The Roles of Laparoscopic Liver Resection and Hypoxia Inducible Factor in the Pathophysiology of Liver cancer

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Declaration

Except where acknowledgement is made, all work contained in this thesis was performed by myself in the Faculty of Medicine (Renal Section) and Department of Surgery, Hammersmith Hospital Campus, Imperial College London. The work reported in this thesis has not been submitted for any other degree in this or any other university or institute of learning.
Abstract

In the last 20 years laparoscopic liver resection has been increasingly practised. However its role in the treatment of liver cancer remains under scrutiny. I performed a pilot study at a specialist HPB unit assessing the results of the laparoscopic resections of one surgeon and comparing them to the results of matched cases on whom he had performed open resection. The resection technique was radiofrequency assisted resection pioneered in this unit. I also analysed the resected tissue to investigate any differential effect on cell characteristics of the 2 operative techniques. Operative time was significantly longer in laparoscopic cases and time to recurrence of R0 resections significantly shorter. Resected tissue demonstrated significantly higher levels of the hypoxia inducible factor-2 and CD10, a recognised poor prognostic marker in primary colorectal tumours.

I hypothesised that livers resected laparoscopically are under a relative hypoxia because of the increased intraabdominal pressure associated with the pneumoperitoneum and tumours cells therefore have a positive selection advantage. In the setting of longer resection times this may compromise the oncological result of the surgery causing earlier recurrence. Using a established model of HIF activation, I showed that poor prognostic marker CD10 may be a function of hypoxia inducible regulation. Certainly I was able to replicate data from cervical squamous epithelia demonstrating that both in benign, dysplastic and malignant tissue, HIF expression corresponded to a reduced cell E-cadherin expression that may allow a more malignant potential.

I also analysed the effect of RF ablation on circulating tumour cells in palliative irresectable cancers and in the context of both open and laparoscopic liver resection. This showed only a transient rise in both resectional techniques, (open and lap) that would unlikely count for the differential oncological outcome previously demonstrated in the pilot study.

In keeping with current international opinion, further work is required to verify the role of laparoscopic liver resection in liver cancer.
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CHAPTER 1

INTRODUCTION
1.1 Liver surgery: a brief history

Liver surgery has grown over 2000 years from the mystic hepatoscopy of the Babylonians to the ultimate of orthotopic transplantation by Starzl in 1968 (Starzl, Groth et al. 1968). The first successful liver resection was for trauma by Hildanus in the 17th century. The first successful planned resection was by Langenbuch in 1888 and the first hemihepatectomy by Wendel in 1911 (Foster 1991). The principles of liver regeneration and liver haemostasis were determined in the period 1880-1900; Ponfick, Mayer, Kousnetzoff, Pensky and Pringle making significant contributions. Couinaud, Healey and Schroy popularized segmental anatomy and Lortat-Jacob and Robert performed the first inflow ligation resection starting the modern era (Foster 1991; Ellis 2009). Starzl brought liver transplantation to its current status and Bismuth introduced hepatobiliary units as a means of optimizing treatment of liver disease (Gugenheim, Castaing et al. 1987).

1.2 The current role of liver surgery in cancer

1.2.1 Hepatocellular carcinoma (HCC)

Primary liver cancer or hepatocellular carcinoma (HCC) ranks fifth in frequency in the world with an estimated number of 437,000 new cases in 1990 (Bosch, Ribes et al. 1999). The incidence of HCC is rising (El-Serag and Mason 1999) owing to a large pool of people infected with hepatitis C virus, hepatitis B virus or both (Nagasue, Yukaya et al. 1984; Schafer and Sorrell 1999; Okuda 2000). HCC is the most common primary cancer of the liver and accounts for 90% of all primary liver malignancy (Kaczynski, Hansson et al. 1996). Within this population more than 80% of patients have cirrhosis (Nagasue, Yukaya et al. 1984; Schafer and Sorrell 1999; Okuda 2000). The prognosis of untreated patients with HCC is poor (Nagasue, Yukaya et al. 1984; Okuda, Ohtsuki et al. 1985) although early detection through the wide use of ultrasonography (US) and improved management of patients with liver function impairment have improved the outcome (Llovet, Bustamante et al. 1999).

Different methods of non-surgical local treatment for these tumours have been devised. These are particularly important in cases of multiple tumours involving both lobes of the liver, poor hepatic reserve in cirrhosis, in tumours within the liver which are not easily accessible and patients unfit to withstand surgery (Hargreaves, Adam et al. 2000).
CHAPTER 1: INTRODUCTION

When there is no evidence of extrahepatic disease, partial or total liver resection (the latter followed by orthotopic liver transplantation (OLT)) is the only potentially curative treatment for HCC. It appears from different reports that OLT may lead to a better outcome than liver resection in carefully selected cirrhotic patients with HCC. For example, those with small tumours, uninodular or binodular tumours and the absence of portal vein tumoural thrombosis (Bismuth, Chiche et al. 1993; Mazzaferro, Regalia et al. 1996; Bismuth, Majno et al. 1999; Yamamoto, Iwatsuki et al. 1999; Figueras, Jaurrieta et al. 2000).

In patients with early stage HCC in whom liver transplantation is contraindicated and in those with good liver function, partial hepatectomy is associated with excellent survival. Tumour spread and the frequent coexistence of underlying cirrhosis may limit the cases amenable to surgical resection. Resectability rates in large series of HCC range from 17 to 54% (Lai, Fan et al. 1995; Cance, Stewart et al. 2000; Takano, Oishi et al. 2000). However, progress in imaging, surgical procedures and perioperative care have turned hepatectomy for HCC into a safe operation with low mortality (Fan, Lo et al. 1999; Torzilli, Makuuchi et al. 1999).

1.2.2 Colorectal carcinoma

Colorectal cancer is the commonest gastrointestinal malignancy and the second commonest cause of cancer death, comprising 11% of new cancer diagnoses and 10% of all cancer deaths in the UK. Approximately 32 000 cases are diagnosed each year and 17000 deaths are attributed to the disease (Weiss, Grundmann et al. 1986). Surgery is the treatment of choice for patients with localised disease but over half of all patients will develop metastases.

The liver is often the first site of metastatic disease and may be the only site of spread in as many as 30–40% of patients with advanced disease. Of new cases, 20–25% of patients will have clinically detectable colorectal liver metastases (CLMs) at the time of the initial diagnosis and a further 40–50% of patients will eventually develop liver metastases after resection of the primary, most commonly within the first three years of follow up (Scheele, Stangl et al. 1990; Sugarbaker 1990; Stangl, Altendorf-Hofmann et al. 1994; Scheele, Stang et al. 1995). It has been postulated that the principal mode of tumour dissemination is via the portal system and therefore that surgical resection of isolated hepatic metastases
from colorectal cancer may be curative in a number of cases (Weiss, Grundmann et al. 1986).

The natural history of metastatic colorectal cancer is variable. Median survival without treatment is less than eight months from presentation (Lahr, Soong et al. 1983; Seymour, Stenning et al. 1997; Simmonds 2000). Patients with isolated hepatic metastases have a better prognosis than those with more extensive metastatic disease (Goslin, Steele et al. 1982; Lahr, Soong et al. 1983; Stangl, Altendorf-Hofmann et al. 1994; Rougier, Milan et al. 1995) suggesting biological differences in the two settings. However, few patients with limited liver-only metastases survive greater than 5 years (Goslin, Steele et al. 1982; Stangl, Altendorf-Hofmann et al. 1994).

Approximately 20–30% of patients with metastatic colorectal cancer have disease that is confined to the liver and is potentially resectable (Stangl, Altendorf-Hofmann et al. 1994). Several large series on resection for colorectal liver metastases (CLM) have reported five year survival ranging from 25% to 44%, with operative mortality of 0–6.6% (Cady and Stone 1991; Scheele, Stang et al. 1995; Choti and Bulkley 1999; Fong, Fortner et al. 1999). Given that approximately 18 000 patients will develop hepatic metastases annually in the UK, approximately 3600 patients maybe suitable for hepatic resection although many more patients may benefit from hepatic resection with less restrictive criteria of resectability being employed in many centres (Fong, Fortner et al. 1999). It seems unlikely that a randomised study assessing outcome following resection compared with other treatment modalities will be undertaken in patients with resectable liver metastases. However, a variety of therapeutic approaches have been used in the treatment of metastatic colorectal cancer including radiofrequency ablation, cryotherapy, new chemotherapy regimens or some combination of these that allow safe ablation of liver metastases (Bismuth, Adam et al. 1996; Giacchetti, Perpoint et al. 2000; Adam, Avisar et al. 2001; Primrose 2002; Tournigand, Andre et al. 2004).

It has been postulated that because haematogenous spread usually occurs in a stepwise fashion, initially to the liver, with subsequent intrahepatic spread via the portal vein and further spread to the systemic circulation, surgical resection of isolated hepatic metastases from colorectal cancer may be curative.

The selection criteria for surgery are usually controlled primary tumour, no extra hepatic metastases and a resection that is technically feasible with tumour-free margins (Hugh,
Kinsella et al. 1997). Rarely, a small proportion of patients with completely resectable extrahepatic disease may become long-term survivors (Scheele, Stang et al. 1995).

Chemotherapy used alone is palliative but may prolong the survival of patients with unresectable disease (de Gramont, Figer et al. 2000; Douillard, Cunningham et al. 2000; Saltz, Cox et al. 2000; Simmonds 2000). Used in combination with surgery it may prolong the time to recurrence after resection of hepatic metastases (Kemeny, Adak et al. 2002) or downsize to resectability patients previously judged inoperable (Giacchetti, Itzhaki et al. 1999). The role of other treatments is poorly defined (Armstrong, Anderson et al. 1994; Adam, Akpinar et al. 1997; De Baere, Elias et al. 1999; Primrose 2002).

1.2.3 Feasibility of hepatic surgery

Operative morbidity and mortality following liver resection are related to the development of hepatic failure. The risk of this occurring is a function of the extent of resection (Wigmore, Redhead et al. 2001; Shoup, Gonen et al. 2003; Stewart, O'Suilleabhain et al. 2004) and the presence of coexisting liver disease (Kooby, Fong et al. 2003). Techniques for determination of the hepatic functional reserve in patients with chronic liver disease such as the plasma retention rate of indocyanine green at 15 min (ICG15) (Caesar, Shaldon et al. 1961; Matsumata, Kanematsu et al. 1987) and preoperative portal pressure assessment (Bruix, Castells et al. 1996) are usually all considered together when deciding whether to proceed to surgery. Other complications that may contribute to or be related to postoperative liver failure include haemorrhage, bile leak, intra-abdominal sepsis, and cardiopulmonary dysfunction (Scheele, Stangl et al. 1990; Rees, Plant et al. 1996; Fong, Cohen et al. 1997; Kooby, Fong et al. 2003). Laparoscopic liver resection may have some advantage in the short term over open surgery but there are no data to indicate the impact of this procedure on long term outcome (Vibert, Perniceni et al. 2006). Duration of survival is shortened by the presence of inadequate or involved resection margins (Pawlik, Scoggins et al. 2005). Previous data from the Registry of Hepatic Metastases, a multi-institutional database of liver resections, suggested that a margin >1cm was associated with 45% five year survival, but only 23% survived five years if the margin was less (Hughes, Simon et al. 1986). A number of other studies have supported the view that poorer overall survival and disease free 5 year survival is associated with resection margins <1 cm although others have produced evidence to suggest that a lesser margin may be acceptable as long as the tumour pseudocapsule is resected during dissection (Yamamoto, Sugihara et al. 1995; Rees, Plant et al. 1996; Rees, Plant et al. 1997).
Studies have shown that long term survival is achieved in patients whose primary colorectal cancer has been managed by radical resection and appropriate local adjuvant treatment. Long term survival may be affected adversely by the presence of more than three metastases but multivariate analysis has provided inconsistent results as to whether the number of resected metastases has a significant effect on long term survival (Hughes, Simon et al. 1986; Scheele, Stang et al. 1995; Fong, Cohen et al. 1997; Fong, Fortner et al. 1999; Schindl, Wigmore et al. 2005).

Resectability of liver tumours requires assessment by a radiologist in conjunction with a liver surgeon experienced in the management of colorectal metastases as there is a need to define the acceptable residual functioning volume. It is accepted that prediction of liver dysfunction following liver resection is difficult to quantify for the individual patient. Previous studies have suggested that that liver volume can be readily calculated by CT volumetry (Wigmore, Redhead et al. 2001; Shoup, Gonen et al. 2003). Based on liver transplant and liver resectional experience, acceptable residual functioning volume is thought to consist of approximately one third of the standard liver volume or the equivalent of two liver segments. Such decisions are again best made by the hepatobiliary team.

As for most surgical procedures, the operating surgeon and the anaesthetist are best able to assess the patient’s fitness for intervention. Increasing ASA and POSSUM grade have been shown to be of value for several operative procedures (Pol, Campan et al. 1999). Experience of the managing team is likely to have an effect on outcome and it has been demonstrated that patients who undergo liver resection at low volume hospitals are at a higher risk of postoperative complications and death than those who have the same operation at high volume hospitals (Dimick, Cowan et al. 2003).

For those patients not considered fit for operative intervention, radiofrequency ablation has been shown to be a safe and effective treatment (Oshowo, Gillams et al. 2003; Oshowo, Gillams et al. 2003). However, its precise role in the management of hepatic colorectal metastases has yet to be defined and no study has addressed its potential superiority over other treatment modalities in the setting of a randomised controlled trial. The NCRN/EORTC CLOCC trial (EORTC 40044-Chemotherapy and local ablation versus chemotherapy (5-FU and oxaliplatin) alone) trial aims to address this issue in part.

Surgical resection for HCC is technically demanding but may be considered after careful selection of patients with normal or subnormal preoperative liver function. Two different
populations of patients are involved: in 90% the tumour arises in a cirrhotic liver (and in these cases good preoperative liver function, classified as Child grade A, is necessary) whilst 10% have non-cirrhotic livers. The former have a higher operative mortality, lower regeneration capacity (Yamanaka, Okamoto et al. 1993) and higher intrahepatic recurrence rate.

In summary, careful patient selection for resectional surgery is paramount as very often the requirements and the objectives of these resections are contradictory: it is important to balance need to conserve functional liver parenchyma so as to prevent postoperative liver failure with the need to ensure a sufficient surgical margin so as to avoid local recurrence.

1.3 Liver resection techniques

1.3.1 Classical liver resection technique

Liver resections as performed today are the result of the practical application of segmental surgery to the liver as described by Bismuth (Bismuth 1982). This approach is based on precise knowledge of the segmental anatomy as described by the French anatomist and surgeon Couinaud (Couinaud 1957) and on the other hand on the development of advanced imaging techniques. Intraoperative ultrasound (IOUS) ‘has become the eyes of the surgeon’ to operate upon a segmental organ with no external landmarks (Bismuth, Castaing et al. 1987).

**Figure 1**: Couinaud’s segmental anatomy of the liver.
1.3.2 Surgical aids and resection tools

Technological innovations such as an ultrasonic dissector that skeletonises portal and hepatic vein ramifications free from hepatocytes (particularly useful in fine intraparenchymatous dissections) have facilitated the task of liver surgery (Andrus and Kaminski 1986).

Haemostasis of the exposed raw surface of the liver at the end of the operation can be improved with the argon beam coagulator and biological glues. However, use of the argon beam coagulator (a modified, powerful means of diathermy based on the concentration of current through argon gas) instead of conventional contact coagulation and/or biological glues (fibrin tissue adhesive: tissucol, biocol) can help and enhance haemostasis that depends mainly on selective surgical ligation and coagulation of vessels.

Together with these technological advances, evolving surgical techniques have contributed to better safety and improved results after surgical resection. Methods of vascular control to stop blood inflow to the liver during parenchymal transection have contributed to lowering perioperative blood loss and the need for blood transfusion. The Pringle manoeuvre, initially developed for liver trauma, which consists of intermittent clamping and unclamping of the whole hepatic pedicle is still used today (Pringle 1908).

More selective ways of limiting vascular inflow to the liver permit perfusion of the liver that is to remain, either by using suprahilar vascular control (Takasaki, Kobayashi et al. 1990) or a more precisely demarcated exclusion area by means of balloon occlusion of segmental portal branches (Castaing, Garden et al. 1989) which can be helpful when a limited segmentectomy is necessary (Bismuth, Houssin et al. 1982).

1.3.3 Indications for surgical resection

As previously mentioned, HCC usually occurs in association with chronic liver disease and cirrhosis. When HCC arises in non-cirrhotic liver it is often diagnosed when the tumour becomes large and symptomatic. In the absence of diffuse disease involving both lobes or metastases, aggressive surgical management of HCC is indicated in a non-cirrhotic liver. In these patients resection of the tumour may be considered no matter its size, since they are
usually in good general condition and surgical resection usually involves the tumour mass rather than the functional parenchyma (Bismuth, Chiche et al. 1995).

Since most HCCs occur in patients with cirrhosis or chronic liver disease, important changes in portal haemodynamics and a reduction in the functional liver parenchyma must be anticipated. The Pugh–Child Classification (Child and Turcotte 1964; Pugh, Murray-Lyon et al. 1973) is useful and decisive for evaluating cirrhotic patients with impaired liver function.

Liver resection for HCC in a cirrhotic liver is contraindicated in the presence of severe hepatic impairment (such as ascites, jaundice, Child–Pugh B and C, liver atrophy). In these settings, there is an increased risk of decompensation of the liver disease or liver failure in the postoperative period. However, although cirrhotic patients presenting with jaundice due to hepatic insufficiency have a dismal prognosis, a few cases of obstructive jaundice due to the tumour itself may benefit from surgical resection (Lau, Leung et al. 1997).

Other situations that preclude resection are the presence of portal vein thrombosis reflecting extensive disease, lymph node metastases, extrahepatic localisations and intrahepatic multiple, diffuse disease. All these situations, in the absence of currently effective adjuvant treatments, render any treatment palliative. Recently a reduction of up to 60% in the number of laparotomies done for HCC is evident in patients in whom laparoscopic US is employed first (Lo, Lai et al. 1998).

1.3.4 Strategies to increase resectability

HCC is mainly fed by branches of the hepatic artery, and this is used to selectively deliver a combination of lipiodol (iodised poppy seed oil) and a chemotherapeutic agent (adriamycin or cisplatin) followed by embolisation with absorbable gelatin particles. The so-called chemoembolisation is effective in producing tumour necrosis and reducing the size of the tumour, improving surgical access and resectability (Majno, Adam et al. 1997).

Although no survival benefit has been obtained in randomised trials many HPB teams favour (Figueras, Jaurrieta et al. 2000) the use of preoperative transarterial chemoembolisation (TACE) not only to downstage the tumour but for accurate staging of the intrahepatic disease. Lipiodol fixation to tumoural tissue increases the possibility of detection of small sized lesions on CT scan and US (Novell, Dusheiko et al. 1991).
The remnant liver appears to be an important predictor for postoperative liver failure, particularly after major hepatectomies (Shirabe, Shimada et al. 1999). It is possible to accurately determine the volume of the potential remnant liver by means of CT. In some cases when a large hepatectomy is planned, compensatory hypertrophy of the future remnant liver can be induced by preoperative portal vein embolisation of the liver to be resected (Azoulay, Raccuia et al. 1995) using Histoacryl™ (enbucrilate, Braun Melsungen Lab, Germany) or ethanol to overcome potential postoperative liver failure. This was first reported to be feasible by Makuuchi (Makuuchi, Thai et al. 1990) who reported that fatal liver failure did not occur after major liver resection for cancer when the portal vein of the resected liver was obstructed. The same technique has been shown to offer satisfactory results even when used in chronic liver disease (Lee, Kinoshita et al. 1993; Azoulay, Castaing et al. 2000).

1.3.5 Results of surgical resection

1.3.5.1 Hepatocellular carcinoma (HCC)

Aside from the underlying functional liver parenchyma, other aspects of the general condition of the patient are directly linked to the outcome of surgical resection for HCC. The importance of the nutritional balance of the patient undergoing surgical resection for HCC has been well established (Fan, Lo et al. 1994). Diabetes mellitus (Shirabe, Shimada et al. 1999) and pre-existing cardiovascular disease (Miyagawa, Makuuchi et al. 1995) have also been shown to intervene as morbidity co-factors. Although old age has long been regarded as an adverse factor for major surgery including liver resections, several groups report on the feasibility and good results obtained after hepatic resection for HCC in elderly patients (Ezaki, Yukaya et al. 1987), even in those with underlying cirrhosis (Lui, Chau et al. 1999). Improvements in perioperative technique including reduction in blood loss and blood transfusion (Fan, Lo et al. 1999; Torzilli, Makuuchi et al. 1999) intermittent warm ischemia (Miyagawa, Makuuchi et al. 1995; Torzilli, Makuuchi et al. 1999) and shortening the operation time have made considerable contributions to safer procedures and improved outcomes (Miyagawa, Makuuchi et al. 1995).

Through application of all the above-mentioned patient selection criteria, surgical principles and techniques and postoperative care, overall mortality has been much improved after surgical resection for HCC from 58% to less than 10% reported in recent series (Belli,
Limongelli et al. 2009). Moreover, it has been found that the results of liver resection for HCC are related to the hospital volume (Begg, Cramer et al. 1998).

The long-term prognosis after surgical resection of HCC is limited by the high rate of postoperative recurrence. At the present time, tumour recurrence is the major cause of death following resection of HCC both in cirrhotic (Nagasue, Kohno et al. 1993) and non-cirrhotic livers (Bismuth, Chiche et al. 1995). Without some form of treatment most patients with recurrent HCC will die within 1 year, (Hu, Lee et al. 1996). The frequency of tumour recurrence depends on the duration of the time of follow-up. In different series between 43% and 65% of the patients had recurrences within 2 years of removal of the first tumour (Lee, Lin et al. 1995; Hu, Lee et al. 1996) and up to 85% within 5 years (Lai, Fan et al. 1995). The disease is confined to the liver in 30–91% (Matsuda, Ito et al. 1993; Bismuth, Chiche et al. 1995). However, up to 50% of the intrahepatic recurrences are of multiple or diffuse pattern (Nagasue, Kohno et al. 1993).

Intrahepatic recurrence after resection of HCC may arise through two different mechanisms; firstly, growth of residual satellite tumours, not identified at the time of the initial surgery, from intrahepatic metastasis through the portal vein (Shirabe, Takenaka et al. 1996), and secondly, multicentric occurrence from synchronous and metachronous multicentric carcinogenesis. It is difficult to establish the role of each type of process and this is very much a matter of controversy for hepatologists. Further studies based on clonal analysis of both recurrent and primary tumours may help to clarify this issue (Yamamoto, Kajino et al. 1999). Intrahepatic spread would appear, however, to be more frequent (Shimada, Takenaka et al. 1998). This differentiation may have prognostic and even therapeutic implications. Patients whose relapse is due to a solitary recurrence and good liver function, classified as Child grade A, would appear to be good candidates for re-resection, as opposed to patients with intrahepatic metastases, which are often multifocal (Poon, Fan et al. 1999).

Several reports have studied the risk factors for development of postoperative intrahepatic recurrence (Nagasue, Uchida et al. 1993). Factors involved in recurrence are related to the tumour characteristics such as: the size of the tumour, with tumours larger than 5 cm associated with a higher risk of recurrence (Bismuth, Chiche et al. 1995); multiple nodules whether due to intrahepatic metastasis or to multicentric carcinogenesis (Ikeda, Saitoh et al. 1993; Bismuth, Chiche et al. 1995; Hu, Lee et al. 1996); the presence of satellite nodules associated with tumour invasiveness (Nagasue, Uchida et al. 1993; Fuster, Garcia-Valdecasas et al. 1996); vascular involvement (Nagasue, Uchida et al. 1993; Bismuth,
Chiche et al. 1995; Vauthey, Klimstra et al. 1995; Fuster, Garcia-Valdecasas et al. 1996; Yamamoto, Ikai et al. 1999), evidence of the importance of intrahepatic metastasis through the portal vein circulation (Yamamoto, Kosuge et al. 1996); the absence of capsule formation (Nagasue, Uchida et al. 1993; Bismuth, Chiche et al. 1995); preoperative AFP (Chen, Hwang et al. 1994); and DNA aneuploid content (Okada, Shimada et al. 1994), nuclear DNA content possibly reflecting the malignant potential of some tumours.

Other risk factors for recurrence are related to the patient and the underlying liver status (Kakazu, Makuuchi et al. 1993). A higher intrahepatic recurrence rate has been found in cirrhotic patients (Sasaki, Imaoka et al. 1992). It is likely that this is due to the presence of more cases of multicentric tumours and more cases of positive surgical margins as a result of limited resections due to poorer residual liver function (Nagasue, Kohno et al. 1993). Active hepatitis with increased inflammatory activity, as reflected by high levels of transaminase (Shirabe, Takenaka et al. 1996) and alcohol abuse (Okada, Shimada et al. 1994) has also been linked to an increased recurrence rate.

Another group of risk factors is related to surgery and include distance from the tumour to the resection margin of less than 1cm. However, this is controversial as some authors found it to be an unfavourable prognostic factor (Chen, Hwang et al. 1994) whereas others have found no correlation (Fuster, Garcia-Valdecasas et al. 1996; Poon, Fan et al. 2000). A wide resection margin during hepatectomy for HCC has been reported not to be an effective means of reducing postoperative recurrence (Poon, Fan et al. 2000). Although, 1cm of clear margin from the edge of the tumour appears desirable to ensure tumour clearance and avoid local recurrence (Poon, Fan et al. 2000), particularly for tumours larger than 4cm. Perioperative transfusion, another example of risk factors related to surgery, has been shown to be associated with increased intrahepatic recurrence (Matsumata, Ikeda et al. 1993).

While the precise role played by each of these risk factors is difficult to determine, it appears from most studies that factors related to tumour invasiveness and vascular involvement are the key risk factors for recurrence of HCC.

Although no effective adjuvant treatment is yet available, the recurrence rate may be reduced by careful attention to some of the following technical aspects: avoiding manipulation, especially of large tumours, using an anterior approach to minimise the risk of tumour cell dissemination (Lai, Fan et al. 1996) and minimising intraoperative blood loss so
as to avoid blood transfusion (using portal clamping and lowering central venous pressure (Cunningham, Fong et al. 1994). The treatment modalities in use and available today for primary HCC, namely surgical resection (partial or total and followed by liver transplantation), TACE and percutaneous treatments (Hargreaves, Adam et al. 2000) have also been used in postoperative recurrent HCC. The use of multiple therapies may improve survival rates (Ikeda, Saitoh et al. 1993).

While the use of systemic chemotherapy after curative resection for HCC has been associated with a worse outcome (Lai, Lo et al. 1998), postoperative regional chemotherapy or lipidiolisation may be an effective therapy to reduce recurrence and improve survival (Lau, Leung et al. 1999). However, adjuvant therapies still need to be developed to prevent recurrence. The use of polyprenoic acid, a synthetic retinoid or percutaneous alcohol injection (Muto, Moriwaki et al. 1996) would appear to reduce the incidence of a second primary HCC after resection. More recently the use of interferon beta has been reported to be effective in preventing recurrence of HCC after complete resection or ethanol ablation in patients with HCV-related chronic liver disease (Ikeda, Arase et al. 2000). Other promising lines of investigation may arise from investigation into the role of angiogenesis on recurrence of HCC (El-Assal, Yamanoi et al. 1998) and the potential use of antiangiogenic drugs.

Embolisation or chemoembolisation, performed in most cases for non-operable patients (Lai, Fan et al. 1995; Lee, Lin et al. 1995), may be used to confirm the diagnosis of recurrence and in some cases these techniques may reduce the size of the tumour making it resectable (Majno, Adam et al. 1997). This treatment appears to be the most frequently used for intrahepatic recurrent HCC when unresectable because of multinodularity or inadequate functional liver reserve.

Although randomised studies are not available, repeat hepatectomy appears to be the best treatment for recurrent HCC. This is possible with solitary, anatomically resectable recurrences and no extra-hepatic disease in patients with sufficient functional liver parenchyma to withstand a re-resection. The resectability rate of recurrent intrahepatic HCC is variable, ranging from 10 to 77% according to the different series (Table 1). This procedure has proved to be safe in selected patients, with mortality ranging from 0% to 8%.
Table 1: Summary of the most recent series of documented repeat resections for recurrent HCC and their respective outcomes

<table>
<thead>
<tr>
<th>Authors</th>
<th>Year</th>
<th>Number of first resections</th>
<th>Number of Intrahepatic recurrences</th>
<th>Number of repeat resections</th>
<th>Resectability rate of recurrence (%)</th>
<th>5 year survival after second resection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shimada</td>
<td>1998</td>
<td>312</td>
<td></td>
<td>41</td>
<td></td>
<td>43</td>
</tr>
<tr>
<td>Farges</td>
<td>1998</td>
<td>251</td>
<td>89</td>
<td>15</td>
<td>17</td>
<td>60</td>
</tr>
<tr>
<td>Poon</td>
<td>1999</td>
<td>244</td>
<td>105</td>
<td>11</td>
<td>10</td>
<td>69</td>
</tr>
<tr>
<td>Adam</td>
<td>2000</td>
<td>236</td>
<td>102</td>
<td>22</td>
<td></td>
<td>59</td>
</tr>
</tbody>
</table>

Long term survival rates after repeat resection range from 37 to 86% after 5 years. A longer interval from hepatectomy to recurrence predicts improved survival (Lee, Lin et al. 1995). Patients treated by repeat hepatectomy have been reported to have better survival rates than those treated by other palliative methods (Matsuda, Ito et al. 1993; Lee, Lin et al. 1995; Hu, Lee et al. 1996).

1.3.5.2 Colorectal liver metastases (CLM)

Surgical resection of hepatic metastases from colorectal cancer can be undertaken safely in the majority of patients. The median postoperative (30 day) mortality reported by 24 studies was only 2.8% (0–6.6%) (Simmonds, Primrose et al. 2006). The most frequent causes are hepatic failure, postoperative haemorrhage, and sepsis. It is likely that current surgical and anaesthetic practice is associated with perioperative mortalities nearer to 1% as demonstrated in a large multicentre trial (Nordlinger 2005).

Operative morbidity is more difficult to quantify as many studies report only fatal morbidity or a very limited range of postoperative complications. Some studies present information on outcomes that may be surrogate markers for operative morbidity. The limited reported results suggest that most patients require only a short period of intensive care following hepatic resection for colorectal metastases. The median or mean length of hospital stay reported by 17 studies ranged from 7–21 days indicating that while most patients made a rapid recovery following surgery, some experienced a more prolonged hospital stay.
Disease recurrence is common after resection of colorectal hepatic metastases indicating that in the majority of cases the extent of the metastatic disease is underestimated by pre and intraoperative staging investigations. Around one-third of patients experience disease recurrence.

Studies including only patients who underwent resection reported a survival of around 30% at 5 years for patients undergoing potentially 'curative' resection of isolated hepatic metastases and the majority are disease free at this time. The median survival of patients undergoing R0 resections were substantially better (32% at 5 years) than for patients undergoing R1 resections (7.2% at 5 years) and those who did not undergo resection (0% at 5 years) (Simmonds, Primrose et al. 2006).

Patients undergoing surgery have a better prognosis than other patients with metastatic colorectal cancer as their disease is both confined to the liver and circumscribed within it. The patients are also more likely to be of good performance status and have little or no comorbidity. Identifying a comparable control group in the absence of randomised trials is difficult. The survival of the patients not treated surgically or who have palliative surgery is poor. A small number of retrospective studies have attempted to determine the natural history of patients with isolated liver metastases. In a review of 484 untreated patients with liver metastases from colorectal cancer, those with the best prognosis (25% liver involvement, primary tumour grade 1/2, no extra hepatic tumour and no mesenteric nodal involvement) had a median survival of 21.3 months, compared with 30 months in patients undergoing hepatic resection in the same institution (Stangl, Altendorf-Hofmann et al. 1994).

