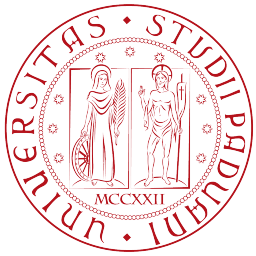

Sede Amministrativa: Università degli Studi di Padova

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CORSO DI DOTTORATO DI RICERCA IN:

SCIENZE CLINICHE E SPERIMENTALI

CURRICOLO:

**Scienze epatologiche e trapiantologiche**

CICLO: 32°

# *TRANSPLANT ONCOLOGY: FROM LABORATORY TO BEDSIDE*

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Supervisore: Ch.mo Prof. Umberto Cillo

Dottorando: Alessandra Bertacco

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# Chapter 1

# INTRODUCTION

## 1.1 The "Transplant Oncology" concept

*Transplant oncology* is a new concept that has developed recently in response to the increasing pressure of oncological indications in liver transplantation (LT). It is based on the multidisciplinary approach to liver cancer diseases, both primitive and secondary, in order to offer to these patients the best chance of treatment. The three 'E' (**Figure 1**) that constitutes the pillars of transplant oncology are:

1. Evolution of a multidisciplinary cancer care by integrating liver transplantation.
2. Extending the limits of safe hepatobiliary resections by applying transplantation techniques to cancer surgery.
3. Exploration of biomechanisms of disease through genomic studies.

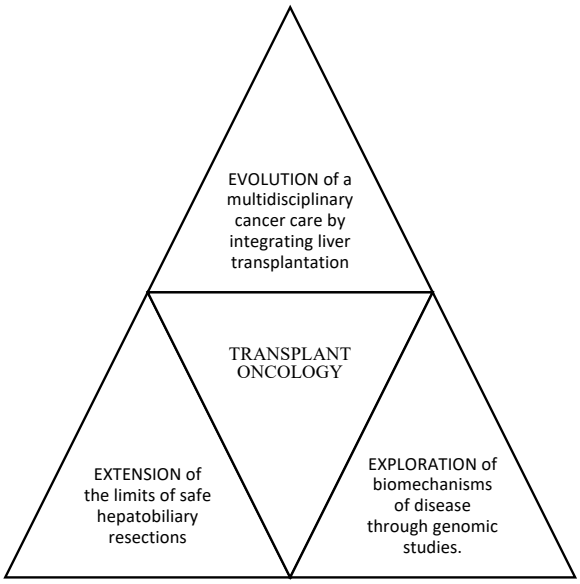



***Figure 1.*** *The three "E" of Transplant Oncology*

This new branch of medicine was born following the great successes in terms of overall survival obtained from studies on liver transplantation for hepatocellular carcinoma (HCC), which is now to be counted among the usual indications. The derived enthusiasm inspired several studies whose aim was evaluating the effectiveness of liver transplantation even in the case of other hepatobiliary tumors. Despite the initial failures, with the advancement of surgical techniques and an ever better multidisciplinary management of patients, to date the

oncological pathologies that can be susceptible to potentially curative treatment through transplantation are numerous. The main ones are:

- Hepatocellular carcinoma
- Peri-hilar cholangiocarcinoma (CCA)
- Hepatoblastoma
- Metastases from Neuroendocrine tumors
- Metastases from Colo-Rectal cancer

It is also to underline that, with the decrease incidence of HCV and HBV among the transplant indication, due to the new antiviral agents and the vaccine policy respectively, the weight of this new oncological indications will be even greater. Among the diseases considered by the transplant oncology, surely colorectal liver metastasis (CRLM)s stands out for its high prevalence.

### 1.2 CRLM: state of art

While liver transplant has traditionally been reserved for HCC and, in select circumstances, hilar angiocarcinoma, there has been increasing interest in utilizing LT for CRLMs. Colorectal carcinoma (CRC) is the third most common malignancy worldwide [1] representing one of the "big killers". It represents a dramatic public health issue, considering that the CRC incidence is rising in developed countries and among people younger than 55 years old [2]. Approximately half of patients will have liver metastasis at the time of diagnosis or during the course of disease; 15–25% of CRC patients will have distant metastases at the time of primary diagnosis, mainly to the liver [3] and another 18%–25% patients will develop distant metastases within 5 years from the first diagnosis [4]

Despite the tremendous progress in the efficacy of chemotherapy, the prognosis of patients with liver metastases from colorectal cancer remains poor, with only about 25% alive at 5 years. Systemic chemotherapy combined with radical surgical resection is the milestone of treatment in these patients [5]. However, only 15-20% of patients are eligible for curative liver resection due to insufficient liver remnant volume or large tumor burden. In this situation, liver transplantation may represent an attractive option allowing R0 resection. Organs shortage and absence of long-term results are the main issues that justify the ethical dilemma rised from this new prospective. Before 2000 the results of LT for CRLM were discouraging with 5-yrs overall survival (OS) range from 12 to 21%. In 1991 the group of Vienna [6] reported 25 cases; 1, 3 and 5-yrs OS were 76%, 32% and 12% respectively with

recurrence rate of 64%. In the same year a report from the University of Cincinnati [7] described 8 cases of LT for CRLM with a recurrence rate of 70%. From the European Liver Transplant Registry (ELTR)[8] 58 cases were described in the period 1977-1995 with 5-yrs survival of 18%.

In 2006 the group of Oslo activated a prospective pilot study (SECA Trial) of LT for CRLM; 25 patients with R0 colorectal resection, unresecable liver metastasis, treated with al least 6 weeks of chemotherapy and no extra-hepatic disease were enrolled and 21 underwent LT. From this study derived the first promising results with 5-yrs OS of 60% [9]. These survival figures were significantly greater if compared with similar patients undergoing only systemic chemotherapy (less than 10% 5-yrs survival in NORDIC VII study [10]). Even though recurrence rate was high (6 months median time); metastasis were more frequently pulmonary-only and were associated to a relatively mild outcome only marginally impacting survival (5 yrs OS 72%). When selecting patients in partial response or stable disease after chemotherapy, CEA<80, maximum diameter 5.5 cm and prolonged time after colon cancer diagnosis (more than 2 years), results post-transplantation are even more relevant.

Currently several prospective trials are ongoing to further address the potential of LT for CRLM. The SECA-II Trial (NCT01479608) by the Oslo group [11] is a randomized controlled trial comparing OS between patients undergoing LT or liver resection . The preliminary published data [11] on 15 LT in super-selected CRLM patients reported 5-yrs OS of 83%. The SECA-III Trial (NCT03494946) is comparing in a randomized matter patients undergoing LT versus standard chemotherapy. The same group conceptualized the RAPID (Resection and Partial Liver Segment 2/3 Transplantation with Delayed Total Hepatectomy) Trial (NCT02215889) using partial graft from deceased donors [12]. The TRANSMET Trial (NCT02597348) from France is a multicenter study recruiting patients to be randomized to receive standard of care chemotherapy or LT plus chemotherapy using cadaveric grafts. Two recent trials are exploring the role of living donation as a source for CRLM. The Toronto study (NCT02864485) using right lobe for LT and the Germany group (LIVER-T(W)O-HEAL NCT03488953)[13] with two-stage hepatectomy in combination with a left-lateral living donor liver transplantation.

Independently from the results of the ongoing studies there is now increasing internationally interest to explore LT as a treatment option in selected unresecable CRC patients. Considering the possity of therapeutic bullets against metastatic CRC the hope of a new role for LT is great.

### 1.3 RAPID: the concept

According to Line et al. [12] the acronimus for RAPID is: Resection And Partial Liver Segment 2/3 Transplantation With Delayed Total Hepatectomy.

RAPID is the results of various surgical technique and physiopathological knowledge: the concept includes conceptual elements deriving on one side from the classic Auxiliary partial orthotopic liver transplantation (APOLT) and on the other from the two staged hepatectomy. **Figure 2**. It is indeed an auxiliary liver transplantation where a small liver partial graft (namely left lateral segments from living or cadaveric donors) is implanted orthotopically after a left hepatectomy of the native liver. Subsequently, in order to implement a fast regeneration of the transplanted segments a portal flow diversion is operated in the direction of the future remnant as in the ALPSS (Associating Liver Partitioning and portal vein Ligation for Stage Hepatectomy) techniques. After obtaining a fast regeneration of the auxiliary future remnant liver the native liver hepatectomy is completed as in a two stage-hepatectomy. The concept represents one of the most fascinating in the whole surgery.

The rationale of this new surgical approach is to enable the transplant of small grafts in adult patients with liver malignancies, expanding the donor pool with little or no negative impact on waiting lists, especially in the case of living donation (LD).

### 1.4 LD-RAPID

The RAPID concept foresees the use of left lateral sector (segment II-III) as partial auxiliary graft. The major source of these are the pool of deceased donor not more suitable for the pediatric list due to age criteria. One of the biggest limits of the procedure is the lack of organs, that is a consistent reality, mostly in Italy. To bypass this issue, the use of left lateral segment provided by living donor has been proposed. As far as living donors are concerned, it has diffusely shown how the donation of left lateral segments (for pediatric recipient) is associated to an extremely low profile of morbidity risk whereas mortality is basically close to 0%. In terms of outcomes, risk and ethical equipoise, left lateral segment donation has been compared to kidney living donation. Moreover, the LD- RAPID option has also the advantage to bring this complex procedure in the context of an elective setting.

From the technical point of view, this new procedure constitutes the sum of the latest developments in liver surgery and liver transplantation that is a combination of the living donor liver transplant (LDLT), APOLT and ALPPS procedures. Therefore, it can and should

be performed only in highly specialized centers having the appropriate technical background and skills.

