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Multidisciplinary perioperative strategies for improving short and long-term outcomes in hepato-biliary and pancreatic cancers

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To Alessandra e Bianca, my source of happiness

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Introduction

Epidemiology and outcomes of liver and pancreatic cancers

Hepatobiliary and pancreatic (HBP) tumors represent a formidable challenge in the landscape of oncology, demanding a nuanced understanding of the intricate anatomy and physiology of the liver, bile ducts, and pancreas as well as of the behavior of these cancers. Their incidence has been on the rise globally, making these pathologies a major cause of morbidity and mortality worldwide and therefore a huge socioeconomic problem. The economic and social implications of these cancers derive moreover from the current interest in their main risk factors, such as chronic viral hepatitis, non-alcoholic fatty liver disease and lifestyle-related contributors, which underscore the urgency of understanding and addressing these malignancies. Incidence rates of liver cancers have more than tripled since 1980, while the death rates have more than doubled during this time.(1) More than 800,000 people are diagnosed with this cancer each year throughout the world, making these cancers the sixth most common neoplasia in terms of prevalence. Liver cancer is also a leading cause of cancer deaths worldwide, accounting for more than 700,000 deaths each year and thus ranking as the third most important cancer for mortality.(2) Numbers are slightly inferior for pancreatic tumors, with about 500,000 new cases in 2020, but with more than 460,000 deaths per year making this cancer one of the first in lethality. Part of the aggressiveness of HBP tumors is related to the advanced stage at diagnosis, with lesions often silent and asymptomatic for months before being discovered. In these cases, and thus in the presence of an unresectable or metastatic disease, care is almost always exclusively oncologic, with the deliverance of systemic therapies and no room for other types of approaches. Outcomes are extremely poor, and no curative option can be proposed although the recent development of new strategies in the therapeutic armamentarium as immunotherapies, or different types of target therapies. Things change in

case of a resectable disease, where a curative pathway can be undertaken and a chance of healing offered to the patient. However, this path is often a multistep and multidisciplinary process in HPB cancers, which represents a distinctive feature of the pathologies of this sphere. Experienced radiologists are needed for a correct diagnosis, with the necessity of having a vascular and biliary expertise in case of jaundice or vascular procedures needed. Endoscopists are an essential figure in the perioperative care, often called into question to improve diagnostic accuracy and for operative managements. Oncologists represents a key step in the curative pathway, tailoring the best treatment according to patient status and tumor genetic background. These figures, together with others of the same importance as hepatologists, onco-geneticists and researchers, are indispensable to a correct and complete approach of these cancers.

Hepatocellular carcinoma

General overview

The most frequent liver tumor is hepatocellular carcinoma (HCC), which accounts for around 80% of all hepatic cancers and is the third most common cause of cancer-related death worldwide. This tumor, which originates from hepatocyte cells, has a poor prognosis, with a 5-year relative survival rate of around 20%.(3) Even in case of surgically-treated resectable disease, outcomes are not significantly different, and the recurrence rate remains high, reaching 70-80% after 5 years. However, surgical resection is only a part of the therapeutic armamentarium of these cancers, and other strategies have to be discussed and proposed in parallel with surgery.(4) Experienced hepatologists and radiologists are in fact necessary in a high-level HBP team both for evaluating alternatives to surgery, as liver transplantation or percutaneous thermal ablation, as well as to prepare the patient for surgery, as in case of underlying cirrhosis and portal hypertension or insufficient future liver remnant. When a curative treatment is not anymore indicated, the same specialists are involved for palliative treatments, together with oncologists. The concept of collaboration and multidisciplinary in

these advanced cases keeps its importance in order to deliver the best protocol according to patient's performance status, liver function and, probably in the next years, tumoral molecular background.(5) Understanding the genetic pathways involved in the development of this disease has in fact improved in recent years, with specific mutations found in around 25% of HCC, but we are still far from delivering specific drugs according to the genetic context.(6) Anyway, the concept of target therapies and personalized medicine is increasingly topical and it will be able to identify the best treatment which perfectly fit for each patient.

Surgical resection

The treatment of choice of HCC is nowadays considered surgical resection. Alternative strategies, as percutaneous ablation or liver transplantations, are proposed as convenient options in certain conditions and sometimes offer a better chance of cure over simple resection,(7) but present the limit of a low global applicability, as in case of larger lesion for ablation procedures or age and HCC on non-cirrhotic liver for liver transplantation.(8) Treatment flow-chart is complex and depending on several variables, which is currently under the guidance of the recently uploaded BCLC criteria (**Figure 1**).(4) The feasibility of liver resection is largely dependent on the quality of the underlying liver parenchyma, as well as the extent of planned resection. Owing to recent advances in surgical techniques and perioperative care, perioperative mortality after HCC resection among patients with cirrhosis is now less than 5%, yet postoperative liver decompensation still ranges from 10% to 12%.(9) Stretching the boundaries in these patients, given the low efficacy of systemic treatments, has led to the surgical treatment of larger lesions which are known to be associated more often with important pathologic variables as micro- or macrovascular invasion and satellite nodules.(10) This translates in a higher risk of recurrence which, in a context of major resection for the large HCC, often preclude any other curative treatment. As it happens in other oncologic scenarios, this issue should be solved by neoadjuvant treatments, which are currently lacking in HCC work-flow.

Some procedures are globally used with a weak scientific background as transarterial chemoembolization or transarterial radioembolization, but their role is always under debate.

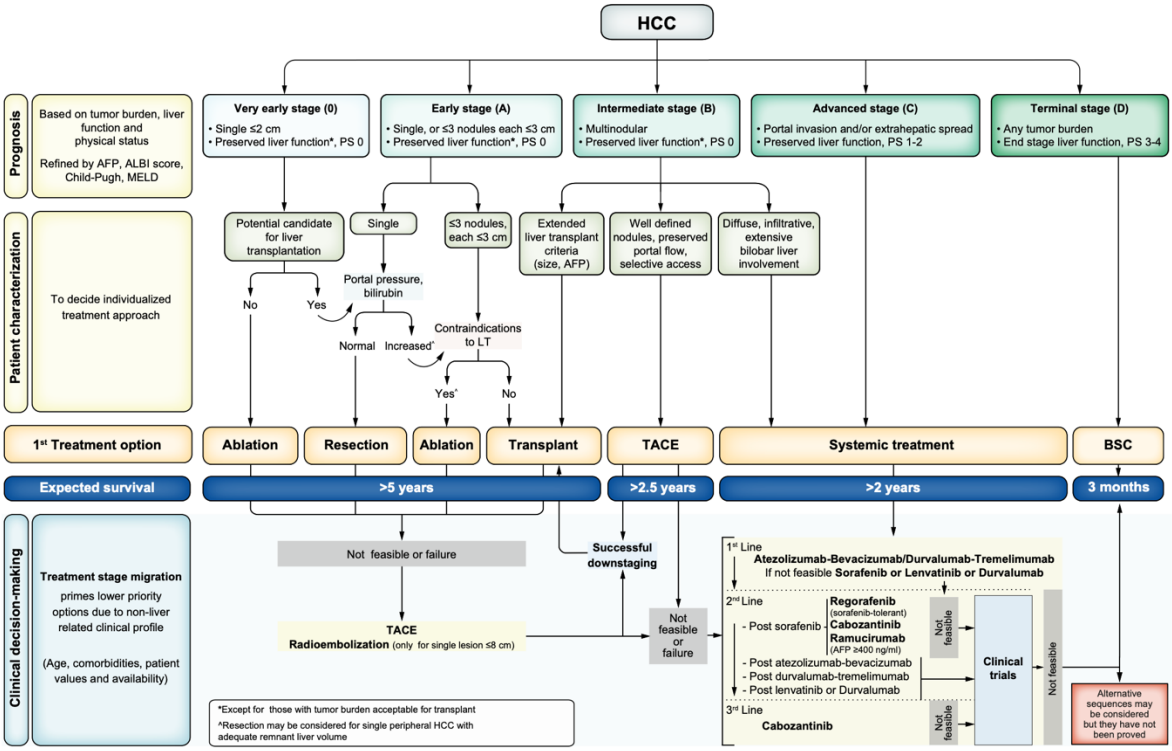

Figure 1. BCLC staging and treatment strategy in 2022. The BCLC system establishes a prognosis in accordance with the 5 stages that are linked to first-line treatment recommendation. The expected outcome is expressed as median survival of each tumor stage according to the available scientific evidence. Individualized clinical decision-making, according to the available data on November 15, 2021, is defined by teams responsible for integrating all available data with the individual patient's medical profile. Note that liver function should be evaluated beyond the conventional Child-Pugh staging. AFP, alpha-fetoprotein; ALBI, albumin-bilirubin; BCLC, Barcelona Clinic Liver Cancer; BSC, best supportive care; ECOG-PS, Eastern Cooperative Oncology Group-performance status; LT, liver transplantation; MELD, model of end-stage liver disease; TACE, transarterial chemoembolization.

Imaging in surgical scenario

Preoperative imaging is essential in HCC, and probably a correct radiologic evaluation acquires greater prominence in this pathology due to the possible cirrhotic background. HCC is in fact one of the rare cancers which diagnosis is based on preoperative imaging, without the need of percutaneous biopsies is underlying cirrhotic liver is presumed.(11,12) Furthermore, CT scan accuracy in detecting satellite nodules or lesions <2 cm is not satisfactory, with the

need to resort to MRI.(13) Imaging techniques are as well useful in the surgical setting, where ultrasound or indocyanine green (ICG) are constantly used for different aims, as for tumor localization, anatomical landmarks or intrahepatic biliary or vascular structures. However, all these methodologies allow an anatomical and "rigid" view of liver parenchyma, without any information on liver function. Prediction of liver failure is in fact currently based on the measure of liver volume, associated to the test of ICG clearance, but they do not take into account surgical events, as for example excessive hepatic clamping. These conditions are at the basis of the liver ischemia and reperfusion injury which could ends in a postoperative liver failure although preoperative volumes were reassuring and surgical gesture uneventful. (14,15) This consideration is particularly important in certain situations such as major liver resections on altered parenchyma and when future liver remnant volume reaches limit values. Intraoperative functional data are therefore necessary and hyperspectral imaging (HSI) has recently been applied to the medical field as a tool for image-guided surgery, with the possibility of quantify tissue perfusion and oxygenation.(16) HSI images are based on the computational analysis of light-tissue interactions through the detection of relative reflectance, giving a quantification of organic compounds such as deoxygenated and oxygenated hemoglobin at different depths (**Figure 2**).(17) Data obtained through HSI in preclinical studies demonstrated how images acquired with this tool and processed with artificial intelligence could be correlated with ischemic liver damage. Clinical studies are therefore needed to explore potentialities of this device.

Systemic treatment

For nearly a decade, sorafenib was the only effective systemic therapy for HCC with trivial improvements in survival compared with best supportive care.(18) Recent development and approval of new systemic therapies have led to many new options to treat patients with advanced HCC as first- or second-line therapy (**Table 1**).(19,20)

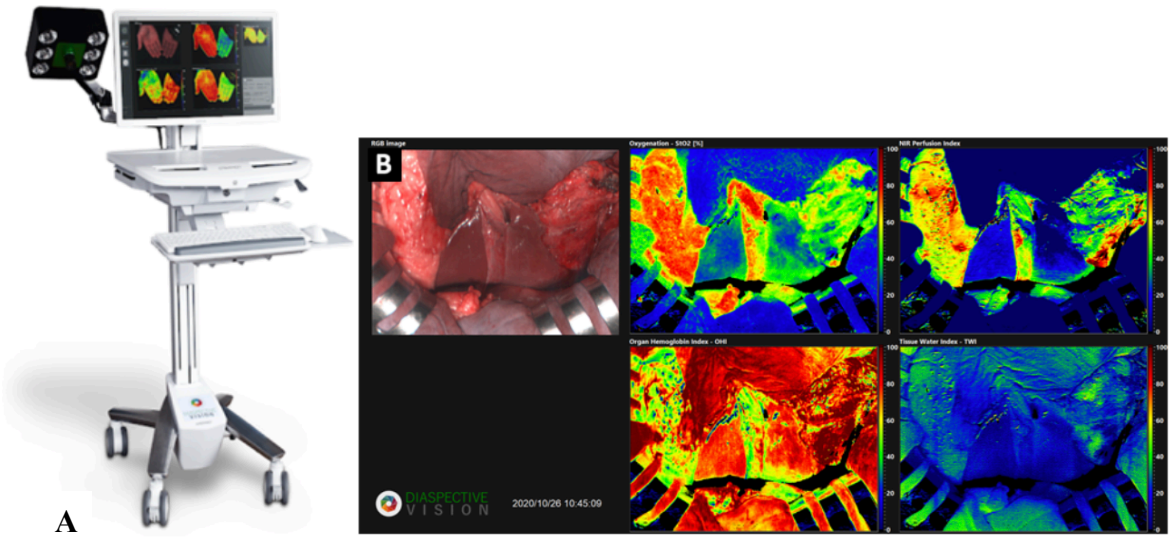

Figure 2. Hyperspectral imaging. A) Intraoperative device for hyperspectral camera (TIVITA, Diaspective Vision). B) Images acquired at the beginning of a liver resection. The RGB (Red-Green-Blue) images and StO2%, NIR, OHI and TWI indexes are showed.

Atezolizumab (anti–programmed death-ligand 1) and bevacizumab (anti–vascular endothelial growth factor) (atezolizumab plus bevacizumab) became standard of care first-line therapy for patients with untreated advanced HCC after demonstrating improved PFS and OS vs sorafenib. Although the improvement in the management of these patients, response to therapy is highly heterogeneous and several factors are implicated in therapy efficacy. These variables are, however, mainly biological or genetic, and molecular landscape or elements of the tumoral microenvironment (TME) are increasingly being studied by researchers to find the key to personalized medicine. In preclinical studies for instance, non-alcoholic fatty liver disease (NAFLD) has been noted to alter the TME, as CD4+ T cells, which alters response to immune based therapies.(21,22) Indeed, a subgroup analysis from the Atezolizumab in Combination With Bevacizumab Compared With Sorafenib in Patients With Untreated Locally Advanced or Metastatic Hepatocellular Carcinoma (IMBrave150) study found an objective response rate for patients with nonalcoholic steatohepatitis–related HCC was 27% vs 35% for individuals with other etiologies of liver disease.(23) As the understanding of resistance to systemic therapies continues to evolve, a personalized treatment approach may be increasingly adopted that is

based on underlying genetic variations thereby hopefully improving response rates. This goal compulsorily passes from the work of researchers, starting from studies on *ex vivo* models.

Drug	Source	Phase	Comparison	Results
First-line				
Atezolizumab plus bevacizumab	Finn et al,[20] 2020 NEJM	3	Sorafenib	Improved OS and PFS
Sorafenib	Llovet et al,[66] 2008 NEJM	3	Placebo	Improved OS and time to radiologic progression
Lenvatinib	Kudo et al,[67] 2018 Lancet	3	Sorafenib	Lenvatinib non-inferior to sorafenib in OS
Durvalumab	Abou-Alfa et al,[68] 2020 J Clin Oncol (Abstract) (HIMALAYA)	3	STRIDE and sorafenib	STRIDE with improved OS compared to survival and non-inferiority of single agent dirvalumab compared to sorafenib
Pembrolizumab	Verset et al,[69] 2022 Clin Cancer Res	2	NA	ORR 16%, duration of response 16 mo, DCR 57%, OS 17 mo
Nivolumab (ineligible for TKI or other antiangiogenic agents)	Yau et al,[70] 2022 Lancet Oncol (CheckMate 495)	3	Sorafenib	OS 16.4 mo with nivolumab and 14.7 mo with sorafenib; *P* = .075
Second-line				
Regorafenib	Bruix et al,[71] 2017 Lancet (RESORCE)	3	Placebo	Improved OS
Cabozantinib	Abou-Alfa et al,[72] 2018 NEJM	3	Placebo	Improved OS and PFS
Ramucirumab	Zhu et al,[73] 2019 Lancet Oncol (REACH-2)	3	Placebo	Improved OS and PFS
Nivolumab plus ipilimumab	Yau et al,[74] 2020 JAMA Oncol (CheckMate 040)	1/2	Variable doses of nivolumab plus ipilimumab	Manageable safety, ORR and durable response
Pembrolizumab	Finn et al,[75] 2020 J Clin Oncol (KEYNOTE-240)	3	Placebo	OS and PFS did not reach statistical significance: risk-to-benefit ratio for treatment
Nivolumab	El-Khoueiry et al,[76] 2017 Lancet (CheckMate 040)	1/2	Dose-escalation and dose-expansion	ORR 20%
Dostarlimab-gxly	Andre et al,[77] 2021 J Clin Oncol (Abstract) (GARNET study)	1	NA	ORR in dMMR 38.7%, CR 7.5%

Table 1. Systemic therapies in HCC. Abbreviations: Clin Cancer Res, Clinical Cancer Research; DCR, disease control rate; dMMR, mismatch repair deficiency; GARNET, TSR-042, an Antiprogrammed Cell Death-1 Receptor Monoclonal Antibody, in Participants With Advanced Solid Tumors; HIMALAYA, Durvalumab and Tremelimumab as First-line Treatment in Patients With Advanced Hepatocellular Carcinoma; J Clin Oncol, Journal of Clinical Oncology; JAMA Oncol, JAMA Oncology; KEYNOTE-240, Pembrolizumab as Second-line Therapy in Patients With Advanced Hepatocellular Carcinoma; Lancet Oncol, Lancet Oncology; NA, not applicable; NEJM, New England Journal of Medicine; ORR, objective response rate; OS, overall survival; PFS, progression-free survival; RESORCE, Regorafenib After Sorafenib in Patients With Hepatocellular Carcinoma; STRIDE, Strategies to Reduce Injuries and Develop Confidence in Elders; TKI, tyrosine kinase inhibitor.
Image from Brown et al.(8)

Intrahepatic Cholangiocarcinoma

General overview

In terms of frequency, the second most common primary liver tumor is intrahepatic cholangiocarcinoma (ICC), with a worldwide incidence and mortality rising in recent years.(24) The 5-year survival rate is estimated at around 9-11%, with a wide variation depending on the stage and treatment offered at the time of diagnosis.(25,26) Surgery, followed by adjuvant therapy with fluoropyrimidine or gemcitabine, is the only curative option available for non-metastatic, resectable disease.(27) Unfortunately, recurrence of ICC occurs in almost half of patients resected within one year.(28) Factors responsible for this unfavorable outcome are definitely the underlying comorbidities, the unquestionable tumor aggressiveness as well as the lack of a demonstrated efficacy of neoadjuvant treatments in the case of resectable diseases. Contrary to the developments in the management of liver metastases from colorectal cancer, in ICC it has not been demonstrated that neoadjuvant treatment, and even adjuvant in some cases, is of any benefit to surgery. ICC tends in fact to systemic dissemination in a very early stage, with a high risk of nodal or hematogenous metastasis at diagnosis. Effective systemic therapies are therefore crucial as well as the knowledge of molecular and biologic features which are at the basis of this aggressive behavior. Pathologic factors are in fact just an expression of the genetic background of the tumor, which is the key to understand the heterogeneous response to systemic treatment. The close collaboration between surgeons, oncologists, researchers and oncogenicities is therefore crucial to deliver a personalized medicine for these tumors, field to be largely explored since the validation of target therapies in clinical practice.

Systemic treatment

<u>Resectable patients</u>

General interest is currently moving to target therapies and immunotherapies in ICC. Since the recent approval of durvalumab, in association with capecitabine and gemcitabine as

a first line treatment for unresectable and metastatic patients,(29) as well as the validation of anti-IDH or anti-FGFR2 drugs in second line, this field has gained much importance among researchers and clinicians.(30) Several possibilities are now available according recent guidelines in these patients but curative options remains limited to surgical resection.(31) New systemic perspectives are in fact not indicated in a surgical scenario and chemotherapy still makes use of an old and not always effective capecitabine regimen. It is paradoxical how the field of personalized medicine makes great strides in advanced cases, where prognosis is extremely poor and no curative options still exits, whereas in treatable patients no changes has been applied in the standard work-flow for several years. If a first step should be taken in this sense, it would involve the understanding of tumor biology. Biliary tract cancers are in fact grouped together in current guidelines and in treatment modalities, but they represent different anatomical, biological and genetic pathologies. ICC for instance show a particular genetic background which is not present in gallbladder or extrahepatic bile duct cancer.(32) Even resectable and advanced ICC could be two different entities, whose aggressiveness is therefore related to a divergent underlying biology. Approaching this field in curative cases needs therefore a state-of-art of the genetic landscape of resected ICC.

Unresectable or metastatic ICC

Even in advanced cases new paths of possible perspectives have been evocated in literature despite the optimism from new therapeutic armamentarium (**Figure 3**). While response to target therapies is directly correlated to mutational status (on which depends the administration of these drugs), variables associated with the heterogeneous response observed to immunotherapy are less known and explored. In other cancers, where immunotherapy have been applied for several years, specific immune cellular populations in TME has demonstrated to be correlated with patterns of response.(33) These aspects have already been demonstrated in liver cancers for HCC.(22,34) However, in ICC main characters could differ compared to

HCC and mechanisms vary, involving dynamic changes of the immune population and of their immune potential. Transversal researches are therefore needed in literature based ideally on *ex vivo* complex models, as organoids, which could reproduce TME, or through Single cell RNA sequencing (scRNA-seq) on tumoral biopsies. Understanding the complex interplay between various cell types in the tumor microenvironment and treatment resistance could in fact pave the way to new developments in personalized medicine for ICC and for all biliary tract cancers

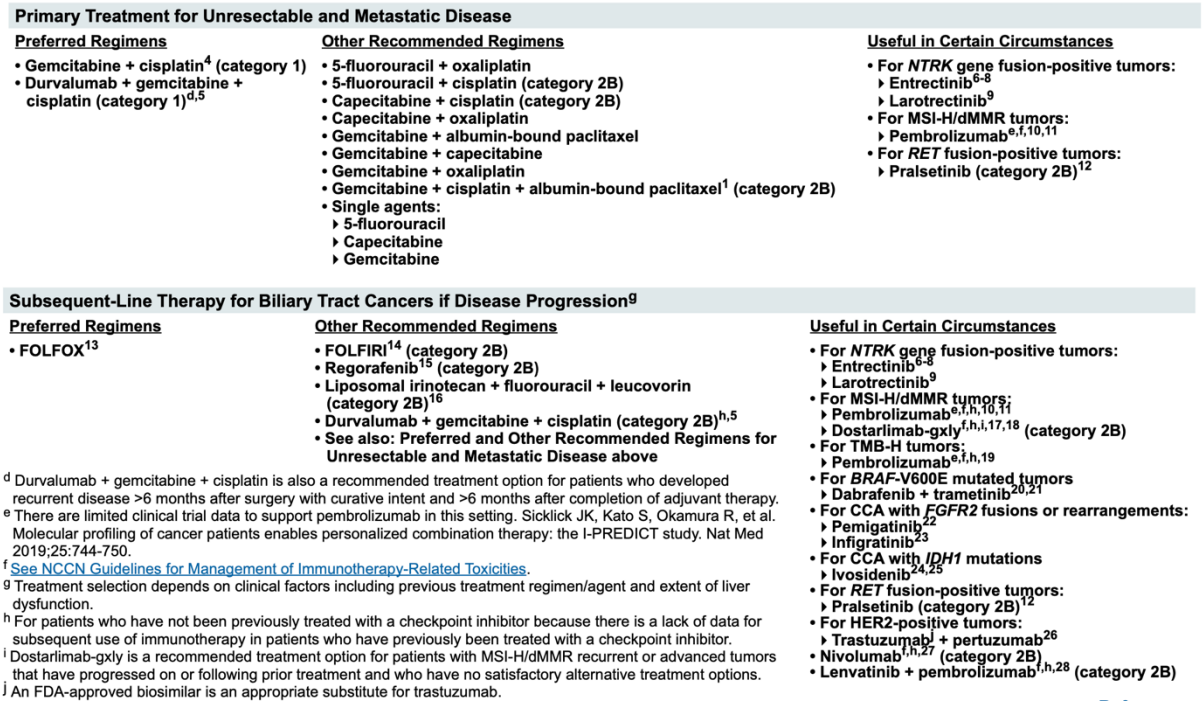

Figure 3. Principles of systemic therapy in biliary tract cancers for unresectable and metastatic patients. *Image from NCCN guidelines for Hepatobiliary Cancers.*(29)

Pancreatic Ductal Adenocarcinoma

General overview

Results are no more optimistic for pancreatic cancer, where the most common histological type is pancreatic ductal adenocarcinoma (PDAC). PDAC is expected to become the second leading cause of cancer-related mortality by 2030.(2) It is an aggressive disease, with an overall 5-year survival rate of less than 10%, and surgical resection represents the most important prognostic

factor associated with long-term survival. Adjuvant chemotherapy has been widely validated in PDAC, irrespective of stage and various prognostic factors.(35) On the contrary, the usefulness of induction chemotherapy is widely debated, mainly because of the risk of progression due to non-response to treatment.(36,37) Preliminary results from some ongoing trials do not demonstrate the efficacy of preoperative systemic therapy in resectable disease in an intention-to-treat analysis. Anatomical criteria are currently used to classify pancreatic disease as resectable, borderline or advanced, according to which chemotherapy should be delivered.(27) However, they do not reflect the aggressiveness and biology of the tumor, and are quite variable depending on the center and the surgeon's technical expertise. Apart from a significant elevation in the tumor marker CA 19-9, there are no other criteria for predicting preoperatively patients who would not benefit from surgery.(38)

Personalized medicine in PDAC

Predict response to systemic therapies

Among the three cancers on which this thesis is developed, PDAC surely represents the most difficult to approach and the one for which fewer innovations have occurred in the field of personalized medicine. Reliable preoperative prognostic factors are lacking and although the recent developments in the oncologic armamentarium, therapies as FOLFIRINOX, Gemcitabine + Abraxane or other drugs are delivered without any molecular or biological indicators. Furthermore, target therapies or immunotherapy are not currently available options. The most frequent mutation is in fact KRAS, associated with poor outcomes and for which promising target drugs do not exist.(39,40) Response to therapy is therefore highly heterogeneous and absolutely unpredictable, without considering that recourse to chemotherapy is based to surgical issues (vascular contact) rather than aggressiveness of the disease. In other gastrointestinal caners, as rectal, gastric or esophageal, this indication is in fact dependent on the size of the lesion or on the nodal invasion, variable this last independently associated with

long-term outcomes and not taken into account in PDAC. By the way, accuracy in detecting nodal metastasis is very low.

Imaging modalities

In pancreatic field, echoendoscopy (EUS) is an essential tool which assumes fundamental relevance in the diagnostic and therapeutic approach of all the type of pathologies. In pancreatic cysts for instance, it presents a higher accuracy rate and reduce the risk of incorrect diagnosis.(41) As regards nodal invasion in pancreatic cancer, EUS is theoretically available to give deeper information compared to CT scan or MRI, thanks to the elastography or the contrast-enhanced EUS,(42) but prospective studies are missing.

In the perioperative setting no imaging modalities are currently available, and ICG has not demonstrated any efficacy in terms of tumoral detection or surgical guidance. New technologies are therefore needed in clinical practice in order to give detailed information on surgical samples and, at the same time, solve the problem of tumoral profiling to guide treatment allocations. Optical coherence tomography (OCT) and its 3D variant Full-field OCT (FF-OCT) are innovative techniques for imaging living tissues based on a low coherence light beam. Their application in the field of oncology has been growing over the past decade, for the imaging of a broad spectrum of malignancies.(43) FF-OCT also allows to reveal information on the tridimensional tissue architecture, which is disorganized in cancerous biopsies, without previous sample preparation.(44) Various studies have been carried out, demonstrating the efficacy of this technology for 3-D imaging of ex vivo specimens. Dynamic FF-OCT (D-FF-OCT) is a novel generation of device that analyzes time dependent FF-OCT images to reveal pixelwise high activity zones. It takes advantage of the intracellular dynamics of cells to add new contrast recognition based on cell motility, metabolism, and cell mitotic state (**Figure 4**). Combining analysis of 3D architecture and cell distribution, D-FF-OCT appears to be a breakthrough technology for real-time non-invasive histological studies without sample

preparation that offers a view of the sample resembling standard H&E histology, which is a major breakthrough for tool adoption in the anatomical pathology community.(45)

In this field our research was focused on this clinical need and on the necessity to apply these tools in clinical practice in order to find more reliable indexes of tumor aggressiveness as well as factors associated with response to systemic treatment. At the same time, we tried to establish new collaborations to develop new perspectives in the translational research of pancreatic cancer.

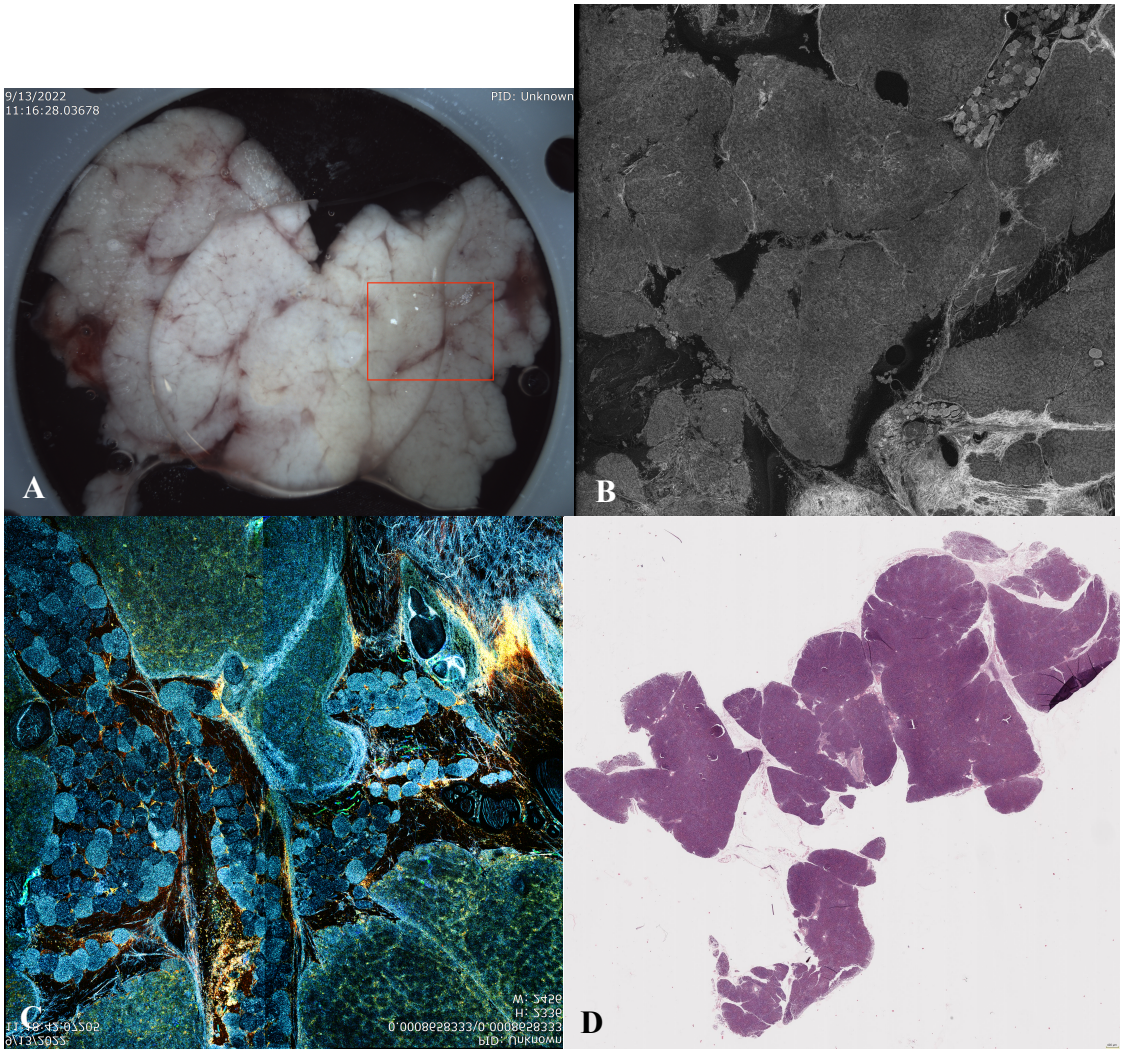

Figure 4. Images acquired through phases of D-FF-OCT. A) Macroscopic image of a pancreatic stump during a pancreatic resection. B) Image of a 13x10 mm portion of tissue at Full-Field OCT. C) Same image at Dynamic Full-Field OCT. D) Classic hematoxylin-eosin staining of the pancreatic slice

The concept of multidisciplinary

The management of primary cancers of the liver and pancreas often goes through complex and sometimes questionable strategies. When faced with the diagnosis of a primary HBP tumor, the best strategy is chosen according to the underlying liver disease and comorbidities, the biology of the tumor, its local or distant extension, and the possibility of considering surgical resection. A close collaboration between researchers, surgeons, endoscopists, hepatologists, radiologists and oncologists is therefore required, both in the clinical context and in proposing cross-disciplinary research. This partnership results in a great benefit to the patient both in the short term, by improving the global approach to the disease and perioperative outcomes, and in the long term, through the construction of new strategies and personalized approaches.

Through development of this concept, the aim of this thesis are i) to evaluate the role of perioperative treatments in the management of primary cancers of the liver and pancreas, and to assess their response by analyzing data from different pre- and intraoperative imaging modalities ii) to determine predictive criteria for response to different treatments, based on clinical data, histopathological data, as well as the tumor's genetic background, and iii) develop new strategies for reading certain intraoperative imaging techniques, including artificial intelligence automation technologies, which could modify the management of these tumors.

Objectives

This thesis is based on this concept of multidisciplinary, and thus on the joint teamwork between different specialties that should be always present and available in a high-level HPB center. Clinical and research activities will merge in this project, with the common and unique aim of the evaluation or the development of different strategies to approach these cancers in order to improve short and long-term outcomes. Radiologic and endoscopic procedures, transversal research, innovative technologies and clinical data will be touched in a continuous interaction by this multifaceted work which, of course, will have its evidence with high-profile manuscripts or projects. Although it will be more difficult to demonstrate in this research scenario, this work will also result in the growth of our unit from a clinical perspective, with the implementation of new tools for taking care of these patients. The thesis is divided into three main parts, each different in type of pathology and aim. As mentioned, these parts will be united by a common thread, which is to improve short and long-term outcomes of these cancers through a multidisciplinary management, but at the same time will be differentiated by theme, type of collaboration and working methodology, in order to better explore this concept of multidisciplinary. This work will be possible through a close collaboration with the IHU - Institut de chirurgie guidée par l'image de Strasbourg, the Pôle Hépato-digestif of the Nouvel Hôpital Civil of the Hôpitaux Universitaires de Strasbourg and the INSERM Unit UMR_S1110. Each part will therefore exploit and benefit from the expertise of these units, with a deep and meticulous collaboration, which are the foundations of this project. In this context, my active role will be twofold, through the direct creation and the consolidation of these partnerships and, at the same time, of developing and finalizing - directly or under the supervision of all the team - several publications.

The first part of the thesis will be mainly focused on the global management of HCC. We started with a pure clinical aim trying to demonstrate if preoperative transarterial

chemoembolization, a radiologic procedure normally performed in patients with a locally advance disease and with a good hepatic function as a palliative treatment, could improve outcomes in patients undergoing liver resection for large HCC. This procedure is in fact used by different centers, including our own, in tumors larger than 5 cm, but its scientific evidence in literature is not debated. Furthermore, given the implementation of the transarterial radioembolization in our hospital, a future comparison between these two procedures could be done in the neoadjuvant setting, considering the current absence of effective perioperative systemic treatments. We then moved to the area of the artificial intelligence thanks to the synergy with the IHU of Strasbourg and IRCAD, two cutting-edge structures in this field. Connecting to the previous subject, and due to the risk of postoperative liver failure in these patients necessitating almost always a major resection in a precarious hepatic function, the second goal will be based on the perioperative use of an innovative imaging system, the hyperspectral imaging, in order to try to predict this complication. Finally, we worked in the translational research in collaboration with the Inserm laboratory (UMR_S1110), with the double aim of i) identifying preoperative tools influenced by tumor molecular background useful to select patients with a poor prognosis and, at the same time, ii) establish a patient-derived spheroid model recapitulating HCC heterogeneity and TME for drug screening in individual patients. This was possible through the creation of the LIVMOD biobank, which is actively and constantly enriched with high quality operative specimens from cirrhotic and non-cirrhotic patients affected by liver tumors.

The second chapter has as its main target ICC. General interest is now focused on the biological background of these tumors, given the approval of target therapies in unresectable cases according to genetic status or immunotherapy. For this reason, we focused on this subject with a strict collaboration with the department of molecular biology of our hospital and the Inserm laboratory (UMR_S1110). The first goal was a comprehensive review of the genetic

background of tumors with a curative prospective, therefore after surgical resection, in order to explore how certain mutations could influence long-term outcomes. This issue is in fact addressed in literature for all biliary tract cancers and no matter their staging, without therefore considering the biologic heterogeneity compared to resectable tumors. A second paragraph of this chapter on ICC will be focused on the recently approved treatment, that is immunotherapy. These drugs have already been validated for other cancers and in these cases, it has been demonstrated how response to treatment is highly heterogeneous, and this could be correlated with the activity of tumor microenvironment (TME). In collaboration with the Inserm (UMR_S1110), we thus built up two projects, on ICC and on biliary tract cancers, respectively, with the goal of exploring TME in biopsies of patients receiving the recently approved durvalumab.

The third and last chapter is dedicated to pancreatic cancer. The management of these tumors is really complex and innovations in biological and genetic fields are still far from being implemented in clinical practice. Our work was twofold, always in strict partnership with the IHU of Strasbourg. The first objective was to assess the role of echoendoscopy in detecting nodal metastasis, the most important prognostic factor after pancreatic resection. Accuracy is in fact rather low and echoendoscopy has the possibility to use a dynamic vision and different tools, as elastography and doppler to create a complex score to improve sensibility and specificity. Secondly, we established a multidisciplinary translational research project aimed to improve real-time diagnosis and subsequent prediction of tumor response to treatments, using cutting edge AI- augmented histological imaging and functional profiling, all in order to foster precision medicine of PDAC. Resected pancreatic tumor specimens will be analyzed through the new technology of the Dynamic Full-Field Optical Coherence Tomography (D-FF-OCT) to assess if features captured by this new technology can predict treatment response. Furthermore,

a collaboration with the Luxembourg Institute of Health, allowed us to create tumoral organoids for drug testing.

Results

First part.

Multidisciplinary approach in Hepatocellular Carcinoma

1.1 Radiologic peri-operative treatments to improve outcomes in large HCC

The first chapter of this thesis is dedicated to HCC. This tumor more closely represents the concept of the multidisciplinarity and the need of a dedicated and expert HBP team. Proposing a curative treatment in patients with large tumors, multiple lesions, altered hepatic function, underlying pathologies or insufficient future liver parenchyma are some of the issues that need to be addressed. The complexity of the perioperative management of HCC is reflected in the articulated therapeutic algorithm of the Barcelona Clinical Liver Cancer (BCLC) flow-chart.(5) Surgical resection and liver transplantation are two alternative curative options whereas thermal ablation has been validated for lesions inferior to 3 cm with comparable outcomes.(46,47) A grey zone is represented by large HCC, that is tumors larger than 5 cm. Proposing a curative treatment in these cases is challenging and not always feasible. Criteria for liver transplantation are in fact more constrained and ablation not more indicated. Resection become therefore crucial but surgeon has to face with patients often needing a major hepatectomy in a delicate liver functional balance. Furthermore, the associated poorer outcomes could be related to the higher risk of incomplete microscopic resections, satellites nodules or progression before surgery.(10) Hence the necessity to find new strategies in the neoadjuvant setting. Starting from this clinical need, and given the poor results of systemic therapies, we explored how radiologic procedures, and transarterial chemoembolization (TACE) in particular (which is normally used as a palliative treatment in unresectable diseases) could help surgeons and be proposed before surgical resection. We built up therefore a multi-institutional database from European high-volume centers and assessed if patients treated preoperatively with TACE showed better long-term outcomes than those upfront resected.

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Original article

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PROGNOSTIC BENEFIT OF PREOPERATIVE TRANSARTERIAL CHEMOEMBOLIZATION IN UPFRONT RESECTABLE LARGE HEPATOCELLULAR CARCINOMA: A MULTICENTRIC PROPENSITY SCORE BASED ANALYSIS OF EUROPEAN HIGH-VOLUME CENTERS

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) has a dismal prognosis and any effective neoadjuvant treatment has been validated to date. We aimed to investigate the role of neoadjuvant transarterial chemoembolization (TACE) in upfront resectable HCC larger than 5 cm.

Methods: This is a multicentric retrospective study comparing outcomes of large HCC undergoing TACE followed by surgery or liver resection alone before and after propensity-score matching (PSM).