In one group of 125 patients with liver-only metastases, most of whom had had no therapy, the median survival was 12.5 months and all patients died within 5 years. Survival correlated with the extent of liver disease. (Garden, Rees et al. 2006). Lahr et al. studied 175 untreated patients with liver metastases from colorectal cancer. Patients with 1–4 liver metastases lived longer than those with >5 metastases (Lahr, Soong et al. 1983). This is consistent with a study of 113 patients with hepatic metastases from colon cancer where a mean survival in patients with widespread liver disease was 3 months (Wood, Gillis et al. 1976).

In the majority of cases the extent of the metastatic disease is underestimated by pre and intraoperative staging investigations. Around one-third of patients experience disease recurrence.
recurrence in the liver alone and may be candidates for repeat resection. The remainder experience recurrence concurrently in the liver and extrahepatic sites or in extrahepatic sites only. It may be that biological features within the tumour may be important however, this requires further study.

Identification of prognostic factors that predict the outcome following surgical resection of colorectal hepatic metastases would assist in the identification of those patients most likely to benefit from this intervention, or more importantly assist in the identification of patients who are unlikely to benefit (Poston, Adam et al. 2005).

Modern chemotherapy using cytotoxic agents alone offers extension of median survival to 2 years in patients with nonresectable disease (Cals, Rixe et al. 2004; Goldberg, Sargent et al. 2004). When monoclonal biological agents are added to cytotoxic chemotherapy, the prospect of median survival now extends beyond 2 years and 20% of patients will still be alive 4 years after detection of unresectable liver disease (Cunningham, Humblet et al. 2004; Hurwitz, Fehrenbacher et al. 2004). It is therefore inevitable that the combination of surgical resection and chemotherapy, which is becoming commonplace, will impact on the postoperative survival (Nordlinger 2005). Secondly, novel surgical strategies such as preoperative portal vein embolisation to increase residual acceptably safe volume, or two-stage hepatectomy to allow compensatory hepatic hyperplasia before completion of R0 resection (Abdalla, Barnett et al. 2002; Poston, Adam et al. 2005) have also increased the number of resectable patients including those with extensive liver-only disease. The changes in the definition of resectability mean that >20% of patients with liver metastases can now be considered for surgery with curative intent at the outset. It is unclear what the long-term outcome of these strategies will be but the results appear encouraging (Abdalla, Barnett et al. 2002; Pawlik, Scoggins et al. 2005; Poston, Adam et al. 2005).

1.3.6 Non-surgical treatment techniques

Surgical resection, hepatectomy or repeat hepatectomy are not always possible for example where there is multicentricity, advanced or decompensated liver cirrhosis or patients unfit to withstand surgery or anaesthesia. Several in-situ methods for destruction of liver tumours have been developed. All these local non-surgical techniques are performed under ultrasound or CT guidance that enables either precise intratumoural delivery of chemical agents (such as ethanol or acetic acid) or accurate delivery of means of thermal ablation (cryotherapy, percutaneous microwave coagulation, hot saline, radiofrequency (RF) or interstitial laser photocoagulation) (Hargreaves, Adam et al. 2000).
Percutaneous ethanol injection (PEI) was first described in 1983 by Sugiura et al. (Sugiura 1983). The technique involves the intralesional injection of absolute alcohol using a fine needle and leads to coagulation and tumour cell destruction. The number of injections for effect needed is variable and the rare side effects and complications include pain and fever. Comparative case series studies have shown good local control of the disease for single tumours of <3cm (Castells, Bruix et al. 1993). However no prospective randomized control trials have been performed.

Hepatic cryotherapy causes the in situ destruction of liver tumours by using subzero temperatures. Once the probe is placed into a lesion under US, CT or MR guidance, an iceball including the tumour is created through multiple freeze–thaw cycles. Technological advances in the design of cryoprobes and delivery systems have made it possible for cryotherapy to be more effective and less invasive, thus extending its use from laparotomy to laparoscopic and percutaneous approaches. Complications related to the technique include hypothermia, haemorrhage as a result of the frozen liver cracking during the thawing phase and ‘cryoshock’ (a syndrome of multiorgan failure, severe coagulopathy, and disseminated intravascular coagulation after treatment). To date, cryosurgery is limited to irresectable lesions, and is used alone or combined with surgical resection (Adam, Akpinar et al. 1997). Although few long term results are available, 5-year survival rates of 27% have been reported following cryoablation in patients with HCC (Zhou, Tang et al. 2002). However, some authors have abandoned its use in favour of RF owing to a higher local tumour recurrence rate with cryoablation compared with RF and a much higher complication rate after cryoablation (Pearson, Izzo et al. 1999).

RF is becoming a widely used ablative method for liver malignancies that can be delivered through an open, percutaneous or laparoscopic approach. The non-insulated tip of a needle electrode probe is positioned within the lesion under US, CT or MR guidance. The probe is attached to a generator delivering high frequency (460–500 kHz) alternating current, which is turned into heat through ionic agitation within the tumour. Advances in probe technology from monopolar to multiple, expanding or cooled-tip electrodes have increased the extent of the thermal lesion (coagulative necrosis) from 1.6 to 3 cm (Buscarini and Rossi 1997). Severe complications or death after RF are rare.

For small HCC nodules (smaller than 3 cm in diameter) RF causes a more complete necrosis than PEI (90% compared with 80%). Moreover, RF has the benefit or only requiring a single session whereas PEI required up to five sessions for tumour ablation on average (Livraghi,
Goldberg et al. 1999). Some authors have proposed percutaneous RF as an alternative to repeat hepatectomy for treatment of liver tumour recurrence since it is much less invasive (Elias, De Baere et al. 2002). A surgical approach, however, allows more accurate assessment of the intrahepatic disease and the abdominal cavity. Furthermore, concerns have been raised recently by a reported 12% incidence of neoplastic seeding along the needle tract after percutaneous RF, particularly if it is to become a ‘bridge’ therapy to liver transplantation (Llovet, Vilana et al. 2001). Longer follow-up and precise indications are needed to assess the place of RF in the treatment of HCC.

With all these procedures, it is essential that treatment of the tumour be complete to be of any benefit, in the absence of widespread intrahepatic or extrahepatic disease.

**1.3.7 The role of liver transplantation**

Total hepatectomy followed by orthotropic liver transplantation (OLT) removes the tumour and the underlying end-stage liver disease and avoids the risk of synchronous nodules or metachronous nodules. Nevertheless, until recently liver resection has been the treatment of choice for small tumours leaving OLT for large and irresectable tumours. This has led to poor initial results after OLT, mainly from high mortality rates of around 24–30% (Iwatsuki, Gordon et al. 1985) and high recurrence rates of up to 70% (O'Grady, Polson et al. 1988). As a result the use of OLT for malignant disease dropped from 29% in 1983-1987 to 15% in 1988-1992 (Pichlmayr, Weimann et al. 1992). Interestingly, a small number of transplanted patients in whom HCC was detected incidentally in the hepatectomy specimen were found to have had a good outcome (Iwatsuki, Starzl et al. 1991). This finding is consistent with a retrospective study in which patients with small HCC had significantly better disease-free survival after liver transplantation when compared with similar patients who had liver resection. This highlights the importance of adequate selection of the patients who are candidates for OLT (Bismuth, Chiche et al. 1995). Patients in whom extrahepatic disease has been ruled out, with fewer than three nodules <3cm in diameter and without portal vein invasion are considered to have the best indication for OLT, according to one unit achieving 83% disease-free survival at 3 years compared with 18% after liver resection (Azoulay, Samuel et al. 2000). Better outcome after OLT for small HCC has shown liver resection to be second best and has restricted the indication for liver resection to the treatment of patients with contraindications to liver transplantation (age, extra-hepatic disease and psycho-social factors or patients with good liver function and easily localised resectable lesions). Similar strict criteria have been followed by other groups such as Mazzaferro et al.
(a tumour of 5 cm or less or no more than three nodules less than 3 cm) (Mazzaferro, Regalia et al. 1996) giving fresh impetus to OLT in patients with HCC. For patients with tumours >3cm and with three or more nodules, poor results after liver transplantation may be due to the fact that the tumour may have already spread outside the liver (Bismuth, Chiche et al. 1993).

A new approach in dealing with intrahepatic recurrence after resection is total hepatectomy followed by OLT. With the present organ shortage this has been studied in a statistical theoretical model only. It appears that the strategy of primary resection followed by "salvage OLT" may be a rational way to cope with lengthening waiting lists (particularly for patients with low risk of recurrence after partial hepatectomy, when results of liver resection are good and stringent follow-up can detect early, "transplantable" recurrences). This approach has been supported by other clinical studies (Poon, Fan et al. 2002).

Improvement in imaging methods and surgical techniques have made it possible to detect HCC at earlier stages, particularly in patients predisposed by pre-existing underlying liver disease. This in turn has lead to an increase in the number of patients in whom treatment is aimed to be curative. Long-term survival after surgical treatment of HCC can be achieved by careful selection of patients presenting with lesions of favourable size, location and number and with good liver function.

The main limit to surgical resection is recurrence, and close post-operative follow-up to detect this early is of paramount importance. Aggressive management of postoperative recurrence may improve the outcome after resection.

1.4 RF-assisted resection: the Hammersmith experience

With improvements in liver surgery and an increased knowledge of liver anatomy, physiology and hepatic cell biology, the mortality and morbidity associated with liver surgery have significantly decreased. Different techniques have been developed to allow safer liver resection (Tranberg, Rigotti et al. 1986; Nuzzo, Giulianite et al. 1996; Hansen, Isla et al. 1999; Yamamoto, Ikai et al. 1999). Surgeons can decrease intraoperative blood loss by using hypotensive anaesthetics, Pringle’s manoeuvre or total vascular exclusion. Parenchymal division can be performed with the scalpel, crushing the tissue with the finger or clamps, ultrasonic dissectors, hydrodissectors or stapling devices. Nevertheless, liver resection remains a formidable surgical procedure and safe performance requires a high level of training and skill. Intraoperative blood loss remains a major concern for surgeons.
operating on the liver (Bismuth, Castaing et al. 1989) and is associated with a higher rate of postoperative complications and shorter long-term survival. In this setting, a novel technique for liver resection using radiofrequency (RF) energy to coagulate the liver resection margins has also been developed. A 2-cm-wide coagulative necrosis zone is created before division of the parenchyma with a surgical scalpel (Weber, Navarra et al. 2002).

1.4.1 The procedure

Under general anaesthesia, a modified right subcostal incision is made and the peritoneal cavity examined for evidence of extrahepatic disease. Intra-abdominal adhesions and the falciform ligament are divided. The liver is then mobilized according to the size and site of the lesion to be resected. An intraoperative ultrasonogram is always performed before liver resection to reveal previously undetected lesions.

In step 1 (Figure 2), an inner line is made on the liver capsule with argon diathermy to mark the periphery of the tumor, assisted by bimanual palpation and intraoperative ultrasound. It is important to do this first, as after RF has been applied the parenchyma is hardened and it becomes difficult to palpate the tumor edge. Also, after RF intraoperative ultrasound fails to visualize the tumor edge due to the increased echogenicity resulting from RF.
**Figure 2:** The sequential steps involved in RF assisted liver resection as pioneered at Hammersmith Hospital

In step 2, a second or outer line, again using argon diathermy, is made on the liver capsule 2cm outside the inner line to mark the site where the probe is positioned to achieve coagulative necrosis.
In step 3, coagulative necrosis is produced along a line that follows the second or outer line using the cooled-tip RF probe and a 500-kHz RF Generator (Model RFG-3D, Radionics Europe, N.V., Wettdren, Belgium) which produces 100W of power and allows measurements of the generator output, tissue impedance, and electrode tip temperature. The probe contains a 3cm exposed electrode, a thermocouple on the tip to monitor temperature and impedance, and two coaxial cannulae through which chilled saline is circulated during RF energy application to prevent tissue boiling and cavitation immediately adjacent to the needle.

The number of probe applications required to obtain a zone of necrosis is related to the depth of the liver parenchyma to be resected. For example, to obtain a zone of necrosis in a core of tissue with a 1cm radius and 3cm in depth, each application of RF energy will need to be applied for about 60 seconds. Thus, for a core of tissue 12cm in depth, four applications will be needed in vertical succession (step 4).

Application of the RF energy begins with the area deepest and farthest from the upper surface of the liver. The surgeon checks that each probe is correctly positioned with ultrasound. The preferred technique is to have the tip of the probe pierce the liver capsule of the inferior surface of the liver and to feel it with the middle finger of the left hand while holding the probe with the right hand. The areas of coagulative necrosis can be monitored using intraoperative ultrasound to show the change in tissue impedance and the formation of microbubbles in the tissue.

Once the deepest 3cm of tissue is coagulated, the probe is withdrawn by 3 cm to coagulate the next cylinder of tissue, and so on until the upper surface of the liver is reached. Each application requires about 60 seconds of RF energy. For example, a cylinder of tissue 12cm in depth will require four applications, each application coagulating 3cm of tissue, and will take about 4 minutes to produce. Once an area is coagulated, the probe is withdrawn completely and placed 1 to 2cm away from the previous application. This allows complete coagulation of a band of parenchyma extending along the second line. The points of entry of each probe are kept close to each other at 1cm to achieve some overlap of the areas to be coagulated and to ensure that the coagulation has been complete. Just before each probe removal, the saline infusion is stopped to increase the temperature close to the electrode. This results in coagulation of the needle tract during withdrawal and reduces the possibility of bleeding from the probe tract and liver capsule. Pringle’s manoeuvre is not needed.
In step 5, the liver parenchyma is divided using the scalpel. The plane of division should be situated midway between the first and second line so as to leave a 1cm resection margin away from the tumor and leave in situ 1cm of burned coagulated surface.

Coagulative necrosis from inside the resection margin is applied to stop any potential point of bleeding and to increase the safety margin. A drain is placed at the site of resection and the abdomen is subsequently closed in layers.

This innovative technique uses RF energy for bloodless liver resection without the use of sutures, surgical knots, clips, or glue. When this procedure is used carefully, the surgeon can perform a liver resection with minimal blood loss. It is easy to teach, and surgeons with a good knowledge of liver anatomy can apply it to segmental resections.

The technique reduces the anesthetic and operative time and also the amount of blood loss. These are significant improvements for both the patient and the surgeon. Liver resection is less hazardous and it reduces the need for intensive care unit facilities with less postoperative mortality and morbidity because of the smaller surgical insult to the patient.

The technique was developed at a time when liver resection had become unpopular because of competitive therapeutic modalities based on novel engineering technologies to achieve local tumor control and improved chemotherapeutic regimens. As it is a simple technique to teach, it may encourage surgeons to perform more liver resections and popularize liver surgery as a ‘safe’ therapeutic modality in the management of liver tumours. Indeed, application of this technique is now widespread and represents a considerable step in making laparoscopic liver resection safer and more feasible for many liver surgeons.

The concept of using heat to cause coagulative necrosis is not new and many authors have published series of RF tumour ablation for malignant tumours (Curley, Izzo et al. 1999; Cuschieri, Bracken et al. 1999; Jiao, Hansen et al. 1999; Gazelle, Goldberg et al. 2000). The innovative feature of this technique is that coagulation of normal liver parenchyma is very fast, in contrast to coagulation of liver tumour tissue. Typically, achieving coagulative necrosis in tumour tissue takes about 20 minutes for one probe application and only 40 seconds to coagulate the same amount of normal liver tissue.

There are two obvious limitations to the technique. The first is that RF energy cannot be applied near the hilus or the vena cava for fear of damaging these structures. The second is
that it sacrifices parenchymal tissue that is usually spared using other resectional techniques.

1.5 Modern surgical advances: laparoscopic surgery

Cholecystectomy (removal of the diseased gall bladder), has been a common major abdominal operation since it was first performed in 1882 by Carl Langenbuch. It was the laparoscopic removal of this organ that heralded the use of this fibre optic instrument in general surgery. The first laparoscopic cholecystectomy was carried out by Erich Muhe, of Boblingen, Germany in September 1985, using a sigmoidoscope, but the operation was met with scepticism. Phillipe Mouret, a gynaecologist in Lyons, performed the next cholecystectomy the following year, using his gynaecological laparoscopic instruments, and was given credit for getting the operation accepted. Thereafter, it was adopted with enthusiasm by Edward Reddick and Douglas Olsen in Nashville Tennessee and in the UK by Professor Cuschieri in Dundee (Ellis 2009).

Today, minimal access surgery, or to give it its popular name, ‘key-hole’ surgery, with fibre optic telescopes, allows many types of operation to be carried out without the extensive ‘surgery of approach’ that was previously required to access the diseased organ. Operations on the gall bladder and gynaecological procedures are the most common example but many abdominal and thoracic operations from appendicectomy to major tumour resections are now routine (Ellis 2009).

1.6 Laparoscopic liver surgery

The first reported laparoscopic liver resection was by Gagner et al. in 1992 (Gigot, Glineur et al. 2002). Since then, over 700 laparoscopic liver procedures have been reported. The vast majority (70%) of procedures performed are for benign lesions. The use in the remaining 30% for malignant tumour resections, such as HCC and liver metastases, remains controversial. There are also concerns over possible tumour cell exfoliation and port site metastases (Gigot, Glineur et al. 2002). Overall, the usual benefits of laparoscopic surgery, such as cosmetic aspect, rapid recovery and short post-operative stay, have to be balanced with overall disease-free survival, the paramount oncological objective.

In benign lesions, the main objectives of laparoscopic surgery are low mortality, low morbidity rate, the absence of heterologous blood transfusions and a satisfactory long term
outcome. More recently, in resections of malignancies, the short term survival rates compare favourably with the open technique for both overall and disease-free survival and furthermore experimental *in vivo* and *in vitro* studies have shown decreased tumour growth after laparoscopy compared to laparotomy (Dahn, Schwalbach et al. 2005). However, there are currently no randomised control trials comparing the two approaches, nor long term oncological follow up in those series to date. Despite early favourable survival data, resection margins <1cm, a poor prognostic marker, are found in up to 30% of patients resected laparoscopically and port site metastases have been described (Gigot, Glineur et al. 2002). These findings support the need for a multicentre randomised control trial.

Radiofrequency-assisted liver resection was originally described for ‘bloodless’ liver resection without the need for vascular clamping or the use of suture, surgical knots, or clips (Weber, Navarra et al. 2002). Because of this unique feature, it would seem ideal to use this device for laparoscopic liver resection.

Careful preoperative staging and selection of patients are the keys to success in laparoscopic surgery for cancer. The size and location of the liver tumour is fundamentally important in determining the feasibility of resection with laparoscopic approach. Although no reports exist on major liver resection (Vibert, Perniceni et al. 2006) it is believed that laparoscopic technique can only be performed safely for reasonably small tumours located peripherally. For resection of large liver tumours, the liver must be mobilized completely and the risk of bleeding is high.

The benefits of the laparoscopic approach over open surgery, such as decreased postoperative analgesia requirement, shorter delay to oral intake after surgery, and a decreased hospital stay, are well known. In early RF series (Jiao, Navarra et al. 2005), patients were mobilized more quickly and had a much shorter duration of hospital stay (6 days) compared with those undergoing open resection (10 to 14 days), which is consistent with later reported series (Lesurtel, Cherqui et al. 2003; Jiao, Navarra et al. 2006).

The risk of tumour seeding during laparoscopic surgery has long been debated in the literature. The potential mechanisms for this event include direct contamination from technical errors during laparoscopic resection, cell exfoliation, and cytokine activation secondary to pressure effects from pneumoperitoneum (Neuhaus, Texler et al. 1998). No patients developed either port-site or intraperitoneal metastases at postoperative follow-up after RF assisted resection (Jiao, Navarra et al. 2005; Ayav, Jiao et al. 2007). Promotion of
tumour growth by carbon dioxide gas insufflations has also been cited as a reason against the application of laparoscopic technique for oncologic surgery (Volz, Koster et al. 1999; Takiguchi, Matsuura et al. 2000; Whelan 2001). However, clinical data suggest that the incidence of recurrence between laparoscopic and conventional open operation for cancer is similar and the immune system may also be better preserved in patients undergoing laparoscopic surgery (Vittimberga, Foley et al. 1998; Whelan 2001). In a recent prospective randomised study, Lacy showed better long-term survival in patients who underwent laparoscopic resection for colonic cancer than those who underwent open resection (Lacy, Garcia-Valdecasas et al. 2002).

Fong et al. (Heredia, Ricart et al. 2002) described a number of problems related to laparoscopic liver resection, including the difficulty in identifying tumour margins and mobilizing the liver, in addition to the inherited problems of dense adhesions from previous surgery for primary cancer. Meticulous surgical technique and care are crucial to prevent any inadvertent damage to abdominal organs during induction of pneumoperitoneum and insertion of ports. Although previous abdominal surgery should not be considered as a contraindication for laparoscopic liver resection, adhesions are a major reason for conversion to open procedure and accounted for 2 out of 3 early converted cases.

Biertho et al. (Biertho, Waage et al. 2002) reviewed 186 laparoscopic liver resections performed between 1991 and 2001 and concluded that it would be feasible and safe to perform laparoscopic liver resection in a group of selected patients at an established hepatobiliary centre with highly trained surgeons in advanced laparoscopic techniques. However, this should only be applied to minor resections involving less than 2 segments. Their reported mortality and morbidity rates were 0.5% and 16% in 1991 and 2001, respectively, with a conversion rate of 7%.

Since our first description of RF ablation for unresectable liver cancer, we have expanded the role of RF in liver surgery from mere ablation to actual liver resection (Jiao, Hansen et al. 1999; Weber, Navarra et al. 2002). This technique has now been applied to laparoscopic liver resection. In our experience, this is a safe and feasible technique for laparoscopic liver resection and has little intraoperative blood loss and zero transfusion of either blood or blood products during or after surgery. Although only a small number of have been reported, RF has established itself as one of the current techniques for laparoscopic liver resection (Bachellier, Ayav et al. 2007).
1.7 The Pneumoperitoneum: what do we know?

One of the key manoeuvres that allows laparoscopic surgery logistical success is the use of a ‘pneumoperitoneum’. This is process involves the insufflation of the abdominal cavity with pressurised gas to enable more space to use the telescopic camera (laparoscope) to visualise the liver.

**Figure 3**: The process of insufflation of the abdomen to establish pneumoperitoneum for laparoscopic abdominal surgery. In this case the use of Veress needle is shown.

1.7.1 Pneumoperitoneum: The Gas

The gas most commonly used for laparoscopy is carbon dioxide (CO$_2$) (Dahn, Schwalbach et al. 2005). It is colourless, non-explosive and has good availability. Carbon dioxide is also easily absorbable allowing increased protection from gas embolism which can occur in laparoscopy with room air. However, it can also lead to metabolic acidosis during longer operation times. The use of inert gases such as helium for laparoscopic surgery is increasingly discussed. Helium has the same positive qualities as carbon dioxide but no metabolic effect. Previous studies indicate that helium pneumoperitoneum used for laparoscopic surgery suppresses postoperative tumour growth and may lead to less metastatic disease. However, the mechanism of decreased tumour growth by helium is unknown. The only known detriment of usage of helium is the increased risk of gas embolism compared to carbon dioxide pneumoperitoneum. Xenon belongs, like helium, to the inert gases and has the same positive qualities. Studies have shown a xenon-induced inhibition of cell cycle (Dahn, Schwalbach et al. 2005) suggesting that xenon also has a suppressive effect on tumour growth. However, these gases are not generally used for laparoscopy because of poor availability and high primary costs. CO$_2$ is the gas used in the Hammersmith Surgical Unit. Room air, the gas with best availability, is no longer in use for pneumoperitoneum because of its negative effects, principally the risk of air embolism.
1.7.2 Pneumoperitoneum: insufflation pressures

There are many studies both in humans and animal models assessing the impact of the increased intra-abdominal pressure (IPP) caused by insufflations (Hashikura, Kawasaki et al. 1994; Eleftheriadis, Kotzampassi et al. 1996; Jakimowicz, Stultiens et al. 1998; Ishida, Murata et al. 2000). One representative study involved 11 patients undergoing laparoscopic procedures for benign disease and used duplex Doppler ultrasonography to demonstrate changes in the portal flow observed at different pressure levels of 0, 7, and 14 mmHg (as outlined in Figure 4). Importantly, at an IPP level of 14 mmHg (routinely used in most laparoscopic procedures) the flow of blood in the portal vein (which supplies >80% of blood to the liver) sustained a mean drop to 440 ± 56 mmHg. This corresponds to a 53% reduction compared to the initial baseline value (Hashikura, Kawasaki et al. 1994). This finding has been replicated in other clinical series and is supported by animal models (Eleftheriadis, Kotzampassi et al. 1996).

Figure 4: Increasing intraabdominal pressure reduces portal venous flow.

Research suggests that the insufflation pressure can significantly alter the growth of cancer cells. For example, one model showed a significant correlation between the insufflation
pressure and area of the liver affected by metastases after portal vein inoculation of colon cancer cells in mice (Ishida, Murata et al. 2001; Ishida, Hashimoto et al. 2002). A rabbit model assessed the effect of the pneumoperitoneum on established liver metastases and demonstrated a similar correlation that was not present in an open laparotomy group (Ishida, Murata et al. 2000; Ishida, Murata et al. 2000).

1.8 Hypoxia and cancer: a poor prognostic marker

The oxygen concentration in solid neoplasms is generally lower than in the adjacent non-neoplastic tissue. As a consequence of increased cellularity and proliferation, as well as enhanced metabolism within a tumour (Harris 2002; Vaupel 2004). Histopathological examination of carcinomas frequently reveals hypoxic areas within the tumour mass, mostly in the form of necrotic regions.

In cancer, invasion and metastasis are key issues in terms of prognosis and the ability of surgery to be curative. This is partly because hypoxic regions are relatively inaccessible to chemotherapy, as they are poorly perfused (Young 1990), and radiotherapy, as oxygen-dependent radical production is required for efficient cell killing (Airley, Phillips et al. 2005; Williams, Telfer et al. 2005). However, prognosis is also poor when patients are treated by surgery alone (Garden, Rees et al. 2006) and this reflects the reaction to hypoxia whereby tumour cells can alter their metabolism and activate survival mechanisms. Associations between microenvironmental hypoxia, activation of hypoxia pathways, and aggressively malignant phenotypes are observed across a range of cancers (Colpaert, Vermeulen et al. 2003; Giatromanolaki, Sivridis et al. 2003; Chang, Qin et al. 2006; Generali, Berruti et al. 2006; Shyu, Hsu et al. 2007; Yasuda, Shimizu et al. 2007; Chai, Chen et al. 2008; Evens, Schumacker et al. 2008; Kolev, Uetake et al. 2008; Rajaganeshan, Prasad et al. 2008; Shimogai, Kigawa et al. 2008; Wigfield, Winter et al. 2008; Xie, Song et al. 2008). These findings have focused attention on the molecular dissection of the hypoxia pathways and how these contribute to tumour biology. Important insights have been gained through the definition of hypoxia-inducible factor (HIF) as a key transcription factor regulating oxygen-dependent gene expression.
1.9 The cellular response to hypoxia: HIF

1.9.1 Introduction

Oxygen is the substrate of multicellular life. Reduced oxygen availability (hypoxia) is sensed and triggers homeostatic responses, which impact on virtually all areas of biology and medicine. Since the purification and molecular cloning of hypoxia-inducible factor 1 (HIF-1) in 1995, the scientific community has witnessed the explosive expansion of this field, (Zhang and Semenza 2008).

1.9.2 HIF-1

HIF-1 is a heterodimeric protein composed of HIF-1α and HIF-1β subunits (Wang, Jiang et al. 1995; Wang and Semenza 1995) which modulates the regulation of hundreds of genes according to the cellular O$_2$ concentration (Manalo, Rowan et al. 2005). HIF-1α levels increase dramatically as O$_2$ concentration declines (Jiang, Semenza et al. 1996). Under normoxic conditions, HIF-1α is subjected to ubiquitination and proteasomal degradation (Salceda and Caro 1997; Huang, Gu et al. 1998; Kallio, Wilson et al. 1999) due to the binding of the von Hippel-Lindau tumor suppressor protein (Maxwell, Wiesener et al. 1999) which is the substrate recognition subunit of an E3 ubiquitin-protein ligase (Kamura, Sato et al. 2000). VHL binds to HIF-1α only when the latter is hydroxylated on proline residue 402 and/or 564 (Ivan, Kondo et al. 2001; Jaakkola, Mole et al. 2001; Yu, White et al. 2001). The hydroxylation reaction is performed by prolyl hydroxylases (PHDs) that utilize O$_2$ and α-ketoglutarate as substrates and generate carbon dioxide and succinate as byproducts (Epstein, Gleadle et al. 2001). Under hypoxic conditions, hydroxylation, ubiquitination and degradation are inhibited, leading to the accumulation of HIF-1α (Figure 5). Under normoxic conditions, asparagine residue 803 is also hydroxylated. This reaction, which is mediated by factor inhibiting HIF-1 (FIH-1), prevents the binding of the co-activators CBP and p300 to HIF-1α (Lando, Peet et al. 2002). Thus, O$_2$-dependent hydroxylation regulates both the stability and transcriptional activity of HIF-1. Once activated, HIF-1 mediates a variety of adaptive responses to hypoxia. Two general classes of responses exist: firstly, those that serve to increase O$_2$ delivery (for example, by stimulating angiogenesis by activation of the gene encoding vascular endothelial growth factor [VEGF]); and secondly, those that serve to regulate O$_2$ utilization. Recent findings in connection with the latter responses are described below.
1.9.3 Intratumoral $PO_2$, lactate, and pH

The mean $PO_2$ in human tumours is significantly reduced compared to surrounding normal tissue and tumours with the greatest reduction in $PO_2$ are most likely to invade, metastasise, and kill the patient (Vaupel and Mayer 2007). Many carcinomas also manifest an increased concentration of lactate which is also associated with increased risk of metastasis (Vaupel and Mayer 2007). The increased lactate production is associated with increased expression of lactate dehydrogenase A (LDHA) (Koukourakis, Giatromanolaki et al. 2006) and the monocarboxylate transporter MCT4 which transports lactate out of cancer cells (Koukourakis, Giatromanolaki et al. 2007). Cancer cells also overexpress the sodium–hydrogen exchanger NHE1 and carbonic anhydrase 9 which maintain an alkaline intracellular pH and an acidic extracellular pH (Fang, Gillies et al. 2008). Expression of the LDHA, MCT4, NHE1, and CA9 genes is induced by hypoxia through the activity of HIF-1 (Kroemer and Pouyssegur 2008). Thus, along with its control of genes encoding glucose transporters and glycolytic enzymes (Iyer, Kotch et al. 1998), HIF-1 coordinately regulates all of the proteins required for glucose uptake and its conversion to lactate (Figure 4). The induction of CA9, MCT4, and NHE1 allows cancer cells to maintain an alkaline intracellular pH and an acidic extracellular pH, critical for cell proliferation and invasion, respectively.