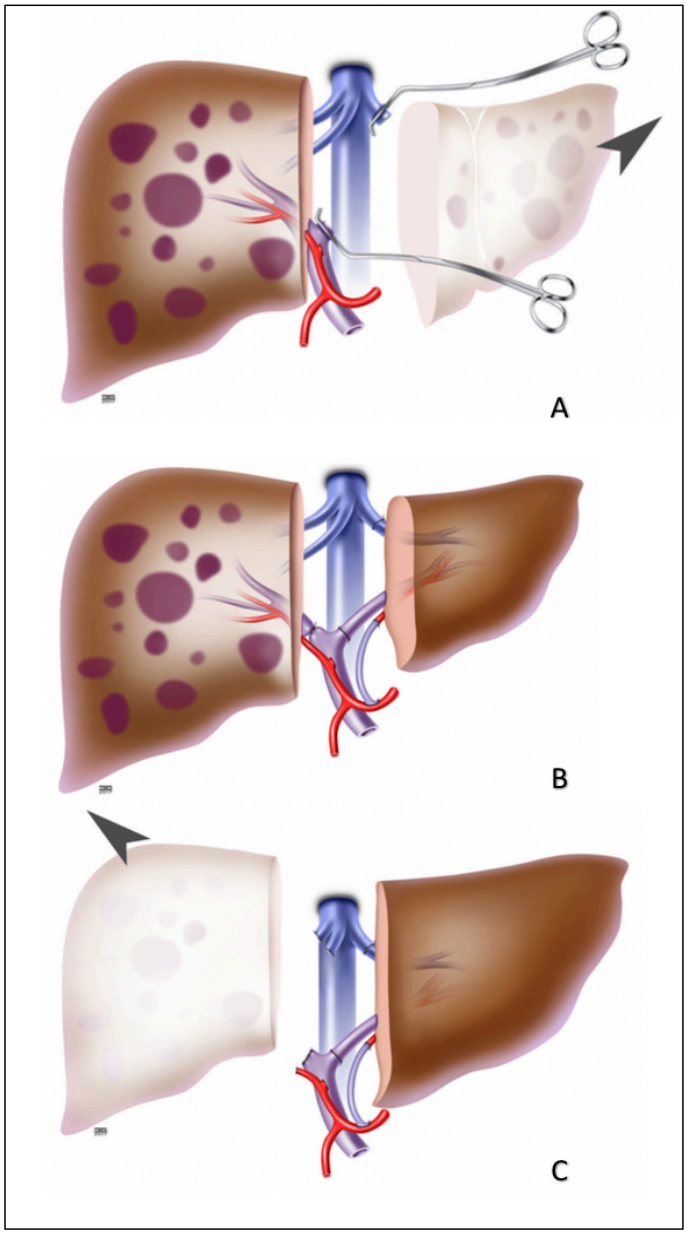



***Figure 2.*** *The RAPID technique*

*A) Step 1: recipient left hemihepatectomy*
*B) Step 1: auxiliary transplantation of left lateral segment*
*C) Step 2: Right hepatectomy*

### 1.5 RAPID: the innovative finding

The RAPID technique answers to some major clinical and pathophysiologic demands:

- *Provides "extra donor resources"*

In Italy waiting list mortality for LT is still around 5-7%; new donor resources are, therefore, needed. Living donor liver transplant have never really took off in Europe due to cultural and logistic reasons. In particular, the donor risk of about 1% mortality is seen as a relevant negative drawback. Patients themselves often refuse the LDLT option not to put at risk the relative life. In turn, donor morbidity and mortality risk is associated to the relevant liver mass needed often overcoming the 60% of liver parenchyma. In the RAPID concept, donor resource may be a conventional (Left lobe) split liver from a cadaveric donor or a live donor. In Italy donors younger than 50 years are offered to the pediatric centers to cover the pediatric national waitlist. However, selected donors older than 50 years may provide excellent left lateral splits available for RAPID without impacting the scarce donor pool. As far as living donors are concerned, it has diffusely shown how the donation of left lateral segments is associated to an extremely low profile of morbidity risk whereas mortality is basically close to 0%. In terms of outcomes, risks and ethical equipoise (**Figure 3**), left lateral liver living donation has been compared to kidney living donation. In this sense RAPID technique represents a potential breakthrough opening liver transplantation to a virtually infinite donor pool. Furthermore, the LDLT option has also the advantage to bring this complex procedure in the context of an elective setting.

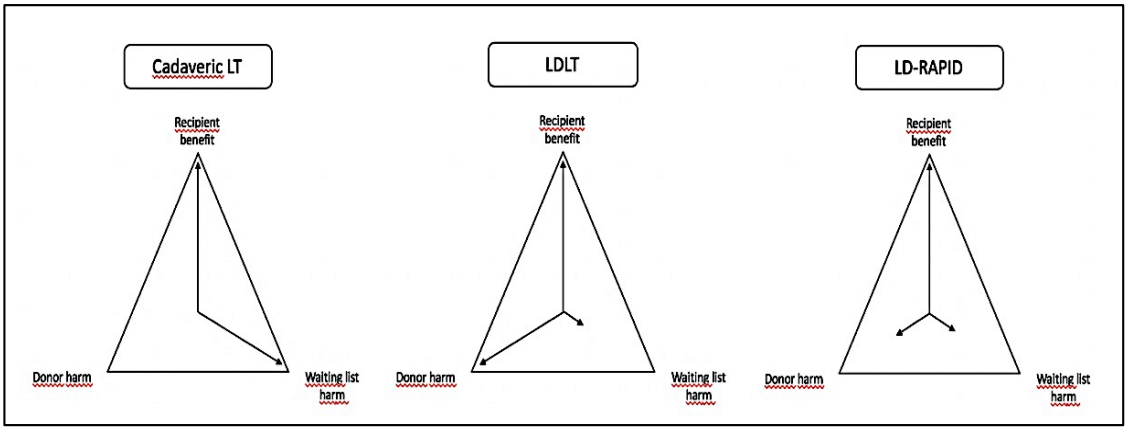



***Figure 3.*** *The donor "equipoise"*

- *Overcome an insufficient metabolic mass typical of that of left lateral segments (with the aim to sustain life).*

Keeping a sufficient functional liver mass in the time during the hypertrophy of transplanted left lobe is essential for patient life. The residual metastatic right liver acts as an additional support until the grafted liver regenerates to sufficient size. The remnant native portal vein ligation is crucial in promote graft regeneration.

- *Prevent the shear stress on portal endothelium associated to an excess of portal pressure in relation to the small vascular bed of a small graft.*

In LDLT a graft to body weight ratio (GRWR) of at least 0.8 is needed to avoid a small for size syndrome after transplantation. For this reason a left lateral liver is never used to transplant ad adult recipient. The only condition in which a GRWR lower than 0.8 is tolerated is when portal pressure is reduced to less than 20 mmHg. This condition has the aim to avoid endothelial shear stress and intrahepatic porto-arterial buffer alteration. Many techniques to achieve such a pressure reduction have been described to date; among the others, splenic artery ligation and partial porto-caval shunt are the most relevant. In the RAPID experience after left lateral segment implantation in orthotopic position, an accurate measurement of portal pressure and flow is mandatory. Several measurements have to be realized before and after clamping of the portal vein to the native liver. If, after clamping the pressure goes above 20 mmHg, a splenic artery ligation or a different portal modulation flow has to be put in place. If it remains stable below 20 mmHg the portal vein of the native right liver can be ligated. In the case described by Line et al. the RAPID technique allowed a transplantation with an astonishing and never presented GRWR of 0.36; after reperfusion portal vein pressure was 14 mmHg so it was decided to close the portal inflow to the native liver using a vascular stapler.

- *Promote a quick future remnant regeneration allowing to complete the native hepatectomy in the context of a safe two stage hepatectomy.*

The regeneration of the graft implanted in orthotopic position is promoted by portal flow divertion to the graft after ligation of the portal to the native liver. The principle has been diffusely tested and demonstrated in the context of the ALPSS procedure. The relevant cytokine release induced by parenchymal transection plays a role in the induction of a rapid regeneration as shown by Schlegel et al [14]. In the Oslo case the interval between auxiliary transplantation and second stage hepatectomy was 23 days.

### 1.6 Aim the study

Aim of my project was to develop and to expand the concept of Transplant Oncology in the Padua Liver Transplant Center and to define potential prognostic factors of recurrence. Patients with unresecable CRLMs were screened and careful selected for potential Liver Transplantation.

To reach this purpose different scenario were investigated:

1.TRANSMET Protocol: we are one of the 2 Italian centers involve in this international multicentric randomized protocol offering LT+CT versus CT alone.

2. Liver transplantation using a RAPID technique: we performed the first Italian case of RAPID in a CRLM patient that refused TRANSMET randomization. Technical refinements were applied compared to the classical RAPID procedure.

3. Molecular studies and liquid biopsy: we are performing liquid biopsy for all patients underwent LT for cancer indication (HCC, CCA, CRLM)

4. Production and submission of new protocols of LT for CRLMs: RAPID Padova and MELODIC (Colorectal metastasis and liver transplantation with organs from deceased donors: an inductive Padova center protocol). We have designed two experimental protocols recently approved by the ethical Committee.

# Chapter 2

# TRANSMET study

TRANSMET is a Phase 3, international multicentric randomized parallel group open trial designed in two arms:

- Chemotherapy followed by LT: Group LT+C
- Chemotherapy alone: Group C

The population is represented by patients with confirmed unresectable liver-only metastases well controlled by chemotherapy (no progression) and extensively explored by modern imaging techniques.

## 2.1 Study aims

The primary objective of the trial is to validate in a large multicentric cohort of selected patients the possibility to obtain at least 50% 5-years survival with LT combined to chemotherapy compared to around 10% with chemotherapy alone.

