AUS DEM LEHRSTUHL FÜR CHIRURGIE
PROF. DR. MED HANS-JÜRGEN SCHLITT
DER FAKULTÄT FÜR MEDIZIN
DER UNIVERSITÄT REGENSBURG

- CAN "RESPONSE TO BRIDGING" PREDICT OUTCOME OF LIVER TRANSPLANTATION FOR HCC? -

An analysis within the *SiLVER Study* population.

- Kann “Response to Bridging” das Outcome von Leber-Tranplantation für HCC vorhersagen?-

Inaugural – Dissertation
zur Erlangung des Doktorgrades
der Medizin

der
Fakultät für Medizin
der Universität Regensburg

vorgelegt von
Tomás Pinheiro Fernandes Neto da Silva

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Dekan: Prof. Dr. Dr. Torsten E. Reichert

1. Berichterstatter: Prof. Dr. Edward K. Geissler, PhD
2. Berichterstatter: Prof. Dr. Gabriele Kirchner

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Abstract (English)

Livertransplantation (LTX) is the therapy of choice for patients with HCC in early stage disease who are not suited for curative Liver-Resection or curative LRT. This dissertation explores the ability of the tumor size variation as a “Response to Bridging” and “Milan Dynamics” to identify the most aggressive HCC tumors and, by extent, those patients with a better/worse prognosis after LTX. In order to do so, a retrospective cohort study with a sub-population of 356 patients that received some form of bridging therapy before LTX was retrospectively analyzed. Concerning the Milan variation, no statistically relevant difference could be found between the patients that downstaged and the patients that were outside Milan at LTX day (DFS 5-year 52% vs 55%, *p-value* = 0.71 & OS 5-year 58% vs 64%, *p-value* = 0.52). Once a tumour reaches the outside Milan stage, a decrease in the tumour size after bridging does not correlate with a better prognosis. Patients that always remained inside Milan, but revealed progress of their HCC size or number of lesions (In-to-Inside Milan with progressive disease) did not show a better prognosis than patients outside Milan (OS *p-value* = 0.76 and DFS *p-value* = 0.49). This led us to conclude that patients with any evidence for tumor progression after Bridging had a prognosis similar to patients outside Milan. In summary this analysis reveals that there are two crucial factors that predict prognosis in HCC after LTX – if the tumor has reached an extent outside Milan, or if it has progressed after Bridging. Once one of these factors is evident, the prognosis considerably worsens.

Abstract (deutsch)

Livertransplantation (LTX) ist die Therapie der Wahl für Patienten mit HCC im Frühstadium der Erkrankung, die nicht für eine kurative Leberresektion oder kurative LRT geeignet sind. Diese Dissertation untersucht die Fähigkeit der Tumorgrößenvariation als "Response to Bridging" und "Milan Dynamics", um die aggressivsten HCC-Tumoren und die Patienten mit einer besseren / schlechteren Prognose nach LTX zu identifizieren. Zu diesem Zweck wurde eine retrospektive Kohortenstudie mit 356 Patienten, die vor dem LTX eine Art Bridging Therapie erhalten hatten, retrospektiv analysiert. In Bezug auf die Milan-Variation konnte kein statistisch relevanter Unterschied zwischen den Patienten festgestellt werden, die sich am LTX-Tag außerhalb von Milan befanden (DFS 5 Jahre 52% vs. 55%, p-Wert = 0,71 und OS 5 Jahre 58% vs 64%, p-Wert = 0,52). Sobald ein Tumor das Äußere Milan Stadium erreicht, korreliert eine Abnahme der Tumorgröße nach Bridging nicht mit einer besseren Prognose. Patienten, die sich immer in Milan aufhielten, aber einen Fortschritt ihrer HCC-Größe oder Anzahl der Läsionen (In-to-Inside-Milan mit fortschreitender Erkrankung) zeigten, zeigten keine bessere Prognose als Patienten außerhalb von Milan (OS p-Wert = 0,76 und DFS p-Wert = 0,49). Dies führte uns zu dem Schluss, dass Patienten mit Anzeichen für eine Tumorprogression nach Bridging eine ähnliche Prognose hatten wie Patienten außerhalb von Milan. Zusammenfassend zeigt diese Analyse, dass es zwei entscheidende Faktoren gibt, die die Prognose des HCC nach LTX vorhersagen - wenn der Tumor außerhalb von Mailand ein Ausmaß erreicht hat oder wenn er nach der Bridging fortgeschritten ist. Sobald einer dieser Faktoren erkennbar ist, verschlechtert sich die Prognose erheblich.

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1 INTRODUCTION

1.1 Introductory Note

This dissertation is an original retrospective cohort study with the primary goal of identifying possible associations between the tumor size dynamics, in particular the "Response to Bridging", and the outcome after Liver-Transplantation (LTX) in patients with Hepatocellular Carcinoma (HCC). The database used comprised a sub-population of the SiLVER Study, kindly made available for this research purpose.

In a moment when transplantable-liver scarcity is worrying the international community - and in particular Germany, where this dissertation was written – it is necessary to understand the factors that influence LTX outcome and how to predict which patients can benefit the most from the few livers available.

In order to keep patients in the waiting-list and extend their survival while waiting for transplantation, several bridging techniques have been successfully developed in the last decades. Although many studies focused on which techniques present the best chances to prolong survival until LTX, not enough studies have been conducted relating the response revealed by the tumors to these bridging modalities and prognosis after LTX.

This dissertation was based on the clinical expertise of the surgeons of the Department of General Surgery at the University Hospital Regensburg, who noticed that the "Response to Bridging" could in many cases reveal in advance the aggressiveness, or tumor biology, of HCC.

Transplantation is often a sensitive topic full of ethical considerations. However, although the Organ Allocation Systems were studied, it was not the purpose of this dissertation to suggest how livers world-wide should be allocated. On the other hand, some models that are the base of these systems (such as the Milan Criteria) were extensively analyzed to give a practical and utilitarian approach of the results here presented.

Although the fruits of science might not always be seen in a short period of time, we are convinced that every piece of research should be oriented to increase the quantity and quality of people`s lives. Our goal with this dissertation is to help the scientific community to better understand how one can predict the prognosis of LTX in the context of HCC.

1.2 Key-Questions – Goals of the Dissertation

This dissertation aimed to answer the following questions:

1) Is the tumor size variation before LTX associated with the prognosis after LTX?

And, in particular:

2) Is the "Response to Bridging" associated with the prognosis after LTX?

2 THEORETICAL BACKGROUND

2.1 Hepatocellular Carcinoma – General Approach

Primary liver cancer is a major health problem worldwide. It is the fifth most common neoplasm in the world, and the third most common cause of cancer-related deaths, with over 80% of cases developing in cirrhotic liver [1]. In Europe and the USA, HCC has gained a major interest because of the rising incidence in the past decade and it is considered to have the fastest growing mortality rate of all cancers.

Geographical differences in incidence reflect variations of the main causal factors. Most cases worldwide occur in Asia and in sub-Saharan Africa owing to the high prevalence of hepatitis B. Characteristically, in developing countries HCC related to hepatitis B virus infection results from acquired infection at birth or early in life, and involves individuals aged 40 years or younger at a symptomatic phase when treatments are not effective (non-cirrhotic in up to 40% of cases). Aflatoxin B1 intake from contaminated food is also a common cause of HCC in Asia and Africa. Developed countries, such as those in Europe, are considered to be a low incidence area; however, even in the European countries, the incidence of HCC has increased over the last 20 years, most likely because of the rising prevalence of hepatitis C and alcohol-induced liver cirrhosis.[2] The contribution of metabolic syndrome and nonalcoholic steatohepatitis (NASH) as a risk factor for cirrhosis and HCC development received more interest recently and a growing number of NASH cases are reported for Europe.[3]

In Germany, a 3-fold increase in mortality for the last 30 years was noted. According to *Weinmann et al.* (2014) [4], in a retrospective study that included 1066 German patients that suffered from HCC in between 1998 and 2008, it was observed that lately the number of older patients (more than 80 years) with HCC has increased (2.3% to 6%) due to chronic alcohol abuse as a main cause in Germany; in contrast, the rest of the European Countries have viral hepatitis as the most common etiology. The authors also stated that there is a relatively low prevalence of chronic viral hepatitis B and C in Germany and high level of *per capita* alcohol consumption.

The Overall Survival (OS) depends on HCC stage. In Germany, a BCLC Stage A patient will have a mean OS of 49 months, 24.2 months in Stage B, 9.7 months in Stage C and 3.2 months in Stage D.[5]

[1] Llovet JM 2003b
[2] Morgan TR 2004
[3] Ratziu V 2010
[4] Weinmann A 2014
[5] Weinmann A 2014

2.2 HCC Risk Factors

HCC can either develop from liver cirrhosis (80% of the cases) or directly as a consequence of Hepatitis B or Aflatoxin, for example[6]. The following figures (Fig.1 and 2) summarize the most common influencing factors that can lead to HCC according to *Monsour Jr et al*[7].

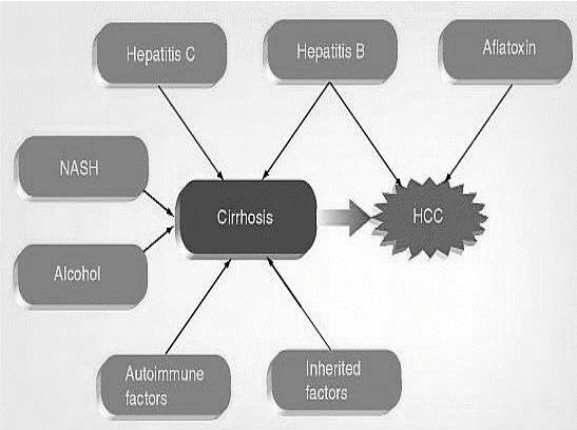

Figure 1 - Risk factors for HCC (Monsour Jr 2013).

Age
Gender (male)
Cirrhosis
HBV
HCV
Fatty liver (NAFLD/NASH)
Obesity
Metabolic syndrome
Diabetes type II
HIV + HBV/HCV
Dietary
Beneficial (negative influence)
Coffee (mod. strong evidence)
Miso soup/tofu
Selenium
Beta carotene
Retinoic acid
Vegetable/fruit intake
Detrimental (positive influence)
Alcohol
Aflatoxin
Abbreviations: NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; HBV, hepatitis B virus; HCV, hepatitis C virus.

Figure 2 - Common influencing factors for HCC (Monsour Jr 2013)

Gender

For almost all countries, males have higher rates of HCC incidence than females, usually with a ratio of 2:1. Higher discrepancies are seen in some European states, such as Switzerland (male:female: 4:1) and Italy (male:female: 5:1). In the developing world, the rates are similar: China [3:1], Gambia [2.8:1] and Zimbabwe [2.4:1].[8]

Age

Reflecting etiological factors, the age of patients diagnosed with HCC varies between sexes and geographical regions. In most regions, female incidence rates peak 5 years later in age compared with males. The change of incidence appears to be affecting the aging population most profoundly. In Europe age-specific tumor incidence has risen 25% and mortality 35% in the 75-years-and-over age

[6] Mohamed IF 2009
[7] Monsour HP 2013
[8] Mohamed IF 2009

group between 1979 and 2004, the greatest rate of increase of any age group. This rise may partially be explained by the increasing burden of HCV amongst the aging population contracted between the 1960s and 1970s.[9]

Cirrhosis

Cirrhosis is defined as the histological development of regenerative nodules, surrounded by fibrous bands, in response to chronic liver injury. Complications of cirrhosis include HCC and portal hypertension. The majority of patients with HCC have underlying cirrhosis. In an autopsy series from Italy and Japan, the prevalence of cirrhosis in patients with HCC is between 80 and 90%. With the exception of HBV and Aflatoxin, all other etiological risks for HCC are associated with cirrhosis. Furthermore, in the USA and parts of Europe, the mortality rate of HCC incidence is increasing, but the rate of mortality due to non-HCC complications of cirrhosis is decreasing or static. This would suggest that the improved management of the complications of cirrhosis has reduced the mortality of patients with cirrhosis, allowing an increased percentage of them to survive long enough to develop HCC.

Hepatitis B Virus (HBV)[10]

HBV is the leading risk factor for HCC globally and accounts for at least 50% cases of HCC. In endemic areas, HBV is mostly acquired by vertical and perinatal transmission with >90% of these cases becoming chronic HBV carriers. In contrast, in areas of low prevalence such as western countries, it is usually acquired in adulthood by horizontal transmission (through sexual and parenteral routes) with >90% of acute infections resolving spontaneously. HBV is a notorious HCC cause in the absence of cirrhosis; however, most (70%-90%) HBV-related HCC develops in cirrhotic livers. Several meta-analysis have demonstrated that the risk of HCC is 15-20 times greater among HBV infected individuals as compared to the uninfected population.

Hepatitis C Virus (HCV)[11]

Hepatitis C virus is the most important risk factor for HCC in Western Europe, North America and Japan. Epidemiological studies from these areas have shown up to 70% of patients with HCC have anti-HCV antibodies in their serum. According to WHO estimates, HCV global prevalence is 2%, representing 123 million infected individuals. [12]

[9] Mohamed IF 2009
[10] Mittal S 2013
[11] Mittal S 2013
[12] Shepard CW 2005

HCV appears to increase the risk of HCC by inducing hepatic inflammation and importantly fibrosis, but also promoting malignant transformation of infected cells. The development of HCC has been shown to occur decades after the initial infection. In an American and Japanese study, HCC development took an average of 28 and 29 years to develop, respectively. The risk is highest among cirrhotics where HCC develops at a rate of 1-4% per year, though rates up to 8% have been reported in Japan.

Viral Co-infection

Follow-up studies have shown that patients co-infected with HCV and HBV have a higher risk of developing HCC than those with a single infection. The same applies for those individuals with HBV and hepatitis D virus co-infection. It is, therefore, recommended that patients infected with HCV should be vaccinated against HBV.[13]

Alcohol

Hutchinson et al showed in 2005[14] that the risk of HCC increased in a linear fashion with heavy alcohol intake (>60g/ day), and this risk doubled in those infected with HCV. Although heavy alcohol intake increases the risk of HCC through the development of cirrhosis, there is no definite evidence to show carcinogenic potential of alcohol. Alcohol acts in synergism with HCV and HBV infection, presumably accelerating the process of fibrosis and progression to cirrhosis. Abstinence does not appear to affect HCC risk once cirrhosis is established. The effect of low or moderate amounts of alcohol intake on risk of HCC is unclear.

Non-Alcoholic Fatty Liver Disease (NAFLD)[15]

Nonalcoholic fatty liver disease (NAFLD) and Nonalcoholic Steatohepatitis (NASH – where there is biopsy-proven hepatic inflammation) have been implicated as risk factors for HCC. Both NAFLD and NASH, similar to other HCC risk factors, promote HCC through advancing liver disease and cirrhosis. NAFLD is now the leading cause of chronic liver disease in the United States. Features of metabolic syndrome such as visceral obesity or insulin resistance are present in virtually all cases of NAFLD.

Aflatoxin

Aflatoxins are carcinogens produced by Aspergillus species (A flavus and A parasiticus) and infest grains, corn, cassava, peanuts and fermented soybeans, particularly when stored in high moisture conditions. There is a high incidence of HCC in regions where Aflatoxin contaminated food ingestion

[13] Montalto G 2002
[14] Hutchinson SJ 2005
[15] Mittal S 2013

is common, such as in parts of sub-Saharan Africa and eastern Asia; however, these regions have high HBV prevalence as well. A study from China demonstrated about a 60-fold increased risk of HCC in patients with chronic HBV and Aflatoxin exposure[16].

Inherited Liver Disorders [17]

Hereditary Hemochromatosis is the most common inherited risk factor for HCC. The risk, as with most other factors, requires the presence of cirrhosis. A Swedish population-based study revealed a 1.7-fold increase in the incidence of HCC among 1800 patients with hereditary Hemochromatosis. Those with HFE ("High Iron Fe"-gene) heterozygous mutations have been found to be at decreased risk for HCC.[18] HCC may also develop in other disorders which are complicated by the development of cirrhosis, such as Wilson's disease, a1-antitrypsin deficiency and type 1 glycogen-storage disease, but this is a rare occurrence.

Coffee – a Protective Factor[19]

Several epidemiological studies have elucidated the link between heavy coffee consumption and low liver enzymes, reduced risk of cirrhosis and reduced risk of HCC. A meta-analysis of studies on risk of HCC among coffee drinkers in European and Japanese studies showed a reduced risk of HCC. They concluded that relative risk of HCC among low or moderate coffee drinkers (defined as 1-2 cups / day) was 0.70, and that for high drinkers (defined as ≥ 3 cups / day) was 0.45, as compared to non-drinkers.[20] Although the mechanism of this possible protective effect is unclear, coffee consumption lowers insulin levels and reduces the risk of diabetes, a known risk factor for HCC.

2.3 HCC Classification

An important step in the management of early HCC is the application of an appropriate and accurate staging system – ideally, one that stratifies patients for treatment and predicts prognosis. The principal system used to score underlying liver function in cirrhotic patients is the Childs-Turcotte-Pugh score (CTP), also known as Child-Pugh score. The Model for End-Stage Liver Disease (MELD), predicts short-term prognosis and is extensively used in liver transplant evaluation. The MELD score is usually used in association with the Milan Criteria (MC), that distinguishes the prognosis of patients according to a two-classification system. Several other classification systems have been used in an attempt to optimize the prognosis-based classification, including the Okuda classification and the TNM score.

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[16] Qian QS 1994
[17] Mohamed IF 2009
[18] El-Serag HB 2007
[19] Mittal S 2013
[20] Bravi F 2007

2.3.1 The Childs-Turcotte-Pugh score (CTP)

The prognostic importance of liver function was first codified in the Child-Turcotte publication in 1964[21], where patients being considered for surgery for portal venous shunting were risk-stratified into three categories. The initial Child-Turcotte staging included clinical assessments of encephalopathy, ascites, nutritional status and laboratory measurements of serum bilirubin and albumin and then was modified by Pugh in 1973[22], with the replacement of nutritional status by prothrombin time.

The CTP score is the simplest and most widely used grading system for liver function. Given that most HCCs arise in the milieu of cirrhosis, and surgical interventions have the highest potential of cure, CTP is ubiquitous in the evaluation of HCC. In addition to routine clinical and research use, the CTP score is referenced routinely by regulatory agencies reviewing new drug applications. However, the drawbacks are many, including inter-laboratory variations, day-to-day fluctuations in the key parameters and the subjective nature of the clinical grading of encephalopathy and ascites[23]. Though the CTP score by itself does not include any HCC-specific parameters, it has been incorporated into multiple contemporary scoring systems including Cancer of the Liver Italian Program (CLIP) and Barcelona Clinic Liver Cancer (BCLC).

2.3.2 The Barcelona Clinic Liver Cancer Staging System

The BCLC staging system (Fig. 3), which has come to be accepted world-wide for clinical practice, divides HCC patients according to five stages (0, A, B, C, and D) depending on tumor-status-related parameters (size, number, vascular invasion, N1, M1), liver function (Child-Pugh grade), and health status (ECOG)[24].

[21] Child CG 1964
[22] Pugh RN 1973
[23] Botta F 2003
[24] European Organisation For Research And Treatment Of Cancer. 2012

Stage	PST	Tumor status		Liver function studies
		Tumor stage	Okuda stage	
Stage A: early HCC				
A1	0	Single	I	No portal hypertension and normal bilirubin
A2	0	Single	I	Portal hypertension and normal bilirubin
A3	0	Single	I	Portal hypertension and abnormal bilirubin
A4	0	3 tumors <3 cm	I-II	Child-Pugh A-B
Stage B: intermediate HCC	0	Large multinodular	I-II	Child-Pugh A-B
Stage C: advanced HCC	1-2*	Vascular invasion or extrahepatic spread	I-II	Child-Pugh A-B
Stage D: end-stage HCC	3-4†	Any	III	Child-Pugh C

PST, Performance Status Test; Stage A and B, All criteria should be fulfilled; *, Stage C, at least one criteria: PST1-2 or vascular invsion/extrahepatic spread; †, Stage D, at least one criteria: PST3-4 or Okuda Stage III/Child-Pugh C.

Figure 3 - BCLC staging classification[25]

2.3.3 The Model for End-Stage Liver Disease (MELD)

The MELD score, initially developed to determine prognosis following a transjugular intra-hepatic shunt (TIPS) procedure for liver failure[26], is now widely used in the liver transplant arena to prioritize donor liver allocation. It is a logarithmic score that is comprised of International Normalized Ratio (INR), serum creatinine, total serum bilirubin and the etiology of cirrhosis. After minor modifications, the resulting MELD model, can be generalized to all patients with end-stage liver disease.

