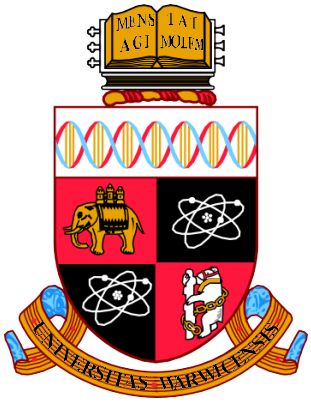

Assessing clinical implications and immunomodulatory changes following liver resection using RF-based device in liver cancer

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Figures

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Appendices

Declaration

I, Kumar Jayant, hereby declare myself as sole author of this thesis. The published work onto which this thesis is based are presented alongside and I confirm my close and substantial involvement in authoring them. Further, I declare that submitted material is not substantially similar to my other published or unpublished documents, for a degree, diploma or certification at any institution or university elsewhere. No part of these works has been submitted for similar qualification. All sources have been specifically acknowledged by means of reference.

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Statement of contribution in published work

Paper	Detail
Paper 1 (Appendix 1)	Reccia I, Kumar J, Kussano T, Giakoustidis A, Zanellato A, Retsas P, Habib N, Jiao L, Duncan S, Pai M. Radiofrequency-assisted liver resection: technique and results Surgical Oncology. 2018:27(3):415-20. Kumar Jayant collected data and conducted the literature search. With Dr Isabella Reccia, he took lead role in writing the paper and responded to reviewers as the corresponding author.
Paper 2 (Appendix 2)	Jayant K, Sodergren MH, Reccia I, Kusano T, Zacharoulis D, Spalding D, Pai M, Jiao L, Huang KW. A systematic review and meta-analysis comparing liver resection with RF-based device Habib™ -4X with clamp-crush technique. Cancer 2018;8:10(11). Kumar Jayant conceived the idea of study, conducted the literature search, collected data and conducted meta-analysis. He took lead role in writing the paper and responded to reviewers as the corresponding author.
Paper 3 (Appendix 3)	Huang K, Lee P, Reccia I, Kusano T, Jayant K, Habib N. Impact of Cavitron Ultrasonic Surgical Aspirator (CUSA) and Bipolar Radiofrequency Device (Habib-4X) based hepatectomy for hepatocellular Carcinoma on tumour recurrence and disease-free survival. Oncotarget 2017;8(55):93644-54. Kumar Jayant conducted the literature search and performed literature review. He supervised and contributed to data collection. He took lead role in writing the paper in liaison with co-authors and responded to reviewers as the corresponding author.

Paper 4 (Appendix 4)	Mazmishvili K, Kumar J, Janikashvili N, Kikodze N, Mizandari M, Pantasulaia I, Paksashvili N, Sodergren MH, Reccia I, Pai M, Habib N, Chikovani T. Study to evaluate the immunomodulatory effects of radiofrequency ablation compared to surgical resection for liver cancer. Journal of Cancer 2018;9(17):3187-95. Kumar Jayant collected data and conducted the literature search. With Dr Nona Janikashvili and Mazmishvili, he took lead role in writing the paper and responded to reviewers as the corresponding author.
Paper 5 (Appendix 5)	Huang K*, Jayant K*, Lee PH, Yang PC, Hsiao CY, Habib N, Sodergren MH. Positive immuno-modulation following radiofrequency assisted liver resection in hepatocellular carcinoma. Journal of Clinical Medicine 2019;8(3). Kumar Jayant conceived the idea and design of study, conducted the literature search, collected data and assessed studies. He took lead role in writing the paper in liaison of co-authors and responded to reviewers as the corresponding author.

Copies of these statements of contribution, signed by all coauthors can be found in Appendix 6.

* denotes joint first authors

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Abbreviations

AFP	Alpha-fetoprotein
ALBI	Albumin-Bilirubin score
ALP	Alkaline phosphatase
BCLC	Barcelona Clinic Liver Cancer
CC	Clamp crush
CD	Cluster of differentiation
CI	Confidence interval
CRLM	Colorectal liver metastasis
CTP	Child-Turcotte Pugh classification
CUSA	Cavitron Ultrasonic Surgical Aspirator
CTLA-4	Cytotoxic T lymphocytes-associated antigen-4
DCs	Dendritic cells
DFS	Disease free survival
DNA	Deoxyribonucleic acid
ECOG	Eastern Cooperative Oncology Group
ELISA	Enzyme-linked immunosorbent assay
FLR	Future liver remnant
GSA	Galactosyl serum albumin
HBV	Hepatitis B
HBsAg	Hepatitis B surface antigen
HCV	Hepatitis C
HLA	Human leukocyte antigen
ICG	Indocyanine green
IFN-γ	Interferon gamma
IL	Interleukin
INR	International normalized ratio
I/R	Ischemic reperfusion
ISGLS	International Study Group of Liver Surgery
ITU	Intensive therapeutic unit
LR	Liver resection
MD	Mean difference

MHC	Major histocompatibility complex
MELD	Model for end-stage liver disease
N	Number
NF-κβ	Nuclear factor kappa-light-chain-enhancer of the activated B cells
NHS	National Health Service
NK	Natural killer cells
NLR	Neutrophil-lymphocyte-ration
OS	Overall survival
PBMC	Peripheral blood mononuclear cells
PD-1	Programmed death
PD-L1	Ligand of programmed death 1
PHLF	Post-hepatectomy liver failure
PLR	Platelet-lymphocyte-ration
PT	Prothrombin time
SD	Standard deviation
SEER	Surveillance, Epidemiology and End Result
TAA	Tumour associated antigen
TACE	Trans-arterial chemoembolization
TAM	Tumour associated macrophages
TCR	T cell receptor
TGF-α	Transforming growth factor alpha
TGF-β	Transforming growth factor beta
Tregs	Regulatory T cells
Yr	Year

Abstract:

Liver cancer is an aggressive tumour and often presents late during the natural course of progression. In spite of advancements in the medical sciences, liver cancer has still remained recalcitrant to treatment. The exclusive treatment approach with curative intents is the resection of hepatic tumour if the disease gets diagnosed in an early stage. Nonetheless, better survival has also been observed following surgical resection in advanced stages, particularly for those who were not found to be apt for other treatment options by virtue of the extent of disease. The main purpose of surgical resections is to achieve complete oncological clearance; however, despite all endeavours, only 10-15% of newly diagnosed patients get a surgical resection. Additionally, in the case of hepatocellular carcinoma micro-metastasis beyond tumour margin and satellitosis are important attributes influencing the patient survival following resection. Five-year recurrence of up to 70% has been reported following curative resection with observed 5-year survival between 47% to 53% for early and small size tumours (<3 cm).

Insofar, various kinds of hepatic resection devices have been evolved to facilitate hepatic tumour resection and are compared with the gold standard methodology of hepatic resection, the clamp-crush (CC) technique. The notable factors outlined in the literature that influence post-hepatic tumour resection outcomes are negatively influenced by the amount of bleeding, higher requirement for blood transfusions, bile leak and hepatic insufficiency. However, the questions as to the clinical benefits of one device over another in terms of minimal blood loss, reduction in transfusion requirement and postoperative complications and survival remain unanswered.

The first paper in the index thesis includes a single-arm study assessing clinical outcomes following radiofrequency (RF) based liver resection and has been comparing it with the existing literature. The findings of the data analysis supports that RF-based device is a suitable and safe device for bloodless hepatic resection with acceptable perioperative morbidity and mortality when compared with contemporary literature. However, findings were limited from being a retrospective single centre study with no comparative group. Thus, a meta-analysis has been performed to evaluate RF-based liver resection device in comparison to the standard technique CC. The findings included lower blood loss with fewer patients requiring blood transfusion when hepatic resection was performed with RF-based devices with equivalent morbidity and mortality.

The third study was to compare two contemporary liver resection devices. Here, retrospective data analysis was done to determine the survival benefits and other peri-operative outcomes following liver resection through a direct comparison of RF-based device with Cavitron Ultrasonic Surgical Aspirator (CUSA). The study has demonstrated significant improvement in disease free survival and tumour recurrence have been reported with the RF-based device. Alongside, a significant reduction in all other parameters, including blood loss, blood transfusion and need for inflow occlusion during liver resection surgeries with the RF-based device as compared to CUSA.

The survival benefits observed following resection of hepatic tumour with RF-based device might be owing to the reinstatement of anti-tumour immune response following the application of RF energy. The assumption has been derived through the analysis of recent publications that demonstrated immunological changes following application of RF energy over hepatic tumour nodules.

In order to prove this assumption, a fourth study was undertaken to understand changes in the serum inflammatory [neutrophil-lymphocyte-ration (NLR) and platelet-lymphocyte-ration (PLR)] and immunological parameters following radiofrequency ablation (RFA) or non RF-based liver resection. The study highlighted that a decrease in NLR, PLR and $CD39^{+}CD4^{+}$ T cells expression not only reciprocates with systemic inflammation and anti-tumour immune status but also act as an explicit marker for improved survival in liver cancer patients.

The final study was performed to assess the changes in pre and post-liver resection levels of immunological parameters (cellular subtypes and cytokines) comparing the RF-based liver resection device with CUSA. Here significant changes in anti-tumour immune cells and cytokine levels were observed following liver resection with RF-based device and together with the analysis of contemporary literature revealed its favourable implications on survival and recurrences.

Taken together, the above findings are suggestive of the need for modification in the current practice and incorporation of RF-based liver resection as the primary surgical technique for the management of very early and early HCC cancers. Moreover, the proof of concept suggests that positive immunomodulatory changes observed following RF-based liver resection can enhance the activity of checkpoint inhibitors. Hence, the synergism of RF-based liver resection along with checkpoint inhibitors does not only remove tumour tissue but also helps in re-establishing anti-tumour immunity which might further aid in improving survival and preventing recurrences. Future studies are required to better understand the applicability of RF-based liver resection with checkpoint inhibitors in liver cancers.

Chapter 1- Introduction, epidemiology and risk factors for liver cancer

1.1 Introduction and epidemiology of liver cancer:

The primary liver tumours comprise mainly of hepatocellular carcinoma (HCC) followed by cholangiocarcinoma, fibrolamellar carcinoma, mixed hepatocholangiocarcinoma, hepatoblastoma and mesenchymal liver cancers [1–3]. In spite of advances made in diagnostic and therapeutic techniques, the survival of these patients has not substantially improved. Based on Global Cancer Observatory 2018 database, HCC is the sixth most commonly encountered malignancy with an approximate annual incidence of 841,000 and the fourth leading cause of all cancer-related deaths [4].

Secondary or metastatic liver tumours are more common than primary and constitute 95% of all hepatic cancers [5,6]. The pathophysiology of liver metastases can be explained via two mechanisms. Firstly, the dual blood supply to the hepatic parenchyma via the portal vein and hepatic artery could heighten the likelihood of metastatic spread. Moreover, fenestrations within the sinusoidal lining epithelium of hepatic vessels permit transmigration of malignant cells to the hepatic parenchyma [7].

1.2 Risk factors:

1.2.1 Risk factors for hepatocellular carcinoma:

The most notable risk factors for the development of HCC include chronic hepatitis B & C infection, cirrhosis, alcohol, obesity, non-alcoholic fatty liver disease, aflatoxin B1, hemochromatosis and Wilson's disease [8,9]. About 40% of cases in developed nations are owing to hepatitis B (HBV) or hepatitis C (HCV) infection. The risk is estimated to be 20 times higher in the population with HBV and/or HCV infection following the development of cirrhosis; however, cancer can also unfold in the absence of cirrhosis [10–13]. The cancer occurrence is highest in the elderly population of age 85-89 years which could be attributed to the accumulation of the damaged deoxyribonucleic acid (DNA) resulting from a cumulative effect of ageing and exposure to the risk factors. Further, it leads to the genesis of inflammatory tumour microenvironment, which lowers anti-tumour immunity and gives rise to malignant cells [14,15].

1.2.2 Risk factors for secondary liver tumours:

The secondary tumours are mostly metastatic and commonly originate through primaries from colon, rectum, breast, lung, pancreas, etc. Almost 50% of patients with a diagnosis of colorectal cancer have liver metastasis at the time of the first presentation or during follow-up [16].

Surgical resection is the recommended treatment modality for colorectal liver metastasis; however, only 10-15% of patients are deemed suitable. The post tumour resection 5-year survival has been 40-50% with a cure rate of 20%. On the contrary, the median survival was 5-10 months in the historical non-resected cohorts [17–20].

Chapter 2 Management of liver tumours

2.1 Introduction:

HCC is an aggressive tumour, which usually develops over the hostile and inflammatory background of chronic liver disease (cirrhosis) and is often diagnosed belatedly during its natural course of progression. The treatment of liver cancer is primarily determined on the basis of tumour stage, underlying liver function and overall functional status of the patient. One of the potentially curative treatment options for early-stage tumours is hepatic resection; however, studies have also outlined better survival for patients with advanced disease patients who underwent major hepatic resection because they were adjudicated not suitable for other treatments options owing to the extent of disease. On this account, hepatectomy should be recommended within bounds of possibility for tumour negative resection for the advanced stage primary or metastatic liver cancers [21].

The optimum decision regarding surgical resection is usually made following contemplation of various attributes like the extent of cancer, applicability and ability to tolerate treatment, functional status of liver and overall functional status of the patient. Advancement in the surgical sciences through the development of novel devices for liver resection and non-surgical technique, including radiofrequency ablation (RFA), trans-arterial chemoembolization (TACE), chemotherapy, and immunotherapy have ushered a conspicuous impact on the management of liver cancer patients [22,23].

2.2 Staging system and management guidelines:

The approach towards the management of HCC patients is outlined in a number of staging systems, for example the Barcelona Clinic Liver Cancer (BCLC), the Cancer of the Liver Italian Program, Japanese Integrated Staging and the Hong Kong Liver Cancer staging system [24,25].

The BCLC is the most common, externally validated staging system and presides over other alternative variants. Here, HCC patients are placed into one of the five categories (very early, early, intermediate, advanced and terminal) depending on tumour size,

number of nodules, portal invasion, lymph nodes status, metastasis, performance status, and underlying liver dysfunction to determine treatment and survival [26] (Figure 1). The performance status of the patient is evaluated through the Eastern Cooperative Oncology Group scoring which assesses progression of the disease, daily living abilities and ascertains congruent management [27].

Similarly, the most widely accepted systems to quantify hepatic functioning are the Child-Turcotte- Pugh classification (CTP) and model for end-stage liver disease (MELD), albeit other measures including Albumin-Bilirubin (ALBI) score, Indocyanine green (ICG) clearance test, scintigraphy based on 99mTc diethylenetriamine-pentaacetic-acid (GSA) are increasingly getting incorporated [28]. Patients with very early-stage HCC [BCLC 0] and early-stage [BCLC A] have a solitary lesion or up to three nodules that are less than 3 cm in diameter (with no macrovascular invasion or extrahepatic spread) and preserved liver function (CTP class A or MELD score <10). Patients with intermediate-stage HCC [BCLC B] have large, multifocal tumours with no vascular invasion or expansion beyond liver. If liver function is preserved, these patients could be candidates for transarterial chemoembolization. Patients with advanced-stage disease [BCLC C] have one or more of the following features: extent of the tumour beyond liver, vascular invasion, and mild cancer-related symptoms [ECOG performance status grades 1-2]. BCLC D patients have end-stage liver disease with poor functioning or marked cancer-related symptoms [ECOG performance status grades >2]; nevertheless, such patients are also not deemed suitable for transplantation owing to dismal prognosis and receive the best supportive care [29].

Therapeutic options with intent to treat must be rendered to all eligible liver cancer patients. Among patients presenting at a very early or early stage should be considered for various potentially curative options such as radiofrequency ablation, surgical resection and liver transplantation. Hepatic resection is the best possible treatment option for HCC patients drawn within the criterion of resection, with improved survival and without any increased risk of severe complications [30–32]. Even though surgical resection is the mainstay of therapy, the majority of patients are not fitting into criterion owing to delayed presentation, tumour location and deplorable liver

function. Further, HCC patients with non-resectable tumours falling within the purview of Milan's criteria should be considered for liver transplantation because of improved disease-free survival (DFS) and decreased remission. Patients deemed suitable for liver transplantation have apparently unresectable disease secondary to poor liver functioning rather than the extent of tumours, although such a proposition is equally dependent on the availability of organ. The shortage of organs not only prolongs the waiting period but also worsen disease, thus, rendering such patient unsuitable for transplantation [33,34].

During the evaluation of resectability of liver tumours, the following determinants are taken into account to ascertain the complete removal of cancer: the extent of disease; size and location of tumour and underlying hepatic function as the preservation of non-cancerous liver parenchyma is associated with decreased morbidity and mortality. The tumour size or multifocality should not be considered as a contraindication to hepatic resection; although, they do worsen the odds of vascular invasion and dissemination [35,36].

Despite all endeavours, only half of the initially considered to be surgically resectable liver tumours have been found to be suitable for surgery, hence merely 10-15% of newly diagnosed patients had a surgical resection. The basic goal of surgical resection is to achieve complete oncological clearance; nevertheless, resection of large liver parenchyma heightens the risks of procedure-specific complications, increases perioperative morbidity and diminishes the disease specific survival rates [37,38].

The liver resection surgery was first undertaken using the clamp-crush (CC) technique and is still considered as a gold standard technique for hepatic parenchymal transection; though, the post-surgery outcomes are negatively influenced by bleeding, increased requirement for blood transfusion, bile leak, hepatic insufficiency and escalated peri-post operative morbidity and mortality [39–41].

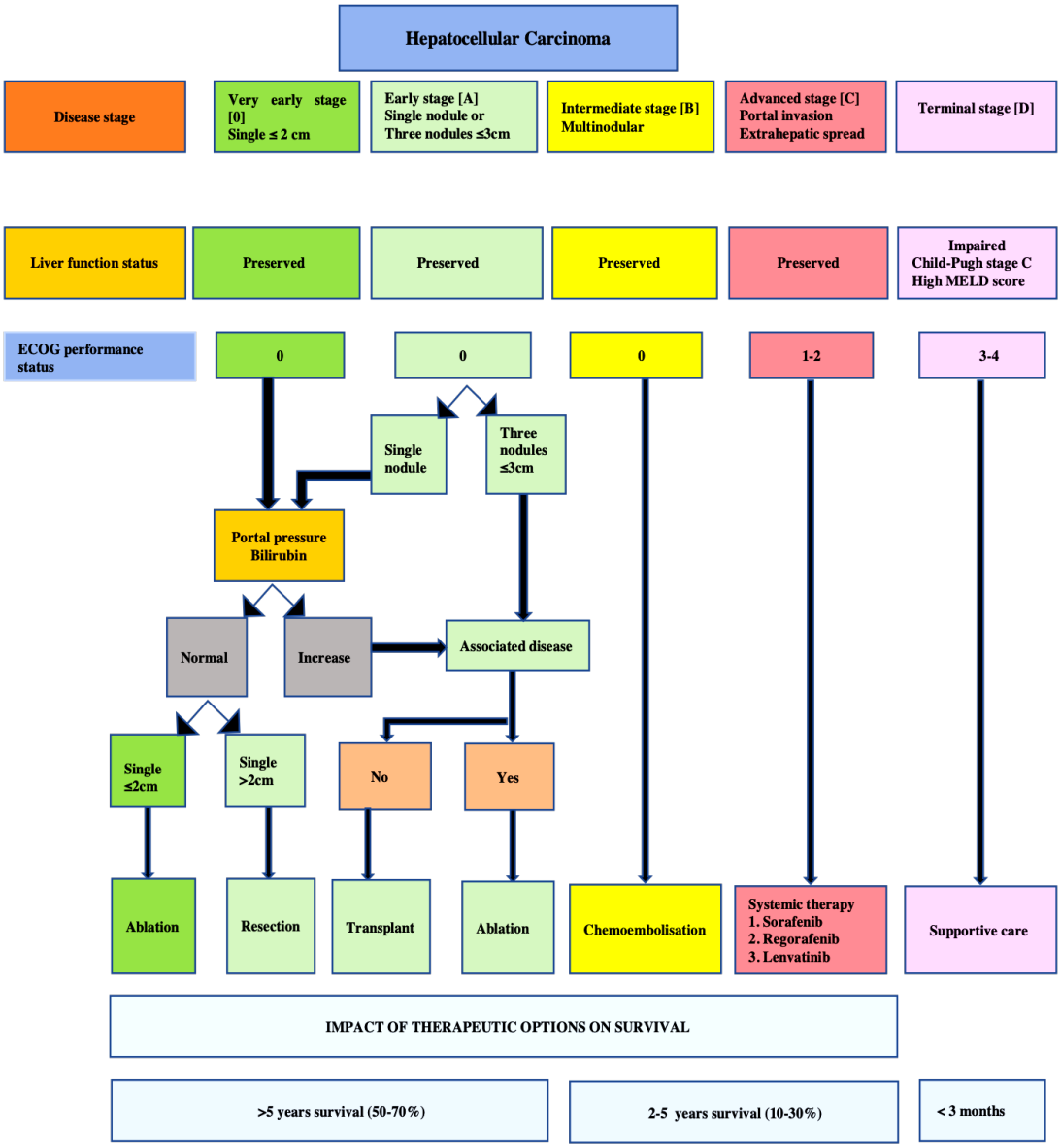

Figure 1: Barcelona Clinic Liver Cancer staging system with management strategy

2.3 Factors influencing the outcomes following liver resection:

The introduction of newer liver resection devices and the development of different hepatic resection strategies have opened a new dimension in the surgical management of HCC patients. However, liver resection surgeries are still considered as a challenging and perilous procedure because of unenviable postoperative complications, including morbidity and mortality of 40% and 5% respectively [42,43]. The factors which directly influence the postoperative outcomes of liver resection are outlined in Table 1.

Table 1: Factors influencing postoperative outcomes following hepatic resection

Increase blood loss
Blood transfusion
Underlying liver status
Hepatic function
Child Pugh Turcot score
Inadequate future liver remnant (FLR)
Extended liver resection

a) Increased blood loss and transfusion requirement:

Increased blood loss and blood transfusions cause greater postoperative complications leading to the heightened risk of bile leak, post-hepatectomy liver failure (PHLF), symptomatic collection, abscess, prolonged intensive care, reduced survival, increased incidence of recurrence and death [44–47]. Numerous studies have demonstrated the negative consequences of the increased intra-operative bleeding and blood transfusion during liver resection and noteworthiness of reduced blood loss in minimizing the morbidity and mortality [48–52].

Poon et al. (2004) studied the impact of blood loss during hepatectomy through the analysis of a database of 1222 patients. The group with median intraoperative blood

loss and blood transfusion requirement of 1450 mL and 68% respectively had morbidity and mortality of 37.0% and 7.5% respectively. This was in contrast to the other group with lower median blood loss (750 mL) and blood transfusion necessity (17%), which had reduced morbidity and mortality of 30.0% and 3.7%, respectively [53].

The observation is further supported by a study from Yang et al. (2011), which mentioned increased bleeding (800 mL) during hepatectomy as an independent high-risk determinant for perioperative morbidity [54]. Additionally, various studies have reported that higher perioperative blood transfusion can undermine the oncological outcomes and increase tumour recurrence following hepatectomy in liver cancer patients [45,49,55-57].

The importance of minimizing perioperative blood loss and transfusion has been well acknowledged, and diverse kind of introductions have been made in terms of modification of surgical techniques and the invention of different resection devices to curtail haemorrhage whilst transection of hepatic parenchyma.

This could be attained through the reduction of central venous pressure or judicious use of hepatic vascular inflow occlusion technique or Pringle manoeuvre [58,59]. The later has got limited applicability in HCC patients with underlying cirrhosis owing to the increased risk of ischemic reperfusion (I/R) injury and ineptness in averting the back-flow of blood through hepatic veins which could further enhance the catastrophe of (I/R) [60]. In addition, a meta-analysis involving eight randomized studies and 558 patients was conducted by Rahbari et al. (2008) [61]. The authors concluded that the routine application of portal triad clamping carries no added benefit over no inflow occlusion and should be practiced with caution.

b) Condition of hepatic parenchyma:

A multitude of studies have outlined the importance of liver parenchyma preservation, functioning of the liver remnant and limiting haemorrhage in ameliorating the postoperative morbidity and mortality in liver cancer patients [26,62,63]. The

functional status of hepatic parenchyma is one of the prime determinants of the regenerative capacity of the liver. All forms of hepatic parenchymal injuries extending from steatosis, steatohepatitis to cirrhosis are associated with reduced regenerative capacity and worse postoperative outcomes [64–66]. The complications following hepatic resection in cirrhotic livers' endanger life with a mortality of 30% [67,68].

Hepatic resection in jaundiced patients is a high risk procedure owing to cholestasis-induced ischemia/reperfusion injury and reduced antioxidant levels leading to enhanced inflammatory response and increased morbidity [69]. Further, with regard to colorectal liver metastasis (CRLM), prolonged duration of neoadjuvant systemic chemotherapy incites steatohepatitis, which would imperil the post-hepatectomy outcomes and pose significant rise in the mortality [70,71].

c) Future liver remnant:

The incidence and severity of PHLF could be minimized by avoiding extensive liver resection as the volume and condition of the remaining liver i.e. the Future liver remnant (FLR), ascertains the regenerative capacity and potential to preserve optimum physiologic function. FLR is a stand-in term for the volume and functioning of the remnant liver for envisaging PHLF and other postoperative complications that is contingent on the degree of liver dysfunction. Studies have shown that liver resection should be directed towards the preservation of maximum liver parenchyma irrespective of surgical resection type i.e. anatomical or non-anatomical [72,73].

The minimum required volume of the liver remnant must allow adequate vascular inflow, outflow and biliary drainage in contiguous segments. With the intent to limit PHLF and other complications, various studies have recommended a safe limit of FLR ≥20% in normal functioning liver, ≥30% in chemotherapy-treated patients and ≥40% in cirrhotic patients [71,74,75].

d) Preoperative liver function:

Preoperative liver function determines the suitability of patients for hepatic resection and quantifies the incidence and severity of PHLF. Various traditional laboratory tests

including albumin, bilirubin, creatinine, prothrombin time/international normalized ratio (PT/INR), alkaline phosphatase (ALP), transaminases are incorporated in scoring

Table 2: Common liver function assessment system to stratify patients for liver surgery

Scoring system	Attributes				
Child-Turcotte-Pugh	Parameters	Point distribution			Score
		1	2	3	Class A= 5-6 Class B= 7-9 Class C= 10-15
	Bilirubin (mg/dL)	<2	2-3	>3	
	Albumin (g/dL)	>3.5	2.8-3.5	<2.8	
	Prothrombin time prolongation (sec) or INR (international normalized ratio)	<4 <1.7	4-6 1.7-2.3	>6 >2.3	
	Ascites	None	Mild/ moderate	Severe	
	Encephalopathy (grade)	None	1-2	3-4	
MELD	Parameters				6-40
	Bilirubin (mg/dL)	INR		Creatinine (mg/dL)	
	$3.8 \times \log_e(\text{bilirubin [}\mu\text{M]}) + 11.2 \times \log_e(\text{INR}) + 9.6 \times \log_e(\text{creatinine [}\mu\text{M]}) - 53.77$				

Abbreviations: INR: International normalized ratio; MELD: Model for end-stage liver disease

systems such as the Child-Turcotte-Pugh or MELD to evaluate the suitability for hepatic resection and predicting the complications [76,77] (Table 2).

e) Estimation of functional hepatic reserve:

The most advanced and dynamic ways to assess the functioning of the liver are Indocyanine green (ICG) clearance test and scintigraphy based on 99mTc diethylenetriamine-pentaacetic-acid galactosyl serum albumin (GSA) [78].

ICG is a water-soluble cyanine dye that adheres to plasma proteins and is cleared by the functioning hepatic parenchyma. The rate of clearance of ICG from plasma at 15 minutes acts as an indicator of underlying liver function and correlates with postoperative morbidity [79,80]. Studies suggest that levels greater than 14% of ICG are associated with the heightened risk for post-hepatectomy morbidity and mortality. Hence, liver cancer patients with normal bilirubin and without ascites can be subjected to extensive liver resection provided the ICG clearance rate is under 10% [80,81].

2.4 Liver resection devices:

According to the oncological principle of hepatic resection, the tumour negative margin should be achieved along with the preservation of adequate functioning liver. A number of devices have been evolved to facilitate hepatic tumour resection whilst preserving maximum residual liver volume and ensuring negative oncological margins to improve postoperative outcomes. The most notable ones' are CUSA (Cavitron Ultrasonic Surgical Aspirator), radiofrequency (RF) based liver resection device Habib-4X (Angiodynamics Inc., Latham, NY, USA), Harmonic Scalpel (Ethicon Endo-Surgery, Cincinnati, OH, USA), Ligasure (Valley Lab, Tyco Healthcare, Boulder, CO, USA), TissueLink (Salient Surgical Technologies, Portsmouth, NH, USA), water-jet dissection device, microwave-based resection devices and vascular staplers [82]. However, the questions related to the clinical benefits of one device over another are still outstanding.

2.4.1 Principle of RF-based liver resection device and understanding of surgical technique:

RF-based devices utilize radiofrequency energy to generate heat which produces coagulative necrosis over normal liver parenchyma and creates a resection margin around the tumour. The zone of coagulative necrosis helps in sealing the blood vessels feeding tumour/s and bile ducts, hence minimizing the blood loss and limiting the requisite for the Pringle manoeuvre and transfusion. This principle was the foundation for the invention of an RF-based liver resection device [83,84].

2.5 Requirement for studies:

The wealth of literature available on the liver resection devices and techniques suggests that haemorrhage and the necessity of blood transfusions are the matter of prime concern during liver surgery. Moreover, they are the foremost in influencing the incidence and magnitude of postoperative morbidity, recurrences, survival and mortality [40,85,86]. The 5-yr recurrence of HCC has been observed in up to 70% of cases following curative resection and 5-yr survival for early, small tumours of size <3 cm has been reported between 47% to 53% [87–89]. In the last two decades, several liver resection devices have been brought into the surgical practice to target the Achilles' heel of post liver resection complications and ease the technical challenges faced during surgery. However, the choice of device that curtails the blood loss, diminishes transfusion requirement and reduce postoperative complication has remained an issue of debate until now.

First paper included in the index thesis was a single-arm study assessing clinical outcomes following RF-based liver resection and had been compared with the contemporary literature [90]. The role of RF-based liver resection was further evaluated in a second paper; here meta-analysis was performed with available literature comparing with the gold standard liver resection technique Clamp-Crush [91]. Further, the third paper compared the survival and recurrences between the two contemporary devices RF-based device with Cavitron Ultrasonic Surgical Aspirator (CUSA) in addition to other outcomes following liver resection through [92].

Recent studies have demonstrated befitting immunological changes following RF energy application which aids in reinstating the anti-tumour immunity back to normalcy in addition to the destruction of tumour cells [93,94]. The above knowledge has helped in contemplating the fourth paper to comprehend changes in the serum inflammatory and immunological parameters following RFA or non RF-based liver resection [95]. Additionally, more evidence for radiofrequency induced immunomodulation has been obtained through the direct comparison of RF-based liver resection device with another modern liver resection technique using CUSA [96].