Results: A total of 384 patients were included of whom 60 (15.6%) received TACE. This group did not differ from upfront resected cases neither in terms of disease-free survival (p= 0.246) nor in overall survival (p= 0.276). After PSM, TACE still did not influence long-term outcomes (p= 0.935 and p= 0.172, for DFS and OS respectively). In subgroup analysis, TACE improved

OS only in HCC ≥ 10 cm (p= 0.045), with a borderline significance after portal vein embolization/ligation (p= 0.087) and in single HCC (p= 0.052).

Conclusions: TACE should not be systematically performed in all resectable large HCC. Selected cases could however potentially benefit from this procedure, as patients with huge and single tumors or those necessitating of a PVE.

INTRODUCTION

Hepatocellular Carcinoma (HCC) accounts for about 80% of all liver cancer and it ranks as the third leading cause of cancer deaths worldwide.[1] As its cholangiocyte-derived counterpart, HCC shows a dismal prognosis with a relative 5-year survival rate of approximately 20%.[2] Even in case of resectable disease undergoing surgical treatment, outcomes do not differ significantly and recurrence rate remains high, reaching 70 - 80% in 5 years. Other curative-intent strategies are validated in the Barcelona Clinical Liver Cancer (BCLC) algorithm in alternative to surgery,[3] as local ablation or liver transplant, but not all of them are always available, with different factors – as size or numbers – limiting the indiscriminate use of this armamentarium. Large HCC, lesions with a maximum diameter equal or superior to 5 cm, represent for instance a real challenge in this context. Although belonging to the early stage of the BCLC classification in case of single localization, these tumors show a poor prognosis if compared to smaller lesions.[4,5] When possible, surgical resection have largely demonstrated to improve long-term outcomes in these patients but risk of recurrence remains high.[6–10] Given the lack of validated neoadjuvant protocols, different authors reported the use of transarterial chemoembolization (TACE) before surgery in large HCC with the aim of inducing tumoral cell death, increasing R0 resection rates and thus improving outcomes.[11–13] However, results are far from being exhaustive with contradictory conclusions and a difficulty in finding those cases who could really benefit from this procedure. A large meta-analysis revealed that neoadjuvant

TACE did not increase disease-free survival (DFS) and overall survival (OS) rates but favorable results were found when assessing exclusively cirrhotic patients.[14] Similarly, a multicentric cohort recently showed improved oncologic outcomes when performing this procedure before surgery in huge HCC (≥ 10 cm).[11] Other unsolved issues derive from the statistical robustness of these studies, with possible selection bias, and not least, that almost all these series come from Asiatic centers, which present different underlying etiology as well as distinct genetic altered pathways.[15]

This study aimed to investigate the utility of preoperative TACE in upfront resectable HCC larger than 5 cm, analyzing cases from European centers gathered in a common database. By setting accurate inclusion criteria, a homogeneous cohort was therefore created in which long-term outcomes were evaluated by a propensity score matching (PSM) and in different subgroup of patients.

METHODS

Study Design

This is a retrospective study conducted on a multicentric international database following the items of the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement.[16] An informed consent was obtained before each procedure and the study was aligned to the ethical standards of the Helsinki declaration. Seven Italian and French centers provided data on patients affected by large HCC (≥5 cm) undergoing TACE followed by surgical treatment (preoperative TACE, cases) or liver resection alone (upfront surgery, control) with a curative intent, from January 2012 and December 2020. Only cases considered as resectable at diagnosis were included, thus without prior systemic or local treatment – except for preoperative TACE followed by a planned surgery – or history of distant metastases. Upfront resectability was based on single institution decision. Due to the likely higher risk of recurrence in case of atypical resection[17,18] and the consequent possible selection bias, one of

the inclusion criteria set before data collection was cases undergoing anatomical resection. Exclusion criteria were surgery for HCC recurrence, adjuvant systemic or local treatment (i.e. post-operative TACE or patients included in an experimental protocol with an adjuvant therapy), more than one preoperative TACE and surgical resection performed later than 10 weeks after the endovascular procedure. Patients with incomplete data, a follow-up inferior to 12 months or lost to follow-up were as well excluded from the analysis. Clinico-pathologic, peri-operative and histologic features were collected from all centers in a common database. Diagnosis of preoperative cirrhosis, its nature and Child-Pugh score were recorded. Portal hypertension was evaluated by platelet count and classified in a binomial variable according to its normal value (150.000 x 10^9/L). Intraoperative blood loss, transfusions and operative time were registered as an indirect marker of surgical complexity. Information on post-operative complications such as post-hepatectomy liver failure (PHLF) and hemorrhage (PHH) were collected and scored according to the ISGLS classification.[19,20] Overall post-operative complications were further graded according to the Clavien-Dindo classification.[21] Among histologic features, satellites nodules were defined as tumors inferior to 1 cm in diameter and located less than 1 cm to the main tumor. If this condition was not fulfilled, tumor was considered as multifocal. The final cohort was then divided according to the preoperative performance of a TACE in order to assess its prognostic meaning in terms of disease-free survival (DFS) and overall survival (OS).

Trans-arterial Chemoembolization

There is no consensus on the use of preoperative TACE in large HCC, therefore the indication of performing this procedure before resection and its modalities were decided case by case according to surgeon and radiologist judgement in each institution. Given the multicentric nature of the study, type (doxorubicin or idarubicin) and dose of drug administrated varied among the centers, as well as the embolization material and the simultaneous combination of

lipiodol. The procedure started by the insertion of a vascular catheter in the femoral artery. Superior mesenteric artery was first cannulated to exclude an accessory or replaced hepatic artery feeding the tumor. Then the coeliac artery was catheterized. The main first-order hepatic artery was chosen or, if possible, a more selective branch vascularizing all the tumor. The emulsion of the selected drug and the embolization agents was therefore injected. A final arteriography confirmed the success of the procedure. When PVE/PVL was further indicated in order to increase future liver remnant (FLR), a minimum delay of two/three weeks was respected between TACE and venous occlusion. Date of TACE, PVE/PVL and surgery as well as data regarding drug, agents and modalities of the two procedures were always recorded.

Statistical analysis

Categorical data were reported as absolute number with relative proportions (%) and compared by the $\chi 2$ test with Yates correction if necessary, or Fischer's exact test if indicated. Continuous data were expressed as median and range and compared using Student's t test or Mann-Whitney U test in case of normal distribution. Kaplan-Meier analysis were performed and survival outcomes compared using log-rank test for categorical variables and through Cox test in case of continuous data. Hazard Ratios and the relative 95% CI were always reported. Significant variables at the univariate analysis were included in the Cox multivariate analysis. A PSM was then performed to create two homogeneous cohorts and thus reduce the bias of treatment selection. Covariates used to create the model included gender, age, ASA, platelets level, preoperative cirrhosis, history of viral infection, AFP at diagnosis, type of approach, type of hepatectomy, PVE/PVL performed, tumor size at diagnosis, number of nodules, microvascular infiltration (MVI), capsular invasion, satellites nodules and margin status. Despite several attempts, undergoing preoperative PVE/PVL was the only feature which could not be balanced between the two groups. A nearest neighbor matching without replacement with a ratio 2:1 was therefore chosen to create the largest sample size as possible preserving, at the same time, the

homogeneity of all the remaining variables. Survival analysis were repeated between the two new groups. Subgroups analysis were further performed to assess a possible benefit of preoperative TACE in a selected group of patients. All tests were 2-tailed and level of significance was set at $p < 0.05$. All statistical computations were performed using SPSS (SPSS Statistics, version 26.0, IBM Corp) or R (R Project for statistical computing, version 4.2.2, R Core Team).

RESULTS

General features and peri-operative outcomes

After data collection, a total of 384 patients resected for a HCC ≥5 cm and respecting all inclusion and exclusion criteria were included in the final cohort. Of these, 324 (84.4%) underwent upfront surgery whereas 60 (15.6%) were previously treated by TACE. **Table 1** shows main features of the whole population. The two groups were extremely heterogeneous in terms of baseline, operative and histologic characteristics. Patients with a neoadjuvant TACE had a significantly higher ASA score ($p= 0.014$) and AFP level ($p= 0.001$). As regards operative data, these patients underwent more often an open ($p< 0.001$), major hepatectomy ($p< 0.001$) requiring preoperative PVE ($p< 0.001$) compared to controls undergoing upfront resection. Histologic data comparison revealed that tumors treated by TACE had a lower differentiation grade ($p <0.001$), presented less frequently a MVI ($p= 0.023$) and a capsular invasion ($p= 0.017$) and were more often multiple ($p= 0.027$). When assessing perioperative outcomes, preoperative TACE was associated with longer operative times ($p= 0.023$), major blood loss and intraoperative transfusions ($p= 0.029$ and $p= 0.037$) and a higher risk of severe post-operative complications ($p <0.001$).

All significant variables were used for the PSM statistical model. The new cohort consisted of 180 patients of whom 120 (66.7%) underwent upfront surgery. Except for the FLR hypertrophy, the two groups were balanced in all baseline, operative and histological features (**Table 1**). No

differences in peri- and postoperative outcomes were found in this new cohort between TACE and upfront surgery group.

Survival analysis in the whole cohort and after PSM

Median follow-up for the whole cohort was 24 months (range: 0-127 months). Death occurred in 112/324 patients (34.6%) undergoing upfront resection and in 15/60 (25%) with preoperative TACE, whereas recurrence was observed in 173/324 (53.4%) and 35/60 (58.3) patients without and with neoadjuvant TACE, respectively. There was no difference in DFS (p= 0.246, **Figure 1A**) and OS (p= 0.276, **Figure 1B**) between the two groups. Univariate and multivariate analysis of all possible prognostic factors for DFS and OS are shown in **Table 2** and **Table 3**. Independent predictors of impaired DFS were AFP ≥400 ng/mL (HR: 1.645, p= 0.046), minimally-invasive vs open approach (HR: 0.725, p= 0.045), extension of hepatectomy (HR: 1.434, p= 0.014), tumor number (HR: 1.507, p= 0.018), MVI (HR: 1.683, p <0.001) and satellite nodules (HR: 1.584, p= 0.003). In OS multivariate Cox regression only severe post-operative complications (HR: 2.151, p= 0.004) and MVI (HR: 2.074, p <0.001) turned out to be significantly associated with decreased patient survival. The same analysis was performed in the PSM cohort. Of the 120 patients undergoing upfront liver resection, 68 (56.7%) experienced disease recurrence and 45 (37.5%) died at follow-up. Despite covariates balancing, preoperative TACE was not associated with improved oncological outcomes, neither in terms of DFS (p= 0.935, **Figure 1C**) nor OS (p= 0.172, **Figure 1D**). After matching, type of approach and extension of hepatectomy lost their independent prognostic role for disease recurrence at multivariate regression analysis (**Table 2**), whereas presence of multiple HCC became an independent predictor of survival (HR: 1.859, p= 0.028. **Table 3**)

Subgroup analysis

Comparison of prognostic outcomes was then performed in specific subgroups of patients in order to assess a potential benefit of preoperative TACE in certain situations as insufficient

FLR or cirrhosis (**Figure 2**). The first analysis was focused on patients undergoing PVE. This cohort included 87 cases of whom 47 (54%) were preceded by TACE. Kaplan-Meier curves showed no differences in this subgroup in terms of recurrence (p= 0.376) whereas a tendency towards an improved survival was observed, although not reaching a statistical significance (p= 0.087). Another class of patients explored was those with an underlying cirrhosis. Of the whole population, 185 (48.2%) showed a cirrhotic liver at pathological report and 27 of these (14.6%) received neoadjuvant TACE. Even in this subgroup, this procedure did not show any benefit when analyzing DFS (p= 0.751) and OS (p= 0.495) curves. Finally, we separately assessed long-term outcomes in HCC between 5 and 10 cm and huge (≥ 10 cm) HCC. Patients with HCC between 5 and 10 cm (n= 305, 79.4%) underwent TACE in 41 cases (13.4%) without any improved outcomes (DFS: p= 0.431; OS: p= 0.952). Analysis of huge HCC (n= 79, 20.6%), by contrast, revealed a prolonged survival in the 19 cases (24.1%) pre-treated with TACE (p= 0.045) with a similar trend in case of single lesion (**Figure 2**), although of borderline significance (p= 0.052).

DISCUSSION

One of the most important lacks in the therapeutic algorithm of HCC is undoubtedly the absence of any effective pre- and post-operative treatment. As it happens in large unresectable diseases converted to surgery thanks to the shrinkage obtained by TACE,[22] this technique has been proposed as well in large upfront resectable HCC with the aim of down-staging the tumor and improving long-term outcomes. Although several series have been published, results are far from being promising with only a few authors reporting a benefit when performing this procedure before liver resection in specific subgroups of patients.[11–14] However, conclusions are difficult to be drawn. The majority of the evidence comes from retrospective heterogeneous cohorts while randomized control-trials are rather dated with limited inclusions.[23–26] Furthermore, some series present evident selection bias as patients included after tumor down-

staging in initially unresectable disease, several TACE sessions or cases with non-anatomic resection, which are known to be associated with a higher risk of disease recurrence.[17,18] To our knowledge, this study represents the largest experience of western centers comparing patients undergoing surgical resection with or without preoperative TACE for upfront resectable HCC larger than 5 cm. Our results suggest that neoadjuvant TACE is a safe procedure with no increased perioperative morbi-mortality, but long-term outcomes analysis showed no associated benefit when combining this treatment prior to surgery even after PSM, neither in terms of disease recurrence nor of overall survival. These findings are not far from those reported in literature. Indeed, several series already concluded that systematic use of TACE before surgery was not recommended because of a lack of real oncologic benefit.[27,28] Similarly, results from three meta-analysis and a RCT revealed comparable OS and DFS between hepatic resection with or without preoperative TACE in large resectable HCC.[14,24,29,30]

In theory, principle behind the benefit of the use of this technique before liver resection lies in the necrosis of a large portion of tumor cells, the destruction of any possible satellite nodules and a consequent reduction in MVI and R1/R2 rates. TACE may additionally limit tumor cell dissemination during surgery and inhibit metastasis of HCC.[31,32] Following these assumptions, Yang et al. recently analyzed HCC cases undergoing liver resection with or without neoadjuvant TACE with the aim of assessing any possible correlation between this procedure and incidence of MVI.[33] Although an initial association with a lower rate of MVI was found in the initial cohort of the TACE group, after PSM this correlation was not confirmed. In this series, neoadjuvant TACE was associated with a lower incidence of MVI and capsular invasion, whereas no differences in satellite nodules and positive margin was observed. Nevertheless, these results come from the initial cohort without covariates balancing. The aim of this study was, in fact, rather prognostic and these pathological variables were later included in the

propensity score model in order to create two groups as homogeneous as possible and thus compare long-term outcomes.

As above mentioned, different issues limit the possibility of drawing consistent conclusions regarding the oncologic benefit of preoperative TACE and literature analysis provides cases in which this procedure was successfully used before surgery.[11,12] Some authors even reported biologic predictive indicators which can filter patients who may benefit from the use of neoadjuvant TACE.[34,35] This means that solution could be found in a possible advantage in selected cases or in specific situations. One of these may be the necessity of increasing FLR by preoperative PVE. Time required to obtain a sufficient FLR vary from 4 to 6 weeks which means a delayed resection with a consequent higher risk of tumor progression. In this context, TACE is used in some centers prior to PVE to induce necrosis and reduce the risk of tumor cells dissemination. This association was already corroborated by a few series in terms of oncologic outcomes,[36–38] but only one study focused on large HCC with an intention-to-treat analysis.[13] Other possible scenario with favorable results reported in literature concern huge HCC[11], intermediate BCLC stage,[30,39] portal vein invasion[40] or cirrhotic patients,[14] although mechanisms are not always clear and results usually not statistically robust with possible selection bias. Larger HCC for example may exhibit a richer arterial blood supply which translates into a massive necrosis and a more effective TACE. In order to confirm a possible benefit in these specific cases, a subgroup analysis was therefore performed which found an improved OS when performing neoadjuvant TACE in case of huge HCC, single lesion (corresponding to early stage BCLC) and in association with PVE, although reaching a statistically significance only in tumor ≥10 cm.

Some limitations have to be reported. Although a strict and well-focused study design, the retrospective and multicenter nature of the study represent undoubtedly a limit of our study. TACE, for instance, was not standardized in terms of technique (more or less selective

procedure), type and dose of drug administrated (doxorubicin or idarubicin), with a consequent heterogeneity and a possible different effect on tumor necrosis. Another drawback was the impossibility of accurately gather some variables, as portal venous invasion, tumor response after TACE or degree of tumor necrosis, which were therefore excluded from the analysis. Furthermore, an intention-to-treat analysis could not be performed and some patients could have progressed after performing TACE or PVE. Finally, it must be considered that some of the criteria used for the PSM are histological and therefore influenced by a possible downstaging by the TACE. Consequently, upfront resected cases were matched with a group which actually contained originally more aggressive tumors.

In conclusion, TACE represents a safe and well-tolerated technique with no increased risk of morbidity and mortality after liver resection. However, our results do not support the indiscriminate use of this procedure in all patients with a large HCC in which surgical resection is validated. Selected cases could benefit from a neoadjuvant TACE, as patients with a huge and single tumor or those with an insufficient FLR necessitating of a PVE.

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TABLES

Table 1. Patients Characteristics in the whole cohort and after PSM (ratio 2:1)

Variable	*Before matching (n= 384)*			*After PSM (n= 180)*		
	Upfront Resection *n*= 324	**Preoperative TACE *n*= 60**	***p***	**Upfront Resection *n*= 120**	**Preoperative TACE *n*= 60**	***p***
	n (%)			***n (%)***		
Age (years), SD	69.89 (9.6)	69 (7)	0.604	69 (9)	69 (7)	0.874
Sex						
Male	252 (77.8)	54 (90)	***0.031***	104 (86.7)	54 (90)	0.520
Female	72 (22.2)	6 (10)		16 (13.3)	6 (10)	
BMI (kg/m^2), SD	25 (4.4)	26.7 (4.1)	***0.024***	25.8 (4.3)	26.7 (4.1)	0.429
ASA						
I	30 (9.3)	-	***0.014***	4 (3.3)	-	0.551
II	175 (54)	27 (45)		50 (41.7)	27 (45)	
III	116 (5.8)	32 (53.3)		64 (53.3)	32 (53.3)	
IV	3 (0.9)	1 (1.7)		2 (1.7)	1 (1.7)	
Preoperative Cirrhosis						
No	164 (50.6)	33 (55)	0.702	64 (53.3)	33 (55)	0.768
A	158 (48.8)	27 (45)		55 (45.8)	27 (45)	
B	2 (0.6)	-		1 (0.8)	-	
Normal platelets count, (≥ 150 x10(9)/L)						
No	81 (25)	12 (20)	0.406	24 (20)	12 (20)	1
Yes	243 (75)	48 (80)		96 (80)	48 (80)	
History of viral infection (HBV/HCV)						
No	191 (59)	44 (73.3)	***0.036***	76 (63.3)	44 (73.3)	0.180
Yes	133 (41)	16 (26.7)		44 (36.7)	16 (26.7)	
AFP at diagnosis, ng/mL						
≤400	307 (94.8)	50 (83.3)	***0.001***	106 (88.3)	50 (83.3)	0.352
>400	17 (5.2)	10 (16.7)		14 (11.7)	10 (16.7)	
HCC median size at diagnosis (mm), SD	70 (41.6)	75 (29)	0.117	72 (43.7)	75 (29)	0.958
PVE/PVL performed						
No	284 (87.7)	13 (21.7)	***<0.001***	94 (78.3)	13 (21.7)	***<0.001***
Yes	40 (12.3)	47 (78.3)		26 (21.7)	47 (78.3)	
Approach						
Open	186 (57.4)	52 (86.7)	***<0.001***	96 (80)	52 (86.7)	0.270
Minimally-invasive	138 (42.6)	8 (13.3)		24 (20)	8 (13.3)	
Extension of hepatectomy						
Minor	186 (57.4)	6 (10)	***<0.001***	22 (18.3)	6 (10)	0.146
Major	138 (42.6)	54 (90)		98 (81.7)	54 (90)	
Intraoperative blood loss (ml), SD	350 (466)	500 (571)	***0.029***	400 (600)	500 (571)	0.076
Operative time (min), SD	289 (92)	330 (85)	***0.023***	300 (106)	330 (85)	0.265
Intraoperative blood transfusion						

No	281 (86.7)	45 (75)	***0.037***	99 (82.5)	45 (75)	0.236
Yes	43 (13.3)	15 (25)		21 (17.5)	15 (25)	
Post-operative complications						
PHLF	36 (11.1)	9 (15)	0.390	19 (15.8)	9 (15)	0.884
PHH	5 (1.5)	-	1	2 (1.7)	-	0.553
Death	3 (0.9)	2 (3.3)	0.131	2 (1.7)	2 (3.3)	0.602
Severe post-operative complications (CD ≥3)						
No	295 (93.9)	47 (78.3)	***<0.001***	105 (87.5)	47 (78.3)	0.110
Yes	19 (6.1)	13 (21.7)		15 (12.5)	13 (21.7)	
HCC median size on pathology (mm), SD	70 (45.2)	80 (37)	0.250	70 (50)	80 (37)	0.429
WHO tumor differentiation*						
Well	46 (17)	20 (35.1)	***<0.001***	27 (27)	20 (35.1)	0.302
Moderately	184 (68.1)	35 (61.4)		64 (64)	35 (61.4)	
Poor	40 (14.8)	2 (3.5)		9 (9)	2 (3.5)	
Tumor number						
Solitary	271 (83.6)	43 (71.7)	***0.027***	97 (80.8)	43 (71.7)	0.163
Multiple	53 (16.4)	17 (28.3)		23 (19.2)	17 (28.3)	
Microvascular infiltration						
No	159 (49.1)	39 (65)	***0.023***	69 (57.5)	39 (65)	0.333
Yes	165 (50.9)	21 (35)		51 (42.5)	21 (35)	
Capsular invasion						
No	261 (80.6)	56 (93.3)	***0.017***	103 (85.8)	56 (93.3)	0.140
Yes	63 (19.4)	4 (6.7)		17 (14.2)	4 (6.7)	
Satellites nodules						
No	242 (74.7)	45 (75)	0.960	93 (77.5)	45 (75)	0.709
Yes	82 (25.3)	15 (25)		27 (22.5)	15 (25)	
Margin status						
Negative	296 (91.4)	55 (91.7)	0.938	111 (92.5)	55 (91.7)	0.844
Positive	28 (8.6)	5 (8.3)		9 (7.5)	5 (8.3)	

* 57 cases missing for the whole cohort and 23 values after PSM

PSM: Propensity Score Matching; TACE: transarterial chemoembolization; SD: Standard Deviation; BMI: Body Mass Index; ASA: American Society of Anesthesiologists; HBV: Hepatitis B Virus; HCV: Hepatitis C Virus; AFP: Alpha Fetoprotein; HCC: Hepatocellular Carcinoma; PVE: Portal Vein Embolization; PVL: Portal Vein Ligation; PHLF: Post Hepatectomy Liver Failure; PHH: Post Hepatectomy Hemorrhage; CD: Clavien-Dindo; WHO: World Health Organisation.

Table 2. Univariate and Multivariate Cox Regression Analysis of Prognostic Factors for Disease-Free Survival in the whole cohort and after propensity score matching.

		Before matching (n= 384)		*After PSM (n= 180)*	
Variable	**Category**	**Hazard Ratio (95% CI)**	***P***	**Hazard Ratio (95% CI)**	***P***
Univariate Analysis					
Age	Continuous data	1.003 (0.989–1.017)	0.685	0.978 (0.956–1)	0.055
Sex	Female vs Male	0.851(0.602–1.202)	0.352	0.896 (0.479–1.676)	0.727
BMI	Continuous data	1.011 (0.982–1.042)	0.465	0.978 (0.935–1.023)	0.332
ASA	III-IV vs I-II	1.350 (1.026–1.778)	***0.029***	0.856 (0.581–1.261)	0.423
Preoperative cirrhosis	Yes vs No	0.977 (0.744–1.284)	0.864	0.924 (0.626–1.363)	0.684
Normal platelets count	Yes vs No	0.962 (0.701–1.320)	0.807	0.723 (0.454–1.152)	0.163
History of viral infection	Yes vs No	1.165 (0.883–1.536)	0.272	1.268 (0.845–1.904)	0.242
AFP level at diagnosis	>400 vs ≤400	2.081 (1.295–3.343)	***0.002***	1.807 (1.020–3.202)	***0.036***
HCC size at diagnosis	Continuous data	1.002 (1–1.004)	0.055	1.003 (0.999–1.006)	0.106
Preoperative TACE	Yes vs No	1.236 (0.859–1.779)	0.246	1.017 (0.676–1.530)	0.935
PVE/PVL performed	Yes vs No	1.371 (1.003–1.875)	***0.044***	1.128 (0.764–1.667)	0.537
Approach	MI vs Open	0.565 (0.420–0.759)	***<0.001***	0.634 (0.360–1.115)	0.105
Extension of hepatectomy	Major vs Minor	1.579 (1.2–2.077)	***0.001***	1.573 (0.894–2.768)	0.107
Severe post-operative complications	Yes vs no	2.221 (1.381–3.572)	***0.001***	1.938 (1.149–3.270)	***0.010***
HCC tumor size on pathology	Continuous data	1.003 (1.001–1.005)	***0.008***	1.005 (1.001–1.008)	***0.012***
WHO tumor differentiation	Moderately vs Well	1.057 (0.720–1.553)	0.776	1.192 (0.722–1.968)	0.492
	Poor vs Well	1.106 (0.647–1.893)	0.712	1.623 (0.689–3.824)	0.268
Tumor number	Multiple vs Solitary	1.715 (1.229–2.392)	***0.001***	1.819 (1.168–2.831)	***0.006***
Microvascular infiltration	Yes vs no	1.640 (1.247–2.158)	***<0.001***	2.115 (1.432–3.125)	***<0.001***
Capsular invasion	Yes vs no	1.199 (0.852–1.687)	0.299	1.618 (0.948–2.761)	0.070
Satellites nodules	Yes vs no	1.760 (1.313–2.357)	***<0.001***	2.335 (1.543–3.532)	***<0.001***
Margin status	R1 vs R0	1.549 (0.986–2.435)	0.052	2.135 (1.108–4.113)	***0.018***
Multivariate Analysis					
ASA	III-IV vs I-II	1.211 (0.902–1.625)	0.204	–	–
AFP level at diagnosis	>400 vs ≤400	1.645 (1.008–2.684)	***0.046***	1.904 (1.049–3.456)	***0.034***
PVE/PVL performed	Yes vs No	0.967 (0.669–1.398)	0.857	–	–
Approach	MI vs Open	0.725 (0.530–0.994)	***0.045***	–	–
Extension of hepatectomy	Major vs Minor	1.434 (1.075–1.914)	***0.014***	–	–
Severe post-operative complications	Yes vs no	1.551 (0.943–2.552)	0.084	1.529 (0.888–2.633)	0.126
HCC tumor size on pathology	Continuous data	1.002 (1–1.004)	0.120	1.004 (1–1.008)	0.056
Tumor number	Multiple vs Solitary	1.507 (1.073–2.116)	***0.018***	1.955 (1.234–3.098)	***0.004***
Microvascular infiltration	Yes vs no	1.683 (1.269–2.233)	***<0.001***	1.887 (1.259–2.828)	***0.002***
Satellites nodules	Yes vs no	1.584 (1.175–2.136)	***0.003***	1.961 (1.277–3.013)	***0.002***
Margin status	R1 vs R0	–	–	1.504 (0.753–3.006)	0.248

PSM: Propensity Score Matching; BMI: Body Mass Index; ASA: American Society of Anesthesiologists; AFP: Alpha Fetoprotein; HCC: Hepatocellular Carcinoma; TACE: transarterial chemoembolization; PVE: Portal Vein Embolization; PVL: Portal Vein Ligation; MI: minimally-invasive; WHO: World Health Organization.

Table 3. Univariate and Multivariate Cox Regression Analysis of Prognostic Factors for Overall Survival in the whole cohort and after propensity score matching

		Before matching (n= 384)		*After PSM (n= 180)*	
Variable	**Category**	**Hazard Ratio (95% CI)**	***P***	**Hazard Ratio (95% CI)**	***P***
Univariate Analysis					
Age	Continuous data	0.990 (0.973–1.008)	0.275	0.976 (0.949–1.004)	0.094
Sex	Female vs Male	0.868 (0.560–1.345)	0.522	0.708 (0.304–1.650)	0.417
BMI	Continuous data	1.017 (0.979–1.056)	0.385	1.011 (0.952–1.073)	0.721
ASA	III-IV vs I-II	1.432 (1.009–2.034)	***0.042***	1.1 (0.661–1.831)	0.711
Preoperative cirrhosis	Yes vs No	1.521 (1.069–2.163)	***0.018***	1.249 (0.753–2.073)	0.384
Normal platelets count	Yes vs No	0.699 (0.480–1.020)	0.063	0.591 (0.337–1.037)	0.061
History of viral infection	Yes vs No	1.446 (1.020–2.052)	***0.036***	1.158 (0.680-1.970)	0.586
AFP level at diagnosis	>400 vs ≤400	1.251 (0.634–2.466)	0.514	1.348 (0.608–2.986)	0.457
HCC size at diagnosis	Continuous data	0.999 (0.995–1.003)	0.699	1 (0.994–1.006)	0.956
Preoperative TACE	Yes vs No	0.744 (0.434–1.275)	0.276	0.670 (0.373–1.202)	0.172
PVE/PVL performed	Yes vs No	1.012 (0.665–1.539)	0.956	0.845 (0.502–1.423)	0.522
Approach	MI vs Open	0.756 (0.520–1.101)	0.140	0.946 (0.479–1.870)	0.873
Extension of hepatectomy	Major vs Minor	1.266 (0.893–1.795)	0.180	1.353 (0.642–2.849)	0.420
Severe post-operative complications	Yes vs no	2.327 (1.395–3.880)	***0.001***	2.340 (1.303–4.201)	***0.003***
HCC tumor size on pathology	Continuous data	1 (0.977–1.004)	0.953	1.002 (0.997–1.007)	0.485
WHO tumor differentiation	Moderately vs Well	1.237 (0.762–2.007)	0.390	1.023 (0.547–1.911)	0.944
	Poor vs Well	0.948 (0.457–1.966)	0.885	0.948 (0.272–3.302)	0.934
Tumor number	Multiple vs Solitary	1.492 (0.980–2.272)	0.059	1.923 (1.115–3.317)	***0.016***
Microvascular infiltration	Yes vs no	1.967 (1.370–2.826)	***<0.001***	2.022 (1.201–3.337)	***0.006***
Capsular invasion	Yes vs no	0.914 (0.577–1.447)	0.698	1.383 (0.681–2.812)	0.364
Satellites nodules	Yes vs no	1.527 (1.055–2.212)	***0.023***	2.098 (1.240–3.550)	***0.004***
Margin status	R1 vs R0	1.555 (0.920–2.628)	0.134	2.3 (1.131–4.673)	***0.017***
Multivariate Analysis					
ASA	III-IV vs I-II	1.379 (0.952–1.997)	0.089	–	–
Preoperative cirrhosis	Yes vs No	1.405 (0.977–2.021)	0.067	–	–
History of viral infection	Yes vs No	1.277 (0.884–1.844)	0.193	–	–
Severe post-operative complications	Yes vs no	2.151 (1.279–3.618)	***0.004***	2.043 (1.131–3.691)	***0.018***
Tumor number	Multiple vs Solitary	–	–	1.859 (1.071–3.228)	***0.028***
Microvascular infiltration	Yes vs no	2.074 (1.439–2.989)	***<0.001***	2.024 (1.211–3.382)	***0.007***
Satellites nodules	Yes vs no	1.288 (0.873–1.899)	0.202	1.581 (0.903–2.770)	0.109
Margin status	R1 vs R0	–	–	1.564 (0.747–3.273)	0.236

PSM: Propensity Score Matching; BMI: Body Mass Index; ASA: American Society of Anesthesiologists; AFP: Alpha Fetoprotein; HCC: Hepatocellular Carcinoma; TACE: transarterial chemoembolization; PVE: Portal Vein Embolization; PVL: Portal Vein Ligation; MI: minimally-invasive; WHO: World Health Organization.

FIGURES

Figure 1. Kaplan-Meier curves of Disease-Free Survival and Overall Survival in patients undergoing upfront resection or preoperative TACE before (A and B) and after (C and D) the propensity score matching.

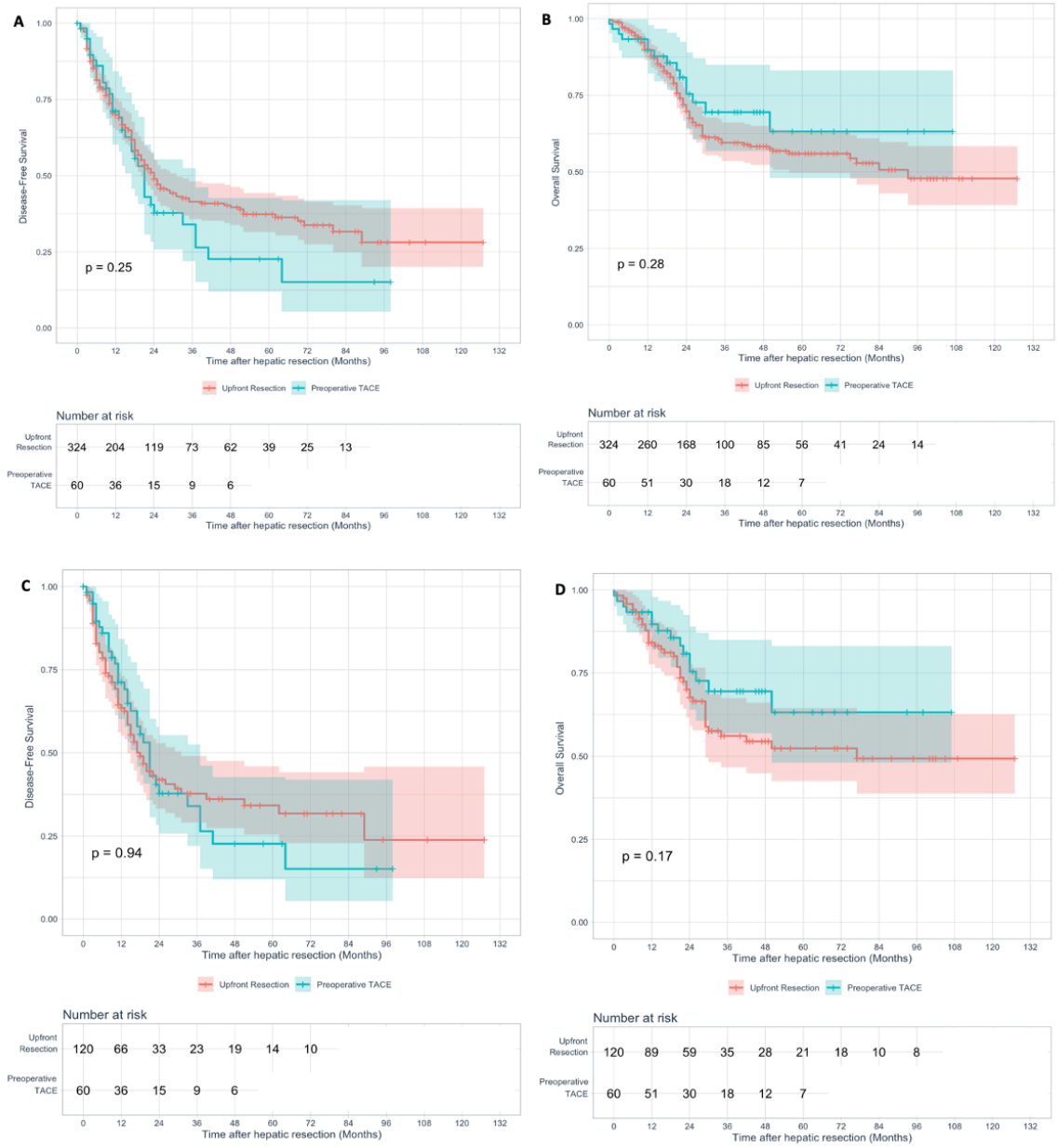
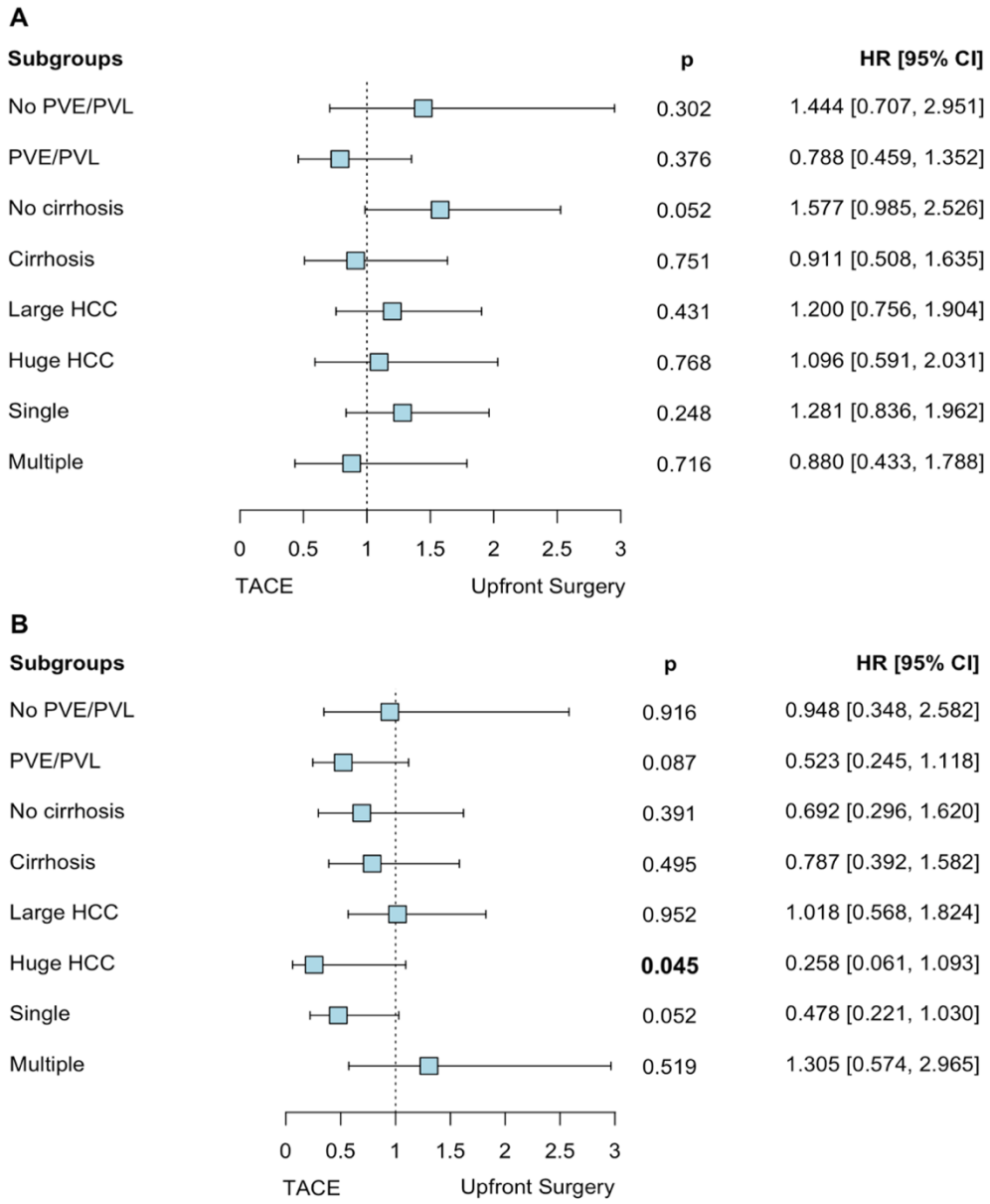


Figure 2. Forest-plot representing subgroup analysis for disease-free survival (A) and overall survival (B).



1.2 Peri-operative imaging techniques to predict outcomes in major liver resections

One of the great issues exposed in the previous paragraph was the approach to large tumors which often need major hepatectomies in a delicate liver functional background. In these patients' indication of surgical resection is conditioned by the risk of post-operative liver failure (PLF), probably the most challenging complications in liver surgery. Morphologic evaluation of remnant liver volumetry through preoperative imaging, as well as functional assessment using indocyanine green clearance test,(48) are useful tools to accurately estimate the volume and function of the future liver remnant. Nevertheless, all these approaches provide a poor snapshot of preoperative conditions and of intraoperative events. For instance, the pringle maneuver results in oxygen deprivation, which is the starting event that induces parenchymal damage, further compounded once blood circulation has been reestablished by the ischemia-reperfusion injury mechanism, which can be the cause of PLF.(49,50). Liver oxygenation impairment and ischemia can be challenging to detect intraoperatively, which is partly due to multiple hepatic vascular inflows.(51–53) Parenchymal disruption in the reperfusion phase mainly depends on ischemic time duration. Consequently, intraoperative localization and quantification of oxygen impairment may be helpful in quickly detecting future reperfusion injury sites. To date, there is no tool which can spatially visualize and quantify liver oxygenation intraoperatively. Currently, hepatic circulation can be evaluated intraoperatively using ultrasound (US). However, US may be time-consuming, especially during laparoscopic surgical procedures, and has a long learning curve.(54) Additionally, US evaluation might be difficult in some patients and the interpretation is strongly operator-dependent.(55) Besides, US aims to analyze and quantify blood circulation in a specific area of interest and does not provide an immediate localization and quantification of oxygenation of the whole liver surface.