1.9.4 The molecular basis of the Warburg effect in renal clear-cell carcinoma

Although many cancer cells utilize the physiological responses to hypoxia described above, in some, genetic alterations can result in a fixed and $O_2$-independent reprogramming of metabolism. Warburg noted increased production of lactate in the tissue culture media of liver tumour explants as compared to normal liver explants cultured under aerobic conditions (Warburg 1930). In renal cell carcinoma lines in which VHL is inactivated by mutation, HIF-1α and HIF-2α are constitutively expressed and mediate glycolytic metabolism. Reintroduction of WT VHL into the cell results in loss of HIF-1α and HIF-2α expression under aerobic conditions and a dramatic increase in mitochondrial mass and $O_2$ consumption (Zhang, Gao et al. 2007). In VHL-deficient renal carcinoma cells, HIF-1 blocks the biogenesis of mitochondria through inhibition of MYC which would otherwise activate transcription of the gene encoding PGC-1β, a transcription factor that controls mitochondrial biogenesis. Loss of VHL activity and the subsequent dysregulation of HIF-1 represents the most well understood mechanism through which cancer cells can be reprogrammed from oxidative to glycolytic metabolism (the Warburg effect) as a result of a single genetic alteration. Many other mutations that either activate oncoproteins or inactivate tumor
suppressors also lead to increased HIF-1 activity (Semenza 2003), suggesting that upregulation of HIF-1 represents a general mechanism underlying the Warburg effect in human cancer.

**Figure 5**: The outline of regulation of stability and transcriptional activity of HIF-1α. In the presence of $O_2$ and cofactors $Fe^{2+}$ and 2OG, PHDs hydroxylate HIF-α, allowing its recognition by the E3 complex that is followed by E2/E1-mediated ubiquitylation and degradation in the 26S proteasome. FIH-1-mediated N-hydroxylation prevents recruitment of p300/CBP transcriptional coactivators. In the absence of $O_2$, PHDs and FIH-1 are inactivated, non-hydroxylated HIF-α translocates to the nucleus, dimerizes with HIF-β, recruits p300/CBP, and induces the expression of its target genes via binding to the HRE.

BTM, basic transcriptional machinery; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; FIH-1, factor inhibiting HIF-1; HRE, hypoxia-response element; 2OG, 2-oxoglutarate; PHDs, prolyl hydroxylases; Ub, ubiquitin; VHL, von Hippel–Lindau protein.
Genetic experiments with tumour models performed by Professor Maxwell and others, have clearly established that the HIF system is important in tumour growth (Maxwell, Wiesener et al. 1999; Mandriota, Turner et al. 2002). Importantly, cancer cells often have an upregulated HIF response, with partial activation of the HIF system when they are supplied with oxygen and a greater level of HIF activation in hypoxia than untransformed cells. Consistent with this, activation of many different oncogenes or inactivation of tumour suppressor genes upregulate the HIF system via variety of mechanisms. A striking example of this process is the loss of VHL tumour suppressor gene associated with a familial cancer syndrome with a very high risk of clear cell renal cancer. According to Knudson’s two hit hypothesis, biallelic VHL inactivation underlies the majority of sporadic clear cell renal cancers, the commonest form of kidney cancer. Indeed, renal carcinoma cells lacking VHL and stably complemented sublines provide a very powerful system to identify the consequences of HIF activation.

1.10 HIF and liver cancer:

1.10.1 HIF and liver metastases

Activation of HIF-1α has been reported in many solid tumours including carcinomas of the gastrointestinal tract (Zhong, De Marzo et al. 1999; Talks, Turley et al. 2000; Jiang, Fan et al. 2003; Kuwai, Kitadai et al. 2003; Yoshimura, Dhar et al. 2004; Koukourakis, Giatromanolaki et al. 2006; Lu, Xing et al. 2006; Yu, Cui et al. 2006; Wincewicz, Sulkowska et al. 2007). However, the role of HIF-1α in tumour progression of colorectal carcinomas is still unclear and the published data so far are controversial. The expression of HIF-1α in some studies was correlated with increased tumour aggressiveness, whereas in other studies a direct contribution to tumour progression was not demonstrated.

HIF-1α is one of the key factors promoting carcinogenesis independently of histogenetical origin. An enhanced expression of HIF-1α from normal tissue through premalignant lesions to carcinomas has already been observed in prostate (Zhong, Semenza et al. 2004), gastric (Griffiths, Pritchard et al. 2007), breast (Bos, Zhong et al. 2001), oral cavity (Costa, Coradini et al. 2001), cervical (Acs, Zhang et al. 2003) and endometrial (Horree, van Diest et al. 2007) carcinogenesis. So far, detailed studies about the role of HIF-1α in the development of colorectal cancer are limited. In the case of colorectal carcinogenesis two
major pathways with different morphological features of the precursor lesions have been described (Vogelstein, Fearon et al. 1988; Longacre and Fenoglio-Preiser 1990): the conventional "adenoma-carcinoma pathway" and the alternative "serrated pathway", in which serrated polyps replace the traditional adenoma as the precursor lesion. The incidence of colorectal cancer did not differ significantly between serrated and traditional adenoma.

In addition to hypoxia, more recent evidence suggests that non-hypoxic pro-inflammatory stimuli, including cytokines and growth factors, can also activate HIF-1α under normoxic conditions and modulate the transcription of hypoxia-associated genes (Frede, Berchner-Pfannschmidt et al. 2007). This phenomenon is well documented in inflammatory cells such as macrophages and monocytes (Blouin, Page et al. 2004; Frede, Stockmann et al. 2006; Peyssonnaux, Cejudo-Martin et al. 2007) but similar observations have also been made in tumour cell lines. In this context, the very potent pro-inflammatory bacterial lipopolysaccharide (LPS) may be of particular importance as a possible stimulator of HIF-1α in the gut under normoxic conditions (Liu, Kirschenbaum et al. 2002; Frede, Freitag et al. 2005). Because LPS is a component of the cell wall of gram-negative bacteria ubiquitously present in the colon, normal and neoplastic intestinal cells are continuously exposed to this factor (Morrison, Lei et al. 1993). Previous studies suggest that LPS could influence the progression of colorectal cancer through its receptor TLR4 (Toll-like receptor 4) (Simiantonaki, Kurzik-Dumke et al. 2007) and the downstream transcription factor nuclear factor-κB (NFκB) (Simiantonaki, Kurzik-Dumke et al. 2007), leading to release of factors from colon carcinoma cells capable of up-regulating endothelial cell adhesion molecules (Simiantonaki, Jayasinghe et al. 2002). Interestingly, a study investigating gut ischemia, has found that LPS can induce HIF-1α expression in enterocytes under normoxic conditions (Koury, Deitch et al. 2004). However, a potential activation of HIF-1α in colorectal carcinoma cells after exposure to LPS has not been reported.

1.10.2 HIF and primary hepatocellular carcinoma

Although hepatocellular carcinomas (HCC) are one of the leading causes of cancer death in the world, the molecular mechanism of hepatocarcinogenesis, especially in the early stage, is still not sufficiently understood. During the early stage of hepatocarcinogenesis, focal lesions called hyperplastic or dysplastic hepatocytic foci emerge. These lesions exhibit a number of altered gene expression and higher proliferating capacity compared with the surrounding normal hepatic tissue and grow into grossly visible lesions called hepatic
adenomas or dysplastic nodules in the later stage. It is thought that, during the development of these lesions, the gradual accumulation of genetic changes may occur in the preneoplastic hepatocytes, leading to the final development of HCC cells. However, how the cellular signalling enabling the early preneoplastic hepatocytes to exhibit the growth advantage occurs, is not exactly known. A recent study showed that overexpression of HIF-1α and activation of its target genes are changes in the early stages of hepatocarcinogenesis. In particular, activation of PI3K/Akt signalling, IGF-II and TGF-α under the influence of HIF-1 activation may be important for the proliferation of preneoplastic hepatocytes (Tanaka, Yamamoto et al. 2006). Therefore, HIF-1 activation may be crucial in the progression of hepatocarcinogenesis by expanding preneoplastic hepatocyte populations, which in turn increase the chance for accumulation of oncogenic mutations within the populations. Intervention of the HIF-1 pathway may then be effective to prevent the development of HCCs.

1.11 HIF target genes

Historically, the HIF target genes have been identified on the basis of one or more of the following strategies: identification of a functional HRE containing a HIF-binding sequence (HBS); comparison of patterns of gene expression in HIF-α wild-type and null cells (or cells treated with siRNA targeting HIF-α); screening for increased gene expression using VHL-null cells or cells transfected with a HIF-α expression vector (Kaluž, Kaluzova et al. 2008). Expression profiling experiments indicated the heterogeneity of gene subsets induced by hypoxia, some of which could be explained by different cell types examined and the relative level or duration of hypoxia (Denko, Fontana et al. 2003). However, the differences in hypoxia-responsive profiles cannot be explained by the genetic instability of the tumour cell lines used, as normal stromal and epithelial cells also display distinct hypoxic profiles (Denko, Fontana et al. 2003). The growing consensus is that there exists a core set of genes that are consistently induced by hypoxia and then there are other genes exhibiting cell-type specific induction. This conclusion underscores the importance of studying hypoxic gene expression in a cell type dependent context (Denko, Fontana et al. 2003). Hypoxia induces HIF activity in almost all cell types and therefore, HIF alone cannot account for the cell-type specific gene expression. Instead, the cell type specific induction by hypoxia appears to be determined by functional interactions of HIF with other transcription factors (Semenza 2003).
Estimates of the total number of HIF target genes induced by hypoxia in one or more cell types vary, from more than 200 (Wenger, Stiehl et al. 2005) to as many as 1–5% of all human genes (Semenza 2003), although not all of them may be regulated directly through HREs. Other transcription factors have also been implicated in hypoxia-inducible gene expression, for example, HIF-activated transcription factors (DEC 1 and 2, ETS-1) also contribute to the pool of hypoxia-induced genes. General stress-responsive transcription factors, such as AP-1, NF-κB, and Egr1 are also up-regulated by hypoxia although their sensitivity to mild hypoxia and the duration of their transcriptional response are much less than that of HIF (Denko, Fontana et al. 2003).

Table 2: Selected HIF target genes grouped according to their function

<table>
<thead>
<tr>
<th>Group</th>
<th>Gene</th>
<th>Protein function</th>
</tr>
</thead>
<tbody>
<tr>
<td>O₂ supply</td>
<td>Transferrin</td>
<td>Iron transport</td>
</tr>
<tr>
<td></td>
<td>Transferrin receptor</td>
<td>Iron transport</td>
</tr>
<tr>
<td></td>
<td>VEGF</td>
<td>Angiogenesis</td>
</tr>
<tr>
<td></td>
<td>Flt-1 (VEGF receptor)</td>
<td>Angiogenesis</td>
</tr>
<tr>
<td></td>
<td>Plasminogen activator inhibitor 1</td>
<td>Blood flow</td>
</tr>
<tr>
<td></td>
<td>Erythropoietin</td>
<td>Erythropoiesis</td>
</tr>
<tr>
<td>Cellular</td>
<td>Enolase 1</td>
<td>Glycolysis</td>
</tr>
<tr>
<td>metabolism</td>
<td>Lactate dehydrogenase A</td>
<td>Glycolysis</td>
</tr>
<tr>
<td></td>
<td>Phosphoglycerate kinase 1</td>
<td>Glycolysis</td>
</tr>
<tr>
<td></td>
<td>GAPDH</td>
<td>Glycolysis</td>
</tr>
<tr>
<td></td>
<td>Phosphofructokinase L</td>
<td>Glycolysis</td>
</tr>
<tr>
<td></td>
<td>Glucose transporter 1</td>
<td>Glucose uptake</td>
</tr>
<tr>
<td></td>
<td>Carbonic anhydrase 9</td>
<td>pH regulation</td>
</tr>
<tr>
<td></td>
<td>MDR1</td>
<td>Xenobiotic metabolism</td>
</tr>
<tr>
<td>Transcription</td>
<td>DEC 1 and 2</td>
<td>Transcription factors</td>
</tr>
<tr>
<td></td>
<td>ETS-1</td>
<td>Transcription factor</td>
</tr>
<tr>
<td></td>
<td>ID2</td>
<td>Transcriptional repressor</td>
</tr>
<tr>
<td></td>
<td>CITED2/p35srj</td>
<td>Transcriptional cofactor</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>NIP3</td>
<td>Pro-apoptotic</td>
</tr>
<tr>
<td></td>
<td>BNIP3</td>
<td>Pro-apoptotic</td>
</tr>
<tr>
<td></td>
<td>Noxa</td>
<td>Pro-apoptotic</td>
</tr>
<tr>
<td></td>
<td>Mcl-1</td>
<td>Anti-apoptotic</td>
</tr>
<tr>
<td>Other</td>
<td>PHD2</td>
<td>O₂ sensing</td>
</tr>
<tr>
<td></td>
<td>PHD3</td>
<td>O₂ sensing</td>
</tr>
</tbody>
</table>

The HIF pathway directly activates an array of at least 70 genes in which functional HREs were experimentally confirmed (Wenger, Stiehl et al. 2005). In general, HIF induces expression of proteins that specifically help meet the metabolic and survival needs of
hypoxic cells. For practical purposes, these proteins are grouped according to their function (Semenza 2003; Wenger, Stiehl et al. 2005) and the major groups with selected representatives are shown in Table 2. Interestingly, HIF activates genes coding for the pro-apoptotic proteins such as BNIP3 (Kothari, Cizeau et al. 2003) as well as the anti-apoptotic protein Mcl-1 (Piret, Minet et al. 2005). This has led to the concept that the ratio of pro- and anti-apoptotic factors could play a role in the differential response of various cell types to hypoxia. In the context of solid tumours, it could contribute to the process in which the most malignant cells are selected (Denko, Fontana et al. 2003). The presence of HREs in the regulatory regions of PHD2 and PHD3 (Metzen, Stiehl et al. 2005) allows activation of corresponding proteins by hypoxia and provides a negative feedback on HIF activity.

1.12 Regulation of target genes by HIF-1α and HIF-2α

The existence of three members within the HIF-α family, HIF-1α, HIF-2α (also called EPAS1, MOP2 or HLF), and HIF-3α, raises questions about the role of individual isoforms in regulation of hypoxic transcription. All α subunits exhibit high conservation at the protein level, domain structure, and hypoxia-dependent mechanisms of regulation, they heterodimerize with HIF-1β and bind to the same cis-element, HBS (Huang and Bunn 2003). Yet, despite similar biochemical properties, distinct patterns of cellular expression appear to be responsible for distinct physiological roles of HIF-1α and HIF-2α (Huang and Bunn 2003). For instance, both isoforms are abundantly expressed in the kidney but in different types of cells: HIF-1α is predominantly expressed in epithelial cells whereas HIF-2α is predominantly detected in interstitial fibroblast and endothelial cells (Rosenberger, Mandriota et al. 2002). The fact that neither HIF-1α−/− nor HIF-2α−/− embryos can survive suggests that HIF-1α and HIF-2α are functionally non-redundant and unable to functionally complement each other (Huang and Bunn 2003). Inactivation of HIF-1α or HIF-2α by siRNA elicits remarkably different cell-specific effects: hypoxia-inducible gene expression is critically dependent on HIF-1α in endothelial and breast cancer cells whereas in renal carcinoma cells it was critically dependent on HIF-2α (Sowter, Raval et al. 2003). HIF-α isoforms display unexpected suppressive interactions in renal cell carcinoma, where up-regulation of HIF-2α suppresses HIF-1α and vice-versa (Raval, Lau et al. 2005).

Several studies have suggested that HIF-1α and HIF-2α differ in their capability to transactivate hypoxia-inducible genes. An earlier study found that some genes were transactivated exclusively by HIF-1α (notably genes coding for glycolytic enzymes), some genes were transactivated by both isoforms but no genes were transactivated only by HIF-
2α (Hu, Wang et al. 2003). In a different study, using siRNA, a small group of genes was found to be preferentially regulated by HIF-2α. Promoter analysis revealed that these genes have binding sites for the ETS family of transcription factors in common and knock-down of ELK-1, the most abundant member of ETS family, significantly reduced hypoxic induction of the HIF-2α-dependent genes (Aprelikova, Wood et al. 2006).

In summary, available data support the notion that, of the two HIF-α isoforms, HIF-2α is the more selective activator of hypoxia-inducible genes. However, the question of how HIF-1 and HIF-2 discriminate between the target genes is far from settled. The observed significant difference in the magnitude and/or specificity of activation of target genes does not appear to depend on the sequence of the relevant HBS. Instead, these differences could be accounted for by preferential cooperation of one of the isoforms with certain subsets of transcription factors, coactivators or corepressors, and/or tissue-specific expression of HIF-α isoforms (Wenger, Stiehl et al. 2005).

### 1.15 E-cadherin

Intercellular adhesion in the context of epithelia is mediated by specialized structures termed intercellular junctions (Esteban, Tran et al. 2006). The adherens junction is mainly composed of transmembrane calcium-dependent glycoproteins called cadherins. E-cadherin is the classic epithelial cadherin, and by dimerizing with other E-cadherin molecules on adjacent cells, it seals the basolateral intercellular space, helping to maintain tissue integrity and architecture (Conacci-Sorrell, Zhurinsky et al. 2002; Esteban, Tran et al. 2006). Besides this structural role, E-cadherin also exerts important effects on cell signaling, through the interaction of its cytoplasmic domain with the catenin family of proteins (Conacci-Sorrell, Zhurinsky et al. 2002; Bienz 2005). Although α-catenin connects E-cadherin to the actin cytoskeleton, γ-catenin and particularly β-catenin have the ability to interact with the T-cell factor family of transcription factors, whose transcriptional activity they increase (Bienz 2005). By controlling the amount of β-catenin available in the cytoplasm, E-cadherin can regulate the expression of critical genes involved in cell death/proliferation (e.g., cyclin D1 and c-myc). Taken together, it is thus not surprising that loss of E-cadherin expression is a hallmark feature of cancers of epithelial origin (Paraskeva, Ridgway et al. 2005). There is a substantial amount of clinical and experimental data indicating that hypoxia within solid tumours has a profound impact on malignant progression by initiating a rapid and progressive down-regulation of cell adhesion molecules (including E-cadherin). Furthermore the process has been shown in colorectal and renal carcinoma cell lines to be (at least in
part) mediated by HIF (Paraskeva, Ridgway et al. 2005; Esteban, Tran et al. 2006). The ability of hypoxic cells to lose and regain homotypic and matrix adhesion, has significant implications for the potential behaviour of tumours in the laparoscopic (hypoxic) surgical environment.

1.14 Aims

It has been postulated that because haematogenous spread usually occurs in a stepwise fashion, initially to the liver, with subsequent intrahepatic spread via the portal vein and further spread to the systemic circulation, surgical resection of isolated hepatic metastases from colorectal cancer may be curative. Nonetheless a majority of patients undergoing curative resection for HCC have intrahepatic recurrence within 5 years and even more patients have recurrence after resection for colorectal liver metastases. I aim to assess the cellular markers of resected liver tumour tissue in order to specifically analyse the role of hypoxia and HIF target genes in the development of liver metastases and in orchestrating further tumour spread.

I will study 10 paired tissue samples from liver resections to analyse whether there is any significant difference in tumour biology attributable to the nature of the resection surgical approach, laparoscopic or open.

I will use a recognised model for analysing potential HIF target genes; specifically CD10 after recent research has shown it may represent a prognostic indicator of liver metastases in primary colorectal carcinoma.

I will assess the effects on circulating tumour cells of liver resection using the new Hammersmith technique, radiofrequency assisted laparoscopic and open liver resection.
2.1 Cell culture

2.1.1 Cell lines

I used three VHL defective human renal cancer cell lines, RCC4, RCC10 and 786-O and the corresponding sublines transfected with wild-type VHL, RCC4/VHL, RCC10/VHL and 786-O/VHL. The RCC10 and RCC10/VHL cell lines were a gift from Dr. K. Plate and Dr. T. Acker (Frankfurt University Medical School, Frankfurt). The RCC4 and RCC4/VHL were previously described by Maxwell et al. (Maxwell, Wiesener et al. 1999). The 786-O and 786-O/VHL cells were a gift from Prof. W. Kaelin (Dana-Farber Cancer Institute, Boston).

2.1.2 Maintenance of cell lines

Tissue culture reagents used for the experiments in this thesis were purchased from Gibco (Invitrogen, UK), unless otherwise stated.

In order to culture the renal cancer cell lines, RPMI 1640 was used as the basal media. It was supplemented with 10% FCS, 2mM L-Glutamine, 50 IU/mL penicillin and 50µg/mL streptomycin. Stock cell cultures were maintained in 100mm tissue culture dishes (TPP, UK) in a GalaxyR CO₂ incubator (Wolf Laboratories, UK) with a humidified atmosphere of 5% CO₂ at 37°C. Cells were passaged using trypsin-EDTA solution. Cells were transferred to a 15ml falcon tube and spun at 1,000 rpm for 5mins to pellet the cells. After spinning cells were resuspended into single cell suspensions in the appropriate volume and re-plated into tissue culture dishes.

2.1.3 Preparation of frozen cell stocks

In order to store the cells for long term purposes, cells from a confluent 100mm tissue culture dish were trypsinised as described in section 2.1.2. Cells were spun to form a pellet and were resuspended in 3mLs of FCS. An equal amount of 2x freezing medium (20% dimethyl sulphoxide (DMSO) and 80% FCS) was added drop wise to the cells. 2mL aliquots of cells were prepared in labelled cryovials. These cryovials were placed in a freezing box containing isopropanol and then transferred to -80°C overnight prior to being transferred to liquid nitrogen for long-term storage.
2.1.4 Manipulating HIF-α protein levels in culture

2.1.4.1 Hypoxia

Cells were plated near to confluence into tissue culture dishes and subjected to hypoxic conditions using a GalaxyR tissue culture incubator (Wolf Laboratories, UK). Hypoxic cells were cultured in an atmosphere of 1% O$_2$, 5% CO$_2$ balanced with N$_2$ at 37°C. The exposure periods pertaining to the individual experiments are quoted in the relevant results section. The same number of control dishes were cultured under normoxic conditions, 20% O$_2$, 5% CO$_2$ balanced with N$_2$ at 37°C.

2.1.4.2 DMOG

Cells were plated at near to confluence. A 1M stock solution of DMOG (Frontier Scientific) was prepared in DMSO. Cells were stimulated in a final concentration of 1mM DMOG, which is sufficient to activate HIF in cell cultures. As comparable controls, the same numbers of dishes at the same density were cultured under normoxic conditions.

2.2 RNA procedures

2.2.1 Extraction of RNA from tissue culture cells

All reagents used to extract RNA were purchased from Sigma unless otherwise stated. During all RNA procedures care was taken to ensure that all reagents and consumables as well as the working environment were RNAse free. Prior to extracting RNA, cells were plated in 60mm tissue culture dishes and were allowed to reach confluence for 24hrs. The monolayers of the cells were trypsinised and pelleted. The supernatant was carefully removed from the cell pellet and then vortexed to loosen the pellet before adding 500µl of RNA-Bee (AMS biotechnology) to the falcon tube. The respective tubes were then vortexed for 15 seconds and aliquoted into 1.5mL RNAse free tubes. Then 100µl (20%) chloroform was added to the tubes and vortexed thoroughly for at least 30 seconds and incubated on ice for 5mins. Next the samples were centrifuged at 13,000rpm for 15mins at 4°C in a chilled microcentrifuge (Eppendorf, UK). Following centrifugation the mixture is separated into three phases. The upper aqueous phase containing the RNA was transferred into fresh RNAse free 1.5ml tubes. Care was taken not to disrupt the lower organic phases. In the
next step, RNA was precipitated by adding equal volumes of propan-2-ol. The samples were mixed gently by vortexing and incubated at room temperature for 10mins. Samples were then centrifuged at 13,000rpm for 10mins at 4°C to pellet the RNA. RNA pellets were washed with 1,000µl of 70% ethanol and the RNA pellet was dislodged by flicking the tube. The samples were centrifuged again at 13,000rpm for 5mins at 4°C. The 70% ethanol was carefully removed from the tube and the pellets were air-dried for 5mins prior to resuspending them in the appropriate volume of RNAse free water. Finally, to ensure that the RNA pellet was dissolved properly, the samples were placed in a hot block (Wolf laboratories, UK) for 5mins at 55°C with intermittent vortexing.

2.2.2 Reverse transcription (RT-PCR)

The RNA extracted was used to synthesise cDNA using an avian myeloblastosis virus (AMV) RT-PCR kit (Roche, Indianapolis). A total of 2µg/µl of RNA was reverse transcribed into cDNA. A master mix excluding the RNA was prepared and aliquoted into the individually labelled tubes. Once the RNA template was added, the tubes were placed on a thermal cycler (MJ Research). The reaction and thermal cycler conditions are summarised in the Table 3. Note, that the samples were incubated at 25°C to allow primer annealing to the RNA template, followed by reverse transcription at 42°C for 60mins. Finally, the samples were incubated at 99°C for 5mins to denature the AMV reverse transcriptase enzyme. The cDNA mixes were then diluted 1:2 in RNAse free water prior to performing quantitative real-time PCR analysis.

<table>
<thead>
<tr>
<th>RT- Reagents</th>
<th>Volume/20µl</th>
<th>RT – PCR Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer 5x</td>
<td>2</td>
<td>Incubate at 25°C for 10mins</td>
</tr>
<tr>
<td>Mg2+ (25mM)</td>
<td>4</td>
<td>Incubate at 42°C for 60mins</td>
</tr>
<tr>
<td>RNase inhibitor</td>
<td>1</td>
<td>Incubate at 99°C for 5mins</td>
</tr>
<tr>
<td>Radom primers</td>
<td>2</td>
<td>Incubate at 4°C for 5mins</td>
</tr>
<tr>
<td>dNTP (10mM)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Enzyme AMV Reverse Transcriptase</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>RNAse free water</td>
<td>8.2</td>
<td></td>
</tr>
<tr>
<td><strong>Total reaction volume</strong></td>
<td><strong>20</strong></td>
<td></td>
</tr>
</tbody>
</table>
Table 3: Reagents and conditions used for the reverse transcription reactions.

2.2.3 Quantitative real-time PCR analysis (qRT-PCR) using SYBR Green

For quantitative real-time PCR analysis, SYBR Green (ABgene) was used. Analysis was performed on an Opticon 2 PCR machine (MJ research). Similar to taqman analysis, samples were analysed in triplicate and the mean presented. Expression of β-actin was not influenced by VHL status and was therefore used for normalisation. All statistical analysis was performed using the Student’s t-test.

Table 4 shows the components mixed together in a single reaction. For the purpose of SYBR Green analysis, all primers were diluted to a 2.5µM concentration. A melting curve was included for the SYBR Green assay to check that a single PCR product was formed and that there was no evidence of primer dimer formation.

<table>
<thead>
<tr>
<th>qRT-PCR Reagents</th>
<th>Volume / 25µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYBR Green mix</td>
<td>13.75</td>
</tr>
<tr>
<td>Primer Mix</td>
<td>0.77</td>
</tr>
<tr>
<td>(2.5µM F+R primer mixed)</td>
<td></td>
</tr>
<tr>
<td>cDNA</td>
<td>10.48</td>
</tr>
<tr>
<td>(diluted in 750µl water)</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>25</strong></td>
</tr>
</tbody>
</table>

**qRT-PCR Conditions**

- **Denaturation:** 95°C for 16mins
- **Amplification:** 95°C for 30secs
  - 60°C for 1min
  - 72°C for 1min

- **Melting Curve:** 65°C to 94°C every 0.2secs

Table 4: Chemical reagents and conditions used for SYBR green qRT-PCR analysis.

The primers used for SYBR Green analysis are listed in Table 5.

Table 5: SYBR Green qRT-PCR primer sequences.
2.3 Protein procedures

2.3.1 Solutions for immunohistochemistry

2.3.1.1 Tris buffered saline (TBS) (pH 7.6)

A 10x stock solution of TBS (50mM $\text{H}_2\text{NC(CH}_2\text{OH)}_3$ and 300mM NaCl) was prepared and stored at room temperature.

The 10x stock solution prepared was diluted in deionised water to give a 1x TBS solution, which was then adjusted to pH 7.6 with 1M HCl. For wash buffer, TBS-Tween (TBS-T) was used. To the 1x TBS solution Tween 20 was added at a concentration of 0.1% to produce TBST.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta$-actin</td>
<td>F 5’-CCCAGAGCAAGAGAGAGGG-3’&lt;br&gt;R 5’-GTCCAGACGCAGGATG-3’</td>
<td>60</td>
</tr>
<tr>
<td><strong>Glut-1</strong></td>
<td>F 5’-TGGCATGGGCGGTTGT-3’&lt;br&gt;R 5’-CCAGGGTAGCTGCTCCAGC-3’</td>
<td>60</td>
</tr>
<tr>
<td>Phd3</td>
<td>F 5’-GATGCTGAAGAAAGGGC-3’&lt;br&gt;R 5’-CTGGCAAAGAGAGTATCTG-3</td>
<td>60</td>
</tr>
</tbody>
</table>

2.3.1.2 Target-antigen retrieval solution (TRS)

The TRS was purchased as 10x concentrate (Dakocytomation, Germany). Therefore, for the purpose of heat induced antigen retrieval (HIER), TRS was diluted in deionised water to 1x TRS solution.
2.3.2 Microtomy and tissue preparation

For the purpose of my experiments, it was important for the tissue sections to be serial sections to enable a direct comparison of between adjacent slides. Tissue blocks were paraffin embedded at the histology service at Cancer Research UK, (CRUK). I cut sections from the formalin fixed paraffin embedded tissue blocks at 3µm thickness using a standard microtome (Anglia Scientific, Cambridge, UK). Sections were then placed in a deionised water bath at 40°C (Lamb, Middlesex, UK). These were then transferred onto labelled Snowcoat Xtra slides (Surgipath, Peterborough, UK). Slides with the tissue section were immediately dried by incubating at 37°C overnight.

2.3.3 Immunohistochemistry

All immunohistochemistry incubations were performed in a humidified chamber at room temperature unless otherwise stated. For all immunohistochemistry protocols, TBST was used as the wash solution.

Prior to performing the respective incubations, sections were always dewaxed in two xylene (VWR) baths for 5mins each and rehydrated in graded ethanol (VWR) washes (100%, 100% and 70%) for 5mins each. Slides were immediately placed in deionised water and washed. For the purpose of antigen retrieval, either protein digestion or heat induced epitope retrieval (HIER) was performed where necessary (Table 2.14). HIER was performed by immersing slides into pre-heated 1x Dako target retrieval solution (Dakocytomation, Germany) and subjected to pressure cooking (Delicio, Tefal, London, UK) for 30mins using a hot plate (Prima Hotplate, Model PDS200, Prima-International, Leeds UK). At the end of the 30mins, once slides had completely cooled down, slides were removed and rinsed with TBST. The relevant immunohistochemistry protocol and incubation periods were then performed (Table 6).

The sections analysed contained both tumour and adjacent normal liver parenchyma. This provided an important internal control. As an additional negative control, the primary antibody was substituted with either antibody diluent or rabbit/mouse IgG (Dakocytomation, Germany).
CHAPTER 2: MATERIALS AND METHODS

2.3.3.1 Dako Envision kit

The Dako Envision kit is a HRP-polymer linked secondary antibody kit which was used for most immunohistochemistry protocols presented in this thesis unless otherwise stated. This kit is based on an immunoperoxidase method of signal detection. To reduce non-specific signal by quenching the endogenous peroxidase activity, slides were incubated with 0.03% hydrogen peroxide (Dakocytomation, Germany) after antigen retrieval. After applying block to the slides, they were washed in TBST and the primary antibody at the appropriate concentration was applied to the sections. For antibody conditions, concentrations and incubation period see Table 6. Primary antibody diluted in antibody diluent (Dakocytomation, Germany) was usually applied to the sections for 1hr at room temperature. Alternatively, if the signal was weak in the positive control slides with high antibody dilutions, I would then progress to a lower dilution together with a 1hr incubation period at 37°C, using the Dako Envision kit. Following incubation with the primary antibody or the appropriate control, sections were washed in TBST (3x 10mins) before incubating the slides with the appropriate peroxidise labelled polymer (rabbit-HRP or mouse-HRP) for 30mins. After this incubation period, slides were washed in TBST (3x 10mins) and the antigen/antibody complexes were detected using 3, 3’Diaminobenzidine tetrahydrochloride (DAB). Each slide was individually developed under the light microscope (Olympus, Hertfordshire) for the same period of time. Sections were then counterstained with Harris haematoxylin for 30secs, followed by a rapid destain in acid/alcohol (1% acetic acid and 70% ethanol) before being dehydrated through graded ethanol washes (70% ethanol, 100% ethanol, 100% ethanol) and mounted in DePX (RALamb, Sussex). Slides were then visualised using a BX-41 optical microscope (Olympus, Hertfordshire).

