary ICI resistance[90-92].

Thus far, key determinants of response and resistance have been most extensively studied within the tumor and biomarkers suggested for patient stratification highlight that both the mutational landscape as well as the transcriptomic tumoral state define outcomes.

2.3.1 Tumor Mutations and TMB

To understand how the presence of mutations shapes antitumoral immunity it is critical to distinguish two broad concepts: first, the overall tumor mutational burden (TMB) that characterizes how many mutations occur in a tumor per megabase of DNA, and second, distinct mutations that affect the antigen-presentation machinery or how immune cells traffic to the tumor site. It is reasonable to assume that the overall number of non-synonymous mutations (mutations that results in a different amino acid if a protein is generated) in a tumor impacts the likelihood of generating an immunogenic peptide that is presented and recognized, eventually generating an immune response. Indeed, a metaanalysis has shown that across tumor entities TMB correlates with the objective response rates of tumors (ORR) after ICI[93]. In keeping with this, tumors with mutated mismatch-repair genes (MMR) displaying microsatellite instability (MSI) are highly amenable towards ICI[94, 95]. In a recent study that tested pembrolizumab in MMR mutated/MSI patients with colorectal cancers all patients responded to treatment. This represents a remarkable therapeutic option in a population that was previously characterized by dismal outcomes[96]. Moreover, through integrative molecular studies, distinct mutations have been identified as biomarkers of either response or resistance. Mutations of CTNNB1, a gene coding for Beta-Catenin which operates both as a transcription factor and a protein stabilizing cell-to-cell interaction has been described as a driver of resistance to ICI by hindering immune cell infiltration into the tumor[97, 98]. Mutations in SERPINB3 and SERPINB4, on the other hand, have been linked to high response rates in melanoma, independent of TMB, although a reproducible mechanism has never been identified[99].

2.3.2 Tumor microenvironment, T cells and beyond

Early studies in melanoma have shown that the tumoral transcriptional state as captured by the tumor microenvironment (TME) assumes a critical role in determining outcomes after ICI. The TME entails all tumoral and non-tumoral cells including stromal and immune cells, their soluble signaling factors as well as the extracellular matrix (ECM). Conceptually, the TME is highly plastic and can assume several phenotypes even within a distinct cancer type. This understanding has led to the development of molecular subclasses of tumors that factor in tumoral mutations, chromosomal aberrations, TME immune cell composition, signaling pathways and more. Some of these features directly impact the ability of the host to boost antitumoral immunity after ICI treatment. Translational studies in melanoma and subsequently other cancer types have revealed that the density of tumor-infiltrating lymphocytes (TILs), particularly CD8+ T cell infiltration and activation are readouts of nascent antitumoral immunity that is leveraged by ICI to improve outcomes[85, 100]. While some studies have suggested that particularly the presence of TILs at the invasive margin of the tumor predicts response to ICI, the applicability of this approach has been constrained by conflicting results across tumors and difficult standardization[101]. TILs as a singular marker fails to account for the severe heterogeneity in infiltrating lymphocytes and their biological role and is thus a crude metric to predict ICI vulnerability. Indeed, transcriptomic and proteomic markers that denote the biological function of effector T cell subsets and their cytotoxic capacity have been validated as surrogates for antitumoral immunity and ICI response[102]. Bioinformatic deconvolution techniques such as CIBERSORT and MCP counter have enabled researchers to infer the presence of these T cell subsets and other TME cells from bulk data with subsequent reports introducing the notion that the overall cellular composition of the microenvironment is a key feature impacting outcomes[103, 104]. This has been confirmed through single-cell sequencing studies in melanoma that have revealed the ratio of two distinct CD8+ subsets, memory-like and exhausted T cells, to be indicative of outcomes[105]. Further studies have suggested natural-killer cells and the presence of tumor-associated macrophages (TAM) to be linked with resistance[106].

Several translational efforts have probed whether distinct transcription programs within the tumor are linked to response. Although there are conflicting reports, even within one cancer type, several studies have suggested high levels of Interferon-gamma (IFNy) signaling, the main effector molecule by which ICI-driven immunity is conveyed,

to facilitate response in melanoma and in NSCLC[78, 84, 85]. Bioinformatic gene set enrichment analyses (GSEA) have shown in these reports that adaptive immune response pathways, antigen presentation signatures and an active IFN response were shared among responding patients prior to therapy. Biopsy series with tissue being obtained before and during treatment, have identified active IFN signaling as the most upregulated pathway among responders in melanoma[86, 107].

In summary, translational research of mechanisms facilitating ICI response stems mostly from patients with NSCLC and melanoma and has linked an inflamed tumor microenvironment, characterized by active antigen-presentation with effector T cell infiltration, to response. Considering the paucity of molecular studies that investigate potential biomarkers of response to ICI in HCC, little is known on whether or not those factors also translate to HCC. Encouragingly, recent years have seen the emergence of high-quality genomic studies that have uncovered the molecular landscape of HCC and have defined tumor subclasses with prognostic implications, as discussed in the following chapter.

2.4 Molecular subclasses of HCC

Integrative analyses factoring in genomic, epigenomic, transcriptomic and histological data have led the understanding that HCC is a highly heterogeneous malignant entity spanning several molecular subclasses. These subclasses entail distinct drivers and signaling pathways that shape the tumoral phenotype and are linked to clinical outcomes. The heterogeneity of HCC is underscored by the mutational landscape of the tumor where several drivers have been identified and no single mutation dominates carcinogenesis[108, 109]. The distinct molecular subtypes are broadly accounted for by the two main classes: first, the *proliferation class*, that represents ~50% of tumors and is associated with poor clinical outcomes and is more frequently observed in patients with HBV as the underlying liver disease[110]. These tumors show more chromosomal instability as well as an upregulation in cell cycle-related genes and increased frequency of TP53 mutations as well as enhanced mTOR and RAS-MAPK signaling. Two molecular subtypes of the *proliferation* group have been identified: (i) the *progenitor class* (30% of all HCCs), which shows high expression of cell proliferation pathways mTOR, RAS-MAPK and MET as well as upregulation of progenitor cell markers (IGF2, AFP and PCAM) and (ii) the *TGF-beta-WNT subtype* (20% of all HCCs) that shows enhanced non-canonical Wnt activation and an activated stroma with an