Hyperspectral imaging (HSI) is a non-invasive technique which has been recently applied to the medical field as a tool for image-guided surgery and specifically for an intraoperative quantification of tissue perfusion.(16,56,57) HSI detects the relative reflectance of light with a wavelength comprised between 500 and 1000 nm, allowing the quantification of organic compounds, such as oxygenated and deoxygenated hemoglobin.(17) The application of HSI has recently gained importance for its non-invasiveness and the accuracy of oxygen quantification at different depths.(58) For that reason, HSI is a promising technology as it allows for the intraoperative quantification and spatial visualization of hepatic oxygenation and to discriminate among different types of liver ischemia.

In collaboration with the IHU, the institute of image-guided surgery and the IRCAD, we therefore applied these evidences into a clinical setting, based on the preliminary results on preclinical models,(59–62) by evaluating the HSI potential to predict postoperative outcomes after major hepatectomies.

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HYPERSPECTRAL IMAGING AND ARTIFICIAL INTELLIGENCE IN MAJOR HEPATECTOMIES: PRELIMINARY RESULTS FROM THE EX-MACHINA TRIAL

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Simple Summary: major hepatectomy may be associated to major morbidity or mortality. At present no intraoperative tools exist to predict the post-operative course. Hyperspectral imaging is a new and promising technology that may potentially help to evaluate real time liver function during and at the end of the operation. As no clinical data exist concerning a major hepatectomies series our aim was to correlate intraoperative images acquisitions and findings with the postoperative course.

Abstract: Major hepatic resections are associated with higher risk of postoperative complications and post-hepatectomy liver failure. After hepatic pedicle clamping ischemia-reperfusion injury may sometimes lead to post-hepatectomy liver failure. Early detection or, ideally, intraoperative prediction of liver dysfunction or failure would be essential for timely treatment. Fifteen patients who underwent major hepatic resections at Nouvel Hôpital Civil (Strasbourg, France) were retrospectively analyzed. Intraoperative acquisition with the hyperspectral camera system was performed at the beginning of the operation, before any liver manipulation, and at the end of hepatic resection after specimen removal. TWI was the only HSI index to correlate with postoperative reintervention. Lower OHI final values showed significant correlation with postoperative sepsis (0.641417 ± 0.0340463 vs 0.704987 ± 0.0712082, p=0.045), while ΔOHI correlated with PHLF (-0.074822 ± 0.0774023 vs 0.022819 ± 0.0220585, p= 0.010), also showing negative correlation with ALT values

observed in POD5 (ρ = -0.813, p=0.001). This is the first clinical application in a monocentric major hepatectomy series, and the reported results suggest that TWI and OHI could be associated to short-term postoperative outcomes. Further experimental and clinical studies are necessary to better explore and evaluate the potential value of this technology in current practice.

Keywords: Hyperspectral imaging; Liver; Hepatectomy

1. Introduction

Liver cancer and metastatic liver disease are a leading cause of cancer mortality world- wide, accounting for more than 700,000 deaths annually [1]. Indications for liver resections have expanded with advances in surgical techniques and chemotherapy, allowing sur- geons to approach lesions previously deemed unresectable [2,3]. However, major hepatic resections are technically challenging and associated with the highest risk of adverse post- operative outcomes [4,5]. Almost all major hepatectomies require pedicle clamping to reduce bleeding during liver transection, which results in oxygen deprivation, the catalyst for parenchymal damage. This is further compounded when blood flow is reestablished from ischemia-reperfusion injury (IRI). IRI commonly results in post-hepatectomy liver failure [6,7]; thus, predicting IRI to avoid post-operative liver disfunction is important. Before surgery, evaluations with magnetic resonance imaging (MRI), computed tomogra- phy (CT) scan, or sequential hepato-biliary scintigraphy can estimate the volume of the future liver remnant (FLR); adding an indocyanine green (ICG) clearance test can provide a functional assessment [8]. Even combined, these modalities provide a poor snapshot of post-operative conditions [9]. In the operating room, ICG fluorescence imaging, microdial- ysis, carbon dioxide sensors, and near-infrared spectroscopy (NIRS) can assess real-time perfusion [10–14]. However, they are limited by the need for exogenous dye, expensive commercial equipment, and lack of standardization in interpretating results. On-table

ultrasonography (US) plays a critical role in detecting inadequate blood supply or outflow obstruction but is operator-dependent and cannot provide a precise real-time map of liver oxygenation [15]. As a result, current technologies inaccurately estimate perfusion or the complication risk following major hepatectomies. Thus, novel optical imaging technologies that could provide real-time intra-operative feedback on oxygenation and localization of ischemic damage are needed.

Hyperspectral imaging (HSI) is a non-invasive technology that detects the relative reflectance of light at a wavelength between 500 and 1000 nm. The science was originally developed for remote sensing, then successfully applied to military, environmental, geol- ogy, agriculture, and global change research, and lately used for quantification of relevant organic compounds [16,17]. Our group recently showed the potential benefits of HSI as an intra-operative tool during image-guided surgery in bowel and liver resections; HSI was able to improve the surgical resection lines via real-time overlay of the hyperspectral image and routine red-green-blue (RGB) captures using augmented reality (HYPER; hy- perspectral enhanced reality) [18,19]. Furthermore, the quantification and discrimination of different types of liver ischemia, including arterial or total vascular inflow occlusion, were demonstrated for providing a liver viability score in a pre-clinical model of IRI [20,21]. Moreover, higher values of water within hepatic tissue could be related to IRI and inflam- mation [22,23]. Given these promising preliminary pre-clinical results, the next step for validation was to translate the optical imaging system into the clinical setting.

The goal of this work was to validate a relationship between HSI parameters and post-operative outcomes after major hepatectomy in human subjects. The hypothesis was that the imaging system would be safe, accurate, and precise in translating from pre-clinical to clinical liver resections.

2. Materials and Methods

2.1. Study Design

The present study was part of the EXMachyna3 project (Intraoperative EXamination Using MAChine-learning-based HYperspectral for diagNosis & Autonomous Anatomy As- sessment, Strasbourg, France), registered at ClinicalTrials.gov (NCT04589884) and approved by the local ethics committee of the Faculty of Medicine of the University of Strasbourg (ID-RCB: 2020-A01896-33). A single-institution, one-arm prospective observational study was performed for this portion of the study.

2.2. Study Population

Adult patients undergoing a major hepatic resection (4 or more segments) through an open approach between 1 September 2020 and 30 June 2021 for malignant hepatic lesions at Nouvel Hôpital Civil (Strasbourg, France), a tertiary urban referral center, were included. Patients were eligible if the liver lesions were primary or metastatic adenocarcinoma. Patients were excluded if under 18 years of age, if the lesions were benign, if undergoing a liver biopsy or smaller resection (less than 4 segments), hepatic resection through an approach other than open laparotomy, or if the procedure was aborted prior to the experimental portion.

2.3. Surgical Procedure

The hepatectomy followed a standard protocol. Two experienced hepatobiliary surgeons performed all cases. In short, a laparotomy was made with a J-shaped Makuuchi incision and full exploration of the abdominal cavity was performed to look for carcinomatosis or other pathology. The liver was mobilized according to the type of hepatectomy per standard of care. The hepatic pedicle was encircled with a loop for vascular control and intermittent clamping. In healthy livers, the hepatic pedicle intermittent clamping was standardized as 20 min clamping followed by 10 min off. Whereas for cirrhotic livers, intermittent clamping consisted in 10 min of clamping followed by 10 min off [24]. Intra-operative ultrasound was performed to confirm the surgical strategy and to define the anatomical landmarks before transection. The portal vein

and hepatic artery branches were ligated prior to hepatectomy. Hepatic vein ligation was not routinely performed. The transection line was scored with electrocautery once devascularization was completed. Hepatectomy was performed using the Cavitronic Ultrasonic Surgical Aspirator (CUSA, Integra Lifesciences Corporation, Plainsboro, NJ, USA). Hemostasis and biliostasis was achieved with 5-0 or 6-0 polypropylene stitches and Hem-o-lock clips. A final ultrasonography was performed to ensure hepatic inflow and outflow. External biliary drainage was left according to the type of hepatectomy and surgeon preference; drainage was not systematically used. The same standardized enhanced recovery pathway was used on all patients post-operatively [25].

2.4. Hyperspectral Imaging (HSI)

The overhead light sources in the operating room were switched off during HSI acquisition. The HSI camera system (TIVITA, Diaspective Vision GmbH, Am Salzhaff, Germany) acquired hypercube (640 × 480 × 100 each) and routine RGB images for ten defined phases during the same acquisition mechanism. The HSI camera is equipped with a pushbroom imaging spectrometer with a slit-shaped aperture (motion that occurs for an HSI system to scan the field of view and acquire spectral and spatial information), an internal stepper motor controlling the slit of the spectrograph (device that breaks up a single full rotation into a number of much smaller part-rotations, mechanically connected to a diffraction grating to easily change the wavelength in the spectrograph), a high performance infrared (IR)-enhanced complementary metal oxide semiconductor (CMOS) sensor (electronic chip that converts photons to electrons for digital processing), and data processing equipment. Each hypercube was acquired in 6 s. The TIVITA hyperspectral camera was perpendicularly adjusted to a 40 cm distance from the surgical surface [26]. The system illuminates the area of interest with six halogen spotlights. The acquisition of a single hypercube was performed with a camera-specific module of the Perception Studio software (Perception Park GmbH, Graz, Austria). The spectral range of this

camera is 500–995 nm. The light source per spot is a 20 W OSRAM Halospot 70 Halogen lamp allowing for intense, broadband, temperature-stable, homogeneous, and fast pulses of radiation. The calibration of the wavelength was performed during camera production. Dark current effects were corrected after the recording of the data cube by the dedicated software component. The camera collects and processes the information from the electromagnetic spectrum, measuring the reflectance spectra generated by the target of study. To convert image data from radiance to relative reflectance, a white reference object with a high diffuse reflectance is used to create a reference cube. The TIVITA® camera system has preset algorithms, which can quantify the relative oxygen saturation (StO2%) of the superficial microcirculation at a depth up to 1 mm and the deeper layers within the near-infrared (NIR) spectrum with a penetration depth of 4–6 mm. The tissue water index (TWI) and the organ hemoglobin index (OHI) can be used to quantitatively assess and image the distribution of water and hemoglobin, respectively, in the observed region of interest (ROI) [27]. The intra-operative setting of HSI and acquired images is shown in Figure 1.

2.5. Data Collection

Pre-operative, intra-operative, and post-operative data were collected in a prospectively maintained electronic database. Pre-operative demographic data included gender, age, body mass index (BMI), comorbidities of dyslipidemia, diabetes mellitus (DM), hypertension, pre-existing hepatopathy, pre-operative procedures (chemoembolization, portal vein embolization, and biliary drainage), neoadjuvant chemotherapy, and American Society of Anesthesiologists classification (ASA) [28]. Laboratory values total bilirubin, aspartate transaminase (AST), alanine transaminase (ALT), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), albumin, hemoglobin, prothrombin time (PT), and international normalized ratio (INR) were collected pre-operatively and post-operatively on post-operative days (POD) 1, POD 2, and POD 5. Intra-operative data included opera- tive time (from skin incision to closure), intra-

operative blood loss, need for transfusion, number of resected segments, clamping/ Pringle maneuver (yes/no), total duration of liver ischemia, and RGB and HSI images, with their quantitative parameters. Image acquisition was done before any liver manipulation (baseline, T0) and at the end of hepatic resection/ after specimen removal (T1).

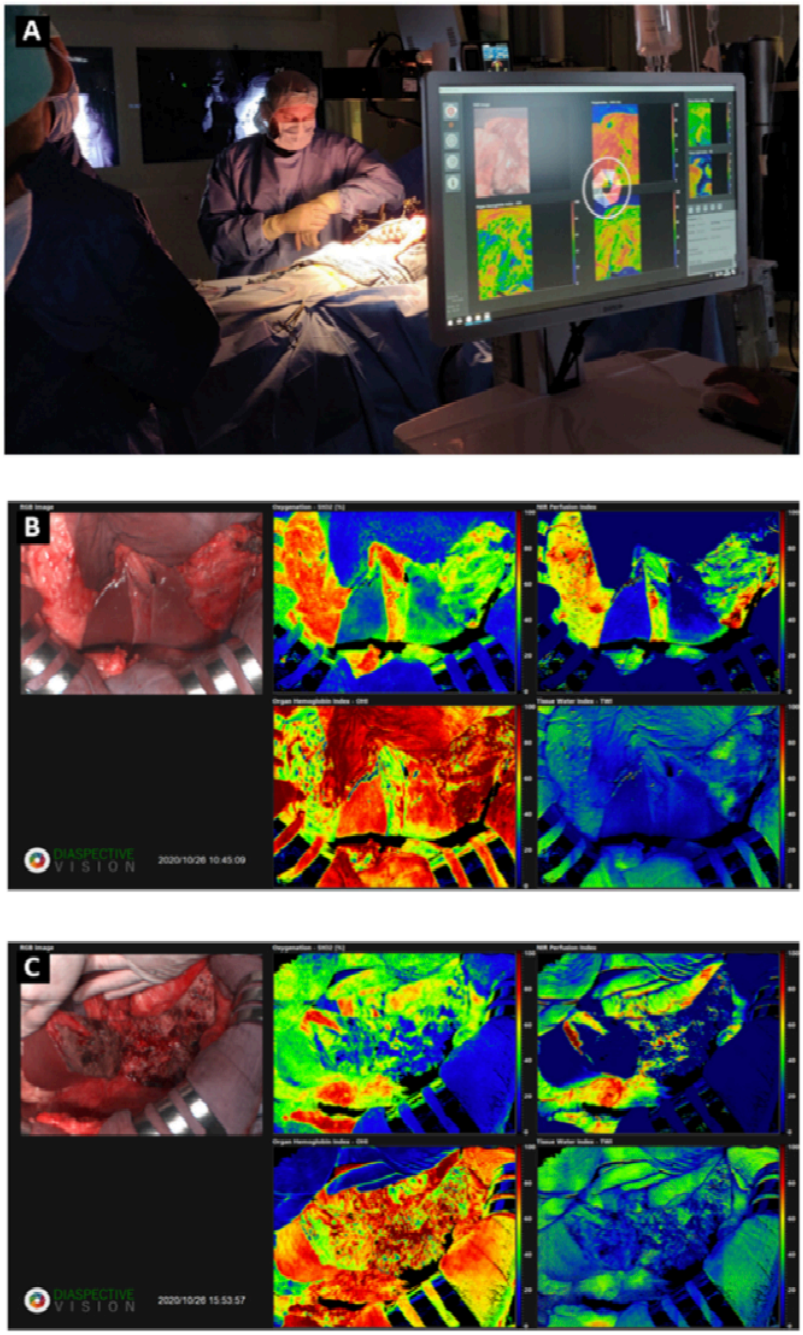

Figure 1. Intra-operative setting of HSI and acquired images. During the acquisition of data, all the lights of the operating room are turned off (**A**). The acquisition was performed at the beginning of the surgical procedure (**B**) and after the liver resection, at the end of surgery (**C**). The RGB (Red-Green-Blue) images and StO_2%, NIR, OHI, and TWI indexes are reported.

The ROIs were identified by surgeons and data scientists together and the procedure was standardized as follows: starting from the last picture (T1), the entire surface of the remaining unresected liver was considered as ROI-1; ROI-0 was exactly the same area on the liver surface at the beginning of the operation (T0). StO2%, NIR, TWI, and OHI values were recorded for the same ROIs at T0 and T1. Post-operative complications were recorded as major (Clavien-Dindo Class 3–5) and minor (Clavien-Dindo Class 1 and 2). Histologic data on steatosis, fibrosis, and cirrhosis from the final pathological report were collected.

2.6. Outcome Variables

The main outcome measure was to assess a relationship between the HSI measures, serum markers, and short-term post-operative clinical outcomes. The absolute values of StO2%, NIR, TWI, and OHI values were analyzed at T0 and T1. Following this, normalized values obtained via subtraction of T1 and T0 measurements (Δ) were used to correlate HSI values and surgical outcomes. Short-term outcome measures analyzed included blood loss, post-hepatectomy liver failure (PHLF), post-hepatectomy hemorrhage (PHH), bile leakage, and re-operation (unplanned return to the operating room within 60 days of the index procedure). These were defined and graded per the latest International Study Group of Liver Surgery (ISGLS) classifications [29–31]. The prothrombin time (PT) < 50 and serum bilirubin >50 μmol/L on POD 5 were analyzed ("50–50 criteria") as markers of liver failure and death after major hepatectomy [32].

2.7. Statistical Analysis

Categorical data were expressed as frequency and percentages. Continuous variables were described as medians, with interquartile range (IQR), and compared using the Student's t-test or the Mann–Whitney U-test, as appropriate. Normality of data distribution was assessed using histogram distribution visual inspection. Two-tailed p-values were considered significant when

alpha was less than 0.05. Statistical analyses were performed using SPSS® for Windows, v28.0 (IBM Corporation, Armonk, NY, USA).

2.8. Ethical Statement

Informed consent was obtained from all subjects involved prior to participation in the study. This study was part of the iEXMachyna3 project (Intraoperative EXamination Using MAChine-learning-based HYperspectral for diagNosis & Autonomous Anatomy Assessment), approved by the local ethics committee of the Faculty of Medicine of the University of Strasbourg (ID-RCB: 2020-A01896-33). The study was designed in accordance with the Declaration of Helsinki and the STrengthening the Reporting of OBservational studies in Epidemiology statement (STROBE) guidelines [33].

3. Results

3.1. Preoperative Variables

During the study period, 15 patients undergoing major hepatectomies for malignant liver tumors met inclusion criteria and were included for experimental HSI analysis. The most frequent pre-operative diagnosis was hepatocellular carcinoma (HCC, 40%), followed by colorectal cancer liver metastasis (CRLM, 33%). Five patients underwent neoadjuvant chemotherapy, and one patient had biliary drainage for pre-operative obstructive jaundice. Full demographic details and biochemical assessment are reported in Table 1.

3.2. Intraoperative and Postoperative Outcomes

Intra-operatively, the median operative time was 382 min. A Pringle maneuver was performed in 10 patients (67%), with a median duration of vascular intermittent clamping of 53 min. Post-operatively, eight patients total had complications (53.3%); five total were major complications. Four patients (26.9%) had liver failure, with one requiring invasive treatment (grade C PHLF). Two patients developed post-operative bile leak necessitating percutaneous drainage, one had acute renal failure requiring temporary hemodialysis, and the other had an intra-abdominal

abscess that required percutaneous drainage. The overall mortality was 13.5% (n = 2). One died from aspiration pneumonia, septic shock and multiorgan failure (POD 21). The other from hemorrhagic shock due to hepatic artery rupture (POD 1). Full intra-operative and post-operative details are reported in Table 2.

Table 1. Descriptive analysis of pre-operative variables.

Variable	Total Cohort *n* = 15
Gender, male (%)	10 (67)
Age, years *	69 (49; 73)
BMI, kg/m^2 *	28.8 (26.6; 30.1)
BMI, >30 (%)	3 (20)
ASA, ≥3 (%)	9 (60)
Dislypidemia (%)	5 (33)
Diabetes (%)	6 (40)
Hypertension (%)	5 (33)
Hepatitis (HBV or HCV) (%)	2 (13.5)
Hepatocellular carcinoma (%)	6 (40)
Colorectal metastasis (%)	5 (33)
Colangiocarcinoma (%)	2 (13.5)
Other (%)	2 (13.5)
Overall comorbidities (%)	11 (73.3)
Neoadjuvant therapy (%)	5 (33)
Pre-operative chemoembolization (%)	5 (33)
Pre-operative portal vein embolization (%)	6 (40)
Pre-operative biliary drainage (%)	1 (6.7)
Pre-operative bilirubin, µmol/L *	9.4 (5.7; 10.7)
Pre-operative serum AST, U/L *	29 (23; 63)
Pre-operative serum ALT, U/L *	46 (21; 60)
Pre-operative serum ALP, U/L *	103 (73; 174)
Pre-operative serum GGT, U/L *	86 (47; 172)
Pre-operative serum Hemoglobin, g/dL*	12.8 (11.3; 13.6)
Pre-operative serum PT, sec *	87 (75; 100)
Pre-operative serum INR *	1.1 (1; 1.21)
Pre-operative serum Albumin, g/dL *	43 (41; 46)

* Expressed as median (interquartile range). BMI body mass index, ASA American Society of Anesthesiologists, AST ASpartate transaminase, ALT ALanine Transaminase, ALP Alkaline Phosphatase, GGT Gamma-Glutamyl Transferase, PT Prothrombin Time, INR International Normalized Ratio.

Table 2. Descriptive analysis of intra-operative and post-operative variables.

Variable	Total Cohort $n = 15$
Number of resected hepatic segments *	5 (4; 6)
Duration of surgery, minutes *	382 (324; 452)
Vascular clamping (%)	10 (67)
Total time of vascular clamping, minutes *	53 (38; 80)
Intra-operative blood loss, mL *	490 (200; 650)
Intra-operative transfusion (%)	2 (13.5)
Post-operative complications, (Dindo–Clavien) ≥ 3 (%)	6 (40)
Post-operative 90 days mortality (%)	2 (13.5)
Liver failure (%)	4 (26.9)
Grade A (%)	1 (6.7)
Grade B (%)	2 (13.5)
Grade C (%)	1 (6.7)
PHH (%)	2 (13.5)
Biliary leakage (%)	2 (13.5)
Sepsis (%)	5 (33)
30-days re-operation (%)	2 (13.5)
POD 1 serum Bilirubin, µmol/L *	25.7 (16.4; 52.1)
POD 2 serum Bilirubin, µmol/L *	24.1 (11.1; 67.1)
POD 5 serum Bilirubin, µmol/L *	25.5 (10.4; 61.6)
POD 1 serum AST, U/L *	788 (438; 1440)
POD 2 serum AST, U/L *	608 (261; 1228)
POD 5 serum AST, U/L *	75 (36; 128)
POD 1 serum ALT, U/L*	541 (380; 1047)
POD 2 serum ALT, U/L*	531 (352; 1383)
POD 5 serum ALT, U/L *	171 (79; 323)
POD 1 serum ALP, U/L *	75 (53; 106)
POD 2 serum ALP, U/L *	87 (57; 117)
POD 5 serum ALP, U/L *	94 (62; 185)
POD 1 serum GGT, U/L *	97 (57; 126)
POD 2 serum GGT, U/L *	74 (34; 112)
POD 5 serum GGT, U/L *	75 (54; 300)
POD 1 serum PT, sec *	52 (42; 67)
POD 2 serum PT, sec *	49 (32; 64)
POD 5 serum PT, sec *	50 (35; 75)
POD5 serum Bilirubin > 50 µmol/L (%)	5 (33)
POD5 serum PT < 50 sec (%)	6 (40)
50–50 criteria (%)	2 (13.5)

* Expressed as median (interquartile range). POD post-operative day, CD Clavien–Dindo, PHH post-hepatectomy hemorrhage, AST ASpartate transaminase, ALT ALanine Transaminase, ALP Alkaline Phosphatase, GGT Gamma-Glutamyl Transferase, PT Prothrombin Time.

3.3. Correlation between HSI and Perioperative Variables

The StO2% values after liver resection were significantly higher in patients who underwent pre-operative biliary drainage for jaundice (0.772 vs. 0.491, p = 0.033) and lower in cases with unhealthy (fatty, fibrotic, or cirrhotic) liver (0.308 vs. 0.545, p = 0.011). Cirrhotic liver presented higher negative ΔStO2% values when compared to healthy liver (−0.223 vs. 0.068, p

= 0.05). For the correlation between HSI and outcome variables, StO2% showed a significant negative correlation with ALT values on POD 5 (r = −0.602, p = 0.030), while ΔStO2% showed a positive correlation with ALP measured on POD 1 (r = 0.594, p = 0.032). NIR measurement at the end of operation presented higher values in unhealthy as compared to healthy liver (0.249 vs. 0.021, p = 0.003). This relationship was maintained when comparing fibrotic and healthy liver (0.439 vs. 0.155, p = 0.028). Pre-operative history of dyslipidemia was associated with lower values of final NIRS (0.072 vs 0.296, p = 0.029). Final NIR values showed a negative correlation with ALT values on POD 2 (r = −0.666, p = 0.013) and POD 5 (r = −0.696; p = 0.008), while intra-operative blood loss was negatively correlated with the ΔNIR (r = −0.629, p = 0.021). TWI was the only HSI index correlated with the rate of post-operative reinterventions. The two patients who presented with biliary leakage and subsequent re-operations showed significantly lower values of final TWI (0.133 vs. 0.270, p = 0.038). Pre-operative chemoembolization showed significant correlation with higher final values of TWI and ΔTWI (p = 0.027 and p = 0.036, respectively). A negative correlation was found between ΔTWI and PT measured on POD 1 (r = −0.567, p = 0.043). Lower OHI final values were significantly correlated with post-operative sepsis (0.641 vs. 0.705, p = 0.045), while ΔOHI was correlated with pre-operative hypertension (−0.125 vs. −0.019, p = 0.013) and PHLF (−0.075 vs 0.023, p = 0.010), as well as a negative correlation with ALT values on POD 5 (r = −0.813, p = 0.001). Full details on the relationships between HSI and peri-operative outcomes are reported in Tables 3 and 4 and Tables S1–S4.

Table 3. Comparisons between final hyperspectral indexes and different peri-operative features.

			TWI Final	*p*	OHI Final	*p*	StO_2 Final	*p*	NIR Final	*p*
Pre-operative	Chemoembolization	No	0.218 ± 0.098	0.027	0.663 ± 0.052	0.085	0.513 ± 0.154	0.930	0.276 ± 0.229	0.364
		Yes	0.307 ± 0.013		0.728 ± 0.078		0.506 ± 0.091		0.106 ± 0.095	
	Biliary drainage	No	0.244 ± 0.090	0.421	0.693 ± 0.065	0.169	0.491 ± 0.112	0.033	0.217 ± 0.213	1.000
		Yes*	0.322		0.594		0.772		0.200	
	Dislypidemia	No	0.240 ± 0.103	0.583	0.673 ± 0.062	0.350	0.528 ± 0.155	0.537	0.296 ± 0.208	0.029
		Yes	0.269 ± 0.058		0.710 ± 0.078		0.480 ± 0.078		0.072 ± 0.101	
	Hypertension	No	0.191 ± 0.074	0.060	0.677 ± 0.050	0.727	0.474 ± 0.165	0.455	0.251 ± 0.274	0.797
		Yes	0.283 ± 0.081		0.691 ± 0.078		0.531 ± 0.114		0.196 ± 0.172	
Intra-operative	Healthy liver	Yes	0.265 ± 0.076	0.814	0.657 ± 0.026	0.531	0.545 ± 0.102	0.011	0.021 ± 0.006	0.003
		No	0.248 ± 0.093		0.691 ± 0.072		0.308 ± 0.118		0.249 ± 0.204	
	Blood loss †		0.357	0.211	0.365	0.199	−0.100	0.735	−0.024	0.934
Post-operative	Liver failure	No	0.247 ± 0.074	0.768	0.681 ± 0.070	0.566	0.517 ± 0.140	0.738	0.198 ± 0.207	0.368
		Yes	0.262 ± 0.153		0.707 ± 0.067		0.487 ± 0.110		0.282 ± 0.225	
	Liver hemorrhage	No	0.270 ± 0.077	0.038	0.690 ± 0.073	0.664	0.513 ± 0.134	0.884	0.214 ± 0.203	0.923
		Yes	0.133 ± 0.061		0.666 ± 0.005		0.498 ± 0.156		0.228 ± 0.306	
	Bile leak	No	0.270 ± 0.077	0.038	0.690 ± 0.073	0.664	0.513 ± 0.134	0.884	0.214 ± 0.203	0.923
		Yes	0.133 ± 0.061		0.666 ± 0.005		0.498 ± 0.156		0.228 ± 0.306	
	Sepsis	No	0.270 ± 0.082	0.190	0.705 ± 0.071	0.045	0.516 ± 0.074	0.826	0.235 ± 0.213	0.635
		Yes	0.200 ± 0.096		0.641 ± 0.034		0.498 ± 0.240		0.168 ± 0.204	
	Re-operation	No	0.270 ± 0.077	0.038	0.690 ± 0.073	0.664	0.513 ± 0.134	0.884	0.214 ± 0.203	0.923
		Yes	0.133 ± 0.061		0.666 ± 0.005		0.498 ± 0.156		0.228 ± 0.306	
	ALP POD1 †		−0.200	0.492	0.007	0.982	0.443	0.113	0.137	0.642
	PT POD1 †		−0.150	0.609	0.253	0.382	−0.026	0.929	0.011	0.970
	ALT POD2 †		−0.325	0.279	−0.127	0.680	−0.517	0.070	−0.666	0.013
	ALT POD5 †		−0.191	0.531	−0.004	0.989	−0.602	0.030	−0.696	0.008

* only 1 case in the present population. † Spearman's rank correlation coefficient. POD post-operative day, ALP Alkaline Phosphatase, PT Prothrombin Time, ALT ALanine Transaminase.

Table 4. Comparisons between differential values of hyperspectral indexes and different peri-operative features.

			ΔTWI	*p*	ΔOHI	*p*	ΔStO_2	*p*	ΔNIR	*p*
Pre-operative	Chemoembolization	No	−0.049 ± 0.077	0.036	−0.064 ± 0.083	0.798	0.024 ± 0.174	0.545	−0.001 ± 0.241	0.435
		Yes	0.059 ± 0.083		−0.052 ± 0.082		0.078 ± 0.092		−0.088 ± 0.109	
	Biliary drainage	No	−0.016 ± 0.093	0.280	−0.060 ± 0.083	0.966	0.024 ± 0.130	0.067	−0.054 ± 0.195	0.368
		Yes *	0.093		−0.056		0.299		0.199	
	Dislypidemia	No	−0.001 ± 0.119	0.773	−0.071 ± 0.070	0.527	0.037 ± 0.168	0.820	−0.039 ± 0.250	0.943
		Yes	−0.018 ± 0.039		−0.041 ± 0.098		0.057 ± 0.119		−0.027 ± 0.099	
	Hypertension	No	−0.032 ± 0.092	0.484	−0.125 ± 0.057	0.013	0.075 ± 0.150	0.579	−0.052 ± 0.266	0.943
		Yes	0.007 ± 0.098		−0.019 ± 0.064		0.026 ± 0.150		−0.024 ± 0.165	
Intraoperative	Healthy liver	Yes	0.065 ± 0.092	0.063	−0.036 ± 0.035	0.879	0.037 ± 0.225	0.841	−0.177 ± 0.165	0.064
		No	−0.013 ± 0.078		−0.030 ± 0.091		0.022 ± 0.123		−0.032 ± 0.146	
	Blood loss †		0.222	0.467	0.507	0.077	−0.427	0.146	−0.629	0.021
Post-operative	Liver failure	No	−0.020 ± 0.077	0.263	−0.075 ± 0.077	0.010	0.069 ± 0.133	0.171	−0.004 ± 0.201	0.154
		Yes	0.063 ± 0.186		0.023 ± 0.022		−0.087 ± 0.191		−0.202 ± 0.067	
	Liver hemorrhage	No	−0.006 ± 0.098	0.861	−0.048 ± 0.072	0.090	0.029 ± 0.141	0.203	−0.038 ± 0.207	1.000
		Yes *	−0.024		−0.189		0.228		0.011	
	Bile leak	No	−0.006 ± 0.098	0.861	−0.048 ± 0.072	0.090	0.029 ± 0.141	0.203	−0.038 ± 0.207	1.000
		Yes *	−0.024		−0.189		0.228		0.011	
	Sepsis	No	−0.018 ± 0.102	0.474	−0.045 ± 0.079	0.246	0.019 ± 0.115	0.257	−0.022 ± 0.167	1.000
		Yes	0.028 ± 0.060		−0.108 ± 0.071		0.132 ± 0.231		−0.077 ± 0.329	
	Re-operation	No	−0.006 ± 0.098	0.861	−0.048 ± 0.072	0.090	0.029 ± 0.141	0.203	−0.038 ± 0.207	1.000
		Yes *	−0.024		−0.189		0.228		0.011	
	ALP POD1 †		0.146	0.635	−0.239	0.431	0.594	0.032	0.160	0.603
	PT POD1 †		−0.567	0.043	0.110	0.720	−0.234	0.442	0.267	0.378
	ALT POD2 †		0.266	0.404	0.140	0.665	0.049	0.880	−0.154	0.633
	ALT POD5 †		−0.105	−0.745	−0.813	0.001	−0.091	0.778	−0.312	0.324

* only 1 case in the present population. † Spearman's rank correlation coefficient. POD post-operative day, ALP Alkaline Phosphatase, PT Prothrombin Time, ALT ALanine Transaminase.

4. Discussion

The present results demonstrate the safety and feasibility of hyperspectral imaging in major hepatectomy, the ability of HSI to precisely and accurately localize ischemic damage during surgery, and the relationship of HSI measures to clinical outcomes and serum markers after this high-risk procedure. From this first human clinical application in hepatectomy, results suggest that HSI is a promising tool which could potentially help translate intra-operative advanced vision to improve short-term post-operative outcomes.

Published work has demonstrated the benefits of intra-operative evaluation of the liver parenchyma. Currently, evaluation of the remnant liver mainly relies on subjective and operator-dependent methods, naked-eye estimation, or ultrasound evaluation. This subjective evaluation becomes more challenging when underlying liver disease is present. The need for an objective, precise, and convenient analysis tool has driven researchers to find alternative solutions. ICG-fluorescence imaging is the most common objective method used to identify segmental boundaries of the liver, for more accurate anatomic resections [34,35]. Several works reported the benefit of ICG as a predictive factor of PHLF [6,36,37]. However, these studies were biased by high heterogeneity of the included population and their retrospective design. In addition, the ICG excretion rate is affected by the functional excretion ability of hepatocytes, hepatic blood flow, shunt volume, and bile flow rate, all which are unreliable in liver dysfunction [38].

HSI has great potential to overcome these limitations. Our group has previously used HSI to assess StO2% and intra-operatively localize preselected ROIs during esophagectomy [39], small bowel ischemia [40], and hepatectomy [18]. When adding an augmented reality function, HSI was successfully able to intra-operatively guide anatomical liver resections [18] and to discriminate between total and arterial liver ischemia [20]. The current work adds to the literature's positive validation of prior pre-clinical studies. Promising relationships with HSI

parameters and post-operative outcomes were demonstrated, as well as the relationship between prognostic laboratory values and HSI parameters.

As expected, StO2% had lower values in unhealthy liver tissue, as it primarily measures oxygenation close to the surface. In addition, NIR spectroscopy showed higher values in unhealthy liver tissue, especially in the presence of fibrosis. This validated the ability of HSI to measure deeper into the tissue (4–6 mm) and provided information about oxygen perfusion differences at different depths. HSI parameters of TWI and OHI were parameters significantly correlated with clinical post-operative outcomes. Specifically, the lower concentration of water inside hepatic tissue at the end of the operation was related to post-operative PHH, biliary leakage, and re-intervention. It is likely this reflects the extensive use of vascular inflow clamping and goal-directed fluid therapy with the aim to reduce intra-operative bleeding, especially in patients who underwent major hepatectomies. In support, hemoglobin concentration recognized by OHI index is affected by Pringle maneuver and a negative ΔOHI% is expected when vascular clamping is performed. Consequently, higher negative ΔOHI% reflects major reductions in hemoglobin and increased liver damage, with the resultant correlation with post-operative liver failure. Furthermore, the negative significant correlation of HSI parameters and ALT in POD 1 and POD 5 adds support to our results, as ALT is the most specific laboratory biomarker of liver injury [41].

We recognize several limitations in this study. HSI technology requires further techno- logical advances for video-rate and time necessary for acquisition. The HSI-based enhanced reality (HYPER software) was previously developed, but not clinically validated [18]. The single center observational design, small sample size, and limited number of post-operative major complications limit the results and clinical implications. Finally, HSI acquisition is feasible only in open surgery currently. Further clinical support would allow greater development of the platform and expansion into other surgical platforms.

5. Conclusions

In the first clinical application of HSI for major hepatectomy, relationships between the HSI parameters and post-operative outcomes variables and laboratory marker ALT were seen. These promising but preliminary results support further clinical studies with larger samples to test the discriminative ability of the platform, independent relationships with serum markers, and clinical outcomes. Predictive models based on machine learning and larger samples could be useful to assess the real clinical applicability of HSI on liver resections. With the drive towards precision surgery, there could be great benefit from HSI guidance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cancers14225591/s1, Table S1: Comparison of final hyperspectral indexes between patients with different perioperative features; Table S2: Comparison of differential values of hyperspectral indexes between patients with different perioperative features; Table S3: Correlation between final hyperspectral indexes and perioperative continuous variables; Table S4: Correlation between differential hyperspectral indexes and perioperative continuous variables.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data presented in this study are available in the article and Supplementary Materials.

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Conflicts of Interest: J.M. is the President of the IRCAD Institute, which is partly funded by KARL STORZ, Siemens, and Medtronic. M.D. is a member of the Board of Diagnostic Green and the recipient of the ELIOS grant, all unrelated to this work. S.G. is an employee and stockholder of Intuitive Surgical Sàrl. The other authors declare that they have no conflict of interest.

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1.3 Molecular tools to predict adverse outcomes in resectable HCC

Despite the constant and progressive evolution of therapeutic algorithms as well as of technical strategies to approach advanced diseases several critical issues persist in the clinical management HCC. First and foremost, the absence of a reliable prognostic clinical marker to predict long-term remains a significant challenge. While the plasmatic alpha-fetoprotein (AFP) is the most commonly used prognostic indicator,(63) its limitations are evident, with 15–30% of HCC cases presenting normal AFP levels.(64) Other tools, as microvascular invasion or capsule rupture are pathological and therefore known only after surgical resection. Secondly, the complex treatment allocation process often falls short of providing a complete therapeutic arsenal. Effective and validated peri-operative therapies are still elusive, and the current inability to precisely detect more aggressive tumors may lead to surgeons endorsing complex, high-morbidity resections in patients at elevated risk of recurrence.(65) In recent years, a growing body of evidence has underscored a robust correlation between systemic inflammation and HCC prognosis, with various systemic and pathological markers linked to survival and recurrence. Notably, markers such as platelet-to-lymphocyte ratio (PLR), neutrophil-to-lymphocyte ratio (NLR), and similar scores have shown promise in predicting poor long-term outcomes post-treatment.(66) This relationship extends to the molecular level, where gene expression alterations underpin inflammatory cell shifts crucial in the development and progression of cancer.(67)

In response to these challenges, a collaborative effort with INSERM (UMR_S1110), a cutting-edge laboratory renowned for its expertise in liver pathologies and translational research, was undertaken. Recognizing the limitations of current preoperative tools, particularly in predicting prognosis before resection, our collaboration aimed to delve into the molecular sphere

leveraging the profound knowledge base of the laboratory. This collaborative endeavor has allowed for a nuanced exploration of these inflammation markers in literature, which ended in the publication of a comprehensive review focused on inflammation-related markers as predictive indicators for poor outcomes in resected HCC.

Review

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INFLAMMATION-RELATED PROGNOSTIC MARKERS IN RESECTED HEPATOCELLULAR CARCINOMA

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Hepatocellular carcinoma is usually detected late and therapeutic options are unsatisfactory. Despite marked progress in patient care, HCC remains among the deadliest cancers world-wide. While surgical resection remains a key option for early-stage HCC, the 5-year survival rates after surgical resection are limited. One reason for limited outcomes is the lack of reliable prognostic biomarkers to predict HCC recurrence. HCC prognosis has been shown to correlate with different systemic and pathological markers which are associated with patient survival and HCC recurrence. Liver inflammatory processes offer a large variety of systemic

and pathological markers which may be exploited to improve the reliability of prognosis and decision making of liver surgeons and hepatologists. The following review aims to dissect the potential tools, targets and prognostic meaning of inflammatory markers in patients with resectable HCC. We analyze changes in circulant cellular populations and assess inflammatory biomarkers as a surrogate of impaired outcomes and provide an overview on predictive gene expression signatures including inflammatory transcriptional patterns, which are representative of poor survival in these patients.

