‘Cholangiocarcinoma: Validation of Surgical Selection and Prognostic Methodologies’

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Medicine by Mr Nicholas Thomas Edward Bird (FRCS; MBBS; MA).

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LIST OF PUBLICATIONS FROM THESIS:

[6] Bird, N., et al., *Role of staging laparoscopy in the stratification of patients with perihilar cholangiocarcinoma.* Br J Surg, 2016.

[7] Bird, N., et al., *Role of a pre-operative radiological scoring system in determining resectability for potentially resectable hilar cholangiocarcinoma.* Eur J Surg Oncol, 2018.

[8] Bird, N.T.E., et al., *Evaluation of the utility of prognostic models for patients with resected hilar cholangiocarcinoma.* HPB (Oxford), 2019.

[9] Bird, N.T.E., et al., *Meta-analysis of prognostic factors for overall survival in patients with resected hilar cholangiocarcinoma.* Br J Surg, 2018.

[10] Jones, R.P., et al., *Prognostic molecular markers in resected extrahepatic biliary tract cancers; a systematic review and meta-analysis of immunohistochemically detected biomarkers.* Biomark Med, 2015.

[11] Bird, N.T., et al., *Immunohistochemical hENT1 expression as a prognostic biomarker in patients with resected pancreatic ductal adenocarcinoma undergoing adjuvant gemcitabine-based chemotherapy.* Br J Surg, 2017.

THESIS; A Reader

This thesis is reflective of research undertaken, during a period of 'Out of Programme' work from Higher Surgical Training, between August 2015 and February 2017.

The Hepato-Biliary research group led by Mr. Hassan Malik, Mr. Stephen Fenwick, Professor Graeme Poston and Professor Daniel Palmer, have been clinically involved in surgically selecting, resecting and palliatively treating cholangiocarcinoma patients at Aintree University Hospital since 2008. Surgical management of cholangiocarcinoma has only become a viable curative methodology over the last 2 decades. This is primarily due to the advancements in peri-operative supportive management. However, concomitant improvements in surgical technology, data management and intensive care facilities have contributed to the viability of surgery.

Despite the increasing viability of curative treatment of cholangiocarcinoma there is little known regarding the patient/case characteristics which enable safe and appropriate treatment allocation. Relatively little is known about prognostic factors which accurately stratify patients and enable the strategic planning of safe and effective management of cholangiocarcinoma patients. Traditional approaches to prognosis for patients following resection for cholangiocarcinoma has been to utilise the American Joint Committee on Cancer (AJCC) to determine survival and plan adjunctive multimodality treatment. This dogmatic approach to patient prognostication has yet to be questioned in the literature.

This thesis attempts to address some of the areas of concern regarding surgical selection and prognostication. The *Introduction* focuses on detailing the current landscape of knowledge regarding cholangiocarcinoma as a disease entity. The majority of the resources included in the *Introduction* are current publications acquired and assessed during the period of research. The *Introduction* ranges from current epidemiological considerations to conceptions of surgical resectability and prognosis. The focus of the research assessed is on peri-hilar and intrahepatic cholangiocarcinoma. This focus reflects the patient population analysed and described in the thesis. The Liverpool University Foundation Trust Liver Unit at University Hospital Aintree (the clinical research group within which the research has been undertaken) manages and cares for patients with peri-hilar and peripheral cholangiocarcinoma primarily due to the referral pathway and expertise contained within the Multidiscipilinary Team (MDT).

The '*Clinical*' chapter focuses upon assessment of the clinical cohort developed from the clinical work undertaken by the Hepato-Biliary group over the last 11 years. The clinical chapter focuses on primarily peri-hilar cholangiocarcinoma and intrahepatic (peripheral) cholangiocarcinoma. In this chapter assessment of the surgical selection techniques utilised by the group to determine resectability is undertaken. Section 2.1 addresses the utility of staging laparoscopy in stratifying patients for resection while Section 2.2 assesses the

validity of current predictive resectability scoring systems. Section 2.3 quantitatively meta-analyses survival characteristics of globally published cohorts to determine if there are objective covariates, which are not accounted for in the AJCC staging system, and which can help explain prognosis. Section 2.4 utilises the clinical cohort to validate the utility of traditional and putative prognostic systems.

The *Basic Science* chapter focuses upon determining a suitable biological tumour prognostic factor for cholangiocarcinoma patients. Section 3.1 quantitatively assesses and systematically reviews the current literature regarding molecular prognostic factors in biliary tract cancer. Section 3.2 quantitatively assesses and systematically reviews a specific molecular marker, hENT1, and its utility as a prognostic marker for response to gemcitabine chemotherapy in resected pancreatic ductal adenocarcinoma (PDAC) patients. PDAC is a biologically aggressive cancer from the hepato-pancreatico-biliary tree with comparative survival characteristics to cholangiocarcinoma. Pancreatic and biliary cells share a common stem-cell progenitor and have similar, but not identical, molecular markers and in vitro characteristics. Accordingly, the chemotherapeutic regimes of neoplastic conditions of both diseases are to a certain degree analogous. Prognostic markers which are have utility in PDAC may also be demonstrated to be prognostic in cholangiocarcinoma. Section 3.3 discusses the construction of a Tissue Matched Array (TMA) of 111 patient samples for immunohistochemical assessment. Through the meta-analytical approach in the previous two sub-sections a set of molecular markers and techniques have been identified to be used on the TMA's for assessment of utility for prognosis.

This research encompasses both clinical and scientific methodologies to assess and further the current knowledge base regarding prognosis for patients with cholangiocarcinoma.

THESIS ABSTRACT

Background:

Cholangiocarcinoma is a rare cancer with a poor prognosis. Radical surgical resection is the only option for curative treatment. Optimal determination of resectability is required so that patients can be stratified into operative or chemotherapeutic treatment cohorts. This thesis sought to validate and augment contemporaneous resectability systems in a large independent European validation cohort. Numerous putative prognostic histo-pathological and demographic characteristics have been reported to effect Overall Survival (OS). This thesis sought to validate a variety of histopathological, clinical and radiological systems and to determine the clinical prognostic utility of these systems. Improved prognostication through biomarkers has been suggested, and direct analysis of tumour may allow the development of a more personalised therapeutic approach. This thesis sought to define the utility of 2 potential prognostic biomarkers, hENT1 and Ki67, via direct immunohistochemical analysis of resected and biopsied patient specimens.

Methods:

Standardised meta-analytical methods were utilised to stratify clinical and biomarker prognostic co-variates. These clinical and biomarker co-variates were then assessed and validated within the context of a large, non-continuous, European, contemporaneous registry of surgically resected cholangiocarcinoma patients at Aintree University Hospital between June 2006 – February 2017. Forty-four resected patient's specimens and 58 non-matched biopsy specimens were acquired from CellNass. Two Tissue Matched Array's (TMA's) were constructed and immunohistochemical assessment of hENT1 and Ki67 abundance was undertaken.

Results:

Regression analyses identified that BC score, MSKCC score, age at diagnosis and left artery involvement were all significant independent predictor's of resectability. The meta-analysis highlighted the significance of clinical prognostic variables affecting OS. The significant prognostic factors which had an effect upon OS were; 'T' status, lymph node involvement, microvascular invasion, peri-neural invasion, tumour differentiation and age. Numerous pre and post-operative co-variates retained prognostic utility when assessed within the validation cohort. Meta-analytical methods demonstrated that Ki67 and hENT1 biomarkers had significant prognostic effects for immunohistochemically assessed patients. Immunohistochemical assessment of the TMA specimens was undertaken. hENT1 and Ki67 abundance did not demonstrate significant survival correlates. However, an alternative commercially available hENT1 antibody was determined to demonstrate selectivity and utility in accurately assessing hENT1 abundance in resected peri-hilar cholangiocarcinoma specimens.

Conclusion:

This thesis externally validated the utility of standardised scoring systems for pre-operatively stratifying patients for potential resection. It has also provided a potential novel anatomical co-variate which could be used to augment scoring systems to increase predictive accuracy. This thesis validated standardised clinical prognostic systems and provided novel and augmented alternative systems which explained variability in OS in the validation cohort. This thesis has validated an alternative commercially available hENT1 antibody which can accurately determine hENT1 abundance in resected peri-hilar cholangiocarcinoma specimens.

INTRODUCTION

1.) Epidemiology of Cholangiocarcinoma

1.1) United Kingdom Epidemiology

Cholangiocarcinoma is a rare cancer arising from the epithelial and peri-biliary gland cells lining the biliary ducts and radicals. The National Cancer Intelligence Network (NCIN) published the *Rare and Less Common Cancers* statement in June 2015 which reported the incidence and mortality rates for all cancers diagnosed in the United Kingdom (U.K.) between 2010 – 2013 with an incidence of less than 6 per 100, 000. There were 7606 cases of cholangiocarcinoma and biliary tract cancers with an incident rate of 3.58 per 100, 000 (See Table 1) [12].

Table 1: Hepato-Pancreatico-Biliary Tract Cancer Incidence Rate U.K. [12]

	Cancer site 2010 to 2013	Number of incident cases	Incidence rate per 100, 000 population
Upper GI	Ampulla of Vater	1,569	0.74
	Biliary tract cancer (or cholangiocarcinoma or bile duct cancer (intra- or extrahepatic))	7,606	3.58
	Duodenal cancer	1,700	0.80
	Gallbladder cancer	2,714	1.28
	Pancreas	29,892	14.05
	Primary liver (excluding intrahepatic bile duct cancer)	9,902	4.66

The mortality rate for cholangiocarcinoma and biliary tract cancers in the report was discovered to be 3.64 per 100, 000. The high rate of mortality was attributed to historical cases diagnosed prior to 2010 dying from the disease after the initiation of the collation of data for the report (See Table 2).

Table 2: Hepato-Pancreatico-Biliary Tract Cancer Mortality Rate U.K. [12]

	Cancer site 2010 to 2013	Number of deaths	Mortality rate per 100, 000 population
Upper GI	Ampulla of Vater	352	0.17
	Biliary tract cancer (or cholangiocarcinoma or bile duct cancer (intra- or extrahepatic))	7,743	3.64
	Duodenal cancer	912	0.43
	Gallbladder cancer	1,698	0.80
	Pancreas	27,869	13.10
	Primary liver (excluding intrahepatic bile duct cancer)	6,899	3.24

While it is the 3rd most common hepato-biliary (HPB) malignancy, accounting for between 10 – 15 % of all HPB cancers, it represents only approximately 2 % of all cancer diagnoses in the United Kingdom. However, the data from the *Rare and Less Common Cancers* report demonstrated a substantial increase from the previously reported incident rates from the U.K. which were approximately between 1 – 2 per 100, 000 head of population. Taylor-Robinson *et al* [13], in 2001, demonstrated an increase in all primary liver tumours, with particularly intrahepatic cholangiocarcinoma incidence increasing. Part of the increased incidence was attributed to improved radiological ascertainment of disease. However, it was determined that the increase appeared to occur before the advent of computed tomography (CT) and endoscopic retrograde cholangiopancreatography (ERCP). West *et al* [14] supported the findings of Taylor-Robinson *et al* in a 2006 analysis of the Office of National Statistics cancer diagnostic registry database. The authors noted that particularly intrahepatic cholangiocarcinoma incidence had dramatically increased, with a 1200 % rise in incidence between 1971 – 2001 (See Table 3).

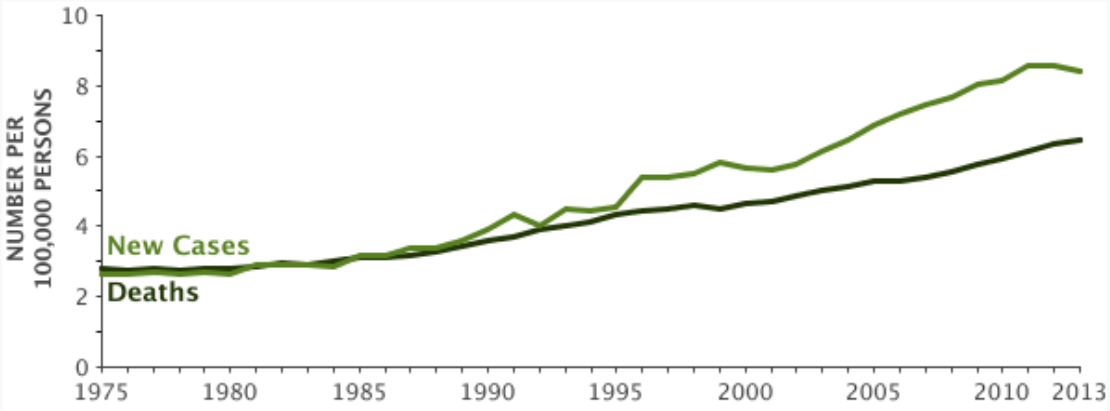
Table 3: Variance of Incidence of Hepato-Biliary Cancers by Sub-site between 1971- 2001 in the U.K.

	Males				Females			
Subsite	**1971 –73**	**1999 –2001**	**Rate ratio**	**95% CI**	**1971 –73**	**1999 –2001**	**Rate ratio**	**95% CI**
Liver, gallbladder and biliary tract (total)	3.93	6.13	1.56	1.46–1.66	3.06	3.89	1.27	1.18–1.37
Liver cell cancer	1.84	2.68	1.46	1.31–1.60	0.84	0.91	1.08	0.91–1.26
Intrahepatic bile ducts	0.11	1.33	12.19	10.92 –13.46	0.09	1.06	11.94	10.68 –13.20
Liver unspecified	0.27[a]	0.58	2.15	1.74–2.56	0.13[a]	0.28	2.07	1.58–2.57
Gallbladder	0.70	0.45	0.65	0.45–0.84	1.19	0.79	0.67	0.54–0.80
Extrahepatic bile ducts	0.86	0.42	0.48	0.32–0.65	0.67	0.36	0.53	0.37–0.69
Other parts of the biliary tract	0.42	0.68	1.61	1.31–1.91	0.27	0.50	1.85	1.50–2.20

An international consensus statement on intrahepatic cholangiocarcinoma by Bridgewater *et al*, from 2014, noted a potential incidence rate increase in both the U.K. and the United States (U.S.) concomitant with an increase in the Age Standardised Mortality Rates (ASMR) in both countries [2]. The United Kingdom guidelines for the '*Diagnosis and Management of Cholangiocarcinoma'*, by Khan *et al*, provided a more nuanced assessment of the epidemiological considerations of cholangiocarcinoma [15]. The authors stated that the incidence and mortality rate rises, particularly with regards to intrahepatic cholangiocarcinoma, may be attributable to inconsistencies in the '*International Statistical Classification of Diseases and Related Health Problems'* (ICD - 10) coding system used in the U.K and the U.S [16]. Data for all cause liver and biliary tract cancers from the Surveillance Epidemiology and End-Results (SEER) program in the U.S. indicated that incidence rates and mortality rates increased between the years 1975 and 2013 [1]. However, there was an increase in the 5-year survival rates of patients undergoing surgical resection (See Figure 1; p. 21).

The ICD-10 coding system, published in 2016, has attempted to address this issue and simplify the codes for biliary tract cancers and cholangiocarcinoma. The ICD-9 had 4 codes for extrahepatic biliary tract cancer (156; 156.1; 156.8; 156.9) which in the ICD-10 has been reduced to 3 codes (C24.0; C24.8; C24.9). The ICD-9 also had 4 codes for intrahepatic cancer (155; 155.1; 156.9; 751.69) which has also subsequently been reduced to 3 codes in the ICD-10 manual (C22.1; C22.8; C22.9). While this reduction in applicable classificatory codes may have gone someway to addressing the discrepancies and inconsistencies in coding it is unlikely to have sufficiently clarified a disease which is inherently anatomically and histologically difficult to classify.

Figure 1: SEER Data for Incidence; Mortality and Survival Trends [1]



Year	1975	1980	1985	1990	1995	2000	2004	2008
5-Year Relative Survival	3.0%	3.2%	7.0%	5.3%	5.7%	11.7%	14.5%	18.5%

1.2) Global Epidemiology

While cholangiocarcinoma is classified as a rare cancer in modern Western populations the incidence is dramatically higher in China and the Far East. China has an incidence of approximately 7 per 100, 000. This is still significantly less than the incident rate for patients in Thailand which has been determined to be as high as 85 cases per 100, 000 per year (See Table 4) [5, 17, 18].

Table 4: Age Standardised Incident Rate for Males and Females in Thailand [5]

Age-standardized incidence rates (ASR) of all cancers in males, 2001-2003, National Cancer Institute and 13 Population based cancer registries in Thailand. [Estimated Rates]	
Liver and Bile Duct :	38.6
Trachea, Bronchus and Lung :	24.9
Colon and Rectum	11.3
Prostate :	5.5
Non-Hodgkin Lymphoma :	5.0
Leukemia(s) :	4.9
Urinary Bladder :	4.6
Oral Cavity :	4.5
Stomach :	4.1
Esophagus :	3.2
All Sites :	143.3

Age-standardized incidence rates (ASR) of all cancers in females, 2001-2003, National Cancer Institute and 13 Population based cancer registries in Thailand. [Estimated Rates]	
Breast :	20.9
Cervix Uteri :	18.1
Liver and Bile Duct :	14.6
Trachea, Bronchus and Lung :	9.7
Colon and Rectum :	7.9
Ovary :	5.1
Leukemia(s) :	3.7
Oral Cavity :	3.7
Thyroid :	3.7
Non-Hodgkin Lymphoma :	3.2
All Sites :	118.6

There are clearly significantly higher incidence rates in Asia compared to the West [19, 20]. Western countries have incidence rates which vary between 0.3 – 0.45 per 100, 000 [21-23]. The dramatic variation in global incidence rates maybe attributable to the different aetiological risk factors to which people are exposed in different geographical locations. However, the robustness of the global epidemiological data must also be questioned, particularly from lower economically developed countries, given the ICD code variance and lack of formalised national registry databases and reporting systems within these countries.

1.3) Aetiology of Cholangiocarcinoma

Risk factors for the development of cholangiocarcinoma vary between different populations and are partially

Figure 2: Light Microscope Image of Opisthornis Viverrini – Liver Fluke [18]

reflective of the epidemiological variance of the disease. The difference in prevalence/incidence rates between the West and the East reflects the disparity in exposure to certain risk factors. Eastern populations, particularly populations in Asia, are exposed to the *Opisthornis Viverrini* liver fluke parasite (See Figure 2).

O. Viverrini is a liver-fluke with a 3-stage life cycle which initially starts with parasitism of a sea-snail, progressing to parasitism of a species of fish, with the end-stage of the life-cycle culminating in human and mammalian host parasitism [24]. The definitive stage of the parasitic life cycle occurs with the maturation of the fluke in the upper gastrointestinal tract of its host. Following maturation the mature fluke migrates via the Ampulla of Vater in to the biliary tract and adheres to the biliary duct epithelial layer using suckers [25]. The fluke stimulates host cytokine responses with interleukin (IL-10) and interferon (IF-Ȳ) which in turn produces a vigorous regional inflammatory response within the bile ducts [26]. Chronic inflammation subsequently leads to cholangiocarcinoma. The endemic nature of the *O. Viverrini* infection within Asian

populations has led to a concerted public health initiative to reduce the risks of exposure to the fluke. The *'Integrated Opisthorchiasis Control Program'* launched by Khon Kaen University in Thailand has attempted to educate the local populations regarding cooking techniques (particularly with reference to raw fish), hand-washing, and management of local herds of livestock and faecal waste management [27-30]. Pharmacological anti-helminth treatment has been initiated within endemically affected villages with varying degrees of success. Praziquantel (PZQ), utilised and licensed primarily for the treatment of *Ecchinoccus Granulosus*, has demonstrated some utility in treating infection. Re-infection rates following treatment remain high with 10% of the treated populations demonstrating subsequent *O. Viverrini* parasitism within their stools [31]. A recent open-label, randomised, non-inferiority, phase 2 trial comparing PZQ and tribendimidine in 607 *O. Viverrini* infected patients demonstrated non-inferiority in post-treatment re-infection rates and a substantially improved side-effect profile with the use of tribendimidine [32, 33]. Tribendimidine is a derivative of Amidantel and has only been widely available since 2007. The opening up of alternative treatment modalities for this infection raises the hope that the endemic distribution of *O. Viverrini* could be combated by a judicious use of pharmacological treatments and public health initiatives.

Hepatolithiasis, gallstone disease of the supra-hilar biliary ducts, is another known risk factor for cholangiocarcinoma development [34-36]. Hepatolithiasis in Asian populations is often related to chronic biliary duct infection with *Clonorchis Sinensis*, a liver fluke with a similar life-cycle to *O. Viverrini* [37, 38]. Hepatolithiasis can range from isolated stones within a single biliary radicle through to widespread confluent disease throughout the main biliary ducts. Management of hepatolithiasis commonly involves surgical resection of the affected ducts with concomitant hepatectomy [39]. Endoscopic treatment has limited utility due to the proximal location within the biliary ducts of the gallstones [40]. Incidence rates of hepatolithiasis in the West are significantly lower than in Asia (0.6 % in Western centres compared to 10 % in Asian countries) [41]. It has been noted that the incidence rates in Western populations increases in areas with large Asian populations [40]. Hepatolithiasis within Western populations is aetiologically associated with previous surgical procedures such as bile duct injury following laparoscopic cholecystectomy or bilio-enteric diversion. Biliary stasis produced by the mechanical effects of the gallstones causes fibrogenesis within the biliary ducts providing a carcinogenic microenvironment.

Primary sclerosing cholangitis (PSC) is the most significant non-modifiable risk factor for development of cholangiocarcinoma within Western populations. PSC appears to be an autoimmune mediated disease process with analogous genetic predisposing correlates as rheumatoid arthritis [42-44], type 1 diabetes mellitus, and inflammatory bowel disorders [45]. PSC is a rare condition affecting 1 per 10, 000 in Northern Europe [46, 47]. PSC causes a multi-focal inflammatory reaction within both small and large calibre biliary ducts. The chronic inflammation produces fibrogenic changes and stricturing at multiple sites throughout the biliary tree obliterating biliary duct continuity. PSC significantly increases the chance of developing

cholangiocarcinoma with a yearly increased risk of 0.5 – 1.5 % and a lifetime risk of approximately 13 % [48]. A diagnosis of PSC is a potential indication for liver transplant due to the lack of responsiveness to medical management and the risk of cholangiocarcinoma [49]. Cirrhosis of any aetiology appears to increase the risk of subsequent intrahepatic cholangiocarcinoma development. Hepatitis B and C have been putatively implicated in the development of intrahepatic cholangiocarcinoma [50].

Despite these risk factors most cases are considered to be *de novo* in nature with no single obvious aetiological antecedent [51].

2.)Molecular Pathogenesis of Cholangiocarcinoma

A multi-step molecular cascade has been proposed for the development of cholangiocarcinoma. Cholangiocarcinoma develops from cholangiocytes and progenitor stem cells which line the biliary ducts. These epithelial cells modify the bile content and promote the transport of bile to the duodenum. Chronic inflammation and cholestasis place microenvironmental stresses on cholangiocytes and their progenitor stem cells causing a cascade of adaptions within these cell populations. Unlike other gastrointestinal malignancies there is no clear adenoma-carcinoma stepwise process for cholangio-carcinogenesis.

2.1) Inflammation

The initiating event for inflammation causes interleukin-6 (IL-6) production in the biliary ductal micro-environment. IL-6 overexpression induces MAPK pathway activation which stimulates the progenitor cholangiocytes in peri-biliary glands to undergo clonal expansion and proliferation [52, 53]. The increased mitotic rate of the progenitor cells and subsequent turnover of normal cholangiocytes leads to DNA replicative errors and subsequent dysplastic cellular adaption. In cells which have already undergone malignant transformation IL-6 appears to enhance cell survival by impairing the cellular machinery responsible for apoptosis via the Mcl-1 pathway [52]. Tumour necrosis factor alpha (TNFά), a pro-inflammatory cytokine, is also released in response to an inflammatory initiating event and acts by providing chemotactic stimulus to innate and adaptive immune responses [54].

2.2) Immune Response to Inflammatory Mediators

The immune response to inflammatory mediators is an attempt to remove cells damaged by the initiating inflammatory event. T-Cell lymphocytes, Natural Killer (NK) cells and mesenchymal cells are all activated by the inflammatory mediators [55]. The immune cells migrate to the inflamed ducts and participate in fibrogenic processes. Fibroblast, myofibroblast and macrophage activation occur to enable fibrotic change in the biliary ducts [56]. In normal cholangiocyte populations the end-stage of this process is fibrosis, but with cholangiocytes which have undergone malignant transformation these processes potentiate tumour survival by producing a favourable tumour micro-environment.

2.3) Cholestasis and DNA Damage

The inflamed and fibrotic biliary ducts provide increased resistance to bile flow which potentiates cholestasis. Bile acids reduce the pH of biliary micro-environment which induces apoptosis in normal cholangiocytes [57]. The normal cholangiocytes adapt to this altered microenvironment and develop resistance to apoptosis. Adaption occurs in response to cholestasis due to the activation of the COX-2 and Nuclear Factor Kappa Beta pathways [58]. The activation of these pathways also encourages clonal proliferation of the cholangiocyte progenitors in the peri-biliary pits.

Cholestasis promotes direct chemical genotoxic effects on the cholangiocyte DNA. Increased clonal expansion, aberrant cells adaptions in response, and high cell turnover all predispose to susceptibility to cholangiocytic cellular DNA damage. Ras/MAPK/MEK and p53 mutations are high frequency mutations within aberrant cholangiocyte populations [59, 60]. The subsequent dysregulation of apoptosis enables immortalisation of DNA damaged cells. Further dysregulation of DNA repair mechanisms and microsatellite instability, due to ongoing inflammatory effects, produces a population of malignant cholangiocytes capable of indefinite dysregulated growth and expansion [61]. Angiogenic changes occur in the micro-environment of these malignant cells due to the autonomous production of Vascular Endothelial Growth Factor (VEGF), concomitant with increased correlated receptor expression. These angiogenic molecular changes further facilitate the survival of the population of malignant cholangiocytes [62-65].

2.4) Metastasis

Multiple biomarkers have been implicated in the epithelial-mesenchymal transition required for malignant populations of cholangiocytes to be able to invade beyond the biliary epithelia and enable systemic metastasis. Research has primarily focused on the aberrant expression of E-Cadherins and multiple micro-RNA's as the principle drivers of metastasis [66-68].

2.5) A Personalised Future

Cholangiocarcinoma is a molecularly diverse heterogenous entity. There appear to be differences in mutational populations between intrahepatic and extrahepatic cholangiocarcinoma, which may be explained by different progenitor cells and supportive stromal architectures between the intra and extrahepatic ducts [69]. Significant advances in the molecular understanding of cholangiocarcinogenesis have provided multiple potential actionable biomarkers and targeted treatments. There are numerous recently closed, current, and future registered clinical trials (See Table 5). Frustratingly, there are limited evidence of efficacy for these targeted approaches [69]. The lack of efficacy may reflect the molecular heterogeneity and complexity of the cellular cross-talk and interactions of the expressed molecules. Further elucidation of these complex mechanisms is required to ensure that the concept of personalised therapeutic approaches to treating cancer becomes a reality.

Table 5: Table Demonstrating Current Registered Targeted Biological Trials Currently Recruiting Patients

Registry Number	Register Date	Closure Date	Agent	Target	N Patients
NCT02924376	Oct-16	Jul-19	Pemigatinib	FGFR2	140
NCT03951597	May-19	No current date	Lenvatinib	Multiple	30
NCT03656536	Sep-18	No current date	Pemigatinib	FGFR2	432
NCT03230318	Nov-17	Mar-21	Derazantinib	FGFR2	100
NCT02150967	Jul-14	Oct-19	Infigratinib	FGFR	120
NCT03345303	Jan-17	Dec-22	Bortezomib	PTEN	50
NCT03377179	Mar-18	Jan-21	ABC294640	SK2	70
NCT03250273	Nov-17	Nov-20	Nivolumab	Multiple	54
NCT02699606	Jul-16	Aug-21	JNJ-42756493	FGFR	55
NCT02990481	Mar-17	Aug-19	TRK-950	Multiple	75
NCT03212274	Mar-18	No current date	Olaparib	DH1; IDH2	145
NCT03257761	Feb-18	Feb-22	Durvalumab	PD-L1	90
NCT02052778	Jul-14	Jun-20	TAS-120	FGFR	371
NCT03207347	Aug-18	Dec-22	Niraparib	BAP1	57
NCT03111732	Jun-17	Dec 202	Pembrolizumab	PD1	19
NCT02520141	Dec-15	Dec-19	Ramucirumab	VEGFR	50
NCT03684811	Nov-18	Apr-22	Nivolumab	IDH1	200
NCT02834013	Jun-17	Jun-21	Nivolumab	PD-1	707
NCT03602079	Jul-18	May-21	A166	HER2	82

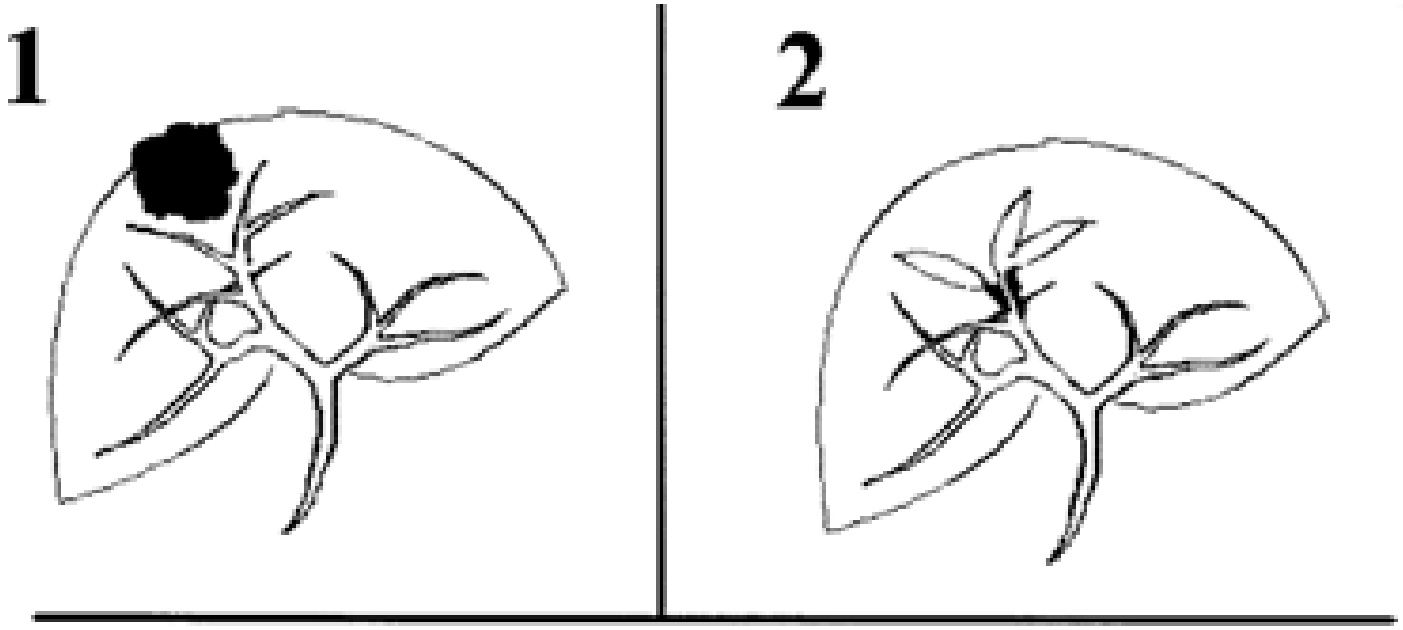
3.) Anatomical Nomenclature and Histological Classification

The ICD-10 codes attempt to classify and differentiate cholangiocarcinoma anatomically in to intrahepatic cholangiocarcinoma and extrahepatic cholangiocarcinoma. The U.K. has traditionally used 3 diagnostic descriptions to differentiate between cholangiocarcinoma diagnoses: intrahepatic cholangiocarcinoma; hilar or peri-hilar cholangiocarcinoma; distal cholangiocarcinoma.

3.1) Intrahepatic Cholangiocarcinoma

Intrahepatic cholangiocarcinoma is commonly described as arising from the epithelial cells of the intrahepatic biliary radicles. They are macroscopically and histologically heterogenous tumours with 3 main macroscopic sub-types classified by the Liver Cancer Study Group (LCSG) of Japan [3]. The three types are mass-forming, mixed-mass forming, and peri-ductal infiltrative sub-types. The mass-forming type tends to arise peripherally and can lead to large solid tumours which are usually macroscopically white/tan in colour with dense desmoplastic capsular reaction and replace the hepatic parenchyma (See Figure 3).

Figure 3: LCSG Schematic Macroscopic Description of Intrahepatic Cholangiocarcinoma [3]



1.) **Mass-forming type**

2.) **Peri-ductal type**

The peri-ductal type can be further sub-divided in to large duct (peri-hilar) or small duct (peripheral) types [70]. Histologically 90 % of intrahepatic cholangiocarcinoma, and cholangiocarcinoma in general, are adenocarcinoma with a variety of cellular sub-types making up the remaining 10 % [71]. Sempoux

et al noted that there is substantial histological cross-over between intrahepatic and peri-hilar cholangiocarcinoma with 45.2 % of tumours forming microscopically mixed sub-types which share features of intrahepatic, hepatocellular and peri-hilar cellular morphology [72].

Histological assessment of intrahepatic cholangiocarcinoma's has demonstrated that there are 4 distinct pleiomorphic dysplastic types (See Table 6; Figures 4 and 5). The categorisation is based upon the percentage of glandular structures present with well-differentiated cholangiocarcinoma containing greater than 95 % glands; moderately differentiated containing 40 – 94 % glands; poorly differentiated containing 5 – 39 % glands; undifferentiated containing less than 4 % glands.

Table 6: Cellular Grading of Intrahepatic Cholangiocarcinoma

Grade 1: Low Grade	Also known as well differentiated. Cells have little deviation from non-neoplastic biliary cells Sheets of columnar or cuboidal epithelia with honey-comb appearance increased nucleus: cytoplasm ratio. Nuclei appear hypochromatic.
Grade 2: Intermediate Grade	Cells demonstrated to be increasingly abnormal. Vacuolar cytoplasm with increasingly disordered growth.
Grade 3: High Grade	Poorly differentiated. Increased pleomorphism with features of squamous cells such as distinct borders and hyper-chromatic nuclei.
Grade 4: High Grade	Completely abnormal cells with loss of polarity and chaotic growth and piling up of cells.

Figure 4: Haemotoxylin and Eosin Histopathological Appearances of Well-Differentiated Cholangiocarcinoma

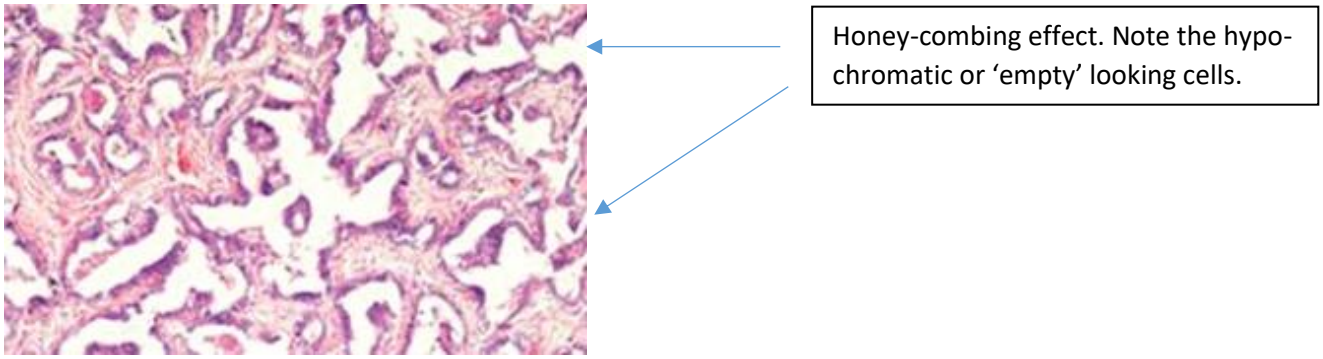
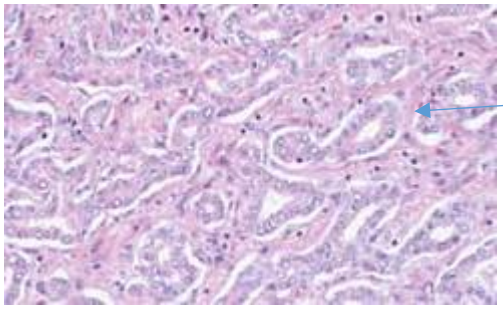


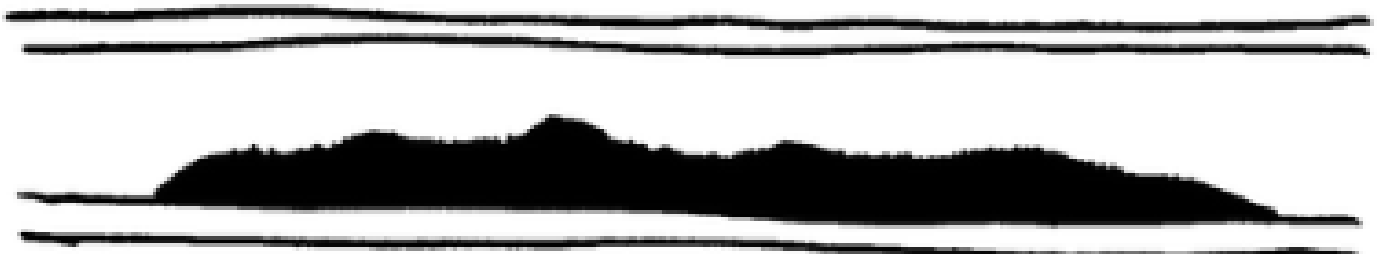
Figure 5: Haemotoxylin and Eosin Histopathological Appearances of Poorly Differentiated Cholangiocarcinoma



3.2) Extrahepatic (Peri-hilar) Cholangiocarcinoma

Hilar cholangiocarcinoma tends towards a macroscopic peri-ductal and intra-ductal growth pattern and are consequently smaller solid tumours compared to peripheral intrahepatic cholangiocarcinoma. DeOlivera *et al* in a large historical case series of 564 resected cholangiocarcinoma specimens, noted that the average size of peripheral intrahepatic cholangiocarcinoma tumours was 5.5 cm compared to 2.5 cm for peri-hilar and 2 cm for distal cholangiocarcinoma (See Figure 6) [73].

Figure 6: Schematic Representation Intra-ductal Growth Pattern Associated with Peri-Hilar and Distal Cholangiocarcinoma [4]



The intra-ductal hilar cholangiocarcinoma sub-type tend to be mucin-producing tumours which cause gross ductal dilatation. They can be further sub-divided in to a superficial spreading type and intraductal papillary (IPNB) or intraductal tubular (ITNB) types. The vast majority, 81.8 %, are IPNB in origin with the remainder made up of the other two types [74-76].

Despite the distinct macroscopic morphological sub-types cholangiocarcinoma uniformly spreads with peri-neural (intra-hepatic 39 %; peri-hilar and distal 75 %), lymphatic (intra-hepatic 61 %; peri-hilar 50 %; distal 73 %) and vascular invasion (intra-hepatic 64 %; peri-hilar 38 %; distal 73 %) [77].

3.3) Differentiation of Cholangiocarcinoma from Hepatocellular Carcinoma

Hepatocellular carcinoma can potentially be confused macroscopically and microscopically with cholangiocarcinoma, especially in cases where tumours have poor differentiation or a sclerosing appearance. Actual 5 year survival rates for hepatocellular cancer vary within case series but have been reported as high as 60 % [78-80], which is considerably better than cholangiocarcinoma (which typically has 5 year survival rates of between 20 – 45 %). Given the difference in 5-year survival the prognostic implications of misdiagnosis and subsequent differences in treatment modalities means accurate histopathological assessment is vital for the patient. Immuno-histochemical analysis facilitates the differentiation of these tumours with cholangiocarcinoma having high expression of cytokeratin 7 (CK7) and cytokeratin 19 (CK19), which are antigens found on glandular and transitional epithelia in the biliary tract but not on hepatocytes [81], with CK19 being highly specific for cholangiocarcinoma [82]. Lau *et al* [83] demonstrated that a panel of immuno-histochemical glycoprotein biomarkers, including HepPar1 (Hepatocyte Paraffin Antigen) and CEA (Carcinoembryonic Antigen) , could accurately differentiate hepatocellular carcinoma from metastatic adenocarcinomas and cholangiocarcinoma. HepPar1 is highly specific for hepatocytes and hepatocellular carcinoma, particularly in combination with CEA and Alpha-Fetoprotein [84]. Furthermore delineation of tumour chromogranin immuno-histochemical profile is used to differentiate cholangiocarcinoma from neuroendocrine tumours [85]. CD-56 (Neuronal Cell Adhesion Molecule, N-CAM) is used to differentiate biliary duct adenomas, benign biliary diseases and Primary Biliary Cirrhosis from biliary tract malignancies [86]. This differentiation panel has subsequently been supported by a number of other publications and is part of the current histopathological practice at University Hospital Aintree NHS Trust [82, 87, 88].

4.) Clinical Presentation, Radiological Assessment, Stratification and Management of Cholangiocarcinoma

4.1) Clinical Presentation of Intrahepatic Cholangiocarcinoma

Patients with intrahepatic cholangiocarcinoma may present with right upper quadrant tenderness due to capsular stretch of the liver. Associated features of weight loss, anorexia and general malaise are also common symptoms due to the disease burden of the cancer. Intrahepatic cholangiocarcinoma rarely presents with jaundice due to its peripheral parenchymal position within the liver, in cases where this occurs the mass is usually an intra-ductal or large duct sub-type close to the hilum and is usually classified as being a peri-hilar tumour. Utilisation of ERCP in intrahepatic cholangiocarcinoma is, as a consequence, relatively rare.

4.2) Radiological Assessment, Stratification and Staging of Intrahepatic Cholangiocarcinoma (IHC) Patients

Radiological assessment with ultrasound (US), magnetic resonance imaging (MRI) and computed tomography (CT), in conjunction with ERCP, provide the basis for investigation and stratification of patients in to non-operative and operative candidates.

Accepted practice in the U.K. is to utilise the American Joint Committee on Cancer (AJCC) TNM classificatory system (See Table 7) for radiological pre-operative assessment and post-resectional histopathological staging for prognosis.

Table 7: 7th Edition AJCC TNM Post-Resectional Histopathological Staging

Primary tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ (intraductal tumor)
T1	Solitary tumor without vascular invasion
T2a	Solitary tumor with vascular invasion
T2b	Multiple tumors, with or without vascular invasion
T3	Tumor perforating the visceral peritoneum or involving the local extrahepatic structures by direct invasion
T4	Tumor with periductal invasion (the pathologic definition of periductal invasion is the finding of a longitudinal growth pattern along the intrahepatic bile ducts on both gross and microscopic examination)
Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis present
Distant metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis present

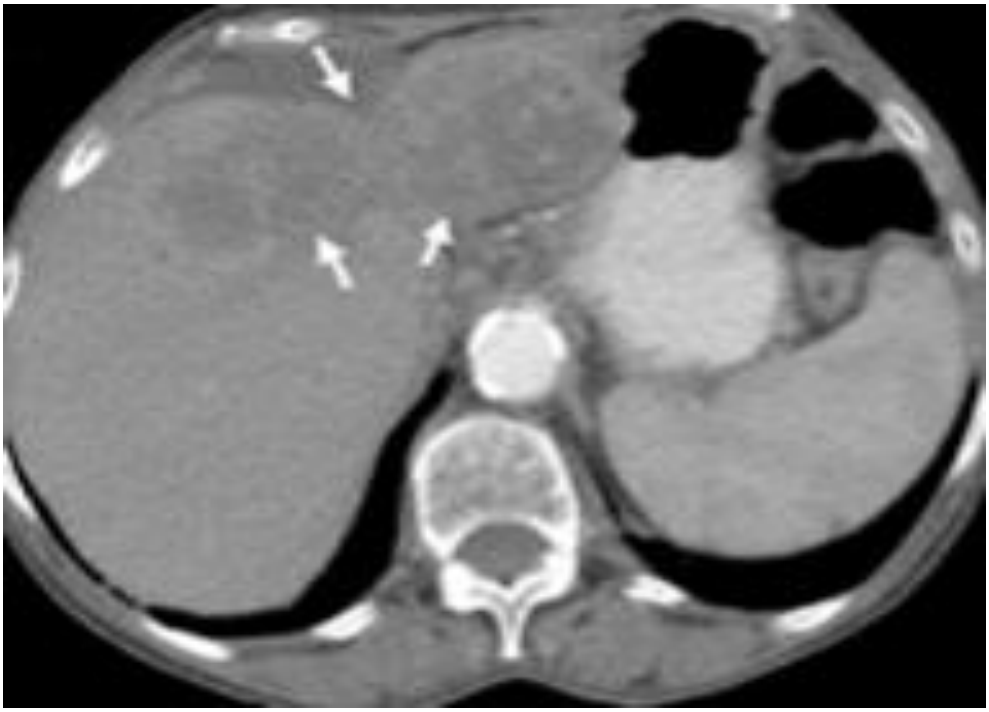
i.) Ultrasonography (US)

Patients may initially be referred for investigation of right upper quadrant pain and undergo a transabdominal ultrasound (US). Transabdominal US is an operator dependent modality but it demonstrates high sensitivity for detection of biliary duct dilatation (89 %) and ability to localise biliary duct dilatation (94 %) [89, 90]. However, transabdominal US is a poor modality for detection and differentiation of intrahepatic cholangiocarcinoma from other hepatic lesions [91]. Contrast enhanced ultrasound (CEUS) has dramatically improved the detection rate of liver lesions with a sensitivity of 95.3 % and is accurate (90.3 %) for differentiating benign lesions and between malignant lesions [92, 93]. In a recent meta-analysis of the 3 main radiological modalities for detection and diagnosis of liver lesions CEUS was demonstrated to not be significantly worse than the other 2 modalities in terms of sensitivity, specificity and diagnosis [94]. However, CEUS demonstrated variable sensitivity in detecting IHC in the setting of cirrhosis and is poorer than both computed tomography and magnetic resonance imaging in this context [95].

ii.) Computed Tomography (CT)

Contrast enhanced CT has a good sensitivity for detection of mass forming intrahepatic cholangiocarcinoma, lobar atrophy and level of biliary obstruction if the mass is peri-hilar [96, 97].

Figure 7: Contrast Enhanced CT Demonstrating Capsular Retraction and Peripheral Rim Enhancement and Satellite Nodules of Intrahepatic Cholangiocarcinoma



Triple phase contrast scans have extremely high (virtually 100 %) sensitivity in detecting intrahepatic

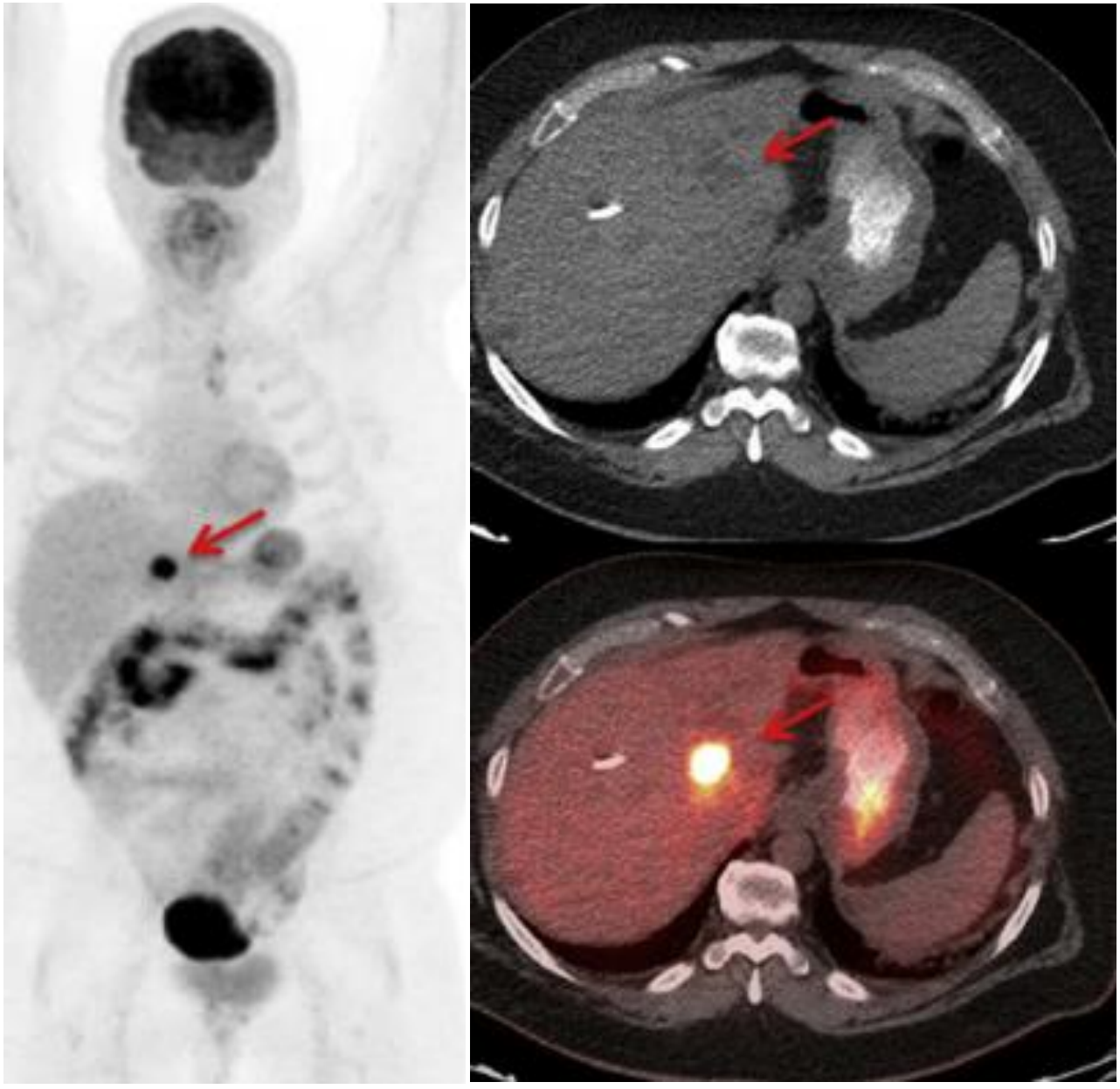
masses greater than 1cm in diameter (See Figure 7) [98]. Triple phase CT can also differentiate accurately between IHC and hepatocellular carcinoma (HCC) by the contrast wash-out characteristics which differs between the tumours [99]. The mass-forming IHC tend to have capsular retraction and display peripheral nodule enhancement with delayed washout of contrast unlike HCC [100]. Differentiation between HCC and IHC becomes problematic when the patient has concomitant cirrhosis because the radiographic features have crossover. Vilana *et al* [101] demonstrated in a retrospective cohort of patients who were subsequently histologically proven to have IHC, that approximately 30 % of patients had features consistent with HCC such as the arterial washout of contrast.

iii.) Positron Emission Tomography – Computed Tomography

The determination of lymph node metastases is key in the accurate staging of intrahepatic cholangiocarcinoma. N1 lymph node status adversely affects prognosis in resected intrahepatic cholangiocarcinoma. Lymph nodes with diameters in excess of 1 cm are considered suspicious for lymph node metastases. However it has been demonstrated that substantial proportions of positive lymph nodes, validated histopathologically post-resection, do not fulfil size-criteria on CT pre-operatively [102]. PET-CT is an alternative radiological modality which utilises radiolabelled tracers, CT, PET, and computer modelling to detect loco-regional lymph node metastases or distant metastases. Radiolabelled fluorodeoxyglucose (FDG) is injected in to the starved patient undergoing PET-CT on the day of the scan. The PET scanner measures the metabolic breakdown of the radiolabelled FDG which occurs preferentially within adenomatous or malignant cells due to aberrant anaerobic cellular respiration (See Figure 8).

Tumour cells are known to be FDG avid compared to non-neoplastic cells due to the alteration of the lactic acid cycle intracellularly. Therefore PET-CT can be used to exploit the differences in cell glycolysis to demonstrate the presence of neoplastic cells in lymph nodes which would otherwise be missed by conventional CT [103]. CT-PET has been demonstrated to be between 94 – 100 % sensitive for distant metastases and recurrent disease post-resection [104-106]. However CT-PET tends to be less sensitive for mucinous tumours as they are less metabolically active than other macroscopic types and therefore produce a less avid FDG trace [91]. CT-PET is considered to be integral to the pre-operative work-up for cholangiocarcinoma as it reduces the number of patients undergoing un-necessary surgical investigation [107].

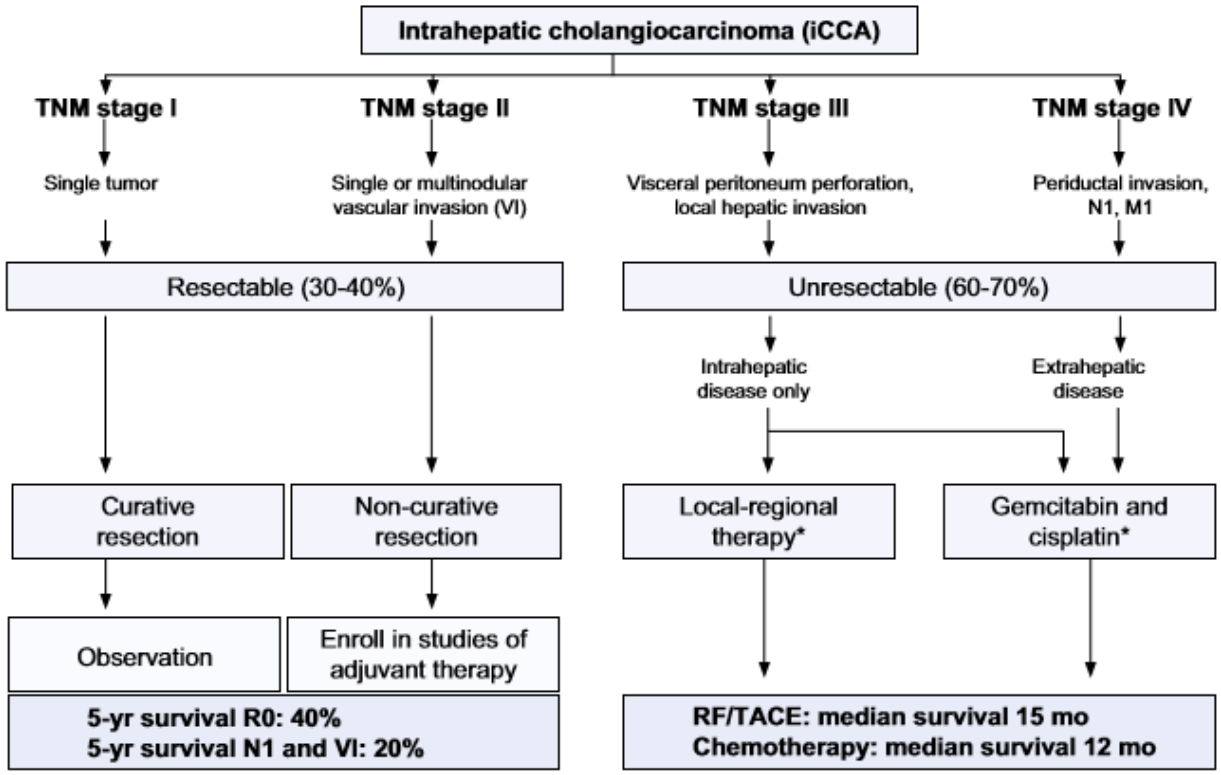
Figure 8: CT-PET Avid Intrahepatic Cholangiocarcinoma Sagittal and Transverse Planes



4.3) Radiological Stratification of Patients with IHC

Patients who have undergone the full pre-operative radiological assessment with CT, PET-CT and MRI are stratified in to potentially resectable and palliative patient groups (See Figure 9).

Figure 9: Pre-operative Stratification of Patients following Radiological Assessment [2]

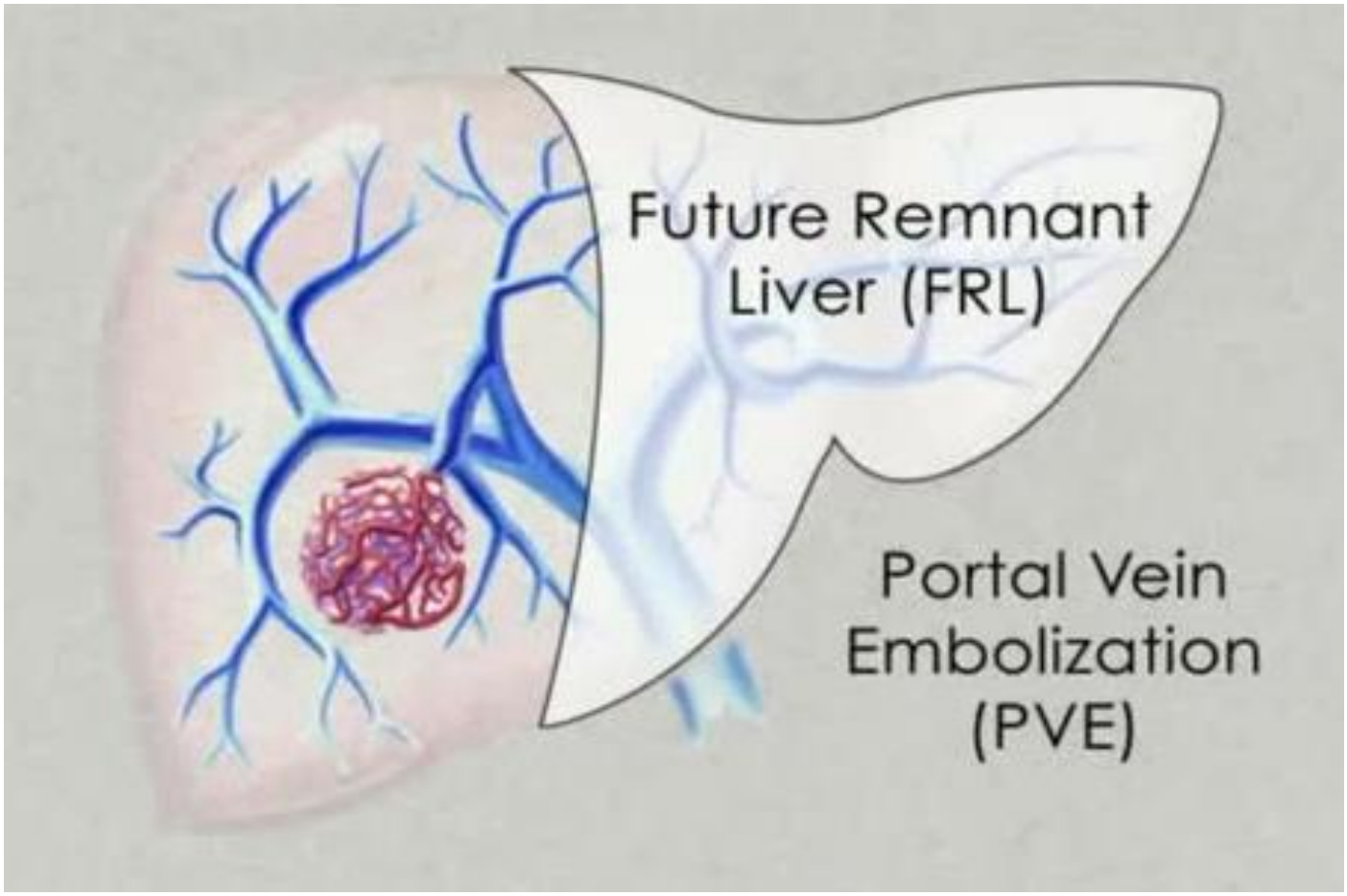


Determination of resectability is dependent upon these factors as well as the functional liver remnant volume left behind after any resection. Functional Liver Remnant (FLR) can be determined pre-operatively by the use of CT volumetry which determines the ratio of FLR to non-tumorous liver volume. The non-tumorous liver volume can be determined either by direct CT measurement or by estimation of body-surface area. Ribero *et al* [108] demonstrated, in a large modern cohort of 243 patients, that CT measurement underestimates the risk of hepatic insufficiency post-operatively and that body surface assessments were more accurate in determining subsequent risk. Retrospective analysis of ex-planted livers correlated to pre-operative CT assessment has demonstrated that the association between liver volume and CT assessment, particularly in diseased cirrhotic livers, is very poor [109]. Intra-operative assessment involving finger spectrometry for indocyanine clearance has been demonstrated, in small prospectively assessed cohorts, to be safe and feasible in determining patients likely to develop post-resectional hepatic insufficiency [110, 111].