A modification of the MELD score formula (Fig. 4), with the variable for etiology of cirrhosis excluded, was adopted by the United Network of Organ Sharing (UNOS) in February 2002 as the standard by which transplant recipients are prioritized. Given that a higher score is associated with shorter survival, priority for receipt of a transplant is logical. The implementation of MELD led to reduction in registration for the waiting list and mortality while on the list, as well as reduced median waiting time to LTX[27].

$$\text{MELD Score} = 9.57 * \ln(\text{Serum Creatinine in mg/dL}) + 3.78 * \ln(\text{Serum Bilirubin in mg/dL}) + 11.2 * \ln(\text{INR}) + 6.43$$

Figure 4 - Modified MELD Score[28]

[25] Subramaniam S 2013
[26] Malinchoc M 2000
[27] Wiesner R 2006
[28] Subramaniam S 2013

The strength of the MELD score is its prediction of short-term mortality and is therefore able to identify the "sickest" patients for graft allocation. However, it fails to correctly classify a portion of patients with advanced cirrhosis[29], and several groups have offered refinements to the score.

Patients with early stage HCC but compensated liver disease may suffer cancer progression while waiting for their MELD score to move them up on the graft allocation priority list. This has been "remedied" by awarding extra points to the MELD score for a diagnosis of HCC; while this has been shown to improve the likelihood of timely transplant in these patients.[30] Only patients with HCC meeting the MC qualify for repeated MELD score upgrading.

Macromorphological tumor progression beyond the Milan size limits results in loss of MELD prioritization. This, however, does not automatically imply drop-out from the waiting list. Tumor-related patient removal is, in the last analysis, a decision of the transplant center. The transplant team might conclude that the patient is still suitable for LTX by rescue allocation or living donor LTX (LDLT).

[29] Al Sibae MR 2011
[30] Yao FY 2004

2.4 HCC Treatment

Managing patients with HCC should be under the care of a multidisciplinary team that typically includes hepatologists, surgeons, radiologists, oncologists and pathologists. Therapy for HCC may include resection (hepatectomy), locoregional therapy, systemic therapy, transplantation or a combination of these modalities.

According to the BCLC staging system, surgical approaches, including surgical liver resection (SR) and LTX, as well as image-guided tumor ablation, such as Radio-frequency Ablation (RFA), are regarded as potentially curative treatments for HCC but are only recommended in patients with early stage tumor[31], being applicable in only 30%-60% of patients having HCC.[32]

Patients diagnosed with intermediate stage HCC are candidates for Trans-arterial Chemoembolization (TACE), which has proven to control symptoms, prolong survival, and is associated with better survival versus using only best supportive care[33]. However, it is important to emphasize that this option is considered as a palliative and not a curative treatment, characterized in most instances by an unsatisfactory long-term outcome due to the inability to achieve complete tumor necrosis. Furthermore, repeated TACE is often required to completely eradicate the residual tumors, but its efficiency is limited and the rate of tumor recurrence or relapse after initial remission or stable disease is very high.[34]

Treatment recommendations for patients with <u>early-stage resectable disease</u>:[35]

- For patients with early-stage HCC, a partial hepatectomy may be curative; however, a patient's overall liver function, tumor assessment, and liver anatomy must be taken into consideration.
- Resection is recommended in patients who have preserved liver function, generally Child-Pugh class A (good operative risk) without portal hypertension.
- LTX also offers patients a potential curative treatment option in early HCC.

Treatment recommendations for patients with <u>unresectable disease</u> in whom local therapy has failed, who are not candidates for local therapy, or who have metastatic disease:

[31] Iezzi R 2016
[32] Llovet JM 2003a
[33] Camma C 2002
[34] Lammer J 2010
[35] Saraswat VA 2014

- For patients who are not candidates for resection, LTX should be offered to those who have tumors that are inside the MC.[36]
- If feasible, loco-regional therapies should be employed before systemic treatment for unresectable limited disease such as ablation, Trans-arterial Chemoembolization (TACE), radioembolization, or stereotactic body radiotherapy and external-body radiotherapy.

Systemic treatment recommendations for <u>unresectable and advanced metastatic disease</u> in patients with Child-Pugh score of A or B (moderate operative risk):

- Patients diagnosed with advanced HCC are often recommended systemic treatment with Sorafenib or, for patients previously treated with sorafenib, with Regorafenib or Nivolumab.

LTX is the ultimate treatment for patients with HCC. Compared to liver resection, LTX bears 2 principal advantages: The removal of the tumor is not restricted by compromised functional capacity of the liver, and tumor recurrence caused by the pre-carcinomatous situation of the cirrhotic liver is eliminated.

2.5 Liver Transplantation Criteria

A question that physicians face when dealing with the complex decision of defining criteria for LTX is: what is the minimum acceptable 5-year survival that patients should achieve after LTX for HCC? This question arises because of donor organ shortage, but it should also be asked if applying a futility approach (whether the risk to the patient after transplantation is higher than offering an alternative or no treatment). An expected 50% survival at 5 years after LTX was proposed as the lowest acceptable cut-off to include a patient on the waiting list for a LTX; however, at an experts' conference in 2010, the consensus was that even a 50% 5-year survival is too low[37]. A 5-year survival figure of 61% was suggested to avoid major consequences to other patients waiting.

[36] Mazzaferro V 1996
[37] Clavien PA 2011

In order to achieve the minimal OS rate proposed, LTX criteria need to be applied and a minimum-prognostic-based filter must be taken into consideration. The following Table 1 summarizes the different LTX criteria that have been proposed[38]. The most widely adapted criteria are the MC (used in the Eurotransplant area) and the UCSF criteria.

Transplantation criteria	Intention-to-treat survival	Disease-free survival	Post-transplantation survival	Comments
Milan criteria[51] • Single tumour ≤5 cm or 3 tumours all ≤3 cm	N/A	92% 4 years	85% 4 years	Based only on size and number
UCSF criteria[39] • Single tumour ≤6.5 cm or 3 tumours all ≤4.5 cm with TTD ≤8 cm	N/A	90.9% 5 years	80.9% 5 years	Based only on size and number
Up-to-7 criteria[49] • The sum of the maximum tumour diameter and number <7	N/A	• Beyond Milan but within Up-to-7 • 64.1% 5 years	• Beyond Milan but within Up-to-7 • 71.2% 5 years	Based only on size and number
Total Tumour Volume (TTV)[47] • Total tumour volume ≤115 cm^3 • AFP ≤400 ng/mL	• Beyond Milan but within TTV/AFP • 53.8% 4 years	• Beyond Milan but within TTV/AFP • 68% 4 years	• Beyond Milan but within TTV/AFP • 74.6% 4 years	Size and number and biological marker (AFP)
Extended Toronto Criteria (ETC)[43] • No limit in size and number • No vascular invasion • No extrahepatic disease • No cancer-related symptoms • Biopsy of largest tumour not poorly differentiated	• Beyond Milan but within ETC • 55% 5 years	• Beyond Milan but within ETC • 30% 5 years • (Cumulative risk of recurrence)	• Beyond Milan but within ETC • 68% 5 years	No size and number limit but biological behaviour (cancer-related symptoms and tumour differentiation)
Kyoto Criteria[55] • Number ≤10 tumours • Size ≤5 cm • DCP ≤400 mAU/mL	N/A	• Beyond Milan but within Kyoto • 30% 5 years • (Cumulative risk of recurrence)	• Beyond Milan but within Kyoto • 65% 5 years	Size and number and biological marker

AFP, α-fetoprotein; DCP, des-γ-carboxyprothrombin; TTD, total tumour diameter; UCSF, University of California San Francisco.

Table 1 -Different LTX Criteria proposed (Clavien PA 2011)

2.5.1 Milan Criteria

The MC is a set of criteria applied in patients with liver cirrhosis and HCC with the aim of distinguishing patients with a better outcome (inside MC) from the others (outside MC). Their significance derives from a landmark 1996 study with 48 patients by *Mazzaferro et al*[39] which showed that selecting cases for transplantation according to specific strict criteria led to improved OS and Disease-free Survival (DFS) at 4-years.

Patients with HCC meeting the MC (one solitary tumor nodule up to a maximum of 5 cm or a maximum of 3 HCC nodules and each of them up to a maximum diameter of 3 cm, without macrovascular invasion and extrahepatic tumor spread) were demonstrated to achieve excellent long-term survival rates (4-year OS 75% and DFS 83%).[40] Therefore, these criteria became the worldwide "standard" for the patient selection process prior to LT. The United Network for Organ Sharing (UNOS) database analysis including 48887 liver transplant patients with HCC between 1987

[38] Clavien PA 2011
[39] Mazzaferro V 2009
[40] Mazzaferro V 2009

and 2001 has shown that implementation of the MC led to a significant improvement of survival from 25.3% (1987–1991) to 61.1% (1996–2001) within two decades.

As a logical consequence, MC were incorporated into the modified liver allocation systems of UNOS in 2001 and Eurotransplant in 2006. Both regions are currently using the model for end-stage liver disease (MELD) score-based prioritization system, which provides exceptional priority upgrades for several indications, such as HCC meeting the MC.

2.5.2 Limitations of the Milan Criteria

Although useful, this widely used criteria is not perfect in predicting prognosis. In fact, a static classification of HCC according to the MC ignores the inherent tumor dynamics. In 2009, *Mazzaferro et al.* [41] stated that at least 28% of the patients originally classified as being outside the MC (from a total number of 1556 patients), were indeed histologically confirmed to be within MC. This incongruence is caused by biological similarities of imaging characteristics in HCC, dysplastic and regenerative nodules. These similarities are especially difficult to distinguish in cirrhotic livers making the correct identification of small HCC lesions impossible and may therefore sabotage the accuracy of the MC.

Moreover, and according to *Otto et al* [42], MC classification based upon the imaging at listing time turned out not to be predictive of HCC recurrence after LTX (*p-value* = 0.58). Nonetheless, if this imaging assessment is done immediately before transplantation, it is highly predictive of the LTX outcome (*p-value* <0.0001).

2.5.3 UCSF Criteria

Several groups have challenged the restrictions imposed by Milan through expanding the size criteria. Most notable has been the group from the University of California San Francisco (UCSF) who have proposed criteria of a single tumor ≤6.5 cm, or up to three tumors, the largest ≤4.5 cm and total tumor diameter ≤8 cm without gross vascular invasion.[43]

[41] Mazzaferro V 2009
[42] Otto G 2013
[43] Yao FY 2001

These numbers were derived from explant tumor characteristics as the authors noted that explant pathology often revealed understaging by preoperative cross-sectional imaging; however, this did not necessarily result in inferior outcome. Of the 168 patients in the initial report, the 5-year DFS was 90% for the 130 patients with a preoperative tumor stage within Milan versus 94% for the 30 patients that met the UCSF criteria but exceeded Milan *(p-value* = 0.58). In several studies that followed the initial proposal of this expanded criteria, results showed that when UCSF criteria were applied at listing, it led to worst OS rates compared to when the MC was applied (for example: 46% vs 61% 5-Year OS, *p-value* <0.001 according to *Decaens et al in 2006[44]).* However, when both criteria were applied to the histological samples, no significant difference could be found in the OS of the two groups (63% vs 70% 5-year OS, *p-value* = 0.30). Similar results have been replicated throughout time, indicating that the UCSF criteria are not a reliable expansion criteria to achieve the minimum OS rate for LTX, defined as 60% OS after 5 years. On the other hand, some researchers argue that once pre-LTX diagnosis accuracy is improved, and the difference to the histological features are minimized, the UCSF criteria might be a reliable way to integrate a greater number of patients in the waiting-lists The Fig. 5 summarizes these both criteria which are most commonly used nowadays.

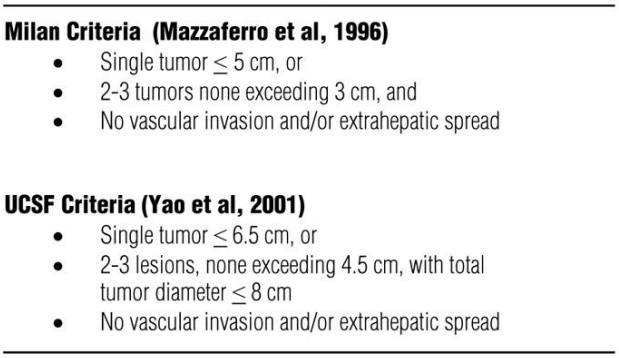

Figure 5 - Milan Criteria and UCSF Criteria

2.5.4 Metroticket 2.0

In January 2018, at the time of writing this dissertation, a new model in this area was presented by the authors of the MC. The *Metroticket 2.0* was presented by *Mazzaferro et al*.[45], who developed a model based on level of AFP, tumor size and tumor number, to determine risk of death from HCC-related factors after LTX. The training set comprised 1018 patients who underwent LTX for HCC from January 2000 through December 2013 at 3 tertiary centres in Italy. In the competing-risk regression, the sum of tumor number and size and of $\log^{10}$ level of AFP were significantly associated with HCC-specific death *(p-value* <0.001). According to the authors, for patients with HCC to have a 70% chance of HCC-specific survival 5 years after transplantation, their level of AFP should be <200 ng/mL and the sum of number and size of tumors (in centimetres) should not exceed 7 cm; if the level of AFP was 200-400 ng/mL, the sum of the number and size of tumors should be ≤5 cm; if their level of AFP was

[44] Decaens T 2006
[45] Mazzaferro V 2018

400-1000 ng/mL, the sum of the number and size of tumors should be ≤4 cm. For the authors, this model based on patients' level of AFP and HCC number and size, outperformed the MC, the UCSF criteria, the Shanghai-Fudan Criteria, the Up-to-7 Criteria *(p-value* <0.001), and the AFP French model *(p-value* = 0.044) to predict which patients will survive for 5 years after LTX.

2.6 Liver Transplantation Demographics and Epidemiology

As the Transplantation System varies widely worldwide, it is important to understand the actual reality in Germany, where this dissertation was conducted. Since the first liver transplant at the University Hospital Bonn in 1969 by Gütgemann and Lie, more than 22,850 transplants have been performed in Germany. According to *Tacke et al*[46], in 2015, 1,489 patients were wait-listed for LTX in Germany, but only 894 transplants were performed at the 23 German transplant centers. The main indication for liver transplant listing was "fibrosis and cirrhosis" (27%) followed by "alcoholic liver disease" (23%) and "hepatic malignancies" (17%).

While the rate of liver cirrhosis and HCC due to viral hepatitis steadily increased over the past years, current projections indicate a potential for decreasing the numbers of patients due to the effective and broadly used antiviral therapies for hepatitis C. However, the relative and absolute numbers of people with pre-obesity (BMI 25-30 kg/m²) and especially with obesity (BMI >30 kg/m²) are projected to dramatically increase until 2030[47], supporting the expectation that nonalcoholic fatty liver disease will become the major cause for end-stage liver disease in the near future in Germany.

Since 2006, the allocation system in Germany was switched from a waiting time-based allocation to an urgency-based system using the MELD Score (exception: "high urgency" status is prioritized and is granted after request for acute liver failure or primary organ failure after transplant). After introduction of the MELD-based allocation policy, the outcomes after LTX have deteriorated in Germany. The average 1-year survival rate is about 75-80% in Germany, which is considerably lower than in the US or the UK (~90%)[48].

Due to the organ shortage and all new implemented guidelines, a Liver Transplant Committee must consider that only patients that are in highest need for a transplant accompanied by the best predicted outcome are listed. This means that many centers evaluate patients not before they reach

[46] Tacke F 2016
[47] Westphal C 2014
[48] Seehofer D 2013

a MELD score of 15-20 or higher. Depending on the blood group, primary organ offers are usually granted at MELD scores between 25 and 35 in Germany[49].

In the Eurotransplant allocation system, HCC patients inside MC receive additional points equivalent to a 10% increase in their theoretical mortality every 3 months. However, this is not the only exception-points allocation system, as can be visualized in the following Table 2[50].

Organ procurement organization (region)	Tumour burden to qualify for exception points	Exception points granted	Exception points progression	Exception point cap	Waiting period before receiving exception points
OPTN/UNOS (USA)	T2	28	First 3 months assignment of MELD score equivalent to 35% mortality risk. Following months additional MELD score equivalent to 10% increase in mortality	Yes: 34	6 months from listing (calculated MELD score)
Eurotransplant (Austria, Belgium, Germany, Holland, Slovakia, Croatia)	T2	22	Add point equivalent to a 10% increase in candidate mortality every 3 months	No	No
Human organ precurement and exchange program (Alberta, Canada)	TTV ≤115 cm^3 & AFP ≤400 ng/ml (T1 excluded)	22	Add 2 points every 2 months	No	No
Human organ precurement and exchange program (Ontario, Canada)	UCSF criteria or TTV≤115 cm^3 & AFP ≤400 ng/ml (T1 excluded)	22	Add 3 points every 3 months	No	No
Brazil	T2	20	Increase to 24 at 3 months and to 29 at 6 months	Yes: 29	No
Organització catalana de trasplantaments (Cataluña, Spain)	Single HCC <3 cm and AFP >200 ng/mL, or single HCC ≥3 cm and <5 cm or 2–3 HCCs ≤3 cm	19	Add one point every 3 months	No	No
Nord Italian transplant (Italy)	None	No exception points	Prioritization according to risk of progression and response to bridging therapies[67] (system under assessment)	No	No

AFP, α-fetoprotein; HCC, hepatocellular carcinoma; MELD, model for end-stage liver disease; OPTN, Organ Procurement Transplantation Network; TTV, total tumour volume; UCSD, University of California San Francisco; UNOS, United Network for Organ Sharing. Modified with permission from Wiley © Toso, C. *et al.* Am. J. Transplant. 14, 2221–2227 (2014).

Table 2 - LTX Criteria in different parts of the world (Toso et al 2014)

2.7 Locoregional Treatments – Bridging Therapies

Due to the common long waiting times, it has become common practice to use Locoregional Therapy (LRT) as a bridging strategy to avoid tumor growth and thereby dropout from the waiting list. It has also been confirmed several times that bridging therapies can even reduce the size and number of lesions in such a way that patients can be included in the MC for LTX (downstaging)[51]. There are a number of different LRTs that can be used in this context which include: TACE, RFA and liver resection. Other LRTs include: brachytherapy, percutaneous ethanol injection (PEI), microwave ablation (MWA), irreversible electroporation (IRE), high intensity focused ultrasound (HIFU) and

[49] Schlitt HJ 2011
[50] Toso C 2014
[51] Otto G 2013

stereotactic body radiotherapy (SBRT). Generalized chemotherapy can also be used as a bridging therapy. A combination of intervention therapies has been widely developed and performed in recent years.

Trans-Arterial Chemoembolization

TACE was first used to treat HCC by *Doyon et al*. in 1974 in Japan. [52] Initially, gelatin sponge particles and anticancer agents were used; in the 1990s lipiodol was introduced. Today, TACE has become the most common approach for the management of HCC without curative options.

A hepatic artery angiography is usually performed through a right common femoral approach to map liver vascular anatomy, check for arteriovenous shunts and identify the arterial tumor supply.[53]

During its progression, HCC exhibits intense neoangiogenic activity and is mostly dependent on the hepatic artery for blood supply, while the rest of the liver is supplied by the portal vein. This provides the rationale to use arterial obstruction as an effective therapeutic option, as it induces ischemic tumor necrosis.

Considering the use of TACE in bridging to LTX, some authors demonstrated that a good response to TACE (necrosis >60%) is significantly related to an improved long-term survival after LTX and a lower recurrence rate[54]. However, several studies did not find any significant advantage in OS and DFS after LTX in HCC patients bridged with TACE[55]. This might indicate that TACE *per se* does not have an influence in the prognosis, although it allows the clinicians to identify patients that have a better "Response to Bridging" and therefore a better survival.

Radio Frequency Ablation (RFA)

RFA is an ablative technique that uses a radiofrequency electrode tip generating alternating current, that induces coagulative necrosis in the target tumor by thermal action, with temperatures of 60 to 100 °C. It can be performed by intraoperative or percutaneous approach. RFA is known to be an effective curative treatment for patients with non-resectable HCC. When used as a bridging treatment, RFA reduces significantly the dropout rate[56]. The success in achieving complete necrosis depends on the size of the target lesion: RFA for HCC with diameter of 2.5 cm or less lead to complete necrosis in up to 90% of cases. For lesions of 5 cm diameter or more, the necrotic effect is

[52] Doyon D 1974
[53] Iezzi R 2016
[54] Allard MA 2015
[55] Decaens T 2005
[56] Pompili M 2005

estimated to be less than 50%[57]. Even if RFA is proven to be a safe procedure, it has some limitations and complications. It should be avoided in subcapsular HCC and in nodules located near bowel loops or the gallbladder, and the tumor should be visualized by ultrasound. Complications of RFA include thermal or mechanical damage, leading to rare but severe complications, such as acute liver failure, liver abscess and haemobilia.[58]

Surgical Resection

Surgical resection (also known as Liver Resection – LR) is commonly used as primary curative treatment for HCC. OS after LR in cirrhotic patients is over 50% at 5 years and perioperative mortality is 2–3%[59]. LR can be considered as a first line bridging treatment to LT. The theoretical advantage of surgery is a better control of tumor growth, as TACE and other LRT do not achieve complete tumor ablation as well as surgery. Moreover, the pathologic analysis of the resected specimen allows an evaluation of tumor biology and provides a selection of patients with risk factors of poor prognosis who are at major risk of early recurrence and should have a priority in the LT wait-list. However, in most transplant Centers, TACE and other LRT are preferred, mainly because LR has higher costs and more complications and can only be performed in well-compensated liver disease[60].