2.3.3.2 Catalysed Signal Amplification (CSA) kit

For the purpose of HIF-1α and HIF-2α detection on human sections, the Dako Envision kit was not sensitive enough to detect the proteins, therefore I used an ultra-sensitive Dako Catalysed Signal Amplification Kit (CSA) which is based on the peroxidise catalysed deposition of a biotinylated antibody followed by a secondary reaction with streptavidin peroxidase (Dakocytomation, Germany). Similar to the Dako Envision kit, all sections were dewaxed and rehydrated in graded ethanol. HIER was performed on the sections. Once sections had cooled and rinsed in TBST, an Avidin/Biotin block is applied to the sections for 10mins each. After each blocking step the slides are washed in TBST. This is an important
blocking step as it quenches all exogenous biotin from the sections. In the next step, a peroxidase block is applied to the sections for 10mins, after which sections are washed in TBST. This is followed by a protein block which was applied for 10mins to suppress non-specific binding of subsequent reagents. Once the protein block was tapped off, the appropriate primary antibody diluted to the required concentration (Table 6) was incubated on the sections for 1hour at room temperature. At the end of the incubation period sections were washed in TBST and the secondary biotinylated link (rabbit or mouse) was applied to the sections for 15mins. Sections were washed in TBST and subsequently the streptavidin-biotin-peroxidase complex and amplification reagent was applied to the sections for 5mins only. Slides were extensively washed in fresh buffer between each step (4x 10min washes in TBST). Finally, sections were incubated with streptavidin-HRP for 10mins and followed by washing with TBST. Sections were developed with DAB chromagen under the light microscope (Olympus, Hertfordshire), counterstained and dehydrated in graded ethanol. Slides were mounted in DePX and analysed.


**Table 6:** Summary of antibodies used and optimised protocols. HIER using pressure cooker method; *using DAKO Target retrieval solution, \(^1\)Na Citrate PH 6, \(^2\)Na Citrate PH 7.

+ CAIX mAb – kind gift from Dr Pastorek.

Some antibodies were not commercially purchased.

Inc\(^n\): Primary incubation period

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Species</th>
<th>Source</th>
<th>Dilution</th>
<th>Antigen Retrieval</th>
<th>Detection</th>
<th>Inc(^n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD10</td>
<td>56C6</td>
<td>Mouse</td>
<td>Novacastra</td>
<td>1:100</td>
<td>HIER*</td>
<td>Envision</td>
<td>30 mins</td>
</tr>
<tr>
<td>ECAD</td>
<td>HECD1</td>
<td>Mouse</td>
<td>CRUK</td>
<td>1:100</td>
<td>HIER*</td>
<td>Envision</td>
<td>1 hour, 37°C</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>SP4</td>
<td>Rabbit</td>
<td>Neomarkers</td>
<td>1:200</td>
<td>HIER</td>
<td>Envision</td>
<td>45 mins</td>
</tr>
<tr>
<td>+CAIX</td>
<td>M75</td>
<td>Mouse</td>
<td>+Non-com</td>
<td>1:50</td>
<td>N/A</td>
<td>Envision</td>
<td>30 mins</td>
</tr>
<tr>
<td>HIF2α</td>
<td>PM8</td>
<td>Rabbit</td>
<td>Non-com</td>
<td>1:20000</td>
<td>HIER*</td>
<td>CSA</td>
<td>25 mins</td>
</tr>
<tr>
<td>HIF1α</td>
<td>Hi67α</td>
<td>Mouse</td>
<td>Neomarkers</td>
<td>1:1000</td>
<td>HIER*</td>
<td>CSA</td>
<td>25 mins</td>
</tr>
<tr>
<td>BNip3</td>
<td>Ana40</td>
<td>Mouse</td>
<td>Sigma</td>
<td>1:200</td>
<td>HIER*</td>
<td>Envision</td>
<td>30 mins</td>
</tr>
<tr>
<td>GLUT-1</td>
<td>-</td>
<td>Mouse</td>
<td>Dako</td>
<td>1:250</td>
<td>HIER*</td>
<td>Envision</td>
<td>30 mins</td>
</tr>
</tbody>
</table>

### 2.3.3.3 Haematoxylin and Eosin (H&E) staining

To examine cell morphology and architecture slides were H&E stained. Sections were dewaxed and rehydrated in graded ethanol. After washing sections in deionised water, they were immersed into H&E solution (Sigma) for 5mins. Sections were immediately destained in acid/alcohol, dehydrated and DePX mounted. Sections were analysed using light microscopy (Olympus BX-41, Hertfordshire).
2.3.4 Solutions for Western blotting and immunodetection

2.3.4.1 Cell lysis buffer

Cells were harvested in an appropriate volume of urea/SDS lysis buffer (8M Urea, 1/10 vol glycerol, 1/20 vol 20% SDS, 1/200 vol 1M DTT, 1/100 vol 1M Tris (pH 6.8)). Prior to cell lysis an appropriate amount of the buffer was placed in a separate tube and 1/100 vol of a 500µM stock (stored at -20°C) of phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor was added.

2.3.4.2 (4x) TrisCl/SDS (pH 8.8)

To make a 4x TrisCl/SDS buffer, 91g of Tris base was dissolved in 300mLs of deionised water. The pH of the solution was then adjusted to 8.8 with 1M HCl. Next deionised water was added to give a total volume of 500mLs prior to filtering the solution through a 0.45µm filter. 2g of SDS was then added and the buffer was subsequently stored at 4°C for a maximum of 1 month.

2.3.4.3 4x TrisCl/SDS (pH 6.8)

To make 4x TrisCl/SDS 12.1g of Tris base was dissolved in 80mLs of deionised water. The pH was then adjusted to 6.8 with 1M HCl and more deionised water was added to give a total volume of 200mLs. The solution was then filtered using a 0.45µm filter, prior to addition of 0.8g SDS. The final solution was stored at 4°C for up to 1 month.

2.3.4.4 4x SDS loading buffer

The sample buffer was prepared by mixing 12.5mLs 4x TrisCl/SDS (pH 6.8), 10mL glycerol, 2g SDS and 1.55g DTT. Deionised water was added to give a total volume of 50mLs. The buffer was then aliquoted and stored at -20°C.
2.3.4.5 (5x) Electrophoresis running buffer

Running buffer was prepared as a 5x stock solution (25mM TrisCl, 200mM glycine, 0.1% SDS) and stored at 4°C. The 1x buffer was prepared by diluting the stock solution with deionised water as required.

2.3.4.6 (10x) Transfer buffer

A 10x transfer buffer stock solution of 100mM glycine and 100mM TrisCl was prepared and stored at 4°C. 1x transfer buffer was prepared as required by diluting the 10x stock solution with deionised water and adding 10% methanol and 0.005% SDS.

2.3.4.7 Phosphate buffered saline (PBS) (pH 7.4)

A 10x PBS stock was made as described in section 2.3.1.5. 1x PBS solution was made by diluting the 10x stock in deionised water. PBS-Tween (PBST) was prepared from a 1x PBS solution by adding Tween 20 to a final concentration of 0.1%.

2.3.4.8 Blocking solution

Blocking solution was prepared by mixing 5g dried skimmed milk (Marvel, UK) and 1g bovine serum albumin (BSA) in 100 mL of PBST.

2.3.5 Preparations of protein extracts

2.3.5.1 Cell extracts

Cell extracts for western blotting were prepared by urea/SDS lysis. The monolayers of the cells grown on 60mm tissue culture dishes were removed and immediately placed on ice and a sufficient volume of ice-cold PBS was added to the cells for 30secs. Subsequently, two other washes in PBS were performed before removing any residual PBS. Cells were harvested by scraping into 150-200μL of urea/SDS lysis buffer. Lysates were sonicated for 15secs (Soniprep 150, Sanyo, USA) and briefly centrifuged at 13,000rpm for 1min and kept on ice. Protein extracts were assayed for protein concentrations and aliquots were stored at -20°C.
2.3.5.2 Protein extracts from tissue

All tissue for protein extraction was snap frozen and stored at -80°C. A small amount of the frozen tissue, between 100-200mg was placed into an eppendorf containing urea/SDS to which an EDTA free protease inhibitor tablet (Roche) was added. Subsequently, protein lysates were prepared by homogenising the tissue (Homogeniser, IKA, UK). Following homogenisation, samples were centrifuged at 13,000rpm for 5mins at 4°C and the supernatants were aliquoted into fresh clean eppendorfs which were stored at -80°C.

2.3.5.3 Determination of protein concentration

A commercially available BCATM Protein Assay (Pierce, USA) was used to estimate the protein concentration in accordance with the manufacturer’s instructions. The protein concentration of the cell lysates was determined by comparing the colour absorbance values of test extracts to the colour values derived from a standard curve of BSA that was run in parallel. 200µl of the BCA Working Reagent (200µl BCA Working solution: Mix 50 parts of BCATM Reagent A with 1 part BCATM Reagent B) was added to 25µl of sample (3.5µl of BSA standard, plus 3.5µl of SDS/urea lysis buffer and 18µl of deionised water or 3.5µl of cell extract plus 21.5µl deionised water) in a 96 well ELISA plate. The plate was then covered put on a shaker for 30secs and incubated at 37°C for 30mins. Once the plate had cooled to room temperature, the absorbance was read at 550nm on a plate reader (Anthos HT-II, Anthos Labtec Instruments, Austria).

2.3.5.4 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoretic separation of proteins was performed using the Mini-Protean apparatus system (BioRad). Resolving gels were prepared using 30% acrylamide: bis-acrylamide (37.5:1) mix and TrisCl/SDS (pH8.8) buffer (350mM Tris-Cl, and 0.1% SDS) to the desired specification e.g. 6% gels were used for HIF-1α and HIF-2α. Ten percent gels were used for α-tubulin (Table 2.16) for formulations used for gel preparation. For stacking gels, the acrylamide concentration was 3.9% in Tris/SDS (pH6.8) buffer (125mM Tris-Cl (pH6.8), and 0.1% SDS). Gels were polymerised by the addition of 0.05% ammonium persulphate (APS) and 0.07% N,N,N’,N’-tetramethylethlenediamine (TEMED). For a typical run, protein extracts at 2 µg/µL were mixed with 4x SDS loading buffer and heated at 95°C for 5mins,
followed by brief centrifugation at 13,000 rpm for 30secs before loading 12µL of protein sample (18µg) onto a polyacrylamide mini-gel. Gel electrophoresis was performed at a constant 120volts in electrophoresis buffer. Coloured molecular weight standards (ProSieve Colour protein markers, Cambrex, USA) were also run on every SDS-PAGE gel to monitor electrophoresis, and were used to calibrate the mobility and hence apparent mass (kDa) of test proteins.

2.3.5.5 Stacking gel

Stacking gel was prepared by mixing 0.65mL 30% Acrylamide/0.8% Bis-acrylamide, 1.25mLs 4xTrisCl/SDS (pH 6.8), 3.05mLs deionised water, 6.7µl TEMED and 33µl 10% APS.

<table>
<thead>
<tr>
<th>% Gel</th>
<th>30% Acrylamide/0.8% Bis</th>
<th>4x Tris-Cl/SDS (pH 8.8)</th>
<th>dH2O</th>
<th>10% APS</th>
<th>TEMED</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>2mL</td>
<td>2.5mL</td>
<td>5.5mL</td>
<td>47µl</td>
<td>9.4µl</td>
</tr>
<tr>
<td>10</td>
<td>3.3mL</td>
<td>2.5mL</td>
<td>5.2mL</td>
<td>47µl</td>
<td>9.4µl</td>
</tr>
<tr>
<td>12</td>
<td>4mL</td>
<td>2.5mL</td>
<td>3.5mL</td>
<td>47µl</td>
<td>9.4µl</td>
</tr>
</tbody>
</table>

Table 7: Resolving gel formulas. Volumes specified are for 3 gels using the BioRad MiniProtean systems.

2.3.5.6 Immunoblotting and immunodetection

Proteins resolved by SDS-PAGE were transferred to PVDF membrane (Immobilon, Millipore, USA) by tank transfer (Mini-Transblot Apparatus; BioRad) in accordance with the manufacturer’s recommendations. The transfer cassette consisting of the pre-equilibrated gel and PVDF membrane was sandwiched between chromatography paper (Whatman, UK) and Scotch-Brite pads. The cassette was then placed in a transfer tank, immersed in pre-chilled transfer buffer, and electrophoresed constantly at 30V overnight at 4°C, followed by 1hr at 85V the following day.

For immunodetection, membranes were transferred to blocking solution for 1hr on a rocking platform (Platform Rocker STR6, Bibby, UK) at ~20rpm at room temperature to prevent non-specific antibody binding. Primary antibodies were diluted in blocking solution to the
dilution specified in Table 8 and applied to the membrane for 1hr at room temperature. After three 10mins washes in PBST solution on a rocking platform set at 40rpm at room temperature, membranes were incubated with secondary antibodies conjugated to horseradish peroxidase (HRP) (Dako) for 1 hr (diluted 1:2000 in blocking solution), followed by at least five 10min washes on a rocking platform set at 40rpm. Bound HRP activity was visualised by enhanced chemiluminescence (ECL for α-tubulin, or the higher sensitivity ECLplus for all other antigens such as HIF-1α and HIF-2α, Amersham Pharmacia Biotech) using Kodak MXB film (GRI autoradiography film, Blue sensitive, GRI, UK).

Equivalent protein loading was verified using two approaches, firstly by immunodetection of α-tubulin (Sigma), and secondly, after immunoblotting PVDF membranes were stained with Coomassie blue (50% MeOH, 10% acetic acid, 0.05% Coomassie Brilliant Blue R-250) for 15mins followed by destaining (5% MeOH, 10% acetic acid) for approximately 20mins.

**Table 8:** Protocol and antibody conditions used for detecting the specified proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Clone</th>
<th>Source</th>
<th>Primary antibody dilution</th>
<th>Relative Molecular weight</th>
<th>Secondary Incubation</th>
<th>% Acrylamide in gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td>NB-449</td>
<td>Novocastra</td>
<td>1:500</td>
<td>120</td>
<td>Goat polyclonal anti-rabbit HRP</td>
<td>6%</td>
</tr>
<tr>
<td>HIF-2α</td>
<td>PM8</td>
<td>-</td>
<td>1:1000</td>
<td>118</td>
<td>Goat polyclonal anti-rabbit HRP</td>
<td>6%</td>
</tr>
<tr>
<td>GLUT-1</td>
<td>GT11A</td>
<td>Alpha Diagnostics</td>
<td>1:500 1:500</td>
<td>55 54</td>
<td>Goat polyclonal anti-rabbit HRP</td>
<td>10% 10%</td>
</tr>
<tr>
<td>CD10 α- tubulin</td>
<td>TAT-1</td>
<td>Gift from Prof Dominic Withers CRUK</td>
<td>1:500 1:500</td>
<td>55 55</td>
<td>Goat polyclonal anti-rabbit HRP</td>
<td>12% 12%</td>
</tr>
</tbody>
</table>
2.4 CTC Detection

The CellSearch system was used to enrich and enumerate the CTCs, as described previously (Slade, Payne et al. 2009). A 7.5mL blood sample was taken in a CellSave preservative tube, kept at room temperature and processed within 72 hours. The system enriched for EpCAM (epithelial cell adhesion molecule) positive epithelial cells by incubating the sample with ferrofluid conjugated to anti-EpCAM antibodies. Cells were stained with the following fluorescent labelled monoclonal proprietary antibodies: CD45-APC to distinguish the CTCs from leukocytes and pan-cytokeratin 8, 18 and 19 (CK-PE) to stain epithelial cells, and epidermal growth factor-receptor (EGF-R) antibodies as we have recently described (in press (Payne, Yague et al. 2009)). Nucleic acids were stained using 4,6-diamidino-2-phenylindole (to exclude red blood cells). Samples were then scanned on the CellTracks analyzer II fluorescent microscope for analysis.
CHAPTER 3

LAPAROSCOPIC AND OPEN LIVER RESECTION:
A CLINICAL PILOT STUDY


3.1 Introduction

Since the introduction of laparoscopic cholecystectomy in 1987, the increased acceptance of laparoscopic strategies in the management of intra-abdominal pathologies has heralded a new philosophy in surgery: one of minimizing the insult delivered by the intervention. Laparoscopy offers significant healthcare benefits over the conventional open approach; decreased post-operative pain and shorter hospital stay resulting in an earlier return to normal activity (Sain 1996). Laparoscopy also provides reduction in the inflammatory and neuroendocrine stress responses (Jakeways, Mitchell et al. 1994), lesser suppression of the cell-mediated immune response (Kloosterman, von Blomberg et al. 1994) and a significant reduction in adhesion formation when compared with conventional surgery (Reissman, Teoh et al. 1996). A reduction in the surgically induced immune paresis and access related trauma encountered after open surgery (Ng, Whelan et al. 2005) should convey an advantage to patients undergoing surgery for malignant disease.

After the introduction of laparoscopic assisted colectomy in the management of colorectal cancer, unexpected reports of port-site and peritoneal metastases raised early concerns about the safety of the approach. The incidence ranged from 1.5% to 21% (Wexner and Cohen 1995) and carried a short-term mortality rate of at least 50%, even with treatment (Paolucci, Schaeff et al. 1999). The maximum rate of wound tumour implantation post standard open laparotomy ranges from 0.6% to 0.8% (Hughes, McDermott et al. 1983; Lacy, Garcia-Valdecasas et al. 2002). These initial reports were personal series and did not constitute high-level evidence to base further practice on. However, this heralded a period of caution in the application of laparoscopic colectomy and the establishment of large mulitcentre prospective randomised trials in the UK, Europe and the USA. Many surgeons believed that the observed incidence of recurrence was a technical issue and subsequently performance of laparoscopic colectomy has often been part of a randomised trial where surgeons operated in recognised laparoscopic centres, having demonstrated their competency by video assessment. Lacy et al. (Lacy, Garcia-Valdecasas et al. 2002) presented the first results of a randomized trial comparing laparoscopic assisted with open colectomy for non-metastatic colon cancer. It was shown that the laparoscopic approach was more effective than open for the treatment of colon cancer from the point of view of morbidity, length of hospital stay, tumour recurrence and cancer related survival. Further randomized control trials have reported their results, for example in the UK the MRCCLASICC trial reported the results of 794 patients in 27 centres randomised to either
open or laparoscopic assisted colectomy. Investigators concluded that laparoscopic colectomy was as effective as open colectomy and was likely to produce similar long-term outcomes (Guillou, Quirke et al. 2005). In light of the evidence from these randomised trials, it would appear that earlier concerns about the safety of laparoscopic colectomy were unfounded and it was likely to be related to the surgical and technological learning curve.

Liver surgeons have been even slower to adopt the laparoscopic technique for liver resections. Laparoscopy was initially used only for minor procedures, such as biopsy and staging of liver tumour or fenestration of non-parasitic liver cysts (Morino, De Giuli et al. 1994). Concerns regarding the difficult mobilization and transection of the liver, the risks of major haemorrhage and of air embolus have been responsible for this initial slow development. However, in the last decade, this technique has been increasingly used for liver resection (Bismuth, Castaing et al. 1989; Gigot, Glineur et al. 2002; Vibert, Perniceni et al. 2006). In the case of hepatic malignancies the same concerns that have been addressed with randomized control trials in laparoscopic resection of primary colonic cancer (oncological clearance and intraperitoneal tumour seeding) persist (Gagner, Rogula et al. 2004; Kaneko 2005). Nonetheless, more than a thousand laparoscopic liver resections have been performed worldwide since 1992, after Gagner et al. performed the first nonanatomical resection of a liver tumour (Gagner, Rogula et al. 2004). Most of these involved resections of only one or two liver segments (Gigot, Glineur et al. 2002; Mala, Edwin et al. 2005; Cherqui, Laurent et al. 2006) although major hepatectomies have also been achieved laparoscopically (O'Rourke and Fielding 2004; Dulucq, Wintringer et al. 2005; Vibert, Perniceni et al. 2006). Improving technology and the development of advanced laparoscopic skills have increased the interest in laparoscopic liver surgery. Subsequently, multiple small series have reported the safety and feasibility of laparoscopic hepatic resection (Gigot, Glineur et al. 2002; Morino, Morra et al. 2003; Robles, Abellan et al. 2005; Belli, Fantini et al. 2007; Dagher, Proske et al. 2007; Lee, Cheung et al. 2007; Aldrighetti, Pulitano et al. 2008; Buell, Thomas et al. 2008; Polignano, Quyn et al. 2008; Topal, Fieuws et al. 2008; Bryant, Laurent et al. 2009; Carswell, Sagias et al. 2009; Ito, Ito et al. 2009; Rowe, Meneghetti et al. 2009; Sarpel, Hefti et al. 2009; Tsinberg, Tellioglu et al. 2009). Indeed, Koffron et al. reported on 300 cases at one academic centre reinforcing the fact that laparoscopic techniques for resection have matured (Koffron, Auffenberg et al. 2007).

One such technique is radiofrequency-assisted liver resection. After development at Hammersmith Hospital it was originally described for ‘bloodless’ liver resection without the
need for vascular clamping or the use of suture, surgical knots, or clips (Weber, Navarra et al. 2002; Ayav, Navarra et al. 2005; Ayav, Bachellier et al. 2007; Ayav, Navarra et al. 2007). This unique feature has deemed it ideal for laparoscopic liver resection. Indeed, recent studies have shown its technical feasibility (Bachellier, Ayav et al. 2007; Ayav, Jiao et al. 2008; Jiao, Ayav et al. 2008; Pai, Navarra et al. 2008). Laparoscopic liver resections are now accepted as a safe and feasible option for selected patients. However, oncological outcome and longer term follow up data are lacking. Against this background, we used a pair-matched design to compare the results of laparoscopic hepatectomy with those of the open approach using radio-frequency assisted liver resection.
3.2 Methods

We reviewed oncological outcome from 10 early consecutive cases of laparoscopic liver resection for hepatic malignancy, performed by the same surgeon at Hammersmith Hospital between June 2003 and March 2005 and matched them to patients who had undergone similar open hepatic resections for the same disease.

3.2.1 Preoperative diagnostic imaging and patient selection

Careful preoperative staging and selection of patients are the keys to success in laparoscopic surgery for cancer (Cherqui, Soubrane et al. 2002). The size and location of the liver tumour is fundamentally important in determining the feasibility of resection with laparoscopic approach. In our unit, all patients under consideration for surgery are discussed at a multidisciplinary team meeting in which a minimum of either CT and/or MR imaging modality was used to characterise the anatomy of the hepatic tumour and suitability for resection. The affected liver segments were agreed by consensus (minimum of 2 radiologists and 1 consultant surgeons) and recorded in the HPB database. In cases where consensus was difficult, 3-D reconstruction software was used to aid interpretation. Indications for laparoscopic resection were patients who required segmentectomy or left lateral hepatectomy without major hepatectomy. Exclusion criteria were extrahepatic disease, (including the unexpected finding of additional tumours on intraoperative ultrasound scan), patients requiring venous or biliary reconstruction, and the presence of dense adhesions precluding both visualisation and intraoperative ultrasound of the liver.

3.2.2 Surgical technique

Laparoscopy was performed under carbon dioxide pneumoperitoneum, with the abdominal pressure maintained between 12 and 14 mmHg. The initial step was visual and ultrasonographic exploration of the liver. The technique employed was radiofrequency assisted liver resection as described previously (Weber, Navarra et al. 2002; Ayav, Navarra et al. 2005; Bachellier, Ayav et al. 2007) and resected liver was placed in an endocatch bag and withdrawn through the most accessible or appropriate port site. A maximum of 5 laparoscopic ports were used (of which no more than 4 were 12mm). In cases in which it was impossible to remove the resected specimen through a laparoscopic port, a single 12mm port site would be extended to accommodate swift exteriorisation.
3.2.3 Routine tissue analysis

Postoperatively, tissue was collected in formalin and sectioned for a consultant pathologist to report. From this report, the macroscopic and microscopic features of the resected tissue, including number of lesions, size of lesion, level of differentiation, positivity of resection margins and histopathological type were input into the surgical database.

3.2.4 Criteria for matching open resections

The patients were matched to equivalent open operations using the Hammersmith RFA Liver Resection database using the following inclusive search criteria: tumour pathological type, grade and Couinaud segment resected. In the absence of an exact match, the closest match was accepted. This prioritised, in the following decreasing order, tumour type, anatomical site and grade.

3.2.5 Clinical Outcome

We compared clinical outcome in terms of operative time, inpatient length of stay, disease-free and overall survival after surgery. Patients were followed up 3 monthly for the first 18 months and 6 monthly thereafter. Clinical suspicion of a disease recurrence resulted in CT/MRI imaging. This was otherwise performed routinely at 6 monthly intervals in the first 2 years and annually thereafter. All radiological imaging was reviewed at an MDT meeting.
<table>
<thead>
<tr>
<th>Patient code</th>
<th>Sex (M/F)</th>
<th>Age (yrs)</th>
<th>Primary pathology</th>
<th>Differentiat(^n) (well/ moderate/ poor)</th>
<th>Liver tumour site (acc. Couinaud)</th>
<th>No.s of lesions</th>
<th>Operative time, (hrs:mins)</th>
<th>RM +ve (Y/N)</th>
<th>In-patient stay (dys)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LapA</td>
<td>F</td>
<td>59</td>
<td>Colonic adenocarcinoma</td>
<td>Moderate</td>
<td>IV</td>
<td>1</td>
<td>03:08</td>
<td>N</td>
<td>10</td>
</tr>
<tr>
<td>LapB</td>
<td>F</td>
<td>56</td>
<td>Colonic adenocarcinoma</td>
<td>Moderate/Well</td>
<td>IV,VII</td>
<td>2</td>
<td>05:15</td>
<td>N</td>
<td>6</td>
</tr>
<tr>
<td>LapC</td>
<td>M</td>
<td>55</td>
<td>Colonic adenocarcinoma</td>
<td>Poor</td>
<td>II,III</td>
<td>1</td>
<td>03:37</td>
<td>N</td>
<td>6</td>
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<td>LapD</td>
<td>F</td>
<td>55</td>
<td>Small cell carcinoma of Lung</td>
<td>Poor</td>
<td>VIII</td>
<td>1</td>
<td>03:00</td>
<td>Y</td>
<td>3</td>
</tr>
<tr>
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<td>63</td>
<td>Colonic adenocarcinoma</td>
<td>Poor</td>
<td>V,VI</td>
<td>1</td>
<td>08:25</td>
<td>Y</td>
<td>23</td>
</tr>
<tr>
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<td>66</td>
<td>Colonic adenocarcinoma</td>
<td>Poor</td>
<td>V,VI</td>
<td>1</td>
<td>06:13</td>
<td>N</td>
<td>14</td>
</tr>
<tr>
<td>LapG</td>
<td>M</td>
<td>69</td>
<td>Colonic adenocarcinoma</td>
<td>Moder ate</td>
<td>VII</td>
<td>1</td>
<td>05:45</td>
<td>Y</td>
<td>7</td>
</tr>
<tr>
<td>LapH</td>
<td>F</td>
<td>64</td>
<td>Colonic adenocarcinoma</td>
<td>Moderate</td>
<td>II,III</td>
<td>2</td>
<td>05:32</td>
<td>N</td>
<td>11</td>
</tr>
<tr>
<td>LapI</td>
<td>M</td>
<td>69</td>
<td>Hepatocellular carcinoma</td>
<td>Moderate</td>
<td>II</td>
<td>2</td>
<td>05:03</td>
<td>Y</td>
<td>15</td>
</tr>
<tr>
<td>LapJ</td>
<td>M</td>
<td>69</td>
<td>Hepatocellular carcinoma</td>
<td>Well</td>
<td>II</td>
<td>1</td>
<td>04:27</td>
<td>N</td>
<td>13</td>
</tr>
<tr>
<td>OpenA</td>
<td>M</td>
<td>57</td>
<td>Colonic adenocarcinoma</td>
<td>Moderate</td>
<td>IV</td>
<td>1</td>
<td>04:05</td>
<td>N</td>
<td>22</td>
</tr>
<tr>
<td>OpenB</td>
<td>F</td>
<td>55</td>
<td>Colonic adenocarcinoma</td>
<td>Moderate</td>
<td>VII</td>
<td>1</td>
<td>04:15</td>
<td>N</td>
<td>13</td>
</tr>
<tr>
<td>OpenC</td>
<td>M</td>
<td>52</td>
<td>Colonic adenocarcinoma</td>
<td>Poor</td>
<td>III</td>
<td>1</td>
<td>03:32</td>
<td>Y</td>
<td>12</td>
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<tr>
<td>OpenD</td>
<td>M</td>
<td>77</td>
<td>Small cell carcinoma of Lung</td>
<td>Poor</td>
<td>V,VI</td>
<td>1</td>
<td>04:45</td>
<td>Y</td>
<td>8</td>
</tr>
<tr>
<td>OpenE</td>
<td>F</td>
<td>65</td>
<td>Colonic adenocarcinoma</td>
<td>Moderate/Poor</td>
<td>V</td>
<td>1</td>
<td>02:15</td>
<td>N</td>
<td>7</td>
</tr>
<tr>
<td>OpenF</td>
<td>F</td>
<td>58</td>
<td>Colonic adenocarcinoma</td>
<td>Moderate/Poor</td>
<td>V,VI</td>
<td>3</td>
<td>04:15</td>
<td>Y</td>
<td>11</td>
</tr>
<tr>
<td>OpenG</td>
<td>F</td>
<td>52</td>
<td>Colonic adenocarcinoma</td>
<td>Poor</td>
<td>VII</td>
<td>1</td>
<td>04:33</td>
<td>N</td>
<td>9</td>
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<tr>
<td>OpenH</td>
<td>M</td>
<td>76</td>
<td>Colonic adenocarcinoma</td>
<td>Poor</td>
<td>II,III</td>
<td>1</td>
<td>04:25</td>
<td>N</td>
<td>7</td>
</tr>
<tr>
<td>OpenI</td>
<td>F</td>
<td>81</td>
<td>Hepatocellular carcinoma</td>
<td>Poor</td>
<td>VI</td>
<td>1</td>
<td>02:20</td>
<td>Y</td>
<td>30</td>
</tr>
<tr>
<td>OpenJ</td>
<td>M</td>
<td>54</td>
<td>Hepatocellular carcinoma</td>
<td>Well</td>
<td>II</td>
<td>1</td>
<td>02:23</td>
<td>N</td>
<td>11</td>
</tr>
</tbody>
</table>
3.3 Results

Demographical data, histopathological classification, resected tumour site, grade, number of lesions, resection margin positivity and operative time are shown for both cohorts of patients in Table 9 above. Table 10 below shows the two groups are well matched in terms of histopathological type, tumour site and procedure. There were no significant differences between the 2 groups in terms of male: female ratio, age and pre-operative Child’s score. Post-operative data shows no significant difference between tumour number, size and resection margins. However, there is a significant difference between the groups when comparing length of operation (p<0.05) There was no difference in inpatient length of stay.

There is notable difference in the Kaplan-Meier survival data for the two groups both for overall survival however, chi squared analysis of the survival curve shows no significant difference (p>0.05).