*Primary aim*

- 5 years overall survival (OS)

*Secondary aims*

- 3-years overall survival (OS) in the 2 arms
- Disease free survival (DFS) at 3 and 5 years (in LT+C arm)
- Progression free survival (PFS) at 3 and 5 years (in C arm)
- Recurrence rate at 3 and 5 years in the 2 arms LT+C arm
- Quality of life (EORCT)

## 2.2 Inclusion criteria

*Inclusion criteria*

- ≥ 18 and ≤ 65 years

- Good performance status, ECOG 0 or 1
- Histologically proved adenocarcinoma in colon or rectum
- BRAF wild–type CRC on primary tumor or liver metastases
- High standard oncological surgical resection of the primary defined by : safe margin of resection; curative resection of primary tumor according to oncological principles; TNM adequate staging
- Absence of local recurrence on colonoscopy (within 12 months before inclusion)
- Confirmed non resectable colorectal liver metastases by the validation committee
- ≥ 3 months of tumor control during the last chemotherapy line: Stable or Partial Response on RECIST criteria
- ≤ 3 lines of chemotherapy for metastatic disease
- CEA < 80 mg/L or a decrease ≥ 50% of the highest serum CEA levels observed during the disease
- Absence of extrahepatic tumor localization according to CT scan and PET-CT
- Renal function should be within the normal limits: no need for extra-renal purification procedure, hemodialysis or kidney transplantation associated (nephrologist assessment)
- A platelet count> 80,000/mm3; White blood cell count> 2500/ mm3
- Eligible for both treatments groups
- Signed informed consent and expected cooperation of the patient for the treatment and follow up

*Exclusion Criteria*

- Participation refusal
- No health insurance facilities
- General contraindication to LT (Severe cardiopulmonary disease or other life-limiting coexisting medical conditions, extrahepatic malignancy, active alcohol or substance

abuse, active infection or uncontrolled sepsis, lack of psychosocial support or inability to comply with medical treatment)

- Other malignancies either concomitant or within 5 years before liver transplantation
- Patients not having received standard treatment for the primary CRC according to recommended guidelines
- Prior extra hepatic metastatic disease or local relapse
- Pregnancy at the time of inclusion

### 2.3 Study design

Study population is represented by adult patients with confirmed unresectable liver-only metastases, extensively explored by modern imaging techniques and well controlled by chemotherapy. At least 3 months of tumor control during the last chemotherapy line defined as Stable or Partial Response on RECIST criteria is mandatory for inclusion. Regards mutation profile only BRAF wild-type patients are eligible. Patient selection is done by a local multidisciplinary tumor board committee including at least one oncologist, one radiologist and one hepatic/transplant surgeon with the aim to confirm the unresecability of CRLM and to proceed with the evaluation for the protocol. If the selection of the patient is confirmed by the local tumor board, a preliminary informed consent form is obtained by the investigator for examination of the case by the validation committee. After the discussion and the approval by the validation Committee the patient will be randomized in one of the two arms. For the two groups, the randomization is considered as Day 0. **Figure 4**

Group LT + CT: as soon as all the results of the assessments are available and confirm that eligibility for liver transplantation, the investigator will complete the registration in the waiting list of transplant center with requirement to the Organ Sharing Organization of a priority for the transplant. The patient will stop the chemotherapy treatment at registration on waiting list for liver transplantation and LT will be performed within 3 months after the inclusion phase.

If extrahepatic tumor or local recurrence are detected during this pre-transplant evaluation, the patient will be considered as a screen-failure and will not be included in this research.

At the time of LT an explorative laparotomy is mandatory in order to excluded any suspicious deposit and pedicular lymph node. In case of extrahepatic disease discovered at laparotomy patient will premature terminate the research treatment.

Group CT: management of these patients will be the same as all patients with unresectable CLM treated by the local team.

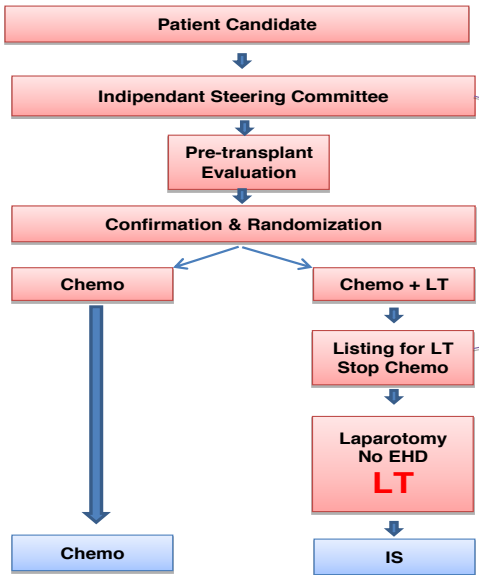



***Figure 4.*** *Study design*

## 2.4 Padova results

The extensive analysis of the TRANSMET study will be pubblished in a few years' time; we are here reporting the ongoing experience at the Padua Liver Transplant Center.

In the period December 2017-July 2019, 11 CRLM patients were screened for inclusion in the study: 6 were excluded during the selection or by the validation Committee due to different reasons:

- Patient 1: diagnosis of concomitant prostate cancer;
- Patient 2: para-rettal nodule at Pet-scan;
- Patient 3: increased size of pulmonary lesions;
- Patient 4: iliac nodes at diagnosis;
- Patient 5: resecable liver lesions;
- Patient 6: disease progression

Five patients were randomized: 2 on Group LT+CT and 3 on Group CT. Patients characteristics are described on **Table 1**. Median age was 50.4 (range 36-60). Primary tumor was located in trasversum colon in 2 cases and rectum-sigma in 3 cases. Liver metastasis were all synchronus; all but one patient received combination chemotherapy as first

therapeutic step, then undergoing high standard R0 resection of the primary tumor. Median time between diagnosis and colon resection was 99 days. The only patient that did no undergo systemic chemotherapy before being operated experienced intestinal obstruction as first disease presentation and underwent thus urgency surgery. The majority of the patients had a (y)p tumor ≥T3 (80%). Mean CEA at diagnosis was 236, mean Ca 19.9 was 1631. All patients were BRAF wt. At the time of diagnosis, the number of liver lesions was >20 in 80% of cases with up to 50 metastases in 1 patient; the median size of the lesions was 50 mm ranging up to 75 mm. At randomization the maximum size of largest metastatic lesions determined by CT/MRI scan was 52 mm; most of patients had more than 20 lesions at the validation discussion.

***Table 1 .*** *Patients characteristics*

| Patient | Gender | Age | Primary tumor | CRLM (n°) | TNM | Nodes + | Adjuvant CT | Random | LT | Status |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | F | 60 | trasversum colon | >50 | T4N1cM1a | nd/11 | FOLFOX Bevacizumab | CT | NA | ***DEAD*** |
| 2 | F | 60 | rectum-sigma | >40 | T3N0M1a | 0/15 | FOLFOXIRI Bevacizumab | LT | No due to PD° | ***DEAD*** |
| 3 | M | 47 | sigma | 12 | T4aN1aM1a | 0/33 | FOLFOXIRI Bevacizumab | CT | Yes* | ***ALIVE*** |
| 4 | F | 36 | rectum-sigma | 20 | T2N1aM1a | 1/23 | FOLFOX Cetuximab | LT | Yes | ***ALIVE*** |
| 5 | M | 49 | trasversum | 20 | T3N1cM1 | 0/60 | FOLFOXIRI Bevacizumab | CT | NA | ***ALIVE*** |

° PD: Progression disease ; * RAPID

**Survival and DFS**

Due to small cohort (n=5) it was not possible to perform a statistical survival analysis. Only two patients underwent LT: one within the protocol and one outside the protocol (RAPID technique). At 1 year from the randomization both patients (100%) are still alive and in good clinical condition; unfortunately both had tumor recurrence. Hilar nodes, liver and lung in one case at 4 months and liver and lung in the other at 5 months. One patient was randomized in the Group LT+CT but she had progression in waiting list (**Figure 5**) so she dropped from the

study. She continued chemotherapy but she progressed and died 8 months from the randomization.

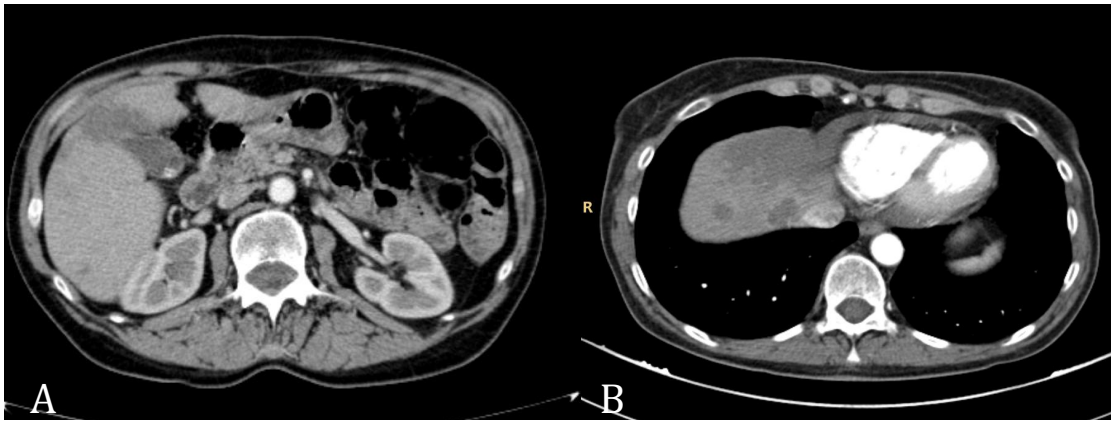



***Figure 5.*** *CT-scan A) At randomization, B) One month from randomization*

For the other due patients randomized in the Group CT the first died 10 months from the randomization due to tumor progression while the other one is still alive and in good condition with stable disease at 8 months from the inclusion. Overall survival of patients is represented in **Figure 6**.

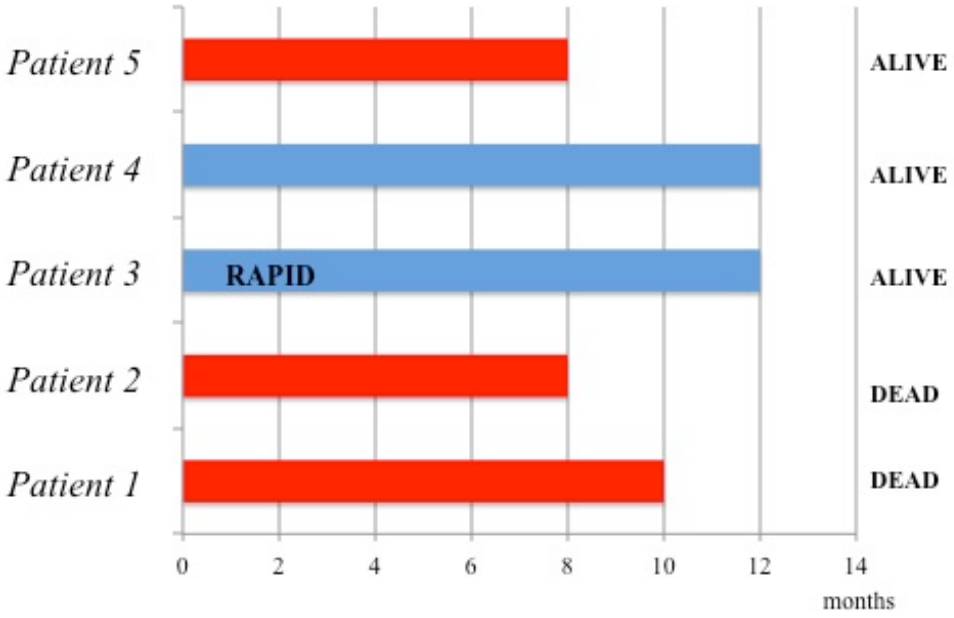



***Figure 6.*** *Survival figures. Red square Group CT, Blue square Group LT+CT*

The only patient that underwent LT within the protocol is a young female, 36 yrs. On December 2017 she had diagnosis of neoplastic ulcerative lesion of sigma. She underwent

conversion chemotherapy with Cetuximab – FOLFOX with partial response and then to left colo-proctectomy. Istology confirmed adenocarcinoma ypT2 ypN1a (1 lymphonode positive on 23) cM1a; IVA. After surgery she had CT with FOLFOX+cetuximab (7 cycles). Liver bilobar metastatic lesions were considered unresecable by local tumor board. **Figure 7**