[57] Pompili M 2005
[58] Coletta M 2017
[59] Chang YJ 2016
[60] Coletta M 2017

2.8 Outcome Predicting Factors

The factors contributing to HCC recurrence after LTX can be divided into: donor-related and graft-related factors; pre-transplant recipient and tumor factors; and tumor factors at explant pathology (Fig. 6).[61]

2.8.1 Donor-related and graft-related factors

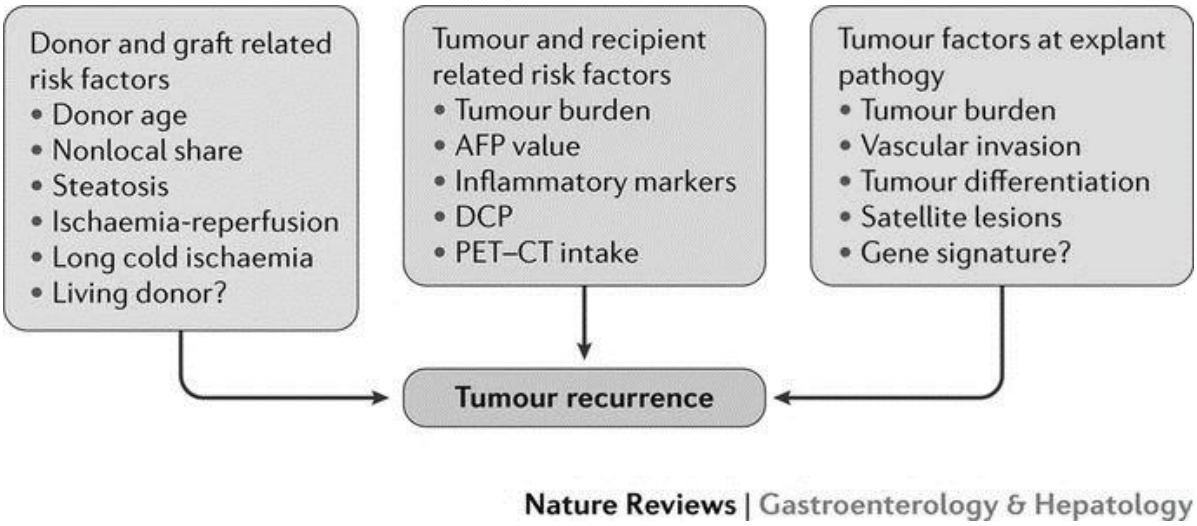

Figure 6 - Outcome Predicting Factors in LTX (Sapisochin G 2017)

Donor factors might be relevant to the recurrence of HCC. A study using the UNOS–Organ Procurement Transplantation Network (OPTN) database found an association between donor age ≥60 and nonlocal shared organs, and HCC recurrence.[62] Others have found donor age or prolonged cold ischemia as relevant. Experimental studies suggest that increased tumor recurrence is related to the magnitude of ischemia–reperfusion injury as suggested for increased HCC recurrence when using steatotic livers[63]. All these factors are difficult to take into account when allocating an organ to a patient; nevertheless, they might be useful when considering a patient with other high-risk factors for tumor recurrence.

2.8.2 Pre-transplant recipient and tumor factors

Tumor factors are the most relevant to the recurrence of HCC. Size and number of tumors on preoperative imaging studies have been related to recurrence and form the base of most of the LTX criteria systems applied[64].

[61] Sapisochin G 2017
[62] Sharma P 2011
[63] Orci LA 2016
[64] Toso C 2015

Pre-transplant tumor markers such as AFP serum levels are increasingly being used to exclude patients from the waiting list due to their association with tumor recurrence[65]. Different AFP cut-offs (20 ng/ml, 100 ng/ml, 400 ng/ml, 500 ng/ml, 1,000 ng/ml) have been proposed as predictors for HCC recurrence. The evolution of AFP while waiting has also been shown to be predictive. Studies have demonstrated a better outcome for patients in whom AFP could be downstaged to <400 ng/ml[66]. Increased des-gamma-carboxyprothrombin (DCP) serum levels have also emerged as a recurrence predictor. One study reported that a DCP level >400 mAU/ml was a risk factor for HCC recurrence and incorporated it into their selection criteria[67]. In 2016, *O´Connor et al* [68] defended a pre-operative cut-off AFP of 100 ng/ml as an independent risk-factor for recurrence (p=0.036). Inflammatory status has also been evaluated. Patients with tumors within MC and a neutrophil-to-lymphocyte ratio ≥ 5 had an increase in tumor recurrence with a Hazard-Ratio (HR) of 19.9 (95% CI 2.5–161)[69]. A subsequent analysis was unable to confirm those results, but in a 2014 study with 865 LTXs for HCC, neutrophil-to-lymphocyte ratio was found to predict tumor recurrence even though the HR was not as high as previously reported[70]. Another study combined response to treatment, AFP and inflammation markers, and argued that these inflammatory markers might help to predict post-transplant outcomes[71]. However, despite being promising, inflammatory factors have not yet reached conventional clinical decision-making usage.

2.8.3 Tumor factors at explant pathology

Most studies have analyzed the effect of tumor factors in the explanted livers on the development of HCC recurrence, but these factors cannot be used for pre-transplant decision-making. The most important factors are macroscopic and microscopic vascular invasion, satellite lesions and tumor differentiation[72]. A meta-analysis analyzing factors contributing to post-transplant HCC recurrence in nine studies and 1,198 cases showed that vascular invasion, poor tumor differentiation, tumor size >5 cm and tumors outside MC were related to tumor recurrence[73].

[65] Toso C 2015
[66] Merani S 2011
[67] Fujiki M 2009
[68] O'Connor DB 2016
[69] Halazun KJ 2009
[70] Agopian VG 2014
[71] Lai Q 2016
[72] DuBay D 2011
[73] Sotiropoulos GC 2007

2.9 Tumor Recurrence after Transplantation

Even with careful selection the HCC tumor recurrence rate after LTX is still between 8–20%[74]. Furthermore, any expansion of selection criteria in terms of size and nodule number increases the risk of HCC recurrence. A large international multicenter study using explant pathology staging (not radiology) showed that the further criteria for size or number of HCCs are expanded, the higher the risk of tumor recurrence after LTX[75]. Tumor recurrence is frequently extrahepatic, particularly in the lungs and bones[76] (Fig. 7). Tumor recurrences after transplantation are likely to the growth of occult metastases months or years after transplantation (most likely) or due to engraftment of circulating HCC cells released at the time of surgery (less likely)[77].

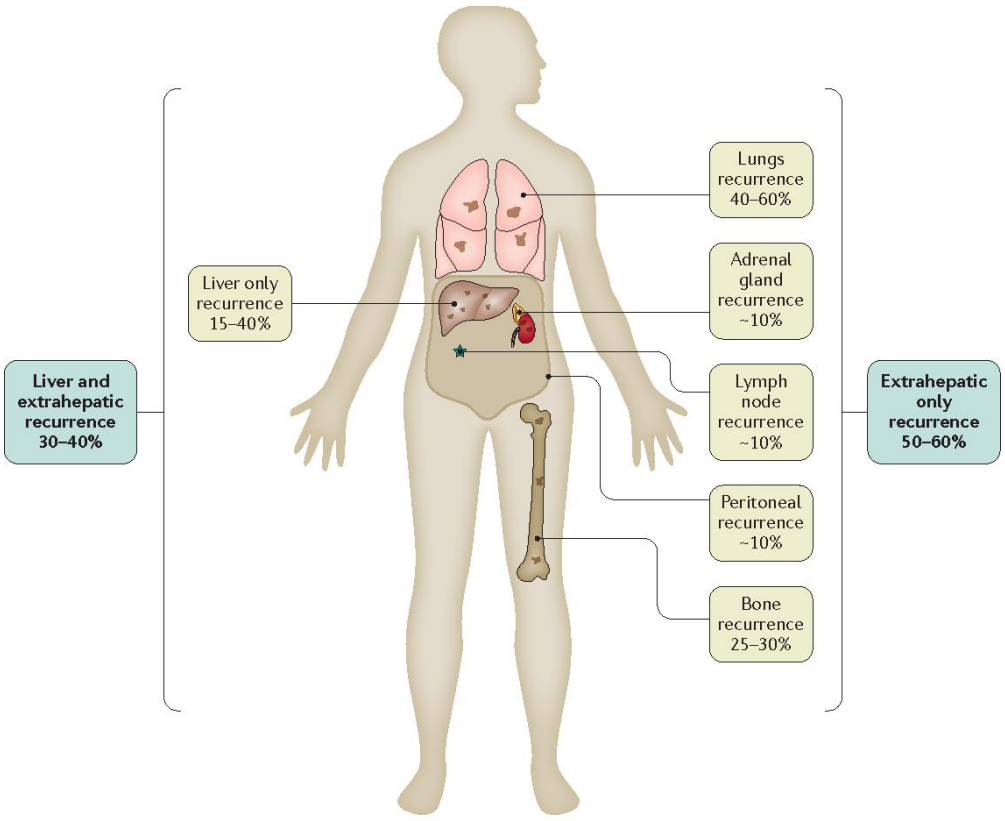

Figure 7 – Common sites of tumor recurrence after LTX for HCC (Sapisochin G 2017)

[74] Toso C 2015
[75] Mazzaferro V 2008
[76] Kornberg A 2009
[77] Toso C 2011

2.10 Tumor Biology and "Response to Bridging"

Variation in the tumor characteristics during the waiting time to LTX should not be considered as irrelevant. In 2013, *Otto et al*[78] showed that after receiving TACE as a bridging therapy, 16% of the patients who were initially outside MC turned out to be inside these criteria when the histological findings were analyzed, while 7% shifted from inside to outside MC. This analysis reveals how inaccurate it might be to propose prognosis based on MC at listing time, especially in patients that undergo a bridging therapy while on the waiting list. More recently it has also been defended that the response of HCC to these therapies gives important information about the tumor biology of the patient. Since not all HCC progress at the same rate, the concept of "Tumor Biology" has been used ever more as an additional tool to predict HCC activity and LTX prognosis. Several methods have been proposed to assess the tumor biology of HCC. Some authors focus on the molecular level (DNA Index, for example[79]), while others focus on the macroscopic tumor size evolution. A tumor with an unfavorable biology will most likely be less sensitive to radio-chemotherapy, grow at a faster rate and recur at a higher rate than an HCC with favorable biology.

Several publications have defended the assessment of tumor biology as a more accurate way to predict outcome in HCC LTX than through the traditional MC. In 2013 Gerd Otto presented a prospective study after 12 years of data collection showing that both MC and "Response to Bridging" could predict LTX outcome. However, MC could only predict outcome when assessed on the day of transplantation (and not at the day of listing) and more importantly, the "Response to Bridging" had a greater predictability of outcome versus MC. The authors concluded that the "Response to Bridging" may reflect HCC tumor biology, which in-turn allows clinicians to predict LTX outcome more accurately than simply through MC[80]. Still in this context, in 2017 Agopian and his collaborators published a North American retrospective study that included 3600 patients over 11 years in 11 USA hospitals, which concluded that although bridging *per se* did not change the patient prognosis, the degree of "Response to Bridging" could accurately predict the outcome after LTX.[81]

[78] Otto G 2013
[79] Andreou A 2014
[80] Otto G 2013
[81] Agopian VG 2017

3 HYPOTHESIS

It is still difficult to predict which patients with HCC benefit the most from LTX, which remains a procedure with a high recurrence and mortality rate. In this context, bridging with LRTs is known to avoid drop-out of LTX waiting lists and allow downstage of tumor size in order to fit MC. Most of the studies failed to prove a prognostic advantage of LRTs in the OS and DFS after LTX. However, there is lack of data studying the "Response to Bridging" as a prognostic factor after LTX.

This dissertation was designed based on the hypothesis that a favourable "Response to Bridging" leads to a better prognosis after LTX and that this tumor behaviour after bridging might reveal the aggressiveness of HCC biology.

4 METHODS

4.1 Population

4.1.1 The SiLVER Study

The population analyzed in this dissertation compromised an original group of 525 patients within the "Sirolimus in Liver Transplant Recipients with HCC study *(SilVER Study)"*. The *SiLVER Study* (www.silver-study.org) was a prospective, randomized, multicenter, open-label phase 3 trial that aimed to investigate whether sirolimus-based immunosuppression improved outcomes in LTX candidates with HCC. (Geissler EK 2016)[82] It is important to mention that the *SiLVER Study* did not aim to study or compare the effect of the different possible bridging therapies before LTX and therefore the complete data related to these therapies (which are the focus of this dissertation) was not present in all the SiLVER patient reports.

The *SiLVER Study* took to consideration that, despite the constant effort to improve the stratification systems applied to the transplant waiting lists (like MC or BCLC criteria), there is still a high recurrence rate of HCC after LTX of *circa* 1 recurrence per 5 liver-transplants. Interestingly, the immunosuppression needed to prevent organ rejection has long been associated with occurrence of cancer, and the most commonly used conventional immunosuppressive drugs (calcineurin inhibitors, such as *cyclosporine* and *tacrolimus*) have specific tumor-promoting activities.

In this context, researchers from 45 transplant centers in Europe (42), Canada (2), and Australia (1) compared the actual most commonly used immunosuppression therapies after LTX in HCC with an immunosuppressive therapy based on a mammalian target of rapamycin (mTOR) inhibitor which is known to inhibit tumor growth. The mTOR inhibitor used was *Sirolimus*.

In this trial, 525 LTX recipients with HCC initially receiving mTOR inhibitor–free immunosuppression were randomized 4 to 6 weeks after transplantation into a group on mTOR inhibitor–free immunosuppression (group A: 264 patients) or a group incorporating *sirolimus* (Group B: 261).

The primary endpoint was the DFS and a secondary endpoint the OS. After 5 years of follow-up of all patients, an intention-to-treat (ITT) analysis was conducted and the results were published in the official journal of the Transplant Society & International Liver Transplantations Society – *TRANSPLANTATION* – on January of 2016.

The SiLVER Study concluded that the used of Sirolimus in LTX recipients with HCC did not improve long-term RFS beyond 5 years. However, a RFS and OS benefit was evident in the first 3 to 5 years,

[82] Geissler et al. 2016

especially in the patients within MC. This trial provided the first high-level evidence base for selecting immunosuppression in LTX recipients with HCC.

4.1.2 Patient Selection (*SiLVER* Study)

This study was based on a sub-analysis of the *SiLVER Study* patient database. The *SiLVER Study* had roughly a 3-year enrollment period (January 2006 to April 2009) with at least a 5-year follow-up; patients remained in the study for its entire duration, regardless of when they were randomized. In the first year after LTX, patients were followed up at months 1, 3, 6, 9 and 12; Thereafter patients had scheduled visits every 6 months until the end of the study. The first patient was randomized in January 2006 and the last patient visit was conducted in March 2014.

The study included all patients eligible for LTX, with the inclusion criteria being 18 years or older, histologically proven HCC before randomization and signed written informed consent. The main exclusion criteria were the presence of extrahepatic HCC and non-HCC malignancies within the past 5 years (excluding successfully treated nonmelanoma skin cancer). Multiple-organ recipients, patients with a known sirolimus hypersensitivity, hyperlipidemia refractory to management, evidence of infection, platelets less than 75 000/mm^3 and women of child-bearing potential not willing to take contraception were also exclusion criteria.

The LTX recipients from the SiLVER Study were recruited from 45 transplant centers in Europe (42), Canada (2), and Australia (1) in a multicenter, randomized, open-labeled, parallel group trial (EudraCT:2005-005362-36; Clinicaltrials.gov: NCT00355862).

4.1.3 Randomization (*SiLVER* Study)

Patients were randomized into 2 groups. Group A was maintained for the study duration on a center-specific mTOR inhibitor-free, generally calcineurin inhibitor–based, immunosuppressive protocol. This control group of patients was compared to a second group (Group B) that received mTOR inhibitor–free immunosuppression for the first 4 to 6 weeks, at which time sirolimus was incorporated into the regimen (target range, 4-10 ng/mL) either as a monotherapy or as a combination therapy with non-mTOR inhibitor–based drugs. The enrolled population included patients with HCC tumors that demonstrated liver cirrhosis and were within the guidelines of the MC, but also recipients with tumors outside the limits of the MC; in all patients, including those receiving bridging therapies for histologically proven HCC, MC stratification was based on post-LTX histopathological data on the explanted organ. The randomization was performed between day 22 and day 42 after LTX, allowing for confirmation of HCC in the post-LTX pathology assessment, when pre-LTX histological confirmation was not available.

4.1.4 Follow-up (SiLVER Population)

Patients in the SiLVER Study underwent a standardized tumor-specific follow-up at every scheduled visit. These regular examinations included ultrasound, a chest X-ray, as well as α-fetoprotein measurements, along with a clinical examination to detect potentially related symptoms. In case of suspicious findings, a computed tomography scan/magnetic resonance imaging/positron emission tomography/bone scintigraphy was recommended in accordance with existing guidelines; a biopsy was also recommended to further confirm the HCC diagnosis. Confirmation of an HCC diagnosis was doublechecked by on-site monitoring. For the purpose of the study, because of normal delays in establishing a definitive diagnosis of HCC, the first day of tumor suspicion constituted time of recurrence. All mentioned time measurements were calculated based on the day of LTX. In the first year after LTX, patients were followed up at months 1, 3, 6, 9, and 12; thereafter, patients had scheduled visits every 6 months until study end. All primary and secondary endpoint data were monitored on-site by the sponsor for accuracy by verifying source data and electronic case report form entries.

4.1.5 *Bridging Population – the SiLVER* Sub-population analyzed in this Dissertation

From the original 528 patients included in the SiLVER database, 151 did not receive bridging therapy, 374 received some form of bridging therapy, and in 3 cases this information was missing.

As it was not the goal of the SiLVER Study to compare the different bridging modalities, the criteria used by the different hospitals and medical teams to decide between bridging types, or even the inclusion and exclusion criteria for receiving such a therapy, was not available. This is a clear limitation of a retrospective analysis and for this reason it was not possible to compare the efficiency of the different types of bridging. All the LRTs used as bridging were accepted when studying the sub-population analyzed in this dissertation.

4.2 Endpoints

The primary endpoints of this analysis were DFS and OS. DFS was defined as the time interval between the date of LTX and the date of recurrence or death (as first event). Date of recurrence was considered the to be the first day of suspicion of a later confirmed HCC recurrence.

4.3 Grouping for Analysis

4.3.1 Grouping by "Response to Bridging"

In order to analyze and compare the consequences of bridging therapies, a categorization according to the updated RECIST-Criteria[83] would ideally be followed. The Response Evaluation Criteria In Solid Tumors (RECIST) is a set of rules, first published in 2000 and more recently updated in 2009, that categorizes in four groups (complete response, partial response, stable disease and progressive disease) the response of solid tumors, like HCC. According to this classification, the response is assessed by measuring the length (longest diameter) and number of the target lesions. This classification system also takes into consideration the evolution of lymph nodes and other non-measurable lesions (such as ascites, pleural effusion, lesions smaller than 20mm, among others).

Although apparently suited for our purpose, this classification could not be fully applied to the database, because: 1) the non-measurable parameters like ascites were not measured/collected and 2) the data related to the size of the lesions was not collected uniformly in the metric scale, and some hospitals categorized the tumor sizes according to size ranges in a categorical scale. For these reasons, the former RECIST-Criteria was reviewed and adapted to our data-reality in order to be as accurate and reliable as possible. An original and broader categorization was then created dividing the patients in 2 groups:

- **Progressive Disease (PD):** Increase in the sum of diameters of target lesions.
- **Controlled Disease (CD):** Stability or decrease in the sum of the diameters of the lesions; this included complete remission after bridging.

Ideally, tumor lesions should be measured before and after each therapy. However, as the *SiLVER Study* did not aim to assess the "Response to Bridging", there was not a standard regular measurement of the tumors until transplantation. This means, that in most of the patients the size and number of the lesions were reported at the moment of listing and then compared to the final pathological finding. This reveals some limitations in the measurements as it will be exposed in the chapter “Limitations and further Research”.