KEYWORDS

HCC, biomarkers, genetic signatures, inflammation, patient outcome

1 Introduction

Hepatocellular Carcinoma (HCC) is the most common primary liver cancer accounting for about 80% of all cases and it ranks as the third leading cause of cancer deaths worldwide (1). Like cholangiocarcinoma, HCC shows a dismal prognosis with a relative 5-year survival rate of approximately 20% (2). Despite the constant and progressive evolution of the therapeutic algorithms on which decision strategy is based, in clinical practice several issues remain to be addressed. First, a reliable prognostic clinical marker to predict HCC outcome is still missing. Among the prognostic indicators, the most common is plasmatic alpha-protein (AFP), which correlates with tumor behavior and risk of recurrence and survival (3–5). However, in 15–30% of HCC, AFP levels remain in a normal range and the heterogeneity of studies prevents from formulating clear recommendations (6, 7). Secondly, the complex treatment allocation process does not always reflect in a complete therapeutic arsenal. Effective and validated peri-operative therapies are still lacking and the inability to accurately detect more aggressive tumors could lead surgeons to validate complex and high morbidity resections on patients with an elevated risk of recurrence (8). In the last years several authors reported a strong correlation between

systemic inflammation and HCC prognosis with different systemic and pathological markers associated with survival and recurrence. For example, high values of platelet-to- lymphocyte ratio (PLR), neutrophil-to-lymphocyte ratio (NLR) and other similar scores seem to predict poor long-term outcomes after treatment (9–11). This relationship is also evident on a molecular level as gene expression alterations are at the basis of these inflammatory cell shifts on which cancer develops and progresses (12). In this review, we provide a comprehensive overview and update on the prognostic meaning of inflammatory modifications in patients with resectable HCC. We analyze changes in circulant cellular populations and assess inflammatory biomarkers as a surrogate of impaired outcomes and provide an overview on predictive gene expression signatures including inflammatory transcriptional patterns, which are representative of poor survival in these patients.

2 Inflammatory microenvironment in HCC carcinogenesis and prognosis

A large body of knowledge has demonstrated that a dysregulation in tumor microenvironment (TME) contributes to carcinogenesis and tumor progression (13). Chronic inflammation is considered as an excessive, abnormal, and prolonged form of cellular immune responses interacting with other factors in the development of the neoplastic process (14). A large panel of innate immune cells in the tumor microenvironment (macrophages, neutrophils, dendritic cells, innate lymphoid cells, myeloid- derived suppressor cells, and natural killer cells) as well as adaptive immune cells (T cells and B cells) are linked to tumor progression and outcome (15). Tumors control their microenvironment by a large number of tumor-associated factors promoting its establishment, growth, survival, and spread by shaping a pro-tumoral local cytokine milieu (15). This cause- effect relationship is well described in HCC patients and several mechanisms have been shown to be related to tumor development, progression, and overall survival. The majority of HCCs occur in injured liver after stimulation with different inflammation- triggering agents, as viruses, alcohol, drugs, toxins, or obesity (16). Alterations

in inflammatory cell populations and a dysregulation of genes and protein expression pattern have been correlated with long-term outcomes in HCC patients. Among many others, these involve an upregulation of several metalloproteinases (MMP) and downregulation of C-type Lectin-like Receptor 2 (CLEC2) which were found to be associated with impaired survival (17). Similarly, hyperexpression of PD-1 and PD-L1 in neoplastic hepatocytes and lymphocytes infiltrating the tumor is a marker of poor survival, while in slowly growing HCC these markers are barely expressed (17). Other authors demonstrated that TNF, IL6 and CCL2 mutations are those most significantly associated with outcomes and considerably longer survival was seen in patients with higher levels of both TNF and IL6 (18, 19). To our knowledge, out of the mentioned markers, targeted therapies have been developed for PD-1 and PD-L1, while the clinical trials targeting the other mentioned markers have so far been unsuccessful, at least in the context of HCC (20–27). The above- mentioned markers have been summarized in Table 1. In regard to cell populations (Figure 1), Kuang and co-workers found that peritumoral stroma of HCC tissues was enriched with neutrophils and their levels could serve as a powerful predictor for poor survival in HCC patients (32). Accordingly, high inflammatory cytokine levels in the tumor can promote local and systemic neutrophilia (33). Lymphocytes are at the same time involved in tumor progression, and an enhanced infiltration of specific subtypes within the tumor samples, as CD8+ and CD3+ T cells, CD20+ B cells and CD56+ NK cells, was found to be present in patients with longer survival (18, 34). A recent study (28) identified a structure formed by specific cell populations and its role in immunotherapy resistance. It was found that a subpopulation of macrophages with high expression of osteopontin (SPP1), in combination with CAFs (cancer-associated fibroblasts) mediates resistance to immune checkpoint inhibitors. Blocking SPP1, a phosphoprotein with a previously identified regulatory role in the TME (35), rendered the tumors more responsive to immunotherapy in an animal model. It was therefore marked as a target for further clinical studies in the context of HCC, but

to our knowledge, no such trials are currently in progress. It is also worth noting that this study focused on a restricted number of cases and did not explore the potential of SPP1 as a serum inflammatory marker.

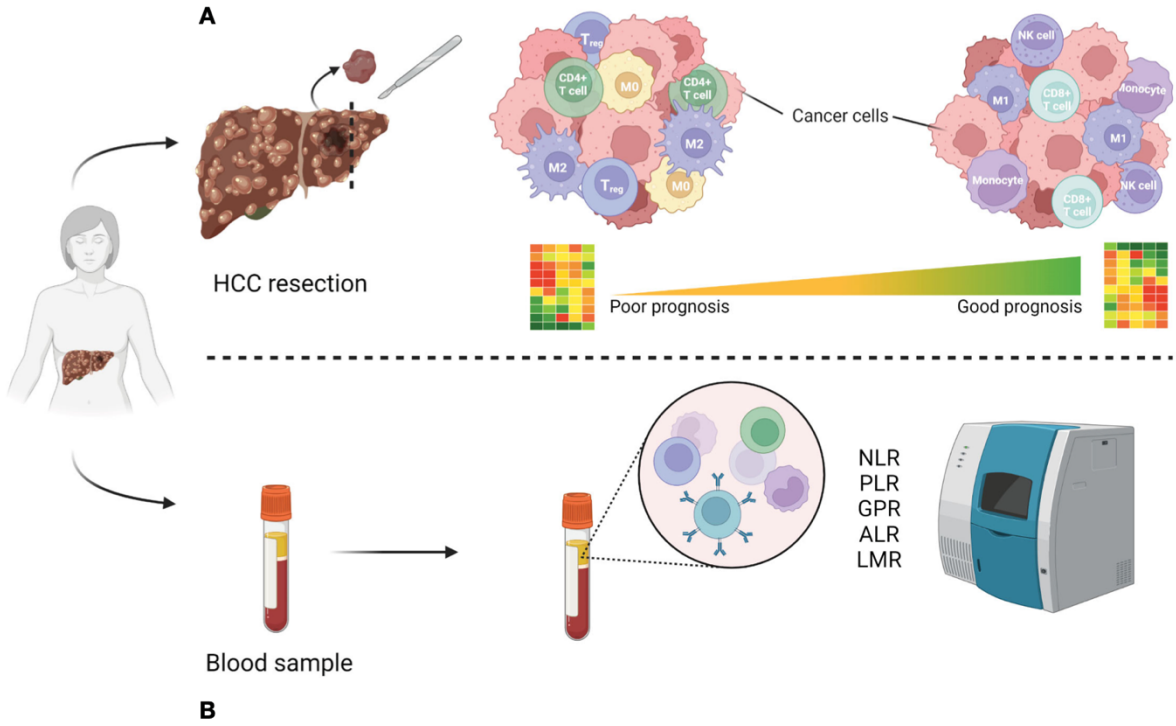

Figure 1. Immune cell population difference analysis in poor vs good prognosis patients. (A) High-risk resected patient tissue with poor prognosis tends to be enriched with regulatory immune cells (Treg, CD4+ T cell), type 2 macrophages (M2) as well as non-activated macrophages (M0), as opposed to natural killer (NK) cells, CD8+ cytotoxic T cells, type 1 macrophages (M1) and monocytes in good prognosis patients (29–31). (B) Most used inflammatory markers analyzable from patient blood samples. Created using BioRender.

3 Serum inflammatory markers

Based on the strong association between tumor microenvironment and natural history of tumors, modifications in circulating inflammatory markers highlight more aggressive diseases and therefore predict poor outcomes. These patterns have been implemented in clinical practice as scores, which have the advantage of being easy to approach, calculated with routine laboratory tests, thus with limited costs, and available before surgical treatment. The most diffused and described serum inflammatory marker in resected HCC is undoubtedly the NLR (10, 11, 36–40).

Table 1. Markers of the inflammatory microenvironment of HCC patients

Type of marker	Study	Expression change	Prognostic meaning
MMP1, MMP10, MMP12	Critelli et al 2017;	Upregulation	Decreased Survival
CLEC2	Critelli et al 2017;	Downregulation	Decreased Survival
PD1	Critelli et al 2017;	Upregulation	Decreased Survival
PDL1	Critelli et al 2017;	Upregulation	Decreased Survival
TNF	Chew et al 2010; Chew et al 2012	Upregulation	Increased Survival
IL6	Chew et al 2010; Chew et al 2012	Upregulation	Increased Survival
CCL2	Chew et al 2010; Chew et al 2012	Upregulation	Increased Survival
SPP1	Liu et al. 2023	Upregulation	Decreased Survival

An increased NLR, despite the different cut-offs used by the authors, seems associated with reduced overall survival and disease-free survival rates after curative resection. Neutrophil count, rather than reduced lymphocytes, could probably explain these results, knowing that elevated neutrophils associated independently with poorer survival and impaired performance status in HCC (41). Although other publications did not support the prognostic value of NLR at univariate or multivariate analysis (11, 36, 40, 42), two meta-analyses confirmed the significant correlation with impaired prognosis in resected patients (43, 44). Another well-established immunity-related score found to be predictive of long-term outcomes in resected HCC is the PLR. Several studies confirmed a strong association between oncologic outcomes

and an elevation of this index and, unlike NLR, this biomarker has almost always confirmed its prognostic role at multivariate analysis (10, 11, 38–40, 44, 45). Other less explored scores are the gamma-glutamyl transpeptidase-to-lymphocyte ratio (GLR) (11, 36), the aspartate aminotransferase-to-lymphocyte ratio (ALR) (11, 46) or the lymphocyte-to-monocyte ratio (39, 40), all more or less related to long-term outcomes. A summary of these inflammatory biomarkers as well as studies assessing their prognostic role is shown in Table 2. In order to increase the accuracy of these biomarkers, some authors developed new scores by combining these aforementioned values together or by adding other non-inflammatory variables in the formula. The first group includes indexes as the A-G-P score, a predictive model to accurately predict survival by analyzing at the same time the ALR, the GLR and the PLR (11). This equation demonstrated to be an excellent independent predictor of OS in resected patients and, at the same time, being able to stratify patients with HCC according to the resulting score well (11). On the other hand, other formulas have been developed starting from these inflammatory markers and other serum values, as nutritional indexes. This is the case of the Glasgow prognostic score (GPS) and the modified GPS, calculated from the CRP and the albumin level (47, 48), the prognostic nutritional index (PNI) combining lymphocyte count and serum albumin (29, 49) or the inflammation-immunity- nutrition score (IINS), a combination of CRP, lymphocyte count and serum albumin level (30). All these equations, although not systematically integrated in clinical practice, have been widely described as factors of impaired survival in literature.

4 Gene signatures

An emerging toolset potentially complementing the classical predictive markers in the clinics are transcriptional gene signatures (GS). They refer to expression values of a group of genes, and are mostly representative of a condition, healthy, diseased or both.

Table 2. Prognostic meaning of different serum inflammatory markers in resected hepatocellular carcinoma in aforementioned studies.

Type of marker	Study	Cut-off assessed	Number of patients	Prognostic meaning	Role at multivariate analysis
Neutrophil to lymphocyte ratio (NLR)	Sullivan et al., 2014	-	75	Not predictive of OS	-
	Lu et al., 2016	2.81	963	Shorter OS and RFS	Independent risk factor for OS and RFS
	Zheng et al., 2017	-	370	Shorter OS and RFS	Lost
	Wang et al., 2019	2.92	239	Shorter OS and RFS	Independent risk factor for OS and RFS
	Dai et al., 2020	2.5	302	Shorter OS and DFS	Lost
	Wu et al., 2021	2.33	347	Shorter OS, no differences in DFS	Lost
	Silva et al., 2022	1.715 for OS 2.475 for DFS	161	Shorter OS and DFS	Lost
	Zhou et al., 2022	4.191 for OS 2.271 for RFS	91	Shorter OS, no differences in RFS	Lost
Platelets to lymphocyte ratio (PLR)	Zheng et al., 2017	275 for RFS a 298 for OS	370	Shorter OS and RFS	Independent risk factor for OS and RFS
	Wang et al., 2019	128.1	239	Shorter OS and RFS	Independent risk factor for OS and RFS
	Wu et al., 2021	117.09	347	Shorter OS, no differences in DFS	Independent risk factor for OS
	Kim et al., 2022	132	159	Shorter OS and RFS	Independent risk factor for OS
	Silva et al., 2022	115.05 for OS 100.25 for DFS	161	Shorter DFS	Independent risk factor for DFS
	Zhou et al., 2022	302.104 for OS 228.644 for RFS	91	Shorter OS and RFS	Independent risk factor for OS and RFS
Gamma-glutamyl transpeptidase to platelet ratio (GPR)	Dai et al., 2020	0.35	302	Shorter OS and DFS	Independent risk factor for OS and DFS
	Wu et al., 2021	0.48	347	Shorter OS and DFS	Independent risk factor for OS and DFS
Aspartate aminotransferase to lymphocyte ratio (ALR)	Chen et al., 2021	26.6 for OS 27.9 for RFS	983	Shorter OS and RFS	Independent risk factor for OS and RFS
	Wu et al., 2021	31	347	Shorter OS and DFS	Independent risk factor for OS and DFS
Lymphocyte-to-monocyte ratio (LMR)	Zheng et al., 2017	-	370	Shorter OS and RFS	Lost
	Zhou et al., 2022	3.785 for OS 4.633 for RFS	91	No differences	-

OS: Overall Survival, RFS: Recurrence-Free Survival, DFS: Disease-Free Survival

The expression pattern of genes is often correlated with the activity of their products and can therefore infer on the cell processes these genes are a part of. Recent technological advancements enable the collection and analysis of large quantities of biological data, as in cases of gene expression values across the genomes of multiple cells. This kicked off the development of gene signatures in several diseases and cancer. Majority of GS have been assessed as predictive tools and are derived from data obtained using techniques such as quantitative PCR (qRT-PCR), hybridization arrays (oligonucleotide, cDNA), RNA sequencing etc., that all have the analysis of levels of RNA production in common. Most signatures focus on messenger RNA transcription, while some of them are based on microRNA (miRNA) (31), long non-coding RNA (lncRNA) (50) or protein expression (51). Contrary to most classical prognostic pathological or clinical features, the analysis of gene signatures allows a profound molecular profiling of the tumour environment. As cancer is a multicellular disease often involving several systems within the body, analysing gene expression patterns from multiple cell types facilitates identification of dysregulated pathways and their comprehension. Gene signatures provide a list of differentially expressed genes (DEG), upregulated or downregulated between the compared groups, usually diseased and non-diseased or healthy conditions. Tissues that are presumably not affected but surrounding the cancer area are usually considered as non-diseased, while healthy tissue is obtained from regions distant from the affected area. Out of the selected genes, some are linked to a poor prognosis or high risk while others are marked as good-prognosis or low risk genes. Therefore, the combination of both poor and good prognosis gene expression pattern allows a classification of patients into high and low-risk groups. The predictive capacity of a signature is mostly measured using machine learning-derived ROC (Receiver Operating Characteristics) and AUC (Area Under the ROC Curve) values, while some authors also use confidence intervals. The closer the AUC value is to one, the more accurate the predictive signature is (52). Recent analyses have studied the drawbacks of gene

signatures, notably their redundancy and possibilities of improving them (53). Even with drawbacks, these signatures can be efficient for a statistically important number of patients and therefore their use in clinical practice should not be ignored.

4.1 Gene signatures predicting HCC recurrence and survival in resected patients

To date, most signature-based studies focus on predicting recurrence as well as survival in HCC patients. A study from 2020, found that 66% of patients experienced HCC recurrence over a period of 8 years emphasizing the drastic recurrence rates of HCC (54). Although still debated, the classification of tumor recurrence into early and late recurrence is strongly linked to the tumour origin. Secondary tumours originating from leftover cancer cells of the resected tumour within two years after surgery are defined as early recurrence, whereas tumours originating from novel cancer cells of the same organ (de novo tumour) more than two years after surgery are considered as events of late recurrence (55). As early as 2008, the first collection of 186 genes was published in the pioneering work from Hoshida et al., highlighting 73 poor and 113 good prognosis genes being predictive for survival in liver disease (56–59). The authors established a robust signature of DEGs from tissues surrounding HCC of 106 resected patients which was then validated in another cohort of 234 patients. They managed to overcome the technical difficulty to analyse more commonly available formalin- and paraffin-treated (FFPE) tissues instead of depending on snap frozen tissues. This signature has since been further studied and validated in additional cohorts. A 5-gene signature from frozen liver tissues was reported (TAF9, RAMP3, HN1, KRT19, and RAN) predicting survival from HCC in 314 HCC patients (60). Depending on the differential expression of these five genes, patients were stratified into poor and good prognosis groups, and the signature was validated in external cohorts of patients. As reported by Nault and co-workers (60), the comparison of the two signatures described above validated the findings from both articles, also as the signatures provide similar output,

i.e., a comparable stratification of patients in their corresponding poor and good survival groups. More recently, a signature specific for early recurrence in HCC has been described, which was not based on coding genes but on 25 lncRNAs, another type of RNA relevant in HCC development (50). This signature had better predictive performance than multiple other factors, including serum AFP. Interestingly, the high and low-risk groups correlated with the immune characterization of the tissue of these patients; for example, the low-risk group showed higher levels of tumour- infiltrating lymphocytes. Another 9-gene survival signature with links to immune microenvironment was derived from the analysis of 274 resected HCC patient tissues by another group (61). Of the four upregulated (C2HC1A, MARCKSL1, PTGS1, CDKN2B) and five (CLEC10A, PRDX3, PRKCH, MPEG1, LMO2) downregulated genes in poor prognosis patients, several signature genes have direct or indirect roles in cancer immune environment (CLECL10A, PTGS1, C2HC1A). Even though they focused on data from HCC patients of viral aetiology, their established signature is seemingly outperforming the previously established ones (61). Finally, a more recurrence-specific gene signature had been identified by comparing recurrence and non-recurrence HCC tissues from 85 patients (62). Within the selected genes, two (HMGA1 and RACGAP1) were found to be particularly relevant for recurrence in HCC patients. Interestingly, both genes have recently been studied for their role in cancer immunity (63, 64). However, while some of the signature genes are known to have roles in HCC, they are generally parts of unrelated pathways and do not necessarily interact with each other.

4.2 Inflammatory gene signatures

As single-cell resolution in transcriptomic analysis boosted our understanding of the HCC microenvironment (65, 66), signatures derived from immune cell populations or linked to immunity in HCC have been increasingly explored in the recent years. However, most of these studies tend to use a variety of patient tissues as source, including results from not only resected

patients, but also biopsies of advanced HCC or data found in online databases, mainly from The Cancer Genome Atlas (https://www.cancer.gov/ tcga). To our knowledge, resection-specific immune gene signatures have yet to be established. A study from 2021 established a robust immune-related gene signature containing seven genes from TCGA-derived data of 372 patients with a variety of backgrounds (histological grade, clinical stage, survival rate etc.) (67). Six out of seven genes (S100A8, BIRC5, CACYBP, NR0B1, RAET1E, SPP1) were associated with high-risk of survival, while SPINK5 was identified as a low-risk factor. On the cellular level they found that immunosuppressive cell groups such as CD4+, Treg cells, M0 and M2 macrophages, as well as neutrophiles were more abundant in the high-risk groups compared to the low-risk ones (Figure 1). This signature, however, needs further testing before it can be confidently applied in patients. Another recent study used a similar but more focused approach, as they report developing an eight gene signature based on M2-like tumour associated macrophages from both patient biopsies and resections (68). Similar findings were reported by two independent studies, whose 6 and 8 immune- related gene signatures had an AUC of 0.71 and 0.68, respectively (69, 70). Finally, Shi and co-workers reported a non-invasive immune signature for early-stage HCC based on the analysis of cells from patient blood samples using single cell cytometry (65). In this dynamic immune atlas, they identify mainly lymphocyte (sub) types characterizing advanced stages of HCC using only patient blood samples. In general, most recent immune signatures tend to have less than 10 genes and their AUC values vary from 0.65 to 0.75. These have been summarized in Table 3. Of note, all the listed studies report the tendency of presence of contrasting immune cell types within the high-risk compared to low-risk group: the high- risk patient group tends to be enriched with macrophages and Tregs while B, NK, cytotoxic T cells and mast cells are less represented.

Table 3. A summary of immune-related predictive signatures in HCC: their predictive power, data origin and the defined genes.

Signature	Study	AUC	Good/Poor prognosis genes	Data origin	Patients
An Inflammatory Response-Related Gene Signature Can Impact the Immune Status and Predict the Prognosis of HCC	Zhuo et al., 2021	0.685, 0.626, 0.605 at 1, 2, and at 3 years	SERPINE1 ADORA2B, MEP1A, P2RX4, ITGA5, NOD2, RIPK2, SLC7A	TCGA LIHC&ICGC	>400
Survival prediction and response to immune checkpoint inhibitors: A prognostic immune signature for HCC	Ying et al., 2021	0.71 at 5-year survival	FYN, IGF1, MASP1, NR3C2, TGFBR3 BIRC5	TCGA&GEO	>400
Identification of a prognostic and therapeutic immune signature associated with HCC	Peng et al., 2021	0.77, 0.73, and 0.74 in predicting 1-, 3-, 5-year overall	SPINK5 RC5, CACYBP, NR0B1, RAET1E, S100A8, SPP1	TCGA, GEO & ICGC	>400
M2-like tumor-associated macrophage-related biomarkers to construct a novel prognostic signature, reveal the immune landscape, and screen drugs in HCC	Qu et al., 2022	1, 3, and 5 years was 0.728, 0.689, and 0.663,	KLF2 LIM3, PAM, PDLIM7, FSCN1, DPYSL2, ARID5B, LGALS3	TCGA, GEO & ICGC	>400
Single-cell immune signature for detecting early-stage HCC and early assessing anti-PD-1 immunotherapy efficacy	Shi et al., 2022	-	Cells, no genes specified	PBMC at resection	~50

TCGA: The Cancer Genome Atlas, LIHC: Liver Hepatocellular Carcinoma, ICGC: International Cancer Genome Consortium, GEO: Gene Expression Omnibus, PBMC: peripheral blood mononuclear cells

5 Challenges & future directions

Inflammation is a key player in the natural history of HCC and thus the relationship between some inflammatory-based tools and patients' prognosis are closely linked by the disease biology of hepatocarcinogensis (71–74). This observation offers an opportunity to predict long-term outcomes as precise as possible if compared to current markers. Although some of the biological markers above cited clearly show a direct and independent connection with

recurrence and survival after liver resection for HCC, they are far from being extensively implemented in clinical practice. Limitations of the currently available serum biomarkers are the difficulty in standardizing reliable cut-offs as well as the universal validation of their prognostic role, regardless of underlying patient pathologies or cancer aetiology. When assessing the above-mentioned ratio (NLR, PLR, etc.), cut-off values are determined by the AUC and therefore always different among all the studies. As a result, we found that authors use various values to define cases with impaired outcomes and, sometimes, these values are significantly different if considering the type of outcome assessed, as recurrence or survival (38, 40). A recent meta- analysis assessing the role of NLR, found that among 13 included studies the cut-off values ranged between 1.505 and 5.0, and only a few studies used the same ratio (43).

Another issue to solve is the large-scale applicability of these markers in clinical practise. This review focusses on resected patients which represent a large minority of all diagnosed HCC. This type of lesion often develops on an immunity-altered host which can distort the results and thus the direct correlation between serum markers and prognosis. Furthermore, authors usually analyze specific subgroups of HCC patients in order to create a homogeneous cohort, as tumors in well-compensated cirrhosis (40). In 2016, Lu et al. assessed the utility of the NLR and used subgroup analysis to examine this potential relationship separately in patients in BCLC stages 0/A, B, or C (37). The authors found that this marker may be a good predictor of survival in early/intermediate stage, whereas it was not associated with risk of overall survival (OS) or tumor recurrence in patients with stage C disease. Similar limitations are found when comparing the potential of transcriptional signatures. Despite the very promising results from a decade of development, no predictive transcriptomic signature is used in a clinical setting. Like the serum biomarkers, the AUC values used to quantify the power of GS vary significantly, and do not have confirmed utility until the signatures are confirmed by other teams or in clinical settings.

Also, as we mentioned earlier and for the purpose of this review, resection- specific immune/inflammatory gene signatures have been scarce. However, a promising immune signature has been recently identified using artificial intelligence on transcriptomics of resected patients (75). The authors argue their approach would allow for the bypass of technical bias and restriction induced with a more "classical" gene signature approach. Moreover, patient samples used are often restricted to small numbers, a single country, patient population or aetiology, potentially affecting the applicability of these signatures without validation in other cohorts (61, 62, 68). An additional important limitation of the transcriptional signatures is their dependency on patient liver tissues. Non-invasive methods, such as described by Shi and co-workers (65), should thus be prioritized in the future. Initiatives to translate transcriptional signatures into minimal-invasive blood surrogates have already been taken with a recently published eight-protein signature termed PLSec (76). It is based on the 186-gene PLS (56, 58) and is predictive for survival, as well as recurrence of HCC in advance fibrosis patients. The very encouraging data are based on the analysis of 400 patients in total and pave the wave for further consolidation in larger cohorts. Out of the eight, 6 proteins were marked as high-risk, including vascular cell adhesion molecule 1 (VCAM-1), insulin-like growth factor-binding protein 7 (IGFBP-7), gp130, matrilysin, IL-6, and C-C motif chemokine ligand 21 (CCL-21), and 2 were defined as low-risk-associated serum proteins, angiogenin and protein S. Collectively, new combinations of classical and novel blood-based biomarker signatures will likely have the biggest impact in transforming patient care.

Finally, beyond the pure prognostic meaning, another non- negligible potential of these biomarkers lies undoubtedly in the possibility of guiding therapeutic approaches in advanced disease. Finding a biomarker which could accurately predict tumor progression or response to specific treatments would mean opening the door to precision medicine in HCC, as already established in other cancers (77). Although immunotherapy is the first-line option in these

patients, with drugs targeting different checkpoints of the immune system, no correlation between tissue and serum inflammatory markers and chemotherapy sensibility have been demonstrated in literature to date. Other non- inflammatory biomarkers have been tested with usually poor or not significant results (78). Currently, there is no established role or indication for molecular or genetic testing in HCC due to the absence of specific benefit. Only a few mutations can influence the therapeutic algorithm in HCC but exclusively in case of progression after first-line administration, and in certain circumstances (79).

Similarly, ramucirumab, another second-line option, has shown better outcomes in advanced HCC with AFP > 400 ng/ml previously treated with sorafenib, leading international drug agencies to approve this anti-VEGF drug in this setting (80). However, the restriction of ramucirumab to patients with AFP > 400 ng/ml does not mean that this should be the agent of choice for that population (81). Further trials are therefore urgently needed to identify new biomarkers for precision medicine in HCC.

Author contributions

FG: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. NS: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. PP: Validation, Writing – review & editing. CS: Supervision, Writing – review & editing. TB: Conceptualization, Funding acquisition, Methodology, Supervision, Validation, Visualization, Writing – review & editing. JL: Conceptualization, Methodology, Supervision, Validation, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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1.4 Comprehensive molecular profiling of HCC to define mechanisms of resistance to immunotherapy

We previously focused on cases with a localized disease and for which a surgical option was possible. Following the *fil rouge* of this thesis, and therefore working under this concept of multidisciplinarity, we then decided to explore the cumbersome subject of HCC under a different light. The collaboration with the Inserm laboratory (UMR_S1110), a cutting-edge structure in the field of liver pathologies, was therefore the perfect scenario through which to continue this work.

Assessing response to systemic treatments in *in-vitro* models in relation to specific molecular patterns is one of the main concerns of the lab, and in HCC these treatments are reserved for advanced or metastatic cases. Starting with the approval of sorafenib 15 years ago,(18) marked progress has been made in this field and for the moment seven treatment regimens showed efficacy in phase III trials.(68) Target therapies and immunotherapies are currently recommended by latest AASLD guidelines as a first line.(69) Among all the validated drugs, atezolizumab, durvalumab and tremelimumab are immune checkpoints inhibitors (ICIs), the first two targeting programmed cell death ligand 1 (PD-L1) and the latest cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). The two above mentioned combinations showed improved survival over sorafenib, with a median progression-free survival of 6.8 and 7.2 months respectively.(20,70) However, regardless of the poor survival rate of these patients under treatment, the response rate is limited, with an objective response rate (ORR) of only 25% in both studies and a complete response in only 5% and 2% of patients respectively. Hence, despite this significant improvement in patient survival, the response rate of ICIs remains unsatisfactory for HCC patients. It is critical to unveil the mechanisms of resistance to

immunotherapy to identify new therapeutic targets in order to address this urgent unmet medical need. Thanks to the partnership between the Inserm (UMR_S1110) and the surgical department of the Nouvel Hopital Civil, we established a simple and robust patient-derived tumor spheroid model combining HCC tissue specimens and patient autologous serum. We then explored the known mechanism of resistance to ICIs in literature and how specific molecular expression influences response to these drugs.

From this subject, two manuscripts have been written. The first one, on the standardization of liver spheroid models for evaluating treatment response has been submitted on *Journal of Hepatology Reports* (IF: 8.3) and it is currently under review. The second one, a review on the mechanisms of resistance to immunotherapy, is currently under review among all authors.

Original article

Under review in: **JHEP Reports** (2024)

A PATIENT-DERIVED HEPATOCELLULAR CARCINOMA SPHEROID SYSTEM HARNESSING PATIENT SERUM TO MODEL THE TUMOR MICROENVIRONMENT AND TREATMENT RESPONSE

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Authors contributions:
E.C., T.F.B., and C.S. initiated and coordinated the study. N.A., S.D., M.P., N.R., and E.C. designed and performed the experiments and/or analyzed the data. S.D., A.S., E.C., C.S. organized the collection of liver tissues. F.G., E.F., P.P. K.C. provided liver tissues. F.G. and A.S. provided clinical information. E.C., T.F.B. and C.S. conceptualized and wrote the manuscript.

Abstract

Background & Aims: Hepatocellular carcinoma (HCC) is the third leading and fastest rising cause of cancer-related death worldwide. The discovery and preclinical development of compounds targeting HCC is hampered by the absence of authentic tractable systems recapitulating the heterogeneity of patient HCC tumors and the tumor microenvironment (TME).

Methods: Here, we established a novel, simple and robust patient-derived multicellular tumorspheroid model based on clinical HCC tumor tissues. HCC tissues were processed using enzymatic and mechanical dissociation. Cells were grown in 3D in optimized medium for the culture of tumorsphere and in presence of autologous patient serum. Characterization of the

tumorspheroid cell populations was performed by flow cytometry and functional assays. As a proof of concept, we treated patient-derived spheroids with FDA approved anti-HCC compounds.

Results: The model was successfully established independently from cancer etiology and grade. We show that the use of autologous patient serum was essential for the TME function and to maintain cell viability. The tumorspheroids comprised the main cell compartments including epithelial cancer cells as well as all major cell populations of the TME (cancer-associated fibroblasts, macrophages and T cells, endothelial cells). Cell type proportions were variable between tumorspheroids reflecting HCC heterogeneity. Moreover, we observed differential responses to FDA HCC approved drugs (including tyrosine kinase and checkpoint inhibitors as well as VEGF-targeting agents) between donors as in patients.

Conclusion: This patient HCC serum-tumorspheroid model provides novel opportunities for drug discovery and development as well mechanism of action studies including compounds targeting the TME. This model will likely contribute to improve the dismal outcome of patients with HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide and the leading cause of death in cirrhotic patients.[1] The HCC incidence is increasing most rapidly in Europe and the USA.[1] The major causes of HCC are chronic hepatitis B and C, alcohol abuse, and non-alcoholic fatty liver diseases (NAFLD) or termed recently also metabolism-associated steatotic liver disease (MASLD). While viral hepatitis has been a major cause of liver disease and HCC in the past, epidemiologists estimate that metabolic liver disease will be the major cause of HCC in the future due to changes in the lifestyle with increasing obesity and diabetes.[2]

Current treatment options are still unsatisfactory. Early-stage tumors can be treated using surgical approaches, radiofrequency ablation or liver transplantation, however less than 30% of HCC patients are eligible because they are often diagnosed at an advanced stage[3,4]. Despite recently approved combination of VEGF-targeting agents with immune checkpoint inhibitors targeting programmed cell death 1 (PD-1) have changed standard of care, overall response rates and improvement of survival remains limited and prognosis of patients with advanced HCC is poor.[3,4]

A roadblock in HCC drug discovery and development is the HCC heterogeneity among patients.[5] Moreover, preclinical assessment of anti-HCC candidate compounds efficacy is hampered by the absence of tractable systems recapitulating heterogeneity of the tumors and the tumor microenvironment (TME) playing a key role in treatment response.[6] Two-dimensional (2D) or planar cell culture remains one of the most applied model in drug discovery because it is simple to use, low cost, and easily applied in high- throughput screening. However, the 2D culture does not effectively mimic activity, function, and behavior of cells in an organ as well as the complex cell-cell, cell- extracellular matrix and cell-tissue interactions.[7] In the past decade, organoids emerged as a major breakthrough in cell biology and drug discovery by recapitulating functional features of in vivo tissues in 3D culture. However, they must be grown by stem cells through a complex induction process that hampers the success rate of organoid cultures.[8] Their growth relies on rigid extracellular matrices that create biochemical forces on cells reducing drug penetration.[9] Moreover, liver organoids are epithelial cultures lacking tumor stroma, thus they do not allow drug testing that target host–tumor interactions.[8] Finally, the use of animal models is also limited due to their complexity, cost and differences between species as well as their limited translatability to patients.[10] To address these limitations, we established a simple and robust patient-derived

spheroid model recapitulating HCC heterogeneity and TME for drug screening in individual patients.

METHOD

Human subjects

Human liver tissues and serum were obtained from liver disease patients undergoing liver resection with informed consent from all patients, for de-identified use at the Center for Digestive and Liver Disease of the Strasbourg University Hospitals, University of Strasbourg, France (DC-2016-2616 and RIPH2 LivMod IDRCB 2019-A00738-49, ClinicalTrial NCT04690972) or at Hiroshima University, Japan (approval number HI-98-21). The protocols were approved by the local Ethics Committee of the University of Strasbourg Hospitals and Hiroshima University ethical committee, respectively. All material was collected during a medical procedure strictly performed within the frame of the medical treatment of the patient. Informed consent is provided according to the Declaration of Helsinki. Detailed patient information and informed consent procedures are implemented by the Strasbourg University Hospital Biological Resources Center (HUS CRB). Patients were given an information sheet which outlines that their left-over biological material (liver resection and blood samples) that was collected during their medical treatment is requested for research purposes. All patients received and signed an informed consent form in order to provide authorization or refuse the use of their biological samples (protocols DC-2016-2616 and RIPH2 LivMod IDRCB 2019-A00738-49 ClinicalTrial NCT04690972). While there was clinical descriptive data available, the identity of the patients was protected by internal coding. A brief summary of clinical characteristics is provided in **Table 1**. Healthy patient serum was collected at « Etablissement français du sang » EFS, Strasbourg (Maison du don).

Tissue processing and tumorspheroid generation from fresh tissue

A protocol “from patient bed to the bench” was established with Strasbourg University Hospital to preserve tissue integrity and cell viability. Tissue resections (HCC tumor tissues and non-tumoral adjacent tissues) were immediately preserved after surgery in cold transplantation medium (HypoThermosol®, Sigma-Aldrich), kept on ice to avoid warm ischemia and processed maximum 30 minutes after resection.

- **Material**

– gentleMACS™ Octo Dissociator with Heaters, Milteniy Biotec

– Tumor dissociation kit, human, # 130-095-929, Milteniy Biotec

– gentleMACS™ C Tubes # 130-093-237, Milteniy Biotec

– Gibco HBSS, calcium, magnesium, no phenol red

– MACS® SmartStrainers, 100 µm, Miltenyi Biotech

– Red blood cell lysis buffer

– Forceps and scalpels

– Dead Cell Removal Kit, # 130-090-101, Milteniy Biotec

- Complete Mammocult (MammoCult™ Human Medium Kit, STEMCELL Technologies #05620). Medium is supplemented with human proliferation supplement (3.4%), hydrocortisone (0.056%), heparin (0.011%) and 20 % patient autologous serum.
- 96-well plates or 384 well plates Ultra-Low Attachment (Corning® microplates, #4515 or #4516)

- **Tissue dissociation**

- For fresh tissue, mince the tissue in small pieces in a petri dish and transfer 0.2 to 1 g of tissue in C tube and proceed with dissociation protocol according to manufacturer instructions.
- After dissociation, briefly spin down the cells in the C tube. Filter the dissociated cells using 100 µm strainer to eliminate the debris. Centrifuge at 700 g for 7 min.

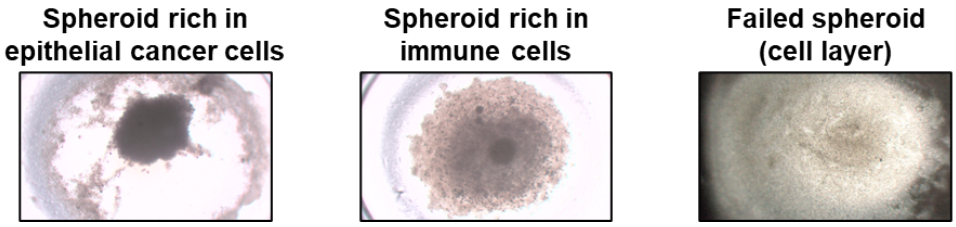
- Gently resuspend the pellet in HBSS to wash the cells and centrifuge again.
- Eliminate erythrocyte using erythrocyte lysis buffer according to manufacturer instructions and wash again with HBSS.
- Resuspend the pellet in complete Mammocult medium.
- Count the cells and assess cell viability using trypan blue or cell counter. For optimal counting, nuclear staining + cell counter may be required to differentiate small cell from debris.

Go/no go step: for fresh tissue, expected cell viability is in average 60 to 80 %. If viability is approximatively 40%, dead cell removal step is required using Dead Cell Removal Kit according to manufacturer instructions. Under 30%, the advantage of dead cell removal vs cell viability is lost. Poor-viability will lead to tumorspheroid generation failure.

- **Culture and tumorspheroid generation**

- For 96 well plates (characterization), between 100,000 and 150,000 cells are required per well. More cells will lead to formation of an important necrotic core at the center of the spheroids. For 384 well plates (FAD-drug screening), between 30,000 and 50, 000 cells are required per well.
- Estimate the number of cells required for the experiment.
- Add the patient serum in cell suspension and distribute 50 µL of cell suspension in 96 well low attachment plates or 25 µL for 384 well plates.
- Centrifuge the plates at 300 g for 3 min in one direction, and 300 g 3 min in other direction to improve cell distribution in the middle of the well.
- Two days after seeding, add 50 or 25 µl of complete medium. Low amount of medium at the beginning of the culture allows cell concentration in the middle of the well and improve cell migration.
- Observe tumorspheroid formation between 4- and 7-days post seeding.

- Spheres can be very dense if the cell suspension was rich in epithelial cells, or more diffuse if the tissue was rich in infiltrated immune cells. Spheres formation failure may appear if cell viability is low.



- Proceed with perturbation studies when spheres start to form to impact the maximum of cells.
- Extra-cells can be frozen at -80°C in CryoStor® cell cryopreservation media (Sigma-Aldrich).

Tumorspheroid generation from frozen tissue or frozen cells

Thaw the tissue pieces or frozen cells at 37°C, rinse/wash with HBSS to eliminate the cryopreservative medium and proceed with dissociation protocol for tissues or to dead cell removal for frozen cells as described above. Erythrocyte lysis is not needed for frozen samples.

Flow cytometry analysis

Flow cytometry analysis was performed on dissociated tissues using the cell suspension obtained as described above and on dissociated tumorspheroids. Tumorspheroids were dissociated using AccuMax (#07921 Stemmcell) following manufacturer's instructions. Cells were washed and filtered using 70 µm strainer. Then, 200 000 cells per conditions were used for each stainings. A panel of antibody was purchased from Miltenyi Biotech (REAfinity technology, recombinant antibodies for flow cytometry). The stainings were performed according to manufacturer's instructions. Data were acquired using Cytoflex B2R2U0 cytometer (Beckman Coulter, BA47394) and CytExpert 2.3 software and then analyzed using FlowJo V10.5.3.