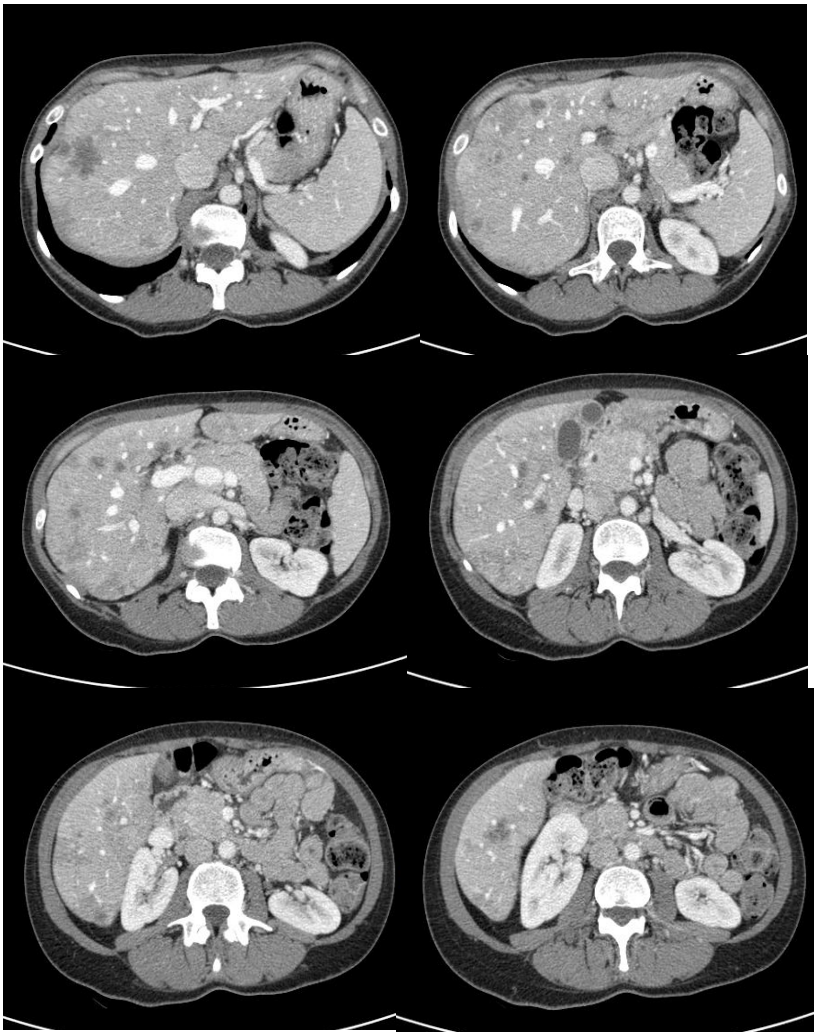
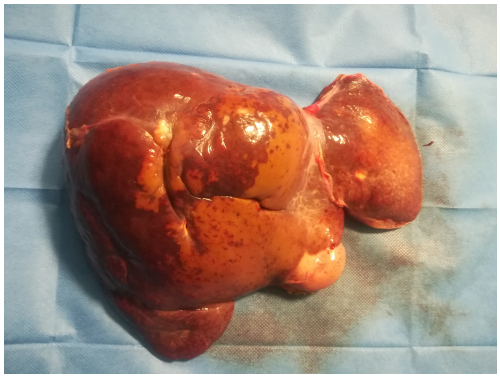


***Figure 7.*** *CT-scan at diagnosis*

On October 2018 she was randomized to the Group LT+CT and one month later she had liver transplant from a deceased donor. The maximum number and size of largest metastatic lesions determined by CT scan before LT were more than 20 and 29 mm. At the explant liver 21 metastatic lesions were confirmed with no vascular invasion (larger lesion 25 mm). **Figure 8** . Post-transplant course was uneventful and patient was discharge on POD14.



| CARATTERIZZAZIONE FENOTIPICA DELLA NEOPLASIA | |
|---|---|
| **Variabile** | |
| Numero di noduli | 21 |
| Diametro del nodo maggiore | cm 2,5 |
| Grading istologico | G2 |
| Necrosi | 0% |
| Fibrosi | ~60% |
| Neoplasia vitale | ~40 |
| TRG | 3 |
| Invasione macrovascolare | assente |
| Invasione microvascolare | assente |
| Invasione endobiliare | assente |
| Invasione perineurale | assente |
| Satellitosi | assente |
| Status linfonodale | negativo |

| DANNO EPATICO ASSOCIATO A CHEMIOTERAPIA | |
|---|---|
| **Variabile** | |
| Steatosi | basso grado (~10%) |
| Steatoepatite | assente |
| VOD/SOS | grado lieve/moderato |
| Iperplasia Nodulare Rigenerativa | assente |

***Figure 8.*** *Explant liver*

On March 2019 hilar nodes uptake (SUV 6.24) was found at the Pet-CT. One month later she started chemotherapy with the scheme FOLFIRI-Bevacizumab. A CT-scan after 3 months revealed progression disease with liver and lung metastatic lesions. **Figure 9.** She in still doing chemotherapy.

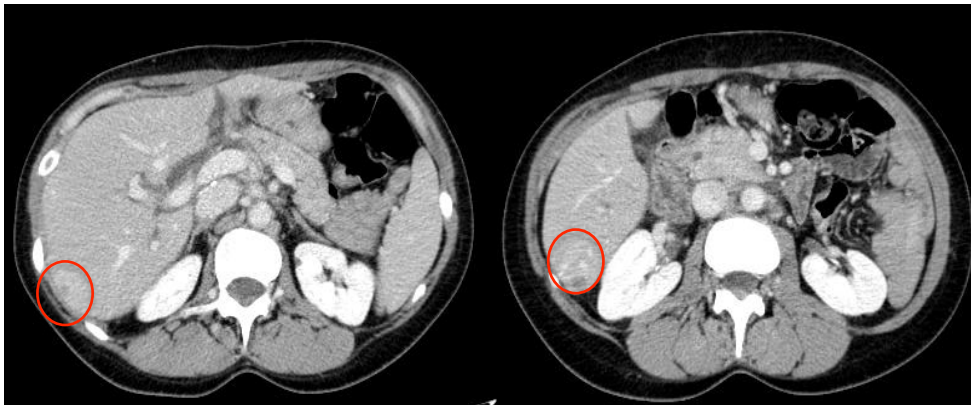


***Figure 9.*** *CT scan: recurrence disease*

## 2.5 Discussion

For liver transplant in CRLM patients the Oslo group (SECA I and II) reported 5-yrs overall survival of 60% and 83%. Our cohort is too small to produce a Kaplan Meier survival curve, then, currently, we can reports only a descriptive analysis. With a median follow up of 10 months (range 8-12 months) we notices that all transplanted patients (n=2) are still alive at 1 year from randomization; only one patient in CT are currently alive while the others two died within 1 year.

Also the recurrence profile is comparable with the one reported by the SECA group; 90% in the SECA I with a median time of recurrence of 6 months. We had relapse in both transplanted patients (100%) within 6 months: recurrence are on lungs, liver and hilar nodes.

Metastasis are millimetric and both patients are actually on chemotherapy. Differently from the TRANSMET study that allow CT post-transplant, in the SECA studies investigators decided to not propose chemotherapy. The reason are the "confounding factor" potentially represented by the CT and the assumption that patients had yet received chemotherapy for an extensive time pre-transplant. Same attitude is adopted by the RAPID group: in this context post-transplantation chemotherapy might hamper liver regeneration precludig successful tranplant. Differently in the Toronto trial, patients receive a post-transplant standard-of-care chemotherapy based on FOLFOX or FOLFIRI with or without bevacizumab. The Oslo approach for recurrence is surgical resection if possible. Considering the factors affecting survival derived from SECA I as time from CRC surgery to liver transplantation < 2 years, CEA levels pre-LT >80ug/L, tumor diameter >5.5 cm, progression disease at the time of LT our patients were all stable at the time of LT with low CEA level (20ug/L). Time from primary tumor resection and liver transplant was < 2 years for both (mean 7 months): largest lesion was 75 mm in one and 35 in the other with important reduction in volume before LT.

From the TRANSMET experience some ethical aspects needed to be discussed. First, differently from Scandinavian countries (especially Norway) that have a surplus of deceased donor organs, with median waiting time less than a month, Italy experiences a dramatic organ shortage. In this scenario offering deceased donor organs for CRLM-LT recipients, especially when hardcore evidence in its favour is yet lacking and relapse profile is significant, remain debated. The alternative, represented by the use of live donor LT may help ease this situation; ongoing clinical trials (Toronto study and LIVER-T(W)O-HEAL) are now recruiting in this manner. Even with LDLT, the principle of double equipoise (balancing donor safety with recipient outcomes) need to be addressed conclusively before LT for CRLM can become part of its evidence-based treatment algorithm. Second, despite randomization represents the gold-standard in medical research is many times challenging in the field of transplantation. The liver transplant for HCC itself has been never validated by a randomized study, but is represent a recognized gold standard treatment. Probably studies in the field of Transplant Oncology may just need to focus on comparability rather than randomization.

## Chapter 3

## RAPID technique

We performed the first case of RAPID technique in Italy in December 2018. It was the sixth RAPID procedure ever worldwide, the second case using a living donor [15] and the first case with a minimally invasive approach for the second stage hepatectomy.

PATIENT – M.F.

### 3.1 Pre-operative setting

A 47-year old previously healthy man was diagnosed in November 2017 with advanced colon cancer and synchronous liver metastases. At the time of diagnosis, the liver metastases were evaluated as inoperable, due to number (> 10) and bilobar distribution. **Figure 10** The unresectability was first evaluated by the Padua multidisciplinary oncologic meeting and subsequently confirmed by an international multidisciplinary board. The CEA level was 195,6 µg/L.

