Title: Advanced immune gene and cell therapies for liver metastases

Acronimus: LiMeT

Version 7.0
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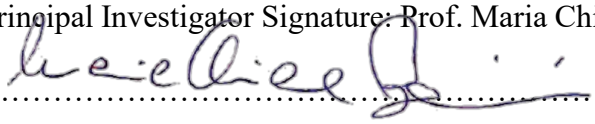
Principal Investigator Signature: Prof. Maria Chiara Bonini



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Note: This version (Protocol v7.0 – LiMeT) integrates the FRRB (Fondazione Regionale per la Ricerca Biomedica) 2024-funded Substudy reported in paragraph 10.

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6. Synopsis

Colorectal (CRC) and pancreatic ductal (PDAC) adenocarcinomas are the second and fourth most common cause of cancer death, respectively. Patients affected by these cancers die of liver metastases (MTS), which therefore represent the major unmet clinical need for these malignancies.
Based on preliminary data and published observations from our and other groups, we have hypothesized that innovative immune gene and cell therapy approaches might overcome the tolerogenic liver microenvironment and represent powerful therapeutic tools for CRC- and PDAC-derived liver MTS.
The prerequisite to develop new advanced therapy medicinal products (ATMP) is the fine profiling of the immune and non-immune liver microenvironment of liver metastases and the identification of tumor associated antigens (TAAs) and local immune suppressive and regulatory pathways.
The development, validation and implementation of clinical testing with innovative immunotherapeutic ATMPs for liver MTS of PDAC and CRC is the goal of the 7-year AIRC5x1000 translational program "Advanced immune gene and cell therapies for liver metastases" awarded to Prof. Maria Chiara Bonini by AIRC funding agency from 2019 to 2026. The proposal is built on 4 highly integrated programs, conceived in hypothesis generating and hypothesis testing activities, the first of which (Program 1) is at the basis of the current clinical protocol.
Specifically, Program 1 is a discovery, observational, non-interventional clinical study aiming at the characterization, at high resolution, of the immune infiltrate and cancer cell profiles in different types of samples harvested from patients with liver MTS from CRC and PDAC.
This program will allow also to collect primary tumor/blood samples of patients who undergo primary tumor surgery, to follow longitudinally these patients and to collect subsequent metachronous liver metastases/blood samples. To reach Program 1 outcomes, shared state-of-the-art -omics, including at single-cell level, flow cytometry and histological platforms will be used to identify TAAs and local immune suppressive and regulatory pathways.
Experimental results obtained from biological samples will be correlated: i) with clinical data from patients at the time of enrollment and along their follow-up; and ii) for a subgroup of patients, with biochemical and immunological data from peripheral blood samples routinely collected as a part of their clinical follow-up. This might lead to the identification of systemic parameters able to discriminate differential disease evolution, stratify patients and better tailor their treatments, including future therapies with the ATMPs developed by the AIRC5x1000 program.
In this regard, the TAAs and suppressive pathways identified by Program 1 studies will be then targeted through state-of-the-art cell therapies and gene transfer/editing tools, assessed on suitable mouse models and *in vitro* cultures of patient-derived samples, by the other 2 translational programs: program 2 will focus on retargeting T cells against cancer cells, while ensuring resistance to the immunosuppressive tumor microenvironment (TME); program 3 will focus on enhancing both spontaneous and exogenous immune responses against the tumor. Finally, Program 4 will prioritize the best performing candidate ATMPs, independently developed by Program 2 and 3, for further preclinical development and, eventually, file an application for the first-in-human testing of the selected ATMPs.
It is expected that the results from Program 1 activities and, as a whole, of this AIRC5x1000 project may impact not only the management and cure of CRC and PDAC patients, but also of other solid tumors.

7. Background and rationale

CRC and PDAC are the second and fourth most common cause of cancer death, respectively. Patients affected by these cancers die of liver metastases [1]. Conventional therapies active in either primary tumors fail in metastatic diseases. Immunotherapy by immune checkpoint blockade proved effective only in a minority (≤5% of patients) of metastatic CRCs and PDACs [2]. Hence, there is pressing need for more efficacious therapeutic approaches. Adoptive cell therapy (ACT) with tumor-specific T cells has proven successful in controlling, at least temporarily, disseminated metastatic tumors also of epithelial origin [3]. However, ACT efficacy requires to be further enhanced by identifying and counteracting local immunosuppressive mechanisms [3]. This is particularly relevant for hepatic MTS, in which a natural tolerogenic milieu may further sustain immunosuppression induced by cancer cells and infiltrating immune cells, such as tumor associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), neutrophils, and regulatory T or B cells [4].

We hypothesize that a combination of: *i)* direct cancer cell killing by adoptively transferred tumor-specific T cells with *ii)* indirect control of tumor progression via reprogramming of the tumor microenvironment, could control hepatic metastases of CRC and PDAC. However, whereas primary PDAC and CRC are well-characterized cancer types exhibiting distinct molecular, cellular and clinical behavior, their metastatic microenvironments remain poorly defined. Therefore, we propose a thorough characterization of the metastatic milieu in these two cancers types to rationally shape, on the basis of similarities and differences, innovative immune and gene therapy approaches.
Such characterization will be carried out by the dedicated observational, non-interventional clinical study on CRC and PDAC patients described herein, corresponding to the discovery Program 1 of the 4 Programs of a translational proposal proposed by Prof. Bonini and funded by AIRC5x1000 grant.
Program 1 aims at unraveling, at high resolution, the immune infiltrate and cancer cell profiles in liver MTS from CRC and PDAC, to identify TAAs and immune suppressive and regulatory pathways to be targeted in these two diseases. Results of this discovery program will progressively shape two independents but potentially combinatorial translational Programs relying on mouse models: Program 2 will aim at harnessing T cells with genetic tools that allow their retargeting against cancer cells, while ensuring resistance to the immunosuppressive TME; Program 3 will modify the immunosuppressive TME through novel targeted in vivo gene therapies, aimed at enhancing spontaneous innate and adaptive immunity against the tumor, as well as the efficacy of exogenous T cell responses. Each candidate ATMP will be stringently assessed for efficacy and toxicity in suitable experimental models and *in vitro* cultures of patient-derived samples and the best performing one prioritized for further preclinical development according to the activities outlined in Program 4 and translated into early phase clinical trials to be launched towards the end of the funding period. Meanwhile, the monitoring of the clinical evolution of patients enrolled in the current observational study, including both the retrieval of clinical data and, for some patients, the study of peripheral blood samples along their routine clinical follow-up, is expected to point at novel biologic parameters and/or signatures to help stratify patients and better tailor their treatments, including future therapies with the ATMPs developed by the AIRC5x1000 program.

8. Objectives

This clinical protocol aims at deeply characterizing MTS tumor cells and their microenvironment, taking advantage of possibly informative dynamic modifications from pro-tumor to anti-tumor profiles that may follow chemotherapy [5], which is administered pre-surgery (neo-adjuvant) to substantial fractions of patients. In addition, it pursues a parallel characterization of hepatic MTS compared to matched/not matched, previously collected, primary not metastatic tissues, in order to highlight dynamic qualitative modifications in cancer cells, immune infiltrates and not-immune microenvironment that could facilitate disease progression toward metastatic spread. Evidence suggests that progression of primary or metastatic CRC correlates with poor immune infiltration and inflammation [6][7], while our published results in primary PDAC support a correlation between tumor progression and a strong type 2 anti-inflammatory/immunosuppressive signature [8]. In addition, efficacy of chemotherapy relies also on reactivation and/or repolarization of tumor targeting immune responses [9]. We hypothesize that the pre-surgical chemotherapy applied to CRC or PDAC MTS may induce beneficial effects and potentially targetable changes in the immune profile through activation of de-novo immune responses and/or repolarization of tumor-promoting Th2/M2 inflammation. In turn, these modifications may offer a window of opportunity for the optimal application of our advanced therapy medicinal products (ATMPs). Moreover, through the systematic and long-term follow-up of enrolled patients, we aim at identifying early predictors of differential prognosis and patients' categories eligible for tailored therapies.

We will define:

a) *the tumor mutational burden and composition of the metastatic TME in the liver;*
b) *the inhibitory pathways that constrain immunity in liver MTS;*
c) *the antigenic and clonal breadth of anti-tumor T cell responses;*
d) *the spatial-temporal variations of effector and regulatory immune cells upon therapy.*

We will also:

e) compare tumor cells and their microenvironment in liver MTS versus primary tumor (longitudinal study of primary PDAC-cohort and comparative analyses on paired primary and MTS tumor samples from metastatic CRC patients undergoing synchronous surgical resections);
f) monitor longitudinal variations of clinical parameters as well as blood-derived biological and immunological markers during the long-term medical follow-up of patients;
g) collect and biobank patients'samples to support the validation and selection of newly-developed ATMPs.

Our primary objectives will be:

1. to identify immune suppressive/exhaustion pathways to be harnessed in Program 2 and 3.
2. to develop a library of tumor-specific TCR (N≥5/tumor type) and identify new CAR targets (N≥1/tumor type) in CRC and PDAC MTS.

4. Study Design and Justification of Methods

This is a monocentric prospective observational study aimed at revealing the relationships between cancer cell genetics and the dynamic and functional profile of the immune infiltrate in liver metastatic lesions of PDAC and CRC patients. To this purpose, liver MTS will be characterized by either cross-sectional or longitudinal investigation on chemotherapy naïve vs treated patients. Moreover, a subgroup of PDAC MTS samples will be also characterized and compared to their matched/not matched, previously collected, not metastatic primary PDAC samples. Also, in CRC MTS patients undergoing synchronous resection of primary CRC and hepatic metastases, comparative analyses will be carried out on both types of tumor material, allowing intra- and inter-patient profiling of both tumor niches. Analyses will be carried out on different biological samples, as better detailed below, and correlated with clinical data collected from all patients since the time of enrollment for a 2-year follow-up period, in case of PDAC patients, and for 3-year follow-up period, in case of CRC patients. Finally, for metastatic CRC patients, the 3-year clinical follow-up will be associated with concurrent collection of blood samples at serial timepoints, that will be prospectively biobanked for further analyses aimed at: i) studying biological and immunological correlates of the patient's clinical status; ii) evaluating key discriminants previously identified in blood samples analyzed at the time of liver MTS surgery, to serve as early predictors of liver progression or recurrence and possibly as indicators for therapeutic stratification.

