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Translational Study of Liver Cancer and Hypertrophy: TranSLiCH

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Surgery

Abstract

Background: Associating liver partition with portal vein ligation for staged-hepatectomy (ALPPS) is a technique for inducing accelerated hypertrophy in patients with insufficient future liver remnant (FLR). It remains unknown whether this hypertrophy may lead to rapid cancer cell dissemination and/or alteration of immune cell/function reconstitution in the FLR. We aimed to determine if the rapid hypertrophy during ALPPS procedure results in more circulating tumour cell (CTCs) dissemination and whether the FLR remains immunologically competent in patients with CRLM.

Methods: In our prospective, observational, 2-arm study, we assessed the utility of CTCs as an evaluation tool for disease dissemination. Moreover, mucosa-associated invariant T (MAIT) cells were used as a marker of liver immune competency of the FLR in patients undergoing to ALPPS (Arm-1) or single stage liver resection (Arm-2; control) from July 2015-June 2016. Blood samples and liver tissue were collected at different time points. CTCs were measured by the CellSearch System. CTC positivity was defined as > 1 CTCs in a 7.5-ml blood sample. Frequency of MAIT cells were measured in both groups (blood, liver and tumour) using flow cytometry.

Results Among 24 potential patients, 17 met the criteria and underwent curative hepatic resection: 7 in Arm-1 and 10 in Arm-2. Baseline demographics were similar between groups**.** In stage-1 ALPPS, CTCs were present in two patients (28.6%), one of whom continued to be positive after completion of both stages, whereas four patients (44.4%) in Arm-2 were positive, p=0.289**.** Patients with positive CTCs (one each-Arms) at follow up developed early recurrence and died, p=0.0083. In addition, we found a trend towards an increase in MAIT cells within the liver in Arm-1 compared to Arm-2 (28 %vs17.42%; respectively), (p=0.067), and within the tumor (17.42%vs10.42%, respectively, p=0.308).

Conclusion: Accelerated and extensive liver hypertrophy during ALPPS was not associated with CTC dissemination. Persistent positivity of CTCs at follow-up was significantly associated with disease progression and cancer-related death. Presence and upward trend in the frequency of the MAIT cells in the ALPPS group suggest immune cell restoration in the FLR. Nevertheless, given the small sample size, a larger cohort is needed to validate these findings.

Keywords: Circulating tumor cells (CTCs), Associating liver partition with portal vein ligation for staged hepatectomy (ALPPS), colorectal liver metastases (CRLM), CellSearch System, the mucosa-associated invariant T (MAIT) cells.

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List of abbreviations

ALPPS: Associating Liver Partition with Portal Vein Ligation for Staged Hepatectomy

APCs: Antigen Presenting Cells

CEA: Carcinoembryonic Antigen

CTCs: Circulating Tumour Cells

CRC: Colorectal Cancer

CRLM: Colorectal Liver Metastases

DCs: Dendritic Cells

DFS: Disease Free Survival

DL: Deportalized Liver

ECOG: Eastern Cooperative Oncology Group

FOLFOX: Folinic acid, **F**luorouracil (5FU), **Ox**aliplatin

FOLFIRI: Folinic acid, **F**luorouracil (5FU), **IRI**notecan

FLR: Future Liver Remnant

HMCs: Hepatic MAIT cells

IFN: Interferon

IL: Interleukins

LHSC: London Health Sciences Centre

LSEC: Liver Sinusoidal Endothelial Cells

LRCP: London Regional Cancer Program

NKc: Natural Killer cells

OS: Overall Survival

PBC: Primary Biliary Cirrhosis

PBMCs: Peripheral Blood MAIT cells

PHLF: Post Hepatectomy Liver Failure

PSC: Primary Sclerosing Cholangitis

PVE: Portal Vein Embolization

PVL: Portal Vein Ligation

PVO: Portal Vein Occlusion

MAIT cells: Mucosal-Associated Invariant T cells

RECIST: Response Evaluation Criteria In Solid Tumors

TranSLiCH: Translational Study of Liver Cancer and Hypertrophy

TGF: Transforming Growth Factor

VEGF: Vascular Endothelial Growth Factor

CHAPTER 1

1. INTRODUCTION

1.1. Colorectal Cancer Incidence

Colorectal cancer (CRC) represents the third most common cancer world-wide with about ~1.4 million new cases in 2012 and the fourth leading cause of death, ~700,000 annually (8.5%). (1,2) The countries with the highest incidence rates are Australia, New Zealand, Canada, the United States, and some countries in Europe such as the UK; whereas China, India, Africa, and South America have the lowest incidence. (1,3) In Canada, CRC is the second most common type of cancer among males (13.9%), and the third most common type of cancer in females (11.5%), representing the third most common overall types of cancer (12.7%). **(**4)

1.2. Colorectal Liver Metastases (CRLM)

The liver is a common site for hematogenous metastases from CRC. (5–8) Up to a fifth of patients with primary colorectal carcinoma have synchronous hepatic metastases, and nearly half of patients who undergo resection of their primary CRC will develop with will develop metachronous liver metastases at some time during the course of their disease.(9)

1.3. Treatment

1.3.1. Systemic Treatment for CRLM

The outcome of untreated colorectal liver metastases (CRLM) has been well documented in the literature. (6,10) The median survival of untreated synchronous CRLM is just 5 to 10 months, and 3-year survival is very unusual, limited only to those patients

who initially have solitary liver metastases arising in a metachronous manner.(6) Over the past 3 decades, 5-fluoracil (5-FU) has been used either alone or in the combination with other regimens for the treatment of colorectal liver metastases. (6,11) The tumor response rates with 5-FU alone are reported to be 10-15%, and can reach up to 30-39% when Leucovorin (LV) is added. The median overall survival (OS) with 5-FU alone is reported to be ~12.6 months, improved to 14.8 months when combination therapy is used. (6,11)

Currently, with the development and improvement of new chemotherapy regimens such as irinotecan (FOLFIRI) and oxaliplatin (FOLFOX), the tumour response rate has improved from 22% to ~ 50%, allowing these therapies to emerge as the first line regimen for treatment of stage IV colorectal cancer (CRC).(5,6,12) The overall survival outcomes reported using these regimens has been superior to single agent therapy (19.5 months versus 15 months) (11) without a significant difference with respect to patient tolerance or toxicity. Overall survival following FOLFIRI and FOLFOX treatment is similar at 21.5 and 20.6 months respectively. (11,13) The FOLFOX regimen is complicated by an elevated rate of neurotoxicity and neutropenia, while FOLFIRI therapy is associated with other symptoms such as nausea, vomiting, mucositis, and alopecia. (6,11)

Other therapies have emerged to target growth factors or cell surface receptors involved in colorectal cancer liver metastases biology. These biological therapies might stabilize disease and improve patient disease-free survival by causing tumour necrosis. (11,12) Bevacizumab (Avastin), is a monoclonal antibody against vascular endothelial growth factor (VEGF), approved to be used in combination with 5-FU as first-line chemotherapy for patients with CRLM. These regimens have demonstrated a higher tumour response rate and longer patient overall survival, when compared to therapies without Avastin therapy, 44.8 vs 34.8% and 20.3 vs 15.6 months, respectively. (12) Moreover,

additional monoclonal antibody therapies such as cetuximab and panitumumab have also shown benefit for tumors with KRAS mutation by improving the response rate and patient survival. (14)

In summary, modern systemic chemotherapy has improved outcomes for metastatic CRC treatment and plays an important role in the absence of resection (median survival ~18 to 20 months) (11). However, notable and superior results are achieved when combined with liver resection (5-year survival ~55-67%). (15)

1.3.2. Surgical Management of CRLM

During the last 3 decades, hepatic surgical treatment has evolved and become the gold standard for patients with primary or metastatic liver malignancies. Moreover, the actual role of the multidisciplinary tumour board committees in the management of this complex group of patients, with the aim of surgical resection has achieved the most satisfactory results, benefiting the overall course of the patient during the disease process. (5,6,16). The actual 5-year survival following a margin-negative hepatic resection with the combination of chemotherapy treatment is ~ 40-70%, with 10-year survival approaching 20%. (6,17,18) Recurrence and progression is reported in 70–80% patients undergoing surgery. (5) In addition, the notable ability of the liver to regenerate after hepatic resection has enabled aggressive surgical approaches in patients with insufficient hepatic reserve.

Resectability criteria for CRLM patients have been widely revised and expanded over the last 15 years. (5) Previous clinic pathological parameters such as number or metastases, presence of extra-hepatic disease or width of resection margin are no longer used to exclude patients from consideration for surgical resection. (5–7) The current hepatic resection criteria should be defined by: the preservation of vascular inflow, vascular outflow, and biliary drainage, with the objective of preserving an adequate future liver

remnant (FLR). Those patients with extensive liver disease or borderline liver parenchyma reserve should undergo a technique to increase the volume of their FLR such as portal vein embolization (PVE), two-stage hepatectomy, or a chemotherapy modality to downsize the disease and convert the liver tumor burden into resectable disease.(5)

As described in the literature, for the best outcomes, surgical resection should only be considered if the following criteria are met: (6–8,19)

1. Adequate patient health performance condition (normal baseline lung, heart, kidney and liver function).
2. Primary tumour resectable or already resected.
3. Absence of extra-hepatic disease, or if present and localized, disease is planned for resection (e.g. lung metastases).
4. Solitary nodule or multiple nodules technically feasible for liver resection (attempt to get negative resection margins “R0”).
5. Vascular inflow and outflow with adequate biliary drainage in the remaining liver must be preserved.
6. Adequate volume/function of the FLR after resection (at least 20% of the total FLR if normal parenchyma and 30-70% when a history of chemotherapy or underlying liver disease is present).(19,20)

1.3.2.1. Future Liver Remnant (FLR)

Over the past two decades, there has been an inclination to expand what is defined as "resectable" disease, pushing the boundaries and leaving a narrow line between complete success and liver failure. Therefore, development of new multidisciplinary modalities has been explored to improve the resectability rate while avoiding catastrophic post hepatectomy liver failure (PHLF). Reports recommend an estimated FLR to body weight ratio of greater than 0.5 to avoid liver failure. (20,21)

To achieve an adequate volume of the FLR after resection and thus, decrease the risk of developing PHLF, patients with normal liver parenchyma or undergoing a short course of chemotherapy (< 12 weeks) usually require a FLR of at least >20 %, while a FLR >30-60% is needed for patients after >12 weeks of chemotherapy, steatosis or hepatitis, and a FLR > 40-70% is mandatory in the presence of underlying liver disease (e.g. cirrhosis, cholestasis depending on the degree of underlying hepatic dysfunction). Thus, chemotherapy duration and adequate FLR volume are the critical determinants of hepatic reserve which directly affect morbidity and mortality following hepatic resection for CRLM. (20–23)

1.3.2.2. Techniques to increase the FLR

During the last 40 years, several surgical and non-surgical techniques have emerged in order to increase the size of the FLR and avoid PHLF, in those patients with primary and metastatic liver cancer undergoing to extensive liver resection. Examples of these procedures are: portal vein occlusion (either by embolization or ligation), two staged hepatectomy, and ALPPS (**A**ssociating **L**iver **P**artition with Portal Vein Ligation for **S**taged Hepatectomy**). (7,8,13,24)**

1.3.2.2.1. Portal Vein Occlusion (PVO)

Makkuchi et al. introduced the use of portal vein embolization (PVE) in the early 1980s as a non-surgical technique to stimulate hypertrophy of the FLR before extensive liver resections. (25) Currently, two methods of portal vein occlusion (PVO) can be applied and are considered as standard therapy for patients with unilobar disease and insufficient FLR: either radiologically by portal vein embolization (PVE) or surgically by portal vein ligation (PVL). The PVO technique has been shown to increase the size of the FLR from 8% to 27% over an interval of 2-8 weeks, depending on the underlying liver disease. Unfortunately, these same hypertrophy results have been difficult to replicate at other centers. PVO is safe, with a complication rate of 5-8%(19). However, its main disadvantages include insufficient hypertrophy of the FLR and disease progression after the PVO, preventing curative liver resection in 20-30% of patients. (26)

1.3.2.2.2. Two-Stage Hepatectomy:

The concept of two-stage hepatectomy was introduced in the early 2000s. (26) This surgical hepatic technique was designed for patients with primarily bilobar unresectable liver metastases, which typically cannot be resected in a single stage while preserving an adequate FLR. The strategy of the two stage hepatectomy is to attempt a curative resection through a sequential approach. The goal of the first stage is to clear the future liver remnant by removing the highest number of metastases, making the second hepatectomy feasible and potentially curative after proper FLR regeneration. (26) Thus, a two-stage hepatectomy offers the potential of disease free survival to patients that otherwise would have a poor outcome. Patients who undergo a two-stage hepatectomy with combination chemotherapy have a 3-year survival rate of 35% and a median survival of 31 months. (5,26) Nevertheless,

initial experience without PVE was associated with high incidence of PHLF from insufficient functional volume of the FLR a high mortality rate of 9-15%. (5,27)

1.3.2.2.3. Two-Stage Hepatectomy with PVO

Liver failure due to insufficient functional liver volume of the FLR represents the main cause of postoperative mortality in patients undergoing two-stage hepatectomy. Thus, the combination of PVO (either PVE or PVL) and staged hepatectomy was proposed as a modality to induce compensatory hypertrophy of the FLR.(27–29) After introduction, initial reports showed no operative mortality, low postoperative morbidity (~ 15 to 30%), and 1-and 3-year overall survival rates of 70% and 54%, respectively. (5,27) Nevertheless, insufficient liver regeneration of the FLR and tumor progression during the long waiting period (6-8 weeks) was again the biggest limitation, making this procedure only feasible in 70% of the cases. (30)

1.3.2.2.4. Associating Liver Partition with Portal Vein Ligation for Staged Hepatectomy (ALPPS)

During a planned extended right hepatectomy for a perihiliar cholangiocarcinoma, ALPPS was created by chance in 2007. Due to insufficient FLR, the right portal vein was ligated to induce hypertrophy of the left lateral segment of the liver, after the liver parenchyma had already been transected. On postoperative day 8, abdominal computed tomography imaging showed extensive hypertrophy of the left lateral segment prompting the decision to complete the resection of the right liver. (23) Therefore, the new technique consisted of adding a liver parenchymal partition during the first stage of a two-staged hepatectomy, facilitating an inter-stage time of only 1 week.

The initial experience of this innovative technique was presented in 2011 during the European Hepato-Pancreato-Bilairy meeting held in South Africa.(30) Soon after, during the inaugural study in 2012, the authors reported their experience in 25 patients. Initially the procedure was named as "two-staged extended right hepatectomy with initial surgical exploration, right PVL, and in situ splitting" (22), and later renamed with the acronym "ALPPS" (Associating Liver Partition with Portal Vein Ligation for Staged Hepatectomy).(31)

1.3.2.2.4.1. ALPPS Results Worldwide:

Since the first description of the ALPPS procedure, refinements to both the technique, and patient selection criteria have been explored. The most promising results have been reported in the field of CRLM, and thus, establishing the main indication for ALPPS to be when metastases are primarily unresectable due to a very small predicted FLR. (7,8,30,32) Other indications to consider are in specific situations such as hepatocellular carcinoma (HCC) or cholangiocarcinoma, if resection is oncologically possible .(7)

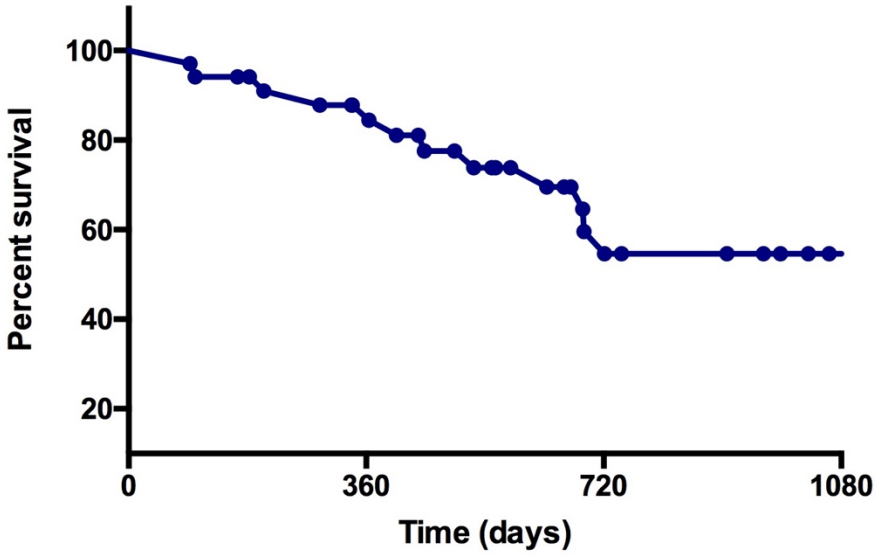
In terms of volumetric growth, rapid liver regeneration remains the main advantage of ALPPS: total liver volume growth of FLR is reported around 50-84% in 1 week and 120-200% after 14 days. (7,32,33) Nevertheless, principle limitations are related to high morbidity (53–90%), high mortality (0–28%), and no reports on long-term oncologic safety and outcome.(8) The largest studies from the ALPPS registry report the procedure has a 97% feasibility rate with a morbidity rate of 44%, and 90-day mortality rate of 8-11%.(33,34) From the oncological standpoint, long-term outcomes with respect to the disease free and overall survival are areas that need further investigation.

1.3.2.2.4.2. ALPPS Results at the London Health Sciences Centre

The first ALPPS procedure in Canada was performed in London, Ontario at the London Health Sciences Centre (LHSC) in April 2012 by Dr Roberto Hernandez-Alejandro. (35) Ever since, LHSC has been the pioneer and leader overall in North America. Despite the high morbidity and mortality earlier described, (30) refinements in the technique and a better understanding of patient selection has been paramount to success at our institution, in addition to our strict selection criteria: patients with colorectal liver metastases (CRLM), extensive bilobar CRLM necessitating an extended hepatectomy, a technically feasible resection (planned R0), a predicted FLR <30%, no evidence of extra-hepatic disease, good functional capacity defined as ECOG (Eastern Cooperative Oncology Group) 0 or 1, and complete or partial tumour response after 6-cycles of neo-adjuvant therapy assessed by the Response Evaluation Criteria In Solid Tumors (RECIST). (35)

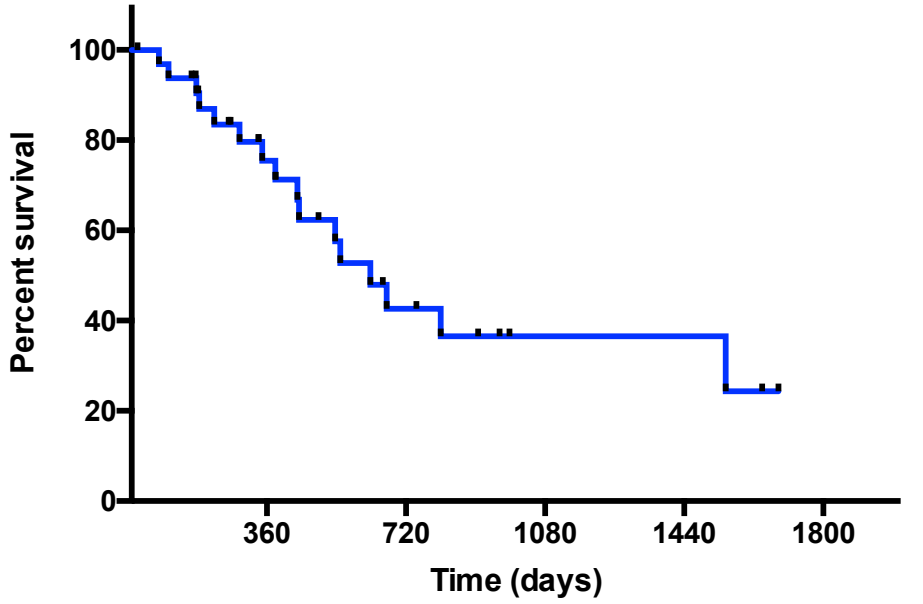
Furthermore, we have paid special attention to the improvement of the surgical technique by maximizing the preservation of the middle hepatic vein and minimizing the dissection of the hepatoduodenal ligament to avoid ischemia and congestion of segment 4 and to decrease the potential for ischemic injury to the biliary tree. Taken together, these points might explain our lower morbidity rate (14%) and postoperative 90-day mortality rate (0%) compared with other centers.(35) To date, our center has already performed 34 ALPPS procedures and our latest data shows a 1-year OS of 84% and 3 years OS of 55% (median 1264 days), as well as 1-year DFS of 71%, and 3-year DFS of 36%. **(Figures 1 and 2)**

Figure 1. ALPPS Overall-Survival, LHSC 2012-2016



Median Overall Survival: Median 1264 (95% CI 890-1339) 90 days mortality-0

Figure 2. ALPPS Disease-Free Survival, LHSC: LHSC 2012-2016



Median Disease Free Survival (DFS) 628 days (95% CI 298-958)
1-year DFS 71%, 2-year DFS 41%, 3-year DFS 36%

1.4. Liver immunology

The liver is considered the largest solid organ in the body involved in non-immunological activities such as metabolic functions, nutrient storage, and detoxification. Also, it is a place of complex immunological properties, such as induction of immunological tolerance and innate immune responses, which are possible through the production of diverse acute phase proteins, complement components, cytokines and chemokines, and by different populations of resident immune cells.(24,36,37)

The liver functions impressively as an important filter for gut contents and blood systemic circulation via the Porto-systemic interface (70-80% of the liver blood supply comes from the portal vein, with the remaining 20-30% from the hepatic artery). As a low pressure blood system, liver venous blood flows in close contact with the hepatic sinusoids. Hepatocytes compose ~60-80% of overall liver cells (38). Their main role is in metabolism, and production of 80–90% of the circulating innate immunity proteins in the body.(6) Moreover, the liver capillary microenvironment consists of different specialized cells (liver sinusoidal endothelial cells [LSEC], intravascular liver resident macrophages "Kupffer cells", and liver dendritic cells) which have an important role in initiating and organizing hepatic immune responses.