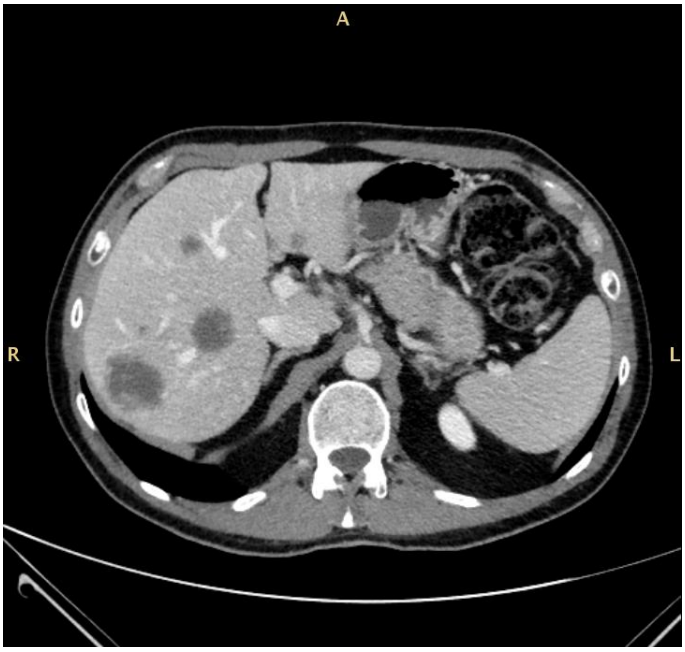



***Figure 10.*** *CT scan at the diagnosis: liver metastasis*

He was initially treated with systemic chemotherapy according to the protocol FOLFOXIRI + Bevacizumab from December 2017 to March 2018, for a total of 6 cycles without any significant side effects. The chemotherapy led to partial response, in accordance with

RECIST criteria. The CEA level fell to 39,7 µg/L.

On April 2018 he underwent to a laparoscopic sigma resection and lymphadenectomy, radically confirmed. Pathological examination of the primitive tumor revealed a well differentiated (G1) colon adenocarcinoma, infiltrating perivisceral fibro-adipose tissue, subcutaneous tissue and with initial infiltration/overcoming of serous membrane; specifically: pT4, pN1a (1/33 lymph nodes), cM1a (liver), stage IVa. The mutation profile revealed:

| | |
|---|---|
| kRAS | Wild type |
| nRAS | Mutated → Eson 2, Codon 12, G12D |
| BRAF | Wild type |
| MLH1 | ++ |
| PMS2 | ++ |
| MSH2 | ++ |

Postoperatively, no surgical complication occurred. The CEA level fell to 11,6 µg/L. Adjuvant chemotherapy was administrated from May to August 2018, according to FOLFOXIRI + Bevacizumab protocol for 6 cycles. Then, in September, the treatment was depotentiated to maintenance capecitabine.

The radiological re-evaluation by CT-scan, highlighted the stability of the disease, confirming unresectability Therefore, the patient was evaluated for inclusion in the TRANSMET protocol. After random assignment to the chemotherapy arm, the patient refuses and decided to drop the study in total autonomy.

Considering his optimal performance status, prior discussion into a multidisciplinary meeting, we proposed the two-stage hepatectomy in accordance with the RAPID technique with a living donor auxiliary liver transplant. In several informative conversation were discussed with the patient and his family the risks and potential benefits. After repeated consultation, the patient and his family accepted the innotive procedure we proposed, signing the informed consent. After the evaluation of the eligibility of the donor, identified in the brother-in-law, under both the physical and psychological profiles, the surgery was approved by ethics committee.

So, a clinical-instrumental evaluation for inclusion in LT waiting list was completed. The administration of Bevacizumab was suspended in the middle of October, leaving only Capecitabine as maintenance chemotherapy. At this time, the CEA level was 46,4 µg/L. The CT scan confirmed no apparent signs of extra-hepatic disease. **Figure 11**

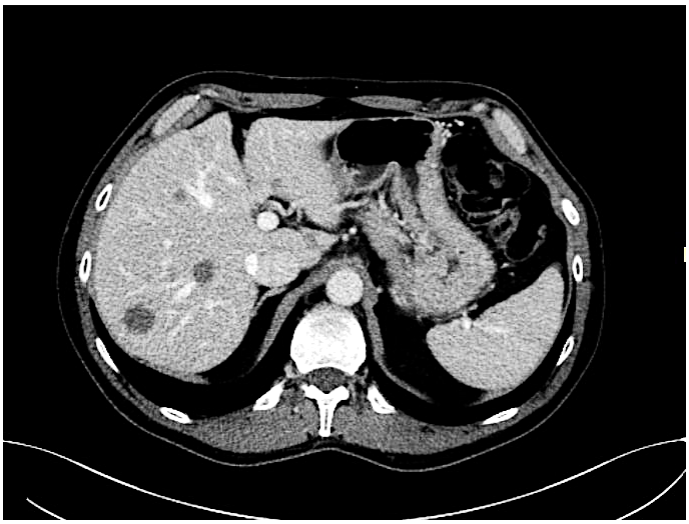


***Figure 11.*** *Pre-operative CT scan*

A liver biopsy was performed to histologically define the liver lesions; the result was totally compatible with primitive diagnosis. The patients was then considered eligible for the RAPID procedure scheduled for December 2018.

A related-donor was evaluated to provide his left lateral segments (LLS). He was an healthy 43 yrs-old man, BMI 25, normal lab test, no previous surgery. At the volumetric CT scan with 3D reconstruction total liver volume was 1983 ml; left liver volume was 392 ml (19.8%). Recipient weight was 68 kg, GRWR on volume was 0.56. At pre-operative assessment a weak positivity at lupus anticoagulant (LAC) was found. Antithrombotic prophylaxis was planned as follow: low-molecular weight heparin (LMWH) 4000 UI the day before the surgery, intermittent pneumatic compression (IPC) during the surgery and LMWH 4000 UI from day 1 to day 20 post-operative.

### 3.2 Surgery

*RECIPIENT - 1^stage: left hepatectomy*

Patient underwent transplantation on December 2018 with a left lateral graft from living donor.

***Table 2.*** *Pre-operative blood test*

| **Parameters** | **Patient's value** |
|---|---|
| Haemoglobin >10 g/dL | 14,0 g/dL |
| Neutrophils >1000/mm$^3$ (after any G-CSF) | 1680 /mm$^3$ (no G-CSF) |
| Thrombocytes >75.000/mm$^3$ | 188.000/mm$^3$ |
| INR | 1,67 |
| Bilirubin < 2 x upper normal level | 0,48 mg/dL [0,1-0,99] |
| AST < 5 x upper normal level | 24 U/L [10-45] |
| ALT < 5 x upper normal level | 17 U/L [10-50] |
| GGT | 62 |
| Creatinine < 1,25 x upper normal level | 0,69 mg/dL [0,65-1,2] |

The surgery starts with the laparoscopic exploration of the abdominal cavity: it confirmed diffuse and bilobar liver metastatic disease; no evidence of extra-hepatic abdominal disease. Due to the absence of macroscopic contraindications, we proceed with surgery both on donor and recipient.

We performed a bilateral subcostal incision prolonged in supra-umbilical median. The left liver was mobilized and dissected free from the retrohepatic vena cava. We proceed with binding and section of left portal vein and left hepatic artery; an accessory left hepatic artery originating from gastric artery was detected and sectioned.

Cholecystectomy and intraoperative cholangiography were executed. Then, a thermoablation of the whole future transection plan (passage between Sg IV/V-VIII) was performed. MWA antenna was infixed every 3 cm along the plan, being careful not to get close to the bile duct. 5-minutes ablation cycle at 60W was executed for single infixion. Then a left hepatectomy enlarged to median suprahepatic vein and enbloc with S1 was completed with the use of Cavitron (CUSA) and monopolar forceps. During the procedure, we sectioned the left bile duct and the left and median suprahepatic veins, in order; that left lobe was extracted and left portal vein, left gastric artery and cava vein were prepared for the future anastomosis. Recipients graft weight was 390 gr.

At the pathology examination 11 metastatic lesions were detected (size 0.4-2 cm).

*DONOR: left lateral resection*

Donor operation started after recipients extrahepatic disease was ruled out at the explorative laparotomy We started with a supraumbilical median laparotomy. The future transection plan was marked on the right of falciform ligament. The hepatic hilum was isolated, and a cotton

ribbon was prepared for Pringle maneuver. After the identification and isolation of all the vascular structures, cholecystectomy and intraoperative cholangiography were performed to check biliary system.

Then, we completed the left resection; the parenchymal transection was executed with Cavitron (CUSA) and monopolar forceps with water-drip. **Figure 12** The liver remained transected without division of hilar structures until the recipient's left lobe was resected and the feasibility of the procedure was completely guaranteed. After that, we proceed with cold-section of graft biliary duct and cross-clamp and section of left hepatic artery, left portal vein and left supra-hepatic vein, in order. Single dose of LMWH 4000 UI was infused i.v before the division of hepatic artery and protamine sulfate i.v was subsequently administrate to reverse the anticoagulant effect. EBL was minimal, no PRBC transfusions were needed.

*BACK TABLE*

The graft weight was 423g with a GBWR of 0.62%, compatible with the estimate one by pre-operatory volumetry. It was placed in a sterile bag inside a container. The latter was filled with sterile ice, which has been replaced regularly to maintain the temperature at 4°C. The graft was then flushed with preservation solution (Celsior) at 4° through the artery, the portal and suprahepatic vein (2L in the portal vein, 1L in the supra- hepatic vein) and biliary duct to test leakage on the vessels and at the cut surface. Further cut-surface sutures were performed to achieve satisfactory hemostasis. Supra-hepatic vein extension with a 'ring' made from saphenous vein of the recipient was implanted with continues suture using prolene 5/0. **Figure 12**

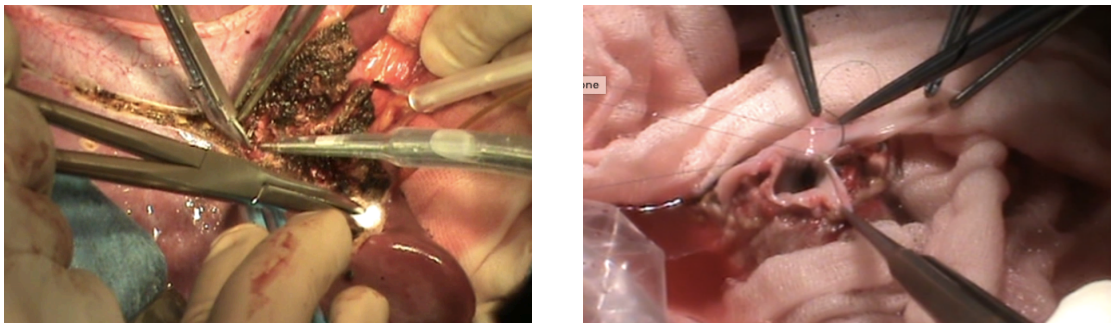


***Figure 12.*** *Intraoperative phase and back table*

*RECIPIENT – 1^stage: auxiliary transplant*

At the end of back table, we proceed with the auxiliary Sg II-III liver transplant. We first fashioned the supra-hepatic vein anastomosis: the graft hepatic vein was anastomosed to recipient cava vein with an end-to-side fashion in continues suture with 4/0 prolene. Then, the

graft left portal vein was anastomosed to the recipient left portal vein with an end-to-end fashion in continues suture with 6/0 prolene. Subsequently, we reperfused the graft. In the end we fashioned the arterial anastomosis with the help of the operating microscope (ZAISS). In particular, the graft left hepatic artery was anastomosed to the recipient left gastric artery with an end-to end fashion by detached points with 8/0 prolene.

