An Investigation of the Clinicopathological and Biochemical Factors Influencing Survival in Ampullary Adenocarcinoma

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Author's Declaration

I am the sole author and the work presented within this thesis is all my own work except where otherwise stated. All citations have been reviewed by myself in preparation of this body of work. This thesis has not been previously submitted to this or any other institution for a degree or diploma.

Craig R. Forrest

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Definitions/ Abbreviations

γ GT	glutamyl transferase
5FU	5-fluorouracil
95% CI	95% confidence interval
AA	ampullary adenocarcinoma
AJCC	American Joint Committee on Cancer
AKI	acute kidney injury
ALP	alkaline phosphatase
ALT	alanine aminotransferase
APC	argon plasma coagulation
AST	aspartate aminotransferase
BCL-2	b-cell lymphoma 2 protein
BJS	British Journal of Surgery
CA 19-9	carbohydrate antigen 19-9
CBD	common bile duct
CDX2	caudal type homeobox 2
CEA	carcinoembryonic antigen
CK7	cytokeratin 7
CK20	cytokeratin 20
CPEX	cardio-pulmonary exercise test
CT	computed tomography
DA	duodenal adenocarcinoma
DPD	dihydropyrimidine dehydrogenase
DNA	deoxyriboneucleic acid
EA	endoscopic ampullectomy
EDTA	ethyleneidiaminetetracetic acid
EGFR	epidermal growth factor receptor

EpCam	epithelial cell adhesion molecule
ERCC1	excision repair cross complementing gene 1
ERCP	endoscopic retrograde cholangiopancreatography
ESPAC	European Study of Pancreatic and Ampullary Cancer
EUS	endoscopic ultrasound
FAP	Familial Adenomatous Polyposis
FFPE	fresh frozen paraffin embedded
FNA	fine needle aspiration
FNB	fine needle biopsy
GDA	gastroduodenal artery
GO	gene ontology
GRI	Glasgow Royal Infirmary
GSK3β	glycogen synthase kinase 3β
H&E	haematoxylin and eosin
hENT1	human equilibrative nucleotide transporter 1
HER2	human epidermal growth factor 2
HGD	high-grade dysplasia
HR	hazard ratio
IAPN	intraampullary papillary tubular neoplasm
IDUS	intraductal ultrasound
IGF-1R	insulin-like growth factor -1R
IHC	immunohistochemistry
IPMN	intraductal papillary mucinous neoplasm
ISGPF	International Study Group on Pancreatic Fistula
IQR	interquartile range
Kras	Kirsten rat sarcoma viral oncogene homolog
LFT	liver function tests
LGD	low grade dysplasia
LKB1	liver kinase B1

LNR	lymph node ratio
MEN-1	multiple endocrine neoplasia 1
MG	milligrams
MCG	micrograms
MMP7	Matrix Metalloproteinase 7
MRI	magnetic resonance imaging
mTOR	mammalian target of rapamycin
MUC1	mucin 1
MUC2	mucin 2
MUC5AC	mucin 5AC
MUC6	mucin 6
NET	neuroendocrine tumour
NSCLC	non small cell lung cancer
NSP	non-standardised protocol
PCNA	proliferating cell nuclear antigen
PDAC	pancreatic ductal adenocarcinoma
PD	pancreaticoduodenectomy
PET	positron emission tomography
PFT	pulmonary function test
POPF	post-operative pancreatic fistula
PPPD	pylorus preserving pancreaticoduodenectomy
R0	resection margin negative
R1	resection margin positive
RCPath	British Royal College of Pathologists
RCT	randomised controlled trial
RFA	radiofrequency ablation
RNA	ribonucleic acid
SA	surgical ampullectomy
SD	standard deviation

SMA	superior mesenteric artery
SOD	sphincter of oddi
SP	standardised protocol
SRC	proto-oncogene tyrosine-protein kinase
TERT	telomerase reverse transcriptase
TMA	tissue micro-array
TNM	tumour node metastasis
TS	Thymidylate Synthase
UICC	Union for International Cancer Control
US	ultrasound

1.0 INTRODUCTION

Chapter 1

Introduction to Ampullary Adenocarcinoma

1.1 Anatomy of biliary system

The biliary system is consists of the organs and ducts (bile ducts, gallbladder, and associated structures) that are involved in the production and transportation of bile from the site of production in the liver through the pancreas into the duodenum.

These organs are all connected by an intricate ductal system allowing the passage of bile. This begins within the liver where hepatic ducts converge to form the common hepatic duct that in turn combines with the cystic duct from the gallbladder to give rise to the common bile duct (CBD). The ampulla of Vater (or major ampulla) is the name given to the confluence and terminal conduit of the distal CBD and main pancreatic duct, first described in 1720 by the German anatomist Abraham Vater (1684-1751).

It is a relatively firm and nodular structure that is conically shaped and surrounded by muscle fibres of the Sphincter of Oddi (SOD) that are anchored in the duodenal muscularis propria. The ampulla drains pancreatic juice and bile into the second part of the duodenum (approximately 8 cm from the gastric pylorus) via the major duodenal papilla.

From the duodenal lumen the major papilla appears slightly elevated with a nipple like appearance, measuring approximately 1 cm in length; however, much variation does exist with mucosal folds often covering the papilla.

In the majority of patients, lying 2 cm proximal to the Ampulla of Vater lies the minor

ampulla which connects an accessory pancreatic duct to the duodenum. This duct is commonly known as the Duct of Santorini. The minor ampulla is typically very small in size and is often of limited functionality.

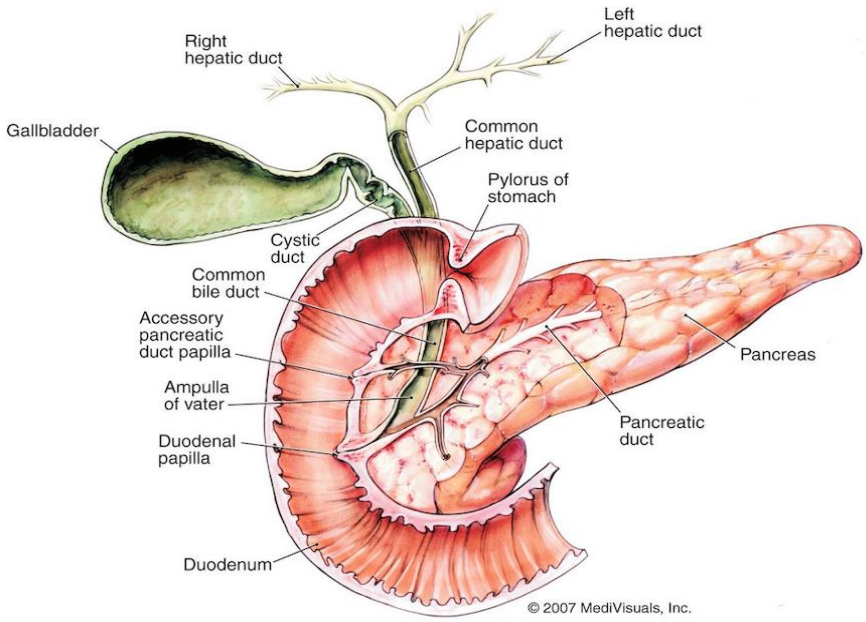


Figure 1.1 Diagram of relationship of ampulla to other biliary structures
Image courtesy of MediVisuals Inc

The Ampulla of Vater is also a key anatomical landmark from a blood supply perspective as it denotes the change from coeliac trunk arterial circulation of the foregut to the superior mesentery artery (SMA) supply of the midgut. The blood supply of the biliary system is derived from a combination of these arteries and their branches with significant anatomical variation seen.

The specific arterial blood supply to the pancreatic head and ampulla comes from the inferior pancreaticoduodenal artery which arises from the SMA. The venous drainage of this region is from a network of small veins which converge together and join the superior mesenteric vein. This then converges together with the splenic vein

to form the portal vein which enters the liver.

1.2 Pathology of the Ampulla of Vater

The ampulla can give rise to a surprising number and variety of different neoplasms due to the presence of several types of epithelia within. The commonest form of ampullary neoplasm is adenocarcinoma and its precursor adenoma(1) although some neuroendocrine tumours and gangliocytic paraganglioma can also affect this region.

Review of the Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute also indicated adenocarcinoma is the most frequently identified histology for ampullary cancer. Adenocarcinoma (not otherwise specified [NOS]) was reported in 65% of cases. Carcinoma (NOS) was reported in 8.1%; adenocarcinoma arising from adenoma (adenocarcinoma in villous adenoma, in tubulovillous adenoma, in adenomatous polyp and villous adenocarcinoma) was third most common in 7.5%. Other pathologic diagnoses reported included papillary adenocarcinoma (5.6%), mucinous adenocarcinoma (4.7%), and signet ring cell carcinoma (2%)(2).

1.2.1 Neuroendocrine Tumours

These tumours can be grossly divided by their differentiation, namely well or poorly differentiated. Poorly differentiated NET's of the Ampulla of Vater are extremely rare(3, 4). There are two distinct histological types called small cell carcinoma(5) and large cell neuroendocrine carcinoma(6) and both are distinguished from well

differentiated NET's by marked necrosis, a high mitotic rate and a high Ki-67 index (>20%, and often >50%)(1) resulting in a highly aggressive nature. With Ki-67 being a cellular marker strictly associated with cell proliferation it is widely used in pathological investigation as a proliferation marker to determine how aggressive a tumour is. In this regard, most patients present with nodal metastases and recur early with a high mortality.

The well differentiated NET's have previously been known as carcinoid tumours(7, 8) and unlike their poorly differentiated counterparts demonstrate a low mitotic rate and Ki-67 index (<20%, usually <5%) with infrequent necrosis seen(1). Most of these tumours are small and are usually identified within the submucosal or muscularis layers of the ampulla. They are associated with several hereditary conditions such as multiple endocrine neoplasia 1 (MEN-1) and neurofibromatosis type 1(9) with tumours arising in these patients sometimes possessing specific morphological features such as gastrinomas or glandular duodenal NET's.

The immunohistochemistry analysis of these tumours demonstrates high expression of the neuroendocrine markers chromogranin and synaptophysin with most expressing keratin also(9). A small NET found within the ampulla is unlikely to harbor nodal spread; however, the tumour can occasionally prove to be more advanced that initially thought with the likelihood of spread increasing as primary tumour size increases. Patients with nodal disease or liver metastases are difficult to cure although the natural history of the disease is protracted and survival for many years in this situation is common.

1.2.2 Gangliocytic Paraganglioma

This tumour type is pathologically distinct from others arising at the ampulla and is predominantly limited to this region. This rare tumour behaves in a benign manner and contains multiple different cell types that have lost cellular regulation. Like glandular duodenal NET's they are located in the submucosa and are associated with neurofibromatosis type 1(10). The three cell types with different proportions involved in tumour evolution are epitheloid, spindle-cell and ganglion(9). The exact origin of the tumour remains unclear due to its possession of both NET and schwannian components(11) Although regarded as a benign tumour cases of regional lymph node metastases have been reported.

1.2.3 Adenoma and Adenocarcinoma

1.2.3.1 Adenomas

Ampullary adenomas can be subdivided into two overlapping categories; namely intestinal type adenomas that arise from the duodenal surface of the ampulla, are almost identical to colonic adenomas microscopically and are associated with familial adenomatous polyposis (FAP) syndrome(1); and intraampullary tubular neoplasms (IAPN) that arise from within the ampulla(12). The progression of periampullary adenomas to adenocarcinoma in FAP patient is between 4% and 12%(13).