The liver immunological homeostasis mechanism is not fully understood yet. The narrow balance between tolerance and liver immunity is controversial. Classical examples of tolerance can be observed in viral hepatitis infections, metastatic cancer (most commonly from colorectal cancer), and transplantation. On the other hand, undesirable immune responses may ensue, for instance, in the case of autoimmune hepatitis, primary sclerosing cholangitis (PSC), and primary biliary cirrhosis (PBC). (6,37) **Figure (3)**

Moreover, the liver has essential immune and metabolic roles that are described and summarized in **Figure 4.** The following sections will provide a basic understanding of the immunological mechanisms orchestrated by the liver, and focus on the role of those liver cells that are implicated in tumour immune surveillance (liver lymphoid cell population). In particular, the role of the "Mucosal-associated invariant T (MAIT) cells" will be discussed.

1.4.1. Human liver cell distribution

The liver contains most of the cellular elements of the innate and adaptive immunity. Their frequency and distribution are observed in **Figure 5**. (39) Each of them has a specific role and effector function, either cytotoxic or regulatory, capable of killing pathogens or suppressing the immune response. Also, differentiation into cells that can produce different cytokines and antibodies, IFN (interferon), IL (interleukins), TGF (transforming growth factor), TNF (tumor necrosis factor) can be observed, which has an important role during antitumor surveillance. (6,39)

Figure 3. Liver Tolerance vs Immunity (6):

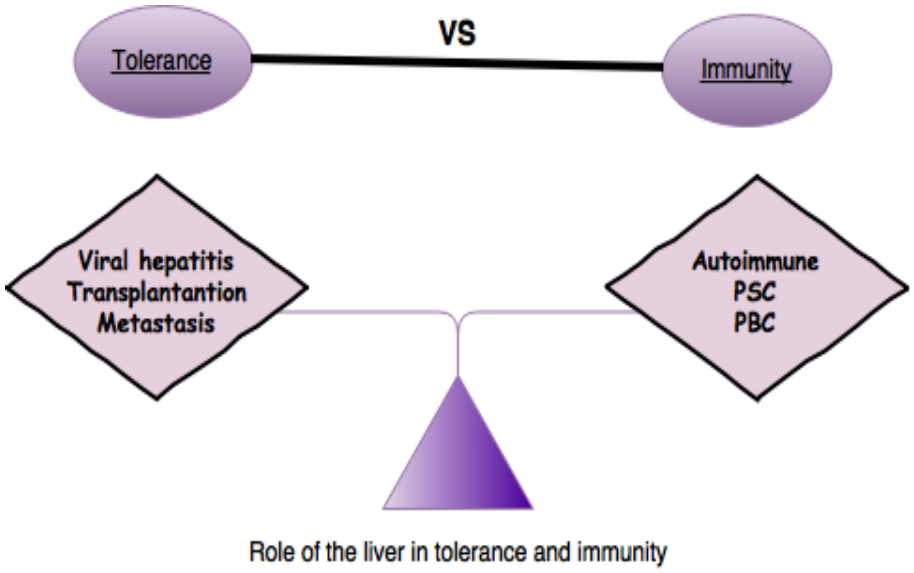


Figure 3. Image representing liver tolerance vs immunity. The liver has tolerogenic characteristics observed in diseases such as viral hepatitis, metastatic cancer and transplantation. On the opposite side, the liver exhibits a high immune response: autoimmune hepatitis, PSC and PBC. *From Jarnagin WR, Blumgart LH, 2012: Blumgart's surgery of the liver, biliary tract and páncreas. 2-Volume set. 5E ed. Copyright 2012,2007,2000,1994,1998 by Saunders, and imprint of Elsevier Inc.*

1.4.2. Antigen-Presenting Cells:

Antigen-presenting cells (APCs) are important in orchestrating or shaping the adaptive immune response. The three most important APC cells are Kupffer cells, LSEC, and dendritic cells. They play a crucial role in the maintenance of tolerance balance on non-inflammatory scenarios. The liver APC cells function in capturing and presenting antigens to T cells. The success of their activation and response, depends directly on the proper molecular co-stimulation. If co-stimulation is successful, proliferation and development of effector T cells will occur, with subsequent release of cytokines. In contrast, if co-stimulation is unsuccessful T cell death or anergy will result. (6,38–40)

1.4.3. Dendritic cells (DCs), liver sinusoidal endothelial cells and Kupffer cell (KCs)

Liver DCs are known as the most potent APCs of the immune system (Figure 6,7). They play an essential role in the antigen capture and subsequent presentation to the immune effector cells. Liver DCs travel from the portal circulation through the space of Disse, finally exiting via lymphatic circulation. Their essential function involves immune regulation, bridging, and adaptive immunity. Nevertheless, liver DCs show tolerogenic characteristics, promoting the production of anti-inflammatory IL-10, which has a suppressive function. (24,37,38)

Liver sinusoidal endothelial cells work as sentinels, and are important during pathogen detection. Their structure in the form of multiple fenestrations selectively allows the passage of antigens through the LSEC to the hepatocytes, establishing close contact among antigens and hepatocytes. This characteristic promotes organ homeostasis, blood detoxification, and inflammatory response regulation.(6,37)

Figure 4. Liver immunological & metabolic roles (37) :

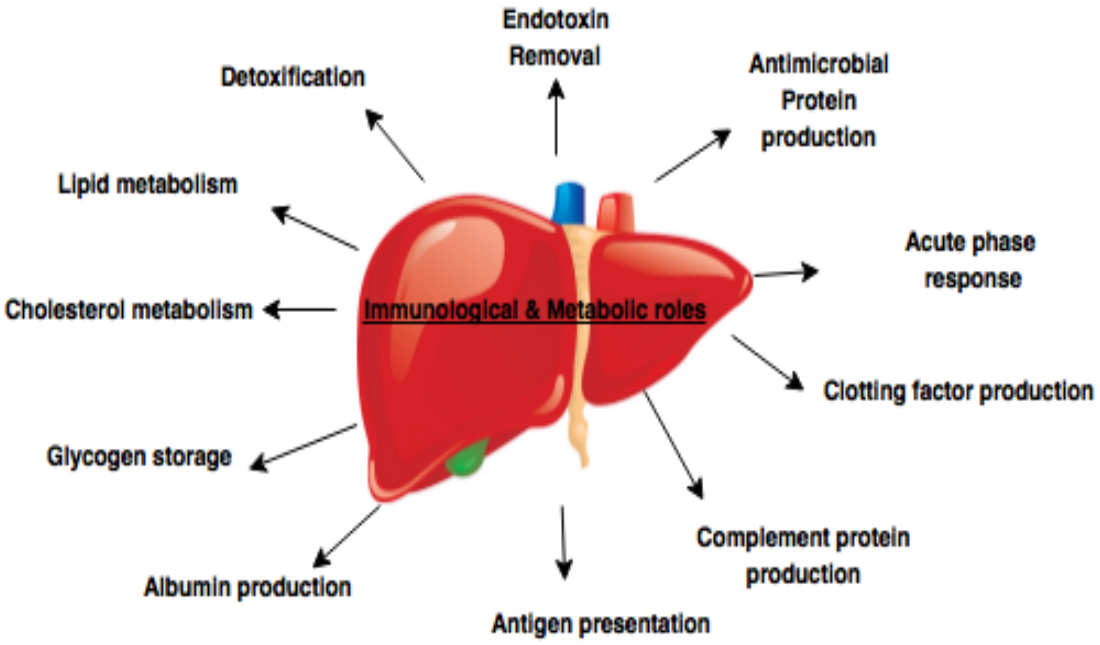


Figure 4. Metabolic and immunological roles of the liver. The liver has an important role in inflammatory regulation via production of clotting factors and acute phase proteins, antigen presenting T cells and anti-microbial proteins. In addition, it has important metabolic roles including lipid metabolism, glycogen storage production, protein production, and others. *From Robinson MW, 2016: Liver immunology and its role in inflammation and hemeostasis. Cell Mol Immunol 13:267-76. Copyright 2016, CSI and USTC.*

Figure 5. Healthy liver cell distribution.

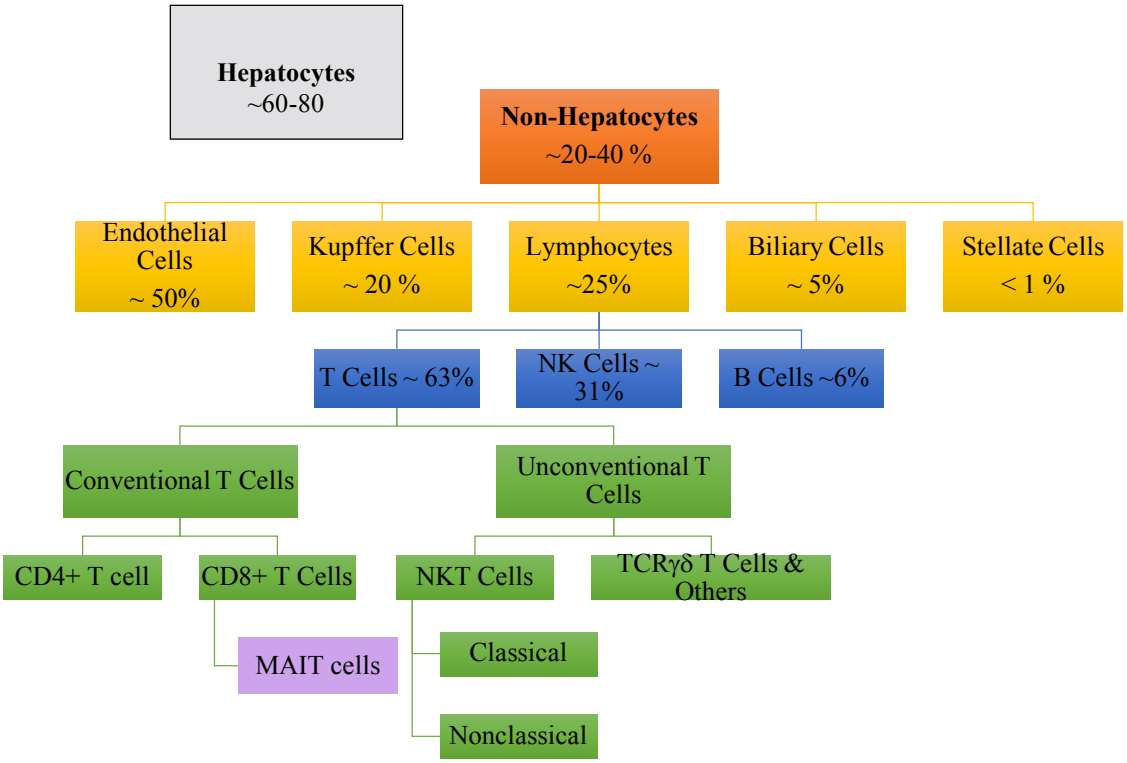


Figure 5. Distribution and frequency of parenchymal and non-parenchymal healthy human liver cells. *From Racanelli V,2006: The liver as an immunological organ. Hepatol Baltim MD 43:S54-62. Copyright 2006, The American Association* for the Study of Liver Diseases.

Liver Kupffer cells originate from monocytic precursors and correspond to the largest group of macrophages in the body. Their phagocyte function and strategic location allow them to travel from the periportal area to the space of Disse, with subsequent elimination of endotoxins and apoptotic hepatocytes. (6,38,39)

1.4.4. Liver Lymphoid Immune Cell Population

The liver lymphoid cell population is composed of T cells, natural killer cells (NK cells), and B cells. (37,39) The main characteristics of these cells include potent cytokine production and mediating the innate and adaptive immune response of the liver. In addition, they contribute to the removal of infected, injured or malignant cells. A subpopulation arising from T cells comprises conventional T cells (CD8+ and CD4+), which identifies antigens in the context of major histocompatibility complex (MHC) class I and II. Typically, CD8+ is higher in proportion to CD4+ (2:1) in the liver, whereas their frequency is inverted in the blood. Unconventional T cells correspond to another subtype of T cells. These are integrated of natural killer T cells (NKT cells), which have a restricted T receptor and recognizes antigens only in the case of MHC I (molecule CDI d), and TCRγδ T cells, that identifies a very limited proportion of antigens.(6,36,37,39)

1.4.5. Natural Killer Cells

Human NK cells comprises most of the lymphocyte liver population and play an important role during liver inflammation, particularly in antiviral defense. Traditionally, NK cells are categorized as either CD56-low-expressing cells with cytolytic activities, CD56-high-expressing with immune-modulatory cytokine-producing cells, and CD49a,

which mainly release high proportions of CD69, granzyme B, interferon-γ (IFNγ), tumour necrosis factor-alpha (TNF-alpha), and granulocyte macrophage colony-stimulating factor (GM-CSF).(24,36,41)

Figure 6. Innate immune liver elements:

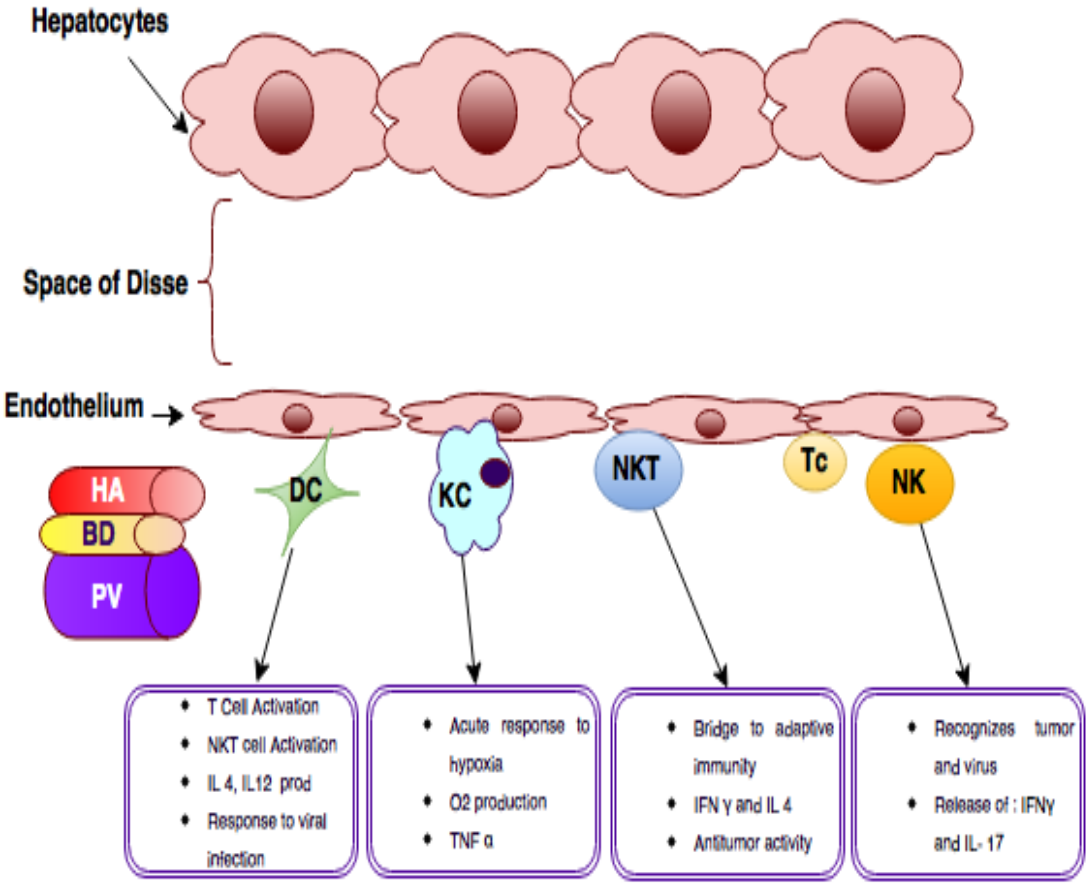


Figure 6. Image representing the microvascular hepatic anatomy**.** Liver sinusoidal endothelial cells shield and divide hepatocytes from sinusoidal blood flow by the "Space of Disse" flow. In addition, DC, KC, NKT cells, NK cells can be found and have close contact with the microcirculation, and had a subsequent specific function in the inflammatory response.

Figure 7. Liver immune response.

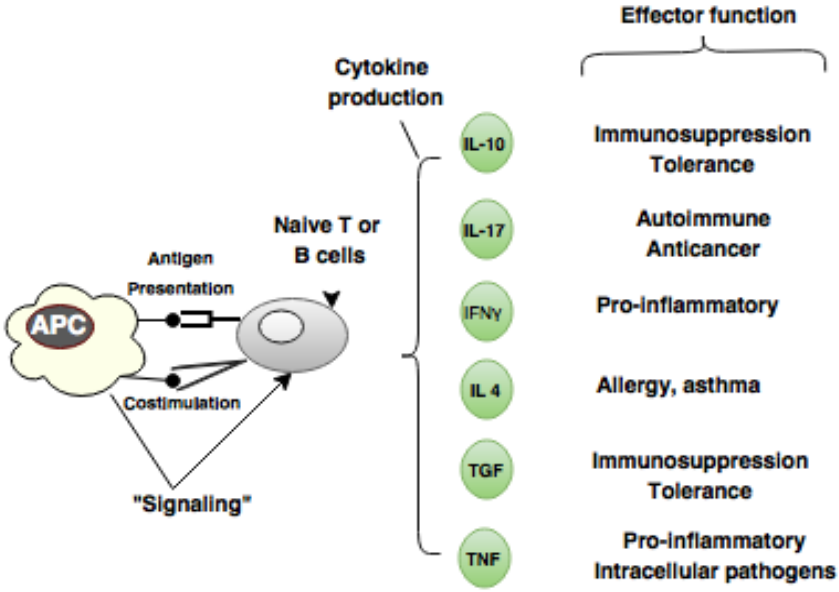


Figure 7. Schematic representation of the adaptive immune response illustrating inflammatory activation, from antigen-presenting cells to T and B cells leading to signaling, costimulation and cytokine production with subsequent effector function, From *Jarnagin WR, Blumgart LH, 2012: Blumgart's surgery of the liver, biliary tract and páncreas. 2-Volume set. 5E ed. Copyright 2012,2007,2000,1994,1998 by Saunders, and imprint of Elsevier Inc.*

1.4.6. Natural Killer T cells (NKT cells)

NKT cells are a specific subgroup of T cells, whose main role is to regulate the immune responses in the environment of autoimmunity, cancer, and microbial infection, through the expression of different cytokines such as interleukin-4 (IL-4) and IFNγ. In the cancer context, tumour-infiltrating human NKT cells express CD56, CD69, and pro-inflammatory cytokines such as TNF-alpha and IFNγ, showing an important antitumor role.(41,36,37)

1.4.7. MAIT cells

The study of mucosal-associated invariant T (MAIT) cells in humans is an emerging field in immunology. Human MAIT cells are known as a subset population of conventional T cells, which are localized preferentially in liver and intestinal mucosa, composing ~30-50% of the liver lymphocytes and 10-15% of the peripheral blood lymphocytes.(42) Circulating and tissue-infiltrating human MAIT cells express a semi-invariant T cell receptor (TCR) of Vα7.2-Jα33 alpha chain rearrangement (CD161 or IL 18Rα) and respond to MHC I like molecule MR1 (MHC-related protein 1). (42–44)

Emerging experimentation has shown that activated MAIT cells can display different cytokines such as interleukin-17A (IL-17A), IFN-γ, and TNF-αlpha. (42,43,45) As a subset of the innate-like T cells, MAIT cells produce an arsenal of cytotoxic effector molecules and pro-inflammatory cytokines that play an important function in defense against infectious pathogens (bacteria and yeasts, but not viruses). (43,46,47) In addition, they play a role in inflammatory diseases and wound healing by their quick reactivity to bacterial infection and injury. (43,46,47) These distinguishing characteristics have prompted the immunology community to question their potential role in certain

circumstances such as cancer. Despite this, yet we know next to nothing about their role in immune surveillance against tumor formation in the liver. Recent evidence has suggested that they may have potential clinical relevance for patients with colorectal cancer.(44) Moreover, further investigations demonstrate their peculiar ability to persist through chemotherapy and xenobiotics secreted by the gut bacteria (42), due at least in part to their high levels of ABCB1 expression, which works as a multidrug efflux protein transporter to remove toxic compounds from the cytoplasm.(42,43)

1.4.7.1. MAIT Cells in Colorectal Cancer

Results presented in early 2016 by a group from China (44) suggest that MAIT cells accumulated and infiltrated in CRC tumour tissue, and thus might play an important role in immune surveillance against CRC patients **(Figure 8)**. Their study showed that the frequency of circulating activated/memory MAIT cells was lower compared with the levels observed in the tumour tissue. Furthermore, serum carcinoembryonic antigen (CEA) levels were found to be highly associated with the rate of infiltrating MAIT cells and negatively associated with the rate of circulating active/memory MAIT cells. In fact, high levels of TNF-α, IFN-γ, IL-2 and IL-17 were positively associated with TCRValpha7.2Jalpha33 mRNA in cancer tissues.(44) This data supports that high levels of infiltrating activated/memory MAIT cells in the tumour tissues might represent anti-tumor immunity against CRC, so, use as a potential biomarker for testing the severity and prognosis of CRC should be considered. Also, some reports demonstrated an important association between IFN-γ levels in the setting of T cell immunity against CRC through p53 modulation to induce tumor cells apoptosis (45), whereas, other studies show an associaciation between IL-17 expression in the tumor and worse prognosis in patients with CRC. (48)

In the present investigation, we further evaluate MAIT cells in terms of their role on hepatic immune surveillance against colorectal liver metastases in patients undergoing liver resection.