Chapter 3 Evaluation of surgical devices and techniques in hepatic cancer

Post liver resection outcomes are determined by tumour negative margin and FLR. Additionally, survival and recurrence are also influenced by intraoperative blood loss, transfusion and need for inflow occlusion. The modern surgical practice is bestowed with several liver resection devices; however, data to support the usefulness of one device over other is limited.

3.1.1 Study 1 (Appendix 1): Reccia I, Kumar J, Kussano T, Giakoustidis A, Zanellato A, Retsas P, Habib N, Jiao L, Duncan S, Pai M. Radiofrequency-assisted liver resection: technique and results Surgical Oncology. 2018:27(3):415-20 [90].

The index study was conducted at Hammersmith Hospital, Imperial College London, through analysis of retrospective data of liver cancer patients following liver resection by RF-based device.

3.1.2 Material and methods:

Data of 857 patients following hepatic resection for different liver tumour was analyzed to assess the incidence of perioperative outcomes including amount of blood loss, transfusion requirement, post- hepatectomy liver failure (PHLF), bile leak, symptomatic collection, pleural effusion necessitating intervention, thoracic empyema, hospital-acquired pneumonia, intensive therapeutic unit (ITU) admission and mortality.

The term PHLF refers to one of the most serious complications occurring in 10% of major liver resections. The International Study Group of Liver Surgery (ISGLS) defines PHLF as “a post hepatic resection deterioration in the ability of the liver to maintain its synthetic, excretory, and detoxifying functions, which is characterized by an increased INR and concomitant hyperbilirubinemia on or after postoperative day 5” [97].

Symptomatic intra-abdominal collection is defined as any postoperative collection which needs radiological intervention or surgical drain placement onto which patient

is discharged from hospital [98,99]. Bile leakage is one of the frequent complications encountered following liver resections, with a reported incidence ranging between 3.6% to 12%. The ISGLS defines bile leak based on the concentration of bile in the intraabdominal fluid, which should be ≥ 3 times the serum bilirubin concentration at or after postoperative day two [100]. Postoperative, 30-day mortality is used to report death directly related to hepatic resection [101–103].

3.1.3 Results and implications:

The resection phase of hepatic parenchyma plays a pivotal role in liver surgery and directly influences outcomes, such as bleeding, bile leak, and survival. In the majority of the cases, 93.1%, (n=798), intraoperative blood loss was <150mL. The need for transfusion was 9.8% (84 patients) whilst re-laparotomy for bleeding was required in 0.6% (5 patients). The reduced requirement for blood transfusions and Pringle's manoeuvre not only minimises the degree of ischemic injury to the compromised hepatic parenchyma but also enhances the chances of early recovery.

Patients with cirrhosis, steatohepatitis and prolonged chemotherapy are disposed to PHLF, sepsis, extended ITU stays and mortality. PHLF occurred in 1.5% (13 patients) with a direct association in single death. Among these, four patients were with limited liver function owing to cirrhosis and nine had extended hepatectomy. Further, if we classify them as per ISGLS classification, five cases were categorised Grade A; three were Grade B; and the remaining five (including one death) were Grade C. Our findings were congruous to the contemporary published literature, where PHLF ranges between 2.0% - 18.9%, with a mortality of 0.6%–2.3% [104–107]. In addition, the preservation of hepatic parenchyma whilst achieving tumours free margin also aid in limiting PHLF as they are mostly encountered with major hepatectomy. Symptomatic intra-abdominal collections were found in 10.6% (91 patients); of these 75 (82.4%) required percutaneous drainage. The remaining 16 were managed by delaying the removal of the surgical drain placed during surgery and/or antibiotics. Bile leak was reported in 4.3% (37 patients), of which 24 were from parenchymal transection margin and 13 due to the leak from the area of bile duct anastomosis. This was in line with

recently published articles, which outlined bile leak incidence between 2.2-15.6% [108-111].

Re-laparotomy was required in four patients because of jejunal perforation, small bowel obstruction, colonic perforation and biliary leak from anastomosis site. The most frequently encountered postoperative complication was pleural effusions in 22% (189 patients) for which chest drain placement was required in 27 patients (3.1%) and thoracic surgery was entailed for five patients as they developed thoracic empyema. About 3.5% (30 patients) had hospital-acquired pneumonia. Among the serious complications, the postoperative mortality was 1.5% (13 patients), including PHLF (1); bleeding from splenic bed varices (1); severe sepsis (3); pulmonary embolism (2); cardiovascular complications (4); right heart failure due to pulmonary hypertension (1) and mediastinitis secondary to Boerhaave's syndrome (1). About 4.2% (36 patients) patients required ITU admission whilst rest of patients were moved to the routine ward from postoperative recovery unit, which not only helped in minimizing the unexpected cancellation of the surgery owing to non-availability of ITU beds but also in limiting the National Health Service's (NHS) expenditure on ITU stay.

3.1.4 Conclusion:

The incidence of outcomes observed in the above study were compared favourably with those reported in the literature, supporting the view that RF-based device is suitable for 'bloodless' liver resection. However, the index study has got certain limitations, being a single centre retrospective study with no comparative group; hence, a meta-analysis was performed to compare RF-based liver resection device with standard technique, Crush-Clamp (CC).

3.2.1 Study 2 (Appendix 2) Jayant K, Sodergren M, Reccia I, Kusano T, Zacharoulis D, Spalding D, Pai M, Jiao L, Huang KW. A systematic review and meta-analysis comparing liver resection with RF-based device Habib™-4X with clamp-crush technique. Cancer 2018;8:10(11) [91].

The index study was possible because multitude of literature have outlined the benefits of various hepatic resection devices and techniques; however, no meta-analysis was undertaken to date to compare an RF-based liver resection device (Habib™ -4X) with the standard clamp-crush (CC) technique. Hence, following an extensive literature search all the available data on the clinical effectiveness of mentioned liver resection technique were combined together and analyzed to deduce a better conclusion.

3.2.2 Material and methods:

The aim was to compare the perioperative outcomes of 'RF-based device Habib™- 4X' with the standard CC technique through a meta-analysis. It was envisaged that the results of this meta-analysis would help in resolving the ongoing contention, about the safest and most efficient technique for liver resection. The outcomes of interest were blood loss, transfusion necessity, operative time and incidence of adverse events including bile leak, PHLF, intra-abdominal collection, pleural effusion, length of hospital stay, morbidity and 30-day mortality.

3.2.3 Results and implications:

The literature search yielded four studies for review and data extraction [112–115]. The data encompassed 543 patients in the CC group and 491 patients in RF-based group. The blood loss (mL) and blood transfusion requirement were significantly lower in the RF-based device group [(MD = 162.12 mL, 95% CI 45.34 to 278.90, P = 0.007, I2 = 53%), (MD = 1.93 times, 95% CI 1.43 to 2.61, P < 0.0001, I2 = 0%) respectively]; however, there were no significant difference in terms of operative time and complications such as PHLF, bike leak, pleural effusion, length of hospital stay, total morbidity and mortality.

3.2.4 Conclusion:

To the best of our knowledge, this was the first meta-analysis performed to compare the outcomes after hepatic resection using RF-based device Habib™-4X and the clamp-crush technique. The study has outlined the benefits of RF-based liver resection device in terms of reduced blood loss and decreased need for transfusion (Figure 2).

However, as this meta-analysis was based on only four identifiable studies, future trials are warranted to determine wider benefits of device. Nevertheless, study has demonstrated the safety and benefits of the RF-based device Habib™-4X in hepatic resection.

Based on these observations further study was performed where two contemporary liver resection devices, RF-based device and CUSA were compared to understand their implications in modern liver surgeries.

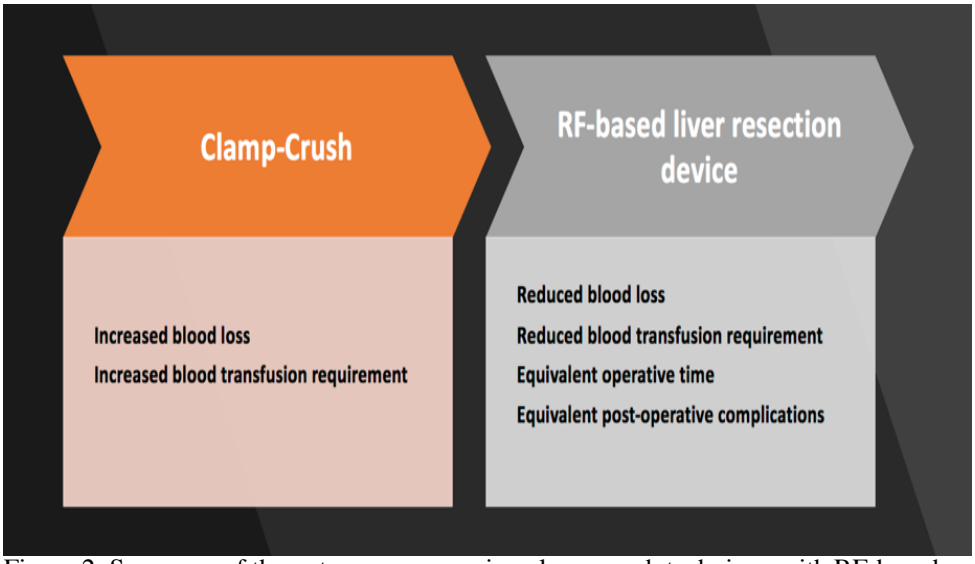

Figure 2: Summary of the outcomes comparing clamp crush technique with RF-based liver resection device
Abbreviations: RF: Radiofrequency

3.3 Assessing short and long term roles of RF-based liver resection device and CUSA in HCC

3.3.1 Study 3 (Appendix 3): Huang K, Lee P, Reccia I, Kusano T, Jayant K, Habib N. Impact of Cavitron Ultrasonic Surgical Aspirator (CUSA) and Bipolar Radiofrequency Device (Habib-4X) based hepatectomy for hepatocellular Carcinoma on tumour recurrence and disease-free survival. Oncotarget 2017;8(55):93644-54 [92].

Cavitron Ultrasonic Surgical Aspirator (CUSA), operates with ultrasonic waves to generate energy to fragment hepatocytes. The approach has advantage that blood vessels and bile ducts are spared owing to sparse water content. However, as it doesn't coagulate blood vessels or bile duct, hence ties or clip are used where required [116,117]. On the contrary RF-based device creates a line of coagulation around tumour margins and facilitates bloodless liver resection [118]. Both devices are routinely practiced to facilitate liver resection, which gave us an opportunity to compare the short and long term outcomes through retrospective data analysis.

3.3.2 Material and methods:

In index paper, 280 patients were assessed following liver resection with CUSA or RF-based device Habib-4X for HCC [92]. The data was collected between January 2010 to August 2016, allowing a follow-up period of 5 years. The primary endpoints of the study were the quantification of blood loss, transfusion required, need for vascular control, whilst secondary endpoints included PHLF, length of stay, complication within 30 days, overall recurrences, local recurrences, interventions needed following recurrences, disease-free survival (DFS) and overall survival (OS).

Differences in continuous variables between the groups (CUSA or RF-based device) were evaluated with Student's t-test, and differences in categorical variables were evaluated with chi-square or Fisher's Exact test. The date of surgery was considered as a landmark to determine period of overall survival (OS) and disease-free survival (DFS). Survival analysis was performed using the Kaplan-Meier method and groups compared using the log-rank test. A P- value of < 0.05 was considered significant.

Data were stored in a Microsoft Excel worksheet and analysis performed through the IBM-SPSS version 24 (SPSS Inc, Chicago, IL, USA).

3.3.3 Results and implications:

The study groups were comparable in respect of demographics, tumour characteristics, tumour stage, cirrhosis, serum bilirubin, serum albumin, serum alpha-fetoprotein (AFP), ICG clearance, HCV status, HBsAg status.

The blood loss was significantly greater in the CUSA group compared to RF-based device group (271.47 ± 214.5 mL vs 150.93 ± 103.6), $P < 0.01$; Student's t-test) (Table 3). Understandably, the requirement for transfusion was also significantly higher in CUSA group than RF-based device group; 21 and 3 cases respectively ($P < 0.01$; $\chi 2$). Additionally, vascular inflow control was required only once with RF-based device group compared to 139 times whilst using CUSA ($P < 0.01$; $\chi 2$). Major complications including PHLF, bile leakage and postoperative haemorrhage were also more frequent in CUSA group than RF-based device group; however, difference was not significant ($P = 0.36$; $\chi 2$).

The observed outcomes in RF-based device group were secondary to coagulation of blood vessels at the tumour margins. Coagulation limits the requirement of hepatic inflow occlusion and minimizes the risk of I/R; thus, improves DFS and OS [73,119–121]. On the contrary, liver resection with CUSA has remained operator dependent owing to the need to ligate the blood vessels and the requirement for inflow occlusion to minimize blood loss during liver parenchyma resection [122,123].

The five-year data revealed a significantly lower recurrence in the RF-based liver resection group (44 patients) compared to CUSA (85 patients, $P < 0.01$; $\chi 2$). This seems comprehensible as an area of 20 mm beyond tumour margins already gets coagulated and ablated during resection with RF-based device, which creates an extra layer of protection in addition to surgical resection of HCC tumour [82]. Studies have outlined that micrometastasis and satellitosis are mostly present within 18-20 mm beyond tumour margin [124,125] which remain unattended in conventional liver

resection surgery; however, get included within ablation margin whilst using RF-based liver resection device, owing to its capabilities to ablate 20 mm area beyond tumour margin [82]. Likewise, significantly higher number of patients needed post recurrence palliative interventions in the CUSA group, i.e. 81.1% compared to 62.4% RF-based group (P <0.01; $\chi 2$). The most common modality for palliation in the event of recurrence in the CUSA group was RF-ablation (47/85; 55.3%), whilst TACE (21/44; 47.7%) was the preferred method of palliation in the RF-based liver resection group.

Median DFS in RF-based group was significantly better than CUSA group (50.80 vs 45.87 months; P = 0.03), whilst the median overall survival was also found to be better in RF-based group than CUSA group, however did not reach statistical significance (60.57 vs 57.17 months, P = 0.12). The observations were akin to the previous literature by Qiu et al. (2017) [112] reporting better DFS and significant other benefits following resection of HCC with the RF-based device over the clamp-crush technique, notably due to minimal blood loss, less requirement of blood transfusion and HCC tumoural characteristics like micrometastasis and satellite nodules.

Table 3: Comparing perioperative attributes following liver resection with CUSA and RF-based liver resection device

Attributes	CUSA (163)	RF-based device (117)	P value
Blood loss (mL) (Mean ± SD)	271.47±214.5	150.93±103.6	0.00*
Blood transfusion required	21	3	0.00#
Requirement of vascular inflow control	139	1	0.00#
Major complications	9	3	0.36
Recurrence (+/-)	85/78	44/73	0.01#
Intervention required following recurrence	85 (81.1%)	44 (62.4%)	0.00#
Disease free survival (Median) (months)	45.87	50.80	0.03$
Overall survival (Median) (months)	57.17	60.57	0.11$

- *#$ marks significant P value.
- *Difference between group was analyzed by the Student's *t*-test.
- #Difference between group was analyzed by the chi-square test.
- $Difference between group was analyzed by the Kaplan-Meier survival plot.

Recent research has demonstrated that RF ablation of HCC nodules generates neoantigens to instigate immunomodulatory changes towards the anti-tumour state to combat micrometastatic disease and recurrences [126–128]. Likewise, the survival benefits outlined in the present paper could be because of the reinstatement of anti-tumour immune cells through RF based liver resection, which does not occur in conventional hepatic resection; however, further randomized trials are required considering the retrospective nature of this study (Figure 3).

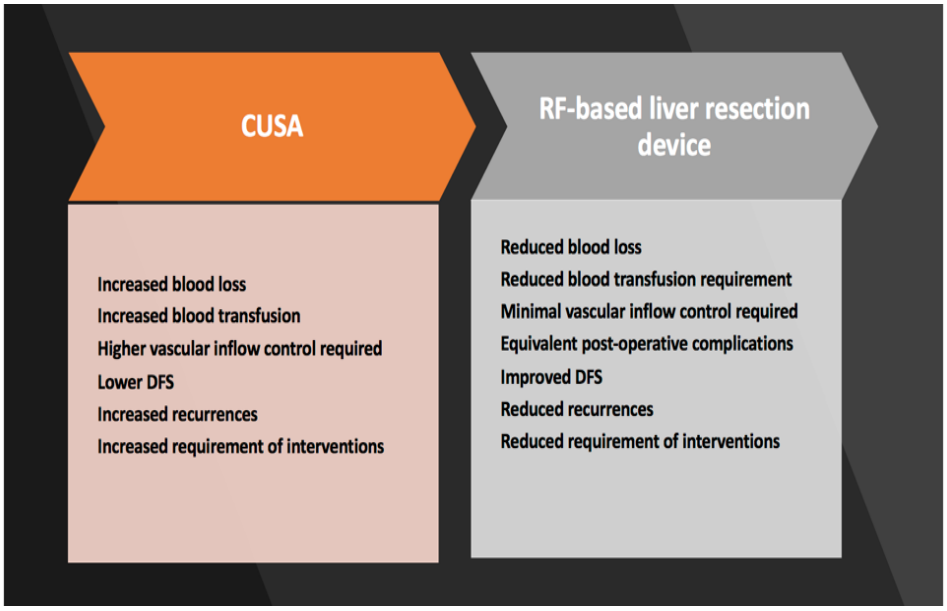

Figure 3: Summary of the outcomes comparing CUSA with RF-based liver resection device
Abbreviations: CUSA: Cavitron Ultrasonic Surgical Aspirator; DFS: Disease free survival: RF: Radiofrequency

3.3.4 Conclusion:

The given paper demonstrated a significant improvement in DFS and tumour recurrence with RF-based device in comparison to CUSA along with reduction in all other parameters including blood loss, blood transfusion and need for inflow occlusion during liver resection surgeries with the RF-based device Habib-4X. Thus, RF-based

device offers excellent short-term outcomes, which get translated into improved long-term outcomes.

In light of notable findings present in recent literature that induction of positive immunomodulatory changes have been implicated as contributing factors for the observed improved survival following radiofrequency ablation (RFA) of liver tumour [93,94]. Further research was conducted to comprehend the implications of RFA induced changes in immunological parameters in comparison to non-RF-based liver resection.

Chapter 4 Changes in immunological parameters and their implications

Evasions on the instinctive and connate anti-tumour immune response are essential prerequisites for the genesis of liver cancer. Liver cancer evolves on the framework of chronic inflammation; proinflammatory cells along with cytokines and other molecules present within the tumour microenvironment not only influence the natural course of the disease but also determine the survival and recurrence following surgical resection [129].

4.1.1 Study 4 (Appendix 4): Mazmishvili K, Kumar J, Janikashvili N, Kikodze N, Mizandari M, Pantasulaia I, Paksashvili N, Sodergren MH, Reccia I, Pai M, Habib N, Chikovani T. Study to evaluate the immunomodulatory effects of radiofrequency ablation compared to surgical resection for liver cancer. Journal of Cancer 2018;9(17):3187-95 [95].

Liver cancer is a state of generalized immunosuppression; thus oncogenesis is not only dependent on the genetics of tumoural cells but is also regulated by the microenvironment. Considerable evidence supports the theory that tumour site inflammatory state fosters malignant growth and progression of liver cancer [130,131]. Studies have outlined that increased neutrophilic and platelet infiltration within the tumour microenvironment contribute towards genesis, proliferation, metastasis and recurrences of liver cancers. The peritumoural accumulation of neutrophils and platelet and the relative increase in neutrophil-lymphocyte-ration (NLR) and platelet-lymphocyte-ration (PLR) are the signature of underlying state of chronic inflammation [132–135]. These ratios typically reflect the balance between neutrophils and platelet-associated pro-tumour systemic inflammation and lymphocyte dependent anti-tumour immune function. A tip in the balance towards increased NLR and PLR provides a nidus for tumour growth whilst, dysregulation of immune system helps cancer cells to elude immune surveillance.

The increase in NLR is associated with tumour invasion and metastasis of liver cancers through paracrine-mediated hepatocyte growth factor and consequently is contemplated as an independent predictor of survival following resection of tumour. Qi et al. [136] (2016) conducted a meta-analysis following review of 90 articles and

20,475 HCC patients, describing not only the role of NLR in recurrence, DFS and OS of HCC patients but also outlining the advantages following reduction in its level during the course of the disease.

Platelets induce an epithelial-mesenchymal-like transition and generate platelet-derived nucleotides which stimulate transendothelial migration and metastasis of tumour cells. One of the best predictors of the inflammatory and thrombotic state is PLR, which also acts as an inflammatory biomarker for liver tumours [137,138]. Lately, Zhao et al. [139] (2017) published a meta-analysis involving 10 articles and concluded that high PLR is an unfavourable predictor of overall survival.

In addition, ectonucleotidase CD39 (nucleoside triphosphate diphosphohydrolase-1 present on Tregs cells) upregulation is poorly associated with the outcome of HCC patients. CD39 overexpression in Tregs cells limits the NK cell activity and promotes the growth of metastatic liver tumours [140–142].

Thus, both of these inflammation based markers (NLR and PLR) and increased expression of CD 39 on CD4+ T lymphocytes influence the onset, proliferation and post-treatment recurrence of liver tumours with higher levels being associated with poor prognosis.

Based on the evidence that decrease in NLR, PLR and reduction in expression of ectonucleotidase CD39 on circulating CD4+ T lymphocytes might lead to improved survival in liver cancer patients. Herein, a prospective study was conducted to determine the extent of change in NLR, PLR and expressions of CD39 on circulating CD4+ T cells following radiofrequency ablation (RFA) and non-RF based liver resection device.

4.1.2 Material and methods:

In given paper, 17 liver cancer patients were prospectively distributed among two groups. RFA was performed in seven patients (RFA group) whilst the remaining 10

had hepatectomy by non-RF based liver resection device (non RF based liver resection group).

a) Pre and post-procedure blood samples were collected a month before and after the intervention to obtain the NLR, PLR ratio. Flow cytometry was used to quantify expression of CD39 on CD4+ T lymphocytes.

b) The NLR and PLR were calculated by dividing the absolute neutrophil and platelet count with absolute lymphocyte count.

Peripheral blood mononuclear cells (PBMCs) were isolated and CD39+ CD4+ subsets were detected using lineage-specific antibodies anti-CD39+. The data were assessed using the software BD FACSArray system.

c) All data were fed into a Microsoft Excel worksheet and analysis was performed with Graph Pad software. The differences between the groups were evaluated with Mann-Whitney U test and Mc Nemar test.

d) A P value of < 0.05 was considered statistically significant.

4.1.3 Results and implications:

The two groups were comparable in terms of tumours number, tumour type, size and stage, HBsAg status and anti-HCV status.

Levels of NLR and PLR were significantly reduced following RFA with respect to pre-ablative levels; however, no such observation was made in liver resection group. Contemporary evidence has marked higher NLR and PLR as predictors of recurrence and indicative of poor survival, likewise post treatment decline is associated with reduced recurrence and improved survival [143–146]. In addition, a significant decline was noted in CD4+CD39+ lymphocytes levels in RFA group, whilst in contrast to the increment in values after liver resection (Table 4). A reduction in CD4+CD39+ lymphocytes act as a marker for improved survival and declined tumour recurrence [147].

Table 4: Observed changes in respective groups before and after interventions

Parameters	Prior to RFA	Following RFA	P value	Prior to LR	Following LR	P value
NLR	4.7±3.3	3.8±1.8	0.283	3.5±2.8	4.5±3.2	0.183
PLR	140.5±79.5	137±69.2	0.386	116±42.2	120.8±29	0.391
CD39+ CD4+	55.8±13.8	24.6±21.1	0.030*	47.6±8.8	55.7±33.2	0.380

Difference between groups were analyzed by Wilcoxon's matched pairs signed rank test; * marks significant P value

Abbreviations: CD: Cluster of differentiation; LR: Liver resection; NLR: Neutrophil-lymphocyte-ration; PLR: Platelet-lymphocyte-ration; RFA: Radiofrequency ablation

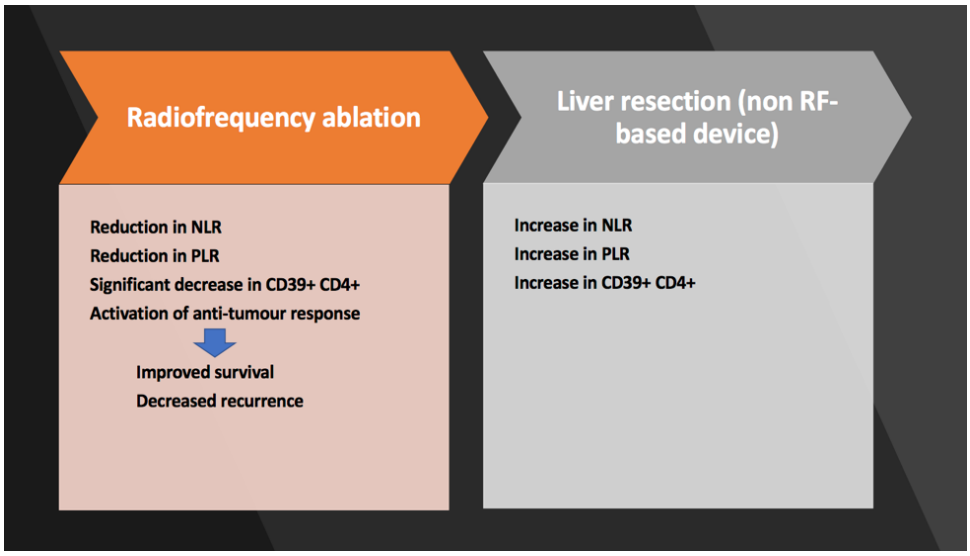

Figure 4: Summary of the immunological changes following RF ablation and liver resection using non RF-based device
Abbreviations: CD: Cluster of differentiation; NLR: Neutrophil-lymphocyte-ration; PLR: Platelet-lymphocyte-ration; RF: Radiofrequency

4.1.4 Conclusion:

Based on the above observations and taking into account of other published literature [146,148–151], the present study highlights that decrease in NLR, PLR and $CD39^{+}CD4^{+}$ T cells expression not only reciprocates with systemic inflammation, anti-tumour status immunity but also act as explicit markers to envisage better survival in liver cancer patients (Figure 4). However, considering the small population size further studies with large sample size is much warranted.

Considering these observations, further study was conducted to understand the immunological changes in terms of circulating immune cells and serum cytokines following liver resection with RF-based device in comparison to the contemporary device, CUSA.

4.2 Immunoediting hypothesis for the genesis of the hepatocellular carcinoma

The immunoediting hypothesis for the genesis of hepatocellular carcinoma points towards the development of malignant cells over the inflammatory framework with ability to circumvent an anti-tumour immune system. Literature suggests various possible mechanisms, of which the foremost is the mitigation in the presentation of tumour associated antigens or class I major histocompatibility complex (MHC) molecules to CD8+ T cells. Along with, enhanced expression of immunosuppressive Tregs cells and accumulation of various immunosuppressive checkpoint factors programmed death 1 (PD-1), programmed death ligand-1 (PD-L1) and cytotoxic T lymphocytes-associated antigen-4 (CTLA-4) [150,152,153]. Hence, the malignant cells induce the genesis of an extremely complicated matrix of immunosuppressive cells and cytokines to outmanoeuvre the anti-tumour immune response (Figure 5).

The dysregulated inflammatory microenvironment of liver includes changes in various immune cells and cytokines, which are worth mentioning here. The dysregulation comprise enhanced expression of Tregs cells, increase in TGF-β (Transforming growth factor beta) and IL-10 levels, debacle of CD8+ T cells and presence of immune checkpoints. The subset of CD4+ T cells, Tregs cells exhibit CD 25 & Foxp3 and have potential to repress anti-tumour immune reaction of host and might initiate the progression of tumour [140,154].

The increased serum Tregs cells levels and the Tregs/CD4+ T cell ratio are linked with poor prognosis and regarded as independent prognostic marker for DFS and OS [155,156]. Tumour associated macrophages (TAMs) pose a pivotal role in tumour microenvironment and along with Interleukin 10 (IL-10), Interleukin 13 (IL-13) and Toll like receptor ligands, get transformed into a subtype which can ensue tumour genesis and growth [157,158]. IL-10 is one of the important cytokines involved in the pathogenesis of HCC through the induction of immunosuppression [159,160]. Enhanced expression of IL-10 vitiates CD8+ T cells functioning and engenders Tregs cells differentiation via Foxp3 [160,161].

Similarly, increased levels of TGF-β in serum and tumour tissue of HCC patients are associated with poor survival [162]. TGF-β has an immunosuppressive property and along with IL-10 is involved in the genesis of Tregs cells and activation of hepatic stellate cells leading to growth and progression of tumour [163]. Considering the molecular pathogenesis of lowered anti-tumour immunity behind the development of HCC, the reinstatement of the anti-tumour immune response could provide better disease control [164,165].

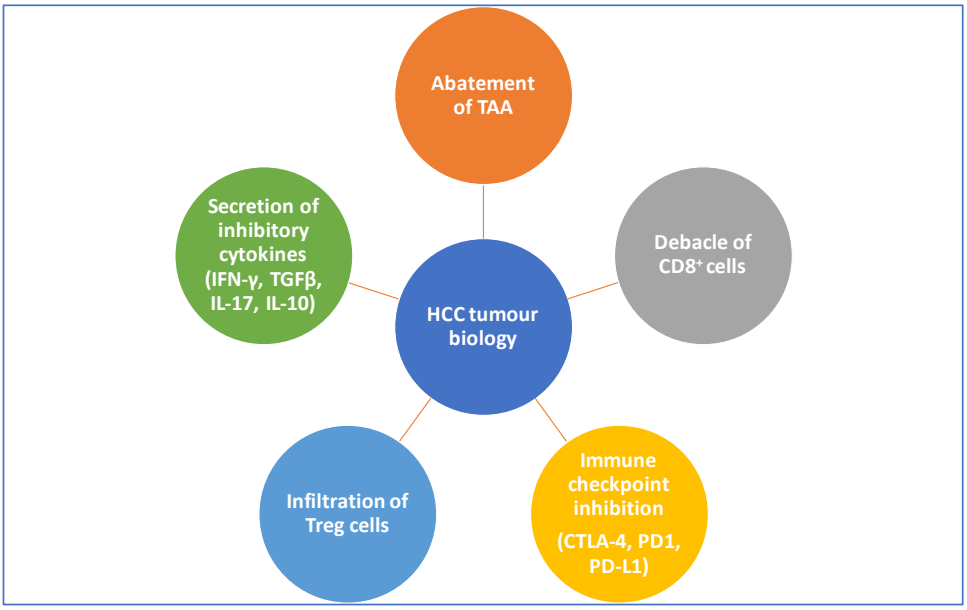

Figure 5: Immunoediting hypothesis for development of hepatocellular carcinoma
Abbreviations- CD: cluster of differentiation; CTLA-4: cytotoxic T lymphocytes-associated antigen-4; HCC: hepatocellular carcinoma; IFN-γ: interferon gamma; IL: interleukin; PD-1: programmed death 1; PD-L1: ligand of programmed death 1; TAA: tumour associated antigen; TGF-β: Transforming growth factor beta

The application of RF waves to HCC nodules heats (150 °C) the tissues and triggers ionic agitation and coagulative necrosis, causing irreparable cellular damage and releasing tumour debris or antigens; which in turn induces the systemic and peritumoural immune responses in an effort to control the development, growth and spread of malignancy [166–168]. Research has deduced that RF induced ablation of HCC nodules not only destroys tumour cells but also induces significant local and systemic immunological changes in the body to limit recurrence and improves survival

[169,170]. A meta-analysis conducted by Xu et al. [171] (2014) involving 31 studies and 16,103 HCC patients (≤2 cm tumours) reported a significantly improved DFS and OS following RFA in contrast to conventional liver resection. Moreover, enlisted following notable independent attributes such as microvascular invasion, blood loss, blood transfusion, micrometastasis beyond tumour margin, and satellitosis, which influence the patient survival after resection of HCC tumours [172,173].