4.3.2 Grouping by Milan Criteria Dynamics

As already mentioned, MC are considered to have a big impact on LTX prognosis. For this dissertation, patients were grouped according to their Milan status at the time of listing and according to the

[83] Eisenhauer EA 2009

histological finding after LTX. This allowed us to understand how many patients had a variation of their Milan status during the waiting time, in which they received bridging therapies. The categorization resulted in the following groups of Milan dynamics:

- **Inside-to-Inside** – Patients considered to be inside MC at the time of listing and inside MC at LTX day;
- **Inside-to-Outside** - Patients considered to be inside MC at the time of listing and outside MC at LTX day;
- **Outside-to-Inside** - Patients considered to be outside MC at the time of listing and inside MC at LTX day;
- **Outside-to-Outside** - Patients considered to be outside MC at the time of listing and outside MC at LTX day;

4.4 Statistical Analysis

All statistical analysis were performed using IBM SPSS Statistics 24 (Chicago, IL.).

5 RESULTS

5.1 Descriptive Statistics

5.1.1 Demographics

Among this SiLVER sub-population, from now on referred to as “Bridging Population”, 87% were male. The mean age at LTX was 57.9 years old (max: 75 years; min: 24 years; SD: 7.11). Mean time on waiting list was 369 days (max: 2649 days; min: 0 days; SD: 302 days). The 68% of patients in the Bridging Population were within the MC at the time of listing, and 32% were considered to be outside Milan. The Figure 8 shows the absolute numbers of patients distributed within the “Bridging Population” according to their Milan status at the time of listing.

Figure 8 - Diagram illustrating the number of patients distributed within the "Bridging Population" according to their Milan status at the time of listing

On the day of transplantation, histological findings revealed that only 62% of the livers removed were within the MC. Further details are summarized in the following table (Table 3).

Bridging Population (n=356)	
Age ([y]; mean, SD)	57.9 (7.1)
Male (%)	87.2%
No. of lesions (mean, SD)	
- Initial	3.4 (4.8)
- Post-LTX	3.7 (6.0)
Initial lesion size (%)	
- <3 cm	58%
- 3.1 to 5 cm	34%
- 5.1 to 7.5 cm	6%
- >7.5 cm	2%
Post-LTX lesion size (%)	
- no lesion	17%
- <3 cm	33%
- 3.1 to 5 cm	38%
- 5.1 to 7.5 cm	8%
- >7.5 cm	4%
Inside Milan (%)	
- Initially	67%
- Post-LTX	62%
Time on waiting list (mean in days; [SD])	369 [302]
Cumulative Disease-free Survival (DFS) (%)	
- 1st Year	90%
- 2nd Year	79%
- 3rd Year	74%
- 4th Year	70%
- 5th Year	65%
Cumulative Overall Survival (OS) (%)	
- 1st Year	95%
- 2nd Year	87%
- 3rd Year	81%
- 4th Year	76%
- 5h Year	71%

Table 3 - Descriptive Statistic of the Bridging Population

In the SiLVER Study patients were randomized in order to form to groups with the same number of patients. Half of the patients were in the Group A and half in the Group B. This distribution was also seen in the "Bridging Population". When this distribution was analyzed according to the Group per

Protocol (which refers to a distribution according to the medication that patients actually received), no substantial changes could be found (Table 4).

Bridging Population (N=356)	
Intention to Treat (ITT)	
- Group A	50%
- Group B (Sirolimus)	50%
Per Protocol (PP)	
- Group A	53%
- Group B (Sirolimus)	47%

Table 4 - Distribution of SiLVER Patients per Intention-to-treat vs. per Protocol

In this dissertation a specific sub-population was particularly important to evaluate the association between "Response to Bridging" and prognosis – the group of patients that permanently stayed inside Milan since listing until LTX ("In-to-Inside Milan" Population). Although this topic will only be described in future chapters, the descriptive statistics can be found in the following Table 5. This sub-population has roughly the same mean age as the "Bridging Population" (57.1 vs. 57.9 years, respectively) a similar percentage of male individuals (86.4% vs. 87.2%) and a similar percentage of distribution within the immunosuppression groups (Group A-B: 48-52% vs. Group A-B: 50-50%). However, in the "In-to-Inside Milan" population there was a shorter mean-time on waiting list (223 vs. 369 days) and, as it was to be expected, a smaller number and size of lesions.

In-to-Inside Milan Population (n=184)	
Age ([y]; mean, SD)	57.1 (6.9)
Male (%)	86.4%
No. of lesions (mean, SD)	
- Initial	1.4 (6.6)
- Post-LTX	1.0 (0.7)
Initial lesion size (%)	
- <3 cm	75%
- 3.1 to 5 cm	25%
Post-LTX lesion size (%)	
- no lesion	27%
- <3 cm	47%
- 3.1 to 5 cm	26%
Time on waiting list (mean in days; [SD])	223 [308]
SiLVER Group (ITT)	
-Group A	48%
-Group B (Sirolimus)	52%

Table 5 - Descriptive Statistics of Inside-to-Inside Milan Population (within the Bridging Population)

5.1.2 Bridging Modalities

The most common single bridging method used was TACE (47%), followed by RFA (19%), liver resection (7%), PEI (4%), brachytherapy (0.5%) and after-loading (0.25%). Around 23% of those who received bridging before LTX had a combination of two or more bridging modalities and 1 patient received four different types of bridging therapies. The Figure 9 illustrates this distribution.

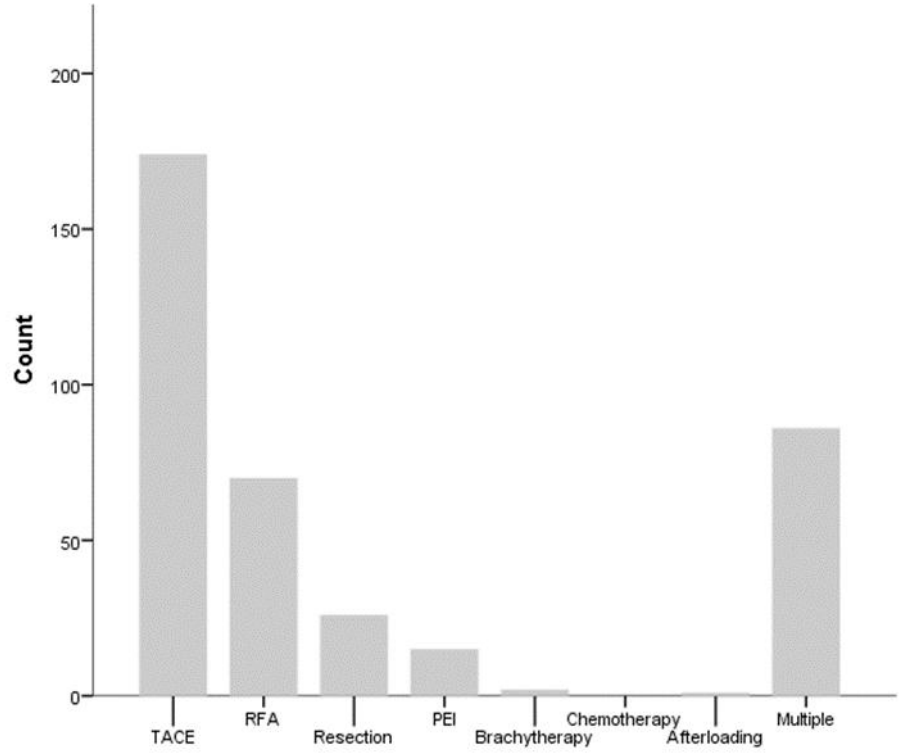

Figure 9 - Modalities of Bridging Therapies used in the Bridging Population.

5.1.3 Bridging Responses

As seen in the Figure 10, when grouped according to the response to bridging, 65% had a "Controlled Disease" while in 36% the tumor progressed when compared the CT imaging at time of listing with the histological finding. According to the ANOVA test there was no significant difference of age between the two groups *(p-value* = 0.80).

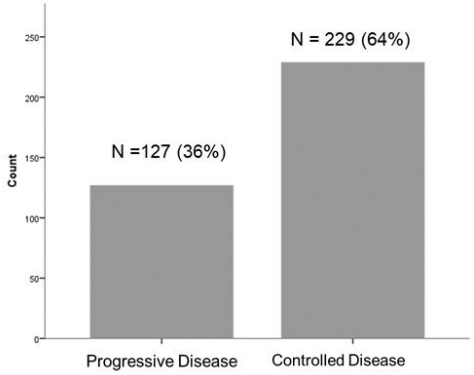

Figure 10 - Distribution of the "Bridging Population" according to the "Response to Bridging"

5.1.4 Milan Dynamics

As previously mentioned in chapter 5.1.1., some patients experienced a tumor size change during the bridging period that preceded LTX. This led to a shift in the Milan status of some patients and was referred to as "Milan Dynamics".

As further detailed in chapter 5.2.2., it became clear that patients that started with an “Inside Milan” classification at the time of listing and remained inside Milan until LTX, had a different prognosis than all the other patients. This group of patients was called “In-to-Inside Milan”. In this context, an analysis of this population was done according to the "Response to Bridging" . This group was divided in two, according to the "Response to Bridging" (Progressive vs. Controlled Disease).

The following graphic (Fig. 10) shows the distribution of patients according to their “Milan Dynamics”.

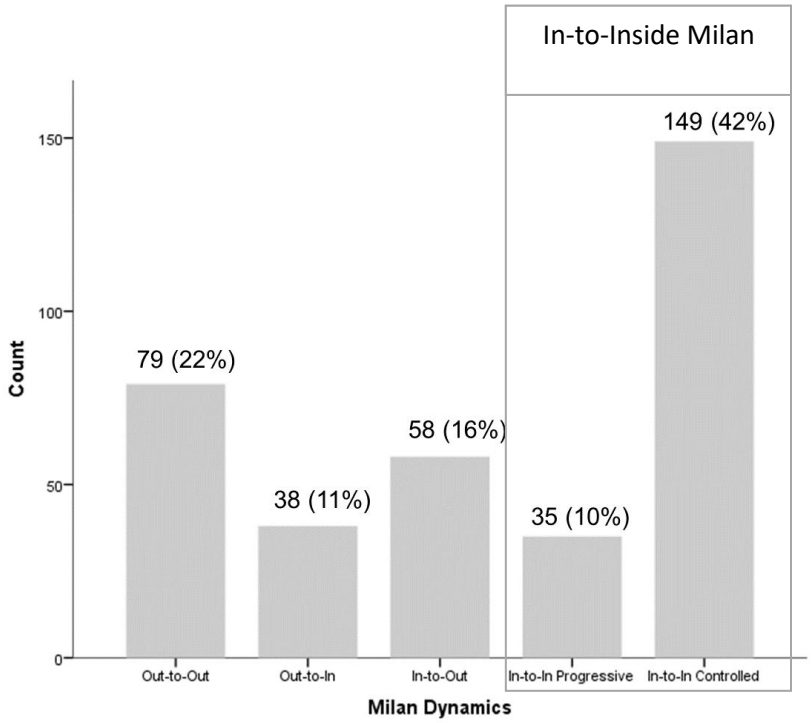

Figure 10 – Distribution of the Bridging Population according to the Milan Dynamics including the division of the Inside-to-Inside Milan Group concerning the "Response to Bridging" within.

Out-to-Out (Outside-to-Outside Milan); Out-to-In (Outside-to-Inside Milan); In-to-Out (Inside-to-Outside Milan); In-to-In Progressive (Inside-to-Inside Milan and Progressive Disease); In-to-In Controlled (Inside-to-Inside and Controlled Disease) - as defined in chapter 4.3.2.

According to the One-Way ANOVA test, the differences of ages of patients at the time of LTX was also not relevant *(p-value* = 0.40).

5.2 Disease-free Survival and Overall Survival

5.2.1 Bridging Response

When assessing the DFS and the OS between the two groups of bridging response there was a clear distinction between the prognosis both in the DSF and the OS, with a Log Rank p-value of respectively 0.05 and <0.01 (Fig. 11-12). This revealed that there is not a random association between the "Response to Bridging" and the prognosis of the patients after LTX. Patients whose HCC did not progress after bridging had a clear tendency to live longer and have a longer period without recurrence of HCC. This included both inside and outside Milan patients.

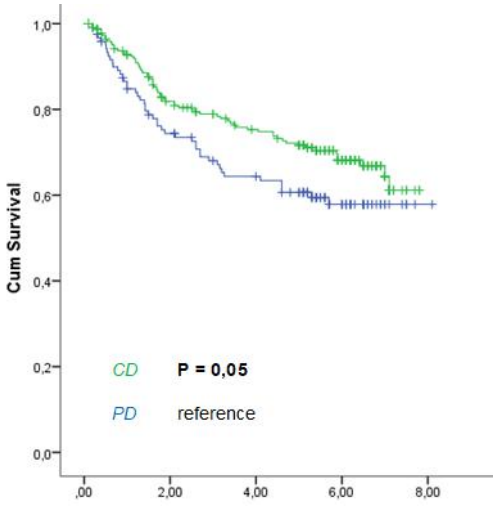

Figure 11 – Impact of "Response to Bridging" in the DFS.

Progressive Disease (PD) vs. Controlled Disease (CD). Kaplan-Meier Curve with log-rank p-value of 0.05. Progressive Disease as reference.

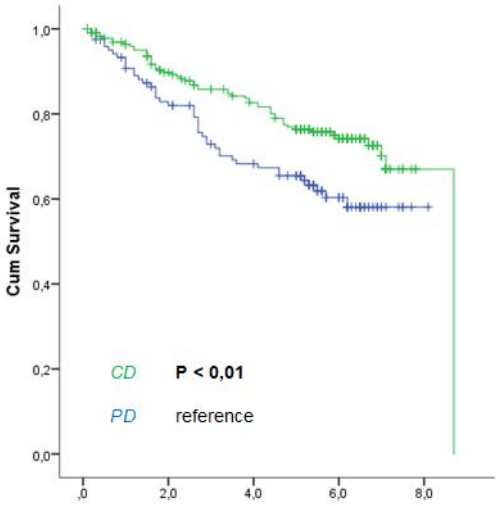

Figure 12 – Impact of "Response to Bridging" in the OS.

Progressive Disease (PD) vs. Controlled Disease (CD). Kaplan-Meier Curve with log-rank p-value <0.01. Progressive Disease as reference.

In order to understand to which extent “Bridging Response” played a role in DFS and OS, Chi-Square analysis were performed for both this parameters for the first five years.

Concerning the impact of the “Bridging Response” in the first five years, according to the Chi-Square test, there was a clear difference seen in the cumulative OS. There was a statistically relevant difference between the “Progressive Disease” and the “Controlled Disease” group in the first four years after LTX (p-value <0.05 - Table 6). When the cumulative DFS was compared, a relevant difference between these groups was seen only in the first year after LTX (*p-value* = 0.01 – Table 7).

Cumulative OS			
	Chi² (p-value)	Controlled Disease	Progressive Disease
1st Year	**0.01**	**96 %**	**85 %**
2nd Year	**0.03**	**92 %**	**78 %**
3th Year	**0.01**	**88 %**	**69 %**
4th Year	**0.03**	**86 %**	**69 %**
5th Year	0.15	81 %	69 %

Table 6 - Cumulative OS per Year in the first five years within the "Bridging Population". Impact of the "Response to Bridging" in the OS according to the Chi-Square Test.

Cumulative DFS			
	Chi² (p-value)	Controlled Disease	Progressive Disease
1st Year	**0.01**	**96 %**	**85 %**
2nd Year	0.28	86 %	78 %
3th Year	0.07	83 %	69 %
4th Year	0.11	82 %	69 %
5th Year	0.30	78 %	69 %

Table 7 - Cumulative DFS per Year in the first five years within the "Bridging Population" Impact of the "Response to Bridging" in the DFS according to the Chi-Square Test.

Interestingly, when both the "Progressive" and "Controlled" groups were analyzed through the Kaplan-Meier Curve according to their initial Milan status, a significant difference in the outcome after LTX was only seen in the patients Inside Milan. In the case of patients outside Milan, there was no significant difference in both DFS *(p-value* = 0.46) and OS *(p-value* = 0.75) – Fig. 13-16.

It is important to note, that the analysis of "Response to Bridging" in patients inside Milan at listing compares the patients that were initially Inside Milan and remained within this criteria without progression until LTX, versus patients that were initially Inside Milan but, because of the progression might have been outside Milan at the day of LTX and therefore were expected to have a worse prognosis. This being stated, it was not surprising that the patients that progressed (and were mostly outside Milan at LTX-day) had a worse prognosis.

Impact of "Response to Bridging" Inside Milan (at listing)

DFS | OS

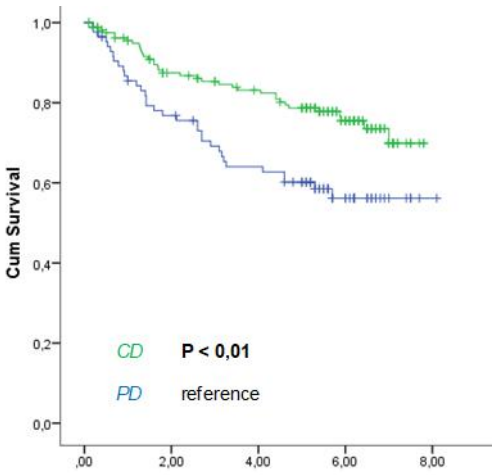

Figure 13 – Impact of the "Response to Bridging" in DFS within patients inside Milan at listing.

Progressive Disease (PD) vs. Controlled Disease (CD). Kaplan-Meier Curve with log-rank p-value <0.01. Progressive Disease as reference.

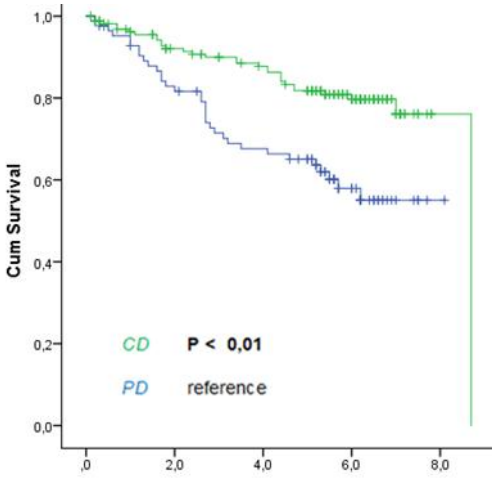

Figure 14 – Impact of the "Response to Bridging" in the OS within patients inside Milan at listing.

Progressive Disease (PD) vs. Controlled Disease (CD). Kaplan-Meier Curve with log-rank p-value <0.01. Progressive Disease as reference.

Impact of "Response to Bridging" Outside Milan (at listing)

DFS | OS

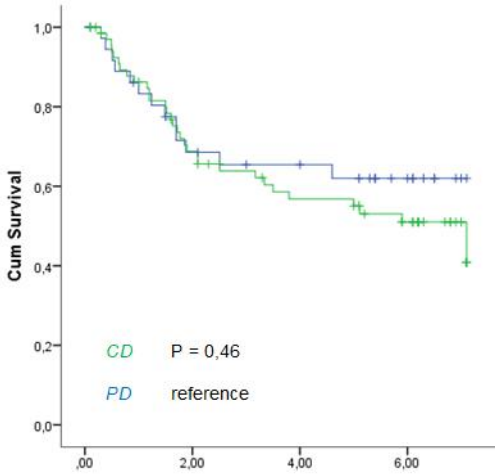

Figure 15 - Impact of the "Response to Bridging" in the DFS within patients outside Milan at listing.

Progressive Disease (PD) vs. Controlled Disease (CD). Kaplan-Meier Curve with log-rank p-value = 0.46. Progressive Disease as reference.

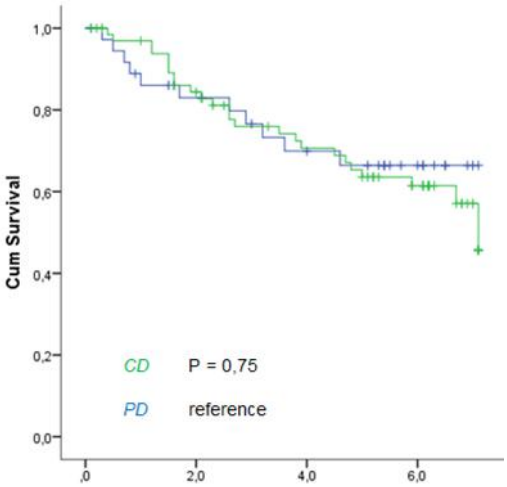

Figure 16 - Impact of the "Response to Bridging" in the OS within patients outside Milan at listing.

Progressive Disease (PD) vs. Controlled Disease (CD). Kaplan-Meier Curve with log-rank p-value = 0.75. Progressive Disease as reference.