From the time to protocol approval until the end of June 2026 we expect to enroll at least 200 patients with CRC and liver metastases, 95 PDAC MTS patients (either synchronous or metachronous) and 30 healthy donors.

As anticipated, a cohort of primary PDAC tumors, candidates for surgical resection with radical intent of the primary tumor (upfront surgery or after neoadjuvant therapy) will be also enrolled in this study and followed-up for monitoring the development of metachronous liver metastases.

Based on previous experience, the rate of surgical resection of resectable PDAC at San Raffaele Hospital (OSR), we estimate that at least 150 localized resectable PDAC patients will be enrolled upon approval of V2.0 amendment of this clinical study (end of October 2020) and until the end of June 2026 and their samples will be collected for further research analysis (as discussed below).
By analyzing 305 PDAC patients who underwent pancreatic resection (upfront surgery or after neoadjuvant therapy) at the Division of Pancreatic Surgery of IRCCS San Raffaele Hospital between 2015 and 2017, 24% and 32% of these patients developed liver relapse within 1 year and 2 years after surgery, respectively. Based on these preliminary data, and considering an indicative number of 100 patients with resected PDAC/year, we can expect about 40-60 patients with liver relapse in about 1-2 years follow-up period.
Considering that some of these patients will not undergo percutaneous liver biopsy because of their poor performance status after relapse or because of a technical difficulty to perform the biopsy (i.e. presence of small metastases not amenable of percutaneous biopsy), we estimate a minimum enrollment number of about 20 patients with liver relapse, from whom blood and, when possible, tissue samples will be collected.

Unlike CRC, the genomic and transcriptional features of PDAC progression from paired (i.e., in the same patient) primary to metastatic lesions has not been studied in large series (Trevidi N. et al, 2018).
The inclusion of the primary PDAC cohort is therefore an important opportunity to harvest primary not metastatic PDAC samples for future comparison with subsequent liver metastases developed by the same patient. This study will allow to longitudinally characterize the differences between paired tumor tissues deriving from the same patient at different timepoints of their clinical history in order to have comparable snapshot pictures of their tumor profile and immune and not-immune microenvironment at different stages of their disease progression (not metastatic primary PDAC versus PDAC hepatic MTS).
Moreover, the tissue/blood samples of primary PDAC samples from:

i) patients that will not develop distant metastasis detected through the described clinical/radiological follow-up testing and
ii) patients that will develop a distant relapse at sites other than the liver

could be a reference control to characterize differences in tumor profile and its immune and not-immune microenvironment compared to patients that will instead experience a tumor relapse at liver.

As anticipated above, patients will be studied (including sample collection and clinical data collection) from the time of enrollment in the study and during their clinical follow-up, whenever possible, for up to 2 years, considering PDAC patients, and 3 years, considering CRC.

The statistical analysis of the collected clinical and experimental data will be carried out all along the duration of the clinical study, starting 6 months after the enrollment of the first patient until the end of patients' follow-up, for a total of 7 years. The study will last 7 years.

The objectives of the clinical study, as better detailed in the AIRC5x1000 grant proposal, will be pursued through activities organized in 6 Work Packages (WPs):

WP0. Collection and biobanking of the material from patients with CRC and PDAC MTS to the liver and of primary not metastatic resectable PDAC;
WP1. Definition of tumor mutational burden, epigenetic and gene expression profile of the CRC and PDAC metastatic liver at bulk and at single cell level;
WP2. Evaluation of the molecular and cellular composition of CRC and, if possible, PDAC liver MTS by spatial transcriptomics technologies (NICHE-seq and Visium);
WP3. Characterization of the immune landscapes of CRC and, if possible, PDAC liver MTS by high dimensional flow cytometry
WP4. Validation of the molecular results obtained in WP1-3
WP5. Definition of the antigenic landscape and TCR repertoire of CRC and PDAC liver MTS

The results obtained by WP1-5 research studies will be crossed with clinical outcome data from clinicians to provide an in-depth characterization of the distinct tumor metastatic niches in treated and untreated lesions and will be instrumental to calibrate the activities of Programs 2 and 3 towards establishing cancer regression.

WP0. Collection and biobanking of the material from patients with CRC and PDAC MTS to the liver

In this protocol we expect to enroll at least 200 CRC MTS patients, 95 PDAC MTS patients, either synchronous (N=75) or metachronous (N=20), 150 primary not metastatic PDAC patients and 30 healthy volunteers. We will collect samples and clinical data from the different cohorts, as detailed below.

Tissue samples

- ***CRC***

Surgery is the only potentially curative treatment for patients with CRC liver MTS and is proposed, as far as surgical resection is technically feasible, either as an upfront treatment or after a course of systemic chemotherapy. Based on incidence and clinical management of the disease at Ospedale San Raffaele, we expect to analyze at least 200 CRC liver metastases (either metachronous or synchronous), of which: ~70%

(N=140) from patients receiving pre-surgical chemotherapy, ~30% (N=60) from naïve patients that received no treatment prior to sampling. In addition to cross sectionally compare pre-treated and naïve patients, we propose to study longitudinally patients undergoing pre-surgical chemotherapy (borderline-resectable) by sampling their metastatic tumor tissue by tumor biopsies preceding the pre-surgical chemotherapy and by surgical resection following the chemotherapeutic treatment. Specimens from CRC liver MTS are collected throughout the standard clinical management of patients, which includes surgical resection. Moreover, in those CRC MTS patients undergoing synchronous resection of primary CRC and hepatic metastases (about 5% of liver MTS-resected patients, according to previous experience) tumor samples will be collected from both the primary and the metastatic sites, in order to provide biologic material for paired study of both tumor microenvironments.

- ***PDAC***

Surgical resection is recommended only for localized not metastatic disease (NCCN guidelines for PDAC). The availability of PDAC liver MTS is poor and, in the majority of cases, limited to percutaneous ultrasound-guided fine-needle aspiration, or core biopsies carried out to obtain material for the cytological or histological analysis of these lesions.

-In order to open a further scenario for our endpoint analysis, we plan to enlarge the collection of WP0 samples by including a cohort of patients affected by not metastatic primary PDAC (at least 150 patients). Based on the aforementioned OSR preliminary data and considerations on possible patients drop out at relapse diagnosis, we plan to harvest longitudinally blood and, whenever possible, metastatic tissue from at least N=20 patients from whom primary PDAC tissue/blood samples have been previously collected after pancreatic resection carried out at our Institution.

We propose to collect the following samples:

1) Samples from synchronous hepatic PDAC MTS will be obtained from the following cohorts of patients by the indicated procedures:

Cohort A:
a) percutaneous ultrasound-guided fine-needle core biopsies of liver MTS in patients with metastatic PDAC at imaging;

Cohort B:
b) Liver MTS resections performed during laparotomy/laparoscopy in patients with radiological resectable PDAC scheduled for pancreatic resection, but with incidental detection of liver metastases intraoperatively
c) Liver MTS resections performed during laparotomy in patients undergoing palliative surgery;
d) Liver metastasectomy in stage IV PDAC patients undergoing chemotherapy followed by pancreatic cancer and liver metastasis resection.

2) Samples from primary PDAC from a cohort of resectable not metastatic patients (Cohort C). These patients will be monitored for early diagnosis of hepatic PDAC MTS by follow-up testing. For those in whom we will be able to detect a hepatic recurrence at imaging, the metastatic tissue will be collected following procedures described for Cohort A patients.

Based on incidence and clinical management of the disease at Ospedale San Raffaele, we expect to collect and analyze liver metastatic samples from at least 75 PDAC patients with synchronous metastasis, of which ~90% (N=65-70) will be core biopsies from cohort A patients and ~10% (N=5-10) will be liver resections/metastasectomies from cohort B patients.

Peripheral Blood samples

-For both CRC and PDAC MTS, paired peripheral venous blood (20 ml) will be collected in parallel with the collection of surgical samples. Tumor tissue sample collection will be done by surgical oncologists, or by radiologists, while the peripheral blood will be collected by a dedicated research nurse, at Ospedale San

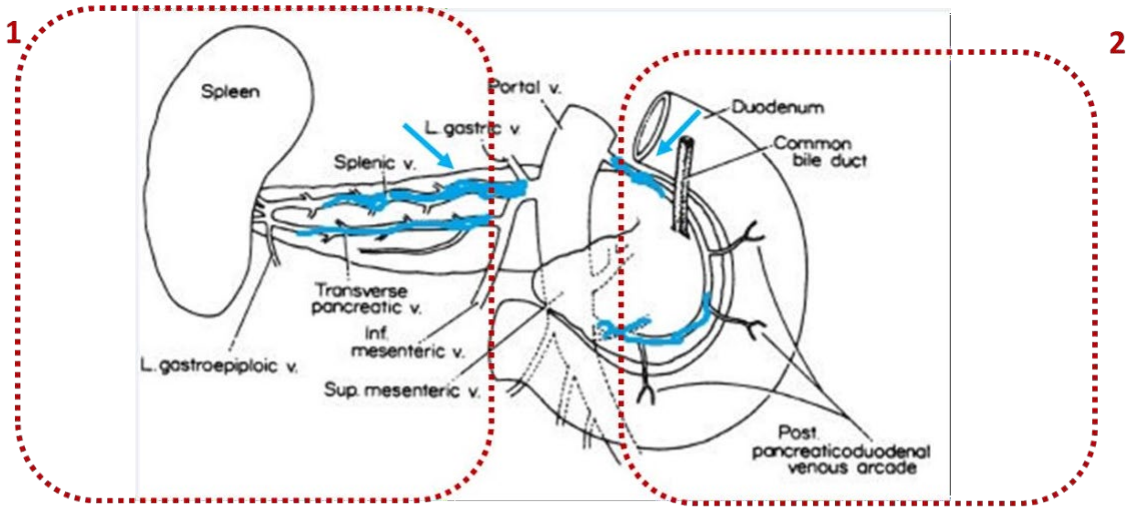
Raffaele during the routine diagnostic or as a part of surgical resection. In addition, after the approval of the 3rd amendment of the protocol, an additional blood sample will be collected, whenever possible, for research purposes from CRC patients undergoing hematochemical testing as part of the follow-up visits, as indicated by standard clinical practice (Colon cancer- NCCN evidence blocks. Version 3-2021), i.e., every 6 months for up to 3 years, and in case of recurrence.