The intestinal type adenomas progress slowly from premalignant lesions and follow a carcinomatous sequence to develop invasive potential as previously described by Fischer et al (14). This adenoma to adenocarcinoma sequence, having been well

described previously in colorectal cancer, describes the stepwise progression from a normal to dysplastic epithelium to carcinoma associated with the accumulation of multiple mutational genetic alterations resulting in oncogene activation and tumour suppressor gene inactivation(15, 16). The transformation of an adenoma to in-situ or invasive carcinoma is in the region of 25-85%(17) and neoplastic transformation occurs more commonly near the ampulla than any other site in the small intestine.

In patients diagnosed with FAP the ampulla and periampullary duodenum are the commonest locations for extracolonic adenomas(1) and they are usually accompanied by multiple other adenomas within the duodenum, sometimes creating a carpet like appearance. In most other settings they are usually solitary.

The microscopic appearances of this intestinal type adenoma show pseudostratification of elongated nuclei with addition cytological atypia depending on the degree of dysplasia. In cases of high-grade dysplasia additional nuclear atypia, complex cribiform glands are evident and epithelial polarity is lost(1).

The IAPN's are commonly intraampullary lesions that develop into exophytic masses within the ampulla. They microscopically demonstrate complex papillary architecture lined by cuboidal cells with round, atypical nuclei that lack the pseudostratification seen in their intestinal-type counterparts(1, 12). The majority of these lesions have high-grade dysplasia present and some show a mixture of intestinal and pancreaticobiliary features(12). The prognosis of these lesions is regarded as very good with rare episodes of recurrence recorded.

1.2.3.2 Adenocarcinoma

Ampullary adenocarcinomas are regarded as those that originate distal to the CBD and pancreatic duct convergence within the ampullary complex and are the commonest malignancies of the ampulla. Similar to the adenomas and papillary neoplasms from which they arise, adenocarcinomas are more common in the ampulla than any other site of the small bowel(1).

There are three main macroscopic appearances of an ampullary adenocarcinoma, ulcerative, intramural and extramural, which are polypoidal and protrude through the ampullary orifice into the duodenum(18). Malignancies can arise from either the ampullary epithelium or from the epithelium covering the distal biliary or pancreatic ducts.

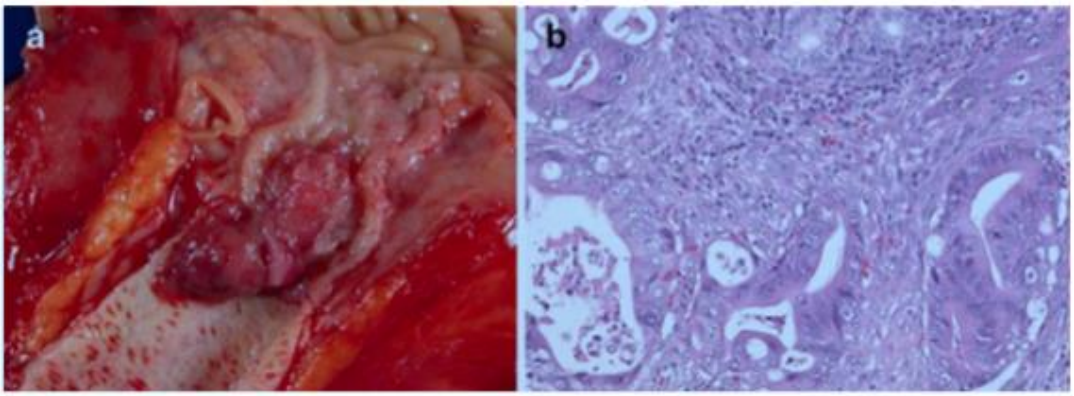


Figure 1.2 a) Gross appearance of ampullary adenocarcinoma; **b)** microscopic appearances of an ampullary adenocarcinoma (x64 magnification) (19)

Invasive disease develops as ampullary epithelial cells incur increasing genetic mutations causing oncogene activation and deactivation of tumour suppressor genes. A KRAS gene mutation, resulting in loss of normal cell signaling and continuous proliferation, is a common abnormality identified and has been seen in

approximately 20% of ampullary tumours(20). However, this figure is significantly less than that seen in pancreatic ductal adenocarcinoma where activating mutations in KRAS are seen in greater than 90% of tumours(21). Owing to the heterogeneity in behavior of these tumours, their histological and molecular characteristics have been scrutinized in an attempt to prognostically stratify them effectively(2, 22-24)

1.2.3.3 Subtypes

Adenocarcinoma of the ampulla of Vater may be classified by morphological appearance, with Kimura and co-workers first describing intestinal and pancreaticobiliary subtypes based on morphological and immunohistochemical (IHC) features(25). The intestinal phenotype originates from the epithelial cell layer overlying the ampulla whereas the pancreaticobiliary type evolves from the ampullary confluence, distal pancreatic duct or distal CBD endothelium(25-29).

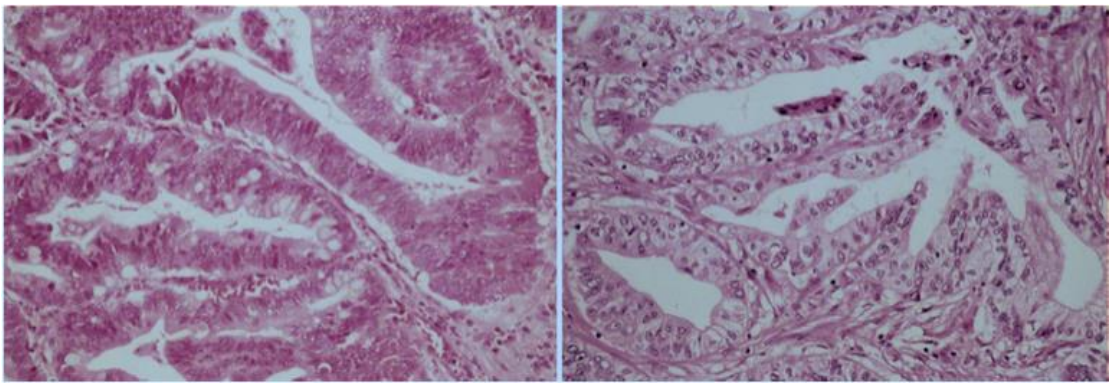


Figure 1.3 a) Adenocarcinoma of the intestinal type, resembling that of a colonic tubular carcinoma; **b)** adenocarcinoma of the pancreaticobiliary type (both x 200 magnification) (27)

Tumours of pancreaticobiliary phenotype are typically more aggressive than those of intestinal origin and are often accompanied with lymph node involvement(25) and are associated with higher T stage, advanced UICC/AJCC stage and perineural invasion(30). In this regard, patients whose tumours are of an intestinal phenotype have a more favourable prognosis than those of the pancreaticobiliary phenotype(29, 31-34).

1.2.3.4 Evaluation of Protein markers in AA using immunohistochemistry (IHC)

Multiple investigators have attempted to further enhance the histological sub-classification described above and several immunohistochemical markers have been identified as being specific in adenocarcinoma of the ampulla such as mucin 1(MUC1), mucin 2 (MUC2), cytokeratin 7 (CK7), cytokeratin 20 (CK20), and caudal type homeobox 2 (CDX2)(14, 20, 29, 34-36). The intestinal phenotype tends to stain for MUC2, CK20 and CDX2 with the pancreaticobiliary phenotype typically positive for MUC1 and CK7; hence when the former markers are positive this correlates towards better overall survival and the opposite is true for the latter markers. MUC1 is a transmembrane glycoprotein that that is expressed in 66-98%(37) of PDACs and cholangiocarcinomas whereas early intestinal differentiation involves CDX2 and is commonly strongly positive in duodenal or intestinal tumours but infrequently in PDAC(5%)(38-41).

A more recent study from Chang(31) in 2013 combined tumour histology, MUC1 and CDX2 to classify patients having undergone resection of ampullary tumours. They defined their pancreaticobiliary histomolecular phenotype as a tumour displaying

pancreaticobiliary histology whilst staining negative for CDX2 and for MUC1. Any tumour not meeting the criteria was classed as an intestinal histomolecular phenotype. Their findings demonstrated that pancreaticobiliary phenotype and lymph node positivity were independent factors of poor prognosis in three separate validation cohorts.

A further study aimed at validating these results in a large patient population in the United States and once again demonstrated the pancreaticobiliary histomolecular phenotype to be an independent factor of poor prognosis with HR 2.26 (95%CI 2.40 – 3.65; p=0.0009)(42). By routinely defining the histomolecular phenotypes that are associated with improved overall outcome will help to facilitate clinical decision making in the future.

Furthermore, recent genomic research has identified that some ampullary tumours display characteristics of intestinal tumours such as microsatellite instability, inactivation of the tumour suppressor gene ELF3 by a high frequency of mutations and disruption of the WNT signaling pathway; however, not homogenous for tumour type or subtype. As greater than 50% of patients demonstrated a WNT pathway mutation, they hypothesise that their findings may impact treatment choice and pave the way for genomic targeted therapies in the future with WNT pathway targeted therapies currently in development(43).

Despite identification of biomarkers that help to categorize the two distinct subtypes of AA, there has been little progress or evolution in the discovery of other markers that aid in the assessment of tumour aggressiveness or spread and overall survival. On review of the literature there has been a few publications that have suggested

that specific markers correlate towards a poorer survival but in the most part have failed to identify any trends to specific clinicopathological factors or survival significance whatsoever. An overview of the current literature is displayed in **Table 1.1**. The studies evaluating IHC markers in AA are generally limited in terms of power and with a failure to consistently control for clinicopathological factors and therefore a need exists for an evaluation of these markers in a mature data set with compete follow and robust histopathological reporting.

Following completion of this work, a meta-analysis of the KRAS biomarker as a prognostic indicator in ampullary cancer was published in 2016(44) and is the only of its kind within the literature. The five included papers are detailed in **Table 1.1**. Their analysis involved 388 patients for overall survival and found that there was no correlation with KRAS mutation. However, two studies were eligible for disease-free survival (175 patients) and they concluded that there was significant association between mutation and shorter disease-free survival (HR 2.74, 95%CI: 1.52-4.92 p=0. 0008).