The cellular infiltrates in the zone of coagulative necrosis following RFA of liver tumour include macrophages, neutrophils, dendritic cells (DCs), and NK cells. DCs induce CD8+ and CD4+ T lymphocytes to bring about a systemic and peritumoural immune response otherwise called as "in-vivo dendritic cell vaccine effect" [174–176]. The theory of anti-tumour response following RF waves application is further supported from literature with spontaneous regression of distant metastatic lesions after ablation of primary lesions [177,178].

4.3.1 Study 5 (Appendix 5): Huang K, Jayant K, Lee PH, Yang PC, Hsiao CY, Habib N, Sodergren MH. Positive immuno-modulation following radio-frequency assisted liver resection in hepatocellular carcinoma. Journal of Clinical Medicine 2019;8(3) [96].

Herein, the change in pre and post-liver resection levels of immunological parameters (cellular subtypes and cytokines) were determined in HCC patients. The liver resection was performed either with RF-based device or CUSA and further implications were drawn through comparison with contemporary literature.

4.3.2 Material and methods:

Here, 11 HCC patients were divided into two groups; 5 liver resections were performed with CUSA whilst in the remaining six patients procedure was accomplished by the RF-based device.

Blood samples were collected on day 0 (pre-procedure) and at day 7 following resection to allow analysis of immunological parameters: circulating cell populations (Panel 1) and serum cytokines (Panel 2).

Panel 1: Tregs cells, CD8+, CD4+, CD3+, CD4+CD45RO+/CD4+, CD4+CD39+/CD4+, NK, NKT cells.

The cell surface markers were detected with fluorescent-labelled antibodies: anti-CD39-FITC, FITC-CD45, PerCP-CD3, PE-CD8, CD161-DX12, anti-CD45RO-ECD, anti-CD45RA-ECD, PE-CD127, APC-CD25 and APC.Cy7-CD4 manufactured by BD Biosciences (San Jose, California). The flow cytometry was carried out using FACSVerse (Becton Dickinson, Mountain View, California). Data were processed utilizing FlowJo software (Ashland, Orlando).

Panel 2: IFN-γ, TGF-α, TGF-β, IL-1b, IL-6, IL-17, IL-10.

Cytokines levels were quantified through enzyme-linked immunosorbent assays (ELISA, R&D Systems, Abingdon, England). The assays did not assess the activity of the cytokines. All the observations were made twice to corroborate findings.

All the data were fed into a Microsoft Excel worksheet and analysed by IBM-SPSS version 24 (Chicago, IL, USA). Difference in continuous variables was assessed using a Student's t-test. Categorical variables were compared using a chi-square or Fisher's Exact Test, as appropriate. Pre- and post-hepatic resection immunomodulatory changes were determined with the paired Student's t-test. P values under 0.05 were considered statistically significant.

4.3.3 Results and implications:

Both intervention groups were comparable in terms of tumour numbers, tumour size, tumour stage, cirrhosis, serum albumin, serum bilirubin, serum AFP, HBsAg, HCV and ICG clearances.

Modulation in Circulating Immune Cells

Pre- and post-intervention levels of various T cells subtypes including CD8+ T cells, helper T cells (CD4 T cells), regulatory T cells (Tregs cells), and natural killer (NK) T cells were assessed and compared utilizing paired Student's t-test (Table 5a). There was a significant increase in CD8+ (P value = 0.050), CD4+ CD45RO+/CD4+ (P value = 0.002) and NKT cells (P value = 0.002) following liver resection in the RF-based device group; however, no such changes were discernible in the CUSA group. A significant decline in Tregs cells (P value = 0.002) and CD4+CD39+/CD4+ cells (P value = 0.002) in the RF-based liver resection group in comparison to the CUSA group. No statistically significant alteration was observed for cellular subtypes, NK cells and CD4+ cells in either group.

Modulation in circulating cytokine levels:

The RF-based liver resection device led to wanted changes in the concentration of cytokines, interleukins and chemokines IFN-γ, TGF-ß, interleukin (IL)-10, and IL-17 in contrast to CUSA group (Table 5b).

Table 5a: Pre & post interventions changes in circulating immune cells

Attributes		CUSA			RF-based device	
	Prior to surgery	Following surgery	P value	Prior to surgery	Following surgery	P value
$CD3^+$	1681.57 ± 384.25	1565.71 ± 459.78	0.819	1632.00 ± 392.68	1700.00 ± 445.35	0.721
$CD8^+$	515.71 ± 255.46	401.42 ± 98.39	0.291	580.0 ± 216.18	732.00 ± 188.31	0.050*
$CD4^+$	1085.71 ± 278.91	1095.71 ± 384.48	0.956	1008.00 ± 283.50	1028.00 ± 331.86	0.886
Tregs	24.57 ± 4.83	25.00 ± 3.36	0.850	27.20 ± 6.17	17.90 ± 5.26	0.002*
$CD4^+$ $CD45RO^+$ / $CD4^+$	44.71 ± 1.98	45.00 ± 4.43	0.879	44.60 ± 1.78	49.50 ± 4.03	0.002*
$CD4^+$ $CD39^+$ / $CD4^+$	36.29 ± 4.92	35.86 ± 4.38	0.866	36.90 ± 4.23	23.70 ± 8.49	0.000*
NKT	7.43 ± 1.90	8.14 ± 2.12	0.519	6.80 ± 1.62	10.60 ± 3.50	0.006*
NK	11.86 ± 3.02	11.57 ± 3.64	0.876	11.60 ± 2.32	10.90 ± 2.51	0.526

All variables represented (Mean ± SD)
Post intervention level of each variable within respective group, was compared with baseline value using paired Student's t-test to determine statistical significance
* marks significant P value
Abbreviations: CUSA: Cavitron Ultrasonic Surgical Aspirator; CD: Cluster of differentiation

Table 5b: Pre & post interventions changes in circulating cytokines

Attributes	CUSA			RF-based device		
	Prior to surgery	Following surgery	P value	Prior to surgery	Following surgery	P value
IL-17	58.00 ± 16.54	63.00 ± 15.35	0.569	52.6 ± 13.92	36.10 ± 13.55	0.010*
IL-1b	7.92 ± 1.47	7.90 ± 1.05	0.970	7.28 ± 1.69	9.39 ± 4.51	0.180
IL-10	7.47 ± 0.69	7.47 ± 0.50	1.000	7.93 ± 0.58	4.47 ± 1.47	0.000*
IFN-ϒ	45.57 ± 9.65	45.28 ± 10.73	0.959	48.20 ± 11.82	57.30 ± 7.41	0.027*
TGF-ß	2191.42 ± 400.43	1978.57 ± 478.83	0.385	2378.00 ± 382.35	1490.00 ± 366.60	0.000*

All variables represented (Mean ± SD)
Post intervention level of each variable within respective group, was compared with baseline value using paired Student's t-test to determine statistical significance
* marks significant P value
Abbreviations: CUSA: Cavitron Ultrasonic Surgical Aspirator; IFN-γ: interferon gamma; IL: interleukin; RF: Radio frequency; TGF-β: Transforming growth factor beta; Tregs: T regulatory cells

The positive changes in immunomodulatory cellular subsets and cytokines levels could be the plausible explanations for the better survival reported in the earlier study where RF-based liver resection was compared to CUSA. The immunomodulation is achieved through generation of neo-antigens following coagulation of resection margin; which are presented by DCs via MHC molecules to CD8+ and CD4+ T cells to engender an anti-tumour immune response [174,176,179].

In the present paper analysis was performed to determine absolute number of several immune cell populations. Statistically significant beneficial changes were observed in Tregs cells, CD4+CD39+/CD4+, cytotoxic CD8+ T cells, CD4+CD45RO+/CD4+ and NKT cells following the liver resection with the RF-based resection device in contrast

to CUSA. The T-cells infiltration on post-surgery day 07 was marked by significant increase in CD8+ T and CD4+CD45RO+/CD4+ cells, significant decline in Tregs cells and CD4+CD39+/CD4+ cells. These changes have been considered to move the scale toward anti-tumour immune response following the RF application [128, 153,155].

Additionally, high levels of TGF-ß and IL-10 have been associated with progressive disease [160,180] and the observed decline in their levels (P value <0.000 & <0.000; paired Student's t-test) not only halts the progress of inflammatory state but also has stifled the induction of Tregs cells and the functioning of NKT cell [181,182].

Cytokine IL-17 is a crucial determinant for the genesis of inflammatory background for HCC growth. The data of the index study reported a significant decline in IL-17 level whereupon liver resection RF based device in juxtapose to CUSA (P value = 0.010). Literature suggests expression of IL-17 acts as a marker to predict disease progression and survival and high levels are associated with worse outcomes [183–185].

CD45RO+ T cells are memory T cells engendered upon the cell-mediated immune response. These cells persist for considerable duration (months to years) and in the ensuing antigen exposure cause an enhanced immune response [186]. Recent studies have reported improved survival in colorectal, pancreatic and gastric cancer in association with increased infiltration of CD45RO+ T cells and marked them as an independent prognostic factor [187–189]. The meta-analysis has outlined increased intra-tumoural CD45RO+ T cells density with improved 5 years DFS [190]; Moreover, studies outlined that the observations such as increased serum CD45RO+ T cells, heightened CD4+CD45RO+/CD4+ ration and IFN-γ release not only lowered the threshold of T cells response to tumour antigens but also contribute towards prolonged anti-tumour immune response and better survival [150,152-154].

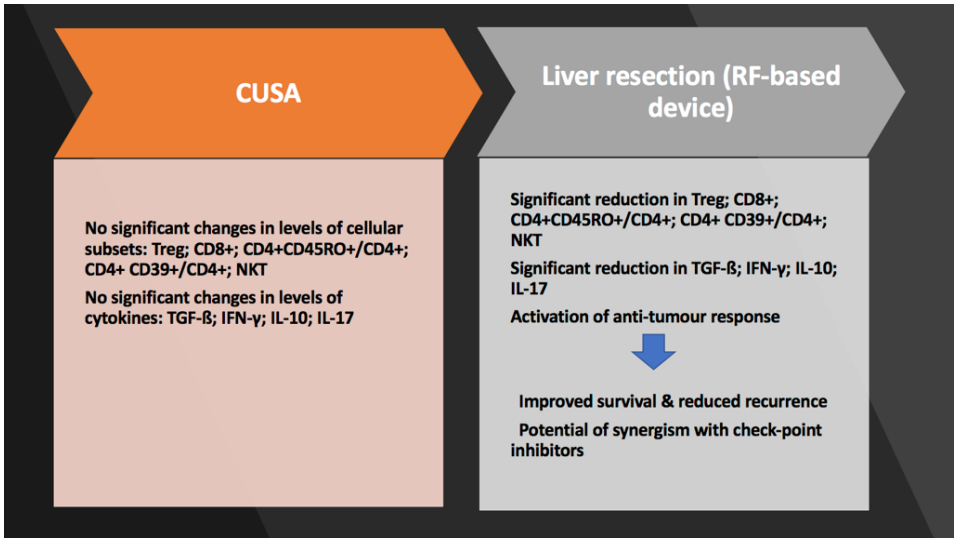

Figure 6: Summary of the immunological changes after CUSA and RF-based liver resection device

4.3.4 Conclusion:

The prognosis and risk of recurrence following resection of HCC tumour are determined by micrometastasis, satellite nodules, blood loss and blood transfusion. Based upon the observed significant changes in anti-tumour immune cells and cytokine levels following liver resection with RF-based device and increased understanding of the immune profile, there are significant positive implication on the survival (Figure 6). Improved DFS and reduced recurrences discerned following liver resection with RF-based device could be because of reinstatement of anti-tumour immunity, minimal blood loss and reduced transfusion requirement. Hence, the evidence is suggestive of the need for modification in BCLC guidelines and incorporation of RF-based liver resection as the primary surgical technique for the management of very early and early HCC cancers (Figure 7). However, further studies on a larger scale are much needed, to confirm the findings from the current study.

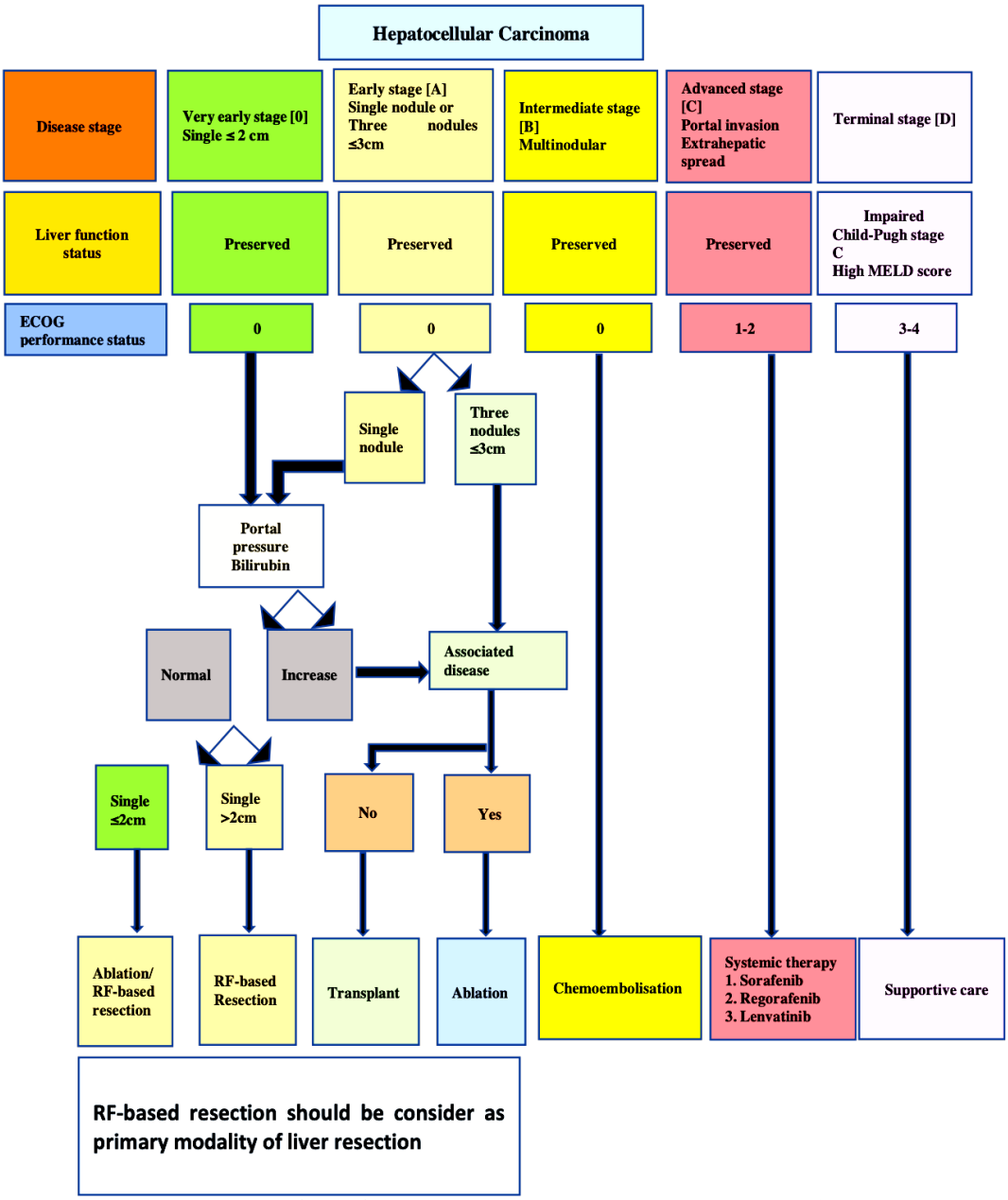

Figure 7: Barcelona Clinic Liver Cancer staging system with proposed changes in management of very early and early HCC tumour

5 Future Directives

The papers included in the index thesis suggest that RF-based liver resection should be offered to liver cancer patients needing hepatic resection as it is not only remove the tumour but also produces positive immunomodulatory changes that helps reinstating anti-tumour immunity towards the pristine state.

Additionally, a better understanding of mechanisms of immunomodulatory cells and cytokines in the genesis of liver cancer and advancement in immunotherapeutic drugs have led to the applicability RF-based liver resection with checkpoint inhibitors. Both CD8+ T cells and Tregs cells are primary regulators of the tumour microenvironment and are amenable to modification with RF-based therapies. RF increases CD8+ T cells infiltration towards tumour nodule and checkpoint inhibitors augment their activity, thus act in synergy to produce superlative effects [191,192].

Checkpoint molecules, CTLA-4, PD-1, and PD-L1 are important attributes involved in the suppression of T cell activation in the tumour microenvironment of liver carcinoma [152]. The expression of CTLA-4 is enhanced following activation of T cells and consequently, the coupling of CTLA-4 and its ligand not only inflict anergy in T cells but also increase the population of inhibitory Tregs cells. The clinical trials with anti-CTLA-4 monoclonal antibodies (Tremelimumab and Ipilimumab) have demonstrated their potential to deplete Tregs cells and reverse the exhaustion of T cells with intent to reinstituting the anti-tumour immune state [191–193].

Similarly, tumour microenvironment induce expression of PD-1 on reactive T lymphocytes and PD-L1 on tumour cells or tumour associated macrophages. The coupling of PD-1 with PD-L1 leads to inactivity of T cells and NK cells functioning. The development of anti-PD-1 (nivolumab and pembrolizumab) and anti-PD-L1 (atezolimumab and avelumab) can reactivate these cells [194,195]. PD-1 and PD-L1 are expressed in up to 30%-60% of HCCs [196,197] and recently published study by Farha et al. [196] have observed 20 months longer survival in this group of patients. Likewise, checkpoint inhibitors are also expressed in metastatic liver cancers secondary to primaries from colorectal, melanoma, lung, oesophageal and breast

carcinoma [198–200]. Pembrolizumab, tremelimumab, nivolumab, durvalumab, and avelumab are few notable anti-PD-1 drugs, currently in various stages of trials for the management of melanoma, Hodgkins lymphoma and various solid tumours such as HCC [201,202].

Combining radiofrequency ablation with checkpoint inhibitors seems a pragmatic approach to invigorate an anti-tumour immune response in HCC cells. Duffy et al. assessed clinical response of combining ablation with anti-CTLA-4 (tremelimumab) in 19 advanced HCC patients and outlined partial response in five patients (26.3%) (95% confidence interval, 9.1%–51.2%) with improved median overall survival of 12.3 months (95% confidence interval, 9.3–15.4months) [203]. Similarly, radiofrequency ablation with checkpoint inhibitors is also being tested presently in clinical trial for advanced CRLM [204].

Moreover, as HCC forms an intricate immunosuppressive network to evade anti-tumour immunity through CD8+ T cells; the proof of concept suggest that synergism of RF-based liver resection and checkpoint inhibitors can not only remove tumour tissue but also help in reinstating anti-tumour immunity to aid in improving survival and preventing recurrences. However, future studies are required to better understand the applicability of RF-based liver resection with checkpoint inhibitors in very early and early stage of HCC and metastatic liver cancers.

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Appendix 1 Study 1

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Radiofrequency-assisted liver resection: Technique and results

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ABSTRACT

Background: Radiofrequency (RF)-assisted liver resection allows non-anatomical liver resection with reduced blood loss and offers the opportunity for a combination of resection and ablation. However, there are still concerns with regard to postoperative complications related to this technique. In the present study, we discuss the technical aspects of RF-assisted liver resections and analyse the rate of perioperative complications, focusing on post-hepatectomy liver failure (PLF), bile leak and abscess, and mortality.

Methods: Between 2001 and 2015, 857 consecutive open and laparoscopic elective RF-assisted liver resections for benign and malignant liver tumours were reviewed retrospectively to assess perioperative outcomes.

Results: Median intraoperative blood loss was 130 mL, with 9.8% of patients requiring blood transfusion. Intra-abdominal collections requiring percutaneous drainage developed in 8.7% of all patients, while bile leak at resection margin developed in 2.8% of the cases. Major liver resection was performed in 34% of patients and the incidence of PLF was 1.5% with one directly related mortality (0.1%).

Conclusion: RF-assisted liver resection has evolved into a feasible and safe technique of liver resection with an acceptable incidence of perioperative morbidity and a low incidence of PLF and related mortality.

1. Introduction

Despite technical advances, liver resection remains a complex surgical procedure requiring great expertise and excellent clinical judgment [1]. Liver resection is associated with perioperative complications, which negatively affects disease-specific survival [1].

Post-hepatectomy liver failure (PLF) remains one of the most serious complications, occurring in up to 10% of cases, with subsequent mortality rate as high as 10% [1]. Post-hepatectomy liver failure is the principal cause of mortality after major hepatectomy and correlates with size and quality of the future liver remnant [2].

As major hepatectomies are still associated with higher morbidity and mortality, parenchymal-sparing liver resection has gained acceptance worldwide, as demonstrated by the large evidence available in the literature particularly for colorectal liver metastases [3–5]. Parenchymal-sparing liver resection can achieve comparable oncological outcomes with fewer perioperative complications and can enhance the chance of future resection [4]. Different techniques have been developed to preserve residual liver volume, whilst maintaining adequate surgical margins [6]. Among them, radiofrequency (RF) assisted liver resection induces coagulative necrosis of the liver parenchyma, reducing the risk of intraoperative bleeding and allowing a combination of resection and ablation during the same procedure [7]. However, concerns about the risk of perioperative complications after RF-assisted liver resection still remains in the surgical community [8].

Since 2001, we have been successfully using RF for open and laparoscopic liver resections [7,9]. RF-assisted hepatectomy requires meticulous application, excellent knowledge of liver anatomy and accurate performance of intraoperative ultrasound. This technique has evolved and improved over the years. The aim of this study was to highlight the incidence of postoperative complications, especially liver-related complications, after RF-based liver resection in our tertiary care centre and to review the resection technique for hepatic tumours located in different lobes of the liver.

Abbreviations: HCC, hepatocellular carcinoma; HDU, high dependency unit; ICU, intensive care unit; ISGLS, International Study Group of Liver Surgery; PLF, post-hepatectomy liver failure; RF, radiofrequency

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2. Methods

2.1. Study population

We prospectively collected our data at our tertiary referral hospital of patients who underwent RF-assisted liver resection for benign and malignant liver tumours from 2001 to 2015. The RF devices used included both monopolar (Radionics Europe N.V., Wettdren, Belgium) and bipolar (Habib™ 4X, Angiodynamics Inc., USA) devices. Resection of three or more segments was considered as a major resection according to Couinaud's classification [10].

2.2. Surgical technique with bipolar device

Following liver mobilisation, bimanual palpation of the liver and intraoperative ultrasound (US), the resection margin was marked with argon diathermy. Ideally, a 2 cm resection margin is produced by two parallel lines of ablation. There were, however, situations where this could not be obtained. For these cases a resection line closer to the tumour was performed with the first line of ablation performed 1 cm away from the tumour, and the second parallel line made between the first line and the tumour edge. The partial interruption of the blood supply allowed a quicker resection and resulted in 12–14 mm area of ablated liver parenchyma. The safe division of portal and hepatic veins was easily accomplished without any application of sutures or clips. Once the two vertical lines of ablation were completed, a transverse line was then applied perpendicular to the parallel tracks (Fig. 1).

For deep-seated tumours, the device was introduced at a 45-degree angle and guided posteriorly by the surgeon's hand, to protect the inferior vena cava. During insertion of the device, the four needles were kept parallel to each other by a push off plate, this prevented the needles from touching each other and producing an electrical short-circuit (Fig. 2).

The probe was moved 3–5 mm in its vertical axis during the coagulation process to avoid ablated liver parenchyma adhering to the probes. Several transverse applications were required to connect the vertical lines and ensure complete ablation, after which resection was commenced with a scalpel outside the four needles on the tumour side, i.e. between the device and the tumour edge. The needles were used as a guide to prevent any deviation from the resection line. At the end of the resection, the device was used to arrest residual bleeding, if any, at the resection margin.

If significant bleeding was seen at the exit point following the removal of the needles, it was important not to re-apply RF at the same site as this led to liver parenchymal desiccation and disintegration while the bleeding continued. In cases of unwarranted desiccation of liver parenchyma, there could be difficulty in controlling haemorrhage as sutures tended to cut through the excessively ablated hepatic tissue. In these instances, RF ablation was applied in different planes away from the bleeding site (Fig. 3). Using this technique, haemorrhage was controlled in almost all cases, Pringle's manoeuvre being required in only 0.6% cases (n = 5).

In cases of challenging hepatic tumours located anatomically close to major vessels and bile ducts, the Habib™ 4X was used to ablate the tumour centrally to cause shrinkage. This helped tumour excision by creating a plane away from the vessel that needed to be preserved thus avoiding a major anatomical resection.

Hilar tumours limited the use of RF energy, as ablation could not be applied within a 1 cm circumference of biliary or vascular structures that had to be preserved. In hilar cholangiocarcinomas the hilum was first dissected, and after the contralateral hilar structures were well delineated RF energy was applied.

RF energy was applied to the anterior aspect of the quadrate lobe, caudate lobe and the adjoining part of the gallbladder bed for cancer of the gallbladder. A swab soaked in cold water was placed over the common bile duct to prevent heat-induced injury. Cold water was used to irrigate continuously during ablation near the hilus.

The resection line for the ablation of quadrate lobe was on both sides of the midline (Cantlie's line), umbilical fissure and then transversally adjoining the two planes. The direction of the needle entry was very important and was made more posteriorly with the tip pointing anteriorly in front of the hilum with guidance from the surgeon's finger. It was important to draw two lines along the transverse plane to give sufficient ablated length of the middle hepatic vein.

For the resection of the posterior part of quadrate lobe and segment VIII, a circular area of liver parenchyma was ablated with the intention of excising the tumour as a “cauliflower”. The ablation proceeded in three planes: the first near the liver surface with the needle inserted vertically, an angled oblique in the second and transverse for the third application. The resection was carried out with extreme caution as the

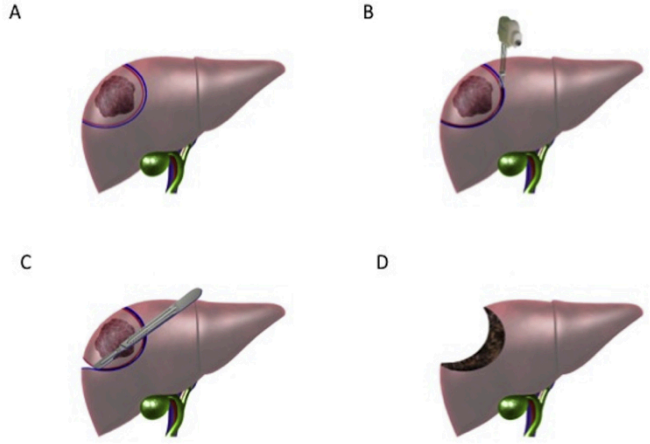

Fig. 1. Radiofrequency-assisted liver resection
A. Pictorial depiction of tumour in the liver
B. Resection technique involves creation of parallel ablation lines following sequential application of probe 1 cm adjacent to tumour
C. Liver resection at proximal margin of 12–14 mm width adjacent to tumour
D. Pictorial representation of post tumour resection liver parenchyma.

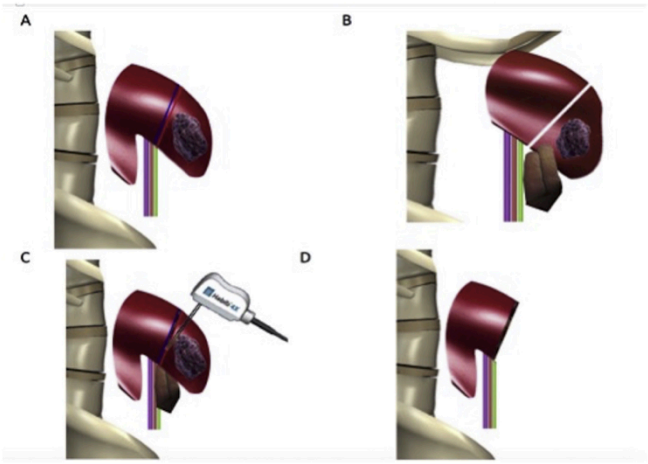

Fig. 2. Radiofrequency-assisted liver resection for deep-seated tumours
A. Pictorial depiction of deep-seated tumour in the segment of liver.
B. Resection technique involves positioning of surgeon's hand in posterior aspect of the involved liver segment.
C. Sequential positioning of probe 1 cm adjacent to tumour margin creates 12–14 mm width necrotic liver parenchyma.
D. Pictorial representation of post tumour resection liver parenchyma.

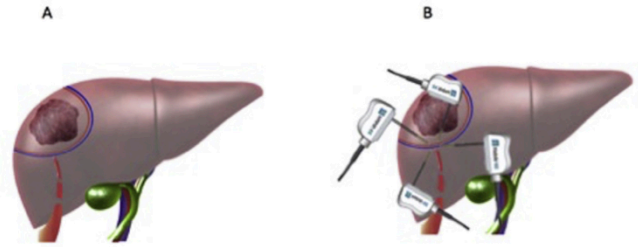

Fig. 3. Control of bleeding after Radiofrequency-assisted parenchymal transection
A. Figure shows blood loss in some cases following the removal of the probe.
B. Pictorial representation of RF ablation application in different planes away from the bleeding site (top, bottom, anterior, posterior, right, left) to stop blood loss.

blood supply invariably derived from the deepest part of the tumour.

In case of R2 resection, the high temperature generated at the probe site obliterates malignant cells of the tumour remnant at the resection margin, minimising risk of tumour spillage and seeding, even in cases of inadvertent rupture of the tumour.