- Moreover, at least 30 healthy donors will be enrolled as negative controls for the analysis of this clinical study. Only peripheral blood (20 ml) will be collected from healthy donor volunteers during blood donation drawings.

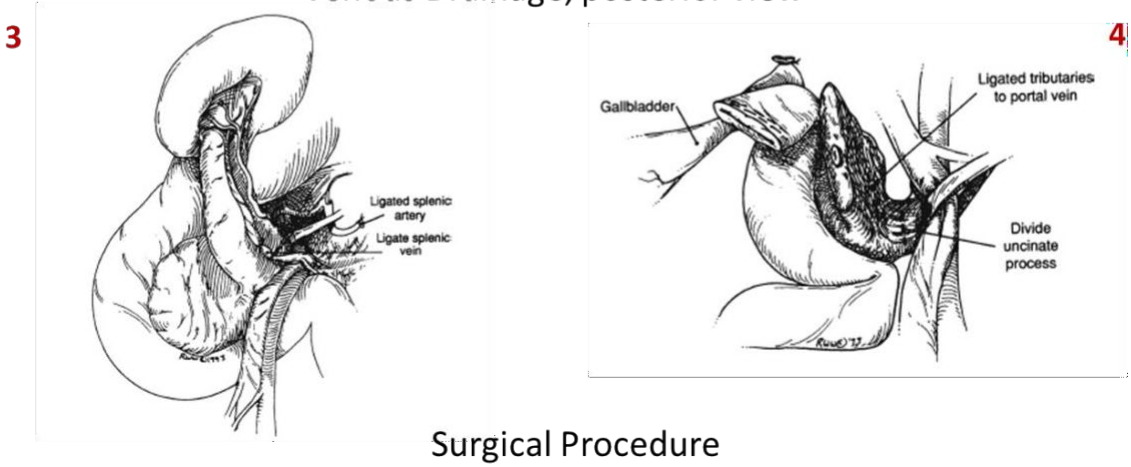
Portal Blood samples

- Portal blood sampling of primary not metastatic PDAC will be performed by collecting blood directly from venous vessels which will be subsequently cut and removed during the standard surgical procedure, but that drain directly into the portal vein.

This procedure will be performed during surgical resection of primary tumor and does not represent an additional risk for the patients. In the case of distal pancreasectomy (figure 1), the sampling is performed on the part of the splenic vein (blue arrow) which is removed with the surgical resected specimen, before splenic vein will be ligated and divided (figure 3). In the case of pancreaticoduodenectomy (figure 2), the collection is performed from the anterior superior pancreaticoduodenal vein branch (blue arrow) which is removed with the surgical specimen, before this vein will be ligated and divided (figure 4). In the case of total pancreatectomy, the collection will be performed with one of these two techniques at the discretion of the surgeon.



Venous Drainage, posterior view



Surgical Procedure

Follow-up and sample collection

- As regards patients who will undergo primary not metastatic PDAC resection, a structured clinical follow-up will be performed according AIOM 2019/NCCN V1.2020 guidelines. The follow-up tests include i) a clinical examination for symptoms assessment (medical history and physical exam), ii) laboratory testing for blood prognostic markers including the measurement of serum cancer-associated antigen (CA19-9) and of carcinoembryonic antigen (CEA) and iii) computed tomographic imaging (with contrast material, when possible) which consists in CT scan of chest and CT scan or MRI of abdomen and pelvis at 3- to 6-month intervals for the first 2 years, and every 6 months or annually thereafter, as needed. This clinical follow-up strategy was associated by different retrospective studies to an increased survival rate (Nordby et al. 2013, Tzeng CW et al. 2012) [10][11]. In particular, early detection of asymptomatic recurrence could allow the patients to benefit from specific oncological treatments (Tzeng CW et al. 2012, Tjaden et al., 2016)[10][12], thus increasing the median post-recurrence survival rate compared to patients with a clinical surveillance activated upon a symptomatic disease evolution (median time to recurrence was comparable between the two groups) (Tzeng et al. 2012)[10].
When follow-up testing will show the presence of liver metastases, patients will be informed about the possibility to adhere to the LiMeT procedures for PDAC Hepatic MTS patients.
Upon obtaining their informed consent, this subgroup of resected PDAC patients, that were longitudinally followed until hepatic metastasis identification, will go through a hepatic tumor core biopsy (as previously described) performed to assess/confirm the metastatic pattern of hepatic lesions (procedure contemplated by NCCN guidelines). Hepatic tumor biopsy will be performed by percutaneous ultrasound-guided fine-needle core biopsies (procedure details described before); moreover, a paired 20 ml peripheral venous blood sample will be collected –as we do for cohort A of patients with synchronous hepatic PDAC MTS at diagnosis.
This procedure will be performed in respect to clinical practice (NCCN guidelines) and according to feasibility criteria based on interventional radiology techniques (i.e., percutaneous biopsy could not be performed in small lesions with size < 1 cm and for this reason CT scan images will be reviewed) and also considering the clinical conditions of the patients (i.e., patients with poor performance status who are not amenable to further chemotherapy treatment but that can only undergo best supportive/palliative care will not be considered for hepatic biopsy). For those patients who will not undergo percutaneous liver biopsy because of their poor performance status after relapse or because of a technical difficulty to perform the biopsy (i.e. presence of small metastases not amenable of percutaneous biopsy), only a peripheral blood sample will be collected.

- As regards patients who will undergo metastatic CRC resection, a structured clinical follow-up will be performed (Colon cancer- NCCN evidence blocks. Version 3-2021), including i) a clinical examination for symptoms assessment (medical history and physical exam), ii) laboratory testing for blood prognostic markers including including the measurement of serum cancer-associated antigen (CA19-9) and of carcinoembryonic antigen (CEA) and iii) computed tomographic imaging and/or MRI, at 3- to 6-month intervals for the first year, and every 6 months for the next 2 years, for a total of 3 years. After the approval of the 3rd amendment of the protocol, at 6 month intervals an additional blood sample will be collected for research purposes from patients during the hematochemical testing performed in the follow-up visits and in case of recurrence. The table below summarizes the described timepoints for clinical tests and biological samples collection during CRC MTS patients' follow-up.

Time point (T)	Timepoint (month)	Clinical follow-up data collection				Biological sample collection	
		Visit	Hematochemical evaluation	Markers (CEA & CA19.9)	CT scan	Peripheral blood	liver MTS
0	Enrollment=liver MTS surgery	face-to-face	+	+	+	+	+
1	1st month (1st year)	face-to-face/telemedicine	+	+	-	-	-
2	3rd month (1st year)	telemedicine	+	+	-	-	-
3	6th month (1st year)	face-to-face	+	+	+	+	-
4	9th month (1st year)	telemedicine	+	+	-	-	-
5	12th month (1st year)	telemedicine	+	+	+	+	-
6	18th month (2nd year)	face-to-face	+	+	+	+	-
7	24th month (2nd year)	face-to-face	+	+	+	+	-
8	30th month (3rd year)	face-to-face	+	+	+	+	-
9	36th month (3rd year)	face-to-face	+	+	+	+	-
10	recurrence (within 3 years)	face-to-face	+	+	+	+	+ (in case of ri-enrollment in LiMeT)

Sample collection and handling

- In relation to tissue sample collection procedures, needle biopsy of focal liver lesions is generally performed percutaneously with an intercostal or subcostal approach. Preemptive local anesthesia is performed with 10 cc of lidocaine hydrochloride injected into the liver capsule, under ultrasound guidance, making the procedure practically painless. Freehand ultrasound guide allows directing the needle by controlling the entire route and avoiding damage to the diaphragm, lung, vascular structures and viscera (gallbladder, colon). Always under ultrasound guidance, the needle's tip is inserted into the focal hepatic lesion with a semi-automatic snap needle of the caliber between 14 and 20 Gauge, depending on the choice of the operator based on the risk of complications in relation to the position and characteristics of the nodule. Ultrasound guidance allows sampling in the central or peripheral portions of the lesion, to obtain a sample as representative as possible: often the center of the lesion is necrotic and the extracted frustum is considered inadequate. In our case where it is necessary to take several samples, it is possible to use a coaxial system by making a single perforation of the hepatic capsule and inserting the biopsy needle inside it as many times as desired. The lashes will be collected in a specific physiological solution (e.g., RNA later or MACS Tissue storage Miltenyi solution, to preserve cell viability and prevent nucleic acid degradation) and sent to the pathologist. Ultrasound performed at the end of sampling allows excluding early signs of complications (peri-hepatic hemorrhage collection). In addition, a blood count check is prescribed after about 2 hours. The patient is followed clinically in day hospital for at least six hours to rule out late bleeding.

- For what concerns the bioptic material, we will always preserve one biopsy for diagnostic purposes, whilst the other biopsy will be rapidly evaluated on site in the intraoperative room at first floor in the surgical block. This will be performed with a "touch imprint" method as described by the American Society of Cytopathology (ASC). Depending on the quality of material we will decide to whom to give the material between the research groups according to priority.