increase in exhausted T cell infiltration[109, 111, 112]. The remaining 50% of tumors are captured by the *non-proliferation class* that is enriched in patients with alcohol- and HCV-related HCC[113-115]. Tumors within this group frequently have CTNNB1 mutations that are linked with canonical WNT signaling and, overall, less immune cell infiltration. As these tumors tend to show more chromosomal stability, less AFP expression and lower frequencies of vascular invasion they are less aggressive and associated with better outcomes[110]. These consensus molecular tumor classes of HCC derive mainly from three separate reports that provide overlapping results. First, the classification by Boyault et al that defines *G1* through *6*, where *G1* aligns with the *progenitor-proliferation* subgroup and is enriched in proteinkinase A (PKA) and AKT signaling. *G2-3* resemble the TGF-beta-WNT subclass, with *G3* showing significantly high frequencies of TSC1-2 mutations and 17p loss[114]. *G4* is mostly present in steatohepatic *non-proliferation* class HCC and shows enrichment in IL6-JAK-STAT signaling and *G5-6* that captures CTNNB1 mutated HCC[114]. Second, the *S1-3* subgroups defined by Hoshida et al, where *S3* summarizes all *non-proliferation class* tumors, *S2 progenitor classes* and *S1* most closely aligns with the TGF-beta-Wnt group[111]. Finally, the classification by Chiang et al has provided further molecular insight confirming the presence of *CTNNB1*, *proliferation*, and *IFN*-related molecular subgroups and defining a novel subgroup, characterized by polysomy of chromosome 7[115].

While these findings have collectively advanced our understanding of pathogenesis and molecular heterogeneity in HCC, these reports predate the immunotherapy era thereby providing limited insight into how the molecular phenotype shapes immune cell infiltration, antitumoral immunity and, conversely, immune evasion. To this end, an *in-silico* analysis of the transcriptomic landscape of HCC has proposed the *HCC Immune Class* that features abundant IFN signaling, CD8+ T cell and macrophage infiltration as well as enhanced expression of cytotoxicity-related gene sets[112]. The mechanisms precluding an active immune response, on the other hand, remain to be understood. Considering the pivotal role of T cells in conveying antitumoral immunity, their absence may compromise the potency of ICI in HCC and new insight is needed to develop therapeutic strategies aimed at overcoming this limitation.

Research aims

The following reports provide clinical and translational data with the overriding aim of honing how surgical and systemic treatments are applied to treat patients with

hepatocellular carcinoma. For early-stage disease, clinical reports target improving patient selection for surgical resection by refining risk assessment. For advanced-stage disease, translational reports uncover immune-based molecular classes of HCC and how these and other factors shape a tumors amenability to immunotherapy with checkpoint inhibitors. The reports featured in this work are:

1. Inflamed and non-inflamed classes of HCC: a revised immunogenomic classification
2. Molecular markers of response to anti-PD1 therapy in advanced hepatocellular carcinoma
3. Evidence-Based Management of Hepatocellular Carcinoma: Systematic Review and Meta-analysis of Randomized Controlled Trials (2002-2020)
4. Predicting the Risk of Postoperative Complications in Patients Undergoing Minimally Invasive Resection of Primary Liver Tumors
5. Laparoscopic liver surgery in cirrhosis - Addressing lesions in posterosuperior segments

3 PRESENTATION OF OWN WORK

3.1 Inflamed and non-inflamed classes of HCC: a revised immunogenomic classification

Montironi C*, Castet F*, Haber PK*, Pinyol R, Torres-Martin M, Torrens L, Mesropian A, Wang H, Puigvehi M, Maeda M, Leow WQ, Harrod E, Taik P, Chinburen J, Taivanbaatar E, Chinbold E, Solé Arqués M, Donovan M, Thung S, Neely J, Mazzaferro V, Anderson J, Roayaie S, Schwartz M, Villanueva A, Friedman SL, Uzilov A, Sia D, Llovet JM. Gut. 2022 Feb 23:gutjnl-2021-325918. Doi: https://doi.org/10.1136/gutjnl-2021-325918 Online ahead of print. PMID: 35197323,

*contributed equally

Original abstract:

*"**Objective:** We previously reported a characterisation of the hepatocellular carcinoma (HCC) immune contexture and described an immune-specific class. We now aim to further delineate the immunogenomic classification of HCC to incorporate features that explain responses/resistance to immunotherapy.*

***Design:** We performed RNA and whole-exome sequencing, T-cell receptor (TCR)-sequencing, multiplex immunofluorescence and immunohistochemistry in a novel cohort of 240 HCC patients and validated our results in other cohorts comprising 660 patients.*

***Results:** Our integrative analysis led to define: (1) the inflamed class of HCC (37%), which includes the previously reported immune subclass (22%) and a new immune-like subclass (15%) with high interferon signalling, cytolytic activity, expression of immune-effector cytokines and a more diverse T-cell repertoire. A 20-gene signature was able to capture ~90% of these tumours and is associated with response to immunotherapy. Proteins identified in liquid biopsies recapitulated the inflamed class with an area under the ROC curve (AUC) of 0.91; (2) The intermediate class, enriched in TP53 mutations (49% vs 29%, p=0.035), and chromosomal losses involving immune-related genes and; (3) the excluded class, enriched in CTNNB1 mutations (93% vs 27%, p<0.001) and PTK2 overexpression due to gene amplification and promoter hypomethylation. CTNNB1 mutations outside the excluded class led to weak activation of the Wnt-*

βcatenin pathway or occurred in HCCs dominated by high interferon signalling and type I antigen presenting genes.

Conclusion: *We have characterised the immunogenomic contexture of HCC and defined inflamed and non-inflamed tumours. Two distinct CTNNB1 patterns associated with a differential role in immune evasion are described. These features may help predict immune response in HCC."*[116]

This first report refines the molecular landscape of hepatocellular carcinoma and describes how the tumor mutational burden, chromosomal aberrations and gene expression modules interplay to shape immunogenicity. Integrating findings from whole-exome sequencing, RNA sequencing, immunohistochemistry and T-cell receptor sequencing we define immune-based molecular subclasses with prognostic implications and develop a liquid-biopsy based test that can capture the molecular phenotype of a tumor. Next we aimed at investigating whether the inflamed molecular subclasses that reflect a viable host immune response against cancer cells are more likely to respond to immune checkpoint inhibitor treatment. We thus gathered tissue from patients with HCC prior to the anti-PD1 treatment to explore whether transcriptome-based molecular subclasses and other gene expression profiles are linked to response ICI.