*Flows*. At the end of the anastomosis, we performed intraoperatory Doppler-US with confirmation of good portal and arterial flows, checked also with flow-meter. In particular, portal flow was about 80 ml/min/100g and arterial flow 27 ml/min/100g.

*Pressure*. After these measurements, we evaluated the left portal pressures using a catheter inserted through splanchnic collateral vein up to the portal trunk. We checked the pressure values both with pervious and clamped right portal vein with several measurements. In both cases, the left portal pression to the graft remained under 20 mmHg, in particular the maximum value was 19mmHg with clamped right portal vein. This value remained stable for almost 3 minutes. Therefore, we decided to tie the right portal vein without diversion maneuvers of the portal flow.

We performed a further ultrasound check that confirmed optimal inflow and outflow. We fixed the graft to the parietal peritoneum. Left biliary stump was then anastomosed on a Roux-en-Y loop.

*INTERSTAGE: POD1-POD18*

The patient was discharge from ICU on POD3. Immunosuppressive therapy consisted of induction therapy with Basiliximab and Steroids and maintenance therapy with Tacrolimus, Mycophenolate Mofetil and Steroids, according to our standard protocol after liver transplantation. We couldn't perform the switch from MMF to mTOR-inhibitor, as recommended in Oslo protocol and Hamburg data, due to the proteinuria (1 mg/die) established after administration of Everolimus. The post-operative course of the first stage was uneventful apart from a right pneumothorax raised during surgery that has been treated with a thoracic drainage, until complete resolution on POD14 (Clavien IIIa).

We overlooked the orthotopic liver growth with CT-scan plus volumetry on POD 1 (480ml – 34,8%), POD 7 (645ml – 41,2%) and POD 14 (672 ml – 43,2%). **Figure 13**

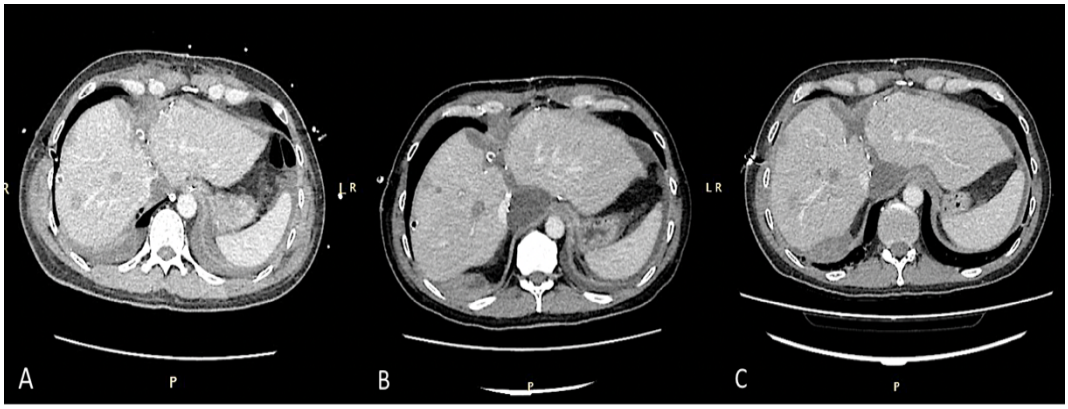



***Figure 13.*** *CT-scan after surgery A)POD1 B)POD7 C)POD14*

For a more thorough assessment of liver function, a hepato-biliary scintigraphy with iminodiacetic acid (HIDA scan) were executed on POD 16, reporting a relative caption of 35% for right lobe (native liver) and of 65% for left lobe (future liver remnant). These results were a further confirmation of the ability of future liver remnant to guarantee the liver function necessary to respond to systemic metabolic demands.

At POD 14 volume and the function confirmed to HIDA were enough for guarantee liver autonomy so second stage was planned on POD 18. Graft volume enlargement was monitored over time. **Figure 14**

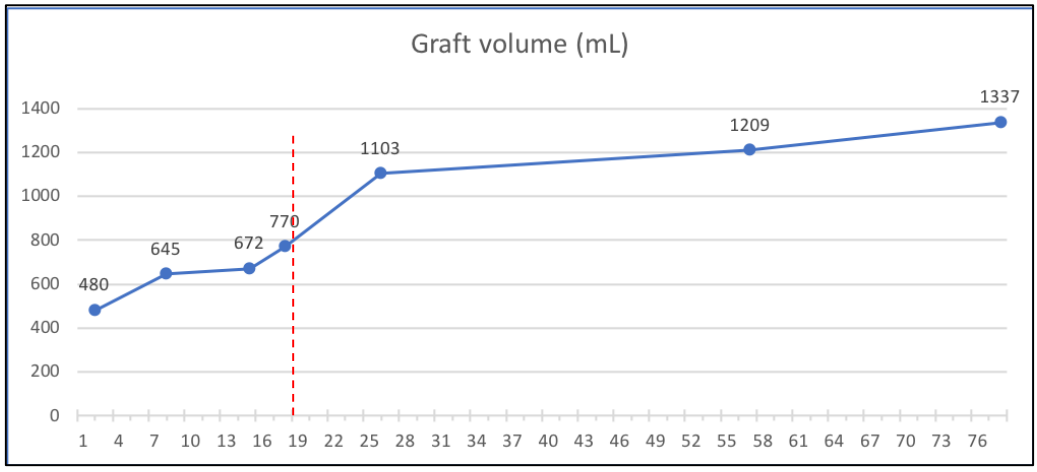



***Figure 14.*** *Graft volume*

*RECIPIENT – 2^stage: laparoscopic right hepatectomy*

On POD 18 we completed the second stage (right hepatectomy) laparoscopically. We started with an accurate exploration of the abdominal cavity: we found minimal imbibition of biliary material and no evidence of extra-hepatic disease or intracavitary collections.

After hepatic artery clamping, we performed the Indocianine Green Test (IGC15=14.9%) for the evaluation of the function of the future liver remnant, in this case of the auxiliary graft. Then we sectioned the right hepatic artery, right portal vein and right biliary duct, in order. We sectioned the right supra-hepatic vein, achieving the complete mobilization of the hemiliver. The extraction of the organ was performed through a little right subcostal laparotomy.

**3.3 Post-operative setting**

DONOR The donor was discharge from the ICU on POD1. His postoperative course was characterized by a biliary fistula at low range treated conservatory. He was discharged from hospital on POD6. No medical or psychological complications have occurred thus far.

RECIPIENT After the 2^ stage, the patient was discharge from ICU after 3 days. The postoperative course has been characterized by the functional recovery of the orthotopic liver with progressive normalization of cytolysis index and progressive lowering of cholestasis index and bilirubin in the range. On POD 27 we performed an US-guided percutaneous liver biopsy due to a suspicion of rejection given by the rise in transaminases: the result was negative for rejection. An a hepatic intra-parenchymal hematoma developed as complication of the biopsy; it was treated with drainage during a re-laparotomy (Clavien IIIb). The further postoperative course was uneventful, and the patient was discharged from hospital on POD 37. On POD 57, the follow-up CT revealed a fluid collection localized in right sub-diaphragmatic region. It was drained on POD67 (Clavien IIIa). On POD76, the patient was readmitted to our ward for hyperpyrexia. It was initially treated conservatively with empiric antibiotic therapy, but the CT-scan show two fluid infected collections. A surgical toilet was performed on POD81 (Clavien IIIb). At the control MRI, a biliary fistula was detected and treated conservatively; in addition, the fluid collection was still present. On POD105, the follow-up CT showed an increase in volume of the know collection; it was treated with percutaneous drainage on POD108. (Clavien IIIa).

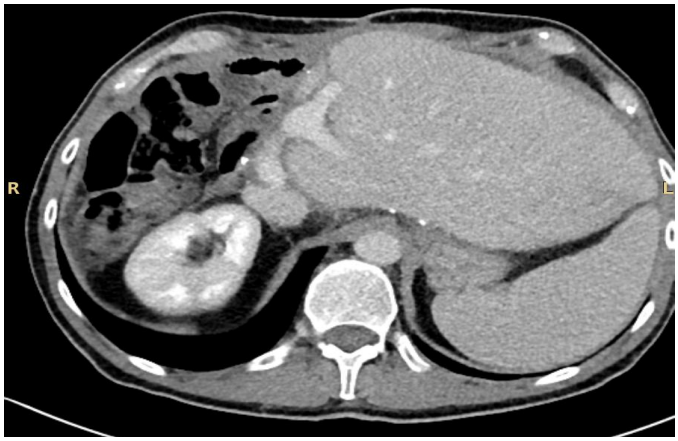



***Figure 15.*** *CT scan graft volume (POD25)*

### 3.4 Follow up

On May, at 5 months after surgery, we found the first radiological evidence of recurrence. The pattern was both pulmonary and hepatic. **Figure 16** In particular, the liver was affected by a single lesion 10 mm in diameter that was treated with a CT-guide MW ablation. A further disease progression was found at CT-scan in June CT-scan: three millimetric hepatic lesions, and lung nodules. After a multidisciplinary meeting, we decided to start the administration of chemotherapy: FOLFIRI + Bevacizumab, still on-going with partial response.