Figure 6: Kaplan-Meier curve comparing overall survival after laparoscopic and case-matched open liver resection for malignant disease.

Table 10: Patient demographics, operative time, resection margin status and tumour details for ten patients undergoing laparoscopic resection and ten matched* open cases, (*see methods above for matching criteria and rules).
### CHAPTER 3: A CLINICAL PILOT STUDY

<table>
<thead>
<tr>
<th>Characteristic/Outcome</th>
<th>Laparoscopic</th>
<th>Open</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M:F)</td>
<td>6:4</td>
<td>5:5</td>
<td>0.67</td>
</tr>
<tr>
<td>Age</td>
<td>62.5 (±1.9)</td>
<td>62.7 (±3.6)</td>
<td>0.96</td>
</tr>
<tr>
<td>Child’s Grading:</td>
<td></td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>A</td>
<td>9</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Tumour Characteristics:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histological type:</td>
<td></td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>HCC</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>CLM</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Other Metastasis (e.g.:)</td>
<td>1</td>
<td>1</td>
<td>0.68</td>
</tr>
<tr>
<td>No. of tumours:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Tumour site:</td>
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<td></td>
<td>1.00</td>
</tr>
<tr>
<td>Segment 2-3</td>
<td>4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Segment 5-6</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Segment 4b</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Segment 7-8</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Tumour size (cm)</td>
<td>3.44 (±0.81)</td>
<td>6.56 (±1.22)</td>
<td>0.068</td>
</tr>
<tr>
<td>Procedures:</td>
<td></td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>Wedge/Subsegmentectomy</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Segmentectomy</td>
<td>4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Bisegmentectomy</td>
<td>3</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Outcome:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resection Margin &lt;1cm</td>
<td>4</td>
<td>4</td>
<td>1.0</td>
</tr>
<tr>
<td>Operative Time (mins)</td>
<td>303 (±30.8)</td>
<td>220 (±18.8)</td>
<td>0.036</td>
</tr>
<tr>
<td>Inpatient Stay (dys)</td>
<td>10.8 (±1.8)</td>
<td>13.0 (±2.3)</td>
<td>0.47</td>
</tr>
</tbody>
</table>
To compare disease free survival for the two groups during the post-operative period, we eliminated all resections with a positive resection margin, as time to ‘recurrence’ would be contentious. 5 matched pairs of RM-ve laparoscopic and open resections remained, shown below in Table 11.

**Table 11: R0 resections laparoscopic vs. open compared**

<table>
<thead>
<tr>
<th>Group</th>
<th>Time to recurrence (months)</th>
<th>Paired Group</th>
<th>Time to recurrence (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LapA</td>
<td>21</td>
<td>OpenA</td>
<td>N/A</td>
</tr>
<tr>
<td>LapB</td>
<td>9.5</td>
<td>OpenB</td>
<td>N/A</td>
</tr>
<tr>
<td>LapF</td>
<td>5.5</td>
<td>OpenF</td>
<td>N/A</td>
</tr>
<tr>
<td>LapH</td>
<td>16</td>
<td>OpenH</td>
<td>N/A</td>
</tr>
<tr>
<td>LapJ</td>
<td>N/A</td>
<td>OpenJ</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Chi-squared analysis of the survival curves shows significant difference between laparoscopic and open disease free survival rates after initial R0 resection (p=0.0455). In pairs, A, B, F and H-there is earlier recurrence of colorectal adenocarcinoma and in pair J, earlier recurrence after laparoscopic resection of hepatocellular carcinoma.

**Figure 7:** Kaplan-Meier survival curve comparing disease free survival in 5 patients undergoing laparoscopic and 5 matched open liver resection patients.
3.4 Discussion

There are currently 7 published individual case-matched series comparing laparoscopic and open liver resection (Rau, Buttler et al. 1998; Morino, Morra et al. 2003; Belli, Fantini et al. 2007; Lee, Cheung et al. 2007; Aldrighetti, Pulitano et al. 2008; Polignano, Quyn et al. 2008; Carswell, Sagias et al. 2009). A number of series compare similar groups of patients and assess the relative benefits of minimal access approach over the resection of hepatic malignancies (Farges, Jagot et al. 2002; Gigot, Glineur et al. 2002; Lesurtel, Cherqui et al. 2003; Robles, Abellan et al. 2005; Dagher, Proske et al. 2007; Buell, Thomas et al. 2008; Topal, Fieuws et al. 2008; Belli, Limongelli et al. 2009; Bryant, Laurent et al. 2009; Ito, Ito et al. 2009; Rowe, Meneghetti et al. 2009; Sarpel, Hefti et al. 2009; Tsinberg, Tellioglu et al. 2009). Many of these series have supported the technical feasibility of laparoscopic resection of hepatic malignancies in selected cases but few have reported on follow-up and oncological outcomes. In this chapter we have performed the first case matched pilot study to compare laparoscopic RF-assisted liver resection to historic matched open resections.

We identified a significantly longer operative time required for laparoscopic resection compared to their matched open cases (p<0.05). Operative time was downloaded from a surgical theatre database and represented time into theatre, after induction of anaesthetic and time and out of theatre, with the patient awake and self-ventilating. There will be an inevitable extra variable, dependent on anaesthetic factors such as ASA, airway, respiratory reserve and perioperative analgesic regime (for example, epidural use). Nonetheless, other series that demonstrated no underlying difference in ASA, have also shown independently a significantly longer operation time in laparoscopic resection (Rau, Buttler et al. 1998; Belli, Fantini et al. 2007). Indeed in the majority of published data, operative time was equivalent or slower. Few studies actually demonstrated a shorter operative time (Koffron, Auffenberg et al. 2007; Buell, Thomas et al. 2008). In these series, an endovascular stapler was the primary resection tool to transect the liver parenchyma. Certainly, one of the more time consuming parts of RF-assisted liver resection is the meticulous positioning of the probe under IOUS guidance prior to application of current and transection of tissue with laparoscopic dissecting scissors, (Weber, Navarra et al. 2002; Bachellier, Ayav et al. 2007). Positioning is less challenging under direct vision at laparotomy.

Laparoscopic liver resection presents several technical challenges which must be factored into the operative time: the liver must be atraumatically retracted and the parenchyma
must be accurately divided so that vascular and biliary structures can be safely divided, thereby avoiding inadvertent injury to the vital structures of the remnant liver. There is growing experience with technicological advances in laparoscopic transection devices, for example, ultrasonic dissectors, articulating vascular staplers, water-jet dissectors, harmonic scalpel and vessel sealing systems (Poon 2007). In several case series a significantly reduced blood loss was witnessed with laparoscopic surgery (Lesurtel, Cherqui et al. 2003; Belli, Fantini et al. 2007; Koffron, Auffenberg et al. 2007; Aldrighetti, Pulitano et al. 2008; Buell, Thomas et al. 2008). There is evidence to suggest blood transfusion is associated with earlier tumour recurrence in resections for malignant disease, so the importance of minimising blood loss should not be underestimated in long term outcome measures (Nielsen 1995; Dionigi, Boni et al. 2009). RF-assisted liver resection has been well received since its introduction in 2002 for similarly significant low blood loss intraoperatively, such that the technique is often described as ‘bloodless’ (Weber, Navarra et al. 2002; Ayav, Navarra et al. 2005; Ayav, Jiao et al. 2007; Ayav, Navarra et al. 2007). Its adaptation and use in laparoscopic resection has yielded similarly encouraging results, and in this respect we have not included blood loss as an outcome measure in this pilot, (Bachellier, Ayav et al. 2007; Jiao, Ayav et al. 2008; Pai, Navarra et al. 2008).

There was no significant difference in length of inpatient stay between our laparoscopic and open resection patients in our study, which is consistent with only a minority of previous reports (Laurent, Cherqui et al. 2003; Lesurtel, Cherqui et al. 2003). Of note, these reports were predominantly in smaller series ((Laurent, Cherqui et al. 2003; Lesurtel, Cherqui et al. 2003)with N=27 and N=38, respectively). More commonly, laparoscopic liver resection results in a significant reduction in length of hospital stay (Rau, Buttler et al. 1998; Farges, Jagot et al. 2002; Morino, Morra et al. 2003; Kaneko 2005; Mala, Edwin et al. 2005; Belli, Fantini et al. 2007; Koffron, Auffenberg et al. 2007; Aldrighetti, Pulitano et al. 2008; Buell, Thomas et al. 2008).

In laparoscopic resection for malignancies, the same oncologic rules of open surgery are applied, including the ‘no-touch’ technique, radical resection and achievement of a 1-cm margin. Resection margins were positive in 4/10 laparoscopic resections. Possible explanations for this include direct contamination from technical errors during laparoscopic resection, cell exfoliation, or cytokine activation secondary to pressure effects from pneumoperitoneum (Neuhaus, Texler et al. 1998). Importantly, this is a small sample size and more recent larger series have shown lower rates (6-14%) of positive resection margins.
(Mala, Edwin et al. 2005). Certainly, in well established laparoscopic centres, resection margin positivity is comparable to matched open resections (Tsinberg, Tellioglu et al. 2009) and an equivalent positive resection margin rate was demonstrated in the open matches in our pilot study. There is significant difficulty in interpreting resection margin positivity when using the RF assisted resection technique because the ‘true’ resection margin will inevitably lie in a cylindrical zone of coagulative necrosis around the tumour deposit and this may not be preserved during tissue preparation, such as paraffin embedding, or in subsequent sectioning.

Laparoscopic liver resection took longer to complete. The risk of tumour seeding during laparoscopic surgery has long been debated in the literature. In this series, none of the patients developed either port-site or intraperitoneal metastases at postoperative follow-up at 32 months. However, clinical data suggest that the incidence of recurrence between laparoscopic and conventional open operation for cancer is similar, and the immune system may also be better preserved in patients undergoing laparoscopic rather than open surgery (Vittimberga, Foley et al. 1998; Whelan 2001). In a recent prospective randomised study, Lacy showed better long-term survival in patients who underwent laparoscopic resection for colonic cancer than those who underwent open resection (Lacy, Garcia-Valdecasas et al. 2002). Two further trials have concluded similarly (group 2004; Guillou, Quirke et al. 2005). Despite several large case series advocating a role for laparoscopic resection of liver malignancies, no randomised control trial exists. In our pilot series there is a markedly different overall survival rate in the 2 groups. For example, at 2 years follow-up, mortality is nearly 40%, almost double that in the open group. Furthermore, when we compared the 5 paired ‘curative’ resections, which were resection margin negative, there was a significantly poorer outcome: those undergoing laparoscopic resection had earlier disease recurrence ($p<0.05$). We chose these patients, and in so doing took an even smaller sample, as it was important to minimise the confounding factors. Surgical resection margin positivity (R1) causes a significantly reduced overall and disease-free survival (Nuzzo, Giuliani et al. 2008). In our pilot we defined any clearance $\geq 1\text{mm}$ as negative, and in other larger series such a narrow clearance has also been significantly correlated to poorer prognosis when compared to groups $>5\text{mm}$ resection margins for example (Nuzzo, Giuliani et al. 2008). Unfortunately the limitations of our sample size precluded a more in-depth breakdown of size of resection margin that may have also offered further insight to the technical challenges of laparoscopic approach in this important oncological prognostic parameter.
So far, we have compared clinical data for 2 matched groups undergoing liver resection. Laparoscopic resection takes significantly longer, results in the same length of time in hospital and for those that achieve curative resections, an earlier disease recurrence.

Although a majority of published data suggests laparoscopic liver resection may have some advantage in the short term over open surgery, this may reflect publication bias. Data on the impact of this procedure on long term outcome is lacking (Vibert, Perniceni et al. 2006). This pilot study supports the need for further studies on the effects of RF-assisted resection. In particular, a randomized control trial is warranted to compare the effects of laparoscopic and open resection in terms of oncological outcome.
CHAPTER 4

LAPAROSCOPIC AND OPEN LIVER RESECTION:

A RETROSPECTIVE IMMUNOHISTOCHEMICAL STUDY
4.1 Introduction

In liver surgery, laparoscopy was initially used only for minor procedures, such as biopsy and staging of liver tumour (Morino, De Giuli et al. 1994) or fenestration of non-parasitic liver cysts (Morino, De Giuli et al. 1994). However, in the last decade, this technique has been increasingly used for liver resection (Bismuth, Castaing et al. 1989; Katkhouda, Hurwitz et al. 1999; Cherqui, Soubrane et al. 2002; Gigot, Glineur et al. 2002; Vibert, Perniceni et al. 2006).

Liver resection remains a high-risk procedure with significant morbidity and mortality rates. Intraoperative bleeding and perioperative blood transfusion are usually considered to be the major reasons affecting these rates (Bismuth, Castaing et al. 1989; Capussotti, Borgonovo et al. 1994; Fong, Cohen et al. 1997). In laparoscopic liver surgery, access to the liver is limited and control of intraoperative bleeding is inevitably difficult. Conversion to an open procedure is often required to achieve haemostasis and complete the resection when bleeding occurs.

RF-assisted liver resection was originally described for ‘bloodless’ liver resection without the need for vascular clamping or the use of suture, surgical knots or clips (Weber, Navarra et al. 2002; Ayav, Navarra et al. 2005; Ayav, Bachellier et al. 2007; Ayav, Navarra et al. 2007). This unique feature has deemed it ideal for laparoscopic liver resection. Recent studies have shown its technical feasibility (Bachellier, Ayav et al. 2007; Ayav, Jiao et al. 2008; Jiao, Ayav et al. 2008; Pai, Navarra et al. 2008).

The benefits of the laparoscopic approach over open surgery, such as decreased postoperative analgesia requirement, shorter delay to oral intake after surgery and a decreased hospital stay, are well known. However, none of the rare articles reporting limited experience with laparoscopic resection for HCC or liver metastases provide information concerning the late outcome of such patients (Gigot, Glineur et al. 2002).

We undertook a study looking at our early experience of 10 cases of laparoscopic liver resection with the main aim of assessing oncological outcome. The pilot clinical data for the 2 matched groups undergoing RF-assisted liver resection showed laparoscopic resection takes significantly longer, results in the same length of time in hospital and for those that achieve curative (R0) resections, an earlier disease recurrence as discussed in Chapter 3. Using resected tissue from this pilot study we attempted to explain these findings at a cellular level. Although this may be a multifactorial cumulative effect, the single most
obvious physiological difference between the 2 techniques is the pneumoperitoneum. The insufflation of the peritoneal cavity necessary to obtain the endoscopic exposure means these patients are being subjected to an artificially elevated intraperitoneal pressure (IPP) for several hours. The adverse effects of this elevated IPP on the systemic circulation and cardiac, pulmonary, and renal functions have been well documented in experimental and clinical studies (Smith, Benzie et al. 1971; Marshall, Jebson et al. 1972; Kotzampassi, Kapanidis et al. 1993). In the last decade, the changes in the splanchnic circulation such as decreased mucosal blood flow and the effect of a sustained elevated IPP on the flow to the solid intraabdominal organs has been reported in both animal and human experiments (Ishida, Murata et al. 2000). It was hypothesised by us that the pneumoperitoneum required in laparoscopic surgery may have an effect on tumour cell biology allowing an enhanced metastatic phenotype.

4.2 Methods

4.2.1 Immunohistochemistry

Immunohistochemical staining of paraffin embedded tissue blocks was performed on all 10 laparoscopic and the paired open resection blocks as described in Chapter 2. Blocks were selected to include ‘normal’ liver as internal controls. Thereafter slides were scored on the strength of the staining on 5 separate random high power fields of view. Scoring was performed by 2 blinded technicians. For nuclear molecules (e.g.: HIF), scoring was as a percentage of total nuclei in the field of view that stained positive and for cell surface proteins, (e.g.: GLUT-1 membrane transporter), scoring was as percentage of the area of whole cells in the field of view staining positively.

Table 12: Scoring system for immunohistochemical staining.

<table>
<thead>
<tr>
<th>Score</th>
<th>Descriptive term</th>
<th>Percentage positively staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No/Weak staining</td>
<td>0-25%</td>
</tr>
<tr>
<td>+</td>
<td>Mildly positive</td>
<td>25-50%</td>
</tr>
<tr>
<td>++</td>
<td>Moderately positive</td>
<td>50-75%</td>
</tr>
<tr>
<td>+++</td>
<td>Strongly positive</td>
<td>&gt;75%</td>
</tr>
</tbody>
</table>
For the purposes of statistical analysis, each group was allocated a number based on the maximum percentage positive staining, for example for the weak/no staining category above a number of 25 was used in each sample. T-test statistical analysis was performed using GraphPad prism and SPSS software thereafter and only $p<0.05$ was considered significant.

### 4.3 Results

Table 13 below summarises all the immunohistochemical staining performed on the 20 resected liver tumours. HIF-1α and HIF-2α, (the active HIF subunits of HIF-1 and HIF-2 respectively) and recognised hypoxia-inducible genes CaIX, GLUT-1 and Cyclin D1 were stained for. CD10 has been used as a marker of hepatocellular cell differentiation and to delineate non hepatocellular tumour margins when these are not easily identifiable. CD10 also has been implicated in adenoma-carcinoma sequences.

#### 4.3.1 Carbonic Anhydrase IX (CaIX)

CaIX was mildly positive in more samples from the laparoscopic group than open, but not significantly ($p>0.05$). Only one colorectal metastasis stained anything more than mildly positive staining (LapB-strongly). Hepatocellular resected tissue was only weakly positive in all 4 cases, whereas both resected lung carcinomas were mildly positive.
**Table 13:** Immunohistochemical staining profiles for 10 laparoscopic and 10 paired open resected liver cancer specimens

<table>
<thead>
<tr>
<th>Patient Code</th>
<th>Primary Pathology</th>
<th>CaIX</th>
<th>GLUT-1</th>
<th>HIF-1α</th>
<th>HIF-2α</th>
<th>CD10</th>
<th>Cyclin D1</th>
</tr>
</thead>
<tbody>
<tr>
<td>LapA</td>
<td>CLM</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LapB</td>
<td>CLM</td>
<td>+++</td>
<td>+</td>
<td>0</td>
<td>++</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>LapC</td>
<td>CLM</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>++</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>LapD</td>
<td>Small Cell Lung</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LapE</td>
<td>CLM</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>++</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>LapF</td>
<td>CLM</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>++</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>LapG</td>
<td>CLM</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LapH</td>
<td>CLM</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LapI</td>
<td>HCC</td>
<td>0</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>0</td>
</tr>
<tr>
<td>LapJ</td>
<td>HCC</td>
<td>0</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>0</td>
</tr>
<tr>
<td>OpenA</td>
<td>CLM</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>OpenB</td>
<td>CLM</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>OpenC</td>
<td>CLM</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>OpenD</td>
<td>Small Cell Lung</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>OpenE</td>
<td>CLM</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>OpenF</td>
<td>CLM</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>OpenG</td>
<td>CLM</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>OpenH</td>
<td>CLM</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>OpenI</td>
<td>HCC</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>0</td>
</tr>
<tr>
<td>OpenJ</td>
<td>HCC</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
</tr>
</tbody>
</table>

**P value* (lap vs. open)**

| | | | | | | |
|---|---|---|---|---|---|
| 0.39 | 0.08 | 0.17 | **0.024** | **0.037** | 0.19 |

*Two-tailed paired t-test
**Figure 8:** Carbonic anhydrase IX expression in a colorectal liver metastatic margin showing the brown staining of the colorectal epithelial metastases at the tumour margin, abutting the normal pink liver parenchyma. (x50)

### 4.3.2 GLUT-1

GLUT-1 is a high affinity glucose transporter and a recognized hypoxia inducible gene (otherwise known as HIF target). All 10 laparoscopic cases demonstrated mild positivity for GLUT-1, but only 7/10 in the open group demonstrated the same level. These included both hepatocellular and the lung carcinoma; 3 openly resected CLM showed weak or no staining. This difference between laparoscopic and open resection specimens only approached significance (p=0.08).

The pattern of GLUT-1 expression in the normal liver demonstrates a canalicular pattern, (control). The expression in the CLMs was more diffuse but most prominent in pseudoclefts, the areas representing luminal surface in normal colorectal architecture and between metastatic columnar crypt cells. In 4/4 resected hepatocellular carcinomas GLUT-1+
staining was conserved in a similar canalicular pattern to the control, despite differing degrees of differentiation (see Table 9).

**Figure 9:** GLUT-1 expression in two colorectal liver metastases with parallel CaIX expression.

What is evident when assessing serial sections for GLUT-1 and CaIX positive staining is that there are areas of complementary staining as shown in Figure 9, although these were mostly found in areas of low differentiation, high mitotic rate (H&E not shown).

### 4.3.3 HIF-1α and HIF-2α

The HIF-2α isotype was mildly positive in all (20/20) resected tumours. Laparoscopically resected tissue demonstrated significantly higher levels of HIF-2α staining when compared to the matched open tissue (p<0.05). For example, 57% (N=7) of CLM resected laparoscopically demonstrated moderately positive staining compared to only 14% in the open group (p<0.05).
Figure 10: Colorectal liver metastatic tissue stains positively for HIF-2α. Tissue resected laparoscopically shows significantly stronger staining for HIF-2α than matched open resection tissue.

The strength of HIF-2α expression was not tumour-specific as both HCC and lung Ca in the lap group showed equivalent or stronger staining. All CLM resected demonstrated weak or no staining for HIF1α (positive control: VHL-defective renal cell carcinoma, see Chapter 2). Only the non-CLM tumours expressed a positive level of the HIF1α isotype- small cell lung carcinoma metastases and primary HCC HIF-2α expression patterns are shown in Figure 11 below. The HCC shows characteristic islands of malignant hepatic parenchyma. The bronchial metastases show fewer features to delineate origin but the deep purple on H+E are likely to represent malignant cells with high mitotic index.
Figure 11: (A-D): Primary HCC (A,B) and metastatic small cell carcinoma of Lung (C,D) express HIF-2 isotype, (x50)

Figure 12: HCC shows concurrent HIF-1α positivity in parallel with HIF-2α.

4.3.4 Cyclin D1

Cyclin D1 is a HIF-2 target gene as characterised by experiments using CCRCC with pVHL- and wt, (Sowter, Raval et al. 2003). In our pilot study it showed mildly positive expression
in 64% of CLM (N=9) and none in either HCC or small cell lung. There was no significant difference in expression between those samples resected laparoscopically vs. open.

4.3.5 CD10

CD10 expression was evident in all HCC resected and to a significantly higher level in CLM resected laparoscopically compared to those samples resected via open technique. It was not expressed in small cell lung carcinoma. The results of CD10 expression are discussed in Chapter 6.
4.4 Discussion

4.4.1 Markers of hypoxia in resected liver tissue

GLUT-1 is a high-affinity glucose transporter (others include GLUT-3 and -4) (Zimmerman, Fogt et al. 2002). It has a Michaeli’s constant (Km) below the normal blood glucose concentration, allowing transporting function at rates close to maximal velocity. Therefore, the level of cell surface expression greatly influences the rate of glucose uptake into the cells. High-affinity transporters are found in almost every tissue but their expression is higher in cells with high glycolytic activity. The basal hepatocyte phenotype is conferred by the expression of liver-specific genes. In the adult liver, the basal hepatocyte phenotype is further modified by transcriptional and post-transcriptional regulation of genes which results in the appearance of specific proteins in selected hepatocytes, these include GLUT-1 (Zimmerman, Fogt et al. 2002). The GLUT-1 protein is detected in the plasma membrane of hepatocytes located at the end of the liver cell plate, contiguous to the hepatic venule, resulting in the characteristic canalicular pattern demonstrated in our liver controls. It has been noted that GLUT-1 is expressed in a variety of carcinomas although its expression in HCC has not been studied extensively (Zimmerman, Fogt et al. 2002). One group studying the cellular mechanism of chemoresistance in HCC has shown that GLUT-1 expression was low in HCCs but was significantly higher in poorly differentiated HCCs compared to moderately differentiated HCCs (Seo, Hatano et al. 2009). All the resected HCC tissue, independent of both level of differentiation or resection technique, demonstrated only mild GLUT-1 positivity although the sample size of purely HCC tissue in our pilot is likely to preclude any significant findings.

Recent microarray analysis of both primary and secondary colorectal disease demonstrated significantly higher GLUT-1 expression at tumour margins when compared to core tissue (Rajaganeshan, Prasad et al. 2009). However, a similar sized study showed the reverse on the basis of immunohistochemical data, with strongest GLUT-1 expression at the centre of primary and secondary foci (Gu, Yamamoto et al. 2006). In our pilot we took random fields of view to quantify intensity of staining. It was felt that for the purpose of assessing the effect of pneumoperitoneum on tumour cells, and given that the 2 groups were matched (mean size of lesions in the 2 groups p>0.05, see Chapter 3), this would likely reflect any significant trend without introducing further bias or confounding factors.
CHAPTER 4: A RETROSPECTIVE IMMUNOHISTOCHEMICAL STUDY

GLUT-1 was present in low levels in all liver metastases of small cell carcinoma of lung and to identical levels of HIF-1α. Data on GLUT-1 expression in small cell carcinoma are scarce. In one series from 1998, 24 resected primary small cell lung carcinomas expression Glut-1 immunostaining was stronger in the central area of tumour cell nests corresponding to the hypoperfused region, although they did not look at HIF-1α expression or indeed use it as a marker of hypoxia (Ito, Noguchi et al. 1998). However, small cell lung carcinoma lines have been demonstrated to upregulate GLUT-1 under hypoxic stress in vitro (Pedersen, Holm et al. 2001), The small cell carcinoma lesions in our pilot were both poorly differentiated but expression of HIF-1α and GLUT-1 were no more than mildly positive.

Carbonic anhydrase isoenzyme IX (CaIX) is a transmembrane protein which is thought to maintain acid-base balance and intercellular communication. Previous studies have demonstrated that Ca IX is expressed in the basolateral plasma membrane of normal biliary epithelial cells but not in hepatocytes (Saarnio, Parkkila et al. 1998; Saarnio, Parkkila et al. 2001). This may explain the absence of CaIX in the resected hepatocellular carcinomas in our study. Increased expression of Ca IX has been observed in certain epithelial tumours. We investigated the expression of Ca IX in CLMs of which there was positive staining in 79% of cases, (N=14). A larger series looked at 69 colorectal neoplasms of varying stages (Saarnio, Parkkila et al. 1998). Dysplastic polyps showed weak or moderate reaction for CaIX only in the cryptal epithelium, as did the normal intestinal mucosa. Adenomas showed immunoreactivity mainly in the superficial part of the mucosa whereas the distribution in the carcinomas and metastases was more diffuse. Comparative immunostaining of serial sections for Ki-67, a well established marker of cell proliferation, confirmed that CaIX is expressed in areas with high proliferative capacity. Marked parallel expression of CaIX and GLUT-1 was noticed in our series. These are both recognized HIF targets therefore they may serve to mark areas of hypoxia. However, although in van Laarhoven’s study he demonstrated the consistent expression of CaIX in primary colorectal resection tissue and its paired resected metastatic tissue, no such continuity was observed for GLUT-1 expression (van Laarhoven, Kaanders et al. 2006). Furthermore, there was no correlation between combined GLUT-1/CaIX positive areas and hypoxic areas measured through pimonidazole binding. This raises the issue of whether CaIX and GLUT-1 are endogenous markers of hypoxia in CLMs. Interestingly, there was no significant correlation between HIF-1α and GLUT-1 or CaIX in either primary or metastatic disease in one study (Rajaganeshan, Prasad et al. 2009).
The two HIF isoforms, HIF-1α (HIF-1) and HIF-2α, (HIF-2) are widely expressed by various cancers and have proved to be significant prognostic markers in selected cancers. Currently, there are no studies assessing HIF expression in metastatic liver disease. We have shown that the isoform HIF-2 is strongly expressed in 14/14 CLMs and also in the 2 bronchial cancer liver metastases analysed. Furthermore, HIF-2 was expressed in the normal tissue in all CLMs to a lesser degree. In contrast, HIF-1 had a much lower expression and was only isolated in HCCs. Yoshimura et al. first investigated the significance of both HIF isotypes in colorectal carcinoma (Yoshimura, Dhar et al. 2004). They compared expression levels of the isotypes in 87 resected primary tumours and correlated their findings to clinicopathological features, including patient prognosis. HIF-1α expression showed a trend towards early tumour stages but had no significant correlation to any of the parameters measured. In contrast, HIF-2 expression had significant correlation with advancement of T stage, Dukes stage, lymphatic/vascular invasion, reduced differentiation status and microvessel density. Essentially, they successfully identified a subgroup of patients with colorectal cancer in whom HIF-2 expression was an independent poor prognostic indicator and marker of aggressive phenotype (Yoshimura, Dhar et al. 2004). In our data series, we have assessed patients with primary curative resection who later developed metastatic liver disease. This introduces a selection bias towards a more aggressive phenotype. Hence, HIF-2 may play a role not only in the invasiveness of primary colorectal carcinoma but also in haematogenous or otherwise spread and development of colorectal liver metastases. In support of preferential expression of one isotype in advanced cancer, an experimental study involving Maxwell et al. has compared the effects of HIF-1 and HIF-2 isoform activation (Raval, Lau et al. 2005). It demonstrates that, despite close similarities between the HIF-isoforms, differential activation of HIF-1 or HIF-2 pathways in VHL-defective RCC cells has non-equivalent or even opposing effects on gene expression and experimental tumour growth. HIF-2 had positive effects on tumour growth and HIF-1 negative effects on tumour growth in this setting. Suppressive interaction between HIF-isoforms was also found therefore, at present, it is not possible to conclude whether suppression of tumour growth by HIF-1 arises from direct effects, indirect effects through downregulation of HIF-2, or a combination of these mechanisms.

Although the majority of published data suggests laparoscopic liver resection may have some advantage in the short term over open surgery, it cannot be discounted that this may reflect publication bias. There are no data to indicate the impact of this procedure on long term outcome (Vibert, Perniceni et al. 2006). Indeed, evidence from this pilot study would
suggest further studies on the effects of RF assisted resection are required, and a randomized control trial is warranted to compare the effects of laparoscopic and open resection in terms of oncological outcome. Nonetheless, as disease recurrence is common after resection of colorectal hepatic metastases, identification of prognostic factors that predict the outcome following surgical resection of colorectal hepatic metastases would assist in the identification of those patients most likely to benefit from this intervention. Importantly, this would assist in the identification of patients who are unlikely to benefit. Clearly, further work characterising HIF’s role in metastatic liver disease would be of significant clinical benefit.
CHAPTER 5

THE INTERRELATIONSHIP OF HIF AND E-CADHERIN IN A STRATIFIED EPITHELIAL MODEL: IMPLICATIONS FOR THE ROLE OF HIF IN CANCER SPREAD
CHAPTER 5: THE INTERRELATIONSHIP OF HIF AND E-CADHERIN

5.1 Introduction

Constant availability of molecular oxygen is crucial for the structure and function of any mammalian cell. Therefore, cellular responses to reduced oxygen tensions (hypoxia) play an important role in development and many aspects of physiological homeostasis. Many important disease processes, including cancer, involve reduced tissue oxygenation and cellular adaptation to this is implicated in disease progression and clinical outcome.