4.4) Pre-Operative Procedures to Improve Functional Liver Remnant (FLR) Volume

Augmentation of the FLR to reduce the likelihood of post-resectional liver insufficiency and increase resectability rates has become a widely-accepted technique [112]. Augmentation typically is utilised in patients with right-sided intrahepatic or hilar cholangiocarcinoma type 3A (Bismuth-Corlette staging). FLR augmentation is typically undertaken on patients whom the FLR post-resection would be; 20 % in patients with normal liver parenchyma [113]; 30 % in post neoadjuvant chemotherapy (a rare cohort in cholangiocarcinoma patients); 40 % in patients with established liver cirrhosis [114]. The main approaches to augmentation are; Portal Vein Embolisation (PVE); Portal Vein Ligation (PVL); and Associated Liver Partition and Portal Vein Ligation (ALPPS).

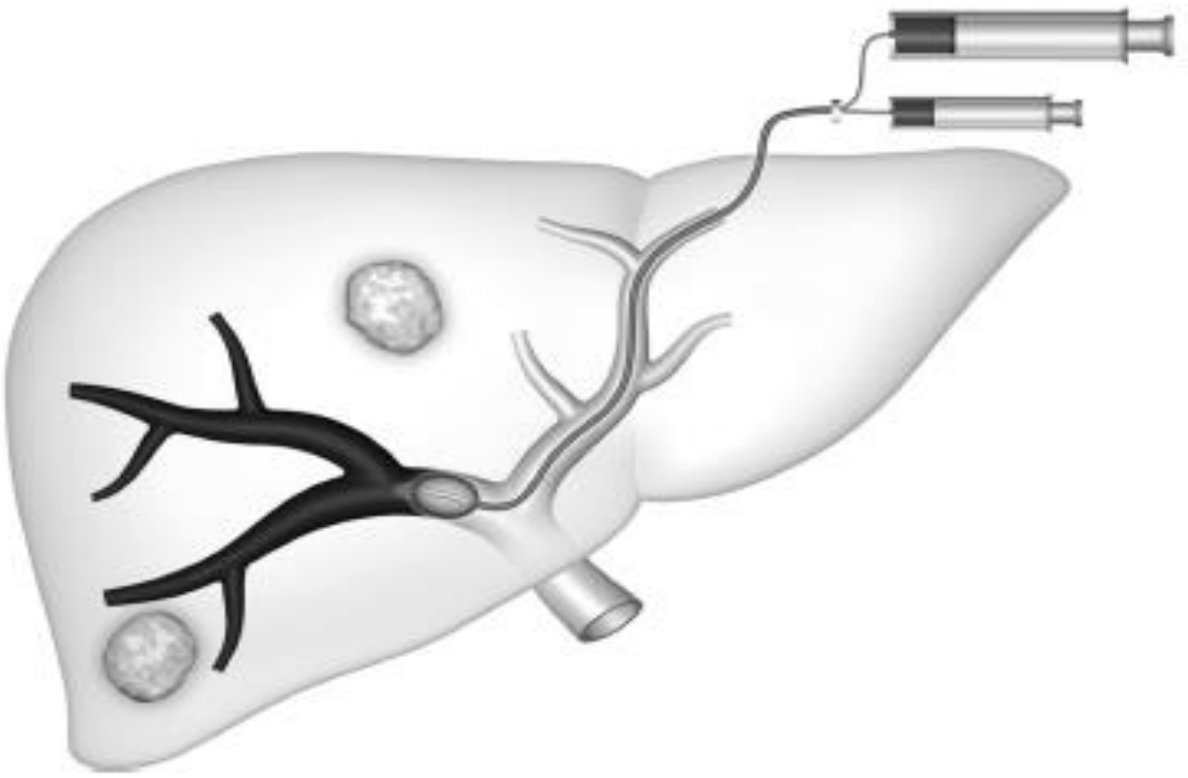
Figure 10: Schematic Concept of the Future/Functional Liver Remnant for Portal Venous Embolisation [131]



Portal Vein Embolisation (PVE) is a widely disseminated minimally invasive interventional procedure (See Figure 10). PVE is performed to re-direct portal venous blood flow, via the use of embolic agents, from the affected tumour bearing hemiliver to the contralateral tumour free hemiliver. The increased blood flow stimulates hypertrophy and hyperplasia of the non-affected post-resectional FLR. FLR response rates are typically 12 – 15 % hypertrophic growth in non-cirrhotic livers and approximately 7 – 9 % in cirrhotic livers [115].

FLR responsiveness to PVE has been demonstrated to be an independent pre-operative predictor of post-operative liver insufficiency [116].

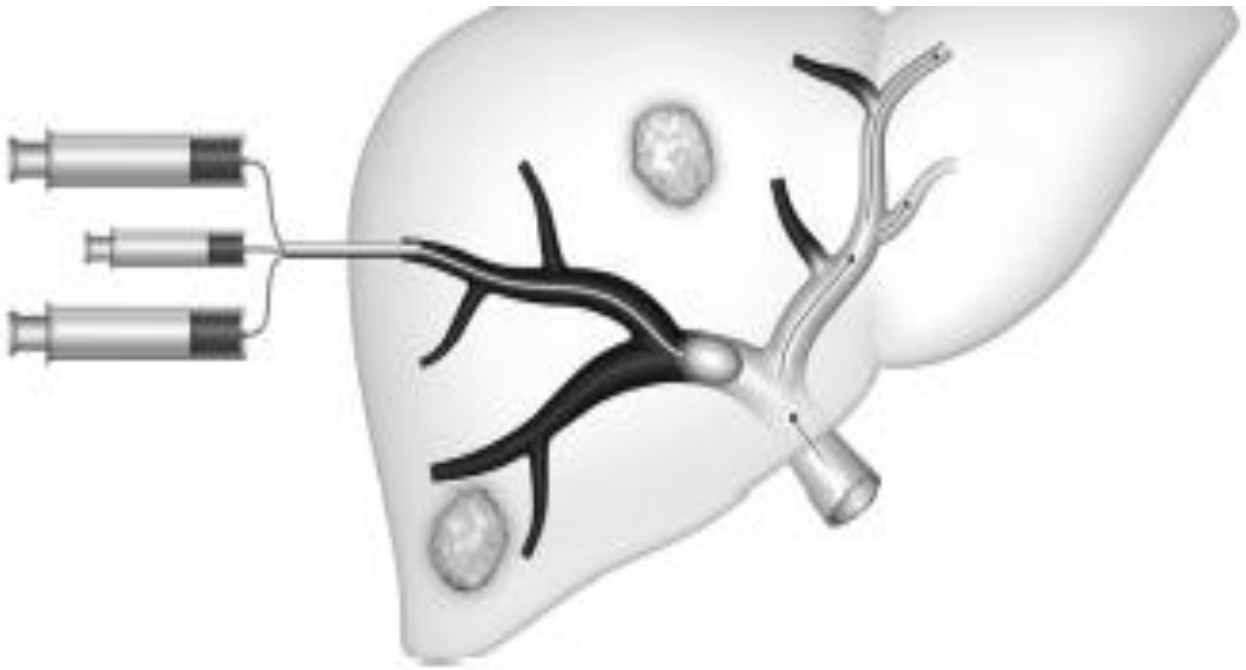
Figure 11: Schematic Representation of the Contralateral Approach for PVE [131]



PVE is a percutaneous procedure undertaken in the Interventional Radiology (IR) suite under either local or general anaesthetic. Access to the right portal venous system is typically achieved via 2 approaches; the ipsilateral approach (i.e. same side as the tumour – See Figure 11) [117]; and the contralateral approach (i.e. via the left portal venous system – See Figure 12) [118]. The contralateral approach is considered the easier technique to access the right portal venous system at its origin and is considered to produce the greatest chance of hypertrophy of the FLR. However, the approach requires instrumentation of the FLR's portal system and therefore risks complete occlusion of the portal system and also injury to the FLR. Comparison of outcomes between the 2 approaches demonstrates that both approaches are safe with low overall risk of complications and that the morbidity and mortality rates are not significantly different [119]. The contralateral approach is preferred when the operator is utilising specific liquid embolic agents, such as cyano-acrylate glue, due to the rapid polymerisation time [120]. The contralateral approach is easier and quicker to navigate to and from the right portal system. This makes withdrawing the catheter quicker and easier. Therefore, if the glue polymerisation time is short, the ease of access and withdrawal of catheter is crucial to prevent manipulation of the polymerised glue by the catheter. The ipsilateral approach is

utilised when the operator is using particulate embolic agents as the effect time of the agent is longer in comparison to liquid-based embolic agents.

Figure 12: Schematic Representation of Ipsilateral Approach [131]



PVL and ALPPS are surgical procedures utilised to produce FLR hypertrophy. PVL involves surgical ligation of the portal vein to ensure re-direction of portal venous blood flow. A recent systematic review comparing outcomes between PVE and PVL determined that there was no difference in FLR hypertrophy [121]. PVL is accordingly not commonly utilised as a modality for increasing the FLR due to the significant morbidity incurred due to surgical access.

ALPPS is a novel concept with a narrow evidence base and significant controversy. The procedure involves surgical access to the abdomen and subsequent splitting of the hepatic parenchyma and ligation of the right portal vein to provide complete partition and reduce the chance of collateralisation of blood supply to the FLR [122]. Following the initial stage of vascular ligation and parenchymal microvascular isolation a second stage right trisectionectomy is undertaken for resection of the primary tumour, following radiographically demonstrated adequate FLR hypertrophy. ALPPS procedures significantly increase the FLR compared to PVE and isolated PVL [123]. The rate of growth of the FLR is also significantly faster with maximal growth occurring at day 9 post-operatively compared to 4 – 6 weeks following PVE [124, 125]. The short interval between the initial portal vein ligation and parenchymal transection and the peak hypertrophic stage potentially increases the likelihood of completion of the second stage of the procedure, the

trisectionectomy. PVE and two-stage hepatectomy require significant periods between the initial procedure and the completing resection of the primary [126]. This interval produces the potential of disease progression occurring thereby precluding completion of resection. ALPPS therefore appears to be a viable alternative for patients with borderline resectable disease. Laparoscopic approaches to ALPPS have also been reported demonstrating the feasibility of the procedure to potentially be adopted as a minimally invasive approach for hypertrophy of the FLR [127-130].

The first ALPPS procedure was undertaken on a patient with peri-hilar cholangiocarcinoma, however adoption of the technique has been cautious primarily due to the substantial morbidity and mortality rates associated with the procedure in patients with cholangiocarcinoma [131]. An international consortium of surgeons has produced a centralised registry to accrue data upon all patients undergoing ALPPS procedures globally (www.ALPPS.net). Olthof and colleagues have subsequently published data from the registry and have demonstrated that ALPSS produces inferior survival outcomes compared to PVE and extended liver resection alone [131]. There is an ongoing multi-institution randomised control trial comparing ALPSS and trisectionectomy versus two-stage hepatectomy (**NCT02758977)** which should provide clarity regarding the post-operative morbidity and mortality of the respective techniques. The primary end-point is assessment of Disease Free Survival (DFS) at 1 year, with the secondary end-points being Overall Survival and Peri-Operative Survival. Further scientific and clinical research is required to determine the utility of this novel procedure for improving FLR, resectability and patient outcomes before wide-spread adoption can be undertaken.

4.5) Post-Resection Liver Insufficiency

Improvements in surgical techniques, intensive care support and pre-operative morbidity and mortality assessment over the last 20 – 30 years have led to an increase in the number of hepatic resections undertaken in high volume centres. The ability to predict post-operative liver insufficiency has become increasingly important. This process enables the multi-disciplinary team to plan further treatment and provide prognostic information to both the patient, family and other colleague's so appropriate ongoing management can be determined in a collaborative fashion.

4.6) Surgical Management and Post-Resection Prognosis of Intrahepatic Cholangiocarcinoma

Anatomical resection of intrahepatic cholangiocarcinoma is classified by the Couinaud classification of the resected hepatic segments. Non-anatomical resections can be undertaken but are considered as producing a higher risk of producing a non-curative R1 resection. R0 resection is defined as a margin clear of neoplastic cells. R1 resection is classified as a resection with microscopic tumour involvement of the margin (in any plane). R2 is defined as macroscopic tumour at the resection margin. Following resection, the AJCC histo-pathological components are combined and a scored 'stage' is allocated to

the case to determine prognosis (See Table 8). The 7th AJCC classification for intrahepatic cholangiocarcinoma was released in 2011 and has been validated by a number of large retrospective case series [132, 133]. The 7th AJCC classification arose out of SEER data published in 2009 by Nathan

Table 8: Table Demonstrating Staging System from AJCC 7th Edition

Stage	T	N	M
0	Tis	N0	M0
I	T1	N0	M0
II	T2	N0	M0
III	T3	N0	M0
IVA	T4	N0	M0
	Any T	N1	M0
IVB	Any T	Any N	M1

et al who noted that the previous 6th AJCC classification did not accurately differentiate between patients when stratified by tumour size [134]. The 'T' component of the score, which was based on a measurement of tumour size of > 5cm, did not offer any prognostic validity.

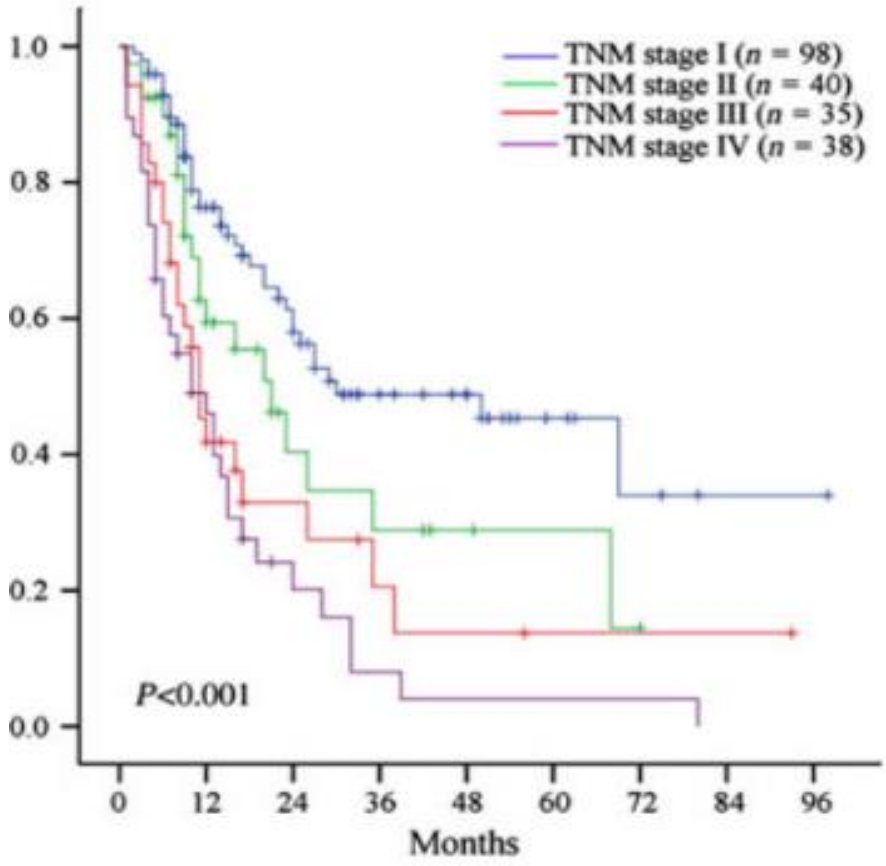
Prior to 2009 there had been considerable debate regarding the utility of a variety of scoring systems which included 2 separate Japanese classifications and the AJCC 6th edition [3, 135].

The LCSG from Japan had proposed an alternative staging system which doesn't specify loco-regional nodal status and has a binary cut-off defining positive nodal status as the presence of any lymph nodes in the resection specimen. The system also utilised tumour size (with cut-off at 2 cm) and the presence of a solitary solid tumour as independent prognostic factors in their system. The authors staging system is yet to have been validated in a large external cohort.

Farges *et al* demonstrated clear significance between the stage stratified patients and comparison with previous staging systems demonstrated that the AJCC most accurately predicted prognosis. Li *et al* [136], in a large Chinese multicentre Chinese cohort of 283 patients, further validated the utility of

the AJCC 7th edition demonstrating again coherent grouping of patients with adequate distribution between the stages for patients undergoing R0 resection (See Figure 13).

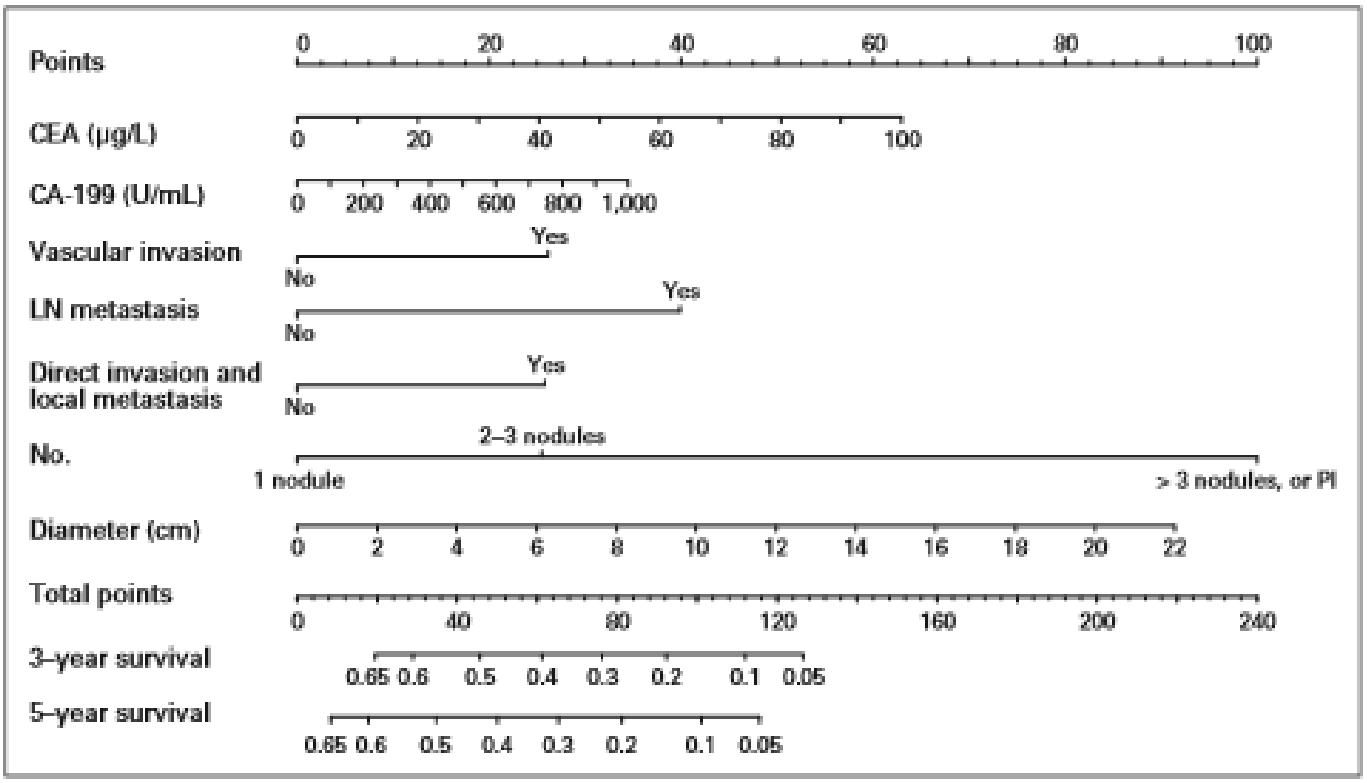
Figure 13: Kaplan-Meier Demonstrating Validation of AJCC TNM System [36]



The main limitation regarding the scoring system concerns the T2 tumour stage. This sub-category includes T2b staging of multifocal disease i.e. multiple primaries located in the same lobe of the liver. Clinical differentiation of multi-focal disease or solitary primary with multiple metastases is difficult. This is a common problem for liver cancers in general [137]. Specifically, in the case of intrahepatic cholangiocarcinoma it is a concern as multifocal disease has been shown to have significant prognostic consequences compared to large solitary intrahepatic tumours [138]. In a large multi-institutional cohort of 557 modern and historical cases multifocal tumours had significantly poorer 5 year survival rates (30.5 % solitary vs 18.7% multifocal; $p<0.05$) and significantly higher associations with nodal disease ($p<0.05$) and vascular invasion ($p<0.05$) [138]. This would suggest that the T2 category requires further refinement and validation in other cohorts with potential amendment or upstaging for multifocal disease. This is particularly pertinent given that multifocal IHC is the predominant pathological type and accounts for approximately 80 % of IHC [3].

A prognostic nomogram has been constructed by Wang *et al* for IHC [139]. The authors retrospectively analysed a cohort of 367 patients with R0 resected IHC's and through multivariate analysis determined that, along with the histopathological components of the AJCC system, the pre-operative serum tumour markers CEA (carcino-embryonic antigen) and CA19-9 (carbonic anhydrase) were independent

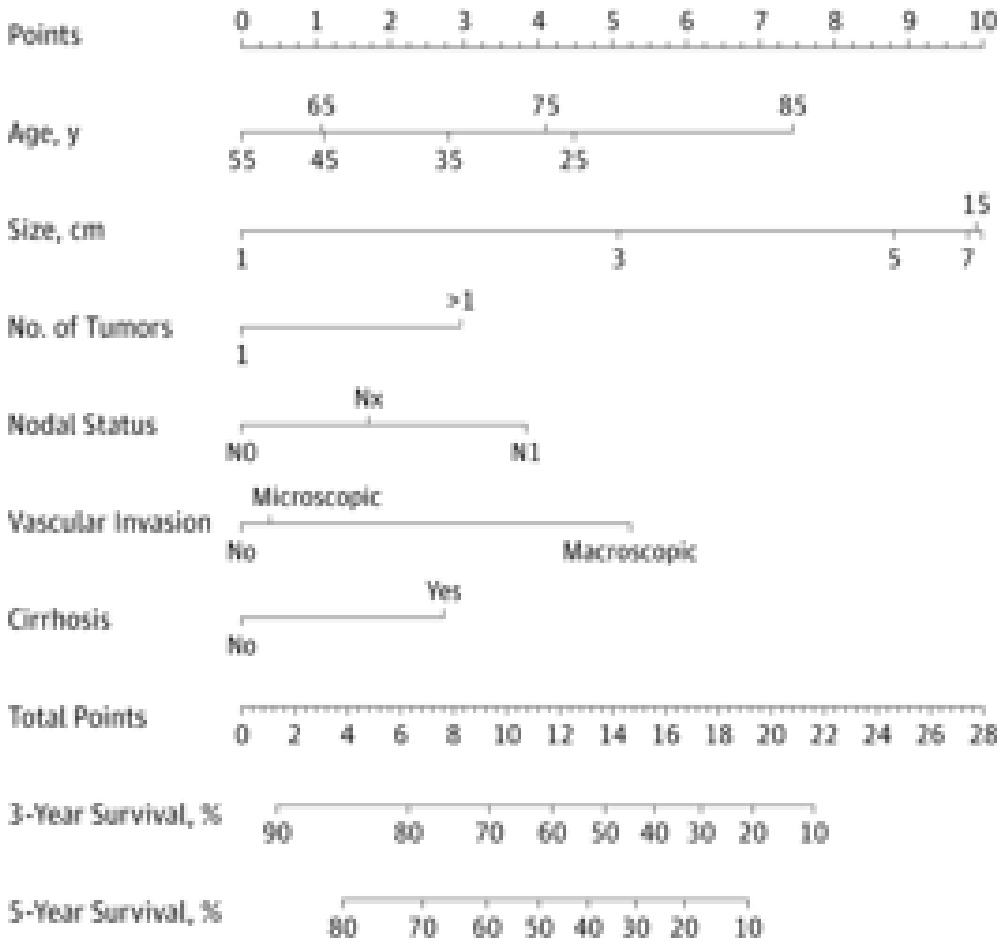
Figure 14: Prognostic Nomogram for IHC [133]



predictors of prognosis. The nomogram (See Figure 14) was then prospectively applied to a cohort of 82 patients at the same institution for validation purposes. The authors found that the proposed nomogram had significantly improved levels of prediction of prognosis following resection compared to the other staging systems.

Hyder *et al*, in a large multicentre international collaborative cohort of 514 patients produced a similar nomogram [161]. The nomogram (See Figure 15) disregarded the pre-operative serum tumour markers and developed a scale to determine correlation between tumour size and prognosis. Comparison with the AJCC 7th edition demonstrated improved prognostic sensitivity. The authors acknowledged the heterogeneous nature of the multi-institutional and transnational cohort. Further validation work is required on this nomogram and it is yet to be externally validated in a large cohort.

Figure 15: Hyder Nomogram for IHC Prognosis



There have been further validations of the AJCC 7th edition in numerous cohorts and further prognostic nomograms have been constructed [140]. The relationship between tumour size and prognosis however remains a controversial one in intrahepatic cholangiocarcinoma. While the AJCC 7th edition did not contain it as a prognostic category, the nomograms did. Spolverato *et al*, in a large multicentre retrospective cohort of 443 patients, determined that tumour size positively correlated with vascular invasion ($p< 0.001$) and cellular grading ($p= 0.04$) indicating a level of multi-colinearity between the variables [141].

The subsequent development of the AJCC 8th edition attempted to address this concern. Tumour size became the explicitly incorporated in the prognostic model (See Table 9).

Table 9: AJCC 8th Edition T-Stage Re-classification

✓	*T Category*	*T Criteria*
	TX	Primary tumor cannot be assessed
	T0	No evidence of primary tumor
	Tis	Carcinoma *in situ* (intraductal tumor)
	T1	Solitary tumor without vascular invasion, ≤5 cm or >5 cm
	T1a	Solitary tumor ≤5 cm without vascular invasion
	T1b	Solitary tumor >5 cm without vascular invasion
	T2	Solitary tumor with intrahepatic vascular invasion or multiple tumors, with or without vascular invasion
	T3	Tumor perforating the visceral peritoneum
	T4	Tumor involving local extrahepatic structures by direct invasion

Furthermore T4 disease and presence of nodal metastasis irrespective of station was down-staged from stage 4 disease to stage 3b disease [142] (Table 10).

Table 10: AJCC 8th Edition TNM Staging System

✓	*When T is…*	*And N is…*	*And M is…*	*Then the stage group is…*
	Tis	N0	M0	0
	T1a	N0	M0	IA
	T1b	N0	M0	IB
	T2	N0	M0	II
	T3	N0	M0	IIIA
	T4	N0	M0	IIIB
	Any T	N1	M0	IIIB
	Any T	Any N	M1	IV

A large retrospective single-centre cohort of 621 patients whom had undergone resection for intrahepatic cholangiocarcinoma compared survival outcomes as stratified by AJCC edition [143]. The AJCC 8th edition demonstrated a high prognostic ability to discriminate between T-stages, especially when accounting for the size of tumour re-classification. However, there was no significant increase in the prognostic ability of the AJCC 8th edition compared to the 7th edition. The failure to produce a significant improvement in prognostic ability indicates either that the size of tumour and number of tumours are multicolinear variables or that there are other variables not accounted for in the AJCC system which are producing significant effects upon survival.

The AJCC 8th edition has been conceived of as a 'bridge' from the assessment of 'population-based' concepts of prognostic variables to a more 'personalised' approach. Multiple other factors which have

been demonstrated to have a significant impact on survival have been incorporated as registry variables in the AJCC 8th edition [144]. Histopathological variables considered include histological grade (Gx – G3), the presence of lympho-vascular invasion and tumoural growth patterns. Serological CA 19-9 antigen, a standardised tumour marker utilised in the management of hepato-pancreatico-biliary cancers, has also been included as a standard registry variable. The utility of the serological test is contested because approximately 5 - 14 % of all patients are non-secretors of the antigen even in the presence of disease [145]. CA 19-9 serological levels also appear to vary due to benign hepato-biliary disease such as primary sclerosing cholangitis (PSC), however elevated levels are significantly more likely to occur in malignant processes [146]. Benign hepato-biliary disease, such as PSC and hepatic fibrosis, presenting concomitantly with cholangiocarcinoma, have also become a registry collected variable. The incorporation of these variables will hopefully allow assessment of the effect these factors have on survival and enable subsequent incorporation of any significant variables in to the AJCC prognostic models.

5.) Radiological Assessment, Stratification and Staging of Extrahepatic (Peri-hilar) Cholangiocarcinoma Patients

The aims of radiological assessment of patients with hilar cholangiocarcinoma are to determine level of biliary duct occlusion, extent of local disease and to demonstrate the presence of any intra-abdominal or distant metastases. This process stratifies patients in to 2 cohorts; potentially resectable patients with disease confined to the surgical field, and patients with distant/systemic.

5.1) Clinical Presentation of Peri-hilar Cholangiocarcinoma

Clinical presentation varies between intrahepatic and extrahepatic cholangiocarcinoma. Hilar cholangiocarcinoma accounts for between 60 – 70% of all presentations for patients with cholangiocarcinoma to medical services in the U.K. [147]. Extrahepatic (peri-hilar and distal) cholangiocarcinoma typically presents with jaundice (95 – 98 %) due to biliary duct obstruction from its intra-ductal and peri-ductal growth patterns. Often concomitant with jaundice is sepsis which occurs due to the transmigration of enteric bacteria proximally up the blocked biliary ducts [148]. Associated features of anorexia, malaise and gastric outflow obstruction may also be present. Late presentation of patients with painless jaundice and/or sepsis is typical. Sixty to 80 % of all patients present with unresectable locally advanced or metastatic disease [149]. Biliary duct decompression via endoscopic or percutaneous methods following suitable radiographic investigations is utilised as temporising measure to complete thorough staging of the patient and to optimise liver function prior to potential resection.

5.2) Drainage Methodologies of the Biliary Tree due to Malignant Obstruction

Endoscopic retrograde pancreaticography (ERCP) for malignant biliary obstruction is a highly utilised technique available in the United Kingdom. The British Society of Gastroenterologists has recently established guidelines for the safe practice, audit and continuing professional development of practitioners and centres providing ERCP [150]. The published guidelines are an attempt to standardise the practice of ERCP in an attempt to improve nationwide outcomes for this procedure by production of a common set of auditable key performance indicators.

Part of the process is an attempt to transform the provision of ERCP services to a hub and spoke system with tertiary referral centres providing the bulk of the service with regards to malignant biliary obstruction (and particularly with reference to emergency relief of cholangitis secondary to malignant occlusion [151]). High volume centres appear to provide safer services with greater efficiency and increased responsiveness to complex clinical presentations [152]. While the transformation of ERCP services within the U.K. is underway there is currently no nationalised registry for ERCP practice unlike other advanced health care economies [153-155].

ERCP has a contested role within the diagnosis and management of malignant biliary obstruction. ERCP has been utilised to acquire brushings and biopsies for cytological and histopathological assessment. However, the sensitivity and specificity rates for detection of malignant biliary obstruction are low for both modalities with only approximately 45 % of malignant biliary obstructions diagnosed due to the acquired tissue [156]. Experimental studies have consistently demonstrated that the presence of jaundice in animals correlates to increased infection rates and a reduction in the liver's ability to compensate for the presence of bacterial endotoxin [157-160]. The experimental data has provided the impetus to relieve the biliary obstruction in palliative and surgical patients to prevent development of biliary sepsis and to improve hepatic function. The role of undertaking ERCP in patients with potentially resectable disease is less clear. Studies have demonstrated no clear survival benefit for pre-operative stenting in non-septic patients and that there may actually be an increased incidence of post-operative infections following stenting of the biliary tree [161].

Early surgery within one week of diagnosis rather than bridging biliary instrumentation appears to be the ideal approach in non-septic biliary obstructed patients. This approach however has inherent logistical difficulties regarding access to appropriately trained and staffed theatres which limits wide-spread adoption. Patient's with malignant biliary obstruction and sepsis however demonstrate a conferred survival benefit and reduced post-operative morbidity when undergoing major hepatic resection, thus providing the only clear indication for pre-operative stenting [162]. Technological advancements have enabled endoscopists to deploy Self-Expanding Metal stents (SEMS). Data

suggests that SEMS have greater patency times and a reduced likelihood of recurrence of biliary obstruction, particularly within the palliative setting [163, 164].

Percutaneous trans-hepatic approaches to alleviate malignant pre-operative biliary obstruction have similar success rates but significantly reduced infective complications [165]. However, logistical concerns regarding access to appropriately trained interventional radiologists and ancillary services, along with the increased rate of haemorrhagic complications post-procedure compared to ERCP, has prevented this modality from becoming the preferred method of pre-operative biliary drainage.

5.3) Radiological Assessment of Peri-hilar Cholangiocarcinoma

i.) Ultrasonography

Ultrasound has limited utility in the diagnostic assessment of hilar cholangiocarcinoma. Ultrasound is effectively limited to the determination of intrahepatic or extrahepatic biliary duct dilatation. The level of obstruction is usually definable but not the characteristics of the obstruction. More complex imaging modalities are required to determine the provenance of the biliary obstruction. Endoscopic US (EUS) has high sensitivity (94 %) and specificity (85 %) for staging hilar cholangiocarcinoma but is only selectively used in the UK due to limited access to appropriately trained staff and resources [166], and so tend to be limited to tertiary hepatobiliary services.

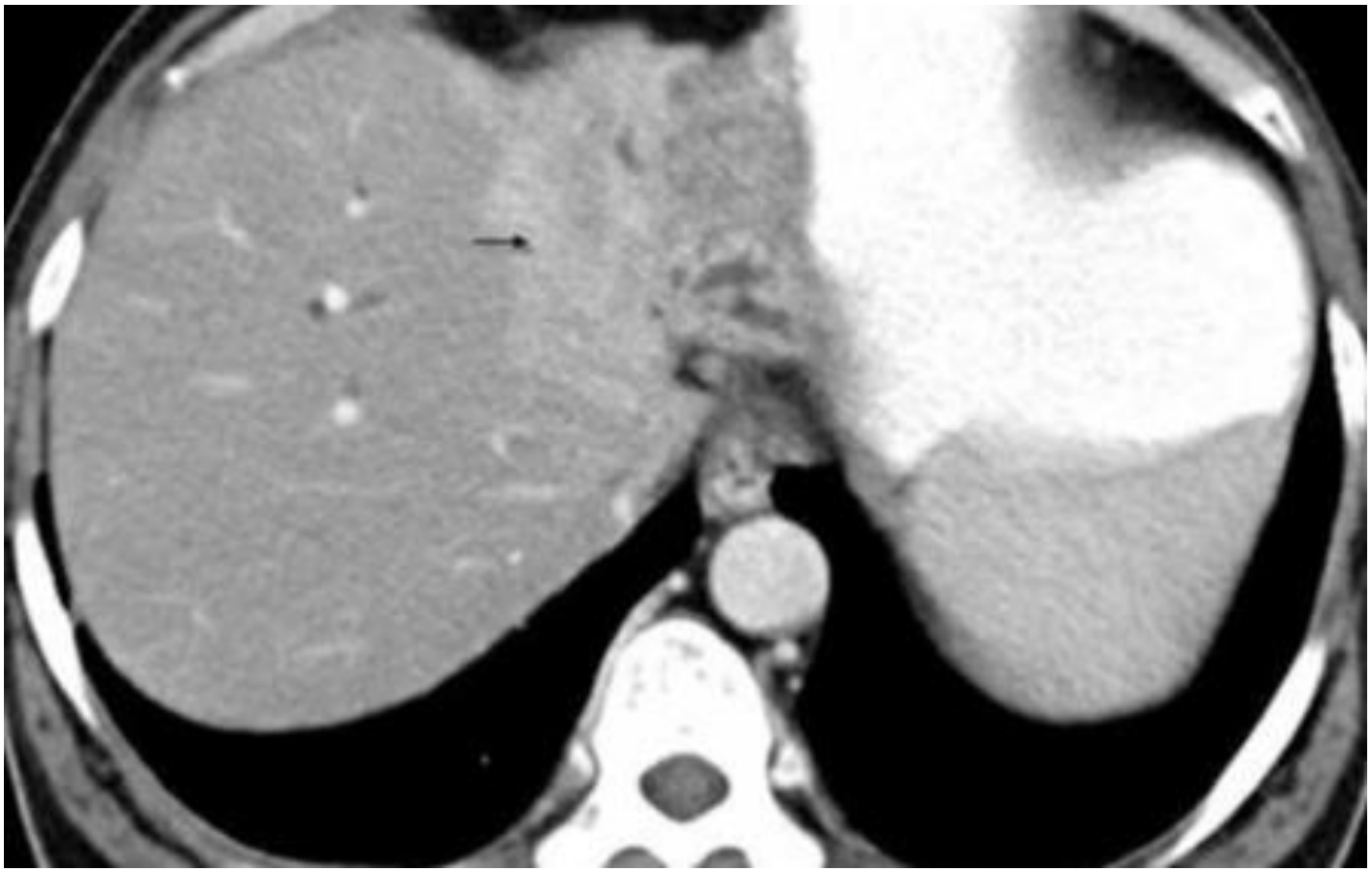
ii.) Cross-sectional Imaging

Prior to the advent of modern cross-sectional imaging ERCP and PTC were considered the gold-standard investigations for diagnosis and determination of tumour extent. These invasive techniques, while having the advantage of potential tissue acquisition, have several disadvantages. Both carry an increased risk of hepatobiliary sepsis and an inability to determine extent of tumour in the presence of complete ductal occlusion [167]. Non-invasive cross-sectional imaging has the benefit of delineating tumour characteristics at the hilum along with radiological features associated with the tumour both proximally and distally to the tumour without introducing any risk of sepsis. Multi-slice CT scans are typically utilised in the UK for initial diagnosis. A recent meta-analysis by Ruys *et al* has demonstrated that CT was able to delineate ductal involvement accurately in 86 % of patients [168]. The mass-forming or ductal morphological types tend to be more easily demonstrable on CT due to their soft-tissue effects and surrogate distension of the biliary ducts proximal to the obstruction. The peri-ductal morphological sub-type rarely has a demonstrable mass on cross-sectional imaging but typically demonstrates proximal ductal dilatation [169].

Multi-slice CT have high sensitivity (86 % portal vein involvement (PVI); 89 % hepatic artery involvement) and specificity (92 % PVI and 93% hepatic artery involvement) for vascular invasion

[168]. Invasion of the peri-ductal vascular structures can also produce surrogate radiological features such as ipsilateral or contralateral lobar atrophy (See Figure 16).

Figure 16: CT scan Demonstrating Ipsilateral Left Lobar Atrophy Secondary to Left Hepatic Duct Cholangiocarcinoma



Arrow demonstrates complete atrophy of left lobe of liver

iii.) PET-CT

PET-CT has a limited utility in the primary assessment of hilar cholangiocarcinoma. PET-CT has been demonstrated to have utility in confirming recurrence or determining distant metastatic spread but has been demonstrated to be inferior to standard multi-slice CT scanning in the context of diagnosis [170]. Surgical assessment via diagnostic laparoscopy is considered to be a more accurate methodology of determining the presence of radiologically occult intra-abdominal metastases.

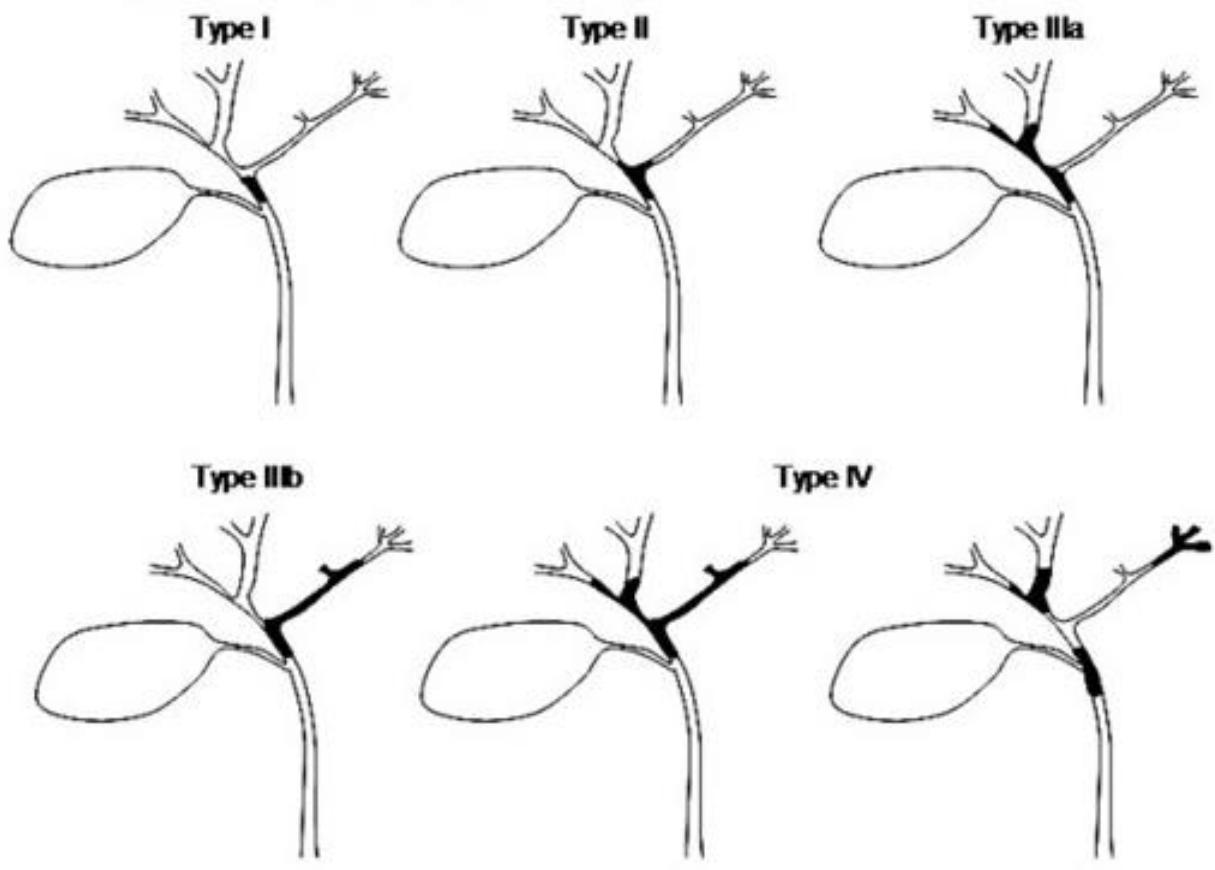
5.4) Surgical Assessment and Stratification of Patients with Potentially Resectable Hilar Cholangiocarcinoma

i.) Pre-Operative Radiological Assessment

Initial assessment of resectability is made based upon the Bismuth-Corlette (BC) classificatory system [171]. The BC system is used to anatomically locate the tumour with reference to the bifurcation of the common hepatic duct (CHD). Tumours located distally in the biliary tree to the CHD are classified as BC Type 1 tumours. Tumours located proximal to the CHD in the biliary tree are further divided in to 4 types (See Figure 17). The BC classification is broadly utilised to describe the longitudinal extension of the tumour from the CHD. Type 2 BC tumours are inclusive of the CHD, type 3a BC tumours incorporate longitudinal extension along the right hepatic main duct, and type 3b BC tumours incorporate extension along the left hepatic main exclusively. Type 4 BC tumour classification incorporates bi-ductal extension and multifocal ductal disease.

While the BC system has been universally adopted as an anatomical descriptive system for tumour location its utility in determining resectability is contested. The BC system is poor at accurately describing longitudinal

Figure 17: Schematic Representation of the Bismuth-Corlette Classificatory System [175]

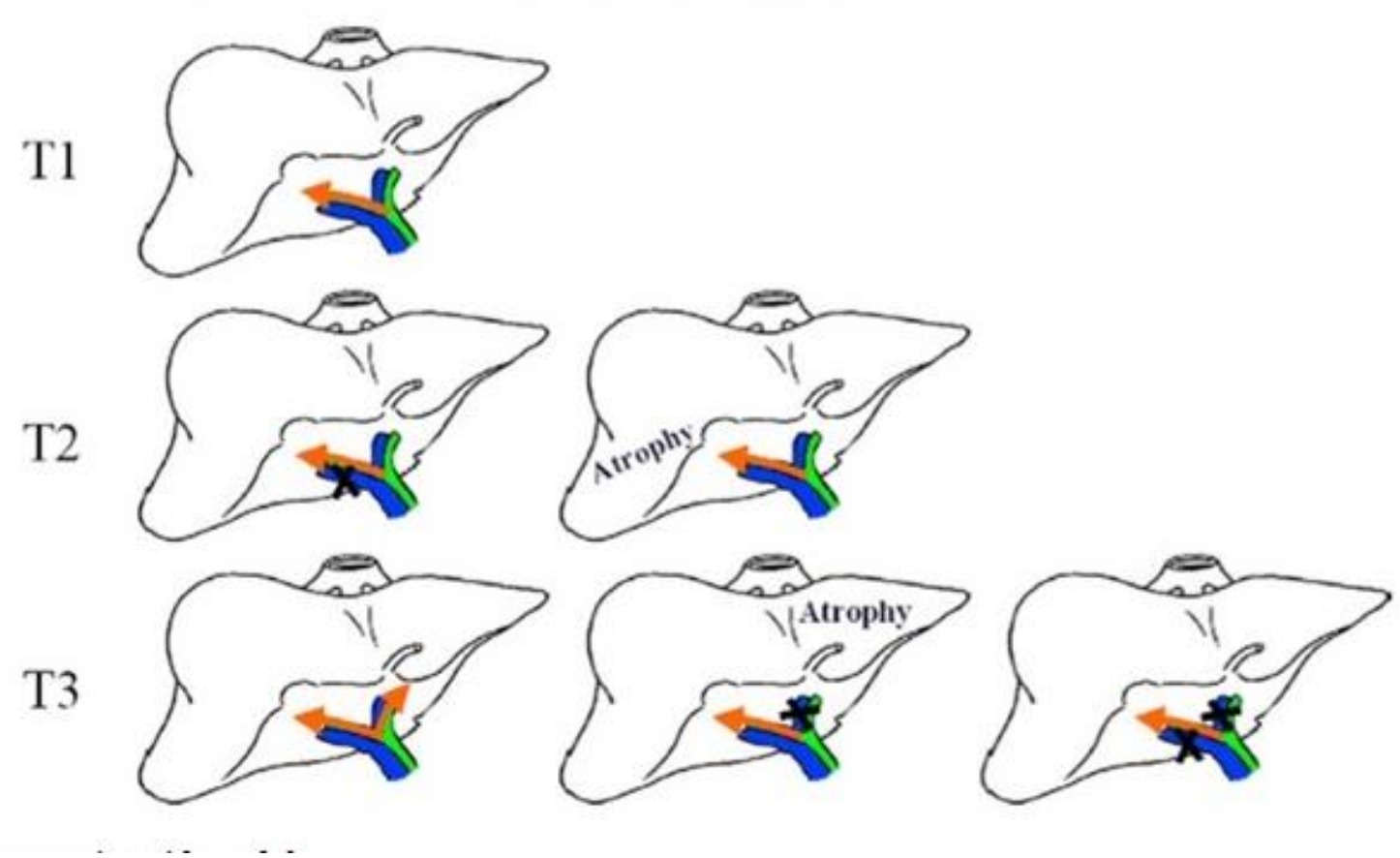


Note the longitudinal extension from biliary confluence along the ductal system

extension and is commensurately poor at distinguishing between left and right duct extension [172, 173]. The BC system has been demonstrated to have a limited utility in determining resectability and no evidence-based role in post-resection prognosis [174].

The Memorial Sloan-Kettering Cancer Centre (MSKCC) hepato-biliary group have proposed an alternative resectability classification system [175-179]. The MSKCC system attempts to account for both longitudinal and radial extension of the primary tumour (See Figure 18).

Figure 18: Schematic Representation of the MSKCC Classificatory System [179]



The MSKCC system primarily incorporates surrogate markers of radial extension as current imaging modalities are not sufficiently sensitive to determine radial tumour spread. Increasing severity of the markers of radial extension produces a corresponding increase in stage and subsequent reduction in potential resectability. Matsuo *et al* internally validated the staging system, in a consecutive cohort of 380 patients, for the purposes of determining resectability and also demonstrated that the MSKCC system retained validity in determining post-resection prognosis and the presence of metastases [177]. The MSKCC system is yet to be robustly validated in an external modern cohort in terms of resectability. The majority of

validation studies are concerned with determining the utility of the system with reference to survival [180, 181].

ii.) Surgical Assessment of Resectability

The utility of staging laparoscopy (SL) in stratifying patients for resection has been contested. Typically routine SL for all potentially resectable patients has been undertaken. A recent meta-analysis by the Amsterdam Medical Centre (AMC) hepatobiliary group claimed that SL in modern cohorts has limited utility due to the improved sensitivity of multi-slice CT and PET-CT in determining the presence of peritoneal and distant metastases [182]. The MSKCC system has been utilised to stratify patients for suitability to proceed to SL or exploratory laparotomy (EL) [178]. The MSKCC group advocate for a selected utilisation of SL for locally advanced MSKCC stage T2 or T3 patients. This approach risks under-staging small surgically resectable tumours which have already spread in the peritoneum beyond the surgical field. The yields of SL for peritoneal disease vary between 10 – 17 % of all patients undergoing surgical assessment precluding un-necessary laparotomy in this group [183-189]. Laparoscopic intraoperative ultrasound (LIOUS) may provide additional information for determining hilar resectability, particularly with respect to defining radial extension in to surrounding vascular structures, however its utility is yet to be demonstrated in a large modern cohort [190]. Russolillo and colleagues, in the largest studied cohort of biliary tract cancer patients undergoing staging LIOUS, determined that the technique retained utility for gallbladder cancer and intra-hepatic cholangiocarcinoma but not for peri-hilar cholangiocarcinoma patients [191]. Current pre-operative radiographic imaging modalities appear to have comparable sensitivity and specificity to LIOUS for determining hilar vascular involvement and intrahepatic metastases, thereby negating the utility of the technique for discriminating between resectable and un-resectable disease in patients whom have undergone standardised staging approaches [191, 192].

iii.) Exploratory Laparotomy

Exploratory laparotomy (EL) is undertaken following exclusion of disseminated intra-peritoneal disease at SL. R0 resection of the primary tumour confers significant survival benefits compared to R1/R2 resection [193]. The focus of EL is to determine and confirm local resectability of the primary tumour with regards to local vascular invasion, distal biliary duct extension, and intra-abdominal nodal spread.

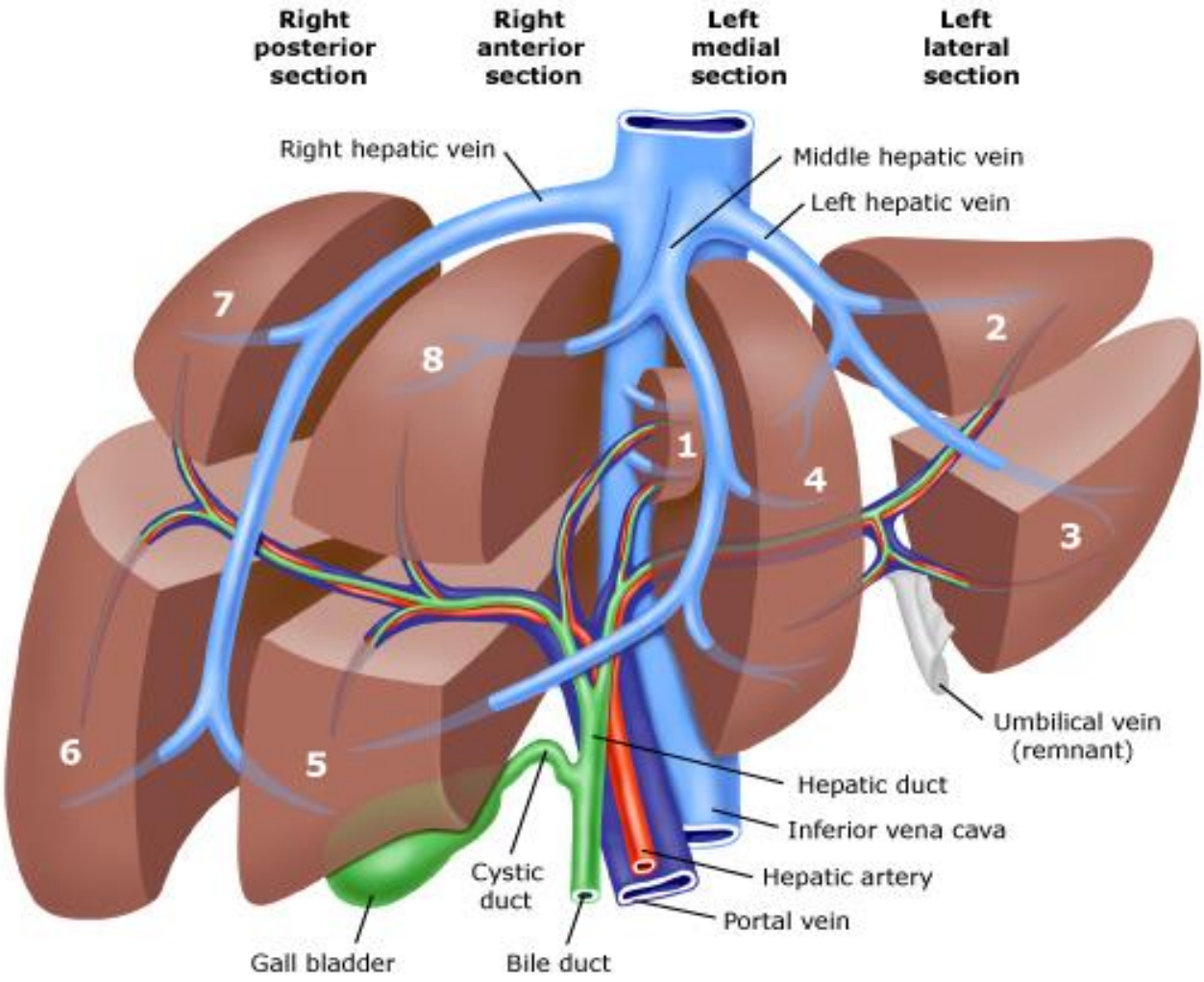
Surgical assessment of vascular invasion includes visual inspection and palpation with intra-operative ultrasound providing evaluation of extension of the tumour in to the hilar vascular structures. Coeliac axis nodes confirmed intra-operatively on frozen section are considered to represent metastatic disease outside of the surgical field and if detected would constitute closure without proceeding to resection.

iv.) Resectional Considerations

Distal bile duct transection occurs early in the resection to ensure adequate access to the hilar vasculature structures. During isolation and transection of the distal margin the specimen routinely undergoes frozen section analysis. Frozen-section analysis is utilised to determine the presence of microscopic disease at the distal resection margin. If there appears to be microscopic invasive disease threatening the resection margin then further excision can be undertaken to ensure adequate R0 resection margins. Patients undergoing re-excision of the distal margin and subsequently achieving a negative frozen section of the new margin appear to have similar survival characteristics to patients who achieved negative margins on the initial frozen section [194]. Carcinoma-in-situ threatening or present at the resection margin does not appear to produce negative effects upon survival and can essentially be considered as being equivalent to a negative frozen section result [195]. Frozen-section has a sensitivity of between 60 - 70 % with a significant number of false-negative and false-positive findings confirmed on subsequent full histopathological assessment [196, 197]. The false-negative results have been putatively linked to the utilisation of pre-operative biliary stenting producing epithelial re-generation at the site of the distal margin [198]. The re-generation of the normal epithelial layer occurs in response to the friction produced by the stent at this site. Frozen-section of the proximal bile duct margin in the hepatic parenchymal resection specimen is not routinely undertaken and appears to confer no added prognostic benefits [199, 200].

Following confirmation of clear distal margins on frozen section attempted resection of the primary tumour can proceed. The hilar bifurcation is located near Couinaud segments 4, 5 and the caudate lobe (See Figure 19).

Figure 19: Schematic Representation of the Couinaud Segmental Anatomy of the Liver [201, 202]



Traditionally, concomitant resection of the caudate lobe is undertaken due to the high proportion of patients with microscopic infiltration of the caudate lobe [203, 204]. Sufficient resection to achieve R0 resection margins are advocated [205, 206]. Extended right hemi-hepatectomy, inclusive of the inferior section of segment 4 (4B) with hilar bile duct excision at the confluence, has been demonstrated to achieve good R0

Figure 20: Schematic Representation of Segments Resected (in black) for Anatomical Right Tri-sectionectomy [145]

resection margins for type 3a disease [207, 208]. The anatomical proximity of the portal vein to the hilar confluence has led to the development of *en bloc* 'no-touch' techniques including resection of the portal vein as necessary [209-211]. *En bloc* resection has been suggested to offer improved survival [210], but may also be associated with increased perioperative mortality [212, 213].

Right-sided trisectionectomy (See Figure 20) is the preferred approach, if feasible, for resecting hilar cholangiocarcinoma. Left-sided approaches for hilar pathology are surgically demanding and reserved solely for predominantly left-sided BC 3b tumours [214]. Principally, the difficulty of the approach relates to the extrahepatic course of the respective portal vein. The right portal vein has a short extrahepatic course which makes reconstruction of the portal vein following left-sided resection difficult [214-216]. In the largest series of left-sided resections currently published left trisectionectomies had significantly superior oncological outcomes and comparative post-operative mortality rates (See Figure 21) [217]. However, the increased complexity of this approach resulted in increased operative times and post-operative morbidity rates.

Left-sided resections for BC 3b tumours are also more likely to involve complex hepatic arterial resection and reconstruction. The right hepatic artery is potentially threatened due to its proximity to the left portal vein and its course within the hilum [218]. Consequently, there is a corresponding increase in potential for post-operative liver insufficiency if the right hepatic artery is encountered during a left-sided resection.

Invasion of the portal vein is reflective of locally advanced cholangiocarcinoma and represents T3/T4 disease. Despite portal vein invasion representing more locally advanced disease, OS in all-comers undergoing resection is comparable to patients undergoing major hepatectomy without portal vein resection (PVR) [193, 219]. However, extent of invasion of the portal vein does appear to impact long-term OS. Post-resectional

Figure 21: Schematic Representation of Segments Resected (in black) for Anatomical Left Tri-sectionectomy [145]

histopathological assessment demonstrating invasion of the portal vein adventitia or intima correlates with OS rates comparable to palliative unresected patients [220]. Increasing the extent of invasion thereby produces a negatively correlating effect on OS. Vascular resection and reconstruction of the hepatic artery appears to confer limited survival benefits although in highly selected patients may be suitable [221, 222].

An important resectional consideration is ensuring an adequate lymphadenectomy field is achieved. Fastidious dissection of the course of the proper hepatic and common hepatic artery, in the hepato-duodenal ligament, to the level 8 lymph node in the retroperitoneum is required to gain an adequate surgical field. Acquisition of lymphatic tissue is technically difficult to achieve and has substantial risk of comorbidity to the patient. Inadequate acquisition of lymphatic tissue, with less than 5 nodes resected, has a detrimental effect on OS due to under-staging of disease [223]. Acquisition of 15 lymph nodes within the resection specimen has been suggested as the optimal lymphadenectomy for accurate staging of disease and subsequent determination of prognosis [224]. However, the optimal number of lymph nodes acquired within the resection specimen rarely reaches this number, with the median number of nodes acquired being between 5 and 10 [225-227]. The ratio of positive lymph nodes to total lymph nodes acquired has been linked to OS and RFS [228, 229].

v.) Post-Operative Staging, Surveillance and Management

Peri-hilar cholangiocarcinoma is histopathologically staged utilising the AJCC 8th edition. The AJCC Tumour, Node, Metastasis definitions are shown below (See Table 11).

Table 11: Tumour Staging Peri-hilar Cholangiocarcinoma

✓	*T Category*	*T Criteria*
	TX	Primary tumor cannot be assessed
	T0	No evidence of primary tumor
	Tis	Carcinoma *in situ*/high-grade dysplasia
	T1	Tumor confined to the bile duct, with extension up to the muscle layer or fibrous tissue
	T2	Tumor invades beyond the wall of the bile duct to surrounding adipose tissue, or tumor invades adjacent hepatic parenchyma
	T2a	Tumor invades beyond the wall of the bile duct to surrounding adipose tissue
	T2b	Tumor invades adjacent hepatic parenchyma
	T3	Tumor invades unilateral branches of the portal vein or hepatic artery
	T4	Tumor invades the main portal vein or its branches bilaterally, or the common hepatic artery; or unilateral second-order biliary radicals with contralateral portal vein or hepatic artery involvement

✓	*N Category*	*N Criteria*
	NX	Regional lymph nodes cannot be assessed
	N0	No regional lymph node metastasis
	N1	One to three positive lymph nodes typically involving the hilar, cystic duct, common bile duct, hepatic artery, posterior pancreatoduodenal, and portal vein lymph nodes
	N2	Four or more positive lymph nodes from the sites described for N1

✓	*M Category*	*M Criteria*
	cM0	No distant metastasis
	cM1	Distant metastasis
	pM1	Distant metastasis, microscopically confirmed

Following resection and histological staging all cases are routinely discussed in Multi-Disciplinary Team Meetings to determine correct post-operative management. During this meeting surveillance and chemotherapeutic approaches are discussed and consensus regarding patient-management reached. Early post-operative recurrence is a common feature of resected peri-hilar patients, particularly if

patients have undergone an R1 resection [230]. R1 resection has been demonstrated to have a comparable survival to patients with borderline resectable disease treated with palliative photodynamic therapy [231]. A significant percentage of post-operative patients develop late recurrence outside the 5 year post-operative period [232]. Long-term follow-up past 5 years is routinely used at the North-West Hepatobiliary Centre.

vi.) Systemic Chemotherapy

Post-operative recurrence can occur in a localised or systemic fashion indicating that radiologically occult micro-metastases occurring outside the surgical field may be common-place in resected patients [233, 234]. Adjuvant chemotherapy is utilised in certain clinical settings to reduce the likelihood of systemic recurrence [235, 236]. The recently reported randomised controlled BILCAP study (for which the North-West Hepato-Biliary Centre was one of the recruiting units) has demonstrated that capecitabine utilised in an adjuvant setting has potential OS and RFS benefits compared to surgery alone [237]. The BILCAP study recruited from March 2006 to December 2014. During recruitment for the BILCAP trial the ABC-02 trial was concluded and reported in 2010 [238]. The ABC-02 trial compared survival characteristics for patients with palliative biliary tract cancer treated with gemcitabine alone versus gemcitabine in combination with cisplatin. Patients were demonstrated to have a significantly increased OS for the gemcitabine-cisplatin group. This protocol has been adapted for the ACTICCA trial to compare survival characteristics of patients undergoing resection and adjuvant chemotherapy (**NCT02170090**). This randomised trial will compare the current adjuvant BILCAP capecitabine protocol to an adapted gemcitabine-cisplatin adjuvant regimen. Despite the promising results from the BILCAP trial the recently reported phase 3 randomised controlled PRODIGE trial comparing a gemcitabine-oxaliplatin (GEMOX) chemotherapy protocol versus surveillance demonstrated no conferred benefit of treatment, despite being well tolerated [239]. Further adequately powered randomised controlled trials assessing adjuvant chemotherapy are required to optimise treatment regimens.