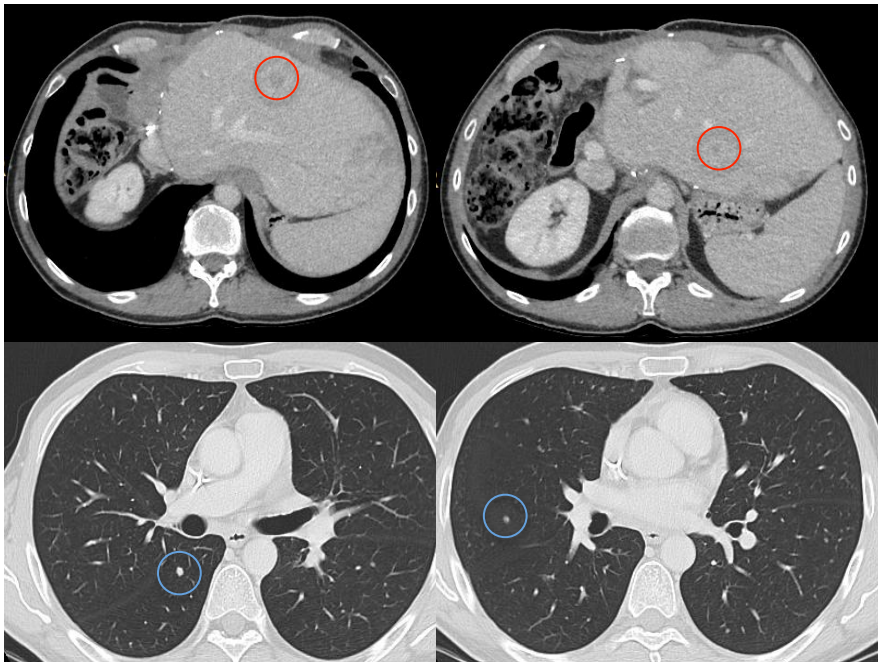


***Figure 16.*** *Recurrence disease*

### 3.5 Discussion

Our case represented a proof of concept; from this single experience we can postulate that RAPID is a safe and feasible procedure. To date, at 10 months from the procedure patient is alive, in good health condition and graft function is normal. Regarding post-operative complication it is not possible to define a complication profile. LOS was 38 days of which 18 days represent the inter-stage period. It could have been shorter if there were no complications following the biopsy. A total of 6 days was spent in the ICU (3 after the first stage and 3 after the second stage). Our patient had several readmission due to biliary fistula but it is in line with the common post-hepatectomy complications.

From an oncological purpose the histological exam of the explant right native liver after 2^ stage confirmed the absence of tumor at the transection (R0) underlining the positive effect of using MW ablation in the section plane and the absence of extrahepatic abdominal dissemination potentially related to the first step procedure. The DFS, defined as the time from liver transplantation to the first evidence of disease recurrence, was about 5 months in our case; this data is lower the median DFS of 10 months reported from the published studies of LT for CRLM. Recurrence pattern was both pulmonary and hepatic. This is in line with the Oslo experience. We know from published reports that in case of lung only recurrence, the prognosis would have suffered the slightest due to a very slow growth of lung metastasis. Hepatic involvement, on the other hand, is a negative prognostic factor and, above all, has a major impact on the donor-recipient balance because it emphasizes very strongly how much transplantation is still a “palliative” therapeutic option. To justify such early recurrence there are several aspects to consider. First of all, the positivity of a lymph node to the definitive post-operative histological examination we had, it means the presence of extra-hepatic disease already present; this certainly played a primary role. Secondly, the patient could not take the m-TOR inhibitor (Everolimus) as immunosuppressive due to associated proteinuria. This drug is known to have both immunosuppressive and anti-tumoral action (cellular growth pathway inhibitor) and it is included in post-transplant immunosuppression protocols for oncological indications (HCC specifically). Currently there is not evidence about its role in liver transplant patients for CRLM. Finally, there are still no definitive results on the RAPID technique, so we cannot know if the "coexistence" of the two livers has had a significant impact.

The donor issue remains a crucial point. In fact, although associated with very low mortality, the risk is always present. In our case, the donor presented as a single complication a low-flow biliary fistula, in line with the classic hepatectomy-related complications. This normal

course, however, becomes significant if it occurs on a healthy patient for the purpose of donation, whose graft remained disease-free for only five months. The donor issue is closely related to the DFS; once this is improved, the donor risk will also become more acceptable.

From a cost-efficacy point it should be noted that the realization of this new cutting-edge technique required three different surgical procedures, with two equipes working simultaneously in the first step. Taking into account also the post-operative management of two patients (donor and recipient), the surgery as a whole cost 150.000€ to the national health system. The problem in this case is not the absolute cost, but the cost-effectiveness ratio. As in the previous case, we are connected to the relationship with the DFS and therefore to the palliative nature of the surgery.

Despite the comment above described in conclusion we believe that the technical refinements we added to the 'classical' RAPID technique demonstrated some potential advanges:

1. *MWA on the transection plan.* First of all, the use of MWA creates an avascular separation and a necrotic groove between the cancer and the future liver remnant in the future transection plan, allowing an easier execution of the left hepatectomy with a limited blood loss during procedure. We believe that this aspect positively affected both the success of a very long and complex operation and the post-operative course. It also improved the oncological radicality on the section shear, confirmed histologically by the analysis performed on the surgical piece after the second stage.

*2. Saphenous ring on the left suprahepatic vein* The saphenous ring implanted on the left suprahepatic vein of the graft avoided the kinking phenomenon ensuring better stability of the anastomosis. The outflow has been greatly improved, with consequent lower probability of complications such as portal thrombosis. In addition, it guaranteed optimum portal pressure for the graft, favoring its rapid hypertrophy.

*3. Minimally invasive right hepatectomy* The choice to perform the second time with a laparoscopic approach, besides representing a world premiere in reference to this new technique, was a must. It has been shown that, whenever possible, laparoscopy should be preferred over the open approach. The second time of the intervention took place in less time than a right hepatectomy with an open approach and minimal blood losses were recorded. Post-operative period was characterized by a rapid functional recovery; in few days, patients started to feed himself again and to move.

# Chapter 4

# LIQUID BIOPSY

## 4.1 The role of liquid biopsy

Although traditional tissue biopsies and imaging studies remain the gold standard in metastatic cancer care, the space-temporal dynamic heterogeneity of cancer limits their utility. Nowadays, it is known that tumor heterogeneity exists not only amongst different patients, but also within individual tumors and among metastatic sites. Multiple cancer sub-clones coexist and evolve simultaneously, with treatment acting as selection pressure. Tumor biopsy at a single site at particular time cannot reflect the entire disease throughout the treatment period. The idea of a minimally invasive way to obtain accurate information from a blood sample, known as liquid biopsy, has gained increasing attention in cancer diagnosis, risk stratification and monitoring/predicting treatment response and acquired resistance. Liquid biopsy refers to the analysis of circulating tumor cells (CTCs) and cell-free circulating nucleic acids, in particular circulating tumor DNA (ctDNA) and exosomes (dtEV), released in the peripheral blood from the primary tumor or metastatic sites. With a simple non-invasive blood sample, the liquid biopsy can provide the genetic and epigenetic landscape of all cancerous lesions and offer the opportunity to track genomic evolution in real-time.

Clinical application of liquid biopsy for precision medicine are:

- Screening and early detection of cancer
- Stratification and therapeutic intervention
- Real-time monitoring of therapy
- Identification of therapeutic target and resistance mechanism
- Prediction of risk for metastatic relapse (prognosis)
- Improvement of drug delivery

### CTCs

The presence of circulating tumor cells was speculated since Recamier coined the term “metastasis” in 1829 and confirmed by Engell’s documentation in 1955. Conventionally isolated CTCs are defined by cell surface expression of epithelial cell adhesion molecule (EpCAM) or cytokeratin (CK), and the absence of the pan- leukocyte marker, CD45. Circulating tumor cells are detectable in blood of patients with metastatic carcinoma. Their role in metastatic disease is generally accepted. High number of CTCs has been showed a correlation with aggressive disease, increase metastasis and lower PFS. Moreover, inverse

correlations between CTCs and PFS/OS have been reported in metastatic cancer of breast [16, 17], colon-rectum [18] and prostate [19]. Therefore, in 2004 the Food and Drug Administration approve finally the IVD use of the CellSearch CTC assay in these three malignancies. CTCs potentially carry valuable information about tumor heterogeneity, invasiveness, drug susceptibility, resistance to therapy [20]. Therefore, they could serve as a real-time biomarker to predict cancer progression and survival in both early-stage and metastatic cancer patients.

**cfDNA**

Circulating-free DNA (cfDNA) originates from both normal and tumor cells that undergo apoptosis or necrosis, and from macrophages that phagocytize necrotic cells, which release naked DNA into the blood circulation and create a residual fingerprint. Although cfDNA was first detected in healthy individuals in the late 1940s, it was not until the 1970s-1980s that neoplastic characteristics were identified and that cfDNA was found to exist in higher concentrations in cancer patients relative to healthy controls. Furthermore, circulating-free tumor DNA (ctDNA) represents the portion of cfDNA specifically derived from apoptotic, necrotic or living tumor cells that actively release DNA in the circulation. It is defined by mutations and other genomic changes that are hallmarks of cancer cell and is a potential surrogate for the entire tumor genome. CtDNA is an informative, inherently specific and highly sensitive biomarker for human cancer. It remains in the circulation for a few hours before being metabolized, which allows real-time monitoring of tumors as they spread and mutate or develop resistance to treatment. Several studies on cfDNA have detected point mutations and microsatellite abnormalities in patients' blood from head and neck cancer, lung cancer and CRC. Many researchers indicated that ctDNA is more sensitive and specificity than CTCs in early detection and presents large dynamic range and considerable relation with changes in tumor burden [21].

### 4.2 Liquid biopsy in CRLM

A strong need exists for a noninvasive tool to improve the prognosis evaluation of colorectal cancer patients, particularly for stratifying stage IV. Metastatic colorectal cancer patients show a broad range of outcomes and no prognosis-validated biomarker is currently available for their management. Several prognostic factors have been reported in literature as predictors of metastatic CRC (mCRC) patient survival: tumor diameter, number of hepatic metastases, node-positive primary, poorly differentiated primary, extra-hepatic disease, positive resection margins, mutational status and carcinoembryogenic antigen (CEA) level [22]. To improve specificity and sensitivity, it has been pursuit a new generation of blood-based biomarkers

that have correlative or biologic value, as well as providing tumor genomic information on which to modify treatment. Although still in development, these new factors, including populations of circulating tumor cells (CTCs), circulating free DNA (cfDNA) and exosomes have the potential from the further development of critical assays to surveil patients with CRC and monitoring drug resistance and response to therapies.