Once these tests were again ran considering the histological Milan status, which reveals the actual status at the LTX day, no statistically relevant difference could be seen in the prognosis of the “Progressive Disease” group against the “Controlled Disease” group. This was seen both in patients inside Milan and outside Milan (Fig 17-20).

These results imply that the "Response to Bridging" would not be a reliable prognostic-tool for LTX. Here it is important to state that the histological “Inside Milan” group included patients that were always Inside Milan and those who downstaged during the Bridging Therapy. Although some investigators have defended that downstaged patients have similar prognosis to patients that always remained inside Milan, this topic is still controversial. For this reason, detailed research that took in consideration the Milan variation needed to be continued.

Impact of "Response to Bridging" Inside Milan (Histology)

DFS | OS

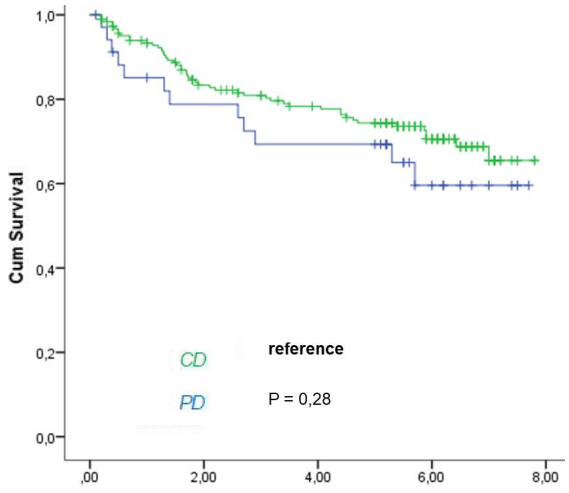

Figure 17 - Impact of the "Response to Bridging" in the DFS within patients inside Milan at LTX day (Histology) .

Progressive Disease (PD) vs. Controlled Disease (CD). Kaplan-Meier Curve with log-rank p-value = 0.28. Progressive Disease as reference.

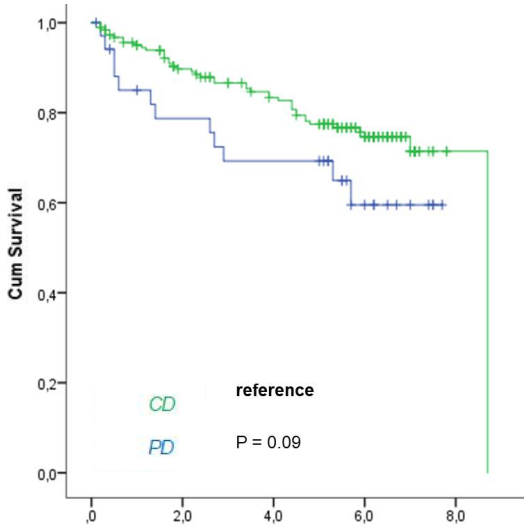

Figure 18 - Impact of the "Response to Bridging" in the OS within patients inside Milan at LTX day (Histology).

Progressive Disease (PD) vs. Controlled Disease (CD). Kaplan-Meier Curve with log-rank p-value = 0.09. Progressive Disease as reference.

Impact of "Response to Bridging" Outside Milan (Histology)

DFS | OS

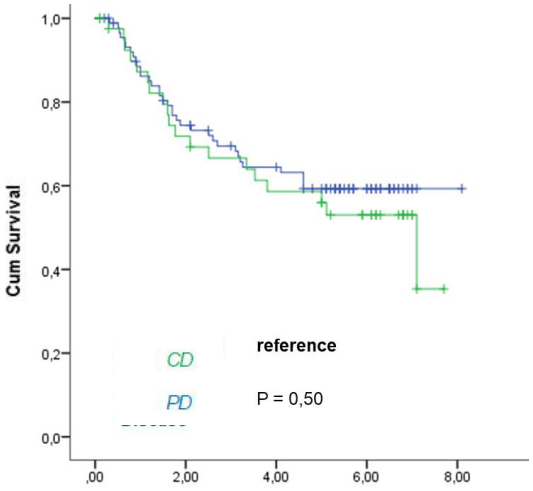

Figure 19 - Impact of the "Response to Bridging" in the DFS within patients outside Milan at LTX day (Histology) .

Progressive Disease (PD) vs. Controlled Disease (CD). Kaplan-Meier Curve with log-rank p-value = 0.50. Progressive Disease as reference.

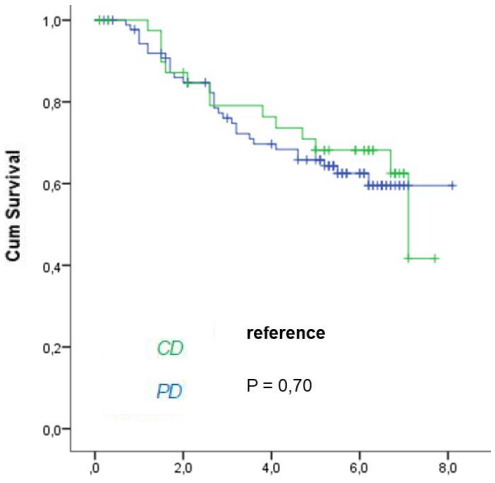

Figure 20 - Impact of the "Response to Bridging" in the OS within patients outside Milan at LTX day (Histology).

Progressive Disease (PD) vs. Controlled Disease (CD). Kaplan-Meier Curve with log-rank p-value = 0.70. Progressive Disease as reference.

5.2.2 Milan Dynamics

When comparing the initial Milan status at the time of listing (done by image assessment) with the Milan status resulting from the histological findings, we found out that 27% of the patients that received some kind of bridging therapy shifted their Milan status when compared to the histological specimen. More specifically: in 16% of the cases, patients were initially radiologically considered to be Inside MC and by the time of LTX were actually outside MC – suggesting a relevant progression during the waiting time - while the opposite happened to 11% of patients, that were outside Milan at the time of listing but showed a decrease in the total size of HCC lesions during the waiting time. This last group revealed a downstaging of the MC. A summary of these Milan variations can be seen in the Table 8.

Bridging Population	**Histology** Inside Milan	Outside Milan
Initial Inside Milan	51%	16%
Outside Milan	11%	21%
(missing = 14)	Downstaging	

Table 8 – Milan Dynamics. Variation of the Milan status at listing (initial) and at Transplantation-day (Histology) in the Bridging Population.

Based on Table 8 it was possible to create 4 groups that gathered patients with different Milan Dynamics. When analyzing the DFS and the OS of these groups through the Kaplan-Meier Curve and applying the Log Rank test, it was clear that two patterns could be distinguished according to prognosis (Fig.21-22): 1) patients that remained inside MC during the time of listing until LTX, and 2) all others. Interestingly, patients who regressed from outside Milan to Inside Milan during bridging, had a similar prognosis to patients that were always outside of Milan or that progressed from inside to outside Milan.

If patients were outside Milan at time of LTX, outcomes did not improve with downstaging (Tab. 9-10).

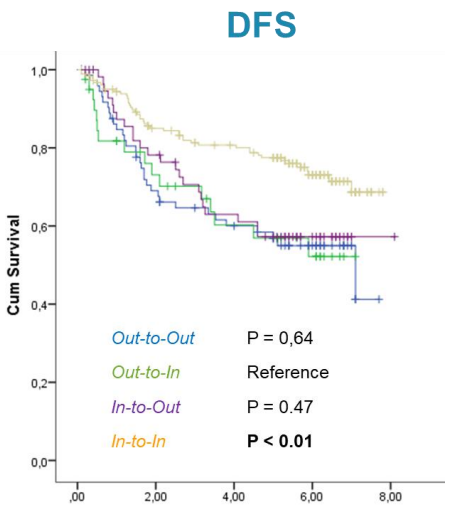

Figure 21 - Impact of the Milan Dynamic in the DFS.

Out-to-Out (Outside-to-Outside Milan); Out-to-In (Outside-to-Inside Milan); In-to-Out (Inside-to-Outside Milan); In-to-In (Inside-to-Inside Milan). Kaplan-Meier Curve with log-rank p-value values. Downstaging Group (Out-to-In) as reference.

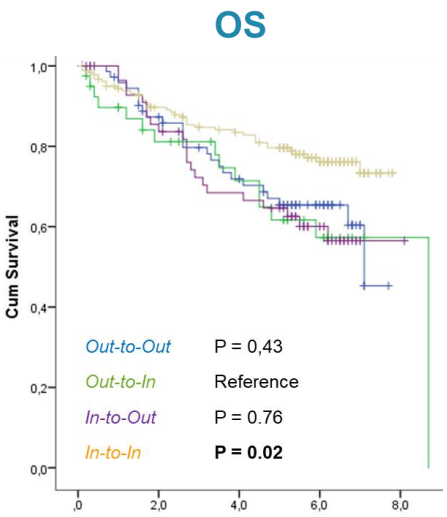

Figure 22 - Impact of the Milan Dynamic in the OS.

Out-to-Out (Outside-to-Outside Milan); Out-to-In (Outside-to-Inside Milan); In-to-Out (Inside-to-Outside Milan); In-to-In (Inside-to-Inside Milan). Kaplan-Meier Curve with log-rank p-value values. Downstaging Group (Out-to-In) as reference.

Cumulative OS

	Chi² (p-value)	Downstaging	Outside at LTx-day
1st Year	0.10	89 %	96 %
2nd Year	0.36	79 %	86 %
3th Year	0.82	78 %	76 %
4th Year	0.79	67 %	70 %
5th Year	0.52	58 %	64 %

Table 9 - Cumulative OS and Chi-Square Analysis with p-value of Downstaging Group (Out-to-In) vs. the patients that were Outside Milan at the day of LTX (Out-to-Out and In-to-Out).

Cumulative DFS

	Chi² (p-value)	Downstaging	Outside at LTx-day
1st Year	0.46	81 %	86 %
2nd Year	0.81	71 %	73 %
3th Year	0.96	66 %	66 %
4th Year	0.59	55 %	60 %
5th Year	0.71	52 %	55 %

Table 10 - Cumulative DFS and Chi-Square Analysis with p-value of Downstaging Group (Out-to-In) vs. the patients that were Outside Milan at the day of LTX (Out-to-Out and In-to-Out).

On the other hand, patients that permanently remained inside Milan revealed a different and much better prognosis than their counter-parts. According to the Chi Square test, included in Table 11 and 12, there is a clear difference in the prognosis between the patients that always remained inside Milan, and the patients that downstaged from outside to inside. The difference was statistically relevant in all the analyzed periods for the DFS (5-year DFS 76% vs. 52%, p-value <0.01) and starting at the 4th year for the OS (5-year OS 78% vs. 58%, p-value <0.01). These results were also confirmed visually and analytically through the Kaplan-Meier Curve and the log-rank p-values (Fig. 23-24). Unexpectedly, patients that downstaged from outside to inside Milan showed an alarming 57% 5-year OS Rate.

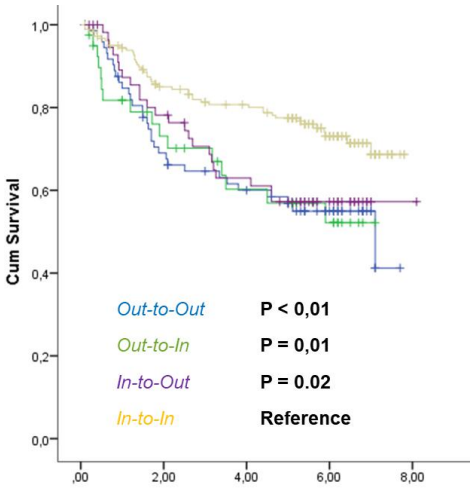

Figure 23 - Impact of the Milan Dynamic Group in the DFS within the Bridging population.

Out-to-Out (Outside-to-Outside Milan); Out-to-In (Outside-to-Inside Milan); In-to-Out (Inside-to-Outside Milan); In-to-In (Inside-to-Inside Milan). Kaplan-Meier Curve with log-rank p-value values. Always Inside Milan Group (In-to-In) as reference.

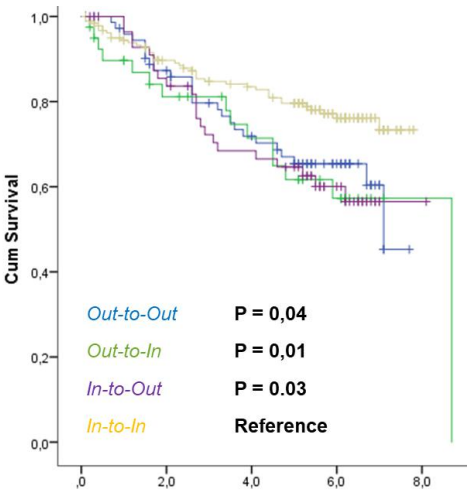

Figure 24 - Impact of the Milan Dynamic Group in the OS within the Bridging population.

Out-to-Out (Outside-to-Outside Milan); Out-to-In (Outside-to-Inside Milan); In-to-Out (Inside-to-Outside Milan); In-to-In (Inside-to-Inside Milan). Kaplan-Meier Curve with log-rank p-value values. Always Inside Milan Group (In-to-In) as reference.

	Cumulative OS		
	Chi² (p-value)	In-to-Inside (In-to-Inside)	Downstaging (Out-to-Inside)
1st Year	0.24	94%	89%
2nd Year	0.10	89%	79%
3th Year	0.37	84%	77%
4th Year	**0.05**	**82%**	**67%**
5th Year	**0.01**	**78%**	**57%**

Table 11 - Cumulative OS and Chi Square analysis with p-values between the group of patients always Inside Milan (In-to-Inside) and patients that downstaged (Out-to-Inside).

	Cumulative DFS		
	Chi^2 (p-value)	In-to-Inside	Downstaging
1st Year	**< 0.01**	**94%**	81 %
2nd Year	**0.04**	**84%**	71 %
3th Year	**0.05**	**80%**	66 %
4th Year	**< 0.01**	**79%**	55 %
5th Year	**< 0.01**	**76%**	52 %

Table 12 - Cumulative DFS and Chi-Square analysis with p-values between the group of patients always Inside Milan (In-to-Inside) and patients that downstaged (Out-to-Inside).

5.2.3 Combining “Milan Dynamics” and "Response to Bridging"

In an attempt to create an integrated model of distribution that could distinguish patients based on based on their prognosis, a series of analyses were performed in order to gather both parameters assessed until now in a single distribution.

First, it was analyzed whether the "Response to Bridging" also played a significant role in the patients according to their Milan Dynamic groups.

This analysis was performed for the patients that permanently remained in the same Milan category (“In-to-Inside” and “Out-to-Outside”). In the other two “Milan Dynamic” groups this analysis did not make sense since “Inside-to-Outside” patients had a progressive disease and “Out-to-Inside” had a controlled disease.

In the patients that remained inside Milan during the whole waiting-list time (In-to-Inside Milan), it was confirmed that the "Response to Bridging" had a clear impact in the DFS *(p-value* <0.01) and in the OS *(p-value* <0.01) – Fig 25 and 26. Once the cumulative DFS and OS *per* year was analyzed in the “In-to-Inside Milan” group according to the "Response to Bridging", it became clear that the "Response to Bridging" had a significant impact at least in the first year of DFS *(p-value* = 0.01) and in the first four years of the OS *(p-value* <0.03) – Table 13 and 14.

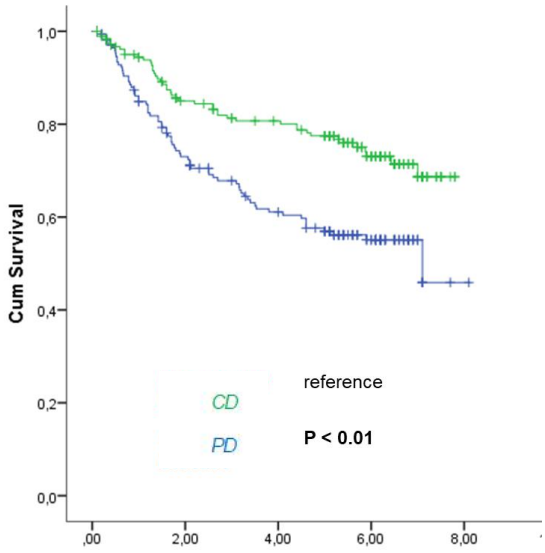

Figure 25 - Impact of the "Response to Bridging" in the DFS within the Inside-to-Inside Milan group.

“In-to-In Progressive Disease” vs. “In-to-In Controlled Disease”. Kaplan-Meier Curve with log-rank p-value <0.01. Progressive Disease as reference.

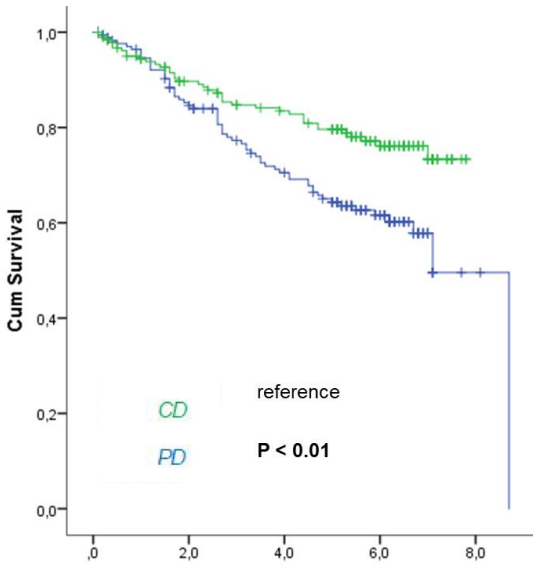

Figure 26 - Impact of the "Response to Bridging" in the OS within the Inside-to-Inside Milan group.

“In-to-In Progressive Disease” vs. “In-to-In Controlled Disease”. Kaplan-Meier Curve with log-rank p-value <0.01. Progressive Disease as reference.

Cumulative DFS
(In-to-Inside Milan)

	Chi² (p-value)	DFS In-to-In Controlled	DFS In-to-In Progressive
CR 1st Year	**0.01**	**96% (135)**	**85% (28)**
CR 2nd Year	0.28	86% (115)	79% (25)
CR 3th Year	0.08	83% (108)	70% (22)
CR 4th Year	0.13	82% (103)	70% (22)
CR 5th Year	0.35	78% (97)	70% (22)

Table 13 - Cumulative DFS in the first five years and Chi-Square Analysis with p-values between the group of patients always inside Milan whose HCC did not progress (In-to-Inside Controlled) and the patients always inside Milan whose HCC progressed (In-to-Inside Progressive).

Cumulative OS
(In-to-Inside Milan)

	Chi² (p-value)	OS In-to-In Controlled	OS In-to-In Progressive
CR 1st Year	**0.01**	**96% (135)**	**85% (28)**
CR 2nd Year	**0.03**	**92% (121)**	**79% (25)**
CR 3th Year	**0.01**	**88% (113)**	**70% (22)**
CR 4th Year	**0.03**	**86% (107)**	**70%(22)**
CR 5th Year	0.18	81% (100)	70% (22)

Table 14 - Cumulative OS in the first five years and Chi-Square Analysis with p-values between the group of patients always inside Milan whose HCC did not progress (In-to-Inside Controlled) and the patients always inside Milan whose HCC progressed (In-to-Inside Progressive).

In this context, it is also important to state that between these two groups ("In-to-In Controlled" vs. "In-to-In Progressive") there was no statistically relevant difference in the age *(p-value* = 0.83) or Time-on-waiting-list *(p-value* = 0.36).

When the same analysis was done for the group whose patients always remained outside Milan, no significant differences in the prognosis could be found (Fig 27 and 28). This seems to indicate, that the "Response to Bridging" can only indicate differences in the outcome of LTX if the patients always remained inside Milan.

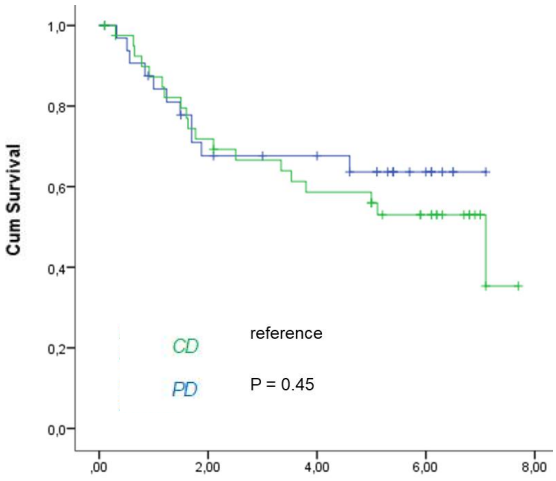

Figure 27 - Impact of the "Response to Bridging" in the DFS within the Out-to-Outside group.