There have been no adequately powered randomised controlled trials for neoadjuvant chemotherapy for treating cholangiocarcinoma. While neoadjuvant chemotherapy has been determined to be safe and well tolerated, these are only sparsely reported non-randomised studies [240, 241]. Neoadjuvant chemotherapy may be utilised as a method of down-staging borderline resectable cholangiocarcinoma facilitating subsequent curative resection, but this should probably be considered as part of a trial protocol [242].

vii.) Palliative Management

The aim of palliative treatment is to manage symptoms and to maintain biliary duct patency to prevent biliary sepsis. Survival in patients with metastatic cholangiocarcinoma is universally dismal. Management of biliary patency is primarily via the use of metal stents which have an improved prolonged patency compared to plastic stents in the palliative setting [164]. Photodynamic therapy (PDT), which utilises focused infra-red light in sensitised patients to prevent primary progression, has been utilised sporadically throughout Europe. However, a phase 3 randomised trial in the United Kingdom did not demonstrate any conferred survival benefits compared to stenting alone [243]. PDT is consequently not practised in the United Kingdom. The ABC-02 trial has demonstrated that Gem-Cis chemotherapeutic protocols produce a 2.7 month OS improvement, with survival for metastatic patients reaching 1 year [238]. Targeted use of biological agents in combination with traditional chemotherapeutic approaches may provide further survival benefits, although there is yet to be any convincing evidence of this at the time of writing [244].

CLINICAL CHAPTER

2.1 Role of Staging Laparoscopy (SL) in the Surgical Stratification of Patients with Peri-hilar Cholangiocarcinoma

2.11) SUMMARY

Background: Cholangiocarcinoma is a rare cancer with a poor prognosis. Radical surgical resection is the only option for curative treatment. Optimal determination of resectability is required so that patients can be stratified into operative or chemotherapeutic treatment cohorts in an accurate and time-efficient manner. SL is utilized to determine the presence of radiologically occult disease that would preclude further surgical treatment. The aim of this chapter is analysing the utility of SL in a contemporary cohort of patients with peri-hilar cholangiocarcinoma.

Methods: Patients diagnosed with potentially resectable peri-hilar cholangiocarcinoma between January 2010 and April 2015 were analysed retrospectively from a prospective database linked to UK Hospital Episode Statistics data. Patients with distal cholangiocarcinoma and gallbladder cancer were excluded from analysis.

Results: A total of 431 patients with peri-hilar cholangiocarcinoma were referred for assessment of potential resection at a supra-regional referral centre. Some 116 patients with potentially resectable disease subsequently underwent surgical assessment. The cohort demonstrated an all-cause yield of SL for unresectable disease of 27.2 per cent (31 of 114). The sensitivity for detection of peritoneal disease was 71 per cent (15 of 21; $P < 0.001$). The accuracy for all-cause non-resection for SL was 66 per cent (31 of 47) with a positive predictive value of progress to resection of 81 per cent (69 of 85). Neither the Bismuth–Corlette nor the Memorial Sloane Kettering Cancer Center preoperative scoring system was contingent with cause of unresectability at SL ($P = 0.461$ and $P = 0.280$ respectively).

Discussion: In the present cohort, SL proved useful in determining the presence of radiologically occult metastatic disease in peri-hilar cholangiocarcinoma.

2.12) Background

Cholangiocarcinoma is a rare cancer arising from epithelial and peri-biliary gland cells lining the biliary ducts and radicals. Mortality rates remain high despite the increasing use of radical surgery [12]. Currently the only curative option is R0 surgical resection of the primary tumour, as systemic chemotherapy has no proven role in the treatment of non-metastatic disease.

Cholangiocarcinoma is staged using the seventh edition of the AJCC staging system [245], which incorporates a standardized TNM classification of the disease [246-249]. Multiple radiological and endoscopic modalities are used to provide an initial determination of both stage and potential resectability of the primary tumour. Case series indicate that 40 per cent of all borderline resectable primary tumours are truly resectable at exploratory laparotomy (EL) [174, 177, 178, 250-254].

SL is important in determining the resectability of the primary tumour, to exclude the presence of radiologically occult peritoneal disease obviating the need for EL and trial dissection. SL, in this context, prevents unnecessary exploratory laparotomy with its associated increased recovery period and delayed return to normal activities of daily living. It also helps stratify patients with un-resectable metastatic disease from those with potentially resectable disease, and expedites prompt referral for palliative chemotherapy.

This study analysed the role of SL in the assessment of resectability of cholangiocarcinoma by assessing a prospective database of a supra-regional tertiary referral centre serving a population in excess of three million people.

2.13) Methods

Patients presenting with potentially resectable peri-hilar cholangiocarcinoma between January 2010 and December 2015 were analysed retrospectively from a prospective database linked to UK Hospital Episode Statistics data. Patients with distal cholangiocarcinoma and gallbladder cancer were excluded from analysis. All case notes were retrieved from an electronic patient database. Radiological images were assessed at the tertiary referral hospital where the supra-regional multidisciplinary team (MDT) meeting was held.

Staging and laparoscopy

All patients were assessed at the authors' centre, as previously described by Gomez and colleagues [255]. The MDT consisted of consultant oncologists, hepatologists, radiologists, liver surgeons and specialist nurses. All previous blood tests, imaging and pre-referral management were discussed on a case-by-case basis. Ongoing investigation and management was explored and further radiological and endoscopic investigations were ordered. Standard staging investigations included transabdominal ultrasonography, multi-slice helical liver CT, magnetic resonance cholangiopancreatography, MRI of the liver, endoscopic retrograde cholangiopancreatography, endoscopic ultrasonography and PET–CT.

The BC classification was used in patients with radiologically proven hilar disease. Discussion of resectability incorporating assessment of longitudinal and radial extension of the tumour, involvement of hilar vasculature and presence of radiologically proven metastases was undertaken. Only patients with radiologically proven metastatic disease were assigned to palliative chemotherapy rather than further surgical investigation. All cholangiocarcinoma's demonstrated to be potentially resectable subsequently underwent SL. In all patients who underwent SL the relevant CT images re-reviewed by a consultant hepatobiliary surgeon blinded to the outcome of staging laparoscopy. The purpose of the radiological review was to determine resectability based on the involvement of vascular structures. No further assessment of peritoneal disease was undertaken at this stage. The prospective MDT radiological assessment of the presence of metastatic disease was deemed to be sufficient for detection of disseminated disease.

SL was performed under general anaesthesia with a two-port approach involving a Hasson open cut-down for the 11-mm infraumbilical port with a left upper quadrant 5-mm port placed to help manipulate intra-abdominal viscera. General inspection of the entire abdominal cavity was performed to exclude peritoneal metastases. A thorough visual inspection of the liver and diaphragm followed by focused assessment of the hepatoduodenal ligament was undertaken. The lesser sac was not routinely opened to inspect the coeliac lymph nodes. All suspicious lesions were biopsied and sent for full histopathological assessment. Histopathological evidence of metastatic disease was used to stratify patients and precluded further exploratory laparotomy and trial dissection. Intraoperative ultrasound imaging was not used in the cohort assessed, and therefore had no impact on approach to staging. No patients underwent peritoneal cytological washings as this technique has limited utility in determining the presence of radiologically and laparoscopically occult disease [256, 257]

Exploratory laparotomy and trial dissection

A thorough visual inspection of the abdominal cavity was undertaken at subsequent EL to determine whether there were peritoneal metastases missed at SL. If peritoneal lesions were discovered, biopsies were taken and assessed by frozen-section examination. If the lesion was proven adenocarcinoma, the abdomen was closed. If the biopsy was determined to be benign, further visual inspection of the liver, hepatoduodenal ligament and coeliac lymph nodes was undertaken. If coeliac lymph nodes were enlarged, biopsies were taken and specimens sent for frozen-section analysis. An examination of the hilum was then undertaken to determine local resectability, with emphasis on viability of the hilar vasculature being the main determinant of progression to resection. All decisions to abandon resection were made in this stepwise manner. All resections at this stage were undertaken with a curative intention-to-treat. All patients deemed to have unresectable disease at this stage were referred for palliative chemotherapy using ABC-02 gemcitabine–cisplatin regimen [239].

Statistical analysis

Patient demographic data, radiological investigations, therapeutic interventions, complications of treatment and operative findings were collated from the collected cholangiocarcinoma patient database and analysed. Yield and accuracy of SL, as defined by Ruys and co-workers [186], were determined. Yield was defined as the total avoided laparotomies, given as a percentage of all laparoscopies undertaken. Accuracy was defined as the total avoided laparotomies, given as a percentage of all unresectable cases. The sensitivity of staging laparoscopy was defined as the total laparotomies avoided for peritoneal metastases over total unresectable cases due to peritoneal disease. The positive predictive value of SL was defined as the percentage of patients completing curative intention-to-treat resection of a lesion considered to be suspicious for cholangiocarcinoma from the total undergoing laparotomy (patients undergoing resection who were correctly deemed by laparoscopy to have truly surgically resectable tumours). χ^2 contingency tables were used for groups containing 30 or more patients, and Fisher's exact test with Freeman–Halt extension for groups with fewer than 30 patients. Yates' corrections were employed in conjunction with χ^2 tests where appropriate. Hypergeometric assessments without replacement were utilized to determine the probability of events occurring following staging laparoscopy. Tests of proportions were used for univariable analysis where appropriate. Prism® version 7.0 (GraphPad Software, San Diego, California, USA) and Excel® 2013 (Microsoft, Redmond, Washington, USA) were used for statistical analysis.

2.14) Results

Radiological assessment and demographics

A total of 431 patients with perihilar cholangiocarcinoma were referred for assessment for resection at a supra-regional referral centre. Some 116 patients considered to have potentially resectable disease underwent surgical assessment of resectability (See Figure 22). One hundred and fourteen patients (98.3 per cent) underwent staging laparoscopy, with two proceeding straight to laparotomy owing to hostile abdomens from previous surgery. Forty-seven of the 114 patients were defined as having unresectable disease; 69 (59.5 per cent) of the 116 patients underwent resection with curative intent, of whom 55 were proven to have cholangiocarcinoma on final histopathological assessment. Two patients were found to have neuroendocrine tumours and 12 had cholangiopathy or benign disease on final histopathological assessment.

Figure 22: Flow Diagram for Potentially Resectable Cholangiocarcinoma Patients Undergoing Staging and Determination of Resectability

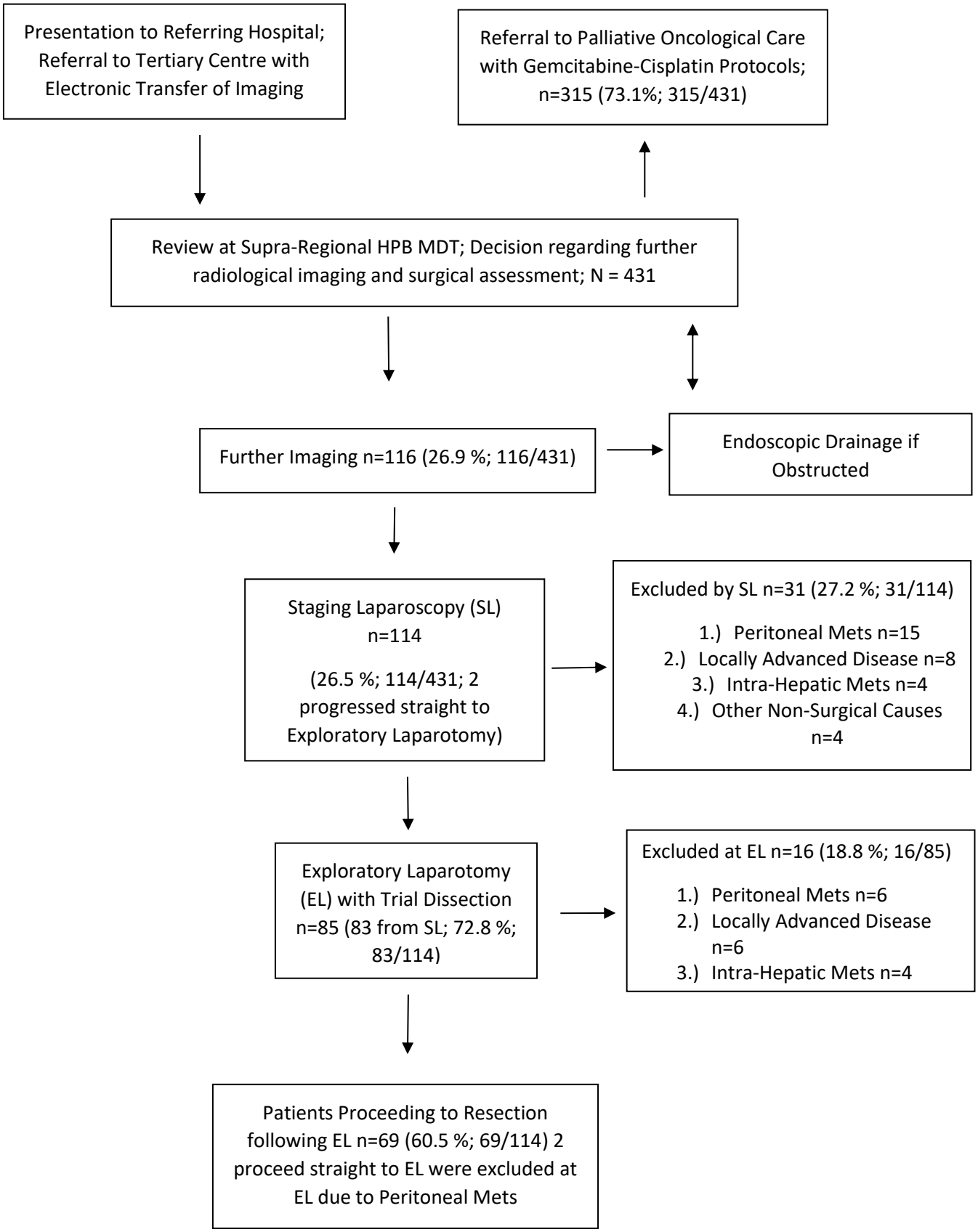


Table 12: Patient Demographics and Presentation to University Hospital Aintree MDT

CHARACTERISTICS	PATIENTS (N=116)	PERCENTAGE
MALE	58	50
FEMALE	58	50
CT	108	93.1
MRI/MRCP	67	57.8
US	43	37.1
PET-CT	3	2.6
ERCP	74	63.8
PTC	2	1.7
JAUNDICE	70	60.3
ABDOMINAL PAIN	20	17.2
DERANGED LFT'S	15	12.9
CACHEXIA	2	1.7
ABDOMINAL MASS	2	1.7
INCIDENTAL RADIOLOGY	2	1.7
GASTRIC OUTFLOW OBSTRUCTION	2	1.7
MEAN AGE	59.7	
95 % CI	56.6 - 62.8	
RANGE	32 - 83	

The patient demographics demonstrated equipoise for sex (See Table 12). Some 108 (93.1 per cent) of the 116 patients had multi-slice helical liver CT (See Table 13). Sixty-seven patients (57.8 per cent) had MRI images available as an adjunct to CT. For 43 patients transabdominal ultrasound images were available as an adjunctive investigatory modality. Only three patients had PET–CT data; they underwent staging laparoscopy, which confirmed the PET–CT findings.

Table 13: Radiological Data Acquired Prior to and Following Full Electronic Regional System Integration

	Pre-2012	Post-2012	P-Value
Patients Surgically Assessed (N)	47	69	0.041
CT Images Available (N)	39	69	0.0004
CT Written Reports (N)	47	69	1.0
MRI Images Available (N)	17	50	0.0001
MRI Written Reports (N)	29	60	0.0002
No MRI Information on System (N)	18	9	0.002

Surgical assessment of the abdomen

Staging laparoscopy (SL)

One hundred and fourteen patients underwent staging laparoscopy, of whom 31 were deemed to have unresectable disease, producing a yield of 27.2 per cent. Fifteen of these patients were found to have peritoneal metastases, eight were deemed to have locally advanced disease, and four patients were demonstrated to have bilobar intrahepatic metastases (See Table 14).

Table 14: All-Cause Distribution of Cases Precluded from Undergoing Resection

Characteristics	Laparoscopy Only	Laparotomy Open/Close	Total	Sensitivity Laparoscopy (%)
Peritoneal Mets	15	6	21	71.4
Locally Advanced Disease	8	6	14	57.1
Intra-Hepatic Mets	3	4	7	42.9
Other	5	0	5	n/a
Intra-Hepatic Cholangiocarcinoma	11	2	13	84.6
Hilar Cholangiocarcinoma	20	14	34	55.9

All 15 of the patients with peritoneal metastasis observed at SL had this confirmed on histopathological assessment. EL found six patients with peritoneal metastases confirmed on frozen-section analysis,

producing a sensitivity for detection of peritoneal metastases at staging laparoscopy of 71 per cent (15 of 21) ($P < 0.001$, χ^2 test with Yates' correction).

Eight patients were deemed to have locally advanced disease precluding exploratory laparotomy. Three of these patients had liver atrophy resulting from portal vein encasement, two of which were demonstrable on helical liver CT and MRI, with one patient demonstrating a mass but no encasement of vessels or lobar atrophy on helical liver CT. One patient had invasion of the inferior vena cava, which was demonstrated partially on the helical liver CT, with the lesion also encasing the right hepatic vein. Two patients had invasion into the omentum and colon, both of which were suspicious findings on preoperative helical liver CT. One patient had invasion into the duodenum that was not visible on preoperative imaging. Laparoscopic biopsies from two patients with bilobar metastases showed positive histopathology for adenocarcinoma. There was one infective complication of staging laparoscopy, presenting with an infected infraumbilical port site and classified as a Clavien–Dindo grade II postoperative complication [258].

EL (open/close)

Forty-seven of the 116 patients were deemed to have unresectable disease after both SL (31 patients) and EL (16), producing an accuracy for staging laparoscopy of 66 per cent (31 of 47). Sixty-nine patients completed curative resection, producing a positive predictive value for laparoscopy of 81 per cent (69 of 85).

Of the 16 patients deemed to have unresectable disease at EL, six had peritoneal metastases and six were demonstrated to have locally advanced disease. Of the six patients with peritoneal disease demonstrated on exploratory laparotomy, two also had coeliac node disease, two had isolated hepatoduodenal ligament metastases and two were found to have small-bowel mesentery metastases. In two of the patients with metastases detected at EL, the metastases had been visualized at SL by the surgeon; both of these lesions had been biopsied, but at histopathological assessment were considered to be benign. The two patients with small-bowel mesentery metastases were deemed to have had metastases undetectable at SL owing to their anatomical location. The two patients with concomitant coeliac node disease had metastases throughout the abdomen; these were determined as true failures of SL. The six patients with peritoneal metastases found at EL had a hypergeometric probability of unresectability owing to peritoneal disease following SL of 15 per cent. The presence of radiologically occult peritoneal disease in the present cohort was detected in 21 of 116 patients, accounting for 21 of the 47 unresectable cases ($Z = 1.71$, $P = 0.088$).

SL had a failure rate of six of 14 for detection of locally advanced disease, and deemed only one of the six patients detected at EL as having suspicion of hilar vascular compromise. Two of the unresectable cases related to the lack of palpability of a sufficient vascular supply, and one was related to an inability to kocherize the duodenum. The failure of SL to detect locally advanced disease produced a hypergeometric probability of having locally advanced disease precluding resection at EL following SL of 12.8 per cent.

There were two complications in patients who underwent EL without progression to resection. One patient developed a complicated urinary tract infection with sepsis on day 2 after surgery that was secondary to per-urethral catheterization, requiring 5 days of intravenous antibiotics (Clavien–Dindo grade II complication). The other patient had a myocardial infarction on the second postoperative day that required intensive care support, and died on the fourth postoperative day (Clavien–Dindo grade V complication).

Distribution of unresectable hilar cholangiocarcinomas stratified by classification systems

Thirty-four hilar cholangiocarcinomas were deemed unresectable at combined operative staging (SL plus EL). Failure to progress to resection was due to peritoneal metastases in 14 patients, local advancement in 14, and the presence of intrahepatic metastases in four patients (See Table 15).

Table 15: Distribution of Cases Precluded from Resection Stratified by Bismuth-Corlette System

BC CLASSIFICATION	PERITONEAL DISEASE	LOCALLY ADVANCED	INTRA-HEPATIC METS	OTHER
1	4	5	0	0
2	3	2	1	1
3A	4	0	1	0
3B	2	4	2	1
4	1	3	0	0

The BC classification system stratifies patients by longitudinal extension of the tumour along the biliary ducts and radicals (See Figure 17, p. 51). In the present series no contingency was found between the Bismuth–Corlette classification and operative stage (either SL or EL) at which unresectability was determined ($\chi^2 = 4.79$, $P = 0.310$). There was also no contingency between the Bismuth–Corlette classification and reason for subsequent unresectability ($\chi^2 = 11.8$, $P = 0.462$).

The Memorial Sloan Kettering Cancer Center (MSKCC) hilar cholangiocarcinoma scoring system [177, 178] is a preoperative radiological scoring system that predicts resectability on the basis of longitudinal and radial extension of the primary tumour into the hilar structures (See Figure 18, p.52). It is specifically related to unilateral extension along biliary radicals and is applied only to Bismuth–Corlette types II, IIIa, IIIb and IV. The MSKCC score allocates a 'T' score from 1 to 3, with T1 being the least extensive through to T3 being the most extensive. In the present series, 25 (74 per cent) of the 34 hilar cholangiocarcinomas were classified via this scoring system. The ten determined to be T1 tumours included six patients with peritoneal disease, one with local advancement precluding further surgery, and two with intrahepatic metastases.

The three T2 cases consisted of one patient with intrahepatic metastases, one with peritoneal disease, and one patient with locally advanced disease. The 12 T3 cases included six patients with locally advanced, four

with peritoneal disease, and one with intrahepatic metastases (Table 16). In an all-cause analysis of determination of unresectability, there was no contingency with operative stage ($P = 0.280$, Fisher's exact test). Subgroup analysis demonstrated a relationship between T3 status and preclusion from resection by local advancement ($Z = 2.53$; $P = 0.015$). There was also a dependence between T1 status and preclusion from resection owing to detection of peritoneal metastases at laparoscopy ($Z = 1.19$; $P = 0.069$).

Table 16: Distribution of Cases Precluded from Resection by Cause and Blumgart-Jarnagin Score

Characteristics	Peritoneal Disease	Locally Advanced	Intra-Hepatic Mets	Other
T1	6	1	2	1
T2	1	1	1	0
T3	4	6	1	1

2.15) Discussion

This study suggests that SL is useful to stratify patients for further surgical treatment or palliative chemotherapy. In the specific context of detecting radiologically occult peritoneal disease, it had particular utility in the present cohort.

SL appears to be a poor modality for determining locally advanced disease. Contraindications to resection relating to local advancement are unlikely to be determined at SL because the judgement of resectability is dependent upon the operator's 'hands-on' assessment. The decision not to proceed to resection due to local advancement should therefore be made explicitly at EL. This could possibly result in an increase in open-and-close EL. However, potentially this increase could be offset by patients who previously would have been deemed to have unresectable disease due to local advancement at laparoscopy, with no peritoneal disease, progressing to resection at EL following a trial dissection. Intraoperative ultrasound imaging is an alternative modality for detection of locally advanced disease, and may have advantages over the traditional two-stage approach of SL and EL because it may reduce the need for EL. However, intraoperative ultrasonography is yet to be validated formally in a large robust prospective study of hilar cholangiocarcinoma.

This study has demonstrated that the BC classification has no negative predictive value for resectability, supporting the consensus in the literature that this system should be used only to describe the anatomical location of the primary tumour and should have no influence in determining the likelihood of progression to resection [259]. The MSKCC system has utility in predicting the likelihood of progression to resection when stratifying cholangiocarcinoma's by local advancement, and accurately describes features that represent un-

resectable disease. However, the MSKCC score does not incorporate any features that determine the likelihood of the presence of peritoneal metastases, with a considerable number being distributed in the T1 category in the present series. Accordingly, the MSKCC scoring system is useful in patient-centred preoperative planning at the EL stage, but has little relevance to stratifying patients at the SL stage.

A recent meta-analysis [182] raised the possibility of stratifying patients for SL owing to the improved sensitivity of different radiological modalities, particularly PET–CT, in detecting peritoneal disease [182]. The pooled sensitivity of yield for SL was 24 per cent, which is comparable to that found in the present series of 27.2 per cent. The authors noted that, in the more recent series subjected to quantitative analysis, the all-cause yield of SL tended to be significantly lower. The present study has demonstrated that the utility of SL is in determining the presence of radiologically occult peritoneal disease, as SL has poor utility in identifying local advancement or intrahepatic metastasis. In one of the most recent series in which the all-cause yield of SL was demonstrated to have decreased from 40 to 14 per cent, the sensitivity of detection of peritoneal disease remained high at 72 per cent, which compared favourably to that in the present cohort of 71 per cent [183]. SL directly prevented 15 laparotomies due to peritoneal disease, thereby enabling these patients to be stratified to palliative chemotherapy in a time-efficient manner and not to be exposed to the risk of complications associated with EL. Sensitivity of detection of peritoneal metastases is the quantifiable standard by which the utility of SL should be judged.

2.2 Role of a Pre-Operative Radiological Scoring System in Determining Resectability for Potentially Resectable Hilar Cholangiocarcinoma

2.21) SUMMARY

Background:

Case series indicate that 25 - 40 % of all borderline resectable primary peri-hilar tumours are potentially resectable. The Memorial Sloane Kettering System (MSKCC) stratifies patients for resectability by longitudinal and radial extension of the hilar tumour. The Bismuth-Corlette (BC) system describes the longitudinal extension of the tumour within the biliary duct system. This thesis sought to validate and, if possible, augment these two scores within an independent validation cohort.

Methods:

Patients diagnosed with hilar cholangiocarcinoma between January 2009 and December 2016 were analysed from a prospectively held database. Patients with distal cholangiocarcinoma, peripheral cholangiocarcinoma and gallbladder cancer were excluded. Comparison of surgical findings to pre-operative radiological imaging was undertaken at the time of surgery.

Results:

The validation cohort was formed of 198 patients, of which, 55 (27·8 %) patients underwent resection. Logistic regression analyses identified that BC score, MSKCC score, age at diagnosis and left artery involvement were all significant independent predictor's univariately. BC score explained 28 % of the variability in resectability compared to 26 % explained by MSKCC. In combination, the model consisting of BC score, age at diagnosis and left artery involvement explained 39% of variability in resectability compared to the 34 % explained same model including MSKCC score instead of BC score.

Discussion:

In this cohort an augmented BC score, incorporating left hepatic artery involvement, is more discriminative in predicting resectability than the current MSKCC system.

2.22) Background

Cholangiocarcinoma is a rare cancer arising from the biliary tree case-series indicate that 25 - 40 % of all borderline resectable primary tumours are potentially resectable [174, 177, 182, 250-254]. Currently the only curative option is R0 surgical resection of the primary tumour [12].

Two systems are used to describe anatomical location of the primary tumour. The Bismuth-Corlette (BC) classificatory system stratifies patients by longitudinal extension of the tumour along the biliary ducts and radicals (Figure 17; p.51). Series have demonstrated that the BC system has limited utility in determining potential resectability [148, 176, 260]. The Blumgart-Jarnagin Memorial Sloane Kettering System (MSKCC), initially proposed in 1997 and subsequently modified, stratifies patients for resectability by longitudinal and radial extension of the hilar tumour (Figure 18; p.52) [176, 177]. Radial extension is determined by the extension of tumour in to the hilar vascular structures. The MSKCC system also utilises surrogate indicators of radial tumour extension, such as ipsilateral or contralateral lobar atrophy, to infer resectability. The MSKCC system has been internally validated but lacks external assessment of validity.

Primary end-point of study was to determine the utility of the MSKCC system in predicting resectability within a large European cohort of hilar cholangiocarcinoma patients.

Secondary end-point of study was to determine if there were any novel co-variates which could be utilised to augment either of the scoring systems to improve their predictive accuracy.

2.23) Methods:

Inclusion Criteria

Patients diagnosed with hilar cholangiocarcinoma, referred to a supra-regional tertiary referral centre between January 2009 and December 2016, were extracted from a prospectively held database linked to Hospital Episode Statistics data. Patients with distal cholangiocarcinoma, peripheral (true intrahepatic) cholangiocarcinoma and gallbladder cancer were excluded from analysis. Central mass-forming tumours, demonstrated on radiological imaging, which appeared to be predominantly intra-hepatic malignancies were excluded from analysis. All patients with computed tomography (CT) or endoscopic evidence of tumour originating at the biliary confluence and extending in to the biliary radicles were included. Any patients with tumour arising in the common hepatic duct were included if the tumour extended to the confluence or in to the radicles.

Multi-disciplinary Team Assessment

All patients were assessed at our centre as previously described [6, 255]. The Bismuth-Corlette (BC) classification was applied to patients with radiologically proven hilar disease as a means of description of anatomical location. Allocation of the MSKCC resectability score was undertaken independently by a

Consultant Radiologist and a Consultant Hepato-biliary Surgeon retrospectively and blinded to prior outcomes. Comparison of surgical findings to pre-operative radiological imaging was undertaken at the time of surgery.

Discussion of potential resectability incorporating longitudinal and radial extension of the tumour, involvement of hilar vasculature and presence of radiologically proven metastases was undertaken. Encasement of the hilar structures precluded surgical assessment and was defined as representing in-operable locally advanced disease. Patients with these radiological characteristics were allocated to palliative treatment for locally advanced disease. Involvement of any vascular structure was considered to occur when greater than 180 degrees of the vessel were incorporated by the tumour. Qualitative assessment of lobar atrophy was undertaken. Comparison to previous CT scans was undertaken to determine chronology of lobar atrophy. Functional volumetric assessment of liver volume was undertaken on all patients considered to be potentially resectable. Selective portal embolization techniques are undertaken on patients with a functional liver remnant of less than 30 %. No volume augmentation procedures were undertaken due to concerns regarding qualitative function of the hypertrophied liver remnant.

Nodal status was not used to stratify patients to palliation pre-operatively unless there were concomitant metastases present. Patients considered not fit for surgery or chemotherapy were excluded from further analysis. Radiologically proven metastatic disease stratified patients for palliative gemcitabine-cisplatin chemotherapy as per ABC-02 trial [238], rather than further surgical investigation.

Surgical Assessment

All patients considered potentially resectable underwent surgical assessment to stratify patients for resection as described by Bird and colleagues [6]. Patients deemed un-resectable at this stage were referred for palliative chemotherapy [238]. Intra-operative frozen-section was utilised routinely on all attempted resections. Negative margins on frozen section were considered to indicate successful resection of the tumour at the time of surgery. Full histo-pathological assessment of the specimens was undertaken to determine resection status (R0/R1). R1 resection status was defined as microscopic involvement of the longitudinal and circumferential resection margins. Only patients with histo-pathologically proven cholangiocarcinoma were included for analysis.

Statistical Analysis

Resection was treated as a binary outcome, coded as 1 if resection occurred and 0 if it did not. Logistic regression modelling was undertaken to determine correlation between covariates and resection status.

Both the BC and MSKCC scores were treated as factors, with the highest severity of score being set as the reference factor. Demographic covariates included age at diagnosis (measured in years), sex (coded as 1 if

male; 0 if female). Binary indicators were used for the involvement of the following: right artery, left artery, hepatic artery, hepatic veins, main portal vein, right portal vein, left portal veins. Binary indicators were also used for ipsilateral lobar and contralateral lobar atrophy.

All analyses were performed in the software package R (R Foundation for Statistical Computing, Vienna, Austria) with the library packages MASS (Modern Applied Statistics with S. 4th edition) and RMS (Regression Modeling Strategies, https://CRAN.R-project.org/package=rms).

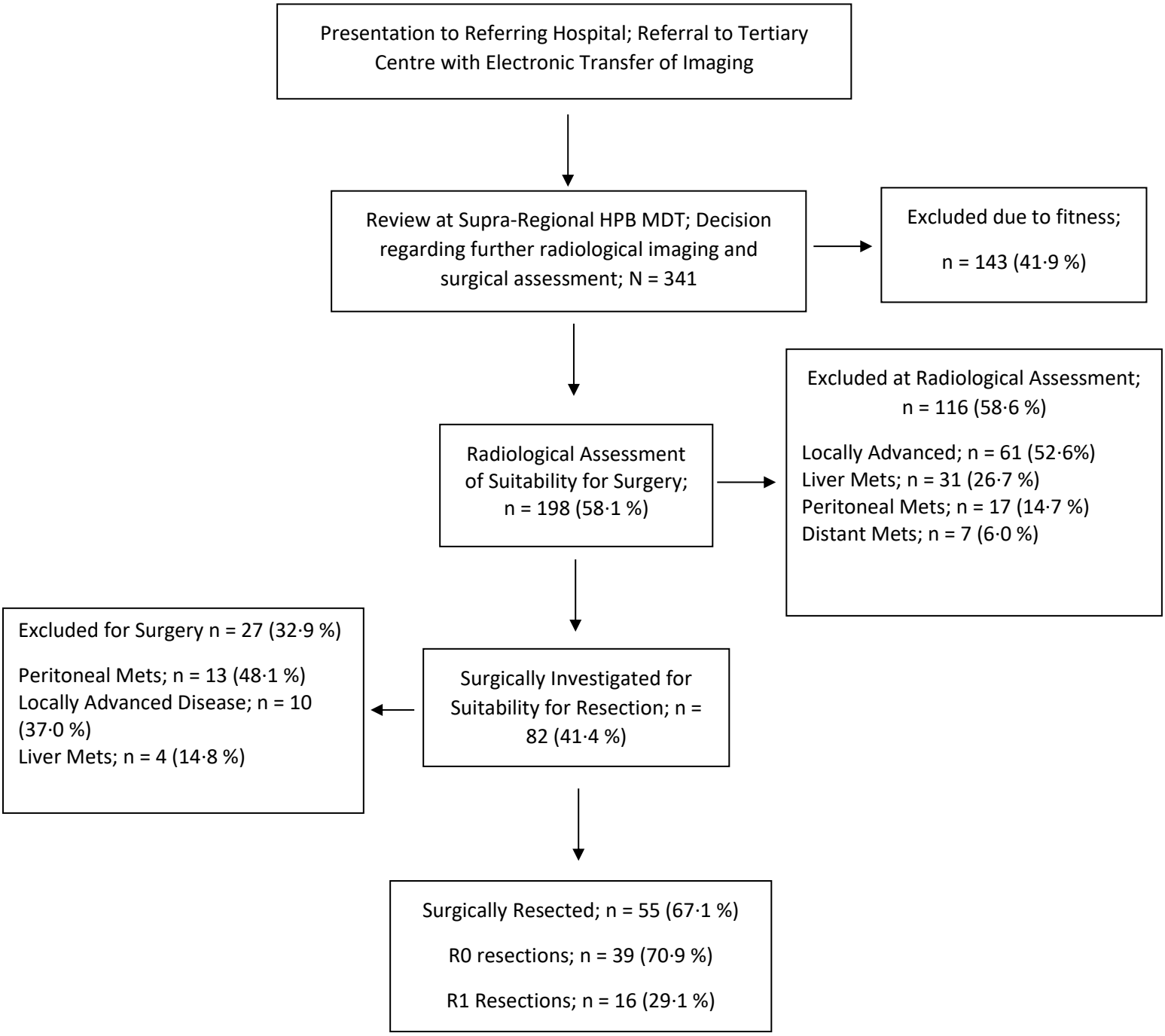
For logistic regression analyses, covariates were investigated against outcomes univariately. Covariates with suggestive significance ($p<0.1$) were combined in a multivariable model and stepwise selection was utilised to derive the maximal model for each outcome. The pseudo-R^2 of models was calculated using the log likelihoods of alternative compared to null models.

2.24) Results

Patient Stratification and Demographics

The results of the MDT assessment are shown in Figure 23. A total of 341 patients records were retrieved of those with peri-hilar cholangiocarcinoma who were referred un-selected (all-comers) for assessment for resection at a supra-regional referral centre. All patients had computed tomography (CT) imaging of the hepato-biliary system.

Figure 23: Flow Diagram Demonstrating MDT Assessment of Hilar Cholangiocarcinoma Patients



Of these 341 patients record, 115 indicated the patient as being medically unfit for either surgical assessment

Table 17: Demographics, resectability scores and anatomical covariates of study population

Covariate	Descriptor
Sex	Male: 104 (52%) Female: 95 (48%)
Age	Mean ± SD: 68.3 ± 10.4 years
BC Classification	1: 29 (14%) 2: 33 (16%) 3A: 37 (19%) 3B: 43 (22%) 4: 57 (29%)
Radiological MSKCC Score	N/A: 29 (14%) T1: 51 (26%) T2: 45 (23%); T3: 74 (37%)
Right Artery	Not Involved: 175 (88%) Involved: 24 (12%)
Left Artery	Not Involved: 152 (76%) Involved: 24 (24%)
Hepatic Artery	Not Involved: 192 (96%) Involved: 7 (4%)
Hepatic Veins	Not Involved: 198 (99%) Involved: 1 (1%)
Portal Vein Main	Not Involved: 191 (96%) Involved: 8 (4%)
Right Portal Vein	Not Involved: 174 (87%) Involved: 25 (13%)
Left Portal Vein	Not Involved: 146 (73%) Involved: 53 (27%)
Ipsilateral Lobar Atrophy	Not Involved: 144 (72%) Involved: 55 (28%)
Contralateral Lobar Atrophy	Not Involved: 189 (95%) Involved: 9 (5%)

or palliative treatment. Further, 36 patient records were identified to be serial entries for those patients who had been referred more than once, for these, the latter entry was removed. Ten patients were removed due to having an incomplete set of covariates. The remaining 198 patients were assessed for suitability for surgery. The demographics, resectability scores and anatomical covariates are presented in Table 17.

Twenty-nine patients with BC class 1 anatomy demonstrated on CT radiography were excluded from MSKCC sub-group analysis due to non-involvement of the biliary duct confluence. One hundred and seventy patients were stratified to the MSKCC sub-group.

Resected Cohort

Eighty-two (41·4 %) patients considered to have potentially resectable disease underwent surgical assessment of resectability (Figure 23; Table 18). Of these 82 patients, 55 (67·1 %) went onto to have attempted curative resections. Potentially curative R0 resection was achieved in 39 (70·9 %) patients. Potentially curative R0 resection was therefore achieved in 19·7 % (39/198) of the unselected MDT cohort. There were no R2 resections. Sixteen (29·1 %) of the patients underwent vascular resection and reconstruction. There were 13 portal vein reconstruction's (PVR) and 3 inferior vena cava re-construction's (IVCR) with 2 of the cases consisting of concomitant PVR and IVCR. Seven PVR's (46·7 %) resulted in R0 resection compared to 32 of the 39 (82·1 %; p = 0·04; χ^2) patients undergoing hepatectomy alone.

Mortalities in Resected Cohort

Table 18: Cases Stratified by System Category

System Category	(N) Discussed at MDT	Explored (n; %)	Resected (n; %)
T1	51	34; 66·7	19; 36.5
T2	45	29; 64·4	27; 60.0
T3	74	12; 16·2	9; 12·2
BC 1	29	7; 17·2	0
BC 2	33	12; 36·3	11; 33.3
BC 3A	37	26; 70·3	15; 40.5
BC 3B	43	27; 62·8	22; 51.2
BC 4	57	10; 17·5	8; 14.0

There were 6 30-day mortality's producing a 30-day mortality rate of 10·9 % (49/55). There was 1 mortality at 33 days, 26 days following initial discharge and 8 days following re-admission, producing an overall 90 day mortality rate of 12·7 % (48/55). Five of the 7 patients who died before 90 day's post-op had PVR's (See Table 19). Of these 5 patients only 1 PVR had pre-operative radiology demonstrating portal vein involvement (PVI). Only 2 of the 40 patients (5·0 %) whom underwent extrahepatic biliary duct resection and hepatectomy without PVR suffered a 90 day post-operative mortality (p = 0.02; χ^2).

Table 19: Table Demonstrating 30/90 Day Mortality for Resected Cohort

Gender	Age at Operation	Radiological MSKCC Score	BC Classification	Operation	Vascular Reconstruction	Complication	Re-Intervention	Survival (Days)	Cause of Death
M	47	T1	3a	Right Tri + Caudate	Portal vein	P.V.* + H.A.** Thrombosis	Laparotomy	8	Liver Failure
M	70	T1	2	Right Tri + Caudate	N/a****	G.A.*** Branch bleed	2 x Laparotomy	14	Liver Failure
M	68	T3	4	Right hemi + Caudate	Portal Vein	P.V. Thrombosis	N/a	6	Liver Failure
F	61	T1	2	Right Tri + Caudate	Portal Vein	Post-op Bleed	Laparotomy	21	Chest Sepsis
F	69	T1	3A	Right Tri + Caudate	Portal Vein	P.V. Thrombosis	Radiological	9	CVA*****
M	53	T1	3A	Right hemi + Caudate	N/a	Intra-operative Haemorrhage	Laparotomy	4	Multi-Organ Failure
M	71	T2	3b	Left hemi + Caudate	Portal Vein	Chest Sepsis	N/a	33	Chest Sepsis

***Abbreviation -** Portal Vein**; **Abbreviation –** Hepatic Artery**; ***Abbreviation –** Gastro-Duodenal Artery**; ****Not Applicable; *****Abbreviation –** Cerebro-Vascular Accident

Factors Influencing Resectability

Univariate logistic regression of demographic, resectability scores and anatomical covariates against resectability demonstrated that contralateral lobar atrophy was significantly negatively associated with resection. Age at diagnosis, BC score, MSKCC score, and left artery involvement were all suggestively associated with resection (See Table 20).

For multivariable logistic regression, we began with two different base models, one with BC score and one with MSKCC score due to the correlation of the two scores (Spearman's rho 0.77, $p<0.001$). The pseudo-R^2 of the BC and MSKCC base models was 0.28 and 0.26, indicating the models explain 28 % and 26 % of the variability in resectability respectively. In comparison to the reference value of BC score of 4, a BC score of 3A ($\beta=2.02$; $p<0.001$) and 3B ($\beta=1.73$; $p<0.001$) were both associated with increased resectability. Similarly, in comparison to the reference value of MSKCC score of 3, a MSKCC score of 2 ($\beta=1.78$; $p<0.001$) and 1 ($\beta=1.36$; $p=0.003$) were both associated with increased resectability.

Using the BC score base model, stepwise selection included age at diagnosis ($\beta=-0.06$; $p=0.004$) and left artery involvement ($\beta=-1.41$; $p=0.006$) in the final model (pseudo-$R^2=0.39$). Using the MSKCC score base model, stepwise selection included age at diagnosis ($\beta=-0.06$; $p=0.001$) in the final model (pseudo-$R^2=0.34$).

Table 20: Univariate Logistic Regression of the Resected Outcome

Covariate		β Effect Size	Standard Error	p-value
Sex		-0.0035	0.063	0.95
Age		-0.014	0.0028	<0.0001
BC Classification (Reference: 4)	**1**	-0.12	0.092	0.18
	2	0.089	0.088	0.31
	3A	0.39	0.085	<0.0001
	3B	0.32	0.081	0.0001
Radiological MSKCC Score (Reference T3)	**T1**	-0.14	0.090	0.14
	T1	0.35	0.074	<0.0001
	T2	0.24	0.077	0.0018
Right Hepatic Artery		-0.06	0.096	0.53
Left Hepatic Artery		-0.12	0.073	0.10
Hepatic Artery		-0.27	0.17	0.11
Hepatic Veins		-0.26	0.44	0.55
Portal Vein Main		-0.14	0.16	0.37
Right Portal Vein		-0.021	0.094	0.82
Left Portal Vein		-0.022	0.071	0.76
Ipsilateral Lobar Atrophy		-0.11	0.070	0.12
Contralateral Lobar Atrophy		-0.28	0.14	0.05

2.25) Discussion

This study validated the use of both MSKCC and BC scores in an external cohort, demonstrating that BC score is the stronger predictor of resectability within our cohort. This study has identified a novel anatomical co-variate, left hepatic artery involvement, to augment the predictive capabilities of the BC score.

Blumgart and co-workers derived the MSKCC resectability score to improve surgical determination of resectability which was initially based on the Bismuth-Corlette system [177, 178]. However, in this cohort both scoring systems demonstrate utility in pre-operatively stratifying patients for likelihood of resection. Both systems also explain a comparable percentage of the variability in resectability in this cohort.

The augmented BC system incorporating left hepatic artery involvement substantially increases the quantification of variability in resectability. Left-sided tumours appear to represent more challenging disease to surgically resect than right-sided disease [209, 261]. Typically the extrahepatic course of the right hepatic duct, portal vein and hepatic artery are comparatively shorter than the left-sided biliary duct/vasculature complex. Resection of the left-sided system leaves a shorter right-side vascular pedicle to isolate, resect and re-construct. The associated increased complexity of resection of the left-sided hilar anatomy reduces the resectability of tumours involving these structures. Left hepatic artery involvement may potentially be a surrogate indicator of radial extension due to its typically medial spatial relation to the left portal vein [262].

Limitations of radiological sensitivity affect the utility of both systems by potentially pre-operatively under-staging disease [263-266]. This is a particularly important consideration when un-expected PVI is determined at exploratory surgery. PVR is a technically demanding procedure undertaken to ensure adequate R0 resection margins in patients with locally advanced disease [220, 267]. While R0 resection is feasible in this subset of patients, comparison with patients undergoing R0 resection without PVR demonstrates that it is significantly less achievable [268]. Patients undergoing hepatectomy and PVR compared to hepatectomy alone are historically considered to have significantly increased risk of mortality [193]. This correlates with the experience of patients within this cohort. Four mortalities occurred in patients undergoing PVR and hepatectomy with no PVI demonstrated on pre-operative imaging. Unexpected PVI in potentially surgically resectable patients appears to thereby confer a substantial risk of mortality. For patients without obvious PVI on pre-operative imaging utilising the augmented BC-left hepatic artery system as an adjunct stratifying system may improve predictability of resection and improve peri-operative outcomes.

Age has been identified as a factor predicting un-resectable disease within this series. Age can act as a surrogate marker for frailty and co-morbidities. A clinical review of oncological practice has suggested that chronological age alone has been used to inappropriately limit treatment offered to older patients [269]. In this series we excluded patients deemed unfit for surgical and chemotherapeutic treatment, following MDT discussion, from analysis (Figure 23; p.75). Age retained significance in the multivariate model despite this

exclusion criteria. Older people may potentially be presenting to MDT with later-stage or more aggressive disease precluding surgical treatment.

Hilar cholangiocarcinoma is a rare cancer with the majority of patients presenting with locally advanced or metastatic disease precluding surgical treatment. Many of the reported cohorts are small and historical with even relatively high volume centres rarely operating on more than 1 patient per month with hilar cholangiocarcinoma [270]. DeOliveira and colleagues have established a European multinational registry in an attempt to develop a significant international cohort and to standardise prospective reporting of hilar cholangiocarcinoma management and outcomes [271]. Provisional analysis of the data collated since inception indicates that increased co-operation and concordance is required to validate the utility of the registry [31]. The Extrahepatic Biliary Malignancy Consortium (EBMC), a multi-institutional collaborative group in the United States, has attempted to pool independent cholangiocarcinoma registries to facilitate and co-ordinate robust clinical research and has published extensively predominantly on post-surgical prognostic factors [32, 33]. Cholangiocarcinoma patients in the United Kingdom, with its unified National Health Service (NHS) and nationally standardised cancer referral pathways, could potentially benefit from a co-ordinated and collaborative approach to cholangiocarcinoma treatment and research.

This study has externally validated the utility of both the MSKCC and BC scoring systems for pre-operatively stratifying patients for potential resection. It has also provided a potential novel anatomical co-variate which could be used to augment scoring systems to increase predictive accuracy. However, acknowledgement of both systems limitations is required, particularly regarding the limitations of radiological imaging sensitivity which affect its utility by potentially pre-operatively under-staging disease.

2.3 Prognostic Factors for Overall Survival in Resected Hilar Cholangiocarcinoma Patients: A Systematic Review and Meta-analysis

2.31) SUMMARY

Background

Hilar cholangiocarcinoma is staged using the American Joint Committee on Cancer staging system. Numerous other prognostically important histo-pathological and demographic characteristics have been reported. The objective of this meta-analysis is to statistically assess the effect of post-resectional tumour characteristics upon the overall survival (OS) for patients undergoing attempted radical curative resection for hilar cholangiocarcinoma.

Methods

Search terms were employed on OVID MEDLINE and PUBMED search engines articles capture. Temporal limitations for study eligibility were defined as any studies published between 2009 and 2017. Papers referring to intrahepatic or distal cholangiocarcinoma were excluded from review. Data extraction utilised standard Parmar modifications to determine pooled univariate hazard ratios (HR's).

Results

Twenty-four articles, containing 4599 patients, were quantitatively assessed. Significant prognostic factors (Pooled HR [95% CI]) were: Age (HR 1.16 [1.04 - 1.28]), 'T' stage (HR 1.49 [1.30 - 1.70]), lymph node involvement (HR 1.78 [1.65 - 1.93]), microvascular invasion (HR 1.49 [1.34 - 1.68]), peri-neural invasion (HR 1.54 [1.40 - 1.68]) and tumour differentiation (HR 1.54 [1.38 - 1.72]) had significant effects with low heterogeneity. Portal vein resection (HR 1.54 [1.38 - 1.72]) and resection margin status (HR 1.77 [1.57 - 1.99]) had significant effects with high heterogeneity. Sex, tumour size and pre-operative CA 19-9 levels did not have a statistically significant ($p<0.05$) effect on post-resectional prognosis.

Discussion

Several tumour biological variables, not included in the AJCC 7th edition, affect OS and require incorporation in to prognostic models to ensure a personalised approach to prognostication and treatment.

2.32) Background

Cholangiocarcinoma is a rare cancer arising from epithelial and peri-biliary gland cells lining the biliary tree. Prognosis for patients remains dismal, with only R0 surgical resection of the primary tumour considered potentially curative treatment [12].

Cholangiocarcinoma is routinely staged utilising the American Joint Committee on Cancer (AJCC) staging system which incorporates a standardised TNM classification of the disease [245, 247, 248]. Staging provides prognostic information and allows comparison of survival rates between reported series. The AJCC staging system 7th edition published in 2009 amended the 6th edition primarily by establishing hilar cholangiocarcinoma as a separate diagnostic category from distal cholangiocarcinoma. The 7th edition also stratified and refined the criterion for radial depth of tumour invasion. Radial extension of tumour beyond the bile duct (T2) was upstaged from Stage 1b to Stage 2. Vascular invasion of the portal vein or hepatic artery (T3) and positive lymph node status were also upstaged from Stage 2 to Stage 3 disease. The AJCC staging system 8th edition, published in January 2017, amended the 7th edition by down-staging T4 tumours from Stage 4a to 3b due to new considerations of the achievability of R0 resection by undertaking caudate hepatectomy and concomitant vascular resection and reconstruction. The 8th edition is yet to be validated in a large resectional cohort.

Apart from the AJCC 7th edition several other prognostically important histopathological and demographic characteristics have been reported, but their precise impact on outcome after surgery is unclear with many seemingly contradictory reports. This study aimed to systematically review and statistically assess the effect of reported post-resectional tumour characteristics upon overall survival (OS) for patients undergoing attempted radical curative resection for hilar cholangiocarcinoma.

2.33) Methods

Search Strategy and Inclusion Criteria

Meta-Analysis of Observational Studies (MOOSE) guidelines were used to identify eligible studies [272]. Search terms were employed on OVID MEDLINE and PUBMED search engines to capture relevant articles. The search terms employed were 'hilar cholangiocarcinoma', 'prognosis, 'survival', 'surgical' and 'resection'. Combination of the terms on the relevant search engines were incorporated to guarantee a broad spectrum acquisition of papers.

Searches were completed independently by 2 of the study authors with disagreement resolved with discussion. Temporal limitations for study eligibility were defined as any studies published between August 2009 and April 2017. This criterion was incorporated so that only studies published utilising the standardised AJCC 7th edition could be considered for review. Preferred Reporting Items for Systematic Reviews and Meta-

Analyses (PRISMA) protocols were utilised for study identification purposes [273]. The prognostic factor status was determined by the respective authors and were classified as adhering to AJCC 7th edition definitions where stated within the article manuscript.

Only articles reporting prognostic characteristics for hilar cholangiocarcinoma primary tumours which had undergone resection were considered as eligible. All papers referring to intrahepatic or distal cholangiocarcinoma were excluded from review. All papers referring to metastatic disease were excluded. The diagnosis of hilar cholangiocarcinoma was determined by the study authors. Papers referring solely to Disease Free Survival (DFS) were excluded from review. Only articles containing robust data for Overall Survival (OS) were included for analysis.

All grey literature and abstracts were excluded from review and analysis.

Data Analysis

Tumour characteristics considered relevant for the review were; tumour size; AJCC 7th edition 'T' stage ; lymph-node involvement; microvascular invasion; peri-neural invasion; resection margin (R0 vs R1); tumour cellular differentiation, portal vein resection and pre-operative CA 19-9 levels. Hepatic artery resection and reconstruction was not considered for review due to the relatively small number of patients that undergo this specific aspect of resection surgery. Demographic data considered relevant for analysis were age and sex of the patients.

The end-point under analysis was the effect of these prognostic factors upon OS determined by appropriate univariate hazard ratio's (HR) with associated 95 % confidence intervals. OS was defined as time from surgery to death or censuring of data. If HR's were provided by the articles they were used directly in the quantitative meta-analysis. Standard errors (SE's) for the HR's were calculated from the confidence intervals if they were provided in the paper.

If papers which did not report the HR's and confidence intervals then the p-value provided from log-rank tests or univariate Cox Models was used along with number of deaths, Kaplan-Meier survival curve, or 5 year survival estimates to compute estimated HR's and SE's. Univariate Cox models were utilised with the prognostic factor relevant to improved survival of the patients uniformly reported the results thereby ensuring that the HR's were >1 to report a decreased survival. The data extraction methodology utilised was based upon standard Parmar modifications [274]. All statistical analysis was undertaken on Stata 14th Edition Software Package, www.stata.com, StataCorp LP.

Funnel plots were produced for all variables considered in 10 or more studies, as per Sterne and colleagues [275].

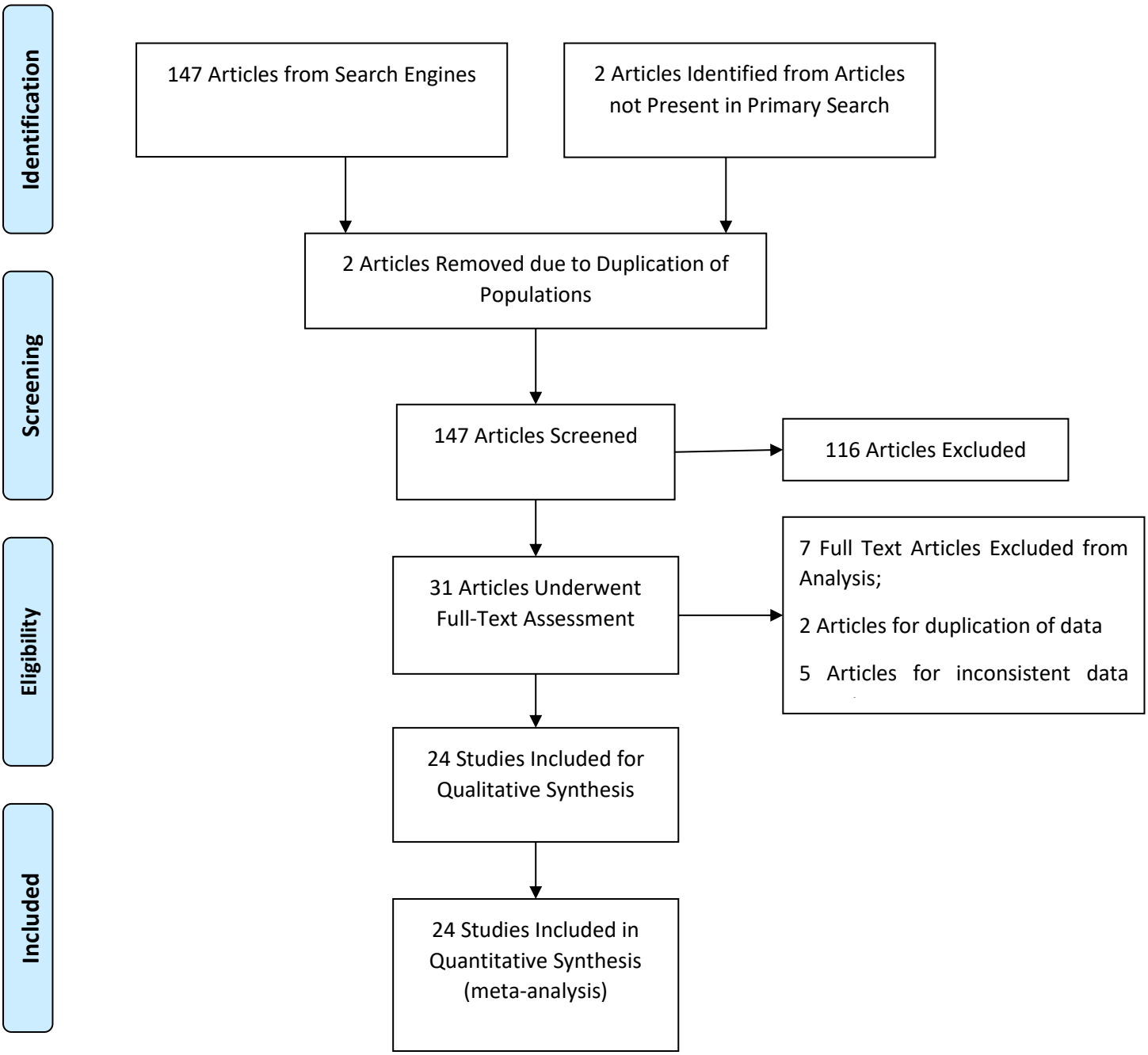
2.34) Results

One hundred and forty seven articles were found through utilisation of the primary search terms. Two citations were identified in the literature which were not present on the initial search. Two were excluded for being duplicated articles. One hundred and sixteen articles were excluded as they did not fit the stated criterion for article analysis (See Figure 24).

Thirty-one articles underwent initial qualitative assessment with 7 articles being subsequently excluded. Two articles were excluded due to data duplication [276, 277]. Five articles were excluded due to variable data reportage; 1 article had trichotomisation of data which resulted in non-acquisition of meaningful data [267]; 1 article did not report a primary series [268]; 1 article did not provide prognostic variables for OS [178]; 1 article only provided survival data for patients with N0 disease [278]; and 1 article did not provide consistent numbers for overall survivors [279].

The 24 articles included for analysis contained 4599 patients (range 42 – 457 patients per study) who had undergone attempted curative resection for hilar cholangiocarcinoma [177, 180, 193, 210, 220, 280-298]. Prognostic factors were reported variably throughout the studies (See Table 21). Nineteen of the 24 series meta-analysed had populations of at least 100 (Range 100 – 457; 93.3 % of total population; See Table 21). The size of the pooled cohorts, the number of resections contained within individual series, and the global distribution of the populations provides high generalizability of this meta-analysis' results to other populations.