2.3. Data collection and statistics

Parameters examined included age, gender, co-morbidities, and characteristics of the tumour. Perioperative morbidity outcome data included blood loss, blood transfusion, high dependency unit (HDU)/intensive care unit (ICU) admission, PLF, bile leak, symptomatic collection, pleural effusion needing intervention, thoracic empyema, hospital acquired pneumonia and mortality. Post hepatectomy liver failure was classified according to the classification of the International Study Group of Liver Surgery (ISGLS) [2] and symptomatic collection was defined as any postoperative intra-abdominal collection requiring intervention or surgical drains being left in situ after discharge [9]. Postoperative 30-day mortality was considered a better indicator of mortality in patients undergoing liver surgery, as accepted by the scientific community [11]. The data was analysed by using MedCalc_ 2004 software (ver. 7.4.3.0).

3. Results

3.1. Patients and tumour characteristics

Eight hundred fifty-seven patients underwent RF-assisted liver resection between January 2001 and December 2015. There were 468 males and 389 females with a median age of 61 (range 19–89) years. The most common diagnosis was colorectal metastases (57.4%, n = 492), followed by cholangiocarcinoma and gallbladder cancer (9%, n = 77). Liver resection for hepatocellular carcinoma (HCC) was performed in 9% (n = 77) of the cases. Several other liver lesions were also resected, as shown in Table 1. (15.8%, n = 136).

Minor liver resections (wedge resection and segmental resection) were performed in 565 patients (66%), predominantly for anterior, left lateral and right posterior lesions. Major liver resection was performed

Table 1
Patient, tumour characteristics and type of hepatic resections performed.

Characteristics	Finding
Total number	*857*
Age (median, range, years)	61 (19–89)
Sex, M:F	1.2:1
Diagnosis	*No. (%)*
Hepatocellular	77
Colorectal liver metastases	492
Other metastases	136
Cholangiocarcinoma and gallbladder	77
Benign lesions	75
Resections	No. (%)
Total number	*857*
Major liver resections	*292 (34.0)*
Right hepatectomy	161
Left hepatectomy	60
Extended right/left hepatectomy	18
Three or more segmentectomies	53
Minor liver resections	*565 (66.0)*
One or two segments	433
Wedge resections	132

in 292 patients (34%), mostly for metastatic liver disease, and either due to the large size of the tumour or to multiple metastases in the same hemi-liver (Table 1).

3.2. Peri-operative morbidity and mortality

Intraoperative blood less was than150 mL in the majority of the cases (93.1%, n = 798), with a median of 130 mL (Table 2). Major bleeding (> 1500 mL) occurred in 59 cases (6.9%), with an estimated blood loss of above 2000 mL in only 19 cases (2.2%). Blood transfusion was required in 84 cases (9.8%) and re-laparotomy for bleeding occurred in 5 cases (0.6%). ICU/HDU admission was required in 36 cases (4.2%), with the majority of patients directly transferred from the recovery room to the surgical ward.

Symptomatic collections developed in 91 patients (10.6%). Among these patients, 75 out of 91 (82.4%) required percutaneous drainage (8.7% of all patients) and 16 out of 91 (17.6%) were managed with antibiotics and/or keeping the surgical drain in situ for longer (1.9% of all patients) (Table 2). Bile leak was seen in 37 cases (4.3%). Of those, 24 occurred at the parenchymal resection margin (2.8%). The remaining 13 cases occurred at biliary anastomotic sites, which were performed in addition to parenchymal resection. The incidence of PLF was 13 cases (1.5%) with one mortality directly related (0.1%). This was a 74 year-old patient who underwent right hepatectomy for colorectal liver metastases, and who developed PLF and died from septic shock. Amongst patients who developed PLF, 4 had underlying cirrhosis and 9 had extended hepatectomy. Liver failure developed in only 4 minor resection cases (0.5%). According to the classification of the ISGLS, we had 5 cases of Grade A PLF; 3 cases of Grade B; and 5 cases of Grade C, including the patient who died from PLF.

Table 2
Postoperative morbidity and mortality following hepatic resection.

Postoperative outcome	No. (%)
Total number	*857*
ITU Admission, No. (%)	36 (4.2)
Blood loss, median (range) mL	130 (0–4300)
Blood transfusion, No. (%)	84 (9.8)
Morbidities	No. (%)
Symptomatic collection, No. (%)	91 (10.6)
Bile leak, No. (%)	37 (4.3)
Liver insufficiency, No. (%)	13 (1.5)
Pleural effusion needing intervention, No. (%)	27 (3.1)
Thoracic empyema (VATS), No. (%)	5 (0.5)
Hospital acquired pneumonia, No. (%)	30 (3.5)
Re-laparotomy (%)	9 (1.0)
30-Day mortality, No. (%)	13 (1.5)

Postoperative complications have been classified also according to Clavien-Dindo Classification, as shown in the supplemental table [12].

Four (0.4%) patients had a re-laparotomy, for small bowel obstruction, colonic perforation, jejunal perforation and leak from the biliary anastomosis. Pleural effusions were the commonest postoperative complication (22%, n = 189), percutaneous chest drainage required in only 3.1% (n = 27) and thoracic surgery necessary in 5 cases (0.5%) for thoracic empyema. Hospital acquired pneumonia developed in 3.5% (n = 30) of cases. Overall, there were 13 postoperative deaths (1.5%). One death was due to PLF, as already discussed; another one was due to major haemorrhage from splenic bed varices following hospital discharge. The other 11 deaths were due to cardiac complications (n = 4); sepsis and multiorgan failure (n = 3); pulmonary embolism (n = 2); mediastinitis following Boerhaave's syndrome (n = 1); and right heart failure secondary to pulmonary hypertension (n = 1).

4. Discussion

Experienced liver surgeons are able to perform liver resections with good results using different surgical techniques and devices, but the choice of the technique depends essentially on the surgeon's preference. [6].

The parenchymal transection phase has a considerable impact on bleeding, bile leak, and survival [13]. Cirrhotic patients and patients who had prolonged courses of chemotherapy, and those with steatohepatitis, are at a higher risk of PLF, prolonged ICU stay, sepsis and mortality following a major hepatectomy [14]. Post-hepatectomy liver failure is a major prognostic factor in patients undergoing hepatic resections [1]. In our series, the incidence of PLF was 1.5%, with a single directly related mortality (0.1%). This compares well with other large series in the literature, where incidence of PLF ranges from 2.0% to 18.9%, with related mortality of 0.6%–2.3%. [15–18] Other studies have reported that major liver resection, intraoperative blood loss and blood transfusion are independent risk factors for developing PLF. [19] Our data supports the logical approach of non-anatomical, parenchymal preserving resection as PLF was mostly observed after major hepatectomy. These findings are particularly relevant as they underline the importance of preserving functional liver parenchyma [20].

In line with our earlier published studies we reported reduced blood loss (130 mL average loss per case), decreased need for blood transfusions (9.8%), and decreased use of Pringle's manoeuvre (0.6%), all of which helped minimise the level of insult to the functioning liver remnant and thus offered a rapid recovery. [9,21] Occasionally persistent bleeding was encountered when withdrawing the probe. This was effectively dealt with by applying the RF probe from “different directions” around the bleeding site, instead of placing it in the “same area”.

Only 4.2% patients (n = 36) needed ICU/HDU admission in the post-operative period, and this was often a pre-planned event due to other co-morbidities. The remaining 95% of patients routinely returned to the surgical ward on the same day following the operation, thus significantly limiting NHS expenditure on ICU/HDU stay or risk of cancellation of the procedure due to non-availability of ICU/HDU bed.

Furthermore, RF assisted hepatectomy could carry an increased risk for postoperative bile leak and abscess formation due to the necrosed tissue left at the resection margin. [8,22–26] However, this evidence is based on few randomized controlled trials on other different RF devices and on articles with smaller numbers compared to our current series.

We reported an incidence of bile leak of 4.3%, but in 13 cases a biliary reconstruction was also performed. Therefore, our incidence of bile leak from the resection margin was 2.8%. The incidence of bile leak in recent large series (above 500 cases, similar percentages of major and minor resections) ranges from 2.2% to 15.6%, with most series reporting an incidence above 4.8%. [16,17,27–34] Therefore, RF-assisted

liver resection does not increase the risk for bile leak despite the number of minor, non-anatomical resections performed.

In our series, the incidence of symptomatic intra-abdominal collections was 10.6%, which were managed by either keeping the surgical drain in situ for longer (1.9%) or with additional percutaneous drainage (8.7%). Our results are comparable to other techniques, as in recent large series the incidence of intra-abdominal collections ranged from 2.9% to 12% [17,28–30,32–34].

Whilst there are few studies with inconclusive results comparing the outcomes of RF-assisted hepatectomy with other techniques, RF seems to be potentially superior to clamp-crush technique in cirrhotic patients, as Li et al. reported in a randomized trial that there was less blood loss, no requirement for Pringle manoeuver and lower morbidity in the RF group. [26,35–37] Recently, Qiu et al. demonstrated that patients with HCC who underwent liver resection with the Habib 4X had shorter operative time, less blood loss, and less blood transfusions in comparison with patients in whom the clamp-crush technique was used, with no difference in complication rates. [38] However, the Habib 4X group showed better survival, especially for larger tumours [38].

Although our study has some limitations, including the fact that it is a single centre retrospective study, with no statistical comparison with other techniques, our results show that RF is safe, with low incidence of PLF and comparable rates of bile leak and intra-abdominal collections. Therefore, we believe that there is insufficient evidence to conclude that RF carries higher risks of liver-related complications in comparison with other techniques.

5. Conclusion

RF-assisted liver resection technique has evolved into a safe and efficient technique for liver resection. The meticulous application of the RF-assisted technique helps in preserving the maximum future liver remnant and in minimising perioperative complications, in particular PLF.

Conflicts of interest

Nagy Habib is a shareholder and director of EMcision Limited, which developed the Habib 4X. This device is no longer manufactured or currently marketed by this company.

Phil Retsas is working for EMcision Limited, which has developed the Habib 4X, one of the devices cited in this article which is not manufactured or currently marketed by this company.

All other authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.suronc.2018.05.024.

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Appendix 2 Study 2

A systematic review and meta-analysis comparing liver resection with the Rf-based device habib™-4X with the clamp-crush technique [91].

cancers

MDPI

Review

A Systematic Review and Meta-Analysis Comparing Liver Resection with the Rf-Based Device Habib™-4X with the Clamp-Crush Technique

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Abstract: Liver cancer is the sixth most common cancer and third most common cause of cancer-related mortality. Presently, indications for liver resections for liver cancers are widening, but the response is varied owing to the multitude of factors including excess intraoperative bleeding, increased blood transfusion requirement, post-hepatectomy liver failure and morbidity. The advent of the radiofrequency energy-based bipolar device Habib™-4X has made bloodless hepatic resection possible. The radiofrequency-generated coagulative necrosis on normal liver parenchyma provides a firm underpinning for the bloodless liver resection. This meta-analysis was undertaken to analyse the available data on the clinical effectiveness or outcomes of liver resection with Habib™-4X in comparison to the clamp-crush technique. The RF-assisted device Habib™-4X is considered a safe and feasible modality for liver resection compared to the clamp-crush technique owing to the multitude of benefits and mounting clinical evidence supporting its role as a superior liver resection device. The most intriguing advantage of the RF-device is its ability to induce systemic and local immunomodulatory changes that further expand the boundaries of survival outcomes following liver resection.

Keywords: liver cancer; liver resection; radiofrequency; Habib™-4X; clamp-crush technique

1. Introduction

A central tenet to liver surgery lies in the complete oncological resection with minimal morbidity [1,2]. Advancement in liver resection techniques over the last few decades has resulted in improved morbidity, mortality and long-term survival. Consequently, the speciality of hepatobiliary surgery has experienced tremendous growth; however, liver resections are still contemplated as a high-risk surgical procedure with a mortality of ~5% and a morbidity of up to 40% [3–5]. Currently, liver resections are considered pertinent in the management of a wide variety of benign and malignant liver tumours including haemangiomas, colorectal liver metastases, hepatocellular carcinoma and hilar cholangiocarcinomas, with significantly reduced mortality in comparison to the earlier medical care available for such ailments and have added a significant number of productive years to patients' life-span [6–9]. This improvement can be attributed to a number of factors including, increased use of

parenchyma-sparing resections, lower intraoperative central venous pressure, better patient selection, ipsilateral portal vein embolization, staged resections in advanced diseases, the advent of newer devices for parenchymal transection, further improvements in perioperative patient management, and so forth [10–14].

Moreover, the development of non-surgical treatment modalities such as chemotherapy regimens, trans-arterial chemoembolisation (TACE), percutaneous radiofrequency ablation (RFA), microwave ablation, electroporation and cryotherapy could serve as an adjunct to the surgical management and produce a positive impact on the survival of liver cancer patients [15–20]. In particular, attention has turned towards liver resection owing to its ability to achieve oncological clearance; however, that comes with the price of several procedure-specific complications and increased perioperative morbidity, which may influence the disease-specific survival rates [21,22]. The foundation of liver resection surgery is the clamp-crush (CC) technique, which is regarded as a gold standard method of liver parenchymal transection, albeit that the post-resection outcomes are often limited by excessive bleeding, massive blood transfusions, bile leak and increased postoperative morbidity and mortality [23,24].

The advent of specialized liver resection devices with newer techniques for liver resection has introduced a new era in the surgical management of liver tumours [25,26]. Despite the continuous efforts to improve the surgical outcomes of liver resections, the intraoperative bleeding during liver parenchyma transection has remained a matter of prime concern and is often implicated in poor postoperative outcomes. The increased haemorrhage and blood transfusions have a negative implication of morbidity with increased bile leak, post-hepatectomy liver failure (PHLF), symptomatic collection, abscess, prolonged ICU stay, poor survival and mortality [27–29]. A vast majority of clinical studies have outlined the importance of limiting the intra-operative bleeding and blood transfusion in improving the morbidity and mortality of patients following hepatectomy [30–34].

Poon et al. demonstrated a significant decline in the morbidity and mortality from 37.0% to 30.0% and 7.5% to 3.7%, respectively, in a group of patients following hepatic resection with a median intra-operative blood loss of 750 mL and blood transfusion in 17% of cases, in contrast to a group with a median intra-operative blood loss of 1450 mL and blood transfusion in 68% of cases [35]. In addition, Yang et al. outlined increased intra-operative bleeding (≥800 mL) during hepatectomy as an independent risk factor of perioperative morbidity [36]. Furthermore, the present evidence suggests compromised oncological outcome and increased recurrence of hepatocellular carcinoma (HCC) in patients receiving blood transfusion perioperatively during hepatectomy [29,37]. The pre-eminence of decreasing perioperative blood loss and blood transfusion has been recognised, and different strategies have been implemented to limit the blood loss during parenchymal transection in liver surgery [38]. The hepatic vascular inflow occlusion technique or Pringle manoeuvre has been described, although it has limited applicability in patients with underlying liver disease secondary to heightened risk of ischemic reperfusion injury, incompetence to impede the back-flow bleed from hepatic veins and enhanced risk of ischaemic reperfusion (I/R) injury [39,40].

Radiofrequency (RF) energy can create an avascular plane for liver resection, this principle being first introduced by our group at Imperial College London, where later, a liver resection device, the bipolar Habib™-4X (Angiodynamics Inc., Latham, NY, USA), was developed [41]. Liver resection surgery has been transformed following the introduction of this device, which has facilitated the bloodless techniques of hepatic resection [41–43]. The radiofrequency-generated coagulative necrosis onto normal liver parenchyma creates a resection margin adjacent to the tumour. The coagulative necrosis helps in sealing of the blood vessels and bile ducts before transection of the parenchyma, hence limiting the requirement of the Pringle manoeuvre and blood transfusion [44,45]. The device is simple, cost effective, facilitates safe and efficient liver resection and fosters the practice of parenchymal sparing liver resections with a significant reduction in bleeding and subsequent perioperative complications [46,47]. To date, several retrospective and prospective (randomized or non-randomized studies) have been reported comparing various techniques of liver resections' however, a meta-analysis

comparing "bloodless technique of liver resection" (Habib™-4X) with the standard clamp-crush (CC) technique is lacking [48–52].

In spite of much literature available on liver resection, intriguingly, the quest to find the best technique and tool is still on. The present meta-analysis was undertaken to analyse the available data on the clinical effectiveness or outcomes following liver resection with Habib™-4X in comparison to the clamp-crush technique.

2. Materials and Methods

2.1. Search Strategy

We performed this meta-analysis following completion of registration (CRD42018085616) in PROSPERO, an international database of prospectively-registered systematic reviews. The search strategy was fundamentally designed according to the guidelines mentioned in the Cochrane Handbook for Systematic Reviews of Interventions and reported as per the guidelines proposed by the Meta-analysis of Observational Studies in Epidemiology [53,54].

A detailed literature search was completed on MEDLINE, EMBASE, Cochrane, Crossref, Scopus and clinical trial registries assessing the role of the RF-based device Habib™-4X in liver resection as an alternative to traditional the clamp-crushing (CC) technique. The search covered the period 2001 (the year of the first reported use of monopolar (Radionics Europe N.V., Wettdren, Belgium) and bipolar (Habib™-4X, Angiodynamics Inc., Latham, NY, USA) devices) to 4 July 2018 [41]. The medical subject headings (MeSH) 'Liver resection' OR 'Radiofrequency device' OR 'Habib-4X' 'clamp-crush' OR AND 'Hepatocellular Carcinoma' OR 'Colorectal liver metastases' OR 'Cholangiocarcinoma' OR 'Hepatic metastasis' were searched, adapting to each database without any limitation, to complete the analysis. The last search was completed on 10 September 2018. Further, all available conference abstracts, bibliographies and citation lists of the relevant articles were searched manually for additional studies.

2.2. Inclusion Criteria

The prospective or retrospective studies comparing the RF-based device (Habib™-4X) with the clamp-crush (CC group) technique of liver resections were selected for this meta-analysis. Further, all other available literature including editorials, reviews and letters were excluded. The primary outcomes of interest were blood loss, the requirement of blood transfusion and operative time. The secondary outcomes were adverse events, bile leak, post-hepatectomy liver failure, abdominal abscess, pleural effusion, overall hospital stay, morbidity and 30-day mortality (Table 1).

Table 1. Criteria for the inclusion of studies.

Study Design	Retrospective, Prospective, Randomized or Non-Randomized
Study group	Liver resection
Study size	Any
Length of follow-up	Any
Source	Peer-reviewed journals
Language	Any
Outcome measure	Primary: blood loss, blood transfusion, operative time; secondary: bile leak, post-hepatectomy liver failure, abdominal abscess, pleural effusion, overall hospital stay, morbidity and 30-day mortality

2.3. Data Extraction

The initial screening for the study was done independently by two separate physician reviewers, Kumar Jayant and Mikael H. Sodergren, who employed a two-stage method, the first stage involving scrutiny of titles and abstracts while excluding obviously ineligible studies. At the second stage, the full texts were considered in explicit detail to exclude ineligible studies. In the event of disagreement, disputes were resolved via consensus, and matters for which consensus could not be made were settled

after much deliberation with the senior author NH. The complete search strategy and study selection were contemplated, performed and outlined according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines (Figure 1).

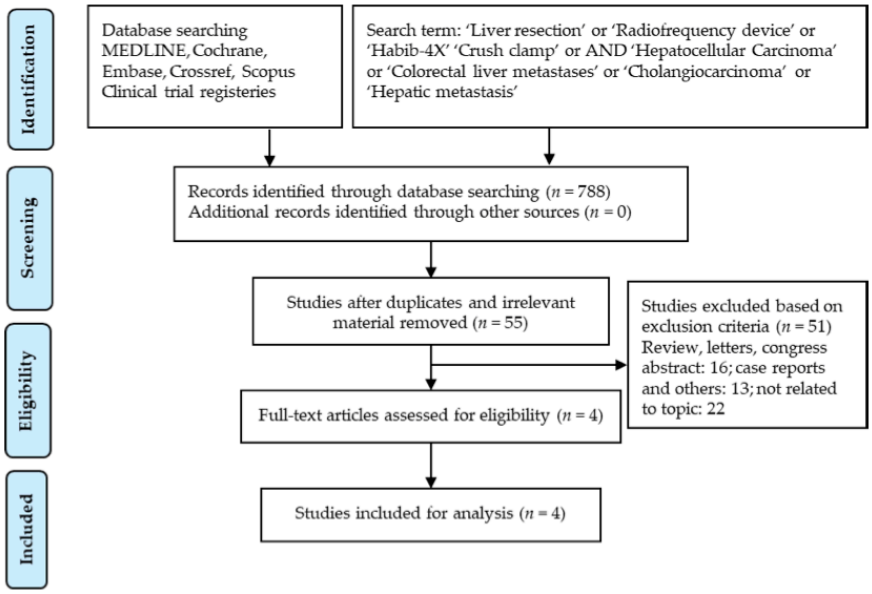


Figure 1. Search strategy and study selection used in this systematic review as per the PRISMA protocol.

2.4. *Statistical Analysis*

The validity of pre-specified inclusion and exclusion criteria of the included studies was determined by using the Cochrane Risk of Bias tool. Each study was thoroughly analysed to evaluate the above-mentioned parameters (Table 2). The Cochrane Collaboration Review Manager (RevMan) Version 5.3 can analyse a minimum of two trials with the available continuous and dichotomous data. The effect measures used were mean difference (MD) for continuous data and odds ratio (OR) for dichotomous data, with 95% confidence intervals (CI). In the case of continuous data presented as median and range, the statistical methods described by Hozo et al. were applied to calculate the mean and standard deviation [55].

The heterogeneity (I^2) between the trials was considered low with an I^2 value $\leq$25%, moderate with an I^2 value >25%, but <75% and higher with an I^2 value of $\geq$75%. An I^2 statistic of more than 30% was determined to be significant. In the stance of significant heterogeneity, the random effects model assessment was used following the evaluation of the forest plot while the fixed-effect model was applied in the situation of low heterogeneity [56,57]. Unfortunately, publication bias could not be assessed in the present study, as it requires at least 10 trials to assess it, and our current meta-analysis involved only four trials [58].

Table 2. Characteristics of studies included in the meta-analysis.

Study	Publication Year	Study Design	(Clamp-Crush) (CC) Group	Habib™-4X Group	Liver Disease	Operative Time (Minutes) (CC vs. Habib™-4X)
Li et al. [51]	2012	Randomized (Prospective)	37	38	HCC	188.7 ± 62.1 vs. 193.7 ± 50.5 (p = 0.28)
Guo et al. [52]	2015	Retrospective	325	272	HCC	295.9 ± 107.3 vs. 211.2 ± 63.2 (p = 0.00)
Zhang et al. [50]	2015	Retrospective	79	100	HCC	245.6 ± 75.5 vs. 230.5 ± 77.9 (p = 0.19)
Qiu et al. [48]	2017	Retrospective	102	81	HCC	196.0 ± 54.0 vs. 160 ± 61.0 (p = 0.00)

Abbreviations: CC, Clamp-crush technique; HCC: hepatocellular carcinoma.

2.5. Surgical Technique

The liver resection techniques involving the RF-based device Habib™-4X have been outlined in our previous publication [59].

3. Results

3.1. Search Results

The primary literature search yielded a total of 788 manuscripts; of these, 784 articles were excluded following careful evaluation of the previously described selection criteria. After resolution of differences between reviewers, a total of four studies were retrieved for further review and data extraction [48,50–52]. These include three published papers on retrospective studies [48,50,52] and one with a prospective randomized study [51] (Table 2). The detailed data of all the studies related to the duration of surgeries' adverse events, blood loss, requirement of blood transfusion, bile leak, post-hepatectomy liver failure, liver abscess, pleural effusion, length of hospital stay and 30-day mortality and are summarized in Tables 2 and 3. All attributes of the analysed outcomes are structured further in this section.

3.2. Blood Loss and Quantity of Blood Transfused

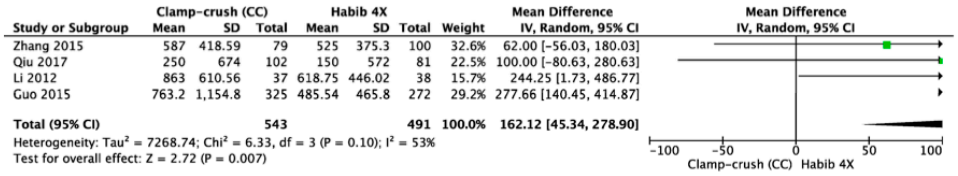
The blood loss (mL) was significantly lower in the Habib™-4X group (MD = 162.12, 95% CI 45.34 to 278.90, p = 0.007, I^2 = 53%) (Figure 2a). This outcome was determined by four studies [48,50–52], with a moderate heterogeneity between them. The Habib-4X and CC group included 491 and 543 patients, respectively.

Table 3. Post-hepatectomy analysis of outcomes in included studies.

Study	Blood Loss (mL) (CC vs. Habib™-4X)	Blood Transfusion (CC vs. Habib™-4X)	Bile Leak (CC vs. Habib™-4X)	PHLF (CC vs. Habib™-4X)	Pleural Effusion (CC vs. Habib™-4X)	Abdominal Abscess (CC vs. Habib™-4X)	Total Morbidity (CC vs. Habib™-4X)	Mortality 30 Days' (CC vs. Habib™-4X)
Li et al. [51]	863.0 ± 610.5 vs. 618.7 ± 446.0 (p = 0.001)	10 vs. 6 (p = 0.23)	6 vs. 4 (p = 0.46)	NA	5 vs. 4 (p = 0.69)	2 vs. 1 (p = 0.54)	16 vs. 11 (p < 0.001)	0 vs. 1 (p = 0.321)
Guo et al. [52]	763.2 ± 1154.8 vs. 485.54 ± 465.8 (p = 0.003)	103 vs. 52 (p = 0.000)	21 vs. 22 (p = 0.44)	12 vs. 16 (p = 0.44)	27 vs. 13 (p = 0.40)	16 vs. 20 (p = 0.21)	88 vs. 83 (p = 0.35)	NA
Zhang et al. [50]	587.0 ± 418.6 vs. 525.0 ± 375.3 (p = 0.01)	19 vs. 17 (p = 0.24)	2 vs. 7 (p = 0.31)	0 vs. 6 (p = 0.04)	4 vs. 6 (p = 1.0)	3 vs. 3 (p = 1.0)	14 vs. 28 (p = 0.11)	0 vs. 3 (p = 0.23)
Qiu et al. [48]	250.0 ± 6 74.0 vs. 150.0 ± 572.0 (p = 0.005)	25 vs. 10 (p = 0.03)	3 vs. 2 (p = 0.89)	2 vs. 1 (p = 1.0)	NA	NA	9 vs. 4 (p = 0.39)	0 vs. 0 (p = 0.99)

Abbreviations: CC, clamp-crush technique; PHLF, post-hepatectomy liver failure; NA, not available.

(**a**)



(**b**)

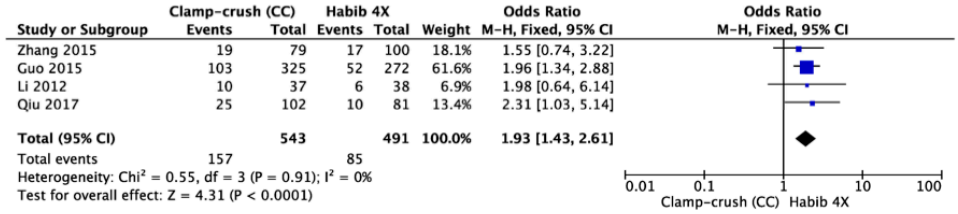


Figure 2. Forest plot representing the (**a**) total blood loss (mL), (**b**) number of patients requiring blood transfusion during liver resection comparing the control group (clamp-crush) with the study group (Habib™-4X). Squares' size depicts the effects while comparing the weight of the study in the meta-analysis. The diamond shows the significant favour towards the study group (Habib™-4X) following the analysis. The 95 percent confidence interval is represented as horizontal bars.

Four studies reported the number of patients requiring blood transfusion in the two groups with low heterogeneity between the studies [48,50–52]. The Habib™-4X group received a statistically lesser amount of transfusion (MD = 1.93, 95% CI 1.43 to 2.61, $p < 0.0001$, $I^2 = 0\%$) (Figure 2b).

3.3. Operative Time

Four studies reported operative time in minutes in the two groups with high heterogeneity between studies [48,50–52]. The random effects model revealed no statistical difference in terms of duration of surgery (MD = 33.59, 95% CI −6.32 to 73.51, $p = 0.10$, $I^2 = 94\%$) (Figure 3).

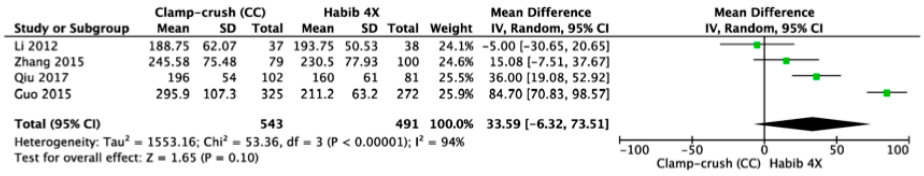


Figure 3. Forest plot representing the operative time (minutes) during liver resection comparing the control group (clamp-crush) with the study group (Habib™-4X). Squares' size depicts effects while comparing the weight of the study in the meta-analysis. The diamond shows no favour towards any study group following the analysis. The 95 percent confidence interval is represented as horizontal bars.

3.4. Serious Adverse Events

The rate of bile leakage was studied by four studies [48,50–52], with 543 patients in the CC group and 491 patients in the Habib™-4X group and a heterogeneity of 0%. The pooled data showed no difference between the groups (OR = 0.82, 95% CI 0.50 to 1.35, $p = 0.43$, $I^2 = 0\%$) (Figure 4a).