Table 1.1 Overview of biomarker analysis in AA from current available literature since 2001

Biomarker	Papers	Year	Author	Findings
MMP7	1	2015	Kumari et al(45)	Correlates with reduced survival
EGFR	1	2015	Xia et al(46)	Over expression correlates with reduced survival
IGF-1R	1	2015	Xia et al(46)	IGFR correlates to PB subtype
ERCC1	1	2015	Kawabata et al(47)	No survival correlation
hENT1	2	2015	Kawabata et al(47)	No survival correlation
		2008	Santini et al(48)	Over expression correlates with reduced survival
DPD	1	2015	Kawabata et al(47)	Expression Independent prognostic factor for survival
Kras	5	2016	Kwon et al(49)	No overall survival correlation, correlations to reduced disease free survival
		2015	Valsangkar et al(50)	No overall survival correlation
		2014	Mikhitarian et al(51)	No overall survival correlation
		2012	Schultz et al(52)	No overall survival correlation, correlations to reduced disease free survival
		1997	Howe et al(53)	No overall survival correlation
HER2	2	2015	Ata et al(54)	No survival correlation
(c-erbB-2)		2001	Ajiki et al(55)	No survival correlation
Nestin	1	2015	Shan et al(56)	Over expression promotes invasiveness
P53	3	2014	Guo et al(57)	No survival correlation
		2004	Zhou et al(29)	No survival correlation
		2001	Ajiki et al(55)	No survival correlation
P21	1	2014	Guo et al(57)	No prognostic value
Cyclin D1	1	2014	Guo et al(57)	Expression correlates with reduced survival
Bcl2	1	2014	Guo et al(57)	No survival correlation
E-cadherin	1	2014	Sung et al(58)	Expression correlates to cell grade & TNM stage
B-catenin	1	2014	Sung et al(58)	Expression correlates to cell grade, TNM stage & lymphatic/perineural invasion
S100A4	1	2014	Sung et al(58)	Expression correlates to TNM stage and lymphatic invasion
CK7	6	2013	Bronsert et al(59)	High CK7 and low CDX2 predicts PB subtype and poor survival
		2013	Morini et al(60)	No survival correlation
		2015	Yun et al(61)	Expression with CK20 loss independent factor of poor survival
		2010	Haddad et al(34)	High expression in pancreaticobiliary subtype
		2004	Le Pessot et al(62)	Prognostic value in terms of subtyping
		2004	Zhou et al(29)	No survival correlation

Biomarker	Papers	Year	Author	Findings
CK20	7	2013	Bronsert et al(59)	High expression in intestinal subtype
		2013	Morini et al(60)	Expression independent factor of improved prognosis
		2015	Yun et al(61)	Loss with CK7 expression independent factor of poor survival
		2010	Kawabata et al(63)	Subtypes based on this and Muc1 correlate with survival
		2010	Haddad et al(34)	High expression in intestinal subtype
		2004	Le Pessot et al(62)	Prognostic value in terms of subtyping
		2004	Zhou et al(29)	No survival correlation
CDX2	2	2013	Bronsert et al(59)	High CK7 and low CDX2 predicts PB subtype and poor survival
		2010	Haddad et al(34)	Muc1, Muc2, CDX2 allow most accurate subtyping
Caspase3	1	2013	Xue et al(64)	Assoc differentiation only
Livin	1	2013	Xue et al(64)	Correlates with differentiation, LN metastases and reduced survival
Ki67	2	2013	Xue et al(64)	Assoc differentiation only
		2004	Zhou et al(29)	No survival correlation
TERT	1	2012	Sakabe et al(65)	No survival correlation
Telomerase	1	2012	Sakabe et al(65)	Expression independent prognostic factor for survival
CD44	1	2012	Piscuoglio et al(66)	No survival correlation
CD166	1	2012	Piscuoglio et al(66)	No survival correlation
CD133	1	2012	Piscuoglio et al(66)	No survival correlation
EpCam	2	2012	Piscuoglio et al(66)	Loss in aggressive tumour, correlated with survival
		2008	Feng et al(67)	Over expression correlates with poor survival
Muc1	4	2011	Wang et al(68)	No survival correlation
		2010	Kawabata et al(63)	Subtypes based on this and CK20 correlate with survival
		2010	Haddad et al(34)	Muc1, Muc2, CDX2 allow most accurate subtyping
		2004	Zhou et al(29)	No survival correlation
Muc2	3	2011	Wang et al(68)	No survival correlation
		2010	Haddad et al(34)	Muc1, Muc2, CDX2 allow most accurate subtyping
		2004	Zhou et al(29)	No survival correlation
Muc5AC	3	2011	Wang et al(68)	No survival correlation
		2010	Haddad et al(34)	No survival correlation
		2004	Zhou et al(29)	No survival correlation
Muc6	2	2011	Wang et al(68)	No survival correlation
		2010	Haddad et al(34)	No survival correlation
CEA	1	2012	Kim et al(30)	Expression Independent prognostic factor for recurrence

Biomarker	Papers	Year	Author	Findings
Osteopontin	2	2011	Zhao et al(69)	No survival correlation
		2010	Hsu et al(70)	Expression predicts recurrence
Osteonectin	1	2007	Bloomston et al(71)	Expression correlated with nodal disease and poor survival
KL-6 Mucin	1	2005	Tang et al(72)	Expression correlates to LN mets, local invasion and poor prognosis
PCNA	1	2001	Ajiki et al(55)	Over expression correlates with poor survival
TS	1	2001	Ajiki et al(73)	No survival correlation
COX2	2	2006	Perrone et al(74)	Expression associated with intestinal subtype
		2005	Santini et al(75)	Over expression correlates with improved survival

1.3 Epidemiology of ampullary adenocarcinoma

Ampullary adenocarcinoma is rare with an incidence of approximately four to six new cases per million population (76). Between 1973 and 2005, 5,625 new cases of ampullary cancer were recorded in the Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute. Ampullary cancer now accounts for approximately 0.5% of all gastrointestinal tract malignancies with the incidence having increased since 1973 at an annual percentage rate of 0.9%. Prior to the recently reported increase, the quoted incidence for ampullary carcinoma was 0.2% of all gastrointestinal malignancies and 6% of all periampullary tumors (77). It has been suggested that men are more frequently affected than women and is also more commonly seen in Caucasians than Afro-americans (2). The incidence of ampullary malignancy increases with advancing age, particularly over 50 years with mean age at diagnosis 65 years(2). The occurrence of ampullary adenocarcinoma is typically random; however, in patients with hereditary conditions, most notably familial adenomatous polyposis (FAP) syndrome the risk of periampullary cancer is increased by 100-200% (78). Following panproctocolectomy, the ampulla is the commonest site for malignant lesions in FAP patients(14).

1.4 Clinical Features and Presentation

Patient features of ampullary adenocarcinoma are generally the same as the more recognized pancreatic ductal adenocarcinoma (PDAC) although tends to present earlier in its natural history. This is due to their proximity to the CBD and means fewer patients are seen with disseminated disease at presentation(79) and as such

are associated with a more favourable prognosis following surgical resection with a 5-year survival approaching 65% having been previously described(19, 80, 81).

At early stages of the disease process ampullary adenocarcinomas are often asymptomatic and found incidentally during endoscopic or radiological investigations for other reasons. It has been previously reported that up to 50% are diagnosed in this manner(82). Later, the most common symptom is jaundice seen in 80% of patients(83) and is associated with itch, pale, greasy stools and dark urine due to intrinsic biliary obstruction causing conjugated hyperbilirubinaemia. Recurrent episodes of acute pancreatitis can occur if pancreatic duct obstruction is present. Other more general symptoms suggestive of malignancy such as weight loss, nausea and vomiting, abdominal or back pain; early satiety, anorexia and vomiting can all be present.

Clinical examination may be unremarkable but again, in larger, more advanced lesions jaundice may be accompanied with a distended palpable gallbladder (Courvoisier sign) in keeping with CBD obstruction or hepatomegaly. Right hypochondrial or epigastric masses may suggest advanced local disease and palpable lymphadenopathy or ascites may suggest disseminated metastases.

1.5 Investigative Options

After a thorough history and examination has raised suspicion of a biliary tree abnormality the assessing clinician will then request routine blood tests. It is important to accurately stage tumours pre-operatively to avoid exploring inoperable tumours. There are many investigative modalities that are utilized in the

confirmation of an ampullary tumour with conventional ultrasound (US), computed tomography (CT), magnetic resonance imaging (MRI), endoscopy, endoscopic ultrasound (EUS), endoscopic retrograde Cholangiopancreatography (ERCP) and positron emission tomography (PET) having all been utilized in diagnosis and subsequent tumour staging. The following is a brief overview of the frequently utilized investigations.

1.5.1 Blood Tests

As with many cancers, a patient with ampullary adenocarcinoma may have non-specific laboratory blood tests which point towards potential causes but are not diagnostic. Liver function tests (LFT's) are a group of biochemical blood tests that provide information about the condition of a patients liver. The bilirubin, alkaline phosphatase (ALP) and γ glutamyl transferase (γGT) show preferential abnormal elevation in the presence of cholestasis or biliary obstruction whereas alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are elevated in cases of hepatocellular damage. The prothrombin time and albumin are associated with liver synthetic function. In the presence of a large ampullary tumour causing jaundice the patient is likely to have an obstructive picture to their LFT's with disproportionately elevated ALP and γGT compared to the transaminases with raised bilirubin.

There are serological markers that have been as adjuncts to conventional investigation to aid in a diagnosis of ampullary cancer. The most commonly used is carbohydrate antigen 19-9 (CA 19-9) and when elevated may help guide the

diagnosis towards an ampullary adenocarcinoma, although 37% of patients with an adenocarcinoma have been found to have a normal CA 19-9(19).

It has been reported that pre-operative elevated CA 19-9 levels are associated with poor survival following AA resection(83-85). The main issue with this marker is that it is extremely non-specific and is expressed in many gastrointestinal malignancies as well as benign conditions including liver cirrhosis and pancreatitis(86). Carcinoembryonic antigen (CEA) has also been measured in various centres; however when compared to CA 19-9 it failed to demonstrate any prognostic power (85). However, more recent studies of greater power have reported that elevated pre-operative CEA is a significant indicator of a poor prognosis(30, 87, 88). Again, this marker can be elevated for other reasons, the most common being cholangitis and is therefore non-specific.

There are currently no serological markers that reliably detect early stage ampullary adenocarcinoma or in fact any other biliary based cancer that could form the basis of a screening test which would potentially improve survival from earlier diagnosis and treatment.

1.5.2 Ultrasound

Ultrasound is often the initial investigation for patients with ampullary adenocarcinoma. This is because it is readily available and a radiation free test; however, is limited in its use for the detection of ampullary lesions due to difficulty in identifying small focal lesions from ultrasonic images. This modality is highly operator dependant and it has been proposed from several studies within the past

15 years that it is diagnostic in 12-27% of cases (89-91). Where ultrasound is more useful is in the detection of more generic findings in keeping with CBD obstruction such as intra and extrahepatic biliary dilitation, enlarged CBD diameter and a distended gallbladder indicating the need for further investigation. It can also readily identify gallstones as an alternative diagnosis and liver metastases or peritoneal ascites as an indicator of disease stage.

1.5.3 Computed Tomography (CT)

The use of CT scanning in the diagnosis and staging of ampullary adenocarcinoma is widespread due scanners being easily accessible, non-invasive, quick to perform and non operator dependant all of which makes it the first line investigation for staging patients with obstructive jaundice(92). It also allows for the quick detection of disseminated disease and its use in staging should therefore be routine(93). A triple phase 'Pancreatic Protocol' scan should be undertaken with both oral and intravenous contrast during both arterial and portal-venous enhancement stages. Examples of key images obtained following CT are displayed below with **Figure 1.4a** demonstrating 'double duct' sign with both CBD and PD dilatation due to external compression in an axial plane and **Figure 1.4b** showing an ampullary mass lying within the second part of the duodenum, again in the axial plane.

The last 15 years has seen an increase in detection rates due to technological improvements in CT imaging quality such as higher resolution and reduced slice thickness(89, 91). However, accurate TNM staging remains challenging and generally tumours are understaged (89, 90, 93, 94) with the main pitfall being the

precise assessment of lymph node metastases, particularly out with the loco-regional zone. From recent studies, accurate primary tumour staging by CT scanning has demonstrated a sensitivity between 82% to 90% and specificity between 66% to 87% whereas lymph node staging has a reported low sensitivity of 0% to 40% in the literature(92). A meta-analysis from Tseng et al(95) reviewed the diagnostic accuracy of CT scanning in assessing extra-regional lymphadenopathy in peri-ampullary cancer with only 25% of histopathologically proven lymph node metastases having been correctly diagnosed on CT. Conversely, a further 14% of patients were incorrectly labeled as having metastases according to CT imaging.