Figure 24: PRISMA Diagram for Article Selection



Author	Year	Patients	T size (large)	Age (old)	LN Status	Microvascular Invasion	Perineural Invasion	Portal Vein	Resection Margin	Sex	T Status	Tumour Diff.	CA19-9
Buettner	2016	242	✓(2.5cm)	✓(>65)	✓	✓	✓		✓	✓	✓	✓	
Chauhan	2011	51			✓				✓			✓	
Chen	2009	138			✓			✓					
Cheng	2012	171	✓(>3cm)	✓(>65)	✓		✓		✓	✓		✓	✓ (196.2 U/ml)
Cho	2012	105				✓	✓	✓	✓				✓(37 U/ml)
de Jong	2012	305			✓	✓	✓	✓	✓	✓			
Dumitrascu	2013	82		✓(>58)	✓		✓	✓	✓	✓			✓(200 U/ml)
Furusawa	2014	144		✓(>70)	✓	✓	✓	✓	✓	✓	✓		
Hu	2016	381			✓	✓			✓		✓		
Igami	2010	252			✓				✓				
Kang	2016	260			✓				✓		✓		
Lee	2010	302		✓(>70)	✓		✓	✓	✓	✓	✓	✓	
Li	2011	187			✓	✓	✓		✓			✓	
Matsuo	2012	144	✓(2.5cm)		✓				✓			✓	
Miyazaki	2010	107			✓				✓				
Nagino	2013	457		✓(>65)	✓	✓	✓	✓	✓	✓		✓	
Nakanishi	2016	168			✓		✓	✓	✓				✓(50 U/ml)
Neuhaus	2012	100		✓(>60)	✓	✓	✓			✓	✓		
Nuzzo	2012	376		✓(>60)	✓		✓		✓		✓	✓	
Oguro	2014	224		✓(>66)	✓	✓	✓		✓			✓	✓(64 U/ml)
Saxena	2011	42	✓(4cm)	✓(>61)	✓		✓	✓	✓	✓		✓	✓(37 U/ml)
Song	2013	230	✓(3cm)	✓(>60)	✓				✓				✓(37 U/ml)
Young	2010	51	✓(2.5cm)	✓(>60)	✓	✓	✓	✓	✓	✓		✓	
Zaydfudmin	2013	80			✓				✓			✓	

Table 21: Summary of Numbers and Prognostic Variables Stratified by Article for Resected Cholangiocarcinoma Patients

Twenty-three studies, containing 4494 patients in total (97·7%), reported lymph node status as a prognostic variable. The pooled HR and 95 % confidence intervals were 1·78 (1·65 – 1·93). The pooled HR demonstrated small and non-significant, heterogeneity (I^2 5·2 %; p= 0·390) indicating reasonable concordance of effect between studies. Only 2 studies had HR's where the lower limit of the 95 % confidence interval was <1 (See Figures 25 and 26).

Figure 25: Forest Plot Demonstrating Effect of Positive Lymph Status on Overall Survival

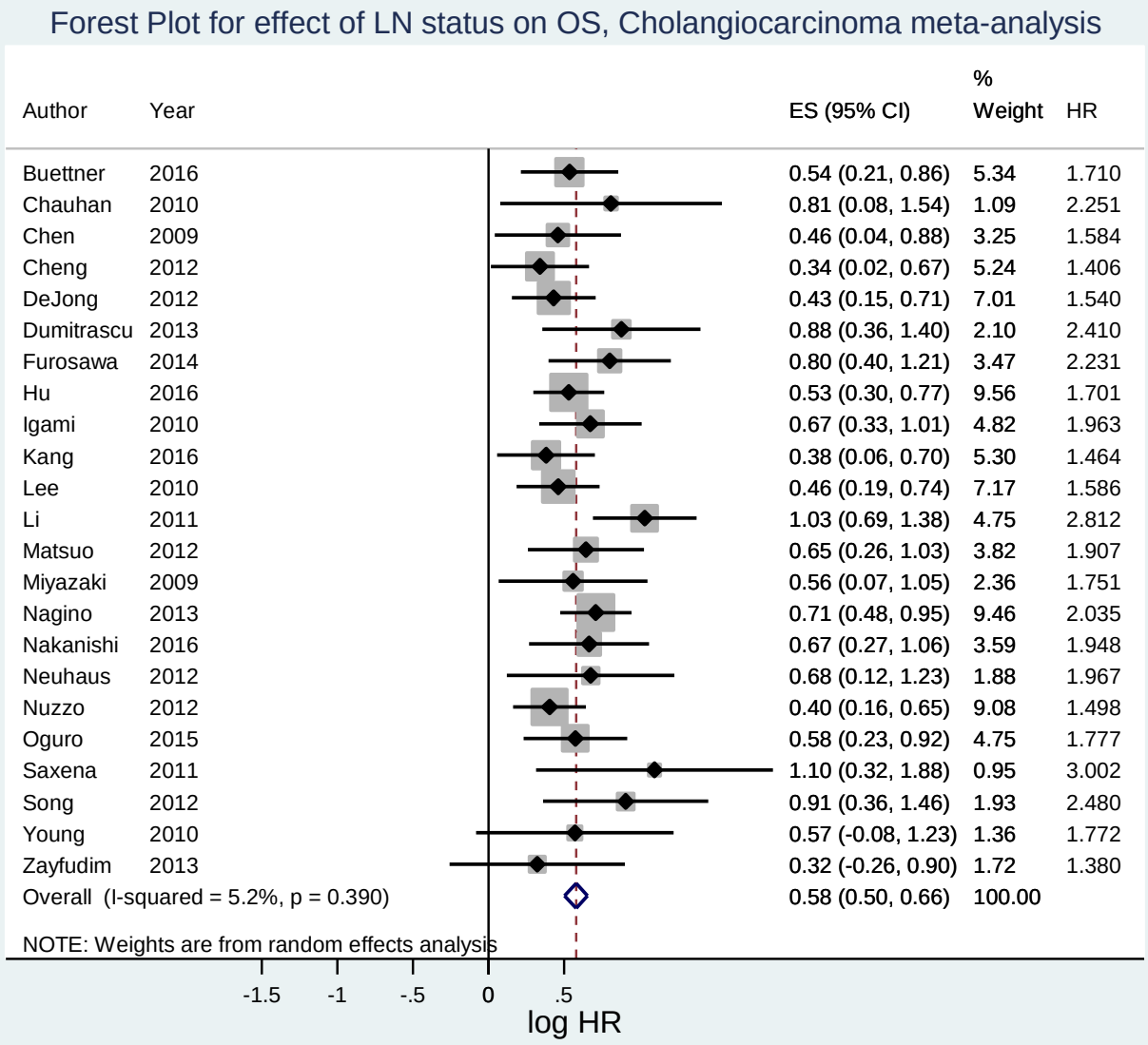
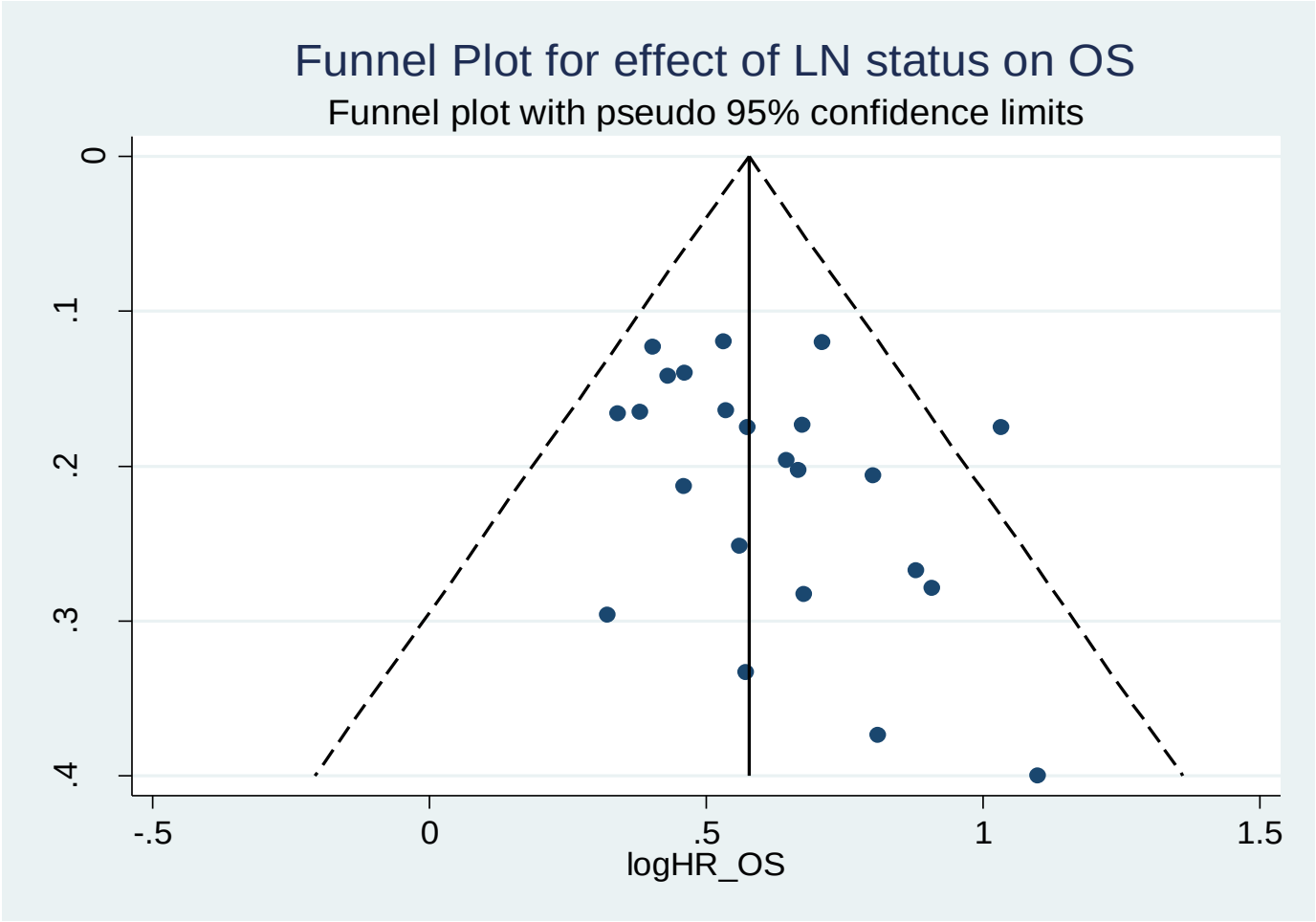
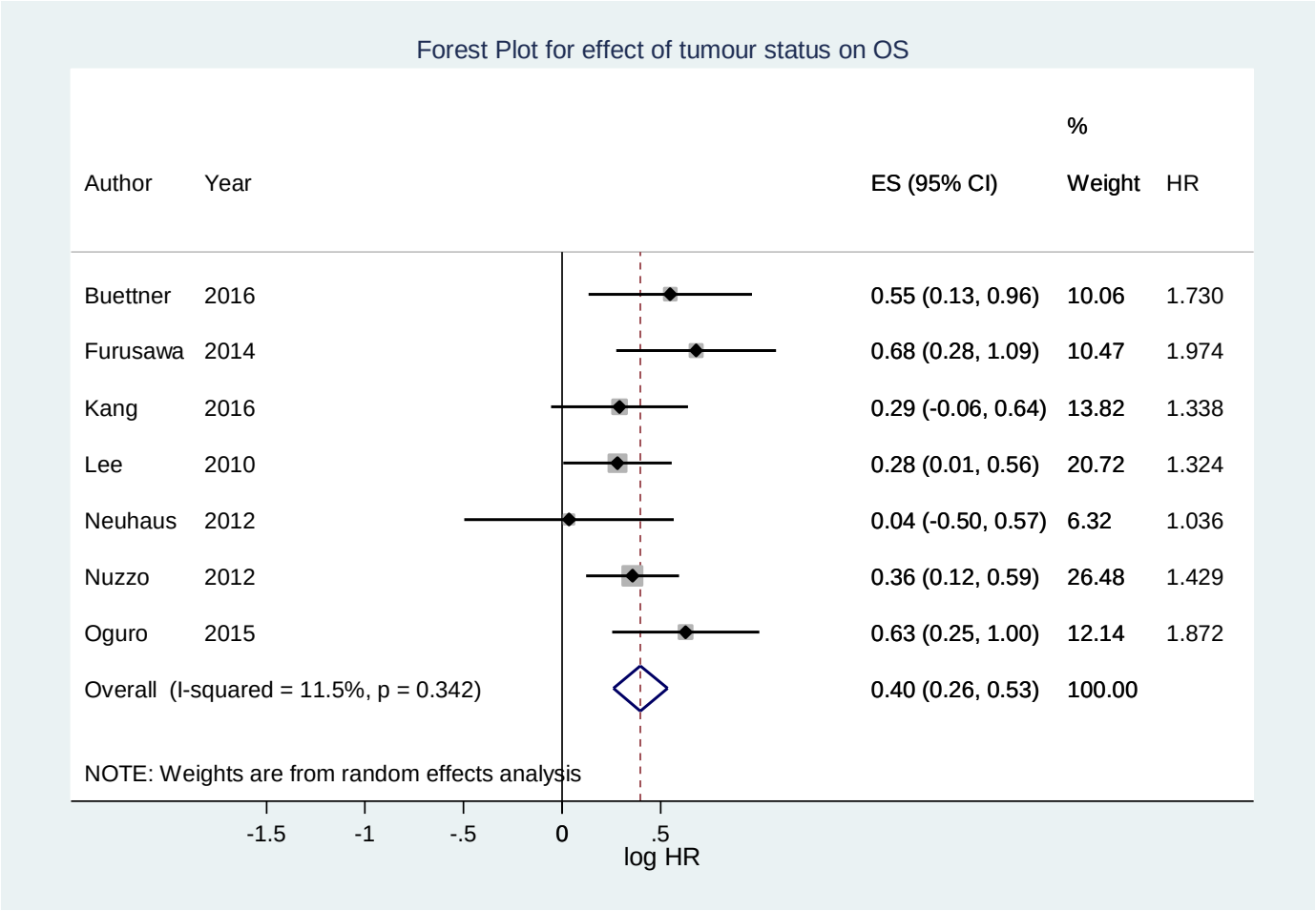


Figure 26: Funnel Plot for Pooled Hazard Ratios for Positive Lymph Node Status



Seven studies, containing 1805 patients, reported 'T' status as dichotomised variables (T1 and T2 versus T3 and T4). The pooled HR and associated 95 % confidence intervals were 1·49 (1·30 – 1·70). The HR demonstrated small but non-significant levels of heterogeneity (I^2 11·5 %; p= 0·345; See Figure 27).

Figure 27: Forest Plot Demonstrating Pooled Hazard Ratios for 'T' Status



Fifteen studies, containing 2956 patients (65·7 %), reported peri-neural invasion as a prognostic variable. The pooled HR and 95 % confidence intervals were 1·54 (1·40 – 1·68). The pooled HR demonstrated minimal heterogeneity (I^2 0·0 %; p= 0·680) indicating homogeneity of effect of the variable on survival between studies. Six studies, with a combined weighting of 25·9 %, had HR's where the lower limit of the 95 % confidence interval was <1 (See Figures 28 and 29).

Figure 28: Forest Plot Demonstrating Effect of Peri-neural Invasion Status on Overall Survival

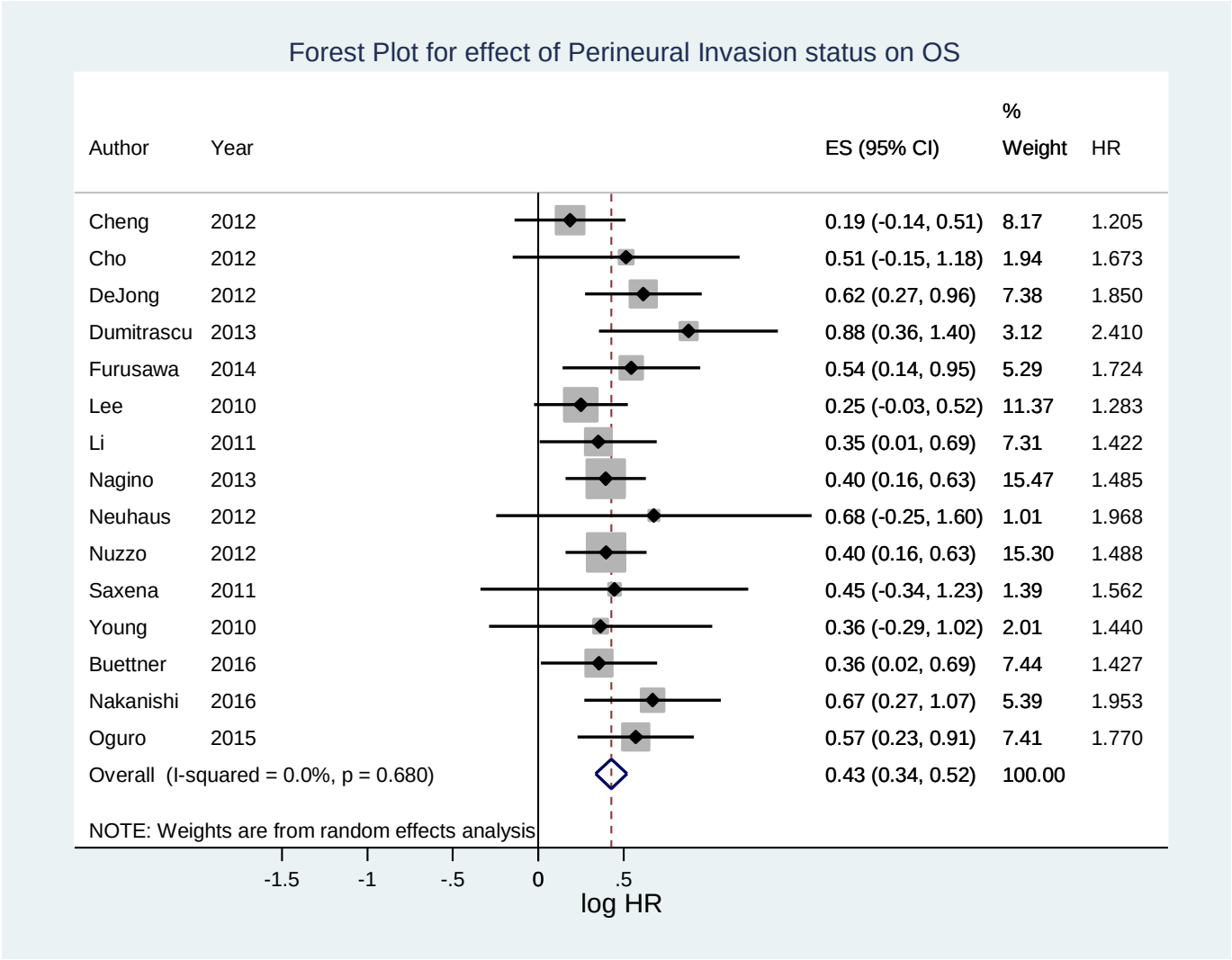
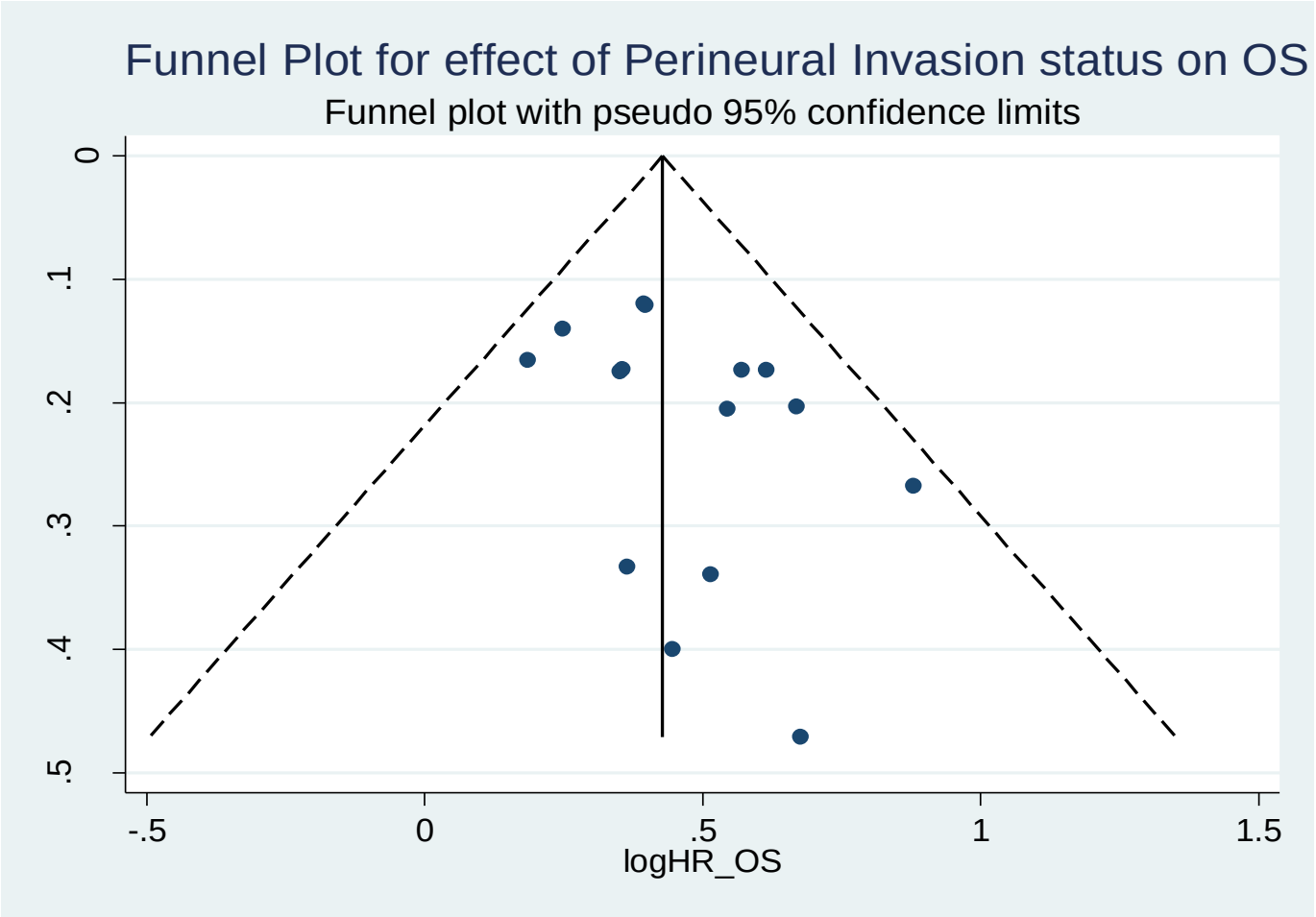


Figure 29: Funnel Plot of Effect of Peri-neural Invasion Status on Overall Survival



Ten studies, containing 2196 patients in total, reported microvascular invasion as a prognostic variable. The pooled HR and 95 % confidence intervals were 1·49 (1·34 – 1·68). The pooled HR demonstrated minimal and insignificant heterogeneity (I^2 0·0 %; p= 0·659) indicating homogeneity of effect of the variable on survival between studies. Four studies with a combined weight of 32·5 % had HR's where the lower limit of the 95 % confidence interval was < 1 (See Figures 30 and 31).

Figure 30: Forest Plot of Effect of Microvascular Invasion Status on Overall Survival

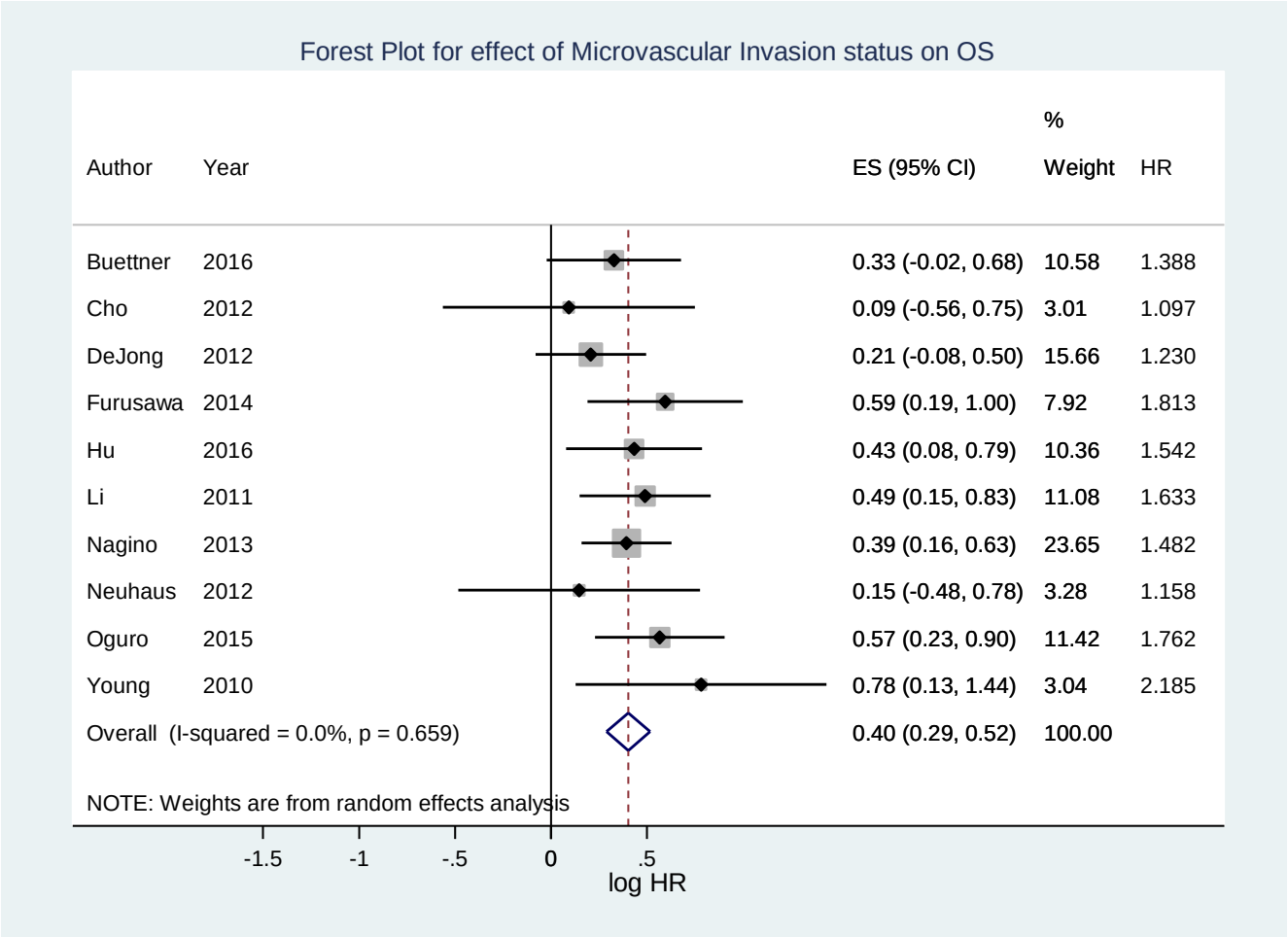
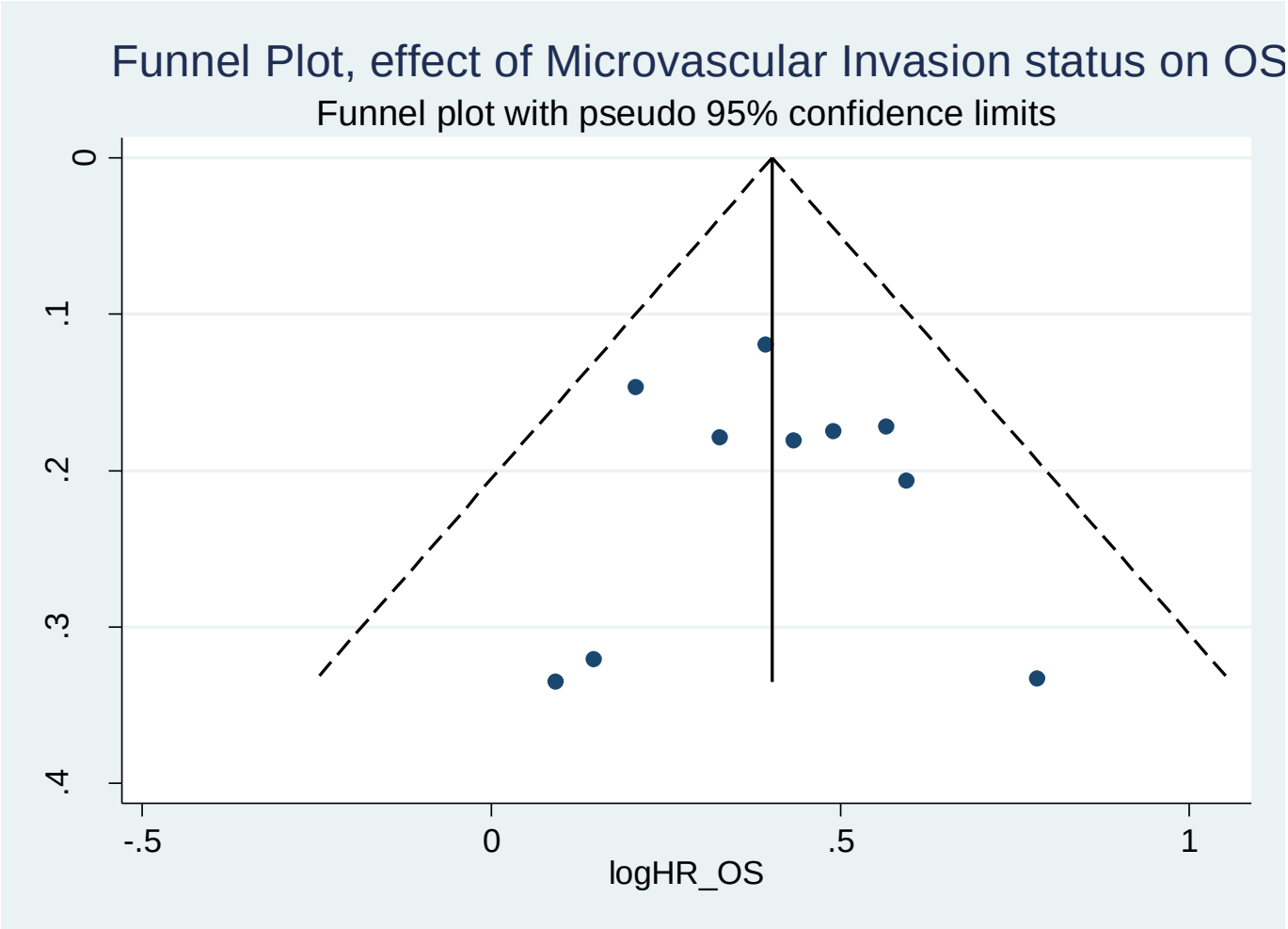


Figure 31: Funnel Plot Effect of Microvascular Invasion Status on Overall Survival



Twelve studies, containing 2327 patients in total (50·6 %), reported tumour differentiation in a dichotomised manner ('well' vs 'others'). The pooled HR and 95 % confidence intervals were 1·54 (1·38 – 1·72). The pooled HR demonstrated low, and insignificant, heterogeneity (I^2 15·2 %; p= 0·291) indicating a high level of inter-study concordance in reporting the effect of this variable. Four studies, with a pooled weighting of 16·9 %, had 95 % confidence intervals where the lower limit was <1. (See Figures 32 and 33).

Figure 32: Forest Plot Demonstrating Pooled Hazard Ratios for Tumour Differentiation Status

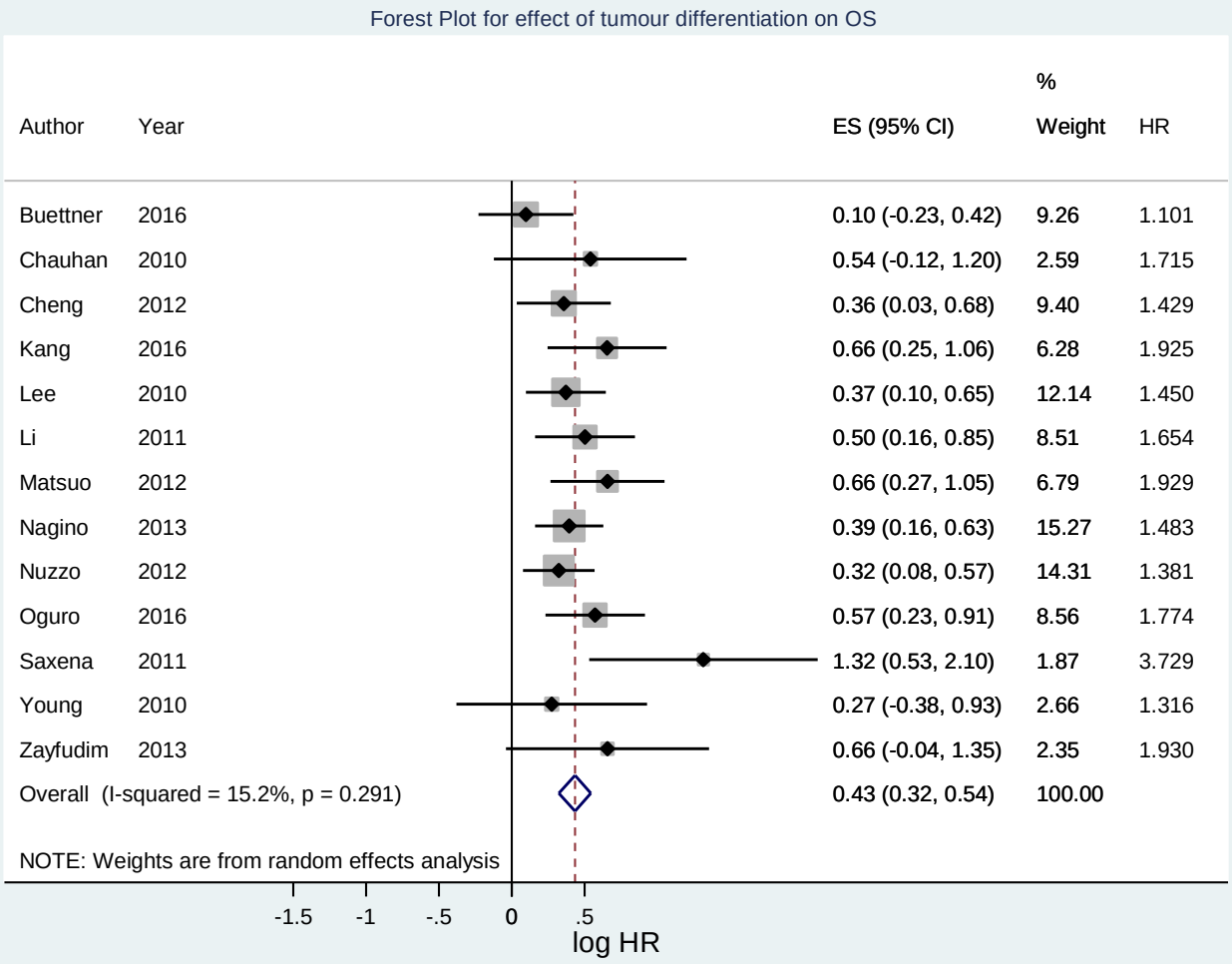
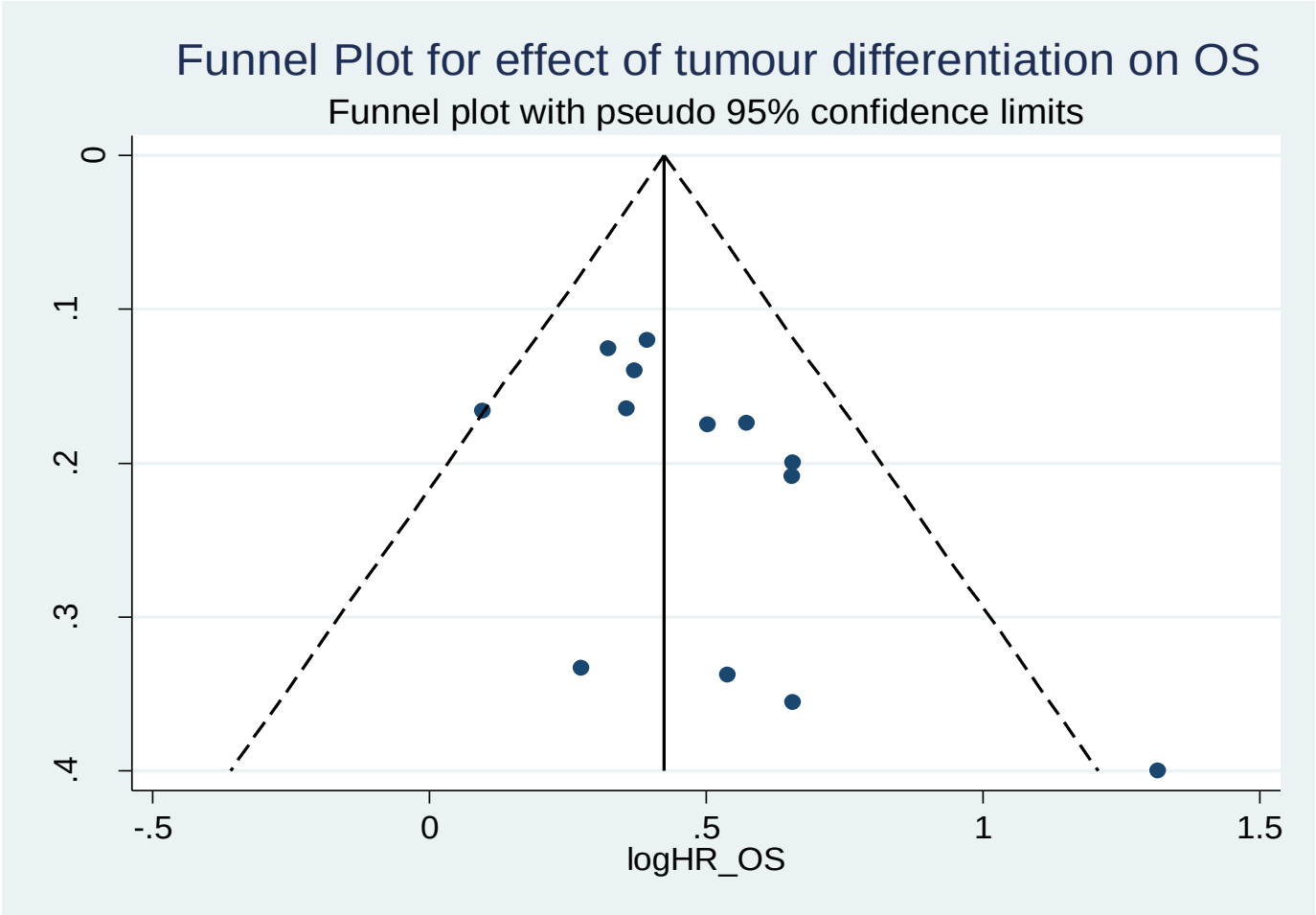
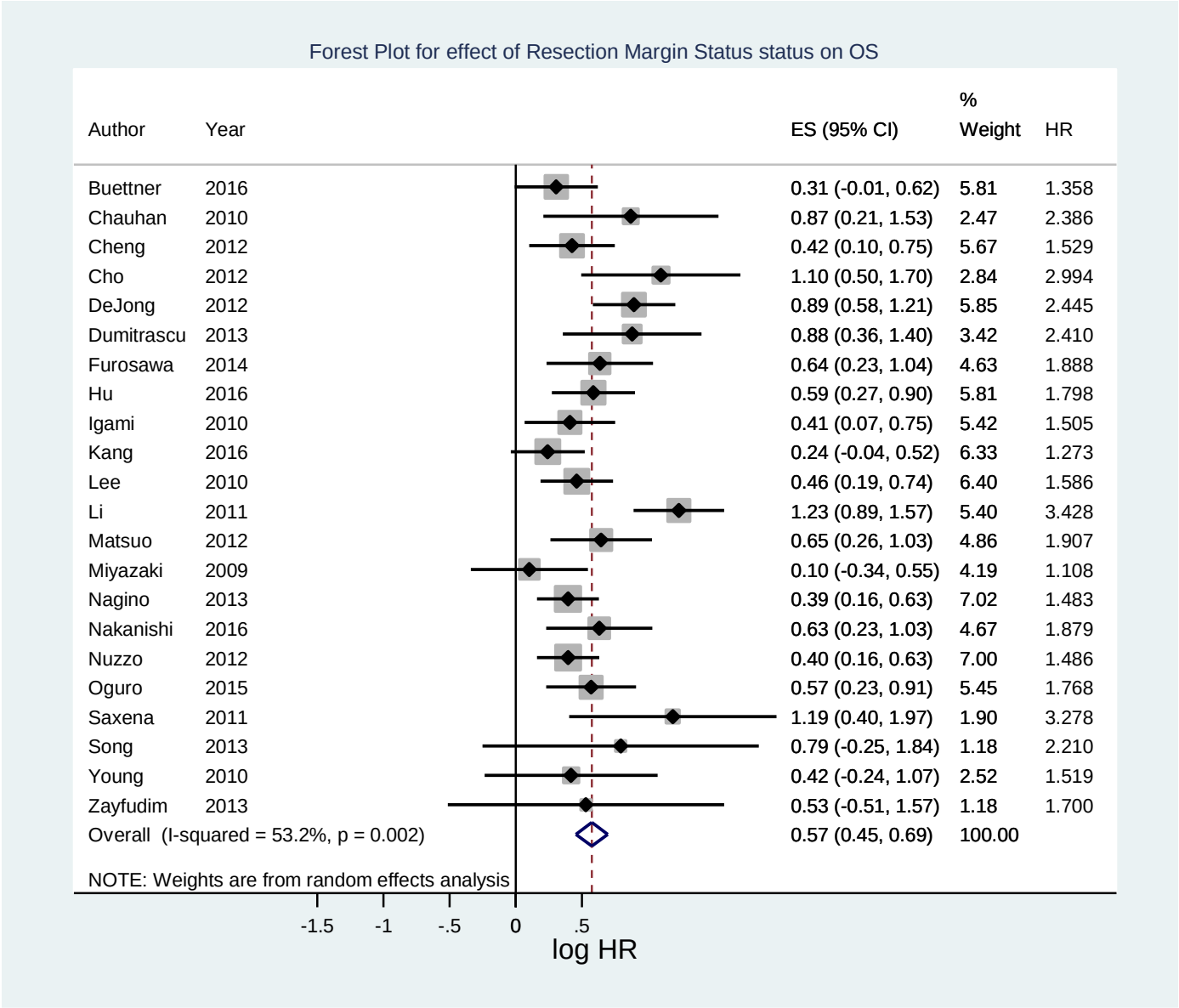


Figure 33: Funnel Plot Demonstrating Hazard Ratio Dispersal for Tumour Differentiation Status



Twenty-two papers, containing 4361 patients in total (94·8%) reported resection margin as a prognostic co-variate. Quantitative meta-analysis was used to determine the prognostic implications of microscopic margin involvement (R0 v R1). The pooled HR and 95 % confidence intervals were 1·77 (1·57 – 1·99). However, the pooled HR had large, and significant, heterogeneity associated with it (I^2 53·2 %; p= 0·002) indicating high levels of variability of effects for the prognostic variable. Six of the papers, with a pooled weight of 21·2 %, had HR's where the lower limit of the 95 % confidence interval was <1. The pooled series contained 3164 curative resections producing an R0 rate of 72·6 % (Figures 34 and 35).

Figure 34: Forest Plot Demonstrating Pooled Hazard Ratios for Resection Margin Variable



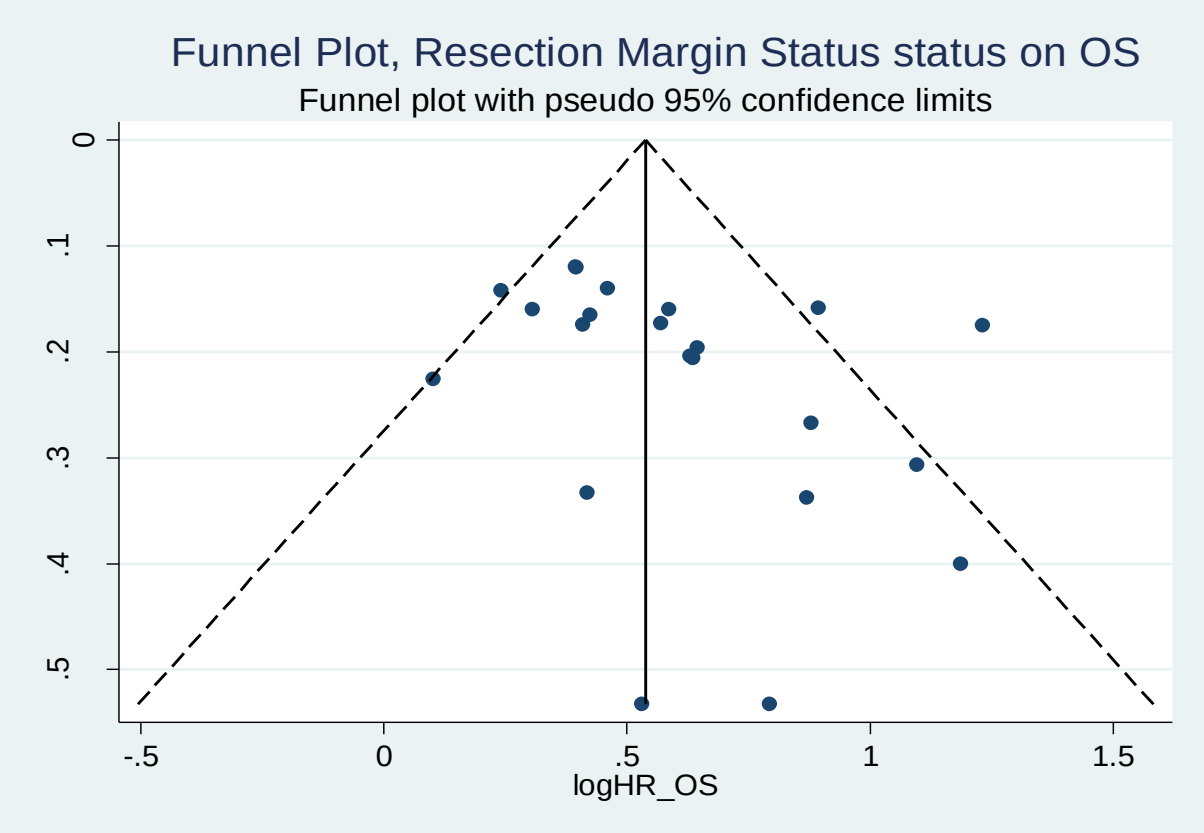


Figure 35: Funnel Plot Demonstrating Hazard Ratio Dispersal for Resection Margin variable

Ten studies, containing 1794 patients, reported portal vein resection as a prognostic variable. There were 459 portal vein resections undertaken in these studies. The pooled HR and 95 % confidence intervals were 1·54 (1.15 – 1·70). The pooled HR demonstrated large, and significant, heterogeneity (I^2 52·6 %; p= 0·025) indicating high levels of variability of effect for this prognostic variable. One paper reported a positive association of portal vein resection and prognosis, although the upper limit of the 95 % confidence interval was >1. Five papers had HR's > 1 but with the lower limit of the 95 % confidence interval <1. Four populations demonstrated convincing negative prognostic associations between portal vein resection and OS (Figures 36 and 37).

Figure 36: Forest Plot Demonstrating Pooled Hazard Ratio for Portal Vein Resection Variable

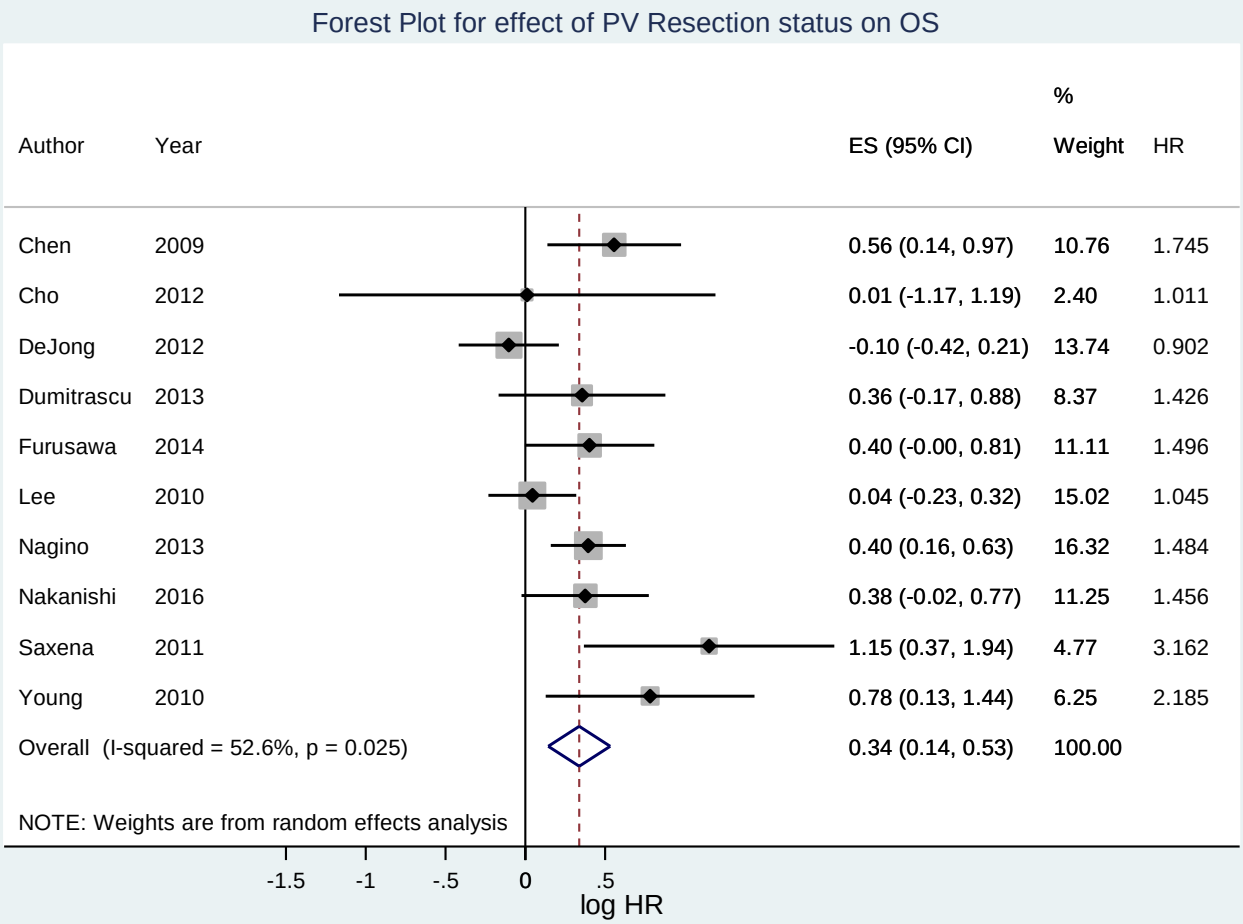
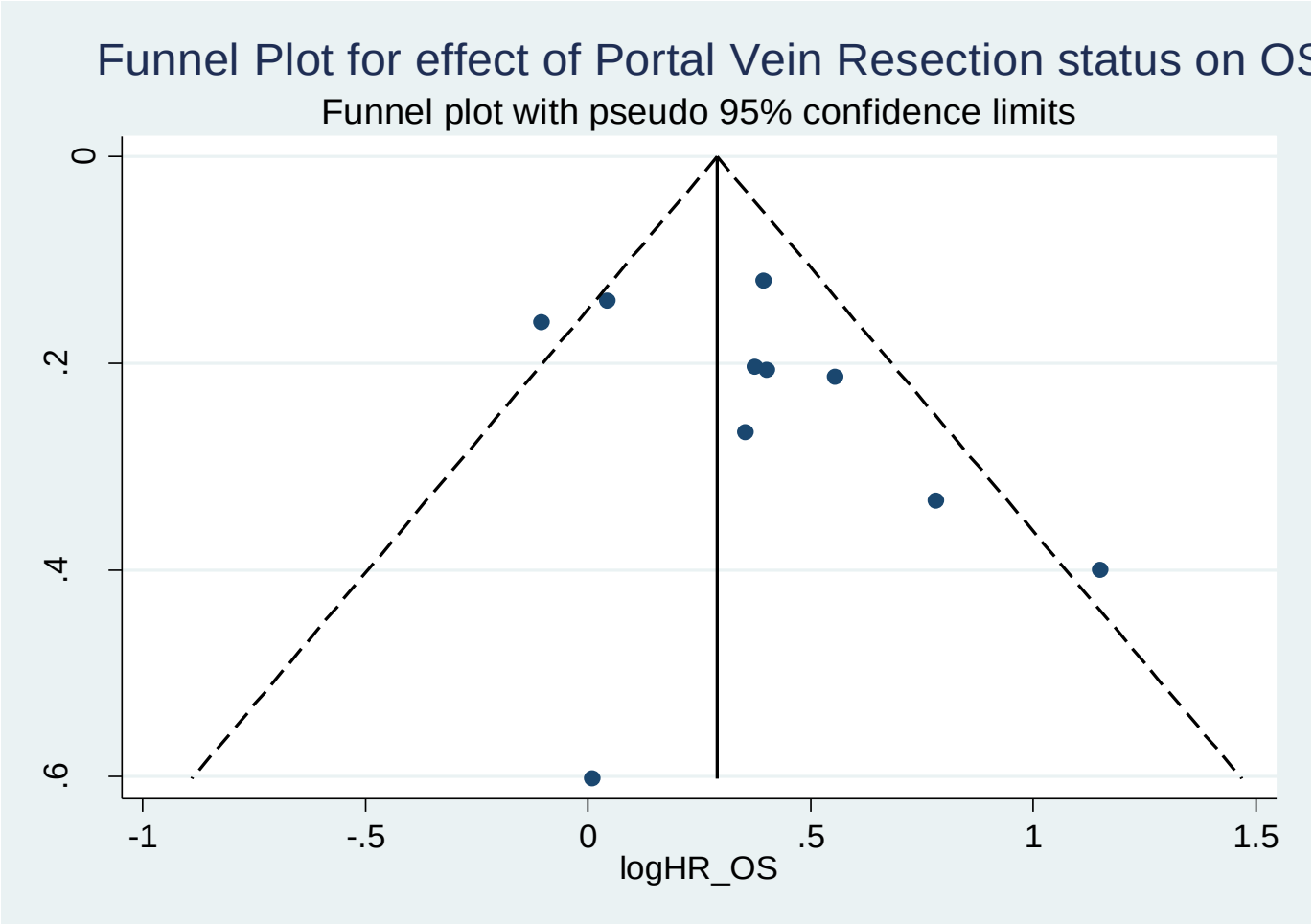
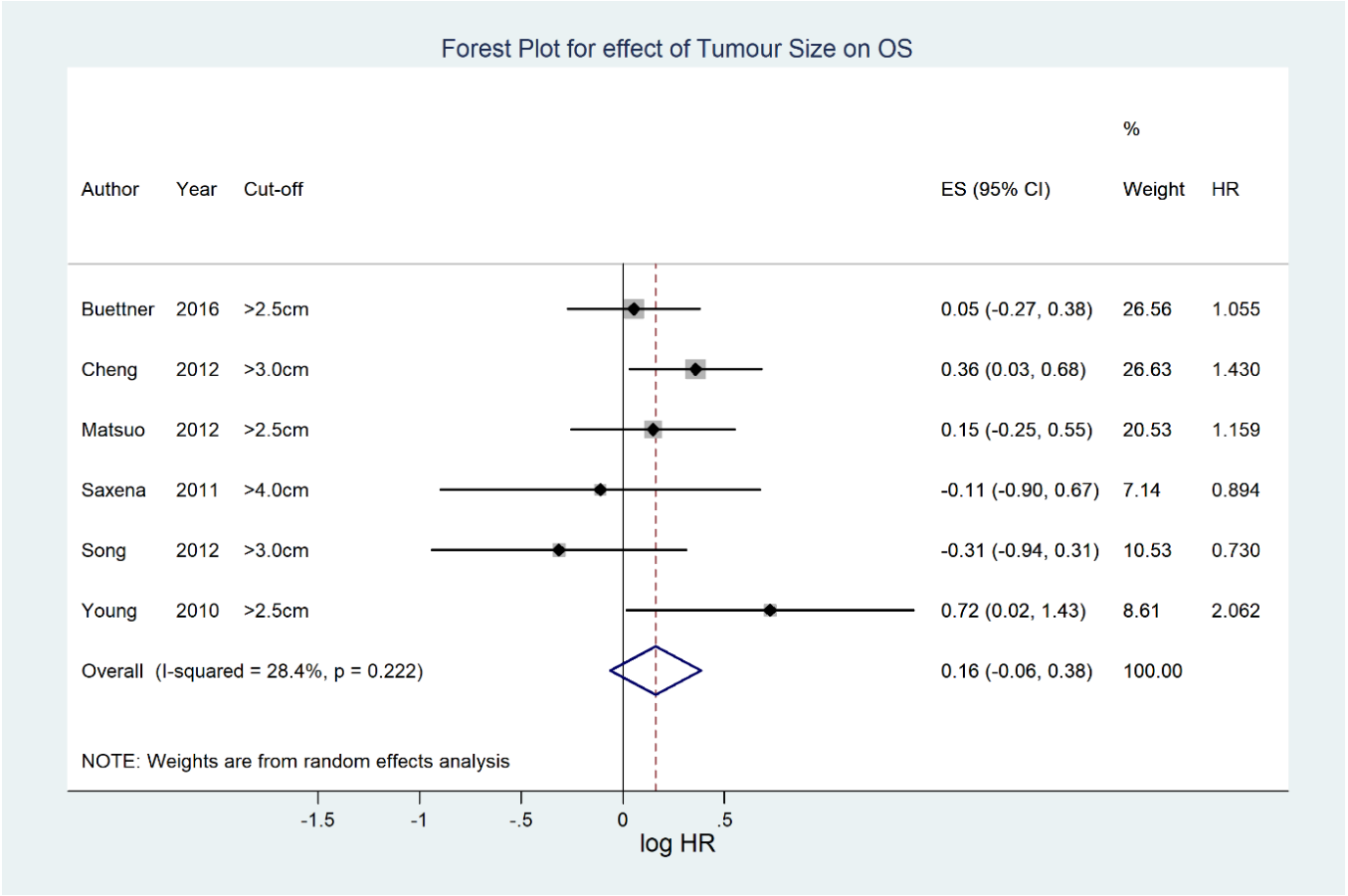


Figure 37: Funnel Plot Demonstrating Hazard Ratio Dispersal for Portal Vein Resection Variable



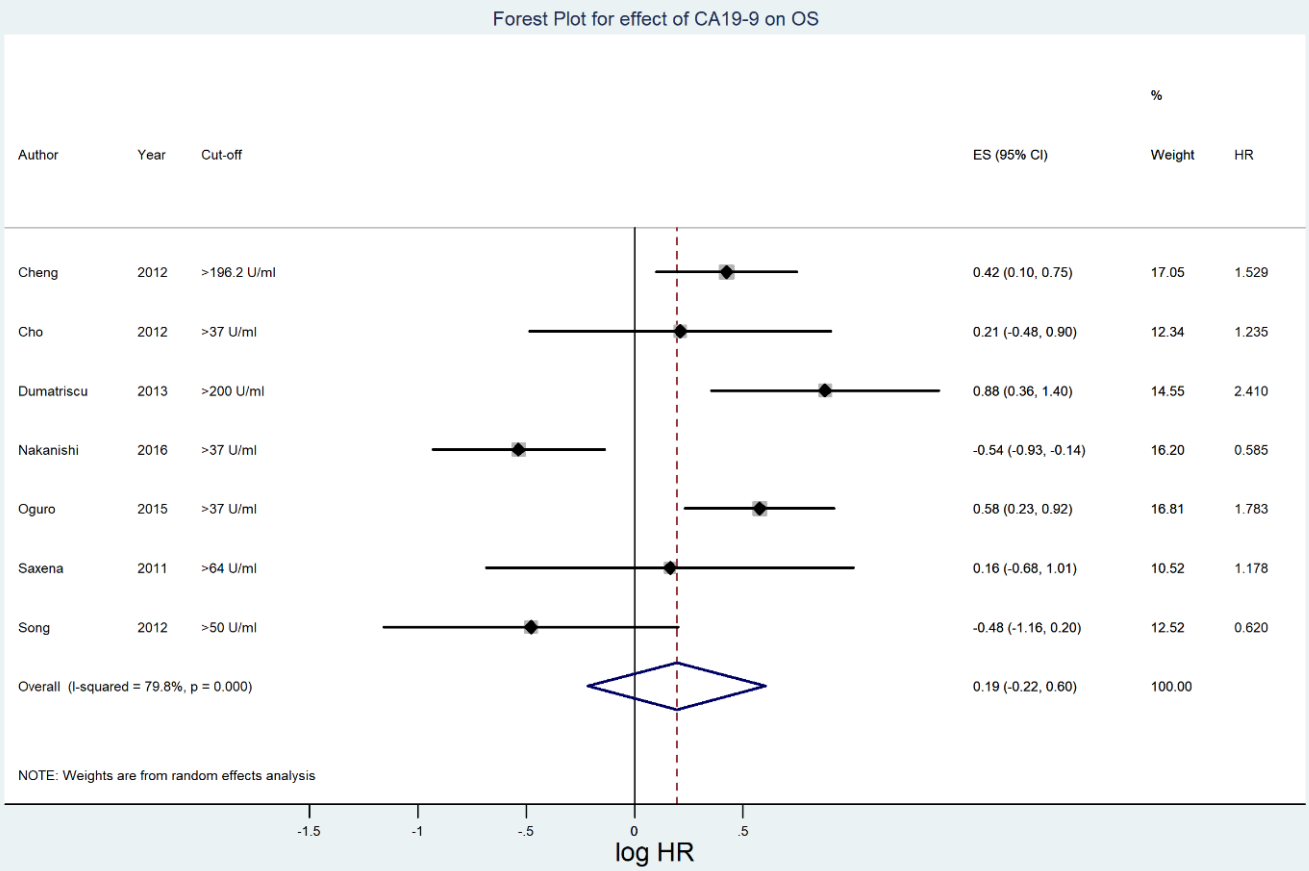
Six studies, containing 880 patients in total, reported tumour size as a potential prognostic variable. The tumour size cut-off was not reported consistently between studies. Three studies utilised a cut-off of 2·5 cm in largest dimension measured; 2 studies utilised 3 cm in largest dimension measured; 1 study defined 4 cm as the largest dimension measured. The pooled HR for tumour size as a prognostic factor was calculated as 1·17 (95 % CI 0·94 – 1·46) indicating that tumour size was not a significant prognostic variable. The I^2 value of 28·4 % demonstrates moderate, but not significant (p= 0·222), heterogeneity, indicating that there was consistency in the reporting of non-significance of this variable between studies (Figure 38).