Figure 8. Hypothetical MAIT cell role during CRLM.

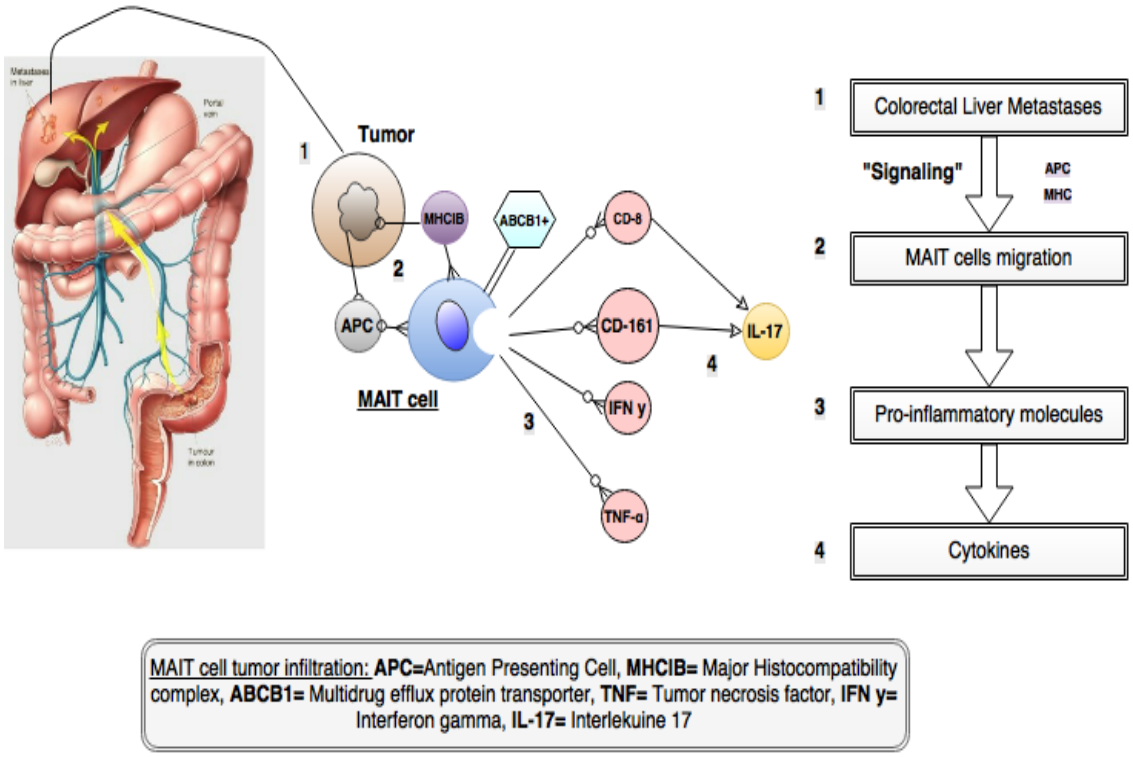


Figure 8. Schematic representation of the hypothetical role of MAIT cells during colorectal liver

1.5. Circulating Tumour Cells (CTCs)

Cancer metastasis is the principal cause of death in patients with cancer, and dissemination of tumour cells into the systemic blood circulation is an essential step. Therefore, early identification of this dissemination sequence is crucial for monitoring and impeding metastatic disease. The mechanisms by which circulating cancer cells disseminate from the tumour to the blood circulation is an area under intensive investigation. (49–52)

1.5.1. Natural History of Metastases from Colorectal Cancer

Tumor metastasis can be defined as a series of biological processes whereby tumor cells from the primary neoplasm migrate to a distant location.(9,49,52) (**Figure 9**) Invasive CRC cells can metastasize through two pathways:

1. Regional lymph nodes → through central lymphatics → the systemic circulation.
2. Portal venous drainage → Liver.

Circulating tumour cells (CTCs) are defined as a group of cells that are shed from primary tumour and from metastatic deposits into the blood circulation.(49,53) CTCs are found in the blood of patients with many cancers, however, their presence is extremely infrequent in healthy people.(49,52) The use of CTCs as prognostic and predictive tumour markers has been explored in patients with metastatic cancers such breast (54–57), prostate (58,59) and colorectal (60,61) carcinoma. On the basis of these studies, the number of CTCs (≥5 for breast/prostate; ≥3 for colorectal) were predictive of progression-free survival (PFS) and overall survival (OS). Moreover, the analysis of CTCs at different time-points during treatment has been shown to be useful for determining treatment efficacy and therapy

response. These promising findings suggest that CTCs may represent a “liquid biopsy” that could inform clinical decision-making, potentially allowing for the discontinuation of ineffective chemotherapy regimens earlier than current practice, thereby avoiding morbidity due toxicity and enabling a change to other potentially more beneficial treatment options. (54,58)

Metastatic colonization includes the following process:

1. Tumor cells invade the tissue surround the primary tumor.
2. "Invasion" into either the lymphatics or blood circulation.
3. Intravasion → Extravasion → Growing → Angiogenesis.

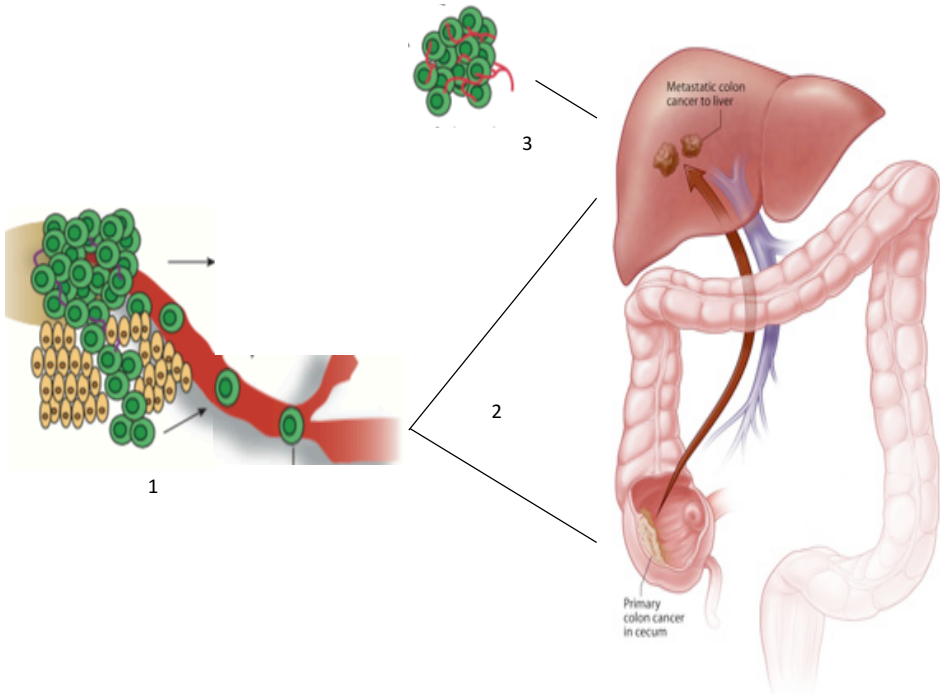


Figure 9. Schematic representation of biological process of metastatic colorectal dissemination. The term **'colonization'** is used herein to reflect the combined influences of tumor cell proliferation, apoptosis, dormancy and angiogenesis in the formation of a progressively growing lesion in a distant site.(9)

Multiple methods for CTC enrichment and identification have emerged during the last decade, either label-dependent such as immunomagnetic, where the main target has been epithelial cell adhesion molecule (EpCAM), or label-independent enrichment based on size or density. Other approaches characterize CTCs by immunocytochemistry, using PCR approaches or mRNA/DNA sequences. Additionally, others can detect viable tumour cells or tumor-specific chromosomal abnormality **(Figure 10)**. (49,62–64)

1.5.2. CellSearch System

Originally developed by Immunicon, the CellSearch system (Janssen Diagnostics, Raritan, NJ, USA) is the first and currently the only method clinically approved assay by the Food and Drug Administration (FDA) for capturing and enumerating CTCs in advanced breast, prostate and colorectal cancer.(54,55,59,60,64)

The epithelial cell adhesion molecule (EpCAM) is a cell adhesion molecule overexpressed in many carcinomas and has been used as a marker for CTC enrichment. The CellSearch is a semi-automated system based on immunomagnetic technology that isolates and enumerates CTCs by targeting EpCAM-positive cells. Through this approach, CTC numbers have been correlated with disease free survival and overall survival in primary and metastatic carcinoma. (54,56,57,59,60) Due to its reported clinical evidence, high reliability, and FDA clearance, we chose the CellSearch system as a method for CTC detection in this research study.

CTC enrichment and identification approaches for CTCs (63):

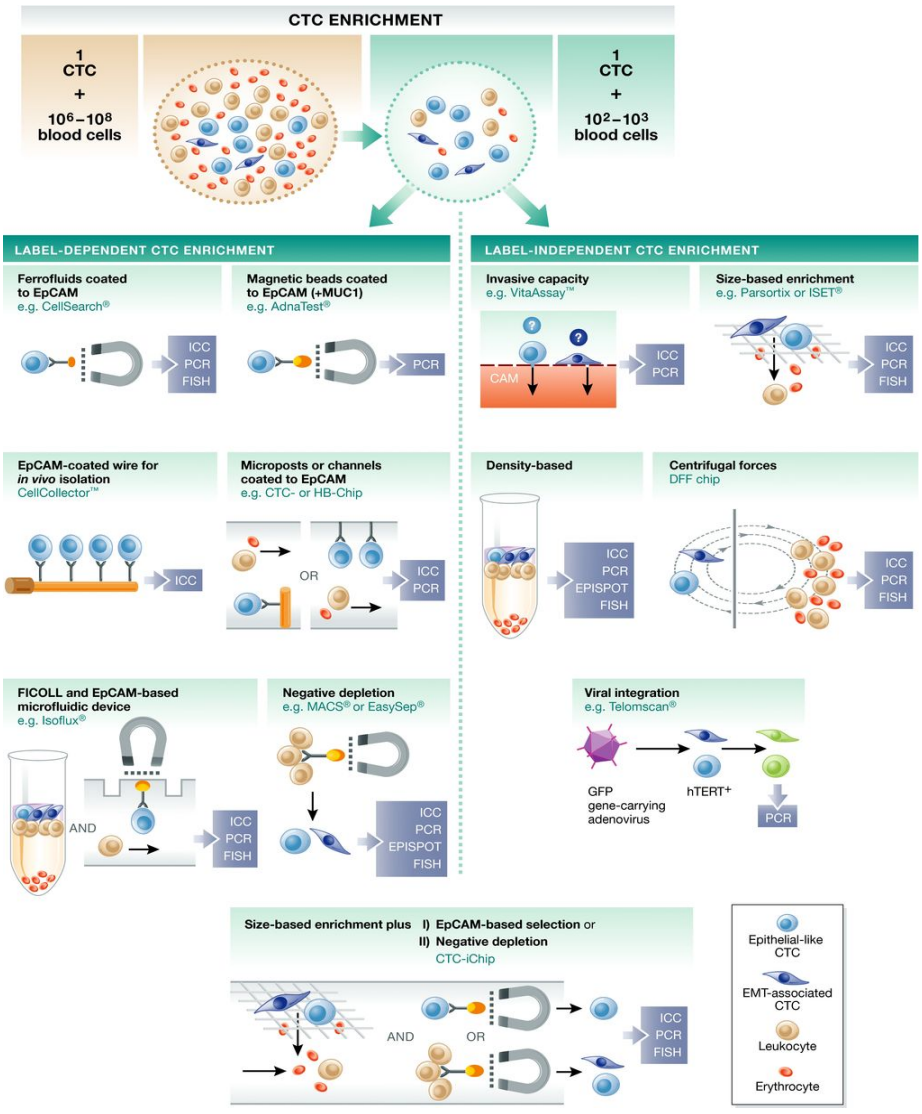


Figure 10. Schematic representation of different strategies to separate and capture circulating tumour cells: label-dependent and label-independent methods. Immunomagnetic based methods focusing on EpCAM protein are the most common used among label-independent CTC enrichment methods. *From Joosse SA, 2015: Biology, detection and clinical implications of circulating tumor cells. EMBO Mol Med 7:1-11. Copyright The Authors.*

1.5.3. Use of CTCs in Colorectal Malignancies

Novel technology platforms using immunomagnetic separation have permitted the accurate identification, isolation, enumeration, and characterization of CTCs from many epithelial carcinomas. (49,52,64–66) In a multicenter study, Cohen et al reported, that patients with colorectal liver metastasis had unfavorable survival prognosis when levels of CTCs of CTCs were ≥ 3 in 7.5 ml of blood, and showed that this was an independent predictor of inferior disease free survival and overall survival in patients with colorectal liver metastases. (60)

These results provide a promising basis from which to investigate different scenarios of the applicability of CTCs in colorectal cancer patients; including screening in earlier stages of colorectal cancer, evaluation of treatment response and analysis of progression during the time off chemotherapy **(Figure 11)**. Recent studies have also evaluated the impact of the presence of CTCs at the time of liver resection in metastatic colorectal cancer. (2,67–69) A Norwegian group reported the association between positive CTCs and high recurrence rate in patients with colorectal liver metastases undergoing two-stage hepatectomy, suggesting another potential utility for postoperative CTC assessment.(2) This current study will assess the utility of measuring the number of CTCs as an evaluation tool of disease progression using the CellSearch system in patients with colorectal liver metastases undergoing for liver resection; either ALPPS or single stage liver resection.

Pathways of circulating tumour cells in patients with colorectal cancer (62):

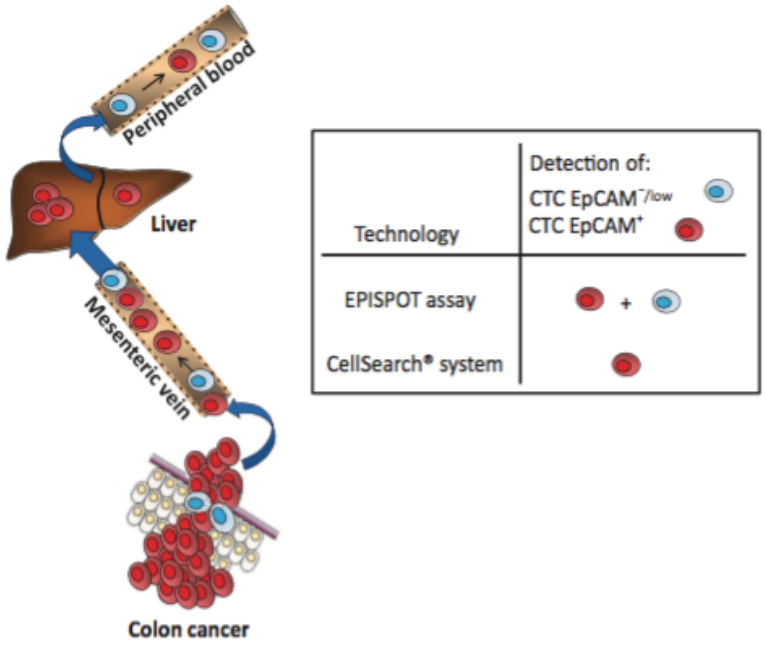


Figure 11. Diagram showing the pathway of CTCs, through the mesenteric-porto-systemic circulation in colorectal cancer patients. Circulating tumour cell (red cells) flows from the primary tumour to the systemic blood, then are captured using the CellSearch system technology. *From Deneve e, 2013: Capture of viable circulating tumor cells in the liver of colorectal cáncer patients. Clin Chem 59:1384-92. Copyright American Association for Clinical Chemistry.* **(62)**

CHAPTER 2

2. RATIONALE, HYPOTHESES, AND STUDY OBJECTIVES

2.1. Rationale

Since the first description of ALPPS procedure, refinements to both the technique and patient selection criteria have been developed. ALPPS has been demonstrated to be a valid treatment option in selected patients who were previously considered unresectable, especially in those with colorectal liver metastases.(7,30,35,70) Nevertheless, several important aspects of the procedure remain unknown, including long-term oncological outcomes. Moreover, concerns arise with respect to the impact of this rapid hypertrophy in the liver microenvironment, in particular related to potential repercussions for tumour biology and disease progression. In addition, the immunological composition of the liver during the ALPPS approach and whether the rapid hypertrophy of the FLR is accompanied by equally rapid immune cell/function reconstitution remains essentially unexplored.

Circulating tumour cells are defined as a group of cells that are shed from primary tumour and metastatic deposits into the blood circulation.(49,53) Their use as a prognostic and predictive tumour marker has been explored in patients with colorectal liver metastases (60,61). On the basis of these studies, the number of CTCs (≥3/7.5 mL blood) were predictive of inferior disease free survival and overall survival in patients with colorectal liver metastases. Moreover, the analysis of circulating tumour cells at different time points during the treatment was useful to determine treatment efficacy and therapy response, thus supporting the use of this novel biomarker as a tool for decision making before liver resection in patients with CRLM. This study aims to investigate the utility of measuring the number of circulating tumour cells as an evaluation tool of disease progression using the CellSearch system in patients with colorectal liver metastases undergoing two stage

hepatectomy; ALPPS or single stage liver resection.

The liver represents a specialized immunological environment that promotes tolerance in normal conditions, but also contains immune cells that can potentially combat cancer. While tumor-specific T cells are detectable in many cancer patients including those with primary liver cancers or liver metastases, they typically fail to eradicate cancer. The liver harbors many natural killer T (NKT) cells that constitute an attractive therapeutic target for adjuvant immunotherapy of cancer. In addition, the liver is highly enriched in mucosa-associated invariant T (MAIT) cells, which can comprise up to 50% of all hepatic lymphocytes; yet we know next to nothing about their role in immune surveillance against tumors forming in the liver. MAIT cells express an arsenal of cytotoxic effector molecules as well as pro-inflammatory cytokines (interferon [IFN]-gamma, interleukin [IL]-17) known to favor antitumor immunity. It is currently unclear whether MAIT cells infiltrate tumor masses in the liver.(42,45) Due to these characteristics mentioned, it has prompted the immunology community to hypothesize on their potential role in certain circumstances, such as cancer. In the present investigation, we further explore the role of MAIT cells, in terms of immune surveillance against colorectal liver metastases in patients undergoing liver resection.

2.2. Hypotheses

We hypothesize that an elevation of ≥3 CTCs/7.5 ml in the blood will indicate disease progression during the rapid liver hypertrophy in patients with colorectal liver metastases. Moreover, we believe that the presence of MAIT cells with their cytotoxic effector molecules and pro-inflammatory cytokines such as (interferon [IFN]-gamma, interleukin [IL]-17) in the future liver remnant will represent the immunological

competency of the liver during the rapid liver regeneration process in ALPPS patients with colorectal liver metastases. Finally, we predict that chemotherapy will not affect the liver immune functions.

2.3. Objectives

1. To determine if rapid hypertrophy in colorectal liver metastases induces disease progression.
2. To determine if the liver in patients with colorectal liver metastases undergoing ALPPS is immunologically competent.
3. To identify if chemotherapy affects the liver immune response in patients with colorectal liver metastases undergoing for liver resection.

CHAPTER 3

3. Methodology

3.1. Experimental Design

We designed a prospective, observational, 2-arm feasibility study assessed the utility of blood biomarkers of cancer progression (Circulating tumour cells, "CTCs"), and immunological competency of the liver (MAIT cells) in patients with colorectal liver metastases (CRLM) undergoing ALPPS (Arm 1) or single stage liver resection (Arm 2; control).