- For what concerns patients with primary not metastatic PDAC, who give their consent to participate in this study, the following procedures will be performed: i) collection of an additional volume of 35 ml peripheral venous blood, which will take place on the same day of surgery, before induction of general anesthesia; ii) withdrawal of a volume of 15 ml of portal blood. This procedure (discussed in details below) will be performed during the surgical removal of the primary tumor and does not represent an additional risk for patients; iii) collection of the pancreatic tumor and peritumoral tissues that will take place within standard and oncologically safe surgical procedures, which involve the surgical removal of pancreatic adenocarcinoma for histological analysis for diagnostic/therapeutic purposes. If possible, a portion of these tissues will be analyzed for research purposes described in the following discussion. These procedures do not involve additional risks for patient health.

- The tissue samples of liver metastasis or PDAC primary tumors, either collected by the surgical oncologists or by radiologists, will be sent as soon as possible to an experienced pathologist that will manage the surgical specimen. Following the operative procedures, the pathologist will open the specimen to evaluate properly tumor size and he/she will decide the amount of tumor tissue to be dedicated to research aims, as the priority will be the histopathological diagnosis, molecular evaluation and tumor staging. If possible, not-neoplastic tissue will be isolated from adjacent not-neoplastic parenchyma removed during surgery resection as part of the standard procedure - together with the tumor tissue- according to clinical practice associated with surgical techniques. Then the specimens will undergo routine histopathological processing.
When part of the sample is not necessary for patients' diagnosis this material will be made accessible for being directly analyzed by the involved Research groups or stored in our institutional Biobank, i.e. the "Centro Risorse Biologiche – Ospedale San Raffaele" (CRB-OSR) for subsequent analysis.

- Regarding surgical samples, when possible, according the aforementioned discussion, pieces of tissues will be transferred to DMEM /F-12 (Dulbecco's modified eagle medium/nutrient mixture F12) GlutaMAX ™ Supplement (Thermo Fisher Scientific) or to a specific physiological solution (e.g., PBS/Tissue storage solution, RNA later or MACS Tissue storage Miltenyi solution), to preserve cell viability and prevent nucleic acid degradation) and distributed to one or more research groups, according to a pre-established priority coordinated by the program manager, either embedded in OCT (O.C.T. Compound 4853 Tissue-Tek), snap frozen, cut in a LEICA CM1950 microtome (at -22°C) for HE staining and pathological evaluation to define the quality of the material and number of neoplastic cells before storing at -80 ˚C in the institutional Biobank. Multiple IHC and IF will be performed on sections derived from paraffin blocks utilized for diagnostic purpose, paying special attention not to exhaust the material. Paraffin blocks will be stored in the archive of the Pathology Unit.

In line with project needs and amount of available material, liver tissues or primary PDAC or CRC tissues will be collected for:

1) formalin-fixation and paraffin embedding (diagnostic and research purpose)
2) fresh use (research groups)
3) OCT-based embedding and Cryopreservation in liquid nitrogen (research groups and Biobank storage).

In conclusion, tissue sample collection will be performed within dedicated protocols upon approval by the Institutional Ethical Committee and patient informed consent. Tissue sample processing will be supervised by a dedicated pathologist, with immediate distribution to coordinated research groups and the remaining material will be stored in our institutional Biobank, i.e. the "Centro Risorse Biologiche – Ospedale San Raffaele" (CRB-OSR), headed by Prof. Fabio Ciceri, in collaboration with the Department of Pathology headed by Prof. Claudio Doglioni.
Likewise, the blood samplings destined to research purposes will be collected for:

1) fresh use (research groups)
2) cryopreservation in liquid nitrogen (Biobank storage)

Biobanked samples will be studied with the advanced analytic platforms described in the next paragraphs, but possibly also with new state-of-the-art technologies developed in the next future.

Moreover, they will be instrumental to set up *in vitro* and *in vivo* systems (e.g., patient-derived organoids, tissue chips, possibly patient-derived xenograft models) to validate the efficacy and safety of the novel ATMPs developed by Program 2 and 3, and finally select, by Program 4 activities, the best candidate for first-in-human trials to be launched at the end of the AIRC5x1000 translational research program.

WP1. Definition of tumor mutational burden, epigenetic and gene expression profile of the CRC and PDAC metastatic liver at bulk and at single cell level

MTS CRC and PDAC samples will be dissected and analyzed by:

1. Whole exome DNA sequencing (WES), with >120x coverage, to ascertain, on tumor-derived samples containing >70% cancer cells, the clonality of the somatic mutations and HLA loss of heterozygosity

in cancer cells [13] and tumor DNA mutation burden (TMB) defined by non-synonymous and indel (insertion/deletions) per genome area[14]. Raw Illumina Reads will be quality checked and trimmed to remove sequence adapters and noisy base calls. High quality reads will be aligned to human reference genome with BWA-MEM algorithm for DNA. Alignments will be pre-processed following the Broad-GATK best-practices. Genomic variants will be called in a tumor-normal setting with Mutect2, or in single sample mode with GATK-Haplotype caller. Only passing-filter somatic variants will be processed further. Variants will be annotated with dbSNP, COSMIC, dbNSFP, 1KG and Clinvar. Ensembl-VEP will be used to predict variant effects. Copy number changes and LOH events will be analysed through CNVKit software.

2. RNA-sequencing, on the same sample, which will determine the gene expression levels and define local oncogenic and immune-related molecular pathways that will be compared between the naïve vs chemotherapy treated groups. Library preparation will be performed using the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Clontech), starting from 1-10 ng mRNA. Libraries will be sequenced on Illumina NovaSeq 6000 platform to obtain 30M reads/sample. Raw Illumina Reads will be quality checked and trimmed to remove sequence adapters and noisy base calls. High quality reads will be aligned to human reference genome hg38 with STAR aligner algorithm. Transcript abundance will be quantified by Bioconductor Subread FeatureCounts function, normalized with TMM algorithm and quantified as CPM. Samtool Mplieup will be used to counts reads mapping at single variant level. Moreover, some PFFE tissue samples will be studied with high-plex gene expression panels (e.g., the Tumor Signaling 360TM Panel) from the Nanostring nCounter platform, to obtain transcriptomic profiles from different areas of the tissue specimens (i.e., intratumoral, peritumoral, and distal).

3. ATAC sequencing, to determine the dynamic variations in the chromatin accessibility and overall epigenetic signatures of cancer cells. ATAC-seq libraries will be prepared following Buenrostro *et al* protocol (DOI:10.1038/nmeth.2688). Briefly, 100'000 cells will be permeabilised using a detergent-based buffer, then nuclei will be transposed using Nextera enzyme (Illumina) and resulting library of accessible fragments will be amplified with PCR and sequenced on NovaSeq6000, 1x75 bp. Raw Illumina Reads will be quality checked and trimmed to remove sequence adapters and noisy base calls. High quality reads will be aligned to human reference genome hg38 with bwa-mem aligner algorithm. Regions of read accumulation (peaks corresponding to open chromatin) will be detected with the MACS2 took and annotated with the R/Bioconductor package ChIPPeakAnno.

4. Single cell RNA sequencing (scRNA seq), by available 5' RNA and TCR sequencing kit, on sorted myelomonocytic (CD45+CD11b+) and T (CD45+CD3+) cells infiltrating CRC and PDAC MTS samples, to delineate the immunosuppressive gene signatures of myeloid cell populations as well as the clonality of T cell populations. scRNA seq will be performed on 5,000-10,000 single FACS-sorted cells, by a droplet-based digital 5' RNA seq on a chromium single cell controller (10x Genomics). Current microfluidics-based scRNA seq allows reliable detection of ca. 1,500 genes/cell. Data will be processed with Seurat (v2.1; http//Satijalab.org/Seurat/) as described by [15][16] applying graph based clustering and gene signature analysis to define cell subsets. In validation experiments with selected groups of patients, we plan to perform full-transcriptome scRNA seq on individually sorted cell using the Smart-Seq (PMID 24385147), which is already set up in the group.

Naïve vs chemotherapy treated lesions will be compared to evaluate chemotherapy-induced modifications. Likewise, primary vs MTS lesions will be compared to evaluate the molecular evolution in the distinct tumor sites.

WP2. Evaluation of the molecular and cellular composition of CRC and, if possible, PDAC liver MTS by spatial transcriptomics technologies (NICHE-seq and Visium)

Dr. Iannacone team recently participated in the description of NICHE-seq, which allows the sorting and analysis of cells from visually selected anatomical areas in transgenic mice expressing photoactivatable GFP [17], thus elucidating high-order spatial organization of immune cell types and their molecular pathways, at the same time. The team will adapt NICHE-seq to work with human samples by taking advantage of two photoactivatable ("caged") fluorophores (Thermo Fischer Scientific), that are capable of entering cells and be photoactivated in freshly isolated human tissues (i.e. tonsils). The first one is a non-targeted caged fluorescein, in which a cell permeable form of the dye is incubated in the tissue of interest, and then uncaging photolysis is performed to localize the activated fluorophore only in the 2-photon uncaging volume. The second one is a caged version of fluorescein in a reactive ester form, for attachment to targeting antibodies (e.g. anti-CD45, anti-CD8). After setting the technique with human samples, we will aim at applying the new NICHEseq to ≥5 naive and ≥5 chemotherapy treated CRC MTS and ≥3 PDAC MTS. Classification of the immune cell types contained in the NICHE-seq area will be done as described above for scRNA seq. NICHE-seq and conventional scRNA seq obtained in comparable MTS samples will proceed side-by-side and thoroughly compared. In addition to Niche-seq, we will probe specimens also with more advanced spatial transcriptomics techniques developed in recent years, that we are currently testing and setting. Among them, *Visium* Spatial Gene Expression (10X Genomics).