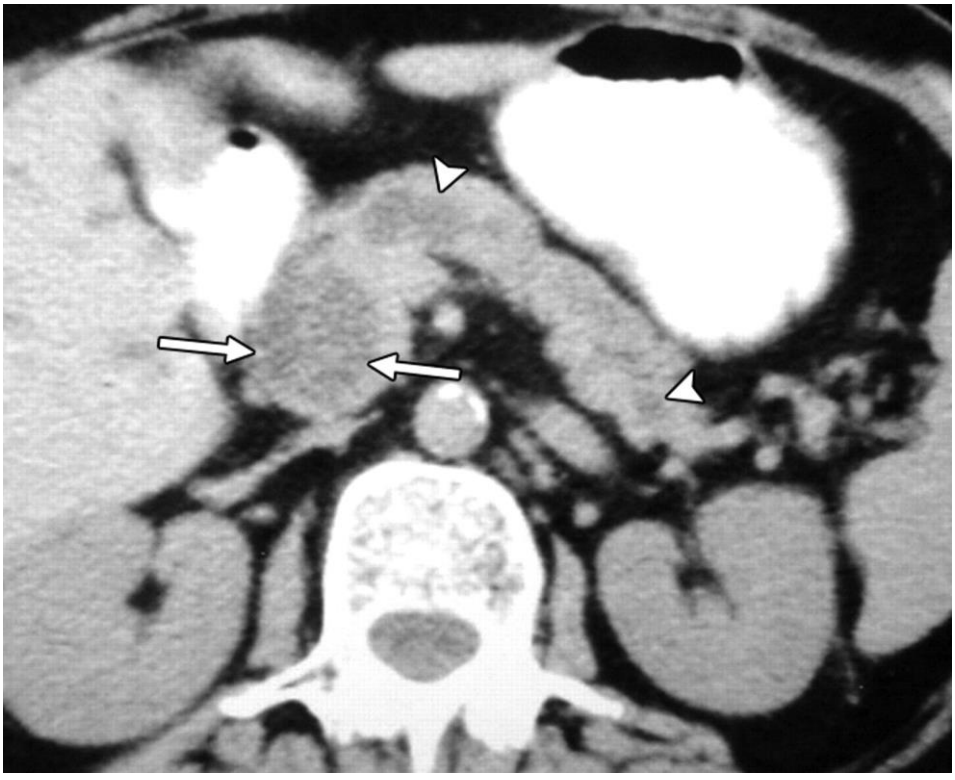


Figure 1.4a Non contrast axial CT demonstrating dilatation of CBD (arrows) and pancreatic duct (arrowheads)(96)

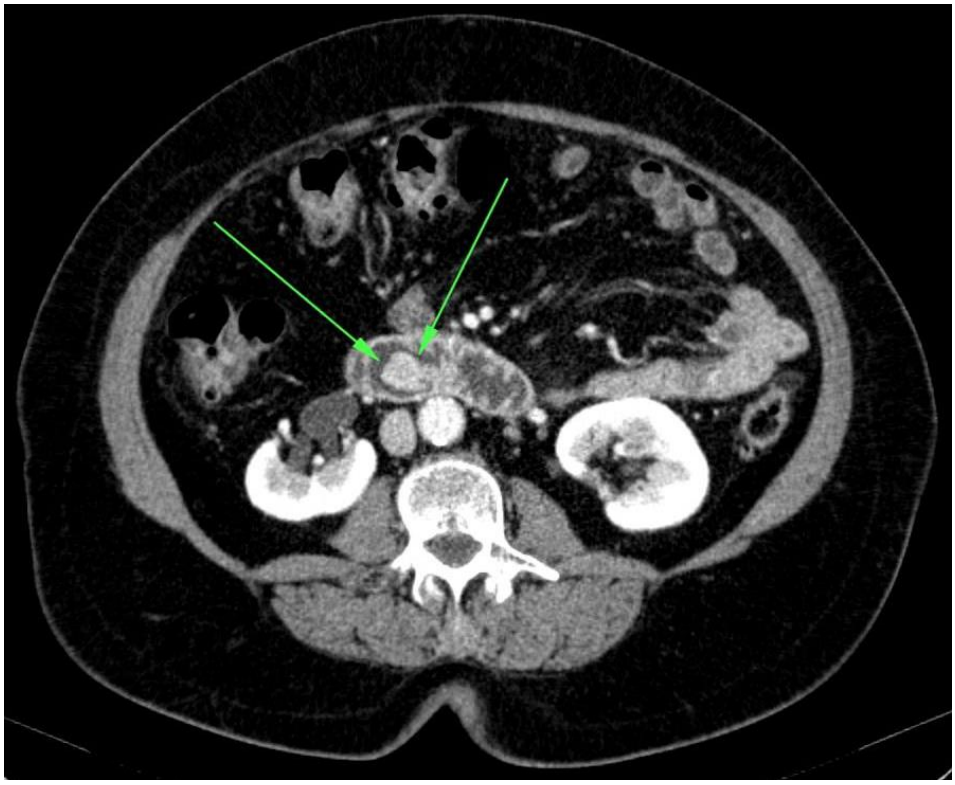


Figure 1.4b Axial CT image demonstrating a solid ampullary mass lying within the duodenum (Image courtesy of Dr Bruno Muzio radiopaedia.org rID:19429)

1.5.4 Endoscopic Biopsy

A tissue diagnosis is vital to enable informed treatment decisions therefore biopsy of all ampullary lesions is mandatory. The visual appearance at endoscopy often allows differentiation between a benign and malignant process where ulceration, irregular margins, firmness, spontaneous haemorrhage and necrosis are all suggestive of adenocarcinoma(97-99). Granular or villiform exophytic lesions are most commonly seen and are usually benign(100); whereas smooth elevated lesions have a greater risk for invasive disease(101). There is no agreed correlation between increasing lesion size and predicted malignancy with many papers publishing conflicting findings (19, 79, 102). Forceps biopsy accuracy has also shown marked heterogeneity and range from 47% to 95% (97, 103).

1.5.5 Endoscopic Ultrasound (EUS)

EUS has been utilised in the staging of periampullary tumours since its inception by DiMagno et al in 1980(104). The use of a specialised endoscope with an ultrasonic transducer at the tip when positioned within the duodenum allows for detailed assessment of the periampullary region and biliary tree. It is highly operator dependent but useful in identifying intraductal extension and provides the ability to undertake ultrasound guided fine needle aspiration (FNA) and more recently fine needle biopsy (FNB) via the endoscopic lumen of suspicious lesions for histological diagnosis. This not only includes primary lesions around the ampulla and also loco-regional lymph nodes to aid in disease staging. Currently the American Society for Gastrointestinal Endoscopy guideline recommends using EUS on a case by case basis in the work-up of ampullary lesions due to a paucity of high powered evidence(105).

A small study of 35 patients found the diagnostic accuracy of endoscopic FNA to be in excess of 90%(106). The main advantages of this modality is that it negates fat and gas lying between a traditional US probe and the lesion allowing for better images although due to its invasive nature is not without complications, predominantly haemorrhage(1.3-4%)(107, 108) and pancreatitis(0-2%)(109-111) . EUS has an excellent tumour detection rate with sensitivity above 90%(92) whilst also providing precise assessment of local and distant invasion with T and N staging accuracy having been documented between 63-82% and 60-81% respectively(112). The accuracy of identifying loco-regional lymph node metastases is comparable to that of CT(113). The overall positive predictive value for detecting primary tumour has been reported as 93%(92).

This modality is currently evolving further with the introduction of intraductal ultrasound (IDUS) where the ultrasound transducer is placed within the bile ducts for assessment. With this technique being in its infancy there is a paucity of information regarding its use at present; however, there are encouraging results with its use in accurately determining duodenal submucosal invasion(114, 115) and hence identifying advanced tumours that would benefit from surgical excision.

1.5.6 Endoscopic Retrograde Cholangiopancreatography (ERCP)

ERCP is an endoscopic technique that allows for direct visualization of the ampulla, CBD and pancreatic duct using a side viewing endoscope. It also has the ability to obtain cytological and histological specimens with brushings and allows for the deployment of biliary stents when clinically appropriate. The classical appearance suggestive of an ampullary or pancreatic head tumour at ERCP ('double duct' sign) is evident when both CBD and pancreatic ducts are dilated (Figure 1.5)

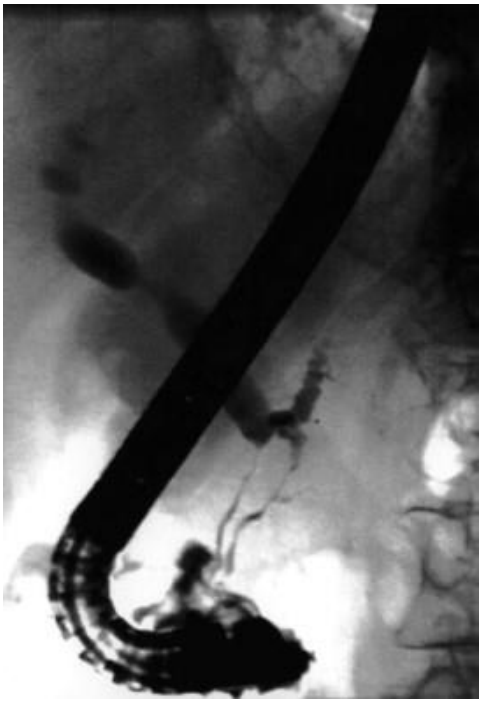


Figure 1.5 ERCP film demonstrating double duct sign(116)

Pre-operative biliary stenting for jaundice patients with resectable peri-ampullary disease is controversial with a recent systematic review from 2014 by Lai et al(117) showing no benefit of pre-operative drainage in surgical outcome(118). Instead there were concerns regarding post drainage complications and an increase in positive intra-operative bile cultures and subsequent infective complication rate post resection(117). This is reinforced in the GUT guidelines on pancreatic and ampullary cancer management from 2005 that state it is reasonable to obtain internal biliary drainage only if definitive surgery has to be delayed by more than 10 days. The guideline also state that self-expanding metal stents should not be placed in this situation as surgical resection is made more challenging due to the tissue reaction provoked by these stents(119).

However, this statement is now regarded as incorrect with plastic stents no longer being recommended for use. The evidence base for this stems from the multicentre, randomised trial by van der Gaag et al who assigned 202 patients with cancer of the pancreatic head and obstructive jaundice with a bilirubin level of 40 to 250μmol/l to either early surgery or preoperative biliary drainage. They found the rate of serious complications was higher (74% versus 39%; $p<0.001$) in patients undergoing routine pre-operative plastic biliary stenting compared to those proceeding straight to resection(120). Furthermore, 46% of patients having preoperative biliary drainage experienced a complication and patients who had undergone pre-op drainage had more surgery related complications than those having not (47% versus 37%; $p=0.14$) resulting in a longer hospital stay and more readmissions(120).

Further work by this group compared pre-operative biliary stenting with metal stents in patients with a resectable tumour who could not undergo early surgery for

logistical reasons against the previous cohort stented with plastic stents. They found that biliary drainage related complications (24% versus 46%) and stent related complications (6% versus 31%) were both less in the metal stent group whilst there was no difference in complication rate following their definitive surgical procedure(121).

They concluded that early PD without pre-operative biliary drainage remains the treatment of choice but metal stents should be preferred over plastic stents in the occasion pre-operative stenting is indicated(121).

1.5.7 Positron Emission Tomography (PET) & Magnetic Resonance Imaging (MRI)

MRI has the benefit over CT in that it does not expose the patient to ionising radiation whilst PET scanning is a newer imaging technique that works on the basis that malignant cells absorb and metabolise a radio-labeled glucose analogue administered intravenously quicker than normal tissue allowing for identification(122).

Currently both modalities have a limited role in the diagnosis of ampullary tumours and are predominantly used as adjuncts to the previously described investigations in select cases, often to clarify the presence of disseminated disease. A small study by Sugita et al demonstrated an accuracy of 96% for MRI in detecting tumour invasion from autopsy specimens(123). A further study by Kim et al evaluated MR imaging of 29 patients with pathologically proven ampullary carcinomas and found that 62% had a discrete nodular mass, a positive double duct sign in 52% and only bile duct dilatation seen in the remaining 48% of patients(124).