Hypoxic conditions have been detected in several human malignancies, including cancers of the breast, prostate, lung, pancreas, rectum, uterine cervix, vulva, head and neck, brain tumours, melanomas, soft tissue sarcomas, non-Hodgkin lymphomas, metastatic liver tumours and renal cell cancer (Vaupel and Mayer 2007). Hypoxic or anoxic areas, usually found as heterogeneously distributed areas within solid tumours, may depend either on poor/altered vascularisation (perfusion-limited $O_2$ delivery), deterioration of diffusion geometry (diffusion-limited $O_2$ delivery) or to a lesser extent to conditions of tumour-or therapy-associated anaemia (Vaupel and Mayer 2007; Dellas, Bache et al. 2008). Although low oxygen tension may even contribute to kill some tumour cells (Vaupel and Mayer 2007) hypoxia more probably provides a strong selective pressure able to regulate tumour growth and eventually favour survival of the most aggressive malignant cells (Graeber, Osmanian et al. 1996). Hence, hypoxia within a neoplastic mass is considered as an independent prognostic indicator of poor outcome with a significant risk to develop metastasis that may escape therapy (Vaupel and Mayer 2007; Dellas, Bache et al. 2008). Indeed, neoplastic cells surviving hypoxia exhibit enhanced invasive propensity, suggesting that hypoxia may favour cancer progression (Ishikawa, Sakurai et al. 2004; Le, Denko et al. 2004). However, the precise mechanisms by which this occurs remain incompletely understood (Cannito, Novo et al. 2008). Both E-cadherin downregulation and increased EMT have been shown to be hypoxia-inducible and a role for HIF in this promalignant adaptive response is likely.

HIF is a central mechanism responding to low cellular oxygenation and mediates a variety of systemic and local adaptive responses (Maxwell, Wiesener et al. 1999). In most normal tissues in vivo and in vitro under standard cell culture conditions, activation of the HIF pathway is minimal (Esteban, Tran et al. 2006). One of the first tumours studied to demonstrate a mechanism for hypoxia mediated tumourigenesis and metastatic potential was clear cell renal cell carcinoma (CCRCC), which is the commonest form of kidney cancer. The great majority of CCRCC involve biallelic inactivation of VHL. As a consequence of pVHL
inactivation, the HIF pathway is constitutively active (please refer to Chapter 1, Introduction). pVHL knockdown experiments have allowed analysis of the effects of HIF-1 activation. Indeed down-regulation of the cell adhesion molecule E-cadherin both in vivo and in vitro has been shown to be mediated, at least in part, by HIF (Esteban, Tran et al. 2006; Krishnamachary, Zagzag et al. 2006; Evans, Russell et al. 2007). Since this discovery, further experiments specifically demonstrating HIF-1-mediated downregulation of E-cadherin have been performed in ovarian (Imai, Horiuchi et al. 2003), prostate (Luo, He et al. 2006) and breast (Chen, Imanaka et al. 2010) cancer cell lines in vitro.

One of the tumours most studied for the role of hypoxia in pathophysiology is uterine cervical cancer (Jeffers, Paxton et al. 1997; de Boer, van Dorst et al. 1999; Acs, Zhang et al. 2003; Hutchison, Valentine et al. 2004; Kaplanis, Kiziridou et al. 2005; Rodriguez-Sastre, Gonzalez-Maya et al. 2005). Tissue hypoxia is a characteristic property of cervical cancers that makes tumours chemo and radiotherapy resistant (Jeffers, Paxton et al. 1997; de Boer, van Dorst et al. 1999; Acs, Zhang et al. 2003; Hutchison, Valentine et al. 2004; Kaplanis, Kiziridou et al. 2005; Rodriguez-Sastre, Gonzalez-Maya et al. 2005). Following established work on VHL-deficient renal cell carcinomas this mechanism is at least in part mediated by HIF. However the consistency of this relationship and role in benign or premalignant epithelial conditions is less well characterised. Research has used HIF as a marker of hypoxia in these tissues although it shows only weak correlation with low pO₂ (Hutchison, Valentine et al. 2004). The multistage nature of carcinogenesis in cervical epithelium makes it possible to follow the events for a number of intermediate stages leading from a disturbance in proliferation and differentiation of squamous metaplasia, via low and high grade intraepithelial lesions, to carcinoma. The aim of this experiment is to evaluate differences in the distribution of HIF-1α and the cell adhesion molecule E-cadherin during various stages of neoplastic progression in cervical cancer. Using immunohistochemistry, we examined the expression of E-cadherin and HIF-1 in both benign and dysplastic cervical epithelium.
5.2 Materials and Methods

5.2.1 Cervical tissue

Formalin-fixed paraffin-embedded sections of human uterine dysplastic/carcinomatous cervical tissue from 27 patients were a kind gift of Dr M.Esteban, (Servicio de Inmunología, Hospital de la Princesa, Departamento de Medicina, Universidad Autónoma de Madrid, Diego de León 62, 28006 Madrid, Spain) and 21 premenopausal benign formalin-fixed paraffin-embedded sections were kindly provided by Human Biomaterials Resource Centre, Department of Histopathology, Hammersmith Hospital, Imperial College, London.

Table 14: Summary of clinicopathological features of banked cervical tissue used to examine HIF expression in increasing dysplasia of CIN.

<table>
<thead>
<tr>
<th></th>
<th>Benign</th>
<th>CIN I</th>
<th>CIN II</th>
<th>CIN III</th>
<th>ISCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of samples (n)</td>
<td>21</td>
<td>6</td>
<td>8</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Median age of Patient</td>
<td>44.3 (42.1)</td>
<td>26.1 (27)</td>
<td>31.0 (31)</td>
<td>37.5 (38.3)</td>
<td>43.4 (45)</td>
</tr>
<tr>
<td>Mean size of tumour (cm, range)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>3.5 (0.4-11)</td>
</tr>
<tr>
<td>Mean depth of invasion:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depth (cm)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>1.42 (0.4-2.8)</td>
</tr>
<tr>
<td>Percent of cervical wall</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>82 (20-100)</td>
</tr>
<tr>
<td>Grade of tumour</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intermediate</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>3</td>
</tr>
<tr>
<td>High</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>4</td>
</tr>
</tbody>
</table>

5.3 Methods

5.3.1 Immunohistochemical Staining
CHAPTER 5: THE INTERRELATIONSHIP OF HIF AND E-CADHERIN

Immunohistochemical assays were performed on formalin-fixed paraffin-embedded sections as described previously (Esteban, Tran et al. 2006). Five-µm-thick sections were cut and deparaffinised in xylene and rehydrated in graded alcohols. SurePath cytological slides were de-cover slipped in xylene, rehydrated in graded alcohols, and postfixed in 10% phosphate-buffered formaldehyde for 30 minutes. All slides were steamed in 0.01 mol/L of sodium citrate buffer (pH 6.0) for 20 minutes. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide in methanol for 20 minutes. Slides were incubated with the antibodies against E-cadherin and HIF-1α (clone H1alpha67, mouse monoclonal, 1:10,000; Neomarkers) overnight at 4°C and incubated for 30 minutes at room temperature with horseradish peroxidase-labelled dextran polymer coupled to anti-mouse antibody (DAKO Envision + System HRP, DAKO). Slides were then washed three times with TBST, developed with diaminobenzidine for 10 minutes, and counterstained with haematoxylin. For HIF-1α the DAKO CSA signal amplification kit was used according to the manufacturer’s recommendations. For HIF-1α slides of VHL deficient renal cell carcinoma, known to show HIF-1α overexpression were used as positive control (Esteban, Tran et al. 2006). A negative control was done in each case by omission of the primary antibody.

5.3.2 Interpretation of immunohistochemical stains

Immunohistochemical stains for E-cadherin and HIF-1α were interpreted semi quantitatively by assessing the intensity and extent of staining on the entire tissue sections present on the slides according to a four-tiered (0 to 3) scale. For E cadherin membrane immunoreactivity was considered positive. For HIF-1α nuclear staining was considered positive. In the case of dysplasias or in situ carcinomas, first, the percentage of total epithelial thickness showing positive staining was determined (e.g., 50% if the basal half or 75% if the basal three-fourths of the squamous epithelium showed positive immunostaining, and so forth). In the case of invasive tumors, first the total percentage of positively staining tumor cells was determined. Then the percentage of weakly (1), moderately (2), and strongly (3) staining cells was determined, so that the sum of these categories equated with the overall percentage of positivity. A staining score was then calculated as follows: score (out of maximum of 300) = sum of 1 x percentage of weak, 2 x percentage of moderate, and 3 x percentage of strong staining. To assess the immunostaining for HIF-1α adjacent to areas of tumour cell necrosis and keratinization, immunostaining was scored as described above in at least 10 high-power fields of viable tumor cells within 1 mm of necrotic or keratinized areas, and in tumor regions away from such areas. Immunohistochemical stains were
CHAPTER 5: THE INTERRELATIONSHIP OF HIF AND E-CADHERIN

evaluated independently by two pathologists and slight differences in interpretation were resolved by simultaneous viewing.

5.3.3 Statistical analysis

The Wilcoxon signed rank test was used for the comparison of median HIF-1α and E-cadherin immunohistochemical expression levels in dysplastic epithelia and benign squamous epithelium, and for the comparison of the normal versus abnormal distribution of HIF-1α expression in benign and dysplastic epithelia. Statistical significance was determined if the two-sided \( P \) value of a test was less than 0.05. Graphpad Prism software was used for statistical analysis.

5.4 Results

5.4.1 Immunohistochemistry

To further investigate the potential role of HIF and E-cadherin expression in benign cervical tissue, squamous dysplasia and carcinoma we examined their expression by immunohistochemistry in a series of clinical samples. The results of the immunohistochemical assays are summarized in Table 15.

**Table 15:** Expression of E-Cadherin and HIF-1α in Benign Squamous Epithelium, Squamous Dysplasia (CIN I-III), and Invasive Squamous Cell Carcinoma (ISCC)

<table>
<thead>
<tr>
<th></th>
<th>Benign</th>
<th>CIN I</th>
<th>CIN II</th>
<th>CIN III</th>
<th>ISCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.pos</td>
<td>21</td>
<td>6</td>
<td>8</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Median</td>
<td>48</td>
<td>75.50</td>
<td>138</td>
<td>177</td>
<td>190</td>
</tr>
<tr>
<td>Mean</td>
<td>47.3 (1.5)</td>
<td>72.8 (4.6)</td>
<td>151.8 (11.3)</td>
<td>174.7 (5.18)</td>
<td>191 (5.03)</td>
</tr>
<tr>
<td>( p^\dagger )</td>
<td>&lt;0.0001</td>
<td>0.0156</td>
<td>0.0039</td>
<td>0.0156</td>
<td>0.0078</td>
</tr>
<tr>
<td>E-cadherin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.pos</td>
<td>21</td>
<td>6</td>
<td>8</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Median</td>
<td>134</td>
<td>84</td>
<td>45.0</td>
<td>33.0</td>
<td>22</td>
</tr>
<tr>
<td>Mean</td>
<td>136.5 (4.71)</td>
<td>84.8 (3.38)</td>
<td>45.4 (3.817)</td>
<td>45.6 (3.82)</td>
<td>19.6 (5.55)</td>
</tr>
<tr>
<td>( p^\dagger )</td>
<td>P&lt;0.0001</td>
<td>0.016</td>
<td>0.0039</td>
<td>0.015</td>
<td>0.0078</td>
</tr>
</tbody>
</table>
† Wilcoxon signed rank test, with two-sided \( P \) value of a test was less than 0.05

**Figure 13:** Expression of HIF-1\( \alpha \) (A) and E-cadherin (B) in benign cervical squamous epithelium, cervical dysplasia (CIN, grades I to III), and ISCC, determined by immunohistochemistry. Bars indicate median immunostaining score values

All of the 21 benign squamous and dysplastic epithelia showed expression of nuclear HIF-1\( \alpha \), although it was more widely expressed with increasing degree of dysplasia. HIF-1\( \alpha \) expression was significantly increased in all grades of dysplasia (\( P = 0.0156 \) for CIN I, \( P = 0.0039 \) for CIN II, and \( P = 0.0156 \) for CIN III, see Table 15, Wilcoxon signed rank test).
Whereas strong membrane immunostaining for E-Cadherin was present in all benign samples and early dysplastic epithelia (CIN I and II), membranous expression was only evident in 5 of 6 and 5 of 8 CIN III and ISCC tissues respectively, (Table 2 and Figures 1B and 2). Furthermore, membranous E-Cadherin expression decreased significantly with increasing degree of dysplasia, (Table 1, Figures 1(B)).

**Figure 14:** E-Cadherin and HIF-1α expression in benign, moderate dysplasia (CIN II) and ISCC (X50)

HIF-1α immunostaining was predominantly nuclear, although in a few cases of ISCC weak cytoplasmic reactivity was also observed (Figure 14). In benign squamous epithelia, HIF-1α immunostaining was seen in the parabasal cells and in the middle third of the epithelium (Figure 14). In addition, in dysplastic epithelia HIF-1α immunostaining was not restricted to the middle third of the epithelium as in benign cases but was also present in the basal and/or the superficial portions. In invasive carcinomas, HIF-1α expression was seen in all cases but expression was more focal (less than 50% of tumour cells). When focal, HIF-1α immunostaining was concentrated to areas surrounding necrotic regions and the infiltrating edge of tumours.

In benign squamous epithelia membranous E-Cadherin expression was confined to the basal and parabasal cell layers. In contrast, in squamous dysplasia, membranous E-Cadherin expression was significantly reduced. Indeed the E-Cadherin expression was confined to the
basal cell layer where increasingly the staining was cytoplasmic, in contrast to HIF-1 expression for the same level of dysplasia, which demonstrated expression in all layers of the epithelium.

The mean E-Cadherin staining score decreased with increasing grade of dysplasia (slope -27.22 ± 5.410, $r^2 = 0.894, P = 0.0151$, linear regression).

The mean HIF-1α staining score increased with increasing grade of dysplasia (slope 38.93 ± 6.108, $r^2 = 0.9312, P = 0.0078$, linear regression).

5.5 Discussion

The major finding of this study is that HIF-1α expression is inversely related to the membranous expression of the cell-cell adhesion molecule E-cadherin in stratified cervical epithelium. Interestingly, the expression and role of HIF-1α in benign cervical epithelium has, until now, been poorly characterised (Mayer, Hoeckel et al. 2010). The presence of HIF-1α in the middle third of benign epithelium may have a physiological role in downregulating cervical E-Cadherin to facilitate cell migration in normal epithelial homeostasis. We have shown that in increasing degrees of dysplasia HIF-1α expression is upregulated, although which of these two factors represents the primary stimulus is unclear. A similar HIF-1α expression pattern has been demonstrated previously (No, Jo et al. 2009). The relatively dynamic expression of E-Cadherin has been demonstrated in a number of epithelia as a result to injury, for example, ulceration or fibrosis (Hanby, Chinery et al. 1996; Zhou, Dada et al. 2009). The subsequent phenotypic change with its downregulation EMT is thought to be integral in the early reparatory ability of such epithelia. The role of HIF in this context has not previously been investigated. In this study HIF-1α expression may reflect a local hypoxic stimulus although even in early ISCCs (using the ‘gold standard’ Eppendorf histography system for clinical assessment of tumour oxygenation) there is no correlation between pO$_2$ and HIF-1α expression (Dellas, Bache et al. 2008; Mayer, Hoeckel et al. 2010). Furthermore, Vaupel et al., in a recent experiment on large uniformly hypoxic solid uterine leiomyomas, showed that neither HIF-1α or HIF-2α or their respective target genes were upregulated and their non-expression was not a function of elevated prolyl hydroxylase activity. Conversely, their malignant tumoural form leiomyosarcomas, show abundant expression of HIF-related markers despite a similar microvascular density. The results indicate that the strong activation of the HIF system observed in solid malignant
tumours may be linked to their transformed phenotype, rather than being a physiological reaction activated in a pathological context.

The role of E-Cadherin in EMT and its importance in neocarcinogenesis and the ability of primary tumours to metastasise is well documented. The importance of HIF-1α in orchestrating an adaptive response to the common environmental stimulus of hypoxia in such tumours is also well recognised (Dellas, Bache et al. 2008). However, the interrelationship between HIF-1α and E-Cadherin and its combined role in carcinogenesis remains complex and a fertile area of research (Chuang, Sun et al. 2008; Bendinelli, Matteucci et al. 2009; Sun, Ning et al. 2009; Du, Huang et al. 2010). In premalignant conditions, such as cervical dysplasia, the exact roles of HIF-1α and E-Cadherin remain unclear. One novel paradigm for HIF-1α in multistage carcinogenesis stems from evidence that HIF-1α facilitates cervical cancer progression in HPV type 16 infected transgenic mice. This suggests that it interacts with viral oncogenes to induce multiple genomic networks in premalignancy and fosters the development of advanced cervical cancer (Lu, Wright et al. 2007). Conversely, another group silenced E7 oncogene in transformed keratinocytes and restored functional E-Cadherin expression (Caberg, Hubert et al. 2008). Unfortunately, the viral status of our cervical tissue was unknown and this may represent a significant weakness in our ability to extrapolate the true significance of the HIF profile.

As HIF response to hypoxia is seen in all cell types examined to date, this study raises the interesting possibility that E-cadherin expression in settings other than renal epithelium and kidney cancer may also be influenced by HIF. Importantly, recent studies have shown that hypoxic incubation was able to suppress E-cadherin protein expression in pancreatic, colon and hepatoblastoma cell lines (Imai, Horiuchi et al. 2003; Esteban, Tran et al. 2006; Krishnamachary, Zagzag et al. 2006; Cannito, Novo et al. 2008). The mechanism by which hypoxia and/or HIF represses E-cadherin expression is not clear. Genetic, epigenetic, and transcriptional changes have been implicated (Peinado, Olmeda et al. 2007). For example, the HIF target gene lysyl oxidase (LOX) has been implicated in involvement in E-cadherin regulation (Erler, Bennewith et al. 2006). Cannito et al. exposed 4 human cancer cell lines to hypoxia and suggests that early EMT-related events, such as down regulation of E-Cadherin, are dependent on transient intracellular increase in ROS, whereas late migration and invasiveness were sustained by HIF-1α. Other experiments have highlighted the relevant Notch intracellular signalling molecules involved, for example the increased expression of Slug and Snail, Mitochondrial ROS, HIF-1α induces Twist (Sun, Ning et al.
2009), NF-kappa B, TNFα (Chuang, Sun et al. 2008), Snail and SIP1 (Evans, Russell et al. 2007) correlated with the decreased expression of E-cadherin (Chen, Imanaka et al. 2010) in 6 breast carcinoma cell lines. In their series of intraepithelial and invasive cervical squamous lesions, Carico et al. (Carico, Atlante et al. 2001) also describe a statistically significant correlation between the site at which E-cadherin localized and the histological diagnosis. This alteration in the site at which these molecules are detected in benign and neoplastic endocervical epithelia may reflect an alteration in cell–cell interactions associated with disease progression and ultimately invasion. This possibility could offer an insight into why mutations in E-cadherin are infrequent in cancers in general, because HIF activation is very common in solid tumours and may thus provide a reliable epigenetic route to down-regulating E-cadherin efficiently. It would also explain why expression of E-cadherin in tumours is frequently patchy (as it commonly happens with areas of hypoxia) rather than homogeneous. Our study demonstrates that during the development of cervical lesions substantial qualitative (subcellular localization-membrane to cytoplasmic) and inverse quantitative alterations (changes in expression) occur in the protein expression of E-cadherin and HIF1 in cervical cancer.
CHAPTER 6

CD10: A HIF TARGET GENE?
CHAPTER 6: CD10 - A HIF TARGET GENE?

6.1 Introduction

6.1.1 What is CD10/CALLA/NEP?

Common acute lymphocytic leukemia antigen (CALLA) is a 100-kD glycoprotein and an important cell surface marker in the diagnosis of human acute lymphocytic leukemia (ALL) (Letarte, Vera et al. 1988).

6.1.2 Where is it expressed?

CALLA is present on leukemic cells of pre-B phenotype, which represent 85% of cases of ALL, and is absent from normal PBMC. However, it is not restricted to leukemic cells and is found on a variety of normal tissues (Metzgar, Borowitz et al. 1981) including the kidney (Platt, LeBien et al. 1983). There it is present on the brush border of proximal tubules and on glomerular epithelium (Metzgar, Borowitz et al. 1981; Platt, LeBien et al. 1983).

6.1.3 What does it do?

It was thought CD10 had no functional activity until Letarte et al. cloned a cDNA coding for CALLA and showed that the amino acid sequence deduced from the cDNA sequence is identical to that of human membrane-associated neutral endopeptidase (NEP), also known as enkephalinase (Letarte, Vera et al. 1988). NEP cleaves peptides at the amino side of hydrophobic residues and inactivates several peptide hormones including glucagon, enkephalins, substance P, neurotensin, oxytocin, bradykinin, and the chemotactic peptide fMLF (Kerr and Kenny 1974; Malfroy, Kuang et al. 1988). Letarte et al.’s observation opened new avenues for testing the function of this molecule in leukemic cells and its potential role in the process of malignancy.

6.1.4 Why is it of interest in carcinogenesis?

CD10 belongs to a ‘superfamily’ of more than 20 zinc matrix metalloproteases (MMPs). MMPs are critical for maintaining tissue allostatics. MMPs are active at neutral pH and can therefore catalyze the normal turnover of extracellular matrix (ECM) macromolecules such as the interstitial and basement membrane collagens and proteoglycans as well as accessory ECM proteins such as fibronectin (Malemud 2006). Given the importance of their
role in maintaining cell-matrix structure, they have been the subject of extensive research into the pathology of carcinogenesis and development of metastases. One example is matrix metalloproteinase-9 (MMP-9), which promotes key processes underlying tumour cell propagation, from migration and invasion to angiogenesis and tumour growth (Coussens, Fingleton et al. 2002). MMP-9 cleaves specific factors constituting or present in the ECM, including collagen IV in basement membranes and angiogenic molecules (Fridman, Toth et al. 2003), thereby creating a physical and biochemical path for tumour invasion and neovascularisation (Curran and Murray 1999). Although significant, the role of MMP-9 in metastasis remains controversial. On one hand, MMP-9 regulates most of the rate-limiting steps underlying metastasis and could represent an ideal target for effective disruption of the metastatic cascade. However, MMP-9 also mediates wound healing and inflammation (Okada, Kita et al. 1997; Baker and Leaper 2003) and opposes tumour growth by mobilizing angiogenetic inhibitors angiostatin and endostatin (Overall and Kleifeld 2006). MMP-9 is not the only MMP implicated in the development of certain cancers, for example, transin is induced by oncogenes and is expressed more abundantly in malignant than in benign tumors (Matrisian, Leroy et al. 1986).

### 6.1.5 What do we know about CD10 in colorectal carcinogenesis?

CD10 expression has been reported to correlate with the histologic features and biologic behaviour of colorectal carcinomas (Yao, Takata et al. 2002). Yao et al. suggested that patients with colorectal adenocarcinoma with CD10 expression are at increased risk of liver metastasis (Yao, Takata et al. 2002). More recently Fujimoto et al. confirmed by multivariate analysis that there was a significant association between CD10 expression in colorectal carcinoma and liver metastases (Fujimoto, Nakanishi et al. 2005). The reason for this association is unknown. CD10 expression tends to be seen more frequently at the luminal surfaces of the cribriform glands in moderately differentiated adenocarcinoma than that in well-differentiated adenocarcinoma and a roles in vascular invasion has subsequently been postulated (Fujimoto, Nakanishi et al. 2005).

### 6.1.6 CD10: a HIF target gene?

It is hypothesised in this chapter that the significant association of CD10 expression in colorectal carcinoma and liver metastases may be explained by a HIF mediated mechanism and that CD10 may therefore be a HIF target gene. This hypothesis has been in part
supported by the observation of close concurrent expression of HIF-1 isotype and CD10 in the proximal renal tubule in humans, (Tran, M., Maxwell, P. et al.-private correspondence). Furthermore, following Houda et al.’s sequencing of the CALLA (or CD10) gene in 1995, (Haouas, Morello et al. 1995) it is possible to identify the prerequisite hypoxia response element (HRE) segment. HREs are composite regulatory elements, comprising the conserved HIF binding site (HBS) with a core A/GCGTG sequence, and a highly variable flanking sequence (Wenger, Stiehl et al. 2005) (Figure 15).

[Figure 15: Alignment of 5’ UTR sequences from CALLA/NEP/CD10 cDNAs, (reproduced with permission from D’Adamio et al.).]

There are many more sites with the core A/GCGTG sequence in the regulatory regions of mammalian genes than are actually used by HIF to regulate gene expression and at the moment it is not understood what distinguishes HBSs in the functional HREs from the putative, non-functional HBSs that contain the identical core sequence. However, encouragingly subsequent analysis of the CD10 promoter region (Ishimaru and Shipp 1995) demonstrates that following gene splicing many of these HRE segments and HIF binding sites are conserved and expressed at transcription. Our aim to identify whether HIF mediates expression of CD10 may help elucidate the respective roles of HIF and CD10 in colorectal carcinogenesis.

6.2 Methods

6.2.1 Cell culture, chemicals, and antibodies

RCC4, RCC10, and 786-O cell lines and the corresponding stable transfectants with wild-type pVHL have been described previously in Chapter 2. RCC10 cells were generously provided by K. Plate and T. Acker (Institute of Neurology/Edinger Institute, Frankfurt University Medical School, Frankfurt, Germany). Cells were cultured in RPMI 1640 (Life
Technologies, Gaithersburg, MD) with penicillin/streptomycin, glutamine, and 10% fetal bovine serum. Each of the 3 cell lines and their respective paired transfected wild type VHL cell line were cultured as described in Chapter 2. mRNA was extracted, cDNA and RT-PCR (using the CD10 primer, and β actin control) was performed to assess respective levels of CD10 mRNA expression in VHL knockout and wild-type renal cell cancer cell lines. For this we used the CD10 primer sequence (5′GGGATCCTCACCAAACCGCGACCTTTTT3′).

Desferrioxamine (DFO) and cobalt chloride (CoCl₂) were purchased from Sigma (St. Louis, MO); dimethyl-oxalylglycine (DMOG) was purchased from Frontier Scientific (Logan, UT). For hypoxic experiments, cells were exposed to 1% oxygen for the indicated times using either a hypoxic workstation (INVIVO₂ 100, Ruskin, Leeds, United Kingdom) or a hypoxic incubator Galaxy R (Biotech, Palo Alto, CA). DMOG is a chemical induction agent that acts to upregulate HIF in the absence of hypoxia. Its mechanism is demonstrated in the diagram below.
Figure 16: Schematic diagram showing the mechanism by which DMOG inhibits HIF hydroxylation

To assess the effects of physiological and chemical HIF stabilization on levels of CD10 mRNA expression, the 3 renal cell cancer cell lines (wild type transfectants) were kept in hypoxia (1% oxygen) for 16 hours, or normoxia and 1 mM DMOG for 16 hours. CD10 mRNA was extracted thereafter and levels measured as described already.

HT-29 colorectal cells were cultured as described previously, and similarly were stimulated by physiological hypoxia and DMOG to assess CD10 response. Concurrent HIF-1/2 isotope expression was measured and results described below.

6.3 Results

6.3.1 CD10 expression in colorectal liver metastases

We first verified the reported distribution of CD10 in colorectal carcinoma. Figure 17 below shows the high level of CD10 positive staining at the margins of colorectal liver metastases, (see Chapter 4). The canalicular pattern of the liver tissue adjacent is also evident but more weakly positive. CD10 showed significantly greater positive staining in colorectal liver metastases resected laparoscopically as did the HIF-2 isotype, although the mechanism remains unknown.

Figure 17: CD10 expression in colorectal liver metastases margin, (x50).

6.3.2 The effect of constitutively active HIF on CD10 mRNA expression in CCRCC model
Figure 18 below, shows the relative fold induction of CD10 mRNA detected by RT-PCR in 3 RCC lines (RCC4, RCC10 and 786-O) defective for the pVHL expression. PHD3 was used as a control HIF-responsive gene, (data not shown). Means were taken from 3 independent experiments. 786-O shows the greatest upregulation in transcription in pVHL negative cells, with a mean of 8 fold induction. RCC10 was next greatest and then RCC4 (means of 5 and 4 respectively, \( p<0.05 \)-paired Student t-test).

![Graph showing relative CD10 mRNA levels in pVHL negative CCRCC cell lines compared to wild type (pVHL competent).](image)

**Figure 18**: Relative CD10 mRNA levels in pVHL negative CCRCC cell lines compared to wild type (pVHL competent).

### 6.3.3 CD10 protein expression in CCRCC lines with defective pVHL

We next examined CD10 expression in all 3 pVHL-defective cell lines derived from sporadic CCRCC (RCC4, RCC10 and 786-O) and corresponding sublines in which pVHL was stably re-expressed (referred to here as RCC4/VHL, RCC10/VHL and 786-O/VHL). Notably, whereas RCC4/VHL and RCC10/VHL cell lines expressed little or no CD10, pVHL-defective RCC4 and RCC10 showed substantial levels of CD10 protein on immunoblotting.
To further investigate the link between pVHL status and CD10, we then analyzed CD10 mRNA by quantitative real-time RT-PCR in RCC10 and RCC10/VHL. The level of CD10 mRNA was higher in RCC10 as were mRNA levels of VEGF and PHD3 (two well-characterized targets of HIF1 and 2 respectively). Thus, eliminating expression of pVHL (stabilisation of HIF expression) in CCRCC cells can increase CD10 expression, and this operates at least in part through increasing the level of CD10 mRNA.

6.3.4 CD10 mRNA Expression in pVHL Competent Cells

The above results may be consistent with HIF mediating the expression of CD10 in VHL-negative CCRCC cells. To further explore this, we tested whether activating HIF in RCC10/VHL (i.e.: VHL competent RCC cell line) would upregulate CD10 mRNA. Firstly, we used hypoxia (1% oxygen). As expected, this activated the HIF pathway potently, as evidenced by the induction of expression (measured by real-time RT-PCR) of VEGF,

Figure 19: wtVHL abrogates the expression of CD10 in all three renal cancer cell lines. (* indicates a much shorter exposure time) (Films developed with kind assistance of Dr M. Tran)
(recognised HIF-1 target in CCRCC) (data not shown) and PHD3 mRNA, (recognised HIF-2 target gene in CCRCC, Figure 20).

**Figure 20:** Effects of different stimuli on PHD3, (HIF2 target gene) mRNA expression in RCC10

In parallel, hypoxia significantly increased mRNA as measured by real-time RT-PCR. Secondly, we used three different chemical agents that activate the HIF pathway by inhibiting HIF hydroxylation (Schofield and Ratcliffe 2004): (a) the iron chelator

**Figure 21:** Effects of different stimuli on CD10 mRNA expression in RCC10
desferrioxamine (DFO), (b) the 2-oxoglutarate analogue dimethyl-oxalylglycine (DMOG) and (c) CoCl₂. The results show that all 3 upregulate CD10 mRNA expression, the greatest fold increase associated with DMOG. Thereafter we used DMOG as the inhibitor of prolyl hydroxylase in HT-29. The results are shown in Figure 22 below alongside hypoxia as a stimulus. The results are from 3 independent experiments. Hypoxia results in almost a mean 4-fold increase in CD10 mRNA (paired Student t-test) whereas inhibiting HIF hydroxylation results over a 5-fold increase (p<0.05).

6.3.5 Effects of hypoxia and DMOG on CD10 mRNA expression in HT-29 colorectal carcinoma cell line

We stimulated the HT-29 cell line, which has similar culture requisites to the RCC lines, with hypoxia and mmOG. The results are shown below. HT-29 demonstrates an upregulated CD10 mRNA expression when compared to HT-29 untreated cells (control).