WP3. Characterization of the immune landscapes of CRC and, if possible, PDAC liver MTS by high dimensional flow cytometry

Single cell suspension of CRC and PDAC MTS and paired autologous PBMCs and portal blood harvested before and after pre-operatory chemotherapy, will be analyzed by a series of parallel polychromatic high dimensional 28 color flow cytometry panels, using a recently acquired BD Symphony cytometer. Known membrane and intracellular marker combinations, and new ones that will be inferred from the above molecular analysis, will be utilized to study:

1. conventional MHC-restricted Tαβ cells, the main anti-tumor effectors, in terms of:
    a) effector/memory/activation phenotypes (CD3, CD4, CD8, CD45RA, CD45R0, CD62L, CCR7, CD95, CD103, CD27, CD28, CD127, 41BB, ICOS, GITR, OX40, HLA-DR, CD25, CD69, CD161,IFNAR1);
    b) effector functions (Perforine, Granzymes, IFNg, TNFa, IL-2, IL-4, IL-13, IL-10, IL-17, IL-21);
    c) T follicular helper (TFH) cell subsets (TFH1, TFH2, TFH17) implicated in the generation of tumor-associated ectopic lymphoid follicles that may impact disease progression (CD4, CXCR5, PD1, ICOS, BCL-6, CXCR3, CCR6);
    d) exhaustion, defined by the upregulation of inhibitory receptors and transcription factor expression (CTLA4, PD1, TIGIT, TIM3, 2B4, LAG3, BTLA, CD39, Eomes, T-bet);
    e) cancer tissue homing molecules (CXCR3, CCR5, CXCR6, CXCR4);
    f) suppression by Treg (CD4, CD25, Foxp3, CD127);
2. innate-like T cells, such as invariant Natural Killer T (iNKT), Mucosal-Associated Invariant T (MAIT) and Tγδ cells. In particular, a high dimensional flow panel has been set to characterize Tγδ cells (that represent up to 50% of total hepatic T lymphocytes and lose IFNγ expression in CRC MTS[18]) with CD1d and MR1 tetramers (provided by the NIH Tetramer Service) and TCRγδ mAbs, and include the above "conventional T cell panels" plus specific markers such as: CD161, CD62L, CD122, IL-12Rb2, IL-17Rb, IL-23R, CCR6, Tbet, PLZF, Rorγt;
3. B cells, in terms of naïve, memory, unswitched memory, regulatory B cell and plasmablast phenotypes (CD19, IgM, IgD, IgG, CD10, CD24, CD27, CD25, CD95, CD86, CD1c, CD1d, CXC3, CD38, CD138);
4. myelomonocytic populations such as monocytes, TAM, DC and MDSC, involved in the stimulation or suppression of anti-tumor responses, identified by "myeloid cell panels": CD14, CD15, CD23, CD33, CD115, CD11c, CD11b, CD32, HLADR, CD80, CD86, CD40, CD66b, CD68, CD124, CD163, CD117, CD200R, CD205, CD1d, CD1c, PD-L1, PDL2.

High dimensional data will be analyzed by Cyt software to generate bh-SNE maps as described by us[19] [20]. Whenever available, matched primary vs MTS lesions will be compared to evaluate the immune landscapes in the distinct tumor sites. Clinically relevant immunological markers and/or signatures emerged from the high-dimensional characterization of peripheral blood samples from CRC MTS patients will be longitudinally monitored in blood samples collected during the 3-year routine follow-up of patients, to validate their predictive value for the clinical evolution of the metastatic disease.

WP4. Validation of the molecular results obtained in WP1-3.

The expression of relevant markers and molecular pathways defined in WP1-3 will be validated at the histological level, i.e., in terms of anatomical distribution, by polychromatic IF (up to 6 colors) and IHC (up to 4 colors) on MTS CRC and PDAC biopsies and, whenever available, matched primary tumor samples.
All samples will be fixed in 10% neutral buffered formalin before being dehydrated and paraffin embedded. For histopathologic evaluation, 5-µm sections of formalin-fixed, paraffin-embedded liver biopsies will be stained by a standard method with hematoxylin and eosin (HE). HE-stained slides will be histopathologically assessed for tissue quality and morphology and then we will be able to perform multiple polychromatic immunofluorescence (up to 6 colors) and immunohistochemistry (up to 4 colors) of both liver metastases from CRC and PDAC on routinely available formalin fixed paraffin embedded samples of the tumor and perilesional parenchyma. We will use Ventana Discovery ULTRA machine for multiple IHC and IF.
The specimens will be collected within 10 min after the tumor resection and fixed in formalin for 24 hr. Dehydration and embedding in paraffin will be performed following routine methods such as the staining for histological reports. For research purposes we will apply at least the following panel of markers: CD3, CD20, CD4, CD8, CD68, CD163, FOXP3, T-bet, GATA3, IFNAR, PDL1, MERTK, MLH1, MSH2; MSH6, PMS2. Additional markers to be assessed on the tumor sections will be defined by the molecular and cellular analysis outlined above.
We will scan multiple immunohistochemistry with Aperio technology available in the pathology unit, whilst the multiple immunofluorescence will be acquired with the confocal microscope thanks to the ALEMBIC facility in San Raffaele Hospital. In addition to multiplex IF and IHC, some specimens we will be characterized more deeply with advanced spatial proteomics technologies, that we are currently testing on some selected samples. Among them, GeoMx Digital Spatial Profiler (Nanostring).

WP5. Definition of the antigenic landscape and TCR repertoire of CRC and PDAC liver MTS

Identification of tumor-reactive TCRs will be pursued through different and complementary strategies:

1) The most relevant inhibitory checkpoint genes/pathways expressed by MTS-TILs will be used to enrich for tumor specificities by cell sorting as already performed in the context of hematological malignancies [20]. We will start with TILs because, compared with blood cells, they are highly enriched with tumor specificities, as reflected by the upregulation of activation and/or exhaustion markers (i.e. PD1, CD137) [21]. Combined WES and RNA seq analysis of autologous CRC and PDAC MTS (WP1) will be interrogated with epitope prediction algorithms (MHC-I: NetMHCpan, SYFPEITHI, IEDB; MHC-II: Propred) to predict putative immunogenic peptides for patients expressed HLA-A, B, DR alleles and, for MHC-I epitopes, their probability to be processed by the immunoproteasome. TILs sorted for PD1/CD137 expression (or surface markers identified throughout WP1-3) will be activated with mutated or unmutated peptide pools pulsed on autologous antigen presenting cells[22]. Based on standard criteria in the field, candidate CD8 neoepitopes with IC50<150nM, will be considered strong binders. Responding T cell cultures will be subject to bulk and single-cell TCR seq according to an already established protocol[23].

2) In samples characterized by a low percentage of MTS-TILs, single cell immune profiling will be performed in order to simultaneously determine gene expression and correctly pair α/β chain TCR sequences from individual T cells.

3) T cells harvested from portal, hepatic and peripheral blood will be also sorted based on PD1/CD137 or the

surface marker expression profile identified throughout WP1-3. Sorted cells will be stimulated as described in 1) with the peptides predicted from WES and RNA seq of the autologous MTS. TCR sequencing will be performed on responding T cell cultures and results will be compared to the ones obtained in 1) to identify the presence of T cell clone overlapping among metastatic lesions, portal, hepatic and peripheral blood repertoires.

4) The library of tumor-specific TCRs identified in 1)-3) will be transiently expressed in reporter Jurkat cells, expressing an IL-2-promoter driven luciferase, following a protocol available in Dr. Dellabona laboratory. To confirm their tumor antigen specificity, we will exploit peptides derived from putative overexpressed and cancer-testis antigens or neo-epitopes defined in 1). Selected peptides will be pulsed on autologous APC (i.e., immortalized B cells [33]) to upregulate luciferase in Jurkat cells transiently expressing these TCRs.

5) The combined WES and RNA seq of CRC and PDAC MTS performed in WP1 will be also interrogated to identify transmembrane molecules enriched in cancer cells that could be new surface antigenic targets for CARs. These putative Ags will be functionally validated for proper selection of new targets based on cell surface localization, high expression, tumor specificity, oncogenic role of the candidate molecule and availability of antibody sequences to be used for CAR design.

We have recently set up a protocol for TCR gene hunting from patients affected by acute myeloid leukemia and from healthy donors, by which we built a library of ~20 TCRs, specific for overexpressed tumor antigens (CCNA1, cathepsin, WT1, Ny-Eso1) and restricted by common HLA alleles. Other TCRs relevant for this proposal (i.e., mesothelin, survivin, Her2, EGFR, EPCAM-specific TCRs) are currently in pipeline.
Based on average mutation load of MSS CRC and PDAC, we expect to find between 50-200 neoantigens/sequenced tumors[24][25], with a frequency of neoantigen-specific T cells in the range of 1-10%[21].

Longitudinal PDAC primary tumor immune microenvironment characterization

The hypothesis of the study is that the factors produced by ductal pancreatic adenocarcinoma (PDAC) cancer cells, present in the primary tumor or metastasis, alter the profile of gene expression and the function of cells of the immune system. Therefore, we ask to be able to analyze, in addition to liver biopsies, also primary PDAC tumor tissue and possibly healthy peritumoral tissue and a portal blood sample taken during surgery and a paired venous peripheral blood sample in patients who will develop liver metastasis after pancreatic resection (primary PDAC and liver metastasis of the same patients). On these samples we will be able to carry out the following analyses:
a)
- phenotypic analysis by cytofluorimetry on tumor infiltrate and on circulating cells;
- genomics and transcriptomics analysis on tumor infiltrate and on circulating cells;
- analysis of the soluble factors secreted by the tumor and present both in the tumor tissue and in the plasma.
b)
- detection of selected tumor-related molecules secreted by PDAC that can contribute to the hepatic pre-metastatic niche formation;
- detection of PDAC associated DNA mutations, cell-free DNA methylation and miRNAs in free circulating and exosomes-associated nucleic acids.
c)
- evaluation of the level of immunocompetence and immunosuppression within the immune contexture in primary PDAC and to correlate the expansion/reduction of given immune cell subsets with other relevant new or already described immune cell subsets and with the clinical characteristics/outcome;
- evaluation of the impact of neoadjuvant chemotherapy in the induction of de-novo immune responses and/or in the repolarization of pre-existing immune phenotypes;
- comparation in longitudinal cases of patients with relapse in the liver the immune contexture in the two tumor microenvironments (primary versus liver metastases).

d)
In parallel analysis of PDAC hepatic MTS and their matched/not matched, previously collected, primary not metastatic PDAC tissues with the aim to:
- detect the effect of the immunosuppressive tumor microenvironment (TME) on transferred T cells;
- analyze the immunosuppressive pathways active in tumor-specific TILs;
- perform single cell immune profiling.