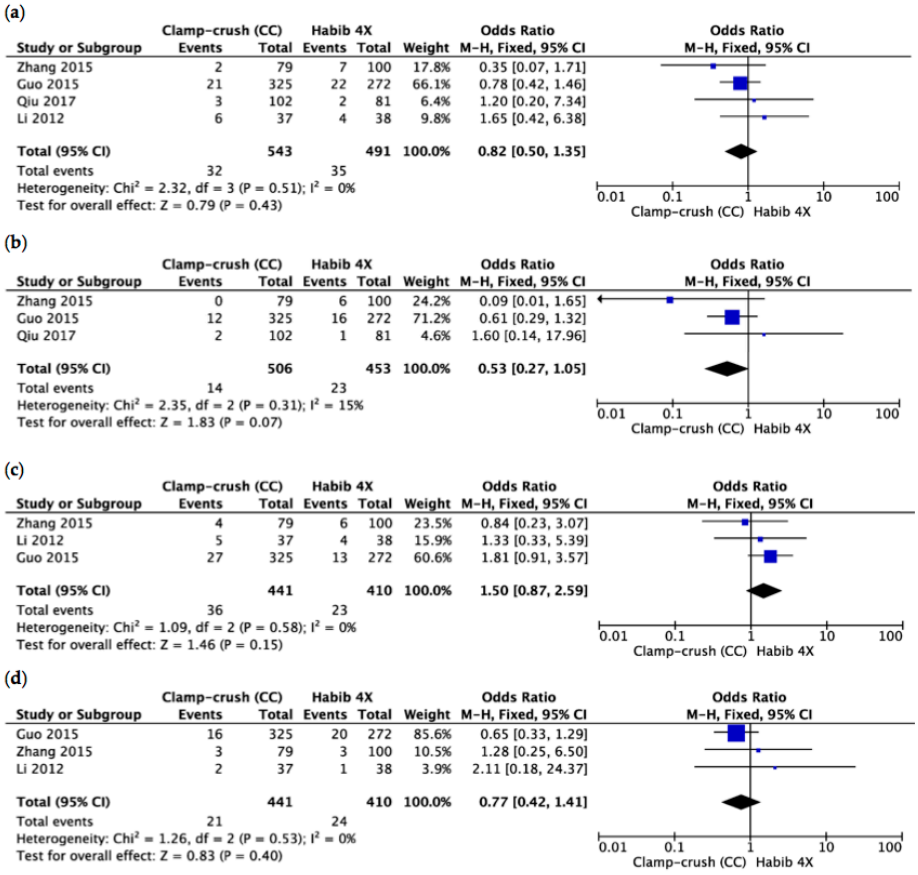


Figure 4. Forest plot representing the (**a**) bile leakage, (**b**) post-hepatectomy liver failure (PHLF), (**c**) pleural effusion and (**d**) abdominal abscess following liver resection comparing the control group (clamp-crush) with the study group (Habib™-4X). Squares' size depicts effects while comparing the weight of the study in the meta-analysis. The diamond shows no favour towards any study group following analysis. The 95 percent confidence interval is represented as horizontal bars.

The PHLF incidence was reported in three of four included studies with low heterogeneity between them [48,50–52]. Both groups were statistically equivalent in term of given complications (OR = 0.53, 95% CI 0.27 to 1.05, $p = 0.07$, $I^2 = 15\%$) (Figure 4b).

We analysed pleural effusion rate given in three studies with low heterogeneity and found no difference between the Habib™-4X group and the CC group (OR = 1.50, 95% CI 0.87 to 2.59, $p = 0.15$, $I^2 = 0\%$) (Figure 4c). Similarly, there was no difference regarding abdominal abscess rate between them (OR = 0.77, 95% CI 0.42 to 1.41, $p = 0.40$, $I^2 = 0\%$). This outcome was reported by three studies with low heterogeneity between them (Figure 4d).

3.5. Length of Hospital Stay, Total Morbidity and 30-Day Mortality

The length of hospital stay was reported by four studies with low heterogeneity between them. In the fixed-effect model, there was no difference between the Habib™-4X group and the CC group (MD = 0.60, 95% CI −0.04 to 1.24, $p = 0.07$, $I^2 = 0\%$).

Three studies reported total morbidity, with 543 patients in the CC group and 491 patients in the Habib™-4X group. There was moderate heterogeneity between studies. The random effects model showed that both groups were comparable (OR = 0.89, 95% CI 0.67 to 1.19, $p = 0.44$, $I^2 = 45\%$) (Figure 5a). Similarly, there was no difference in terms of 30-day mortality between them (OR = 0.23, 95% CI 0.03 to 1.99, $p = 0.18$, $I^2 = 0\%$). This outcome was outlined by three studies with low heterogeneity between them (Figure 5b).

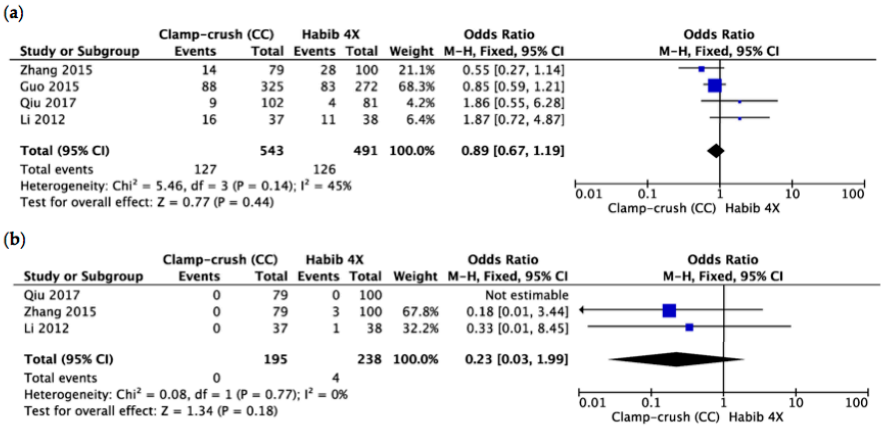


Figure 5. Forest plot representing (**a**) total morbidity and (**b**) 30-day mortality during liver resection comparing the control group (clamp-crush) with the study group (Habib™-4X). Squares' size depicts effects while comparing the weight of the study in the meta-analysis. The diamond shows no favour towards any study group following analysis. The 95 percent confidence interval is represented as horizontal bars.

4. Discussion

Operative blood loss and blood transfusion are common complications of any surgical procedure; however, these are rather concerning in liver resection and are often associated with increased perioperative morbidity and mortality [29,37,60]. Intraoperative bleeding more commonly occurs during the hepatic parenchymal transection phase; hence, newer surgical modalities have been developed to target the real Achilles' heel of liver resection and facilitate optimal transection with minimal blood loss [61]. Recognizing the vital role of minimizing the bleeding during liver resection, the "bloodless hepatectomy technique" was developed by Habib et al. [41]. Since, multiple publications have reported the benefit of this technique [48,49,59]; however, some researchers believe that the clamp-crush technique with inflow occlusion is still a reliable methodology of hepatic parenchymal transection [62–67].

The Pringle manoeuver and clamping of the hepatic pedicle is the most traditional and effective methodology to minimize blood loss during liver surgery [68–71], albeit the pathophysiological implications in the form of induction of liver ischaemia and ischaemic reperfusion injury are concerning and particularly unpredictable in patients with decreased hepatic reserve [62,72]. The intermittent vascular occlusion technique has been introduced as an alternative to lessen the degree of ischaemic injury to the liver parenchyma during hepatic resection [73]. Studies demonstrated equivalent bleeding control with similar or less deterioration in postoperative hepatic function and comparable operative time [74,75]; however, they did not result in a statistically-significant decrease in adverse event, morbidity, length of hospital stay and mortality, leading to a blunted enthusiasm to use this modality in hepatic resection. In addition, a meta-analysis by Rahbari et al. (2008) included eight randomized control trials containing 558 patients and concluded that the routine application of portal triad clamping

does not add any benefit over no portal triad clamping and ought not to be practiced as a standard procedure [76].

In the presence of ongoing contention, over the safest and most efficient technique for liver resection, the present meta-analysis combines and quantifies the direct evidence present in studies and provides a systematic evaluation and statistical analysis of all the available outcomes with RF-based resection in comparison with the clamp-crush technique of hepatic resection. To our knowledge, this is the first meta-analysis comparing the outcomes of liver resection performed by the RF-based device Habib™-4X with the clamp-crush technique and eloquently outlines the broader picture of this practice with primary focus on blood loss and blood transfusion and subsequent complications. The data analysis was conducted using a rigorous methodology, which led to a sample size of 543 patients, who underwent liver resection with the CC technique, to 491 patients with the RF-based device Habib™-4X and demonstrated intriguing results. Further, in the discussion, we have highlighted our findings and the impact of both surgical modalities in liver resection.

Blood loss and blood transfusion have been frequently implicated in the increase in morbidity and mortality subsequent to hepatic resection. The pooled data of our meta-analysis not only demonstrated a significant reduction in the blood loss in the Habib™-4X group, but also showed the decreased requirement of blood transfusion. One of the major benefits of the Habib™-4X device is reduced blood loss without portal triad clamping, which is invariably often required in CC techniques and approximately one third of liver resections performed with the Cavitron ultrasonic surgical aspirator (CUSA) [26].

In an attempt to investigate the role of three different hepatic resection techniques: CC, CUSA and bipolar device (LigaSure), Doklestic et al. (2012) conducted a randomized clinical trial and demonstrated no differences in terms of intraoperative blood loss, blood transfusion, postoperative complications and mortality; however, all the patients involved in the trial had ischemic preconditioning and intermittent inflow occlusion [77]. Therefore, the decrease in operative blood loss and blood transfusion requirement observed here was considered secondary to inflow occlusion, which did not get translated into improved postoperative outcomes or reduced rate of complications. The plausible explanations for the observed findings could be ascribed to the higher degree of ischaemic insult to the hepatic tissue and increased risk of I/R, which may be more pronounced in livers with underlying disease such as cirrhosis or fatty changes [78–80]; however, this has no value in the case with the RF-based device Habib™-4X, as reduced blood loss observed during liver resection was owed to RF-induced coagulation of the liver tissue.

The postoperative infectious complications are considered as one of the important reasons for the morbidity and mortality observed following hepatic resection and remain as a matter of prime concern during recovery. Previous single centre's experience reported increased rates of abdominal abscess following RF-based liver resection compared to the CC technique [81]. In contrast, Li et al. (2013) reported the incidence of abdominal abscess as 2.6% and 5.4% in the RF-Habib™-4X and CC group, respectively [51].

The review of the available literature in the present meta-analysis has demonstrated no difference in terms of the abdominal abscess in the compared groups. The data analysis of all the included studies in the index meta-analysis reported bile leak; however, no statistically-significant difference was observed in the present meta-analysis, which is in accordance with the reported incidence of bile leak of ~10% seen the literature.

Further, pooled data analysis outlined no statistically-significant differences in terms of PHLF, pleural effusion, hospital stay, total morbidity and mortality. The effectiveness of the RF-based device Habib™-4X has been demonstrated in several published papers, and the outcomes are remarkable with both cirrhotic and non-cirrhotic patients; the plausible explanations of these observed benefits are inscribed in the basic tenets on which this device was built. The RF-induced coagulation not only limits the blood loss and the requirement of blood transfusion, but also prevents any ischemic insult to the hepatic parenchyma.

The present meta-analysis has a few limitations, which need to be acknowledged, and caution ought to be exercised whilst interpreting these results, especially owing to the observed clinical heterogeneity between the included studies. The random effects model for pooled data analysis was used to limit the shadow of heterogeneity. Publication bias could not be excluded because of the limited number of included studies. Here, we could only identify four trials, and thus, further large-scale trials would provide much-needed data to allow firmer conclusions and elucidate the role of the RF-based device Habib™-4X in liver resection. Despite these limitations, this meta-analysis has outlined the safety and benefits of the Habib™-4X liver resection device in terms of reduced blood loss and decreased requirement of blood transfusion.

A recent study by Qiu et al. (2017) demonstrated better survival benefits following resection of liver tumour with the RF-based device Habib™-4X and outlined significant other benefits over the clamp-crush technique, notably due to minimal blood loss and less requirement of blood transfusion [48]. Similarly, Huang et al. (2017) compared the RF-based liver resection device Habib™-4X with CUSA and outlined significantly better disease-free survival [82]. The survival benefits observed in these studies could be a translation of better oncological outcomes associated with the interaction of the RF-assisted device with T-cells in the tumour microenvironment. The proliferative and energetically-dysregulated nature of tumour cells bring a metabolic dearth of the tumour microenvironment, which inflicts T-cells and creates "metabolic checkpoints" afflicting their endurance to survive, proliferate and function explicitly. The direct receptor-ligand interactions, expressing co-inhibitory ligands like programmed death-ligand 1 (PD-L1), inhibit CD8+ tumour-infiltrating lymphocytes' function through programmed death-1 (PD-1). In addition, there are certain other checkpoint molecules in the tumour microenvironment, which negatively regulate T-cells' functionality and are worth mentioning here, including cytotoxic T lymphocyte-associated protein-4 (CTLA-4), lymphocyte activating gene 3 (Lag3), mucin domain containing-3 (Tim-3), T-cell immunoglobulin and T-cell immunoreceptor with Ig. A "checkpoint inhibitor" such as anti-CTLA-4 and anti-PD/PD-L1 binds with these co-inhibitory checkpoint molecules and counter-inhibits the downregulation of T effector function, hence reinstating anti-tumour activity.

The exact reasons are not explicitly well defined, but could be explicable after the systemic and local immunomodulatory effect generated following the application of radiofrequency energy over tumour mass, causing T-cells' infiltration into the tumour microenvironment [83].

The induced systemic antitumour immunity overcomes the challenges of micrometastases, which often escape destruction and are held responsible for the recurrence of hepatocellular carcinoma. The debris produced following RF-induced coagulative ablation during liver resection generated tumour antigens and chemokines, enticing the immunoprotective infiltrates, macrophages, neutrophils, dendritic cells (DCs) and NK cells. Dendritic cells activate the nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κβ) pathway, which stimulates CD8+ and CD4+ T-lymphocytes and promotes a systemic immune response also known as the "in-vivo dendritic cell vaccine effect" [84–87]. The increased understanding of the immunological behaviour of CD8+ T-cells has added a new perspective in the management advanced tumours and led to the development of newer drugs as checkpoint inhibitors, which augment the CD8+ T cells [88,89]. The potential effect is superlative as checkpoint inhibitors act in synergy with the RF-based device. Here, RF induces the infiltration of CD8+ T-cells at the resection margin, while checkpoint inhibitors augment the activity. In accordance, Duffy et al. (2017) conducted a study and demonstrated the activation of the immune system following introduction checkpoint inhibitors and the accumulation of intratumoral CD8+ T-cells after RF ablation, thus establishing the synergism of the combined use of checkpoint inhibitor tremelimumab and ablation in the management of advanced hepatocellular carcinomas [90,91]. The advantages of combining the checkpoint inhibitor with RF-energy continue to be unravelled. In particular, further knowledge and research are required to elucidate the effects induced through the combined application of RF-energy with immunotherapies that escalate the antitumour adaptive immune system exponentially. Nevertheless, the enhanced anticancer immune response exhibited

through the synergism of RF-energy with immunotherapies has enormous potential for oncologic treatment for the coming years ahead.

5. Conclusions

To recapitulate, the RF-assisted device Habib™-4X is considered a safe and feasible modality for liver resection in reference to the clamp-crush technique owing to a multitude of benefits and mounting clinical evidence supporting its role as a superior liver resection device (Figure 6). The most intriguing advantage of the RF-device is its ability to induce systemic and local immunomodulatory changes that further expand the boundaries of survival outcomes following liver resection. Furthermore, recent studies have outlined that the synergism of RF-energy and checkpoint inhibitors could have role in the management of advanced HCC tumours; however, further studies focusing on RF-energy and checkpoint inhibitors are required to ascertain the applicability of this combination.

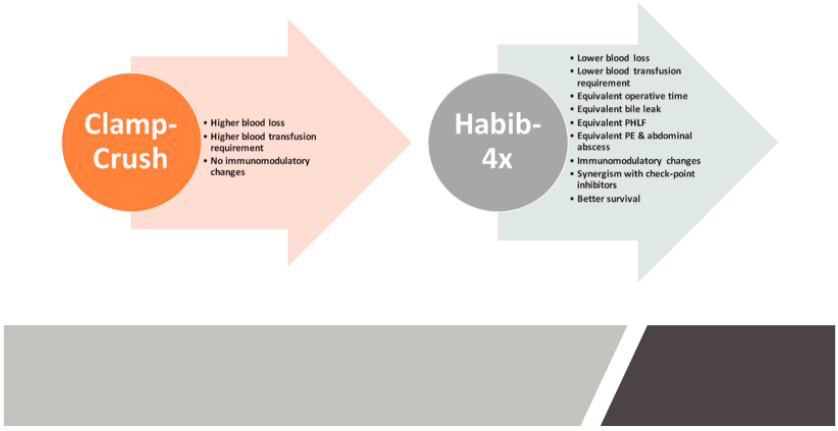


Figure 6. Comparative summary of the benefits of Habib™-4X based liver resection over the crush-camp technique. PHLF: Post hepatectomy liver failure; PE: Pleural effusion.

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Appendix 3 Study 3

Impact of cavitron ultrasonic surgical aspirator (CUSA) and bipolar radiofrequency device (Habib-4X) based hepatectomy for hepatocellular carcinoma on tumour recurrence and diseasefree survival [92].

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Research Paper

Impact of cavitron ultrasonic surgical aspirator (CUSA) and bipolar radiofrequency device (Habib-4X) based hepatectomy for hepatocellular carcinoma on tumour recurrence and disease-free survival

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ABSTRACT

Background: The aim of this study was to evaluate the oncological outcomes of hepatocellular carcinoma patients undergoing liver resection using cavitron ultrasonic surgical aspirator (CUSA) or radiofrequency (RF) based device Habib-4X.

Study Design: We prospectively analyzed the data of 280 patients who underwent liver resection for hepatocellular carcinoma at our institution from 2010–2012 with follow up till August 2016. The CUSA was used in the 163 patients whilst Habib-4X in 117 patients. The end points of analysis were oncological outcomes as disease recurrence, disease-free survival (DFS) and overall survival (OS) were estimated by the Kaplan–Meier method, which has been compared with all other existing literature on the survival study.

Results: Compared with CUSA the reported incidence of recurrence was significantly lower, in Habib-4X group; $p < 0.01$. The median DFS was significantly better in Habib-4X group than CUSA group (50.80 vs 45.87 months, $p = 0.03$). The median OS was better in Habib-4X group than CUSA group (60.57 vs 57.17 months, $p = 0.12$) though the lesser difference in OS between the groups might be explained by the use of palliative therapies as TACE, percutaneous RFA, etc. in case of recurrence.

Conclusions: RF based device Habib-4X, is safe and effective device for resection of hepatocellular carcinoma, in comparison to CUSA with better oncological outcomes, i.e., significantly lesser tumour recurrence and better DFS. This could be explained on the basis of systemic and local immunomodulatory effect involving induction of kupffer cells and effector CD-8 T cells that help in minimizing postoperative complications and bring more advantageous oncological outcomes.

INTRODUCTION

Hepatocellular carcinoma (HCC) has been reported as the fifth most common malignancy and considered as one of the aggressive cancer of mankind. In 2012, the worldwide incidence of HCC was 782,000 with mortality of 746,000 per year [1]. In the present era, surgical resection of the tumour has been considered as the standard treatment option for early Barcelona classified HCC patients. Moreover, the indication for surgical resection has widened owing to improved knowledge of liver anatomy, anaesthetic techniques, intraoperative ultrasounds and other imaging techniques [2, 3]. In the last two decades surgical world has witnessed the introduction

of many devices to ease the technical challenges imposed with liver resection, though the device of choice has remained an issue of debate till date.

The initial prototype technique of liver resection is clamp crush or finger fracture is associated with high incidence of intraoperative bleeding during parenchymal transection and is the major obstacle to surgical success [4]. Excessive blood loss and blood transfusions have further ratified the perioperative morbidity, infections and mortality. Furthermore, it is associated with an increased risk of HCC recurrence [5, 6]. To limit the blood flow during parenchymal transection an infamous hepatic vascular inflow occlusion technique or Pringle manoeuvre has been introduced into the practice although its applicability is limited particularly in patients with underlying liver disease owing to an increase risk of ischemic reperfusion injury and inability to control back-flow bleed from hepatic veins [7, 8].

Early era of liver surgery has evidenced a mortality of 10-20%, which significantly reduced to 5% with further advancements in the medical science, equipment and operative techniques [9]. Various equipments' of liver resection, such as the CUSA (Cavitron Ultrasonic Surgical Aspirator), RF based Habib-4X, Ligasure (Valley Lab, Tyco Healthcare, Boulder, CO, USA), Harmonic Scalpel (Ethicon Endo-Surgery, Cincinnati, OH, USA), TissueLink (Salient Surgical Technologies, Portsmouth, NH, USA), Water-jet dissection, microwave assisted resection, vascular staplers, and others have been introduced to facilitate easy and safe resection of liver parenchyma. Despite that, the question regarding the clinical benefit of using one over the other still remains unanswered [10].

Cavitron Ultrasonic Surgical Aspirator (CUSA), also known as Ultrasonic Dissector has been first popularized by Hodgson et al. in 1979 [11]. Here, the ultrasonic waves generate energy to fragment and aspirate parenchymal tissue. The contact of oscillating titanium tip causes fragmentation of hepatocytes owing to the high water content while, selectively sparing the blood vessels and bile ducts because of poor tissue water content. As CUSA doesn't coagulate, one needs additional help of ties, clips or staplers as per the surgeon's disposition to achieve haemostasis and biliostasis. Consequently, it results in an explicit line of transection by safeguarding the normal hepatic tissue, though the benefit obtained in terms of reducing the blood loss is not significant [12–14]. The ability of CUSA to selectively modulate tissue dissection depends upon the mechanical resistance offered by the tissue itself, e.g., hepatocytes contain less fibrous tissue than vessels and thus extend less resistance to crushing during parenchymal division. This is a particularly important point for consideration in a cirrhotic liver by virtue of an increased fibrous component [14–16].

Habib's technique, first introduced by Habib in 2002, has received well acceptance as "Bloodless Hepatectomy Technique". The Habib-4X is a bipolar device introduced perpendicularly into the liver in a serial fashion to create a parallel lines of ablation [17, 18]. This RF based device permits hepatic resection with minimal blood loss [19]. It coagulates all vessels and bile ducts in its field of application thus minimizing the need of Pringle Maneuver and blood transfusion [20].

The present study is the first and largest study done so far to compare the oncological outcomes of CUSA and Habib-4X based liver resection. Based on the prospective analysis of our database, we specifically compared the recurrence rates, DFS and OS following use of these two modalities.

RESULTS

Demography

A total of 280 patients with hepatocellular carcinoma who underwent hepatic resection were included in the present study. CUSA based hepatic resection was performed in 163 patients while 117 patients were treated with Habib-4X. Patients' demographic characteristics of each group has been tabulated (Table 1) and compared. The mean age of patients in CUSA and Habib-4X group was 58.39 ± 11.9 years and 58.18 ± 11.37 years respectively ($p > 0.05$). There were 31 women (19.0%) and 132 men (80.9%) in the CUSA cohort whilst, 13 women (26.4%) and 86 (73.5%) men in the Habib-4X group. Along with that, we didn't observe any significant differences between groups regarding serum albumin, serum bilirubin, serum AFP, tumour numbers, tumour size, tumour stage, cirrhosis, HBsAg, HCV, ICG clearances (Tables 2 and 3).

Procedure and complications

No significant difference was observed in the number and size of tumours in the CUSA and the Habib-4X resection groups (Table 2). Major liver resection was done for 45 patients in CUSA group and 27 patients in Habib-4X group with no significant differences ($p = 0.43$). Anatomical resection was accomplished in 63 cases with CUSA while 43 cases were done with Habib-4X ($p = 0.84$) (Table 3)

The operative blood loss was significantly higher (271.47 ± 214.5 mL) in the CUSA group than Habib-4X group (150.93 ± 103.6 mL; $p < 0.00$). Furthermore, our results demonstrated single event of vascular inflow control in Habib-4X group compared to 139 in the CUSA group ($p < 0.00$; Table 3).

The length of postoperative hospital stay was comparable for patients in both the groups. The mean length of stay was 7.88 ± 1.25 days (5–12 days) in the Habib-4X group compared to 7.84 ± 2.04 days (6–21days) in CUSA group ($p = 0.77$). We reported 0% mortality as in-patient or within 30 days of hospital admission in both groups (Table 3).

Table 1: Demographics and clinical characteristics of patients in the study groups

Parameters	CUSA (163)	Habib-4X (117)	*p* value
Mean age ±SD (yrs)	58.39 ± 11.9	58.14 ± 11.37	0.85
No. male/female	132/31	86/31	0.14
Albumin (g/dl)	4.28 ± 0.88	4.05 ± 1.20	0.08
Bilirubin (mg/dl)	1.06 ± 0.86	.97 ± 0.49	0.27
ICG Clearance (15 mins)	9.41 ± 7.2	10.62 ± 9.4	0.22
AFP (ng/ml)	1790.02 ± 7623.98	1901.17 ± 9763.70	0.91
Cirrhosis	64	51	0.47
HBsAg	112	78	0.72
HCV	46	40	0.29

• Statistical significance was analyzed by the chi-square test.
• Statistical significance was analyzed by the Student's *t*-test

Table 2: Tumour characteristics of patients in study groups

Parameters	CUSA (163)	Habib-4X (117)	*p* value
Tumour Numbers			
1	144	106	0.68
2	13	9	0.93
3	4	1	0.58
4	2	1	0.76
Tumour Stage			
T1	106	79	0.76
T2	53	34	0.63
T3	4	4	0.91
Tumour Size (cm)	4.66 ± 3.43	4.43 ± 3.27	0.56

• Statistical significance was analyzed by the chi-square test.
• Statistical significance was analyzed by the Student's *t*-test.

The major complications as post hepatectomy liver failure, bile leakage, bleeding were higher in CUSA group than Habib-4X although not reached any statistical significance ($p = 0.36$; Table 3).

The data analysis of tumour histology showed that free resection margins were comparable in both groups i.e., 91 cases with CUSA than 57 cases in Habib-4X group ($p = 0.29$) also vascular invasion were comparable (Table 5).

Recurrence

We registered significantly lower recurrence in the Habib-4X group (44 patients) as compared to the patients treated with CUSA (85 patients; $p < 0.01$) (Table 4). Similarly, the percent of patients who received other palliative interventions post recurrences were significantly higher for the CUSA group i.e., 81.1% compared to 62.4% in Habib-4X group ($p < 0.01$) The further analysis of the data showed RF ablation as the most common modality of palliation in CUSA group (47/85; 55.3%) compared to Habib-4X (8/44; 18.1%); The TACE was the second most common modality of palliation required in 23/85; 27% cases of CUSA group compared to 21/44; 47.7% cases in Habib-4X group (Table 4).

Survival

The median duration of disease-free survival was significantly longer in the Habib-4X group (50.80 months) than CUSA group (45.87 months; $p = 0.03$). Five-year disease free survival was 56% in the Habib-4X group compared to 54% in the CUSA group while the corresponding overall survival rates for the Habib-4X group and CUSA group were 61% and 51.30% respectively (Table 5; Figure 1). Although, the median

Table 3: Operative and postoperative characteristics of patients in study groups

Parameters	CUSA (163)	Habib-4X (117)	*p*-value
Anatomical resection	63	43	0.84
Non-anatomical resection	100	74	0.84
Major resection	45	27	0.43
Minor resection	116	90	0.43
Blood Loss (mL) (Mean ± SD)	271.47 ± 214.5	150.93 ± 103.6	0.00*
Blood transfusion received	21	3	0.00#
Vascular Inflow Control	139	1	0.00#
Hospital Stay (Days) (Mean ± SD)	7.84 ± 2.04	7.88 ± 1.25	0.77
Post hepatectomy liver failure	4	0	
Bile leakage	4	1	
Sepsis	0	2	
Bleeding	1	0	
Major complication	9	3	0.36

- *#$ Showing Significant *p*- value.
- #Statistical significance was analyzed by the chi-square test.
- *Statistical significance was analyzed by the Student's *t*-test.
- $Statistical significance was analyzed by the Mann-Whitney *U* test.

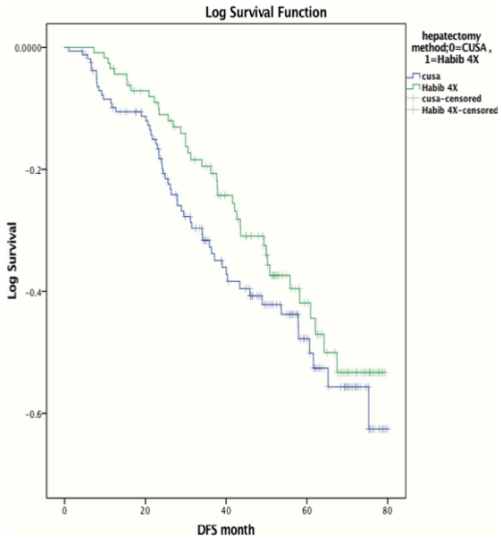

Figure 1: Disease-free survival (DFS) estimates. Kaplan-Meier survival plot of DFS comparing CUSA group (Blue line) with the Habib-4X group (Green line) ($p = 0.03$).

Table 4: Comparison with number of interventions post-recurrence within studied groups

Parameters	CUSA (163)	Habib-4X (117)	*p* value
Recurrence (−/+)	78/**85**	73/**44**	0.01[#]
Intervention done in cases of recurrence (−/+) (%)	78/**85** **(81.1%)**	73/**44** **(62.4%)**	0.00[#*]
RFA	47	8	
TACE	23	21	
Re-operation	9	3	
Sorafenib	5	12	
Radiotherapy	1	0	

- [#*]Showing Significant *p*-value.
- [*]Degree of freedom = 5.
- [#]Statistical significance was analyzed by the chi-square test.

Table 5: Postoperative tumour characteristics of patients in study groups

Parameters	CUSA (163)	Habib-4X (117)	*p*-value
Resection margin			
Free	91	57	0.29
Free within 1 cm	63	52	0.39
Involved	9	6	0.82
Vascular invasion	42	27	0.67
Local Recurrence	5	3	0.80[#]
Recurrence (−/+)	78/85	73/44	0.01[#]
Disease Free Survival (Median) (months)	45.87	50.80	0.03[$]
Overall Survival (Median) (months)	57.17	60.57	0.11

- [*#$]Showing Significant *p*- value.
- [#]Statistical significance was analyzed by the chi-square test.
- [*]Statistical significance was analyzed by the Student's *t*-test.
- [$]Statistical significance was analyzed by the Mann-Whitney *U* test.

overall survival was better in Habib-4X group than CUSA group but was not statistically significant (60.57 vs 57.17 months, $p = 0.12$) (Table 5).

DISCUSSION

Studies have proved that surgical resection confers better likelihood of cure and survival in patients suffering from early stage of HCC [21, 22]. Nevertheless, disagreement exists regarding the most efficacious and safest modality of liver resection. Current evidence indicates that the stage of parenchymal transection during surgery has maximum impact on blood loss, blood transfusion, post-operative complications and bile leak. Furthermore, this has also influenced the oncological outcomes such as survival and tumour recurrence [23–26]. Studies have shown that Habib-4X provides favourable operative outcomes in terms of blood loss, post-operative recovery, hospital stay, postoperative morbidity and mortality [17, 27, 28]. Till date, many studies have looked into the safety and efficacy of various modalities for hepatic parenchymal resection, however very few studies have compared the long-term outcomes of these techniques [29]. The disease recurrence and survival are considered as the most important factors determining the therapeutic success in management of malignant disease. To the best of our knowledge, this is the first study to investigate into the oncological outcomes following the use of CUSA or Habib-4X for liver resection. Here we matched and compared both groups to minimize confounders as serum albumin, serum bilirubin, serum AFP, tumour numbers, tumour size, tumour stage, cirrhosis, HBsAg, HCV, ICG clearances, type of resection (major/minor and anatomical/non-anatomical), resection margin, tumour number, size and stage, and vascular invasion. The data analysis showed

that both groups were comparable against above outlined parameters with no significant difference ($p < 0.05$) and has increased significant strength to our study.