Target protein	Target cell	Conjugate	Catalog #
Isotype CTRL 1 APC (S)	/	APC	130-113-434
Isotype CTRL 2 APC (I)	/	APC	130-120-709
Isotype CTRL FITC	/	FITC	130-113-437
ASGR1	Hepatocytes	APC	130-120-705
EPCAM	Epithelial cancer cells	APC	130-111-000
CD45	Immune cells	APC	130-110-633
CD3	T cells	APC	130-113-135
CD3	T cells	FITC	130-113-138
CD31	Endothelial cells	APC	130-110-670
CD68	Macrophages	APC	130-114-461
PD-1	Exhaustion immune cell marker	APC	130-117-694
CD69	Early activation immune cell marker	APC	130-112-614
Anti-desmin	CAFs	APC	130-119-491

Cell viability assay

Cell viability was performed using CellTiter3D Glo (Promega). Results are expressed as RLUs mean ± s.d. or as percentage to control mean ± s.d. A minimum of three or for replicates were performed for each culture or treatment condition.

Effect of patient serum in Huh7/LX2 spheroids

Human hepatocarcinoma Huh7 cells (gift from Prof. Gerhard Cristofori, University of Basel) and human stellate LX2 cells (purchased from Merck) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-decomplemented fetal bovine serum (FBS), gentamycin (0.05 mg/mL) and non-essential amino acids (complete DMEM) at 37°C with 5% CO2. All cell lines were certified mycoplasma-free. For spheroid formation, cells were detached and washed two times to remove serum. A total of 75,000 cells (LX2 20%) were

resuspended in DMEM and seeded in 96-well Ultra-Low Attachment plates in presence of increasing concentrations of FBS or HCC patient serum (from 0 to 50%). After 72h, spheroids were imaged and lysed to measure ATP levels (cell viability) or extract RNA. Stellate cell activation was assessed by measuring *ACTA2* and *COL1A1* expression. The primer sequences were as follows:

Gene	Forward	Reverse
ACAT2	5'-TGAAGAGCATCCCACCCT-3'	5'-ACGAAGGAATAGCCACGC-3'
COL1A1	5'-CCTCAAGGGCTCCAACGAG-3'	5'-TCAATCACTGTCTTGCCCCA-3'
GAPDH	5'-GTCTCCTCTGACTTCAACAGCG-3'	5'-ACCACCCTGTTGCTGTAGCCAA-3'

Collagen secretion assay

Collagen secretion was assessed on tumorspheroid culture medium using Total Collagen Assay Kit (Abcam # ab222942) according to manufacturer's instructions. The collagen assay protocol is based on the alkaline hydrolysis of samples to yield free Hydroxyproline.

Effect of patient serum on macrophages

THP1 (purchased from ATCC) cells were cultured in RPMI 1640 Medium with GlutaMAX™-I supplement and HEPES and supplemented with 10% FBS and gentamycin (0.05 mg/mL). To induce macrophage differentiation, 5.10^5 THP-1 cells were treated with PMA 320 nM in 24 well plates according to Yeung et al[34]. After 2 days, cells were washed two times with PBS and incubated with RPMI medium supplemented with 10% FBS (CTRL) or 10% HCC patient serum. Atfer 72h, cells were lysed, and RNA was purified. Macrophage phenotype was assessed by measuring tumor associated macrophage markers expression by qRT-PCR[34]. The primer sequences were as follows. CD163: TaqMan gene expression assay # 4331182, assay ID Hs00174705_m1, and Human GAPDH Endogenous Control (VIC®/MGB) #4326317E.

Gene	Forward	Reverse
CD206	5'- GCAGGGCCCTCTTAAGATCA-3'	5'- AACACGGGAACCAAAGTCAT-3'
CD204	5'- CAACAGCGGTTGGCAGT-3'	5'- CTGATGGACTTCCTGGTAACCAG-3'
IL10	5′-TCTCCGAGATGCCTTCAGCAGA-3′	5′-TCAGACAAGGCTTGGCAACCCA-3′
IL12	5′-TGCCTTCACCACTCCCAAAACC-3′	5′-CAATCTCTTCAGAAGTGCAAGGG-3′

TNF	5'-GAGGCCAAGCCCTGGTATG-3'	5'-CGGGCCGATTGATCTCAGC-3'
GAPDH	5'-GTCTCCTCTGACTTCAACAGCG-3'	5'-ACCACCCTGTTGCTGTAGCCAA-3'

FDA-approved drug treatments

Sorafenib tosylate (10 µM), lenvatinib (10 µM), regorafenib (10 µM), cabozantinib (10 µM), nivolumab (10 µg/ml), atezoluzimab (10 µg/ml), and bevacizumab (10 µg/ml) were from Selleckchem. CTRL antibody was from Evitria. DMS0 was from Sigma-Aldrich (#D8418). Tumorspheres were treated after beginning of sphere formation (in average 3-4 days post-seeding) for 3 days. Cell viability was assessed by measuring ATP levels.

Real-Time qRT-PCR

cDNAs were synthetized by reverse transcription using SuperScript III First-Strand Synthesis SuperMix (Life Technologies). Gene expression was analyzed using iTaq™ Universal SYBR® Green Supermix (Bio-Rad), excepted for CD163 TaqMan Gene Expression Assays (Thermo Fisher Scientific), on Applied Bioscience 7900HT Fast Real Time PCR system. The $2^{-\Delta CT}$ method was applied for relative quantification of mRNA with normalization to GAPDH mRNA.

Immunoblotting

Total protein extraction was performed from liver tissues and from spheroids in a lysis buffer containing 100 mM NaCl, 50 mM TRIS pH 7.5, 1 mM EDTA, 0.1% TX-100, 10 mM NaF, supplemented with proteinase inhibitors (Roche cOmplete™ Protease Inhibitor Cocktail) and phosphatase inhibitor cocktail (Sigma-Aldrich). After SDS-PAGE and transfer onto PVDF membrane, proteins were detected with specific antibodies (Cell Signaling).

Antibody target	Supplier	Catalogue #
VEGF Receptor 2	Cell signaling	9698S
Phospho-VEGF Receptor 2 (Tyr951)		2471S
Phospho-MET(Tyr1234/1235)		3126S
MET		4560S
Phospho-p44/42 MAPK (Erk1/2)		4370S
p44/42 MAPK (Erk1/2) (137F5)		4695S
Beta tubulin	GeneTex	GTX101279
GAPDH	Abcam	ab9485
Anti-rabbit antibody secondary antibody	Jackson IR	111-006-45

Statistics and reproducibility

For experiment performed on patient-derived tumorspheroids and with patient serum, the limited amount of HCC tissue and blood restricted the number of repeated experiments for the same donor. To ensure the reproducibility of our findings, and for arresting conclusions, we therefore performed independent experiments several times but on different tissues (minimum of 3 HCC tissues). The number of technical replicates was 3 or 4 for each experiment (except otherwise stated). The precise number (n) of biologically independent samples is indicated in the figure legends. The data are presented as the mean ± SD. No statistical analyzes were performed if $n<4$. Otherwise, data were analyzed by parametric tests (unpaired Student's t-test) or non-parametric tests (two-tailed Mann-Whitney U test or Kruskal-Wallis test) as indicated in figure legends, after determination of distribution by the Shapiro-Wilk normality test. $p < 0.05$ (*), $p < 0.01$ (**) were considered statistically significant.

RESULTS

A simple and robust protocol to establish patient-derived HCC tumorspheroids. As described in detail in Materials and Methods, a novel protocol “from patient bed to the bench” was established to generate high quality patient-derived spheroids using HCC tumor tissues and autologous patient serum (**Fig. 1A**). The clinical characteristics of all the patients enrolled in this study are reported in **Table 1**. Tumor tissues were dissociated using mechanical and enzymatic digestion and tumorspheroids were generated using a scaffold-free approach using

ultra-low attachment spheroid microplates in presence of patient autologous serum (**Fig. 1A**). Using this protocol, we observed that cell migration and compaction started on average 4 days after culture, with a complete cell agglomeration into compact tumorspheroids after 7 days (**Fig.1B**). Optimal culture conditions allowed to preserve high cell viability up to 7 days after seeding (**Fig.1C**).

Next, we applied the protocol to a large panel of surgical resections from a cohort of HCC patients with varying etiologies and tumor grades (**Table 1**). Tumorspheroids were successfully generated independently of etiology and tumor grades, with nearly an overall success rate of 90%. The failed tumorspheroid generation in about 10% of patient samples was due to poor-quality of the tissue or necrotic tissue for which only a low fraction of living cells was recovered after dissociation (see Material and Method). Interestingly, we observed shape variations of the spheres depending on the characteristics of the original HCC tissues. Indeed, tumorspheroids generated from HCC tissues rich in infiltrated immune cells lead to less compact structures (HCC 544) than tumorspheroids generated from HCC tissues with low infiltration (HCC 576 and HCC 577) (**Fig.1D**). These data indicate that the shape of the tumorspheroids may reflect cold and hot HCC tumor subtypes.

Tumorspheroids contain the main HCC cell compartments and functional TME

The TME is known to play a critical role in tumor growth and response to standard-of-care therapies [11]. Since the presence of a functional TME is essential for drug discovery and development, we investigated the different cell populations in this model. The tumorspheroids were dissociated and analyzed by flow cytometry using the markers classically used to characterize liver cells[12,13] (**Fig. 2A**). Our analysis demonstrated that the tumorspheroids contain epithelial cells and different non parenchymal cells including $CD45^+$ immune cells, $CD3^+$ T cells, $CD31^+$ endothelial cells and $CD68^+$ macrophages (**Fig. 2B-C**). Importantly, flow cytometry analysis of the original tissue after dissociation showed that the proportion of the

different cell compartments are preserved in tumorspheroids (**Supplementary Fig. 1**). A summary of the tumorspheroids characterization is presented in **Table 2**. These results indicate that our approach does not lead to enrichment of a particular cell population but includes all cell populations that were present in the original liver tissue at sampling time. Moreover, we observed that the presence of numerous immune cells leads to variation in tumorspheroid size and shape and less compaction (**Fig. 1B**). Interestingly, tumorspheroids generated from different donors harbors cell populations that are specific to each individual at collection time, reflecting HCC tissue heterogeneity among patients (**Fig. 2B-C; Table 2, Supplementary Fig. 2**).

Cancer-associated fibroblasts (CAFs) are a central component of the TME with several functions including extracellular matrix (ECM) secretion and remodeling as well as cytokine/growth factor production[14]. Flow cytometry analysis using desmin as marker for activated CAFs showed that tumorspheroids also comprise fibroblasts (**Fig. 3A**). To demonstrate that these CAFs are functional, we stimulated tumorspheroids with TGFβ and assessed sphere morphology and collagen secretion. We observed cell proliferation at the periphery of the spheres associated with an increase of collagen secretion in the culture supernatant (**Fig. 3B and Fig. 3C**), indicating that tumorspheroids indeed comprise functional CAFs. Moreover, measurement of collagen secretion by tumorspheroids showed that functional CAFs produce collagen up to one week after sphere generation (**Fig. 3D)**. ECM protein secretion by fibroblasts is most likely responsible for aggregation of the different cell types and generation of stable tumorspheroids in the absence of scaffold. Collectively these results show that our tumorspheroid model includes a functional TME and recapitulates original tissue and HCC inter-individual heterogeneity.

Autologous HCC patient serum is essential to model a functional TME in tumorspheroids and preserve cell viability.

Next, we investigated the effect of autologous patient serum on the phenotype and function of patient-derived tumorspheroids. First, we observed that the presence of autologous patient serum facilitates cell aggregation into spheres (**Supplementary Fig.3**) and is essential to preserve high cell viability in patient-derived tumorspheroids generated from different donors with different HCC etiologies (**Fig. 4A**). Next, we investigated the effect of HCC patient serum on hepatic fibroblast activation, by generating tumorspheroid based on the HCC cell line Huh7 co-cultured with hepatic stellate cells (LX2). The spheroids were incubated with increasing concentrations of HCC patient serum or fetal bovine serum (FBS) as a control (Fig. 4B). As shown in **Fig. 4 C**, the presence of HCC patient serum induces cell proliferation in spheroids, independently from the etiology. Furthermore, HCC patient sera triggered stellate cells activation as showed by the increase of *ACTA2* (coding for alpha smooth muscle actin) and *COL1A1* (coding for collagen 1) expression in the cell line derived-spheroids (**Fig. 4D**). Our results also showed that 10 to 20% of patient serum in the culture medium is sufficient to obtain this phenotype (**Fig. 4B-D**). Importantly, this phenotype is only observed with HCC patient serum and not with patient serum without liver disease etiology or cancer, indicating that soluble factors specific from HCC lead to fibroblast activation **(Supplementary Fig. 4 A-F)**.

Next, we assessed the effect of HCC patient serum on immune cell phenotype. As a model, we used the well-characterized monocyte-derived cell line (THP1) differentiated into macrophages and incubated these cells with different HCC serum (10%). HCC sera induced phenotypic changes in macrophages (**Fig. 5A**). The further investigation of HCC serum-induced macrophage phenotype indicated a differentiation of THP1-derived macrophages into tumor-associated-macrophage (TAM)-like cells, as demonstrated by an increase in *CD204*, CD206 and CD163 expression. Interestingly, the expression of these markers is often associated

with a poor prognosis in patients, and production of IL10, a strong immunosuppressive cytokine promoting tumor growth[19–21] (**Fig. 5B-D**). Interestingly, macrophage differentiation was only observed in presence of HCC patient serum and not serum from healthy patients, demonstrating the specific effects of HCC serum (**Supplementary Fig. 4**). Collectively, these results indicate that the use of HCC patient serum is essential for robust tumorspheroid and functional TME generation.

HCC tumorspheroids show heterogeneous responses to FDA-approved drugs.

Next, we investigated whether our model can be applied to investigate therapeutic response. For proof-of-concept, we treated HCC tumorspheroids with a panel of FDA-approved compounds (see Method) (**Fig. 6A**). Tumorspheroids were generated from 17 HCC patient tissues and treated with multi-target tyrosine kinase inhibitors (TKI), namely sorafenib, lenvatinib, regorafenib and cabozantinib, as well as nivolumab, an anti-programmed death-1 (PD-1) monoclonal antibody (mAb) and the combination of atezolizumab (anti-PD-1 mAb) and bevacizumab (anti-VEGF mAb) and their corresponding controls. Prior to their application in the patient tumorspheroid model, the drug concentrations inhibiting targeted signaling pathways and inducing cancer cell death had been determined a Huh7 3D spheroid model (**Supplementary Fig. 5**). The optimal concentrations for mAb was selected according to the literature[22,23].

First, we observed a heterogeneous response to TKI, likely reflecting inter- and intra-individual tumor phenotypes (**Fig. 6B-C**). As an example, HCC 577 was sensitive to TKI whereas HCC 557 was resistant to all the tested TKI molecules. HCC 551 harbored an intermediate phenotype and showed a decrease in cell viability after cabozantinib treatment but resistance to sorafenib (**Fig. 6B-C**). The response rate obtained with sorafenib was 13.33%, which reflects response rates observed clinically in patients[3] (**Fig. 6C**). Moreover, we observed that in the tumorspheroid model, lenvatinib showed a superior response compared to sorafenib,

as observed in patients[24] (**Fig. 6C**). Finally, a robust response rate was observed for cabozantinib, which correlates with recent meta-analysis study demonstrating superiority of cabozantinib for second line setting[25] (**Fig. 6C**).

To further confirm the validity of the patient-derived HCC model for the investigation of chemotherapeutic agents, we generated spheroids from adjacent non-tumoral liver tissues using the same approach. We then compared treatment responses in liver spheroids of the non-tumorous adjacent tissue to the response in corresponding tumorspheroids for the same patients (**Fig. 6D**). As a proof-of-concept study, we selected HCC tissues responding to cabozantinib (**Fig. 6C**). Cabozantinib strongly decreased cellular viability only in tumorspheroids, with minor or absent effect on the adjacent non tumoral spheroids (**Fig. 6D**), demonstrating the specificity of the tumorspheroid model for tumor-targeting drugs.

Finally, tumorspheroids were treated also with standard of care consisting of two mAbs targeting PD-1 and/or VEGF pathway (atezolizumab and bevacizumab) and with mAb targeting PD-1 alone (nivolumab). Interestingly, a low level but detectable response was observed for 4 out of 15 tested tumorspheroids (**Fig. 6C**). Moreover, flow cytometry analysis after nivolumab treatment demonstrated target engagement on CD3+ T cells (Fig.7). Collectively, our data demonstrate that the tumorspheroid model retains HCC inter- and intra-heterogeneity, recapitulates the TME and enables to investigate differentiated responses to TKIs, targeted- and immune-based HCC therapies.

DISCUSSION

In this study, we describe a simple multicellular patient-derived HCC spheroid model recapitulating tumor heterogeneity and TME for HCC drug discovery and development. While HCC spheroid systems have been described,[23-27] the conceptual advancement and innovation of the model established in this study is the combination of patient-derived tumorspheroids with autologous serum enabling a novel simple and robust platform modeling patient heterogeneity,

treatment response as well as the TIME. Collectively, in contrast to previous models, our approach allows generation of functional tumorspheroids for drug proof-of-concept studies within only a few days, with high and stable viability, without bias and enrichment of particular cell types.

In the present study, we used a matrix-free approach to generated tumorspheroids, based on cell's self-aggregation capabilities. These tumorspheroids include CAFs and produce their own ECM allowing appropriate ECM- and cell-cell interactions that mimic the original tumor tissue without addition of artificial matrix. Therefore, our model overcomes the limitations of previously described matrix-based methods such as the variability of matrix batch, issues in reproducibility, high costs, and limitation in drug or large molecule diffusion.[28,29]

The other innovation is the use of autologous patient serum to generate spheroids. Hepatocarcinogenesis is accompanied by a modification of the secretome. The secretome is present in the patient serum including tumor-associated proteins and immune mediators playing key roles in tumorigenesis ([30-33]). We therefore generated tumorspheroids in presence of patient autologous serum to mimic the HCC secretome. Our data demonstrate that HCC patient serum facilitates cell migration and aggregation and is essential to preserve high cell viability in patient-derived tumorspheroids. Moreover, the use of patient serum contributes to activation of fibroblasts and maintenance of macrophage phenotype, most likely through different soluble factors (i.e., cytokines, chemokines, extracellular vesicles). These features offer a unique opportunity to study the cellular communication within the TME. Moreover, our model will enable the investigation of the secretome to decipher cell-to-cell communication in the pathogenesis of HCC.

By using FDA-approved drugs in a large series proof-of-concept studies, we demonstrate that the serum-tumorspheroid system enables to study the efficacy of anti-HCC therapies in authentic patient material. Moreover, our data indicate that HCC tumorspheroids

are a valuable tool for the understanding of drug mechanism of action, drug screening and drug repositioning. The model offers also an opportunity to assess novel combination therapies in an authentic patient model helping to identify synergistic or additive effect of molecules to accelerate HCC treatment drug development[3] including the investigation of approaches targeting the TME.

A possible limitation of the model was the observation that responses to CPI were detectable but at a low magnitude. It is conceivable that CPIs do not induce robust cancer cell mortality in tumorspheroids due to the limited number of immune cells present in the spheres (no infiltration/recruitment of new cells is possible in this model) or the absence of tertiary lymphoid structures. Nevertheless, flow cytometry analysis after nivolumab treatment demonstrated target engagement on CD3+ T cells (Fig. 7), suggesting that the tumorspheroid model enables to detect and investigate T cell responses to immune based therapies in a cancer tissue context.

The next step will be a prospective study to compare treatment responses in tumorspheroids with clinical responses in the patients, where the material is derived from. Since in this study patient material was used from liver resections in a surgical curative approach, retrospective data on the response to systemic therapies of the patients, from which the resection were obtained, are not available. The opportunity to assess treatment responses of tumorspheroids from liver biopsies of patients undergoing future treatment may harness the model for future precision medicine.

In conclusion, the HCC serum-tumorspheroid system based on the authentic patient tissue and autologous serum offers new perspectives to improve HCC drug discovery and development including compounds targeting the TME. Following validation of prediction of therapy response in a prospective study, the model likely also will provide a valuable perspective to identify patient-tumor specific therapies as a component of precision medicine.

Based on these opportunities and perspectives, the serum-tumorspheroid model will likely contribute to improve the dismal outcome of patients with HCC.

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FIGURES

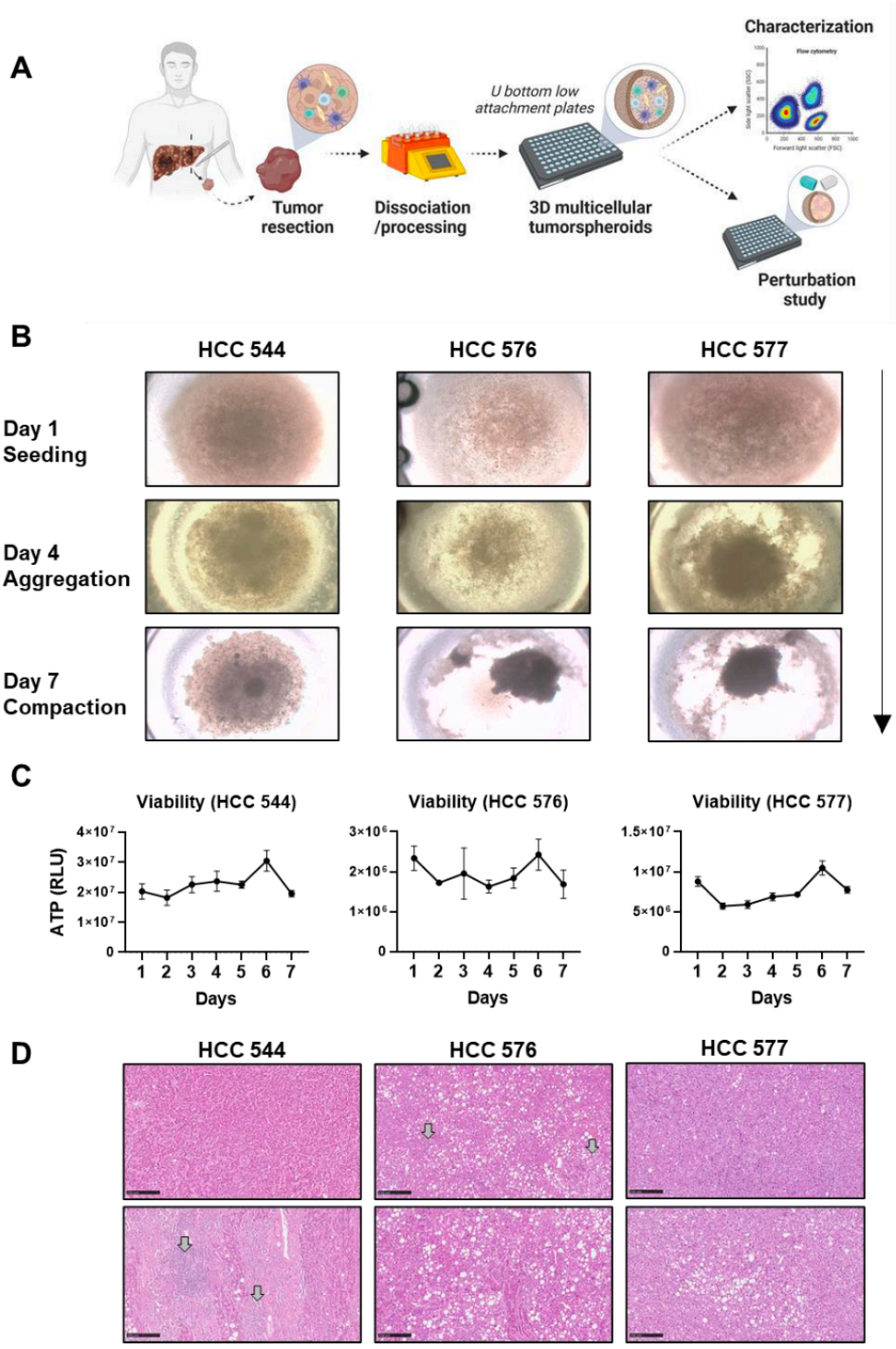

Figure 1: A simple and robust protocol to establish a patient-derived HCC tumorspheroids. A. Experimental approach. A protocol "from patient bed to the bench" was established to preserve HCC tissue and maintain high cell viability. Tissues were immediately processed maximum 30 minutes after resection using mechanical

and enzymatic digestion. Tumorspheroids were generated using a scaffold-free approach in ultra-low attachment spheroid microplates and used for characterization and perturbation study. **B. Kinetic of tumorspheroid formation.** Cells were imaged after seeding (day 1). Aggregation of cells was observed in average 4 days after seeding. Cell compaction and tumorspheroid generation was observed 7 days after seeding. Tumorspheroids were generated using HCC tissues. Three representative examples are shown (MOTIC AE2000 Lordill, X10). **C. Kinetic of tumorspheroid viability.** Viability was assessed by measurement of ATP levels and is stable at least for 7 days. Graph shows mean ± SD (n = 4, 3 independent experiments). RLU = relative light unit (RLU). **D. H&E staining of the original tumor specimen.** Tumor 544 is a well differentiated HCC on cirrhotic liver with an important infiltration of immune cells. Tumor 576 is a steatotic well differentiated HCC on fibrotic liver with less important immune cell infiltration. Tumor 577 is a steatotic moderately differentiated HCC on cirrhotic liver with low infiltration. Arrows show immune cell infiltrates. Scale bar = 250 µm.

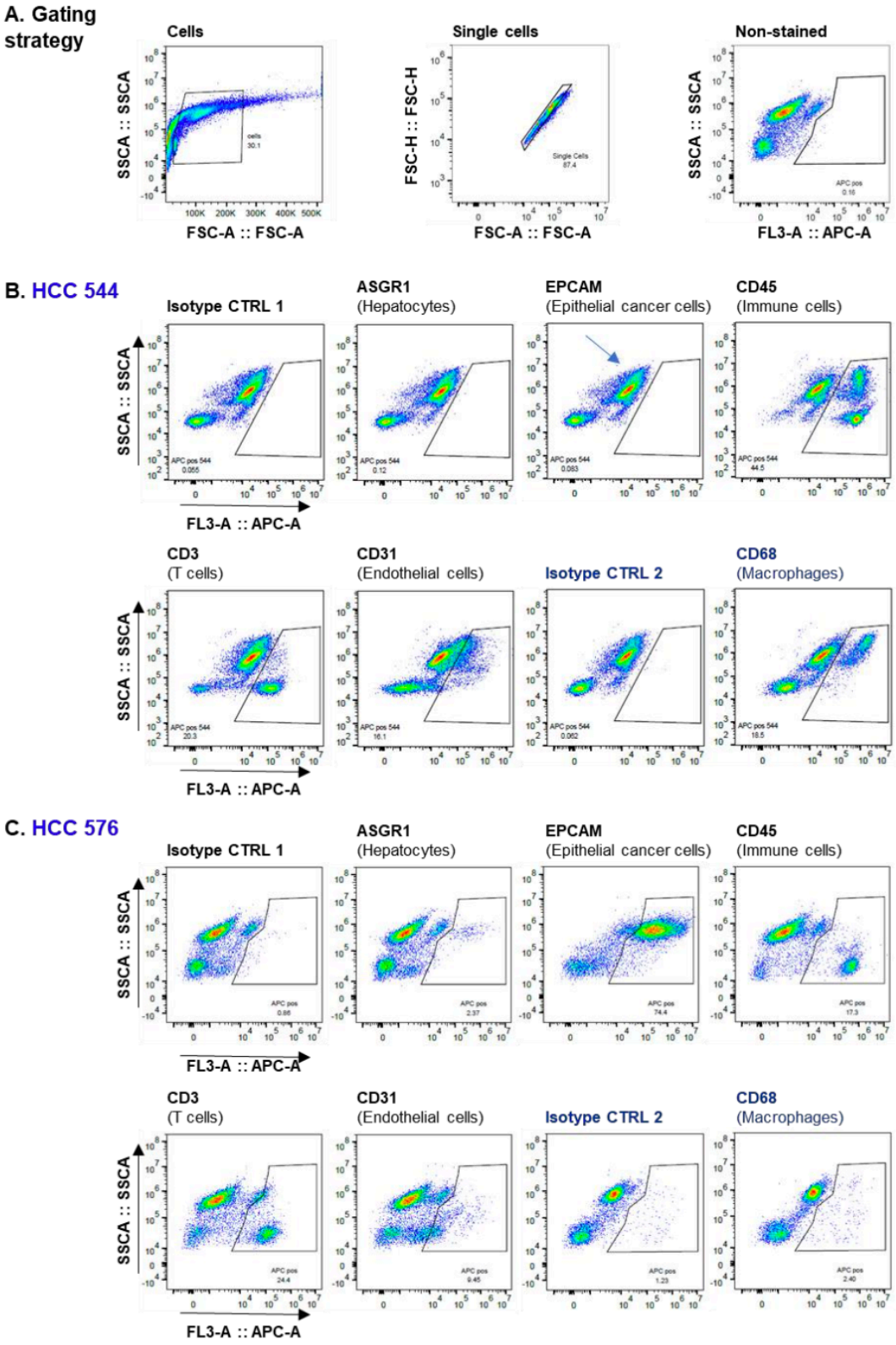

Figure 2: Tumorspheroids contain the main HCC cell compartments. Tumorspheroids were dissociated and analyzed by flow cytometry. **A. Gating strategy.** The gating was performed on total cell population using FCS/SSC dot plots

to remove cell debris. The cells were then gated to isolate “singlets” and exclude “doublets” using plot through FSC-H and FSC-A parameters. From the singlet gate, negative cells were determined using non-stained samples. One representative example (HCC 576) is shown. **B-C Analysis of different cell compartments in tumorspheroids 544 and 576**. ASGR1 (asialoglycoprotein receptor 1) = hepatocyte marker; EPCAM (epithelial cell adhesion molecule = marker of epithelial cancer cells), $CD45^+$ = immune cells marker, $CD3^+$ = T cells marker, $CD31^+$ = endothelial cells marker and $CD68^+$ = macrophages marker. Arrow shows epithelial cell population $EPCAM^-$. Flow cytometry analysis of the original tissue is shown in **Supplementary Fig. 1**. Other HCC characterization is shown in **Table 2** and **Supplementary Fig. 2**.

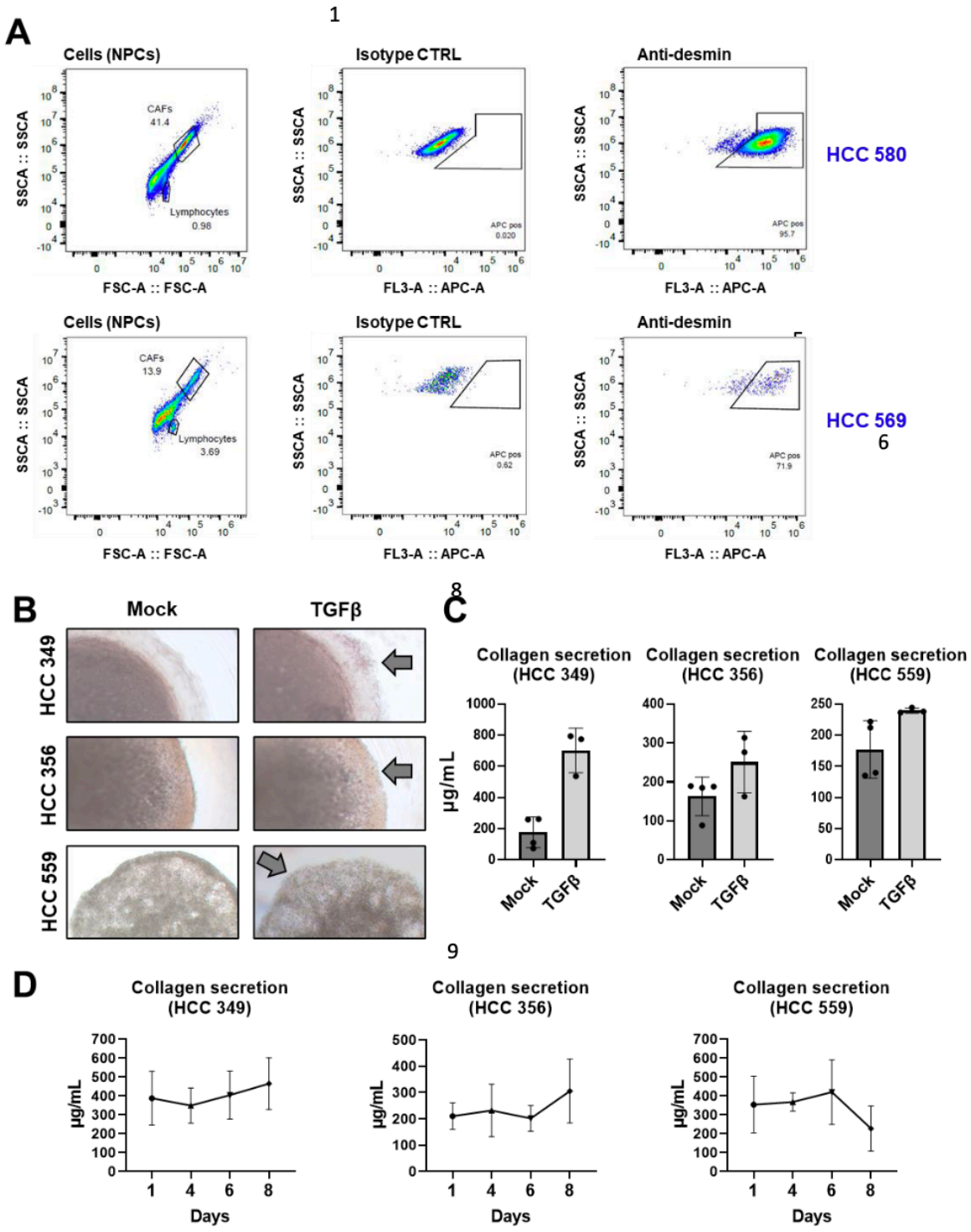

Figure 3: Tumorspheroids contain functional CAFs. A. Tumorspheroids contain fibroblasts. Spheres from HCC 580 and HCC 569 were dissociated and analyzed by flow cytometry using anti-fibroblast antibody. Gating shows selection of a specific cell population among total cells. **B-C. Effect of TGFβ treatment in tumorspheroids. (B)** HCC tumorspheroids were generated from 3 tumor tissues and were treated with TGFβ

for 24h and then imaged using MOTIC AE2000, Lordill (20X). Arrow shows morphological change in sphere proliferative area suggesting cell proliferation upon treatment. **(C)** TGFβ induces an increase in collagen secretion from tumorspheroids. In parallel culture supernatants were collected, and total collagen secretion was measured. Graph shows mean ± SD of collagen secretion in µg/mL (n = 3 from 3 HCC tissues). **D. Collagen secretion from tumorspheroids is stable for at least 7 days.** A kinetic experiment was performed using the same HCC tumorspheroids. Graph shows mean ± SD of collagen secretion in µg/mL (n = 3 for each day, 3 HCC tissues, 3 independent experiments).

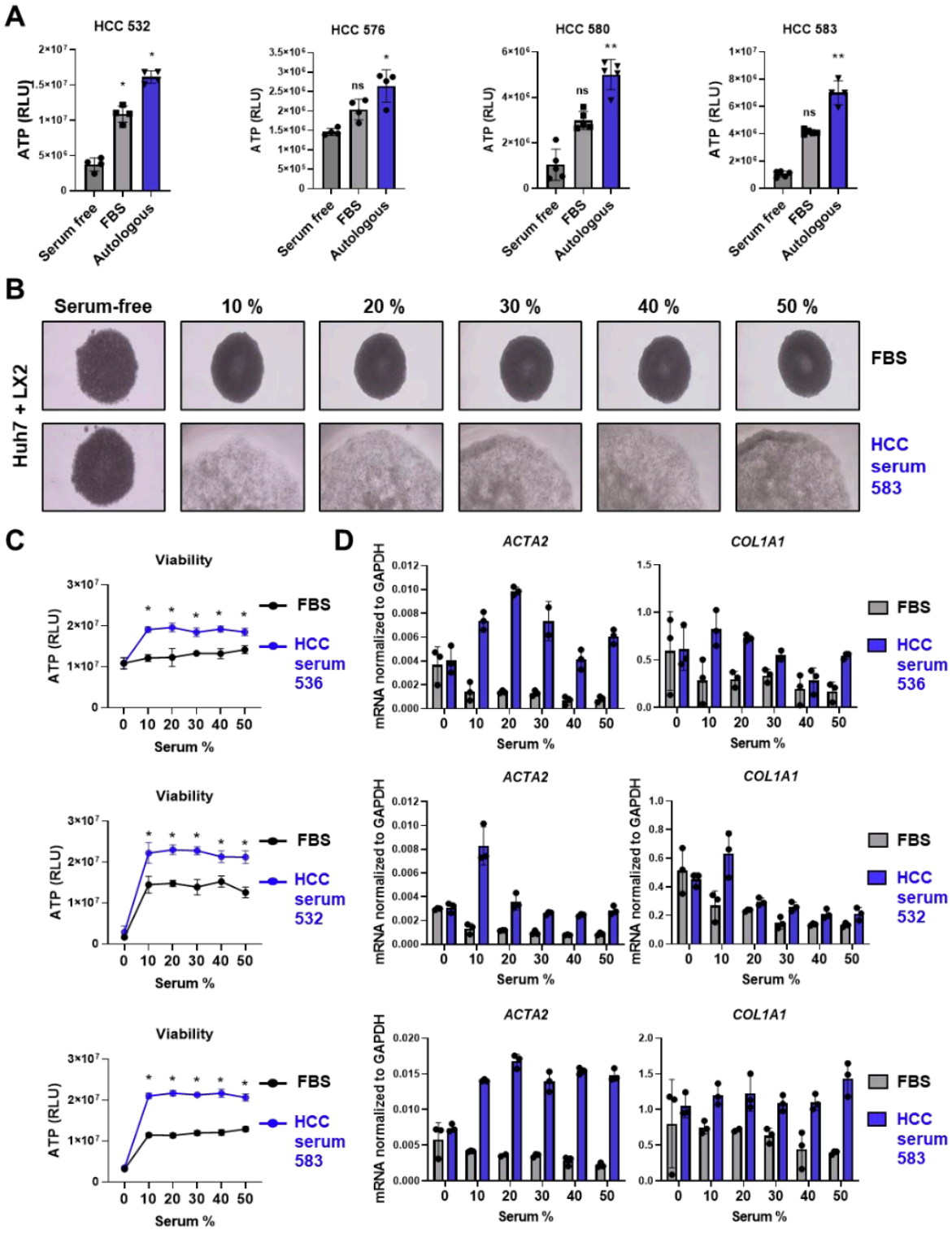

Figure 4: HCC patient serum preserves tumorspheroid viability and activates fibroblast. A. HCC patient autologous serum improves tumorspheroid viability. Tumorspheroids were generated from 4 different HCC tissues without serum or in presence of fetal bovine serum (FBS) or patient autologous serum. Viability was

assessed by measurement of ATP levels (day 4). Graph shows mean ± SD (n = 4 or 5, 4 independent experiments). RLU = relative light unit (RLU). * = $p < 0.05$; ** $p < 0.01$, Kruskal-Wallis test). **B-D. Effect of HCC patient serum on Huh7/LX2 spheroids.** Tumorspheroids from Huh7/LX2 (20%) cells were generated in absence of serum or increasing concentrations of FBS or HCC patient serum. After 72h, tumorspheroids were imaged using MOTIC AE2000, Lordill (4X) **(B)**, viability was assessed by measuring ATP levels (C) and stellate cell activation was assessed by measuring ACTA2 and COL1A1 expression by qRT-PCR (D). **(B)** one representative experiment out of 3 is shown. **(C)** Graph shows mean ± SD (n = 4 for each condition, 3 HCC serum, 3 independent experiments). RLU = relative light unit (RLU). * = $p < 0.05$; two-tailed Mann-Whitney U test. **(D)** Graph shows mean ± SD of mRNA relative quantity normalized to GAPDH (n = 3 for each condition, 3 HCC serum, 3 independent experiments).