Longitudinal analyses on peripheral blood follow-up samples from CRC MTS patients

The collection and biobanking of peripheral blood samples, in parallel with clinical data retrieval, at different timepoints (detailed above in chapter 4) during medical follow-up of patients, will allow the longitudinal monitoring of those biologic and immunological markers/signatures emerged as candidate prognostic predictors from experimental analyses on samples collected at the time of patients' enrollment. This experimental monitoring might include:

- cytofluorimetry, genomics and transcriptomics analyses on PBMCs;
- dosages of plasmatic soluble factors and tumor-related molecules that can contribute to the hepatic progression or recurrence;
- detection of CRC-associated DNA mutations, cell-free DNA methylation and miRNAs in free-circulating and exosomes-associated nucleic acids.

Biobanked samples will be studied with the advanced analytic platforms described but possibly also with new state-of-the-art technologies developed in the next future.

5. Study Population and Inclusion Criteria

In this study we will enroll CRC and PDAC patients with hepatic MTS, primary not metastatic PDAC patients and healthy volunteers.

- ***CRC***

Based on incidence and clinical management of the disease at Ospedale San Raffaele, we expect to analyze a total of N=200 CRC liver metastases (either metachronous or synchronous) in 4 years, of which: ~70-80% from patients receiving pre-surgical chemotherapy, ~20-30% from naïve patients that received no treatment prior to sampling. In addition to cross sectionally compare pre-treated and naïve patients, we propose to study longitudinally the majority of the patients undergoing pre-surgical chemotherapy (borderline-resectable) by sampling their metastatic tumor tissue by tumor biopsies preceding the pre-surgical chemotherapy and by surgical resection following the chemotherapeutic treatment. Moreover, in those CRC MTS patients undergoing synchronous resection of primary CRC and hepatic metastases (about 5% of liver MTS-resected patients, according to previous experience), tumor samples will be collected from both the primary and the metastatic sites, in order to provide biologic material for paired study of both tumor microenvironments. CRC tumor specimens (both from liver MTS and primary CRC) are collected throughout the standard clinical management of patients, which includes surgical resection.

- ***PDAC***

Based on incidence and clinical management of the disease at Ospedale San Raffaele, we expect to analyze a total of at least 95 PDAC liver metastases (either synchronous and metachronous). Surgical resection is recommended only for localized not metastatic disease (NCCN guidelines for PDAC). The availability of PDAC liver MTS is poor and, in the majority of cases, limited to percutaneous ultrasound-guided fine-needle aspiration, or core biopsies carried out to obtain material for the cytological or histological analysis of these lesions. Identification and selection of metachronous liver MTS PDAC patients will be conducted starting from an additional cohort of 150 primary PDAC not metastatic resectable patients.
In particular we propose to collect:

1) Samples from hepatic PDAC MTS, obtained from the following cohorts of patients by the indicated procedures:

Cohort A:
a) percutaneous ultrasound-guided fine-needle core biopsies of liver MTS in patients with metastatic PDAC at imaging;

Cohort B:
b) Liver MTS resections performed during laparotomy/laparoscopy in patients with radiological resectable PDAC scheduled for pancreatic resection, but with incidental detection of liver metastases intraoperatively
c) Liver MTS resections performed during laparotomy in patients undergoing palliative surgery;
d) Liver metastasectomy in stage IV PDAC patients undergoing chemotherapy followed by pancreatic cancer and liver metastasis resection.

2) Samples from primary PDAC from a cohort of resectable not metastatic patients (Cohort C). These patients will be monitored for early diagnosis of hepatic PDAC MTS by follow-up testing. For those in which we will be able to detect a hepatic distant recurrence, the metastatic tissue will be collected following procedures described for Cohort A patients.

- ***Healthy donors***

At least 30 healthy donors will be enrolled as negative controls for the clinical study. Only peripheral blood (20 ml) will be collect from healthy donor volunteers during blood donation drawings.

Inclusion and exclusion criteria – CRC patients

Inclusion criteria:

1. Patients with histologically or cytologically confirmed diagnosis of CRC metastatic to the liver (stage IV disease, AJCC)
2. Patients with indication to surgical resection and/or chemotherapy treatment
3. Age ≥18
4. ECOG PS 0-1 at enrollment
5. Written informed consent
6. Patients will be treated in OSR

Exclusion criteria:

1. Pregnancy or lactation
2. Inability to provide a written informed consent
3. Extrahepatic disease with the exception of selected cases in which the coexistence of extrahepatic disease does not constitute an exclusion criterion for hepatic resective surgery (for example in patients with extrahepatic lesions in remission or in any case stabilized by chemotherapy)
4. Severe comorbidities (e.g. cardiac diseases, history of psychiatric disabilities, HIV, autoimmune disorders)
5. Concurrent or previous other malignancy within 5 years of study entry, except cured basal or squamous cell skin cancer, superficial bladder cancer, prostate intraepithelial neoplasm, carcinoma in-situ of the cervix, or other noninvasive or indolent malignancy
6. Other conditions (medical or psychiatric) that in the judgment of Investigators would make the patient an inappropriate candidate for the study

Inclusion and exclusion criteria – PDAC patients

Inclusion criteria:

1. Patients with clinical/radiological diagnosis/suspicious of pancreatic adenocarcinoma metastatic to the liver, with subsequent cytological/histological confirmation (stage IV disease, AJCC)
2. age ≥18
3. Karnofsky performance status ≥50
4. Metastatic pancreatic adenocarcinoma patients with histological specimens from whole liver metastasis biopsy or core liver biopsy collected at San Raffaele Hospital and stored in the OSR Biobank.
5. Written Informed consent
6. Patients with clinical/radiological diagnosis of not metastatic primary PDAC that will undergo pancreatic resection in OSR

Exclusion criteria:

1. Severe comorbidities (e.g., cardiac diseases, history of psychiatric disabilities) representing an absolute contraindication for whole or core liver metastasis biopsy
2. Pregnancy or lactation
3. Inability to provide a written informed consent
4. Metastatic pancreatic adenocarcinoma patients enrolled in other research trials entailing the analysis of the liver metastasis histological sample

6. Sample Dimension, recruiting period and justification

From time to protocol approval until the end of June 2026 we expect to enroll, collect samples and clinical data from at least N=200 CRC patients, 95 - either synchronous (N=75 patients,) or metachronous (N=20 patients) - PDAC MTS patients. An additional cohort of at least N=150 primary resectable not metastatic PDAC patients will be enrolled after version 2.0 LiMeT protocol amendment approval until the end of June 2026.
Each PDAC patient will be followed-up possibly for 2 years from the time of enrollment, each CRC MTS patient possibly for 3 years. Since protocol amendment V4.0 approval, an additional blood sample will be drawn, whenever possible, for research purposes from patients undergoing ematochemical testing as part of the follow-up visits indicated by standard clinical practice.

- In detail, based on incidence and clinical management of the disease at the Ospedale San Raffaele, we expect to analyze at least a total of 200 CRC liver metastases (either metachronous or synchronous), of which: ~70-80% from patients receiving pre-surgical chemotherapy, ~20-30% from naïve patients that received no treatment prior to sampling. According to previous experience, about 5% of liver MTS-resected CRC patients will undergo synchronous resection of primary CRC and hepatic metastases.

- Concerning PDAC patients, based on incidence and clinical management of the disease at the Ospedale San Raffaele, we expect to collect and analyze: i) synchronous liver metastatic samples from patients from at least 75 patients, of which ~90% (N=65-70) will be core biopsies from cohort A patients and ~10% (N=5-10) will be liver resections/metastasectomies from cohort B patients; ii) metachronous PDAC MTS and/or blood samples from at least 20 patients. Identification and selection of the latter subgroup of patients will be conducted starting from a iii) cohort of at least 150 primary PDAC not metastatic resectable patients. These patients will undergo clinical data and samples collection at the moment of the surgery asportation of the primary tumor. A clinical/radiological follow-up will be performed in order to monitor patients for early diagnosis of liver distant relapse occurrence. Bases on the aforementioned OSR statistical analysis and considerations on possible patients drop out reasons, at least 20 of these patients are expected to develop liver metastases within 1-2 years after surgical resection of primary adenocarcinoma, We decided to plan the recruiting period of this cohort of patients from protocol amendment V2.0 approval (end of October 2020) until the end of June 2026. In this way we can expect that at least 20 patients would develop PDAC liver MTS within the clinical follow-up period of 1-2 years from their enrollment.
Thanks to follow-up testing, we will be able to early diagnose PDAC liver recurrence. Upon patient informed decision to adhere to protocol procedures intended for PDAC MTS patients (a dedicated informed consent form was predisposed), a core biopsy of the metastatic tumor samples (PDAC hepatic MTS) will be collected in order to assess the hepatic lesion derivation from the pancreatic primitive tumor (NCCN guidelines for PDAC).

At least 30 healthy donors will be enrolled as negative controls for the clinical study.

The study is exploratory in nature, with the aim to identify tumor associated antigens (TAAs) and local immune suppressive and regulatory pathways associated to liver MTS from CRC and PDAC that will be then targeted by state-of-the-art cell therapies and gene transfer/editing tools, first assessed on suitable mouse models and *in vitro* cultures of patient-derived samples and then undergoing further preclinical development and, eventually, filing an application for the first-in-human testing of the selected ATMPs. The identification of local and systemic factors predictive of liver progression or recurrence will be pursued with the prospective follow-up study of CRC patients after liver MTS resection.