The analysis of immediate outcomes in our study showed that the mean blood loss, need for blood transfusion, and requirement for vascular inflow control was significantly lower in the Habib-4X group than the CUSA group. This might be explained by the fact that Habib-4X utilizes radiofrequency energy to coagulate the blood vessels while the ability of CUSA has been limited by the need of manual activation to coagulate the blood vessels [30–33]. In accordance with a previous study, we also found that surgeon's experience in using CUSA has an influence on the surgical outcome [34].

The length of hospital stay did not reach statistical significance in either study group, however this appeared similar to other existing reports. The present study didn't report any mortality in either group which could be attributed to the recent advances in the surgical techniques and better peri-operative and post-operative management making hepatic resection a reasonably safe treatment option.

As, recurrence following curative surgery in patients with HCC remains a major clinical hurdle while deciding the best treatment strategy. We demonstrated significantly lower recurrence with the RF based device Habib-4X. One of the reasons accounting for the present oncological outcomes are based on the coagulating property of RF ablation which minimises blood loss and the need for blood transfusion; prevents any tumour spillage and micro metastasis. The lower recurrence could also be attributed to the favourable systemic and local immunomodulatory changes induced by the RF resection [34–36].

The further data analysis of long term outcomes has demonstrated significantly better disease-free survival for the patients who underwent RF based liver resection for HCC. The disease-free survival rates were 95% at 1 year, 96% at 2 years, 88% at 3 years, 67% at 4 years and 56% at 5 years, (Table 6) which was not only better than the CUSA group but also stands in accordance with the previous notable studies [23, 37–53]. We also evidenced better overall survival in the Habib-4X group, although, it did not reach statistical significance. This might be because the recurrence of cancer did affect the disease free survival though this was not reflected in terms of overall survival. The inability to reach statistical significance could be explained by the higher use of palliative treatment modalities like percutaneous RF ablation, TACE, sorafenib etc. in the CUSA group in instances of recurrence which helps in prolonging life despite recurrence of the disease. This has facilitated the importance of Habib-4X in providing better disease free survival and this further supports the recently published study Qiu et al. where they reported significantly better recurrence free survival and overall survival with Habib-4X compared to the clamp-crush technique [54].

Following initial hepatic resection, 81.1% patients in CUSA group while 62.4% in Habib-4X required retreatment. Further analysis of data showed that RF ablation was the most common modality of palliation (55.3%) in the CUSA recurrence group while it constituted only 18.1% of palliation in the Habib-4X group. The findings further strengthened the role of Habib-4X as a better modality of treatment over CUSA in HCC management.

The favourable oncological outcomes with Habib-4X could be explained by the virtue of systemic and local immunomodulatory effect of radiofrequency. The debris produced following RF-induced coagulative necrosis during HCC resection releases tumour antigens and chemokines. These chemokines attract inflammatory infiltrates; neutrophils, macrophages, NK cells, dendritic cells (DCs), as well as CD4+ and CD8+ T lymphocytes. The cellular influx at the ablated resection margin phagocytoses the debris and tumour cells. Tumour antigens also drain to nearby lymph nodes, and stimulate immature DCs and naive T-cells thus provide systemic immunomodulation [55–58].

The liver is a unique organ which maintains intricate balance between not over-reacting to the antigens absorbed by the gut and mounting accurate immune responses to eliminate the tumor antigen. HCC is characterized by chronic inflammation and immune suppression. Immunosuppression due to inhibitory checkpoints appear to be an important contributor to the induced immune suppression in this setting and subsequent development and progression of HCC. Tregs and myeloid-derived suppressor cells are thought to play an important role in protecting the tumour from eradication by activated cytotoxic CD8+ T cells. High level of Tregs in tumor has been linked to poor prognosis in HCC [59–62].

Over the last two years, major breakthrough in immunological understanding in the tumour management has led to the development of newer drugs as checkpoint inhibitors which boost CD8+ T cell functioning. The discovery of these drugs have brought significant improvement in the survival of patients with cancers like leukaemia, lymphoma and melanoma, although the same has not been translated for HCC. One of the great potentials of RF based therapies are that they can dovetail with immune modulating therapies. RF ablation induces CD8+ T cells infiltration at the ablation site and further addition of a checkpoint inhibitor might act as a booster. The potential effect on the immune system is further advantageous in terms of better survival as it acts in synergy with checkpoint inhibitors. In addition, recent trials have shown that combined use of RF and checkpoint inhibitors could bring more beneficence towards the long-term survival [63–66]. This has been further strengthened by the study of Duffy et al., 2017, where they reported the activation of the immune system through checkpoint inhibitors and accumulation of intratumoral CD8+ T cells

Table 6: Detailed comparison of disease free survival for the study groups

Parameters (Time in Months)	CUSA (163)	Habib-4X (117)
12 months	93%	95%
24 months	87%	96%
36 months	86%	88%
48 months	66%	67%
60 months	54%	56%

following RF ablation, thus demonstrating the synergism of combined use of checkpoint inhibitor tremelimumab and ablation in aggressive hepatocellular carcinomas [67].

The present study has certain limitations owing to its retrospective design and unintended biases of patient selection which might influence the analysis. In spite of these limitations, this study has outlined significantly better disease free survival and lesser tumour recurrence with the Habib-4X group compared to the CUSA group.

MATERIALS AND METHODS

Study design

In this multicentric study, the data from two centers of National Taiwan University Hospital were prospectively collected and analyzed after obtaining approval from the Institutional Review Board. The data included 280 patients with confirmed diagnosis of HCC on histopathology who underwent liver resection with CUSA or RFA based device Habib-4X from January 2010 till December 2012 and were followed-up till August 2016. The data was collected for the amount of blood loss, vascular inflow control, length of hospitalization, complication within 30 days, post hepatectomy liver failure (PHLF) overall recurrences, local recurrences and interventions done in cases of recurrences. PHLF was measured according to the definition set by International Study Group of Liver Surgery (ISGLS) 2011 [68]. The endpoints of the study as overall survival and disease free survival were estimated by the Kaplan–Meier method.

Procedure

All patients with a diagnosis of HCC underwent open surgical hepatectomy under guidance of intra-operative ultrasound. Both the lobes of liver were mobilized and gall bladder was removed if needed. Selective inflow control was performed in cases of excessive parenchymal bleeding. In cases where CUSA had been used by the surgeon, help of an assistant surgeon was needed to control the bleeding with bipolar coagulation, while no assisting haemostatic instrument was required during resection in the Habib-4X group. The Habib-4X, a bipolar device was introduced perpendicularly into the liver in a serial fashion to create parallel lines of ablation. The third line of ablation was created perpendicular to the parallel track, following which liver was resected by scalpel. The probe was moved swiftly in see-saw fashion for 3-5 mm in its axis of application. The movement of probe helped in averting any adherence of the liver tissue. The device effectively created a 1 cm thick area of ablated and coagulated tumour free margin [16, 17]. A meticulous hemostasis was assured, and raw surface covered by cellulose hemostatic agent. In the present study, a major hepatectomy was defined as resection of three or more liver segments.

Statistical analysis

Overall survival and disease-free survival were calculated from the date of surgical intervention. Continuous variables were analyzed with Student's *t*-test, and categorical variables were analyzed with chi-square or Fisher's Exact Test where appropriate. Survival and recurrence rates were calculated using the Kaplan- Meier method and comparison between groups were done with the log-rank test. Predictors of overall and disease-free survival were analyzed by performing a Cox Proportional Hazards regression model using a backwards selection process. A p- value of < 0.05 was considered significant in this study. Data were fed into a Microsoft Excel worksheet and analyzed by the IBM-SPSS version 24 (SPSS Inc, Chicago, IL, USA).

Literature review

A comprehensive systematic literature review was performed to search all the published articles on National Library of Medicine Database (PUBMED), EMBASE, Cochrane, CrossRef, and Scopus databases on 15th November 2016 describing the outcomes of hepatic resection in hepatocellular carcinoma. The search covered the period from January 1st, 1981 to November 15th, 2016. The search was carried out by using the medical subject headings (MeSH) terms: 'Hepatocellular Carcinoma', 'Liver Neoplasm', 'Hepatectomy open', 'Hepatectomy laparoscopic'. The initial search yielded a total of 154 manuscripts. Following the careful evaluation of inclusion, exclusion criterias and demography characteristics, 137

Table 7: Literature review of survival outcomes of hepatic resection in hepatocellular carcinoma

Author	Year	Study Sample	Hospital Mortality (%)	Median Survival (month)	Overall Survival (%)		
					1 year	3 year	5 year
Heng-Jun et al. [37]	2014	151	-	61.8	99	68	52
Jin et al. [40]	2014	62	11.1	-	83.2	75.7	65
Jianyong et al. [38]	2014	433	2.3	-	91.8	84.2	70.8
Nojiri et al. [41]	2014	107	-	-	-	62	38.1
Lim et al. [42]	2014	172	1	-	-	-	58
Yin et al. [43]	2014	88	11.3	41	76.1	51.5	-
Zhong et al. [21]	2014	660	2.6	-	91	67	44
Zhong et al. [44]	2013	257	3.1	42.9 ± 26.1	84	59	37
Cheng et al. [45]	2012	104	7.3	-	90	-	66
Hsu et al. [46]	2012	268	2.7	-	81	63	43
Lin et al. [52]	2010	93	5.4	29.9±20.1	83	49	-
Ho et al. [47]	2009	294	-	37.9	77.4	51.9	36.6
Wang et al. [48]	2008	243	-	60.4±6.1	81.5	64.4	50.5
Lee et al. [49]	2007	100	2	-	66	44	31
Pandey et al. [53]	2007	166	3	-	-	-	29
Ng et al. [50]	2005	380	2.7	-	74	50	39
Poon et al. [51]	2002	120	5	-	61	38	28
Present Study	2016	117	1.5	60.6	95	86	56

articles were excluded. The remaining 17 papers, were considered, and full-text obtained (Table 7).

CONCLUSIONS

The present study reported better oncological outcomes including significantly lesser tumour recurrence and better disease free survival following Habib-4X based tumour resection for hepatocellular cancer as compared to CUSA.

The small difference in terms of OS between the two groups could be explained by the use of palliative therapeutic modalities in the patients with tumour recurrence. Habib-4X is a feasible, promising and safe liver resection device with excellent short and long term results and with a potential to be used with checkpoint inhibitors. Nevertheless, these findings need to be confirmed with more prospective and randomized controlled trials.

CONFLICTS OF INTEREST

Prof. Nagy Habib is a shareholder and Director of the company, EMcision Limited, that originally developed the Habib™ 4X. Since 2005 the Habib™ 4x has not been marketed or sold by EMcision Limited. All the other authors have no conflicts of interest, including specific financial interests or relationships and affiliations relevant to the subject matter or materials discussed in the manuscript.

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Appendix 4 Study 4

Study to evaluate the immunomodulatory effects of radiofrequency ablation compared to surgical resection for liver cancer [95].

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Research Paper

Study to evaluate the immunomodulatory effects of radiofrequency ablation compared to surgical resection for liver cancer

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Abstract

Introduction: Hepatic cancer is a highly lethal tumour with increasing worldwide incidence. These tumours are characterized by the proliferation of malignant cells, generalised immunosuppression and chronic inflammation marked with an increase in inflammatory markers as a neutrophil-to-lymphocyte ratio (NLR), the platelet-to-lymphocyte ratio (PLR) and overexpression of CD4+CD39+ on T lymphocytes. The studies have outlined immunomodulatory changes in liver cancer patients as the plausible explanation for the better survival. The aim of this pilot study was understand the possible immunomodulatory effect of radiofrequency (RF) energy and liver resection (non-radiofrequency based devices; non-RF device) in relation to NLR, PLR and expression of CD4+CD39+ T lymphocytes and compare the magnitude of these changes.

Material and Methods: In the present study, 17 patients with hepatic cancer were prospectively divided into treatment groups radiofrequency ablation (RFA group) and Liver resection using non-RF devices (LR group). A blood sample was collected from each patient, one month before and after the procedure and compared with the blood samples of age-matched healthy volunteers for group wise comparison. The Mann-Whitney U test, Mc Nemar test and Wilcoxon rank test were used for statistical comparisons as appropriate.

Results: A decrease in NLR was reported after RFA from 4.7±3.3 to 3.8±1.8 (P=0.283), in contrary to an increase from 3.5±2.8 to 4.5±3.2 (P=0.183) in LR group. Likewise, a decrease was discerned in PLR following RFA from 140.5±79.5 to 137±69.2 respectively (P=0.386) and increase in the LR group from 116±42.2 to 120.8±29 respectively (P=0.391). A significant decrease in CD4+CD39+ lymphocytes from 55.8±13.8 to 24.6±21.1 (P=0.03) was observed in RFA group whilst a significant increase was reported in LR group from 47.6±8.8 to 55.7±33.2 (P=0.38).

Conclusion: Studies have shown that decrease in the NLR, PLR and expression of CD4+CD39+ on T lymphocytes as the marker of better survival in hepatic cancer patients and our findings have confirmed that these changes can be induced following application of RF energy. Moreover, this could be the explanation of better survival observed in different studies using RFA or other RF-based devices in comparison to non-RF based liver resection techniques. However, further larger studies are needed to confirm these findings.

Key words: liver cancer, Hepatocellular carcinoma, Radiofrequency ablation, liver resection, Immunomodulation

Introduction

Liver cancer is considered as one of the aggressive malignant tumour, a National Cancer Institute and SEER (Surveillance, Epidemiology and End Result) Program 2016, reported an incidence of 39,230 and a mortality of 27,170 per year [1]. Throughout the world, males are more prone to these

cancers and disease is 2.1 to 5.7 times more frequent than females [2]. In comparison to the declining death rates observed for various other cancers (including breast, colon, lung), the mortality rates in males for hepatocellular carcinoma arose by 2.8% per year and for females increased by 3.4% [1]. The majority of primary liver cancers are hepatocellular carcinomas (HCC), followed by cholangiocarcinoma, gall bladder cancer and so on [3]. It is the third most common cancer of digestive system and the fourth most common cause of cancer-related death following colon and pancreatic cancer [1]. An advancement in the non-surgical and surgical techniques, including radiofrequency ablation (RFA), trans-arterial chemoembolization (TACE), chemotherapy, novel devices for liver resection, liver transplantation and so forth have produced a significant positive impact on the management of patients with liver cancer [4–7]. Nevertheless, prognosis of liver cancer is largely depending on the stage of presentation, and potentially curative treatment options are feasible only at early tumour stages [8,9].

According to Barcelona Clinic Liver Cancer (BCLC) staging system [10,11], liver resection (LR), liver transplantation and percutaneous local ablation including RFA, microwave ablation and so on are recommended as curative therapies for patients with early-stage primary and limited secondary liver tumours (<3cm) with a 5-year survival rate of 50-75% [12–17]. However, in patients with intermediate HCC or liver metastasis >3 cm, transarterial chemoembolization (TACE) or transarterial embolization (TAE) in combination with liver resection (LR) may offer a median survival ranging from 16 to 22 month [18–20] (Figure 1). Hence, the use of non-surgical modalities either alone or in combination with surgical resection are often required owing to inadequate functional hepatic reserve, unfavourable anatomical location, stage of liver cancer and multifocal involvement [21,22]. Studies demonstrated better long-term survival in the 5 and 10 years' longitudinal studies following RFA for HCC and colorectal liver metastasis [23,24]. In a meta-analysis conducted by Xu et al., (2014) included 31 studies and total of 16,103 patients concluded that the overall survival and disease free survival was significantly better in RFA group than LR in HCC patients with tumour of ≤ 2 cm [25]. A recent study by Qiu et al. (2017) has highlighted the benefits of RF based device and reported significantly less morbidity, mortality and better survival over the clamp-crush technique [26]. In addition, Huang et al.

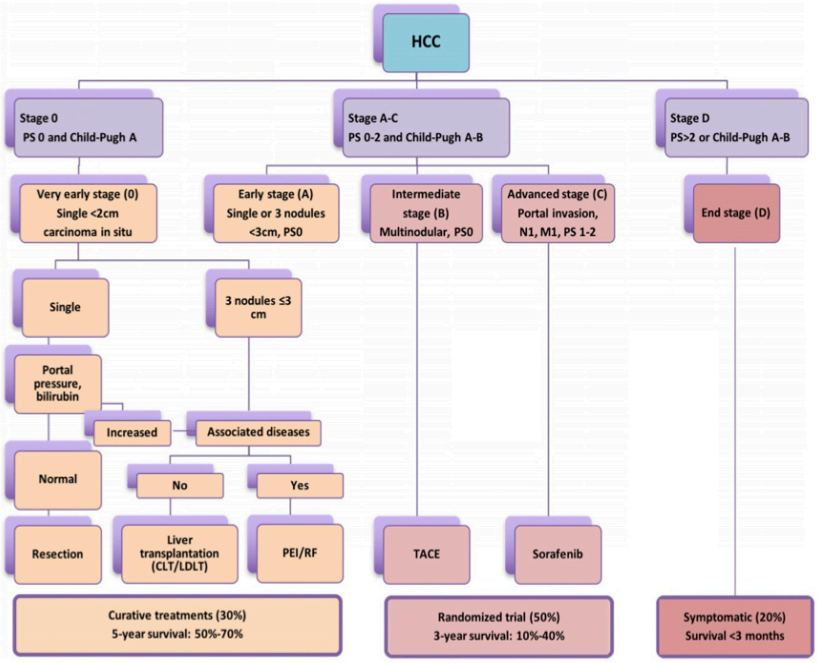

Figure 1. The widely accepted Barcelona Clinic Liver Cancer (BCLC) staging system stating treatment options according to cancer stage, degree of liver dysfunction (Child-Pugh Score) and performance status (PS); CLT: Cadaveric liver transplant; LDLT: Living donor liver transplant.

(2017) compared RF based liver resection device with cavitron ultrasonic surgical aspirator (CUSA) and outlined significantly better long duration disease-free survival [27]. Further, studies have implicated local and systemic immunomodulatory changes following application of radiofrequency as primary reason behind better survival achieved through RF based approach. The present pilot study was planned to understand the liver tumour biology, observe the immunomodulatory changes following application of RF energy and completed literature analysis for its potential role in advanced liver cancer as an adjunct to check-point inhibitors.

The tumour biology is a systematic concept according to which the behaviour of cancer is not only determined by the genetics of tumour cells but influenced through the microenvironment. Considering the fact that cancer is a state of generalized immunosuppression, considerable evidence evinced, that the tumour site inflammatory reaction can foster its growth and progression. Lately, inflammation-based markers as neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR) have been detected in liver cancer patients and received acknowledgment as an important marker of the tumour surveillance system [28–30].

Studies have outlined that increased numbers of neutrophils provide a sustainable microenvironment for liver tumour growth and metastasis through angiogenesis. The relative increase in NLR is considered as the marker of chronic inflammation, and it accounts for tumour characteristics such as vascular invasion and chronic inflammation. The accumulation of neutrophils in the peritumoural stroma enhances tumour invasion through the regulation of a paracrine-mediated hepatocyte growth factor [31–33]. Consequently, proinflammatory cells present in the peritumoral stroma promote the release of IL-17 and other chemokines (CXC) from epithelial cells, which in turn facilitate additional neutrophil and chemokines receptor positive B cells trafficking [34–36]. As mentioned, increased NLR has been found to be associated with poor outcomes in liver cancer patients and considered as an independent predictor for survival following liver resection in HCC [37]. Recent evidence showed that post liver transplant HCC patients have decreased postoperative NLR in comparison to the preoperative NLR with significantly higher serum and peritumoural IL-17 level in the increased NLR group [38]. Concordantly, an inflammatory tumour microenvironment provided by tumour associated macrophages and IL-17 producing cells correlated with HCC recurrence following post liver transplant [39]. In addition, the tumour recurrence following liver transplantation was higher in splenectomised recipients with higher preoperative NLR than those who had not undergone splenectomy. Hence, systemic inflammatory response through upregulation of cytokines, not only promoted hepatic tumour development and growth, but also determined recurrence [40–42]. A recent meta-analysis published by Qi et al. (2017) included 90 papers with 20,475 HCC patients, concluded the importance of NLR in recurrence, disease free survival and overall survival. The changes in NLR during disease course have considerable implication on prognosis too [43].

The platelet-lymphocyte-ratio (PLR) has been explored as a predictor of thrombotic and inflammatory conditions and regarded as an attractive inflammation-related biomarker for liver tumours. Platelets have been shown to induce an epithelial-mesenchymal-like transition and platelet-derived nucleotides and promote tumour cell transendothelial migration and metastasis [44,45]. In a meta-analysis by Zhao et al., (2017) including 10 studies concluded that HCC patients with high PLR have worse overall survival and described them as unfavourable predictors for overall survival [46]. Therefore, both high baseline NLR or PLR have been not only considered as the marker of poor prognosis in patients with liver cancer but also a predictor for cancer invasion, metastasis and survival outcomes [46].

Recognizing that high NLR and PLR are typically associated with chronic inflammatory states, which are linked to immune dysregulation, disordered metabolism, and aberrant cell proliferation in liver cancer. These ratios typically reflect the potential balance between neutrophil and platelet associated pro-tumour systemic inflammation and lymphocyte dependent anti-tumour immune function [47,48]. Hence, inflammatory cell and cytokines in tumour microenvironment establish suppressive milieu for cancer growth whilst, dysregulated immune cells help tumour cells in escaping from immune surveillance system.

The immunological perspective of tumour development is a rough house of two wings: an antitumour immunity group i.e CD 8 cells, natural killer (NK) cells, and protumour group including regulatory T cells (Tregs) and tumour derived repressing substances. The tumour cells escape immunological surveillance by diminished recognition by immune cells, increased resistance by tumour cells, or instigation of an immunosuppressive microenvironment.

An ectonucleotidase (CD 39) which is nucleoside triphosphate diphosphohydrolase-1 has been discovered on the surface of various cell types

such as normal leukocytes, Tregs and endothelial cells. Ectonucleotidase CD39 catalyses the hydrolysis of extracellular adenosine triphosphate (ATP) to adenosine. Thus, favours tumour growth as degradation of ATP lowers the immune response [49,50]. The upregulation of Tregs has been considered a predictor of poor outcome in HCC patients along with, intratumoural balance of Tregs and cytotoxic T cells determines the prognosis of HCC patients following resection [51,52]. Furthermore, CD39 overexpression in Tregs limits the NK cell activity and promotes the growth of metastatic liver tumours [53,54]. Cai et al. demonstrated increased recurrence and poor overall survival in liver cancer patients with overexpression of CD 39 [55].

In light of the current evidence that decrease in immunomodulatory serum markers including NLR, PLR and expressions of ectonucleotidase CD39 on circulating CD4+ T lymphocytes have potential role in the better survival of liver cancer patients. We decided to conduct a pilot study, to understand the degree of immunomodulatory changes following radiofrequency ablation (RFA) and liver resection (LR) using non-RF device. The pre- and post-procedure, serum NLR, PLR and expressions of ectonucleotidase CD39 on circulating CD4+ T lymphocytes were analysed to demonstrate the immunomodulatory changes in both treatment groups and in comparison with healthy population.

Material and Methods

Study Design:

We conducted a single centre prospective pilot study at Tbilisi State Medical University hospital in accordance with the Declaration of Helsinki from 2014 to 2017. The study was approved by the Ethic Committee of Tbilisi State Medical University (# 44/3). The primary endpoint of study was to assess the immunomodulatory changes in the NLR, PLR and expression of ectonucleotidase CD39 on circulating CD4+ T lymphocytes following RFA or liver resection in the liver cancer patients.

Subjects

A total of 17 patients with primary (HCC and Cholangiocarcinoma) and secondary (metastatic) liver cancer were enrolled in this prospective study. Out of 17 patients, 7 were referred to the RFA procedure and another 10 underwent surgical liver resection using non RF based devices. All decisions regarding procedures were made following the MDT (Multidisciplinary Team) discussion. Surgical resection was carried out under the general anaesthesia using an upper middle incision. In three patients anatomical segmental hepatectomy while non-anatomical resection was done in rest seven patients. All liver resections were accomplished by using non-RF based liver resection devices. Healthy age-matched volunteers without history of cancer, recent acute or chronic infectious disease, or autoimmune disease, were used as controls for the comparison.

Inclusion and Exclusion criteria

The inclusion criteria for the RFA patients selection were as follows: a) extensive liver disease or medical co-morbidities associated with tumor vascular invasion and thromboses, b) fewer than three nodules without extrahepatic metastasis, c) largest tumour size of 3-4 cm in diameter, d) visualization of the nodule during the planning of RFA by ultrasonography (US). The exclusion criteria were chemotherapy or TACE, RFA or LR within previous one month.

Technique

Radiofrequency ablation (RFA): An image-guided RFA was performed by senior interventional radiologist at our centre. The tumour was localized and RF antenna introduced into the target tissue under US guidance. RF processing increases temperature into the target tissue up to 102^0C leading to the irreversible damage by coagulative necrosis. Abdominal contrast computed tomography (CT) was performed to document completeness of the procedure. Peripheral blood samples were obtained at one month before and after the procedure.

Liver resection (LR): Surgical resection, anatomic segmental hepatectomy and non-anatomical liver resection were carried out under the general anaesthesia using an upper middle incision, using non-RF based liver resection devices.

NLR and PLR counting

NLR and PLR, known as index systemic inflammatory markers, have proven prognostic role in many types of cancers. Blood samples were collected from each patient in an EDTA anticoagulant-treated tube as per the study protocol. They were further analysed for routine peripheral blood cells, Neutrophil/lymphocyte ratio (NLR) and Platelet/lymphocyte ratio (PLR). The NLR and PLR were defined as the absolute neutrophil count and the absolute platelet count, respectively, divided by the absolute lymphocyte count.

PBMC isolation and Flow cytometry

Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll gradient centrifugation. Freshly isolated PBMCs were immediately stained for

the surface markers using anti-CD4 PE/Cy7 and CD4 subsets were detected by the lineage specific antibodies anti-CD39 PE. Data were acquired on a FacsArray cytometer and analyzed with BD FACSArray system software.

Statistical data analysis

All data were entered into a Microsoft Excel™ database and analysed using Graph Pad Prizm software. The Mann-Whitney U test and Mc Nemar test were used to compare data between RFA and surgical resection group. Furthermore, both study groups were compared with healthy control subjects. Wilcoxon's matched pairs signed rank test were used to compare patients before and after therapy. *P* values less than 0.05 were considered statistically significant.

Results

Demography

A total of 17 patients with liver cancer were included in the study. Patients' demographic characteristics of each group has been listed and compared in Table 1. The mean age of patients in RFA and LR group was 55.1±11.2 years and 58.6±8.1 years respectively (*P*>0.05). There were three women (43%) and four men (57%) in the RFA cohort whilst, five each women and men in the LR group. We didn't observe any significant differences between groups regarding number of tumours primary or secondary, tumour size, tumour stage, HBsAg, Anti-HCV positive.

Table 1: Demographics and Clinical Characteristics of Patients in the Study Groups

Parameters	Radiofrequency Ablation	Liver Resection (non-RF device)	*P*- value
Age(years) ± SD	55.1±11.2	58.6±8.1	>0.05
No. male/female	4/3	5/5	>0.05
Primary tumours	1	5	>0.05
Secondary tumours	6	5	>0.05
HBsAg-positive, n (%)	1 (14.2%)	1 (10.0%)	>0.05
Anti HCV-positive n (%)	0 (0%)	2 (20%)	
Tumour size (mm) ± SD	22.4±5.6mm	47.6±23mm	<0.05
Treatment	7 (41.10%)	10 (58.80%)	>0.05
Localization	5R, 1L, 1L/R	7R, 3L	
Complication	Hydrodissection (4)	Haemorrhage(1)	>0.05

Statistical significance was analyzed by the Mc Nemar Test
Statistical significance was analyzed by the Mann-Whitney U test
Statistical significance was analyzed by the Wilcoxon's matched pairs signed rank test

Neutrophil/lymphocyte ratio (NLR) and platelet/lymphocyte ratio (PLR) in the peripheral blood of patients before and after RFA or Liver resection.

The post-RFA, NLR assessed in peripheral blood sample were lower but not significantly lower in comparison to pre-RFA levels (3.8±1.8 and 4.7±3.3; *P*=0.283). However, no such decrease was observed in LR group; pre- and post- procedures levels were 3.5±2.8 and 4.5±3.2 respectively (*P*=0.183). Similarly, post-RFA, PLR value (137±69.2) was lower but not significantly lower than the pre-RFA value (140.5±79.5), (*P*=0.386), on the other hand opposite trend was observed in LR group. Here the post-LR group level was higher (120.8±29) than pre-LR (116±42.2) but not statistically significant (*P*=0.391) (Figure 2) (Table 2).

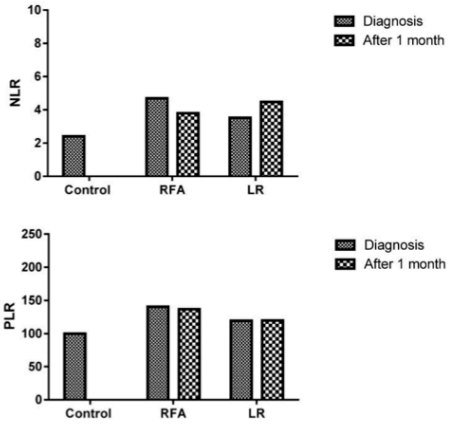

Figure 2. Decreased NLR and PLR in the patients who were treated with RFA (Radiofrequency ablation)

Table 2: Paired comparison of NLR, PLR and CD39+ in the Study Groups

Parameters	NLR	P value	PLR	P value	CD39+	P value
Before RFA	4.7±3.3	p=0.283	140.5±79.5	p=0.386	55.8±13.8	p=0.03
After RFA	3.8±1.8		137±69.2		24.6±21.1	
Before LR	3.5±2.8	p=0.183	116±42.2	p=0.391	47.6±8.8	p=0.38
After LR	4.5±3.2		120.8±29		55.7±33.2	

Statistical significance was analyzed by the Wilcoxon's matched pairs signed rank test
Neutrophil/lymphocyte ratio (NLR)
Platelet/lymphocyte ratio (PLR)
CD39+ (CD39+CD4 T Lymphocytes)

Circulating CD4+CD39+ lymphocytes in the peripheral blood of patients before and after RFA or Liver resection.

Peripheral blood samples were obtained before and after 1 month of RFA or LR procedure in patients with liver cancer to assess CD4+CD39+ lymphocytes. The significant decrease was observed in RFA group following the procedure while no such difference was seen in LR group.