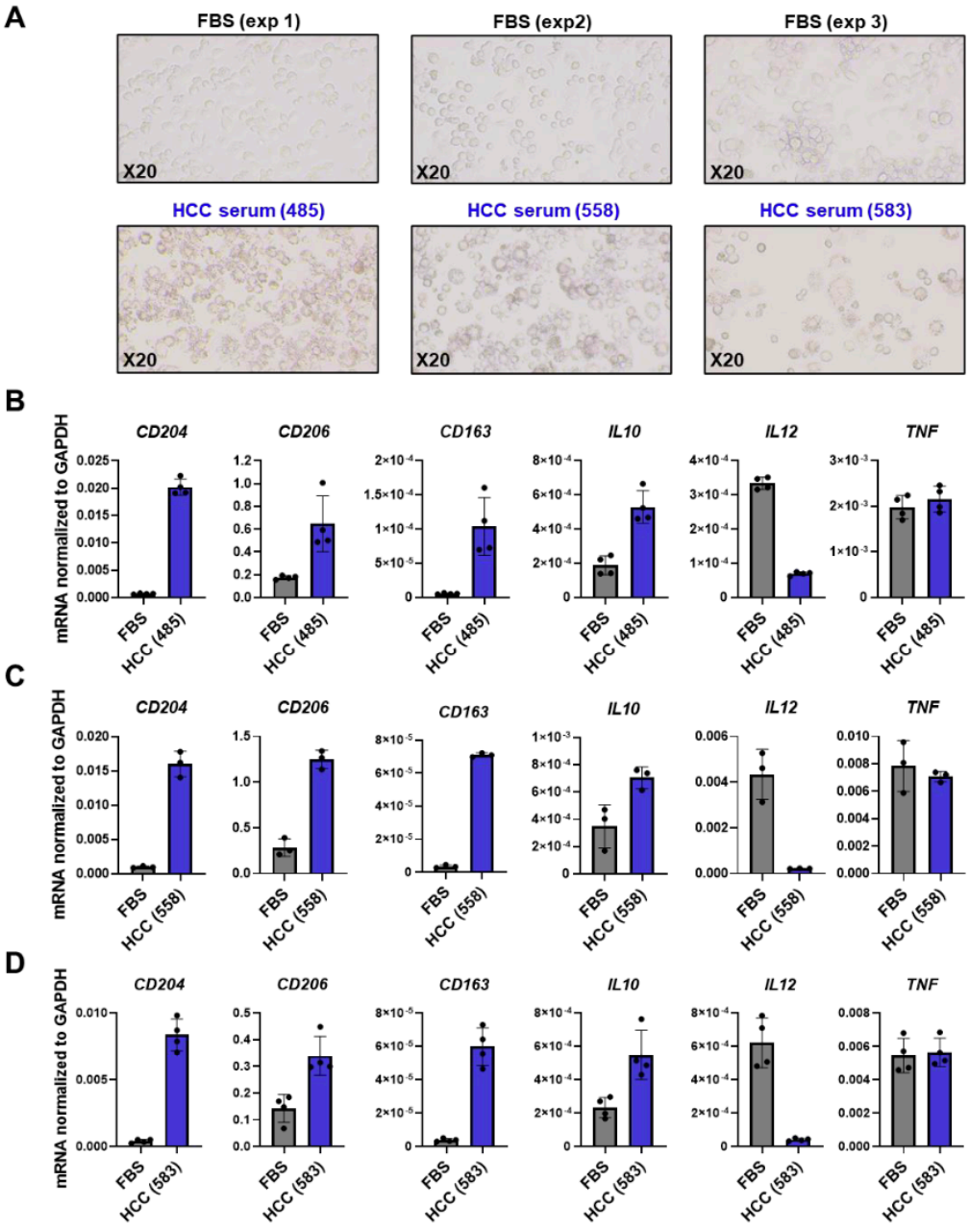

Figure 5: HCC patient serum induces macrophage differentiation in TAM like cells. THP1-derived macrophages were incubated in presence of 10% fetal bovine serum (FBS) or HCC patient serum. After 3 days, cells were imaged using MOTIC AE2000, Lordill (20X) **(A)** and macrophage phenotype was characterized by measuring different TAM markers by qRT-PCR **(B-D)**. Graph shows mean ± SD of mRNA relative quantity normalized to GAPDH (n = 3 or 4 for each condition, 3 HCC serum, 3 independent experiments).

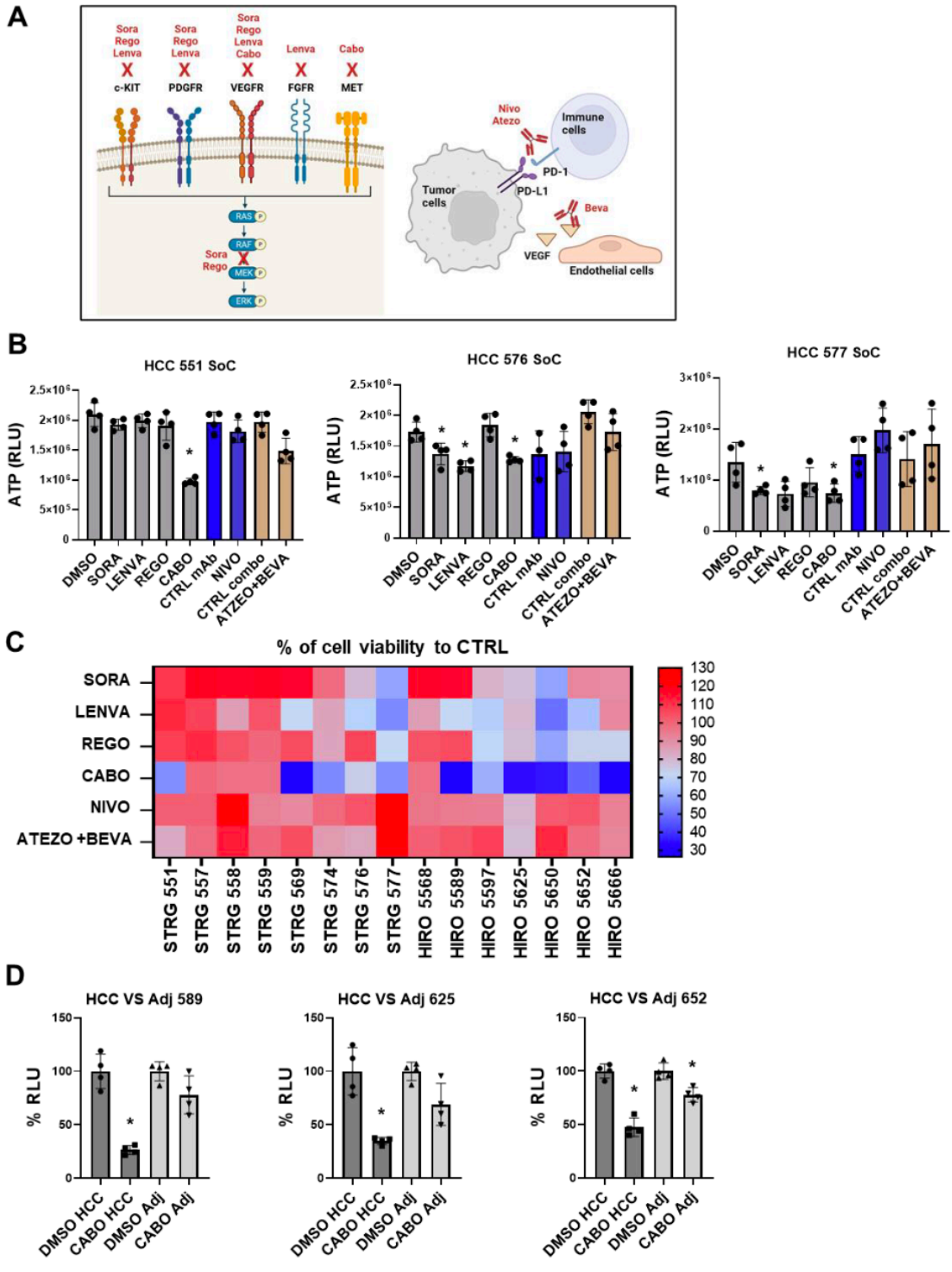

Figure 6: HCC tumorspheroids show heterogeneous responses to FDA-approved drugs. A. Scheme showing the molecular target of selected FDA-approved anti HCC drugs. **B-C Drug screening in tumorspheroids.** Tumorspheroids were generated from 17 HCC patient tissues (Strasbourg and Hiroshima biobank) and

treated (day 4) with different FDA-approved drugs for 3 days. Viability was assessed by measurement of ATP levels. **(B)** Graph shows mean ± SD (n = 4) for 3 representative HCC tumorspheroids. RLU = relative light unit (RLU). * = $p < 0.05$, two-tailed Mann-Whitney U test (MKI VS DMSO, mAb VS CTRL mAb). **(C)** Results are presented as heatmaps showing percentage of cell viability compared to control (DMSO or CTRL mAbs). n = 4 for 17 HCC tumorspheroids. **D. Anti-HCC drug response is higher in HCC tumorspheroids compared to spheroid generated from adjacent non tumoral tissues.** Graph shows mean ± SD of percentage of cell viability VS respective controls (n = 4, 3 independent experiments). * = $p < 0.05$, two-tailed Mann-Whitney U test (Cabo VS DMSO). Sora = sorafenib, Rego = regorafenib, Lenva = Lenvatinib, Cabo = cabozantinib, Nivo = nivolumab, Atezo = atezolizumab, Beva = bevacizumab, DMSO = dimetylsulfoxyde, mAb = monoclonal antibody.

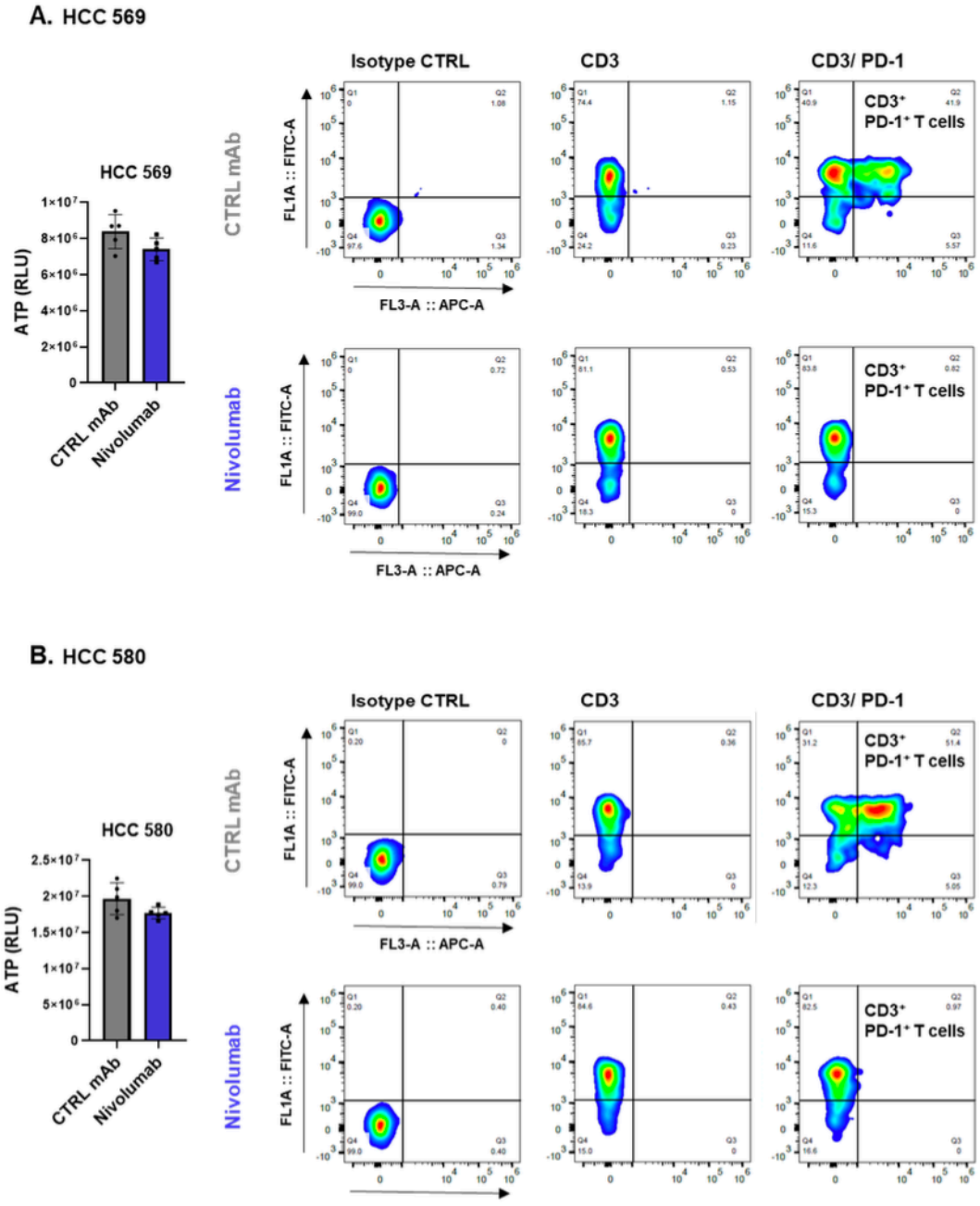

Figure 7: HCC tumorspheroids respond to immunotherapy. Tumorspheroids from HCC 569 and HCC 580 were treated with Nivolumab or CTRL mAb for 6 days. At day 6, they were dissociated and analyzed by flow cytometry. Gating shows selection of a specific $CD3^+$ T cell population among total cells. PD-1 was detected as T cell exhaustion marker.

Second part.

Genetic and molecular landscape in Biliary Tract Cancers

2.1 Influence of genetic background in intrahepatic cholangiocarcinoma

The second chapter of this thesis concerns ICC. We wanted to explore the issue of poor outcomes of this tumor from a different angle, and in particular how the genetic background may influence survival in these patients. Assessing the mutational context of cholangiocarcinoma can be useful for two reasons. The first, partially explored in the literature, is related to the aggressiveness of the disease per se. Indeed, some series report that specific mutations are associated with more aggressive tumors, and others with a more favorable prognosis.(71) This concept is mainly reported in the literature for metastatic or locally advanced patients, for whom different lines of therapy are available and for whom, among other things, there is no curative treatment. In surgical patients, on the other hand, this association has been little explored and is mainly found in small series, explored rather from a molecular point of view. The second reason is the recent authorization of certain targeted therapies as second-line treatments.(72–75) However, this option is reserved exclusively for unresectable patients, whereas surgical patients can only benefit from adjuvant treatment with capecitabine.(76) In collaboration with the Inserm laboratory (UMR_S1110) and with the department of molecular biology we carried out a systematic literature review and meta-analysis to collect a large sample of ICCs and analyze the prevalence of the most frequent mutations as well as their impact on the patient's oncological evolution. This would pave the way in the creation of targeted pathways for patients according to their mutational profile and, at the same time, lay the foundations for a possible extension of targeted therapies to surgical patients.
The manuscript has been submitted to the *British Journal of Surgery* (IF: 9.6) and it is currently under review after a first round with major revisions.

Review

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IMPACT OF GENETIC ALTERATIONS IN RESECTABLE INTRAHEPATIC CHOLANGIOCARCINOMA ON SURVIVAL: A META-ANALYSIS

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Conflict of interests: Inserm, the University of Strasbourg, IHU Strasbourg and Alentis Therapeutics have filed patent applications for the use of anti-claudin-1 monoclonal antibodies for the treatment of fibrosis, HCC and CCA. TFB is a founder, shareholder and advisor of Alentis Therapeutics developing monoclonal antibodies for treatment of fibrosis and cancer.

ABSTRACT

Background: Intrahepatic cholangiocarcinoma (ICC) is a public health threat because of its aggressivity. Its genetic background differs from other biliary cancers and it is strictly correlated with tumour natural history. A systematic review and meta-analysis of the literature was therefore performed focusing on the genetic assessment of resected ICC and their impact on long-term outcomes.

Methods: A systematic search of the literature was conducted in June 2022 using PubMed, Medline, Scopus and Cochrane Library databases looking for studies assessing long-term outcomes of resected ICC according to genetic mutational. Effect size set was Hazard Ratio (HR) with its corresponding 95% confidence interval (CI). Publication bias was determined by the construction of funnel-plots and asymmetry assessed using Egger's regression test. Main outcome was to assess the impact of genetic alterations in survival these patients.

Results: Twenty-four retrospective studies were eligible. KRAS, IDH1/2 and TP53 were identified as the only three genes whose status is significantly correlated with overall survival (HR: 2.476, 95% CI: 1.67-3.671 for KRAS; HR: 0.624, 95% CI: 0.450-0.867 for IDH1/2 and HR: 2.771, 95% CI: 2.034-3.775 for TP53). The prevalence of KRAS and IDH1/2 significantly differed between western and eastern studies ($p < 0.01$ for both genes).

Conclusion: KRAS, TP53 and IDH1/2 genes were associated with patient prognosis and with defined clinico-pathological tumour features. Furthermore, the investigation of the overall prevalence revealed the most common actionable and undruggable mutations in these patients. This knowledge will be useful to expand target therapy indications in the adjuvant setting.

INTRODUCTION

Intrahepatic cholangiocarcinoma (ICC) ranks as the second most common primary liver tumour, with a global increasing incidence and mortality in the last years.[1,2] Estimated 5-year survival rate stands at around 9-11% , with a wide gap depending on the stage and treatment proposed at the moment of diagnosis.[3,4] Surgery, followed by fluoropyrimidine or gemcitabine-based adjuvant therapy, is the only curative option available in case of non-metastatic and resectable disease.[5] Unfortunately, recurrence of ICC occurs in almost half of the resected patients within one year.[6] In contrast to the well-established and validated role of surgical treatment, the positioning of standard of care in the adjuvant setting remains still to be

determined. Dedicated randomized controlled trials are limited and retrospective series, reviews and meta-analysis do not yet support the indiscriminate use of adjuvant chemotherapy.[7–12]

In biliary tract cancers (BTC), well described specific genetic mutations have been identified as disease drivers and independent prognostic factors for overall outcome.[13] For this reason, defined targeted therapies are already approved by regulatory agencies as a valid second-line option, such as neurotrophic tyrosine receptor kinase (NTRK) inhibitors,[14,15] fibroblast growth factor receptor-2 (FGFR2) inhibitors[16] or isocitrate dehydrogenase-1 (IDH1) inhibitors.[17] However, their administration is currently limited to those patients with an unresectable or metastatic disease, whereas studies addressing the use of targeted therapies after hepatic resection are largely absent.

In this regard, it is important to note that cholangiocarcinoma is a highly heterogeneous disease, with genomic differences among all subtypes and with various drug actionable pathways.[18–24] ICC is in fact a biologically distinct entity with a specific mutational pattern compared to all BTC. One could speculate that even those cases who can benefit from a surgical treatment are a subset with a less aggressive behaviour and with well-defined molecular pathways compared to those who are diagnosed as unresectable or with a metastatic spread. However, most of the studies investigating the genomic profiling of these tumours and their association with patient's survival, include in their samples all types of BTC with different stages and treatments, and only a few of them focus on ICC undergoing curative resection.

This systematic review aims to summarize the current knowledge on the mutational status of resected ICC and their prognostic significance. A meta-analysis was performed to assess the risk of recurrence and death in these patients according to the genetic background. At the same time, the prevalence as well as clinico-pathological differences of mutated ICC have been investigated.

METHODS

Literature Search

A systematic search of the literature was conducted in June 2022 for all articles published until 31st May 2022 following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines.[25–27] PubMed, Medline, Scopus and Cochrane Library databases were used to look for original English articles providing prognostic data of resected ICC in which a genetic mutational profiling has been performed. Relevant systematic reviews were also explored to look for potentially pertinent references not found in the screening process. After discussion among all authors, the following terms and combinations were used for the initial search: ((cholangiocarcinoma) OR (bile duct cancer) OR (biliary tract cancer) OR (cholangiocellular carcinoma)) AND ((genetic) OR (mutation)). Following this primary search, a screening process was independently performed by two authors (FG and FDZ) according to specific eligibility criteria.

Inclusion and exclusion criteria

The systematic review included English language articles with a full-text available, focusing on genetic alterations of resected ICC. Only studies reporting survival and/or recurrence data of ICC treated with a curative intent and in which a genetic profiling on the main specimen has been performed were included in all the meta-analysis. Exclusion criteria were as follows: not curative resections, preclinical studies and absence of follow-up data for the mutated/wild-type cohort. Results coming from studies analysing public datasets were excluded as well. Although protein expression analysis by immunohistochemistry has been demonstrated to strongly correlate with the mutational status for some genes, only studies directly analysing genetic mutations by next-generation sequencing (NGS) or sanger sequencing and copy number variations (as amplifications) and fusion transcripts through FISH technique were considered. Papers that failed to fulfil these criteria, as well as articles not available as full-text, studies

written in a language other than English, letters to the editor, case reports or series with less than 10 patients and reviews were also excluded.

Reviewing process, data extraction and quality assessment

Based on the result of the main search process, articles were extracted as title and abstract and reviewed. All papers not related to the topic or not meeting the eligibility criteria were excluded. After the first screening, all full-text of the selected articles were obtained. Manuscripts relevant to the main topic and fulfilling the inclusion and exclusion criteria were selected and included in the review. Any disagreement between the two authors was solved through discussion and reassessment of the data among all the authors. Studies coming from the same centre were further investigated and in case of similar aims and targeted genes, only the most recent publication was included. Outcomes of these screened papers as well as main clinic-pathological information were collected and analysed. When outcomes were reported by a minimum of 8 studies, publication bias was determined by the construction of funnel-plots and asymmetry assessed using Egger's regression test.[28] In case of evidence of small-study effects, a sensitivity analysis was performed through the trim and fill method to identify and correct the cause of the funnel-plot asymmetry.[29] Methodological quality of the included studies was assessed through the Newcastle-Ottawa scale.[30] Each study was judged through a "star system" based on three items: i) selection of the cohort, ii) the comparability of the groups and iii) the ascertainment of either the exposure or outcome. Overall score was converted to Agency for Healthcare and Quality standards according to number of stars on each item.

Outcome measures and statistical analysis

The primary aim of the study was to evaluate the prognostic significance of the mutational status in ICC after curative surgery. Survival data of these patients were recorded according to the mutational landscape of the most important genes. Secondly, prevalence and clinico-pathological variables were always collected, when reported, for all the genes assessed in the

included studies, in order to find any possible correlation between genetic profiling, geographic distribution and tumor aggressiveness. A meta-analysis of proportions was conducted using a random effect model and the logit transformation.[31] Subgroup analysis was conducted according to geographical location of recruiting centers when sufficient number of studies were available. As regards prognostic data, Hazard ratios (HR), p-value and Kaplan-Meier curves were gathered from all the included studies and examined. Effect size set in this meta-analysis was HR with its corresponding 95% confidence interval (CI) and inverse variance method was used for pooling. *Juicr* package on R (version 0.1) was used to extrapolate raw data from Kaplan-Meier curves and reconstruct all survival figures when these were not directly reported in the studies. The method described by Tierney et al. was chosen to estimate HR and 95% CI when not directly specified by the authors.[32] In case of given patients at risk, these were entered into the spreadsheet together with reconstructed Kaplan-Meier raw data. When patients at risk were not reported, HR was obtained starting from the number of patients analysed in the two groups, the number of events and the p-value between the two curves. Considering that all of the included studies were retrospective with a potential between-study heterogeneity, the random-effects model was used. Variance distribution was calculated by the restricted maximum likelihood estimator or the Paule-Mandel estimator when the number of studies was less or equal to four. Statistical heterogeneity was evaluated using the I^2 statistic and prediction intervals (PI) were always assessed. Thresholds of 25, 50 and 75% of I^2 were used to identify low, moderate and substantial heterogeneity, respectively. Two-side p-value <0.050 was considered as statistically significant. All statistical analyses were performed using R (R Project for statistical computing, version 4.2.2, R Core Team) and *Meta* and *Metafor* packages were used for the meta-analysis.

RESULTS

The initial search identified 6075 articles suitable for the reviewing process. After the first screening based on title and abstract revision, a total of 235 manuscripts were selected for full-text review. Finally, 24 papers met the inclusion criteria and were examined in this study.[13,21,33–54] One study was further excluded from the survival meta-analysis for the impossibility of extracting prognostic data.[42] PRISMA flow-chart of the screening process and PRISMA checklist are represented in **Figure S1** and in **Table S1**. A detailed and completed report of all the studies and genes examined is shown in **Table 1.** Quality assessment according to the Newcastle-Ottawa scale and conversion to Agency for Healthcare and Quality standards is showed in **Table S2**.

Prevalence of mutated genes in resected ICC

Forest-plots representing meta-analysis of proportions conducted for all the genes are shown in **Figure S2** and **Figure S3.** The most explored mutation was the *KRAS* status, with 2399 patients examined, followed by *IDH1/2* mutation, with 1632 samples. For these two genes estimated prevalence were respectively 14.8% and 13.6%, which makes these genetic mutations two of the most commonly altered. With a related proportion of 16.3% mutations among 983 patients assessed, *TP53* presented the highest prevalence in this review. Other frequently tested genes were *BRAF* (641 cases and a corresponding 6.6% proportion) and *FGFR2* (623 cases with a proportion of 11.4%). *NTRK* gene fusions was poorly examined and analyzed only in one study.[51] A summary of the mutational status of all the genes found in the included studies is represented in **Figure 1**. Subgroup analysis according to studies' geographical distribution was possible exclusively for some genes (**Figure S2**). A statistical difference between western and eastern series was found for *KRAS* ($p < 0.001$) and *IDH1/2* ($p < 0.001$) but not for *BRAF*, *FGFR2* and *PIK3CA*. For *TP53*, this meta-analysis revealed a different prevalence of 12% against 23.8% for western and eastern studies, respectively, but without a statistical significance ($p = 0.14$).

Analysis of survival according to the mutational status

For *KRAS* mutations nine studies were selected and included in the meta-analysis involving 1833 liver resections for CCI (**Figure 2A**).[13,33,35,36,38,41,43,44,53] The 258 (14.1%) mutated patients showed a worse survival compared to wild-type cases which was statistically significant (HR: 2.476, 95% CI: 1.67-3.671; p <0.01). Heterogeneity tests revealed a moderate to substantial heterogeneity (I^2= 74%, 95% CI: 50-87%) with a PI ranging from 0.663 to 9.251. Survival data associated with *IDH1/2* mutations were extracted from 8 studies with 1266 patients, of whom 189 (14.9%) mutated (**Figure 2B**).[13,37,39,43,47,49–51] Mutated IDH1/2 CCI were associated with a decreased risk of mortality with a HR of 0.624 (95% CI: 0.450-0.867, p <0.01). The between-study heterogeneity was estimated at I^2= 60% (95%CI: 14-82%) with a PI of 0.274-1.422. For *TP53* mutations 6 studies and 583 patients (122 mutated, 20.9%) were evaluated (**Figure 2C**).[13,33,34,43,46,48] As for *KRAS*, analysis of these cases revealed a significantly impaired overall survival (OS) (HR: 2.771, 95%CI: 2.034-3.775; p <0.01) for mutated cases. A lower between-study heterogeneity was found (I^2= 37%, 95%CI: 0-75%) and PI confirmed an association between *TP53* mutation and worse outcomes (1.362-5.627). *FGFR2* genetic alterations were examined in 4 studies with 568 cases (65 mutated, 11.4%) of which 3 assessed *FGFR2* fusions[13,52,54] whereas one evaluated gene amplification (**Figure 2D**).[45] Overall, a slight trend towards an improved survival was seen, although not significant (HR: 0.702, 95%CI: 0.397-1.242: p= 0.22). However, between-study heterogeneity was moderate to substantial (I^2= 61%, 95%CI: 0-87%) an PI rather wide (0.074-6.692), indicating a possible lack of survival benefit in mutated patients. For *BRAF* and *ARID1A* mutations, two other important genes, four[13,35,38,43] and three[13,37,46] studies were analysed with 428 and 307 patients, respectively. Results showed a HR= 1.112 for m*BRAF* (p= 0.66) and HR= 1.493 (p= 0.36) for m*ARID1A*, with a between-study heterogeneity of 0% and 70% (**Figure 2E and 2F**). Other less frequently involved genes were *BAP1* and *PBRM1*, whose oncological impact was assessed in two studies in both

cases.[13,37] After meta-analysis, respective HR was 1.558 (95%CI: 0.617-3.935, p= 0.35) and 0.821 (95%CI: 0.506-1.331, p= 0.42), with a I^2= 42% for *BAP1* mutations and I^2= 0% for *PBRM1* mutations (**Figure 2G and 2H**).

Analysis of recurrence according to mutational status

Correlation between disease recurrence and genetic mutations was less explored and only a few studies reported data on this type of outcome. Five studies were identified for IDH mutations including 848 patients of whom 133 (15.7%) mutated.[13,39,47,49,50] A reduced risk of recurrence was observed with a p <0.01 (HR= 0.549, 95%CI: 0.430-0.701), with no heterogeneity and a PI confirming the association with a better prognosis (0.370-0.816) (**Figure 3A**). Association between FGFR2 fusion and recurrence was evaluated in three studies and 477 cases (52 mutated, 10.9%).[13,52,54] Pooled HR was 0.683 (95% CI: 0.294-1.597) but not statistically significant (p= 0.38) and a moderate to significant heterogeneity (I^2= 71%) (**Figure 3B**). KRAS and TP53 mutated ICC have likely higher recurrence rate (HR= 1.810 with p <0.01 and HR= 1.748 with p= 0.03, respectively) but this correlation was assessed only in 2 studies (**Figure 3C and 3D**).[13,40]

Correlation between mutational status and clinico-pathological features

Comparison of clinical and pathological features according to mutational status was available only for some studies. Data on main pathological report and significative differences found in mutated cases are shown in **Table S3**. Regarding underlying liver disease, a direct correlation between HBV infection and TP53 status was found in two eastern series.[33,48] In contrast, KRAS mutations were more frequent in resected ICC without former viral infection,[33,40,48] although this relationship was not significant in other studies.[38,53] No associations between other genetic alterations and confirmed tumoral aetiologies, such as hepatolithiasis or parasitosis were reported.[36,38,47,52] Comparisons of pathological reports confirmed an association with negative prognostic factors for some genes, as perineural invasion (KRAS),[13,36] higher stage (KRAS and

BRAF),[38] nodal metastasis (TP53, KRAS and BRAF),[13,38,40] or tumour number (KRAS).[44] On the other side, IDH1/2 and FGFR2 alterations were more frequent in less aggressive ICC ,[45,47,49,54] although a higher rate of bilobar and less differentiated lesions was found in IDH1/2 mutated ICC in two studies.[43,50]

Bias assessment

Funnel-plot were calculated for survival analysis in KRAS and IDH mutations (**Figure S4**), and no asymmetry was found after Egger's test (p= 0.104 and p= 0.977, respectively). Possible sources of bias in this meta-analysis may be attributable both to publication bias, and bias in measurement of the outcomes.

DISCUSSION

It is well known that genetic background analysis in BTC is essential to understand the natural history of these tumours and that some mutations are associated with distinct aetiologies, histological patterns and, above all, long-term outcomes.[19,55–61] However, when assessing the prevalence of genetic mutations and related long-terms outcomes, the authors often analysed all BTC regardless their subtypes or both surgical patients and metastatic diseases. Even after selecting only series with resected ICC, results are not always consistent, because of small sample size and cohort heterogeneity.[13,33,37,43,46,47,54] In this meta-analysis the impact of each mutation in patients' survival and recurrence in resected tumours was assessed and a large dataset in terms of prevalence created. In this study only mutations in three specific genes among all those assessed in literature and thus analysed were found to be significantly associated with patient outcomes. As it happens in colorectal cancer,[62] ICC harbouring a mutation in KRAS show an impaired prognosis with a mortality and recurrence rate of respectively 2.5 and 1.8 times higher compared to those wild-type. Despite this does not currently represent an actionable gene, new prospective in KRAS target therapies[63] as well as the possible association with intrinsic resistance to some specific drug in mutated cases, as it

happens in colorectal cancer,[64] could stand out the importance of this date. Similarly, a reduced OS and disease-free survival was found in resected ICC exhibiting a TP53 mutation whereas IDH1/2 mutations were associated with a favourable outcome. All the other genes analysed, among which stand out BRAF, FGFR2 and ARID1A, were non significantly correlated with outcomes in the present meta-analysis. Similar results are found in literature for metastatic ICC in terms of prognostic association, with, however, a different prevalence due to the advanced stage of the disease.[24,61] Regarding the prevalence of genetic alterations, TP53 and KRAS were found to be the most altered genes in this specific population, followed by other important actionable genes as IDH1/2 and FGFR2. At the same time, KRAS mutations were substantially and significantly higher in eastern studies compared to western cases and, by contrast, IDH1/2 was altered mainly in western series. If considering the prognostic meaning of these patterns, a logical consequence would be a higher risk of recurrence and death in eastern patients, which is also probably in relation with the different underlying aetiologies of ICC in these countries. Furthermore, given the actionability of IDH1/2, this data opens to larger target therapeutic possibilities in the western world. It is worth to note that in resected specimen, with a high percentage of tumour cells, sanger sequencing and next generation sequencing have similar accuracy with a low risk of false negatives.

Results of this meta-analysis show us that outcomes after liver resection for ICC go beyond clinical and pathological features and that tumour aggressiveness could be rather a proxy of its genetic background. ICC carrying specific alterations are a high risk of recurrence and are therefore those in which therapeutic algorithm should be carefully balanced between its long-term benefit and the risk of a disproportionate surgery. Although preoperative biopsy is not mandatory in case of suspicion of ICC[5] - or even debated for the controversial risk of peritoneal dissemination - this could be necessary in the future to obtain tumour mutational panel and thus stratify patients and guide therapeutic choice. In this regard, the report of the genetic status of

this meta-analysis could contribute to pave the way to the future clinical development of target therapies in the peri-operative setting, currently approved exclusively for unresectable and metastatic tumors.[5,61]Compared to other cancers, as colorectal one, in BTC oncological armamentarium is scarce in resectable diseases with limited therapeutic lines and valid options to adapt according to the altered molecular pathways. Given the well-known chemoresistance of ICC, future prospective could therefore include the application of this type of treatment before or after surgical resection.[5,64]

Some limitations in this systematic review have to be reported. Firstly, although studies included were of good quality, they were all retrospective and between-study heterogeneity observed was sometimes moderate to substantial. Moreover, overall mutations of the most important genes were considered without being able to assess the role of specific variant subtypes and codons modified, as distinct allele mutations in KRAS,[53] different prognosis between IDH1 and IDH2 cases[39] and other genes variants. It is in fact likely that not all the subtypes have the same impact on prognosis. However, while these differences have already been largely reported in other fields as for colorectal cancer,[65] in ICC this topic has been poorly explored for the main genes. In KRAS, for example, only one study among all included reported specific outcomes for variant subtypes.[53] The same issue concerns co-mutations, without the possibility of assess the epidemiologic and prognostic role of combined alterations in resected ICC in this meta-analysis. Finally, most of the studies assessed are translational, lacking of important clinical and pathological variables and making impossible the performance of a meta-regression, which was initially planned.

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manuscript “Genetic Determinants of Outcome in Intrahepatic Cholangiocarcinoma” which were incorporated in the current meta-analysis.

Data availability: data extracted from included studies as well as data used for analysis are available upon request to the corresponding author. Review was not registered and performed without a protocol.

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TABLES

Table 1. Characteristics of the studies included in the review

Study	Study Country	N° patients (n available for survival)	Study Period	Underlying Liver Disease	Gene analyzed	Mutated, n (%)	Technique of mutation identification
Tannapfel et al., 2000	University of Leipzig, Leipzig, Germany	41 (41)	1994-1997	-	TP53	15 (36.6)	Sanger sequencing
Tannapfel et al., 2003	University of Leipzig, Leipzig, Germany	69 (69)	1994-2000	-	KRAS BRAF	31 (44.9) 15 (21.7)	Sanger sequencing
Chen et al., 2012^	Chang Gung Memorial Hospital, Taoyuan, Taiwan	86 (83)	1985-2008	Hepatolithiasis	KRAS	19 (22.1)	Sanger sequencing
Jiao et al., 2013^	Multicentric	32 (32)	-	Cirrhosis	BAP1 ARID1A PBRM1 IDH1/2 FGFR2* PIK3CA* PTEN* PIK3C2G* PIK3C2A* TP53* CDKN2A* KRAS* NRAS*	8 (25) 6 (18.7) 5 (15.6) 6 (18.7) 4 (12.5) 2 (6.2) 2 (6.2) 2 (6.2) 1 (3.1) 2 (6.2) 1 (3.1) 1 (3.1) 1 (3.1)	Exome DNA capture (Agilent SureSelect)
Robertson et al., 2013	The Johns Hopkins University School of Medicine, Baltimore, USA	54 (54)	1990-2011	HBV, Cirrhosis	KRAS BRAF	4 (7.4) 4 (7.4)	Pyrosequencing
Wang et al., 2013^	Multicentric	325 (298)	-	Cirrhosis	IDH1/2	33 (10.1)	Whole exome sequencing + Sanger sequencing + Pyrosequencing
Gao et al., 2014^	Liver Cancer Institute, Zhongshan Hospital, Shanghai, China	124 (124)	2008-2011	HBV, liver fluke, Cirrhosis	PTPN3 KRAS TP53	51 (41.1) 9 (7.3) 39 (31.4)	Whole-exome sequencing + Sanger sequencing
Jang et al., 2014^	ASAN Medical Center, Seoul, Korea	81 (81)	1998-2008	Viral infection, Cirrhosis, Autoimmune	KRAS MLH1* NRAS* GNAS EGFR* BRAF* PIK3CA* CDKN2A* APC* PTEN* TP53*	11 (13.6) 7 (8.6) 7 (8.6) 6 (7.4) 6 (7.4) 3 (3.7) 2 (2.5) 1 (1.2) 2 (2.5) 1 (1.2) 1 (1.2)	Maldi-ToF (MassARRAY)
Liau et al., 2014*	National Taiwan University Hospital, Taipei, Taiwan	189 (189)	1993-2012	Viral infection	KRAS* IDH1/2*	24/174 (13.8) 18/171 (10.5)	Sanger sequencing
Zhu et al., 2014^	Multicentric	200 (162/200)	1973-2013	-	BRAF IDH1/2 KRAS MAP2K1 NRAS PIK3CA TP53 PTEN*	8/162 (4.9) 40/200 (20) 14/162 (8.6) 3/162 (1.9) 5/162 (3.1) 7/162 (4.3) 4/162 (2.5) 1/162 (0.6)	Sanger sequencing
Zou et al., 2014^	Eastern Hepatobiliary Surgery Hospital, Shanghai, China	102 (101)	2009-2011	Viral infection, Cirrhosis, liver fluke	KRAS TP53 IDH1* PTEN*	17 (16.7) 39 (38.2) 5 (4.9) 6 (5.9)	Whole-exome sequencing

					ARID1A* PIK3CA* RB1* EPPK1* SMAD4*	7 (6.9) 4 (3.9) 5 (4.9) 6 (5.9) 4 (3.9)	
Ruzzenente et al., 2015	University Hospital G.B. Rossi, Verona, Italy	35 (35)	1990-2012	-	PIK3C2G STK11 TGFBR2	1 (2.8) 1 (2.8) 1 (2.8)	NGS
Ikeno et al., 2018^	Kyoto University, Kyoto, Japan	50 (50)	2009-2016	Viral infection, Cirrhosis	KRAS	16 (32)	Multiplex mutation analysis, Luminex xMAP
Pu et al., 2018^	Nanjing Drum Tower Hospital, Nanjing, China	114 (91)	2005-2015	Viral infection, Cirrhosis	FGFR2 *(Amplification)*	15 (13.2)	FISH
Simbolo et al., 2018	University Hospital G.B. Rossi, Verona, Italy	66 (66)	1990-2013	Viral infection, Autoimmune diseases, Cirrhosis	ARID1A TP53 BAP1* BRAF* CHD4* FGFR3* IDH1* IDH2* KRAS* NRAS* PBRM1* PIK3C2A* PIK3C2G* PIK3CA* PTEN* TGFBR2*	6 (9.1) 8 (12.1) 9 (13.6) 2 (3) 3 (4.5) 2 (3) 11 (16.7) 2 (3) 11 (16.7) 5 (7.6) 10 (15.2) 4 (6.1) 3 (4.3) 7 (10.6) 3 (4.5) 3 (4.5)	NGS, FISH
Wang et al., 2018^	Tongji Hospital, Wuhan, China	85 (85)	-	Viral infection, Cirrhosis	IDH1	13 (15.3)	Sanger sequencing
Huang et al., 2018^	Huashan Hospital, Shanghai, China	166 (70)	-	Viral infection, Cirrhosis	TP53 RAS/RAF	35 (21.1) 24 (14.5)	Sanger sequencing
Lee et al., 2020^	Seoul National University Hospital, Seoul, Korea	172 (172)	2005-2012	Chronic liver disease	IDH1/2	16 (9.3)	Pyrosequencing
Ma et al., 2020^	Tianjin Medical University Cancer Institute and Hospital, Tianjin, China	130 (102)	2012-2017	Cirrhosis	IDH1/2	21 (16.1)	DNA Sequencing
Pu et al., 2020^	Nanjing Drum Tower Hospital, Nanjing, China	140 (140)	2005-2017	-	IDH1/2 NTRK1* MDM2* HER2* MET*	10 (7.1) 11 (7.9) 8 (5.7) 6 (4.3) 3 (2.1)	Sanger sequencing, FISH
Boerner et al., 2021	Memorial Sloan Kettering Cancer Center, NY, USA and Erasmus Medical Center, Rotterdam, Netherlands	209 (209)	1993-2018 and 2005-2015	Viral infection, Cirrhosis	TP53 KRAS CDKN2A BRAF IDH1/2 TERT ARID1A BAP1 FGFR2 PBRM1	37 (17.7) 19 (9.1) 28 (13.4) 11 (5.3) 51 (24.4) 8 (3.8) 37 (17.7) 29 (13.9) 31 (14.8) 29 (13.9)	NGS capture
Pu et al., 2021^	Nanjing Drum Tower Hospital, Nanjing, China	173 (139)	2005-2017	Viral infection, Cirrhosis, liver fluke	FGFR2 *(Translocations)*	9 (5.2)	FISH
Buckarma et al., 2022	Mayo Clinic, Rochester, USA	95 (95)	2008-2014	-	FGFR2	12 (12.6)	FISH
Zhou et al., 2022^	Liver Surgical Oncology of Zhongshan Hospital, Shanghai, China	1024 (1024)	2009-2016	Viral infection, Cirrhosis	KRAS	127 (12.4)	NGS + Sanger Sequencing

FIGURES

Fig. 1. Estimated prevalence of mutations and number of cases evaluated. Number of patients included in this meta-analysis (blue columns) with relative estimated prevalence (grey columns). Number in brackets next to the name of the gene indicates the total number of studies assessing prevalence for that specific mutation.