7. Data Collection and Management

Only patients signing the Informed Consent to the specific clinical study and to the institutional Biobank "Centro Risorse Biologiche – Ospedale San Raffaele" (CRB-OSR), regulation will be enrolled in the study. Informed Consent will be obtained from all subjects for their clinical and genetic data to be recorded anonymously. Every patient of the study will be assigned a Unique Identifying Code (UIC) (pseudoanonimization). All data on patients participating in the study will be held in dedicated Case Report Forms (CRFs) in which every patient will be indicated by his/her UIC. The tracking-back of the collected data to personal identities will be possible only for clinicians involved in the study.

8. Statistical Analysis

WP1 analyses

Whole Exome DNA Sequencing (WES): Genomic variants will be called in a tumor-normal setting with Mutect2, or in single sample mode with GATK-Haplotype caller. Only passing-filter somatic variants will be processed further. Variants will be annotated with dbSNP, COSMIC, dbNSFP, 1KG and Clinvar. Ensembl-VEP will be used to predict variant effects. Copy number changes and LOH events will be analysed through CNVKit software.

RNA-seq: Raw Illumina Reads will be quality checked and trimmed to remove sequence adapters and noisy base calls. High quality reads will be aligned to human reference genome hg38 with STAR aligner algorithm. Transcript abundance will be quantified by Bioconductor Subread FeatureCounts function, normalized with TMM algorithm and quantified as CPM. Samtool Mplieup will be used to counts reads mapping at single variant level.

ATAC-seq: Raw Illumina Reads will be quality checked and trimmed to remove sequence adapters and noisy base calls. High quality reads will be aligned to human reference genome hg38 with bwa-mem aligner algorithm. Regions of read accumulation (peaks corresponding to open chromatin) will be detected with the MACS2 took and annotated with the R/Bioconductor package ChIPPeakAnno.

scRNA-seq: Data will be processed with Seurat (v2.1; http//Satijalab.org/Seurat/) as described by [15][16] applying graph based clustering and gene signature analysis to define cell subsets. The immune cell composition of the liver MTS will be mined from RNA seq data by deconvolution algorithms that use expression signature matrices to infer specific cell proportions from expression data of cell mixtures (ie: CIBERSORT[26]).

WP2 analyses

Classification of the immune cell types contained in the NICHE-seq area will be done as described for scRNA seq. NICHE-seq and conventional scRNA seq obtained in comparable MTS samples will be compared.

WP3 analyses

High dimensional data will be analyzed by Cyt software to generate bh-SNE maps as described by us[19] [26].

WP4 analyses

We will scan the slides, analyze them and apply statistical analysis thank to Definiens image software analysis (Parra ER, J Cancer Treat and Diagnosis 2018; 2(1): 43-53).

WP5 analyses

According to our published procedures [27][28], the results obtained in WP1-4 will be combined with computational prediction of shared unmutated and mutated peptide T cell epitopes.
Combined WES and RNA seq analysis of autologous CRC and PDAC MTS (WP2) will be interrogated with epitope prediction algorithms (MHC-I: NetMHCpan, SYFPEITHI, IEDB; MHC-II: Propred) to predict putative immunogenic peptides for patients expressed HLA-A, B, DR alleles and, for MHC-I epitopes, their probability to be processed by the immunoproteasome.

Other analyses
All the analyzed variables will be considered in multivariate analyses, together with the typical clinical assessments of PDAC and CRC patients. According with the aims of this study, the best multivariate Generalized Additive Model and Cox proportional-hazard model will be fitted. Statistically significant differences between groups with will be evaluated by Wilcoxon Mann Whitney, Kruskal Wallis or Chi-squared tests, as appropriated. P-values less than 0.05 will be considered statistically significant.

9. SUBSTUDY 1: ERC Synergy Grant 2023

Background and rationale
Colorectal liver metastases (CRLM) represent a major unmet clinical need, as the 5-year survival rate of patients with unresectable disease does not exceed 2%. New therapies that promote antitumor immunity have been recently developed, mostly focusing on enhancing T cell responses. Although these therapies have led to unprecedented successes, only a minority of patients benefit from these treatments, highlighting the need to identify new cells and molecules that could be exploited in next generation immunotherapies. Given the crucial role of innate immune responses in immunity, targeting these responses opens up new possibilities for tumour control. We hypothesize that the immunotherapy of liver metastases can be significantly improved through harnessing the biology of innate lymphoid cells (ILC), such as Natural Killer (NK) cells and ILC1s, and myeloid cells such as macrophages and DCs. Our team brings together experts in the biology of tissue-resident myeloid (Florent Ginhoux, Gustave Roussy, France) and lymphoid (Georg Gasteiger, University of Wuerzburg, Germany, cPI) cells, in liver immunology (Valeria Fumagalli, Vita-Salute San Raffaele University, Italy), and in the development of novel immunotherapeutic strategies that modulate immune cells in the fight against cancer (Eric Vivier, Aix-Marseille Université, France). By combining cutting-edge single cell and spatial transcriptomics of human patient samples with cross-species analyses in advanced genetic mouse models, we aim (1) to identify cellular interactions defining the metastatic tumor microenvironment across murine and human tissue specimens, (2) to investigate immune cell functions regulating metastatic disease using a unique combination of advanced genetic mouse and human tissue models, and (3) to harness the anti-tumoral functions of innate immune cells via next generation cell engagers.

Study design
We aim at extending the duration of the project LiMeT for the cohort of patients with CRC and liver metastases and the cohort of healthy control from June 2026 to June 2030. The European funding ERC Synergy Grant (101118936), lasting 6 years starting from September 2024, includes an observational study aimed at characterizing the innate immune cells infiltrating liver metastases from colorectal cancer. As described in the existing protocol, samples obtained from patients will be used for flow cytometry analysis, single-cell transcriptomics, spatial transcriptomics, and histology. The goal is to identify interactions between innate immune cells that, in the context of liver metastases, could be reprogrammed through new platforms for immunotherapy.

Population:
This observational sub-study is both retrospective and prospective. The samples previously collected during the LiMeT study and those that will continue to be collected until 2030 will be used, offering a unique perspective on disease progression and treatment effectiveness over time.
This will significantly enrich the scientific and clinical value of the study, contributing to improved treatment strategies and outcomes for CRC patients.
Additionally, the collected samples will be fundamental for deepening our understanding of the cellular and molecular variables associated with CRC. Using existing samples along with future ones will ensure continuity in the quality and quantity of the data collected, allowing for a more thorough and complete analysis.

We anticipate enrolling a minimum of 300 patients and 30 healthy controls.

Collaboration with other centers (Gustave Roussy, France; University of Wuerzburg, Germany; Aix-Marseille Université, France) within the Grant will not only promote greater resource sharing but will also enable us to obtain a broader and more diverse view of the results, thanks to the different expertise and technologies available at each center. This collaborative approach is essential for enhancing the robustness and relevance of our findings and for accelerating the transfer of knowledge from research to clinical practice.

Study objectives

AIM1. To identify cellular interactions defining the metastatic tumor microenvironment using spatially resolved transcriptomics across murine and human tissue-specimens.
Success will be measured by identifying specific cell types, their spatial organization, and their gene expression profiles within the tumor microenviroment, providing insights into the cellular dynamics that support metastasis.

AIM2. To investigate immune cell functions regulating metastatic disease using a unique combination of advanced genetic mouse and human tissue models.
Success will be measured by identifying key immune cell populations and their molecular pathways that either promote or inhibit metastasis. This will include functional assays to determine the impact of specific genetic modifications on immune cell behavior and metastatic progression.

AIM3. To harness the anti-tumoral functions of liver immune cells via next-generation cell engagers.
Success will be measured by demonstrating the efficacy of these cell engagers in preclinical models, including their ability to activate liver immune cells, target metastatic tumor cells, and reduce tumor burden. The end-point will also include safety and toxicity profiles to ensure the feasibility of potential clinical translation.

Impact
The sub-study aims to significantly enrich the scientific and clinical value of the original project by:

- contributing to improved treatment strategies and outcomes for CRC (Colorectal Cancer) patients;
- deepening our understanding of the cellular and molecular variables associated with CRC.

By combining existing samples with future collections, we will ensure continuity in the quality and quantity of the data. This will allow for a more thorough and complete analysis of disease progression and treatment efficacy. Notably, while the original protocol included PDAC (Pancreatic Ductal Adenocarcinoma) samples, this amendment will focus solely on CRC patients.

Statistical analysis and sample size
Based on our experience with the LiMeT protocol, we expect to enroll 6 CRC patients per month. On a more experimental level, usable, non-degraded tissue for research is available from approximately 2/3 of the patients.We anticipate enrolling a minimum of 300 patients and 30 healthy controls. According to statistics from the past 4 years, about 66% of the enrolled patients are synchronous with the diagnosis, and 34% are metachronous.

10. SUBSTUDY 2 – Deciphering and targeting the immunological niche in PDAC (Fondazione Regionale per la Ricerca Biomedica 2024, FRRB 2024)

Rationale
Pancreatic ductal adenocarcinoma (PDAC) remains a largely incurable malignancy, resistant to conventional and advanced immunotherapies. Building on the LiMeT framework, this substudy applies immune gene therapy principles and multi-omic analyses to pancreatic cancer, mirroring the technological and conceptual strategies of the original project. The integration of high-resolution spatial multi-omics and machine learning (ML)–based tissue analysis will help elucidate the immune microenvironment (TME) and identify novel targets for

engineered immune cells.

Study objectives

AIM1. Perform single-cell multiome and spatial gene expression analyses of pancreatic tumor and pre-neoplastic lesions (PDAC and IPMN), to define immune-stromal cell interactions and identify disease-driving niches.