The CD4+CD39+ lymphocytes levels in RFA group decreased significantly from pre-procedure

55.8±13.8 to after-procedure 24.6±21.1 (P=0.03) whilst, in LR group it was 47.6±8.8 and 55.7±33.2 before and after procedure respectively (P=0.38). The observed value of CD4+CD39+ lymphocytes in healthy controls was 10%.

The short-term effect of the treatment of liver cancer with RFA was decreased expression of CD39 on circulating $CD4^+$ cells however we did not observe the same in the patients treated with surgical resection (Figure 3).

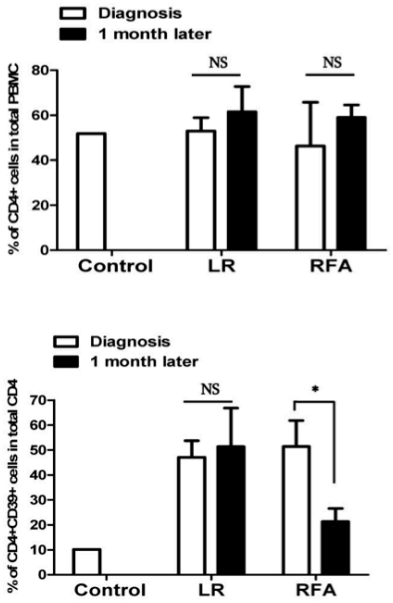

Figure 3. The percentage of $CD39^+CD4^+$ cells in total $CD4^+$ T cells was decreased after RFA, but was not modified after LR.

Discussion

Regardless of the host's underlying condition, chronic inflammation combined with intrinsic cell factors driven by oncogene act as a rheostat in all stages of tumour development; initiation, neoplastic cell growth, proliferation and metastatic spread. A vast majority of experimental work has demonstrated the mechanistic underpinning of host-derived proinflammatory cytokines as inducer of the release of reactive oxygen and nitrogen species, which promote mutational effects and cooperate to instigate epigenetic changes that nurture tumorigenesis [56–60]. This hold true for primary or secondary liver cancers too, which arises and progress in association with chronic inflammation, often linked with immune dysregulation, disordered metabolism and aberrant cell proliferation. Tumour progress and metastasis are not only related to the intrinsic characteristics of cancer cells but also to the cancer microenvironment [61,62].

In a recent meta-analysis done by Qi et al. and Zhao et al. shown that higher NLR and PLR are correlated with adverse survival outcomes in liver cancers patients irrespective of the treatment modalities [43,46]. Recognizing that decrease NLR and PLR are associated with better survival, various studies have reported better disease free survival when radiofrequency based techniques were used in the management of liver cancer patients [27,63–66]. The present study was aimed to assess the immunomodulatory changes in terms of NLR, PLR and percentage of $CD39^+CD4^+$ cells as a possible explanation behind better survival in liver cancer patients receiving RF based treatment.

The present study has showed decrease in post procedural NLR in RFA group however LR group evidenced increase in its level. This was in line to previous studies where higher NLR has been considered as a predictor of cancer recurrence, whilst post treatment lower NLR has been labelled as favourable prognostic factor [43,67–69]. The higher PLR is also considered as an independent prognostic factor and unfavourable predictor of survival in patients with liver cancer [44,70–72]. Here, we observed decrease in post procedural PLR in RFA group in comparison to LR group, which explicate their relationship and usefulness in improving the patient survival and decreasing the risk for tumour recurrence. Studies have elucidated role of anti-inflammatory or anti-platelet medicines in improving the survival of liver cancer patients with higher PLR. The similar attribute may hold plausible explanation for RF either, the post RFA decrease in PLR, not only considered as a better marker for survival but can also bring potential benefit in the management of liver cancer patients with RF based treatment modalities [73,74].

The literature review has demonstrated most intriguing observation of molecule $CD39^+CD4^+$ T cells subsets regarding its ability in inhibition of anti-tumour immune responses and promotion of angiogenesis [75–77]. Cai et al. reported that, CD39 expression in liver cancer can predict postoperative recurrence and survival time in liver cancer patients, thus highlighted the important prognostic value of $CD39^+$ CD4+T cells [55]. In the present research, we studied the changes in CD39 expression of CD4+ T cells and demonstrated significantly lower expression following RFA in contrary LR group.

Therefore, the NLR, PLR and $CD39^{+}CD4^{+}$ T cells expression must be considered as a panel of potential biomarkers and its level not only reciprocates with systemic inflammation, immune function but also act as an explicit marker to envisage the survival in liver cancer patients. Further, studies demonstrated platelet as a complement of neutrophils in the creation of milieu for cancer angiogenesis and metastasis. The studies have outlined the changes in the tumour microenvironment induced by platelets which help in escaping tumour cells from being phagocytosed by natural killer cells [75,78,79].

However, we didn't assess changes in this cell subtype, but owing to it potential impact on tumour micro environment and immunomodulatory role, it is worth mentioning here. RF produces CD8+ T cells infiltration at the ablation site which helps in phagocytosis of tumour cells debris. The advancement in immunological understanding has led to the development of checkpoint inhibitors drugs tremelimumab which boost CD8+ T cell functioning. The potential effect of these drugs on the immune system is further advantageous in terms of better survival as it acts in synergy with checkpoint inhibitors. In addition, recent trials have shown the synergism in combined use of RF and checkpoint inhibitors could bring more beneficence towards the long-term survival [80–82]. Duffy et al., 2017, conducted a study, where they reported the better survival following combined application of RFA and checkpoint inhibitors. The increased margination of intratumoral CD8+ T cells at ablation site following RF application and augmentation of their activity by the checkpoint inhibitors demonstrate the synergism of these two therapeutic modalities in the management of advanced aggressive hepatocellular carcinomas [83].

The present study has certain limitations which need to be kept in mind. First, owing to its small sample size and unintended biases of patient selection which might have influenced the analysis. In addition, we didn't assess immunological changes involving other cell types as CD8 and NK cells, which also get modulated following RF application. Nevertheless, we believe that our study has outlined the positive immunomodulatory changes following RFA, which may be suggestive of the better survival observed in liver cancer patients following application of other radiofrequency based techniques too.

Conclusion

To recapitulate, the decrease in NLR, PLR and significant decrease in CD39+ CD4 T lymphocytes represent a picture of immunomodulatory changes as a possible explanation to the better survival reported by studies using RFA or other RF based techniques in the management of liver cancer patients. However further studies with larger numbers of patients are needed to confirm these preliminary findings.

Acknowledgments

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Authors' Contributions

NJ and TC conceived and designed the study. NJ, KJ, IR and TC screened the abstract and full text, extracted data, assessed studies and drafted the manuscript. NJ and TC performed statistical analyses. NK, MM, KM, IP, NP, MS, MP and NH revised the manuscript. All authors read the manuscript and approved the final version.

Competing Interests

Professor Nagy Habib is the inventor of the Habib™ 4X device, which is a radiofrequency based device. None of the other authors have a conflict of interest, including specific financial interests or relationships and affiliations relevant to the subject matter or materials discussed in the manuscript to declare.

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Appendix 5 Study 5

Positive immuno-modulation following radiofrequency assisted liver resection in hepatocellular carcinoma [96].

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Article

Positive Immuno-Modulation Following Radiofrequency Assisted Liver Resection in Hepatocellular Carcinoma

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Abstract: Introduction: Hepatocellular carcinoma (HCC) often develops on a background of chronic inflammation and a complex immunosuppressive network with increased regulatory T cells, impaired $CD8^+$ T cells and the secretion of immunosuppressive cytokines. Previous clinical studies have reported a superior disease-free survival (DFS) following a radiofrequency-based ablation or resection in HCC tumours compared to conventional liver resection techniques. The aim of this study was to investigate whether there is any correlation with the use of a radiofrequency-assisted liver resection and clinical outcome. Material and Methods: Patients' peripheral blood was collected prior and 7 days following surgery from patients undergoing a liver resection for HCC. There were 5 liver resections performed using CUSA and 6 liver resections with the RF-based device, Habib^{TM} 4X. The primary endpoint of the study was to assess the immunological parameters of circulating immune cell populations as well as serum cytokines. The Student's *t*-test, chi-square or Fisher's Exact test were applied for statistical comparisons, as appropriate. Results: Patients undergoing an RF-assisted liver resection with Habib^{TM} 4X had a significant decrease in the inhibitory Treg cells ($p = 0.002$) and a significant increase in $CD8^+$ T lymphocytes ($p = 0.050$) and $CD4^+CD45RO^+/CD4^+$ memory T cells ($p = 0.002$) compared to those patients undergoing a liver resection with CUSA. It was also noted that the RF-assisted liver resection group had a significant decrease in circulating TGF-ß ($p = 0.000$), IL10 ($p = 0.000$) and a significant increase in IFN-gamma ($p = 0.027$) and IL-17 compared to the CUSA group. Conclusion: A liver resection with RF-based device Habib^{TM} 4X was associated with positive immunomodulatory changes in circulating immune cells and circulating cytokines which could explain the significant improvement in DFS.

Keywords: liver cancer; radiofrequency based device; liver resection

1. Introduction

Hepatocellular carcinoma (HCC) is a primary tumour associated with increasing incidence and mortality [1,2]. The Surveillance, Epidemiology and End Results (SEER) Database of the National Cancer Institute in the States, outlined a ≈3% annual increase in the HCC incidence during the period of 2008–2012 and a 3% increased annual mortality [3,4]. The contemporary advancement in the surgical and non-surgical techniques such as radiofrequency ablation (RFA), trans-arterial chemoembolization (TACE), chemotherapy, liver resection, and liver transplantation have brought a significant impact on the management of patients with liver cancer [5,6]. A considerable amount of vexation observed in the management of HCC is mainly determined by the cancer stage and feasible treatment options available at that stage. In the contemporary world, percutaneous local ablations such as RFA, liver resection and transplantation are recommended therapies of curative intent in patients presenting with early stage primary liver tumours (<3 cm) with an observed 5-year survival of 50–75%; whilst patients with HCC tumours of size >3 cm have a median overall survival of 16 to 22 months [7–9]. Hence, the observed clinical outcomes are the consequence of the distant micro metastasis observed with HCC, and tumours often escape the loco regional destruction offered by conventional surgical resection(s) [10]. The tumour biology is a systematic concept according to which the behaviour of cancer is not only determined by the genetics of tumour cells, but also by the microenvironment. The tumour cells escape immunological surveillance by diminished recognition by immune cells through $CD8^+$, $CD4^+$ T cells and natural killer (NK) cells; the increased resistance by tumour cells; or the instigation of an immunosuppressive microenvironment via regulatory T cells (Tregs) and cytokines. An increased recurrence and metastatic dissemination in HCC patients during a post-surgery period further attests that, even though the systemic antitumor immunity is discernible in cancer patients, the steady-state immune response is ineffectual for delivering reasonable tumour control [11,12].

In accordance with the immunoediting hypothesis for the cancer development and progression, the immune system favours tumour cells which are less immunogenic or release immunosuppressive factors. The immune system eludes an anti-tumour response; in addition, by the time HCC tumours become apparent they have already unfolded several other getaways of immunological recognition and elimination [13–15]. Notably, three aspects of tumour biology are considered main line determinants for eluding the tumours cells from the immune system, including the abatement in the expression of tumour associated antigen (TAA) and major histocompatibility complex (MHC) class I, leading to the debacle of $CD8^+$ T cells to discern cancer cells; immune checkpoint inhibitions that accrue myriads of immunosuppressive molecules, including cytotoxic T lymphocytes-associated antigen-4 (CTLA-4 or CD152), programmed death 1 (PD-1) or its ligand PD-L1, T cell membrane protein 3 (TIM-3), killer cell immunoglobulin-like receptors (KIR), and lymphocyte activation gene 3 (LAG-3); and the induction and infiltration of immunosuppressive cells like regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs) and tumour-associated macrophages to stifle the anti-tumour immune response [16,17].

HCC eludes the anti-tumour immunity by fostering an intricate network of immunosuppression pathways involving tumour and stromal cells by instigating a response opposing the priming of T cells and immune effector functions through the secretion of multitude immunosuppressive cytokines such as IL-10, IFN-γ, TGF-β, IDO, and so on [18]. Moreover, the constraints of immunosuppressive forces and the constant exposure to tumour antigens result in T-cells exhaustion, a process partially conciliated through the intramolecular expression of immune-inhibitory factors [19,20]. One potential way to overcome the challenge of the tumour microenvironment is to induce and augment the systemic antitumor immunity by activating the body immune system [21,22]. The energies used in various thermal ablative techniques such as cryoablation, radiofrequency (RF), microwave, and focused ultrasound (FUS), have the potential to trigger an anti-tumour immune response, which can minimize the tumour recurrence risk by eliminating micrometastatic residual disease [23–25]. The ablation of HCC nodules give rise to tumour antigens as an in-situ cancer vaccine, which can lead to the initiation of a systemic antitumor immune response that can potentially eliminate occult, metastatic tumours. The phenomenon of activation of the immune system with a distal antitumor response is

known as the abscopal effect [26]. The RF energy delivered through radiofrequency based (RF-based) devices initiates ionic agitation and generates high-temperature focal hyperthermia (150 °C), thereby producing irreparable cellular damage and coagulative necrosis. In addition to the cytoreductive antitumor activity, various preclinical and clinical studies have ascribed the potential of RF in fostering an anti-tumour immune response by virtue of its immunomodulatory effects. The debris produced following RF-induced coagulative necrosis during liver resection generates tumour antigens and chemokines, which enticed the immunoprotective infiltrates, macrophages, neutrophils, DCs, and NK cells. DCs activate the nuclear factor kappa-light-chain-enhancer of the activated B cells (NF-κβ) pathway, which stimulates $CD8^+$ and $CD4^+$ T lymphocytes and promote a systemic immune response also known as the "in-vivo dendritic cell vaccine effect" [27–30]. A strikingly dense $CD3^+$ T cell infiltration has been demonstrated by studies at tumour locations following radiofrequency ablations (RFA), consistent with the local antitumor immune response. Further evidence of a RFA-induced systemic immunity stems from preclinical and clinical reports of abscopal effects involving the spontaneous regression of distant metastatic lesions following the ablation of primary lesions [31–33].

The RF-based device Habib^{TM} 4X, introduced the bloodless technique of liver resection and has transformed liver surgery for last two decades. Here, RF generated coagulative necrosis on the hepatic parenchyma creates an avascular plane for resection adjacent to the tumour mass. Most HCC resections are completed on livers with an underlying cirrhosis or fibrosis that diminishes the hepatic functional reserve and heightens the likelihood of a hepatocellular failure. Consequently, resections have to be as conservative as achievable in the ablation of non-tumoural hepatic parenchyma. [30,34–36]. Qiu et al. (2017) [37] have outlined the benefits of a liver resection with Habib^{TM} 4X and demonstrated significantly less morbidity, mortality and better survival than with the conventional clamp-crush technique.

In our previously published study (2017) [34], we compared the oncological outcomes following a liver resection in HCC patients using an RF-based device Habib^{TM} 4X with a cavitron ultrasonic surgical aspirator (CUSA) and reported a significantly longer disease-free survival in the Habib 4X group (50.80 vs. 45.87 months, $p = 0.03$). Herein, we are presenting the immunomodulatory changes in the HCC patients, following a liver resection in HCC patients using an RF-based device Habib^{TM} 4X with CUSA, which are based on the fact that anti-tumour immune responses following radiofrequency applications in HCC tumours mark better oncological outcomes.

2. Experimental Section

2.1. Study Design

We prospectively analysed the data from two centers of National Taiwan University Hospital following the approval from the Institutional Review Board. The data included 11 patients with a proven diagnosis of HCC, who underwent a liver resection with a CUSA or RF based device Habib^{TM} 4X from July 2017 to May 2018. The primary endpoint of the study was to assess pre- and post-liver resection immunological parameters: circulating cell populations and serum cytokines.

2.2. Subjects and Procedures

A total of 11 patients with HCC were included in this study, 5 liver resections were performed using CUSA whilst the RF-based device Habib^{TM} 4X was the modality of choice in 6 patients. An open surgical hepatectomy was completed under the guidance of an intra-operative ultrasound. In this study, the resection of three or more liver segments was considered a major hepatectomy whilst fewer than that was considered a minor hepatectomy. Both the lobes of the liver were mobilized and, if needed, the gall bladder was removed. Inflow control was sought in selective cases where excessive parenchymal bleeding was envisaged. In situations where a hepatic parenchymal resection was accomplished with CUSA, an additional help from an assistant surgeon was required to curb the risk of haemorrhage utilizing bipolar coagulation; however, no assisting haemostatic device was

obligated to perform such a task in the Habib^{TM} 4X group. An RF-based bipolar device was applied perpendicularly onto the hepatic parenchyma in a sequential manner to create parallel lines of ablation. An additional line of ablation was fashioned in a perpendicular manner to join the parallel track. Throughout, the application probe was moved in and out in a sequential fashion for 3–5 mm along its axis, which helped in warding off the adherence of the liver tissue. Once a 1 cm thick area of ablated and coagulated tumour free margin was achieved, the hepatic parenchyma was transected using a surgical scalpel [35,36,38]. A haemostasis was attained and the raw surface was covered with a haemostatic agent.

2.3. Cellular Subsets

Peripheral blood samples were collected from each patient in an EDTA anticoagulant-treated tube on day 0 (pre liver resection) and day 7 following the tumour resection. The immunophenotypic analysis was accomplished within 24 h of the sample collections.

Panel 1: Treg cells, $CD8^+$, $CD4^+$, $CD3^+$, $CD4^+CD45RO^+/CD4^+$, $CD4^+CD39^+/CD4^+$, NK, NKT cells;
Panel 2: IFN-γ, TGF-α, TGF-β, IL-1b, IL-6, IL-17, IL-10.

2.3.1. Lymphocytes Isolation

The 20 mL of blood were collected 7 days following the liver resection through a central venous catheter. To isolate the immunocyte, buffy coats were collected and then separated on a Ficoll-Hypaque gradient and used for further analysis.

2.3.2. Flow Cytometry

The cells were processed, brought to single cell suspensions in PBS with 0.5% BSA. and stain at 4 °C for 30 min. The cell surface markers were stained with fluorescent-labeled antibodies: FITC-CD45, anti-CD39-FITC, PE-CD8, PerCP-CD3, anti-CD45RO-ECD, anti-CD45RA-ECD, CD161-DX12, APC-CD25, PE-CD127 and APC.Cy7-CD4 from BD Biosciences (San Jose, CA, USA), $CD4^+CD45RO^+$ cells are considered an activated and short-life memory helper T cell subset.

The cells were then washed twice and fixed by fixation buffer (BD Biosciences, San Jose, CA, USA). The total numbers of individual leukocyte subsets were determined using 123count eBeads counting beads (eBioscience, San Diego CA, USA). A flow cytometry was performed by FACSVerseTM (Becton Dickinson, Mountain View, CA, USA), and the data were processed using FlowJoTM software (Ashland, OR, USA).

2.3.3. Data Analysis and Absolute Count Determination

(1) Use normal gating strategies to identify the cell population to be enumerated (i.e., FSC/SSC lymphocyte gate $CD3^+CD4^+$ gate);
(2) In the same sample, draw a gate on 123 count eBeads in an ungated plot displaying two blue (488 nm) or violet (405 nm) laser excited parameters;
(3) Using the count statistics from these two gates, the concentration of the original cell sample may be determined by the equations:

$$\text{Absolute cell number (cells/}\mu\text{L)} = (\text{cell count} \times \text{eBead volume})/(\text{eBead count} \times \text{cell volume}) \times \text{eBead concentration (1000/}\mu\text{L)}$$

2.3.4. Serum Assay

Circulating immunoreactive IFN-γ, TGF-α, TGF-β, IL-1b, IL-6, IL-17 and IL-10 levels were measured using commercially available quantitative enzyme-linked immunosorbent assays (ELISA, R&D Systems Europe, Abingdon, UK). The assays did not measure the biological activity of the

cytokines. All the measurements were made by a single trained individual to avoid any interobserver variation. All the samples were assayed in duplicate to ensure accuracy and validity.

2.4. Statistical Data Analysis

All the data were entered into a Microsoft Excel™ database and analysed using SPSS_24.0 software (version 24, IBM, Armonk, NY, USA). Continuous variables were analyzed with a Student's *t*-test, and categorical variables were analyzed with a chi-square or Fisher's Exact Test where appropriate. Furthermore, the paired Student's *t*-test were used to compare data between pre- and post-liver resection immunomodulatory changes in the respective groups. p values under 0.05 were considered statistically significant.

2.5. Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

3. Results

3.1. Demography

A hepatic resection was performed in 11 HCC patients, of which a CUSA-based resection was accomplished in 5 patients, whilst in 6 patients a resection was performed using the RF-based device Habib^{TM} 4X. The demographic parameters for each group are outlined in Table 1. The mean age of patients in the CUSA and Habib^{TM} 4X group was 66.00 ± 17.00 years and 62.00 ± 12.80 years respectively. There were 4 women (80.0%) and 1 man (20.0%) in the CUSA cohort, and 1 woman (16.66%) and 5 (83.3%) men in the Habib-4X group. Along with that, we didn't observe any significant differences between the groups regarding serum albumin, serum bilirubin, serum AFP, tumour numbers, tumour size, tumour stage, cirrhosis, HBsAg (hepatitis B surface antigen), HCV (hepatitis C virus), ICG (indocyanine green) clearances and tumour characteristics (Tables 1 and 2).

Table 1. Demographics and clinical characteristics of patients involved in the respective groups.

Attributes	CUSA	Habib^{TM} 4X	*p*-Value
Number of patients	5	6	NS [#]
Mean age, Mean ± SD (years)	66.00 ± 17.00	62.00 ± 12.80	NS [$]
No. male/female	1/4	5/1	NS [#]
Albumin, Mean ± SD (g/dL)	4.45 ± 0.26	4.40 ± 0.59	NS [$]
Bilirubin, Mean ± SD (mg/dL)	0.95 ± 0.60	1.04 ± 0.30	NS [$]
Prothrombin time, Mean ± SD (sec)	11.5 ± 1.8	12.1 ± 2.10	NS [$]
Ascites	0	0	NS [#]
Encephalopathy	0	0	NS [#]
ICG clearance, Mean ± SD (15 min)	7.23 ± 3.56	11.77 ± 4.04	NS [$]
AFP ± SD (ng/mL)	79.40 ± 151.40	52.60 ± 105.30	NS [$]
Cirrhosis	2	3	NS [$]
HbsAg	3	1	NS [$]
HCV	1	5	NS [$]

AFP: alpha-fetoprotein; CUSA: cavitron ultrasonic surgical aspirator; HbsAg: hepatitis B surface antigen; HCV: hepatitis C virus; ICG: indocyanine green; NS: not applicable; SD: standard deviation. [#] Statistical significance was analyzed by the chi-square test. [$] Statistical significance was analyzed by the Student's *t*-test.

Table 2. Pre and postoperative tumour characteristics of patients in study groups.

Attributes	CUSA	Habib^{TM} 4X	*p*-Value
Tumour Numbers	1–3	1–4	NS $
Tumour Stage			
T1	3	4	NS $
T2	2	2	NS $
T3	0	0	NS $
Tumour Size (cm)	3.30 ± 2.04	3.65 ± 10.60	NS $
Anatomical resection	4	5	NS $
Non-anatomical resection	1	1	NS $
Major resection	1	1	NS $
Minor resection	4	5	NS $
Blood loss (mL), Mean ± SD	300.00 ± 316.00	223.00 ± 150.00	NS $
Major complication	0	0	NS $
Resection margin			
Free	2	2	NS $
Free within 1 cm	3	4	NS $
Involved	0	0	NS $

AFP: alpha-fetoprotein; CUSA: cavitron ultrasonic surgical aspirator; NS: not applicable. # Statistical significance was analyzed by the chi-square test. $ Statistical significance was analyzed by the Student's *t*-test.

3.2. Pre- and Post-Liver Resection Modulation of Circulating Immune Cells

We evaluated the absolute number of several immune cell populations i.e., cytotoxic T cells (CD8 T cells), helper T cells (CD4 T cells), regulatory T cells (Treg cells), and natural killer (NK) T cells (Table 3; Figure 1).

The data demonstrated a significant decrease in Treg cells (p-value = 0.002) and $CD4^+CD39^+/CD4^+$ cells (p-value = 0.002) following surgery in the Habib^{TM} 4X group whilst no such observation was made in the CUSA group (Figure 2). Furthermore, our study reported a significant rebound in $CD8^+$ (p-value = 0.050), $CD4^+$ $CD45RO^+/CD4^+$ (p-value = 0.002) and NKT cells (p-value = 0.002) after the liver resection in the Habib^{TM} 4X group whilst no such modulation was noted in the CUSA group (Figure 3). On the contrary, NK cells and $CD4^+$ cells alone were not significantly modulated after the resection in any of the study group.

The data of interest in the present immunological analysis was pertinent to two main T cell subpopulations: the Treg cells and the cytotoxic CD8 T cells. Both subsets showed significant alterations following the liver resection with an RF-based device Habib^{TM} 4X, suggesting the activation of the adaptive immune response. Moreover, the study exhibited a considerable decrease in immunosuppressive Treg cells which play a crucial role in tumour growth and metastasis; hence, the decline in these subsets of cells following the liver resection in Habib^{TM} 4X group confirms the ability of RF to promote the systemic immune response.

Table 3. Observed immunomodulatory changes in respective groups before and after interventions.

Parameters	CUSA			Habib™ 4X		
	Before Surgery (Mean ± SD)	After 7 Days of Surgery (Mean ± SD)	*p*-Value	Before Surgery (Mean ± SD)	After 7 Days of Surgery (Mean ± SD)	*p*-Value
Treg	24.57 ± 4.83	25.00 ± 3.36	0.850	27.20 ± 6.17	17.90 ± 5.26	0.002 *
$CD3^+$	1681.57 ± 384.25	1565.71 ± 459.78	0.819	1632.00 ± 392.68	1700.00 ± 445.35	0.721
$CD4^+$	1085.71 ± 278.91	1095.71 ± 384.48	0.956	1008.00 ± 283.50	1028.00 ± 331.86	0.886
$CD8^+$	515.71 ± 255.46	401.42 ± 98.39	0.291	580.0 ± 216.18	732.00 ± 188.31	0.050 *
$CD4^+CD45RO^+/CD4^+$	44.71 ± 1.98	45.00 ± 4.43	0.879	44.60 ± 1.78	49.50 ± 4.03	0.002 *
$CD4^+$ $CD39^+/CD4^+$	36.29 ± 4.92	35.86 ± 4.38	0.866	36.90 ± 4.23	23.70 ± 8.49	0.000 *
NK	11.86 ± 3.02	11.57 ± 3.64	0.876	11.60 ± 2.32	10.90 ± 2.51	0.526
NKT	7.43 ± 1.90	8.14 ± 2.12	0.519	6.80 ± 1.62	10.60 ± 3.50	0.006 *
TGF-ß	2191.42 ± 400.43	1978.57 ± 478.83	0.385	2378.00 ± 382.35	1490.00 ± 366.60	0.000 *
IFN-γ	45.57 ± 9.65	45.28 ± 10.73	0.959	48.20 ± 11.82	57.30 ± 7.41	0.027 *
IL-10	7.47 ± 0.69	7.47 ± 0.50	1.000	7.93 ± 0.58	4.47 ± 1.47	0.000 *
IL-1b	7.92 ± 1.47	7.90 ± 1.05	0.970	7.28 ± 1.69	9.39 ± 4.51	0.180
IL-17	58.00 ± 16.54	63.00 ± 15.35	0.569	52.6 ± 13.92	36.10 ± 13.55	0.010 *

Statistical significance was analyzed by the paired Student's *t*-test in all scenarios. CD: cluster of differentiation; IFN-γ: interferon gamma; IL: interleukin; TGF-β: Transforming growth factor beta; Treg: T regulatory cells. * denotes statistical significance.

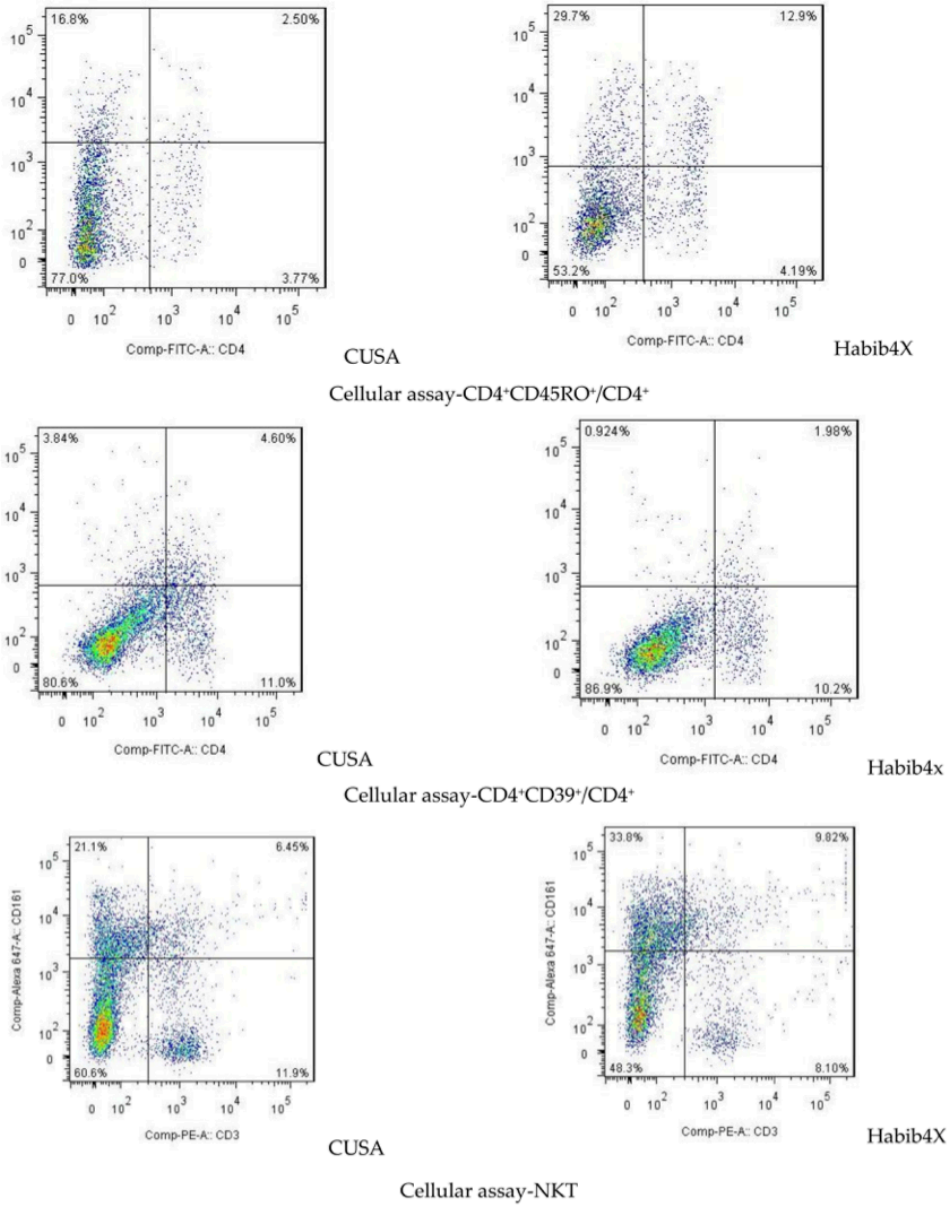

Figure 1. Flow cytometric analysis of immune cells in peripheral blood 7 days after liver resection with CUSA or Habib^{TM}-4X.