Figure 38: Forest Plot Demonstrating Pooled Hazard Ratio for Effect of Tumour Size on Overall Survival



Seven studies, containing 1022 patients, reported pre-operative CA 19-9 as a dichotomised value. The dichotomised categories varied between articles. Three articles [282, 291, 292] utilised 37 U/ml as the dichotomised cut-off. One study used 50 U/ml [220]; one article utilised 64 U/ml [295]; one used 196.2 U/ml [281]; and one study used 200 U/ml [283]. The pooled HR and 95 % confidence intervals for this prognostic factor were calculated as 1·21 (95 % CI 0·80 - 1·82) indicating that pre-operative serum CA 19-9 was not a significant prognostic variable. The I^2 value of 79·8 % demonstrates large, and significant heterogeneity (p= 0·0001), indicating that there was inconsistency in the reporting of non-significance of this variable between studies (Figure 39).

Figure 39: Forest Plot Demonstrating Pooled Hazard Ratio for Effect of CA 19-9 on Overall Survival



Twelve studies, containing 2421 patients in total (52·6%), reported age as a dichotomised value. The dichotomised cut-off value for age was reported inconsistently between studies. One study used an age of 58 years-old as a cut-off for this variable; 4 studies utilised 60 years-old for dichotomisation; 1 study used 61 years-old as a cut-off; 1 study used 66 years; 3 used 65 years and 2 used 70 years-old as the dichotomisation point. The pooled HR and 95 % confidence intervals for this prognostic factor were calculated as 1·16 (1·04 – 1·28) indicating a small improvement in prognosis for those patients defined as 'young' in the articles. The I^2 of 2·9 % demonstrates a minimal level of heterogeneity between studies indicating a low and insignificant (p= 0·416) variability of effect between series (Figures 40 and 41).

Figure 40: Forest Plot Demonstrating Pooled Hazard Ratio for Effect of Age on Overall Survival

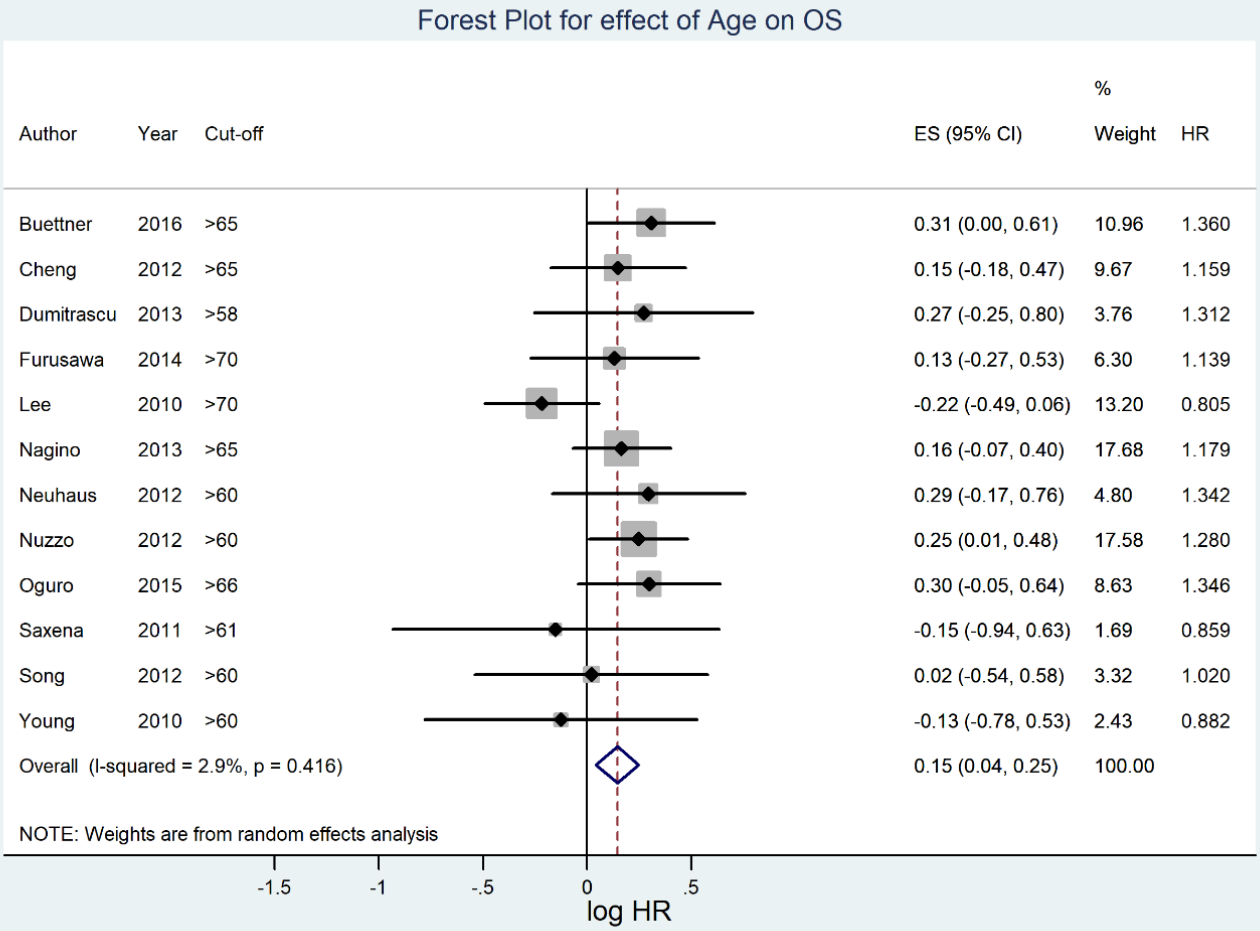
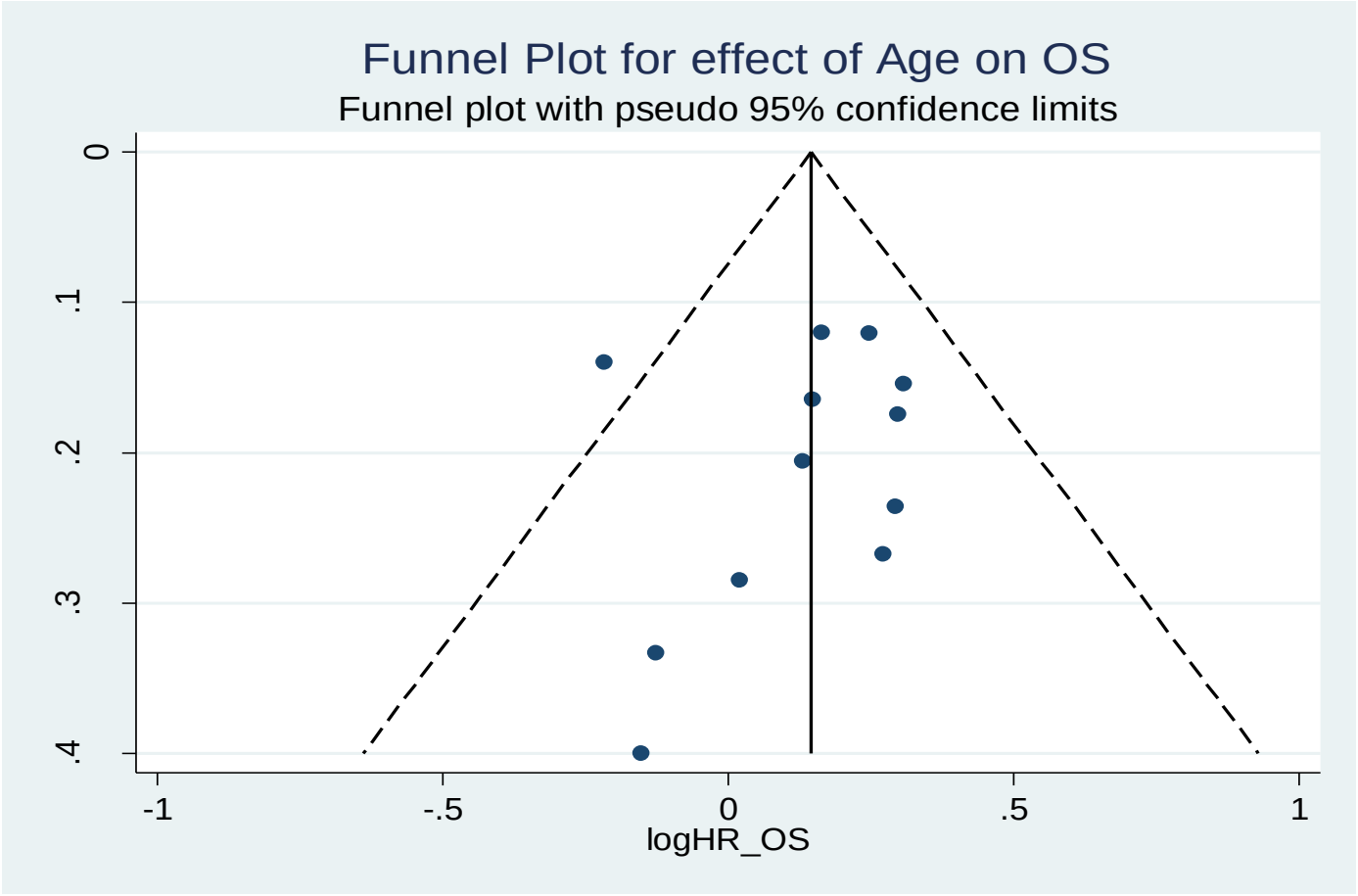


Figure 41: Funnel Plot Demonstrating Hazard Ratio Dispersal for Age Variable



Ten studies, containing 1896 patients, reported sex as a prognostic variable. The pooled HR and associated 95 % confidence intervals were 1·04 (0·91 – 1·19) which indicates a non-significant relationship between sex and OS. The HR demonstrated small and insignificant inter-study heterogeneity (I^2 2·9 %; p= 0·416) indicating reasonable concordance between studies (See Figure 42 and 43).

Figure 42: Forest Plot Demonstrating Effect of Gender on Overall Survival

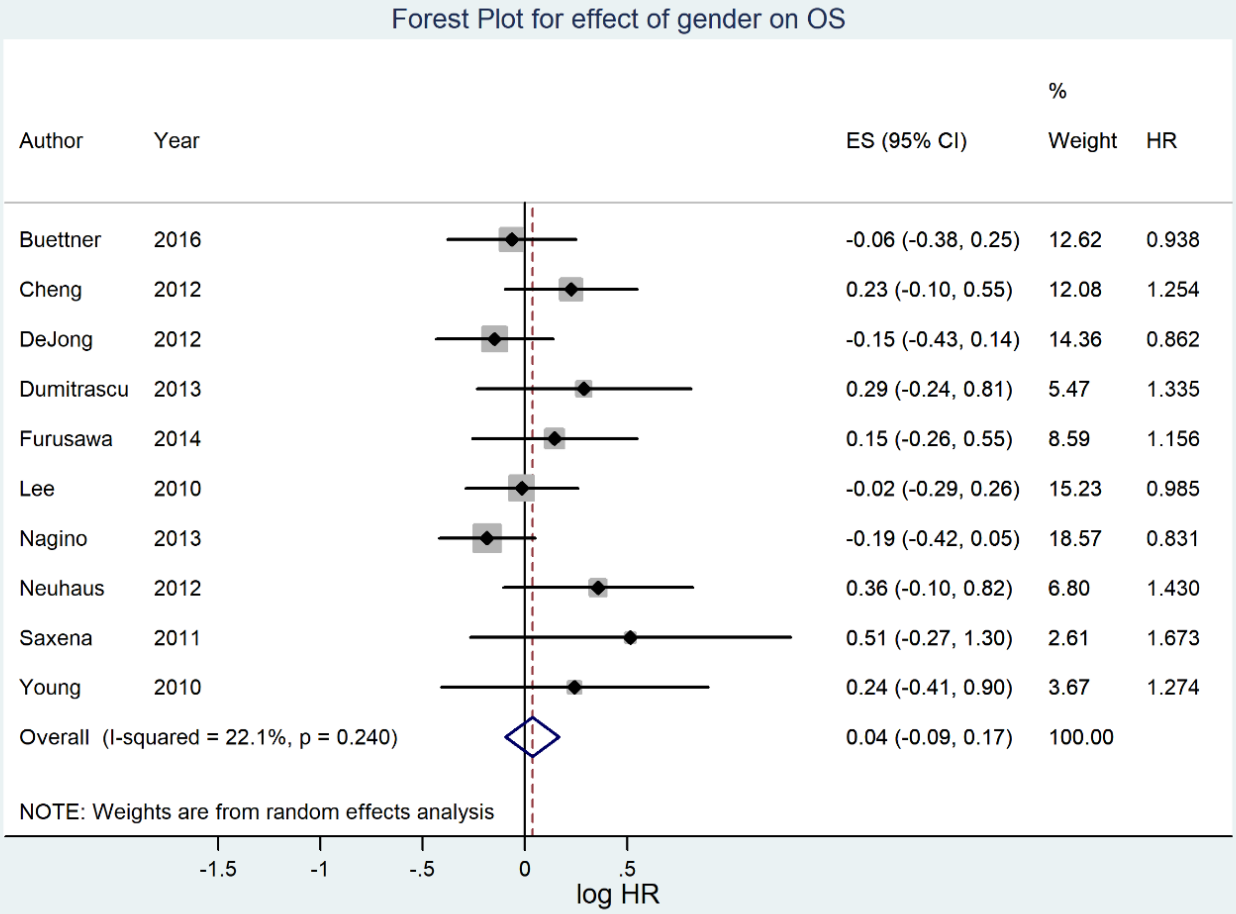
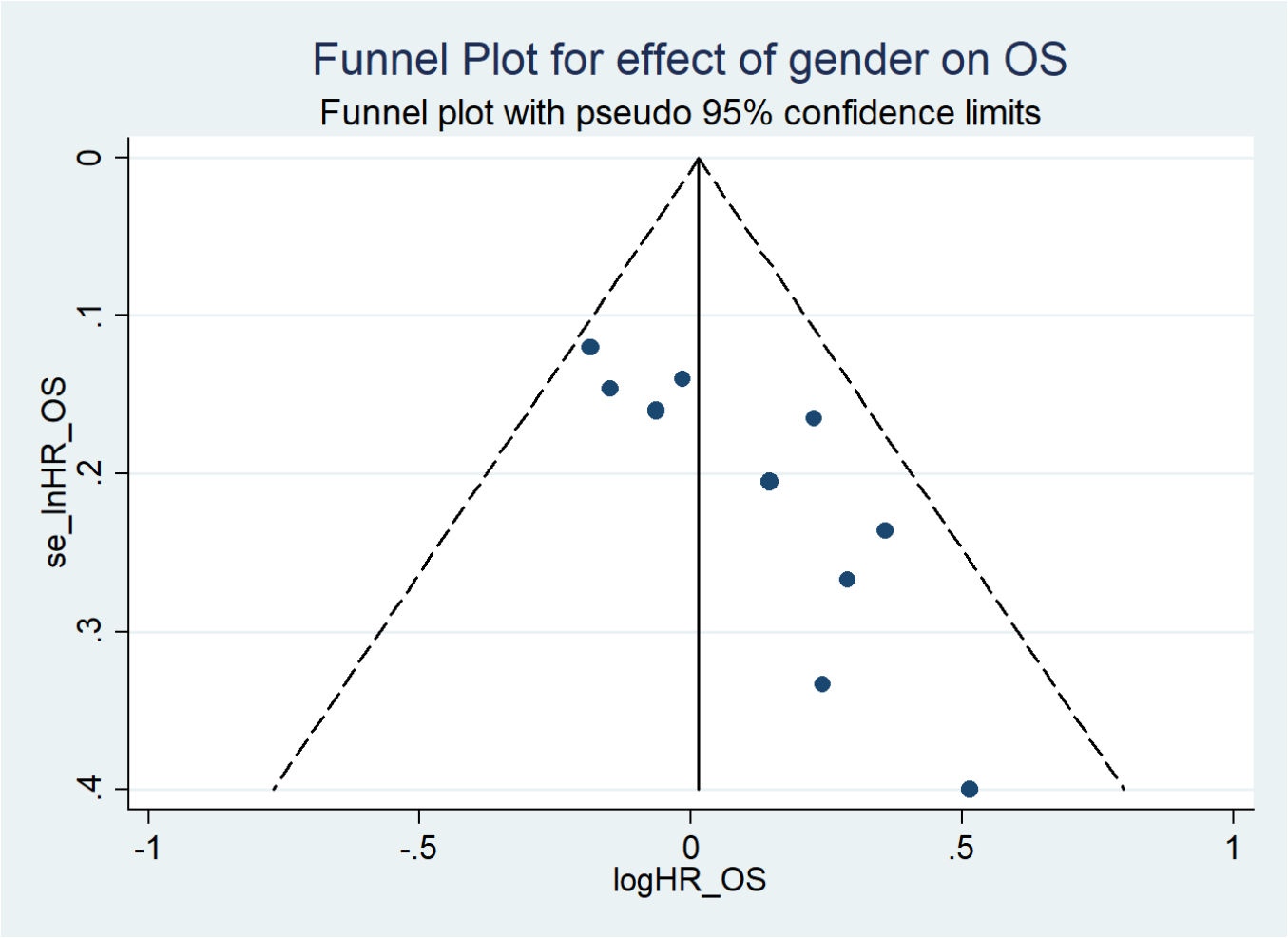


Figure 43: Funnel Plot for Effect of Gender on Overall Survival



Discussion

This meta-analysis has highlighted the significance of prognostic variables not accounted for in the AJCC 7th edition affecting OS. The prognostic factors which had a significant effect upon OS were; 'T' status, lymph node involvement, microvascular invasion, peri-neural invasion, tumour differentiation and age. Resection margin and portal vein resection, despite having a large pooled population of patients, had highly significant effects but also concomitant high heterogeneity. Tumour size and gender had insignificant effects on pooled analysis with insignificant heterogeneity indicating that these prognostic factors can be considered as having negligible effect upon OS. Pre-operative serum CA 19-9 levels demonstrated insignificant effects on pooled analysis with significant heterogeneity indicating that the prognostic utility is yet to be defined for this variable.

This meta-analysis validates the AJCC 7th edition TNM system with the 2 prognostic variables of 'T' status and lymph node involvement demonstrating significant effects with insignificant heterogeneity upon OS. Lymph node involvement has a particularly marked effect on reducing OS. Spread to the loco-regional nodes indicates that this prognostic factor could be a surrogate marker for radiologically and surgically occult

systemic micro-metastases. Given this rationale adjuvant chemotherapy would potentially be a beneficial treatment modality for these resected patients. However the recently published PRODIGE-12 trial, comparing adjuvant GEMOX (gemcitabine-oxaliplatin) in treated R0 patients to un-treated R0 patients, did not demonstrate any conferred survival advantages for the treatment group [239]. The BILCAP multicentre prospective phase 3 randomised control trial investigating the utility of adjuvant fluoropyrimidine (capecitabine) chemotherapy has recently completed in the United Kingdom and has published its findings [237]. The study compared adjuvant fluoropyrimidine chemotherapy to observation alone in patients undergoing potentially curative (R0) resection. Significant survival benefits were demonstrated by the fluoropyrimidine-treated group. This has led to the widespread adoption of Gem-Cis (Gemcitabine-Cisplatin) as the standard of care for adjuvant treatment. The ACTICCA-1 phase 3 randomised control trial comparing standard of care, fluoropyrimidine, and non-adjuvant treatment is currently recruiting and is expected to report in April 2022.

This meta-analysis has demonstrated that elevated pre-operative levels of the biomarker CA 19-9 does not impact survival in patients undergoing attempted curative resection. Hu and colleagues have demonstrated that CA 19-9 levels can be utilised to stratify radiologically resectable patients for surgical assessment [299]. Elevated levels correlate with a reduction in resectability rate due to radiologically occult metastatic disease. Elevated levels of CA 19-9 in all-comers has been reported to correlate to the presence of metastatic disease and reduced overall survival [300]. CA 19-9 serum blood levels appear to be useful within the context of serial measurements in a clinic setting to determine the potential presence of metastasis and have limited utility in the pre-operative predictive assessment of overall survival according to this meta-analysis.

Tumour-specific prognostic variables, not accounted for in the AJCC 7th edition, have profound effects upon OS. Microvascular and peri-neural invasion, along with tumour differentiation, are prognostic variables which could be considered non-surgical variables, given their effect upon OS is independent of surgical treatment if R0 resection is achieved. In this meta-analysis all 3 variables produced significant effects upon OS with no inter-study heterogeneity. However, none of these tumour biological factors are incorporated in the AJCC 7th edition. Groot and colleagues demonstrated that there was negligible difference between the AJCC 6th edition, 7th edition and 2 alternative systems which also utilised primarily anatomical histo-pathological variables to stratify patients [301]. The lack of significant difference between systems indicates that defining prognosis solely by anatomical variables ignores the significance of prognostically important variables which can only be assessed after resection (such as peri-neural and microvascular invasion).

Resection margin status is considered the primary prognostic factor for determining survival [220]. This consideration has provided the impetus to utilise concomitant hepatectomy with bile duct resection as the gold standard for resectable hilar cholangiocarcinoma [193, 285, 289, 302]. This meta-analysis provides convincing evidence that there is a significant effect on OS for microscopically positive (R1) resections. The

high heterogeneity may be due to the variability of reporting resection margin status between institutions or access to adequate frozen section analysis. The use of frozen section to intraoperatively determine sufficient margins to affect OS has been questioned. Typically 60 % of patients with negative margins on frozen section have sufficient margin to affect OS, with around 10 % of patients actually having microscopically involved margins on full histopathological assessment [196, 303]. The high heterogeneity may also be explained by the impact of positive nodal status upon OS for patients with R0 resection. Univariate analysis of OS frequently subsumes the R0N0 and R0N1 resection status categories in to 1 combined category when comparing R0 vs R1 resectional status. Kobayashi and colleagues have demonstrated that positive nodal status reduces OS in patients undergoing R0 resection compared to node negative disease [276]. The AJCC 8th edition has harmonised the nodal status category for hilar cholangiocarcinoma with other extra-hepatic hepato-pancreatico-biliary cancers [304]. The new nodal category, based upon research into Pancreatic Ductal Adenocarcinoma (PDAC) and distal cholangiocarcinoma, quantitatively stratifies patients in to N1 or N2 categories dependent upon the number of positive regional lymph nodes present in the lymphadenectomy field. N1 disease is now classified as a resected specimen containing 1 – 3 positive lymph nodes, and N2 disease is defined as a resected specimen containing 4 or more positive lymph nodes. The effect of this re-classification upon survival for patients undergoing attempted curative resection is yet to be quantified in a significant hilar cholangiocarcinoma cohort.

Portal vein invasion (PVI) occurs when the primary tumour extends radially beyond the duct in to the adjacent vascular structure. PVI is incorporated in to the AJCC 7th edition staging system as being indicative of T3 (invasion of unilateral branch) and T4 (main branch) disease [245]. PVI is also incorporated as a key determinant of resectability in the Memorial Sloane-Kettering Cancer Centre (MSKCC) resectability criteria [177, 178, 305, 306]. Portal vein resection (PVR) is undertaken to ensure adequate R0 resection margins. In a recent meta-analysis, published in 2014 which incorporated studies prior to 2009, PVI and associated PVR was a significant negative predictor of OS compared to non-PVR and demonstrated no inter-study heterogeneity [10]. This current meta-analysis also demonstrated PVR negatively affects OS compared to patients undergoing resection without PVR. However, this meta-analysis demonstrated that there is significant inter-study heterogeneity of the effect of PVR. PVR is a technically demanding procedure undertaken to ensure adequate R0 resection margins in patients with locally advanced disease and is therefore potentially reflective of inter-dependence with T-stage. While R0 resection is feasible in this subset of patients, comparison with patients undergoing R0 resection without PVR demonstrates that it is significantly less achievable [268]. While PVR is less achievable it is considered to be a safe procedure in experienced hands, with comparable post-operative morbidity and mortality rates to patients undergoing

hepatectomy without PVR [307]. The reduced achievability of R0 resection with PVR potentially produces an undefined effect in this subset which may explain the reduced OS.

The limitations of the study primarily are related to the variability of reporting of prognostic independent variables. Selective outcome reporting of these variables introduces publication bias within the meta-analysis. This variability partially explains the moderate heterogeneity for the pooled hazard ratios for some of the prognostic factors. However 8 out of the 11 prognostic variables were reported in more than 10 studies which enabled funnel plot analysis. The variables were; lymph node involvement, peri-neural invasion, tumour differentiation, resection margin status, microvascular invasion, portal vein invasion, age and sex.

The lymph node status funnel plot contains 22 of the 23 studies within the 95 % confidence interval and does not demonstrate significant asymmetry. The microvascular invasion funnel plot demonstrates article distribution within the expected parameters with all points within the 95 % confidence interval. The tumour differentiation, resection margin status and portal vein resection funnel plots also demonstrated minimal asymmetry.

The age funnel plot demonstrated asymmetry with small studies demonstrating only positive HR's in association with OS and large studies demonstrating negative HR's. This may possibly be due to the arbitrary selective reporting of the age cut-off for dichotomisation which ranged from 58 - 70 years. Assessments of OS for continuous independent variables, by producing dichotomised cut-off points, should ideally be avoided when reporting outcomes for populations. Standardisation and harmonisation of reporting prognostic variables could potentially overcome this selection bias when reporting survival data.

The funnel plot for sex demonstrates significant asymmetry with no small studies demonstrating positive HR's associated with male sex indicating potential publication bias in these articles. Hilar cholangiocarcinoma demonstrates a preponderance in the female sex and the smaller studies may not have adequate power to accurately detect the impact of male sex upon survival.

The prognostic factors of resection margin status, tumour size, portal vein resection and 'T' status may be inter-dependent variables. Literature-based meta-analysis cannot determine the level of effect of inter-dependence. The articles included in the systematic review universally attempted to undertake multivariate analysis to determine which prognostic factors could be modelled to explain OS. However, due to inter-prognostic factor multicollinearity and inter-dependence, there was minimal consistency of prognostic factors selected for modelling between cohorts. DeOliveira and colleagues have established a multinational registry in an attempt to develop a significant international cohort and to standardise prospective reporting of hilar cholangiocarcinoma outcomes [271]. Provisional analysis of the data collated since inception

indicates that increased co-operation and concordance is required globally to validate the utility of the registry [52].

2.4 Evaluation of the Utility of Prognostic Models for Patients with Resected Hilar Cholangiocarcinoma

2.41) SUMMARY

Background:

Several prognostic systems have been proposed to guide management strategies post-resection for patients with hilar cholangiocarcinoma. The objective of this study was to evaluate the efficacy of these conventional prognostic models, with respect to Overall Survival (OS), on patients in a modern single-centre resectional cohort.

Method:

Patients diagnosed with hilar cholangiocarcinoma, referred to a supra-regional tertiary referral centre between February 2009 and February 2016, were retrospectively analysed from a prospectively held database linked to Hospital Episode Statistics and Somerset Cancer Registry data.

Results:

Two-hundred and one patients were assessed for suitability for surgery. Eighty-three (41 %) patients considered to have potentially resectable disease underwent surgical assessment of resectability. Fifty-six (68 %) patients proceeded to resection. Multivariate analysis demonstrated that pre-operative Serum CA 19-9 (p= 0·007), Radiological Arterial Involvement (p= 0·005) and Amsterdam Medical Centre (AMC) prognostic model score (p= 0·032) retained significance in association with OS. Multivariate models developed from this cohort out-performed the conventional prognostic systems for OS.

Discussion:

The cohort-derived multivariate models demonstrated significantly improved prognostic capability compared to conventional systems in explaining OS.

2.42) Background

Hilar cholangiocarcinoma resectability rates are low with patient series indicating that approximately 25 - 40 % of all borderline resectable primary tumours are potentially resectable [177, 250-252]. Post-resectional prognosis remains poorly understood. The American Joint Committee on Cancer (AJCC) staging system is utilised to provide stage-specific prognostication for the patient and to guide collaborative multi-disciplinary team management strategies. The eighth edition of the AJCC staging system aimed to act as a bridge to the development of a personalised approach to prognostication by incorporating approved tumour biological factors as collectable registry data for potential future incorporation in to prognostic models [245, 308]. Two other putative prognostic systems for patients undergoing resection for hilar cholangiocarcinoma have been proposed [180, 309].

The Amsterdam Medical Centre (AMC) Hilar Cholangiocarcinoma Group have proposed a nomogram which utilises post-resectional tumour biological characteristics, not incorporated in the standard AJCC model, as an alternative system for prognosis [309]. This system is yet to be validated in an external cohort. The Memorial Sloane-Kettering Cancer Centre (MSKCC) system, primarily a clinical pre-operative system utilised for assessment of potential resectability, has been putatively demonstrated to be able to prognostically stratify patients [180]. The MSKCC system, initially proposed in 1997 and subsequently modified, stratifies patients for resectability by longitudinal and radial extension of the hilar tumour [176, 177]. Radial extension is determined by the extension of tumour in to the hilar vascular structures. The MSKCC system also utilises surrogate indicators of radial tumour extension, such as ipsilateral or contralateral lobar atrophy, to primarily infer resectability.

The objective of this study was to critically assess the effect of putative prognostic variables, in a modern single-centre resectional cohort, upon OS for patients with hilar cholangiocarcinoma and to compare the efficacy of the above proposed prognostic systems.

2.43) Methods

Patient Selection and Surgical Stratification

Patients diagnosed with hilar cholangiocarcinoma, referred to a Hepato-Biliary tertiary referral centre between February 2009 and February 2016, were retrospectively analysed from a prospectively held database linked to Hospital Episode Statistics and Somerset Cancer Registry data. Patients with distal cholangiocarcinoma, peripheral cholangiocarcinoma and gallbladder cancer were excluded from analysis. Central mass-forming tumours, demonstrated on radiological imaging, which appeared to be predominantly intra-hepatic malignancies were excluded from analysis. All patients with radiographic or endoscopic evidence of tumour originating at the biliary confluence and extending in to the biliary radicles were

included. Any patients with tumour arising in the common hepatic duct were included if the tumour extended to the confluence or in to the radicles.

Discussion of potential resectability incorporating longitudinal and radial extension of the tumour, involvement of hilar vasculature and presence of radiologically proven metastases was undertaken. Encasement of the hilar structures precluded surgical assessment and was defined as representing in-operable locally advanced disease. Patients with these radiological characteristics were allocated to palliative treatment for locally advanced disease. Involvement of any vascular structure was considered to occur when greater than 180 degrees of the vessel were incorporated by the tumour. Qualitative assessment of lobar atrophy was undertaken by comparing bi-lobar volume. If possible comparison to previous abdominal computed tomography (CT) scans was undertaken to determine chronology of lobar atrophy. Nodal status was not used to stratify patients to palliation pre-operatively unless there were concomitant metastases present. Patients considered not fit for surgery or chemotherapy were excluded from further analysis. Endoscopic Retrograde Pancreaticography (ERCP) was routinely undertaken to provide pre-operative biliary drainage on all patients. Serological CA 19-9 tests were undertaken at pre-operative assessment following adequate biliary drainage post-ERCP.

All patients considered potentially resectable underwent surgical assessment to stratify patients for resection as described by Bird and colleagues [6]. Only patients with histo-pathologically proven cholangiocarcinoma were included for analysis. The type of surgical procedure was dependent on the resection of all macroscopic disease and achieving a clear resection margin whilst preserving sufficient remnant liver. Resection of the caudate lobe was always performed when liver resection was undertaken. Intermittent Pringle manoeuvre was used during liver transection if required. Lymphadenectomy was performed routinely and consisted of removal of all lymph nodes and connective tissue in the hepato-duodenal ligament and the retro-duodenal area. The presence of tumour at these margins resulted in an extension of the resection within the hepatic parenchyma to achieve clearance. Where necessary, multiple segmental biliary-enteric anastomoses were performed. Roux-en-Y biliary enteric reconstruction was performed using a 70 cm segment of proximal jejunum. Vascular reconstruction of portal vein or hepatic artery was performed when required to achieve R0 resection.

Follow-up

Following surgical treatment and discharge the patients were reviewed in the clinic setting 4 weeks post-operatively. Subsequent to this the patients were routinely reviewed every 4 months. Review involved clinical examination, serial blood serum measurements and yearly abdominal CT scans. If serial CA 19-9 measurements demonstrated a threshold rise then patients underwent positron emission tomography-computed tomography (CT-PET) investigations to determine the presence of loco-regional recurrence or

metastatic disease. Time to death was calculated from intervention to patient case-note recorded death. Any deaths not attributable to disease or patients lost to follow-up were right censored. All patients with a survival time of less than 30 days were excluded from all subsequent analyses.

Statistical Methodology

Univariate analysis of continuous variables with a dichotomisation cut-point about their median values except where clinically relevant cut-points were pre-specified. Categorical variables with less than 10 events per grouping were removed from subsequent analyses [310]**.** Aggregation of multiple grouping levels was utilised where clinically justified. Dichotomisation of continuous variables occurred about clinically relevant cut-off points where appropriate. CA 19-9 levels were dichotomised around the threshold level for a positive test (46 units per milli-litre of serum). Dichotomisation of continuous variables about the median was undertaken where appropriate. The aggregated scaled AMC nomogram score was dichotomised around the median result (100 points).

Kaplan Meier curves and log rank tests were used to assess univariate survival distributions per variable. Median survival and 95% confidence intervals provided a measure of each variables effect size and range of expected values. Global log rank p-values were determined to achieve statistical significance at the 0.05 level.

All variables which achieved statistical significance in the univariate model were considered for use in the multivariable model. Systems which incorporated significant univariate variables were excluded from the multivariate models to prevent analysis of multi-co-linear data. Variables which were deemed non-significant were included if clinical evidence supported this. Pairwise correlation was calculated for each combination and displayed in matrix form. Combinations with high correlation coefficients (>0.5) were removed.

A multivariate model was constructed for 'Overall Survival' (OS). Stepwise backwards selection was used to identify an optimal model [311]. Hazard ratios for each covariate were provided with 95% confidence intervals. P values were calculated after backwards selection. P values below 0.05 were considered for inclusion within the model. Concordance Indices were calculated for the AMC, AJCC, and MSKCC staging/scoring systems. A Pre-operative Cohort (PC) model was constructed utilising the pre-operative variables found to be significant in the multivariate analysis. A Combined Cohort (CC) model was constructed utilising significant pre and post-operative covariates found from multivariate analysis. The concordance indices of the AMC, AJCC, and MSKCC systems were then contrasted with the optimal CC and PC models found in the multivariate analysis. Gonen and Heller's coefficients were used to measure the proportion of concordant pairings. The Akaikes Information Criterion (AIC) was calculated for each staging/scoring system

as an indication of 'Goodness of Fit'. Differences of greater than 3 in the scale were considered as significant [312].

2.44) Results:

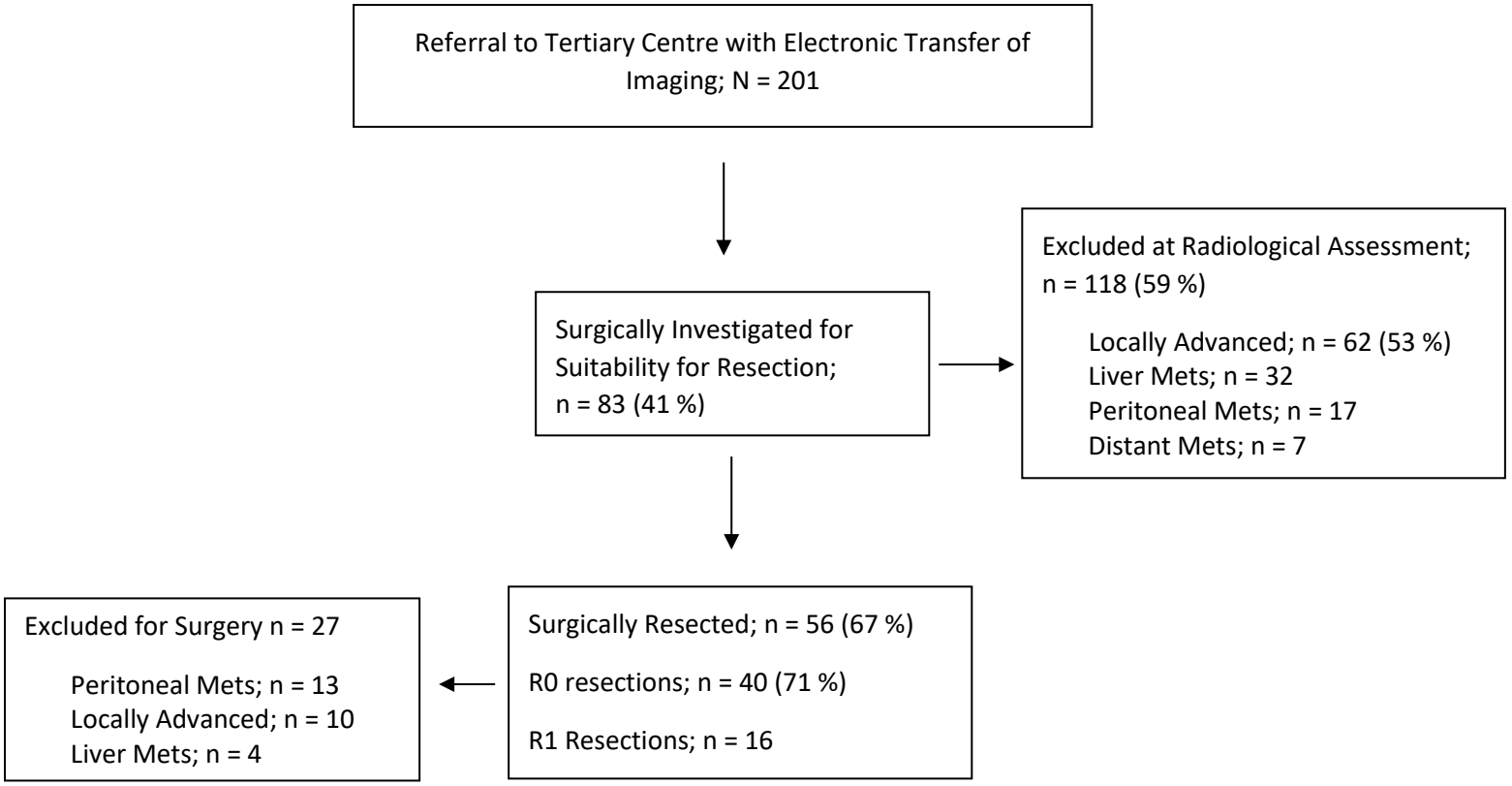
A flow chart of patient selection and outcome are shown in Figure 44. Median OS (95 % Confidence Interval) for all resected patients was 3.65 (2.43–7.01) years. Univariate analysis of standard pre-operative serum, radiological and pathological characteristics of the patients are demonstrated in Table 22.

Table 22: Univariate analysis for Overall Survival of Patient Covariates using Cox PH models

Variable	Level	N deaths (N patients)	Median Overall survival years (95% CI)	P value (log rank test
Pre-Operative Variables				
Serum CA 19-9	Negative	14 (26)	5.55 (2.81-.)	**0.024**
	Positive	13 (22)	3.44 (1.36-4.47)	
Serum NLR (Neutrophil; lymphocyte ratio)	<3	12 (24)	4.47 (3.44-.)	0.423
	>=3	14 (23)	2.81 (1.74-7.74)	
Serum MLR (Monocyte; lymphocyte ratio)	<3	9 (16)	5.55 (2.07-.)	0.480
	>=3	17 (31)	3.43 (1.74-.)	
Radiological Portal Vein Involvement	PV <= 180	11 (24)	4.47 (2.37-.)	0.452
	PV > 180	16 (24)	2.83 (1.65-7.74)	
Radiological Arterial Involvement	HA <= 180	17 (37)	5.55 (3.44-.)	**0.010**
	HA > 180	10 (11)	2.24 (1.36-3.65)	
Radiological Lobar Atrophy	Negative	11 (24)	4.47 (2.37-.)	0.441
	Positive	16 (24)	2.83 (1.65-7.74)	
Post-Operative Variables				
N stage (7th edition)	N0	16 (34)	4.47 (2.83-.)	**0.025**
	N1	11 (14)	2.37 (1.43-.)	
N stage (8th edition)	N0	16 (34)	4.47 (2.83-.)	**0.025**
	N1	11 (14)	2.37 (1.43-.)	
Tumour size (mm)	<30	11 (25)	5.55 (2.81-.)	0.188
	>=30	16 (23)	2.83 (1.40-4.47)	
Grading	Well	5 (9)	7.74 (1.40-.)	**0.018**
	Moderate	12 (24)	5.55 (2.37-.)	
	Poor	10 (15)	2.24 (1.19-.)	
Lymphovascular Involvement	Negative	16 (27)	3.65 (2.24-.)	0.562
	Positive	11 (21)	4.47 (1.74-.)	
Resection Status	R0	19 (35)	4.39 (2.37-7.74)	0.477
	R1	8 (13)	2.81 (1.12-.)	
Vascular Recon	Negative	20 (35)	3.65 (2.07-.)	0.997
	Positive	7 (13)	4.47 (2.43-.)	

Highlighted text for significant variables. Peri-neural Invasion not utilised due to insufficient positive cases.

Figure 44: Flow Diagram Demonstrating MDT Assessment of Hilar Cholangiocarcinoma Patients



Kaplan-Meier curves were constructed of significant variables from the pre-operative univariate analysis (See Figures 45 and 46).

Figure 45: Kaplan-Meier Curve Demonstrating Effect of Serum CA 19-9 on OS

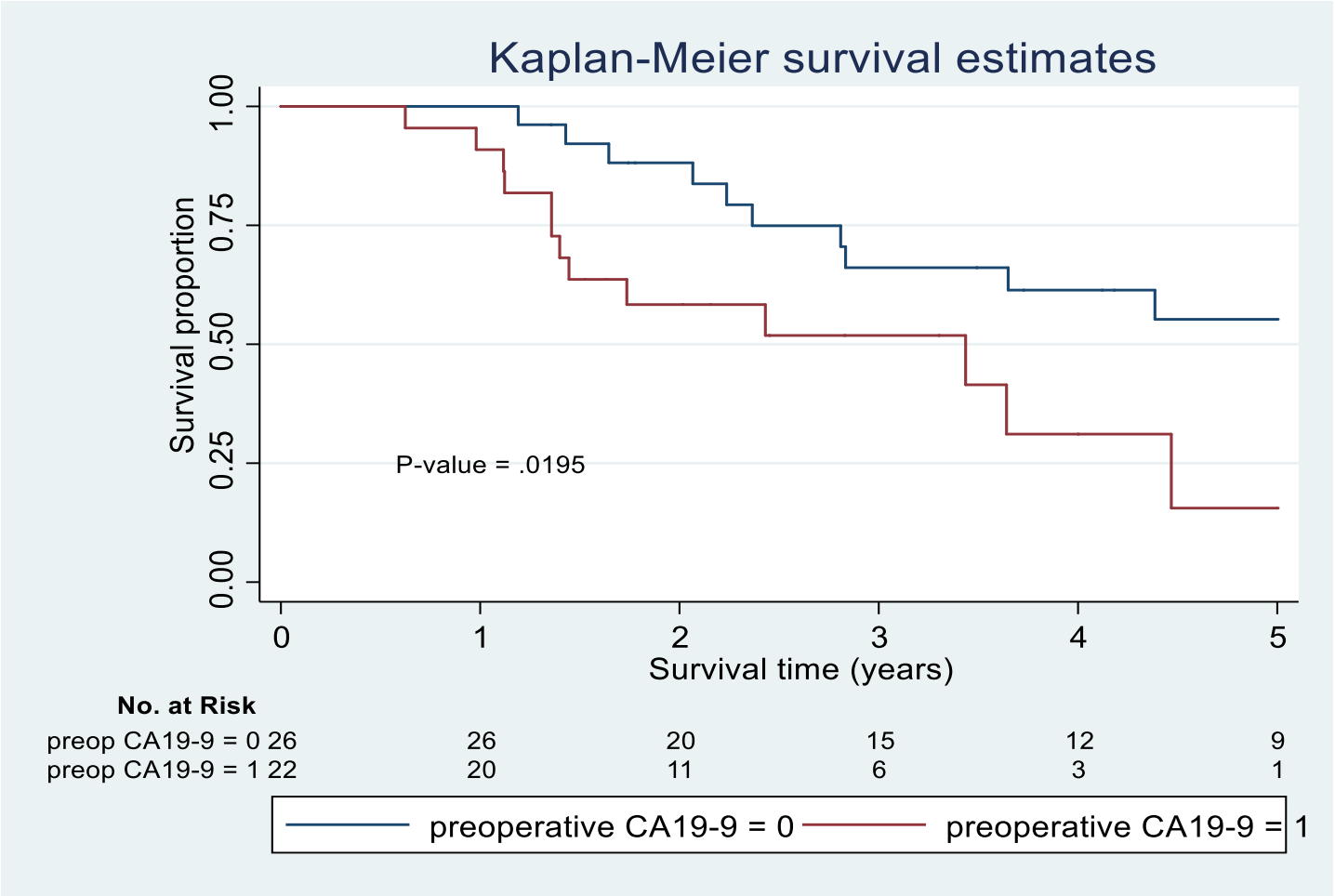
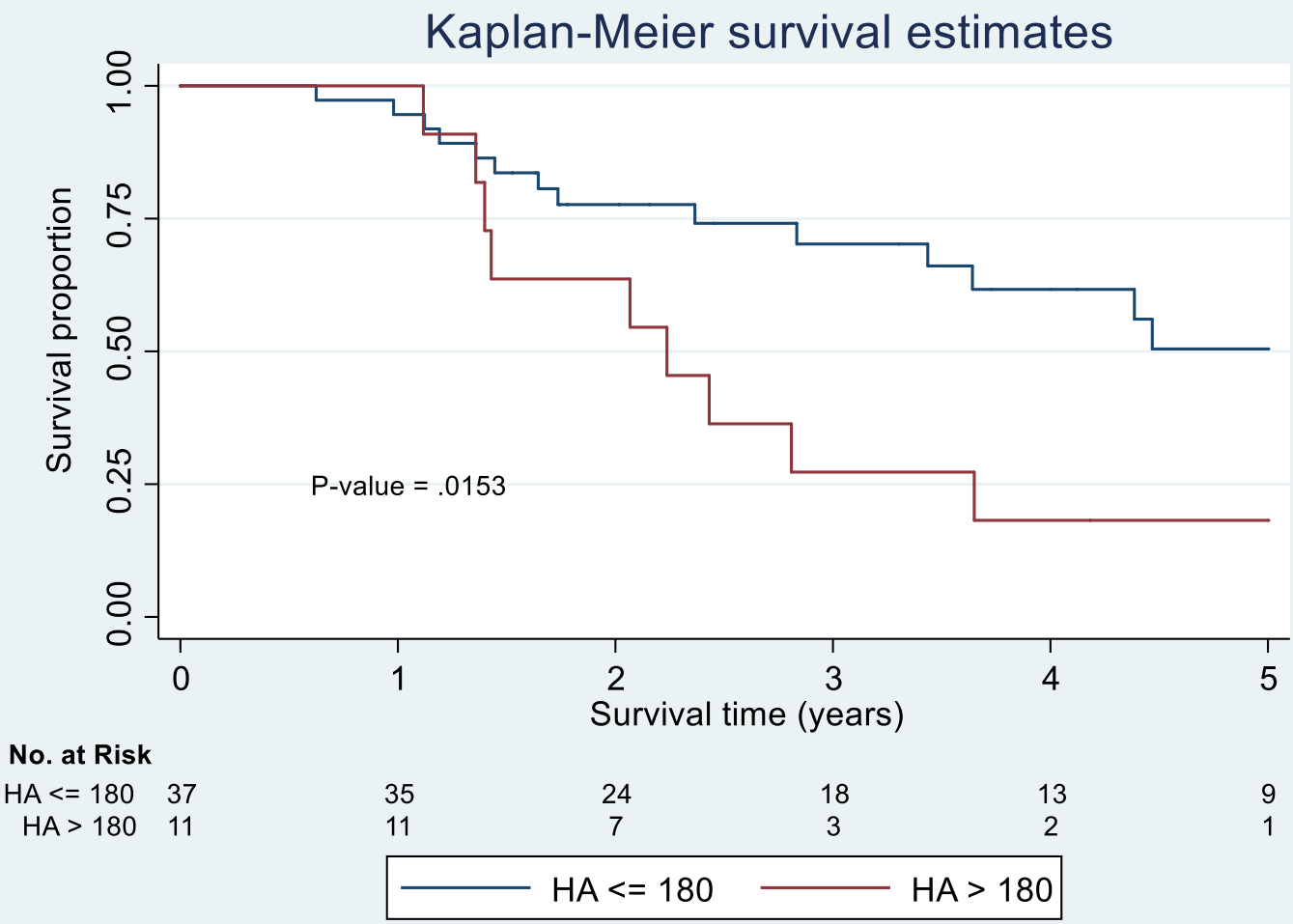


Figure 46: Kaplan-Meier Curve Demonstrating Effect of Radiological Hepatic Artery Involvement on OS – Dichotomisation of Patients in to less than, or greater than, 180 Degree Involvement



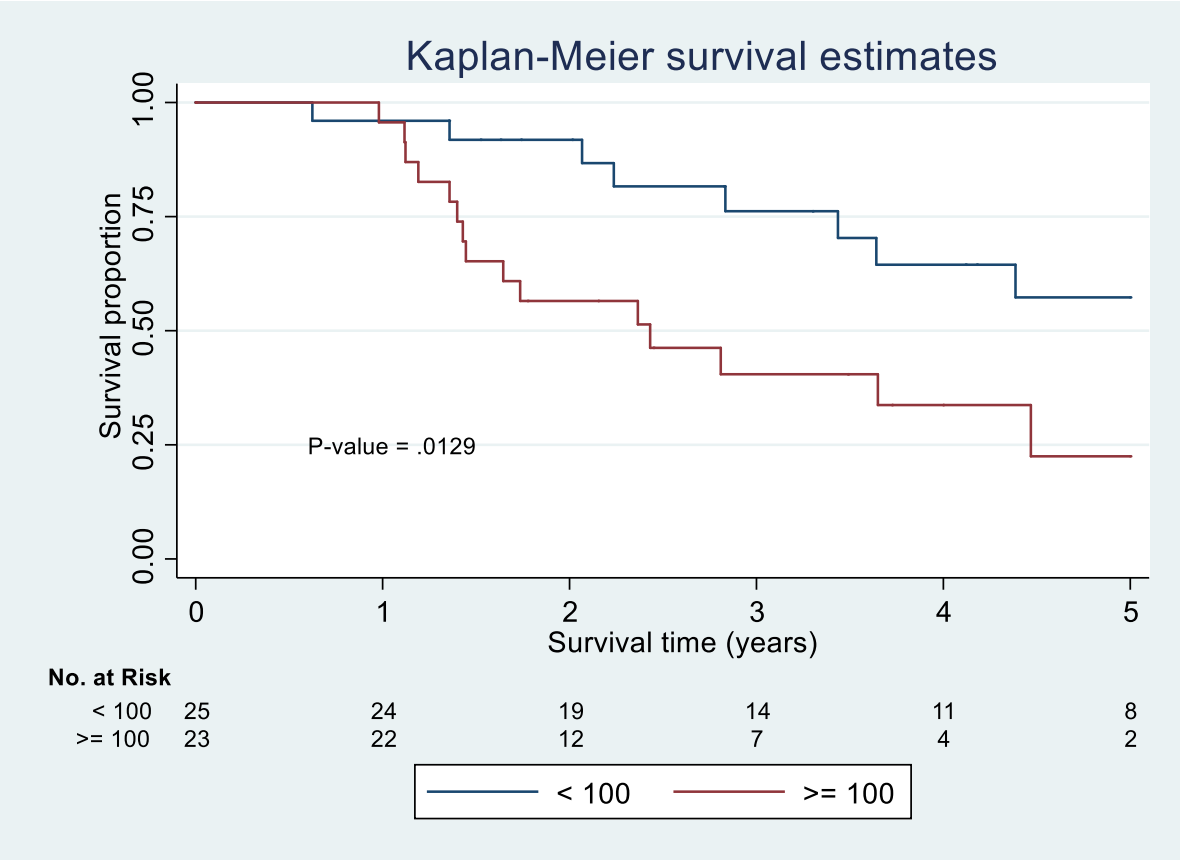
Univariate survival analysis of dichotomised outcomes from the various proposed prognostic systems are demonstrated in Table 23 and Figure 47.

Table 23: Univariate analysis for Overall Survival of Staging Systems using Cox PH models

Variable	Level	N deaths (N patients)	Median Overall survival years (95% CI)	P value (log rank test
MSKCC System	T1	9 (15)	2.37 (1.19-.)	**0.023**
	T2 & T3	18 (33)	4.47(3.44-7.74)	
Bismuth Corlette score	3A	5 (13)	7.01 (2.83-.)	0.099
	3B	13 (20)	3.43 (1.65-.)	
Staging (7th Edition)	1 & 2	13 (30)	7.01 (3.44-.)	0.054
	3 & 4	14 (18)	2.37 (1.45-5.55)	
Staging (8th Edition)	1 & 2	13 (30)	7.01 (3.44-.)	0.054
	3 & 4	14 (18)	2.37 (1.45-5.55)	
AMC Nomogram	<100	10 (25)	7.01 (3.44-.)	**0.011**
	>=100	17 (23)	2.43 (1.43-4.47)	

Highlighted text for significant variables

Figure 47: Kaplan-Meier Curve Demonstrating Association between Dichotomised AMC Nomogram Score and OS



Outcomes of multivariate analysis are shown in Table 24. Multivariate-derived OS models were used to compare concordance indices with the various other prognostic systems for OS.

Table 24: Multivariate Modelling for OS

Model	Covariate	Hazard Ratio (95% CI)	P value
Overall Survival			
Cohort OS	**Pre-op CA 19-9**	**3.24 (1.37-7.69)**	**0.007**
	AMC Nomogram Score	**1.01 (1.00-1.01)**	**0.032**
	MSKCC Score	0.54 (0.20-1.43)	0.254
	Nodal Stage 8th Edition	1.49 (0.33-6.72)	0.659
	Grading	1.12 (0.53-2.36)	0.771
	Radiological Arterial Involvement	**3.33 (1.44-7.71)**	**0.005**

Highlighted text for significant variables

The PC and CC model, constructed utilising Serum CA 19-9 and radiological arterial involvement covariates, significantly outperformed the MSKCC, AMC and AJCC models for OS (See Table 25).

Table 25: Staging/Scoring System Comparison for Prediction and 'Goodness of Fit'

Staging/scoring system	Concordance Index (se)	AIC
Overall Survival		
MSKCC System	0.60 (0.04)	164.5
AMC Nomogram	0.62 (0.05)	163.3
AJCC 7th Edition	0.59 (0.04)	165.4
Stage 8th Edition	0.59 (0.04)	165.4
PC* Model	**0.67 (0.04)**	**157.9**
CC Model**	**0.71 (0.04)**	**155.2**

Highlighted text for significant augmented models; * Pre-operative Cohort; ** Combined Cohort.

The Concordance Index is the weighted pairwise probability of the lower risk patients surviving within the specified system.

The AI Criterion provides an estimate of information loss from over-fitting or under-fitting models to explain OS. The less information lost by a specific model, relative to other models used to explain the OS, the more accurate the model under investigation is.

2.45) Discussion:

This study has demonstrated that the multivariable PC model, constructed utilising pre-operative radiological and serological variables exclusively, demonstrated a significantly improved prognostication system compared to all other conventional standardised systems. The cohort-derived CC model, which combined both the PC and AMC models, demonstrated significant optimal concordance with OS. This study has demonstrated that the AMC nomogram has an improved prognostic capability compared to the AJCC staging system and has been externally validated for the first time in this cohort. The proposed optimal cohort-derived models (CC and PC models) and validated AMC model could provide improved alternative prognostic models, compared to the AJCC system, to guide the care of patients with resected hilar cholangiocarcinoma.

The PC model demonstrated that pre-operatively determined radiological arterial vasculature involvement and serological CA 19-9 levels have a significant impact on OS for patients with hilar cholangiocarcinoma who subsequently undergo resection. Radiologically determined arterial vasculature involvement may act as a surrogate marker of local advancement and has been demonstrated to impact tumour resectability [7]. Arterial involvement by the primary tumour usually occurs concomitantly with the involvement of the portal venous vasculature due to the anatomical relationship of the vascular structures within the hilum. The effect of radiologically determined arterial involvement on OS could be indicative of multi-colinearity of effect between these covariates. However, in this cohort neither radiologically proven portal vein involvement (PVI) nor lobar atrophy (variables considered in conventional systems to be markers of local advancement)

proved to have significant effects on OS. PVI requiring resection and reconstruction to gain adequate R0 resection status, undertaken in this cohort and considered a widely accepted practice, has been demonstrated to not significantly impact OS and may partly explain the lack of effect that radiologically determined PVI has on OS in this cohort [9].

Raised pre-operative serum CA 19-9 levels, in this cohort, demonstrated significantly negative univariate OS associations which were retained within the cohort-derived multivariate models. However, a recent meta-analysis has demonstrated that pre-operative serum CA 19-9 measurements have limited prognostic utility in relation to OS in patients who have undergone resection [9]. Some studies have demonstrated that CA19-9 levels can be used in stratifying patients with radiologically resectable disease for surgical assessment [298, 299]. Raised levels of CA19-9 in patients, not stratified by treatment, have been reported to correlate with the presence of metastatic disease and reduced OS [300]. Raised levels appear to correlate with a reduction in resectability rate owing to radiologically occult metastatic disease [313]. The effect of elevated CA 19-9 levels on OS in this cohort may in fact be due to the presence of systemic micro-metastases which are surgically occult and have spread outside the operative field at the time of surgery.

The AMC nomogram, which incorporates a combination of resectional status, tumour grading and nodal status to produce a scaled score for prognosis, demonstrated significant association with univariate and multivariate OS. The post-resectional variables of resection margin status and tumour grading are not currently incorporated in the AJCC staging system 8th edition despite multiple studies demonstrating the prognostic significance of both post-resectional variables [276, 281, 286, 287, 296]. Prognostic considerations regarding resection margin have provided the impetus for use of concomitant hepatectomy with bile duct resection as the gold-standard for resection of hilar cholangiocarcinoma, however resection status is not currently considered an independent registry collection variable [220]. Tumour grading, a biological characteristic of the primary tumour, is currently considered a registry collection data variable which may lead to incorporation of this data into future staging models for improved patient stratification. The AMC model outperformed the AJCC staging systems in explaining OS, enabling external validation of the model for the first time in an independent cohort.

The CC model, derived from the multivariate analysis, significantly outperformed the other conventional systems in explaining OS within this cohort. The CC model augments the AMC nomogram with the cohort derived PC model. The only common covariate contained within the model which is accounted for in the AJCC system is the category of nodal metastases. The CC model places greater emphasis on biological covariates and contains pre-operative tumour characteristics which may demonstrate less multi-colinearity of effect on OS than the exclusively post-resectional anatomical classifications incorporated by the AJCC. Further validation in external independent series is required to determine the utility of the augmented

model, particularly with reference to prognosis and subsequent clinical-management decisions for patient specific care.