3.2 Molecular markers of response to anti-PD1 therapy in advanced hepatocellular carcinoma

Haber PK, Castet F, Torres-Martin M, Andreu-Oller C, Puighvehí M, Maeda M, Radu P, Dufour JF, Verslype C, Czauderna C, Marquardt JU, Galle PR, Vogel A, Bathon M, Meyer T, Labgaa I, Digklia A, Roberts LR, Mohamed AM, Minguez B, Citterio D, Mazzagerro V, Finkelmeier F, Trojan J, Özdirik B, Mül-ler T, Schmelzle M, Bejjani A, Sung MW, Schwartz ME, Finn RS, Thung S, Villanueva A, Sia D, Llovet JM; Gastroenterology. 2022 Sep 12:S0016-5085(22)01039-3. https://doi.org/10.1053/j.gastro.2022.09.005. Online ahead of print. PMID: 36108710

Original abstract:

*"**Background and aims:** Single-agent anti-PD1 checkpoint inhibitors convey outstanding clinical benefits in a small fraction (~20%) of patients with advanced hepatocellular carcinoma (aHCC) but the molecular mechanisms determining response are unknown. To fill this gap, we herein analyze the molecular and immune traits of aHCC in patients treated with anti-PD1.*

***Methods:** Overall, 111 tumor samples from patients with aHCC were obtained from 13 centers before systemic therapies. We performed molecular analysis and immune deconvolution using whole-genome expression data (n = 83), mutational analysis (n = 72), and histologic evaluation with an endpoint of objective response.*

***Results:** Among 83 patients with transcriptomic data, 28 were treated in frontline, whereas 55 patients were treated after tyrosine kinase inhibitors (TKI) either in second or third line. Responders treated in frontline showed upregulated interferon-γ signaling and major histocompatibility complex II-related antigen presentation. We generated an 11-gene signature (IFNAP), capturing these molecular features, which predicts response and survival in patients treated with anti-PD1 in frontline. The signature was validated in a separate cohort of aHCC and >240 patients with other solid cancer types where it also predicted response and survival. Of note, the same signature was unable to predict response in archival tissue of patients treated with frontline TKIs, highlighting the need for fresh biopsies before immunotherapy.*

Conclusion: *Interferon signaling and major histocompatibility complex-related genes are key molecular features of HCCs responding to anti-PD1. A novel 11-gene signature predicts response in frontline aHCC, but not in patients pretreated with TKIs. These results must be confirmed in prospective studies and highlights the need for biopsies before immunotherapy to identify biomarkers of response."*[117]

The analysis above was based on tissue-based molecular features that shape response patterns to ICI. Given the low number of patients with tissue available prior to systemic treatment our analysis was not powered to reveal clinicopathological features that may have a smaller, yet meaningful impact on outcomes after ICI. Hence, we next aimed at investigating whether through metaanalysis from phase III trials that incorporated ICI for HCC treatment we could identify factors driving response or resistance to ICI. Interesingly, increasing evidence from other reports, has unveiled that HCCs associated with HBV might be more immunogenic leading us to hypothesize that these tumors may be more responsive to anti-PD1. Conversely, evidence from other cancer types indicates that obesity and metabolic syndrome compromise T cell function which may have a detrimental impact on patients with non-viral hepatitis and specifically NASH-associated HCC.

3.3 Evidence-Based Management of Hepatocellular Carcinoma: Systematic Review and Meta-analysis of Randomized Controlled Trials (2002-2020)

Haber PK*, Puigvehí M*, Castet F, Lourdusamy V, Montal R, Tabrizian P, Buckstein M, Kim E, Villanueva A, Schwartz M, Llovet JM. Gastroenterology. 2021 Sep;161(3):879-898. doi: https://doi.org/10.1053/j.gastro.2021.06.008. Epub 2021 Jun 12. PMID: 34126063,

*contributed equally

Original abstract:

*"**Background & aims:** Hepatocellular carcinoma (HCC) is a leading cause of cancer-related mortality, with a rapidly changing landscape of treatments. In the past 20 years, numerous randomized controlled trials (RCTs) have aimed at improving outcomes across disease stages. We aimed to analyze the current evidence and identify potential factors influencing response to therapies.*

***Methods:** We conducted a systematic review of phase III RCTs (2002-2020) across disease stages. A meta-analysis was designed to examine the relationship between etiology and outcome after systemic therapies with either tyrosine-kinase inhibitor (TKI)/antiangiogenic or immune checkpoint inhibitor (ICI) therapy.*

***Results:** Out of 10,100 studies identified, 76 were phase III RCTs. Among them, a rigorous screening algorithm identified 49 with high quality including a total of 22,113 patients undergoing adjuvant (n = 7) and primary treatment for early (n = 2), intermediate (n = 7), and advanced (first-line, n = 21; second-line, n = 12) stages of disease. Nine of these trials were positive, 6 treatments have been adopted in guidelines (sorafenib [2 RCTs], lenvatinib, atezolizumab+bevacizumab, regorafenib, cabozantinib and ramucirumab), but 2 were not (adjuvant CIK cells and sorafenib plus hepatic arterial infusion with FOLFOX). Meta-analysis of 8 trials including 3739 patients revealed ICI therapy to be significantly more effective in patients with viral hepatitis compared with nonviral-related HCC, whereas no differences related to etiology were observed in patients treated with TKI/anti-vascular endothelial growth factor.*

Conclusions: *Among 49 high-quality RCTs conducted in HCC during 2002-2020, 9 resulted in positive results. A meta-analysis of systemic therapies suggests that immunotherapies may be more effective in viral etiologies. "*[118]

The first three reports provide an understanding of which factors precipitate antitumoral immunity in hepatocellular carcinoma and which molecular and clinicopathological features are linked to outcomes after ICI treatment. Eventually, these findings may contribute to furthering precision oncology in clinical practice through unveiling factors that may render ICI treatment ineffective and by providing biomarkers that identify patients with a putative benefit.

ICI treatment is increasingly incorporated in clinical trials for early-stage disease to reduce postoperative recurrence rates. However, once protocols for adjuvancy are established, efficacious treatment will be contingent on uneventful postoperative courses to ensure that therapies will not be delayed. This rationale prompted us to conduct an in-depth analysis of predictors of postoperative complications in patients with primary liver cancer in a cohort of patients undergoing minimally-invasive liver resection at our center.