Up to 10 eligible patients per arm were accrued from the London Health Science Centre-University Hospital (LHSC-UH) and the London Regional Cancer Program (LRCP). Sample size was determined based on the pilot nature of the study and our demonstrated ability to recruit ~14 eligible ALPPS patients/year. (35)

Ethics approval for this study was obtained from the Western University Research Ethics Board for Health Sciences Research Involving Human Subjects (Approval number: REB106937; **Appendix 1**) and the Lawson Health Research Institute (R-15-360). All work involving the use of human samples was done in accordance with ethics guidelines approved by the institutional research ethics review board at Western University. Subjects were provided a letter of consent outlining the study details and informed written consent was obtained.

3.1.1. Patient inclusion criteria:

- Male and female patients over 18 years of age
- Confirmed diagnosis of colorectal liver metastasis (CRLM)

- Systemic chemotherapy treatment response
- No evidence of extra-hepatic metastatic disease
- Intent for curative liver surgery treatment

  Technically feasible resection (planned RO)

3.1.1.1. Specific inclusion criteria for ALPPS (Arm 1):

- Diagnosis of CRLM with predicted future liver remnant less < 30%
- Extensive bilobar CRLM necessitating an extended hepatectomy
- Good functional capacity (Eastern Cooperative Oncology Group [ECOG] 0 or 1)
- Complete or partial response to systemic chemotherapy after 4-6 cycles evident by CEA and radiology
- Consented for ALPPS

3.1.1.2. Specific inclusion criteria for single stage liver resection (Arm 2):

- Diagnosis of CRLM with predicted future liver remnant more than > 50 %
- Single or oligo CRLM consented for single stage resection

3.1.2. Exclusion Criteria:

- Age more than 75 or less than 18 years
- ASA risk score more than 3
- Eastern Cooperative Oncology Group (ECOG) score 2 or more
- End-stage liver disease

3.2. Chemotherapy treatment

All patients in this study were reviewed by a multidisciplinary tumor board (consisting of HPB and colorectal surgeons, medical oncologists, radiation oncologists, pathologists, and radiologists). Following discussion, the consensus recommendation of the multidisciplinary tumour board was followed regarding liver surgical approach and type of neo-adjuvant treatment regimen. The choice of chemotherapy regimen was based on tumour load, tolerance, and comorbidities by the multidisciplinary tumour board. Tumour responses and progression were classified with the use of the Response Evaluation Criteria in Solid Tumour (RECIST) after 4 cycles.(71)

3.3. Surgical technique

The technical aspects of single or two staged hepatectomy "ALPPS" have been previously described **(Figures 12-14)**. (7,8,72–74) Major liver resection was defined according to the Brisbane 2000 terminology (resection of ≥ 3 segments of the liver). (75)

Single staged hepatectomy was performed in all patients with curative intent with predicted future liver remnant (FLR) more than ≥ 50 %, whereas ALPPS was performed in those patients with predicted FLR < 30 % that met full inclusion criteria. In both scenarios a general laparotomy was performed to rule out extrahepatic disease, an intraoperative ultrasound was performed to assess the technical resectability and presence of metastases not visualized prior to surgery. The liver was mobilized according to the location of the tumour and segments to be respected. (7)

The technical aspects of ALPPS procedure are summarized in two major surgical stages and one interval phase. The key points of stage-1 include complete mobilization with ligation and division of the retro-hepatic veins draining into the inferior vena cava (IVC), tumour clearing of the FLR if disease was present in the FLR, right portal vein ligation with hepatic arterial flow preservation and biliary drainage of the deportalized liver (DL), and liver in situ split of the parenchyma between the FLR and deportalized liver. After sufficient growth of the FLR is obtained (generally within 7 to 10 days) measured by volumetric computed tomography (CT) volumetric, a stage-2 procedure was completed with a right trisectionectomy by a transection of the right hilar plate, as well as transection of the right and middle hepatic veins followed by the removal of the DL.

Complications were scored according to the Dindo-Clavien grading system. (76) Minor complication was defined as a complication grade ≤ II, major complication as a grade ≥ IIIa, and severe complications as grade ≥IIIb.

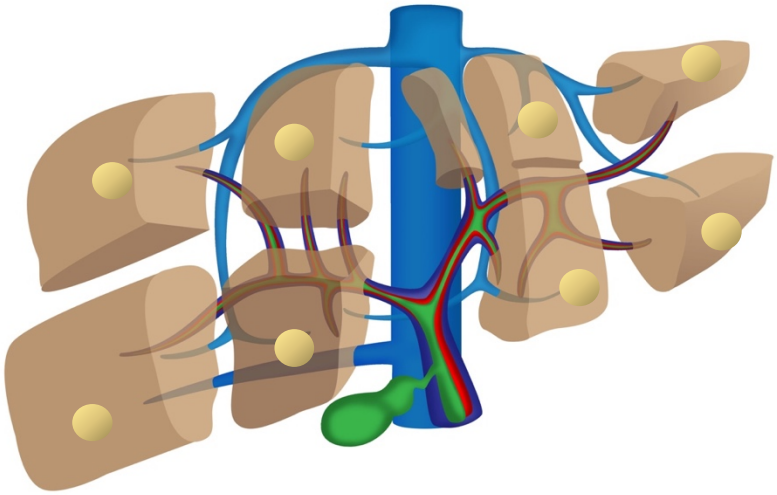


Figure 12. Representation of extensive metastatic bilobar disease from colorectal cancer patients.

Associating Liver Partition with Portal Vein Ligation for Staged Hepatectomy

"ALPPS" concept, surgical technique description:

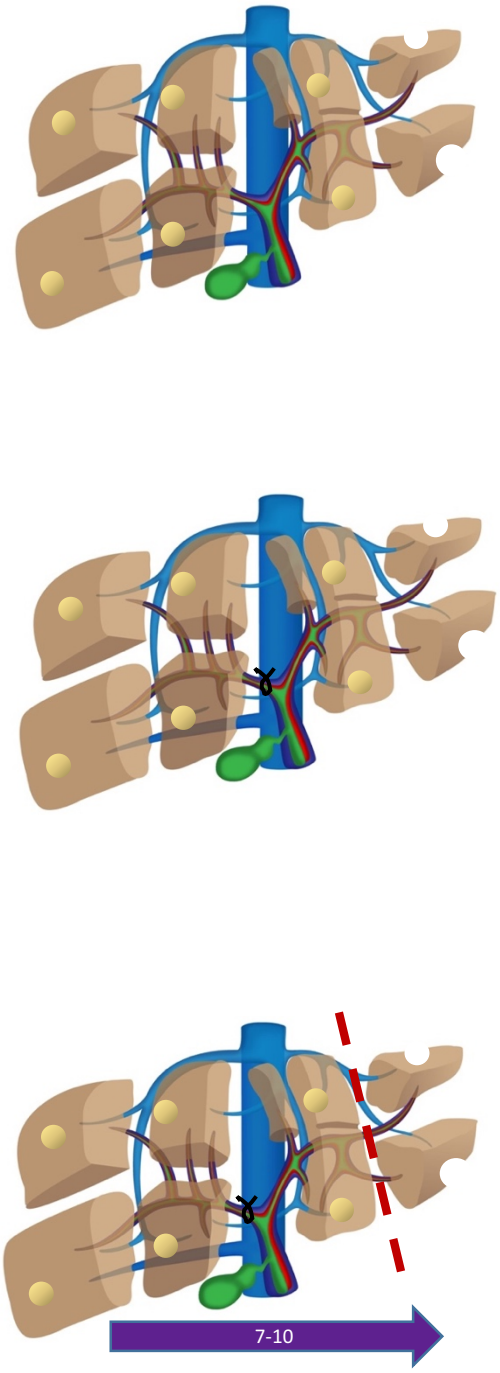
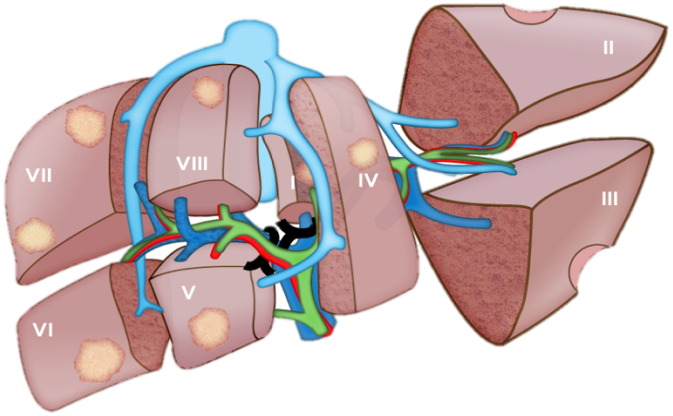


Figure 13. **Stage 1 ALPPS**: **(A)** Clearing of the future liver remnant (resection of CRLM in segment II and III of the liver) **(B)** portal vein ligation in order to redirect the portal blood flow through the FRL with preservation of the right hepatic artery; (C) parenchymal transection along the deportalized liver and future liver remnant. Following by 7-10 days



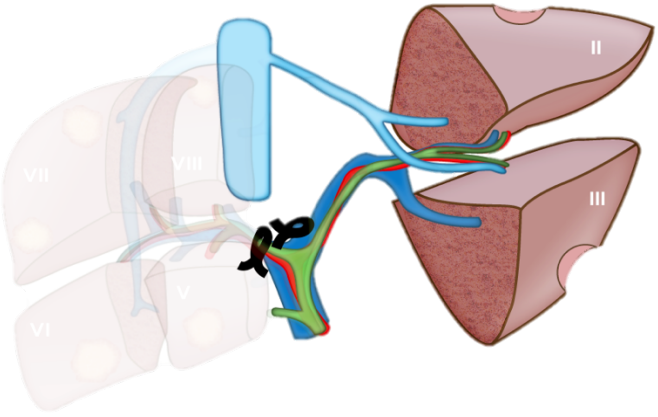


Figure 14. (A) **Inter-stage ALPPS**: volumetric liver of the FLR is reassessed at day 7-10 by abdominal computed tomography for enough hypertrophy**; (B)** **Stage 2 ALPPS**: ALPPS finalized with an extended right hepatectomy, keeping a growth FLR.

3.4. Circulating tumour cells (CTCs)

Blood samples (7.5 ml) were collected by routine phlebotomy, and measured at the following endpoints:

- Arm 1: baseline (pre-chemotherapy), pre- stage 1 ALPPS, inter-stage ALPSS, post 2 stage ALPPS and prior to re-starting chemotherapy (follow-up). **(Figure 15)**
- Arm 2: baseline (pre-chemotherapy), pre-resection surgery (single stage liver resection), 3-4 days' post-resection surgery and prior to re-starting chemotherapy (follow-up). **(Figure 16)**

3.4.1. Blood sample preparation for CellSearch® platform analysis

Samples were collected into CellSave blood collection tubes (Janssen Diagnostics), which stabilize CTCs for 96 hours at room temperature. 1 x 10 ml sample per patient was collected at each time point. Blood samples were analyzed for the presence and number of CTCs using CellSearch® platform (Janssen Diagnostics) located in the clinical flow cytometry facility at LHSC. An automated and standardized immunomagnetic cell enrichment of CTCs in blood samples (7.5. ml) was carried out using antibodies targeting epithelial cell adhesion molecule (EpCAM), and subsequent labeling with fluorescent antibodies specific for epithelial cells (CK 8,18, and 19), DAPI, and leukocytes (CD45/DAPI). Samples were imaged using the CellSearch Analyzer II, and CTCs were identified by two trained (independent and blinded) reviewers based on positive CK staining, negative CD45 staining, cell size (> 4um), cell morphology (round to oval), and staining with the DNA stain 4', 6-diamino-2-phenylindole (DAPI).

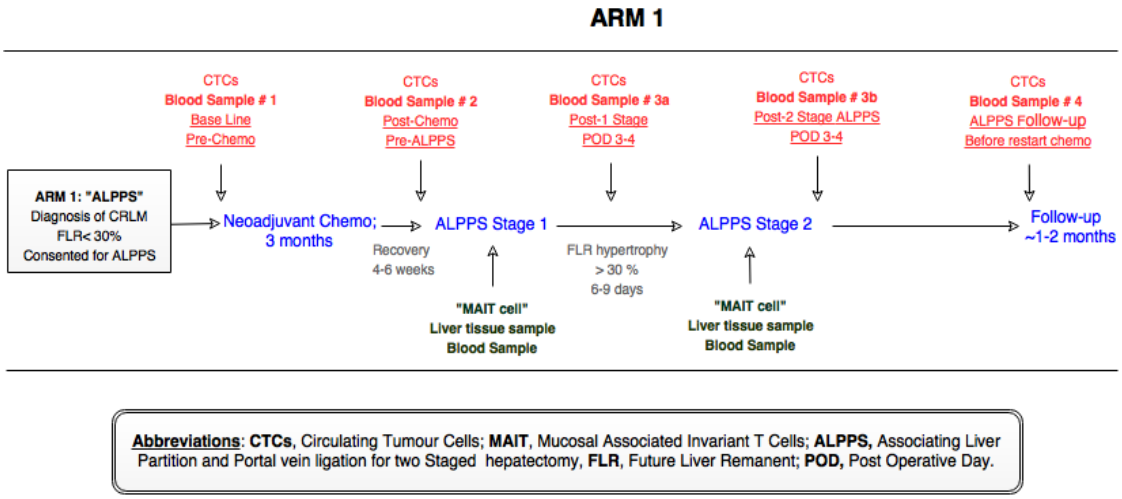


Figure 15. **ARM 1:** ALPPS group, with the sequential description of the time-points, where the CTCs blood samples and the liver tissue with respect to the MAIT cells were taken.

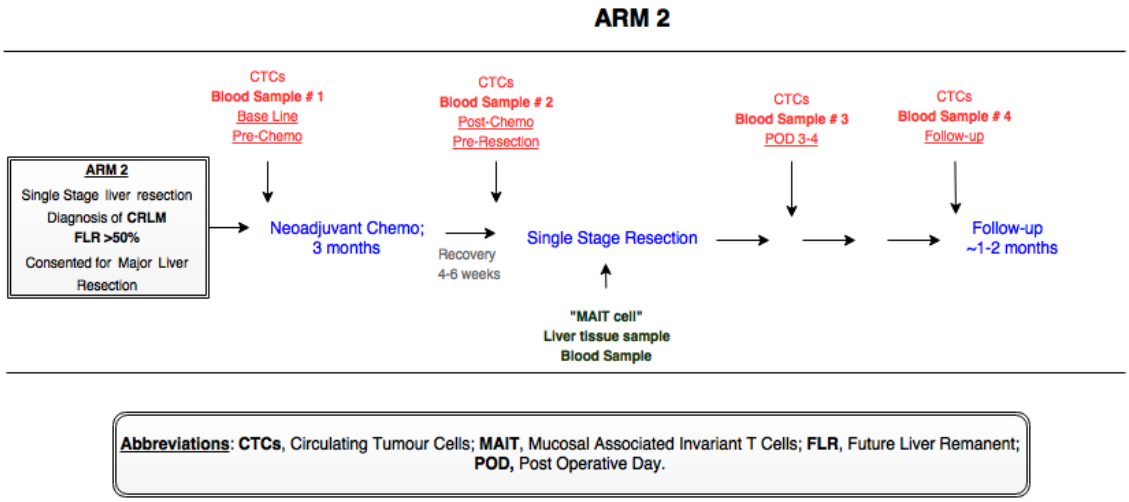


Figure 16. ARM 2: Single stage liver resection group, with the sequential description of the time-points, where the CTCs blood samples and the liver tissue with respect to the MAIT cells were taken.

3.4.2. Circulating tumour cell data analysis

CTCs # at each time point were categorized as being either unfavourable (≥3 CTCs) or favourable (<3 CTCs) as defined previously. (60) The relationship between CTC # and clinical outcome was investigated and were observed and compared during the rapid liver regeneration process and the single liver resection. **(Objective 1)**

3.4.3. Statistical analysis

The distribution of continuous variables was tested for normality using the Shapiro-Wilk test. Continuous variables with normal distributions were express as mean (± standard deviation) and were compared using the independent sample t-test. Variables with non-normal distributions were expressed as median (interquartile range) and were compared using the Mann-Whittney-U test. Categorical variables were compared using the Fisher's exact test. Statistical significance was accepted at $p< 0.05$. Data was analyzed using SPSS 20 (IBM SPSS, Chicago, IL., USA).

3.5. Mucosa-associated invariant T (MAIT) cells

For the immunological studies, liver tissues and blood samples were collected from each Arm of the study at the time of the surgery. **(Figure 15 and 16)**

3.5.1. Human peripheral blood mononuclear cell isolation

Peripheral blood was collected prior to administration of anesthesia in 5 x standard heparinized BD vacutainers (BD Biosciences, San Jose, CA, USA) for a maximum volume of approximately 50mL). Following collection, whole blood was diluted 1:1 in 2% Fetal Calf Serum (FCS) /PBS and applied to a Ficoll-Paque Plus (GE Healthcare Life Sciences,

Mississauga, ON, CAN) gradient in 50mL SepMate tubes (Stem Cell Technologies) and processed at room temperature as per the manufacturers protocol. Following isolation, cells were resuspended in complete RPMI and seeded at approximately $1x10^6$ cells/well in 200µL, and indicated stimuli were added in a volume of 50µL for a total volume of 250µL per well.

3.5.2. Human liver mononuclear cell isolation

During the surgical resection, a 1-5 g sample of healthy liver tissue and 1-5 g sample of tumour tissue were collected in saline in a 15 mL tube and placed on ice. Samples were taken to the laboratory of Microbiology & Immunology located in the SDRI building at Western University. Immune function and profiling experiments were undertaken to determine the status of the immune cells residing within and distal to the tumour, and within the healthy liver tissue as well.

Following collection, liver tissue was cut into small pieces using a razor blade, after which they were pushed through a 400 µM filter and washed with 2%FCS/PBS. Collected cells were spun at 400Xg for 5 minutes, the supernatant decanted, and the pellet washed with an additional 50 mL 2% FCS/PBS. Following the second wash, cells were applied to a 33% Percoll Plus (GE Healthcare Life Sciences, Mississauga, ON, CAN) mixture and spun for 12 minutes at 700Xg (no brake). Following the spin, the supernatant was discharged and the mononuclear cells were isolated from the pelleted fraction. Following isolation, cells were resuspended in cRPMI and plated at approximately $1x10^6$ cells/well in 200µL, and indicated stimuli were added in 50 µL of media for a total volume of 250µL per well.

3.5.3. In vitro stimulation

Recombinant IL-12 (Peprotech, Quebec, CAN) and IL-18 (Peprotech, Quebec, CAN) were used at a concentration of 5 ng/mL for the *in vitro* activation of isolated MNCs. The bacterial superantigen staphylococcal enterotoxin B (SEB) was used at 100 ng/mL. Isolated MNCs were plated at $10x10^6$cells/mL and left untreated or stimulated with a combination of recombinant IL-12 (rIL-12) and rIL-18, SEB, or *Klebsiella pneumoniae* lystate for 24 hrs. For the last 5 hours, a combination of 1 μM brefeldin A (BFA) (Sigma-Aldrich) and 2 μM monensin (eBioscience) was added to the cells.

3.5.3.1. Staphylococcal Enterotoxin B (SEB)

Recombinant SEB was generated using an approved institutional biosafety protocol adhering to the Public Health Agency of Canada regulations. SEB was cloned from *S. aureus* (strain COL), expressed in BL21 (DE3) competent *Escherichia coli* (*E. coli*), and purified by nickel column chromatography.

3.5.3.2. Preparation of Klebsiella lysate

A stock of *Klebsiella pneumoniae* lysate was generated by growing a *Klebsiella pneumoniae* clinical isolate, Parkwood-18 (a gift from Dr. Miguel Valvano (Queen's University Belfast, Belfast, United Kingdom)), overnight in Luria broth at 37°C. Following overnight culture, the culture was washed three times with cold PBS and the OD_{600} adjusted to 6.5. Cells lysis was achieved by subjecting the cells to a pressure at 30,000 pounds per

square inch (PSI) for 5 min to induce membrane rupture. The resulting lysate was stored at -80°C until use. The lysate was at 1:50 for in vitro cell stimulatio

3.5.4. Flow cytometry

Cells were labeled using various panels of anti-human antibodies, as has previously been described.(77) Cells were then stained extracellularly with a panel of anti-human antibodies at 4°C cells diluted in 2%FCS/PBS. After 30 minutes, cells were washed 3 times with 2 mL of 2% FCS/PBS. In cases of intracellular cytokine, 5 hrs prior to the end of culture a mixture of BFA diluted 1:10000 and Monensin A diluted 1:1000 (eBioscience) were added to the cultures to allow for the intracellular accumulation of cytokines. Additionally, for intracellular antigen or transcription factor analysis determination, following extracellular staining cells were re-suspended in 150 μL cytofix/permeabilization buffer (eBioscience) and left at room temperature in the dark for 20 minutes. Following fixation, cells were washed with 100 μL of 1x Permwash (eBioscience) and re-suspended in 100 μL of a panel of intracellular antibodies diluted in 1x Permwash. In the case of transcription factor determination, the *foxP3* transcription factor permeabilization kit was used (eBioscience) in place of the standard intracellular cytofixation kit. A BD FACSCanto II flow cytometer and FlowJo v9 software (Tree Star, Ashland, OR, USA) were used for data acquisition and analysis.