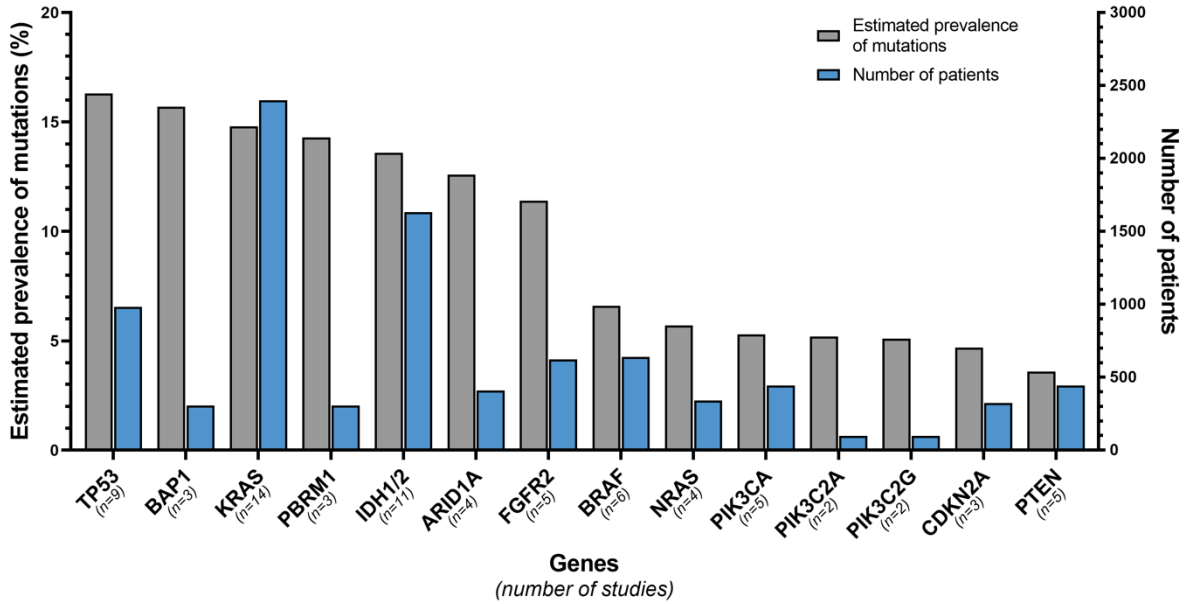


Figure 2. Forest-plots exploring overall survival according to mutation of specific genes. KRAS (A), IDH1/2 (B), TP53 (C), FGFR2 (D), BRAF (E), ARID1A (F), BAP1 (G) and PBRM1 (H)

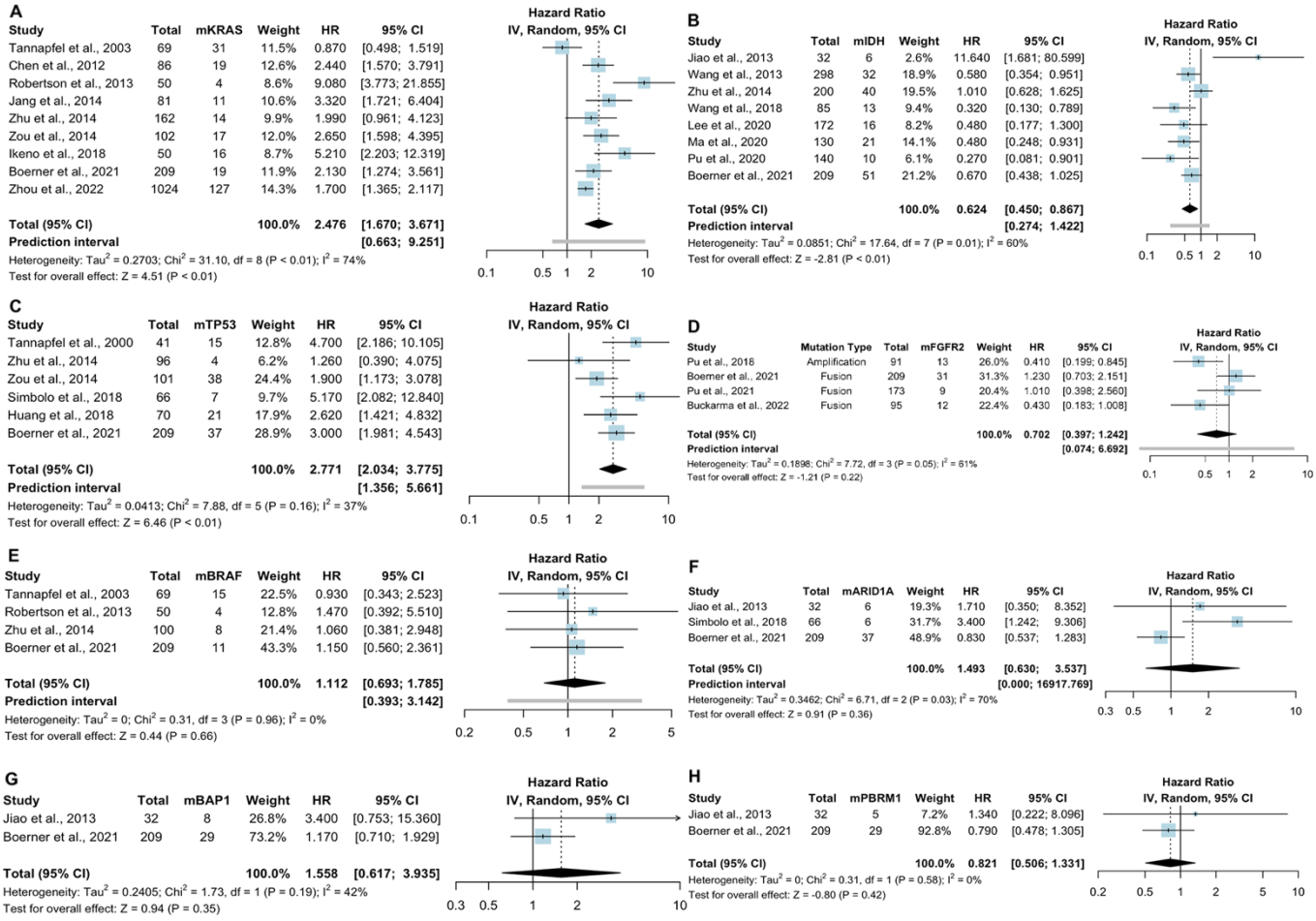
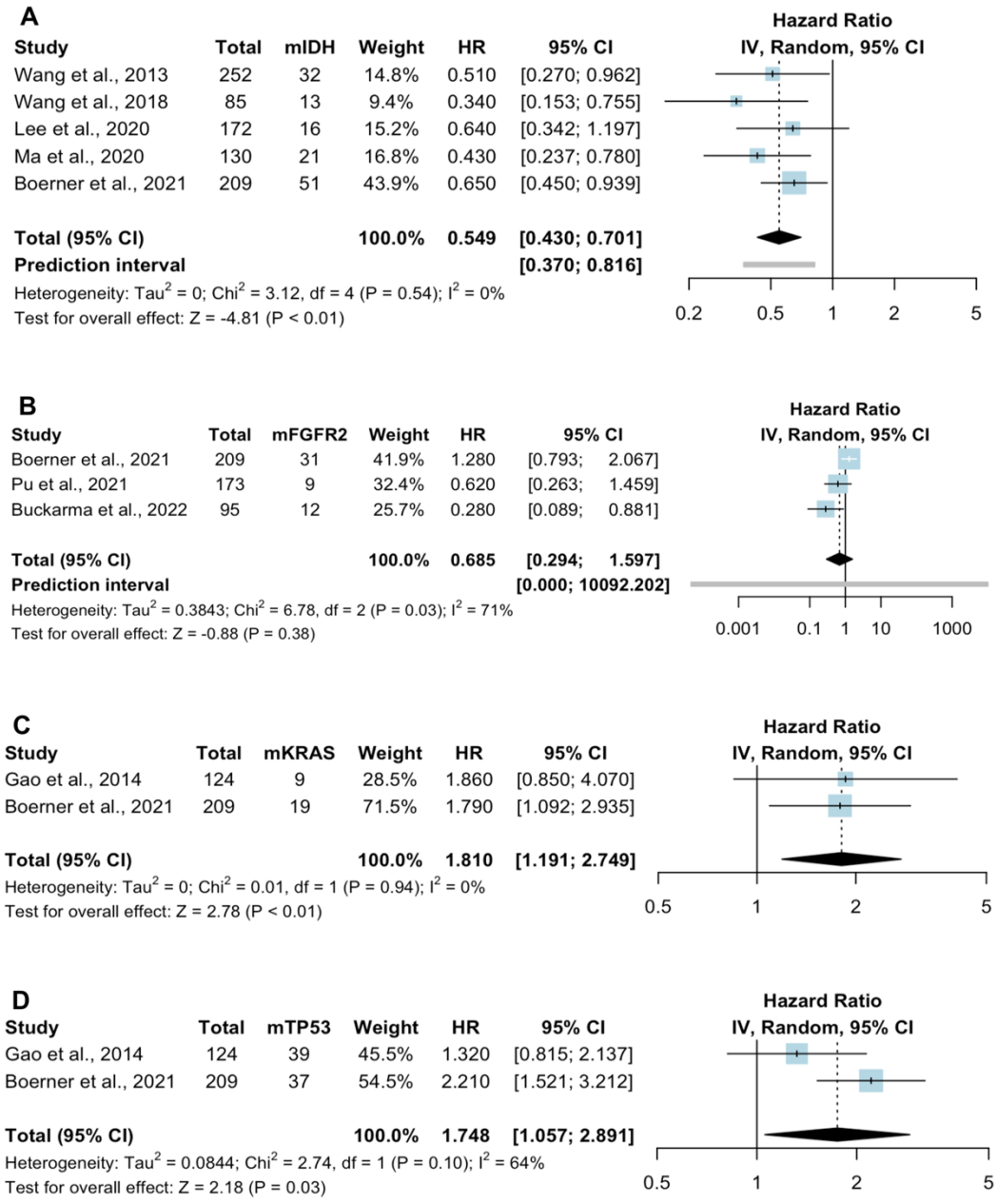


Figure 3. **Forest-plots exploring disease-free survival according to mutation of specific genes.** IDH1/2 (A), FGFR2 (B), KRAS (C) and TP53 (D).



2.2 Patterns of tumor microenvironment cell population in unresectable biliary tract cancers to predict response to immune checkpoint inhibitors

Our review highlighted the importance of precision medicine in intrahepatic cholangiocarcinoma (ICC) and in all biliary tract cancers (BTC). These tumors are known to be associated with chronic inflammation, and preclinical evidence demonstrated an increased expression of immune checkpoints, such as programmed cell death ligand 1 (PD-L1) and cytokine T-lymphocyte-associated protein 4 (CTLA-4) in the tumor microenvironment (TME). Recently, results from the phase III randomized controlled TOPAZ-1 trial(29) have suggested an increased OS in unresectable BTC treated with durvalumab in combination with standard therapy leading the international medical agencies to approve this approach as a first line standard treatment.(77) Immune checkpoint inhibitors (ICI), and anti PD-L1/PD-1 in particular, have been largely explored and validated in other cancers, as hepatocellular carcinoma (HCC), head and neck squamous cell carcinoma (HNSCC) or in melanoma. The antitumor activity of these drugs relies on the disruption of inhibitory signals in tumor-infiltrating lymphocytes (TILs), leading to their functional reinvigoration. However, although some excellent responses have been reported in these cancers, the exact immune mechanisms underlying tumor response and mechanism of resistance remain to be defined. Previous works have demonstrated that these mechanisms could differ on the basis of cancer tissue of origin and include proliferative burst of circulating exhausted T cells, “clonal revival” of pre-existing TILs or “clonal replacement” of intratumoral T cells.(33,34) In order to explore deeper this issue, another interesting point of view proposed is the analysis of TME cellular dynamics in patients experiencing pathological response vs those non-responders. Single cell RNA sequencing (scRNA-seq) has been performed for example in HNSCC tissue showing that responding tumors had specific clonally

expanded CD8+ TILs with a high cytotoxic potential within the baseline TME. By contrast, non-responder baseline TME exhibited a relative absence of these TILs and subsequent accumulation of highly exhausted clones. Similarly, in HCC, CD8+ TILs exhaustion level in tumor microenvironment has been demonstrated it may predict better efficacy of ICI therapy. Information given by molecular analysis before treatment administration could potentially be more useful, being able to find specific patterns or TME population associated with clinical response and therefore drive therapeutic strategy. Immunotherapy in BTC has been recently approved and evidence of molecular mechanisms of tumor response as well as TME tumoral dynamics are still missing in literature.

In collaboration with the Inserm laboratory (UMR_S1110) we therefore elaborate a double project to start the assessment of TME in these cancers. The first part of our work is a retrospective research project already proposed to the association “l’Alsace contre le cancer” (**Appendix 1**) and it is focused on the analysis of advanced ICC samples of different French centers who were treated with Durvalumab. Subsequentially, we will present a prospective protocol to the ethic committee which will be extended to all BTC based on the same methodology.

Third part. Image-guided strategies for a personalized medicine in pancreatic cancer

3.1 Predictive and personalized medicine in pancreatic cancer

In collaboration with the IHU of Strasbourg, a pioneering institute in the image-guided surgery, we developed *CancerProfile* (NCT: 03997617), a multidisciplinary clinical and translational research project (**Appendix 2**) aimed at improving real-time diagnosis and subsequent prediction of tumor response to treatment in pancreatic ductal adenocarcinoma (PDAC). This project will be based on the analysis of histological imaging and functional profiling augmented by cutting-edge artificial intelligence, as well as on the construction of tumor organoids, all to foster precision medicine for PDAC. The project will lay the foundations for a large-scale clinical study to evaluate the implementation of personalized treatment for PDAC patients. It will lead to the establishment of a unique biobank and database, which can serve as a model for other European basic and translational research projects. This study will be carried out in collaboration between the IHU, the Hepatodigestive Pole of the Nouvel Hôpital Civil of the University Hospitals of Strasbourg and the Luxembourg Institute of Health. In practice, tumor tissue is removed during pancreatic resection for PDAC and analyzed by D-FF-OCT, a new perioperative imaging method little explored in pancreatic pathology and mainly during endoscopic procedures. The aim will be to analyze the morphological and metabolic characteristics of tumor cells, which will then be compared with the chemotherapy resistance and prognosis of these patients. At the same time, tumor tissue will be sent to the LIH to develop technologies for creating organoids on which to test different approved drugs.

The protocol is currently recruiting and deadline is expected by the end of 2025.

3.2 Ex-vivo models to predict sensitivity to traditional therapies in advanced cases

Continuing our journey on this delicate topic aimed at recreating models and tools for predicting response to therapies, an additional area of application has been advanced PDAC cases. In these patients, systemic therapy represents the only option and validated drugs are limited, with FOLFIRINOX or gemcitabine (alone or combined with nab-paclitaxel) chosen exclusively based on physical examination. Owning tools to predict response to these treatments would mean personalizing systemic options in order to deliver to the patient the best and the most performing agent. The answer surely lies in biological rather than clinical factors, and the work of translational research is therefore indispensable. Through the collaboration with the Inserm of Marseille, institute constantly applied in translational research of pancreatic tumors, we worked on the establishment of these models. The basis of this project was the recent demonstration that RNA signatures derived from organoids could predict gemcitabine sensitivity.(78) Some model have already been reported by their group, as the *GemPred*, the *improved GemPred* and the *GemCore*, gemcitabine RNA signatures containing thousands of transcripts already validated retrospective cohorts of resected patients.(79–81) Starting from these already developed and published models, we tried to apply them on advanced or metastatic cases, never tested before. A solid multicentric partnership was therefore created and a common databased used for this aim. A paper has been drafted after obtaining robust results and published in Gastroenterology (IF: 33.8).

Research letter

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A TRANSCRIPTOMIC-BASED TOOL TO PREDICT GEMCITABINE SENSITIVITY IN ADVANCED PANCREATIC ADENOCARCINOMA

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Pancreatic ductal adenocarcinoma (PDAC) incidence has increased over the last 30 years.[1] When diagnosed at advanced stages, representing approximately 85% of cases, systemic therapy is the only treatment able to improve patient outcomes. For patients with a good performance status, FOLFIRINOX is the preferred choice, but has a high level of toxicity.2 For unfit patients, gemcitabine administrated alone or combined with nab-paclitaxel remains the standard treatment.[3,4] Treatment choice is currently based on physician evaluation; using tumor molecular analysis to select the most effective and least toxic chemotherapy regimen would represent major progress.

In recent years, we and others have described RNA signatures associated with gemcitabine sensitivity. Tiriac et al[5] found that RNA signatures derived from organoids could determine chemotherapy sensitivity. We reported GemPred, a gemcitabine RNA signature containing

thousands of transcripts and validated in a retrospective cohort of 435 patients.[6] As GemPred predictions were associated with the basal-like and classical PDAC subtypes that relate to patient prognosis, organoid models were included in the signature identification strategy. This allowed us to overcome the prognostic limitations of GemPred and generate an improved GemPred signature.[7] Finally, using a strategy based on the selection of a reduced number of transcriptomic-concordant in vitro and in vivo PDAC models, we identified GemCore, a gemcitabine sensitivity signature that has the advantage of containing fewer than 100 transcripts and that has been validated in 2 clinical cohorts of 80 and 305 patients.[8] As these signatures were all validated in retrospective cohorts of localized tumors on resected formalin-fixed, paraffin-embedded tissues samples, we decided to analyze their ability to predict gemcitabine sensitivity in advanced PDAC on formalin-fixed, paraffin-embedded microbiopsies from primary tumors and metastatic sites.

One hundred and seven patients with advanced PDAC were retrospectively included from 3 hospitals. All patients were treated with gemcitabine as monotherapy in the first line. One hundred and one assessable samples were obtained from 93 patients before treatment (57 unpaired from primary tumors, 28 unpaired from metastatic sites and 16 paired samples).

First, we analyzed primary tumors from 65 patients. Five patients (7.7%) had locally advanced disease and 60 (92.3%) had metastatic disease. Median overall survival (OS) was 5.7 months (95% CI, 4.62–8.52 months) and median progression-free survival (PFS) was 2.3 months (95% CI, 1.38–3.44 months). Gem-Tiriac et al, GemPred, and improved GemPred performed poorly in identifying gemcitabine sensitivity (Figure 1A–F). Improved GemPred revealed a significant association between PFS and gemcitabine sensitivity, with a hazard ratio (HR) of 0.57 (95% CI, 0.34–0.95; P = .032) (Figure 1F). Of all signatures, GemCore achieved the best performance, classifying 29 patients (44.6%) as GemCore+ and 36 (56.4%) as GemCore– (Figure 1G and H). GemCore+ patients displayed a median OS of 13.9 months (95% CI, 9.51–

17.18 months) and a median PFS of 4.85 months (95% CI, 4.29–8.07 months). GemCore– patients had a median OS of 3.1 months (95% CI, 2.33–4.79 months) and a median PFS of 1.15 months (95% CI, 0.49–1.87 months). GemCore was also the only signature to show a significant association with objective response in primary tumors (Table 1 and Supplementary Table 1). In the univariate Cox model, GemCore+ patients showed an OS HR of 0.19 (95% CI, 0.10–0.34; $P < .001$) and a PFS HR of 0.12 (95% CI, 0.06–0.25; $P < .001$). When we contrasted the GemCore signature prediction with clinicopathological variables and transcriptomic RNA biomarkers, we found that 5 variables were statistically significant predictors of OS and PFS ($P < .05$) (Supplementary Table 2): World Health Organization performance status score ≥2, presence of hepatic metastasis, carbohydrate antigen 19-9 levels 59 times higher than the upper limit and poor differentiation were significant for both OS and PFS, whereas number of metastases was only significant for OS and weight loss only for PFS. GemCore was significantly associated with hepatic metastases and the degree of tumor differentiation (Table 1). Despite the observed enrichment of the GemCore stratification with the clinicopathological variables mentioned, GemCore+ remained a predictor of OS (HR, 0.18; 95% CI, 0.09–0.35; $P < .001$) and PFS (HR, 0.11; 95% CI, 0.04–0.26; $P < .001$) in a Cox multivariate model (Supplementary Table 2). When possible, biopsies from metastatic sites are frequently used for diagnostic purposes. Therefore, we analyzed the 4 signatures in 36 biopsies from PDAC metastases. Median OS was 3.5 months (95% CI, 2.39–6.00 months) and median PFS was 1.15 months (95% CI, 0.66–2.39 months). As in primary tumors, GemCore was better able to stratify gemcitabine sensitivity in metastasis samples. GemCore+ patients (n = 19 [52.78%]) had a median OS of 6.6 months (95% CI, 4.72–16.13 months) and a median PFS of 2.95 months (95% CI, 1.38–4.36 months). GemCore– patients (n = 17 [47.22%]) displayed a median OS of 2.1 months (95% CI, 1.64–3.48 months) and a median PFS of 0.36 months (95% CI, 0.00–1.34 months). The univariate Cox model confirmed the predictive capability of GemCore to

discriminate gemcitabine-sensitive patients. GemCore+ showed an HR of 0.14 (95% CI, 0.06–0.35; P < .001) for OS and 0.17 (95% CI, 0.07–0.42; P < .001) for PFS. Among the clinicopathological variables and transcriptomic RNA biomarkers, tumor thickness was the only variable to predict OS in a univariate Cox model (HR, 1.03; 95% CI, 1.00–1.06). In addition, tumor thickness was significantly lower in GemCore+ than in GemCore– patients (33.9 ± 11.8 vs 44 ± 12.4; P = .017) (Table 1). There was a significant association between GemCore– patients and the number of metastases being ≥2 (P = .048) (Table 1). Finally, our analysis of the paired primary tumor and metastasis samples revealed that the GemCore signature gave a matched prediction in 87.5% of cases (57% of samples were GemCore–, 43% were GemCore+).

A weakness associated with drug-response RNA signatures is that they frequently capture the basal-like or classical transcriptomic landscape that is related to the patient's prognosis. However, GemCore did not correlate with any PDAC subtype and was the main OS and PFS predictor in the multivariate Cox analysis (Supplementary Table 2 and Table 1). These observations suggest that GemCore has a predictive, not prognostic, capacity.

Gemcitabine is the main drug used in unfit patients with metastatic PDAC because it has reduced infusion times and fewer adverse effects than polychemotherapy regimens (ie, FOLFIRINOX and gemcitabine/nab-paclitaxel). To avoid any potential biases derived from a combined treatment, here we focused on patients treated with gemcitabine alone. However, further validation of GemCore is needed in patients treated with gemcitabine plus nab-paclitaxel to enlarge the scope of this signature.

We noted that the median OS of GemCore+ patients with biopsied primary tumors was longer than that of those with biopsies from metastatic sites. Although GemCore was able to identify responders to gemcitabine in both, the difference in the median OS is suspected to be because of the small number of patients in the metastatic group and/or because the biopsies of metastatic

tissue correspond to those patients with the most advanced disease; further validation on larger metastasis cohorts is needed to elucidate this discrepancy.

Development of predictive signatures is challenging and in permanent evolution. These predictors depend on the technology used for RNA sequencing and even more on the site from which the biopsy is taken. In this work, we challenged in a multicentric cohort of advanced PDAC patients the GemCore signature alongside 3 other signatures previously validated for gemcitabine as adjuvant treatment for patients who have undergone surgery. GemCore represents the RNA-based signature best able to predict gemcitabine response not only in resected but also in advanced PDAC patients and in all types of samples (ie, resections or microbiopsies from primary tumors and metastatic sites).

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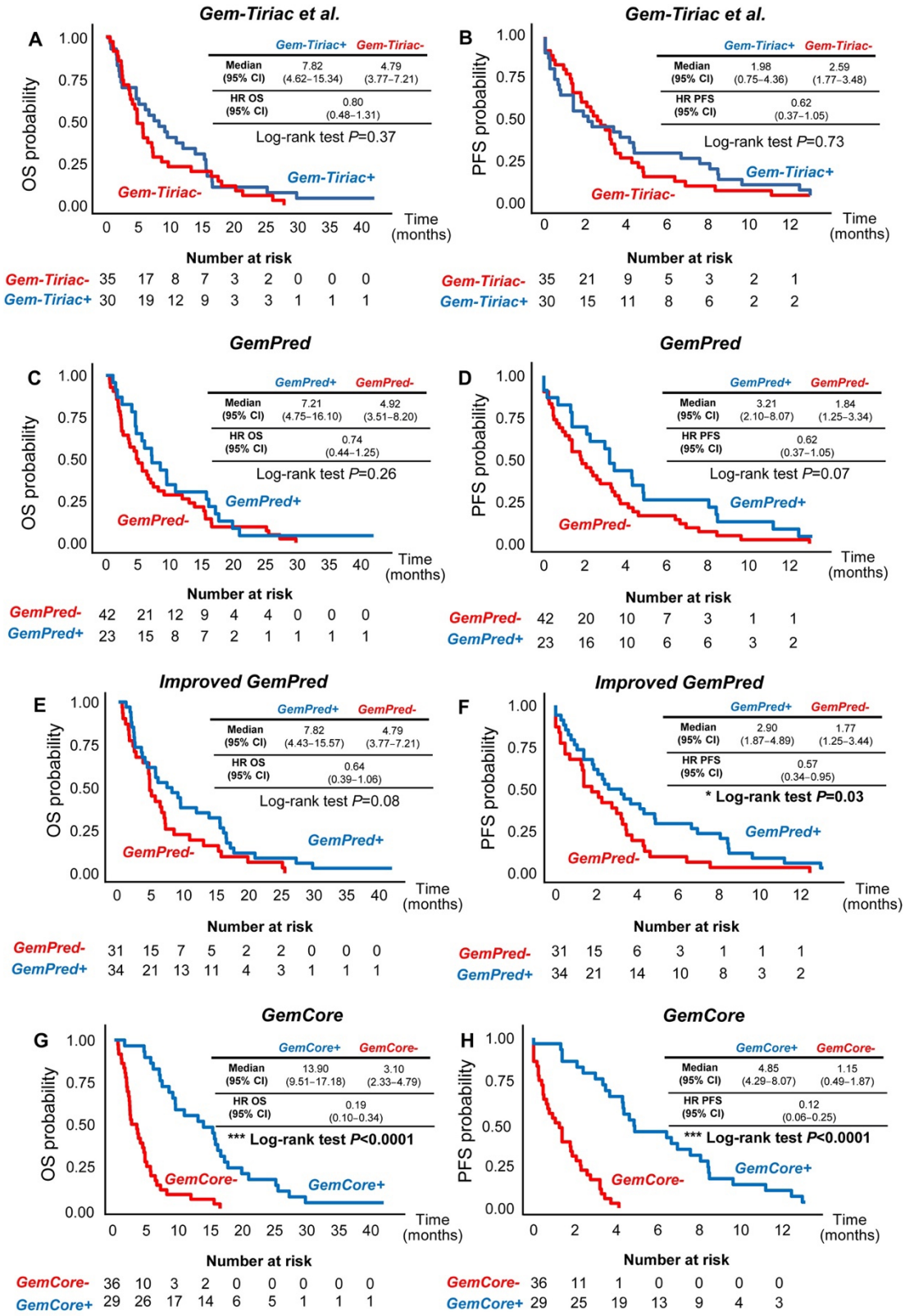

Figure 1. Comparison of gemcitabine sensitivity signatures in patients with advanced PDAC on samples obtained by endoscopic ultrasound fine-needle aspiration biopsy from primary tumors. Kaplan–Meier curve for OS and PFS stratified by gemcitabine sensitivity prediction for the different signatures: (A, B) Gem-Tiriac et al, (C, D) GemPred, (E, F) improved GemPred, and (G, H) GemCore signatures.

3.3 Early detection of occult metastasis in pancreatic cancer through endoscopic ultrasound

The poor outcomes of pancreatic cancer are well known: median survival for locally advanced disease is only 10-12 months; however, in patients with metastatic disease, it falls to 6 months.(82,83) In resected cases numbers are slightly better but risk of recurrence remains high with high-volume centers reporting a 5-years survival rate of 10-20%.(84) One of the most valuable independent prognostic factors in resected patients is undoubtedly nodal status.(84) Patients presenting nodal metastasis show up to three times higher risk of recurrence than N0 cases at multivariate analysis. Detecting N1 cases could be helpful to stratify patients according to their local aggressiveness and propose them a neoadjuvant chemotherapy, as it happens in other gastrointestinal tumors. However, recourse to preoperative chemotherapy currently depends on the vascular contact of the tumor, which i) is not a direct index of aggressiveness but a matter of "bad" location of the lesion and ii) its resectability highly depends on the capability of the surgeon. However, despite the improvements of preoperative imaging modalities, including transabdominal ultrasound, CT, magnetic resonance imaging or PET, the accuracy of predicting tumoral deposits in lymph nodes is very low. Because of its unique ability to obtain refined images of the pancreas and the structures in the vicinity, echoendoscopy (EE) is considered one of the greatest advances in this difficult task.(85) EE provides detailed, high-resolution images that surpass those of CT or MRI due to the proximity of its high-frequency transducers to the pancreas.(86) However, due to the inflammation and edema of surrounding tissues caused by the tumor itself and associated pancreatitis, the accuracy of EE in assessing the lymph nodes of the digestive tract is limited in tumoral settings. Imaging-based technologies, such as elastography, can be used to enhance its diagnostic utility.(87) As

malignant lesions are generally stiffer than adjacent tissues, elastography can help to classify lymph node lesions more accurately. This is added to the diagnostic capacity of EE, thanks to which peripancreatic lymph nodes can be exactly located, characterized by size, shape and/or sharpness of border and, finally, biopsied.(88–90)

In collaboration with the IHU, we therefore proposed a prospective study called *Echosurg*, whose hypothesis is that the implementation of a simple lymph node classification, based on non-invasive ultrasonographic criteria, would facilitate the localization and qualification of peripancreatic and distant lymph nodes, and thus tumor staging. As part of this protocol (**Appendix 3**), we localize and characterize peripancreatic lymph nodes, as well as those remote from the tumor, using Doppler to study their vascularization, elastography to study their elasticity and high-quality endoscopic recording just before surgical resection. At the same time, the video data obtained could be collected in a computer database with a view to creating an artificial intelligence tool for lesion detection and qualification. Ultrasonographic data will then be compared with pathological sampling and accuracy of EE therefore tested.

The protocol is currently recruiting and its end scheduled by December 2024.

Discussion

The most intriguing aspect of HBP surgery is undoubtedly its multifaceted nature and its intricate nature, necessitating a multidisciplinary approach for a correct and high-level approach. Treatment flow-chart is, in fact, sometime cumbersome and the boundaries between one treatment and another are not always well delineated by scientific evidence. As it happens in clinical practice, with the wide-spread diffusion of multidisciplinary meetings and the need for medical and surgical collaborations, this interaction is equally present in the field of research, both clinical and translational. The project of this thesis was based on this concept, and the final goal was twofold. As a first aim, we worked in constant and strict collaboration with hepatologists, researchers, oncologists, and other medical figures in order to develop several scientific projects. These projects were grouped by the common target of refining the management of HBP oncologic patients and improving their outcomes. Although the final goal and the main characters were well-defined, these projects were not clear at the beginning, and they have been created or outlined during these three years through the constant dialogue between different specialists. We tried to touch all the HBP fields not only in terms of the type of pathology, but also on the type of treatment or approach and on methodology, to enhance this dynamic concept of multidisciplinary. Furthermore, this work of this thesis was not an end in itself, but had as its long-term goal to strengthen the collaborations of our unit from a clinical point of view through tangible evidence of the importance of this teamwork. Multidisciplinary meetings, scientific reunions, developing new treatments or accelerating and improving the diagnostic/therapeutic path of our patients were some examples of this clinical aim. The partnership with the researchers of the Institute for viral and hepatic diseases (Inserm U1110) was the first proof. Since the creation of the LIVMOD (UMR_S1110) biobank in 2020, this partnership has continued with the collection of fresh surgical and blood samples with the objective of refining the study of liver parenchyma and hepatic tumors. This efficient and dynamic work led us to collect a great number of operative specimens with the creation of a

personal biobank used for translational research. This allowed, for instance, high quality cultures used for many projects, especially the assessment of the role of the anti-Claudine 1 antibody, developed at the Institute for Viral and Hepatic Diseases, for the prevention of liver fibrosis and HCC development. Ex vivo models like tumor or liver spheroids and patient-derived xenografts (PDX) have been also used to test antitumoral activity of new drugs compared to routinely used therapies to establish “chemograms”, with the objective of a personalized medicine. This last aim passed not only from new therapeutic possibilities, but also by the real comprehension of the deep and molecular mechanisms which regulate tumor response or progression to standard treatment. The tumor microenvironment was in fact reproduced in our spheroids to have a practical, rather than simply theoretical, approach. We explored this complex subject both in HCC and in ICC, complementing in the latter case the research through an in-depth study of the genetic background and its impact on the patient's prognosis. Two research projects started from my constant activity at the Inserm (U1110) during these years and focusing on the investigation of immunotherapy activity in ICC. Beside this main research program, other projects addressed hepatic virus host interactions, especially for HBV and HDV projects, as well as proof-of-concept studies for new molecules as new therapeutic agents for fibrosis and or HCC development and new cellular targets for chemoprevention of HCC development.(91,92)

The multifaceted collaboration between my team and the IHU, the institute of image-guided surgery, stands as a pivotal element in the intricate tapestry of my thesis. This partnership, rooted in the IHU's profound expertise in imaging and extensive knowledge in the HBP domain, has become a testament to the significance of multidisciplinary engagement in advancing medical research and patient care. In this synergistic venture, a diverse cohort of professionals, including surgeons, radiologists, endoscopists, and researchers, coalesced their skills and insights. The collective effort resulted in the inception of two projects that center around the

profound goal of delivering precision medicine to patients grappling with pancreatic cancer. The first prospective project aims to evaluate the accuracy of EUS in detecting nodal metastasis of pancreatic cancer. Serving as a co-Principal Investigator, I am actively involved in steering this initiative towards its anticipated completion by the end of 2024, with a preliminary analysis slated for June. Simultaneously, a second prospective project is currently underway, embarking on a dual mission of incorporating D-FF-OCT during pancreatic resections and fostering drug testing projects on pancreatic spheroids. This innovative approach is made possible through collaboration with the Luxembourg Institute of Health, solidifying our commitment to pushing the boundaries of medical research. The symbiotic relationship with the IHU extends beyond these flagship projects, permeating into other prospective initiatives and informing our clinical approach to daily patient care for those with HBP tumors. A notable testament to our commitment is the "*diagnostic en 1 jour*" project, which revolutionizes the expeditious and multidisciplinary care provided to patients following an HBP tumor diagnosis. This groundbreaking initiative has seamlessly integrated into my personal outpatient practice, underscoring its vital role in transforming patient outcomes. "*Diagnositc en 1 jour*" aims to enhance the quality of patient care by providing complete, specialized, and fast medical opinions all in one day. This type of collaboration has become a catalyst for ongoing and published studies, reaffirming the importance of sustained engagement and shared expertise in navigating the complexities of hepatobiliary and pancreatic disorders.(93)

The continuous pursuit of excellence within our HBP unit has prompted concerted efforts to fortify collaborations within the hospital, underscoring the pivotal role of multidisciplinary approaches in elevating both research activities and everyday clinical practices. Beyond the spectrum of ongoing and listed research projects, our commitment to enhancing patient care and fostering innovation is reflected in various strategic initiatives. Firstly, the establishment of a weekly multidisciplinary tumor board stands as a testament to our dedication to

comprehensive patient management. This dedicated forum provides a dynamic platform for the collective deliberation and analysis of intricate HBP imaging and cases, sharing the collective wisdom of diverse specialists such as surgeons, radiologists, endoscopists, and researchers. The synergy of minds in this multidisciplinary setting not only enriches our understanding but also cultivates innovative solutions for complex clinical challenges. Secondly, recognizing the paramount importance of staying up-to-date on scientific evidence, a dedicated departmental staff for literature reviewing has been instituted. This proactive measure ensures that our HBP unit remains at the forefront of evolving medical knowledge, thereby enriching our research pursuits and augmenting the quality of patient care through evidence-based practices. Furthermore, in a groundbreaking step towards expanding treatment modalities, our unit try to offer the best solutions for oncologic HBP cases, witnessed by the implementation of transarterial radioembolization within our own institution (before shared with another hospital) or the application of multicentric projects for advanced cases. These multidisciplinary interventions, involving collaboration between interventional radiologists, oncologists, and surgeons, exemplifies our commitment to offering cutting-edge therapies for HBP conditions, thereby advancing the standard of care available to our patients. Furthermore, recognizing the paramount importance of structured data management for future research projects, we have established a prospective dedicated common database. This initiative ensures the seamless integration and standardized collection of data across various research projects within the HBP unit. In practice, the already discussed research projects combined with the multifaceted initiatives within our HBP unit, ranging from collaborative tumor boards to cutting-edge treatment implementations and robust data management strategies, underscore the transformative power of multidisciplinary collaboration. These efforts not only amplify the impact of our research but also enhance the daily clinical experience for both patients and

healthcare professionals, positioning our unit as a beacon of excellence at the intersection of research and patient-centric care.

In conclusion, after these 3 years of thesis we can demonstrate how a solid and constant teamwork is crucial in the approach of HBP tumors and how this multidisciplinary, including the expertise of different medical specialties, basic science, and engineering with cutting-edge technologies have a key role in the decision-making process and will be able to improve outcomes in these patients.

Conclusion and perspectives

The culmination of this doctoral journey reveals a comprehensive exploration into the multidisciplinary management of HBP tumors with a focus on enhancing long-term outcomes. In each chapter, the collaborative efforts between surgeons, oncologists, researchers, and various medical stakeholders emerge as a cornerstone in the advancement of HBP tumor management. The integration of diverse expertise not only enriches the depth of understanding but also underscores the significance of a unified approach in tackling the multifaceted challenges posed by these malignancies. Some results have already been achieved but the work done presupposes important new goals with projects already delineated.

In the first chapter, the meticulous investigation into the management of HCC has highlighted the indispensability of collaborative decision-making. The synergy between surgical interventions, locoregional therapies, and systemic treatments necessitates seamless cooperation among medical professionals, ensuring a patient-centric paradigm that goes beyond traditional disciplinary boundaries. Future researches, already outlined to a large extent, will be the consequence of the work already done in recent years, with analysis of new neoadjuvant methods, including transarterial radioembolization, the creation of preoperative clinical scores do predict extrahepatic and early recurrence in these resectable patients, as well as a translational research work aiming to predict response to systemic treatments, based on organoids models.

This collaborative ethos extends into the second chapter, where the intricate landscape of ICC is navigated with a genetic and molecular lens. The interplay between genetic insights, targeted therapies, and immunotherapies underscores the collaborative momentum propelling personalized medicine into the forefront of ICC management. Research works on TME to predict sensitivity to immunotherapy on both ICC and BTCs, based on the RNA sequencing technique, will be one of my most ambitious projects in the coming years in this pathology.

The third chapter ventures into PDAC, where collaborative efforts manifest in the assessment and integration of cutting-edge technologies. The collaborative synergy between researchers and clinicians accelerates the translation of research findings into clinical applications, fostering a dynamic exchange that propels the field forward. These collaborations bridge the gap between bench and bedside, positioning the research not only as an academic pursuit but as a catalyst for tangible improvements in patient care. Also in this fields, some projects are currently ongoing, with results that will be published within the next two years. The use and the application of the D-FF-OCT in preclinical and clinical activity, both in oncologic and non-oncologic scenarios, will definitely be key elements in my future projects on pancreatic cancer. In clinical practice, current results and future perspectives of this research are profound. The refined treatment strategies and personalized medicine approaches outlined in this thesis are not theoretical constructs but tools poised for a possible integration into patient care. The collaborative spirit championed throughout this work resonates in the enhanced caretaking of patients with HBP tumors, ensuring that advancements in research translate into palpable improvements in clinical outcomes. As the thesis sets sail towards broader dissemination, the imperative of continued collaboration remains essential, promising a future where the multidisciplinary fabric of HBP tumor management is woven seamlessly into the fabric of compassionate, precise, and enduring patient care.