3.4 Predicting the Risk of Postoperative Complications in Patients Undergoing Minimally Invasive Resection of Primary Liver Tumors

Haber PK, Maier C, Kästner A, Feldbrügge L, Ortiz Galindo SA, Geisel D, Fehrenbach U, Biebl M, Krenzien F, Benzing C, Schöning W, Pratschke J, Schmelzle M. J Clin Med. 2021 Feb 10;10(4):685. doi: https://doi.org/10.3390/jcm10040685. PMID: 33578875

Original abstract:

"Minimal-invasive techniques are increasingly applied in clinical practice and have contributed towards improving postoperative outcomes. While comparing favorably with open surgery in terms of safety, the occurrence of severe complications remains a grave concern. To date, no objective predictive system has been established to guide clinicians in estimating complication risks as the relative contribution of general patient health, liver function and surgical parameters remain unclear. Here, we perform a single-center analysis of all consecutive patients undergoing laparoscopic liver resection for primary hepatic malignancies since 2010. Among the 210 patients identified, 32 developed major complications. Several independent predictors were identified through a multivariate analysis, defining a preoperative model: diabetes, history of previous hepatectomy, surgical approach, alanine aminotransferase levels and lesion entity. The addition of operative time and whether conversion was required significantly improved predictions and were thus incorporated into the postoperative model. Both models were able to identify patients with major complications with acceptable performance (area under the receiver-operating characteristic curve (AUC) for a preoperative model = 0.77 vs. postoperative model = 0.80). Internal validation was performed and confirmed the discriminatory ability of the models. An easily accessible online tool was deployed in order to estimate probabilities of severe complication without the need for manual calculation."[119]

While the report above defines risk factors for postoperative complications after laparoscopic liver resection for the broad population, we next sought to interrogate whether in high-risk scenarios safety of an operative procedure might be compromised. In this regard, lesions in the posterosuperior liver segments have been consistently described as the most difficult location to perform laparoscopic liver surgery. We thus moved on to assess in the final report whether in this patient population, liver cirrhosis may add another impediment towards achieving favorable outcomes.

3.5 Laparoscopic liver surgery in cirrhosis - Addressing lesions in posterosuperior segments

Haber PK, Wabitsch S, Krenzien F, Benzing C, Andreou A, Schöning W, Öllinger R, Pratschke J, Schmelzle M. Surg Oncol. 2019 Mar;28:140-144. doi: https://doi.org/10.1016/j.suronc.2018.12.001. Epub 2018 Dec 14.

PMID: 30851889

Original abstract:

*"**Background:** Minimal-invasive liver resection has gained considerable attention in recent years, assuming a weighty position in the field of HPB surgery. Even lesions in posterosuperior segments, the technically most challenging localization, have been resected while achieving comparable outcomes to laparotomy. The objective of this study is to evaluate whether the similar beneficial results can be conveyed through minimal-invasive techniques for patients with liver cirrhosis.*

***Materials and methods:** We retrospectively analyzed all consecutive patients undergoing laparoscopic liver resection with at least one lesion in the posterosuperior liver segments (IVa, VII, VIII) at our center between January 2012 and July 2018. Patients were separated in two groups based on the presence (n = 43) or absence (n = 115) of liver cirrhosis.*

***Results:** Preoperative patient characteristics showed that patients with cirrhosis were older (p < 0.001), had more frequently diabetes (p < 0.005) and a history of alcohol consumption (p < 0.0005). Preoperative liver function, as assessed by LiMAx score was markedly decreased in patients with liver cirrhosis (p < 0.005). While a similar percentage in both groups had anatomical resection, significantly more major resections were performed in patients without cirrhosis (cirrhosis: 23.3% vs. no cirrhosis 55.7%; p < 0.0005). Consequently, surgeries were markedly longer in the no cirrhosis group (p < 0.0005). There was no difference with regard to the need for perioperative transfusion or conversion to laparotomy. There was no differences found between both groups with regard to the postoperative course showing similar ICU- and hospital stays. Complication rate,*

both with regard to minor and major complications, as well as rate of clear resection margins were similar between the two groups as well.

Conclusion: *Patients with liver cirrhosis and a lesion in the posterosuperior liver segments are amenable to the minimal-invasive approaches as no significant differences can be observed with regard to safety and oncologic sufficiency. As these procedures are from a technical perspective challenging, they should be performed in specialized centers."*[120]

4 DISCUSSION

Over the past decade, treatment of primary liver cancer has made considerable strides across disease stages to improve patient outcomes. While several of the key developments that have facilitated this progress were developed separately, they are set to synergize given the increasingly multidisciplinary approach in clinical oncology. For example, in early disease stages, minimally-invasive approaches have led to a significant reduction of morbidity and mortality, paving the road for a broader application of potential adjuvant treatments that are currently under investigation in randomized controlled trials[121, 122]. As protracted clinical courses after resection are an impediment for adjuvant treatment, reducing this morbidity will be key not only for safety but also for oncologic outcomes once adjuvancy is available. Liver transplantation is the best therapeutic alternative to resection for tumors >3cm and provides superior oncologic results evidenced by markedly lower recurrence rates[13, 14]. However, the shortage of donor organs results in a shift of treatment allocation towards resection, even in those patients with more compromised hepatic function. Here, the establishment of minimally-invasive resection has provided a safe approach, markedly reducing the risk of post-hepatectomy liver failure[54]. As the tumors progress, patients default to other treatment options such as transarterial chemoembolization (TACE) for patients with intermediate stage HCC. Indeed, such patients have seen hardly any progress for nearly 20 years now since TACE was established. As a notable trend in both clinical practice and the trial landscape, treatments traditionally applied to treat either early-stage (resection) or advanced stage disease (systemic treatment) are increasingly explored in intermediate stage either as stand-alone or auxiliary therapies. In this context, new investigations are exploring whether the tumoral necrosis induced by TACE may be leveraged to enhance neoantigen presentation to T cells that can subsequently be stimulated by checkpoint-inhibitors to improve outcomes[123]. Finally, in advanced stage disease, a near decade long drought of futile RCTs following the establishment of sorafenib as frontline treatment has ended with multiple new drugs entering the clinical space in both first and second line[18, 19, 67-69, 71]. Importantly, combination treatments have been established to harness synergies mostly between checkpoint inhibitors and anti-angiogenics to prolong survival. This approach has yielded a considerable benefit for the patient population with median overall survival extended from ~12 to ~19 months[18, 19]. While the increase may appear modest to some, it should be noted that those patients that display radiological objective

response after treatment (27-33%) have an extraordinary benefit with expected median OS beyond three years. Consequently, the translational field has zeroed in on developing biomarkers of response and resistance to treatment and uncovering therapeutic vulnerabilities to enhance the treatment-sensitive population.