Human MAIT cells were defined as CD3+ Vα7.2+ $CD161^{high}$. Briefly our gating strategy was as follows: From total PBMCs, lymphocytes (defined by their forward and side scatter properties) were gated and divided into CD3+ and CD3-. Of the CD3+ cells, MAIT cells were defined as CD3+ Vα7.2+ $CD161^{high}$, while conventional T cells were considered CD3+ Vα7.2-. For specific analytic analysis (i.e. cytokine, transcription factor

expression, granzyme, etc.), appropriate isotype controls were used when determining the gate locations. Unless otherwise stated, antibodies were obtained from eBioscience. Vα7.2 -PercpCy5.5 and granzyme A - Alexa 700 were obtained from Biolegend (San Diego, CA, USA).

3.5.5. MAIT cells data analysis

Comparison of the MAIT cells present within and outside metastatic lesions in terms of their frequency and function (i.e. IFN- and IL-17 production) within the peripheral blood and liver were performed. Moreover, we evaluated the liver immunological competence regarding the activity of the MAIT cells among patients undergoing for rapid hypertrophy (ALPPS) and single liver resection was also made. **(Objective 2)**

A correlation between the type of chemotherapy and the activity of the MAIT cells in the peripheral blood and the liver was performed in order to analyze the effect of the chemotherapy into the liver immune response in both groups. **(Objective 3)**

3.5.6. Statistical analyses

Statistical assessments were made with the aid of GraphPad Prism 6 software. Comparisons were performed using Student's *t*-test or ANOVA, as appropriate, and differences with $p<0.05$ were deemed significant. *, **, *** and **** denote $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively.

CHAPTER 4

4. RESULTS

The objectives of the current study were to determine if the rapid hypertrophy that occurs during ALPPS induces disease progression, to evaluate if the liver of ALPPS patients was immunologically competent, and to investigate whether chemotherapy affects the liver immune response in patients with colorectal liver metastases undergoing for liver resection; either ALPPS or single stage hepatectomy.

In our prospective, observational, 2-arm feasibility study we were able to assess the utility of CTCs as an evaluation tool of disease progression and the immunological competency of the liver through analysis of MAIT cells in patients with colorectal liver metastases undergoing to ALPPS (Arm 1) or single stage liver resection (Arm 2; control). In the present investigation (From August 2015 to June 2016), 24 patients were recruited for the study and 17 patients full met our study criteria; 7 patients in Arm 1 (ALPPS group) and 10 patients in Arm 2 (Single stage liver resection group). Patient selection and management flow chart is shown in **Figure 17.**

Patient characteristics from the 17 patients that completed liver resection are summarized in **Table 1.** Median follow up was 139 days (interquartile [IQR] range 46 to 379 days). All patients completed follow-up evaluation to assess CTCs at time point 4 (1-2 month after surgery). Demographic characteristics were comparable in both groups, including age, gender and body mass index. Preoperative risk index was also similar in both groups.

Flow chart of the 24 patients recruited in the study for CTC and MAIT cells

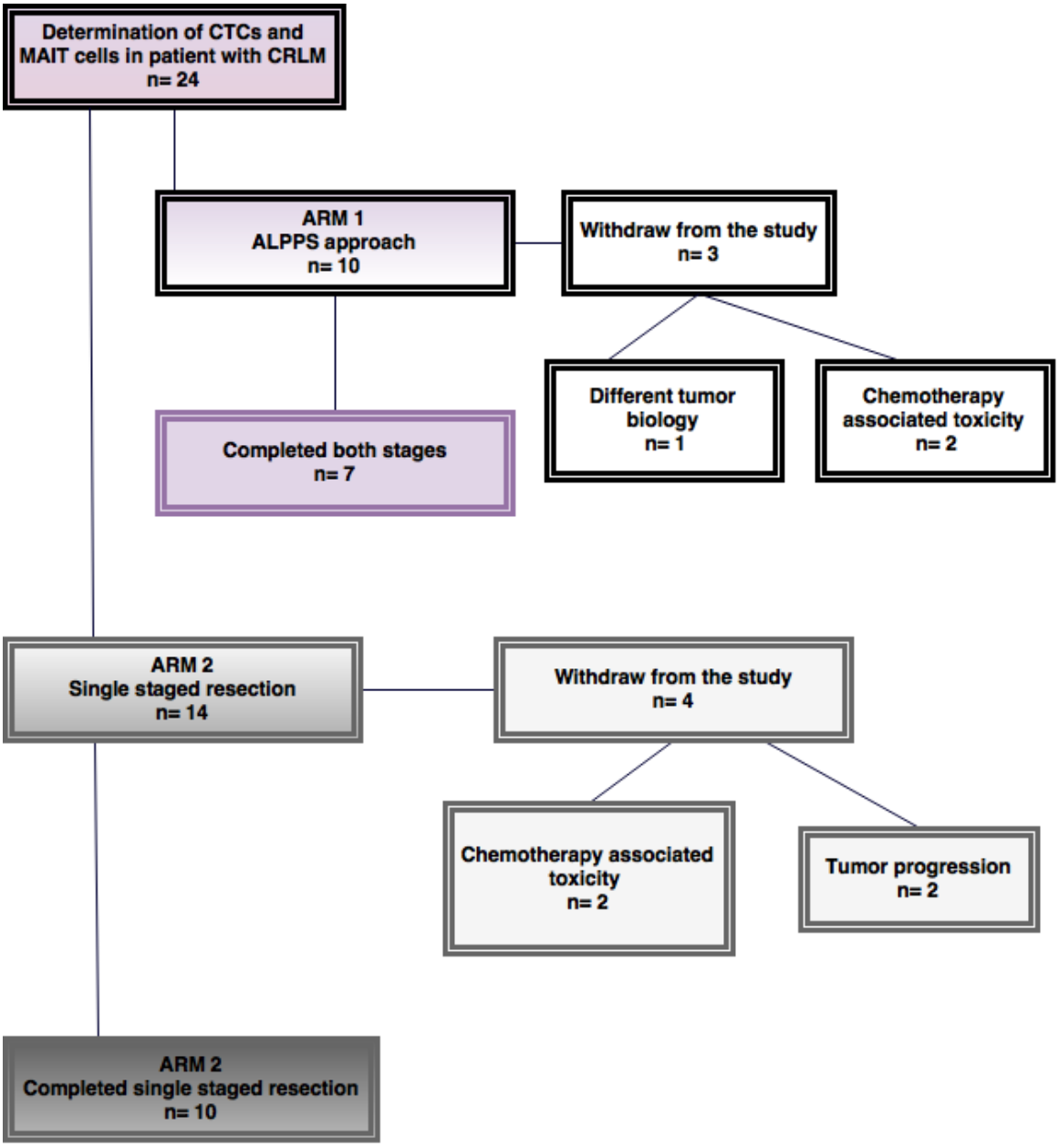


Figure 17. Flow chart summarising the 24 patients recruited in the study for investigation of CTCs and MAIT cells in patients with CRLM undergoing for curative liver resection treatment, either; ALPPS (ARM1) or single staged resection (ARM2). Seven patients completed both stages in ARM 1, while 10 patients completed single staged resection in ARM 2.

TABLE 1. TRANSLICH STUDY POPULATION CHARACTERISTICS			
VARIABLE	**ARM 1 ALPPS N= 7**	**ARM 2 SINGLE STAGE LIVER RESECTION N= 10**	***p= 0.05***
Demographics			
Age, (years) **μ**	57 ± 4	55± 3	0.598
Male Gender [n(%)]	6 (85%)	4 (40%)	0.134
BMI (Kg/m^2) **μ**	25 ± 2	24.7 ± 1.3	0.969
ASA operative risk, n(%)			
< 3	2 (29%)	6 (60%)	
> 3	5 (71%)	4 (40%)	0.334
Primary tumour location, n (%)			
Colon	7 (100%)	7 (70%)	0.227
Rectum	0	3(30%)	
Stage of primary disease, n (%)			
T-4	3 (43%)	4 (40%)	
T-3	3 (43%)	6 (60%)	0.434
T-2	1 (14%)	0	
Lymph Nodes Positive	4 (0-18)	0.5 (0-8)	0.183
Grade, n (%)			
High grade	2 (29%)	1 (10%)	0.536
Low grade	5 (71%)	9 (90%)	
Metastatic tumour disease, n (%)			
Synchronous	6 (85%)	8 (80%)	1..0
Bilobar tumour involvement	4 (40%)	4 (67%)	0.608
Number of lesions	4 (2-9)	3 (2-8)	0.599
Size, largest lesion (mm) before chemotherapy	45 (20-200)	34.5 (10-86)	0.215
Size, largest lesion (mm) after chemotherapy	25 (17-150)	27(10-49)	0.43
Carcinoembryonic Antigen (CEAμ,mL)	23 (23-80)	4 (1-4220)	**0.047**

Continues variables are expressed as median (**IQR**). Abbreviations: **μ**, mean; **Kg**, Kilograms, **BMI**, body mass index

4.1. Demographics Characteristics

4.1.1. Primary Tumor Characteristics

Primary tumour location was as follows: Arm 1; 100% of the patients had the primary tumour in the colon, and Arm 2; 70% (7 patients) had the primary tumor in the colon and 30% (3 patients) in the rectum, p= 0.334. Based on the TNM score classification for malignant tumours, the primary cancers in both Arms were comparable (**Table 1).**

4.1.2. Metastatic Tumour Disease

Synchronous metastatic disease was present in 6 patients (85%) in Arm 1 vs 8 patients (80%) in Arm 2, p= 1.0. Bilobar tumour involvement was observed in 4 patients (57%) in Arm 1 compared to 4 patients (40%) in the Arm 2, p= 0.608. The median number of liver metastatic lesions in Arm 1 was 4 (interquartile range [IQR] 2-9) and 3 in Arm 2 (2-8), p= 0.599 (**Table 1).**

The median size of the largest lesion (mm) before chemotherapy treatment was 45 mm (20 mm-200 mm) in Arm 1 and 34.5 mm (10 mm-86 mm) in Arm 2, p=0.215. After chemotherapy treatment, there was a decrease in the median size of previous largest lesion; 25 mm (17 mm-150 mm) in Arm 1 and 27 mm (10 mm-49 mm) in Arm 2, p= 0.430 (**Table 1).**

The levels of Carcinoembryonic Antigen (CEA μ mL) before chemotherapy treatment was significantly higher in the Arm 1, 23 (23-80) compared to Arm 2, 4 (1-4220), p=0.0478 (**Table 1).**

4.1.3. Chemotherapy Characteristics

Of the 17 patients that underwent liver resection, all received neo-adjuvant chemotherapy treatment. Patients received either single regimen with FOLFOX or combined regimen FOLFOX or FOLFIRI with Bevacizumab. Most of the patients in Arm 1 had combined regimen 5/7 (71%), while most of the patients in the Arm 2 had single regimen with FOLFOX 7/10 (70%), p= 0.095. The median number of chemotherapy cycles was 6 (5-11) in Arm 1 vs 5 (1-7) in Arm 2, p=0.439. The median time off (days) chemotherapy prior liver resection was 50 days (32-72 days) in Arm 1 and 51 days (27-184 days) in Arm 2, p=0.830 (Table 2). Of the 7 patients in Arm 1, 6 (86%) showed radiologically chemotherapy response, compared to 7 of 10 patients (70%) in Arm 2, p=0.338 (**Table 2**).

4.1.4. Surgical approach

One patient in Arm 1 (14%) underwent an extra hepatic simultaneous resection (low anterior resection, LAR), whereas 3 patients (30%) in Arm 2 (2 patients had LAR and 1 patient had an abdominal perineal resection, APR), p= 0.198 (**Table 3**). The estimated blood loss in Arm 1 was significantly higher compared with Arm 2, median of 1000 ml (400ml-2500ml) vs 205 ml (50ml-7000ml), respectively, p= 0.013. However, the number of patients that received blood transfusion was similar in both Arms, 2 each, p =1.0. The range of red blood cells packs per patient was .8 (0-4) in Arm 1 vs 0.9 (0-7) in Arm 2, p=0.933.

TABLE 2. TRANSLICH STUDY POPULATION CHEMOTHERAPY CHARACTERISTICS			
VARIABLE	**ARM 1 ALPPS N= 7**	**ARM 2 SINGLE STAGE LIVER RESECTION N= 10**	***p*= *0.05***
Chemotherapy characteristics			
Chemotherapy prior surgery, n (%)	7(100%)	10 (100%)	**NS**
≥ 6 Cycles chemotherapy	6 (5-11)	5 (1-7)	0.439
Type of chemotherapy			
Single Regimen			
FOLFOX	2 (29%)	7 (70%)	
Combined regimen			0.095
FOLFOX or FOLFIRI /Bevacizumab	5 (71%)	3 (30%)	
Time off chemotherapy prior to liver resection (days)	50 (32-72)	51 (27-184)	0.830
Chemo-response, radiology n (%)	6 (86%)	7 (70 %)	0.338

Continues variables are expressed as median (**IQR**). Abbreviations: **single regimen,** refers to single regimen either FOLFOX, FOLFIRI; **combined regimen,** refers to combination either FOLFOX or FOLFIRI with Bevacizumab.

TABLE 3. TRANSLICH STUDY POPULATION SURGICAL CHARACTERISTICS			
VARIABLE	**ARM 1 ALPPS N= 7**	**ARM 2 SINGLE STAGE LIVER RESECTION N= 10**	***p= 0.05***
Type of liver resection, n (%)			
Extended hemihepatectomy	7 (100%)	0	
Hemihepatectomy	0	5 (50%)	**0.002**
≥ 3 wedges resected	0	5 (50%)	
Extrahepatic simultaneous procedures, n (%)			
Low Anterior Resection	1 (14%)	2 (20%)	
Abdominal perineal resection	0	1 (10%)	0.198

4.1.5. Future Liver Remnant (ALPPS group)

The median interval phase in days between the two stages in the Arm 1 was 9 (7-40 days). Of the 7 patients in Arm 1, the mean %FLR/total liver volume prior the stage one was 20 ± 2.2. The percentage hypertrophy of FLR/Total liver volume after stage 1 was 35 ± 1.6.**(Table 4)**

4.1.6. Surgical Outcomes

4.1.6.1. Complications

Six of 7 patients in Arm 1 (85%) had post-surgical complications versus 4 of 10 in Arm 2 (40%), p=0.050. Of these 6 complications in Arm 1, 66% were minor complications and 17% were major or severe complications, while in Arm 2 these values were, 25%, 50%, 25%, respectively, p=0.405. The total length of hospital stay was significantly higher in Arm 1 due the fact of two stage procedure, 17.5 days (14-33) in Arm 1 compared with 7 days (4-62 days) in Arm 2, p=0.020 (**Table 5**).

4.1.6.2. Liver histopathology

R0 resection was presented in 85% of the cases in Arm 1 and 100% of the cases in Arm 2. The median resection margin was significant higher in Arm 1, 20 mm (8mm -55 mm) compared with Arm 2, 5 mm (1mm-14mm), p= 0.009. Based on the histopathology, all 7 patients in Arm 1 (100%) showed chemotherapy response, versus only 5 patients (50%) in Arm 2, p=0.026. Also, the rate of tumour necrosis related with the chemotherapy response was higher in Arm 1; 80 (75-100) vs 5 (0-100) in Arm 2, p=0.009 (**Table 5**).

TABLE 4. LIVER VOLUME ALPPS GROUP	
VARIABLE	ARM 1 ALPPS N= 7
Interval Phase in days, median (range)	9 (7-40)
% Future Liver Remnant/Total liver volume (SD)	20 ± 2.2
% Future Liver Remnant/Total liver volume after stage 1, mean (SD)	35 ± 1.6

TABLE 5. TRANSLICH STUDY POPULATION POSTOPERATIVE OUTCOMES			
VARIABLE	**ARM 1 "ALPPS" N= 7**	**ARM 2 SINGLE STAGE LIVER RESECTION N= 10**	***P= 0.05***
Complications			
Any complications, n (%)	6 (85%)	4 (40%)	0.050
Minor complications, Grade ≤ II	4 (66%)	1 (25%)	
Major complications, Grade IIIa	1 (17%)	2 (50%)	0.405
Severe complications, Grade ≥ IIIb	1 (17%)	1 (50%)	
90 days' mortality	0	0	
Mortality	3/7 (43%)	2/10 (20%)	NS
Total length of hospital stays in days, Median (range)	17.5 (14-33)	7 (4-62)	**0.020**
Histopathology liver			
Margins (mm)	30 (8-55)	5 (1-14)	**0.009**
Chemotherapy response, n %	7 (100%)	5 (50%)	**0.026**
Chemotherapy, tumour necrosis % (range)	80 (75-100)	5 (0-100)	**0.009**
R0	6 (85%)	10 (100%)	0.411
R1	1 (15%)	0	

4.2. Circulating tumour cells

Evaluation of CTC status in the 24 patients recruited in the study is summarized in **Table 6**. Due to logistical challenges, CTCs were only assessed in 2/10 patients prior to the start of chemotherapy in Arm 1 and in 5/14 patients in Arm 2. The findings showed positive CTCs in 1 (50%) patient of Arm 1 (1-4 cells), and 3 (50%) patients in Arm 2 (1-6 cells), p= 0.999. Moreover, assessment of CTC status was performed after completed chemotherapy for all of the 24 patients recruited in the study; 10/10 patients in Arm 1 and 14/14 in Arm 2. At this time-point, none of the 10 patients in Arm 1 showed positive CTCs cells, whereas 2/14 (15%) patients in Arm 2 had positive CTCs (1-5 cells), p= 0.266. Furthermore, one patient with positive CTCs, ≥3 (5 cells) in Arm 2 after completion of chemotherapy was found to have progression of disease on imaging and was therefore withdraw from the study, p=0.0012 (**Table 6**).

From the 24 patients that initiated the study, 17 completed liver resection, 7 patients in Arm 1 and 10 patients in Arm 2, and CTC status was evaluated after stage 1 (time-point 3a) and stage 2 (time-point 3b) in the case of Arm 1, and compared with the control group of single stage liver resection in Arm 2. Among the 7 patients with CTCs available after stage 1, two (28.6%) patients had positive CTCs (1-2 cells), and one (17%) patient presented with positive CTCs (1 cell) after completing both stages. On the other hand, 4 (44%) patients in Arm 2 presented with positive CTC status (1-2 cells), after completed single staged resection, p=0.286 (**Table 6**) **(Figure 18)**.

TABLE 6. Circulating tumour cell assessment of the 24 patients recruited for the study.

Group	Liver metastases							CTCs Time-Points					Outcome
ARM 1	Sync	N of lesions	Size prior chemo (mm)	Size After chemo (mm)	CEA (**µ/ML**)	# Chemo Cycles	Type of Chemo	1	2	3a	3b	4	**Death**
1	Yes	4	20	18	80	11	F/B	-	0	0	0	0	Yes
2	Yes	9	22	22	6.2	6	FX	-	0	2	-[d]	1	**Yes[R]**
3↓[a]	No	4	32	32	2	3	Cap	-	0	↓	↓	↓	↓
4↓[b]	No	3	26	26	5.4	12	FX	-	0	↓	↓	↓	↓
5	No	2	96	65	-	5	FR/B	-	0	0	0	0	No
6	Yes	2	90	46	26	6	FX	-	0	0	0	0	No
7	Yes	7	45	25	65	5	FX/B	-	0	0	0	-[d]	Yes
8↓[b]	Yes	4	20	15	102	6	FR	-	0	↓	↓	↓	↓
9	Yes	3	200	150	80	6	FX/B	4	0	1	1	0	No
10	Yes	3	20	17	8.3	6	FX/B	0	0	0	0	0	No
ARM 2								1	2		3	4	
11	Yes	5	14	10	4.3	5	FX	-	0		0	0	No
12	Yes	3	13	18	4.1	6	FX/B	-	2		2	6	**Yes[R]**
13	Yes	8	34	19	28.1	6	FX/B	-	0		1	0	Yes
14↓[c]	No	3	85	120	16	3	FX	6	0[c]		↓[c]	↓[c]	↓[c]
15	Yes	4	35	35	-	1	FX	-	0		0	-	No
16	Yes	2	45	35	2.3	7	FX	1	-		0	0	No
17	Yes	2	16	14	2.1	5	FX	0	0		0	0	No
18	Yes	6	86	49	4220	6	FX/B	-	0		0	0	No
19	Yes	4	10	15	4.9	1	FX	-	0		1	0	No
20↓[b]	Yes	2	30	40	6.6	2	Cap	0	0		↓[b]	↓[b]	↓[b]
21↓[b]	Yes	4	40	40	50	6	FX	2	0		↓[b]	↓[b]	↓[b]
22	No	4	47	45	2.0	6	FX/B	-	0		0	0	No
23	No	2	48	48	1.6	1	FX	-	0		1	0	No
24↓[c]	Yes	2	58	58	.	1	FX	-	5[c]		↓[c]	↓[c]	↓[c]

ARM 1, ALPPS group; **ARM 2**, Single stage liver resection; **Sync**, synchronous colorectal liver metastases (CRLM); **n**, number of CRLM lesions; **size**, diameter of the largest CRLM; **CEA**, carcinoembryonic antigen; **# Chemo**, number of chemotherapy cycles before surgery; **Type of chemo**, FX FOLFOX, FR FOLFIRI, F/B FOLFOX and Bevacizumab, and Cap; Capecitabine. ↓ withdraw from the study: ↓[a] Different tumour biology, ↓[b] Associated chemotherapy toxicity, ↓[c] Tumor progression. [d] Insufficient blood sample, [R] recurrence and cancer related-death.