A study on the staging of biliary cancer that compared PET and CT accuracy found no significant difference in the detection of loco-regional lymph node metastases with overall accuracy of 69%. PET did however detect 100% (4/4 patients) of distant lymph node metastases(125).

1.6 Treatment Options

1.6.1 Pancreaticoduodenectomy

Having been first described by the Italian surgeon Alessandro Codivilla in 1898(126), the first periampullary cancer resection was performed in 1909 by Walther Kausch(127) and subsequently improved on by Allen Whipple in 1935(128) from whom the procedure often takes its name.

Currently, following a diagnosis of ampullary adenocarcinoma the gold standard and mainstay of treatment remains radical surgical resection by either conventional pancreaticoduodenectomy (PD) or by the pylorus-preserving (PPPD) method with the latter becomingly increasingly utilized in Europe. The perceived benefits of PPPD over PD as concluded in several reviews and meta-analyses are reduced operative time and blood loss although there was no significant difference in gastric emptying seen(129-131). However, this did not translate into reduced morbidity or mortality, reduced complication rates or long-term survival. There also remains debate that the relative reduced dissection in PPPD is adequate from an oncological perspective(129). There has also been discussion over the years with regards to whether standard or extended lymphadenectomy should be performed at the time

of resection although a randomized trial has demonstrated no significant difference in long-term survival (56% versus 60%)(132).

Both methods are major surgical procedures and involve the en-bloc removal of the pancreatic head, duodenum, proximal jejunum, CBD, gallbladder, local lymph nodes and the gastric antrum depending on method. As the gastroduodenal artery (GDA) supplies the duodenum and the pancreas as it runs through its head both organs require to be resected should the GDA be ligated. A full description of the surgical technique used at the West of Scotland Pancreatic Unit at Glasgow Royal Infirmary is described later in Chapter 3.

The decision to proceed to surgery should be evaluated thoroughly as the procedure possesses a highly significant risk profile. Despite the improvements in surgical instruments, technique and post-operative care reducing the 30 day mortality to 2-5% the procedure remains high risk with significant complications and morbidity seen in approximately 20-50% of patients(112). Complications and subsequent morbidity are particularly common in patients with AA, due to adverse surgical conditions intraoperatively, namely a 'soft' pancreas with normal parenchyma and a bile duct of small caliber, increasing the anastomosis difficulty. The commonest complications include post-operative pancreatic fistula (POPF), bleeding, anastomotic leak, intra-abdominal collection and delayed gastric emptying. Notably a significant reduction in morbidity and mortality has occurred with the centralization of PD to high volume tertiary referral hospitals(133, 134).

The International Study Group on Pancreatic Fistula (ISGPF) classification defined a POPF as a failure of healing of a pancreaticoenteric anastomosis or a parenchymal

leak not directly related to an anastomosis with three different grades of POPF defined according to the clinical impact on the patient's hospital course(135). The classification is seen in **Table 1.2** below.

Pancreatic Fistula	**GRADE A**	**GRADE B**	**GRADE C**
Clinical conditions	Well	Often well	Ill appearing/bad
Specific treatment (antibiotics,TPN,somatostatin)	No	No/yes	Yes
US/CT (if obtained)	Neg	Neg/Pos	Pos
Pesistent drainage at 3 wks[a]	No	Usually yes	Yes
Reoperation	No	No	Yes
Death related to POPF	No	No	Possibly yes
Signs of infection	No	Yes	Yes
Sepsis	No	No	Yes
Readmission	No	Yes/no	Yes/no

[a]With or without a drain in situ

Table 1.2 ISGPF classification and grading of POPF

PD resection for AA is of much higher risk for the development of POPF than that encountered during resection of a PDAC and is attributable to the intra-operative conditions encountered in the presence of an AA. The presence of a pancreatic duct under 3mm; soft pancreatic parenchyma; ampullary, duodenal or cystic pathology and intra-operative blood loss of more than 1000ml have been described as predictive factors for POPF following PD(136). At multivariate analysis, a PD for AA pathology possessed an HR of 2.98 (95%CI 1.36 – 6.54 p<0.001) against PDAC or

pancreatitis for POPF. Similarly, a soft pancreas gland, regularly seen in AA surgery, demonstrated a HR of 5.02 (95%CI 1.97 – 12.81 p=0.007) for POPF compared to firm gland texture at time of PD(137).

1.6.2 Surgical Ampullectomy (SA)

Halsted performed the first transduodenal resection of the ampulla in 1899(138) and currently there are two recognised approaches in this regard by either laparoscopy or open surgery. The procedure itself requires a duodenotomy and then full thickness excision of the ampulla with subsequent local lymph node dissection. Initial results regarding this procedure were promising, with 30 day mortality of <1%(139) and morbidity much lower than PD(79); however, high positive resection margin (R1) rates of 60%(140) and disease recurrence approaching 80-100% has been reported following excision of malignant lesions (140-144).

These issues have restricted SA to benign lesions and with the diagnostic uncertainty of forceps biopsy in determining invasive disease alongside the improvements in EA, both in refining diagnosis and management, its role has become limited with few indications. When comparing local surgical and endoscopic options, morbidity was significantly higher in the surgical arm at 42% versus 18%. There was also a longer hospital stay associated with local surgical excision although no significant difference in recurrence was seen suggesting that EA should be the primary method of local excision(145) in the correct clinical setting.

1.6.3 Endoscopic Management

1.6.3.1 Endoscopic Ampullectomy (EA)

Endoscopic ampullectomy with curative intent was first described in 1993 (146). In the recent past there has been an increasing role for this technique as an effective alternative to surgical resection for certain ampullary lesions and is associated with good outcomes(147, 148). The procedure is undertaken using a side-viewing endoscope to visual the ampulla within the duodenum. The tumour mass is then regularly raised from the deep tissues using saline injections with or without adrenaline to create a plane for resection similar to the technique used in endoscopic mucosal resection for colonic polyps. The lesion is then snared and excised with diathermy assistance. The failure of a lesion to rise from the surrounding mucosa is associated with malignancy(149, 150). A detailed description of our EA technique is described in chapter 4.

It is a less invasive procedure with minimal trauma that reduces overall morbidity and mortality compared to PD although it remains controversial as to whether ampullary adenocarcinoma can be optimally managed in this manner. A lymphadenectomy is not performed using this technique and therefore accurate pre-procedural staging is essential(151) although the performance of CT in this area remains poor as described earlier. EA is not without risk and the commonest complications associated are pancreatitis, bleeding, cholangitis and perforation at a frequency of 27.9%(152). It is however, an attractive option for patients unfit for radical resection or those with low-grade disease, as it does not expose them to the risk of major abdominal surgery.

Not only be EA be used as an effective definitive treatment option but also it serves as a vital component in the diagnostic work-up of patients with ampullary lesions whereby large specimens, following snare excision, can provide refined diagnostic pathological grading of each lesion in addition of biopsy specimens.

The accuracy of traditional forceps biopsy has been questioned due to several factors. Firstly, variability in interpreting histological specimens by pathologists and secondly, small forceps biopsies may not evaluate the degree of dysplastic change within an ampullary lesion correctly and thirdly the presence of a potentially missed foci of invasive adenocarcinoma within global dysplastic change must be considered. Previous studies have shown a false-negative rate of 17%-40% for forceps biopsy in the detection of infiltrating carcinomas(153-155) and Bellizzi et al(156) recently reported a diagnostic agreement of only 64% when comparing biopsy samples to the eventual resected specimen.

1.6.3.2 Ablative Therapy

Endoscopic management options are not limited to ampullectomy with ablative therapies such as argon plasma coagulation (APC), laser and bipolar cautery commonly used in patients whose ampullary lesion is not safely resectable by snare excision or in surveillance procedures following excision to destroy any residual tissue that may be left. APC is the most frequently utilised technique for this purpose(97). Recently radiofrequncy ablation (RFA) has began to play a role as an ablative therapy in this area; particularly in cases with the presence of intraductal extension. A recent retrospective multicentre study evaluated the use of RFA in

ampullary neoplasms with intraductal extension with a total of 14 patients being evaluated. With a median of one RFA catheter session complete intraductal ablation was achieved in 91.6% of patients at a median follow-up period of 16 months(157). Another small case series of four patients from Suarez et al(158) found similar results with three patients(75%) achieving complete eradication of the intraductal adenoma extension. The main adverse risk of this procedure is stricture formation that may require subsequent stenting(157-159).

The increased use of these techniques has been demonstrated to provide both significantly lower morbidity and mortality in selected patients with Ceppa et al(145) reporting 0% and 18% associated mortality and morbidity.

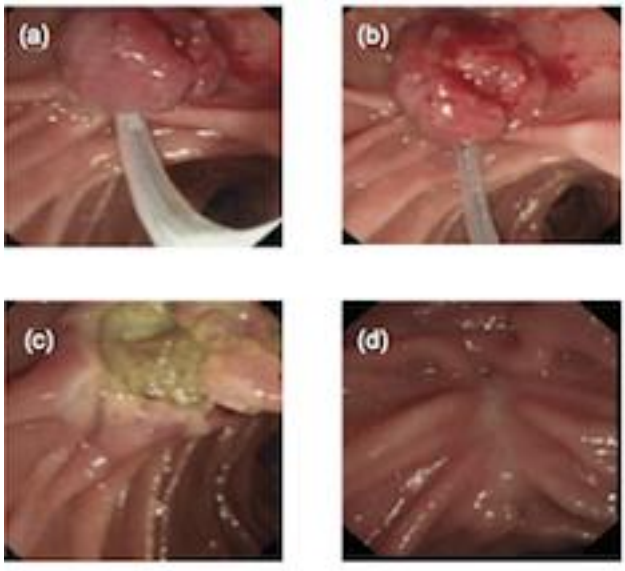


Figure 1.6 a-c) Endoscopic appearances at time of ampullectomy; d) follow-up surveillance

1.6.4 Adjuvant Therapy

1.6.4.1 Chemoradiotherapy

There is currently no standardised guideline for the use of adjuvant chemotherapy in the treatment of ampullary adenocarcinoma(25, 160-163). Previous randomized

controlled trials (RCTs)(164-166) and institutional cohort studies(167, 168) have failed to demonstrate a survival benefit for chemotherapy.

The ESPAC-3 trial(169) is the only prospective RCT evaluating the effects of adjuvant chemotherapy on survival in resected peri-ampullary cancer. The trial was a three group, randomised, open-label phase 3 study with 434 patients included with patients receiving either 5-fluorouracil or gemcitabine in the two adjuvant chemotherapy arms or observation only in the third arm. In the primary analysis, there was no survival benefit seen for 5-fluorouracil or gemcitabine compared to the observation group (45.7 months vs. 38.9 months vs. 35.2 months P=0.23)(169). On secondary analysis, when adjusting for prognostic variables the HR for chemotherapy compared with observation was 0.75 (95% CI: 0.57-0.98; P=0. 03). On subgroup analysis, there was no significant benefit for intestinal or pancreaticobiliary phenotypes although the subgroup of patients undergoing R0 resection demonstrated improved overall survival with adjuvant chemotherapy(169).

When considering adjuvant chemoradiotherapy in resectable ampullary cancer two large series from the Mayo clinic reported that there may be a survival benefit seen in node positive patients following surgery(168, 170). The latter study from 2011 by Narang et al, consisting of 186 included patients undergoing 5-fluorouracil based chemoradiation, demonstrated a mean overall survival of 32.1 months versus 15.7 months (5 yr OS: 27.5% vs. 5.9%; RR=C.47, P=0.004)(168).