The presented work represents an effort to improve the outcome of patients with primary liver cancer in general and specifically HCC, incorporating translational and clinical studies. As the treatment landscape of HCC evolves it is worth noting that the therapeutic landscape will be increasingly interwoven in the future, adding to the complexity of clinical courses. Hence, it is paramount to establish sound rationales for patient selection to a given treatment to maximize therapeutic efficacy while maintaining acceptable drug/procedure safety. A central premise of this work is that immunotherapy with ICI is set to revolutionize the field and that much of the translational and clinical efforts in the future will revolve around broadening the relatively small proportion of the population that benefits from ICI. This is likely to be achieved through combination treatments for advanced stage, through synergizing with intraarterial treatments in intermediate stage and through reducing postoperative recurrence rates via perioperative application in early-stage. Four strategic avenues are pursued to further these developments through the work presented in this report: (I) First, by providing an immune-based molecular classifications of HCC that refines our understanding why some tumors retain key features that precipitate immunogenicity and, conversely, how other tumors evade the hosts immune system. Through integrative analysis on a mutational, transcriptomic and protein level we uncover novel subclasses and disentangle the molecular traits of the tumor that shape the ability of the immune system to identify and combat them. (II) Second, by identifying molecular drivers of response and resistance to anti-PD1 immunotherapy in a cohort of HCC patients with tissue available that precedes the initiation of systemic treatment and therefore can provide clinically informative predictive biomarkers that enable precision medicine in this space. (III) Third, by examining how clinical characteristics of the patient population may impact their responsiveness to ICI through a meta-analysis of randomized controlled phase III trials. (IV) Finally, by optimizing patient selection for minimally-invasive liver resection and understanding how the presence of liver cirrhosis and the technical difficulty of a specific resection impact outcome, this effort contributes to reducing postoperative morbidity. This work hypothesizes in this regard that prudent patient selection will, in the future, compound with ICI-based

systemic treatment to push the envelope in terms of allocating patients to curative treatment and reducing recurrence rates.

The first report, 3.1, disentangles the immune landscape of HCC[116]. Herein, the report builds on the identification of the *HCC Immune Class* that was previously uncovered based on transcriptomic features using a non-negative matrix factorization consensus clustering approach[112]. This subset of tumors accounts for up to 25% of HCCs across discovery and validation cohorts and is defined by a rich immune infiltrate with severe effector cell infiltration and molecular features that resemble melanomas responding to anti-PD1 checkpoint inhibition. Since the study did not examine the immune landscape of the remaining 75% of tumors and specifically, what molecular features shape the absence of a potent immune infiltrate in the majority of these cases, the present report was aimed at refining the immune-based classification by sharpening our definition of tumoral immunogenicity and unveiling mechanisms that prohibit the ensuing phenotype of the tumor microenvironment. The report 3.1. defines overall 35% of HCC tumors to be immunogenic, confirming the presence of the *HCC Immune class* with its subclasses *Immune active* and *Immune exhausted* on the one hand but also identifying a subset of tumors coined *immune-like subclass* on the other hand. This subset shows some features resembling the *Immune Class* such as high expression of immune checkpoints PD-L1, CTLA4, LAG3, active interferon signaling with enrichment in *IFNG*, *IRF1* and *IDO1* and expression of genes related to lymphocyte chemotaxis such as *CXCL9*. Analysis of Whole-Exome sequencing data revealed, however, that CTNNB1 mutations were significantly enriched among the *immune-like subclass*. RNA-seq analysis provided corroborating evidence as Wnt-βcatenin signaling was upregulated among these cases. We validated the presence of this novel subclass and its molecular features in validation cohorts using a specifically designed 20-gene expression signature that reliably identifies immunogenic HCCs. Expression of this signature was linked to response to ICI in an independent cohort of patients with tissue available prior to the initiation of treatment[124]. Moreover, leveraging a highly sensitive protein immune-oncology assay that is capable of simultaneously measuring ~80 plasma proteins, we were able to predict the presence of immunogenic HCCs using peripheral venous blood from the patients. Given the concerns regarding biopsy in patients with HCC, where tumor cell seeding is observed in 1-3% of patients, a blood-based biomarker that captures the immunogenicity of a tumor and may predict therapeutic success entails promising clinical utility, although this finding requires

further independent validation[125]. Aside from immunogenic HCCs, this report is also the first description of non-immunogenic HCC tumor classes and its molecular features. Since it stands to reason that, overall, these tumors are more likely to display resistance to ICI, uncovering therapeutic vulnerabilities to broaden the immune-sensitive population requires a detailed account of mechanisms by which these tumors either inhibit the formation a potent intratumoral immune infiltrate or how an immune infiltrate may be incapacitated towards exerting antitumoral immunity. The presented analysis revealed that 65% of tumors are non-inflamed and two subclasses are identified with molecularly distinct features that explain the phenotype: (I) the *intermediate class*, which is enriched in TP53 mutations that have been previously shown to tamper cytotoxicity of immune effector cells by recruiting suppressive stromal cells to the tumor microenvironment[126]. Moreover, genomic analysis unveiled that this subclass shows a high frequency of deletions in genes related to Interferon signaling which earlier studies have indicated is the key vehicle by which anti-PD1-boosted T cells initiate antitumoral cytotoxicity. Overall non-immunogenic tumors showed higher chromosomal instability than immunogenic tumors as accounted for by the broad score[127]. This finding is rather counter-intuitive as an increasing number of genomic alterations is thought to increase the odds of recognition of resulting neo-antigens by the immune system. Further studies are required to explain this finding. One explanation could be that observed instability fails to generate neoantigens but rather impedes antigen presentation and chemotaxis through the absence of pivotal chemokines and HLA molecules. In this regard, previous studies have highlighted the co-dependency of antigen presentation and IFN signaling and overcoming deletion-based defects through uncoupling these pathways may represent an attractive approach to sensitize these tumors towards ICI[128]. (II) The *Immune excluded class* that is characterized by a near absence of CD8+ Tcell infiltration, low expression of IFN-response genes and high frequency of CTNNB1 mutations accompanied by Wnt-βcatenin signaling. As indicated above these mutations also occur in the *immune-like* subset, although not linked to an immune desert phenotype. Evidence from several murine models have suggested that strong Wnt-βcatenin evokes an immune excluded phenotype through defective lymphocyte and, critically, dendritic cell recruitment[97]. Interestingly, this was reverted after experimental overexpression of CCL5, a chemoattractant, in the mice. The data obtained through the present study provides corroborating evidence for this as CCL5 was markedly downregulated in tumors within

the CTNNB1-mutated *immune excluded class* compared to CTNNB1-mutated *immune-like* tumors. Epigenetic analysis also revealed *excluded* tumors with strong Wnt-βcatenin signaling to feature demethylation of MHCI genes, that are critical for effective antigen presentation. Overall, this data reveals a heterogeneous tumoral transcriptomic state among CTNNB1-mutated tumors where the relative activation of immunosuppressive Wnt-βcatenin signaling in relation to immune cell recruitment dictates the phenotype. In the context of emerging studies in the field, this data prompted the hypothesis that CTNNB1 mutations as a binary factor are not applicable to predict resistance to ICI but that rather the transcriptomic state outweighs the mutation. This would reconcile conflicting reports that have shown CTNNB1 mutations to be linked to resistance to anti-PD1 in a biopsy series and in murine models and, conversely, to not impact response or survival in a recent liquid biopsy report[97, 129, 130]. Confirming this and exploring other molecular features of HCC subclasses as predictors of response in a translational study of HCC patients treated with ICI was a key strategic goal of the following efforts.