AIM2. Develop and benchmark ML algorithms integrating histomorphological and omics data to detect spatial immune signatures predictive of tumor evolution.
The goal of this aim is to develop, together with pathologists at the Pathology Unit of OSR, sophisticated segmentation algorithms that we can apply to H&E imaging of PDAC and IPMN, to automatically identify and quantify diagnostically and prognostically relevant phenotypes. H&E WSI will be acquired from 200 donors. A set of these (n=50) will be used as training set by histopathologists to identify and annotate relevant features such as degree of dysplasia (low-grade or high-grade), presence of invasive cancer, cancer grading (well- moderate- poor differentiation), nerve and vessel infiltration and characterisation of immune cells within the lesions. Segmentation models will be used to then infer the annotations in the held-out 150 slides.

AIM3. Design and validate gene-engineered immune cells (TCR-T, CAR-T, and CAR-macrophages) targeting PDAC immune niches, using the same platforms validated within LiMeT

Population and study design
The present study will span the 3-year FRRB finding period.
Patient cohorts: for aim 1: at least 100 total patients (50 PDAC and 50 IPMN) recruited at OSR Pancreas Center. For aim 2: 200 donors
Patients will be enrolled over the 3-year duration of the research project (refer to the project timeline section below), starting immediately after clinical protocol approval.
Sample collection: fresh peripheral blood and plasma; frozen cystic fluid (CF); fresh and FFPE-fixed biopsies from disease (PDAC or IPMN) and control non-tumor adjacent tissue (NAT) tissues
Samples will be collected by surgeons and immediately transferred to OSR biobank for anonymization, distribution and storage. Fresh samples will be transferred to research laboratories within 2 hours from collection, according to internal SOPs. Fixed samples will be distributed to the Pathology Unit for histopathological analysis and research laboratories for experimental activities.
Methodology:
- *Analyses on patient samples:* scRNA-seq, scATAC-seq, spatial transcriptomics (Visium, MERSCOPE), multiplex-immunofluorescence/immunohistochemistry (multiplex- IF/IHC).
- *Data integration (clinical, immunohistochemical, and omics data):* computational pipelines using Cell2Location, Seurat, and ML-based pattern recognition.
- Genetic engineering: lentiviral and genome-editing (CRISPR, base/prime editing) approaches for TCR-T cell, CAR-T cell, and CAR-macrophage development.

Organization strategy
IRCCS Ospedale San Raffaele (OSR) is the Coordinator of the FRRB project (PI: Prof. Maria Chiara Bonini, Co-PI: Prof. Stefano Crippa) and will be in charge of: patient recruitment, immunophenotyping and spatial omics analyses, and immune cell engineering.

Access to patient material will be ensured by close collaboration, within OSR, among clinicians of the Pancreas Translational and Clinical Center (Prof. Massimo Falconi) and Medical Oncology Unit (Prof. Michele Reni), and coordinated within the intramural Comprehensive Cancer Center, headed by Prof. Fabio Ciceri). Importantly, OSR has already established workflows for the collection and storage of samples from PDAC/IPMN patients, matched with anonymized clinical data, medical history, exposome, radiological/endoscopic features and pathology reports.Patient samples will be processed and biobanked by the institutional biobank (Center of Biologic Resources, CRB). Pathologists at the Pathology Unit of OSR will collect tissue samples for research study, perform pathologic evaluations and of multiplex IHC/IF stainings.

Fondazione Human Technopole is a Partner of the project and will carry out computational and spatial omics

analyses.

Timeline (Years 1–3)
Year 1 – Patient recruitment; scRNA-seq, scATAC-seq, and spatial data generation; initial ML model development.
Year 2 – Patient recruitment; Data integration and refinement of immune niche targets; validation of ML models; generation and in vitro testing of the first engineered immune cell products.
Year 3 – Patient recruitment; in-vivo preclinical validation of newly-developed immune cell therapies; finalization of ML models and prognostic biomarker identification.

Expected Outcomes
The FRRB substudy will produce a spatial and molecular atlas of PDAC and IPMN immune niches, predictive models of disease progression, and preclinical validation of next-generation engineered immune cell therapies for pancreatic cancer. These outcomes align directly with the LiMeT program's translational goals, reinforcing a unified strategy for advancing immune gene therapy in solid tumors. The AIRC5x1000-funded LiMeT project and the FRRB-funded study share the same overarching aim: to develop and validate advanced immune gene therapies for solid tumors resistant to current treatments. Both initiatives are grounded on the integration of cutting-edge single-cell and spatial omics, immunogenetic engineering, and preclinical validation of TCR-T and CAR-T cell products. While LiMeT focuses on liver metastases as a clinical model, the FRRB project extends these objectives to pancreatic ductal adenocarcinoma (PDAC) and intraductal papillary mucinous neoplasms (IPMN). The scientific alignment ensures the sharing of infrastructures, methodologies, and regulatory frameworks across both programs, with coordinated activities between Units and Partners.

11. Ethical Aspects

The responsible investigators will ensure that this study is conducted in agreement with either the Declaration of Helsinki (Tokyo, Venice, Hong Kong, Somerset West and Edinburgh amendments) or the laws and regulations of Italy. This study is non-interventional, and by definition, no additional procedures will be carried out on the enrolled subjects beyond the normal clinical practice. Only patients signing the Informed Consent to the specific clinical study and to the institutional Biobank "Centro Risorse Biologiche – Ospedale San

Raffaele" (CRB-OSR) regulation will be enrolled in the study. Informed Consent will be obtained from all subjects for their clinical and genetic data to be recorded anonymously. Subjects will also be informed of their right to withdraw their consent at any time during the study. Collected data will be treated in accordance with the current legislation on treatment of personal data [Reg. UE n.679/2016 (DGPR) e D. Lgs. n. 196/2003].

12. Relevance to cancer patients

There is a pressing need for more efficacious therapeutic approaches to offer to CRC and PDAC patients with liver MTS. Under both physiologic and pathologic conditions, including cancer, the liver is an organ peculiarly biased toward inducing a state of immune unresponsiveness known as "liver tolerance". Building upon preliminary data and published observations from our groups, we posit that overcoming liver tolerance by innovative cancer immunotherapies might represent a powerful mean to interfere with PDAC- and CRC-derived liver MTS. Adoptive cell therapy (ACT) with tumor-specific T cells, when feasible, has proven successful in controlling disseminated metastatic tumors also of epithelial origin [29][30]. The transfer of very high numbers of activated T cells can even, though temporarily, overcome some of the biological hurdles imposed by the neoplastic mass, such as the tumor induction of immunosuppressive microenvironments. Indeed, ACT efficacy can be further enhanced by identifying and counteracting local immunosuppressive mechanisms [31]. This is particularly relevant for hepatic MTS, which receive tolerogenic signals from both the liver and tumor cells. Therefore, the combination of: i) direct cancer cell killing by adoptively transferred tumor-specific T cells, with ii) indirect control of tumor progression via reprogramming of the microenvironment, become particularly attractive as therapeutic approaches for CRC and PDAC hepatic MTS. The high-risk character of this proposal is compensated by the benefit that would derive from its successful completion, i.e. the initiation of innovative gene/cell therapy clinical trials within the funding period.

13. Organization strategy

The project is built on a solid, long-standing and multidisciplinary collaboration between several groups operating at the Università Vita-Salute San Raffaele and IRCCS Ospedale San Raffaele. Harvest and histological profiling of selected material from patients will be guaranteed by surgical oncologists under the direction of Dr. Aldrighetti and Prof. Falconi, by pathologists headed by Prof. Doglioni and medical oncologists supervised by Dr. Reni and headed by Prof. Reni. For those MTS-resected CRC patients undergoing synchronous resection of primary CRC and hepatic metastases, the collection of tumor samples will be organized in collaboration with surgeons of the Gastrointestinal Surgery Unit, headed by Prof. Riccardo Rosati. A research nurse will be responsible for the delivery of the collected blood and surgical/bioptic samples to the institutional Biobank. Sample collection will be performed within dedicated protocols upon approval by the Institutional Ethical Committee and patient informed consent. Sample processing and storage will be centralized through our institutional Biobank, i.e. the "Centro Risorse Biologiche – Ospedale San Raffaele" (CRB-OSR). Once collected and processed by the Biobank personnel, samples will be partly stored for subsequent analyses and partly divided for fresh use by Prof. Bonini, Dr. Protti, Dr. Casucci, Dr. Dellabona, Prof. Iannacone, Dr. Ostuni, and Dr. Tonon research teams, who will employ the most advanced technology platforms to carry out WP1-5 studies. A dedicated data manager will be responsible for organizing and storing data, upholding agreed-upon security standards.

The research team gathers highly recognized San Raffaele Scientific Institute scientists with complementary expertise in advanced gene and cell therapies and extensive experience in combining high-quality products, and professional risk management in well-designed clinical trials.

The institutional Biobank, i.e. the "Centro Risorse Biologiche – Ospedale San Raffaele" (CRB-OSR) is accredited by the national BBMRI (https://www.bbmri.it/lista-biobanche/centro-risorse-biologiche-ospedale-san-raffaele/).

14. Trial Sponsorship and Financing

There are no additional costs for the National Health System related to sample collection, storage and analysis. All costs of the study will be covered by the AIRC5x1000 "Advanced immune gene and cell therapies for liver metastases" grant to Prof. Chiara Bonini.

Starting from 2026, the costs of the observation substudy will be covered by the ERC Synergy "Treating Liver Metastasis TREATLIVMETS" grant to Dott.ssa Valeria Fumagalli.

15. Data ownership

Data ownership will be of the Finantial Sponsor: Università Vita-Salute San Raffaele and of the Scientific Promoter: Ospedale San Raffaele.

16. Trial Insurance

No insurance policy has been engaged for this observational non-interventional study.

17. Publication Policy

The Principal Investigator will make data of the study publicly available through communications at meetings and publications in peer-reviewed journals.

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