Kim et al reported their series of 118 patients, 41 of whom received adjuvant chemoradiation therapy with 5-fluorouracil and total radiation dose up to 40 Gy. Their results revealed improved locoregional relapse-free survival, and possibly also

an overall survival advantage, although statistical significance was not achieved(167). Similarly, Willett and colleagues reported their experience with adjuvant radiotherapy, with or without concurrent 5-fluorouracil for high-risk tumours. Compared to surgery alone, the radiotherapy group demonstrated a trend toward better locoregional control; however, no advantage in survival was seen(171). However, the only prospective, randomized study into the benefits of chemoradiation in ampullary adenocarcinoma by Klinkenbijl et al failed to demonstrate any survival benefit(164).

Despite the lack of evidence, theoretically those patients with an intestinal subtype tumour may benefit from fluorourocil based chemotherapy regimes whereas pancreaticobiliary subtypes may derive improved survival from treatment with gemcitabine based regimes(172). Further research is required in this area before any definite conclusions can be made.

1.6.4.2 Pathological staging of AA

The majority of patients are staged pathologically after examination of the resected specimen and associated lymph nodes following surgery. The classification is predominantly based upon the extent of local disease with T and N stage indicative of this. The status of resection margins is not included in the conventional staging system applied today. An R0 resection is defined as one with grossly and microscopically negative margins, R1 possess grossly negative but microscopically positive margins and R2 margins have gross tumour evident. Although not included in conventional staging, the extent of resection is of great significance prognostically.

1.6.4.3 Guidelines AJCC 7th Edition

The most recent staging guidelines were published in the American Joint Committee on Cancer staging manual 7th edition and are unchanged from the previous edition. They are similar to those implemented in PDAC with T1 disease being confined to the ampulla or sphincter of Oddi and T4 disease involving structures out with the pancreas. The nodal and metastasis classification is generic to all other gastrointestinal malignancies. The classification is shown in **Table 1.3**.

AJCC 7th Edition Guidelines on Ampullary Cancer Staging				
Tx	Primary tumour cannot be assessed			
T0	No evidence of primary tumour			
Tis	Carcinoma in situ			
T1	Tumour limited to ampulla of Vater or sphincter of Oddi			
T2	Tumour invades duodenal wall			
T3	Tumour invades pancreas			
T4	Tumour invades peripancreatic soft tissues or adjacent organs/ structures			
Nx	Regional lymph nodes cannot be assessed			
N0	No regional lymph nodes involved			
N1	Regional lymph node metastases			
M0	No distant metastases			
M1	Distant metastases			
				Approximate 5 year survival
Stage 0	Tis	N0	M0	>90%
Stage 1A	T1	N0	M0	>75%
Stage 1B	T2	N0	M0	~60%
Stage 2A	T3	N0	M0	~50%
Stage 2B	T1-3	N1	M0	~35%
Stage 3	T4	Any N	M0	25%
Stage 4	Any T	Any N	M1	<10%

Table 1.3 AJCC 7th Edition Guidelines for Ampullary Adenocarcinoma staging(173)

1.7 Overall Survival of Periampullary tumours

Despite a partially shared embryological origin tumours of the biliary tree possess marked variation in overall survival(174). Overall, ampullary adenocarcinoma is regarded to have a poorer prognosis than duodenal tumours but better than both cholangiocarcinoma and PDACs with 5-year survival rates of 37-68% having been reported(19, 140, 151). A large study from Tol et al reaffirmed this with survival rates of 13.9% for PDAC, 27.3% for cholangiocarcinoma, 44.4% for AA and 73.0% for duodenal adenocarcinoma (DA) by tumour location documented at 5 years following PD respectively(175). Approximately 76.5% to 89.4% of ampullary adenocarcinomas are suitable for resection with curative intent(88, 176) which is significantly higher than PDAC and due to their relatively early presentation.

1.7.1 Prognostic factors for Ampullary adenocarcinoma

1.7.1.1 Pathological factors

When considering the TNM staging classification, advancing tumour stage, positive resection margins and most strongly lymph node involvement have all been shown to worsen prognosis and survival. Some other pathological factors such as the presence of lymphovascular and perineural invasion, despite not being a part of routine staging, are associated with poorer overall outcome(33, 83, 177) and all will be further evaluated in the next chapter.

1.7.1.2 Serum Markers

An elevation in pre-operative CA 19-9 levels and associated with poorer survival following surgical resection (83-85, 178). High serum levels of CEA pre-surgery have also been implemented as a factor of poor prognosis in several studies(30, 87, 88) and identified as an independent factor after multivariate analysis in one study(87). In terms of subtype, it has been suggested that CEA levels predict recurrence in the intestinal based tumours(30). The study by Todoroki found that both CEA and CA 19-9 correlated with distant recurrence following an R0 resection with CEA being associated with liver and distant nodal disease and CA 19-9 with lung metastases(88).

A poor prognosis following surgical resection is also true of elevated total bilirubin and patients undergoing pre-operative biliary drainage(83) with the latter likely due to be related to the physiological effects of high serum bilirubin and more advanced disease rather than the drainage procedure itself.

1.7.1.3 Molecular factors

As mentioned previously, Kimura et al first described further subdivision of biliary based adenocarcinomas into two distinct phenotypes, pancreaticobiliary and intestinal, with the latter having demonstrated a significant survival benefit in the majority of studies(30, 31, 83, 179). The two IHC markers MUC1 and CDX2 are closely related to and involved in subtype differentiation with high levels of MUC1 seen regularly in pancreaticobiliary phenotypes and expression of CDX2 almost exclusively expressed in intestinal epithelium.

1.8 Pattern of Recurrence

Despite earlier diagnosis and improved surgical technique, tumour recurrence following radical resection with curative intent remains problematic with rates of 28% to 44% reported within the literature(30, 88, 180). Following radical resection by PD the time to recurrence from index surgery ranged from 13 months to 21 months in studies(30, 88). The common sites of recurrence are regarded as being the liver, aortocaval lymph nodes and lung alongside loco-regional recurrence(88, 180-182). In patients with recurrent disease following PD liver metastases were reported in 55%, 59% and 67% of cases and distant lymph node disease in 43% and 54% respectively(30, 88, 182). There has also been documented recurrent disease found within the peritoneum, bones, brain and mesocolon although in small numbers.

It is uncommon for patients to represent with locoregional failure alone following PD with one study reporting no cases of this in all 54 recurred patients(88) and a further study finding that 49.3% of patients with recurrent disease had more than one site involved(182). Recurrence is more frequently seen in the patients with pancreaticobiliary subtype but no significant difference has been observed between histological subtype and the site of recurrence(30).

The overall pattern of recurrence is different following definitive endoscopic ampullectomy compared with PD described above. When undertaken for LGD or HGD adenomata there has been a reported local recurrence of 6% to 33% with no evidence of distant disease(97, 146, 152, 183-186). These lesions in the most part are usually benign and amenable to repeat endoscopic treatment. Those lesions

recurring due to intraductal extension were most likely to require surgical intervention(186-188).

There is limited data on recurrence patterns in patients who have been treated by EA for an invasive adenocarcinoma as until recently this has been seen as a palliative management strategy. A single paper found reported no recurrence in patients with Cis or T1 disease who were unfit for definitive surgery at 27 months and 32 months respectively(151).

2.0 AIMS AND HYPOTHESIS

Chapter 2

Aims and Hypothesis

2.1 Introduction

Much is known regarding the molecular pathology and clinicopathological factors influencing survival in pancreatic cancer with work previously undertaken in Glasgow to understand the molecular pathology pathways of LKB1, p21, p53 and WNT signaling in PDAC(43, 189, 190). Focus has now turned to extensive pancreatic genomic analysis to provide clarity in the understanding of molecular pathways in tumorigenesis(191). However, in ampullary adenocarcinoma these factors and pathways are less well established partly due to a paucity of research on the topic. At the inception of this project only limited work with isolated tumour markers has been undertaken.

There have been several studies that have looked at outcome following radical surgical resection of AA with no real overall consensus with regards to what are poor prognostic indicators and their weighting. Certainly, like all other gastrointestinal malignancies, lymph node involvement and positive resection margins have been implicated but how detrimental are their presence to outcome and in relation to other routinely evaluated factors? There has yet to be any systematic reviews or meta-analyses undertaken on this subject to the best of knowledge at this time.

Endoscopic ampullectomy has developed in recent years and has gained an increasing role in the diagnosis and management of ampullary adenoma and adenocarcinoma. It has been suggested that EA should be used exclusively in benign

disease except in exceptional circumstances due to the in ability to perform lymphadenectomy amongst other reasons but how does survival compare between definitive EA and PD and is there patient groups who can be adequately treated with only EA?

There has been limited progress in the development and identification of useful tissue markers in AA. Tumour markers such as CA 19-9 and CEA, when elevated pre-op are thought to be suggestive or a poorer prognosis although have no significant value following resection. The identification of MUC1 and CDX2 and their role in subtype differentiation in resected specimens has further allowed for improved prediction of survival in an extremely heterogeneous group of patients. However, to date, no markers have been identified that show strong correlation towards disease progression or invasion which can be applied in the clinical setting.

2.2 Hypothesis

Ampullary adenocarcinoma has a better prognosis than its PDAC counterpart. Lymph node involvement, resection margin status and histological subtype are likely to be the strongest factors determinant of survival following surgical resection. There will be protein markers that, when up regulated are indicative of an aggressive, metastatic phenotype that warrants more aggressive treatment.

2.3 Aims

We will test the hypothesis by the following strategies:

1/. Establish an ampullary cancer patient database with basic demographics, pathological and survival data and maintain prospectively.

2/. Undertake a systematic review and meta-analysis of the literature to establish true clinicopathological predictors of poor prognosis in AA following radical surgical resection.

3/. Simultaneously evaluate the created West of Scotland Pancreatic Units database of resected AA and compare results to meta-analysis.

4/. Interrogate patients pathway who underwent EA from database and assess its role in the diagnosis and management of AA with consideration of survival, complications and outcome.

5/ Create tissue micro-arrays using tissue from the ampullary cancer database to enable the efficient examination of relevant protein markers using standard immunohistochemical techniques.

6/. Using immunohistochemistry, stain with multiple protein markers to establish whether a metastatic phenotype or poor survival in these patients with ampullary cancer is associated with up regulation of these pathways.

3.0 REVIEW AND META-ANALYSIS OF FACTORS INFLUENCING SURVIVAL

Chapter 3

Systematic Review and meta-analysis of clinicopathological factors influencing survival in ampullary adenocarcinoma

3.1 Introduction

Ampullary adenocarcinoma is a rare condition that accounts for 0.2% of all gastrointestinal malignancies(77). It is generally regarded that they have a better overall prognosis than some of their biliary counterparts, namely PDAC and cholangiocarcinoma, whilst possess a poorer prognosis than DA(19, 175). Part of the reason for this may be due to a typically earlier diagnosis than other peri-ampullary tumours resulting in an increased frequency of resectable lesions(192). Having said this, up to 50% of AAs recur following surgical resection(193) and it is therefore important to identify factors implicated in overall survival so that high-risk patients can be identified and treatment strategies tailored accordingly.

In direct contrast to PDAC, which continues to be widely and extensively researched, there is a paucity of published data regarding AA that is further limited by small patient cohorts(32, 80, 88, 194-197). From the current literature there is no consensus regarding the most important clinicopathological features determining outcome with marked heterogeneity in the significance of routinely reported histological features. A landmark paper published in 2004 by Kimura and co-workers first described the two distinct histological subtypes of AA, pancreaticobiliary type and intestinal type whilst demonstrating in their cohort that patients with the latter

had a much better overall prognosis(27). These differences suggested two diverse underlying disease processes which are now routinely recognised today.

There have been multiple, mostly small cohort studies published within the literature in recent years that document both clinical and pathological factors that influence survival in patients with resected AA; however, no systematic reviews or meta-analyses exist on this subject. This chapter aims to combine and appraise the relevant literature and clarify the key clinical and histopathological factors influencing survival in AA following resection with curative intent.