In the second report, 3.2, efforts shifted towards investigating how molecular features of a tumor define response status after ICI treatment with single-agent anti-PD1. The paucity of molecular studies developing predictive biomarkers to ICI in HCC and the prospect of integrating the molecular tumor classes that have hitherto only been shown to have prognostic relevance, prompted the establishment of a well-annotated cohort of patients treated with anti-PD1 for whom archived or fresh tissue was available. Integrating transcriptomic analysis with mutational assessment for CTNNB1 and immunohistochemistry the analysis provides a granular picture of drivers of response and resistance to anti-PD1 in advanced stage HCC, drawing cases from 13 referral centers in the US and in Europe. Differential expression analysis revealed genes related to IFNy-signaling and MHCII-dependent antigen presentation to be significantly upregulated among patients with radiologic objective response and longer OS and PFS. Indeed, gene set enrichment analysis confirmed these pathways to be highly differentially activated based on response status. Our analysis revealed that the composition of the tumor microenvironment is a key determinant of outcomes after anti-PD1 as a strong presence of proinflammatory M1 macrophages and CD4 memory T cells was linked to response, whereas immunosuppressive regulatory T cells precipitated primary resistance. Integrating our analysis with molecular classes of HCC, we found the *inflamed subclass* to be more likely to exhibit response than the

non-inflamed subclasses. Intriguingly, we confirmed that CTNNB1 mutational status was outweighed by the tumoral transcriptomic state in guiding response patterns, as CTNNB1 mutated *immune-like* tumors responded, whereas CTNNB1 mutated *immune excluded* tumors were resistant to anti-PD1. Overall, our findings regarding the key determinants of response are consistent with emerging studies that also underscored the importance of active IFN signaling at baseline in facilitating response and the detrimental effect of regulatory T cells[79, 124, 131]. A biomarker study from the CheckMate040 phase II trial investigating nivolumab has also contributed to this understanding and highlighted the importance of the composition of the immune cell infiltrate[131]. Our study entailed the development of a 11-gene expression signature that was reliably identifying responders and was predicting longer survival after anti-PD1 in our own cohort as well as in an independent HCC and four cohorts of other solid cancer types (melanoma, non-small cell lunger cancer, head and neck squamous cell cancer)[84, 124, 132-134]. As a major point of concern, the molecular features guiding response were only consistently identified in patients treated with anti-PD1 in frontline, whereas in the patients treated in 2nd or 3rd line after prior TKI therapy, any biomarker's predictive ability was compromised. This finding is particularly noteworthy as it collaterally implies the necessity of fresh biopsy to enable tissue-biomarker driven clinical decision making. Moreover, since the tissue originated in samples prior to the initiation of any frontline treatment, it stands to reason that TKI therapy may modulate response to subsequent anti-PD1 opening up the compelling prospect of expanding the ICI-sensitive population. The feasibility of this approach has been probed and confirmed through recent phase III clinical trials that have expanded the response rate from 15-20% after single-agent anti-PD1 to beyond 30% after combination treatment of ICI with anti-angiogenics[18, 135]. Indeed, the number of trials testing ICI alone or in combination in advanced stage HCC has jumped markedly in recent years enabling the thorough analysis of patients' characteristics and their relationship with response status.

The third report, 3.3., draws on the rich landscape of randomized controlled trials in HCC across tumor stages to provide a detailed analysis of reasons for success and failure. Through a metaanalysis of eight RCTs for advanced stage HCC we identify a marked heterogeneity in outcomes between drug classes according to the etiology of the underlying liver disease. Specifically, we find that in patients with chronic viral hepatitis patients have a statistically significant benefit from treatment with either an

ICI or an ICI-containing combination treatment as opposed to patients with non-viral hepatitis. Substantiating the notion that patients with viral hepatitis might draw particular benefit from ICI, no such trend was observed when patients were treated in trials with TKIs. Sensitivity analysis further confirmed this observation. While the report 3.2 lays out which molecular mechanisms govern response patterns, this metaanalysis indicates that the etiology of the underlying liver disease at least affects outcomes after ICI. Considering that this observation required >3700 patients to be identified, it can be speculated that the impact is comparatively mild. Interestingly, the amenability of HCC to ICI appeared consistently most pronounced among patients with HBV. Previous reports have indicated HBV-derived neoantigens to be a source of tumoral immunogenicity, which may prime ICI-boosted CD8+ T cells against tumor cells[136]. On the other hand, several independent reports have presented evidence that patients with non-alcoholic steatohepatitis (NASH) may derive little to no benefit from ICI[137-140]. In line with this, evidence from other cancer types has suggested the presence of metabolic syndrome, which is also linked to NASH, to be associated with T cell dysfunction and futility of ICI[141]. In HCC, one notable report has associated defective ICI-response with the rise of an exhausted T cell subset under anti-PD1 that would in fact promote carcinogenesis[137]. Moreover, a recent study out of NIH has revealed NASH to be linked to lower T cell motility and compromised effector function, which was reverted upon treatment with metformin in murine models[139]. Overall, it appears likely that the etiology-related heterogeneity in outcomes after ICI is fueled both by a more active immune infiltrate in patients with viral hepatitis as well as a by the detrimental effect of NASH. RCTs to date have failed to present subanalysis for the two most prevalent non-viral etiologies alcoholic liver disease and NASH[18, 19, 24, 25]. Providing this data will be paramount in future trials to deepen our understanding of how separate etiology-related effects compound to cause this heterogeneity.