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***Fabio Giannone**, Aina Venkatasamy, Antonin Fattori, Thomas Baumert, Catherine Schuster, Patrick Pessaux*

Currently being drafted

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Fabio Giannone, *Emanuele Felli, Federica Cipriani, Bruno Branciforte, Rami Rhaiem, Bader Al Taweel, Raffaele Brustia, Ephrem Salame, Fabrizio Panaro, Daniele Sommacale, Tullio Piardi, Guido Torzilli, Luca Aldrighetti, Catherine Schuster, Patrick Pessaux*

Currently being drafted

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***Fabio Giannone**, Charles Lagarrigue, Oronzo Ligurgo, Catherine Schuster, Thomas F. Baumert, Paul M. Mertes, Olivier Collange, Patrick Pessaux*
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Robotic left pancreatectomy with perirenal tissue excision and left adrenectomy (posterior ramps) for pancreatic ductal adenocarcinoma infiltrating the adrenal gland
***Fabio Giannone**, Oronzo Ligurgo, Patrick Pessaux*
Under review on Annals of Surgical Oncology

PARTECIPATION IN RESEARCH PROJECTS

TheraCAN - Targeting Claudin-1 for Precision Medicine in Hepato-Pancreato-Biliary Cancers
Submitted to the ARC foundation

CHAPTER IN A PRINTED BOOK

Réalité augmentée et virtuelle en chirurgie pancréatique
***Fabio Giannone**, Arne Kock, Zineb Cherkaoui, Patrick Pessaux*
Rapport du congrès français de chirurgie 2023 sur la chirurgie pancréatique laparoscopique et robot-assistée. Arnette, Éditions John Libbey Eurotext 2023, 8 pages

Positions and access in liver surgery
***Fabio Giannone**, Oronzo Ligurgo, Patrick Pessaux*
The SAGES Manual of Contemporary Indications and Management for Hepatic and Biliary Diseases. Springer nature (submitted to the editor), 25 pages

CONGRESS PRESENTATIONS

Combining radiofrequency ablation with hepatic resection in the setting of a parenchymal-sparing strategy: a retrospective analysis of long-term outcomes

Aurelien Grollemund, ***Fabio Giannone****, Emanuele Felli, Zineb Cherkaoui, Theo Mayer, Patrick Pessaux*

Oral presentation at E-AHPBA, 6th-9th June 2023, Lyon

Redefining prognostic factors in potentially upfront resectable pancreatic head cancer after neoadjuvant chemotherapy

Fabio Giannone*, Stefano Crippa, Giulio Belfiori, Giulia Gasparini, Paolo Camisa, Marco Schiavo Lena, Diego Palumbo, Stefano Partelli, Gianpaolo Balzano, Domenico Tamburrino, Nicolò Pecorelli, Francesco De Cobelli, Claudio Doglioni, Massimo Falconi*

Abstract presentation at E-AHPBA, 6th-9th June 2023, Lyon

Prognostic benefit of preoperative transarterial chemoembolization in upfront resectable large hepatocellular carcinoma. a multicentric propensity score-based analysis of european high-volume centers

Fabio Giannone*, Emanuele Felli, Federica Cipriani, Bruno Branciforte, Rami Rhaiem, Bader Al Taweel, Raffaele Brustia, Ephrem Salame, Fabrizio Panaro, Daniele Sommacale, Tullio Piardi, Guido Torzilli, Luca Aldrighetti, Patrick Pessaux*

Oral presentation at E-AHPBA, 6th-9th June 2023, Lyon

Robotic left pancreatectomy with perirenal tissue excision and left adrenectomy (posterior RAMPS) for pancreatic ductal adenocarcinoma infiltrating the adrenal gland.

Fabio Giannone*, Oronzo Ligurgo, Arne Kock, Valere Barlerin, Zineb Cherkaoui, Patrick Pessaux*

Video presentations at the 1st Consensus conference on robotic hepato-pancreato-biliary surgery, 7th-9th December 2023, Paris

APPENDIXES

Appendix 1. Tumoral TME assessment to understance resistance to immunotherapy

L'Alsace contre le cancer

Nom : Thomas Baumert
Laboratoire : Inserm U1110 - Institut de Recherche sur les Maladies Virales et Hépatiques

Mots Clés (5 au maximum) : Cholangiocarcinoma, resistance to therapy, immunotherapy, Single cell RNAseq, Bioinformatics

Titre du projet : Investigation of the tumor microenvironment in advanced intrahepatic cholangiocarcinoma to understand resistance to immune checkpoint inhibitors

Résumé :
Background: Intrahepatic cholangiocarcinoma (iCCA) is a fatal primary hepatobiliary cancer with growing incidence and mortality rates. Most patients are diagnosed with advanced stages of the disease and are not eligible for surgery. Current treatment involves chemotherapy, but the overall survival remains very low. Recent trials with the immune checkpoint inhibitor (ICI) durvalumab, a recombinant antibody targeting programmed cell death ligand 1 (PD-L1), combined with standard-of-care, have shown improved survival rates, prompting its approval for advanced biliary tract cancer. However, response rates remain unsatisfactory. There is a growing need to understand resistance mechanisms and predict ICI response in patients.

Objective: Using single-cell RNA sequencing (sc-RNAseq), this project aims to elucidate the cellular mechanisms associated with patient response to anti-PD-L1 in advanced iCCA. By analyzing tumor biopsies from responders and non-responders, the goal is to: 1) Identify resistance mechanisms to immunotherapy enabling to uncover new therapeutic targets. 2) Identify biomarkers predicting immunotherapy response and resistance, thus facilitating personalized treatment options.

Previous Work: Our laboratory has successfully employed sc-RNAseq to understand the pathogenesis of liver disease and cancer, including the first human liver atlas at single-cell resolution (Aizarani Nature 2019). We have also a long-standing track record and internationally recognized expertise to investigate pathogenesis and resistance mechanisms in liver cancer (Crouchet Nature Com. 2021, Roehlen Science Transl. Med. 2022, Roehlen J. Hepatol. 2023).

Methods: Specimens from iCCA patients treated with Durvalumab will be categorized into responders and non-responders. A minimum of 6 samples from each group will be studied (sample size was calculated based previously published

studies in other cancers, Oliveira Sci Immunol. 2023). Single cells from these samples will be captured, barcoded, and subjected to sc-RNAseq analysis. Using various computational tools, the team will identify different cell populations, study signaling pathways, and use bioinformatics to pinpoint durvalumab response biomarkers. These findings will further be validated through immunohistochemistry.

Expected Outcomes: The project will provide a comprehensive understanding of the tumor immune microenvironment in iCCA under treatment with ICI. By unraveling novel mechanisms of resistance, it will contribute to improve treatment strategies including a personalized approach to treat iCCA patients. Given the recent approval of ICIs for iCCA and the advent of sc-RNAseq technology, this project provides a unique opportunity to address this urgent unmet medical need in a timely manner. Understanding of the mechanisms of response and resistance to recently extended standard of care will contribute to improve the dismal outcome of patients with advanced CCA.

Appendix 2. *CancerProfile*

OBJECTIFS ET FINALITÉS

Contexte, objectif(s) et justification de l'étude

Avec une survie à 5 ans qui reste à un chiffre, l'adénocarcinome du canal pancréatique (PDAC) a le plus mauvais pronostic de tous les cancers digestifs en raison de l'absence de diagnostic précoce et de la réponse limitée aux traitements [(1)].
Il s'agit du cancer le plus meurtrier au monde, avec un taux de mortalité qui devrait augmenter dans les pays occidentaux avec le vieillissement des populations et l'augmentation des niveaux d'obésité. Il se développe souvent sans symptômes apparents, et le diagnostic est généralement établi tardivement dans l'évolution de la maladie. À ce stade, seuls 15 à 20 % des patients peuvent bénéficier d'une résection chirurgicale, qui reste le seul traitement curatif [(2)].

Ainsi, la chimiothérapie palliative reste un pilier de la gestion de cette maladie [(3)].
Malheureusement, la forte résistance aux agents chimiothérapeutiques actuellement utilisés représente un goulot d'étranglement majeur dans le traitement, et le développement d'approches thérapeutiques efficaces pour combattre le PDAC reste un besoin médical urgent. La caractérisation moléculaire des tumeurs a permis de développer considérablement la médecine de précision [(4)].
Néanmoins, seul un petit sous-ensemble de patients (13 %) recevant des médicaments à visée moléculaire présentent une réponse objective [(5)].
Les essais fonctionnels peuvent pallier les limites de la prédiction de la réponse aux médicaments basée sur la génétique s'ils sont réalisés dans des conditions in-vivo. En effet, le faible taux de réussite (5 %) des essais cliniques des médicaments anticancéreux développés à partir de criblages de lignées cellulaires standard dans des cultures conventionnelles en 2D appelle à l'utilisation de modèles tumoraux plus précis, capables de représenter l'environnement architectural des cancers solides [(6,7,8,9)].
Ceci est particulièrement pertinent dans le cas du PDAC, une tumeur caractérisée par un microenvironnement unique constitué d'un stroma fibrotique dense, qui est en grande partie responsable de la résistance accrue aux médicaments [(10,11)].

L'automatisation et la personnalisation sont deux concepts centraux autour desquels se développent les diagnostiques et traitements du futur. Ces concepts se situent à l'interface entre le diagnostic en temps réel, la chirurgie et la médecine moléculaire, conduisant à réaliser un diagnostic intégré et à élaborer un traitement médical de précision et personnalisé. Cependant, l'innovation en médecine moléculaire n'est pas homogène. Par exemple, dans le traitement contre le cancer, l'utilisation de nouvelles thérapies ou de nouvelles molécules n'a pas encore intégré les dernières découvertes permises par la réalisation de profilages génétiques ou fonctionnels tumoraux systématiques.

Pour ce faire, l'objectif principal de ce projet est donc d'améliorer le diagnostic et la prédiction ultérieure de la réponse de la tumeur aux traitements, en utilisant l'imagerie histologique et le profilage fonctionnel personnalisé assisté par intelligence artificielle (IA) afin de favoriser la médecine de précision dans le domaine de l'adénocarcinome du canal pancréatique (PDAC).

Les objectifs secondaires du présent protocole sont :

- - Établir une technologie augmentée par le diagnostic précis « on site » du PDAC en accroissant la

  précision globale de la caractérisation des échantillons de biopsie de PDAC par l'introduction d'un découpage optique innovant basé sur la tomographie à cohérence optique plein champ (D-FF-OCT) tout en assurant la mise en place d'un système de diagnostic automatique à l'aide de la D-FF-OCT et l'IA.

- - Mettre au point une procédure standard pour développer des organoïdes du PDAC à partir d'une FNB comprenant des cellules cancéreuses et stromales avec un ratio similaire à celui des tissus d'origine.

- - Etablir la faisabilité technique du profilage fonctionnel personnalisé (PFP) sur les organoïdes générées pour évaluer in fine leur potentiel prédictifs et leur adaptabilité au criblage éventuel afin d'identifier et valider les options alternatives de traitements personnalisés.

Justification du respect de l'éthique

Le présent projet ne pose pas de problème éthique, pas de stigmatisation d'un groupe spécifique et ne va pas à l'encontre de la morale.

La procédure chirurgicale permettant de récupérer des pièces réséquées ou de biopsies cancéreuses pancréatiques n'est pas modifiée par l'étude.

Nous respectons la législation en vigueur :

- - L'information individuelle et la non-opposition du patient
- - L'engagement de conformité à la méthodologie de référence MR004
- - Nous nous engageons à ne réaliser que l'étude décrite dans cette saisine

Justification de l'intérêt public

Notre étude cherche à montrer l'intérêt de l'emploi du développement de structure 3D dérivées de pièces réséquées ou de biopsies cancéreuses pancréatiques en vue d'un profilage des tumeurs de façon automatisée et personnalisée afin de trouver, in fine, le traitement le plus adapté et efficace pour le patient.

La finalité de notre projet n'entre pas dans une finalité interdite :

- - Il n'y a pas d'identification possible des personnes sur le fondement des données les concernant,
- - Il n'y a pas de promotions des professionnels de santé ou d'établissements des produits de santé.

  L'intégrité scientifique est garantie par les membres coordonnateurs, qui se réuniront de façon récurrente pour faire l'état d'avancement du projet.

Publication des résultats et valorisation

Notre projet ayant pour objet l'évaluation de la faisabilité de développement de structures 3D dérivées de pièces réséquées ou de biopsies cancéreuses pancréatiques, aucune communication orale ou écrite n'est prévue par le présent protocole par l'un ou l'autre des responsables du traitements et de mise en œuvre du projet (IHU Strasbourg ou LIH).

A l'issue de ce projet, en fonction des résultats obtenus, une étude interventionnelle pourra être envisagée afin de valider la technique de PFP sur biopsies pancréatiques.

Appendix 3. *Echosurg*

Titre de l'étude	Étude prospective non randomisée de l'échoendoscopie diagnostique préopératoire pour le diagnostic des lésions métastasiques occultes du cancer du pancréas opérable
Promoteur de la recherche	IHU Strasbourg
Investigateur principal	Pr Patrick PESSAUX
Type d'étude	RIPH catégorie 2 - Hors produit de santé
Nombre de centres	Un centre : Service de Chirurgie Viscérale et Digestive, Nouvel Hôpital Civil de Strasbourg, France
Méthodologie de l'étude	Prospective Monocentrique Non randomisée
Population étudiée	Patients adultes, homme ou femme, présentant une tumeur pancréatique solide ou kystique et pour lesquels une résection chirurgicale (en première intention et après traitement néoadjuvant) est prévue
Objectif principal de l'étude	L'objectif principal est l'estimation de la sensibilité et de la spécificité d'une classification simple « bénin/malin » des ganglions, établie par l'endoscopiste, par rapport au gold standard (anatomopathologie)
Critère de jugement principal	Taux de ganglions bien classés (sensibilité) et taux de ganglions mal classés (spécificité) par l'endoscopiste, en comparaison avec le gold standard (anatomopathologie).

Critères d'inclusion et de non-inclusion	**Critères d'inclusion :** 1. Patient(e) âgé(e) de plus de 18 ans 2. Patient(e) présentant une tumeur solide ou kystique dégénérée du pancréas devant bénéficier d'une chirurgie à titre curative 3. Patient(e) avec un examen clinique complet réalisé 4. Patient(e) ne présentant pas de contre-indication à l'anesthésie et à la réalisation d'une endoscopie digestive haute et d'une chirurgie du pancréas 5. Patient(e) capable de recevoir et comprendre les informations relatives à l'étude et de donner son consentement éclairé écrit 6. Patient(e) affilié(e) au régime national de sécurité sociale **Critères de non-inclusion :** 1. Patient(e) présentant une maladie hémorragique avec trouble de l'hémostase et de la coagulation (TP < 60%, TCA > 40 s et plaquettes < 60000/mm^3) 2. Patient(e) sous traitement anticoagulant ou antiagrégant ne pouvant être temporairement interrompu 3. Patient(e) porteur(se) d'un shunt droit-gauche, d'une hypertension artérielle pulmonaire sévère (pression artérielle pulmonaire > 90 mm Hg), d'une hypertension systémique non contrôlée ou atteint d'un syndrome de détresse respiratoire. 4. Patiente enceinte ou allaitante 5. Patient(e) en période d'exclusion (déterminée par une étude précédente ou en cours) 6. Patient(e) sous sauvegarde de justice 7. Patient(e) sous tutelle ou curatelle
Objectifs secondaires	1. L'estimation du taux de métastases réellement diagnostiquées. 2. La détermination de l'emplacement des métastases ganglionnaires lymphatiques cachées identifiées par échoendoscopie préopératoire chez les patients atteints d'un cancer du pancréas résécable. 3. Nombre total de ganglions à distance détectés lors de l'EE préopératoire 4. Nombre de ganglions à distance détectés lors de l'EE préopératoire et dont la malignité a été confirmée par le gold standard 5. Taux de contre-indications pour la chirurgie, révélées par l'EE préopératoire 6. L'utilité diagnostique de l'élastographie lors de l'échoendoscopie préopératoire pour l'identification des métastases ganglionnaires cachées à distance du champ opératoire chez les patients atteints d'un cancer du pancréas résécable, évalué en nombre de patients. 7. L'évaluation du temps opératoire lié à l'utilisation de l'élastographie préopératoire chez les patients atteints d'un cancer du pancréas résécable. 8. L'évaluation des coûts liés à l'utilisation de l'élastographie préopératoire chez les patients atteints d'un cancer du pancréas résécable. 9. Impact du marquage à l'encre noire stérile des ganglions à distance lors de l'EE préopératoire sur la procédure chirurgicale qui s'en suit (questionnaire chirurgien) 10. Établissement d'une base de données et création d'un outil dans la détection et la qualification des lésions – Machine learning / Intelligence Artificielle (IA) à partir des données vidéos de l'EUS récupérées
Critères de jugement secondaires	1. La sensibilité de l'imagerie par comparaison entre l'identification préopératoire par échoendoscopie des ganglions suspects et les résultats de l'analyse histologique de ces ganglions réséqués. 2. Description de l'emplacement des métastases ganglionnaires lymphatiques identifiées par échoendoscopie chez les patients atteints d'un cancer du pancréas résécable. 3. Nombre de ganglions à distance détectés lors de l'EE préopératoire

	4. Nombre de ganglions à distance détectés lors de l'EE préopératoire et dont la malignité a été confirmée par l'analyse histologique 5. Nombre de patients chez qui l'EE a permis de détecter une contrindication à la chirurgie sur le nombre total de patient inclus. 6. Le taux de patients pour lesquels l'élastographie a été nécessaire pour identifier des métastases ganglionnaires cachées à distance du champ opératoire chez les patients atteints d'un cancer du pancréas résécable. 7. Mesure du temps opératoire (en minutes) nécessaire à l'élastographie préopératoire chez les patients atteints d'un cancer du pancréas résécable. 8. Mesure des surcoûts (en euros) des matériels nécessaires à l'élastographie préopératoire chez les patients atteints d'un cancer du pancréas résécable. 9. Analyse de l'impact du marquage à l'encre noire stérile des ganglions à distance lors de l'EE préopératoire sur la procédure chirurgicale qui s'en suit par le biais d'un questionnaire rempli par le chirurgien. Ce questionnaire sera évalué sur une base d'un score de Likert variant entre 1 (pas satisfait) et 5 (très satisfait). 10. Analyse des données vidéos de l'EE dans la détection et la qualification des lésions par Machine learning / Intelligence Artificielle (IA)
Déroulement de la recherche	**Modalités de recrutement** Le recrutement s'effectuera au sein du Service de Chirurgie Digestive et Endocrinienne du Nouvel Hôpital Civil (NHC) de Strasbourg. L'étude sera présentée aux patients nécessitant une résection chirurgicale pour cancer du pancréas. Au cours d'une consultation préparatoire à la chirurgie, le patient sera informé de l'étude et des analyses supplémentaires par rapport à une prise en charge classique. Une notice d'information ainsi qu'un formulaire de consentement lui seront remis. **Chronologie des visites** INFORMATION : Information sur l'étude dispensée lors d'une consultation préparatoire à la chirurgie. VISITE D'INCLUSION : Recueil du consentement du patient. ECHOENDOSCOPIE PREOPERATOIRE (30 à 7 jours avant l'intervention chirurgicale) : Anesthésie Évaluation des ganglions lymphatiques péripancréatiques par échoendoscopie. Relevé de l'emplacement anatomique et des caractéristiques des ganglions lymphatiques. Si nécessaire, évaluation par élastographie des ganglions lymphatiques péripancréatiques et à distance du pancréas. Tous les ganglions suspects de maladie métastatique seront marqués à l'aide d'une aiguille de ponction 25 gauges endoscopique par de l'encre noire stérile. Suivi et recueil des éventuelles complications postopératoires. Décision thérapeutique par le chirurgien, en aveugle de la cartographie faite par l'échoendoscopie préopératoire. INTERVENTION (J0) : résection de la tumeur pancréatique et des ganglions résécables. A la fin de l'intervention, les groupes de ganglions lymphatiques seront divisés et envoyés pour une analyse histologique définitive. VISITE DE SUIVI J30 : Visite à 30 jours postopératoires. VISITE DE SUIVI M6 : Visite (consultation ou par téléphone) à 6 mois postopératoires. Fin d'étude. *Voir le calendrier de l'étude pour le détail des examens*
Analyse statistique	<u>Nombre de cas prévisionnel</u> Le critère principal étant défini par l'estimation de la sensibilité et de la spécifié du critère de malignité des ganglions observée par le chirurgien contre la réponse du gold standard, l'hypothèse d'une sensibilité (et d'une spécificité) d'au moins 75%, et pour une estimation de cette valeur dans un intervalle de confiance dont la demi largeur est inférieure à 10%, après simulations de

	données corrélées par réplications bootstrap, le nombre de patients à considérer est de 45 patients, en considérant une moyenne de 6 ganglions malins par patient. <u>Analyse statistique</u> L'analyse statistique comportera en premier lieu une étude descriptive de la population de l'étude et des paramètres étudiés avec évaluation des effectifs et fréquences, et évaluation des moyennes et écart-type, médianes et interquartiles pour les variables quantitatives. Le critère principal, l'estimation de nombre de patients avec ganglions métastatiques observées par l'échoendoscopie préopératoire sera calculé et présenté sous forme de proportion dans son intervalle de confiance à 95% (Wald avec correction de continuité). Cette estimation sera réalisée à l'aide d'un modèle de régression logistique mixte afin de prendre en compte les données des ganglions corrélés pour un même patient. En deuxième lieu seront présentés différents taux, comme par exemple la sensibilité de l'imagerie étudiée ou encore le taux de patients pour lesquels des techniques complémentaires ont été employées. Une analyse univariée ou multivariée des temps opératoires ainsi que des coûts engendrés pourra également être réalisée. Les analyses statistiques seront réalisées à l'aide du logiciel R dans sa version la plus récente, munie de tous les packages additionnels nécessaires aux analyses.
Nombre de cas prévisionnel	45 patients
Planning de l'étude	Durée totale de l'étude : 30 mois Durée individuelle de participation à l'étude : 7 mois Période d'inclusion : 24 mois

RESUME DE THESE

Les tumeurs de la sphère hépato-bilio-pancréatique (HBP) sont une cause majeure de morbidité et de mortalité dans le monde, ils représentent un problème socio-économique majeur. Le carcinome hépatocellulaire (CHC) représente environ 80 % de tous les cancers hepatique et constitue la troisième cause de décès par cancer dans le monde. Cette tumeur qui provient des cellules hépatocytaires présente un pronostic sombre avec un taux de survie relatif à 5 ans d'environ 20 %. Même dans le cas d'une maladie résécable soumise à un traitement chirurgical, les résultats ne diffèrent pas de manière significative et le taux de récidive reste élevé, atteignant 70 à 80 % après 5 ans. En termes de fréquence, la deuxième tumeur hépatique primaire la plus fréquente est le cholangiocarcinome intrahépatique (CCI), avec une incidence et une mortalité mondiale en augmentation ces dernières années. Le taux de survie à 5 ans est estimé à environ 9-11%, avec un écart important en fonction du stade et du traitement proposé au moment du diagnostic. La chirurgie, suivie d'une thérapie adjuvante à base de fluoropyrimidine ou de gemcitabine, est la seule option curative disponible en cas de maladie non métastatique et résécable. Malheureusement, la récurrence de l'ICC survient chez près de la moitié des patients réséqués dans un délai d'un an. Les résultats ne sont pas plus optimistes en ce qui concerne le cancer du pancréas, où le type histologique le plus fréquent est l'adénocarcinome excreto-canalaire pancréatique (PDAC). Le PDAC devrait devenir la deuxième cause de mortalité liée au cancer en 2030. Il s'agit d'une maladie agressive, avec un taux de survie global à 5 ans inférieur à 10 %, et la résection chirurgicale représente le facteur pronostique le plus important associé à la survie à long terme.

La prise en charge des cancers primitifs du foie et du pancréas fait appel à des stratégies le plus souvent complexe et parfois discutables à cause des comorbidités sous-jacentes, d'une agressivité tumorale non négligeable et de l'absence de démonstration d'efficacité des traitements néoadjuvant en cas de maladie résécable. Dans les cancers primitifs du foie,

contrairement aux évolutions ces dernières années dans la prise en charge des métastases hépatiques des cancers colorectaux, il n'a pas été démontré l'intérêt d'un traitement néoadjuvant, ni parfois adjuvant, à la résection chirurgicale. La résection chirurgicale, qui représente toujours la seule option curative, n'est pas toujours faisable malgré l'absence de localisations métastatiques. De plus, en cas de résection, les facteurs biologiques de réponse à un éventuel traitement adjuvant sont peu connus. La chimiothérapie adjuvante a été par contre largement validée dans le PDAC, indépendamment du stade et des différents facteurs pronostiques. Cependant, l'utilité d'une chimiothérapie d'induction est largement débattue, principalement en raison du risque de progression dû à la non-réponse au traitement. Les résultats préliminaires de certains essais en cours ne démontrent pas l'efficacité d'un traitement systémique préopératoire en cas de maladie résécable dans une analyse en « intention de traiter ». Des critères anatomiques sont actuellement utilisés pour classer la maladie pancréatique comme résécable ou avancée, mais ils ne reflètent pas l'agressivité et la biologie de la tumeur et ils sont assez variables en fonction du centre et de l'expertise technique du chirurgien. En dehors d'une élévation significative du marqueur tumoral CA 19-9, aucun autre critère ne permet de prédire les patients chez qui la chirurgie n'apporterait aucun bénéfice.

Face au diagnostic de tumeur primitive HBP, la meilleure stratégie est choisie en fonction de l'hépatopathie et des comorbidités sous-jacentes, de la biologie de la tumeur, de son extension locale ou à distance et de la possibilité d'envisager une résection chirurgicale. La collaboration étroite entre chercheurs, chirurgiens, gastroentérologues, radiologues et oncologues est indispensable tant dans le contexte clinique que dans la proposition d'une recherche transversale multidisciplinaire.

Le projet de ma thèse est basé sur ce concept de multidisciplinarité, et donc sur la collaboration entre les différents spécialités, indispensable dans un centre clinique et de recherche HPB de haut niveau. Le projet s'articule en trois grandes parties, chacune inhérente à l'une des trois

tumeurs primaires HPB. Ces parties seront unies par un fil conducteur, qui est l'amélioration de la résultats oncologiques de ces cancers grâce à la prise en charge multidisciplinaire, diffèrent par thématique, type de collaboration et méthodologie de travail, afin d'explorer au mieux ce concept de multidisciplinarité. Il a été réalisé en collaboration étroite avec l'IHU - Institut de chirurgie guidée par l'image de Strasbourg, le Pôle Hépato-digestif du Nouvel Hôpital Civil des Hôpitaux Universitaires de Strasbourg et l'Unité INSERM UMR_S1110. Les objectifs primaires sont i) d'évaluer la place des traitements peropératoires pour la prise en charge des cancers primitifs du foie et du pancréas et l'évaluation de leur réponse par l'analyse des données de différentes modalités d'imagerie pré- et per-opératoire ii) de déterminer des critères prédictifs de réponse aux différents traitements sur la base des données cliniques, histopathologiques ainsi que du terrain génétique de la tumeur et, également, iii) de développer de nouvelles stratégies de lecture des certaines techniques d'imagerie peropératoire, incluant des technologies d'automatisation par intelligence artificielle, qui pourront modifier la prise en charge de ces tumeurs.

Ce travail a donné lieu à deux articles originaux signés en premier auteur, résumés ci-dessous, à deux études prospectives actuellement en cours d'inclusion ainsi que plusieurs articles cliniques et de recherche translationnelle dont je suis auteur principal ou co-auteur.

1. ARTICLES ORIGINAUX

1.1 Place de la chimioembolisation transartérielle comme traitement neoadjuvant dans le carcinome hépatocellulaire (CHC) de grande taille

Background

Le premier chapitre de la thèse concerne le CHC. Au vu des chiffres exposés plus haut, qui témoignent de l'agressivité de la maladie et de l'importance d'un traitement pér-opératoire (qui n'existe pas à ce jour), il est nécessaire de trouver des alternatives viables au traitement systémique. Dans ce contexte un rôle important pourrait être joué par la chimbioembolisation

transartérielle lipiodolé (CEL), un traitement radiologique qui injecte un agent chimiothérapeutique après canulation de l'artère hépatique. Depuis plus de trente ans ce traitement a été utilisé en association avec la chirurgie, en pré- et post-opératoire, principalement dans les tumeurs de grande taille et dans les pays de l'Est. Les grands CHC, c'est-à-dire les lésions dont le diamètre est égal ou supérieur à 5 cm, représentent un véritable défi dans ce contexte. Bien qu'appartenant au stade précoce de la classification Barcellona Clinic Liver Cancer (BCLC) en cas de localisation unique, et donc potentiellement soignable, ces tumeurs présentent un mauvais pronostic par rapport aux lésions plus petites. Lorsque cela est possible, la résection chirurgicale a largement démontré qu'elle améliorait les résultats à long terme chez ces patients, mais le risque de récidive reste élevé. La CEL a été utilisée chez ces patients dans le but de réduire la taille de la tumeur, d'éviter la progression en attendant la chirurgie et d'augmenter le taux de résection R0. Cependant, les résultats sont loin d'être exhaustifs, avec des conclusions contradictoires et une difficulté à trouver les cas qui pourraient réellement bénéficier de cette procédure. Une vaste méta-analyse a révélé que la CEL néoadjuvante n'augmentait pas les taux de survie sans maladie (SSM) et de survie globale (SG), mais des résultats favorables ont été trouvés en évaluant exclusivement les patients cirrhotiques. De même, une cohorte multicentrique a récemment montré une amélioration des résultats oncologiques en pratiquant cette procédure avant la chirurgie dans le cas d'un énorme CHC (□10 cm). D'autres questions non résolues découlent de la robustesse statistique de ces études, avec un biais de sélection possible, et surtout, le fait que presque toutes ces séries proviennent de centres asiatiques, qui présentent une étiologie sous-jacente différente, comme la cirrhose virale, ainsi que des voies génétiques altérées distinctes.

Objectifs

Mon objectif était d'explorer le rôle pronostique de ce type de traitement chez les patients atteints d'un CHC résécable de grande taille en créant une vaste base de données multicentrique. L'analyse de survie en termes de SG et SSM a été réalisée.

Méthodes

La collaboration avec 6 centres italiens et français, à Milan, Rome, Paris, Reims, Tours et Montpellier, a permis de collecter une collection d'environ 400 patients atteints d'un CHC de grande taille et dont la maladie était résécable au moment du diagnostic. Les patients ont été divisés en deux groupes selon le type de stratégie : ceux qui ont été réséqués d'emblée et ceux chez qui le chirurgien a indiqué un traitement par CEL avant l'opération. Au vu de la nature rétrospective de l'étude et afin d'équilibrer les deux groupes et de réduire autant que possible les biais, l'analyse de survie a été réalisée avant et après un appariement par score de propension (PSM).

Résultats

Après la collecte des données, un total de 384 patients ayant subi une résection pour un CHC de 5 cm et respectant tous les critères d'inclusion et d'exclusion ont été inclus dans la cohorte finale. Parmi eux, 324 (84,4 %) ont subi une intervention chirurgicale d'emblée, tandis que 60 (15,6 %) avaient déjà été traités par TACE. Les deux groupes étaient extrêmement hétérogènes en termes de caractéristiques initiales, opératoires et histologiques. Toutes les variables significatives ont été utilisées pour le modèle statistique PSM. La nouvelle cohorte comprenait 180 patients, dont 120 (66.7 %) ont subi une chirurgie d'emblée. Le groupe CEL ne différait pas des cas réséqués d'emblée, ni en termes de survie sans maladie (p= 0.246), ni en termes de survie globale (p= 0.276). Après le PSM, la CEL n'a toujours pas influencé les résultats à long terme (p= 0.935 et p= 0.172, pour la survie sans maladie et la survie globale respectivement). Une comparaison des résultats pronostiques a ensuite été effectuée dans des sous-groupes spécifiques de patients afin d'évaluer un bénéfice potentiel de la CEL préopératoire dans

certaines situations telles qu'un futur foie restant (FFR) insuffisant ou une cirrhose sous-jacente. Dans l'analyse des sous-groupes, la CEL a amélioré la SGS uniquement dans le CHC □ 10 cm (p= 0.045), avec une signification limite après l'embolisation/ligation de la veine porte (p= 0.087) et dans le CHC unique (p= 0.052).

Conclusions

La CEL est une technique sûre et bien tolérée qui n'augmente pas le risque de morbidité et de mortalité après une résection hépatique. Cependant, nos résultats ne soutiennent pas l'utilisation indiscriminée de cette procédure chez tous les patients atteints d'un CHC de grande taille pour lesquels la résection chirurgicale est validée. Des cas sélectionnés pourraient bénéficier d'une CEL néoadjuvante, comme les patients avec une tumeur unique et □ 10 cm ou ceux avec un FFR insuffisante nécessitant une embolisation.

Ce travail est soumis à publication dans le Journal HPB et il est actuellement en cours de révision.

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1.2 Influence du contexte génétique dans le prognostic des CCI

Background

Le deuxième chapitre de ma thèse concerne le cholangiocarcinome intrahépatique (CCI). Nous avons voulu explorer la question du pronostic de cette tumeur sous un angle différent, et notamment comment le contexte génétique peut influencer la survie chez ces patients. L'évaluation du contexte mutationnel du cholangiocarcinome peut être utile pour deux raisons.

La première, partiellement explorée dans la littérature, est liée à l'agressivité en soi de la maladie. En effet, certaines séries rapportent que des mutations spécifiques sont associées à des tumeurs plus agressives et d'autres à un pronostic plus favorable. Ce concept se retrouve principalement dans la littérature pour les patients métastatiques ou localement avancés pour lesquels il existe différentes lignes thérapeutiques et pour lesquels, entre autres, il n'existe pas de traitement curatif. Chez les patients chirurgicaux, en revanche, cette association est peu explorée et se retrouve principalement dans de petites séries, explorée plutôt d'un point de vue moléculaire. La deuxième raison est l'autorisation récente de certaines thérapies ciblées en deuxième ligne. Cependant, cette option est réservée exclusivement aux patients non résécables, alors que les patients chirurgicaux ne peuvent bénéficier que d'un traitement adjuvant par capécitabine, une stratégie inchangée depuis plus de 10 ans. En collaboration avec l'Inserm, et grâce à leurs connaissances dans le domaine biologique et moléculaire, nous avons réalisé une revue systématique de la littérature et une méta-analyse afin de collecter une large collection de CCI et d'analyser la prévalence des mutations les plus fréquentes ainsi que leur impact sur l'évolution oncologique du patient. Cela permettrait de créer des parcours ciblés pour les patients en fonction de leur profil mutationnel et, en même temps, de jeter les bases d'une éventuelle extension des thérapies ciblées aux patients chirurgicaux.

Objectif

La revue systématique vise à résumer les connaissances actuelles sur le statut mutationnel des CCI réséqués et leur signification pronostique. J’ai réalisé une méta-analyse pour évaluer le risque de récidive et de décès chez ces patients en fonction de leur bagage génétique. Parallèlement, la prévalence ainsi que les différences clinico-pathologiques des CCI mutés ont été étudiées

Méthodes

Une recherche systématique de la littérature a été effectuée pour tous les articles publiés conformément aux lignes directrices PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses). Seules les études rapportant des données de survie et/ou de récidive de CCI traités avec une intention curative et dans lesquelles un profil génétique a été réalisé sur le spécimen principal ont été incluses dans l'ensemble de la méta-analyse. Les critères d'exclusion étaient les suivants : résections non curatives, études précliniques et absence de données de suivi pour la cohorte de type muté/sauvage. Dans notre méta-analyse, l'impact de chaque mutation génétique sur la survie et la récidive des patients a été évalué. En même temps, la prévalence de ces mutations ainsi que l'association avec des caractéristiques clinico-pathologiques bien définies ont été évaluées.

Résultats

Vingt-quatre articles répondaient aux critères d'inclusion et ont été examinés dans le cadre de cette étude. Pour ce qui concerne la prévalence, la mutation la plus explorée était le statut KRAS, avec 2399 patients examinés, suivie par la mutation IDH1/2, avec 1632 échantillons. Pour ces deux gènes, la prévalence estimée était respectivement de 14,8 % et 13,6 %, ce qui fait de ces mutations génétiques deux des plus courantes. Avec une proportion de 16,3 % de mutations parmi les 983 patients examinés, TP53 a présenté la prévalence la plus élevée dans cette étude. Une analyse de sous-groupe en fonction de la répartition géographique des études a révélé une différence statistique entre les séries occidentales et orientales pour KRAS ($p < 0.001$) et IDH1/2 ($p < 0.001$), mais pas pour les autres. KRAS, IDH1/2 et TP53 ont été identifiés comme les trois seuls gènes dont le statut est significativement corrélé au risque de récidive et de décès (SG: $p < 0,001$ pour les trois gènes; SSM: $p < 0,001$ pour KRAS et IDH1/2, $p = 0,003$ pour TP53). Seules les mutations IDH1/2 étaient associées à un pronostic favorable.

Conclusions

La présente méta-analyse rapporte le plus grand ensemble de données concernant l'impact du paysage mutationnel sur les résultats à long terme dans les CCI réséqués. En outre, l'étude de la prévalence globale a révélé les mutations actionnables et non actionnables les plus courantes chez ces patients. Ces connaissances seront utiles pour élargir les indications des thérapies ciblées dans le cadre du traitement adjuvant.

Ce travail est soumis à publication dans le Journal of Hepatology Reports et est actuellement en cours de révision.

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2. ETUDES PROSPECTIVES

Deux études prospectives sont actuellement en cours de recrutement dans le cadre de mon projet de thèse et dans lesquels je suis co-investigateur principal.

2.1 Le premier, CancerProfile (NCT03997617), est un projet de recherche translationnelle multidisciplinaire visant à améliorer le diagnostic en temps réel et la prédiction ultérieure de la réponse de la tumeur aux traitements, en utilisant l'imagerie histologique et le profilage fonctionnel augmenté par l'Intelligence Artificielle de pointe, le tout afin de favoriser la médecine de précision de la PDAC. Le projet jettera les bases d'une vaste étude clinique visant à évaluer la mise en œuvre d'un traitement personnalisé pour les patients atteints de PDAC. Il conduira à l'établissement d'une biobanque et d'une base de données uniques, qui pourront servir de modèle à d'autres projets européens de recherche fondamentale et translationnelle. Cette étude sera réalisée en collaboration entre l'IHU, le Pôle Hépato-digestif du Nouvel Hôpital Civil des Hôpitaux Universitaires de Strasbourg et le Luxembourg Institute of Health (LIH). En pratique, du tissu tumoral est prélevé lors de la résection pancréatique pour PDAC et analysé

par tomographie en cohérence optique à plein champ (D-FF-OCT), une nouvelle méthode d'imagerie périopératoire peu explorée en pathologie pancréatique et principalement lors des procédures endoscopiques. Cette analyse visera à analyser les caractéristiques morphologiques et métaboliques des cellules tumorales, qui seront ensuite comparées à la résistance à la chimiothérapie ainsi qu'au pronostic de ces patients. Dans le même temps, du tissu tumoral sera envoyé au LIH pour développer des technologies permettant de créer des organoïdes sur lesquels tester différents médicaments.

2.2 Le deuxième, EchoSurg (NCT : 04899739), est une étude prospective non randomisée ayant pour objectif d'évaluer la précision de l'échoendoscopie diagnostique préopératoire dans le diagnostic des lésions ganglionnaires métastasiques occultes du cancer du pancréas opérable. En effet, les critères de résécabilité de cette tumeur sont actuellement basés sur le contact avec les vaisseaux péripancréatiques, un critère largement subjectif, et non sur des critères biologiques ou d'agressivité de la maladie. Le facteur pronostique pathologique le plus important, par exemple, l'envahissement ganglionnaire, n'est pas pris en compte, principalement en raison de l'incapacité des méthodes préopératoires à évaluer la présence de cellules tumorales dans les différentes stations ganglionnaires. En collaboration avec l'IHU nous avons donc mis en place une étude prospective qui a comme objectif principal l'estimation de la sensibilité et de la spécificité d'une classification simple « bénin/malin » des ganglions, établie par l'endoscopiste, par rapport au « gold standard » (anatomopathologie). Les patients bénéficieront immédiatement avant la chirurgie pancréatique pour PDAC d'une échoendoscopie qui analysera toutes les stations lymphatiques drainant normalement cet organe, et les données seront comparées au résultat anatomopathologique.

Direction de la **recherche**
et de la **valorisation**
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AVIS DU JURY

SUR LA DIFFUSION

DE LA THÈSE

TITRE DE LA THÈSE : **Prise en charge multidisciplinaires pour améliorer les résultats à court et à long terme dans les cancers hépato-bilio-pancréatiques**

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Date de la Soutenance : **22/02/2024**

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Signature du Président du Jury