3.2 Materials and Methods

3.2.1 Systematic Search Strategy

The literature search aimed to identify all research manuscripts that assessed the clinicopathological factors influencing survival or outcome in ampullary adenocarcinoma following formal surgical resection. A search of the PubMed database on the 21st February 2016 used the following search criteria:

- [ampulla*] AND [survival] OR [ampulla*] AND [outcome] OR [ampulla*] AND [prognosis]

The title and abstract of each citation was then reviewed to identify studies reporting on survival in ampullary cancer and the full text of each was obtained. A further search through the identified manuscripts bibliographies was conducted to ensure identification of any papers that may have been missed in the primary PubMed search. The search was limited to English language publications and those

reporting on human subjects. The study was performed in accordance with the guidelines of preferred reporting items for systematic reviews and meta-analyses (PRISMA) 2009(198).

3.2.2 Inclusion and Exclusion Methodological Assessment

Studies were deemed eligible for inclusion according to the criteria listed below (Table 3.1). As ampullary tumours have been shown to have a different natural history to other biliary tract malignancies any studies reporting combined results (including PDAC, DA or cholangiocarcinomas) as 'periampullary tumours ' were excluded from further review. Conference abstracts, review articles and case series of less than 35 patients were also excluded. A study was also removed if it did not allow for the extraction or calculation of survival data from its published results.

Table 3.1 Criteria for inclusion of ampullary adenocarcinoma outcome studies

1. **Prospective or retrospective cohort design with justification for excluded cases from 2000 onwards**
2. **Patients with AA undergoing surgical resection with curative intent**
3. **Statistical analysis using multivariate proportional hazards modeling**
4. **Documentation of HRs, 95% CIs and P values**

3.2.3 Data Extraction

The author (C.R.F) reviewed and appraised all manuscripts obtained from the PubMed search and extracted the number of ampullary adenocarcinomas studied, the methodology and all results documented in each manuscript. The surgical procedure was either PD or PDDD and the data collected included authors' names,

institution, year of publication, number of cases and the clinicopathological factors evaluated using univariate and multivariate analysis with overall outcome. The multivariate Hazard Ratio (HR) with 95% Confidence Interval (CI) including P value was obtained and in manuscripts reporting results without CIs the P value was used to estimate the standard error using the Z statistic.

3.2.4 Statistical Analysis

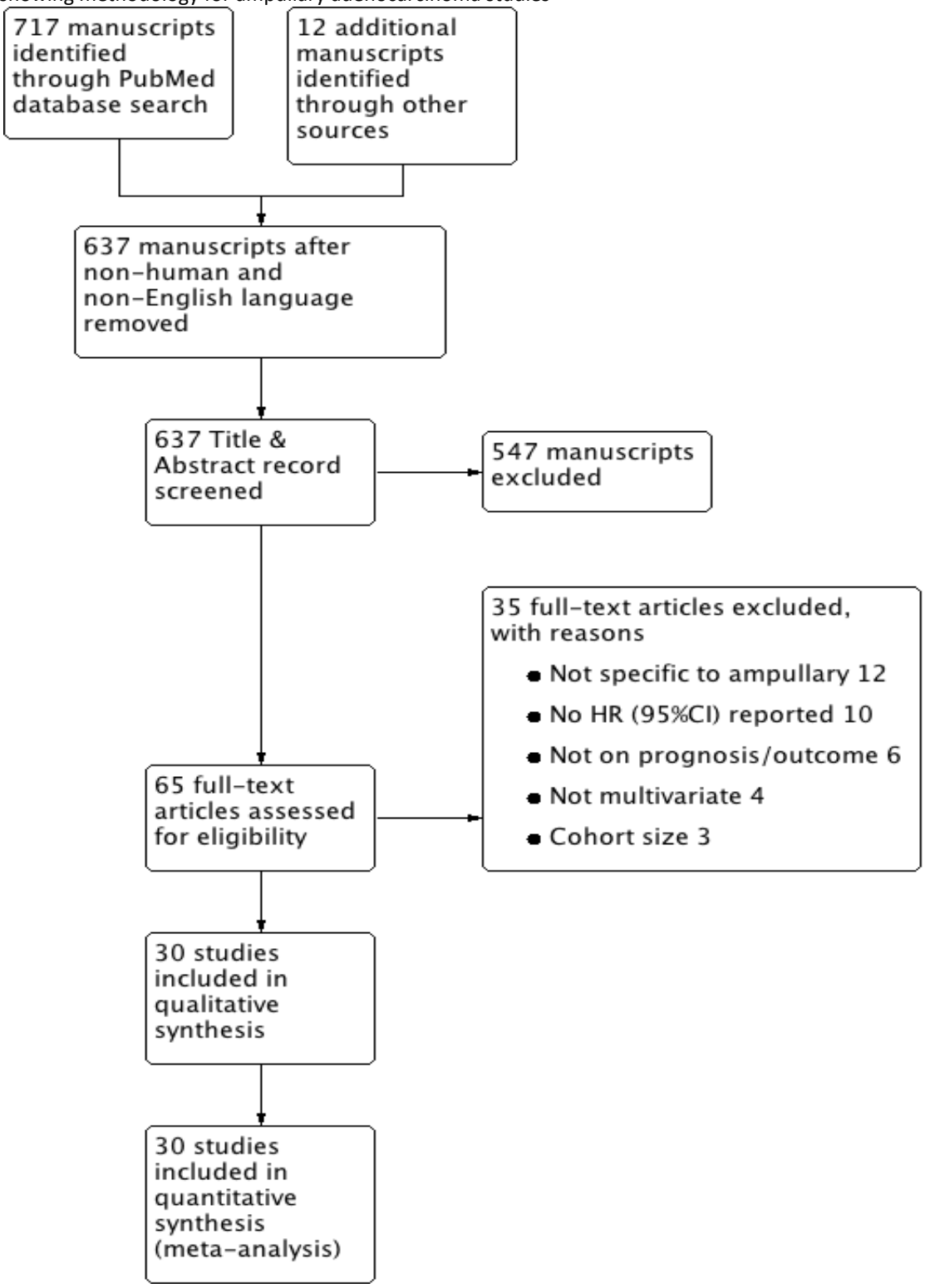
The clinicopathological factors selected were those routinely reported upon in histopathology reviews based upon standardised TNM staging and those evaluated in the current scientific literature. The overall outcome data was presented as a HR with a 95% CI. For factors assessed appropriately in a single study, the HR (including 95% CI) represents the value reported from that study; whereas when reported in multiple studies, a fixed effects summary HR and 95% CI were calculated using the generic inverse variance method and the random effects model as per the DerSimonian-Laird method. An observed HR greater than 1 indicated a worse outcome for the study group compared to the reference group and would be considered as statistically significant when $P < 0.05$ and the 95% CI did not overlap 1. All meta-analyses were performed using the REVMAN systematic review and meta-analysis software package version 5.1 (The Cochrane Collaboration Software Update, Oxford, UK).

3.3 Results

3.3.1 Excluded Studies

The search of AA outcome literature initially identified 717 articles for consideration and the PRISMA diagram (Fig 3.1) summarises the systematic literature search undertaken. An initial 31 manuscripts were immediately excluded as they were not related to human subjects and a further 61 were not published in English language. There were 65 full text articles deemed appropriate for eligibility following initial title and abstract evaluation alongside additional bibliography reviews. Each full text was obtained and the criteria for inclusion were applied to each with 12 studies not exclusively reporting on AA; instead the study design allowed for inclusion of other periampullary tumours including PDAC, cholangiocarcinoma or DA so were excluded. A further six studies did not report on overall survival or outcome. Of the remaining 47 cohort studies, all reporting on AA survival post surgical resection, 11 did not report HRs or 95% CIs in their results with a further four limiting their analysis to univariate log-rank analysis only. Three studies were excluded as they did not meet the minimum cohort size leaving 30 high-quality eligible studies.

Figure 3.1 PRISMA (preferred reporting items for systematic reviews and meta-analyses) flow chart showing methodology for ampullary adenocarcinoma studies



3.3.2 Included Studies

A total of 30 high-quality cohort studies met the eligibility criteria for this systematic review by presenting multivariate survival estimates of independent prognostic variables affecting survival in ampullary adenocarcinoma. A list of all the included retrospective studies is detailed in Table 3.2. Overall there were 3290 patients included with a mean age of 64 years. The frequency of positive lymph nodes ranged from 15% to 67% (median 43.6%) and negative resection margins from 75% to 100% (median 96.8%). Seven studies included only R0 resections.

The 5-year overall survival following resection ranged from 33% to 68% (median 52.6%) and the median survival from 31 to 87 months (median 54 months). Subtype classification was undertaken in seven studies only. 17 of the 30 studies reported on microscopic invasion with perineural involvement present in 10% to 74.6% (median 25%) resection specimens. Lymphatic and venous invasion was seen in 14% to 72.1% (median 47.7%) and 3% to 47.8% (median 36%) respectively.

Of the 30 included studies, only 15 provided any data regarding the use of adjuvant treatment following surgical resection. The percentage of patients who undertook any form of adjuvant treatment ranged from 6.3% to 67% with an overall median of 37.9%. There were 5 studies that specified between the use of chemotherapy and radiotherapy or a combination of both with the remainder referring to adjuvant treatment only. There were no P values that showed statistical significance within any of the manuscripts when presented in univariate analysis or median overall survival.

Table 3.2 Baseline characteristics and clinicopathological features of the 30 included studies

Study	Year	n (m/f)	Age (y)	PD (n)	LN +ve (%)	R0 (%)	Subtype (Int/Pb)	5yr surv. (%)	Median surv. (months)	PI (%)	LI (%)	VI (%)
Allema et al (194)	1995	67(42/25)	61	67	52	75		50				
Beghelli et al (195)	2002	89(55/34)	59	89	41		56/28		46			
Bettschart et al (180)	2004	88(49/39)	63	70	40	97.2		37.9§	45.8	12.9		
Bourgoin et al (199)	2015	55(20/35)	66	55	36	98		51	66	27		36
Brown et al (80)	2005	51(25/26)	69	51	47	100		58				
Carter et al (200)	2008	157(90/67)	64	115	33	86	54/53/11			74.6	72.2*	
Choi et al (201)	2011	78(43/35)	61	70	31.4	95.7		59.9	70	10		
DiGiorgio et al (202)	2005	94(53/41)	65	63	28	100		64.4	54			
Duffy et al (196)	2003	55(34/21)	67	55	41.8	98.2		67.7		21.8	16.4*	
Falconi et al (203)	2008	90 (51/39)	63	90	48	100		61		33.3		47.8
Hsu et al (182)	2007	127(71/56)		127	40.8	94.3						
Iacono et al (204)	2007	59(42/17)	57	59	37.3			46	31			
Lazaryan et al (177)	2011	72(42/30)	72	72	34	96		61		29	39*	
Morini et al (60)	2013	72(42/30)	65	72	48.6		31/35/6	50	68			
Ohike et al (205)	2010	244(145/99)	65	244	38	95		33		23	64*	
Pomianowska et al (206)	2013	61	67	61	48	75		46		33		36
Qiao et al (81)	2007	127(71/56)	62	124	35	98.4		43.3				
Robert et al (179)	2014	319(173/146)	65	301	41.7	97.5	106/105/10	56.4		27.9		23.5
Sakata et al (207)	2007	62(34/28)	62	62	50	96.7		62		14.5	67.7	21
Schueneman et al (42)	2015	163(96/67)	65	163	55.9		70/93	54.4	87.7	28.6	35.5*	
Sellner et al (208)	1999	34	62	34	15	100						
Sessa et al (32)	2007	53(30/23)	68	53	59.2	80.7	23/30					
Showalter et al (209)	2011	61(38/23)	70	61	67	95		44	50			
Sierzega et al (210)	2009	111(61/50)	59	106	47	100	38/59/14	52	61.8	20	14*	
Sommerville et al (197)	2009	39	61	39	62. 0	97		32	35			
Todoroki et al (88)	2003	66(32/34)	64	59	40. 0	93		52.6			56.4	41.8
Tol et al (175)	2015	227(138/89)	65	227	43.6	81.1		44.4				
Winter et al (19)	2010	347(199/148)	68	347	54.5	96.1		45		41.4		43.6
Woo et al (211)	2007	163(84/79)	59	163	31. 0	100		68		15	30	3
Yokoyama et al (212)	2005	59(35/24)	62	58	53.5	100		61		13.6	67.8	16.9

M=male; F=female; PD=pancreaticoduodenectomy; PI=perineural invasion; LI=lymphatic invasion; VI=venous invasion.