Treatment of HCC with ICI is set to expand to intermediate- and early-stage disease. At intermediate stage the rationale behind designing trials in essence mimics the established approach from advanced stage, where ICI treatment efficacy is thought to be augmented by co-treatment. Thus, the drug's efficacy is tested against the active tumor burden. At early-stage disease this becomes more convoluted: traditionally, systemic treatments have been applied adjuvantly to reduce recurrence rates which may be less effective with ICI given the mechanism of action. Indeed, contrary to TKIs and traditional chemotherapy, ICI exerts its antitumoral cytotoxicity via another cell

type and are contingent on immune cell priming by tumoral neoantigens to boost antitumoral immunity[72]. The absence of a large tumor burden post-resection might thereby compromise the efficacy of ICI in an adjuvant setting although some trials across solid cancer types have shown ICI to be effective in reducing recurrence rates even when given only after resection[142-144]. In recent years, however, several trials have increasingly opted to apply ICI neoadjuvantly ± postoperatively or when a residual tumor burden remains after surgery, an approach that has been shown to elicit a meaningful benefit in terms of enhancing recurrence-free survival[145-147]. The rationale behind this appears sound: the tumor burden prior to surgery is leveraged to prime ICI-boosted T cells, that can perform immunosurveillance after resection and effectively combat any remaining tumor load and micrometastasis. Adjuvant maintenance treatment with ICI may lead to a sustained reduction in recurrence rates. Early evidence in HCC supports this approach as trials administering neoadjuvant cemiplimab, nivolumab+ipilimumab or cabozantinib+nivolumab have all shown promising early efficacy data[28, 81, 82]. From a surgical perspective, this evolving approach underscores the need to safeguard a patient's eligibility post-resection to continue the neoadjuvantly initiated systemic treatment. A major obstacle in this regard are postoperative complications that severely protract hospital stay and jeopardize timely recovery. Particularly in HPB surgery, where complications can lead to weeks-to-months long clinical courses, these patients may no longer be candidates for adjuvancy after recovery. Given the availability of percutaneous ablative treatment modalities that deliver acceptable oncologic outcomes, although inferior to resection, this highlights the need for prudent patient selection for surgery to minimize postoperative morbidity. The onset of laparoscopic liver surgery has already contributed to reducing complication rates but the complex nature of these procedures, that can entail technically more challenging resections, demand a separate analysis of feasibility and safety[39, 53, 55]. The clinical aspect of this report encompasses two separate studies, 3.4 and 3.5, that aim to refine current practice by providing analysis into factors precipitating the occurrence of complications and exploring the safety of minimally-invasive liver surgery for technically challenging resection types complicated by the presence of cirrhosis[119, 120]. Reports to date have established the notion that laparoscopic liver resection is at this point regarded as mature for distinct resection types such as left lateral sectionectomy[45]. Consistently, case series have shown LLR to be associated with overall lower morbidity, but little evidence is available regarding

LLR-specific risk factors for morbidity. Given the shift from open to minimally-invasive resection in the HPB field it can be hypothesized that LLR will be, as it is in our center, the default option in the near future, substantiating the need to identify risk factors for these procedures. Available studies have key shortcomings such as using subjective interpretation by individual surgeons, failing to account for the patient's general health or even neglecting patient-derived factors and focusing exclusively on the procedure[56, 148]. In report 3.4, a scoring system is established for the prediction of postoperative complications in patients undergoing LLR for primary liver tumors (HCC and iCC). Herein, a more holistic approach is taken, giving credit to patient history, the surgical technique applied and liver function. Focusing on the occurrence of major complications as accounted for by grades III-V of the Clavien-Dindo classification[149], uni- and multivariate logistic regression analysis identified five preoperative variables as predictors of postoperative morbidity: (I) diabetes, (II) high levels of alanine aminotransferase (ALT), that infers liver inflammation and compromised hepatic function and (III) iCC, which was associated with more complications than HCC, potentially linked to the need for extended resection and lymphadenectomy, (IV) the surgical approach, where standard multiport (MILL) had better outcomes than the hand-assisted (HALS) approach and (V) whether or not the procedure was a repeat procedure, which was linked to higher morbidity. The model derived from this analysis was able to predict morbidity at an AUC of 0.73 and 0.72 after bootstrapping validation (random sampling with replacement). Integration of two intraoperative variables, the length of surgery and the need for conversion, boosted the predictive ability to 0.85. As these variables represent surrogates of technical complexity, the scoring system may not only help in identifying patients at risk for postoperative morbidity but also define which patients require closer and longer post-surgical monitoring. Critically, the work may also benefit structured surgical training. In this scenario, procedures conducted in patients at risk, where the potential margins of error are narrow, may be reserved for later training stages[150]. As the use of logistic regression models generally entails complicated formulas, the herein developed models were deployed as readily available online tools providing risk estimation after input variables are provided.

Finally, the work presented in report 3.5 was based on the premise that LLR may attenuate previously reported differences in outcomes based on the presence of liver cirrhosis in patients undergoing open resection (OLR). Analysis was confined to patients undergoing LLR for lesions in the posterosuperior segments, that have been

established through scoring systems as the technically most challenging localizations with higher postoperative morbidity. Interestingly, we found that despite cirrhotic patients being older and having higher rates of diabetes and alcohol consumption as well as lower hepatic function defined by LiMAX (maximum liver function capacity) score[151], clinical outcomes were equivalent both in terms of safety as well as oncologic radicality. Overall, the study establishes that patients with liver cirrhosis undergoing technically challenging resections are amenable to LLR without compromising outcomes, which has since been acknowledged by current German HCC guidelines[152].

5 FUTURE DIRECTIONS

The progress made in different disease stages in the treatment of HCC are set to compound to improve patients' outcomes in the years to come. Thus far, the clinical course of an HCC patient has always been thought of as a linear trajectory, where therapeutic windows exist but are narrow and once missed, patients irreversibly default to the next option. Particularly in palliative disease stages, which in case of HCC are intermediate stage beyond extended criteria for LT and advanced stage, the treatment rationale has always been to only slow down disease progression to extend survival. This central paradigm is being modified by the onset of immunotherapy. In those patients responding to treatment, clinical outcomes are outstanding, even for patients with advanced stage disease. Importantly, there is increasing evidence showing that tumors downstaged by ICI may be subsequently treated with curative approaches. This would constitute a major change in clinical practice where therapeutic windows for curative procedures may be restored through precision oncology as recently demonstrated[82]. Excitingly, even LT has been successfully applied to treat patients with tumor burden downstaged by ICI to within the Milan criteria[153, 154]. While evidence here is still limited at this point, this may also have implications for donor organ wait-list management, where the therapeutic delta in terms of overall survival is likely higher between LT and intraarterial or systemic treatment compared to LT and resection/local ablation, which are the most frequent contingency options for HCC patients currently on the wait list.

Aside from adding layers of complexity to disease trajectory in HCC, ICIs are being introduced to earlier disease stages, where studies attempt to leverage their efficacy to reduce recurrence rates after resection or ablation. As referenced in the discussion, combined neoadjuvant/adjuvant treatment may in this regard be the most promising

approach, where the tumor burden can in essence serve as a priming load for T cells, that are subsequently boosted by ICI to conduct immunosurveillance and eliminate microscopic residual disease following resection. Early experience has demonstrated the feasibility of this approach[28, 81]. Reducing postoperative morbidity through minimally-invasive procedures to enable timely systemic treatment will therefore remain a priority in the field to ensure that a broad patient population benefits from emerging treatment protocols. Finally, further enhancing the ICI-sensitive population remains a challenge: while combination treatment of anti-PD1/PD-L1 with anti-angiogenics has improved response rates to 27-33%, a similar proportion of the population has no tangible benefit, exhibiting primary progressive disease[18]. Defining molecular rationales and designing clinical trials to find suitable combination drugs that rescue ICI-amenability in these patients is critical and will be the subject of several studies in the following years.