ARM1, Time points blood samples ALPPS: # 1, Pre-Chemotherapy; **# 2** Post-Chemotherapy; **# 3a,** Inter-Stage ALPPS, **# 3b**, Post- 2 Stage, **# 4**, Follow up**.**

ARM2, Time points blood samples Single liver resection: # 1, Pre-Chemotherapy; **# 2**, Post-Chemotherapy; **# 3,** Post-Liver resection; **# 4**, Follow up.

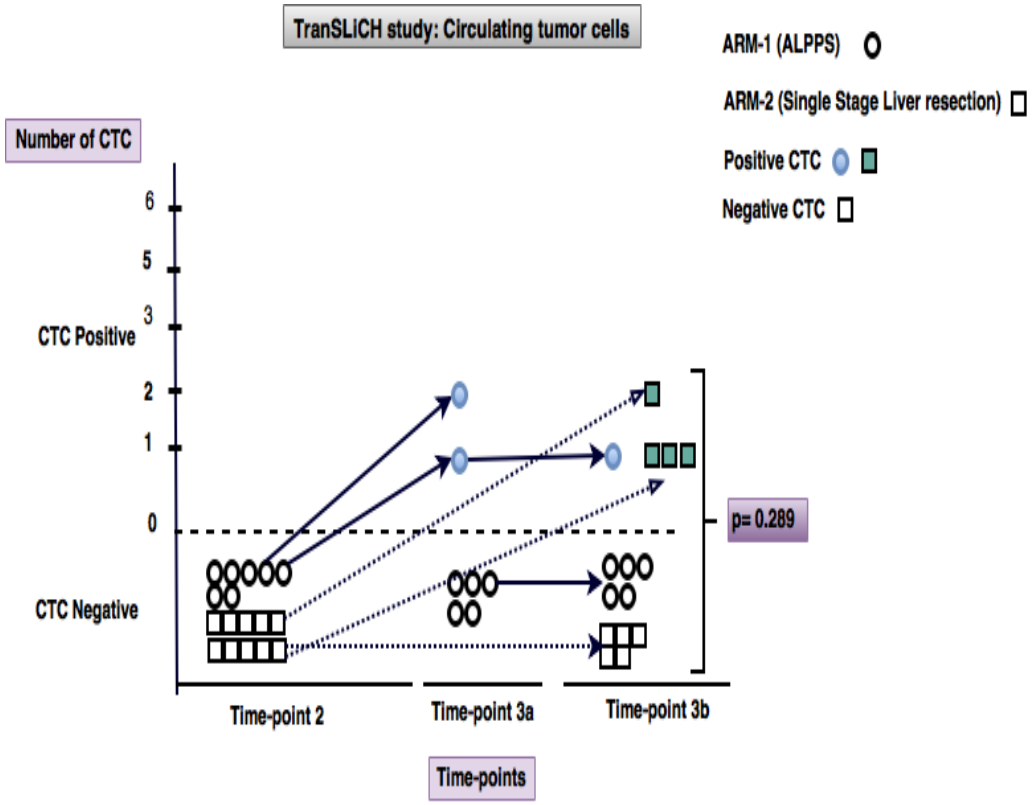


Figure 18. Comparison of Circulating tumor cells (CTC) status after chemotherapy (time-point 2) and after liver resection (time-point 3a and 3b). CTC status was available in 17 patients that underwent for liver resection (7 in ARM-1, and 10 in ARM-2). After stage 1 ALPPS, two patients had CTC positive status (1-2 cells). Among the 6 patients with CTC status after 2 stage ALPPS, none of the patients with prior CTC negative status were found positive after completed both stages. On the other hand, from the 10 patients in AMR-2 with CTC status after completed single stage resection, 4 patients were found with CTC positive status. Number of CTC and status (positive or negative) (y-axis). Time-point of blood sample collection for (x-axis). Open circles represent ARM-1, whereas open squares ARM-2.

The presence of CTCs was evaluated at follow-up (1-2 months after surgery) in the 16 patients with CTC status available. Of the seven patients in Arm 1, one patient who had positive CTCs after surgery remained positive at follow-up (1 cell). Of the 4 patients in Arm 2 with positive CTCs after liver resection, only one (12.5%) patient remained with positive CTC status during follow-up assessment (1-6 cells), p=0.999. In both cases where CTC positive status remained at follow-up (one patient in each Arm), these patients developed tumor recurrence, p=0.0083 **(Figure 19)**. Among the 16 patients with CTC status available at follow-up, eleven (69%) with negative CTC status were found alive without recurrence and three (19%) patients died. Two (12%) patients with positive CTC status were found with recurrence and cancer related-death, p=0.0249 (**Table 6**). Of the five deaths in the present study, three (43%) deaths were in Arm 1 and two (20%) were in Arm 2, p=0.592.

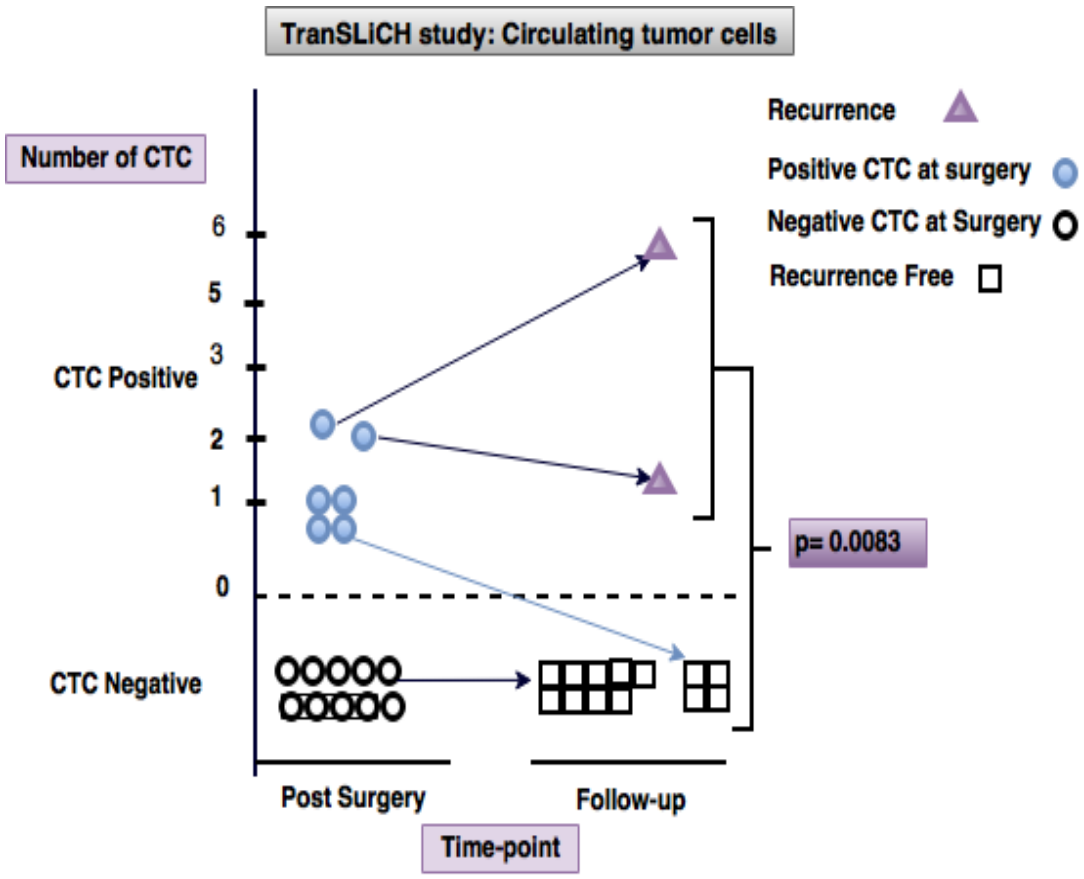


Figure 19. Circulating tumor cells (CTC) assessment after surgery and follow-up. Number of CTC and status (positive or negative) (y-axis). Time-point of blood sample for both groups (Post-surgery, and Follow-up) (x-axis). CTC status was available in 16 patients after surgery (circles), open circles; negative CTC, whereas, blue circles; positive CTC, (1-2 cells). At follow up, CTC status was available in 15 patients, open squares; negative CTC and recurrence free, while, purple triangle, positive CTC with recurrence (1-6 cells).

4.3. MAIT Cells

4.3.1. MAIT cells frequency differences between healthy volunteer's and colorectal liver metastases patients.

Initially, to assess whether our population with metastatic cancer presents with a different frequency of MAIT cells, we decided to compare our colorectal liver metastatic population with a healthy volunteer. We observed that our patients with CRLM had a reduced frequency of PBMCs a mean frequency of 3.3% MAIT cells was found, while the healthy volunteers had a mean of 5.3%. **(Figure 20a)**. This observation was later verified by $MR1^{Tet}$ staining **(Figure 20b)**. Moreover, we compared the frequency of MAIT cells in the healthy liver population with the frequency of MAIT cells within the tumour in our CRLM population. We found a generalized trend towards a reduction in the frequency of MAIT cells within the tumour, 28% vs 21%, however, this difference did not reach statistical significance ($p = 0.073$) (**Figure 20c,d**).

Figure 20 a

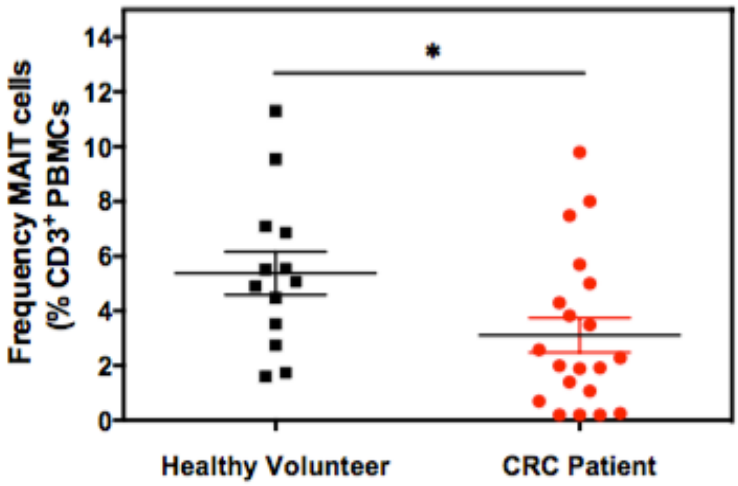


Figure 20 b

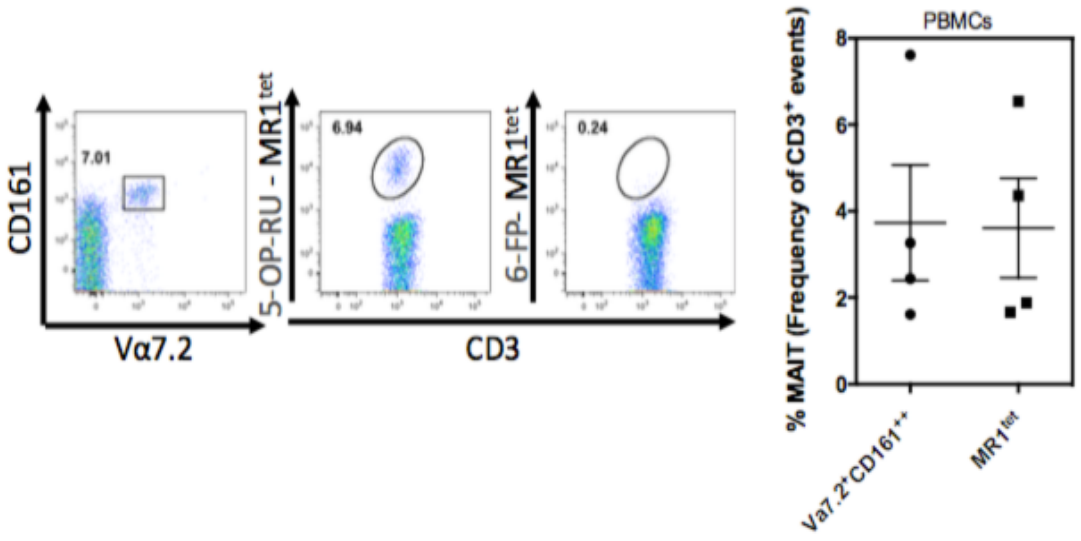


Figure 20 a-b. Graphics comparing the frequency of MAIT cells in the peripheral blood (PBMCS) among healthy 13 volunteers, and CRLM patients. A series of representative dot-plots depicting two equivalent gating strategies for identifying MAIT cells by $CD3^{+}V\alpha7.2^{+}CD161^{++}$ or 5-OP-RU $MR\text{-}1^{tet}$.

Figure 20 c

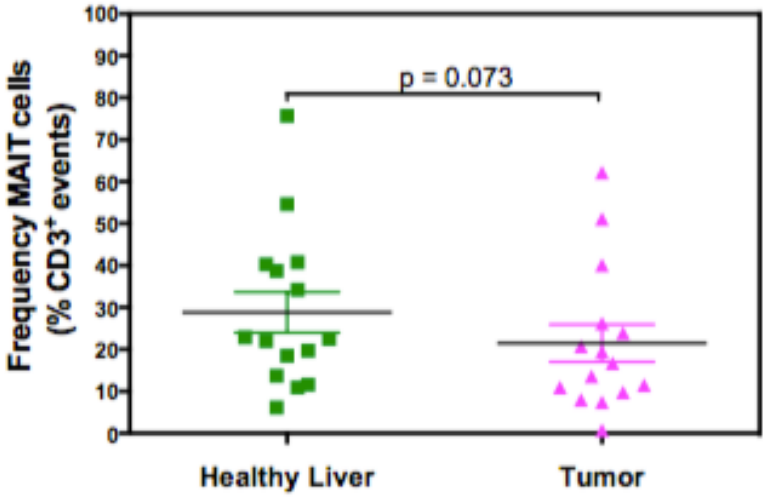


Figure 20 d

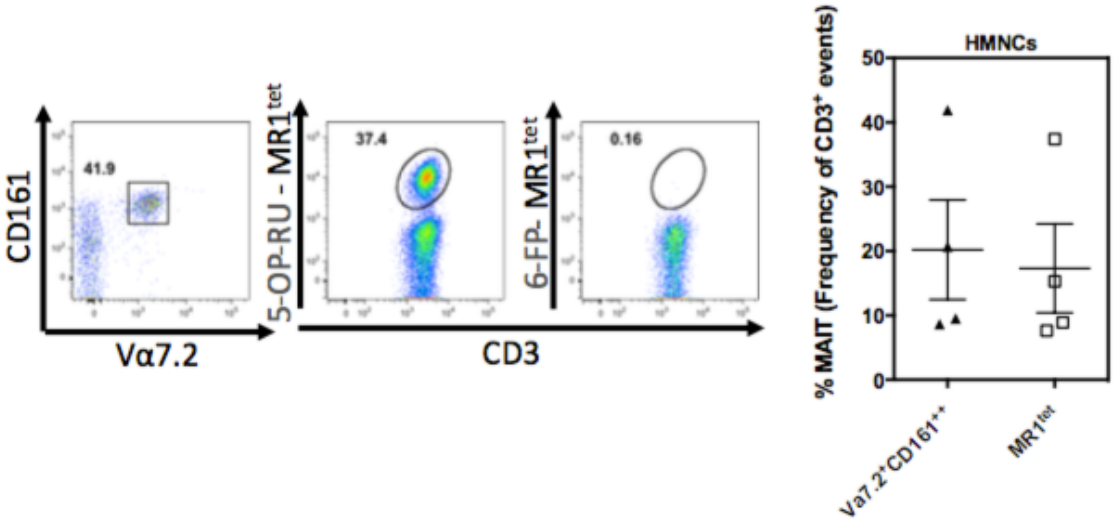


Figure 20 c-d. Graphics describing the frequency of MAIT cells in the healthy liver volunteers, and tumor from CRLM patients. A series of representative dotplots depicting two equivalent gating strategies for identifying MAIT cells by $CD3^{+}V\alpha7.2^{+}CD161^{++}$ or 5-OP-RU $MR\text{-}1^{tet}$.

4.3.2. Immunological differences between ALPPS group and single stage live resection.

To assess whether the liver in the patients undergoing ALPPS (ARM 1) with CRLM remains immunologically competent, we evaluated the frequency of MAIT cells among both groups. Thus, we analyzed the frequency of MAIT cells from the total CD3+ within the peripheral blood (PBMCs), liver, and tumor. Samples were taken during the liver resection, as described previously **(Figure 15-16)**. Among the 17 patients underwent for liver resection, 7 blood samples were available in ARM 1, whereas, 9 blood samples were available in ARM 2 for comparison. We found comparable frequency of MAIT cells in peripheral blood in both groups. Patients in ARM 1 had a mean frequency of 2.5% MAIT cells while the patients in ARM 2 had a mean frequency of 2.7 %, p= 0.870. (**Figure 21)**

Furthermore, we evaluated the frequency of MAIT cells in the liver, as well as the tumor. Interestingly, there was a trend towards an increase in MAIT cells in the ARM 1 within the liver (28 % vs 17.42%), however, this difference was not statistical significance (p=0.067), **(Figure 22).** Then, we observed the frequency of MAIT cells within the tumor. ARM 1 had a mean frequency of 17.42%, while, ARM 2 had a mean of 10.42 %, p=0.308. **(Figure 23).** Altogether, we evaluated and compared whether the frequency of the MAIT cells was different among both groups (the three compartments), but no significant difference was observed, p=0.271. **Figure.24**

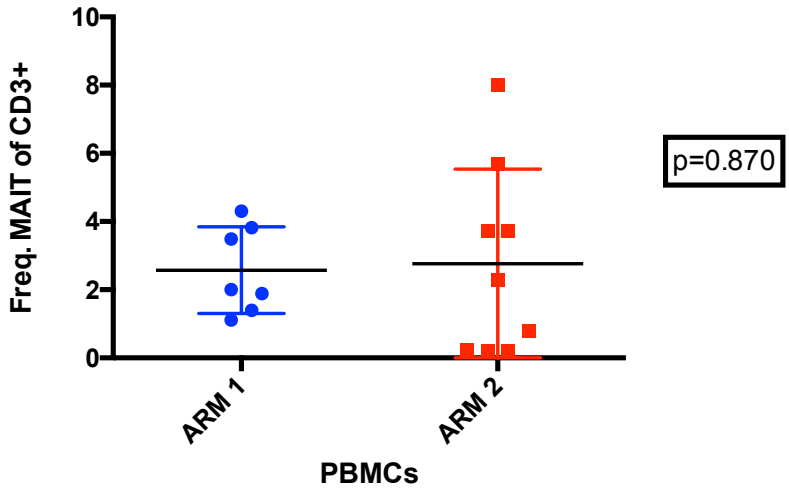


Figure 21. Graphic describing the frequency of MAIT cells in the peripheral blood (PBMCs) in ARM-1 and ARM 2.

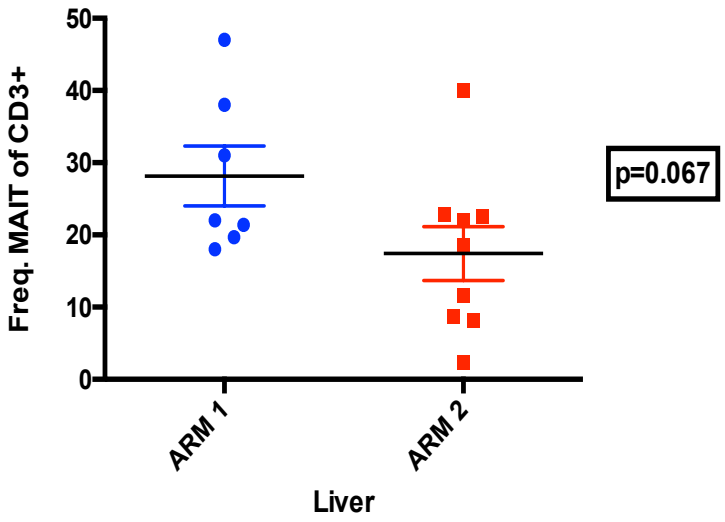


Figure 22. We evaluated the frequency of MAIT cells in the liver in ARM-1 and ARM-2

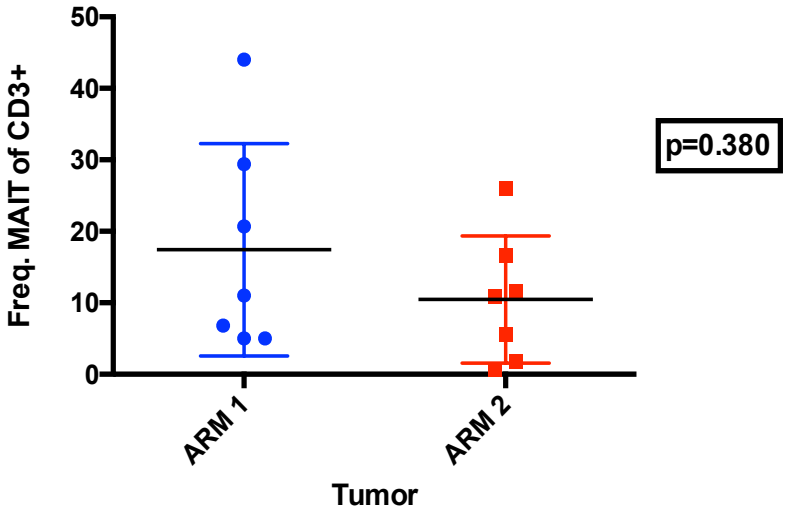


Figure 23. Graphic describing the frequency of MAIT cell within the tumor in ARM-1 and ARM 2 of CRLM patients.