It's demonstrated that circulating tumor cells (CTCs) can be isolated from the blood of patients with mCRC and their presence is a strong and independent prognostic marker in both the metastatic and non-metastatic setting, predicting impair survival [23, 24]. In a multicenter study [25], the detection of CTCs has been showed as both prognostic and predictive factor for patients with mCRC. The presence of 3 3CTCs/7,5 mL at baseline (pretreatment) and follow-up represents the strongest independent prognostic marker compared with others clinical factors for PFS and OS. CTCs enumeration has also been established as an indicator of treatment response: conversion of high to low CTC counts for patients on therapy is associated with improved outcomes [26, 27]. Based on the results of several studies [28-30], CTC enumeration may be a useful tool in stratifying patients and their response to therapy. Patients could be divided based on changes in their CTC counts during treatment to represent different prognostic subgroups:

- Patients without any detectable CTCs prior, to and during treatment
  ➔ longest PFS and OS
- Patients with high CTC counts at baseline which decreases during therapy
  ➔ intermediate PFS and OS
- Patients with persistently elevated CTC counts at baseline and during treatment
  ➔ shortest PFS and OS

The role of CTCs in early relapse detection is currently under investigation. Since CTCs can be collected through serial blood samples, it as an attractive method for obtaining longitudinal molecular and genetic analyses of the tumor and aids in targeted therapy investigations. KRAS mutation in exon 2 (codons 12 and 13) are established as a negative predictive marker for treatment with EGFR inhibitors and several studies have successfully detected mutations in KRAS from CTCs isolated from patients with CRC..

Several studies have shown that ctDNA can serve as a potential molecular marker for poor clinical outcomes in CRC patients and as a tool for early detection of recurrence or metastatic disease [31-33]. Patients with advance CRC and high level of ctDNA had a poor OS [33]. When compared ctDNA and CEA levels it was reported that detectable ctDNA is more sensitive than CEA (ctDNA 100% vs CEA 56%; $p$=0.008) [34]. Detection of ctDNA is a

better predictive marker of disease recurrence than CEA in the postoperative.

CfDNA might used to test RAS status in metastatic CRC, to monitor the efficacy of anti-EGFR agents by tracking early mechanisms of acquired resistance during anti-EGFR-containing treatments [32]. Moreover the early change in the ctDNA level might serve as a biomarker to predict the chemotherapeutic efficacy and clinical outcomes in mCRC patients [35].

The overall concordance of KRAS detection in tumor and plasma was reported to 85% in the IRON study [36] and 100% in the CORRECT trial [37]. Misale et al. [38] detected the KRAS mutant alleles in the circulation as early as 10 months before radiographic evidence of disease progression, underling how qualitative ctDNA analysis could be an earlier predictive marker for development of drug resistance to EGFR-inhibitor and, consequently, of disease progression.

The ability to perform whole genome analysis from CTCs and cfDNA has the potential to uncover biomarkers predictive of sensitivity or resistance to available targeted agents, allowing for personalized therapies and appropriate patient selection to guide management of CRC. Thus, liquid biopsy may become an important companion diagnostic in drug development, especially given the recently reported successful establishment of cell cultures and permanent cell line from CTCs derived from colon cancer patients that allows *in vitro* and *in vivo* drug testing and a variety of functional studies in the cancer biology [39].

### 4.3 Padova experience

MATERIAL AND METHODS

From November 2018 at the Hepatobiliary surgery and Liver Transplant center of Padova in all patients underwent liver transplantation for oncological indications (HCC, CCA, CRLM) a liquid biopsy for the detection of circulating tumor cells (CTCs) is performed at the time of LT and subsequently.

The main objective is to determine the presence and the number of CTC-positive in patients at the time of transplant and at 1 month. The aim is to better stratify patients and to find potential prognostic recurrence factors.

Samples are collected from peripheral vein before the abdominal incision at the day of liver transplant, after one month and in event of recurrence. For CTC measurements, peripheral blood samples is drawn into 10 ml CellSave® Preservative Tubes (Janssen Diagnostic,

Raritan, NJ) and process within 96 hours after collection. The enumeration of CTCs in whole blood is performed with the CellSearchTM Circulating Tumor Cell Kit. An event is classified as a tumor cell when its morphological features are consistent with that of a tumor cell and it exhibits the phenotype EpCAM+, CK+, DAPI+ and CD45-. **Figure 17** Results are expressed as the total number of CTCs.

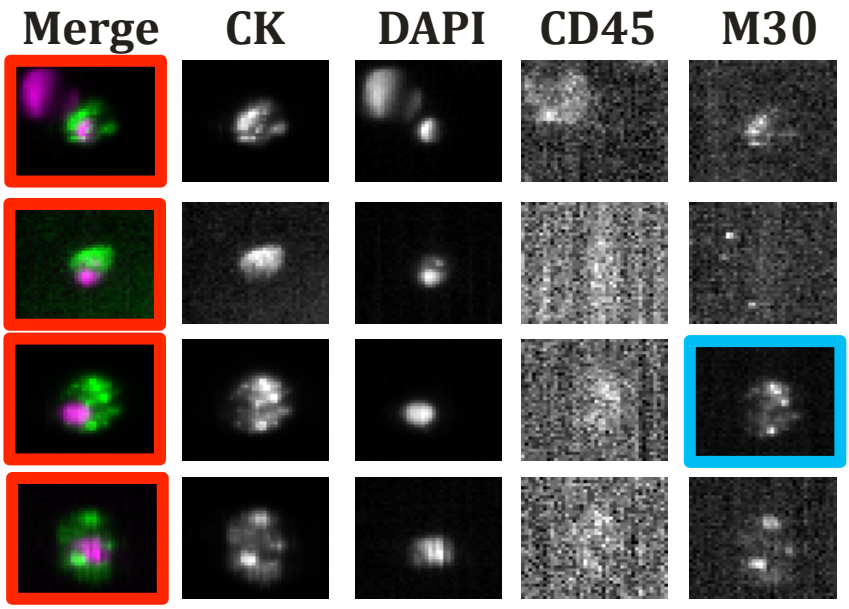



***Figure 17.*** *CTC profile. Red square EpCAM+ (CK+,DAPI+,CD45-). Blue square apoptotic CTC (M30+)*

RESULTS

In the period November 2018-July 2019 we collected 32 samples from 15 patients. Indications for LT were the follow: CCA (n=2), CRLM (n=2), HCC (n=11). Results from the 2 patients reported in my thesis and underwent to LT for CRLM are the follow:

TRANSMET: Patient had 2 CTCs at the time of LT that dropped at 1 CTC three months after LT. At the time of recurrence a blood sample was repeat and we did no detect CTC.

RAPID: Patient had no CTCs detection at LT and at 2^stage hepatectomy. However, at the time of radiological recurrence, we repeated blood sample and 1 CTC was detected. For this patient we tested also the presence of cfDNA at the same times to gain further information about the presence of 'druggable' genomic alterations that might be the basis of treatment after surgery. The detection of ctDNA has been done using a specific primer for the NRAS exon 2 G12D mutation. All the three analysis of ctDNA resulted positive.

### 4.4 Discussion

For the TRANSMET patients the detection of CTC at T0 and T1 has corresponded with an early recurrence that the patient had four months after the transplant. Although CTC number was low (CTC 1 and 2), under the cuf-off defined in the literature for prognostic activity, the recurrence profile was premature and significant. Also if we correlate the CTC finding and the pathological examination of the explant liver (no micro/macrovascular invasion, no nodes involvements) the data remains surprising.

For the RAPID patient the absence of CTC detection at transplant and at 2^ stage hepatectomy may provide a perception of liver-only involvement. In fact, the presence of CTC is usually associated with tumor burden or extrahepatic disease. So the absence of CTCs detected in the operation setting should have been a positive prognostic factor for tumor recurrence. However patient had recurrence within 6 months from the transplant and at time 1 CTC was detected. This kind of detection is not reported to be significant in the literature for prognostic purpose and early recurrence but in our case it represents a new finding in the history of patient: he became positive to CTC only at the recurrence. On the other hand cfDNA resulted positive in all samples. This contrasting finding could firstly be explained by the different sensitivity of the two different methods. Since we already knew tumor mutation is was possible to directly search the NRAS exon 2 G12D mutation with a specific primer, increasing the sensibility of dd-PCR. The ctDNA detection after the second stage might have advised us to start adjuvant chemotherapy in order to destroy the micrometastic disease potentially present but the discrepancy of results between CTCs and ctDNA and the recent patient's surgical stress and post-operative complications guide us to a conservative approach. Additionaly it is not yet clear whether and when a chemotherapy treatment has to be started in these patients. Research on CTCs may provide addition result to guide protocol definition also for this clinical aspect.

# Chapter 5

## CONCLUSION

Globally, published results and preliminary experience confirm the anticipated hypothesis that liver transplant for selected patients with unresectable CRLM, survival results similar to commonly accepted indications can be obtained. What remain to establish is the optimal selection criteria; the ongoing trials are investigating different ways but there are not now univocally accepted evidences. Despite LT for CLM may provide an improved OS, the high recurrence and the failure of LT to achieve a durable "cure" calls into question the use of LT for most patients with CLM.

In general, detection of CTCs/cfDNA provides a unique opportunity to achieve a 'liquid biopsy' for cancer testing, monitoring, and prognostication; it needs to be apply also in the field of liver transplantation. The combination of clinical, pathological and molecular factors may guarantee to better select patient. In the era of organ shortage the achievement of a greater DFS may be the goal of transplant oncology.

The RAPID technique will assure a potentially unlimited donor resource (low risk living donation) for selected oncologic cases dramatically increasing the number of liver transplants performed yearly. In this sense RAPID technique represents a potential historical breakthrough opening liver transplantation to a virtually infinite donor pool and solving the problem of organ source for patients with oncologic indications to transplant.

In conclusion we believe that LT has the potential to become an alternative to current standards of practice for CRLM but the definition of the patient population with the optimal tumor biology that would benefit from LT for CLM is still critical. In the future, genetic profiling to better define tumor biology should be useful for patient selection. Future studies should focus on patient selection criteria to achieve lower recurrence rates.

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