6 SUMMARY

Hepatocellular carcinoma is a leading cause of cancer related mortality globally and rising incidence rates are leading to an expected ~1.000.000 cases annually starting in 2025. Recent years have seen major improvements in the treatment landscape. Proven to be resistant to conventional chemotherapy, no consensus systemic treatment was available until 2008 when the TKI sorafenib was demonstrated to be superior to placebo in the seminal SHARP and AP trials. After nearly a decade of futile results from randomized controlled trials, new agents have entered the clinical space in the past five years. Most notably, the introduction of immune checkpoint inhibitors marks a turning point in the treatment of HCC: for patients responding to ICI-based treatment combinations, overall survival can extend beyond three years, but the vast heterogeneity in outcomes means that nearly two thirds of the patient population retain little to no clinical benefit and thus predictive biomarkers have been an unmet need in the field. Evidence from other solid cancer types suggests that the level and composition of pretherapeutic immune cell infiltration is key in determining outcome.

The present work includes a detailed unraveling of the immune landscape of HCC and delineates which factors impede immune cell infiltration into the tumor and thereby antitumoral immunity. Through an integrative multi-omics approach featuring RNA-seq, whole-Exome sequencing, TCR sequencing, immunohistochemistry and cytokine analysis we uncover the distinct immune-based subclasses of HCC: the inflamed

subtype that accounts for ~35% of cases and consists of the *Immune active*, *Immune exhausted* and *Immune-like* subgroups. These are characterized by strong effector T cell infiltration as well as the presence of M1 macrophages and enriched interferon signalling. As a standalone feature, the *Immune exhausted* subgroup shows strong stromal activation and exhausted T cell infiltration with heavy presence of PD1+ Tcell subsets as defined by multiplex IHC. The *Immune-like* subgroup in turn is enriched in CTNNB1 mutations and WNT-signalling, which is generally a feature of non-immunogenic HCCs. Here, however, other mechanisms appear to be at play that aid in overcoming such a phenotype. The non-inflamed class, which is an umbrella term for the *immune intermediate* and the *immune excluded* subgroup accounts for the majority of tumors and are defined by increased chromosomal instability with deletions in regions coding for key chemoattractants that are necessary to facilitate an immune response and CTNNB1 mutations, respectively. Our analysis reveals that on a prognostic level, immunogenic tumors appear to have better clinical outcomes with longer median overall survival. Next, we interrogated the relationship between the immune subclasses and outcomes after anti-PD1 therapy in HCC and established an international retrospective cohort of 13 centers from which we gathered tissue of patients pre-systemic therapy to develop predictive biomarkers. Transcriptomic analysis via microarray revealed the *Inflamed subclass* to be enriched among patients responding to anti-PD1 alongside strong IFNy signalling and MHCII-dependent antigen presentation. A predictive gene signature, recapitulating these pathways, was developed that predicted response to ICI in the discovery as well as several validation cohorts including HCC patients and those with other solid cancer types. Interestingly, in patients pretreated with a TKI between sample acquisition and anti-PD1 the gene signature and other reported biomarkers lost their predictive ability, highlighting the need for fresh biopsies to facilitate precision oncology in HCC.

HCC can develop on a multitude of different underlying liver diseases that promote the formation of liver cirrhosis. Chronic viral hepatitis B and C, alcohol consumption and non-alcoholic steatohepatitis (NASH) are the most common risk factors. NASH is associated with metabolic syndrome and hyperlipidaemia, two conditions that may undermine ICI efficacy according to recent reports. To evaluate this in HCC we performed a metaanalysis of high-quality phase III trials testing ICI and found that the presence of viral hepatitis was linked to superior clinical outcomes after ICI but not after TKI, whereas non-viral hepatitis (alcoholic liver disease, NASH) was linked to

poorer overall survival. This piece of data is supported by several preclinical studies highlighting the futility of ICI in patients with NASH although the lack of data specifying causes of non-viral hepatitis in randomized trials remains a drawback of the analysis. Overall, this data suggests a modification in trial design towards stratifying for NASH when randomizing patients to treatment arms.

The clinical application of ICI in HCC is gradually moving beyond advanced stage tumors. At intermediate stage ongoing trials are aiming at enhancing the efficacy of TACE, the default treatment, through combination with anti-PD1/anti-PD-L1. The rationale for this approach is to enhance T cell priming by inducing tumor cell necrosis and thereby setting cell-intrinsic neoantigens free. At early-stage disease, ICI is applied either neoadjuvantly, adjuvantly or combined to reduce tumor recurrence after resection or ablation which occurs in 50-70% of patients within five years. Paramount for safe and efficacious application is that the timeframe between surgery and systemic treatment remains short. In a scenario where adjuvant treatment is available, the occurrence of postoperative complications can interfere with adjuvancy and thereby render patients more susceptible towards recurrence. In the last decade, minimally-invasive procedures for liver resection are increasingly applied and have contributed to a reduction of the operative trauma with lower postoperative morbidity while retaining high-quality oncologic outcomes. The clinical studies of the present work aimed at further optimizing patient selection for minimally-invasive resection and exploring predictive factors of postoperative morbidity as well as feasibility of challenging resections in liver cirrhosis. Through logistic regression analysis a predictive model is developed and validated consisting of five preoperative variables that predict severe complications and mortality. This model consists of liver transaminase levels, the specific minimally-invasive approach, indication, prior liver resections and the presence of diabetes. The model outperformed previously published scores that failed to regard the occurrence of complications as a multifactorial process involving patient-, liver- and surgery-related factors. Finally, in a retrospective case series the feasibility of minimally-invasive liver resection for lesions in highly-challenging locations, the posterosuperior liver segments, was explored concerning the impact of underlying liver cirrhosis. Herein, we found that despite higher overall preoperative morbidity, high quality safety outcomes were achieved in cirrhotic patients, underscoring that this patient collective, traditionally more prone to complications, may benefit disproportionally from the minimally-invasive approach.

Overall, this work highlights therapeutic vulnerabilities in patients with HCC across treatment stages, drawing on translational and clinical aspects to improve patient selection and optimizing treatment allocation. As the treatment landscape is evolving substantially, validating how surgical concepts, novel drugs and inteventional procedures can be integrated will be the subject of future studies.

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9 ERKLÄRUNG

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