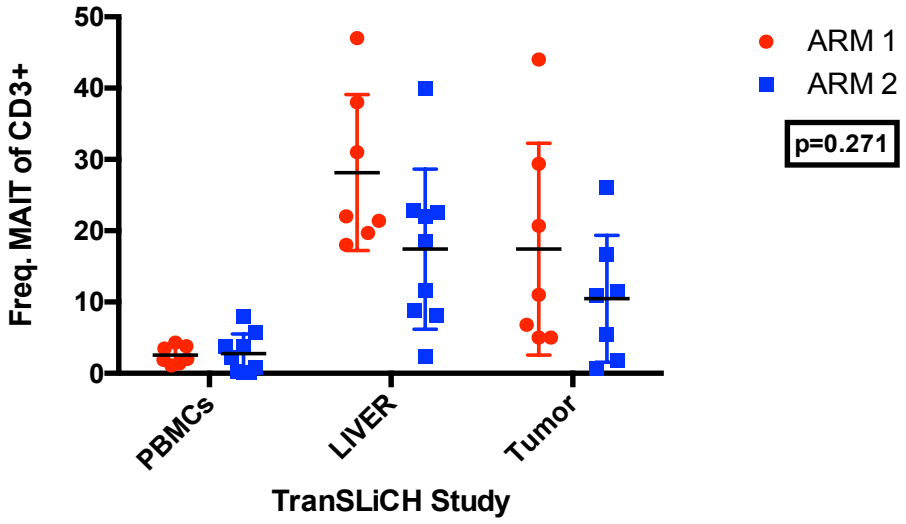


Figure 24. Graphic describing the frequency of MAIT cells in: the peripheral blood, liver, and tumor, between ARM-1 and ARM-2. No significance difference regarding the frequency of MAIT cells was observed, p=0.271.

4.3.3. Immunological competency of the liver after chemotherapy treatment

Having noted a large degree of variability in the frequency of MAIT cells, we wondered what impact the perioperative chemotherapy regimen was having on the frequency and functionality of MAIT cells. With the majority of patients receiving either single regimen of chemotherapy with FOLFOX, or combined chemotherapy regimen with FOLFOX plus bevacizumab **(Table 2)**, thus, we categorized patients based on the regimen of chemotherapy and evaluated the impact of the number of cycles they received on the frequency of MAIT cells. To evaluate this, we grouped patients as follow: 1) those that received FOLFOX (9 patients); 2) those that received FOLFOX + bevacizumab (Avastin) (8 patients); and 3) a group that received no chemotherapy (5 patients).

Although there was a slight upward trend in the patients who received FOLFOX alone, there was no correlation between the number of cycles received and the frequency of MAIT cells in blood (PBMCs), Hepatic MAIT cells (HMNCs), or tumor tissue. **(Figure 25a)** In contrast, there was a strong negative correlation between the cycle number and frequency of MAIT cells in the peripheral blood and healthy liver tissue of patients treated with FOLFOX + bevacizumab (PBMC: $r^2 = 0.944$, $p = 0.0057$; Liver $r^2 = 0.915$, $p = 0.011$**) (Figure 25b).** While a similar trend was observed in the tumor, this did not reach statistical significance **($r^2 = 0.73$, $p = 0.067$) (Figure 25b).**

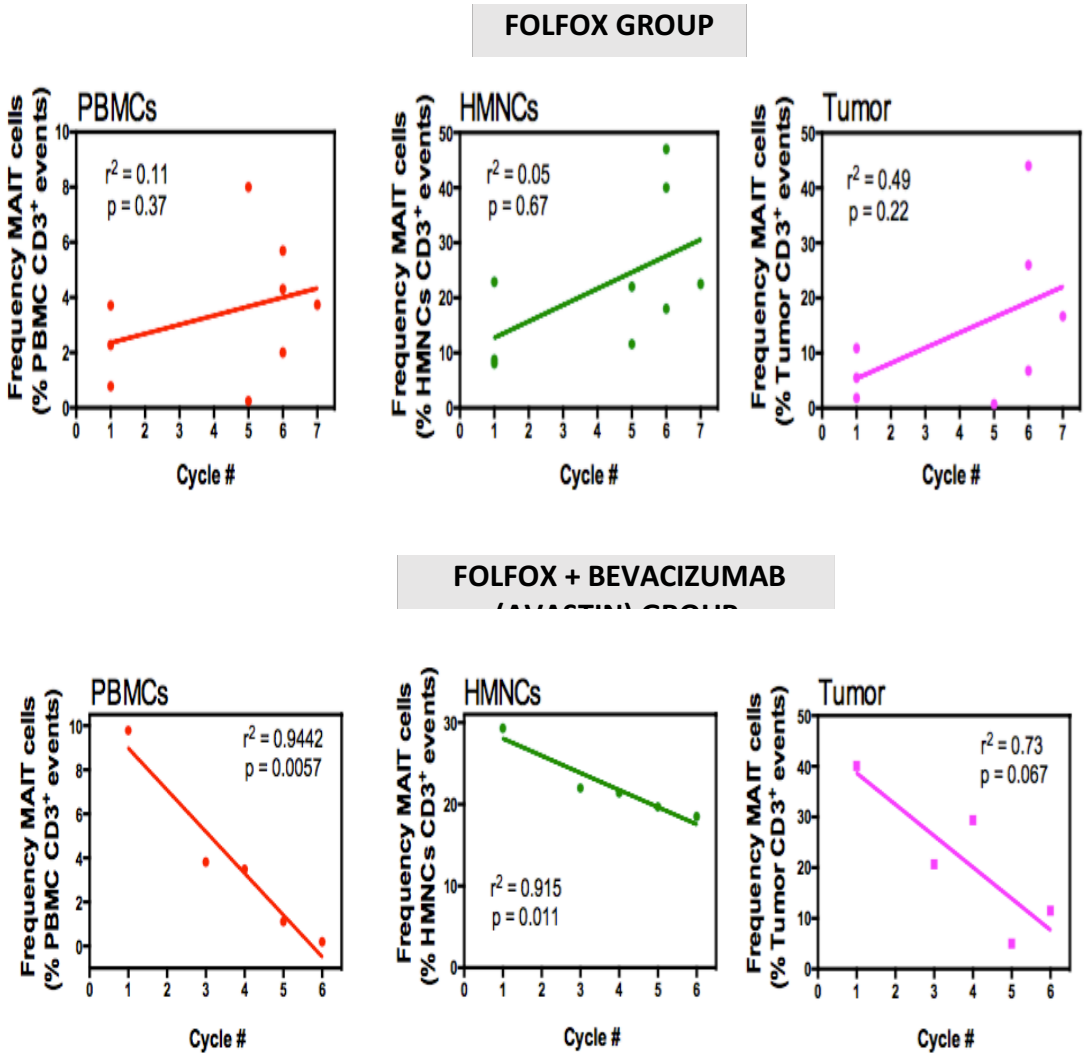


Figure 25a-25b. The frequency of MAIT cells depicted compared to the number of cycles of Folfox received **(Figure 25a),** and Folfox and bevacizumab received **(Figure 25 b)**. r^2 and p values were generated by Pearson analysis. Analyzes in the PBMCs, liver (HMNCs), and tumor was performed. Frequency of MAIT cells (y- axis), number of cycles (x-axis)

4.3.3.1. Perioperative chemotherapy fundamentally alters MAIT cell populations on CRLM patients.

Given the influence of the perioperative chemotherapy on the frequency of MAIT cells, and high variability of activation observed following stimulation, both inside, and outside the tumor. Interestingly, while the frequency of MAIT cells varied depending on the perioperative chemotherapy regimen in the peripheral blood and liver, the responsiveness to stimulation (rIL-12 + rIL-18, SEB, and *Klebsiella* lystate) was highly similar in each case **(Figure 26 A-C)**. Moreover, the degree of responsiveness of MAIT cells within the tumor was highest in the group that received FOLFOX, with individuals who did not receive chemotherapy showing low levels of activation, and the those who received FOLFOX + Avastin showing virtually no responsiveness **(Figure 26 C)**. Unexpectedly, while not significant, both chemotherapy regimens appeared to enhance the responsiveness of MAIT cells to stimulation with rIL-12 combined with rIL-18, as noted by the general increase in IFN-γ by peripheral blood and liver MAIT cells **(Figure 26 A and B)**. While a number of individuals that received FOLFOX alone showed some degree of responsiveness in the tumor, in contrast, MAIT cells within the tumor (with only one exception) of those who received FOLFOX + bevacizumab demonstrated a complete lack of a response **(Figure 26 C)**. Interestingly, this patient was the only individual to receive FOLFOX + Avastin that did not achieve any tumor necrosis, which was typically greater than 75% (data not shown). While this provides a likely explanation for the increased responsiveness in this individual, it remains to be seen what impact tumor necrosis has on the activation or survival of MAIT cells.

Figure 26 A-C. Differential effect of chemotherapy on the function of MAIT cells in CRLM patients.

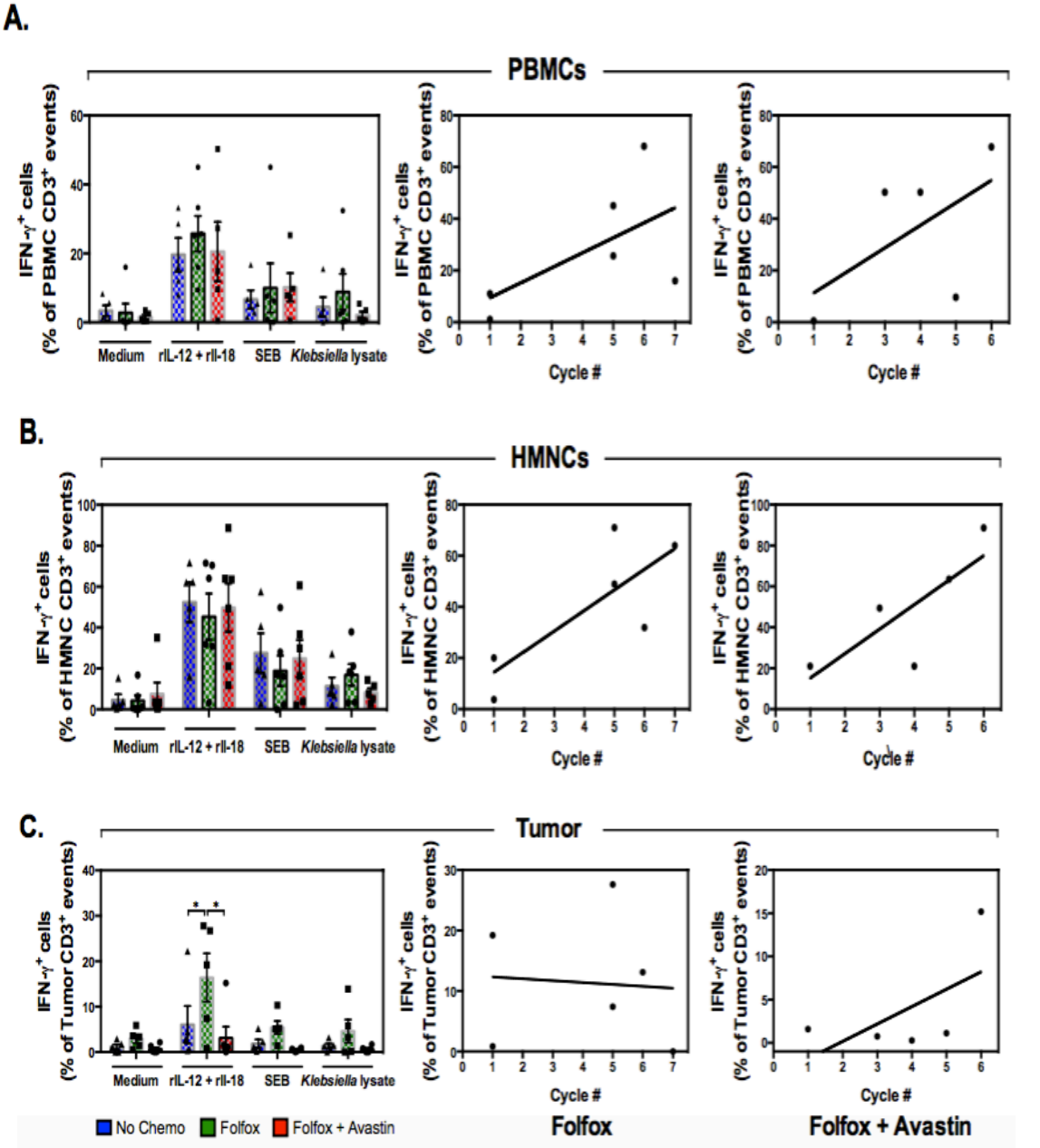


Figure 26 A-C. Describes how patients who had received no chemotherapy, Folfox alone, or Folfox +bevacizumab responded to stimulation with a panel of MAIT cell mitogens. **(A)** Left - the frequency of IFN-γ production by PBMC MAIT cells in response to medium, rIL-12 +rIL-18, SEB, or *Klebsiella* lysate. **Right** – The frequency of PBMC MAIT cells producing IFN-γ in response to rIL-12 +rIL-18 is depicted compared to the number of cycles of Folfox or Folfox + bevacizumab received; **(B)** Left - the frequency of IFN-γ production by HMNCs MAIT cells in response to medium, rIL-12 +rIL-18, SEB, or *Klebsiella* lysate. Right – The frequency of HMNCs MAIT cells producing IFN-γ in response to rIL-12 +rIL-18 is depicted compared to the number of cycles of Folfox or Folfox + bevacizumab received; **(C)** Left - the frequency of IFN-γ production by tumor MAIT cells in response to medium, rIL-12 +rIL-18, SEB, or *Klebsiella* lysate. Right – The frequency of tumor MAIT cells producing IFN-γ in response to rIL-12 +rIL-18 is depicted compared to the number of cycles of Folfox or Folfox + bevacizumab received.

CHAPTER 5

5. DISCUSSION

5.1. ALPPS

ALPPS has emerged as a novel surgical approach with the potential to improve the likelihood of resectability, specifically in those patients with extensive cancer disease and a small FLR. The two major ALPPS advantages are: rapid liver kinetic growth, and surgical complete resection of all disease compared to the conventional two-staged hepatectomy with PVE. (70)

These proposed advantages of ALPPS have been counterbalanced by reports of high perioperative morbidity and mortality. The first published cohort described a 12% perioperative mortality rate (78), with other studies reporting similarly high perioperative mortality as well as severe morbidity in up to two-thirds of patients (79). Our single-center experience with ALPPS demonstrates consistently acceptable morbidity and no perioperative mortality throughout the study period. Our group has previously published an early experience with ALPPS, demonstrating no 90-day mortality in a pilot series of 14 patients (80). In this short cohort, we again report no 90-day mortality in 7 patients, with a 66% minor complication (Clavien-Dindo ≤ II) rate.

5.2. Circulating tumor cells “CTCs”

Circulating tumour cells have been shown to represent the concentration of cancer cells in the blood circulation.(81,82) Furthermore, the presence of ≥3 CTCs has been associated with poor prognosis in patients with metastatic colorectal cancer. (81–83) Therefore, the research community has investigated their utility to serve as potential surrogate markers for early treatment response, by associating their presence and

elevation with disease recurrence or progression, with promising results. (83,84) Accordingly, in our current investigation, we chose it as an evaluation tool to assess if the rapid and extensive hypertrophy that occurs during the ALPPS procedure for treatment of colorectal liver metastasis results in increased tumor cells dissemination and disease progression.

We evaluated the positivity and levels of circulating tumour cells at different time-points during the treatment. After completion of chemotherapy the presence of CTCs was assessed, and of the two patients with positive CTCs, the one who had ≥3 CTCs was withdrawn from the study due to disease progression. This finding correlates with other reports and confirms the prognostic value for disease progression at this CTC cut-off.(61,82) Moreover, CTCs were evaluated at the time of surgery (after first and second stage ALPPS) and compared with the control group (single stage major liver resection) to whether rapid hypertrophy results in tumor cells dissemination. The present results show that intraoperative mobilization of the liver and tumor, portal vein ligation, and liver partition with subsequently accelerated, and extensive liver hypertrophy, do not release or increase the CTCs after first and second stages of ALPPS, based on no significant difference in CTC compared with the control group. Since the 2 Arms were comparable with respect to the tumour load, these results should be considerable. However, no recent report has evaluated ALPPS at such situation that can reinforce and validate our information.

Two staged hepatectomy with PVE has been used as an standard procedure to provide increased hypertrophy in patients with insufficient FLR requiring extensive liver resection. However, 20-30% of the patients do not reach the second staged due to tumor progression.(26) Interesting observations were made by a group of Norway (2), when CTCs

were assessed at surgery after two-staged liver resection with PVE in CRLM patients, it was observed that levels of CTCs where higher after the second staged, and these patients has worse DFS and OS in their population of study. Contrary to their findings, our results showed that ALPPS might provide better outcomes and less tumour cell dissemination. This might be explained by the shorter period of inter-staged waiting time, 7-10 days for ALPPS, compared with the 6-8 weeks during the classical two-staged hepatectomy with PVE, which could increase the time for tumor cell growth. (85–87)

In our study, we also evaluated the presence of CTCs at 1-2 months' follow-up post-surgery, observing that all patients that remained with negative CTC status did not present with tumour recurrence, whereas the two patients who had showed positive CTCs had detectable recurrence. Interestingly, both remained positive during all time-points of the study. Furthermore, both patients with persistence and positivity of CTCs had cancer-related death. This interesting association supports what other groups have reported in other types of cancers, (88) and suggests that persistence of positivity of circulating tumour cells might have an impact on prognosis and patient outcomes.

Another important observation in our results is related to the levels of CTCs. Whereas others groups have reported a cut-off for better or worse outcomes (<3 or ≥3 CTC in a 7.5 mL CTC/blood sample blood sample)(60,82). We observed that the presence and persistence of ≥1 circulating tumour cells was correlated with worse prognosis. This indicates that, an ideal cut-off in metastatic colorectal cancer remains undetermined.

In conclusion, in our present investigation detection of CTC serves as an important tool of prognostic value in patients undergoing for ALPPS for colorectal liver metastasis. These findings suggest firstly that accelerated and extensive hypertrophy was not

associated with more release of cancer cells and subsequently disease progression. Secondly, persistence and positivity of CTC at follow-up was significantly associated with disease progression. Nevertheless, due the small sample size and short follow-up, our results should be taken carefully. Further follow-up combined with serial analysis of CTCs should be considered for future investigation.

5.3. MAIT cells

The ALPPS approach results in an accelerated and extensive hypertrophy of the FLR. However, limited evidence exists regarding the immunological competence of the FLR, particularly in patients with CRLM. The liver represents a unique immunological organ dominated by innate-like T lymphocytes. Recently, it has been shown that a mucosa-associated invariant T cell population comprise 30%-50% of the total hepatic T lymphocytes. (42,45) MAIT cells are innate-like T lymphocytes that express the invariant TCRα (*i*TCRα) chain TRAV1-2-TRAJ33 (Vα7.2-Jα33 in humans) (89,90) and produce a plethora of pro- and/or anti-inflammatory cytokines (*e.g.*, IFN-γ, TNF-α, IL-17, IL-4, and IL-10) and various granzymes (A, B, K ,and M) when activated (91,92). Given this particularity of MAIT cells, their high proportion within the liver microenvironment, and their potential role during the liver immune response, thus, we wondered whether their frequency and functionality was affected during ALPPS and perioperative chemotherapy.

Despite their abundance both in the liver and at other mucosal sites, the role of MAIT cells in tumor surveillance remains largely unknown. Initially, to elucidate the role of MAIT cells in CRLM patients, we compared our CRLM patients with a healthy group of volunteers. We evaluated the MAIT cells frequency from the total $CD3^+$ PBMCs, and their capacity to be activated by a panel of agonists in both groups. Interestingly, and in

contrast to the findings of other studies, in which the frequency of the MAIT cells in the peripheral blood of cancer patients did not change (93), we observed a significant reduction in MAIT cells frequency in the peripheral blood. Moreover, we observed a trend towards a reduction in the frequency of MAIT cells within the tumor.