![Figure 22: Effects of hypoxia and DMOG on CD10 mRNA expression in HT-29 colorectal carcinoma cell line](image)

6.4 Discussion

Years of research looking at hereditary and sporadic pVHL-defective CCRCC has shown that as a consequence of pVHL inactivation, the HIF pathway is constitutively active. Expression of pVHL in otherwise defective CCRCC-derived cell lines restores normal degradation of HIF-α subunits and also suppresses tumour growth in xenograft models (Kondo, Kim et al. 2003). The three VHL-/- RCC cell lines used in this chapter (and their VHL transfected wild type RCC (VHL+/+), have been used extensively in the characterisation of HIF target genes.
Most recently they were used to highlight HIF dependent control of E cadherin expression in CCRCC (Esteban, Tran et al. 2006). Paraf et al. characterised CD10 as a marker in the renal nephron as a marker of the proximal tubule cell type (Paraf, Chauveau et al. 2000). Analysis by Tran et al. (private correspondence) in VHL deficient CCRCC showed that CD10 expression and proximal tubule cell type corresponded significantly to areas of HIF positivity. In Chapter 2, I analysed stained CLMs for CD10 and found significant upregulated expression. Furthermore, similarly high levels of HIF-2 isotype in the same tissues were demonstrated (see Chapter 4). A correlation between CD10 positivity and HIF expression in primary colorectal carcinoma has been demonstrated (Fujimoto, Nakanishi et al. 2005) and previous studies have suggested that HIF-1 is the predominant isotype in colorectal primaries (Yoshimura, Dhar et al. 2004). In this clinical context, we hypothesised that CD10 may be hypoxia regulated in expression and more specifically a HIF target gene.

In this chapter we have demonstrated using these 3 CCRCC cell lines that both CD10 mRNA and CD10 protein expression are upregulated in VHL deficient cell lines, which have constitutively active HIF expression. Furthermore, we demonstrated that this upregulation could be replicated in wtCCRCC cell lines by exposure to 16hr hypoxia and inhibiting prolyl hydroxylation, the mechanism of normoxia-mediated HIF downregulation, using DMOG. The level of mRNA of VEGF and PHD3 was analyzed in parallel and showed the expected upregulation pattern for genes whose expression is increased by HIF-1 and 2 isotypes, respectively, in CCRCC (Raval, Lau et al. 2005). Finally, we selected a colorectal tumour cell line HT 29 to subject to culture in hypoxia and inhibition of HIF hydroxylation and demonstrated through RT-PCR upregulation of CD10 mRNA expression. Further work to differentiate more specifically whether the CD10 expression is under HIF-1, HIF-2 or a combination of both HIF-1 and 2, is needed.

Importantly, overexpression of a modified HIF-1α molecule, which cannot be recognized by pVHL, is sufficient to override the tumor suppressor effects of VHL (Kondo, Kim et al. 2003). Furthermore, knock down of HIF-1α in pVHL-negative CCRCC cell lines abrogates their tumourigenic potential (Kondo, Kim et al. 2003). These observations show that suppression of HIF-2α is necessary and sufficient for pVHL's action as a tumor suppressor in fully transformed CCRCC cells. To directly test the role of HIF isotypes in regulating CD10 expression, one might infect RCC10/VHL cells with retroviral vectors encoding constitutively active forms of either HIF-1α or HIF-2α. These engineered HIF molecules contained mutations of the two prolyl residues, which are targets for oxygen-dependent hydroxylation and pVHL capture. A similar experiment with constitutively active HIF1 and 2 isotypes would
allow further insight into potential specific isotype dependent CD10 transcription control. Alternatively, the use of siRNA to HIF 1 and 2 would enable further insight into the role of each and their interplay in CD10 expression. Current challenges are to understand (1) the key downstream targets of HIF involved in tumorigenicity, and (2) the extent to which these HIF-mediated changes in fully transformed cancer cells reflect tumor initiation and progression in the epithelium (Esteban, Tran et al. 2006).

Similarly, both isoforms are abundantly expressed in the kidney but in different types of cells: HIF-1α is predominantly expressed in epithelial cells whereas HIF-2α is predominantly detected in interstitial fibroblast and endothelial cells (Rosenberger, Mandriota et al. 2002). We have demonstrated HIF-2 propensity in colorectal liver metastases, with conserved CD10 expression. The question of how HIF-1 and HIF-2 discriminate between the target genes is far from settled. The observed differences could be accounted for by preferential cooperation of one of the isoforms with certain subsets of transcription factors, coactivators or corepressors, and/or tissue-specific expression of HIF-α isoforms.

CD10 is normally widely distributed in a number of organs including the kidney, small intestine and liver (Haouas, Morello et al. 1995). It has been shown to exhibit a characteristic canalicular pattern in the liver (Lin, Abdallah et al. 2004). This was also evident in our series. Recent studies have demonstrated CD10 expression in a range of neoplasms including carcinomas of the pancreas, kidney, bladder, prostate and uterus. Studies by Lin et al. and Borscheri et al. have shown that the canalicular expression pattern CD10 in the liver is conserved in primary hepatocarcinogenesis (Borscheri, Roessner et al. 2001; Lin, Abdallah et al. 2004) and CD10 has been proposed as a diagnostic marker of HCC in liver biopsies and fine needle aspirates. In our series (in Chapter 4) CD10 was expressed in a canalicular pattern in keeping with the neighbouring normal liver tissue. Of the series comparing CD10 expression in HCC with metastatic disease, only one looked at colorectal liver metastases (a total of two blocks neither positive for CD10). There are two large retrospective series that have suggested a role for CD10 in the colorectal carcinogenesis. Fujimoto et al. looked at the primary colorectal carcinomas in 505 patients and showed that CD10 was significantly higher (52% vs 39%) in patients who subsequently developed liver metastases. Statistical analysis subsequently confirmed a trend, observed previously by Ogawa et al., that CD10 expression in colorectal primary carcinoma is a predictor of subsequent liver metastases (Ogawa, Iwaya et al. 2002). How CD10 confers a more invasive/malignant potential remains to be elucidated. It must be noted that other MMPs (particularly MMP-2 and MMP-9) have been isolated in active isoform in colorectal cancer.
liver metastases (Okada, Ishida et al. 2001). The same limitations of Fujimoto’s work apply to our pilot data set: he has not investigated CD10 expression in liver metastases and we do not have the tissue blocks available to look at the primaries from where the CD10+ CLM originated. Prospective tissue collection would allow better characterization of timing of CD10 upregulation and would allow parallel analysis of HIF expression.
CHAPTER 7

CIRCULATING TUMOUR CELLS AND RADIOFREQUENCY ASSISTED LIVER RESECTION


7.1 Introduction

The selection criteria for liver surgery are usually controlled primary tumour, no extra hepatic metastases and a resection that is technically feasible with tumour-free margins (Hugh, Kinsella et al. 1997). Survival is shortened by the presence of inadequate or involved resection margins (Pawlik, Scoggins et al. 2005). It has been postulated that the principal mode of colorectal tumour dissemination is via the portal system and therefore that surgical resection of isolated hepatic metastases from colorectal cancer may be curative in a number of cases (Weiss, Grundmann et al. 1986). Previous data from the Registry of Hepatic Metastases, a multi-institutional database of liver resections, suggest that a margin >1cm was associated with 45% five year survival, but only 23% survived five years if the margin was less (Hughes, Simon et al. 1986).

The prognostic role of circulating cancer tumour cells (CTCs) is now established (Cristofanilli, Budd et al. 2004; Cristofanilli, Hayes et al. 2005) and data support their role as more reproducible indicators of disease status than current imaging methods (Budd, Cristofanilli et al. 2006). A number of methodologies have been proposed for their measurement, including quantitative real time polymerase chain reaction based assays (Stathopoulou, Vlachonikolis et al. 2002; Benoy, Elst et al. 2004), immunomagnetic separation and laser scanning cytometry (Smith, Slade et al. 2000; Pachmann, Clement et al. 2005; Ring, Zabaglo et al. 2005; Pachmann, Camara et al. 2008). The FDA has now approved the routine use of the flow cytometry based Veridex CellSearch (Warren, NJ, USA) system in a number of clinical settings including breast and prostate cancer, diseases that are considered incurable when metastatic (Apostolopolous, Giamas et al. 2007; Jiao, Apostolopolous et al. 2008; Payne, Yague et al. 2008; Rack, Schindlbeck et al. 2008; Slade, Payne et al. 2008).

In colorectal cancer approximately 50% of patients develop metastatic liver disease and 25% have evidence of this at the time of initial diagnosis (Windsor, Cohen et al. 2008). If untreated, the median survival of these patients is less than 12 months. With curative liver resection the 5-year survival increases to 40-60% (Meyerhardt and Mayer 2005). Increasingly, a variety of loco regional therapeutic modalities have been advocated to treat patients with unresectable liver metastases although intraoperative manipulation of organs has been reported to promote tumour spread (Gutt, Riemer et al. 1999; Gutt, Riemer et al. 2001; Schmidt, Koch et al. 2008).
RFA is designed to induce tumour destruction by delivering a high frequency alternating current through an active needle electrode introduced into the neoplastic tissue. Frictional heating is the result when the ions in the tissue attempt to follow the changing direction of the alternating current, causing cell death by coagulative necrosis (Napoletano, Taurino et al. 2008). Such therapy results in a ‘noxa’ for the body, inducing a strong inflammatory response. Studies in recent years have suggested a survival benefit for patients with colorectal liver metastases undergoing RFA compared to historical controls undergoing chemotherapy alone (Berber, Pelley et al. 2005) and hence RFA has emerged as an accepted method for treatment of liver metastases. However, there have been no prospective randomized trials comparing RFA with other therapeutic modalities (Hur, Ko et al. 2008; Lee, Yun et al. 2008; Nikfarjam, Shereef et al. 2008; Reuter, Woodall et al. 2008).

RF is a novel way of resecting liver parenchyma with minimal blood loss (Ayav, Navarra et al. 2005; Jiao, Navarra et al. 2005; Jiao, Navarra et al. 2006; Ayav, Bachellier et al. 2007; Ayav, Jiao et al. 2007; Ayav, Navarra et al. 2007; Bachellier, Ayav et al. 2007; Ayav, Jiao et al. 2008; Jiao, Ayav et al. 2008; Pai, Jiao et al. 2008; Pai, Navarra et al. 2008). It employs the coagulative necrosis effect on healthy liver tissue to ‘seal’ adjacent liver parenchyma immediately before sharp resection of the tumour itself.

We have shown that when comparing the oncological outcomes of R0 resections in a small pilot population who had undergone RFALR, those that had undergone a laparoscopic approach had a shorter time to recurrence and overall life expectancy. We therefore wished to compare the overall effect of open and laparoscopic RF assisted liver resection (RFALR) on CTC measurements immediately before and after these curative procedures and also assess the impact of open RFA alone on CTCs. Furthermore, we used these surrogate biomarkers to gain insights into the differential localization or compartmentalization of these cells, likely to be en route to further metastasis, an as yet unconfirmed assertion.

### 7.2 Patients and Methods

Consecutive patients with metastatic colorectal liver metastases, confirmed as adenocarcinoma by histology, were recruited to this study from January to November 2008. Appropriate local ethics committee approval was obtained. Samples were blinded for analysis and patients understood that the results would not be made available to them.
The CellSearch system was used to enrich and enumerate the CTCs, as described previously (Slade, Payne et al. 2008). A 7.5mL blood sample was taken in a CellSave preservative tube, kept at room temperature and processed within 72 hours. The system enriched for EpCAM (epithelial cell adhesion molecule) positive epithelial cells by incubating the sample with ferrofluid conjugated to anti-EpCAM antibodies. Cells were stained with the following fluorescent labelled monoclonal proprietary antibodies: CD45-APC to distinguish the CTCs from leukocytes and pan-cytokeratin 8, 18 and 19 (CK-PE) to stain epithelial cells, and epidermal growth factor-receptor (EGF-R) antibodies as we have recently described (Payne, Yague et al. 2008). Nucleic acids were stained using 4,6-diamidino-2-phenylindole (to exclude red blood cells). Samples were then scanned on the CellTracks analyzer II fluorescent microscope for analysis.

In all patients, 7.5mL of blood was taken from peripheral venous (PV) and arterial circulations (PA), and hepatic (HV) and portal veins (PoV) intraoperatively, before and 20 minutes after resection (RFALR) or open RFA (ORFA). The arterial sample was taken from an indwelling cannula in a radial artery inserted during routine anaesthesia for intraoperative monitoring. The portal and hepatic venous blood was obtained from a direct venous puncture after mobilization of portal triads and liver to exposed portal vein and right hepatic vein during operation. For those undergoing laparoscopic liver resections, 7.5mL of blood was taken from peripheral venous and the arterial circulation before and after resection following introduction of pneumoperitoneum with a CO$_2$ pressure of 12mmHg. All patients were followed-up with our standard practice with microbubble ultrasound at 6 weeks following RFA, then a CT scan at 3 months and 6 months following resection.

Statistical analysis was carried out using SPSS for Windows (version 16.0, SPSS Inc. Chicago, IL). Results are expressed as either median (range) or mean ± SD. Comparisons between variables before and after procedures were analyzed using the paired t test. A $p<0.05$ was considered statistically significant.

7.3 Results

A total of 20 consecutive patients requiring intervention for their colorectal liver metastases were recruited into this study. These comprised individuals for open RF ablation (ORFA (N=5)), open and laparoscopic liver resection, (opRFALR (N=11) and lapRFALR (n=4) respectively); there were no significant differences in baseline characteristics between the
groups except patients undergoing RFA were generally older (Table 16). One additional patient with metastatic carcinoid tumour was also recruited to this study as a negative control and no CTCs were detected in this individual. The mean number of CTCs before and after intervention in both the systemic and portosystemic circulations is shown in Table 17.

**Table 16:** Patient characteristics of 20 patients with colorectal liver metastases included in this study. Histology confirmed adenocarcinomas in all patients.

<table>
<thead>
<tr>
<th>Therapeutic modality (n)</th>
<th>Male/ Female</th>
<th>Age in years (median, range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Open resection (opRFALR, n = 11)</td>
<td>8 male, 3 female</td>
<td>53 (30 – 72)</td>
</tr>
<tr>
<td>Laparoscopic resection (lapRFALR, n = 4)</td>
<td>3 male, 1 female</td>
<td>55 (41 – 76)</td>
</tr>
<tr>
<td>Open RFA (ORFA, n = 5)</td>
<td>3 male, 2 female</td>
<td>60 (37 – 78)</td>
</tr>
</tbody>
</table>
Table 17: The Impact of interventions on CTCs at different vascular sites
PV, peripheral vein; PA, peripheral artery; PoV, portal vein; HV, hepatic vein; NA, not applicable. * denotes a p value < 0.05 compared with before the intervention.

<table>
<thead>
<tr>
<th>Therapeutic modality</th>
<th>Before Intervention</th>
<th>After Intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of CTCs:</td>
<td>Number of CTCs:</td>
</tr>
<tr>
<td></td>
<td>Mean±SD (range)</td>
<td>Mean±SD (range)</td>
</tr>
<tr>
<td></td>
<td>PV</td>
<td>PV</td>
</tr>
<tr>
<td></td>
<td>PA</td>
<td>PA</td>
</tr>
<tr>
<td></td>
<td>PoV</td>
<td>PoV</td>
</tr>
<tr>
<td></td>
<td>HV</td>
<td>HV</td>
</tr>
<tr>
<td>Open resection (opRFALR, n = 11)</td>
<td>1±2.4</td>
<td>0.09±0.3</td>
</tr>
<tr>
<td></td>
<td>1.7±2.8</td>
<td>0.5±0.5</td>
</tr>
<tr>
<td></td>
<td>(0-3)</td>
<td>(0-1)*</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>(0-6)</td>
<td>(0-)</td>
</tr>
<tr>
<td></td>
<td>&gt;500</td>
<td>&gt;225*</td>
</tr>
<tr>
<td></td>
<td>&gt;750</td>
<td>112*</td>
</tr>
<tr>
<td>Laparoscopic resection (lapRFALR, n = 4)</td>
<td>2.3±1.8</td>
<td>0.2±0.5</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>(0-7)</td>
<td>(0-1)*</td>
</tr>
<tr>
<td>Open RFA (ORFA, n = 5)</td>
<td>3±2</td>
<td>12.7±9</td>
</tr>
<tr>
<td></td>
<td>5.5±11</td>
<td>6.5±9</td>
</tr>
<tr>
<td></td>
<td>(0-7)</td>
<td>(0-15)</td>
</tr>
<tr>
<td></td>
<td>10±4.1</td>
<td>(0-4)</td>
</tr>
<tr>
<td></td>
<td>(0-18)</td>
<td>(0-86)*</td>
</tr>
<tr>
<td></td>
<td>24±72</td>
<td>14.6±44</td>
</tr>
<tr>
<td></td>
<td>(0-16)</td>
<td>(0-156)*</td>
</tr>
</tbody>
</table>

7.3.1 Localization of CTCs and the impact of chemotherapy

Localization of CTCs in the circulation was examined by measuring them at different sites in both the systemic circulation, as measured with peripheral venous and arterial blood samples, and the portosystemic circulation, as measured with portal venous and hepatic venous blood samples, in patients undergoing an open procedure (N=16). This demonstrated that the mean number of CTCs immediately prior to intervention in PV, PA, PoV and HV measured 1±2.4, 1.7±2.8, 125.7±249.5 and 173.3±298.5 respectively for open
resection and 3±2, 5.5±11, 10±4.1 24±72 for open RFA. Thus, a larger number of CTCs was observed in the liver macrocirculation, compared to peripheral systemic circulation.

Of the 11 patients who had open surgical liver resections, nine with synchronous liver metastases had preoperative chemotherapy, and two with metachronous solitary metastases detected approximately 2 years after the initial resection for the colonic primary did not have preoperative chemotherapy. Irrespective of chemotherapy or of the type of response to systemic cytotoxics, the number of CTCs in the peripheral circulation remained remarkably low (N=9, 1.4 ±3.4; N=2, 3.0±4.2, respectively, in the patients who had preoperative chemotherapy and in the patients who did not). The number of CTCs in PV and HV was significantly lower in patients who received chemotherapy compared with those who did not (0.7±0.9 vs 400.0±141.0; 10±14 vs 500.0±353.5, respectively), indicating, once again, that the liver macrocirculation and the lungs appear to be sites where CTCs are pooled. Chemotherapy reduced the number of CTCs in the portosystemic system reducing, we suggest, the risk of both lung and systemic spread although long-term clinical outcomes are still awaited.

7.3.2 Impact of procedural interventions on CTCs

In the case of RF assisted-liver resection (RFALR-both laparoscopic and open), the number of CTCs in any blood vessel sampled was significantly lower following the procedure than prior to it (p<0.05). Following the open RFA procedure alone (ORFA), the number of CTCs significantly increased (p<0.05). Following surgical resection (either open or laparoscopic), the number of CTCs in the peripheral blood (PV) decreased, whereas it increased following ORFA as shown in Table 17 and Figure 23. There was a decrease in CTCs throughout the circulation following opRFALR and lapRFALR. It is interesting to note that there was no difference between these 2 subgroups although patient numbers and CTC numbers are small. Similarly, no patients underwent laparoscopic RFA alone to compare with those undergoing ORFA (in many of those amenable to laparoscopic RFA, percutaneous is a less invasive alternative and was not included in this pilot cohort).
Figure 23: Changes in peripheral venous blood (antecubital fossa cannulation) before and after surgery (open or laparoscopic) or RFA (open).

The median follow-up period here measured 154 days (range, 70 – 284 days). Although this was a short period of follow-up, two patients developed recurrent liver metastases after open liver resection at 3 and 6 months. Both of them received preoperative chemotherapy and they had a higher number of pre-operative PV CTCs before intervention compared with...
those without recurrence (7.0±1.4 vs 2.0±2.6, respectively), suggesting that CTCs may be used as a surrogate maker for determining who should have postoperative chemotherapy to prevent recurrence.

CTCs throughout the vasculature were stained for EGF-R as described previously (Payne, Yague et al. 2008). Within an individual, when CTCs were obtained from different sites before or after procedures, the percent that stained positive for the EGF-R was consistent, indicating that CTCs were biologically homogenous within a patient.

7.4 Discussion

In this study of a consecutive series of patients with colorectal liver metastases, we show, for the first time, the impact of RFALR or RF ablation the number of CTCs measured in both the systemic and portosystemic circulations. The results demonstrate that surgical resection immediately reduced the number of CTCs throughout the circulation compared with RFA, which was associated with an increased number of CTCs (Table 17 and Figure 23). The effect of RFALR on CTCs was unaffected by the type of resection, laparoscopic or open, although the sample size was small. To properly assess the effects of surgical approach on CTCs, a prospective randomized control trial of patients with disease amenable to laparoscopic RFALR is necessary.

This is the first study to examine the differential localization of CTCs in the vasculature, by measuring them at different sites in both the systemic circulation, measured by peripheral venous and arterial blood samples, and the portosystemic circulation, measured by portal and hepatic venous blood samples. Some of these procedures were difficult to undertake: the hepatic vein is ‘friable’ and obtaining blood samples directly is difficult requiring extensive mobilization of both the liver and vein. These data, however, convincingly show that CTCs are localized to the hepatic macrocirculation, whilst the lungs appear to ‘sieve’ CTCs and significantly fewer enter the peripheral circulation. While these effects may be dilutional in their origin and reflect a concentration gradient close to the main cancer bulk, this provides insights into the clinical picture observed in cancer, in which metastases in the limbs are seldom seen, compared to liver and lung secondaries. While the exact fate of
these CTCs remains unknown, it is clear that they play a role in cancer spread by acting as a tumour reservoir.

Despite years of research and hundreds of reports on tumour markers in oncology, the number of biomarkers that have emerged as clinically useful is very small with initial promise replaced by inconsistent data (McShane, Altman et al. 2005; McShane, Altman et al. 2006). The development of guidelines for the reporting of tumour marker studies will encourage transparent and complete reporting so that relevant information will be broadly available to others and conclusions can be objectively ascertained. We have attempted to biologically characterize CTCs here by measuring their gene expression but the amount of RNA obtained was insufficient for RT-PCR or expression microarray analyses. Further research into the molecular biology of CTCs, their membranous adhesion expression characteristics such as E-cadherin, and HIF expression will establish the processes by which they are shed and their role in tumour spread.
CHAPTER 8

DISCUSSION, CONCLUSIONS AND FUTURE WORK
8.1 Introduction

The boundaries of 21st century surgery including surgical skills, patient safety and surgical technologies, are dynamic. The abdomen, with its range of interesting and diverse pathologies, has always been a fascinating space to a general surgeon. When Phillipe Mouret, a French gynaecologist, successfully performed the first laparoscopic procedure, the world of surgery underwent a renaissance. Suddenly, general surgeons could circumvent the inevitable pain, stress and trauma of a laparotomy by using gas and a telescopic camera. This pivotal innovation firmly established laparoscopic surgery and, since then, there have been significant advances in the scope and applications of laparoscopy. In colorectal surgery, laparoscopic colectomies are well established. As the first 10 year results of randomized trials comparing laparoscopic and open colectomies become available, liver subspecialists have followed this trend and applied laparoscopic resection to liver cancer. The evaluation of laparoscopic resections however, is complex. There remain many aspects of the laparoscopic procedure itself which are not fully understood, such as the effect on the body of the pneumoperitoneum, the relevance of a particular gas and whether there is an optimum pressure. Furthermore, outcome is inextricably linked to surgical expertise and human error, and these must be factored in when comparing different techniques. There are also difficulties related to how accurately patient samples can be matched and how any compromise impacts on findings. The starting point for my research was a simple clinical observation: patients undergoing laparoscopic liver resection appeared to be presenting earlier with recurrent disease than those undergoing open surgery. This raised an important question: is laparoscopic liver resection a safe procedure? The limitations of a two year postgraduate research degree are such that it was not realistic or feasible to set up a “gold standard” trial. This would ideally involve a multicentred multinational database of liver surgery incorporating the blinded randomization of patients into surgical treatment groups carefully matched to minimise variables. All operating surgeons would be peer reviewed and match up to a minimum technical standard. Instead we analysed 10 early consecutive cases of laparoscopic liver resection for hepatic malignancy and matched them to patients who had undergone similar open hepatic resections for the same disease. We applied well established selection criteria for liver surgery: a controlled primary tumour, no extra hepatic metastases and a resection that is technically feasible with tumour-free margins (Hugh, Kinsella et al. 1997). The sample size of our study was small and it must therefore be qualified as a pilot study. I have presented the findings of our pilot study in Chapter 3. This
final Chapter offers a critical analysis of the study design and discuss the insights gained and lessons learnt in a broader clinical context.
8.2 Discussion

Case pathologies

The pathology distribution we observed was the following: colorectal liver metastases (N=7), hepatocellular carcinoma (N=2) and small cell lung carcinoma (N=1). This is broadly representative of modern liver resection surgery where HCC represents 90% of all primary liver malignancy (Kaczynski, Hansson et al. 1996). Of those eligible for surgical resection (as opposed to liver transplantation) more than 70% of patients have cirrhosis and 80% ASA >II often precluding laparoscopic resection at anywhere other than specialist laparoscopic centres (Dagher, Belli et al. 2010). More commonly, laparoscopic resection is performed for colorectal liver metastases. Of new cases, 20–25% of patients will have clinically detectable liver metastases at the time of the initial diagnosis and a further 40–50% of patients will eventually develop liver metastases after resection of the primary, most commonly within the first three years of follow up (Scheele, Stangl et al. 1990; Sugarbaker 1990; Stangl, Altendorf-Hofmann et al. 1994; Scheele, Stang et al. 1995). The liver is often the first site of metastatic disease and may be the only site of spread in as many as 30–40% of those with advanced disease. The inclusion of one case of non colorectal, non neuroendocrine (NCNNLM) metastasis is admittedly an over-representation. Of non-colorectal metastases, neuroendocrine tumours are the most common, thereafter patients with NCNNLM cases the incidence of Lung Ca metastases amenable to liver resection vary but are often as few as 3% of NCNNLM, (and by extrapolation a much lower proportion overall), (Ercolani, Ravaioli et al. 2006).

Single surgeon, single technique

Multicentre randomized control trials validated the use of laparoscopic colorectal resection by finding equivalent long-term oncological results to open resection (Guillou, Quirke et al. 2005). Early case series reporting higher rates of port site metastases and recurrence were attributed to technical issues and developing surgical skills in the context of a novel and technically complex laparoscopic procedure. We have reduced this operator bias by analyzing the results of a single operating surgeon. The surgeon in our study has extensive operative experience of open resection and laparoscopic procedures and was a member of the research team that developed the RF assisted technique and associated technology and resection tools. This degree of expertise allowed us to minimise the risk of human error as far as possible. Larger multicentre case series have recruited laparoscopic surgical experts.
on the basis of peer review of technique. This selection criterion however, introduces an element of human bias as there has been no international consensus statement identifying world authorities in the field until recently (Buell, Cherqui et al. 2009; Ercolani, Ravaioli et al. 2006). Throughout the duration of the pilot study the same equipment for resection was being used open and laparoscopically, Radionics Cooltip electrode. New parenchymal resection devices were introduced at a later stage and therefore do not represent a confounding factor in our study.

Matching criteria and compromise

In chapter 3 we highlighted the basic demographics of the 2 matched samples. These included: sex, age, tumour type, site and grade, number of lesions and size, procedure performed, resection margin positivity, operative time and inpatient stay. We chose tumour type, anatomical site and grade as our inclusion criteria when searching for open cases to match to our laparoscopic sample and used them in decreasing order of importance when ‘an exact’ match was not possible. There was no significant difference in demographics when comparing means. Further parameters which would be clinically relevant in future studies are comorbidities, ASA grade, alcohol history, Hep B and C status (especially in cases of HCC), treatment with neoadjuvant chemotherapy, synchronous/metachronous metastases, need for blood transfusion and post-operative complications. Although we did not specifically measure these criteria, no patients required intraoperative blood transfusion nor were there any post-operative surgical complications, such as biliary leak or abscess formation. This is important to note because of their effect on the accuracy of our measurement of inpatient stay.

By keeping matching criteria narrow in a retrospective study, we may be skewing our sample. For example, colorectal carcinomas that metastasise to the left lobe, (i.e.: those most amenable to laparoscopic liver resection) may in some way represent a pathologically discreet subset of colorectal carcinomas as a whole. Also, patients with isolated hepatic metastases have a better prognosis than those with more extensive metastatic disease (Goslin, Steele et al. 1982; Lahr, Soong et al. 1983; Stangl, Altendorf-Hofmann et al. 1994; Rougier, Milan et al. 1995) suggesting biological differences in the two settings, yet we did not match for number of lesions. While our matching criteria are clinically relevant to the study aims, they remain the result of an artificial selection. Another weakness specific to this study is where well founded assumptions are made, (laparoscopic surgery allows people return to work earlier). This is not something we could assess in a retrospective study.
However it is especially relevant when other assumed benefits of the laparoscopic group, such as shorter inpatient stay, are not supported by our data (Laurent, Cherqui et al. 2003; Lesurtel, Cherqui et al. 2003).

Operative time was measured from a surgical theatre database. It incorporates time to wake from anaesthetic postoperatively and therefore another variable, the effects of anaesthesia, is introduced. However, one may assume that this variable exists for both laparoscopic and open cases and therefore its impact is negligible. A longer operative time for laparoscopic resections has been previously shown (Rau, Buttler et al. 1998; Belli, Fantini et al. 2007). Indeed in a majority of published data, operative time was equivalent or longer. This finding however, must be closely examined in the context of another significant finding in our study: that R0 resections returned earlier than their open matched cases. Despite many of the limitations of our small scale pilot, we hypothesised that the pathophysiological process that might account for the difference is time dependent.

We found no difference in the resection margin positivity between the 2 groups, although we accept that 40% resection margin positivity is a very high finding in the context of open resection. There is a developing consensus worldwide that higher margin positivity in laparoscopic resection may be attributed to a technical issue (Buell, Cherqui et al. 2009). We generously gave ourselves >1mm for a negative resection margin. This is accepted as an adequate margin in RF ablation when a 2-cm-wide coagulative necrosis zone is created before division of the parenchyma with a surgical scalpel (Weber, Navarra et al. 2002), although alternative liver transection techniques might favour alternative resection margin classification. There are technical difficulties with the accurate measurement of resection margins following RF ablation. Firstly, RF-ablated tissue will not resemble normal tissue and characterizing whether there is a native benign or malignant tumour in the ablated tissue is impossible. Secondly, little is known about the degradation of RF-ablated tissue in formalin prior to paraffin fixation. Despite this, the pathologists involved in our study were clearly able to state whether the tumour extended to the edge of the resected specimen or not. In a context where ‘adequate’ resection margin is still a contentious issue, this is of paramount importance. The clinical relevance of resection margins is contentious. Data from the Registry of Hepatic Metastases, a multi-institutional database of liver resections, suggest that a margin >1cm is associated with a 45% five year survival rate, with only a 23% five year survival rate where margin were <1cm (Hughes, Simon et al. 1986). However, more recent reports comparing margins <1cm and >1cm conclude there is no significant difference in long term survival (Lordan and Karanjia 2010).
DISCUSSION, CONCLUSIONS AND FUTURE WORK

When looking at oncological outcome, we excluded all patients with positive resection margins, as it is universally recognized that survival is shortened. (Pawlik, Scoggins et al. 2005; Nuzzo, Giuliani et al. 2008). Some studies however, include positive resection margins in their analysis of oncological outcome and define ‘time to recurrence’ as the earliest radiologically detectable recurrence. We also relied on radiology post-operatively, but chose specifically to analyse the R0 subgroup. The inevitable problem with this criterion is that it only takes one case out of each matched pair to be resection margin positive and both cases are excluded. Hence, by the end of the pilot, we had only 5 pairs with R0 resections in both laparoscopic and their matched open. We found that at 2 years follow-up, laparoscopic mortality is nearly 40%, almost twice that in the open group. Furthermore, there was a significantly poorer outcome with those undergoing laparoscopic resection presenting with earlier disease recurrence (p<0.05).