The main limitation with this study is the potential instability of analysis produced by the relatively small number of patients undergoing resection. The majority of the reported resected single-centre cohorts are small and historical, with even relatively high volume centres rarely operating on more than 12 patients per year with peri-hilar cholangiocarcinoma [9, 180, 283, 288, 293, 314]. Multi-institutional series, for example the Extra-Hepatic Biliary Malignancy Consortium (EHBM) in the United States, have greater power compared to single centre series in determining the effect of independent variables upon OS [290, 315-317]. However, patient selection, management and follow-up approaches may vary between the centres and the patient cohort could potentially suffer from increased heterogeneity and right-censoring of data. There are currently no single-centre series or multi-institutional collaborations in the United Kingdom, looking specifically at surgical outcomes, which can match these cohorts for power or homogeneity. Hilar cholangiocarcinoma patients in the United Kingdom, with its unified National Health Service and nationally standardised cancer referral pathways, would potentially benefit from a co-ordinated and collaborative approach to cholangiocarcinoma treatment and research.

The cohort-derived models demonstrated significantly improved prognostication systems for explaining OS compared to the conventional standardised AJCC system. The AMC nomogram has an improved prognostic capability compared to the AJCC staging system and has been externally validated for the first time in this cohort. Multi-institutional collaboration and assessment of survival outcomes for post-resectional patients could improve prognostic models and help to guide management for patients within the United Kingdom.

BASIC SCIENCE CHAPTER

3.1 Prognostic Molecular Markers in Resected Extrahepatic Biliary Tract Cancers; a Systematic Review and Meta-analysis

3.11) SUMMARY

Background:

Better prognostic information for resected extrahepatic cholangiocarcinoma could guide treatment strategies and potentially improve outcome. This study performed a systematic review and meta-analysis to identify prognostic biomarkers for further investigation.

Methods:

Relevant literature was identified using Medline, EMBASE and Web of Science. Primary end-point was overall survival assessed on univariate analysis. Log hazard ratio and variance were calculated and pooled using a random effects inverse variance approach. Hazard ratio (HR) and 95% confidence intervals (CIs) were calculated.

Results:

37 studies, including 2371 patients, met the inclusion criteria. Subsequently 9 biomarkers predictive of OS were identified.

Discussion:

Meta-analysis has identified a number of prognostic biomarkers for resected extrahepatic cholangiocarcinoma. These markers warrant further investigation as potential therapeutic targets and validation in a prospective setting.

3.12) Background

Biliary tract cancer (BTC) is an uncommon cancer, with approximately 1200 new cases of diagnosed each year in the UK [238, 318]. However, the incidence is increasing. BTCs are believed to arise directly from cholangiocytes, and it has been suggested the increased incidence of chronic inflammatory biliary conditions (parasitic infections in Southeast Asia, and the rise in gallstones and hepatitis C infection in the West) may explain the rising number of cases [319].

Despite improved therapeutic options, the long-term outlook for patients with BTC remains dismal with a median survival of less than 12 months [318]. Surgery is the only potentially curative treatment, but only a minority of patients [10-30%] have technically resectable disease [320]. Of those undergoing resection, only forty percent survive for 5 years [321], with the remainder succumbing rapidly to disease recurrence [322]. Existing strategies to predict long-term outcome after resection rely on pre-operative radiological staging and post-resection analysis of tumour margin and nodal status [323, 324]. Improved prognostication through biomarkers has been suggested, and direct analysis of tumour may allow the development of a more personalised therapeutic approach; patients with aggressive disease biology may benefit from adjuvant systemic chemotherapy, more intense follow-up after resection or be better served by non-operative management. Better prediction of long-term outcome would also allow reproducible risk stratification for clinical trials, an important consideration for a rare disease where large RCTs remain difficult. As well as allowing the optimisation of existing treatments, better understanding of key biological pathways involved the pathogenesis of BTC also offers the potential identification of novel therapeutic targets for further investigation. However, the biological pathogenesis of BTC remains poorly understood.

Existing work identifying prognostic biomarkers has relied heavily on immunohistochemical analysis of resected specimens. However, there is marked disparity in the literature between individual studies as to the relative prognostic impact of such markers [325]. This study aimed to perform a systematic review and meta-analysis of commonly reported immunohistochemical markers in resected extrahepatic biliary tract cancers in order to identify important prognostic biomarkers for future analysis.

3.13) Methods

Search Strategy

The search strategy followed PRISMA guidelines [326]. Medline, EMBASE and ISI Web of Science were searched to identify potentially relevant published literature. No chronological search criteria were applied. Existing systematic reviews and reference lists were checked for any potentially relevant additional studies. The most widely investigated immunohistochemical tissue markers for extrahepatic BTC were selected for meta-analysis. These comprised of B-catenin, CDX2, COX2, cyclin D1, E-cadherin, EGFR/HER2, Fascin, Glut1, ki67, MUC1, MUC2, MVD, p16, p21, p27, p53, SKP2, SMAD4, Syndecan, Thymidine and VEGF.

Selection Criteria+

The following criteria were used to search English language articles and abstracts; '(*marker*)' AND ("cholangiocarcinoma" OR "bile" OR "biliary" OR "gallbladder") AND ('survival' OR 'prognosis' OR 'prognostic'). Each search was repeated by two reviewers independently substituting *"marker"* with the name of the marker of interest. Relevant synonyms and wildcard searches were also performed. Abstracts were checked for relevance and the full article was retrieved for all potentially eligible studies. Where part or all of the same patient series was included in more than one publication, the most recent study was selected for analysis. Inclusion criteria for selected studies included cases of resected extrahepatic cholangiocarcinoma or gallbladder cancer, immunohistochemical expression assessed in resected primary tumour material, dichotomised univariate survival analysis reported (i.e. positive vs. negative staining) and overall survival times used in analysis. Where a combination of intra- and extra-hepatic cancers were reported together, studies were only included if adequate data on the extra-hepatic subgroup could be identified. At least 3 distinct series were required to perform meta-analysis.

End-points

The primary outcome measure was overall survival (i.e. date of resection to date of death). Additional details were collected in order to identify potential sources of heterogeneity. These included the specific primary antibody (and dilution) used for immunohistochemistry as well as clinico-pathological data. An assessment of study quality was made according to previously defined criteria [327]. The eligibility criteria and quality scoring were assessed by two independent investigators. Any disagreement was resolved by discussion.

Statistical analysis

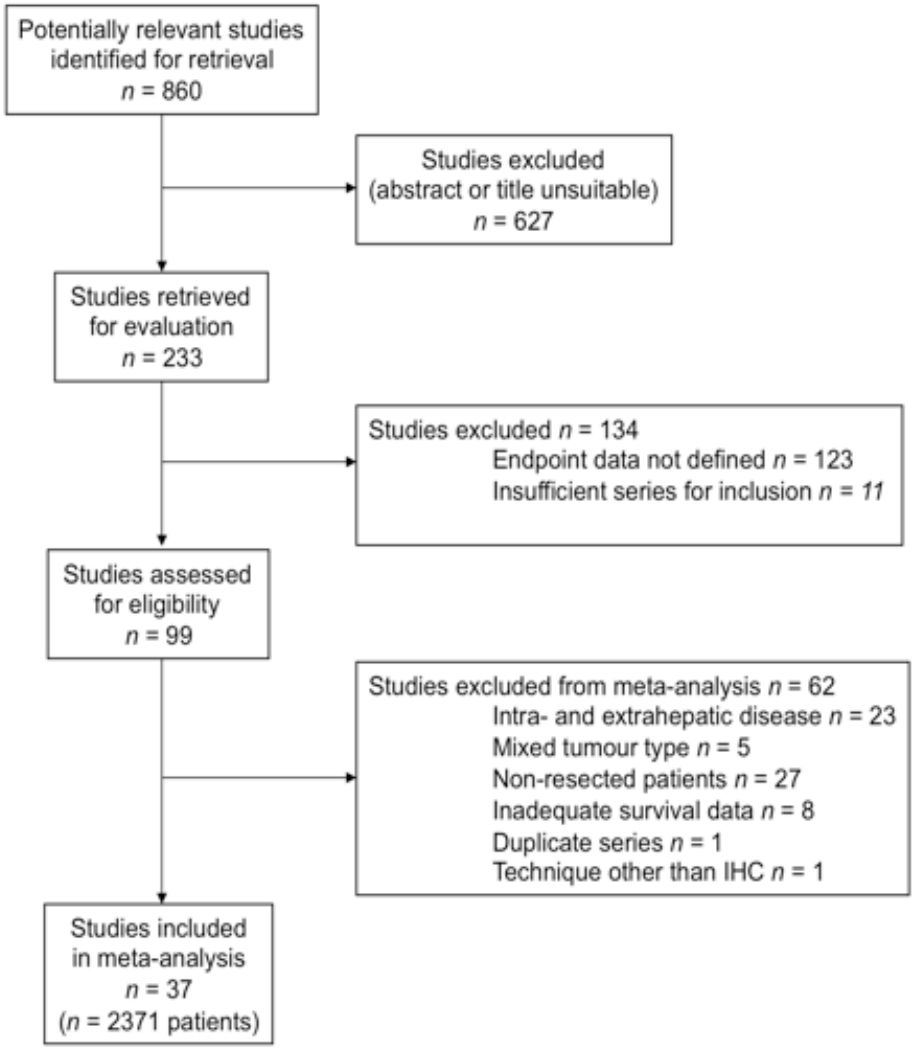
Evaluated markers were sorted according to their major biological function. Previously reported indirect methods were utilised for extracting the log hazard ratio (logHR) and variance due to the paucity of prognostic literature which report these values directly [328-330]. These values were either calculated from the hazard ratio and 95% confidence interval (CI) where quoted, the log rank p-value or from the Kaplan-Meier survival curves directly. The software used for these indirect calculations was designed by the Medical Research Council Clinical Trials Unit, London, UK [328]. The logHR and variance for individual studies were entered into RevMan 4.2 (Cochrane Collaboration, Oxford, UK) and pooled using a random effects inverse variance approach. The overall prognostic effect of positive immunostaining was recorded as a hazard ratio and 95% CI (i.e. a HR > 1 reflecting adverse survival associated with positive immunostaining). Heterogeneity was assessed using a $\chi 2$ test for heterogeneity with a p-value of < 0.10 taken to reflect the presence of significant heterogeneity. The I^2 statistic was calculated to quantify the degree of heterogeneity [331]. A p-value of <0.05 was taken to reflect significance for all other analyses. Funnel plots for publication bias were

assessed where 10 or more studies were included, as test power is considered too low to assess true asymmetry if fewer series are included [332]. Continuous data were compared using Spearman Rank correlation, with two-sided Mann-Whitney for categorical data.

3.14) Results

The initial search strategy identified 860 references, of which 627 were excluded after initial title and abstract review. The remaining 233 were retrieved for further review. Of these, 134 were excluded because the primary endpoint (overall survival) was not defined [n=123] or insufficient series assessing a given biomarker were included (n=11). Of the remaining 99 studies, a further 62 were excluded after further review. 37 studies, assessing 11 distinct biomarkers, were therefore included in the final review with a total study population of 2371 patients (See Figure 48). No studies reported the use of adjuvant chemotherapy after resection. The size of study did not correlate with quality score (Spearman's; p=0.07), nor did the proportion of positive cases (Spearman's p=0.49). Interestingly, study quality was significantly higher for studies reporting a positive rather than negative result (Mann-Whitney; p=0.02).

Figure 48: Flow Diagram Demonstrating Publication Selection



p53

p53 is a perhaps the most important tumour suppressor gene, and regulates cell cycle progression at the G1/S and G2/M checkpoints. Cells with damaged DNA are directed down an apoptotic pathway by p53, and therefore loss of normal p53 is considered a key driver in the development of a number of cancers [333]. Alterations in p53 are frequently detected in BTCs, with overexpression reported more frequently in distal compared to proximal tumours [334].

The 9 eligible studies included a total of 713 patients, with a median quality score of 65% (range 45-75). The median proportion of p53 positive cases was 38% (range 34-57) with significant heterogeneity between series (χ^2=39.26, p=<0.01). The combined hazard ratio was 1.49 (95% CI=0.97-2.29), suggesting a non-significant trend towards worse long term for survival for patients with tumours that stained positive for p53.

VEGF

The development of new blood supply is integral to the development of many cancers. Vascular endothelial growth factor (VEGF) stimulates angiogenesis through the activation of tyrosine kinase receptors (VEGF-1, -2 and -3) bound to the cell surface, and perhaps unsurprisingly VEGF has been shown to be upregulated in a number of different tumour types [335].

Five studies, with a total patient population of 242, were included in the meta-analysis. VEGF comprises of four key ligands (VEGF-A, VEGF-B, VEGF-C and VEGF-D). Three assessed expression of the VEGF-A isoform [336-338], whilst one did not define isoform [339] and one assessed VEGF-C [340]. Median quality score was 60% (range 50-65) and median proportion of VEGF positive cases was 38% (range 29-72). Despite the varied isoforms assessed, there was no evidence of inter-study heterogeneity (χ^2=2.90, p=0.58). The combined hazard ratio was 2.32 (95% CI=1.57-3.44), suggesting that positive staining for VEGF had a negative prognostic value (See Figure 49).

COX-2

Aberrant expression of cyclooxygenase-2 (COX-2) and its principal metabolic product prostaglandin E_2 (PGE_2) are implicated in most of the hallmarks of cancer initiation and progression [341]. The ability of the COX-2/PGE2 pathway to impact on multiple aspects of cellular physiology may explain the apparent chemo-preventative role of COX-2 inhibitors [342]. COX-2 is also upregulated by inflammatory cytokines such as TNF-α and IL-6 via the nitric oxide pathway [343], suggesting a potential pathway of tumour development in pro-inflammatory conditions such as infection or PSC.

Three studies, with 243 patients, were included in the final meta-analysis, with a median quality score of 65% (range 55-70) and 52% median proportion of positive cases (range 33-56). There was no evidence of significant heterogeneity (χ^2=5.12, p=0.08). The combined hazard ratio was 1.94 (95% CI=1.01-3.71), suggesting that positive staining for COX2 was associated with worse overall survival (See Figure 49).

GLUT1

Intracellular glucose transport is necessary for the survival, proliferation and function of cells and this process is mediated by glucose transporter (GLUT) proteins. It has long been recognized that cancer cells have increased rates of glucose metabolism compared with normal cells, with upregulation of GLUT1 reported in many cancers [344].

Four studies assessing GLUT1 were included in the final meta-analysis, containing 357 patients. One gave subgroup analysis for extrahepatic and gallbladder cancer, and so these groups were analysed separately [345]. Median quality score was 75% (range 70-75). The median proportion of GLUT1 positive cases was 42% (range 31-52). No significant heterogeneity was detected using Cochran's χ^2 test (χ^2=4.37, p=0.22). The

combined hazard ratio was 2.09 (95% CI=1.52-2.89), suggesting that positive staining for GLUT1 was associated with worse overall survival (See Figure 50).

Cyclin D1

Cyclin and cyclin-dependent kinases are integral to the progression of cells through the cell cycle and exert their effect through the phosphorylation of the tumour suppressor protein Rb. Overexpression of these molecules results in cell-cycle dysregulation, increased cellular turnover and proliferative activity [346].

Four studies assessing the key cyclin, cyclin D1, were included in the final meta-analysis, and included a total of 248 patients. Median study quality was 75% (range 50-75) and median proportion of Cyclin D1 positive cases was 50% (range 27-69). Significant heterogeneity was detected using Cochran's χ^2 test (χ^2=9.53, p=0.02). The combined hazard ratio was 1.96 (95% CI=1.02-3.76), suggesting that positive staining for Cyclin D1 was associated with worse overall survival (See Figure 50).

p16

p16 blocks the progression of the cell cycle by binding to CDK4/CDK6, inhibiting cyclin D resulting in reduced phosphorylation of RB [347]. Low levels of p16 may therefore result in inappropriate cell cycle progression.

Four studies assessing p16 expression were included in the final meta-analysis, with a median quality score of 65% (range 60-75). The total study population included 364 patients. Median proportion of p16 positive cases was 48% (range 24-70). There was no evidence of significant heterogeneity using Cochran's χ^2 test (χ^2=3.69, p=0.3). The combined hazard ratio was 0.68 (95% CI=0.47-0.98), suggesting that positive staining for p16 was associated with better overall survival (See Figure 50).

p27

p27 is a cyclin dependent kinase inhibitor that belongs to the Cip/Kip family. p27 binds to the cyclin D-CDK4 complex, regulating cell cycle progression through G1 [348]. Loss of p27 can therefore lead to aberrant cell cycling. Six eligible studies were included, with a total of 372 patients. Median quality score for the included series was 75% (range 55-75). Median proportion of p27 positive cases was 54% (range 34-68). There was no evidence of significant heterogeneity (χ^2=11.05, p=0.05) between studies. The combined hazard ratio was 0.48 (95% CI=0.3-0.78), suggesting that positive staining for p27 was associated with improved long-term survival after resection (See Figure 50).

β-catenin

β-catenin is integrally involved in cell-to-cell adhesion [349], as well as being a downstream effector of the Wnt signalling pathway [350]. β-catenin has also been implicated in epithelial-mesenchymal transition [351] and loss of function has been associated with many tumour types [352].

Three studies, containing 235 patients, were considered appropriate for meta-analysis. Median quality score was 55% (range 50-60) and median proportion of β -catenin positive cases was 18% (range 15-20). There was no evidence of significant heterogeneity (χ^2=0.09, p=0.96), with a combined hazard ratio of 0.91 (95% CI=0.55-1.51), suggesting no significant relationship between β–catenin expression and overall survival.

E cadherin

E-cadherin is a calcium-dependent cellular adhesion molecule, integrally involved in cellular migration [353] and loss of function has been implicated in the progression to malignant phenotype [354].

Four studies were included for meta-analysis, and contained 326 patients. Median quality score was 60% [range 50-70]. Median proportion of E cadherin positive cases was 54% (range 45-70). There was no evidence of significant heterogeneity (χ^2=0.63, p=0.89) between the studies selected for inclusion. The combined hazard ratio was 0.47 (95% CI=0.35-0.63), suggesting that positive staining for E Cadherin was associated with improved overall survival (See Figure 49).

Fascin

Fascin is an actin binding protein which regulates cellular adhesion by co-localising with β-catenin [355], and overexpression has been implicated in the development of different gastrointestinal malignancies [356].

Four studies assessing Fascin expression in BTC were included in the meta-analysis, and involved 212 patients. One gave subgroup analysis for extrahepatic and gallbladder cancer, and so these groups (n=4) were analysed separately [357]. Median quality score was 70% (range 45-75) and median proportion of β -catenin positive cases was 52% (range 29-56). There was no evidence of significant heterogeneity (χ^2=4.89, p=0.18), with a combined hazard ratio of 2.19 (95% CI=1.35-3.55), suggesting that positive staining for Fascin was associated with worse overall survival (See Figure 49).

Ki67

Relative rate of tumour growth and therefore aggressive malignant potential can be assessed by measuring rate of cellular proliferation. Ki67 is a protein directly involved in cell division, and degree of expression is commonly used a proliferative index [358].

Five studies, containing 233 patients, were included in the final meta-analysis. Median quality score was 70% (range 55-75) and median proportion of Ki67 positive cases was 45% (range 34-50). There was no significant heterogeneity between series (χ^2=7.20, p=0.13). The combined hazard ratio was 1.69 (95% CI=1.02-2.79), suggesting that positive staining for Ki67 was associated with worse overall survival (See Figure 49).

Figure 49: Forest Plot Demonstrating Prognostic Factors and Associated OS

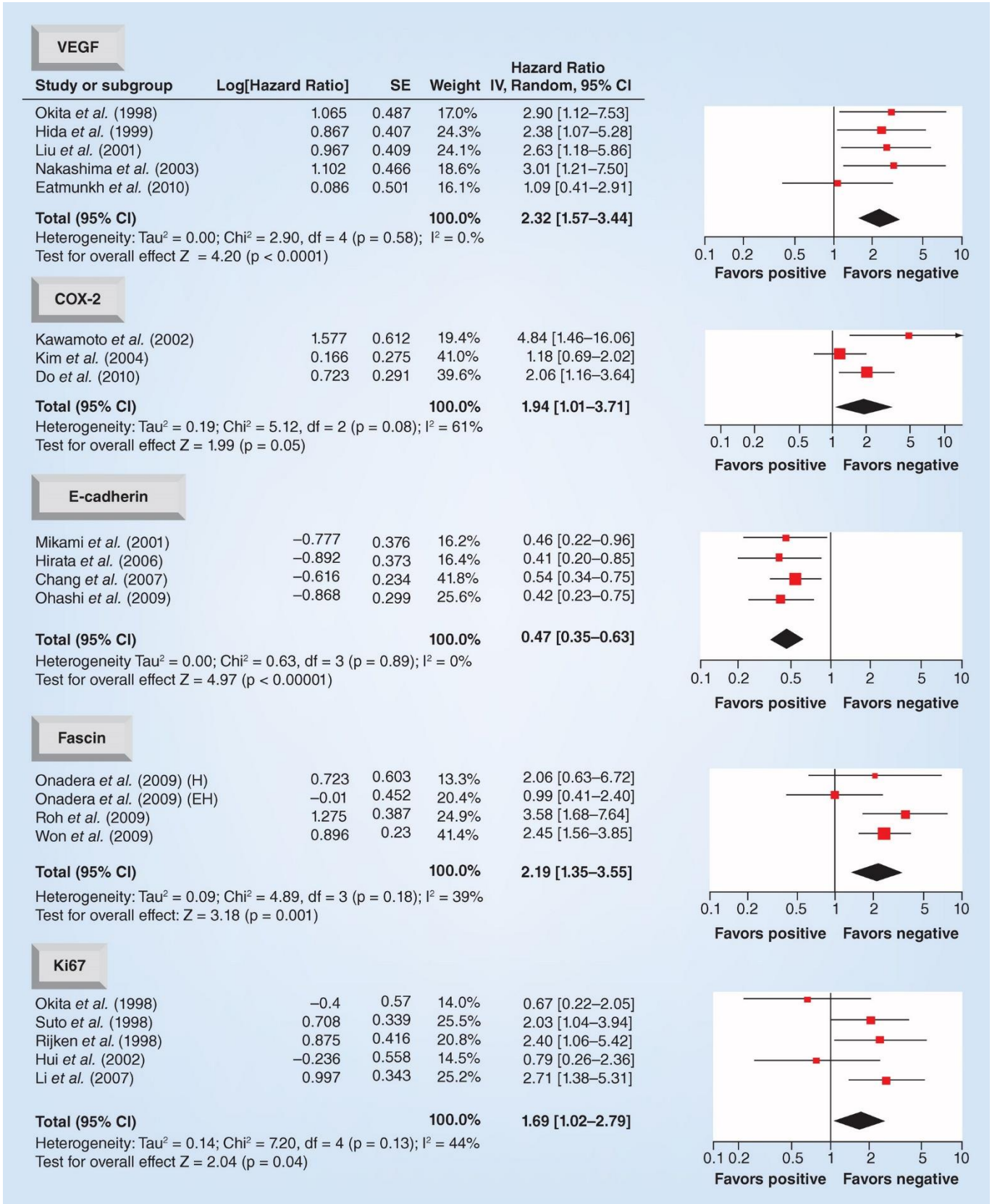
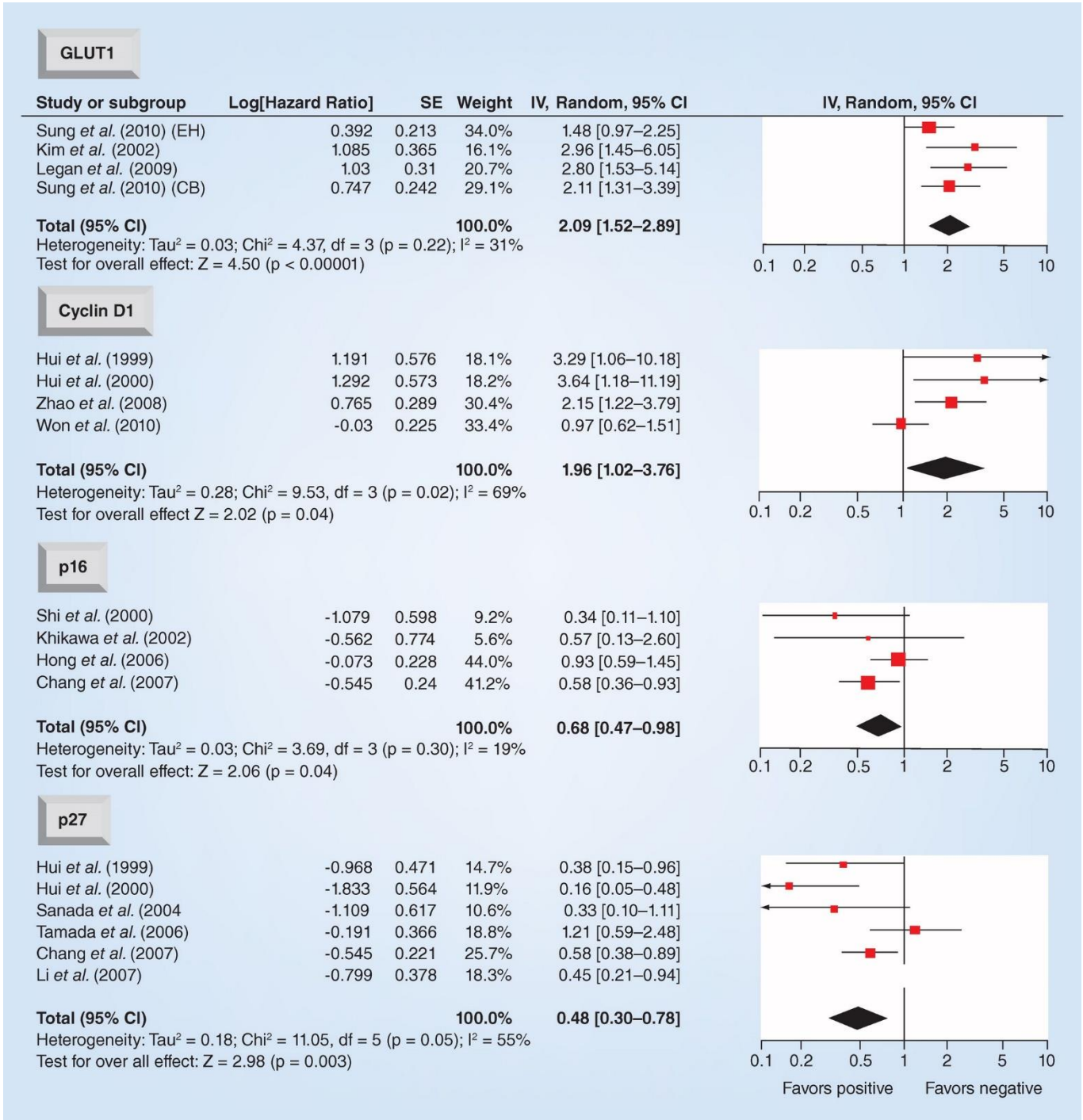


Figure 50: Forest Plot Demonstrating Prognostic Factors and Associated OS



3.15) Discussion

This study has identified a number of biomarkers that offer potential prognostic information for resected extrahepatic BTC. These findings offer an insight into the molecular pathogenesis of extrahepatic cholangiocarcinoma, and suggest potential areas for investigation in future studies.

Meta-analysis as performed in this study has a number of inherent limitations. Data is almost universally retrospective, with study size therefore defined by number of patients treated within a unit. Resected and non-resected patients with different subgroups of disease are therefore often included within the same analysis. Adequacy and reliability of clinical data may also be questionable in this retrospective setting. Because of small study size, many groups do not report multivariable survival data making analysis of subgroups difficult.

Meta-analysis of immunohistochemical analysis also presents challenges. Immunohistochemistry is a semi-quantitative technique, with variations in scoring and cut-off criteria presenting potential confounding factors. Immunohistochemistry also assesses gross changes in protein expression and localisation based on antibody cross-reactivity. Subtle changes in structure of the protein under examination (for example, those caused by genetic mutation) may lead to significant changes in function and are not detected by this technique.

In order to address some these issues, this study applied a number of strict inclusion criteria. BTCs are a heterogenous group of tumours with differences in risk factors and histological features, and there is growing evidence that intra and extrahepatic BTCs are distinct biological entities [359, 360]. This analysis therefore only assessed extrahepatic biliary tract cancers. Only series reporting resected patients were included, and adequate data was required to allow either direct or indirect calculation of $_{log}$HR and variance. Because of these strict inclusion criteria, several markers were analysed using only small numbers of series increasing the risk of publication bias.

In addition, only biomarkers where at least 3 independent series were identified were analysed. Despite this, only 2 analyses (p53, Cyclin D1) demonstrated significant inter-study heterogeneity. There are many potential reasons for this, including different underlying tumour subgroups, scoring criteria, cut-offs and IHC technique. One of the key limitations of immunohistochemistry is such disparate scoring systems, which can limit comparison between series. Predefined cut-offs for immunohistochemical positivity vary, and without centralised pathological review direct comparison may be difficult. Despite this clear limitation, it is striking how variations in cut-off do not appear to directly correlate with proportion of patients considered to be positive for the biomarker of interest nor with the hazard ratio of effect. Whilst acknowledging these limitations, immunohistochemistry remains an important and useful technique which can be easily and reliably performed on historical resection specimens processed using standard pathological techniques i.e.

formalin fixation/paraffin embedding. Prospective collection of tissue with storage techniques allowing more accurate analysis (such as freezing at -80^{o}C) are limited by cost and infrastructure and may present significant obstacles to further research, especially in units outside the Western world where the incidence of cholangiocarcinoma remains significantly higher. Study quality did appear to be higher in studies reporting a positive result but there was no association between study quality and study size. Positive findings are more likely to be published in more prestigious journals [361], and it is possible that the more arduous peer review in these journals resulted in better quality of study.

This meta-analysis suggests a number of potential predictive biomarkers defined in a surgically resected cohort. Further validation in a non-resected cohort is required- it may be that disease that presents as surgically resectable is biologically distinct. ERCP biopsy samples for non-resected patients would offer one potential comparative group. Truly quantitative proteomic techniques, such as mass spectrometry based assays, would also allow more accurate assessment of protein expression.

Despite these limitations, validation of the molecular prognosticators identified in this analysis offers the potential of a stratified management approach. Patients at high risk of recurrence, based on their molecular phenotype, may benefit from more aggressive and potentially toxic adjuvant chemotherapy after curative resection or more intense follow-up after surgery. These biomarkers also provide a biological rationale for targeted therapies in future trials of biliary tract cancer.

In conclusion, this study demonstrates that VEGF, COX-2, GLUT-1, Cyclin D1, p16, p27, E-Cadherin, Fascin and Ki-67 expression are all prognostic for long-term survival in resected biliary tract cancer. These markers warrant further investigation as potential actionable therapeutic targets and validation as prognostic biomarkers in an unselected prospective setting.

3.2 Immunohistochemically Assessed hENT1 Expression in Resected Pancreatic Ductal Adenocarcinoma Specimens is a Prognostic Biomarker in Patients Undergoing Adjuvant Gemcitabine-Based Chemotherapy

3.21) SUMMARY

Background: Human Equilibriative Nucleoside Transporters (hENT) are trans-membranous ubiquitous proteins which facilitate the uptake of nucleosides and nucleoside analogues, such as gemcitabine, into the cell. Research has tentatively inferred that the abundance of hENT1 transporters in resected pancreatic ductal adenocarcinoma (PDAC) is a predictive biomarker of adjuvant chemotherapy. The aim of this meta-analysis and systematic review is to assess whether hENT1 expression, as determined by immuno-histochemistry, is a suitable predictive marker for subsequent treatment with gemcitabine-based adjuvant chemotherapy.

Methods: Databases were searched from January 1997 to January 2016. Articles pertaining to hENT1 immuno-histochemical analysis in resected PDAC specimens from patients who subsequently underwent adjuvant gemcitabine-based chemotherapy were systematically identified. Eligible studies were required to contain survival data, reporting specifically overall survival (OS) and disease-free survival (DFS) with associated Hazard Ratios (HR's) stratified by hENT1 status.

Results: Of 42 articles reviewed 8 were deemed suitable for review with 7 being quantitatively meta-analysed. The total number of patients included in the meta-analysis was 770 (365 hENT1 –ve; 405 hENT1 +ve). Immunohistochemically detected hENT1 expression was found to be significantly associated with both DFS (HR 0.58 [0.42 – 0.79]) and OS (HR 0.52 [0.38 – 0.72]) in patients receiving adjuvant gemcitabine but not fluoropyrimidine-based adjuvant therapy.

Discussion: This meta-analysis demonstrates that hENT1 expression is a suitable prognostic biomarker for outcome in patients undergoing adjuvant gemcitabine-based chemotherapy.

3.22) Background

Gemcitabine is a widely used chemotherapeutic agent in the treatment of a variety of cancers. Randomised trials have established its utility as adjuvant therapy for patients undergoing resection for pancreatic adenocarcinoma. However, although adjuvant gemcitabine improves long term survival, this remains in the order of 20% at 5 years, suggesting that many patients harbour gemcitabine-resistant micro-metastases. Substantial work has been done to determine the pharmaco-genomic interactions of gemcitabine in an attempt to develop a stratified approach for gemcitabine chemotherapy [362-367]. Gemcitabine is a hydrophilic nucleoside analogue requiring human equilibriative nucleoside transporters (hENT) to facilitate its uptake into the cell [368-370].

There are 4 hENT isozymes of which the hENT1 transporter is the primary facilitative transporter of gemcitabine [371]. Low/absent hENT1 expression in cellular membranes correlates with low levels of intracellular gemcitabine [372]. Conversely, overexpression of hENT1 in pancreatic cancer cell lines can increase gemcitabine efficacy [373]. Following uptake into the cytoplasm gemcitabine undergoes sequential phosphorylation steps to generate the active drug. Phosphorylated gemcitabine exerts its effects by mimicking a cysteine nucleoside which becomes inserted into the replicating DNA during S-phase of the cell cycle inducing S-phase arrest and initiating apoptosis through masked chain termination [371, 374].

Accordingly, hENT1 may be a predictive biomarker for adjuvant gemcitabine treatment in the setting of resected pancreatic cancer [374]. This systematic review and meta-analysis focuses specifically on determining if immunohistochemical (IHC) assessment of hENT1 transporter abundance in resected pancreatic adenocarcinoma (PDAC) specimens is predictive for patient survival when treated with gemcitabine adjuvant chemotherapy.

3.23) Methods

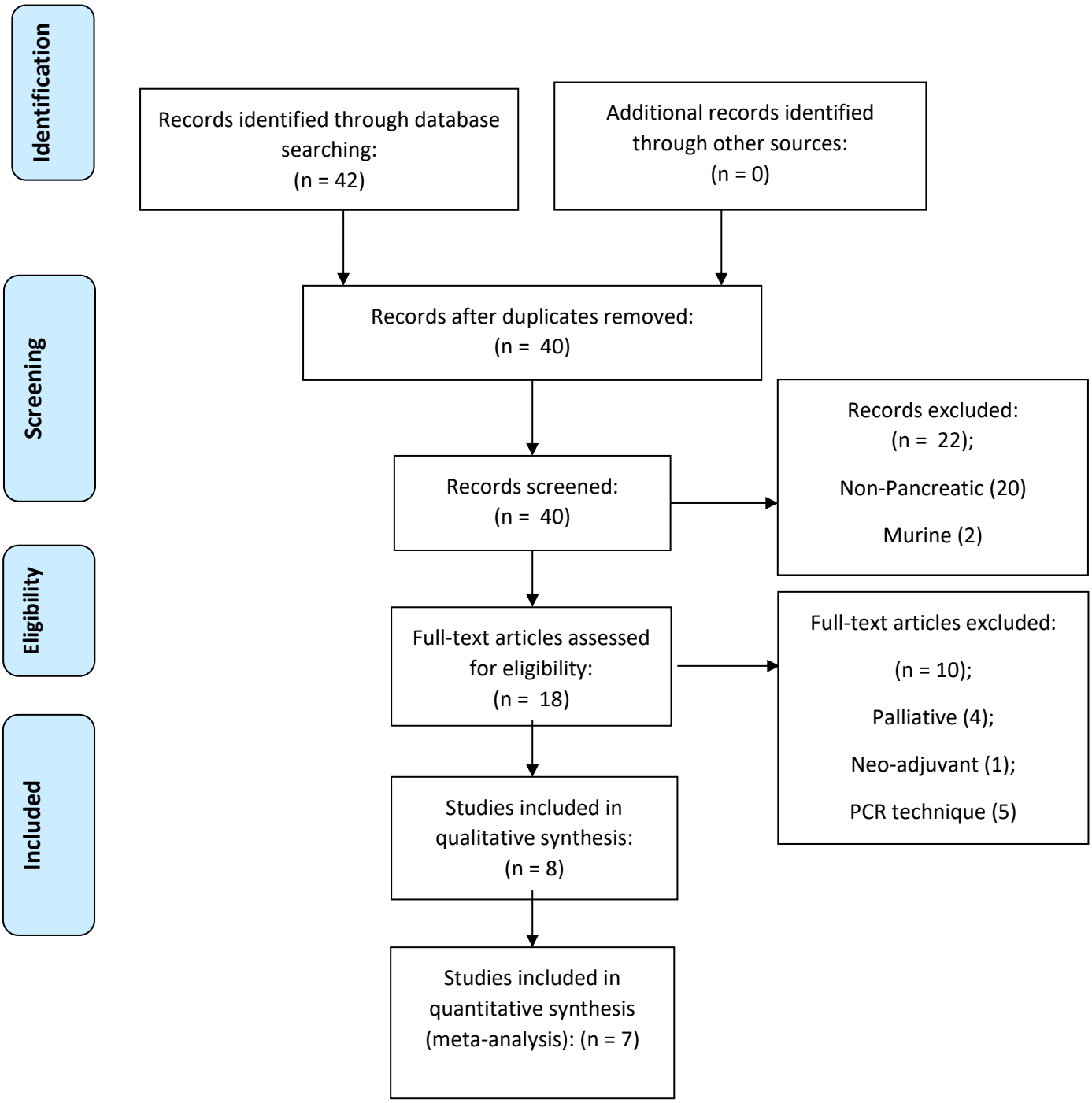
Search Strategy and Study Eligibility

Meta-Analysis of Observational Studies (MOOSE) epistemological guidelines were utilized to identify eligible studies [272, 375]. Exploded and linked combinatorial search terms were used on MEDLINE, PUBMED and EMBASE search engines to ensure full-spectrum acquisition of all relevant articles. The search terms employed were '*pancreatic cancer*', '*hENT1*', '*human Equilibriative Nucleoside Transporter*', '*adjuvant chemotherapy*', '*gemcitabine*', '*prognostic*' and '*predictive*'.

The temporal search parameters were from 1997 – January 2016. The rationale for this time period is that hENT1 was discovered in 1997 and March 2016 was the meta-analysis study inception date. Reviews and other partially relevant studies were analysed to ensure that any further citations could be acquired if necessary. Study inclusion was assessed by the primary authors and any discrepancies were resolved with

discussion. Study identification followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (See Figure 51) [376].

Figure 51: PRISMA Flow-chart for Article Selection



Published articles were considered eligible for meta-analysis if patient survival characteristics were analysed in relation to hENT1 status in resected primary PDAC tissue. The hENT1 status was determined by the respective authors with restriction of laboratory methodology to IHC analysis. No restriction regarding provenance of hENT1 anti-body, the hENT1 epitope status or hENT1 scoring system was enacted. Neo-adjuvant studies were excluded due to the potentially confounding effects of chemotherapy upon intra-tumoural hENT1 expression prior to resection. Palliative studies were also excluded.

Data Extraction

Study characteristics including study date, journal, impact factor of journal, citations, animal antibody origin and survival characteristics were tabulated for an efficient referencing system during data collation (see Tables 26 and 27). Patient numbers and percentages among different sub-groups were calculated from available data when not represented in the articles.

Primary end-point of study was the Overall Survival (OS) of patients determined by the Hazard Ratio (HR), with appropriate Confidence Intervals (CI), and defined as the time from point of diagnosis to point of death or being alive at time the time of the study's analysis. Secondary end-point of study was the Disease Free Survival (DFS) of patients determined by the HR with appropriate CI's and defined as the time from point of diagnosis to point of recurrence.

For most trials the HRs were provided in the paper, and the HR for the univariate Cox model with hENT1 status as the prognostic factor was used. The statistical analysis followed standard meta-analytical procedure using a one-stage approach. The standard error in the HR was calculated from the upper and lower limits of the 95% Confidence Interval (CI) provided. Where the HR was not provided it was estimated from the p-value for the log-rank test comparing the end-points in patients with negative and positive hENT1 status, and the number of events taken from the values for end-points estimated from the Kaplan-Meier curves. The methods used in computing the HR can be found in Parmar et al. [274]. All hazard ratios were calculated with reference to hENT1 –ve patients as the standard. Therefore a HR of <1 indicated that the probability of the assessed event occurring at the next time-point in hENT1+ve patients was less likely to occur than in hENT1-ve patients.

Predictive and prognostic definitions are often used imprecisely and interchangeably therefore the Biomarker Definitions Working Group epistemological considerations of '*prognostic*' and '*predictive*' were utilised to define the '*prognostic*' status of hENT1 as the ability of the anti-body to determine improved survival characteristics of the post-resection patient when treated with gemcitabine-based adjuvant chemotherapy [377-379].

Table 26: Characteristics of Articles Included in Study

Author	Year	Total Patients	hENT1 +ve	hENT1 –ve	% +ve	hENT1 Anti-body	Univariate OS	Univariate DFS	Follow-up (Years)
Farrell	2009	91	73	18	80.2	10D7G2 Mouse Monoclonal	0.51 (0.29 – 0.91	0.57 (0.32 – 1.0)	4
Marechal	2009	45	19	26	42.2	10D7G2 Mouse Monoclonal	0.29 (0.12 – 0.58)	0.28 (0.13 – 0.61)	10
Marechal	2012	234	92	142	39.3	10D7G2 Mouse Monoclonal	0.43 (0.3 – 0.63)	Not Discussed	6
Morinaga	2012	27	16	11	59.3	Rabbit Polyclonal	0.37 (0.15 – 0.91)	0.36 (0.19 – 0.9)	5
Nakagawa	2013	109	78	31	72	Rabbit Polyclonal	0.36 (0.19 – 0.69)	0.52 (0.32 – 0.84)	5
Greenhalf	2014	176	99	77	56.3	10D7G2 Mouse Monoclonal	0.6 (0.43 – 0.83)	0.64 (0.47 – 0.89)	5
Sinn	2015	88	60	28	68.1	SP120 Rabbit Monoclonal	1.05 (0.72 – 1.53)	0.96 (0.66 – 1.41)	13

Table 27: Summary of Immunohistochemistry and Chemoradiotherapy Characteristics of the Studies

Author	Trial	IHC Blinding	IHC Scoring	Positive Control	Negative Control	Chemotherapy Regime
Farrell	RTOG 9704	2 Readers	Trichotomised	Lymphocytes	Not Stated	Gem + Radiotherapy vs 5-FU + Radiotherapy
Marechal (2009)	EORTC	1 Reader	Dichotomised	Lymphocytes	Not Stated	Gem + Radiotherapy
Marechal (2012)	Single Centre	3 Readers	Dichotomised	Lymphocytes	Not Stated	Gemcitabine vs Radiotherapy +/- 5-FU
Morinaga	Single Centre	Not Stated	Dichotomised	Not Stated	Not Stated	Gemcitabine
Nakagawa	Single Centre	2 Readers	Dichotomised	Pancreas	Buffer Replaced Antibody	Gemcitabine
Greenhalf	ESPAC-3	2 Readers	Dichotomised	Array of Cores	Buffer Replaced Antibody	Gemcitabine vs 5-FU
Sinn	CONKO 001	2 Readers	Trichotomised	Not Stated	Not Stated	Gemcitabine Vs Observation

3.24) Results

Eligible Studies

Forty-two studies were initially identified. Twenty studies were excluded without further analysis as the studies were concerned with a variety of solid organ and haematological malignancies or did not have relevance to the associated primary search terms. Two studies were excluded due to replication of data. Two studies were excluded as they assessed murine specimens. Five studies were excluded as the chemotherapy regimens were of a palliative [380-383] or neoadjuvant nature [384]. Thirteen articles underwent qualitative review. Five articles were excluded as they used primarily Polymerase Chain Reaction (PCR) techniques rather than IHC, which was the index method under investigation in this study. Further analysis of Kondo *et al* [385] and Nakagawa *et al* [386] demonstrated them to be replicated PDAC populations resulting in the exclusion of the Kondo *et al* paper, as it was a sub-set of the cohort analysed, to avoid multiplicity errors. Seven articles were deemed suitable for systematic review [386-392].

The primary and the secondary endpoints of this study were reported with varying levels of consistency in the articles. Two studies allocated categories of hENT1 expression to incorporate a '*no hENT*', '*low hENT*' and '*high hENT*' [391, 392] for these studies '*low*' and '*high*' hENT was grouped as hENT1 +ve. The remaining 6 studies dichotomised their categorisation of hENT1 expression to reflect a hENT1 *'low'* or *'high'* sub-division for which hENT1 -ve and hENT1 +ve categories were allocated. Once trial-specific estimates for HRs had been obtained, they were combined to obtain an overall estimate of the HRs and their variances. For the meta-analysis the Metan command in Stata 14 was used. As the HRs are not normally distributed, analysis was performed using log HR and the corresponding 95% CI. Anti-logs of the pooled HR were then reported.

Greenhalf *et al* [390] provided the HR for DFS which was not previously stated in their paper and allowed these data to be grouped in the pooled HR analysis.

Statistical Analysis

The 7 studies analysed contained 770 patients who had undergone attempted curative resection of pancreatic adenocarcinoma with subsequent adjuvant chemotherapy and hENT1 IHC analysis with a median follow-up period of 5 years (Range 4-13 years). Seven papers were used to calculate pooled Univariate Overall Survival (UOS) [386-392] containing a total of 770 patients, 365 (47.4 %) hENT1 –ve and 405 (52.6 %) hENT1 +ve respectively. The pooled hazard ratios (HR's) demonstrate a decreased hazard of death for hENT1+ve patients compared to hENT1-ve patients (0.52 [95% CI 0.38 – 0.72]). The hazard ratio infers that hENT1+ve patients undergoing adjuvant gemcitabine-based chemotherapy have a 48 % reduced probability of dying at the next temporal-point measurement compared to hENT1-ve patients (see Figures 52 & 53). The pooled UOS HR demonstrates high heterogeneity between studies (I^2=65.8%, p=0.008).

Figure 52: Forest Plot of Random-Effects Model for Univariate Overall Survival

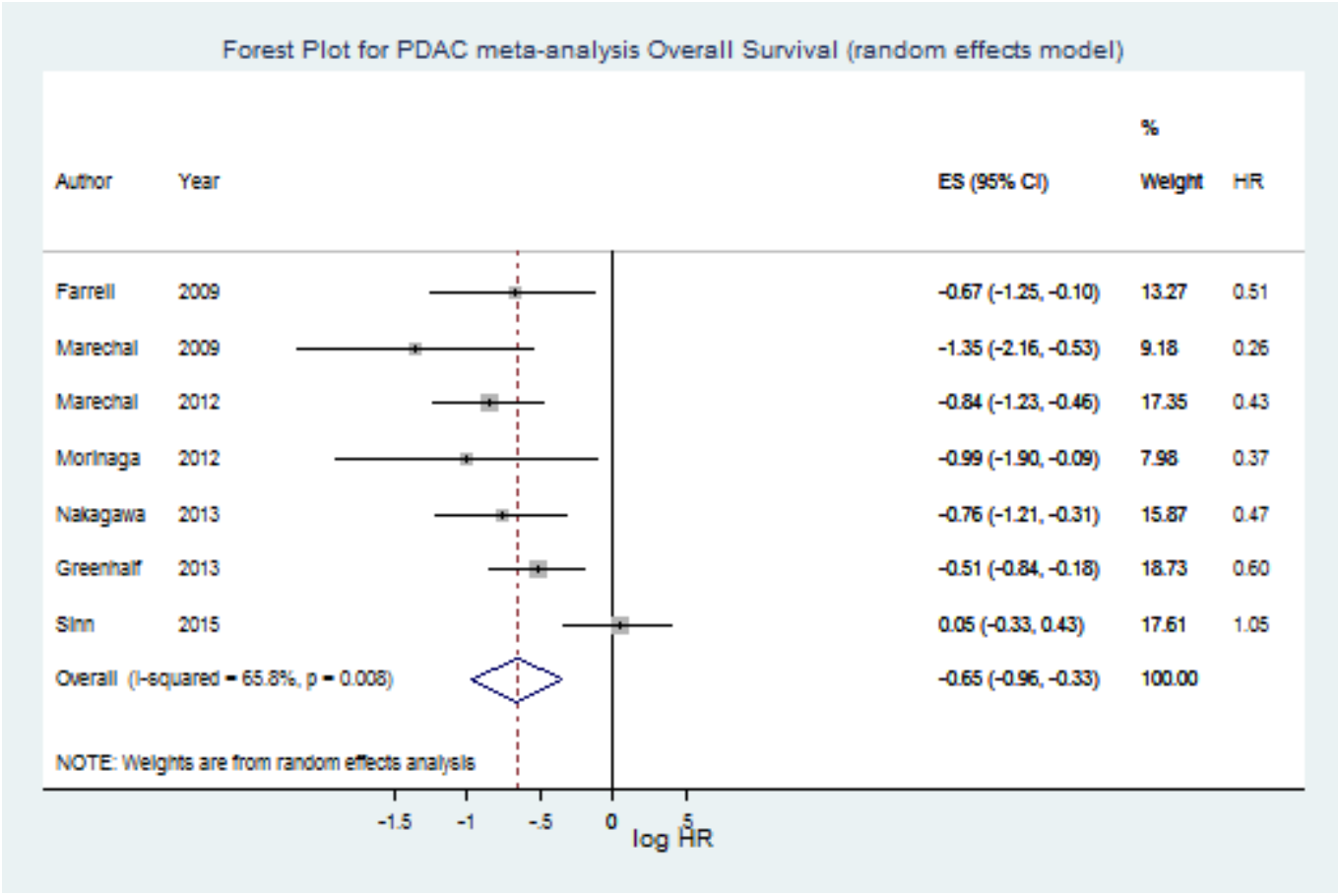
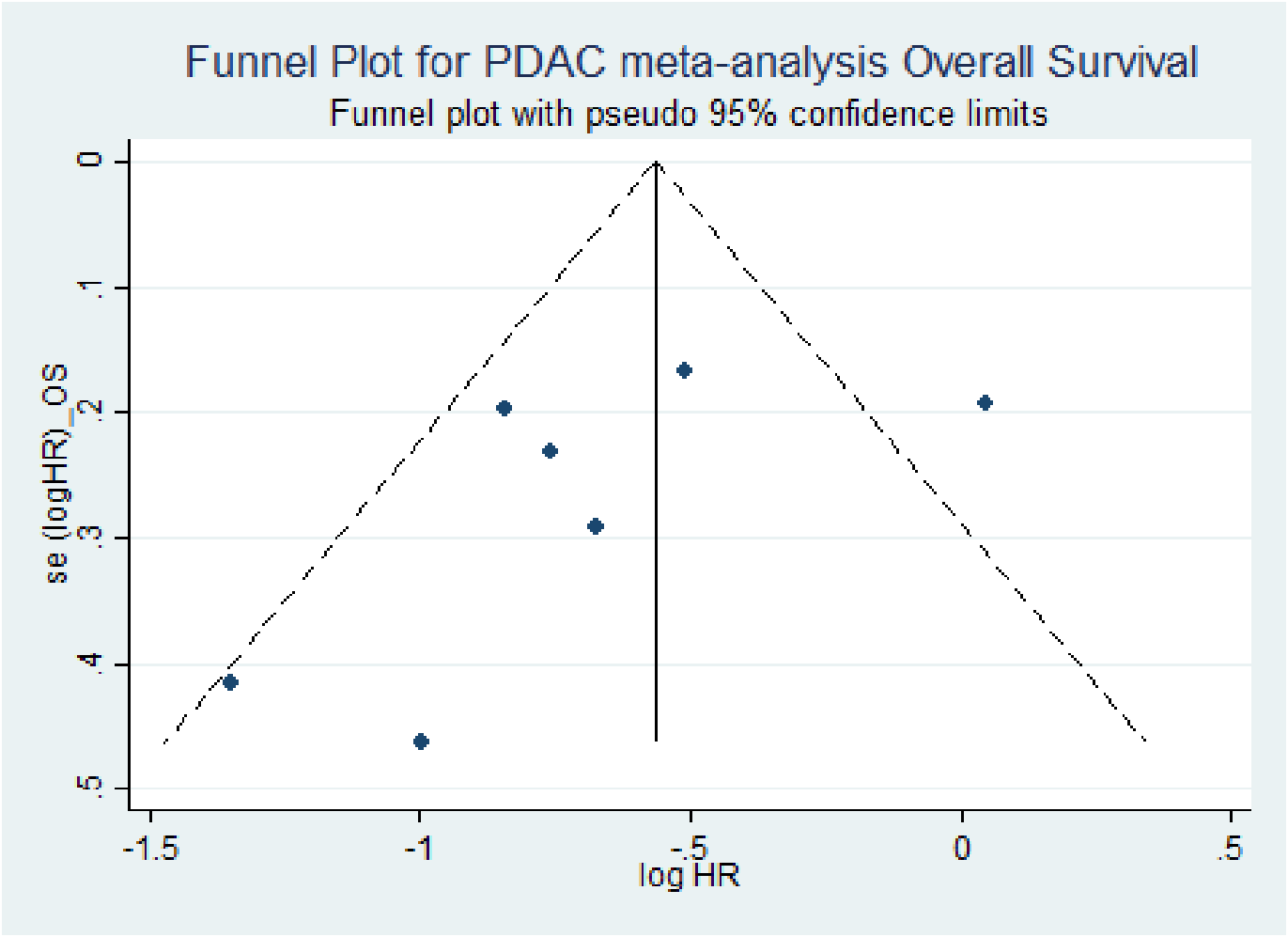


Figure 53: Funnel Plot for Univariate Overall Survival



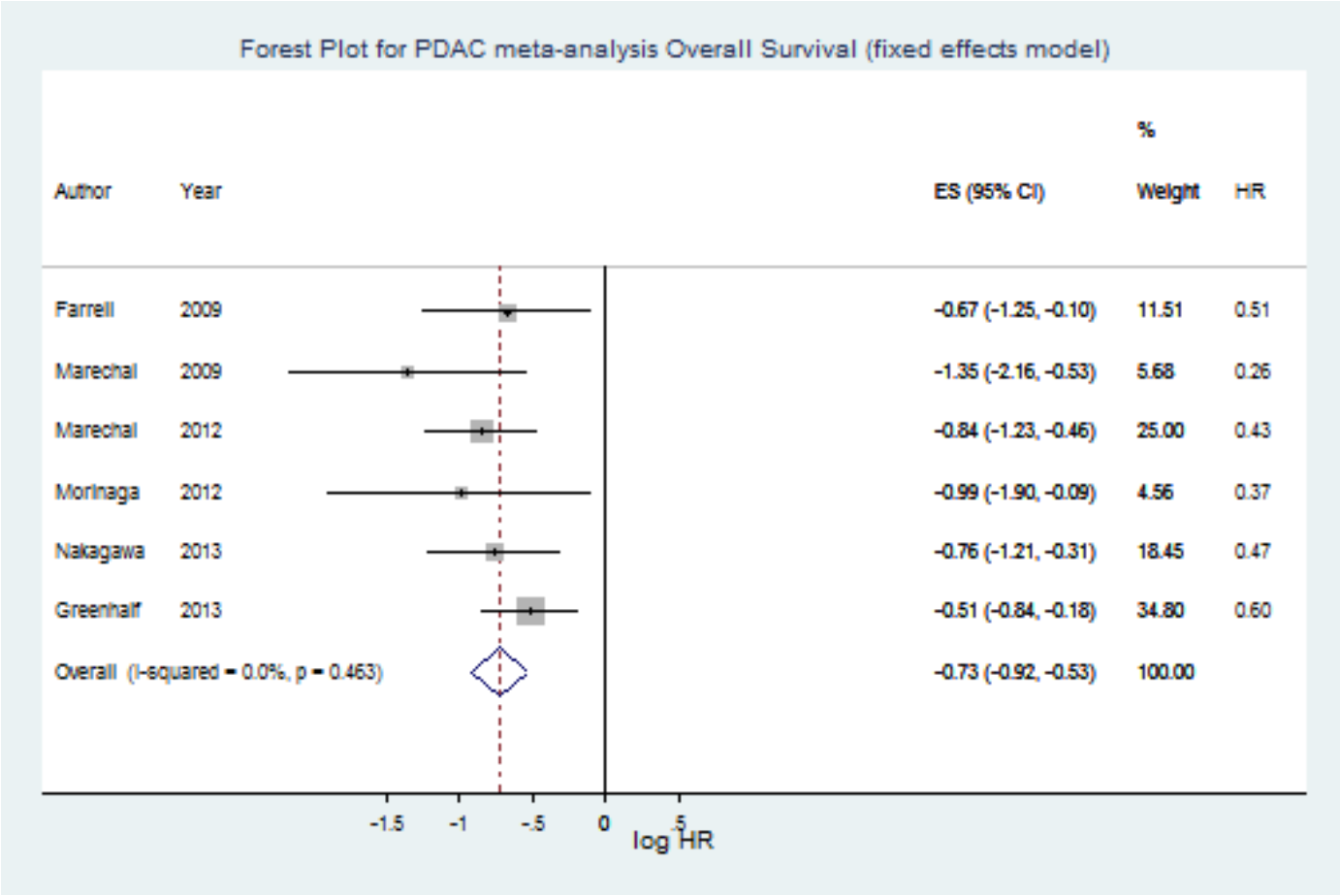
The inter-study heterogeneity was attributed to the Sinn et al. [392] study, and the meta-analysis was re-run with the Sinn data excluded which reduced I^2 to 0% (p= 0.463) indicating that this paper was an outlier in the papers meta-analysed. Accordingly, the UOS improved with exclusion of the Sinn data producing a reduced pooled HR compared to the pre-exclusion pooled HR (0.48 [95% CI 0.4 – 0.59]; See Table 28 and Figure 54).

Table 28: Fixed-effects Analysis of Pooled Hazard Ratios Excluding Data from Sinn and Colleagues [392]

	Log hazard ratio	Hazard ratio
Overall survival	–0.73 (–0.92, –0.53)	0.48 (0.40, 0.59)
Disease-free survival	–0.61 (–0.83, –0.39)	0.54 (0.44, 0.68)

Values in parentheses are 95 per cent confidence intervals

Figure 54: Forest Plot of Fixed-Effect Model for Univariate Overall Survival with Sinn et al. Data Excluded



Six studies were incorporated in analysis of the Univariate Disease Free Survival (UDFS) with a total of 725 patients, 339 (46.8 %) hENT1 –ve and 386 (53.2 %) hENT1 +ve respectively. The pooled hazard ratios demonstrate a reduced hazard of recurrence of disease during the period of follow-up for hENT1+VE patients compared to hENT1-ve patients (0.58 [95 % CI 0.42 - 0.79]). The HR infers that the probability of disease recurrence occurring during the period of follow-up for hENT1+VE compared to hENT1-ve patients is reduced at the next temporal-point measured by 42 % (see Figures 55 & 56). The pooled HR also demonstrates high heterogeneity (I^2=55.5%, p=0.047), which was again attributed to the Sinn et al. [392] paper. Subsequent exclusion of the Sinn study and re-run of the analysis demonstrated a substantial improvement in heterogeneity ($I^{2=}$ 14.8%; p =0.320). The post-exclusion analysis also demonstrated an improved pooled HR for UDFS demonstrating the outlying nature of the Sinn data (HR 0.54 [95% CI 0.44 – 0.68]; See Figure 57).