Observing this variability of the frequency of the MAIT cells, we wondered if the MAIT cells frequency exhibits different proportion in the ALPPS group. In a recent ALPPS cohort published by our group (77), we demonstrate that the ALPPS liver appears to maintain its immune distribution, and that the liver cell composition of the FLR during the extensive and accelerated hypertrophy remains constant. Also, we found the liver cell populations proportion including CD4+ T cells, NKT cells, B cells, Kupffer cells, and including the MAIT cells increased significantly. In our current investigation, we focused on MAIT cells, and found that MAIT cells frequency in the peripheral blood (PBMCs) remains similar, but their proportion in the liver showed a trend towards increasing compared with the control group. These findings enhance, and validate our prior investigation(77), where we concluded that, while accelerated and extensive hypertrophy occurs within the FLR in the ALPPS population, an expansion of hepatic immune cells results as well.

Altogether, MAIT cell frequency in the liver of CRLM population is reduced compare with the healthy volunteers, but higher in those who underwent an ALPPS resection. This might be explained by the immune system activation, as a consequence of the inflammatory mechanism of the ALPPS resulting in rapid hypertrophy of the FLR, therefore suggesting an homogeneous immune cell restoration.

Furthermore, although we observed an increase in MAIT cells frequency in the liver in the ALPPS group, we observed a suppression in their frequency within the tumour in

both groups, thus, we wondered whether this variability may be due the influence of the perioperative type of chemotherapy. As such, we aimed to determine whether the different type of regimens had an impact on the frequency and functionality of MAIT cells, both inside and outside the tumor. We observed a slight upward trend in the frequency of MAIT cells in the patients who received FOLFOX alone, but no correlation between the number of cycles received or the frequency of MAIT cells in PBMCs, liver, or tumor tissue was found. On the other hand, a strong negative correlation between the cycle number and frequency of MAIT cells in the peripheral blood and healthy liver tissue of patients treated with FOLFOX + bevacizumab was observed.

Our observation with respect to the association of responsiveness of the immune response with FOLFOX therapy is similar to what has been reported by other authors (94,95). During the latest report from the randomised control trial EORTC in 2015 (94), whether the immune response in CRLM was influenced by systemic therapy was investigated and was related with patient disease free survival. In particular, the investigators evaluated liver resection alone or with the addition of FOLFOX therapy. Interestingly, in the group treated with FOLFOX, they observed and increase in the CD3+ lymphocytes and mast cells inside the tumor, demonstrating a correlation between high CD3+ response and pathological response. This was also related to an improvement in the disease free survival. A similar association was also reported by *Halama et al,* demonstrating a highly density of CD3+, CD+8, and granzyme B+ T cells within the tumor margins of CRLM patients treated with FOLFOX therapy, and associated this with longer disease free and overall survival.(95)

An explanation of how chemotherapy affects the immune response was offered by Zitovogel et al. Their hypothesis involves transitional mechanisms of lymphopenia,

followed by a high stimulation of more tumour infiltrating T-cells, thereby favoring chemokine attraction into the tumor, with subsequent tumor cell death. (94,96)

In contrary to the findings in the FOLFOX, the combined regimen group experienced a decrease in MAIT cells frequency, and a lack of responsiveness after stimulation in those patients with more than 75 % of tumor necrosis. It is perplexing that the addition of bevacizumab a monoclonal antibody against a vascular endothelial growth factor (VEGF), could so drastically effect the frequency of MAIT cells. One explanation could be related to the regulation of the multidrug resistance gene 1 (MDR1), an ABC transporter known to provide MAIT cells with resistance to chemotherapy (44,93,97). Specifically, there have been reports that VEGF can induce the expression of MDR1 in various cell types (98). It remains plausible that MDRI expression by MAIT cells may be partially dependent on VEGF.

Together, these results showed that chemotherapy affects the liver immune response, depending of the type of chemotherapy regimen received. The findings suggest that FOLFOX therapy has immunostimulatory effects in the liver, while bevacizumab therapy leads to tumor necrosis with immunosuppressive side effects within the tumor. Each regimen seems to act in different pathway, and their therapeutics efficacy should be interpreted independently, giving the concept that chemotherapy should stimulate the immune response. Thus, the liver immune activation or suppression within the tumor may result underestimation of the regimen response.

Although convincing, given the small sample size, a larger cohort is needed to validate these findings. Moreover, longer follow up is necessary to evaluated whether this variability in the responsiveness of the chemotherapy might have a prognostic impact.

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APPENDIX 1:

Research Ethics board HSREB Full Board Approval Notice

Western Research

Research Ethics

Western University Health Science Research Ethics Board
HSREB Full Board Initial Approval Notice

Principal Investigator: Dr. Roberto Hernandez-Alejandro
Department & Institution: Schulich School of Medicine and Dentistry\Surgery,

Review Type: Full Board
HSREB File Number: 106937
Study Title: Translational Study of Liver Cancer and Hypertrophy: TransLiCH
Sponsor:

HSREB Initial Approval Date: July 27, 2015
HSREB Expiry Date: July 27, 2016

Documents Approved and/or Received for Information:

Document Name	Comments	Version Date
Other	Schematic Diagram	2015/06/28
Western University Protocol	Received July 25/15	
Letter of Information & Consent		2015/07/24

The Western University Health Science Research Ethics Board (HSREB) has reviewed and approved the above named study, as of the HSREB Initial Approval Date noted above.

HSREB approval for this study remains valid until the HSREB Expiry Date noted above, conditional to timely submission and acceptance of HSREB Continuing Ethics Review.

The Western University HSREB operates in compliance with the Tri-Council Policy Statement Ethical Conduct for Research Involving Humans (TCPS2), the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guideline for Good Clinical Practice Practices (ICH E6 R1), the Ontario Personal Health Information Protection Act (PHIPA, 2004), Part 4 of the Natural Health Product Regulations, Health Canada Medical Device Regulations and Part C, Division 5, of the Food and Drug Regulations of Health Canada.

Members of the HSREB who are named as Investigators in research studies do not participate in discussions related to, nor vote on such studies when they are presented to the REB.

The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

CURRICULUM VITAE

Name: Mauro Enrique Tun Abraham

Education and Degrees:

Autonomous University of Campeche
Medical Doctor
Campeche, Mexico
2002.2008

National Autonomous University of Mexico
General Surgery
Mexico, D.F,
2010-2014

Western University
Mastery of Surgery
London, Ontario, Canada
2015-Present

Western University
HPB/Liver & Kidney Transplantation
London, Ontario, Canada
2016-2018

Honours and Awards:

Outstanding medical school award,
Autonomous University of Campeche.
2002-2008

Best average award in the internship, "Social Security Mexican Institute, Abraham Farah Hospital", Campeche.
2006-2007.

Best average award in the first year of General Surgery Residency. "Social Security Mexican Institute, Bernardo Sepulveda Hospital, CMN S XXI ", Mexico, D.F.
2010-2011

Participation during the first successful cadaveric donor liver transplantation at the "Social Security Mexican Institute, Bernardo Sepulveda Hospital, CMN S XXI ", Mexico, D.F. 2013

Other Experience:

Group coordinator, Medical School, Autonomous University of Campeche.
2003-2005

Member, Student Society of Medical School, Autonomous University of Campeche.
2004-2007

Observership, Latin American Training program (Colorectal, oncology, general surgery), William J. Harrington, Jackson Memorial Hospital. Miami, Florida (January - April).
2008

Observership, Minimal invasive surgery department, Mayo Hospital Jacksonville Florida (October).
2012

Observership, HPB surgery and MOTS, London Health Science Centre, Western University. London Ontario Canada, (April - June).
2014

Publications:

1. Christopher R. Shaler*, **Mauro E. Tun-Abraham***, Khashayarsha Khazaie, Alexandra J. Corbett, James McCluskey, Tina Mele, Alison L. Allan, Roberto Hernandez-Alejandro, and S.M. Mansour Haeryfar. MAIT cells infiltrate hepatic metastases of colorectal carcinoma but become dysfunctional within and adjacent to tumor microenvironment **(Submitted to PLOS Pathogens).**
2. Al Hasan I, **Tun-Abraham ME,** Kerollos Wanis, Garcia-Ochoa C, Bnadar Al Judabi, Hernandez-Alejandro R. ALPPS. Optimizing ALPPS Outcomes, Surgical experience or Appropriate Patients Selection?. **(Submitted Saudi Journal)**
3. Kerollos N Wanis, Karen Pineda-Solis,**Tun-Abraham ME**, Jake Yeoman, Stephen Welch, Kelly Vogt, MD, MSc1, Julie Ann Van Koughnett, Michael Ott, Roberto Hernandez-Alejandro. Synchronous colorectal cancer and colorectal liver metastases: impact of multidisciplinary case conference review**. (HPB surgery and Nutrition, 2017)**
4. Kerollos N Wanis, Suzana Buac, **Tun-Abraham ME**, Michael Linecker, Victoria Ardiles, Eduardo de Santibañes[3], Pierre-Alain Clavien, Roberto Hernandez-Alejandro. Outcomes of simultaneous ALPPS and colorectal resection for colorectal liver metastases: results from the International ALPPS Registry. **World Journal Of Surgery 2016.**
5. Christopher R. Shaler, Patrick T. Rudak, Joshua Choi, Arash

Memarnejadian, **Tun-Abraham ME**, John K. McCormick, Olivier Lantz, Roberto Hernandez-Alejandro, S.M. Mansour Haeryfar Mucosa-associated invariant T cells launch robust TCR-dependent and -independent responses to bacterial superantigens and quickly acquire an anergic phenotype that impedes their cognate activation. **(Submitted to PLOS Pathogens)**

6. Madeline Lemke, Calvin H.L. Law, Jennifer Li, Elijah Dixon , **Mauro Enrique Tun-Abraham,** Roberto Hernandez Alejandro, Sean Bennett, Guillaume Martel, Paul J. Karanicolas. The Three Point Transfusion Risk Score in Hepatectomy. **(Submitted 2016)**
7. **Tun-Abraham ME**, K. Pineda-Solis, D. Paskar, H.A. Cano, D. Quan, R. Hernandez-Alejandro. The influence of the multidisciplinary cancer conference era on the management of colorectal liver metastases. Can J Surg, Vol. 58, (4 Supple 2), August 2015.
8. K. Pineda-Solis, **Tun-Abraham ME**, D. Mirsattari, H.A. Cano-Gonzalez, D. Paskar, R. Hernandez-Alejandro. How does simultaneous resection of colorectal liver metastases impact chemotherapy administration? Can J Surg, Vol. 58, (4 Supple 2), August 2015.
9. Toledo-Toral C, Guerrero-Franco N, **Mauro Enrique Tun-Abraham**. ” Surgical treatment of pancreatic pseudocyst ". Cir Cir. 2016;84: No 4.
10. **Mauro Enrique Tun-Abraham,** Martinez JL, Vargas A, Sanchez JJ, Perez E, Zaleta O.(2015) L-lactate as a serum marker in intestinal ischemia in patients with complicated intestinal occlusion. Cir Cir 2015;83.
11. **Mauro Enrique Tun-Abraham**, Martinez JL, Obregón G, Romero L.(2015) Acute Pancreatitis associated with hypercalcemia: Report of four Cases. Cir Cir 2015;81.
12. **Mauro Enrique Tun-Abraham**, Martinez JL, Romero T. (2014) Hepatic Artery pseuoaneurysm: Report of two cases. Cir Cir 2014;82:674-679.
13. **Mauro Enrique Tun-Abraham** (2004). Helicobacter pylori infection in patients with gastric cancer review. The gazette university of Campeche, 2004; April 15.

Abstracts 2017:

1. **Mauro Enrique Tun-Abraham**, Lori Lowes, Christopher Ryan Shaler, Kerollos N Wanis, Tina Mele[1], Mansour Haeryfar, Alison Allan, Roberto Hernandez-Alejandro. Does accelerated liver hypertrophy caused by the ALPPS procedure lead to circulating tumour cell dissemination in patients with colorectal liver metastases?. **AHPB 2017**
2. Madeline Lemke, Calvin H.L. Law , Jennifer Li , Elijah Dixon, **Mauro Tun Abraham,** Roberto Hernandez Alejandro, Sean Bennett, Guillaume Martel, Paul J. Karanicolas, The Three Point Transfusion Risk Score in Hepatectomy. **AHPB 2017**

3. H. Sharma, **Mauro Tun-Abraham**, I. Al-Hasan, B. Al-Harbi, Alp Sener, P Luke, D. Quan**.** Modular Training in Organ Procurement, in the Present Era of High BMI Donors: Validation by Results. American Society of Transplant: American Society of **transplant Winter Surgeons Symposium 2017**
4. H. Sharma, **Mauro Enrique Tun-Abraham**, David M. Mikhail, Jingwen Chen,Patrick P. Luke, Alp Sener. Non Inferior outcomes of DCD renal transplant compared to DBD renal transplant from kidneys procured from donors more than 50 years of age. American Society of Transplant: **American Society of transplant Winter Surgeons Symposium 2017**

2016

1. **Mauro Enrique Tun-Abraham**, Kerollos N Wanis, Carlos Garcia-Ochoa, Hemant Sharma, Ibrahim Al Hasan, Bandar Al-Judabi, Mark Levstik, Roberto Hernandez-Alejandro. Can we improve Ischemia Cholangiopathy in Donation after Cardio Circulatory Death Liver Transplantation: A Canadian Single-Centre experience. CTS 2016
2. **Mauro Tun-Abraham**, Cesar Ploneda-Valencia, Ibrahim Al-Hasan, Natalie Sela, Roberto Hernandez-Alejandro. Simultaneous Liver Transplantation and total pancreatectomy for Primary Biliary Cirrhosis and Intraductal Papillary Mucinous Neoplasm. ILTS Seoul Korea 2016.
3. **Mauro Tun-Abraham,** K. Pineda-Solis, Wanis N K, Garcia-Ochoa C, Al Hasan I, Sharma H, Hernandez-Alejandro R. Is there is a learning curve in Donation after Cardiac Death Liver transplantation: a Canadian single-centre experience. AHPBA. 2016 Sao Paulo Brazil
4. Ibrahim Al-Hasan**, Mauro Tun-Abraham**, Hemant Sharma, Roberto Hernandez-Alejandro. Outcomes of Grafts Designated as Exceptional Distribution in Liver Transplantation. ILTS Seoul Korea 2016.
5. Ibrahim Al-Hasan, **Mauro Tun-Abraham,** Garcia-Ochoa C, Hernandez-Alejandro R. ALPPS. Optimizing ALPPS Outcomes, Surgical experience or Appropriate Patients Selection?. AHPBA. 2016 Sao Paulo Brasil.
6. Kerollos N Wanis, Suzana Buac, **Mauro Tun-Abraham**, Michael Linecker, Victoria Ardiles, Eduardo de Santibañes[3], Pierre-Alain Clavien, Roberto Hernandez-Alejandro. Outcomes of simultaneous ALPPS and colorectal resection for colorectal liver metastases: results from the International ALPPS Registry. AHPBA. 2016 Sao Paulo Brasil
7. Al Hasan I, Sharma H, **Mauro Tun-Abraham,** Douglas Quan. A Multi-Modal Approach for Training in Liver Procurement Accelerates Proficiency Gain. American Transplant Congress. 2016 Boston US.
8. H. Sharma, I. Al-Hasan, K. Pineda-Solis, **Mauro Tun-Abraham**, P. Marotta, D. Quan, M. Levstik. London Criteria for Listing Potential Liver Transplant Jehovah's Witness (JW) Patients. A Single Centre Results of Listing and Liver Transplant (LT) in JW Patients in Canada. American Transplant congress . 2016 Boston US

2015

1. **Mauro Tun-Abraham**, Pineda-Solis K, Hernandez-Alejandro R. (2015) Outcomes of multidisciplinary cancer conferences in colorectal liver metastases. Canadian Surgery Forum 2015 Quebec City.
2. **Mauro Tun-Abraham**, Hernandez-Alejandro R. Monosegment ALPPS Hepatectomy: Extending resectability by rapid Hypertrophy. Canadian Surgery Forum 2015. Quebec City
3. **Mauro Tun-Abraham**, Pineda-Solis K, Hernandez-Alejandro R. Simultaneous resection for colorectal liver metastases: 5 years experience. Canadian Surgery Forum 2015. Quebec City
4. **Mauro Tun-Abraham**, Pineda-Solis K, Al-Hassan Ibrahim, Bertens Kimberly, Hernandez-Alejandro R. Two-stage hepatectomy Partition Hepatic and portal vein ligation (ALPPS): Challenging the concept of unresectability: systematic review. International congress of Mexico, Nuevo Leon, October 2015.
5. **Mauro Tun-Abraham**, Pineda-Solis K, Al-Hassan Ibrahim, Bertens Kimberly, Hernandez-Alejandro. The First Assistant experience affects the resectability of periampullary neoplasms? International congress of Mexico, Nuevo Leon, October 2015.
6. Pineda-Solis K, Al Hassan I, **Mauro Tun-Abraham**, Hernandez-Alejandro R.(2015) ALPPS in London: 3 years of experience. 1st International Meeting on ALPPS. February 2015, Hamburg, Germany.
7. Pineda-Solis K, Al Hassan I, **Mauro Tun-Abraham**, Hernandez-Alejandro R.(2015) Segment 6 ALPPS. 1st International Meeting on ALPPS. February 2015, Hamburg, Germany

2014

1. **Mauro Tun-Abraham,** Knowles SA, Pineda K, Croome KP, Bertens K, Hernandez-Alejandro R. Intraoperative Ultrasound During Resection of Colorectal Liver Metastases: Impact on Surgical Strategy, Negative Resection Margins and Perioperative Blood Loss. Oral Presentation: "International congress of surgery", Leon Guanajuato Mexico 2014.
2. **Mauro Tun-Abraham,** Pineda K, Emmerton H, Lelie K, Hernandez-Alejandro R. Surgical resection for pancreatic head cancer: How well are doing? A retrospective institutional experience. Oral Presentation. "International congress of surgery", Leon Guanajuato Mexico 2014.
3. **Mauro Tun-Abraham,** Pineda K, Howe B, Hernandez-Alejandro R. Resection of colorectal liver Metastasis in Elderly. Oral Presentation." International congress of surgery", Leon Guanajuato Mexico 2014.
4. **Mauro Tun-Abraham,** Pineda K, Hernandez-Alejandro R. Associating Liver Partition and Portal Vein Ligation for Staged Hepatectomy (ALPPS) versus Portal Vein Embolization in the Management of Colorectal Liver Metastases. Oral Presentation. "International congress of surgery", Leon Guanajuato Mexico 2014.
5. **Mauro Tun-Abraham,** Martinez J. Acute Pancreatitis secondary to hyperparathyroidism adenoma. October 2014 "International congress of surgery", Leon Guanajuato 2014.

6. **Mauro Tun-Abraham**, Martinez JL, Romero T. (2014) Hepatic Artery pseuoaneurysm secondary to bile duct injure. October 2014 "International congress of surgery", Leon Guanajuato 2014.
7. **Mauro Tun-Abraham**. Surgical management of complications ascariasis: intussusception and Meckel. "International congress of surgery", Leon Guanajuato 2014.
8. **Mauro Tun-Abraham,** Martinez JL. Intussusception jejunum-duodenal in peutz-jeghers syndrome: a case report. October 2014 "International congress of surgery", Leon Guanajuato 2014.

2013

1. **Mauro Tun-Abraham,** Vargas A, Sanchez JJ, Perez E. "Case report: Gallbladder duplication". "International congress of surgery", Acapulco Guerrero 2013.
2. **Mauro Tun-Abraham,** Vargas A, Sanchez JJ, Perez E. "Case report: Perforated Cecal diverticulitis. "International congress of surgery", Acapulco Guerrero 2013.
3. **Mauro Tun-Abraham,** Sanchez P, Roman E. "Case report: Management of cervical esophageal stenosis, secondary to placement of cervical plate. "International congress of surgery", Acapulco Guerrero 2013.
4. **Mauro Tun-Abraham,** Vargas A, Sanchez JJ, Perez E. "Case report: Strangulated internal Hernia ". "International congress of surgery", Acapulco Guerrero 2013.
5. Romero L, **Mauro Tun-Abraham,** Romero T. Presentation of esophagus bronchogenic cyst as dysphagia. "International congress of surgery", Acapulco Guerrero 2013.

2012

1. **Mauro Tun-Abraham**, Mandujano HE. "osteodystrophy aspect renal tumor in a case of secondary hyperparathyroidism. "International congress of Surgery", Cancun Quintana Roo, October 28, to November 2 2012.
2. **Mauro Tun-Abraham,** Mandujano HE (2012). " Wilkie syndrome". International congress of Surgery, Cancun Quintana Roo, October 28, to November 2 2012.
3. **Mauro Tun-Abraham,** Mandujano HE, Avendaño I. "Mesenteric Thrombosis". International congress of Surgery. Cancun Quintana Roo, October 28, to November 2 2012.
4. **Mauro Tun-Abraham**, Mandujano HE, Trujillo Raul. "Hydatid cyst". International congress of Surgery, Cancun Quintana Roo, October 28, to November 2 2012.
5. Ayala Isai, **Tun ME,** Avendaño Ivan, Trujillo Raul (2012). "Upper GI bleeding secondary to Metastasic GIST " International congress of Surgery", Cancun Quintana Roo, October 28, to November 2 2012.