Progressive Disease (PD) vs. Controlled Disease (CD). Kaplan-Meier Curve with log-rank p-value = 0.45. Progressive Disease as reference.

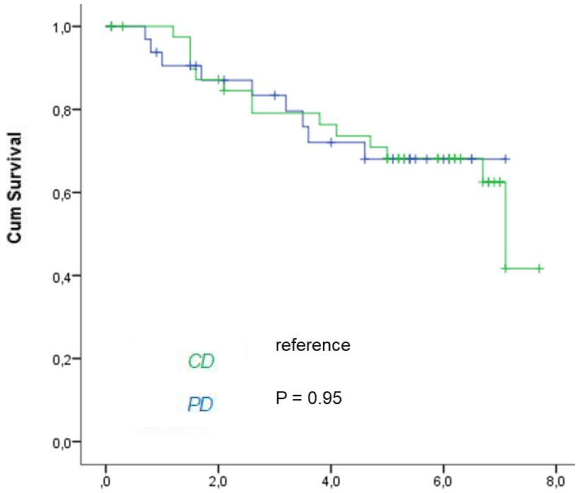

Figure 28 - Impact of the "Response to Bridging" in the OS within the Out-to-Outside group.

Progressive Disease (PD) vs. Controlled Disease (CD). Kaplan-Meier Curve with log-rank p-value = 0.95. Progressive Disease as reference.

Since there was a difference between the patients that remained inside Milan and showed a progressive or controlled disease, and this difference was not evident in patients outside Milan, one could divide the sampled patients into 5 Groups in a distribution methodology that includes both “Milan Dynamics” and "Response to Bridging". The following bar-chart shows the number of patients in each of the new groups formed (Fig 29), as already presented previously in the chapter “Grouping by Milan Dynamics”. Once again, the difference of age or time-on-waiting-list was not relevant between the groups.

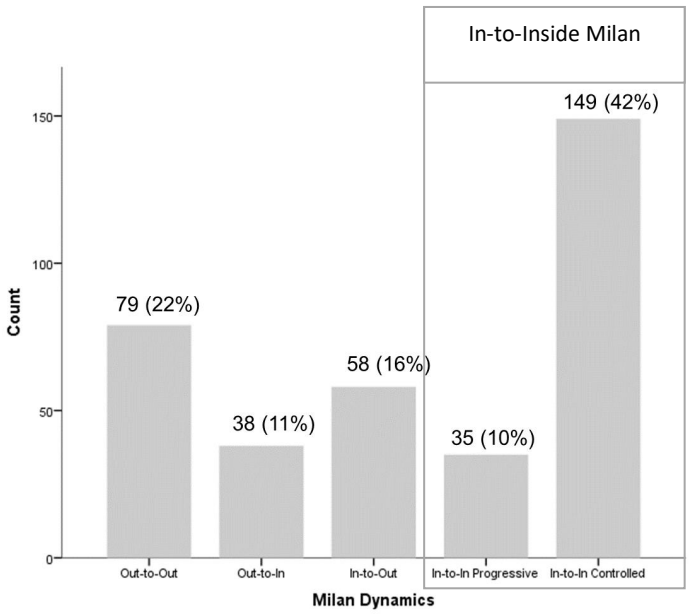

Figure 29 – Distribution of patients based in a 5-Group distribution that includes both “Milan Dynamic” and "Response to Bridging".

A Kaplan-Meier analysis was performed and showed that considering the DFS, there was a relevant difference in the “In-to-In Controlled” group compared to all other patients that were once outside Milan (Fig. 30). Considering the OS, the difference was even clearer and it was also relevant when compared to patients in the “In-to-In Progressive” group. Patients in the “In-to-In Controlled” group showed a better OS than all the other groups (Fig.31).

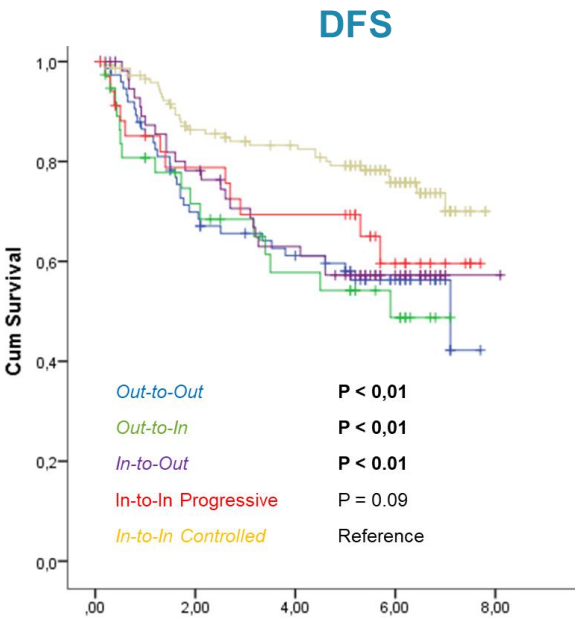

Figure 30 - Impact of the 5-Group Distribution on the DFS within the Bridging population.

Out-to-Out (Outside-to-Outside Milan); Out-to-In (Outside-to-Inside Milan); In-to-Out (Inside-to-Outside Milan); In-to-In Progressive (Inside-to-Inside Milan and Progressive Disease); In-to-In Controlled (Inside-to-Inside Milan and Controlled Disease). Kaplan-Meier Curve with log-rank p-values. Inside-to-Inside Controlled Disease as reference.

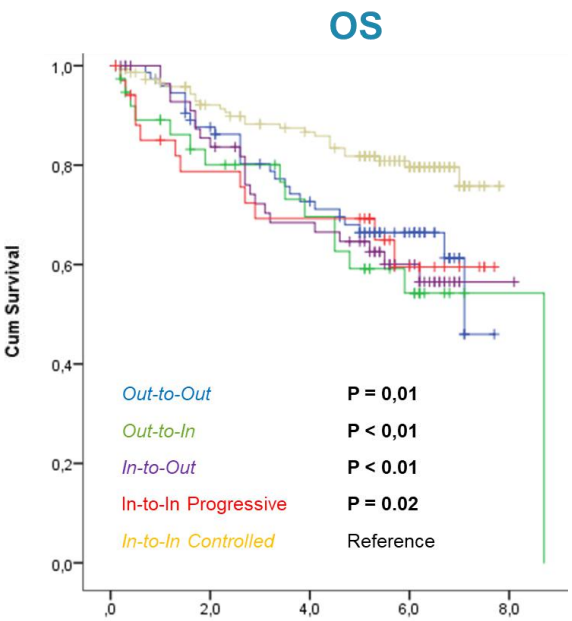

Figure 31 - Impact of the IMTuBio Distribution on the OS within the Bridging population.

Out-to-Out (Outside-to-Outside Milan); Out-to-In (Outside-to-Inside Milan); In-to-Out (Inside-to-Outside Milan); In-to-In Progressive (Inside-to-Inside Milan and Progressive Disease); In-to-In Controlled (Inside-to-Inside Milan and Controlled Disease). Kaplan-Meier Curve with log-rank p-values. Inside-to-Inside Controlled Disease as reference.

Not only was there a clear distinction between the prognosis of the "In-to-In Controlled" group and all others, there was a similar pattern in all the other groups, which included the "In-to-In Progressive" group. Interestingly, these results indicate that patients inside Milan with a progressive disease had a similar outcome in comparison to groups outside Milan. This can be seen in the Table 15, where the "In-to-In Progressive" group is taken as reference for the log-rank tests.

	DFS (p-value)	**OS (p-value)**
Out-to-Outside	0.49	0.76
Out-to-Inside	0.38	0.75
In-to-Outside	0.73	0.97
In-to-Inside Progressive	**Reference**	**Reference**
In-to-Inside Controlled	0.09	**0.02**

Table 15 – P-values of the Kaplan-Meier log-rank test revealing the impact of the "Response to Bridging" in the DFS and OS according to the 5-Group Distribution.

Out-to-Out (Outside-to-Outside Milan); Out-to-In (Outside-to-Inside Milan); In-to-Out (Inside-to-Outside Milan); In-to-In Progressive (Inside-to-Inside Milan and Progressive Disease); In-to-In Controlled (Inside-to-Inside Milan and Controlled Disease). Kaplan-Meier log-rank p-values.

Inside-to-Inside Progressive as reference value for p-values

This could also be demonstrated when the cumulative DFS and OS were compared between the In-to-In Progressive group and the group that gathers all the patients that were at some point outside Milan (Table 16 and 17). These tables show that no differences in the prognosis of this groups could be seen in the first 5 years after LTX, although there was a non-statistical tendency for better outcomes in the In-to-In progressive groups.

	Chi^2 (p-value)	DFS In-to-In Progressive	DFS Not In-to-In
CR 1st Year	0.94	85%	85%
CR 2nd Year	0.46	79%	73%
CR 3th Year	0.70	70%	66%
CR 4th Year	0.26	70%	59%
CR 5th Year	0.11	70%	55%

Table 16 - Cumulative DFS in the first five years and Chi-Square analysis with p-values between the DFS of patients that were always inside Milan whose HCC progressed (In-to-In Progressive) and the group that gathers all the patients that were at some point outside Milan (Not-In-to-In group).

	Cumulative OS		
	Chi^2 (p-value)	OS In-to-In Progressive	OS Not In-to-In
1st Year	0.06	85%	95%
2nd Year	0.44	79%	84%
3th Year	0.44	70%	76%
4th Year	0.96	70%	69%
5th Year	0.44	70%	63%

Table 17 - Cumulative OS in the first five years and Chi-Square analysis with p-values between the OS of patients that were always inside Milan whose HCC progressed (In-to-In Progressive) and the group that gathers all the patients that were at some point outside Milan (Not-In-to-In group).

5.2.4 Integrated Model of Tumor Biology Distribution (*IMTuBio Distribution*)

According to the results just described, the prognosis was analyzed taking into consideration a 2-Group analysis based on an original distribution created in this dissertation in order to form a distribution model that would gather groups of patients according to their prognosis revealed by the "Response to Bridging" and “Milan Dynamics” - the Integrated Model of Tumor Biology (IMTuBio). In practical terms, this distribution tool distinguishes the patients that were always inside Milan and showed no progression during the bridging time (“Inside IMTuBio”), from all the other groups of patients (“Outside IMTuBio”).

Fig. 32 and 33 show a clear difference in the prognosis of the patients that always remain inside Milan and had controlled disease during the bridging period, against the group that gathers all the other patients. The cumulative OS and DFS revealed a relevant difference in the DFS between the groups at all periods and after the first year for OS (Tables 18 and 19).

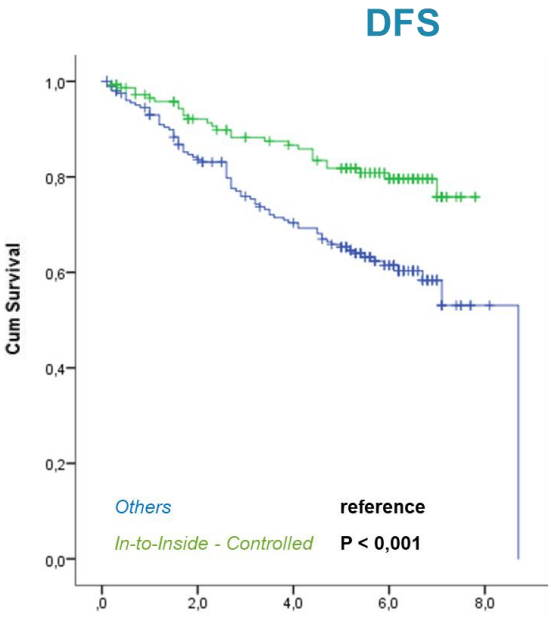

Figure 32 - Impact of the IMTuBio Distribution between Inside-to-Inside Controlled (Inside IMTuBio) and all the group that gather all the other patients (Others – Outside IMTuBio) in the DFS within the Bridging population.

“Others” represent all the patients that were once Outside Milan or whose HCC progressed. Kaplan-Meier Curve with log-rank p-value <0.001. “Others” as reference.

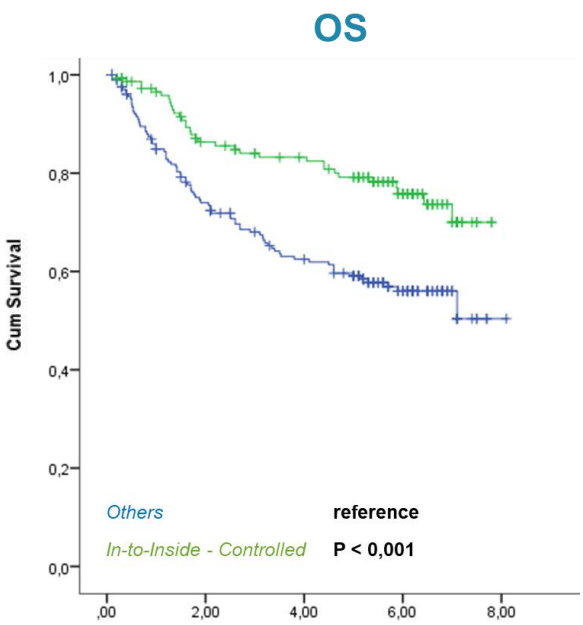

Figure 33 - Impact of the IMTuBio Distribution between Inside-to-Inside Controlled (Inside IMTuBio) and all the group that gather all the other patients (Others – Outside IMTuBio) in the OS within the Bridging population.

“Others” represent all the patients that were once Outside Milan or whose HCC progressed. Kaplan-Meier Curve with log-rank p-value <0.001. “Others” as reference.

Cumulative DFS			
	Chi^2 (p-value)	DFS Inside IMTuBio	DFS Outside IMTuBio
CR 1st Year	**< 0.01**	**96%**	**85%**
CR 2nd Year	**< 0.01**	**86%**	**73%**
CR 3th Year	**< 0.01**	**83%**	**67%**
CR 4th Year	**< 0.01**	**82%**	**61%**
CR 5th Year	**< 0.01**	**78%**	**57%**

Table 18 - Cumulative DFS in the first five years and Chi-Square analysis with p-values between the DFS of the patients inside and outside the IMTuBio criteria.

Cumulative OS			
	Chi^2 (p-value)	OS Inside IMTuBio	OS Outside IMTuBio
CR 1st Year	0.17	96%	93%
CR 2nd Year	**0.03**	**92%**	**83%**
CR 3th Year	**< 0.01**	**88%**	**75%**
CR 4th Year	**< 0.01**	**86%**	**69%**
CR 5th Year	**< 0.01**	**81%**	**64%**

Table 19 - Cumulative OS in the first five years and Chi-Square analysis with p-values between the OS of the patients inside and outside the IMTuBio criteria.

5.2.5 Influence of Sirolimus

As the population in the SiLVER Study was randomized according to the immunosuppression used, the results described above could only be fully validated if there was not a significant variation in the immunosuppression given, and if a significant difference in the prognosis within these groups (of "Response to Bridging" and “Milan Dynamics”) was not attributed to one specific study group (A vs. B).

The SiLVER Study was analyzed based on the *Intention-to-Treat* (ITT) approach. However, to make sure that the immunosuppression did not influence the results presented in this dissertation, a *Per-Protocol* (PP) analysis was also executed.

The data was analyzed through the Kaplan-Meier Curve with log-rank test. No significant difference in group A or B was obtained when the OS and DFS both where compared within the “Controlled Disease” and within the “Progressive Disease” groups of the ITT population for 8 years of the study – Fig. 34, 35, 36 and 37. The PP analysis showed similar results that once more indicated that immunosuppression likely did not affect long-term prognosis in these groups selected by their "Response to Bridging" for the 8-year period – Fig. 38, 39, 40 and 41.

Finally, a sub-analysis of the *Per-Protocol* grouping considering the Milan status was done. None of these tests revealed a difference in the long-term prognosis could attributed to the immunosuppression given – Fig. 42, 43, 44 and 45.

These results were important to validate that the long-term prognostic differences in the sampled patients were likely due to their tumor biology (in this context revealed by the tumor size variation during the bridging period) and not to immunosuppression factors after LTX.

INTENTION-TO-TREAT ANALYSIS - Group A vs. Group B

Disease-Free Survival

Progressive Disease | Controlled Disease

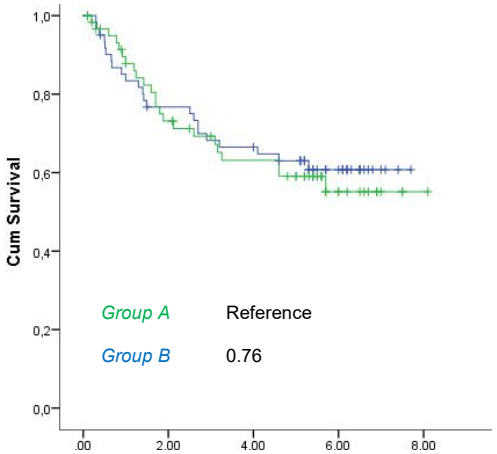

Figure 34 – Impact of the Immunosuppression in the DFS within the "Progressive Disease" group. Intention-to-Treat Analysis.

Group A (Calcineurin-inhibitor) vs. Group B (Sirolimus). Kaplan-Meier Curve with log-rank p-value = 0.76. Group A as reference.

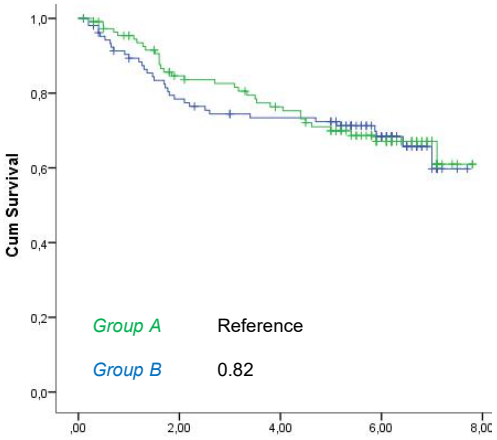

Figure 35 – Impact of the Immunosuppression in the DFS within the "Controlled Disease" group. Intention-to-Treat Analysis.

Group A (Calcineurin-inhibitor) vs. Group B (Sirolimus). Kaplan-Meier Curve with log-rank p-value = 0.82. Group A as reference.

Overall Survival

Progressive Disease | Controlled Disease

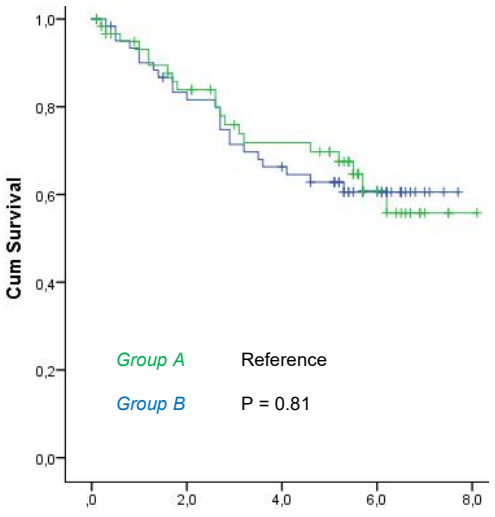

Figure 36 - Impact of the Immunosuppression in the OS within the "Progressive Disease" group. Intention-to-Treat Analysis.

Group A (Calcineurin-inhibitor) vs. Group B (Sirolimus). Kaplan-Meier Curve with log-rank p-value = 0.81. Group A as reference.

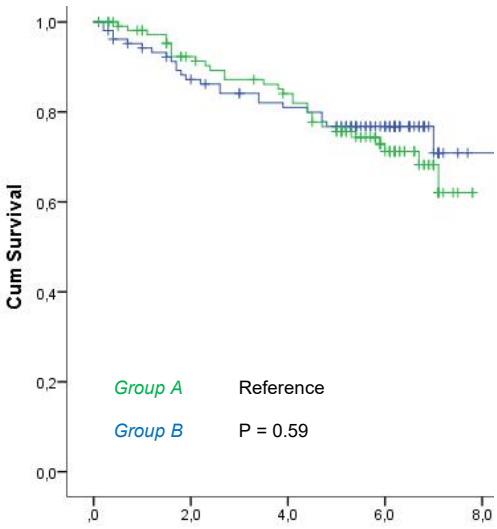

Figure 37 - Impact of the Immunosuppression in the OS within the "Controlled Disease" group. Intention-to-Treat Analysis.

Group A (Calcineurin-inhibitor) vs. Group B (Sirolimus). Kaplan-Meier Curve with log-rank p-value = 0.59. Group A as reference.