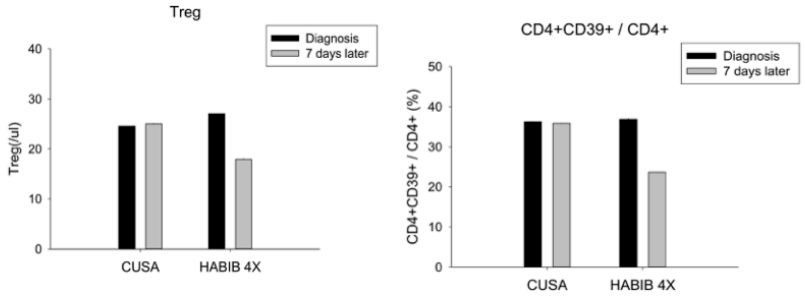

Figure 2. Treg cells and the $CD4^+CD39^+/CD4^+$ cells changes in both study groups. A significant decrease was observed in both cell types in the Habib^{TM} 4X group.

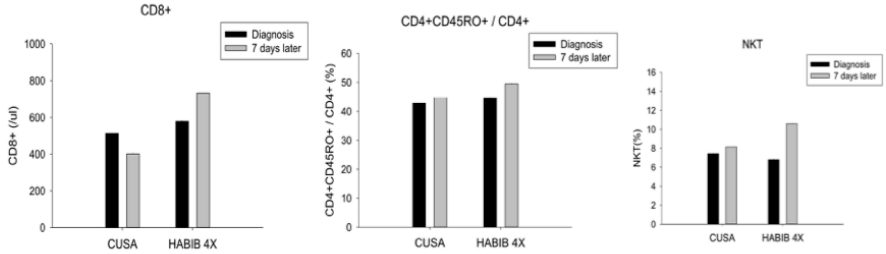

Figure 3. Cytotoxic CD8 T cells, $CD4^+CD45RO^+/CD4^+$ and NKT cells changes in both study groups. A significant increase was observed in all three cell types in the Habib^{TM} 4X group.

3.3. *Pre- and Post-Liver Resection Modulation of Circulating Cytokines and Chemokines*

We analysed the plasma concentration of several metabolites such as cytokines, interleukins and chemokines able to modulate the immune response, at the same time points in which we tested the blood immune cell composition. The study demonstrated that the RF based device Habib^{TM} 4X was associated with marked changes in the plasma concentration of IFN-γ, TGF-ß, interleukin (IL)-10, and IL-17 (Table 2).

The serum IFN-γ level was significantly increased in the Habib^{TM} 4X group in comparison to CUSA (p-value = 0.027), as lower serum levels of IFN-γ were associated with increased Tregs and marked tumour growth and progression (Figure 4).

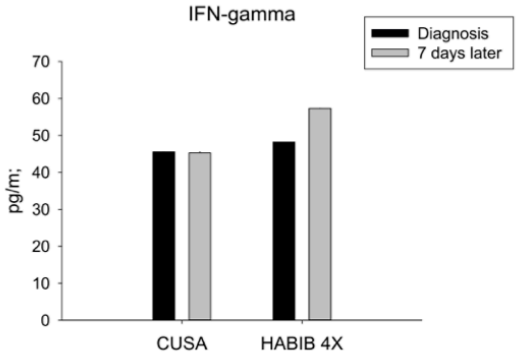

Figure 4. Serum IFN-γ changes in both study groups. A significant increase was noted in the Habib^{TM} 4X group.

The RF-based device Habib^{TM} 4X did significantly decrease in TGF-ß (p-value = 0.002), which normally favours unregulated tumour-growth by sustaining cancer angiogenesis and enhanced tumour associated inflammation (Figure 5a). Similarly, we noted a decline in IL-10 following the resection with the RF-based device Habib^{TM} 4X, which not only directly suppresses cytotoxic T-cells and NK cells but also promotes tumour progression and a poor prognosis (Figure 5b). Both TGF-ß and IL-10 played an instrumental role in the induction of Treg cells and the abolition of NKT cell activity.

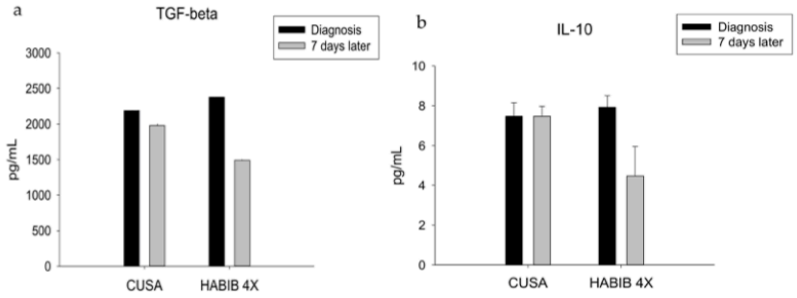

Figure 5. Serum TGF-ß (**a**) and IL-10 (**b**) level changes in both study groups. Significant decrease was noted in Habib^{TM} 4X group.

IL-17 constituted a crucial component of the inflammatory background of HCC, and a high expression was considered as a predictor for disease progression and poor survival. The data analysis outlined a significant decrease in IL-17 following the RF-based liver resection, in contrast to CUSA (p-value = 0.010) (Figure 6).

In addition, we analyzed a few other metabolites, including TGF-α and IL-1b; however, we could not find any significant changes in their levels.

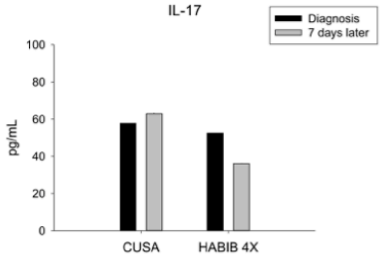

Figure 6. Serum IL-17 level changes in both study groups. Significant decrease was noted in Habib^{TM} 4X group.

4. Discussion

In acts of deception, HCC evades the natural anti-tumour immunity through the formation of an extremely intricate immunosuppressive network. The interactions between the malignant cells with immune and stromal cells instigate the secretion of various immunosuppressive cytokines. The malignant cells of HCC use autonomous and non-autonomous techniques to escape the body's inherent anti-tumour immune response. The selective pressure on transformed cells activates a phenomenon of immunoediting in the immune system, which comes to the aid of cells with less immunogenic potential or who produce immunosuppressive factors. It is reckoned that tumour cells and a multitude of components in the tumour microenvironment conspire to inveigle their own development and progression. The tumour cells circumvent the inherent immunological surveillance

system by limiting the recognition by immune cells, including $CD8^+$, $CD4^+$ and natural killer (NKT) cells [39,40]. The silencing or repressed expression of tumour-associated antigens help tumour cells dodge the immune system despite the persistent expression of antigenic molecules owing to a glitch in antigen processing and presentation. The down-regulated expression pathway involves a proteasomal malfunction leading to a defect in protein fragmentation for the configuring of peptides, or leading to a fault in the antigen peptide transporters 1 and 2 that are involved in the transportation of the peptides to the endoplasmic reticulum to be put onto HLA class I heavy chains and presented over the cell membrane before getting recognized by $CD8^+$ T cells via T-cell receptors (TCR). In addition, the mutation or deletion of beta2 microglobulin results in the complete loss of the HLA class I expression; however, both the absence or reduced expression of the HLA class I undermines tumour antigens recognition by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells [41–43].

The induction and infiltration of immunosuppressive cells such as regulatory T cells, myeloid-derived suppressor cells (MDSCs) and tumour-associated macrophages impede the immune response against tumour cells. Treg cells account for 5–10% of $CD4^+$ T cells and are marked by the presence of the membrane molecules CD25, CTLA-4, CD62L, along with the expression of the transcription factor FoxP3, which play central roles in the maintenance of self-tolerance. FoxP3 is the key regulatory transcription factor for Treg cells, and mutations in the FoxP3 gene result in severe autoimmune disorders and the onset and progression of various cancers [44–46]. Studies have outlined the increased infiltration of $FoxP3^+$ Treg cells in tumour and peripheral blood of HCC patients, their role in anti-tumour immunity and their aide in tumour progression. The activation of a Treg-cell through TCR engendering the inhibition of APC maturation through the CTLA-4-mediated downregulation of CD80 and CD86, repression of CD28 mediated co-stimulatory signaling, decrease of IL-2 via the enhanced expression of the IL-2 receptor with the IL-2 receptor-chain CD25, simultaneous secretion of inhibitory cytokines IL-10 and TGF-β, and ATP (adenosine triphosphate) degradation disposing the diminution of the antitumour immune response, along with the expression of granzyme and/or perforin ushered in the destruction of APCs and effector T cells [47–50]. Various studies described that tumours infiltrating Treg cells are presumed to be activated by neo-antigens released from tumour cells, are present in high concentrations within tumours and manifest the enhanced expression of suppression-related molecules such as CTLA-4. Moreover, the concentration of Treg cells reciprocates with the number of intra-tumoural macrophages and is considered as an independent negative prognostic factor for the overall survival [51]. In our study, we found a significant decrease in Treg cells and $CD4^+CD39^+/CD4^+$ cells following the surgery with the Habib^{TM} 4X. A meta-analysis conducted by Sun et al. included 27 studies with 3854 HCC patients, and demonstrated that high intra-tumoural and peripheral blood levels of Tregs are markers of poor overall survival {OS; HR (hazard ratio) = 1.95, $p < 0.00001$} and disease free survival (DFS; HR = 1.82, $p < 0.00001$). In addition, higher Tregs infiltrations are associated with multiple liver tumours, high AFP levels, poor tumour differentiation, and advanced stage and vascular invasion, and are therefore a measure of poor prognosis [52,53].

Immune checkpoint molecules are coinhibitory in nature, and impede the immune response by steering clear of overactive T cells and averting collateral tissue damage. The important members of this group include CTLA-4, PD-1, ligands of PD-1 and TIM-3 [54–57].

CTLA-4 is constitutively expressed by Treg cells and is also expressed by activated T cells. CTLA-4 is pivotal for the control of the $CD4^+$ T-cell function, and it essentially staged the priming phase of the cell mediated immune response [58,59]. It contends with the actions of the stimulatory protein CD28 by binding to its ligand CD80 and CD86 present on the membranes of APC. Moreover, CTLA-4 imparts an inhibitory signal to the T cell in opposition to conventional TCR signaling [60–62]. In similar fashion within the confines of the tumour, CTLA-4 fosters immunosuppression through the induction and differentiation of Treg cells along with the upregulation of IL-10 and IDO (indoleamine-2,3-dioxygenase) by means of a CD80 and CD86 counter signaling approach [63–65]. The upregulation of IDO in HCC is orchestrated by IFN-γ and other cytokines in HCC which inhibit T-cell activation and proliferation, and induce $CD4^+$ T-cells into $FoxP3^+$Treg cells. Hence,

IDO also favours tumour growth by activating a path in the complex anti-tumour immunity pathophysiology [66,67]. Additionally, proinflammatory cells located in the peritumoral stroma release IL-17 and other chemokines from epithelial cells, which in turn pave the way for additional neutrophil and chemokines receptor positive B cells migrations towards the tumour. Notably, the inflammatory microenvironment produced by tumour-associated macrophages and IL-17 producing cells in HCC are not only correlated with tumour development and growth but also with recurrences following liver transplantation [68–70].

PD-1 is usually present on the membranes of activated $CD8^+$ and $CD4^+$ lymphocytes, B cells, NK cells and is also reported on MDSCs, T_{reg} cells, monocytes and dendritic cells (DCs) [54,71,72]. The expression of PD-L1 is induced by a variety of cytokines of which IFN-γ is the most potent. The tumour microenvironment is a state of chronic antigen exposure where IFN-γ released by antigen-specific T cells instigates PD-1 expression on reactive T lymphocytes and facilitates the binding with ligands (PD-L1) in APC and tumour cells. The coupling of PD-1–PD-L1 burns out T-cells by disrupting TCR signaling pathways, consequently impeding T-cell proliferation and the release of cytotoxic mediators. Additionally, colluded binding between PD-L1 (expressed on other cells) with its receptor PD-1 on macrophages causes IL-10 release and by that means $CD4^+$ T-cell repression. Notably, an intense PD-1 expression on effector $CD8^+$ cells within HCC tumours has been found to be related to disease progression and post-operative recurrence [73–76].

Recently, attention has been given to the inherent ability of RF, which not only kills the HCC cells but also produces favourable immunological change in the tumour microenvironment, minimizes recurrence and improves survival [77]. Studies have implicated local and systemic immunomodulatory changes following the application of radiofrequency during RFA. Consequently, the immunomodulatory properties of RF have emerged as plausible explanations for the improved survival observed in small HCC following ablation [30,78,79]. Xu et al. [80] has conducted a meta-analysis including 31 studies and 16,103 patients, and demonstrated a significantly better overall and disease-free survival of the RFA group than for conventional liver resection for small $\leq$2 cm HCC tumours. However logical this may seem, this theory does not hold much water owing to the underlying field change phenomenon and chronic inflammatory state of the liver over which HCCs developed. Studies have reported better survival in liver resection groups owing to the complete removal of tumour [81,82]; however logical this may seem, this theory does not hold much water owing to underlying field change phenomenon and chronic inflammatory state of the liver over which HCCs develop [83–85].

The immunomodulatory impact of RF in a liver resection device has never been assessed and the present study provides the first detailed analysis of such changes. Here, this study examined the pre- and post-surgery immunological parameters employing either the RF-based device Habib^{TM} 4X or CUSA for a liver resection in HCC.

Our observation demonstrated the significant advantages of immunomodulatory cellular and cytokine changes, which seem to be plausible reasons for the better survival noticed in the Habib^{TM} 4X group. RF energy induces localized coagulative necrosis during liver resection and releases a significant amount of tumour debris including immunogenic particulates, chemokines [Monocyte Chemoattractant Protein-1 (MCP-1) and CXCL16], cytokines (TNF-α, IL-1, IL-6, IL-8 & IL-16) and damage-associated molecular patterns (DAMPs), i.e., DNA and heat shock protein. These debris are taken up by DCs and presented through MHC molecules on $CD8^+$ and $CD4^+$ T cells to induce an immune response. The activation of the nuclear factor kappa-light-chain-enhancer of the activated B-cells (NF-κβ) pathway, stimulates $CD8^+$ and $CD4^+$ T lymphocytes to promote a systemic immune response called the "in-vivo dendritic cell vaccine effect" [23,29,86,87]. In the present study, we did an immune analysis of the absolute number of several immune cell populations and found significant positive changes in Treg cells, $CD4^+CD39^+/CD4^+$, cytotoxic $CD8^+$ T cells, $CD4^+$ $CD45RO^+/CD4^+$ and NKT cells following the liver resection with the RF-based device Habib^{TM} 4X, in contrast to CUSA.

The T-cells infiltration on day 7 was characterised by an increase in $CD8^+$ T and $CD4^+$ $CD45RO^+/CD4^+$ cells, while there was a decrease in Treg and $CD4^+CD39^+/CD4^+$ cells. This resulted in an increase in the ratio of $CD8^+$ T versus Treg cells, indicating a shift of immune balance toward anti-tumour immunity following the RF application. This is in contrast to the contrary evidence, which states that surgical stress brings reduction in $CD8^+$ T; this could be explained by virtue of the RF energy, which has proven to induce significant antigen specific T-cell changes in HCC [88–90].

Furthermore, the enhanced infiltration of $CD45RO^+$ T cells with an increase in $CD4^+$ $CD45RO^+/CD4^+$ cells has been considered a marker for a better clinical outcome. Hu et al. [91] performed a meta-analysis involving 25 studies and 4720 patients to understand the association between the intra-tumoural $CD45RO^+$ T cells density and the overall and disease free survival in patients with HCC, and reported an improved 5-year DFS. Several mechanisms determine the immune response by $CD45RO^+$ T cells in a tumour microenvironment, including a low threshold of activation upon exposure to an antigen; an enhanced capability to proliferate; an increased IFN-γ production and life-long persistence with self-renewal characteristics, all of which established them as a hallmark of adaptive immunity [92].

Tregs, especially $CD4^+CD25^+Foxp3^+$, are one of the most studied immune cells owing to their specific inhibitory influence on HCC tumour growth and progression [93]. Zhao et al. [53] conducted a meta-analysis involving 23 studies and 1279 HCC patients to understand the association between the Tregs cells and HCC, and reported an 87% higher frequency of Tregs in the tumour microenvironment. The plausible explanations indicates towards following distinctives of Tregs cells in regulating the tumour microenvironment, including apoptosis induction of effector cells through the $CD25^+$ mediated diminution of IL-2; cytolysis of effector T cells mediated by granzyme B and perforin; dendritic cells maturation and functioning regulated through CTLA-4 mediated cell-cell contact-dependent mechanism; and alteration in the effector cell immune response through the liberation of inhibitory cytokines such as TGF-β and IL-10 [94,95]. Recently, Tu et al. (2016) [96] reported a significantly poor survival in ($p = 0.006$) HCC patients with high number of intra-tumoural T-cells.

In addition, we observed a significant positive modulation in the plasma concentration of several metabolites including TGF-ß, IL-10, IFN-γ, and IL-17 following a liver resection with the RF-based device Habib^{TM} 4X, as opposed to CUSA. The secretions of these cytokines and the functioning of several immune cell populations are intricately regulated by each other; for instance, a subtype of Tregs, which play a key role in tumour immune escape, is associated with a higher secretion of IL-10 and TGF-ß and is considered a marker of progressive disease and poor survival [96,97]. TGF-β with IL-10 controls the conversion switch of type 1 and type 2 helper T cells, shifting the balance toward Type 2 helper cells. Moreover, it directly suppresses the Type 1 helper cells $CD8^+$ T, NK, DC and M1-type macrophages while enhancing the expression of M2-type macrophages with the increase of Tregs cell functions [98,99]. Studies have shown that the reduction in Tregs cells would prevent the expression of immunosuppressive cytokines or that the targeted therapy against these cytokines, such as CD25, TGF-ß, CTLA-4 and so on, would prevent their functions, thereby controlling tumour growth [100]. In the present study, decreased Tregs following surgery with the RF-based device could be a possible explanation for the better survival in this group of patients, in contrast to the CUSA group, where we neither observed positive immunological changes nor better survival.

The observed immunomodulation is unique to the RF and is different from normal surgical stress or inflammation as both groups were matched in terms of age, sex, number, stages of tumours, and so on, thereby making the patients subject to an equal amount of surgical stress. In addition, the pre-liver resection immune statuses were compared with the one week post-resection statuses, which provided substantial stability in the beneficial changes of T lymphocytes and Tregs.

Furthermore, understanding the anti-tumour immunological properties of $CD8^+$ T-cells and Tregs has not only led to the development of checkpoint inhibitors but also added a new dimension in the management of advanced HCC, and both CTLA-4 and PD-1 are two principles, extensively studied checkpoints, which normally prevent the overstimulation of anti-tumour immune responses.

Increasing the activation of T-cell receptors and proinflammatory cytokines results in an increased CTLA-4 expression, whilst ligands of PD-1 are expressed on many immune T cells, Tregs and B-cells [101,102]. CTLA-4 attaches with costimulatory B7 molecules (CD80/86) with a stronger affinity than CD28. The binding between B7 and CTLA-4 instead of CD28, does not produce a stimulatory signal. Hence, CTLA-4 functions to competitively inhibit T cell functioning, and induce T cell anergy. However, the anti-CTLA-4 antibodies Ipilimumab and Tremelimumab have demonstrated success at overcoming this regulatory blockade. Similarly, the programmed death receptor 1 (PD-1), following activation with PD-L1, a ligand often found on tumor cells, inhibits T cell function and triggers apoptosis. Pembrolizumab, Nivolumab, Durvalumab, and Avelumab, presently available, are all anti-PD-1 drugs approved for the treatment of melanoma, Hodgkins lymphoma and various solid tumors including HCC [103,104]. Here, it is important to understand that $CD8^+$ T-cells and Tregs are the centre of interest for both checkpoint inhibitors and RF; thus combining these therapeutic modalities exerts a superlative effect. Thanks to the synergism between these modalities, RF induces the infiltration of $CD8^+$ T-cells at the resection margin, whilst checkpoint inhibitors enhance their anti-tumour functioning [105,106]. In accordance with this, Duffy et al. [107] conducted a study and demonstrated the activation of the immune system following the introduction of checkpoint inhibitors and the accumulation of intra-tumoral $CD8^+$ T-cells following RF ablation; they thereby presented the first clinical evidence of synergism of the checkpoint inhibitor tremelimumab and RF-ablation in the management of advanced hepatocellular carcinomas. Based on our observation of positive immunomodulatory changes following liver resection with the RF-based device HabibTM 4X, we speculate that combining check-point inhibitors could improve survival or delay recurrence following resection in HCC.

The index study has certain limitations which require attention. First, secondary to the sample size and unintended biases made during the recruitment of patients could have influenced the analysis outcomes. Despite these limitations, we firmly believe that present study has analysed the broad range of circulating cell populations and serum cytokines which are involved in tumour-related immunomodulation and which could be involved in and determine the observed better survival in liver cancer patients following the application of radiofrequency energy.

In this study, we demonstrated positive immunomodulatory changes explicitly in terms of $CD8^+$ T-cells and Tregs, following the liver resection in HCC patients using the RF-based device Habib^{TM} 4X, compared to CUSA, which may account for the observed better survival in the same group. The RF-based device Habib^{TM} 4X not only facilitates a safe and efficient liver resection but also fosters favourable immunomodulatory changes presumably responsible for a better survival in comparison with other modalities of liver resection. The invention of the RF-based device Habib^{TM} 4X has produced a rich array of new visions for HCC cancer treatment, focusing on the surgical resection of liver tumours with RF induced immunomodulatory changes, providing better overall and disease free survival. Further, these anti-tumour cells are a common target for RF and checkpoint inhibitors give an opportunity to combine both treatment modalities. However, future research efforts will further explore the impact of combining the checkpoint inhibitor with RF-energy during the various stages of HCC.

5. Conclusions

RF-based device Habib^{TM} 4X has not only commissioned a safe and bloodless hepatic resection but also persuades appreciative changes in tumour microenvironment. Henceforth, hepatic resection with RF-based device Habib^{TM} 4X in HCC are associated with positive immunomodulatory changes in circulating immune cells and cytokines which could explain the observed improvement in the DFS and decreased tumour recurrence.

Author Contributions: K.W.H. and K.J. have contributed equally and are joint first author. K.W.H., P.-c.Y. and K.J. developed the concept and design of the study. K.W.H., K.J., P.-c.Y., C.-Y.H. and P.-H.L. screened the abstract and

full text, extracted data, and assessed studies. K.J., M.H.S., P.-H.L., C.-Y.H. and P.-c.Y. wrote the manuscript. K.W.H. and N.H. critically revised the manuscript. All authors read and approved the final version of the manuscript.

Conflicts of Interest: Nagy Habib is an inventor of the RF-based device Habib^{TM} 4X. All the other authors have no conflicts of interest, including specific financial interests or relationships and affiliations relevant to the subject matter or materials discussed in the manuscript.

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Appendix 6 Signed statement of candidate's contribution

The individual signatures of all co-authors have been included here.

Paper 1- Reccia I, Kumar J, Kussano T, Giakoustidis A, Zanellato A, Retsas P, Habib N, Jiao L, Duncan S, Pai M. Radiofrequency-assisted liver resection: technique and results Surgical Oncology. 2018:27(3):415-20.

Paper 1

Reccia I, Kumar J, Kussano T, Giakoustidis A, Zanellato A, Retsas P, Habib N, Jiao L, Duncan S, Pai M, Radiofrequency- assisted liver resection: technique and results. Surgical Oncology. 2018;27(3):415-420.

Contribution: Kumar Jayant took a lead role in literature search, data analysis, writing manuscript. He revised manuscipt in liaison with the co-authors and responded as the corresponding author.

I agree that Kumar Jayant made the above mentioned contributions to this publication.

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Tomokazu Kusano		26-6-19
Alexandros Giakoustidis		12-8-2019
Artur Zanellato		26/9/2019
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Nagy Habib		12 April 2019
Long Jiao		12/4/19
Duncan Spalding		26/04/19
Madhava Pai		13/9/19

Paper 2- Jayant K, Sodergren MH, Reccia I, Kusano T, Zacharoulis D, Spalding D, Pai M, Jiao L, Huang KW. A systematic review and meta-analysis comparing liver resection with RF-based device Habib™-4X with clamp-crush technique. Cancer 2018;8:10(11).

Paper 2

Kumar J, Sordegren M, Reccia I, Kusano T, Zacharoulis D, Spalding D, Pai M, Jiao L, Huang K W; Systematic review and meta-analysis comparing liver resection with RF-based device HabibTM-4X with clamp-crush technique in liver cancer. Cancer 2018;8:10(11).

Contribution: Kumar Jayant took a lead role in design of this study, literature search, data analysis and writing. He further revised the manuscript in liaison with the co-authors and responded to the reviewers as the corresponding author.

I agree that Kumar Jayant made the above mentioned contributions to this publication.

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Long R Jiao		12/6/19
Kai Wen Huang		27/9/19

Paper 3- Huang K, Lee P, Reccia I, Kusano T, Jayant K, Habib N. Impact of Cavitron Ultrasonic Surgical Aspirator (CUSA) and Bipolar Radiofrequency Device (Habib-4X) based hepatectomy for hepatocellular Carcinoma on tumour recurrence and disease-free survival. Oncotarget 2017;8(55):93644-54.

Paper 3

Huang K, Lee P, Reccia I, Kusano T, Kumar J, Habib N Impact of Cavitron Ultrasonic Surgical Aspirator (CUSA) and Bipolar Radiofrequency Device (Habib-4X) based hepatectomy for hepatocellular Carcinoma on tumour recurrence and disease-free survival. Oncotarget 2017;8(55):93644-93654.

Contribution: Kumar Jayant was involved in design of this study, literature search, data analysis and writing. He further revised the manuscript in liaison with the co-authors and responded to the reviewers as the corresponding author

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Paper 4- Mazmishvili K, Kumar J, Janikashvili N, Kikodze N, Mizandari M, Pantasulaia I, Paksashvili N, Sodergren MH, Reccia I, Pai M, Habib N, Chikovani T. Study to evaluate the immunomodulatory effects of radiofrequency ablation compared to surgical resection for liver cancer. Journal of Cancer 2018;9(17):3187-95.

Paper 4

MazmishviliK, Kumar J, Janikashvili N, Kikodze N, Mizandari, M, Pantasulaia I, Paksashvili N, Sodergreen M, Ressia I, Pai M, Habib N, Chikovani T. Study to evaluate the immunomodulatory effects of radiofrequency ablation compared to surgical resection for liver cancer. Journal of Cancer 2018;9(17):3187-3195.

Contribution: Kumar Jayant was involved in literature search, data extraction and writing. He did revision in liaison withco-authors and responded to the reviewers as the corresponding author.

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Nagy Habib		12 April 2019
Timatin Chikovani		25/04/2019

Paper 5- Huang K, Jayant K, Lee PH, Yang PC, Hsiao CY, Habib N, Sodergren MH. Positive immuno-modulation following radiofrequency assisted liver resection in hepatocellular carcinoma. Journal of Clinical Medicine 2019;8(3).

Paper 5

Huang K, Kumar J, Po-Huang Lee, Po-chih Yang, Chih-Yang Hsiao, Habib N, Sodergreen M, Positive Immuno-Modulation Following Radiofrequency Assisted Liver Resection in Hepatocellular Carcinoma, Journal of Clinical Medicine 2019;8(3)

Contribution: Kumar Jayant took lead role in literature search, data extraction and writing manuscript. He further revised the manuscript in liaison with the co-authors and responded to the reviewers as the corresponding author.

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Po-chih Yang		2019/9/27
Chih-Yang Hsiao		27/09/2019
Nagy Habib		12 April 2019
Mikael H Sodergren		12/04/19

Appendix 7 Published papers

1. Mauro Podda, Salomone Di Saverio, R. Justin Davies, Jenny Atzeni, Francesco Balestra, Francesco Virdis, Isabella Reccia, **Kumar Jayant**, Agreasta F and Adolfo Pisanu, Prophylactic intra-abdominal drainage following colon and rectal anastomoses. A systematic review and meta-analysis of randomized controlled trials. **The American Journal of Surgery May 2019. (Impact Factor- 2.54)**

2. Huang K, Jayant K, Lee PH, Yang PC, Hsiao CY, Habib N, Sodergren MH. Positive immuno-modulation following radiofrequency assisted liver resection in hepatocellular carcinoma. Journal of Clinical Medicine 2019;8(3). **Journal of Clinical Medicine (Mar 2019) (Impact Factor- 5.68)**

3. **Kumar J,** Reccia I, Virdis F, Shapiro AMJ Systematic review and meta-analysis on the impact of thrombolytic therapy in donation after circulatory death liver transplantation? **Journal of Clinical Medicine** (Nov 2018) **(Impact Factor- 5.68)**

4. **Kumar J,** Sordegren M, Reccia I, Huang K W, Zacharoulis D, Spalding D, Pai M, Jiao L, WH Kai. systematic review and meta-analysis comparing liver resection with RF-based device Habib™ -4X with clamp-crush technique in liver cancer, **Cancer (Nov 2018) (Impact Factor- 6.10)**

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11. Reccia I, **Kumar J**, Kussano T, Kirby S, Pai M, Jiao L, Duncan S, Habib N, Radiofrequency- assisted liver resection: technique and results **Surgical Oncology**. 2018:27(3):415-420 **(Impact Factor- 3.44)**

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14. **Kumar J,** Reccia I, Sordegren M, Kusano T, Zanellato A, Pai M, Spalding D, Zacharoulis D, Habib N Radiofrequency assisted pancreaticoduodenectomy for palliative surgical resection of locally advanced pancreatic adenocarcinoma. **Oncotarget** Feb 2018. PMID: 29644005 **(Impact Factor- 5.16)**

15. Huang K, Lee P, Reccia I, Kusano T, **Kumar J,** Habib N Impact of Cavitron Ultrasonic Surgical Aspirator (CUSA) and Bipolar Radiofrequency Device (Habib-4X) based hepatectomy for hepatocellular Carcinoma on tumour recurrence and disease-free survival. **Oncotarget** Nov 2017. PMID:29212179. **(Impact Factor- 5.16)**

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Kindly Note on **PubMed:** Kumar Jayant may appear as Kumar J or Jayant K