Figure 55: Forest Plot of Random-Effects Model for Univariate Disease Free Survival

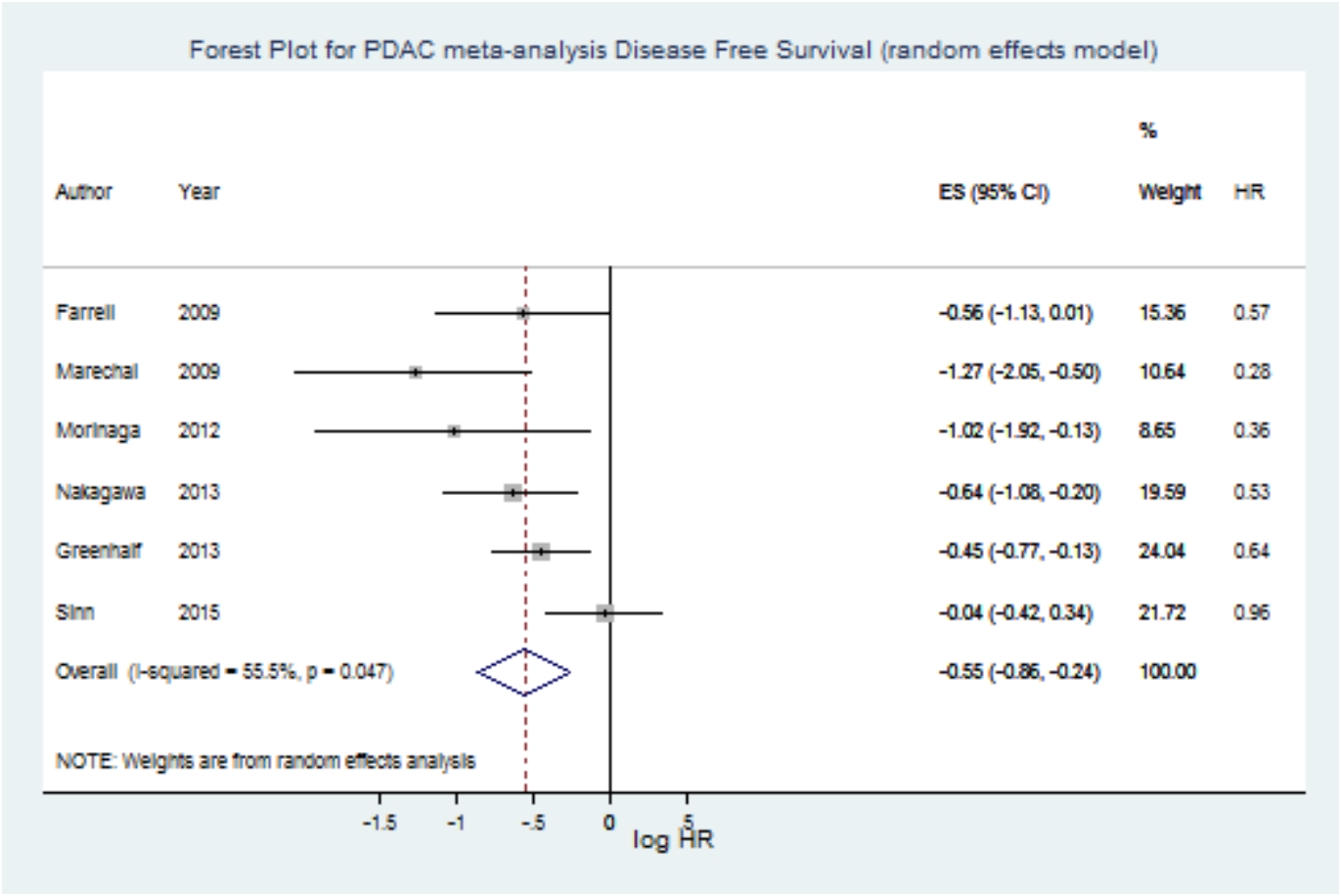


Figure 56: Funnel Plot for Univariate Disease Free Survival

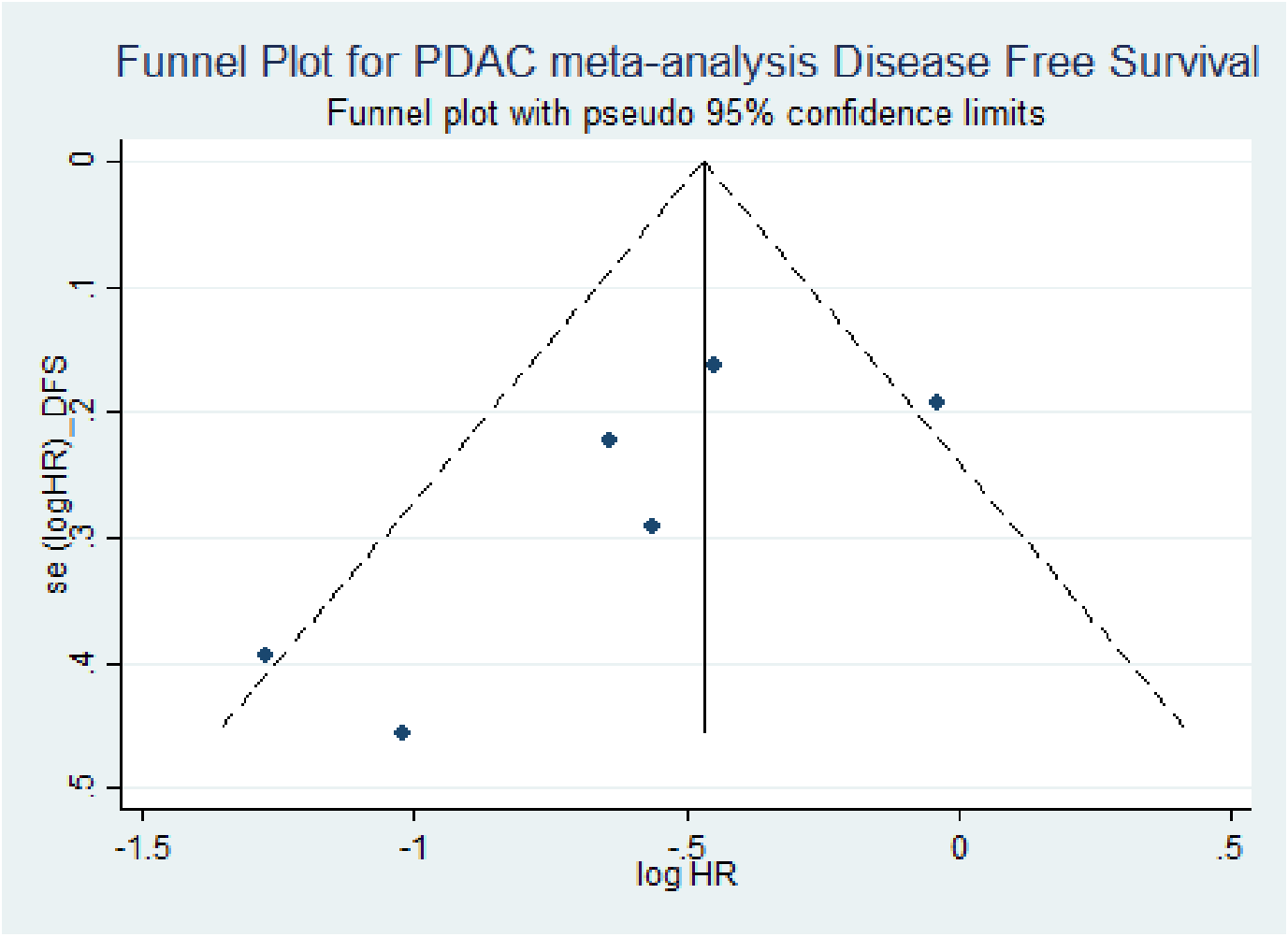
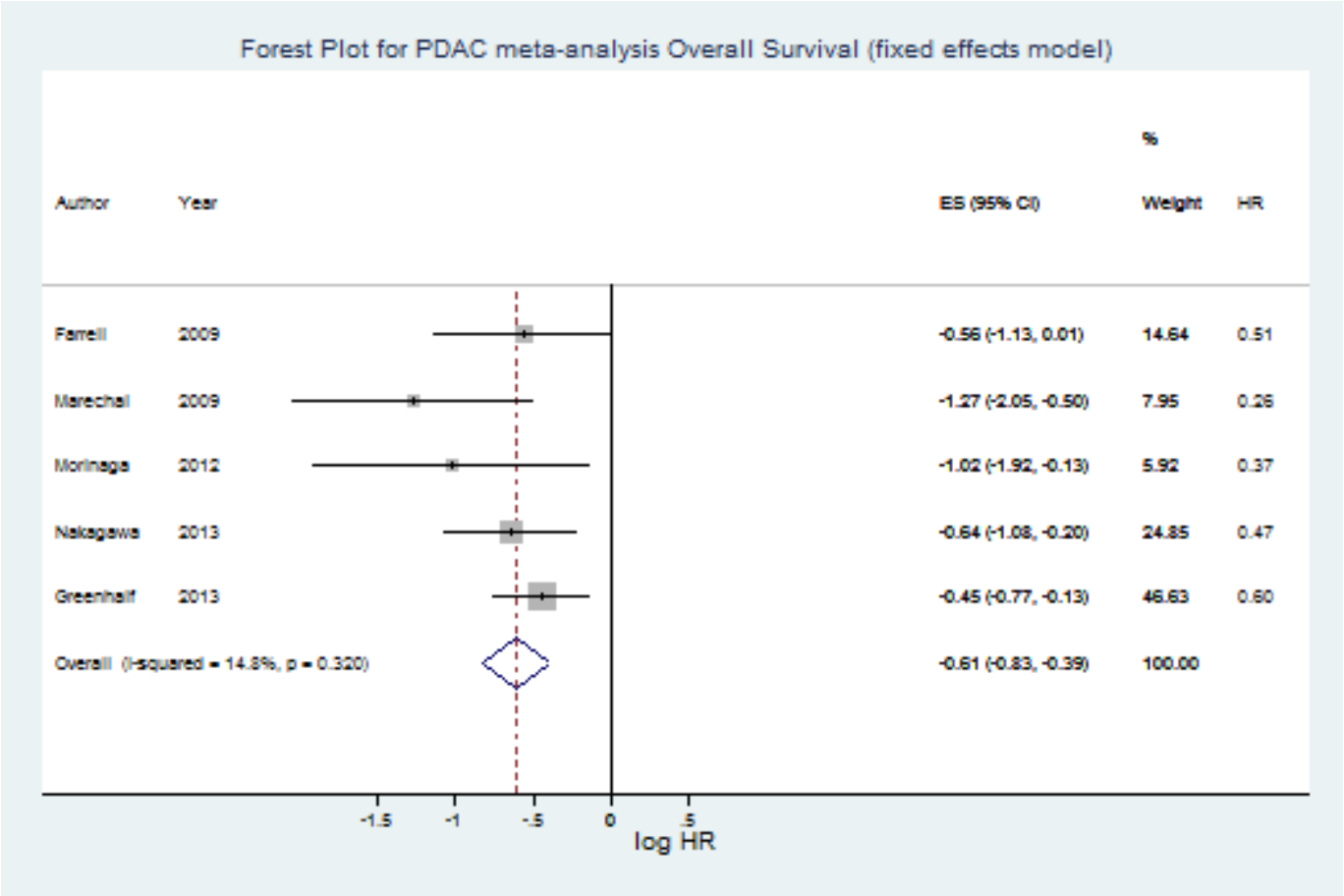


Figure 57: Forest Plot of Fixed-Effect Models for Univariate Disease-Free Survival with Sinn et al. Data Excluded



The Newcastle-Ottawa Risk of Bias Table was fitted to the papers to determine potential areas of bias for the papers undergoing systematic review (See Table 29). Six were deemed as being moderate quality with 1 paper fulfilling criteria for being high quality. As only 7 papers were used for the meta-analysis (6 for DFS), a fixed effects model would usually be deemed the most appropriate model, as there are too few papers to estimate between trial differences. However, because of the outlying results for HR from Sinn et al. a random-effects model was used. A fixed effects model was fitted in both cases as a check (See Table 30).

All clinico-pathological staging was undertaken using the American Joint Committee on Cancer guidelines and the REMARK guidelines for assessment of putative prognostic biomarkers [393]. None of the studies demonstrated a relationship between AJCC staging and hENT1 status, and all of the studies demonstrated an inverse relationship between AJCC staging and outcome in multivariate analysis i.e. as staging increased survival characteristics decreased.

Table 29: Newcastle-Ottawa Risk of Bias Stratification

	SELECTION				COMPARABILITY		OUTCOME			
Study	Representative	Selection of Cases	Ascertainment	Defined End-point	Comparability	Assessment of Outcome	Follow-up	Adequacy Follow-up	Total	Quality
Farrell 2009	*	*	*	*			*	*	6	Moderate
Marechal 2009	*	*	*	*			*	*	6	Moderate
Morinaga 2012	*		*	*			*	*	5	Moderate
Marechal 2012	*		*	*	*		*	*	6	Moderate
Kondo 2012	*		*	*			*	*	5	Moderate
Nakagawa 2013	*		*	*			*	*	5	Moderate
Greenhalf 2014	*	*	*	*	**	*	*	*	8	High
Sinn 2015	*	*	*	*	*		*	*	6	Moderate

Table 30: Table Demonstrating Difference in Pooled HR's for Fixed and Random-Effect Models

	Fixed effects		Random effects	
	ln (HR) (95% CI)	**HR (95% CI)**	**ln (HR) (95% CI)**	**HR (95% CI)**
OS	-0.57(-0.74,-0.39)	0.57(0.48,0.68)	-0.65(-0.96,-0.33)	0.52(0.38,0.72)
DFS	-0.47(-0.66,-0.28)	0.63(0.52,0.76)	-0.55(-0.86,-0.24)	0.58(0.42,0.79)

3.25) Discussion

This meta-analysis demonstrates both overall and disease-free survival advantages for patients positive for hENT1 when compared to patients that lack expression of hENT1 when undergoing adjuvant gemcitabine-based chemotherapy for resected pancreatic adenocarcinoma.

A potential source of the inter-study heterogeneity, prior to the exclusion of the Sinn data, is the variety of different anti-bodies used across the 7 studies. Four of the studies used the non-commercially available 10D7G2 mouse monoclonal anti-body [388-391]. Two studies utilised alternative rabbit polyclonal anti-bodies from 2 separate companies, presumably utilising different methods of generating, purifying and modifying the anti-bodies [386, 387]. Comparison of the UOS from these groups shows that the antibodies demonstrate varying levels of predictivity of survival with gemcitabine-based adjuvant chemotherapy. The Sinn et al. study utilised the SP120 rabbit monoclonal anti-body [392]. This was the only study not to demonstrate a predictive relationship between hENT1 status and subsequent response to gemcitabine-based adjuvant chemotherapy. A recent study investigated the concordance between the SP120 rabbit monoclonal anti-body and the 10D7G2 mouse monoclonal anti-body [394]. This study showed that the SP120 anti-body required amplification techniques to become as sensitive as the 10D7G2 anti-body. However, subsequent to this amplification technique, the anti-bodies demonstrated non-equivalence between individual OS parameters. Further research to address the specific issue of non-equivalence between hENT1 anti-bodies is required with a view to providing a consensus approach for further studies.

Four studies involved retrospective analysis of blinded and randomly allocated patients in multiple treatment arms of randomised control trials (RTC) [388, 390-392]. One RTC compared adjuvant gemcitabine chemo-radiotherapy to 5-FU chemo-radiotherapy [391]. One study compared gemcitabine-based chemotherapy to radiotherapy +/- 5-FU [388]. One study compared gemcitabine chemotherapy to observation alone [392]. One study compared gemcitabine chemotherapy to 5-FU [390]. The three studies which utilised 5-FU in a comparative group did not demonstrate any significant association between UOS and DFS with hENT1 expression in this treatment arm. hENT1 is an ancillary membrane transporter for 5-FU at the basolateral membrane, primarily for re-uptake of 5-FU in to the cell. The primary membrane

transporter at the apical membrane for 5-FU is the Solute Carrier Family 22 Member 7 protein (SLC22A7). This meta-analysis demonstrates that the hENT1 contribution to 5-FU uptake into the cell has negligible clinical effect.

The limitations of the study primarily relate to the methodological approaches undertaken by the studies. Despite the random allocation of patients to treatment modalities in the RCT's, reducing potential sources of bias in these studies, the fact that hENT1 status at this point was not prospectively considered an independent variable under investigation does not entirely obviate these biases and their effect on the inter-study heterogeneity.

The studies show variation in their use of positive and negative controls for IHC expression of hENT1. Three studies utilised lymphocytes as positive controls [388, 389, 391]; one used pancreatic islet cells [386]; two did not state their positive controls [387, 392]; and one used a multi-cellular standard [390]. The documentation of negative controls was also limited with most articles not stating their negative control.

Two studies utilised a trichotomised scoring system based on 50 % of cells staining at author defined categorical intensities of '*zero*', '*low*' and '*high*' [391, 392]. Two studies utilised a verified internationally recognised composite scoring system [389, 390, 395] but with only 1 of the studies actually clarifying the usage of the '*H*' scoring system [390]. The remaining three studies utilised arbitrary composite scoring systems designed by the authors [386-388]. All subsequent scores were dichotomised and survival characteristics were allocated to hENT1 +ve/-ve or *'high/'low*" categories which allowed hazard ratio comparison. However, the lack of a standardised approach to hENT1 scoring criteria raises concerns for inter-study heterogeneity.

The causes for the inter-study heterogeneity are areas that need to be addressed in further work. Particular emphasis on standardisation of species of the hENT1 anti-body should be made with more work to definitively determine the precision of the 10D7G2 antibody. Appropriately powered randomised control studies to prospectively investigate the predictive value of the 10D7G2 monoclonal hENT1 antibody are warranted to delineate whether the relationship between high hENT1 expression and improved prognosis is causative rather than associative. A standardised approach, utilising composite '*H*' scores to define hENT1 signal expression, should also be developed to ensure greater inter-study consistency.

3.3 Method of Construction of Tissue Matched Array for hENT1 and Ki67 Immunohistochemical Assessment of Resected and Biopsied Cholangiocarcinoma Patients

3.31) SUMMARY

Background:

Human equilibriative nucleoside transporter protein 1 (hENT1) is a trans-membranous protein which facilitates nucleoside transport into the cell. Immunohistochemically-detected hENT1 abundance is increased in cholangiocarcinoma tumour cells compared to matched non-tumour cells and increased in highly metabolising cells. The privately-held Mackey 10D7G2 hybridoma has demonstrated prognostic utility in Pancreatic Ductal Adenocarcinoma patients. The commercially available Proteintech Polyclonal hENT1 antibody's prognostic utility has not been previously assessed. Cellular Ki67 expression has been linked to mitotic indices of tumour proliferation. This proof-of-concept study aims to assess the antibodies prognostic utility for hilar cholangiocarcinoma patients undergoing surgical resection.

Methods:

Between February 2009 and February 2016 54 patients underwent resection for peri-hilar cholangiocarcinoma. Formalin-Fixed Paraffin Embedded (FFPE) blocks from a sub-set of 44 resected specimens were retrieved. Appropriate areas of tumour were sampled from the blocks and a Tissue-Matched Array (TMA) was constructed. The TMA underwent staining for each antibody. H-scores were utilised to determine intensity of expression. Correlation of expression between antibodies was determined by Pearson correlation co-efficient and Chi-squared where appropriate. Silencing RNA for transfected HepG2 cell-lines was used to determine accurate hENT1 staining by the Proteintech antibody. Demographic and survival characteristics for the patients were acquired from a prospectively held database linked to Hospital Episode Statistics. Survival characteristics were calculated with global log-rank calculations.

Results:

There was significant correlation between the Mackey 10D7G2 and the Proteintech antibodies ($p<0.001$). There was significant correlation between the Proteintech hENT1 antibody expression and Ki67 expression ($p= 0.02$). Knockdown of hENT1 with silencing RNA transfected HepG2 cells was confirmed by Western blot in a time-dependent fashion over 72 hours for the Proteintech antibody. The antibodies (Mackey; Proteintech; Ki67) did not achieve significance for predicting OS ($p= 0.75$; 0.63; 0.22 respectively

Conclusion:

The Proteintech antibody demonstrates significant concordance with the 10D7G2 antibody in determining hENT1 expression, however the antibodies did not demonstrate significant prognostic ability in this proof-of-concept study. Standard histopathological co-variates retain prognostic utility within the cohort.

3.32) Background:

Cholangiocarcinoma is a malignancy of the biliary epithelial cells. Overall survival (OS) with the disease is discouraging, with surgery providing the only potential of cure [177]. Cholangiocarcinoma is staged utilising the American Joint Committee on Cancer (AJCC) 8th edition. The AJCC system utilises clinico-pathological tumour characteristics to determine prognosis for the patient. The utility of these variables for accurate prognostication has been extensively investigated and questioned [8, 9]. Putative prognostic tumour biomarkers have yet to be thoroughly investigated.

Human equilibriative nucleoside transporter protein 1 (hENT1) is a trans-membranous protein which facilitates purine and pyrimidine nucleoside transport into the cell from the surrounding medium. hENT1 has also been demonstrated to be present on intra-cellular membranes. Immunohistochemically detected hENT1 abundance appears to be increased in cholangiocarcinoma tumour cells compared to matched non-tumour cells [396]. hENT1 abundance has been demonstrated to be increased in highly metabolising cells [397]. hENT1 abundance therefore may act as a surrogate marker for aggressively mitotic cholangiocarcinoma tumours and, by inference, be a putative prognostic biomarker.

hENT1 abundance has been demonstrated to act as an immunohistochemically detected predictive marker for response to gemcitabine-based chemotherapy in resected pancreatic adenocarcinoma patients [11]. Gemcitabine is a nucleoside analogue which requires trans-membranous hENT1 expression for facilitative transport into the target cell. Cholangiocarcinoma patients receive gemcitabine-based chemotherapy in a palliative setting as first line treatment in combination with cisplatin [238]. The actively recruiting randomised controlled phase 3 ACTICCA-01 trial (**UKCRN173496**) aims to compare gemcitabine-cisplatin protocols to the standard of care determined by the BILCAP trial for patients undergoing 'post-surgery' adjuvant chemotherapy [237]. hENT1 abundance in resected specimens may be a predictive biomarker for RFS and OS in patients undergoing gemcitabine-based chemotherapy.

The aims of this study are to determine the utility of immunohistochemically assessed hENT1 abundance as a putative prognostic and predictive biomarker in resected and palliative cholangiocarcinoma patients.

3.33) Methods

i.) Patient Selection

Data for patients diagnosed with hilar cholangiocarcinoma, referred to a regional tertiary referral centre between January 2009 and December 2016, was extracted from a prospectively held database linked to Hospital Episode Statistics data. Patients with distal cholangiocarcinoma and gallbladder cancer were excluded from analysis. All patients with computed tomography (CT) or endoscopic evidence of tumour originating at the biliary confluence and extending into the biliary radicles were included. Any patients with

tumour arising in the common hepatic duct were included if the tumour extended to the confluence or in to the radicles. Multidisciplinary team assessment of cases was undertaken as previously described (p. 90 – 91). Patients determined to have multiple liver metastases underwent subsequent radiologically guided liver biopsy to determine histopathological cell type to target further treatment. All potentially resectable cases underwent surgical assessment of resectability and, where appropriate, underwent surgical resection as previously described (p. 91 – 92).

ii.) Acquisition of Tissue for Tissue Matched Array (TMA)

All cases which followed the specified criteria were selected from the Somerset Cancer Registry linked to the Hepato-Biliary MDT outcomes. An excel datasheet was populated with cases fitting the criteria was created. Patient-specific clinico-pathological and tumour characteristics were incorporated as data. Fixed formalin paraffin embedded (FFPE) resected tumour and biopsy tissue with associated patient-tumour specific histopathological slides were requested from the Aintree University Hospital Biobank and CellNass National Archiving Service.

iii.) Construction of TMA

Two tissue-matched microarrays were constructed using a Tissue Arrayer (Beecher Instruments Inc.) located at the Liverpool Bio-innovation Hub Biobank. One TMA held only tissue from resected cholangiocarcinoma patients (n= 43), the other TMA held only tissue from liver biopsied specimens (n= 68; N= 111). The Tissue Arrayer was fitted with a 0.6mm diameter punch set (Beecher Instruments Inc.) to obtain small cores from a series of paraffin-embedded donor tissue blocks. These tissue cores were transferred into a pre-designed array in a recipient paraffin block (i.e. the new TMA block). The pre-designed TMA maps were encoded and held independently separately from the clinic-pathological data. The tissue cores were taken from tumour regions identified by an experienced Consultant Histopathologist using haematoxylin and eosin (H&E) stained sections.

For the surgically resected TMA 3 cores were obtained from each block with one block per patient. Control cores comprising 3 cores each of colon, kidney, liver, normal pancreas, and chronic pancreatitis, were arranged throughout the TMA. For the biopsy TMA 2 cores were obtained from each block with 1 block per patient. Control cores comprising 2 cores each of colon, kidney, liver, normal pancreas, chronic pancreatitis, and pancreatic ductal adenocarcinoma, were arranged throughout the TMA.

The depth-stop kit controls the depth of the recipient TMA well and the donor tissue core. The resected specimens were larger and had more available tumour for sampling to produce the cores. The depth-stop kit was set at 4mm for the resected specimen TMA and 2 mm for the biopsy TMA. This produced corresponding well depths in the recipient TMA blocks of 4 and 2 mm. Once the TMA blocks were

constructed, they were placed in a 37˚C oven overnight to anneal the cores before being stored at room temperature.

iv.) Antigen Retrieval

The DAKO PT-Link was used to perform de-paraffinization and antigen retrieval on oven-dried slides sectioned from the annealed TMA's. The PT Link can accommodate slide racks used on the DAKO Autostainer staining instruments and is employed to facilitate the efficient transfer of slides from retrieval to staining procedures. DAKO target retrieval solutions (TRS) at varying pH's were utilised to retrieve the antigens from the annealed TMA blocks. The hENT1 antigen was retrieved with a solution at pH 6 and the Ki67 antigen was retrieved with a solution at pH 9.0. The TRS's were pre-heated to the set pre-treatment temperature (65 degrees centigrade) and the oven-dried slides were placed in to the Autostainer racks. The treatment stage was engaged and the run-cycle initiated for 20 minutes with a specified run-cycle temperature (96 degrees centigrade). On completion of the run-cycle the racks were cooled to pre-treatment temperature. Once the racks had sufficiently cooled, they were removed and the slides were immersed in DAKO wash buffer.

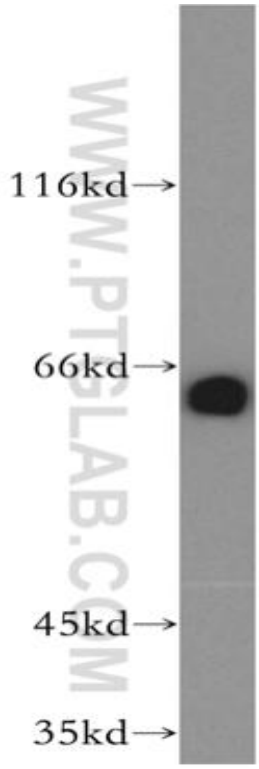
v.) Immunohistochemistry

Following antigen retrieval, the slides were replaced into the pre-heated PT-Link. The run-cycle was engaged and the slides were heated to 96 degrees centigrade for 20 minutes. Environ Flex-Wash buffer was utilised to wash the slides for between 5 and 15 minutes. The slides were cooled and removed and placed upon staining trays and washed in Environ Flex-Wash. The slides were then incubated in 100 micro-litres at room temperature for 5 minutes. Varying dilutions with Environ Flex-Wash were utilised to optimise the antibodies. One hundred micro-litres of primary antibody were added to the slides. The Ki67 primary antibody was incubated for 30 minutes at room temperature and the 10D7G2 hENT1 Mackey primary antibody was incubated at 4 degrees centigrade overnight. The slides were washed with Environ Flex-Wash and 100 micro-litres of linker were added to the slides and incubated for 15 minutes with the Ki67 slides and 60 minutes for the 10D7G2 hENT1 Mackey antibody. Flex-Wash buffer was then utilised to wash the excess linker from the slides. One hundred micro-litres of Horse Radish Peroxidase (HRP) was used to incubate the slides for 20 minutes. Following incubation Flex-Wash was again used to wash the excess of HRP from the slides. Diaminobenzidine (DAB) chromegen substrate was utilised following HRP to produce antigen-linked brown staining of the tissue on the slides. Flex-Wash buffer was again used to wash any excess DAB from the slides. The slides were washed and cleaned in serial steps with haematoxylin, acid, and ammonia. The slides were dehydrated utilising methylating spirits and subsequently finished with mountant. The haemotoxylin sections and stained TMA slide images for hENT1 10D7G2 Mackey antibody and Ki67 are demonstrated in Figures 59 and 60 respectively (p.159 and 160).

vi.) Development of an Alternative hENT1 Antibody to 10D7G2 Antibody

The 10D7G2 'Mackey' antibody has been demonstrated to have predictive utility particularly within PDAC populations. The 10D7G2 privately-held hybridoma antibody manufactured by Professor JR Mackey in Alberta, Canada. Given potential manufacture and supply problems from solitary privately held collections commercially viable alternative antibodies with predictive/prognostic capability would have utility for clinicians involved with managing cholangiocarcinoma. The SP120 monoclonal rabbit antibody has been putatively demonstrated to be predictive of gemcitabine response however results in a variety of studies have been variable [392, 398]. An alternative antibody which has yet to be utilised on TMA's containing cholangiocarcinoma specimens is the Proteintech Rabbit Polyclonal ENT1 Antibody 11337-1-AP. The antibody has been demonstrated to have predictive efficacy in PDAC, leukaemia and glioma malignancies [399-401].

Figure 58: Immuno-Blot Product Information for Proteintech ENT1 Polyclonal Antibody



The Proteintech antibody has a predicted blotting weight of 62kDA (See Figure 58). The antibody was acquired for preliminary investigation on HepG2 cell lines. HepG2 cells are easily cultured using standard culture mediums and are ubiquitously stocked in cellular laboratories in the United Kingdom. Despite HepG2 cell lines being conceived as originating from a hepatocellular cancer primary they demonstrate key immunochemical features consistent with cholangiocarcinoma. The HepG2 cell line demonstrates CK 19

positivity with fluorescence on immunocytochemistry (See Figures 61 and 62; p. 161 Acknowledgement Howell; 2019). CK 19 is a key determinant of cholangiocarcinoma diagnosis on histopathological assessment.

HepG2 cells also have unlimited life spans with stable phenotypes and are therefore easy to maintain and assess in the vitro setting [402]. Given the ubiquity of the cell line and its cholangiocarcinoma characteristics the HepG2 cell line was utilised to immunoblot with the Proteintech antibody for hENT1 expression.

Figure 59: 10D7G2 hENT1 Antibody Immunohistochemical Assessment of 43 Resected Specimens

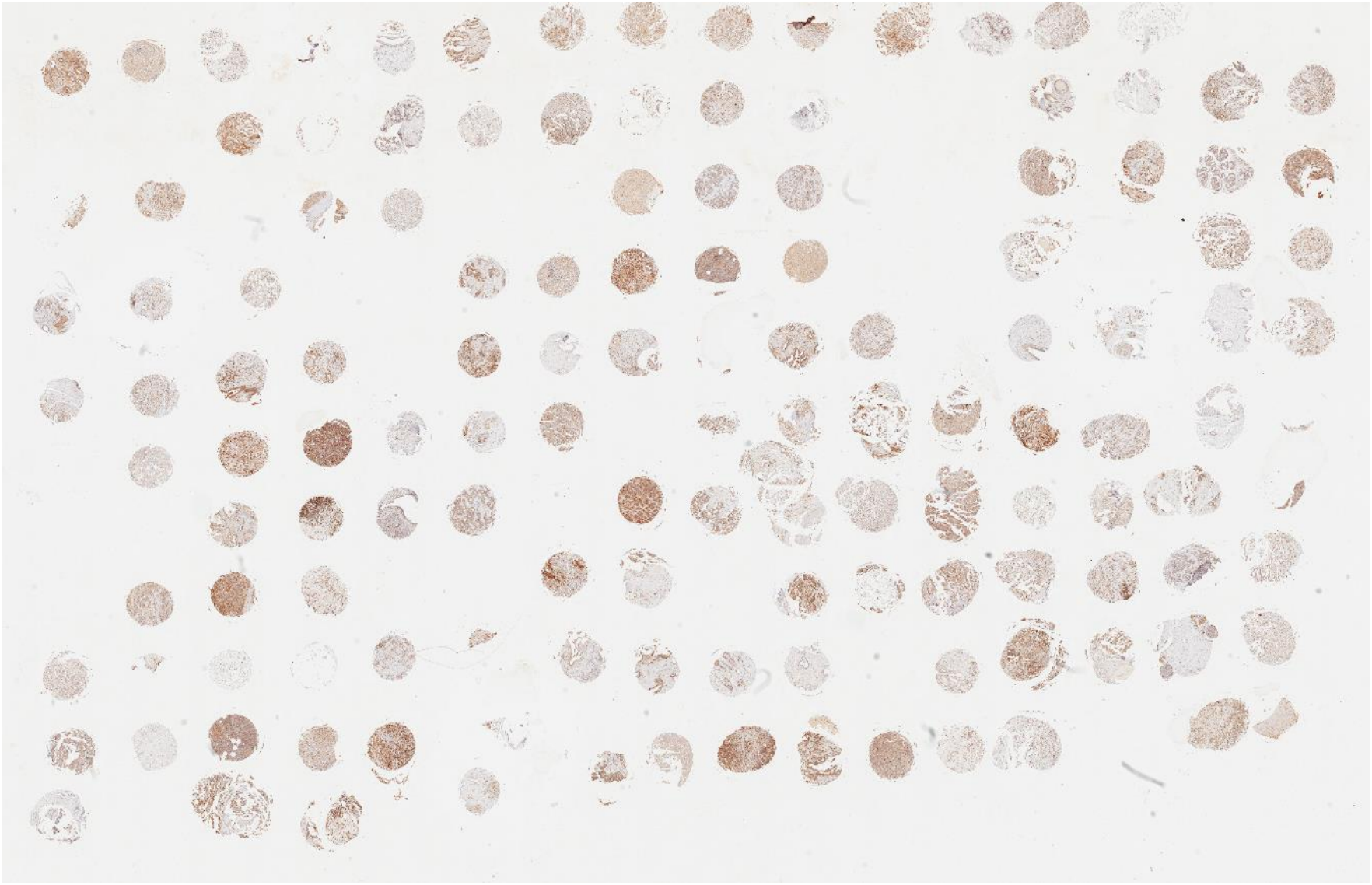


Figure 60: Ki 67 Immunohistochemical Assessment of 43 Resected Specimens

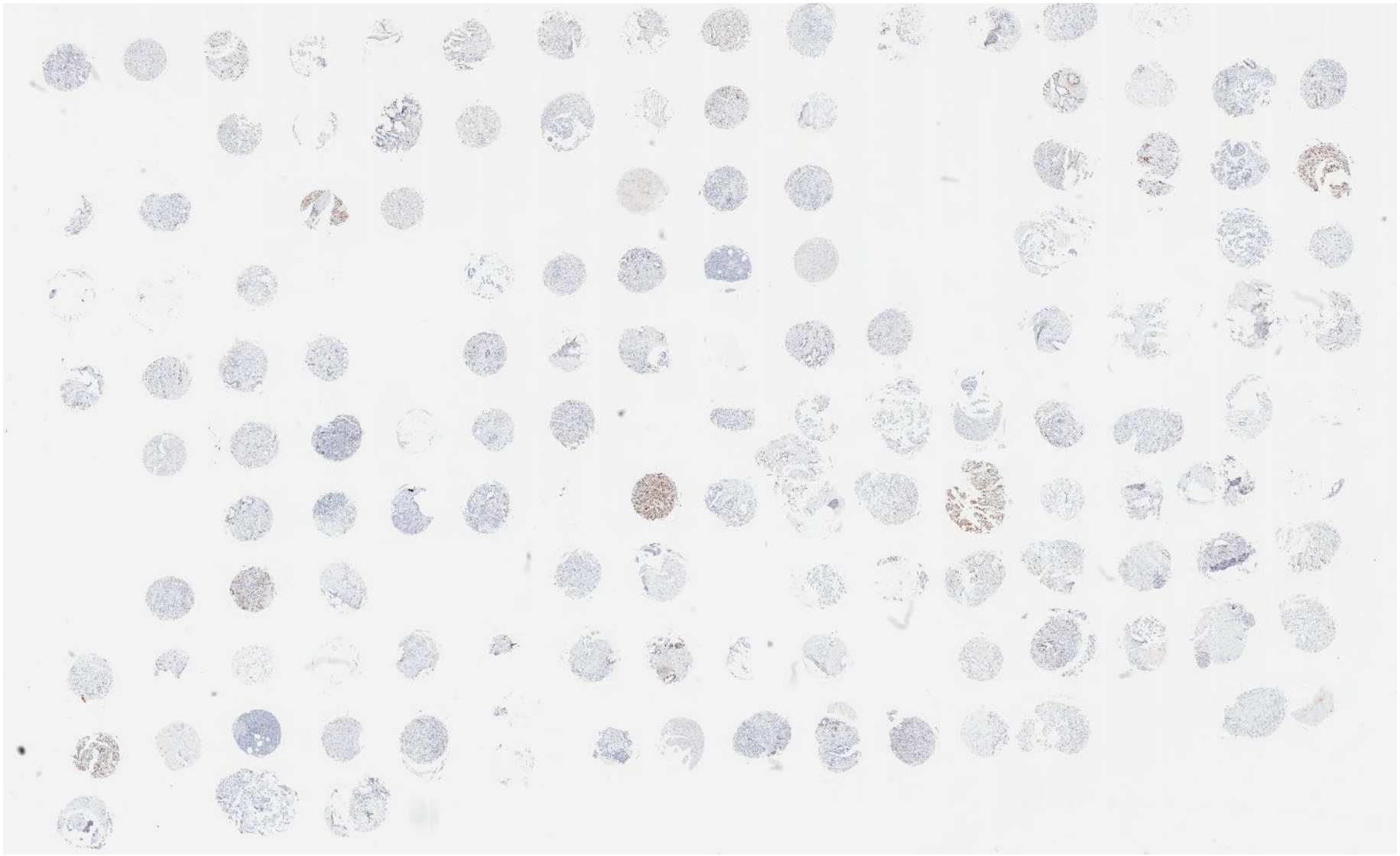


Figure 61: Immunocytochemistry of HepG2 (Blue Represents Nucleus)

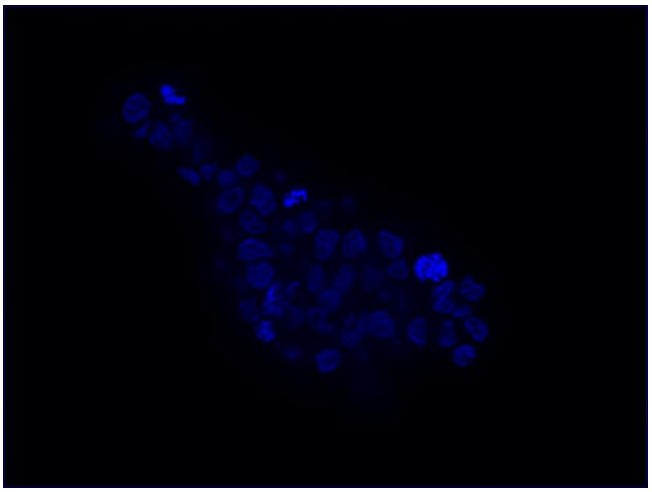
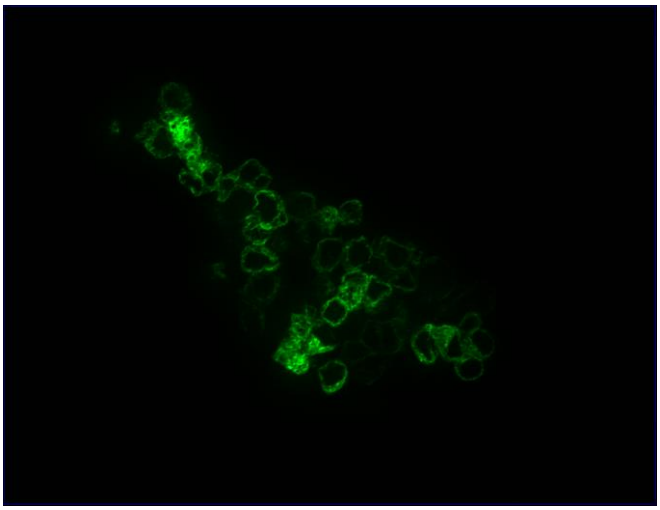


Figure 62: Immunocytochemistry Demonstrating HepG2 CK 19 Positivity (Green Represents CK 19 Positivity)



Courtesy Lawrence Howell – PhD – Translational Medicine; Liverpool University; March 2019

vii.) Culturing and Passaging HepG2 Cells

HepG2 cells were cultured at 37 degrees centigrade with Dulbecco's Modified Eagles Medium (DMEM) with 10 % Foetal Bovine Serum (FBS) in 5 % carbon dioxide (CO^2) atmosphere. HepG2 cells were split when 80 % confluence was reached. The HepG2 cell monolayer was washed with Phosphate Buffered Solution (PBS). Following this 0.05 % trypsin was pipetted on the cell monolayer and placed in the % CO^2 incubator for 5 minutes. The monolayer was examined under a microscope and as detachment occurred the trypsin solution was neutralised with DMEM and 10 % FBS culture medium. The cells were centrifuged and the trypsin and culture medium were removed and the cells were re-suspended with culture medium. The split ratio was 1:8 with culture medium every 6 days.

viii.) Protein Acquisition

Following achievement of cellular confluence of the HepG2 monolayer the protein from the wells was harvested for lysate. Protease inhibitor cocktail (to prevent excessive proteolysis) and RIPA solution (for extraction of protein lysate from the HepG2 cells) from Thermofisher Scientific was applied in a ratio of 1: 100 of RIPA: Protease cocktail inhibitor to 12 well plates containing confluent HepG2 cells. Tissue scraping was used to detach the cell monolayer from the well. The cellular scrapings were collected in to eppendorff containers and sequentially freeze-thawed with vortexing to disrupt the cellular structures. The eppendorff's were then centrifuged at 15, 000 revolutions per minute (RPM) for 15 minutes at 4 degrees centigrade. The supernatant was aspirated and placed in new eppendorff's producing the lysate. A Bichinchoninic Acid Assay (BCA) was ran to determine the protein concentration of the supernatant. Standardised BCA protocols designed by Pierce were utilised with Bovine Serum Albumin (BSA) acting as the standard with copper sulphate as the reagent and sequential dilutions

with sodium docetyl solution (SDS). Photometric analysis of the standards and supernatant was undertaken. Logged transformations of the photometric wavelengths utilising Excel were used to determine the concentration of protein in the supernatant. Varying masses of protein were used in the optimisation process as determined by the BCA concentrations.

ix.) Western Blotting Technique

The following sequential step approach was utilised to produce the Western blots:

- The initial step was to prepare the samples. NuPage sample buffer and reducing agent were mixed in a microtube (70: 30 with a total volume of 5 microlitres).
- The requisite protein volumes of the HepG2 supernatant determined by the BCA protein assay were added to these microtubes.
- The microtubes were centrifuged at 5000 g for 30 seconds.
- The microtubes were then placed in a heating block at 80 degrees centigrade for 10 minutes.

The next steps were to prepare the pre-cast 4 -12 % Bis-Tris gels for the running of the Western:

- The pre-cast gel was removed from the packet and the white tape and comb was removed.
- The gel was locked into the electrophoresis unit.
- Dilution of 50 millitres (mL) of 20 X morpholino propane-sulphonic acid (MOPS) in 950 mL of dH20 to give a 1 X MOPS running buffer.
- This was mixed and poured into the electrophoresis unit. The chamber was filled to approximately half the total volume with the buffer.

The next steps were to initiate running of the Western blot through the pre-cast gel:

- The heated microtubes containing the samples were pulsed to settle the contents.
- 27 microlitres of solution were added to each of the gel wells.
- 3 microlitres of rainbow molecular weight markers into the spare well in the gel.
- The lid was applied to the electrophoresis unit and connected to a power unit. The gel was run for 15 minutes at 90 volts (V) to initiate descent of the protein from the wells into the gel. This was followed by 2 hours at 160 V to run the proteins fully in to the gel.

The next steps were to transfer the proteins from the completed gel to nitrocellulose membrane:

- Transfer buffer was prepared using 100 mL of 10 X Transfer buffer, 200 mL of methanol (MeOH) and 700 mL of dH20.
- Per completed gel 2 sponges, 2 filter papers and 1 nitrocellulose membrane were soaked in transfer buffer.

- The completed gel was removed from the electrophoresis unit and the running buffer was discarded.
- The gel plastic encasement was cracked open and the gel was removed using a spatula and placed on to pre-soaked filter paper. The air bubbles were rolled out of the gel-membrane- paper combination.
- The pre-soaked nitrocellulose membrane was placed on to the gel and covered with filter paper producing a paper-gel-membrane-paper sandwich.
- The sandwich was encased in sponges and placed in a cassette and placed in to the transfer chamber.
- The transfer tank was filled with 1 X transfer buffer to just below the top ridge.
- Ice was added to the transfer chamber and a magnetic stirrer was placed in the tank.
- A 230 milliampere (mA) current was applied for 1.5 hours.

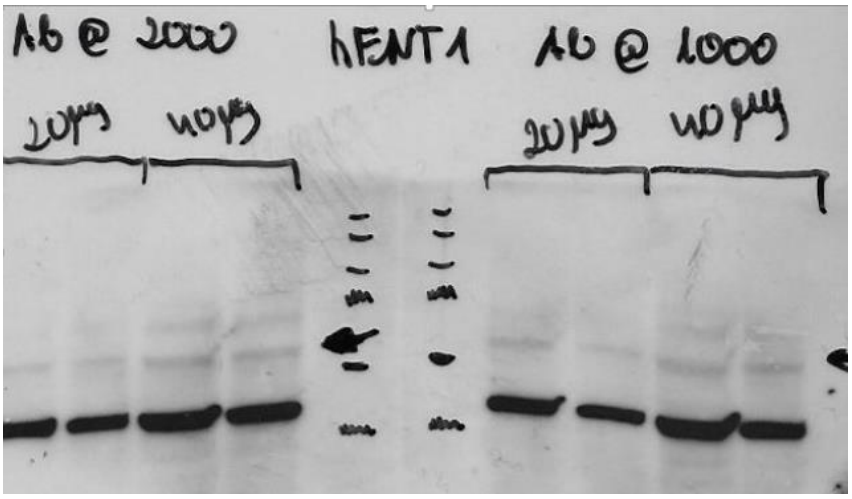
The following steps were undertaken to prepare the nitrocellulose membrane for addition of the antibody:

- The membrane was briefly rinsed in 0.1 % isotonic non-toxic T-Tris-buffer solution (TBS; 50 mL 20 TBS, 10 mL of 10 % Tween and 940 mL of dH20).
- The membrane was stained with penceau red for 10 second. The penceau red stain was then decanted and the membrane was washed with TBS.
- 10 % milk-blocker (1.5 g non-fat milk in 15 ml of TBS solution) was produced. The membrane was rocked in this solution to block non-specific proteins for 30 minutes at 4 degrees centigrade.
- 2 % milk-blocker was produced (0.3 g non-fat milk in 15 ml T-TBS).
- The 10 % milk-blocker solution was decanted and TBS was used to wash the membrane briefly.
- Primary antibody was added to the 2 % milk-blocker and the membrane and antibody were sealed in plastic and left to rock overnight at 4 degrees centigrade.

The following steps were undertaken to prepare the secondary antibody and the chemiluminescence:

- 2 % milk-blocker was prepared.
- The secondary antibody was added to the 2 % milk-blocker and the membrane was incubated at room temperature for 1 hour.
- The membrane was extracted from the plastic sealed pack and washed with TBS.
- The chemiluminescence reagent was prepared (1 mL oxidising agent and 1 mL luminol reagent).
- The membrane was placed on a plastic sheet and blotted dry with tissue.
- The membrane was covered with the reagent and secured in a developing cassette.
- The chemiluminescent covered membrane was exposed to radiographic film in the dark-room and the images fixed in the dark room.
- Multiple iterations and attempts at producing Western blots were undertaken to develop a reproducible technique.

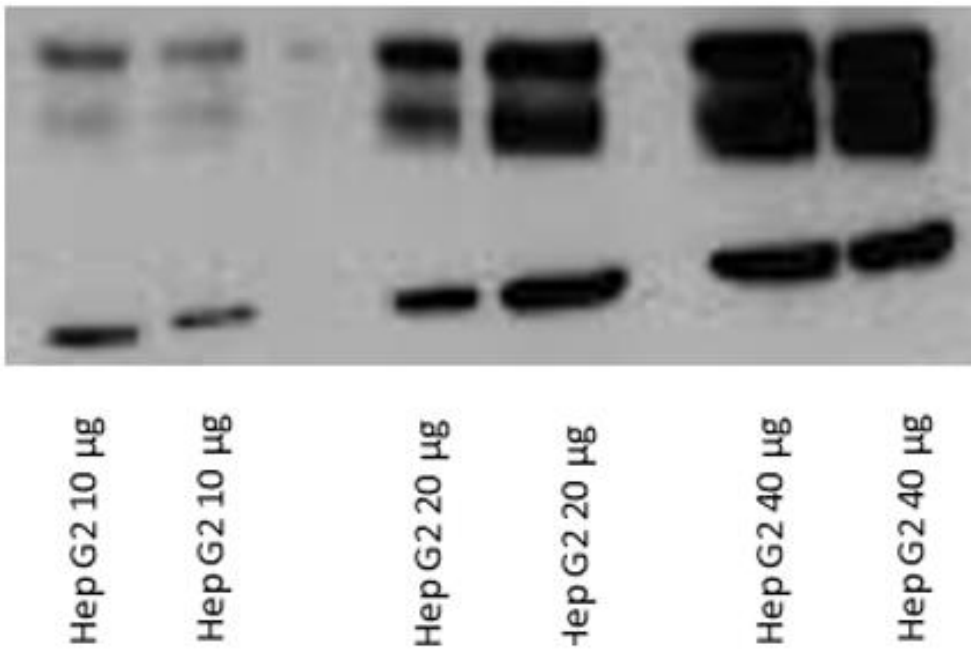
Figure 63: Initial Western Blot for hENT1



Arrow demarcates the 50 kDa molecular weight.

The initial blotting's did not demonstrate strong protein signal other than for the actin standard (See Figure 63). Varying concentrations of protein antibody, protein masses in the gel wells, and lengths of time for transfer were utilised to optimise the protein transfer and signal following chemi-luminescence. Multiple un-successful iterations were produced. However, reproducible methodology and results were achieved utilising a Proteintech hENT1 antibody concentration of 1/500, with 20 microgrammes of protein per well, and a 2-hour transfer time (See Figure 64).

Figure 64: Optimised hENT1 Western Blot with Actin Standard



The 2 protein bands easily demonstrable were blotting at 50 kDa and 62 kDa. The 50 kDa molecular weight is the predicted molecular weight of hENT1. The Proteintech datasheet states that the antibody blotted for hENT 1 at 62kDA. Given that both bands were demonstrable on blotting SiRNA transfection of Hep G2 cells was undertaken to determine which molecular band was associated with hENT1.

x.) SiRNA hENT1 Transfection Methodology

- Four separate SiRNA stocks were acquired from Proteintech for transfection with a scrambled control SiRNA. Lipofectamine and OptiMEM were also acquired from Proteintech.
- The OptiMEM, lipofectamine RNAiMAX and SiRNA stocks were placed on ice.
- HepG2 cells were trypsinised and re-suspended in growth medium in (5mL/T75 flask).
- Cells were counted on an electronic cell counter and density of cells was adjusted to 15 mL of 100, 000 cells/mL.
- The 3.6 mL OptiMEM and 36 microlitres of lipofectamine RNAiMAX were combined to produce 18 wells worth of medium.
- For hENT1 SiRNA 50 nM, 808 microlitres OptiMEM/RNAiMAX with 12 microlitres SiRNA stock was used (to produce 4 wells); for 20 nM SiRNA, 808 OptiMEM/RNAiMAX medium was combined with 4.8 microlitres of SiRNA stock; for 10 nM SiRNA, 808 microlitres of OptiMEM and RNAiMAX was combined with 2.4 microlitres SiRNA stock; for 50nM scrambled SiRNA 808 microlitres of OptiMEM/RNAiMAX was combined with 12 microlitres SiRNA stock.
- Three time points of 24, 48 and 72-hour plates were utilised (See Table 31):

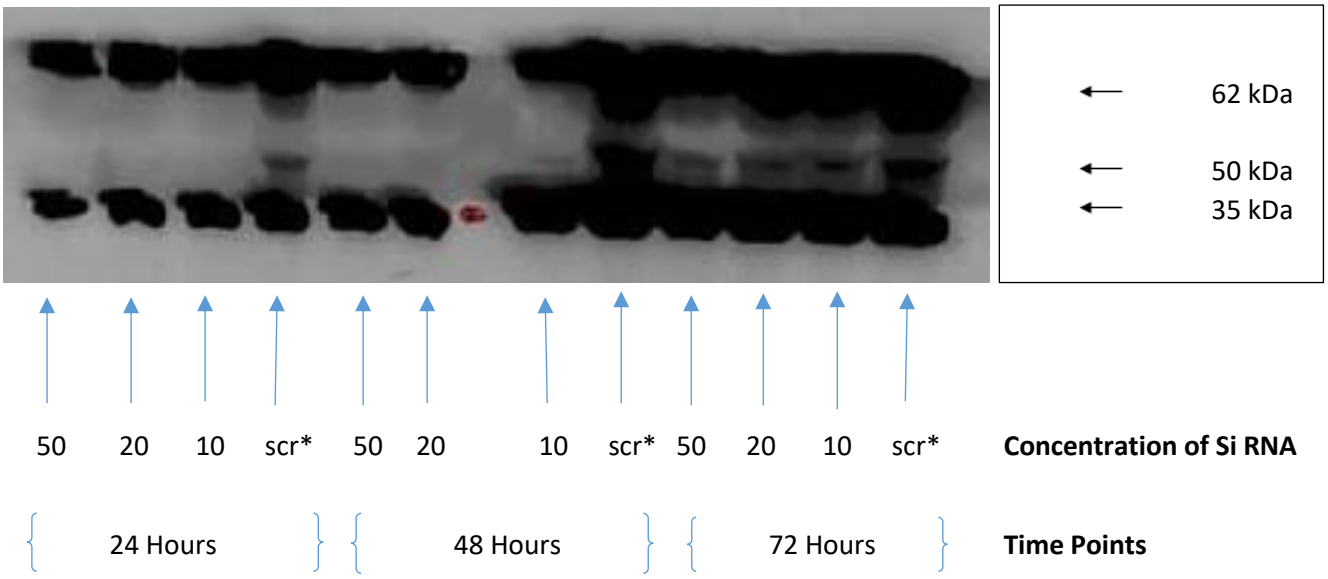
Table 31: Time-Point Plan for SiRNA knockdown of Rabbit Polyclonal hENT1 Antibody

Time-Point	hENT1 SiRNA Conc. (nM)	hENT1 SiRNA Conc. (nM)	hENT1 SiRNA Conc. (nM)	Scrambled SiRNA Conc. (nM)
24 Hours	50	20	10	50
48 Hours	50	20	10	50
72 Hours	50	20	10	50

- HepG2 cells were added from the pre-diluted suspension at 100, 000 cells per well (1mL).
- At the pre-determined time points the cells were washed and lysed with RIPA buffer and the lysates were acquired and stored at -80 degrees centigrade.
- The lysates were centrifuged at 18, 000 g for 5 minutes prior to BCA assessment.
- BCA assessment and protein concentrations were acquired.
- Western blots using the optimised protocols for hENT1 were undertaken.

One of the SiRNA batches demonstrated time and concentration dependent knockdown (See Figure 65). The SiRNA demonstrated knockdown of the 50 kDa molecular band rather than 62 kDa molecular band.

Figure 65: SiRNA Knockdown of Proteintech hENT1 antibody on Western Blot



***scr = scrambled RNA**

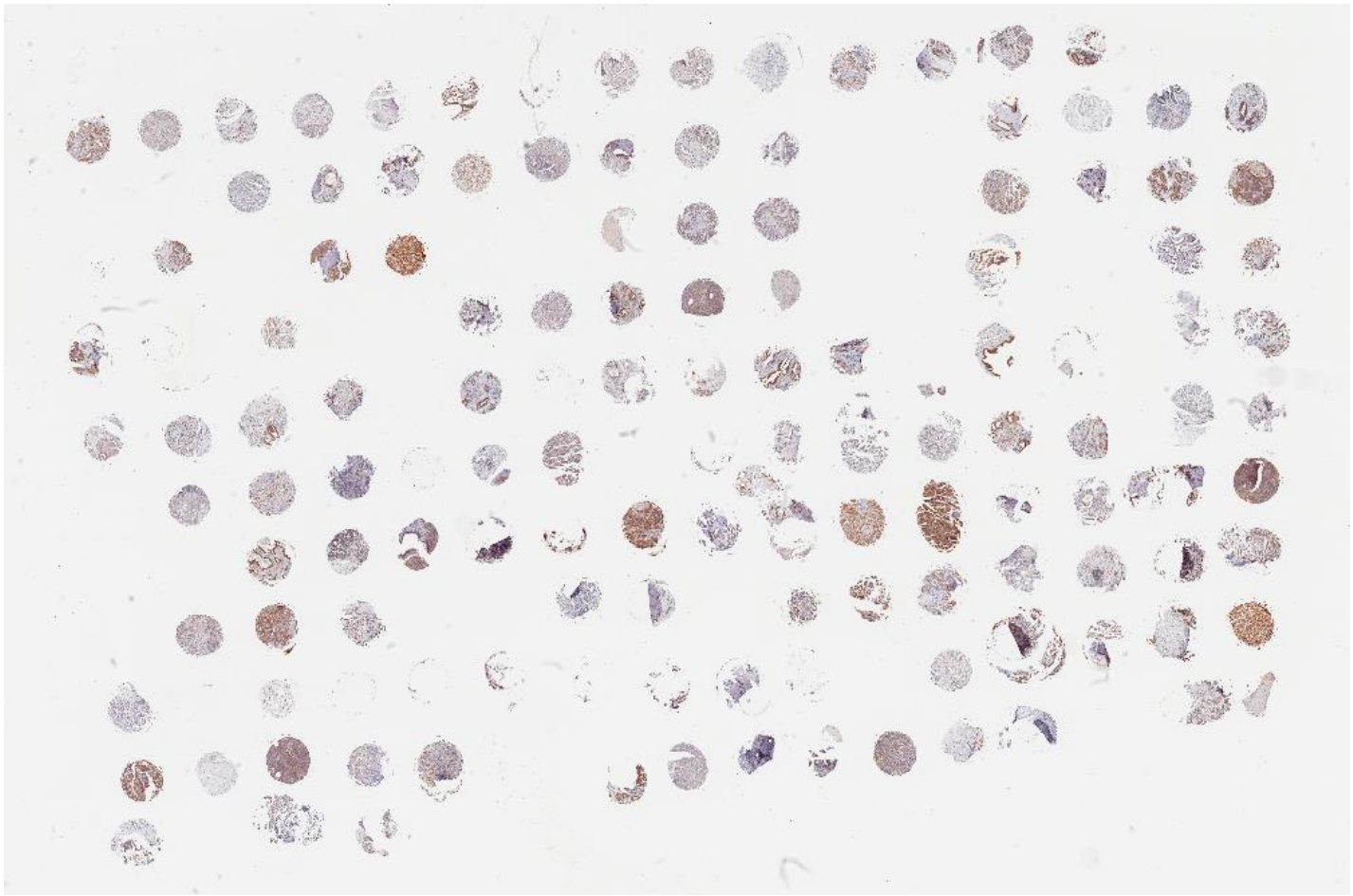
There is demonstrable dose-dependent knockdown of the protein at 50 kDa (the predicted molecular mass of hENT1) at both 24 and 48 hours with minimal effect at 72 hours.

There is no knockdown at the Proteintech stated hENT1 weight of 62 kDa.

The above blot demonstrates a dose-dependent knockdown of the 50 kDa band, i.e. the predicted molecular weight of hENT1 rather than the stated 62 kDa from Proteintech, which remains resolutely in place. The 62 kDa weight may be representative of an oligometric form of the hENT1 molecule. Mass spectroscopy of the different bands would provide a potential mechanism for further determining the provenance of the 2 bands.

Following knockdown confirmation that the Rabbit Polyclonal antibody blotted for hENT1, immunohistochemical assessment of the antibody was undertaken on the resected TMA (See Figure 66). An analogous methodology to the one utilised for the 10D7G2 antibody was used for the Proteintech antibody.

Figure 66: Proteintech Rabbit Polyclonal Antibody Immunostaining



xi.) Assessment of hENT1 Expression

Cores were scored by a specialist pancreas histopathologist (Dr T. Andrews) assisted by the candidate (Mr. N. Bird) to record the findings for each core. Dr T. Andrews was blinded to treatment group when scoring. Both Dr T. Andrews and Mr. N. Bird were blinded to patient outcomes throughout the assessment period. The intensity of cytoplasmic and membrane staining in tumour cells was ranked from 1 to 3. Lymphocyte staining was taken as an internal positive control. 'H' scores were produced for each core by multiplying the standard intensity score, as delineated by Greenhalf and colleagues by the percentage of tumour cells stained [385], and a mean 'H' score was calculated for each patient based on the 3 prognostic cores and 2 predictive cores. Inter-observer discrepancy was settled with discussion. Repeat assessment was undertaken to reduce intra-observer error, any discrepancy was settled with discussion.

xii.) Assessment of Ki67 Expression

For each patient, the overall Ki67 labelling index was obtained from the mean value of 3 TMA cores for the resected specimens and 2 biopsy cores. Ki67 was considered overexpressed when >5% stained nuclei were observed as the mean score for the TMA cores.

xiii.) Statistical Analysis

Skewed numerical data analysed with Mann Whitney U calculations. Categorical data analysed utilising Chi-squared and survival analysis calculated with Log-rank Mantel-Cox calculations. Correlated numerical data analysed with Pearson correlation co-efficient.

3.24) Results

Assessment of the cores demonstrated that the biopsy/predictive biopsy specimens were low in quality with only 5 specimens having sufficient biological material to assess. These samples were not subsequently analysed further due to the low utility of the data.