PER-PROTOCOL ANALYSIS – Group A vs. Group B

Overall Survival

Progressive Disease | Controlled Disease

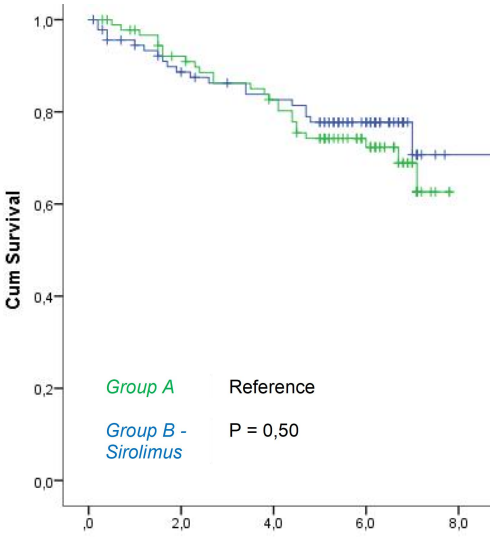

Figure 38 – Impact of the Immunosuppression in the OS within the "Progressive Disease" group. Per-Protocol Analysis.

Group A (Calcineurin-inhibitor) vs. Group B (Sirolimus). Kaplan-Meier Curve with log-rank p-value = 0.50. Group A as reference.

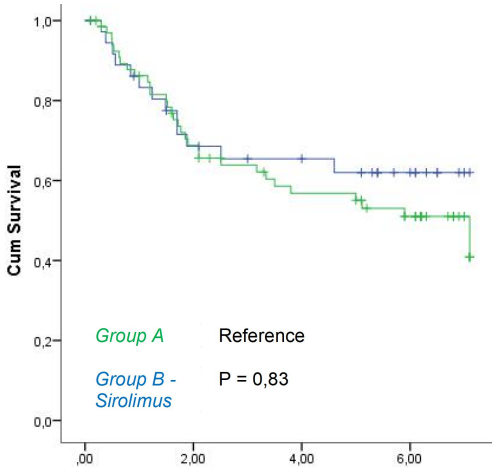

Figure 39 - Impact of the Immunosuppression in the OS within the "Controlled Disease" group. Per-Protocol Analysis.

Group A (Calcineurin-inhibitor) vs. Group B (Sirolimus). Kaplan-Meier Curve with log-rank p-value = 0.83. Group A as reference.

Disease-Free Survival

Progressive Disease | Controlled Disease

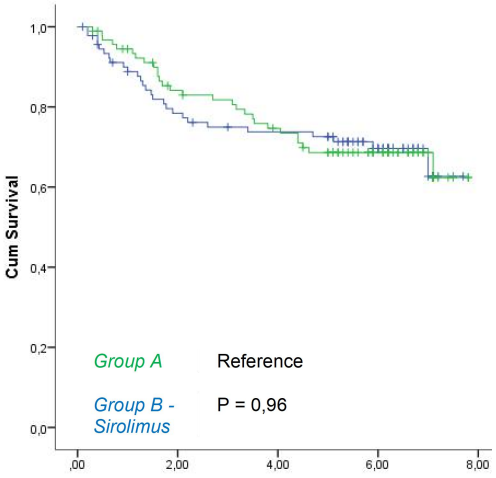

Figure 40 - Impact of the Immunosuppression in the DFS within the "Progressive Disease" group. Per-Protocol Analysis.

Group A (Calcineurin-inhibitor) vs. Group B (Sirolimus). Kaplan-Meier Curve with log-rank p-value = 0.96. Group A as reference.

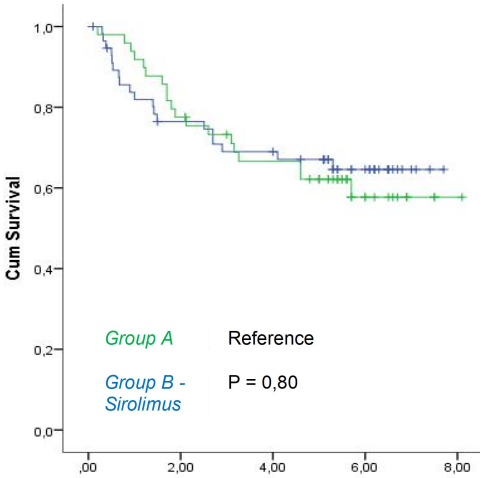

Figure 41 - Impact of the Immunosuppression in the DFS within the "Controlled Disease" group. Per-Protocol Analysis.

Group A (Calcineurin-inhibitor) vs. Group B (Sirolimus). Kaplan-Meier Curve with log-rank p-value = 0.80. Group A as reference.

PER-PROTOCOL ANALYSIS *PER MILAN STATUS*

Overall Survival – Group A vs. Group B

Per-Protocol

Outside Milan

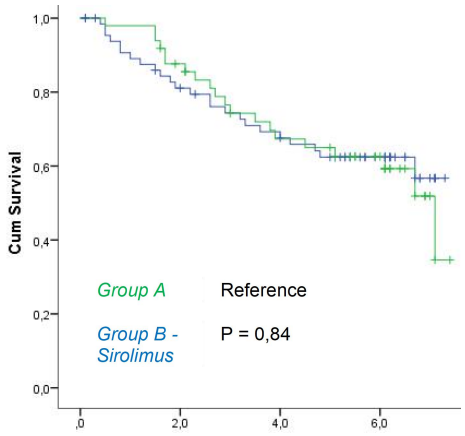

Figure 42 - Influence of the Immunosuppression in the OS within Outside Milan. Per-Protocol Analysis.

Group A (Calcineurin-inhibitor) vs. Group B (Sirolimus). Kaplan-Meier Curve with log-rank p-value = 0.84. Group A as reference.

Inside Milan

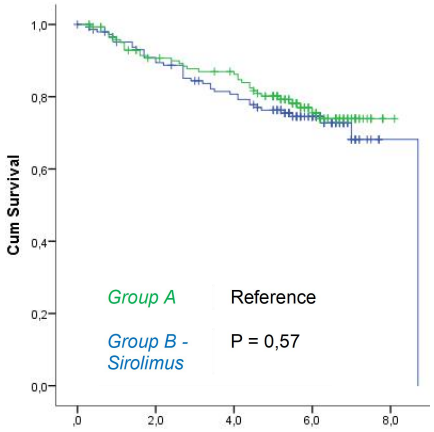

Figure 43 – Influence of the Immunosuppression in the OS within Inside Milan. Per-Protocol Analysis.

Group A (Calcineurin-inhibitor) vs. Group B (Sirolimus). Kaplan-Meier Curve with log-rank p-value = 0.84. Group A as reference.

Disease-Free Survival – Group A vs. Group B

Per-Protocol

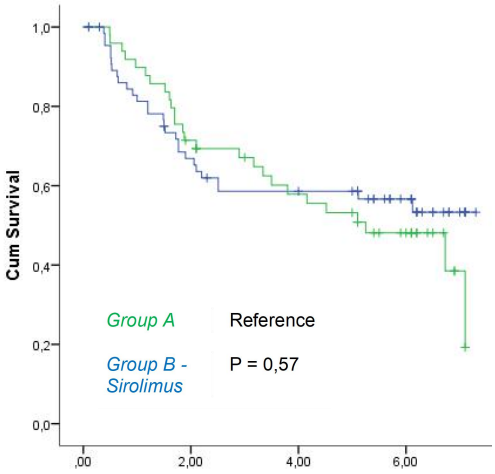

Figure 44 - Influence of the Immunosuppression in the DFS within Outside Milan. Per-Protocol Analysis.

Group A (Calcineurin-inhibitor) vs. Group B (Sirolimus). Kaplan-Meier Curve with log-rank p-value = 0.57. Group A as reference.

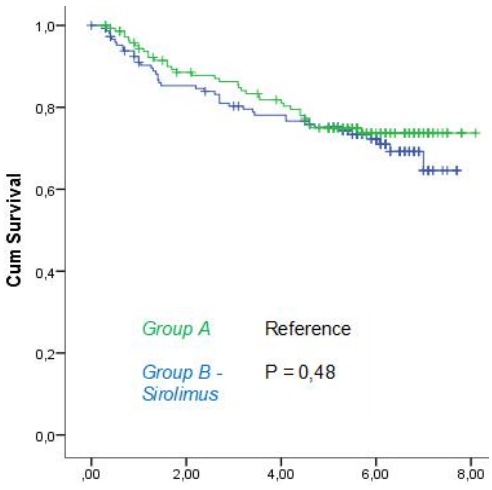

Figure 45 - Influence of the Immunosuppression in the DFS within Inside Milan. Per-Protocol Analysis.

Group A (Calcineurin-inhibitor) vs. Group B (Sirolimus). Kaplan-Meier Curve with log-rank p-value = 0.48. Group A as reference.

6 DISCUSSION

6.1 Introduction

This retrospective cohort study was based on the SiLVER database of patients with HCC that hada LTX and were followed-up for a minimum of 5 years. The general goal was to find-out possible criteria that would predict tumor biology and behaviour, and in this way be able to predict which patients would benefit the most from the LTX. In a more specific manner, it was analyzed whether changes in tumor size and number of lesions, as a "Response to Bridging" during the waiting-list time, were associated with the prognosis after LTX. This was measured by the differences in the DFS and OS in the groups created and compared. In this sense, only patients with an available MC, at time of listing and at LTX-day (through histology), and that received some kind of bridging therapy were included in this analysis. This was called the “Bridging-Population” - a sub-population within the SiLVER Study population.

6.2 "Response to Bridging" as a Prognostic Factor

During the waiting time prior to LTX, the majority (67%) of the patients in the SiLVER Study received some kind of bridging therapy. These therapies are generally indicated to avoid growth/progression of the tumor, or diminish the tumor size to bring the patient within MC..

It was not the goal of my dissertation to analyze the effect of locoregional bridging therapies (LRTs) on the prognosis. Since it was not assessed in the SiLVER Study which criteria were used by the hospitals to choose between LRTs, a prognostic comparison could not be done. However, it is important to state that it has been reported that LRTs per se do not improve the prognosis after LTX. A study published in the Annals of Surgery in 2017[84] compared 747 LTX-recipients not receiving LRT with 2854 receiving LRT and showed that both groups had a similar 1, 3, and 5-year RFS (89%, 77%, 68% vs 85%, 75%, 68%; p-value = 0.49).

Similar results were found by Decaens et al.[85] between 1985 and 1998 when 200 patients that received LTX were divided in two equal groups with and without LRT (in this case TACE) before LTX. The authors concluded that both groups had a similar 5-year OS (59.4% vs 59.3%; p-value >0.05).

Although not focusing on the effects of LRTs, it was of our great interest to understand if the response showed in tumor size after bridging (or in short: the "Response to Bridging") could reveal

[84] Agopian VG 2017
[85] Decaens T 2005

how aggressive the tumor biology of each patient was. In other words, can response to bridging predict the aggressiveness of an underlying tumor biology in LTX patients?

Patients were divided into two groups according to their "Response to Bridging": progressive disease (PD) or controlled disease (CD - which included both the stable disease, the partial remission and complete remission of HCC). It then became clear that patients in the controlled disease group, whose HCC did not progress during the waiting time, had a better OS within the first four years after LTX than the PD-Group (4-year OS - 86% vs. 69%, *p-value* = 0.03). After year 4, no statistically relevant difference was achieved. A clear log-rank p-value of 0.01 was achieved in the Kaplan-Meier Curve that revealed a significant difference between the prognosis of these two groups of "Response to Bridging".

Considering the DFS, although the Kaplan-Meier Curve and log-rank p-value also showed a relevant difference between the two groups (*p-value* = 0.05), this difference was only noticed in the first year after LTX, when the DFS was statistically different (1-year DFS 96% vs 85%, *p-value* = 0.01%). In the following years, no significant p-value was achieved for the DFS.

These data indicate that the "Response to Bridging" plays a role in predicting the prognosis of LTX.

The idea that "Response to Bridging" can predict outcome in HCC LTX is not new and has recently gained more support. In 2017 *Agopian*[86] and his collaborators published a North American retrospective study that included 3600 patients over 11 years in 11 USA Hospitals, which concluded that although bridging *per se* did not change the prognosis of the patients, the degree of "Response to Bridging" could accurately predict the outcome after LTX. Treated patients achieving complete pathologic response (cPR) had superior 5-year RFS (72%) and lower post-LTX recurrence (HR 0.52, *p-value* <0.001) compared with both untreated patients (69%; *p-value* = 0.010; HR 1.0) and treated patients not achieving cPR (67%; *p-value* = 0.039; HR 1.31).

Also considering RFS, *Otto et al*[87] published in 2013 a prospective study that gathered 132 patients from the University Medical Centre in Mainz (Germany), who suffered from HCC with cirrhosis and had a LTX after at least 2 cycles of TACE. The group that revealed progressive disease before LTX had a worse 5-year RFS than the controlled disease group (22% vs 88%, *p-value* <0.001). According to Otto, the "Response to Bridging" could accurately predict the RFS rate with an Area Under the Curve (AUC) of 0.86.

[86] Agopian VG 2017
[87] Otto G 2013

Our studies are consistent with the idea that "Response to Bridging" could be an accurate outcome predictor for LTX.

However, in our analysis when the "Response to Bridging" evaluation was done dividing the patients by their Milan status, we noticed that it only played a role in the patients that were inside Milan at the time of listing (*p-value* <0.01 for both OS and DFS). No relevance could be seen when applied to patients Outside Milan (*p-value* >0.05), or when patients were divided according to their Milan status at the date of LTX.

Although at first intriguing, the fact that "Response to Bridging" did not have the same relevance considering different Milan status was also a matter of publication by *Millonig et al*[88] in *Liver Transplantation 2007*. In a study that gathered 116 patients that received TACE before LTX, the authors reported that patients with complete (CR) or partial response (PR) had a significantly different 5-year OS rates (of 85.1% and 63.9% respectively) compared to the patients whose disease did not respond to therapy (OS 51.4%) – *p-value* <0.001. In subgroup analysis, however, we showed that these benefits were only seen in patients inside MC, but not outside MC.

The difference in the predictivity of the "Response to Bridging" whether inside vs. outside and at listing vs. at LTX day could not be fully understood at first and led us to a deeper analysis of the evolution of the tumor size and number during the waiting time. In a more particular way, the Milan variation ("Milan Dynamics") were then the focus of our investigation.

[88] Millonig G 2007

6.3 Importance of "Milan Dynamics"

The SiLVER Study was an international trial that studied transplanted patients suffering from HCC independently of the Milan status. This means, that the Study database included transplanted patients that were both inside and outside MC. The international LTX regulations world-wide are not uniform but it is common to state as a pre-requisite for LTX that patients ought to be within the MC. The MC were recently introduced in the LTX selection process to guarantee a minimum 5-year OS after LTX superior to 50%-60%. This means, that in most countries, if the tumor size and number of lesions exceed the MC, the patient will no longer be eligible for the LTX due to a worse prognosis. Once the decision of inclusion in the waiting-list is made based on a static Milan evaluation and not on the tumor size dynamics, tumor size changes during LTX waiting period are not considered.

In our study, we found that tumor size variation is relevant and acknowledge that 27% of the patients changed their Milan status during the LTX waiting time. Eleven percent of the patients started outside Milan but downstaged to inside Milan after bridging, while 16% showed HCC progression to a degree that they were finally outside MC (In-to-Out). Because of these changes, it has been previously stated by many authors that the Milan status at the time of listing does not accurately predict prognosis; instead, the Milan status at the time of LTX should be taken into consideration. This was for instance reported by *Otto el al*[89] in the study just described above that prospectively gathered 132 patients with HCC and cirrhosis that received LTX after LRTs. The authors realized that MC assessed at the time of listing did not correlate to them RFS *(p-value* = 0.58). On the other hand, when Milan was applied on the day of LTX (through MRT/CT-scan), it was highly relevant in predicting RFS *(p-value* <0.001). The authors concluded that the tumor dynamics in the waiting time cannot be considered as irrelevant, and that Milan at the time of listing is obsolete and a permanent actualization of the patients´ Milan status should be performed in order to have an accurate prognosis.

Although Otto´s study supports our results that tumor dynamics are not irrelevant for prognosis, it did not fully apply to our findings. The results of our study showed that both the MC at the time of listing and at the time of LTX were inaccurate to predict DFS and OS, mostly because the prognosis varied not only due to the Milan status, but also due to the tumor size change over time. In other words, an assessment of the tumor size variation was crucial for the prognosis .

In particular, our data revealed that being inside MC at the day of LTX did not necessarily translate to a better prognosis versus being outside MC. For instance, according to the Kaplan-Meier Curve, there was no significant difference in the prognosis (OS and DFS) of patients that downstaged from outside

[89] Otto G 2013

to inside Milan (Out-to-In) when compared to the patients that were always outside Milan (Out-to-Out) – OS p-value = 0.64 and DFS p-value = 0.43. The same was observed when downstaged patients were compared to those whose tumor started inside Milan, progressed and exceeded the MC at the day of LTX (In-to-Out) - OS p-value = 0.47 and DFS p-value = 0.76.

Similarly, the group of 40 patients that downstaged (Out-to-Inside) did not have a better outcome than patients that upstaged or remained outside Milan. This could be seen not only in the Kaplan-Meier Curve, as described above, but also in the cumulative OS and DFS. When comparing the downstaged group (Out-to-In) with the patients that were outside Milan at LTX-day (Out-to-Out and In-to-Out), there was no relevant difference compared to OS (OS 5-year 58% vs 64%, *p-value* = 0.52) or to DFS within the first five years (DFS 5-year 52% vs 55%, *p-value* = 0.71).

However, there was a clear difference in the prognosis between the patients that always remained inside Milan (In-to-Inside), versus the patients that downstaged from outside to inside Milan (Out-to-Inside). The difference was statistically relevant in all the analyzed periods for the DFS (5-year DFS 76% vs. 52%, *p-value* <0.01) and starting in the 4th year for the OS (5-year OS 78% vs. 58%, *p-value* = 0.01). Unexpectedly, while "In-to-Inside Milan" patients revealed 78% 5-year OS, patients that downstaged from outside to inside Milan (Out-to-Inside Milan) showed an alarming 58% 5-year OS rate, which is under the 60% 5-year OS mark reported by many authors as the minimum acceptable rate for LTX (*Clavien PA et al. 2011*).

These results took us one step further than *Otto et al*, as they reveal that not only an actual Milan status at LTX is essential for accurate prognostic value, but also that change in the tumor size is key in the patients inside Milan. Only a permanent inside Milan status, from listing until LTX, reveals the expected 5-year OS superior to the 60% minimum accepted level. Milan Dynamics were therefore a critical factor.

Although these results seemed to provide relevant evidence against LTX after downstaging, opposite results have been reported in the few available publications over the last ten years. *Yao et al*.[90] reported, in *Hepatology 2008*, a 92.1% 4-year OS after LTX in 30 patients that downstaged to inside Milan. In the explants of the initial group of 35 patients in which downstaging after LRT was achieved, 13 had complete tumor necrosis, 17 met the MC, and five were actually outside Milan (although considered to be inside Milan by imaging at LTX-day). *Yao et al.* concluded that if downstaging was achieved, it was associated with an excellent prognosis after LTX. This retrospective study led to an increasing interest in including downstaged patients in the waiting-lists for LTX and was, until February 2018, cited by at least 95 PubMed articles.

[90] Yao FY 2008

A systematic review on this subject, published on the *British Journal of Surgery* in 2011[91], found eight studies and included a total of 720 patients who underwent LTX following downstaging after initial presentation outside Milan. The rate of successful downstaging varied from 24 - 69% of patients with reported OS rates ranging from 55 - 94% at 5-years. The authors concluded that successful downstaging of HCC to within the MC is feasible in a proportion of patients and that OS and DFS rates in patients transplanted following downstaging are comparable to those in patients within the MC.

Yao and his collaborators published in this sequence a prospective study in *Hepatology 2015*[92] that reported the outcome of 64 patients after downstaging. The 5-year OS of this downstaging group was 77.8% vs 81% in the always inside Milan group (*p-value* = 0.69), while RFS was 90.8% vs 88% (*p-value* = 0.66).

More recently, *Kim et al*[93] published in October 2017 a single-center report in which 21 patients were downstaged to within MC with an alpha-fetoprotein <400 ng/mL before LTX. The authors reported that recurrence of HCC was higher but acceptable between downstaged and traditional candidates (4.8% vs 1.9%; *p-value* >0.05) at a median follow-up period of 17 months. It was also reported that downstaged candidates had a similar OS compared with those transplanted within MC (9.5% vs 1.9%; *p-value* >0.05) for this period of one and one-half years.