Pilot study IHC Data

Earlier disease recurrence in the laparoscopic resections is an important finding and I looked at the role of the pneumoperitoneum. My working hypothesis was that the pneumoperitoneal environment is one of relative mucosal and visceral hypoxia. There are a number of studies, both in humans and animal models, assessing the impact of the increased intra-abdominal pressure, (IPP) caused by insufflations (Hashikura, Kawasaki et al. 1994; Eleftheriadis, Kotzampassi et al. 1996; Jakimowicz, Stultiens et al. 1998; Ishida, Murata et al. 2000). Under these conditions it is recognized that portal venous flow is significantly decreased and a relative hypoxia is conferred on the liver. In a retrospective study such as ours, the surgical settings cannot be altered and intra-abdominal pressure was a constant parameter with a maximum pressure of 14mmHg under CO\textsubscript{2} insufflation). In any event, the measurement of liver hypoxia in humans presents significant difficulties and actual O\textsubscript{2} probing is a technically challenging and invasive procedure requiring rigorous ethical scrutiny.

An alternative approach to the study of the effects of the pneumoperitoneum is through the analysis of cellular markers of hypoxia. This approach constitutes the basis of my research. We analysed resected tissue for markers, including CaIX, GLUT-1, HIF-1 and HIF-2. We also assessed CD10 positivity as this had already been postulated as a marker of metastatic disease. Within our small pilot sample, we demonstrated a significantly higher expression of the HIF-2 and CD10 within liver tissue resected laparoscopically.
DISCUSSION, CONCLUSIONS AND FUTURE WORK

HIF-1 vs HIF-2

The capacity for mammalian cells to adapt and survive at low oxygen tensions, hypoxia, is a prerequisite for successful development of oxygen-dependent multicellular organisms (Lofstedt, Fredlund et al. 2007). The backbone of this adaptive response is the stabilization of HIF-1 and HIF-2. In well-oxygenated cells and tissues these subunits are degraded via the proteosomal pathway described previously in Chapter 1. With oxygen shortage HIF-α subunits become transcriptionally active and bind to hypoxia response elements (HREs) of target genes. Similar to all mammalian tissues, solid tumours require oxygen to grow. However, the uncontrolled proliferation of tumour cells results in an imbalance between tumour cell oxygen consumption and tumour neoangiogenesis and overall, most tumours are hypoxic relative to the surrounding tissue (Lofstedt, Fredlund et al. 2007). This in turn means that most tumour cells have adapted to and can grow in a hypoxic environment. Furthermore, as part of their adaptation to hypoxic conditions they become proangiogenic and shift towards anaerobic metabolism. In doing so, the hypoxic stimulus is the main driving force behind vascularisation and it promotes the expression of genes associated with increased cell survival, metastasis and invasion (Lofstedt, Fredlund et al. 2007). Tumour cells also seem to lose specialist functions and dedifferentiate during hypoxia, as demonstrated earliest by neuroblastoma and breast carcinoma (Jogi, Ora et al. 2002; Helczynska, Kronblad et al. 2003). More recently, studies looking at EMT have suggested that this occurs in cervical, colon and other tumour types (Lofstedt, Fredlund et al. 2007).

The activation of HIFs frequently observed in tumours seems primarily to be the result of a physiological response and adaptation to oxygen shortage. However, a number of reports have shown that HIF-1α protein also accumulates in tumour cells as a consequence of growth factor signaling and increased HIF-1α translation (Gordan and Simon 2007). We can only speculate about the physiological relevance of this response, but one hypothesis is that growth factor stimulated HIF-1α translation reflects a process that occurs during normal development. Indeed the relationship between E-cadherin and HIF-1α in cervical stratified epithelium, as demonstrated in Chapter 5, may represent an example of how the homeostasis and migration of cells through the epithelium is dependent on increasing localized hypoxia, HIF-1 upregulation and the concurrent E-cadherin downregulation. It is now widely accepted that tumour cells activate and exploit the cellular machinery for adaptation to hypoxia by two different mechanisms and that this adaptation, irrespective of
whether it is driven by hypoxia or abnormal growth factor signaling results in a more aggressive phenotype (Lofstedt, Fredlund et al. 2007).

What makes HIF-2 different?

Despite pronounced similarities between HIF-1 and HIF-2 in structure, function and regulation, knock-out of either HIF-1a or HIF-2a clearly show that they are not redundant and that they serve different functions during development (Lofstedt, Fredlund et al. 2007). HIF-1 remains the most studied factor and is frequently activated by hypoxia and growth factor signaling. Research into prognostic markers for primary colon and hepatocellular carcinoma have identified HIF-1 as a poor prognostic marker (Dai, Gao et al. 2009; Rasheed, Harris et al. 2009). Tumour accumulation of HIF-2 has been less frequently investigated although its induction in hypoxia has been demonstrated *in vivo* and *in vitro* irrespective of the cell background (Wiesener, Jurgensen et al. 2003). An increasing number of reports suggest that HIF-2 is important in tumour progression. Certainly, HIF-2 was highly expressed in all our resected liver tissue, and significantly more so in the laparoscopic subgroup. In contrast, HIF-1 showed weak or no expression in our pilot, irrespective of method of tumour resection. We postulated that the environment in which we have demonstrated a significantly higher expression of HIF-2 is one of lower partial pressure of oxygen relative to those samples resected by open technique. Interestingly, this is not the first time contrasting expressions of the two isotypes have been demonstrated in tumour tissue. Over recent years the role of the HIF-2 isotype has increasingly been viewed as distinct from HIF-1 (Le Bras, Lionneton et al. 2007). A number of reports analyzing the genes that are regulated by hypoxia, by HIF-1 or HIF-2, have shown that HIF-1 is the main inducer of glycolytic genes expression and that it may be considered the main transcriptional regulator of the cell ‘hypoxic response’, while paradoxically HIF-2 only responsive genes are not all regulated by hypoxia (Hu, Wang et al. 2003; Sowter, Raval et al. 2003; Wang, Davis et al. 2005). Wiesener et al. have shown that both isotypes are induced by hypoxia. In the liver, for example, there was a clear zonal distribution with an increase of HIF signal on IHC towards the central vein. This matches the known decline in oxygen tensions from the periphery to the centre of the liver lobule in mice. The authors also used epithelial cell lines to demonstrate that in normoxic conditions HIF-2a protein was detected to a higher degree than HIF-1 and that exposure to mild hypoxia further accentuated this differential expression (Wiesener, Jurgensen et al. 2003). It is interesting to note that HIF-2 transcriptional activity is not strongly activated by hypoxia and may even
be repressed while HIF-1α transcriptional activity is strongly induced under such conditions (O'Rourke, Tian et al. 1999). There are two possible explanations for this. Firstly, 2 of the 3 prolyl hydroxylases responsible for HIF hydroxylation are inducible by hypoxia and this response could form the basis of a negative feedback loop (Epstein, Gleadle et al. 2001). Secondly, HIF-α transcriptional activity is oxygen-regulated independently of protein stability by a cofactor, factor inhibiting HIF (FIH) that works via the C terminal activation domain on HIF-1α. HIF-2a has a relatively higher resistance to this regulation under normoxic conditions relative to HIF-1 (Bracken, Fedele et al. 2006). In short, there is strong evidence that HIF activation in vivo is not invariably linked to a specific oxygen concentration in all cells. Rather, the cellular oxygen response operates at different levels of sensitivity in different cells and tissues and presumably can adapt to local oxygen tensions (Wiesener, Jurgensen et al. 2003). We therefore cannot interpret the expression of HIF-2 in both CLM and HCC as markers of hypoxia. It may be that hypoxia accounts for the significant difference in HIF-2 expression when compared to open resection tissue but in the absence of real-time oxygen monitoring, this remains speculation.

What is the role of HIF2 and what are its targets?

The contrasting levels of HIF-1 and HIF-2 expression in the resected liver tumours represent a significant finding (Stroka, Burkhardt et al. 2001). Stoka’s study of healthy mice inspiring different levels of oxygen showed that HIF-1a induction in normal liver tissue in mice is transient, required a more severe level of hypoxia and only lasted 3 hours, while HIF-2 expression was sustained for more than 6hrs (Stroka, Burkhardt et al. 2001). A molecular mechanism for this has recently been characterised (Uchida, Rossignol et al. 2004; Holmquist-Mengelbier, Fredlund et al. 2006; Luo, Zhong et al. 2010). This time dependent shift in relative expressions of HIF1:HIF2 may explain why little or no HIF-1 was evident in resected liver tissue and why we have demonstrated higher HIF-2 expression. In a retrospective study, such as this, one can only speculate on earlier expression profiles. Following Stoka’s model, it is plausible that HIF-1 may have been evident in the resected tissue. However, as both open and laparoscopic liver resections are lengthy procedures, lasting on average 3hr40mins and 5hours, respectively, HIF-1 would undergo degradation. Interestingly, we did demonstrate GLUT1 expression in the liver and genetic inactivation studies suggest this is predominantly dependent on endogenous HIF-1 (Sowter, Raval et al. 2003). Therefore there are valid reasons to infer that HIF-1 was induced in the first place.
DISCUSSION, CONCLUSIONS AND FUTURE WORK

It is also possible that our hypoxic stimulus may not amount to 'the severe' pro-HIF-1 hypoxia. However, many elements of the resection would generally be regarded as typically 'acute' HIF-1 type stimuli. For example, RF ablation generates heat, a noxa of inflammation, necrosis and, with every application of the radiofrequency probe, increasing devascularisation. Furthermore, the CO$_2$ pneumoperitoneum is known to precipitate a systemic acidosis in 50% of patients and this reinforces the notion that RF resection does induce pro-HIF-1 hypoxia.

An alternative explanation is that HIF-1 may never have been upregulated during the procedure and we are simply resecting a HIF-2 pathology subset indicative of established metastases. The HIF-1 response itself may, in fact, lead to metastasis through its effect on E-cadherin, either as a result of hypoxia or abnormal cell signaling, causing cell shedding and the resultant increase in the number of circulating tumour cells. It has been observed that HIF-1 levels peak at the invasive stage of invasive colon carcinogenesis (Simiantonaki, Taxiades et al. 2008). Thereafter, once shed, the CTCs may change either as a result of being in a bloodstream, (through the lungs and oxygenation) or by default, as they are no longer in a solid tumour mass and therefore no longer in competition for oxygen delivery and relative hypoxia. Consistent with these differential characteristics, Talks et al. showed that HIF-1 protein expression appeared to be more restricted to perinecrotic tumour regions than HIF-2, whereas HIF-2 was more prominent in stromal cells around the tumour (Talks, Turley et al. 2000; Onita, Ji et al. 2002).

HIF-1 and HIF-2 both exert adaptive responses to hypoxia by activating transcription of overlapping, but distinct, batteries of target genes, thereby playing distinct roles in many developmental, physiological, and pathological processes (Hu, Sataur et al. 2007; Mastrogiannaki, Matak et al. 2009; Semenza 2009). One study has shown that when incubating PBLs from 10 healthy humans, significant differences exist between individuals in baseline HIF-regulated gene expression, and that there were also significant differences between individuals in the degree to which these genes are regulated by hypoxia (Brooks, Elvidge et al. 2009). In contrast to basal expression levels, there was a common pattern across the genes for the induction by hypoxia, such that if one gene was strongly induced by hypoxia in an individual then this would tend to be the case for all the other genes within that individual (Brooks, Elvidge et al. 2009). This finding is particularly relevant to our study because it means that while our choice of HIF target genes is limited, it can nevertheless be regarded as representative of HIF regulated transcription. Differences in the amino-terminal
DISCUSSION, CONCLUSIONS AND FUTURE WORK

transactivation domains of HIF-1α and HIF-2α are responsible for target gene specificity. The theory that HIF-2 regulates a different set of genes than HIF-1 is clearly emerging. It must be noted that we skewed our HIF target gene profile to HIF-1 related targets and, in view of our findings, including more HIF-2 target genes would have been wise. It must be noted however, that HIF-2 targets in the liver are not yet fully characterized, (Talks, Turley et al. 2000). As a prognostic marker, high HIF-2a expression relates to an advanced stage and/or poor patient outcome in several tumour forms including breast, non small cell lung cancer and colorectal cancer (Lofstedt, Fredlund et al. 2007; Helczynska, Larsson et al. 2008). Raval et al. used the same VHL and VHL deficient renal cell carcinomas cell lines as I did to demonstrate that HIF-1 positively regulated Bnip3 (no effect on cyclin D1, TGF-a, VEGF) and HIF-2 positively regulated cyclinD1, TGFA and VEGF (Raval, Lau et al. 2005). Another HIF-specific target gene, VE-cadherin gene, is not responsive to hypoxia however HIF-2 specifically activates its promoter while HIF-1 does not. Interestingly, 3 other endothelial cell markers (Tie-2, flk-1, eNOS) are all HIF-2 specific target genes. This mechanism may be involved in the in the process of vascular mimicry by tumour cells, and may suggest a mechanism by which CTCs are able to bind to new sites in endothelia, for example, in liver venules when shed as CTCs from primary colorectal cancer (Le Bras, Lionneton et al. 2007). Certainly, CTCs show altered response to hypoxia both in vitro and in vivo (Ameri, Luong et al. 2010). The importance of HIF-2 expression in colorectal liver metastases and hepatocellular carcinoma both clinically and in terms of therapeutic implications is dependent to an extent on the downstream target genes of HIF-2 in this setting. Given the complexity of the HIF transcriptional cascade and the large number of direct and indirect HIF targets identified in gene expression arrays, such an analysis is a formidable task (Jiang, Lu et al. 2002; Zatyka, da Silva et al. 2002; Hu, Wang et al. 2003). Certainly of the potential HIF targets we investigated, genetic inactivation studies suggest that, outwith the setting of renal cell carcinoma, GLUT1 is predominantly dependent on HIF-1α (Sowter, Raval et al. 2003). The case of cyclin D1 is less clear. Cyclin D1 is one of the main G1-phase cyclins and its expression is associated with G1 to S phase transition in the cell cycle and is upregulated in many types of cancers (Sherr 1996). In VHL deficient RCC it is a HIF-2 target and its HIF-dependent expression in other organs is as yet unclear. In contrast to the high levels of expression of HIF-2 in the resected liver samples, cyclin D1 demonstrated low or no levels of expression. Similarly, colorectal adenocarcinoma with CD10 expression are at increased risk of liver metastasis (Yao, Takata et al. 2002) and for this reason we included this in our IHC study in liver. We used the same in vitro well-established methodology that has led to much of our understanding of the roles of HIF-1α.
and 2α on target genes albeit in VHL+/- RCC cell lines to show that its expression correlates with both hypoxia and HIF-1 expression (Raval, Lau et al. 2005). Unfortunately, we cannot draw any conclusions from the IHC data, presented in Chapter 4, on HIF or hypoxia dependent expression of CD10 or its role in colorectal carcinogenesis. Nonetheless a HIF dependent control over CD10, a member of the MMP family, is plausible, as other members of the same family, such as MMP9, promote key processes underlying tumor cell propagation, from migration and invasion to angiogenesis and tumor growth (Coussens, Fingleton et al. 2002) and key downstream attributes of the HIF, more specifically HIF-1, mediated adaptive response.

The role of CTCs?

In Chapter 7 we prospectively analysed the effects of laparoscopic and open RFALR on circulating colorectal tumour cells to assess whether there is a fundamental difference in tumour behaviour when resected using different techniques. The pneumoperitoneum and laparoscopic set-up were the same as in the pilot study. We also compared RFALR to the effect of RFA alone, without resection. This line of investigation was prompted by the observation that patients undergoing laparoscopic resection present earlier with disease recurrence and by the fact that CTCs are known to have prognostic significance. The methodology for CTC measurement has recently been validated. Although the numbers of patients recruited were small, 11 and 4 for open and laparoscopic resection, respectively, results showed that the level of CTCs was significantly lower following resection, independently of whether this was open or laparoscopic. Interestingly, RFA treatment of the tumour led to significantly higher levels of CTCs. In the context of our earlier findings, one can hypothesise the following: the lapRFALR has equivalent shedding of cells compared to open and poor oncological outcome is an unrelated phenomenon; alternatively, the poorer outcome in the R0 resection resulted from the probes passing through tumour tissue during resection, despite clear margins; or, and this is the most likely explanation, oncological outcome is multifactorial and the sequestration of the mobile pool of CTCs to the liver microcirculation is at least in part, a hypoxia driven process.
8.3 Conclusions

This thesis, commenced in October 2005, stems from a surgical trainee’s interest in the applications of laparoscopic surgery to the treatment of liver cancer. The data and analysis from Chapters 3 to 6 were completed in October 2007. Chapter 8 and the overall critical analysis and discussion were completed as I returned to my surgical training. In November 2009, one year after a consensus conference convened in Louisville, USA, an article was published in The Annals of Surgery entitled “The International Position on Laparoscopic Liver Surgery” (Buell, Cherqui et al. 2009). In summarizing its background and objectives, this article mirrors some of the important points raised in my introduction about the role of laparoscopic liver surgery in the 21st century and also about the limitations that inevitably befall upon a small academic pilot study in what is still recognised as a contentious area of clinical practice. For example, it recognizes that multiple series have reported on the safety and efficacy of laparoscopic liver surgery and its application to major cancer resections in specialist centres without any published randomised control trials comparing it to the more established open resection procedures. Appraising this paper and grasping its findings and recommendations is of paramount importance, not only in the context of clinical research in this field but also to any aspiring academic HPB surgical trainee.

It is important to note that an organizing committee ‘selected 45 recognised experts from around the world with the most extensive published experience in both laparoscopic and open liver surgery’. The committee members’ details are not disclosed, nor the criteria defining “extensive publication”. This is tantamount to an admission of publication bias, suggesting that those who are the most prolific at publishing within the field of liver surgery are those most technically competent and thereby ‘experts’ in the field. Furthermore, 17 of the 45 experts were based at US institutions. This raises the question of whether equally competent liver surgeons, whose native language is not English, are underrepresented because they have fewer publications. Nevertheless, there will inevitably be some correlation between expertise and a strong track record of publications as surgeons with a busy clinical practice are more likely to produce significant data. Furthermore, it should be noted that of the 17 experts recruited from US institutions not all are of US (or primarily English speaking) origins.
DISCUSSION, CONCLUSIONS AND FUTURE WORK

The paper identifies different liver parenchymal transection techniques and concludes that to date ‘no single method has been shown to be superior’. When reviewing safety and efficacy of the laparoscopic approach, it was recognised that publication bias and ‘failure to publish negative events might have contributed to an overly optimistic view of the procedure’. The scope of what is deemed safe in terms of liver volume is 1: left lateral sectionectomy, 2: anterior segmentectomies or 3: wedge resections, (ideally on solitary lesions less than 5cm in maximum diameter in peripheral segments 2-6). In this way, any comparative trial or retrospective analysis hereon would, in a similar way to our pilot, involve comparing non-anatomical resections for which each surgeon will have a preferred parenchymal resection tool. Finally, in the context of patient safety, it was acknowledged that where there is ‘lack of case progress’ conversion to open resection should be performed. However, the paper stopped short of suggesting target resection times in either laparoscopic or open procedures.

When assessing the respective roles in CLM resection vs HCC, there are significant differences. For example, data support the resection of small HCCs laparoscopically in terms of preventing postoperative ascites, yet anatomical resections (technically challenging laparoscopically) are associated with less recurrence in this pathology. Furthermore, the role of radiofrequency ablation is advocated in small HCCs but not recommended in equivalent sized CLMs, due to the high recurrence rates. This may reflect the generally higher ASA for those with HCC or the recognised alternative end-stage treatment of liver transplantation. So despite evidence presented at the conference suggesting similar low resection margin positivity in laparoscopic and open surgery at one centre, it remains that ‘there are currently no trials that clearly demonstrate that laparoscopic hepatic resections have equivalent long-term outcomes to open hepatic resection’. Explanations for this conclusion may be that the negative resection margin rates of open surgery cannot be matched and that small metastases could be missed laparoscopically. It is of course plausible that other specialist centres that have not published their data have found similar results to ours in terms of our high resection margin positivity and earlier recurrence after laparoscopic resection. Furthermore, the passage of the radiofrequency probe during RFALR, as shown in Chapter 8, may account for increased CTC shedding from missed small metastases and the significantly earlier recurrence rate we identified in our R0 group.
The role of a Randomised Control Trial?

Many minimally invasive procedures share common benefits, problems and questions. The Louisville conference examined the North American laparoscopic vs open primary colonic resection trial, which took 10 years to complete, and applied the model to hepatic resection. However, the primary end-point must be carefully considered. If it is patient safety, with 1-2% mortality from liver resection, a substantial trial would be required to find any significant evidence. If the endpoint is oncological outcome then the different pathologies, HCC or CLM, would have to be identified. As both have a lower incidence compared to primary colonic carcinoma, any meaningful results would require over 10 years of follow-up. In short, the paper recommends a prospective centralized registry. This would require broad surgeon participation but would also allow the controlled introduction of the technique, minimising the learning curve effect and enabling the detection of severe negative events. Interestingly, conference experts argue that another trial showing that minimally invasive surgery has benefits on pain control and recovery time is unnecessary. While they acknowledge that the safety of laparoscopic liver surgery remains a valid question, their assertion that there is no need to ‘reproduce the (colon) trial to determine whether laparoscopy per se has a negative effect on oncologic outcome and tumour seeding to incisions’, is questionable. We have shown that laparoscopically resected tissue expresses different cellular markers and may thus be more sensitive or primed to different stimuli within the pneumoperitoneal environment compared to openly resected liver cancer.

While this publication does not support the setting up of RCTs, it advocates the use of liver resection registries and it is clear that future clinical or cellular applied laboratory research in this field will be dependent on these registries and this will involve academic-clinician cooperation, multicentre networking and pooling of data and time.

Summary of conclusions:
1: The role of laparoscopic liver resection is still under review and our data have shown that it involves a longer operative time and a poorer oncological outcome after RO resection for colorectal liver metastases when compared to open surgery.
2: HIF-2 and CD10 may have significant physiological roles under laparoscopic conditions in conferring a poorer prognosis on those patients under resection for colorectal liver metastases, although more research is required to further characterise these markers.
3: HIF-1 and HIF-2 levels are inversely related to E-Cadherin expression in a cervical stratified epithelial model of dysplasia-carcinoma. Further work is required before similar data can be compiled on this inverse relationship in colorectal carcinoma.

4: CD10 may represent a novel hypoxia inducible target in an in vitro clear cell renal carcinoma model regularly used to investigate HIF-target genes. Although clinical data exist suggesting CD10 as a poor prognostic marker and predictor of metastatic disease in primary colorectal carcinoma, more in vitro and prospective clinical trials are required before its role in colorectal carcinoma can be characterised.

5: Radiofrequency ablation causes significant shedding of circulating tumour cells systemically; that is not evident when it is used for liver resection. Hence its role in non resectable liver metastases remains under investigation and review in clinical practice.

6: The future of laparoscopic liver resection lies in international collaboration and prospective collection of liver resection data in secure databases. A randomised control trial is unlikely to yield meaningful and contemporary data that will alter future practice.
8.4 Future Work

My future work will focus on the following areas:

1: HIF-1 to HIF-2 switch
I would like to analyse tissue bank samples of excised precancerous polyps and colorectal carcinoma from patients with accelerated monitored disease for HIF-1, HIF-2 and other potential prognostic markers, including CD10 and other MMPs. I would also investigate E-Cadherin expression in these samples. Key questions are whether there is a change in malignant potential when HIF-2 becomes upregulated and if so, whether this is mediated by MMPs, specifically CD-10. Also, whether cell shedding is a function of downregulation of E-cadherin by HIF-1 or HIF-2 in primary colonic carcinoma.

2: The pneumoperitoneum
I would like to apply for the ethical approval to biopsy human liver under the effects of pneumoperitoneum at different time points and pressures under O\textsubscript{2} probe monitoring of pO\textsubscript{2} of portal vein and hepatic artery. I would prospectively collect samples and snap freeze them in liquid nitrogen to gain a time dependent spectrum of HIF-1/2 nuclear localisation and upregulation.

3: Differing effect of RF on normal liver (control), HCC and CLM
I would like to set up a metabonomic database on patients undergoing radiofrequency ablation of HCC and CLM to investigate whether there is a different inflammatory response and identify the relevant inflammatory markers.

4. CTCs
I would like to isolate CTCs from primary and metastatic colorectal cancer to see whether they have differing responses to hypoxia. In particular, I would look at whether colorectal CTCs primarily target the liver and those that metastasise beyond or to different organs using different stimuli in their propagation.

5. The effect of RF on liver cell biology, architecture and the process of coagulative necrosis.
Using standardised settings, I would like to sample liver tissue at different times of exposure and different radial distances from the RF probe in order to assess whether there is time for cellular adaptation to the stimulus. I would seek to establish whether RF is a
secondary hypoxic stimulus, distinct from the devascularising effect of liver parenchymal resection.
REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


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REFERENCES


APPENDIX I

PUBLICATIONS ARISING FROM THIS THESIS
APPENDIX I: PUBLICATIONS ARISING FROM THIS THESIS


APPENDIX II

ABBREVIATIONS
APPENDIX II: ABBREVIATIONS

ABC kit aidin-biotin-peroxidase complex kit
ADP adenosine diphosphate
ASA American Society of Aesthesiologists
ATP adenosine triphosphate
bp base pairs
CAIX Carbonic Anhydrase IX
CALLA Common Acute Lymphoblastic Leukaemia Antigen
CCRCC clear cell renal cell carcinoma
CD10 Cluster of Differentiation molecule 10
cDNA complementary DNA
CLM colorectal liver metastasis
CO2 carbon dioxide
cRNA complementary RNA
CSA catalysed signal amplification
CT computerised tomography
CTC circulating tumour cell
DAB diaminobenzidine tetrahydrochloride
DAPI 4′,6-diamidino-2-phenylindole·2HCl
dFO desferrioxamine
dH2O deionised water
DMSO dimethyl sulphoxide
DNA deoxyribonucleic acid
dsDNA double stranded DNA
E-cad E-cadherin
ECL enhanced chemiluminescence
EMT Epithelial-Mesenchymal Transition
EORTC European Organisation for Research and Treatment of Cancer
EPAS Endothelial PAS domain protein 1
FCS foetal calf serum
Fe Iron
FIH Factor Inhibiting HIF
G6P glucose-6-phosphate
GAPDH glyceraldehyde 3-phosphate dehydrogenase
GFP green fluorescent protein
GLUT-1 Glucose transporter 1
### APPENDIX II: ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>GLUT-2</td>
<td>Glucose transporter 2</td>
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<tr>
<td>HBS</td>
<td>HIF Binding Site</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B Virus</td>
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<tr>
<td>HCC</td>
<td>hepatocellular carcinoma</td>
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<td>HCl</td>
<td>hydrochloric acid</td>
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<tr>
<td>HCV</td>
<td>Hepatitis C Virus</td>
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<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
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<tr>
<td>HIER</td>
<td>heat induced epitope retrieval</td>
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<td>HIF-1</td>
<td>Hypoxia inducible factor-1 αβ polymer</td>
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<tr>
<td>HIF-2</td>
<td>Hypoxia inducible factor-2 αβ polymer</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Hypoxia inducible factor -1α</td>
</tr>
<tr>
<td>HIF-2α</td>
<td>Hypoxia inducible factor -2α</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia Inducible Factor</td>
</tr>
<tr>
<td>HLF</td>
<td>hepatic leukemia factor</td>
</tr>
<tr>
<td>HPB</td>
<td>hepatopancreaticobiliary</td>
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<tr>
<td>HRE</td>
<td>Hypoxia Response Element</td>
</tr>
<tr>
<td>HV</td>
<td>hepatic vein</td>
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<tr>
<td>IGF</td>
<td>Insulin Growth Factor</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>IOUS</td>
<td>intraoperative ultrasound</td>
</tr>
<tr>
<td>IPP</td>
<td>intra peritoneal pressure</td>
</tr>
<tr>
<td>KM</td>
<td>Michaelis Constant</td>
</tr>
<tr>
<td>LDHA</td>
<td>Lactate Dehydrogenase A</td>
</tr>
<tr>
<td>LOX</td>
<td>Lysyl oxidase</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MCL-1</td>
<td>myeloid leukemia cell differentiation protein</td>
</tr>
<tr>
<td>MCT</td>
<td>Monocarboxylase Transporter</td>
</tr>
<tr>
<td>Mins</td>
<td>Minutes</td>
</tr>
<tr>
<td>MMOG</td>
<td>Dimethyloxalylglycine (also abbreviated to DMOG)</td>
</tr>
<tr>
<td>MMP2</td>
<td>matrix metalloprotease 2</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MR(I)</td>
<td>magnetic resonance (imaging)</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>N</td>
<td>number</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>N₂</td>
<td>nitrogen</td>
</tr>
<tr>
<td>NEP</td>
<td>neutral endopeptidase enzyme</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>NHE-1</td>
<td>Sodium Hydrogen Exchanger</td>
</tr>
<tr>
<td>NS</td>
<td>not significant</td>
</tr>
<tr>
<td>NSCLCs</td>
<td>non small cell lung carcinomas</td>
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<tr>
<td>O₂</td>
<td>oxygen</td>
</tr>
<tr>
<td>OLT</td>
<td>Orth</td>
</tr>
<tr>
<td>ORFA</td>
<td>open radiofrequency ablation</td>
</tr>
<tr>
<td>P53</td>
<td>protein 53</td>
</tr>
<tr>
<td>PBL</td>
<td>peripheral blood leukocyte</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>PBS-Tween</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDH</td>
<td>pyruvate dehydrogenase</td>
</tr>
<tr>
<td>PEI</td>
<td>percutaneous ethanol injection</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PHD</td>
<td>Prolyl Hydroxylase Domain</td>
</tr>
<tr>
<td>PHD2</td>
<td>Prolyl hydroxylase 2</td>
</tr>
<tr>
<td>PHD3</td>
<td>Prolyl hydroxylase 3</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositol 3-kinase</td>
</tr>
<tr>
<td>POSSUM</td>
<td>Physiological and Operative Severity Score for the enUmeration of Mortality and Morbidity</td>
</tr>
<tr>
<td>PoV</td>
<td>portal vein</td>
</tr>
<tr>
<td>PV</td>
<td>peripheral vein</td>
</tr>
<tr>
<td>pVHL</td>
<td>VHL protein</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real-time PCR</td>
</tr>
<tr>
<td>R0</td>
<td>complete resection with no microscopic residual tumour</td>
</tr>
<tr>
<td>R1</td>
<td>complete resection with microscopic residual tumour</td>
</tr>
<tr>
<td>RF</td>
<td>radiofrequency</td>
</tr>
<tr>
<td>RFALR</td>
<td>radiofrequency assisted liver resection</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RPM</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RT</td>
<td>retrotranscription transcriptase</td>
</tr>
<tr>
<td>Secs</td>
<td>seconds</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>siRNA</td>
<td>short inhibitory RNA</td>
</tr>
<tr>
<td>TACE</td>
<td>Transcatheter Arterial Chemoembolisation</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>TBS-Tween</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic Acid</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TGFA</td>
<td>Tumour Growth Factor Alpha</td>
</tr>
<tr>
<td>TLR4</td>
<td>Toll-like Receptor 4</td>
</tr>
<tr>
<td>TRS</td>
<td>target retrieval solution</td>
</tr>
<tr>
<td>US</td>
<td>ultrasound</td>
</tr>
<tr>
<td>3’UTR</td>
<td>3’ untranslated region</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VHL</td>
<td>VHL gene</td>
</tr>
<tr>
<td>Wt</td>
<td>Wild-type</td>
</tr>
</tbody>
</table>
Firstly, I would like to thank Mr Long Jiao, my supervisor for his invaluable support and calm, assured guidance throughout my project, making my time in research enjoyable and without whom, this work would not have been possible. I trust his judgment implicitly and he will remain my mentor in the world of HPB surgery.

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