Thirty-nine of the 43 (90.7 %) resected specimens had sufficient biological tissue in the cores for assessment. There were 12 male patients and 27 female patients in the cohort (p= 0.016). The median age of the cohort was 61 (IQR= 10). The Mackey 10D7G2 antibody median 'H' score was 100, interquartile range (IQR) was 75 (70 to 145), with the range being 0 – 207. The Mackey 10D7G2 group was dichotomised around the median into 'High' and 'Low' hENT1 expressing tumour categories. There were 21 patients categorised as 'Low' and 18 patients were categorised as 'High' hENT1 expressing tumours. The Proteintech commercial antibody median 'H' score was 72, the IQR was 33 (47 to 80), with the range being 0 – 140. The Proteintech group was dichotomised around the median in to 'High' and 'Low' hENT1 expressing tumour categories. There were 20 patients categorised as 'Low' and 19 patients categorised as 'High' hENT1 expressing tumours. The Ki67 stained grouping had 20 patients categorised as 'Low' and 19 patients categorised as 'High' expressing tumours.

Concordance between the mean 'H' scores for the Mackey 10D7G2 and Proteintech antibodies was found to be significant (p< 0.001; See Table 32; See Figure 67).

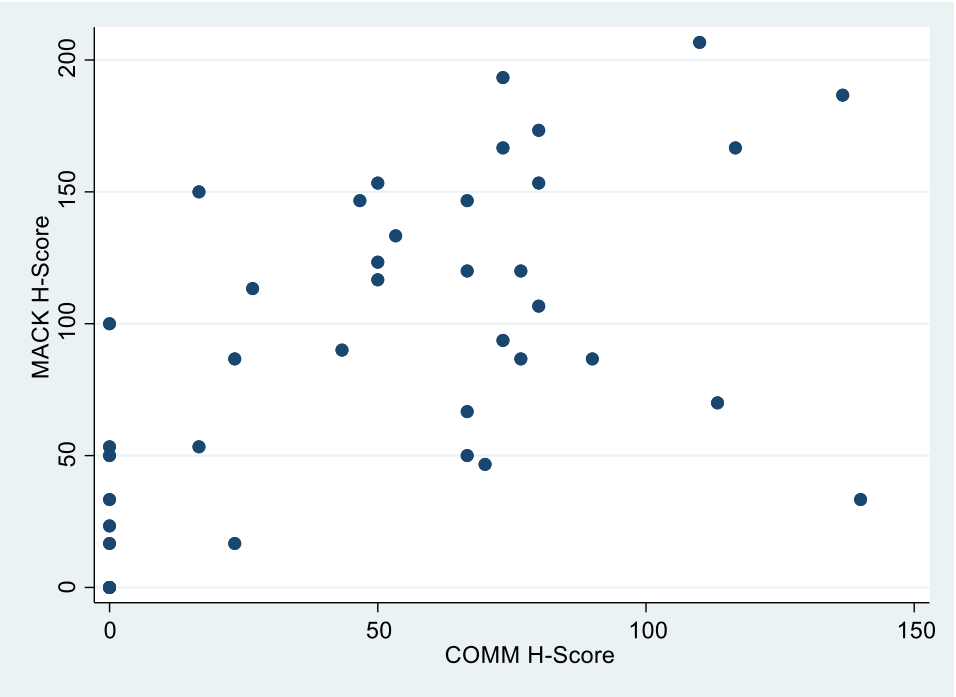
Table 32: Pearson's Correlation Coefficient presented with associated T-test

Antibody	Mean H Score (sd)	range	Correlation (p-value)
Mackey 10D7G2	96 (59)	0-207	0.55 (<0.001)
Proteintech	53 (41)	0-140	

Categorical dichotomisation of the respective antibodies with associated statistical assessment did not demonstrate significant concordance (Pearson Chi-squared value = 2.06; p= 0.152; See Table 33). Assessment of the dichotomised Proteintech hENT1 and the Ki67 antibody groupings demonstrated significant concordance for expression (Pearson Chi-squared value = 5.76; p= 0.016; See Table 34). There

was no significant association between the Mackey 10D7G2 and Ki67b antibody expression (Pearson Chi-squared value = 0.63; p= 0.43).

Figure 67: Scatter Plot Assessing Concordance of Mean 'H' Scores between Mackey 10D7G2 and Proteintech Commercial Antibody



*COMM H-Score = Proteintech Commercial antibody mean 'H' score

MACK H-Score = Mackey 10D7G2 'H' score

Table 33: Categorical Dichotomisation of Mackey 10D7G2 and Proteintech Commercial Antibody Staining

		Mackey	
		high	low
Proteintech	high	11	8
	low	7	13

Table 34: Categorical Dichotomisation of Proteintech hENT1 Antibody and Ki67 Antibody Staining

		Proteintech	
		high	low
KI67	high	13	6
	low	6	14

None of the antibodies demonstrated prognostic utility for OS or DFS (See Table 35). There was no significant difference between the patient, tumour covariates and antibody expression of the cohort (See Table 36).

Table 35: Survival Analysis for Patients Stratified by hENT and Ki67 Tumour Expression

Variables	**hENT1 Mackey**		**hENT1 Protein***		**Ki67**	
Antibody Expression	High	Low	High	Low	High	Low
Overall Survival						
Numbers at Risk at Resection	18	21	19	20	19	20
Numbers at Risk at 5 Years	6	5	6	5	3	8
Log-rank P-Value		0.75		0.63		0.22
Disease Free Survival						
Numbers at Risk at 5 Years	6	5	6	3	2	7
Log-rank P-Value		0.75		0.83		0.19

***Proteintech antibody; referred to in further tables in this manner.**

Table 36: Cohort Patient and Tumour Covariates stratified by Antibody Immunostaining Expression

Variables		hENT1 Mackey		hENT1 Protein		Ki67	
Antibody Expression		High	Low	High	Low	High	Low
Age	Median	61	61	60	61.5	61	59
	IQR	14	11	14	10.5	10	14
	P-value		0.65**		0.80**		0.97**
Differentiation	Well	4	5	4	5	2	7
	Other	15	15	16	15	17	13
	P-Value		0.90		0.75		0.15
Perineural Invasion	Positive	13	14	11	16	15	12
	Negative	5	7	8	4	7	5
	P-Value		0.71		0.25		0.85
LVI *	Positive	6	11	7	10	10	7
	Negative	12	10	12	10	9	13
	P-Value		0.38		0.61		0.43
T-Stage	T1 + T2	15	18	17	16	16	17
	T2 + T3	3	3	2	4	3	3
	P-Value		0.81		0.41		0.95
Nodal Staging	N0	13	15	13	15	12	16
	N1	5	6	5	6	7	4
	P-Value		0.76		0.76		0.42
Resection Status	R0	8	12	10	10	8	12
	R1	10	9	9	10	11	8
	P-Value		0.64		0.88		0.43

***Lymphovascular Invasion; ****MannWhitney U calculations; All other calculations Chi-Squared with Yates correction.

None of the tumour covariates stated in Table 36 had significant association with OS or DFS following Log-rank analysis. The relationship between OS and the presence of lympho-vascular invasion on histopathological analysis approached significance (p= 0.062). The relationship between DFS and adjuvant chemotherapy treatment approached significance (p= 0.061). Fifteen patients underwent gemcitabine-based adjuvant chemotherapy, and 6 patients underwent palliative gemcitabine-based chemotherapy. Subset analysis of patients undergoing adjuvant chemotherapy and tumour hENT1 Mackey 10D7G2 antibody status did not demonstrate significant OS or DFS association (p= 0.58 and 0.57 respectively). Subset analysis of patients undergoing gemcitabine-based chemotherapy and tumour hENT1 Proteintech antibody status did not demonstrate significant OS or DFS association (p= 0.35 and 0.42 respectively). Given the lack of significant findings Kaplan-Meier graphs were not constructed from the hazard ratios as this would not provide additional significant information.

3.25) Discussion

This study has identified a potentially commercially available hENT1 antibody which could provide an alternative to the 10D7G2 Mackey antibody for determining immunohistochemical hENT1 expression in resected cholangiocarcinoma specimens. The Proteintech hENT1 antibody demonstrates hENT1 inhibition following hENT1 knockdown with silencing RNA in HepG2 cells in a dose and time dependent manner. The immunostaining average 'H' scores correlate significantly between the Proteintech hENT1 antibody and the 10D7G2 hybridoma. However, no significant prognostic or predictive associations with antibody expression and OS, or DFS and gemcitabine-based chemotherapy, were demonstrated. No tumour covariates demonstrated significant OS or DFS associations within the cohort undergoing immunohistochemical assessment.

The significant correlation between the Proteintech hENT1 antibody and the 10D7G2 hybridoma is an especially important finding due to the recent cessation of production of the Mackey 10D7G2 hybridoma. The 10D7G2 hybridoma has been the only antibody which has consistently been demonstrated to have a significant prognostic and predictive association with gemcitabine-based chemotherapy for hepato-biliary malignancies [398, 403, 404]. Alternative putative prognostic and predictive antibodies have been discussed within research literature but have demonstrated a lack of concordance and predictive utility [381, 392, 398, 405]. This study failed to demonstrate significant prognostic and predictive associations between hENT1 abundance for either of the hENT1 antibodies. A potential explanation may be due to the relatively small cohort of immunohistochemically assessed specimens which may produce instability of analysis. Further immunohistochemical investigation with an expanded cohort would potentially improve the likelihood of detecting prognostic or predictive associations with hENT1 expression. In the intervening period between construction of the TMA, and subsequent immunohistochemical assessment, and the last assessment of

survival (December 2020) there have been a further 30 resections undertaken at Aintree University Hospital. Construction of an additional TMA with the incorporation of these patient's specimens may provide additional data which could further elucidate any prognostic or predictive association with tumour hENT1 expression.

The lack of prognostic or predictive association with immunohistochemical hENT1 expression may be reflective of the complexity of the ancillary downstream molecular interplay. The hENT1 trans-membranous protein expression is one component of a hugely complex cellular system associated with nucleoside uptake. This system has been elucidated in cellular models but relatively few studies have attempted to determine immunohistochemical tumour expression of these downstream effectors [406, 407]. The relative ratio of immunohistochemical expression of these downstream molecular effectors may provide further elucidation of any prognostic or predictive associations. The hENT1 molecule is also particularly glycosylated and the non-detection of prognostic or predictive associations in the 'high' expression category maybe due to the detection of inactive glycosylated variants which have retained immunoreactivity [403]. The tumour microenvironment, which is a particularly rich, complex and heterogeneous environment for cholangiocarcinoma tumours, may also be affecting our assessment of the link between hENT1 status and prognosis [408]. Further multimodal assessment of the multiple components of each tumour is required to determine the complex interplay between tumour molecular characteristics and prognosis.

Immunohistochemical assessment is a semi-quantitative methodology for determining specimen protein expression. There is potential for error when allocating specimens to 'low' and 'high' protein expression by utilising the median as a measure of central tendency. The median measure may not represent a true measure of discrimination for dichotomisation of patient's specimens into potentially arbitrary 'high' and 'low' expression categories. Specimens which demonstrate expression either side of the median may be incorrectly allocated into either of these categories resulting in potential Type 1 (False Positive) and Type 2 (False Negative) errors. A potential solution to the induction of, particularly Type 2 errors, is to increase the size of the assessed population (the number of specimens immunohistochemically assessed). Power calculations based upon robust meta-analytical data would potentially enable researchers to determine the minimum size of population required for immunohistochemical assessment. This has not been previously attempted and the literature base for hENT1 immunohistochemical analysis of cholangiocarcinoma is, at the time of writing, limited.

A recent meta-analysis which assessed immunohistochemical abundance in resected and palliative patients with biliary tract cancer has demonstrated that there are conferred survival benefits for patients with high hENT1 abundance undergoing gemcitabine-based chemotherapy [409]. While providing a persuasive argument for further investigation this study had significant methodological limitations. Biliary tract cancer

as a diagnostic entity lacks specificity due to the biological heterogeneity of the cancers contained within the criterion [410]. Coalescence of genetically, biologically and clinically disparate cancers at radically different AJCC stages under one subsumed category for analysis produces a significant potential for error to occur. Unfortunately the individual populations meta-analysed are also small, with only 373 patients from 8 studies under analysis, and accordingly prone to Type 1 errors. All of the studies were also retrospective cohort analyses and prone to possible problems with data assurance issues. Six of the studies incorporated in the meta-analysis used the controversial Ventana SP120 antibody. Svrcek and colleagues have demonstrated this antibody has significant applicability issues with respect to detecting hENT1 abundance within immunohistochemical specimens. Only 2 studies used the gold-standard 10D7G2 antibody to determine hENT1 abundance, and none used the Proteintech polyclonal antibody, which raises the possibility of specificity errors regarding the provenance of the immunohistochemically detected signal.

Alternative methods of investigation and analysis may also be more sensitive and specific for determining hENT1 protein expression. Whole tumour RNA assessment of Formalin-fixed Paraffin Embedded specimens, potentially using RNA scope technology, may provide improved sensitivity and quantification of hENT1 expression, within cholangiocarcinoma specimens [411]. Detection thresholds could also be altered allowing further precision by stratification of tumours into multiple expression categories or higher thresholds for assessment of the top 10 % hENT1 RNA producers. Genomic assessment, in parallel with RNA and immunohistochemistry in a multimodal approach, would potentially add further understanding regarding genomic and correlating proteomic expression of hENT1 [412-414]. Genomic assessment of cholangiocarcinoma tumours would also potentially help to elucidate the complex epigenetic factors which may interact with hENT1 expression. Genomic work on fresh tumour specimens and assessment of tumour organoids has been undertaken by a subsequent doctoral student (Mr Marc Quinn) and his report is currently awaited.

Whilst this study has essentially demonstrated no significant association between hENT1 and prognosis, further assessment with the above considerations accounted for could help delineate any potential link. Prognostic systems incorporating molecular biomarkers could provide improved prognostication systems and help guide chemotherapeutic treatment strategies. Further work is required to determine the utility of the hENT1 biomarker for prognosis for overall survival and response to treatment.

3.36) FURTHER CELLULAR WORK

i.) Development of Cellular Models for Gemcitabine Toxicity

Subsequent to development of a reproducible Western blot methodology and confirmation of hENT1 molecular band, in vitro cytotoxic assessments of gemcitabine cellular toxicity were undertaken.

Multiple cholangiocarcinoma cell lines were acquired from The Japanese Collection of Research Bio-Resources (JCRB) cell bank. The KKU M213 cell-line, harvested from a patient with liver-fluke related intrahepatic cholangiocarcinoma, was determined to be viable for cytotoxic assays. The KKU M213 cell line required standard growth media, had a relatively short doubling time (i.e. readily proliferated), grew in a monolayer, and had consistent cellular morphology indicating that experimentation could be reproducible.

Cell viability was assessed using the MTT calorimetric assay [415]. The MTT assay assesses the reduction of yellow tetrazolium bromide salt (thiazolyl blue tetrazolium bromide) by the cells under analysis to measure cellular metabolism as a surrogate indicator of cell viability. The cells reduce the reagent to formazan which emits a purple light wavelength. The formazan crystals are re-suspended in a stabilising solvent and the wavelength is measured on a plate reader at 570 nM. Control wells (containing un-treated cells) and blank wells (containing medium) in the experimental 96 well plate provide the standard for non-reduced cells.

The following methodology was utilised to determine gemcitabine toxicity:

- A stock solution of gemcitabine 500 mM was produced with DMSO (dimethyl sulfoxide) as the carrier. Serial dilutions were completed so that 12 concentrations of gemcitabine were produced and stored in eppendorfs.
- Cells in T75's were trypsinised and re-suspended with 400, 000 cells per mL.
- Initially 20, 000 cells per well were pipetted (50 microlitre of cellular solution). All concentrations previously serially diluted were pipetted in triplicate into 96 well plates.
- Initially 3 time-points at 24; 48 and 72 hours were plated and incubated in 5 % CO^2 atmosphere at 37 degrees centigrade.
- At each time the MTT assay was undertaken. The MTT reagent was made up in Hanks Buffered Salt Solution at 5 mg/mL per assay. Twenty microliters of MTT reagent were added to each well including the 3 blank cells and incubated for 2 hours.
- During the 2 hours lysis buffer was produced from a mixture of: 20 % weight by volume of SDS; 50 % volume by volume of N,N-diethylforamide; 50 % dH_2O. The buffer was warmed in a water bath to dissolve the residual SDS.
- Two hundred microliters of lysis buffer were added to each well at the appropriate time period. Each plate was left over night in the 5 % CO^2 atmosphere at 37 degrees centigrade.

- The plates were then placed in to a microplate reader and assessed at 570 nM absorbance wavelengths.
- Excel programming was used to determine absorbance's relative to the blanks and controls using the following equation:

$$\% \text{ viable cells} = \frac{(abs_{sample} - abs_{blank})}{(abs_{control} - abs_{blank})} \times 100$$

OPTIMISATION CONDITIONS FOR KKU-M213:

Set 1;

1.) Gemcitabine – stock concentration 500 Mm

2.) DMSO as vehicle

3.) Cell density 400, 000 cells per ml

4.) 20, 000 Cells per well (50 micro-litre per well) DMEM/FBS/PenStrep

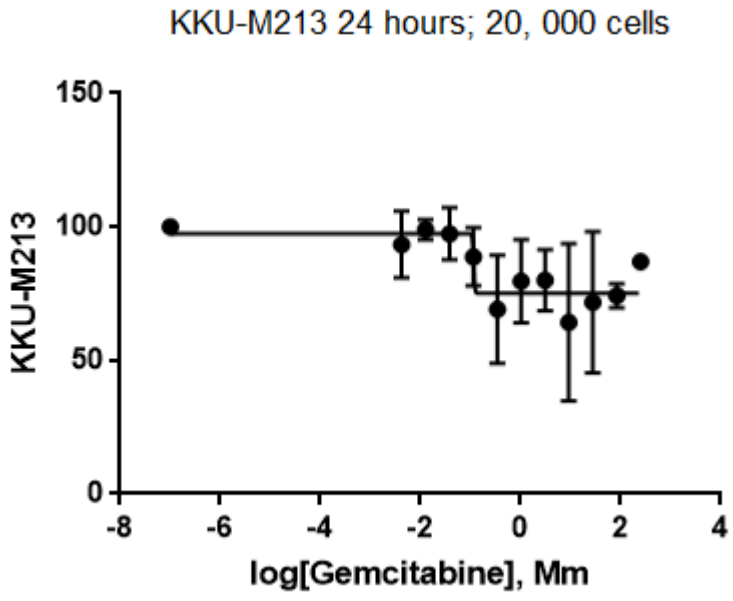
5.) Serial dilution 12 concentrations (50 micro-litre per well)

6.) 3 time points; 24 hours; 48 hours; 72 hours

7.) Graph Pad prism analysis

8.) Dose-response curves with variable fit (See Figures 67, 68 and 69)

Figure 68: Dose-Response Curve at 24-Hour Time Point for Gemcitabine acting on KKU-M213

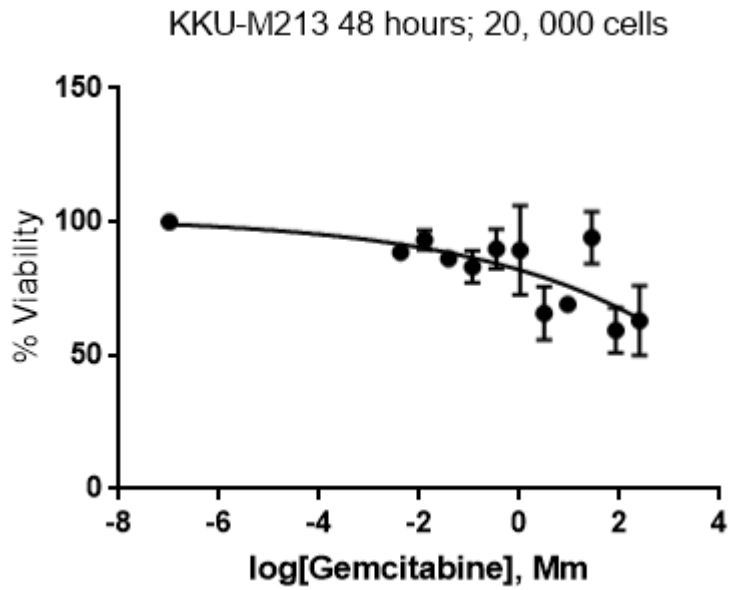


Interpretation

1.) ED 50 not reached

2.) Dose-responsive

Figure 69: Dose-Response Curve for 48-Hour Time Point for Gemcitabine acting on KKU-M213

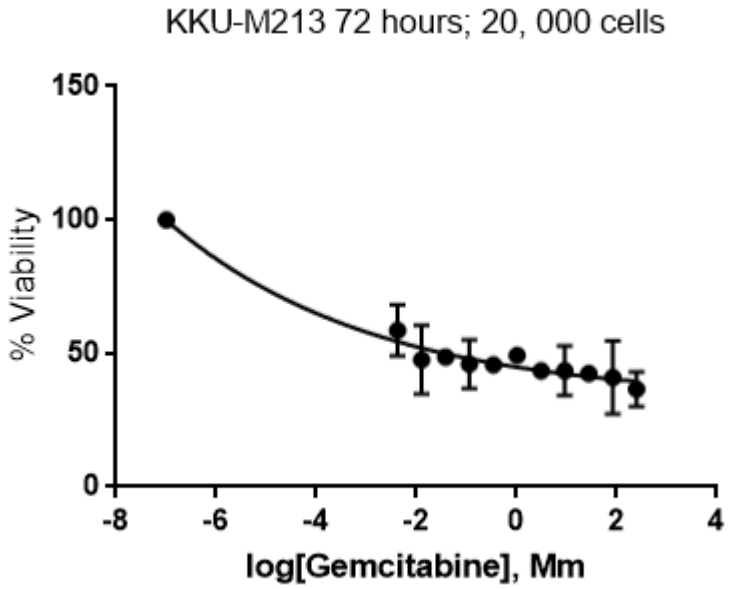


Interpretation

1.) ED 50 not reached

2.) Narrow error bars but not demonstrating sigmoidal response

Figure 70: Dose-Response Curve for 72-Hour Time Point for Gemcitabine acting on KKU-M213



Interpretation

1.) ED 50 reached but confidence intervals wide not calculable

2.) Indication that drug to cell ratio too low

3.) Reduce cell concentration to increase dispersal about cells

OPTIMISATION OF CONDITIONS:

Set 2;

1.) Gemcitabine – stock concentration 500 Mm

2.) DMSO as vehicle

3.) Cell density 400, 000 cells per ml

4.) 10, 000 Cells per well (50 micro-litre per well) DMEM/FBS/PenStrep

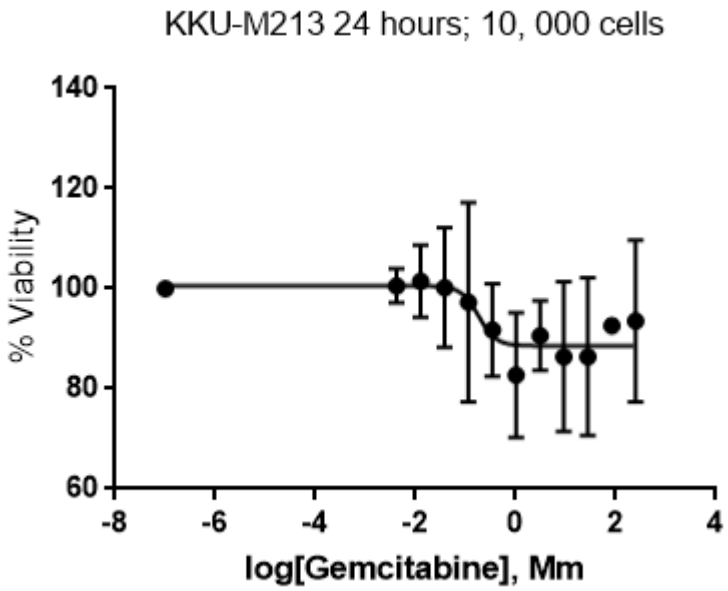
5.) Serial dilution 12 concentrations (50 micro-litre per well)

6.) 3 time points; 24 hours; 48 hours; 72 hours; 96 hours

7.) Graph Pad prism analysis

8.) Dose-response curves with variable fit (See Figures 70, 71, 72 and 73)

Figure 71: Dose-Response Curve for 24-Hour Time Point for Gemcitabine acting on KKU-M213



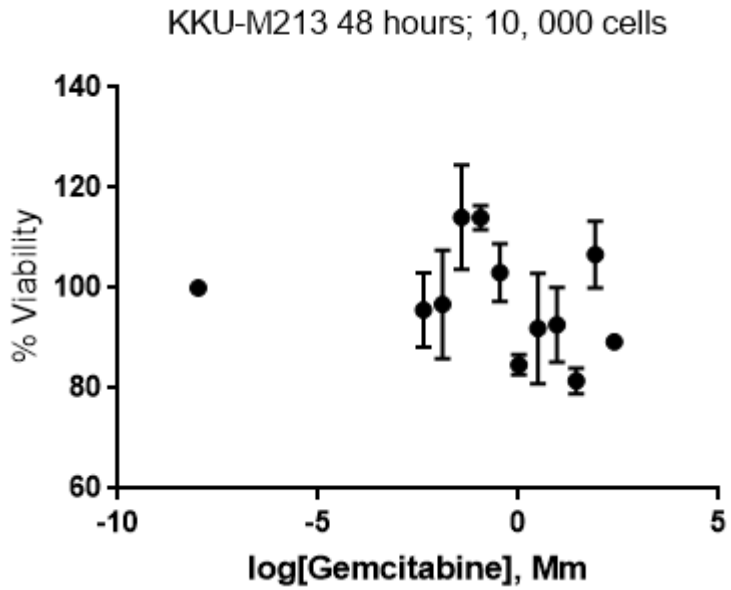
Interpretation

1.) ED-50 not reached

2.) Dose-responsive

3.) Error bars and confidence intervals wide

Figure 72: Dose-Response Curve for 48-Hour Time Point for Gemcitabine acting on KKU-M213



Interpretation

1.) ED-50 not reached

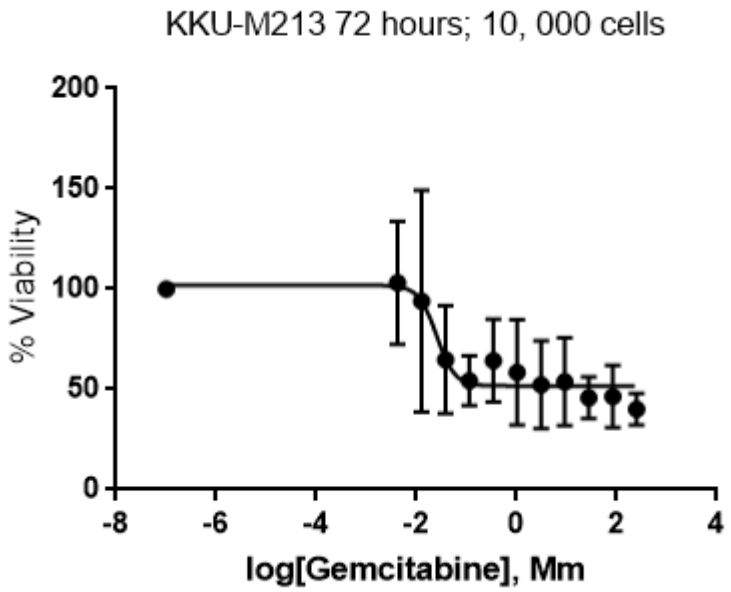
2.) Non dose-responsive

3.) Wide error bars and confidence intervals

4.) Variability in response interpreted to be due to evaporation on the plates

5.) Reduce concentrations and increase non-active wells around the wells undergoing experimentation in next set.

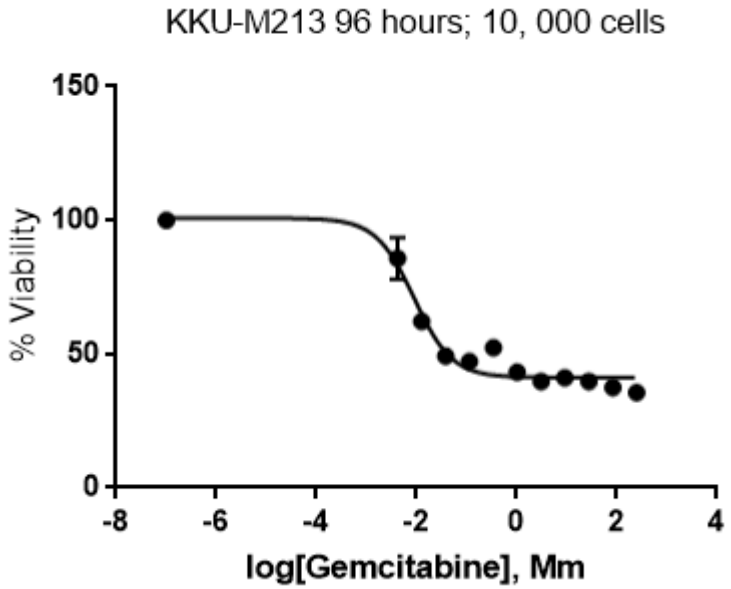
Figure 73: Dose-Response Curve for 72-Hour Time Point for Gemcitabine acting on KKU-M213



Interpretation

1.) Log ED-50: 0.2356
2.) Log 95 Confidence Intervals -2.199 – 1.348
3.) ED-50 0.00633 – 22.27 Mm
4.) Given wide errors and confidence intervals further reduction in cell density to improve accuracy of errors was undertaken.

Figure 74: Dose-Response Curve for 96-Hour Time Point for Gemcitabine acting on KKU-M213



Interpretation

1.) ED-50 reached
2.) Log ED-50 -2.221 to -1.874
3.) ED-50: 0.006009 – 0.01338
4.) More accurate verification of ED-50 over the 96 hour time point.
5.) Possible to use 96 hour time point as time point for further experiments.

OPTIMISATION OF CONDITIONS:

Set 3:

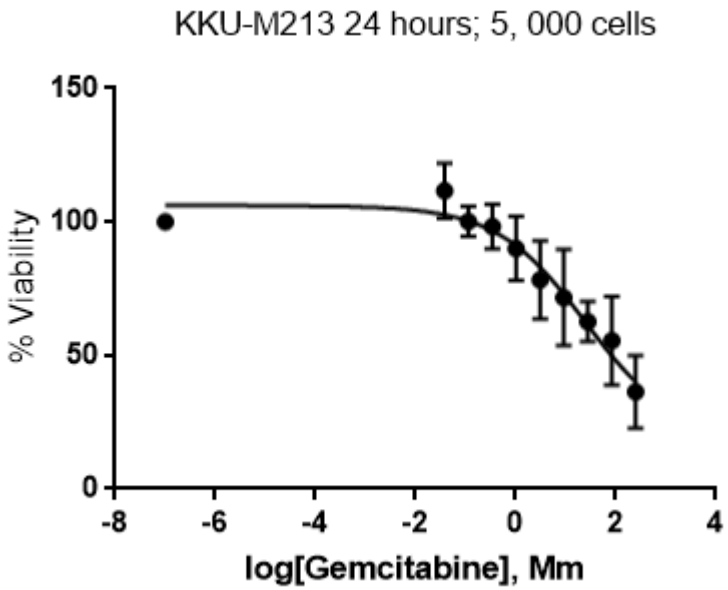
1.) Gemcitabine – stock concentration 500 Mm
2.) DMSO as vehicle
3.) Cell density 400, 000 cells per ml
4.) 5, 000 Cells per well (50 micro-litre per well) DMEM/FBS/PenStrep
5.) Serial dilution 10 concentrations (50 micro-litre per well) reduced from 12 concentrations to reduce evaporation from wells

6.) 3 time points; 24 hours; 48 hours; 72 hours

7.) Graph Pad prism analysis

8.) Dose-response curves with variable fit (See Figures 74, 75 and 76)

Figure 75: Dose-Response Curve for 24-Hour Time Point for Gemcitabine acting on KKU-M213



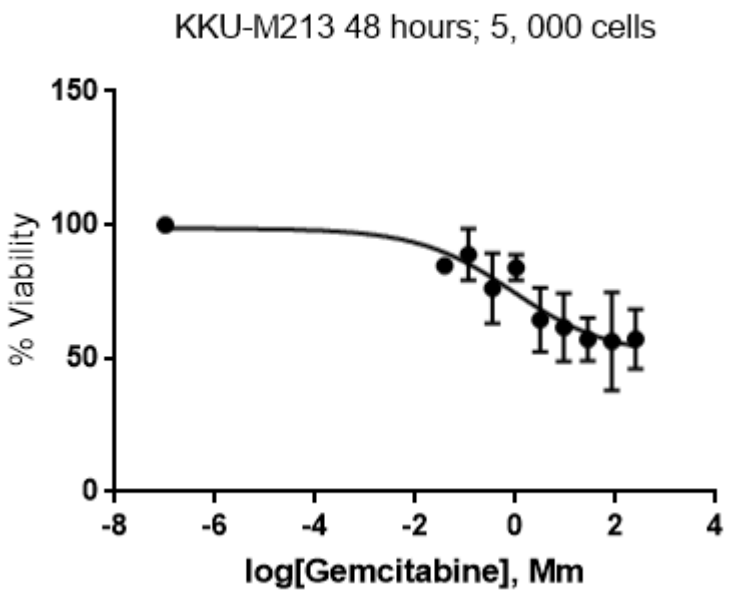
Interpretation:

1.) ED-50 0.7846

2.) Wide confidence intervals with lower confidence interval not reached

3.) Partially sigmoidal and dose-dependent response

Figure 76: Dose-Response Curve for 48-Hour Time Point for Gemcitabine acting on KKU-M213



Interpretation

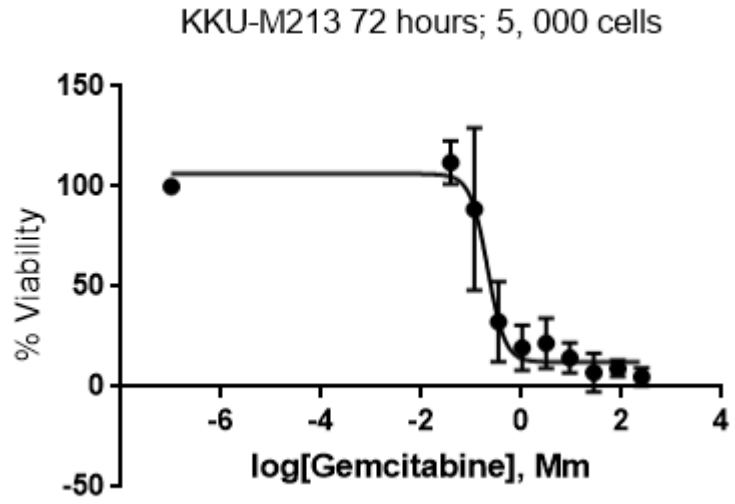
1.) ED-50 not reached

2.) Narrow error bars

3.) Developing sigmoidal curves

4.) Demonstration of dose-dependence

Figure 77: Dose-Response Curve for 72-Hour Time Point for Gemcitabine acting on KKU-M213



Interpretation

1.) Log ED-50 reached 0.08643

2.) 95 % Confidence Intervals -0.8504 to – 0.5182

3.) ED-50 0.1411 to 0.3032 Mm

4.) Convincing ED-50 with narrow error bars and confidence intervals.

5.) Optimised dose-response curves for 5000 cells per well over a standardised time course.

HEP-G2 Cell Line:

OPTIMISATION OF CONDITIONS:

Set 1;

1.) Gemcitabine – stock concentration 500 Mm

2.) DMSO as vehicle

3.) Cell density 400, 000 cells per ml

4.) 20, 000 cells per well (50 micro-litre per well) DMEM/FBS/PenStrep

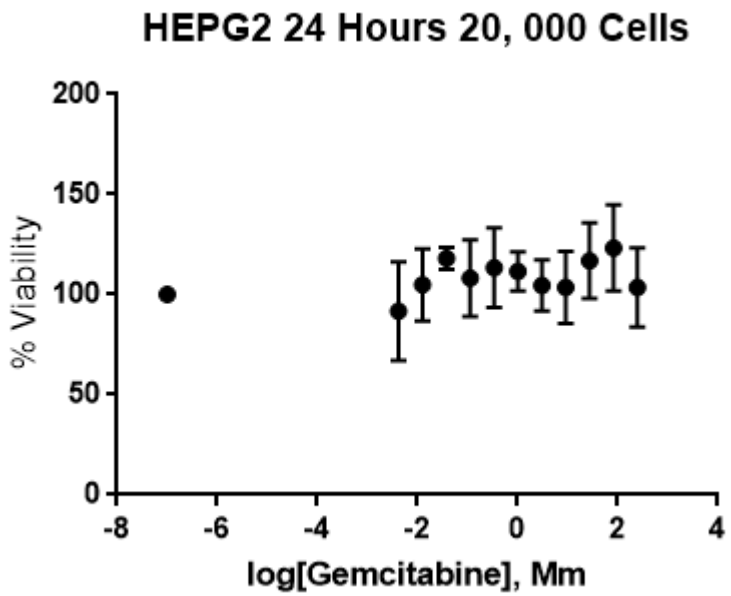
5.) Serial dilutions 12 concentrations (50 micro-litre per well)

6.) 3 Time points; 24 hours; 48 hours; 72 hours

7.) Graph Pad prism analysis

8.) Dose-response curves with variable fit parameters (See Figures 77, 78 and 79)

Figure 78: Dose-Response Curve for 24-Hour Time Point for Gemcitabine acting on HepG2

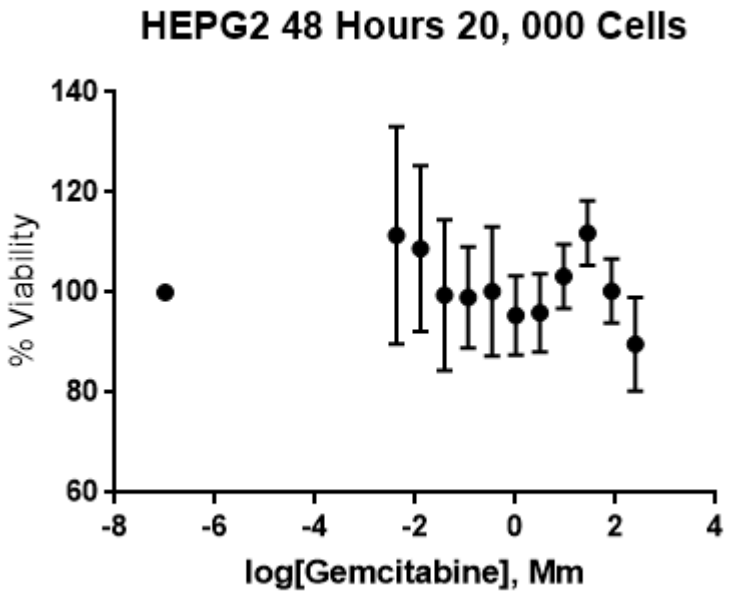


Interpretation

1.) No obvious dose-response

2.) ED-50 not reached

Figure 79: Dose-Response Curve for 48-Hour Time Point for Gemcitabine acting on HepG2



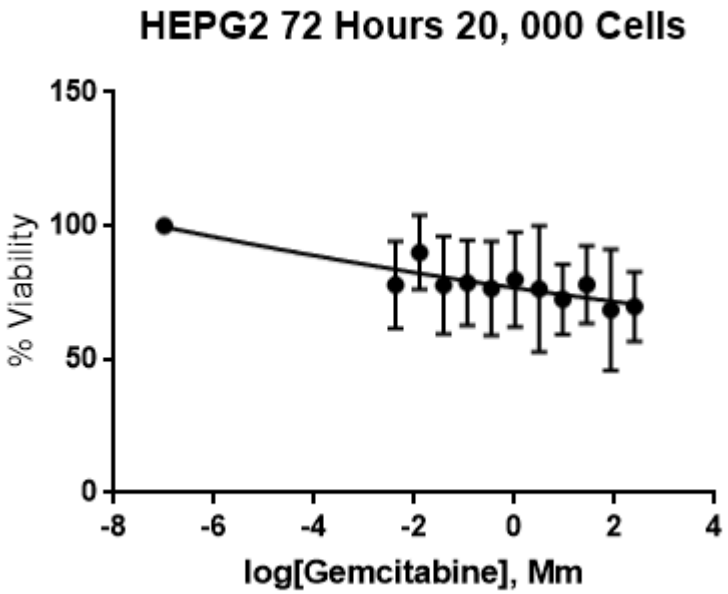
Interpretation

1.) ED-50 not reached

2.) Wide error bars and standard errors

3.) Not apparently dose responsive for these conditions

Figure 80: Dose-Response Curve for 72-Hour Time Point for Gemcitabine acting on HepG2



Interpretation

1.) ED-50 not reached

2.) Cell death occurring but not demonstrating sigmoidal dose-response curves

3.) Change conditions to achieve gemcitabine related cell death

4.) Reduce cell concentration

CONDITIONS:

Set 2;

1.) Gemcitabine – stock concentration 500 Mm

2.) DMSO as vehicle

3.) Cell density 400, 000 cells per ml

4.) 10, 000 cells per well (50 micro-litre per well) DMEM/FBS/PenStrep

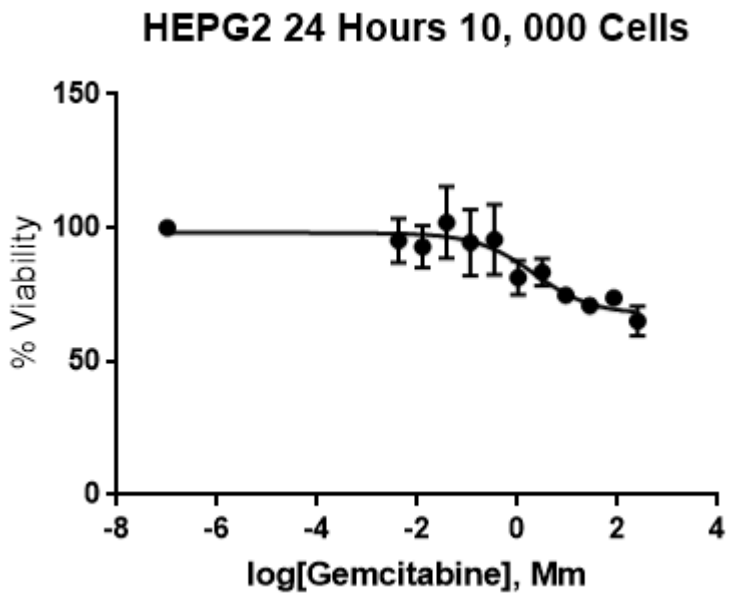
5.) Serial dilutions 12 concentrations (50 micro-litre per well)

6.) 3 Time points; 24 hours; 48 hours; 72 hours

7.) Graph Pad prism analysis

8.) Dose-response curves with variable fit parameters (See Figures 80, 81 and 82)

Figure 81: Dose-Response Curve for 24-Hour Time Point for Gemcitabine acting on HepG2

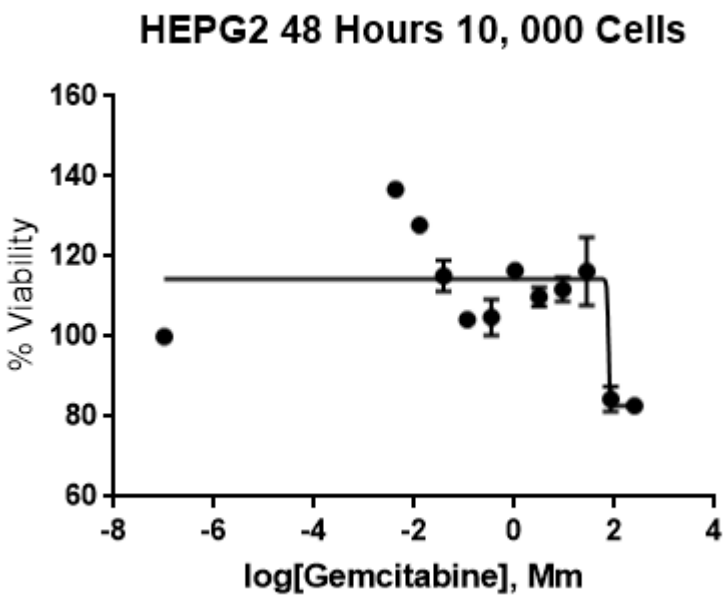


Interpretation

1.) ED-50 not reached

2.) Demonstrating element of sigmoidal dose-response characteristics

Figure 82: Dose-Response Curve for 48-Hour Time Point for Gemcitabine acting on HepG2



Interpretation

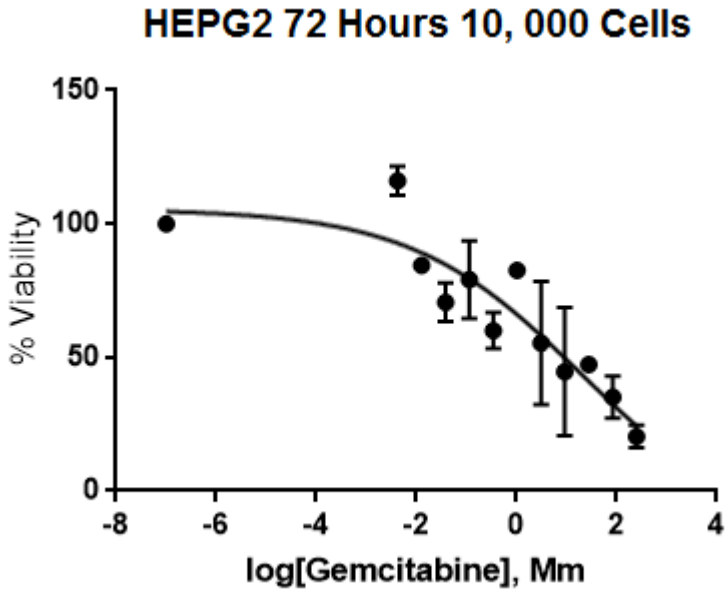
1.) ED-50 not reached

2.) Demonstrates cell growth

3.) High levels of variability demonstrated

4.) Plates showing variable evaporation across the cell wells contributing to inaccuracy of experiment

Figure 83: Dose-Response Curve for 72-Hour Time Point for Gemcitabine acting on HepG2



Interpretation

1.) ED-50 reached

2.) Log ED50 2.214

3.) Wide error bars and upper confidence interval not reached

4.) Demonstration of approach to sigmoidal response

5.) Reduce cell concentration and alter to 10 well approach to reduce evaporation and reduce variability of response in experiment

OPTIMISATION OF CONDITIONS:

Set 3;

1.) Gemcitabine – stock concentration 500 Mm

2.) DMSO as vehicle

3.) Cell density 400, 000 cells per ml

4.) 5, 000 Cells per well (50 micro-litre per well) DMEM/FBS/PenStrep

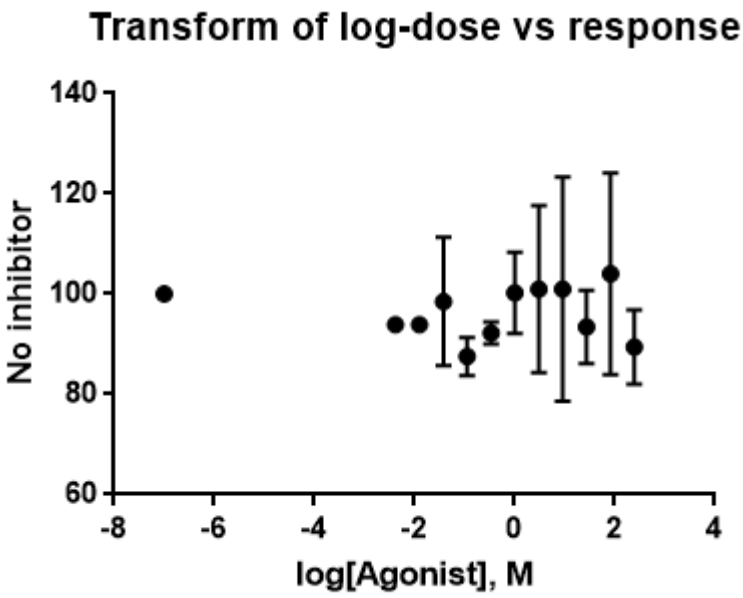
5.) Serial dilution 10 concentrations (50 micro-litre per well) reduced from 12 concentrations to reduce evaporation from wells.

6.) 3 time points; 24 hours; 48 hours; 72 hours

7.) Graph Pad prism analysis

8.) Dose-response curves with variable fit (See Figures 83, 84 and 85)

Figure 84: Dose-Response Curve for 24-Hour Time Point for Gemcitabine acting on HepG2

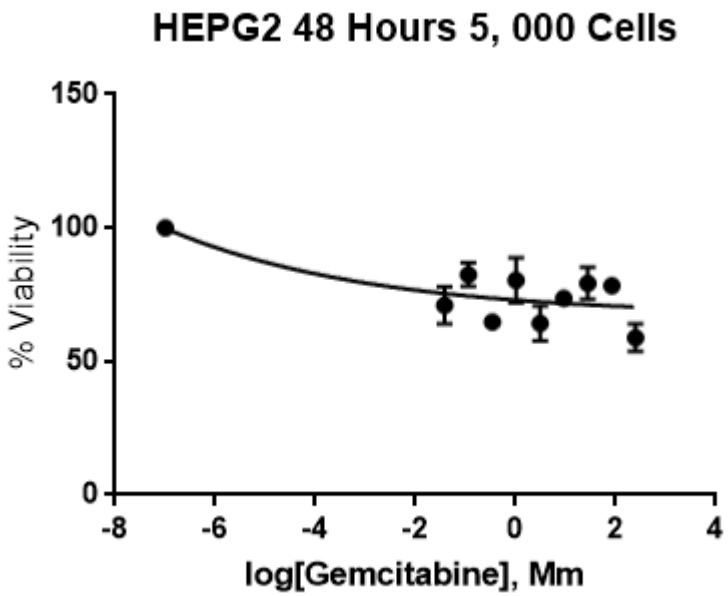


Interpretation

1.) ED-50 not reached

2.) Wide error bars and confidence intervals non-calculable

Figure 85: Dose-Response Curve for 48-Hour Time Point for Gemcitabine acting on HepG2



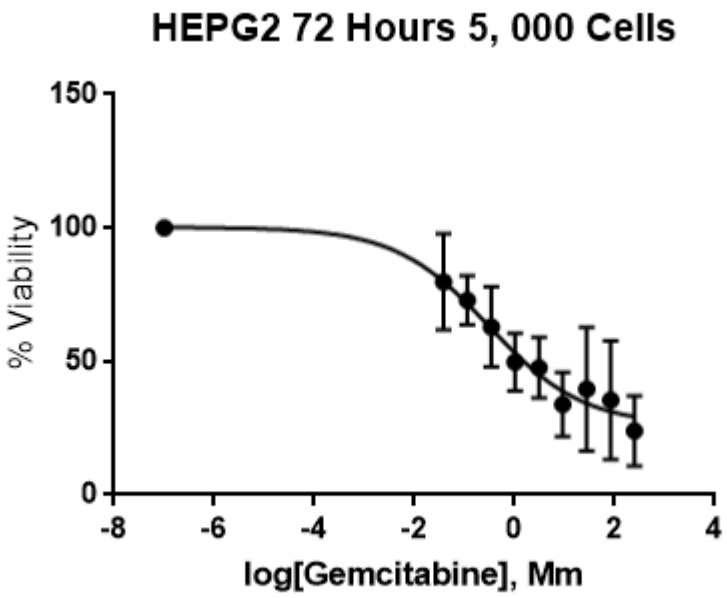
Interpretation

1.) Element of cell death

2.) ED-50 not achieved

3.) Narrow error bars

Figure 86: Dose-Response Curve for 72-Hour Time Point for Gemcitabine acting on HepG2



Interpretation

1.) ED-50 achieved log ED 0.3496

2.) ED-50 0.051

3.) However upper 95 % Confidence Interval were not calculable.

Discussion

The further cellular work has also demonstrated that development of cellular models for determining dose-response relationships for gemcitabine treatment of in vitro cholangiocarcinoma are feasible and easily adaptable from similar HepG2 models. Further cytotoxic models maybe developed to determine development gemcitabine drug resistance in cholangiocarcinoma models.

THESIS DISCUSSION:

This thesis has delineated both clinical and biological factors which affect prognosis for patients with cholangiocarcinoma. The majority of the work has been published by peer reviewed journals indicating the relevance and the novelty of the research. This thesis has validated, for the first time in the medical literature, numerous resectability and prognostic scoring systems and has identified novel pre and post-operative co-variates with significant OS effects. This thesis has identified, via meta-analytical methods, several potential biomarkers for prognosis and predictive response to chemotherapeutics. Further scientific work has been undertaken to produce an immunohistochemical tissue matched array (TMA) platform which has been utilised to initiate assessment of the hENT1 biomarker in resected and biopsied hilar cholangiocarcinoma specimens. This thesis encompasses clinical and scientific approaches to expand the current state of knowledge, within contemporaneous medical literature, regarding hilar cholangiocarcinoma.

The clinical chapter defines the selection processes involved in determining resectability of cholangiocarcinoma. The research suggests that staging laparoscopy is useful to stratify patients for further surgical treatment or palliative chemotherapy. Contraindications to resection relating to local advancement are unlikely to be determined at staging laparoscopy because the judgement of resectability has been demonstrated to be dependent upon the operator's 'hands-on' assessment. The decision not to proceed to resection due to local advancement can only therefore be made explicitly at exploratory laparotomy. Other authors have suggested that laparoscopy is a redundant modality for staging due to the advent of modern multi-slice CT scanning. The research stated within this thesis demonstrates persuasively that staging laparoscopy retains utility within the specific context of determining radiologically occult peritoneal disease. Staging laparoscopy should continue to be part of the surgical selection process for patients with cholangiocarcinoma until imaging becomes sufficiently sensitive to make open and close exploratory laparotomies unlikely.

This thesis has externally validated both the MSKCC and BC scoring systems for pre-operatively stratifying patients for potential resection. Accurate and appropriate surgical selection for patients with peri-hilar cholangiocarcinoma is extremely important given the high perioperative morbidity and mortality attributable to the surgery. Defining and validating putative resectability scores and refining approaches to surgical selection is therefore vital for the development of improved perioperative and long-term overall survival outcomes. This thesis has also identified a novel anatomical co-variate (left hepatic artery involvement) which could be used to augment scoring systems to increase predictive accuracy. Identification of novel co-variates for surgical stratification, not previously demonstrated in other cohorts, expands the

current knowledge base and provides areas for further investigation and potential validation by other cohorts.

This thesis has highlighted the significance of prognostic variables not accounted for in the AJCC 7th edition affecting OS. The prognostic factors which had a significant effect upon OS were; 'T' status, lymph node involvement, microvascular invasion, peri-neural invasion, tumour differentiation and age. The biological co-variates not accounted for in the staging method (microvascular invasion; tumour differentiation; peri-neural invasion) have significant effects upon OS for peri-hilar cholangiocarcinoma patients. The meta-analysis presented within this thesis, which delineates the above findings, was completed in 2017 and published in 2018 shortly after publication of the AJCC 8th edition. The eighth edition of the AJCC staging system aimed to act as a bridge to the development of a personalised approach to prognostication by incorporating several tumour biological factors. All of the co-variates demonstrated by the research in this thesis were subsequently determined to be significant prognostic factors for the AJCC 8th edition publication. These factors have subsequently become collectable registry data for potential future incorporation in to prognostic models.

The AJCC Staging system and AMC Nomogram score, systems based upon a variety of post-resectional tumour characteristics, demonstrated significant associations in prognostication for overall survival. The AMC has an improved prognostic capability compared to the AJCC staging system and has been externally validated for the first time in this modern resectional cohort. Augmentation of the AMC nomogram by addition of the significant pre-operative co-variates demonstrated in this cohort has produced a significantly improved novel prognostication system for OS. Improved overall survival prognostication methods could potentially provide more nuanced frameworks for stratification of patients to treatment and surveillance programmes. The pre-operative prognostic model may also offer a future methodological approach to stratifying patients for selection to neoadjuvant chemotherapeutic treatments. The analysis undertaken in this thesis has potential limitations with regards to the number of resected patients under statistical assessment. Further validation of these pre and post-operative prognostic models within a large, national cohort would provide a potential opportunity to further clarify the relationship between these variables and survival outcomes. It has been proposed that this model is disseminated to the Hepato-biliary Cancer Collaborative at the next meeting of the National Cancer Research Institute Upper Gastrointestinal Group. This could potentially provide a gateway to pooling survival data and assessing the prognostic effects of these variables in large numbers of patients and enable a wide-spread and standardised approach to both stratification of patients by survival and, consequently, potential treatment protocols and strategies.

The thesis has demonstrated that certain independent post-resectional histopathological variables have a significant effect upon OS. High R0 resectability rates are demonstrably achievable from appropriately selected cohorts of patients with a correlated improved OS. The independent post-resectional

histopathological variables of Tumour Grading and Nodal Status are also significantly associated with OS. This research accords with the consensus in current medical literature regarding the effect of these post-resectional co-variates upon OS. However, this thesis has also demonstrated that pre-operative serological and radiological characteristics have significant effects upon OS. Pre-interventional CA 19-9 was demonstrated to have significant effects upon OS for patients undergoing subsequent resection in the examined cohort. Elevated CA 19-9 serum levels are indicative of the degree of extra-hepatic biliary obstruction and may reflect the 'T' staging of the tumour and potentially therefore exhibit multicolinearity of effect with OS. Given that the co-variate retained significance within the multivariate modelling, whereas the 'T' staging did not achieve significance within the study, indicates that the serological measurement of CA 19-9 level may retain higher prognostic utility than 'T' stage. This potentially occurs due to the serum CA 19-9 level reflecting more accurately the in vitro physiological conditions between the patient and the cholangiocarcinoma disease.

This thesis has identified multiple biomarkers, via meta-analysis, that offer potential prognostic information for resected extrahepatic BTC. These findings offer an insight into the molecular pathogenesis of extrahepatic cholangiocarcinoma and suggest potential areas for investigation in future studies. This thesis demonstrates both overall and disease-free survival advantages for patients positive for hENT1 when compared to patients that lack expression of hENT1 when undergoing adjuvant gemcitabine-based chemotherapy for resected pancreatic adenocarcinoma. Given that pancreas and biliary cells have common progenitor stem cells this thesis has hypothesised that there may be correlating effects for patients with cholangiocarcinoma. However, caution must be used when inferring the potential prognostic/predictive utility of biomarkers between genomically, biologically and clinically disparate disease entities despite having common stem cell provenance.

This thesis has identified a commercially available hENT1 antibody which could provide an alternative to the 10D7G2 Mackey antibody for determining immunohistochemical hENT1 expression in resected cholangiocarcinoma specimens. The Proteintech hENT1 antibody demonstrates hENT1 inhibition following hENT1 knockdown with silencing RNA in HepG2 cells in a dose and time dependent manner. The immunostaining average 'H' scores correlate significantly between the Proteintech hENT1 antibody and the 10D7G2 hybridoma. The 10D7G2 antibody is no longer under private production and therefore viable commercially available antibodies are required for further studies. The novel validation of the Proteintech polyclonal antibody as being able to determine hENT1 abundance provides this alternative investigatory avenue. However, no significant prognostic or predictive associations with antibody expression and OS, or DFS and gemcitabine-based chemotherapy, were demonstrated. This is potentially due to the small population under immunohistochemical assessment which may cause instability of analysis and increased

potential for sensitivity error. The resected cohort has significantly increased in size since the TMA construction and immunohistochemical assessment was undertaken in 2018. At the time of writing, there are currently more than 100 patients whom have undergone resection with at least 2 years of survival follow-up. Further TMA construction could be undertaken for relatively little cost, both in time and economically, to increase the number of resected specimens immunohistochemically assessed for hENT1 abundance. The largest study which has assessed hENT1 abundance in resected chemo-naïve patients contained 105 specimens [416], all other studies contained less than 71 patients [396, 417], therefore further assessment of the expanded cohort would produce a globally significant hENT1 immunohistochemically-assessed population and add substantially to the research understanding regarding the hENT1 biomarker. Communication with the Cancer Research UK ACTICCA-1 Trial group (**NCT02170090**) to discuss potential avenues for collaborative research to investigate the effects of hENT1 abundance on overall survival for patients undergoing adjuvant gemcitabine-based chemotherapy is warranted in the context of expanding the population under immunohistochemical assessment.

Alternative methods of investigation and analysis may also be more sensitive and specific for determining hENT1 protein expression. Whole tumour RNA assessment of Formalin-fixed Paraffin Embedded specimens, potentially using RNA scope technology, may provide improved sensitivity and quantification of hENT1 expression within cholangiocarcinoma specimens. Genomic assessment, in parallel with RNA and immunohistochemistry in a multimodal approach, would potentially add further understanding regarding genomic and correlating proteomic expression of hENT1. Genomic assessment of cholangiocarcinoma tumours would also potentially help to elucidate the complex epigenetic factors which may interact with hENT1 expression.

While this study has essentially demonstrated no significant association between hENT1 and prognosis, further assessment with the above considerations accounted for could help delineate any potential link. Prognostic systems incorporating molecular biomarkers could provide improved prognostication systems and help guide chemotherapeutic treatment strategies. Further work, in a larger assessed population, is required to determine the utility of the hENT1 biomarker for prognosis for overall survival and response to treatment.

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