Finally, the larger sample study on this matter was published a month later by *Metha et al.*[94] in the *Clin Gastroenterol Hepatol* in November 2017 which also showed results in favor of LTX after downstaging. This was a retrospective multi-center study done at three liver transplant centers in California (USA Region 5), from 2002 through 2012 and gathered data from 187 adults with HCC enrolled in the downstaging protocol. All patients underwent abdominal imaging 1 month after each LRT, and at a minimum of once every 3 months. LTX was performed after successful downstaging in 109 patients (58%). For these 109 patients, *Metha et al.* reported an 80% 5-year OS and 87% RFS and concluded that this downstaging protocol should be applied on a broader scale. As a consequence, the United Network for Organ Sharing (UNOS) has approved the ”Region 5 Downstaging Protocol” used in this study for receiving automatic Model for End-Stage Liver Disease (MELD) exception listing. This protocol states that candidates are eligible for inclusion in downstaging if they have: 1 lesion >5 cm and ≤8 cm; 2 to 3 lesions each <5 cm, with a total diameter of all lesions ≤8 cm; or 4 to 5 lesions each <3 cm, with a total diameter of all lesions ≤8 cm.

[91] Gordon-Weeks 2011
[92] Yao FY 2015
[93] Kim Y 2017
[94] Mehta N 2017

Although in contrast to the recent publications of Yao, Kim and Mehta, the 57% OS rate showed in our downstaging group of 40 patients might not be an outlier result, since a *BJS* review of 2011 (*Gordon-Weeks et al. 2011*) indicated that OS in downstaging patients could vary between 55 - 94%. Moreover, although most of the studies found on the subject defended downstaging as a way to provide equivalent prognosis after LTX, one study published in 2003 by *Graziadei et al*[95] in *Liver Transplantation* came to the opposite conclusion. This study compared 48 patients with a permanent inside Milan status against a downstaging group of 15 patients concluded that the OS of the downstaging group was significantly less *(p-value* <0.001) with a 5-year survival rate of 41% in the downstaging group versus 93.8% in the selection group (always inside Milan). Considering the lack of data on this topic, and based on our own results, we share the opinion that an equivalent survival between downstaging patients (Out-to-Inside) and patients that constantly remain inside Milan (In-to-Inside) should not be taken for granted and more studies are needed to either confirm or refute this hypothesis.

[95] Graziadei IW 2003

6.4 Combining "Response to Bridging" and “Milan Dynamics”

Taking into consideration that "Response to Bridging" and Milan Dynamics appear to play a role in predicting the LTX outcome in our analysis, a further analysis was done to observe the impact of the "Response to Bridging" in the different Milan Dynamics Groups.

6.4.1 "Response to Bridging" within the Out-to-Outside and In-to-Inside Groups

Our results showed, that the "Response to Bridging" could not predict a difference in the outcome of patients that remained Outside Milan (Out-to-Out) – Kaplan-Meier *p-value* = 0.45 for OS and *p-value* = 0.95 for DFS.

However, in the case of patients that persistently remained inside Milan (In-to-Inside Milan) the "Response to Bridging" had a direct association with the OS and DFS. Patients that remained inside Milan until LTX without revealing any progression (In-to-In Controlled Disease Group), had a clearly better prognosis than their counterparts (In-to-In Progressive Disease Group) – Kaplan-Meier log-rank *p-value* <0.01 for OS; log-rank *p-value* <0.01 for DFS.

Once the cumulative OS and DFS per year was analyzed, it was demonstrated that within this group of patients that remained inside Milan, patients with controlled disease had a significantly better prognosis concerning the OS in the first 4-years (4-year OS 86% vs 70%, *p-value* = 0.03) and in the DFS in the first year after LTX (1-year DFS 96% vs 85%, *p-value* = 0.01). After these periods of time, a *p-value* <0.05 was not achieved. The 5-year OS was of 81% for the controlled-disease group and 70% for the progressive-disease group (*p*-value= 0.18). The 5-year DFS was 78% vs 70% respectively (*p-value* = 0.30).

In short, a favourable “Response to Bridging” predicted a better prognosis only within patients that remained inside MC.

6.4.2 “In-to-Inside Progressive” vs. “Outside Milan”

Also relevant were the results comparing the “In-to-Inside Progressive” group and the groups that were at some point outside Milan. According to the Kaplan-Meier curve, no significant difference could be found in the prognosis between this group and the group of patients that were always outside Milan (Kaplan-Meier log-rank *p-value* = 0.76 for the OS, and *p-value* = 0.49 for the DFS). The same was observed when comparing this “In-to-In Progressive” group to the “Out-to-In”, and to the “In-to-Out” groups. When the cumulative OS and DFS was analyzed, no relevant difference could be

found between the “In-to-In Progressive” group and the “Not-In-to-In” group (which gathered all the patients that were at some point outside Milan). This was true for the first 5 years analyzed.

Considering this data, one can say that the "Response to Bridging" is not only a useful tool to distinguish the patients that were always inside Milan, which would most probably have a longer OS and DFS, but even more importantly, to identify the patients inside Milan that might not have a better prognosis than patients excluded from LTX because of their outside Milan situation.

6.4.3 IMTuBio Distribution – Integrated Model of Tumor Biology Distribution

Acknowledging the importance of “Milan Dynamics” and of "Response to Bridging" in the prognosis, we finally presented an original division of the whole “Bridging Population” within two groups according to their prognosis - a distribution according to an Integrated Model of Tumor Biology (IMTuBio).

When applying the selection according to the IMTuBio, a clear Kaplan-Meier Curve revealed the difference in the prognosis of the "Inside IMTuBio" group versus the "Outside IMTuBio" group (OS and DFS with *p-value* <0.001). The cumulative analysis also showed a clear difference in the 5-year OS (81% vs. 64%, *p-value* <0.001) and DFS (78% vs 57%, *p-value* <0.001). It became clear that this selection based on the tumor dynamics was able to identify the tumors with most favourable biology.

6.4.4 Lack of Literature on the Subject - A Research Opportunity

While reviewing the literature no similar study could be found that joined the assessment of “Milan Dynamics” and "Response to Bridging".

An integrated approach of these two parameters was touched upon by Mazzaferro in a recent opinion paper where the author shared his vision of the future Liver Allocation System. *Mazzaferro et al*[96] proposed in 2016 that in patients inside Milan, bridging therapies should be introduced routinely to all patients in the waiting list, restaged and re-prioritized according to their "Response to Bridging" (progression within MC, “Partial Response” or “Complete Response”) (Fig.40 – A). Considering patients outside Milan, Mazzaferro proposed that a downstaging should be tried through LRTs, and in the cases of patients that achieve the MC, their "Response to Bridging" should be taken into consideration for the prioritization (dividing the patients in “Partial Response” and “Complete Response”) (Fig.40 – B).

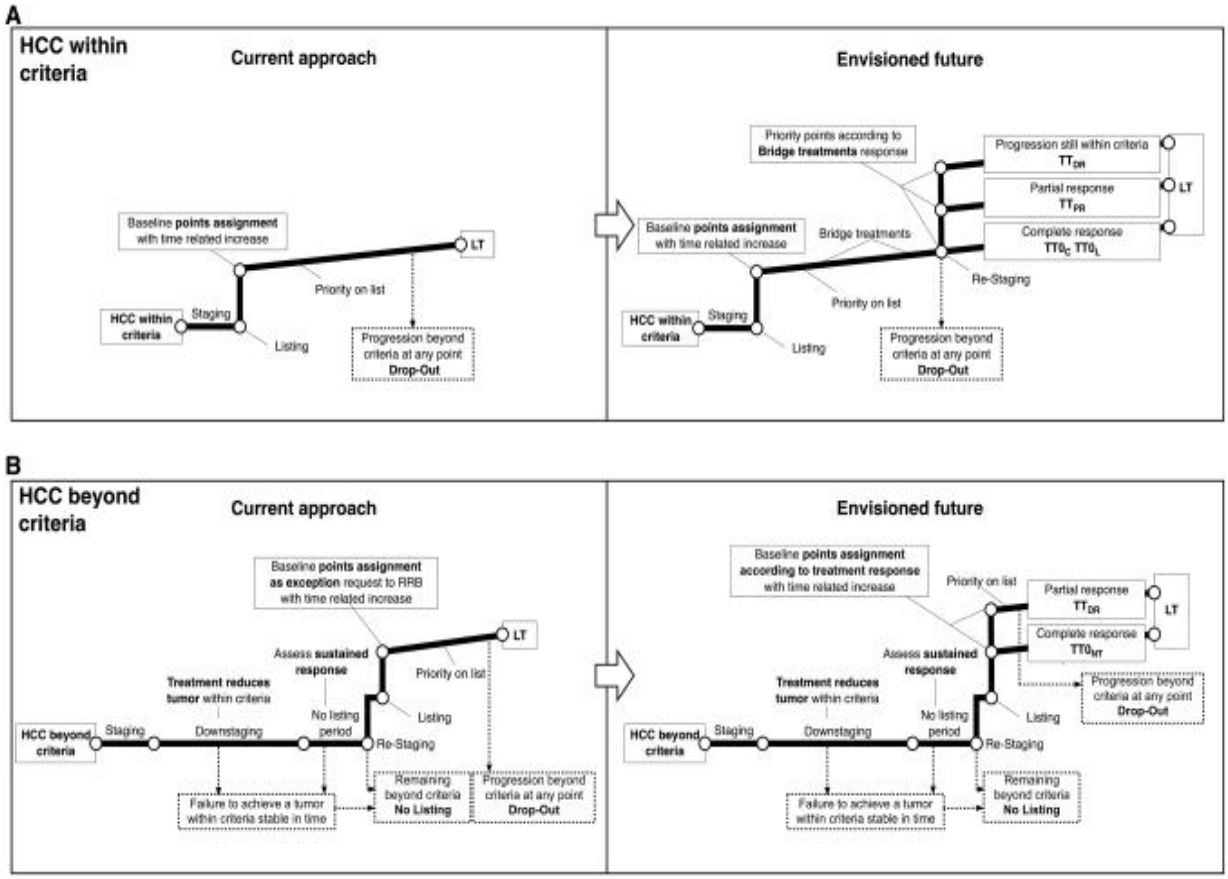

Figure 40 - Paradigm shift in the management of LTX in patients with HCC - as proposed and published by Mazzafero et al 2016.[96]

Although this new management model proposed by *Mazzaferro et al.* has the advantage of recognizing that the "Response to Bridging" predicts the prognosis after LTX, according to our results it should be reconsidered for two main reasons:

1) it considers that patients that downstaged have an equivalent survival rate to patients that were consistently inside Milan; our results showed that this is not the case. As demonstrated

[96] Mazzaferro V 2016

above, in SiLVER, patients that downstaged to fit into MC had a worse prognosis compared to patients always inside Milan, and did not have a better prognosis than patients that remained outside Milan;

2) it does not acknowledge the worse prognosis of patients that were always inside Milan but progressed after bridging. Although Mazzaferro considered a difference in the prognosis between “Complete Response”, “Partial Response” and “Progressive Disease”, the author indicates that the progressive disease group that remains inside Milan is a suitable group to be transplanted. However, according to our data, and as discussed above, patients inside Milan with a progressive disease have a poorer prognosis than those who did not progress within Milan, and do not have a better prognosis than patients outside of MC;

To our knowledge, there is currently no other study available that proposes an integrated assessment of prognosis in LTX for HCC to the extent presented in this dissertation. We did not find any other study comparing the “In-to-Inside Progressive” group with the commonly non-transplantable groups (outside Milan). In the same way, no results have been published assessing the ability of predicting prognosis of "Response to Bridging" in patients outside Milan.

6.5 Influence of Sirolimus

As mentioned before, the SiLVER Study aimed to study the effect of Sirolimus (Group B) when compared to Calcineurin-inhibitors (Group A) on the OS and DFS in 525 patients world-wide. The Study revealed that there was no prognostic difference between both groups on the long-term after 5 years. However, the original analysis did not explore the possibility that Sirolimus had effects in the different "Response to Bridging" or “Milan Dynamic” groups. Ultimately, if an effect was to be confirmed, it could mean that Sirolimus could lead to prognostic changes in patients with a certain "Response to Bridging".

Our data was analyzed through the Kaplan-Meier curve with log-rank test taking into consideration both the Intention-to-Treat Grouping and the Per-Protocol Grouping. When the prognosis of Group A vs. Group B where compared both within the Controlled and within the Progressive Disease Population, no significant difference was obtained. In other words, immunosuppression did not seem to influence the prognosis within the groups of "Response to Bridging" on the long-term.

Similarly, an analysis considering the Milan status was done. The results also showed that the immunosuppression chosen did not seem to play a role in the prognosis within the different Milan groups with regard to “Response to Bridging”.

Finally, the effects of different immunosuppressants was evaluated within the “In-to-In Controlled” and “In-to-In Progressive” population. None of these comparisons showed a relevant difference in the long-term prognosis that could be attributed to the immunosuppression given. These results suggest that the prognostic differences in the sampled patients were mostly due to their tumor biology and not to the immunosuppression that was given after the LTX.

6.6 Possible Future Implications

Currently, patients with a higher MELS-Score have a higher priority for receiving a LTX, and therefore less expected time of survival. Some countries/centers, introduced a system that gives extra-points to patients that consistently remain inside the MC, and therefore have a better expected prognosis than patients outside of the MC. This means, that the patients in the “Out-to-Inside” and the patients in the “In-to-Inside Progressive” group also receive extra-points and are considered to have a better prognosis than the patients outside Milan, which in this dissertation turned-out not to be supported by our data.

Particularly relevant was the data concerning OS of the patients that downstaged from outside to inside Milan. Although these patients are currently treated in many centers similar to those who always remained inside Milan, our analysis showed that their OS after 5 years was much lower, at 57%. This value is considered by many health care professionals as below the minimum OS limit and might indicate that a liver was given to a patient with a limited life expectancy. Although the Liver Allocation System was not particularly the topic of this dissertation, it is a subject worthy of future discussion based in-part on the findings of this dissertation.

This dissertation should also provoke the health care systems and professionals to rethink the way prognosis is assessed for HCC. Should the liver allocation system be more focused on prioritizing the patients according to the benefit that they can take from the donation? As part of the evaluation, perhaps patients with a better biology would benefit more from LTX? A balance between the necessity of LTX measured by the MELD-Score and the profitability of this procedure indicated by the IMTuBio selection is not easy to implement. However, we believe that this selection model might bring to light important parameters to assess the tumor biology of HCC for future patients, towards an ever more effective and efficient LTX system.

6.7 Limitations and further Research

There are important limitations of this retrospective analysis to consider. Incomplete data collection was evident in parameters like AFP values, MC and histological findings. This resulted in a smaller sample within the SiLVER population that, in some cases, provided less clear evidence as expected. For instance, although a group of “In-to-Inside Controlled” group formed by 149 individuals might be a reasonable size sample, the “In-to-In Progressed” group was formed by only 35 patients which naturally decreases the extent of the strength of the results. The MELD-score data of the patients was not available for this dissertation. In studying the association between the "Response to

Bridging" and LTX-prognosis, we acknowledge that the inclusion of MELD data would have been a powerful tool in this setting. Therefore, we realize this limitation and recommend the integration of this important prognostic score in future studies in this area.

Another important limitation of this study is related to the imaging assessment of the HCC tumors and lack of follow up imaging after each bridging therapy. It is known that imaging assessment of HCC, most commonly done with computer tomography, has a relevant margin of error when compared to the actual histological finding. Otto et al[97] found that considering the MC, 26% of the HCC staging assessments done by CT at LTX-day were incorrect when compared to the pathological assessment of the actual explantation specimen. The imaging assessment is particularly difficult for lesions smaller than 3 cm, which can lead to small lesions being unnoticed. If such an error happens, even at a small scale, it can lead ultimately to an erroneous assessment of the HCC evolution and the false assumption that there was, for example, a tumor progression due to the newly identified HCC lesions in the histological finding that were not observed in the imaging analysis. Moreover, and perhaps even more relevant to this study, it is important to state that there were no regular follow-up images after each LRT in most of the hospitals. The vast majority of patients had their HCC lesions staged at the time of listing and this result was compared to the histological finding. This means that there was no accurate assessment before and after each LRT, which would give more accurate and detailed information about the "Response to Bridging".

Considering a hypothetical analysis based on the alpha-fetoprotein (AFP), and its relationship to the "Response to Bridging", it is important to state that although AFP measurement was part of the SiLVER Protocol, only a few patients had a regular measurement before and after bridging. In this matter, it was not possible to form groups big enough to accurately compare this important parameter.

[97] Otto G 2013

7 CONCLUSIONS

LTX is the therapy of choice for patients with HCC in early stage disease who are not suited for curative Liver-Resection or curative LRT. Due to the high prevalence of HCC, considered the fifth most common neoplasm in the world and the third most common cause of cancer-related deaths, the availability of transplantable livers is much lower than the overall demand. This scarcity of organs, as well as the risks inherited in a complex surgical operation such as a LTX, led health care professionals to implement prioritization models such as the MELD-score (that indicates the short-term mortality) and the MC (that defines a set of tumor-characteristics that must not be surpassed in order to achieve an acceptable 5-year OS after LTX). Although world-wide implemented, the MC as been criticized for its inaccuracy. Unfortunately, the scientific community still has not managed to develop a better method of accurately predicting the prognosis after LTX.

Recently it has been theorized that the prognosis after LTX in HCC patients can be directly associated with the tumor biology, which in the clinical setting can be assessed by the aggressiveness of the tumor spreading. This dissertation explores the ability of the tumor size variation as a "Response to Bridging" to identify the most aggressive HCC tumors and, by extent, those patients with a better/worse prognosis after LTX.

In order to do so, a retrospective cohort study was developed based on the SiLVER Study, which includes a large database of 525 patients with HCC that were transplanted and followed-up for at least 5 years. From the original sample, initially prospectively collected to study the effects of mTOR-inhibitor immunosuppression, a sub-population of 356 patients that received some form of bridging therapy before LTX was retrospectively selected and analyzed, having OS and DFS as endpoint values. No distinction was considered regarding the modality or number of bridging therapies used.

Although the actual methodology of organ allocation is based on a static inside vs. outside Milan decision, which allows patients that downstaged from outside to inside Milan to be accepted for LTX, our results revealed that these patients have a significantly worse prognosis compared to those who are permanently inside Milan both for the DFS (5-year DFS 52% vs. 76%, *p-value* <0.01) and for the OS (5-year OS 58% vs. 78%, *p-value* = 0.01). Moreover, no statistically relevant difference could be found between these patients that downstaged and the patients that were outside Milan at LTX day (DFS 5-year 52% vs 55%, *p-value* = 0.71 & OS 5-year 58% vs 64%, *p-value* = 0.52).

We therefore concluded that downstaged patients have a poorer prognosis than patients always inside Milan and show no relevant difference in the prognosis to patients outside Milan.

When patients were grouped by their "Response to Bridging", it was clear that patients whose tumor did not progress had a better prognosis than their counter-parts. However, this was only true for patients that were always inside Milan. In this group, patients with a controlled disease had a significantly better prognosis concerning the OS in the first 4-years (4-year OS 86% vs 70%, *p-value* = 0.03) and in the DFS in the first year after LTX (1-year DFS 96% vs 85%, *p-value* = 0.01). After these periods of time, a *p-value* <0.05 was not achieved. The "Response to Bridging" did not play a role in predicting the prognosis in the patients outside Milan (OS *p-value* = 0.45 and DFS *p-value* = 0.95).

On the other hand, patients that always remained inside Milan, but revealed progress of their HCC size or number of lesions (In-to-Inside Milan with progressive disease) did not show a better prognosis than patients outside Milan (OS *p-value* = 0.76 and DFS *p-value* = 0.49). This led us to conclude that patients with any evidence for tumor progression after LRT had a prognosis similar to patients outside Milan.

We also conclude that once a tumour reaches the outside Milan stage, a decrease in the tumour size after bridging does not correlate with a better prognosis. However, in patients that were never outside Milan, even a small “Response to Bridging” has a significant correlation with the prognosis after LTX.

In summary this analysis reveals that there are two crucial factors that predict prognosis in HCC after LTX – if the tumor has reached an extent outside MC, or if it has progressed after LRT. Once one of these factors is evident, the prognosis considerably worsens.

This dissertation provides original results that should incite the scientific community to better study tumor dynamics as a valuable prognostic parameter to better allocate the ever-limiting number of organs availablle for LTX.

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9 CURRICULUM VITAE

Surname: Pinheiro Fernandes Neto da Silva

Name: Tomás

Parents:

- Maria Teresa Cardoso Pinheiro Fernandes Neto da Silva

- Orlando Jorge Couto Neto da Silva

Birthday: 05.02.1991

Birthplace: Lisbon, Portugal

Marital Status: Single

Education and Professional Carreer:

1991 – 2009: Colégio São João de Brito, Lisbon (Portugal)

2010 – 2016: Master's in medicine (Faculty of Medicine – University of Lisbon)

Since 2017: Doctoral Thesis in the Klinik für Chirurgie, Universitätsklinikum Regensburg

Since 2017: Resident Doctor (Arzt in Weiterbildung), Klinik und Poliklinik für Chirurgie, Universitätsklinikum Regensburg

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