§ includes not resected patients.

* Data as lymphovascular invasion

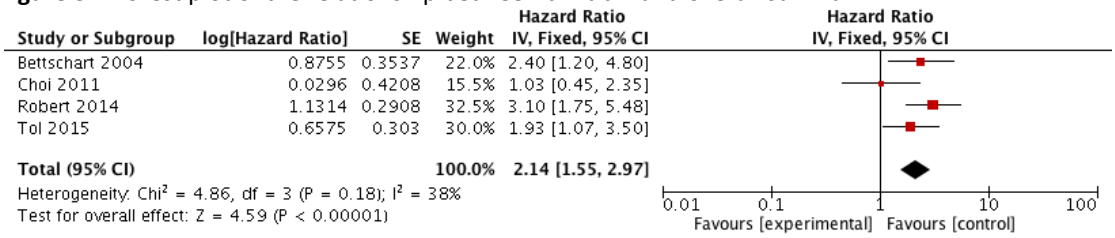
3.3.3 Meta-analysis

Meta-analysis was performed for the following clinicopathological features:

3.3.3.1 Bilirubin

Bilirubin was only the serum marker with data available for evaluation. There were four studies that reported on elevated serum bilirubin within their multivariate survival analysis with two using a value of >50umol/L and one with 75umol/L as cut-offs. The remaining paper provided no numerical values. The average bilirubin from the 3 studies was 87.5umol/L. There were three manuscripts to have found elevated serum bilirubin levels to be an independent factor of poorer outcome with overall HR: 2.14 (95% CI: 1.55 – 2.97; p <0. 00001) (Fig 3.2).

Figure 3.2 Forest plot of the relationship between bilirubin and overall survival

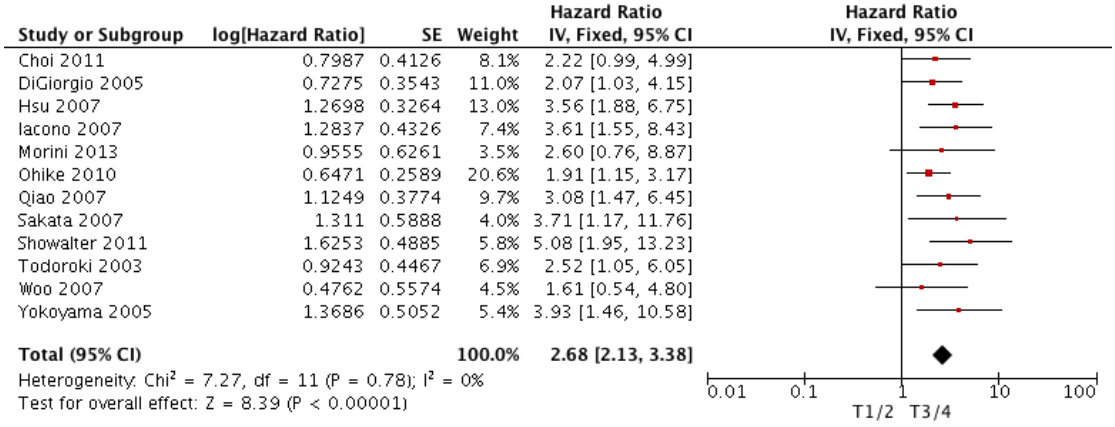


3.3.3.2 T stage

T stage was designated as T1/2 versus T3/4 with a total of 12 manuscripts reporting T stage as an independent prognostic factor when classified in this manner.

Increased T stage was associated with a worse prognosis (HR: 2.68, 95% CI: 2.13 – 3.38; p <0.00001) (Fig 3.3).

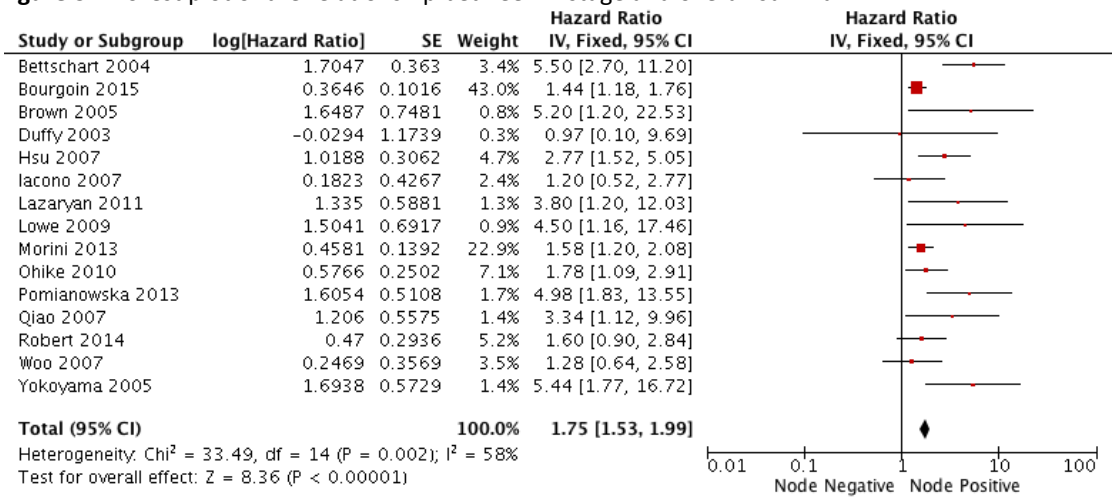
Figure 3.3 Forest plot of the relationship between T stage and overall survival



3.3.3.3 Nodal stage

N stage was measured in a binary fashion as the presence or absence of lymph node metastases and was the most frequently assessed independent prognostic factor influencing survival with 15 of the studies reporting. The combined HR for all of the studies was 1.75 (95% CI: 1.53 – 1.99; p <0.00001) (Fig 3.4).

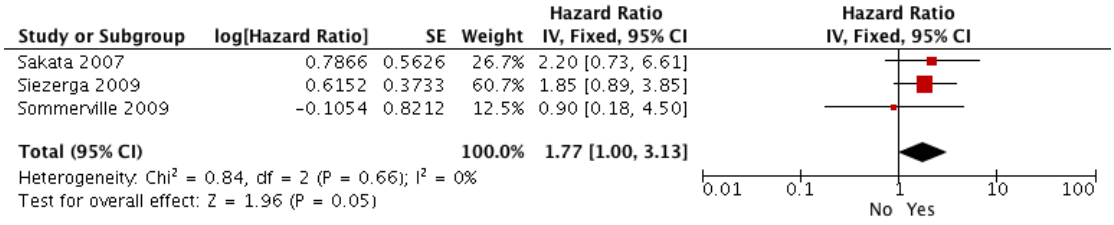
Figure 3.4 Forest plot of the relationship between N stage and overall survival



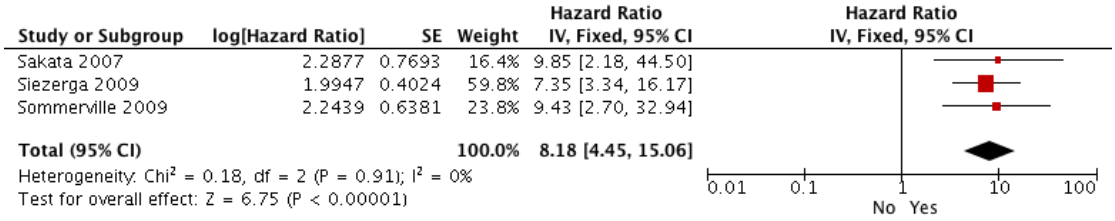
There were 3 different studies that further assessed lymph node involvement by reporting on the total number of nodes involved using the following categories 0, 1 - 3 and >3, demonstrating a modest prognostic disadvantage in the 1-3 positive nodes group (HR: 1.77, 95%CI: 1.00 – 3.13; p=0.05). In contrast, in the > 3 lymph node positive group there was a significantly worse overall survival with HR 8.18 (95% CI: 4.45 – 15.06; p<0.00001) compared to node negative patients (Fig 3.5).

Figure 3.5 Forest plots of the relationship between lymph node number and overall survival

a) 1 – 3 positive LN group

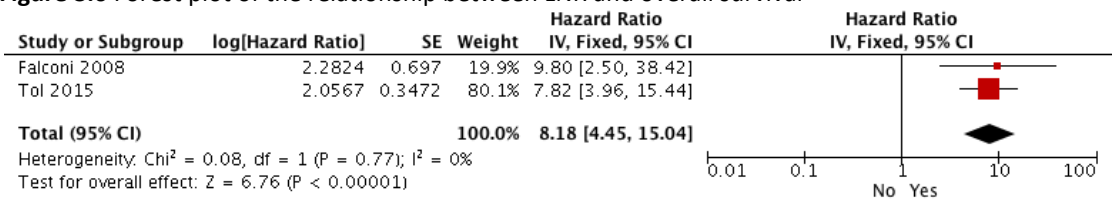


b) >3 positive LN group



Furthermore, lymph node involvement was also reported as a ratio and found to be an independent factor influencing survival in two studies. The lymph node ratio (LNR) is calculated from the number of positive nodes over the total number harvested. The HR from both studies calculated as 8.18 (95% CI: 4.45 – 15. 04; p < 0. 00001) (Fig 3.6).

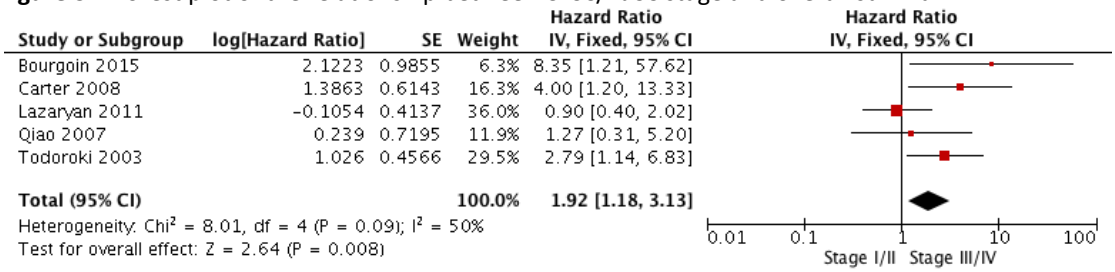
Figure 3.6 Forest plot of the relationship between LNR and overall survival



3.3.3.4 UICC/AJCC Stage

The American Joint Committee of Cancer (AJCC) 7th edition and Union for International Cancer Control (UICC) TNM classification staging systems were combined to maximize numbers. There were two studies reporting on UICC stage and a further three on AJCC stage following their multivariate analyses with three remaining independent predictors of survival. With an overall disease stage of > III the combined overall risk was HR 1.92 (95% CI: 1.18 – 3.13; p=0. 008) (Fig 3.7).

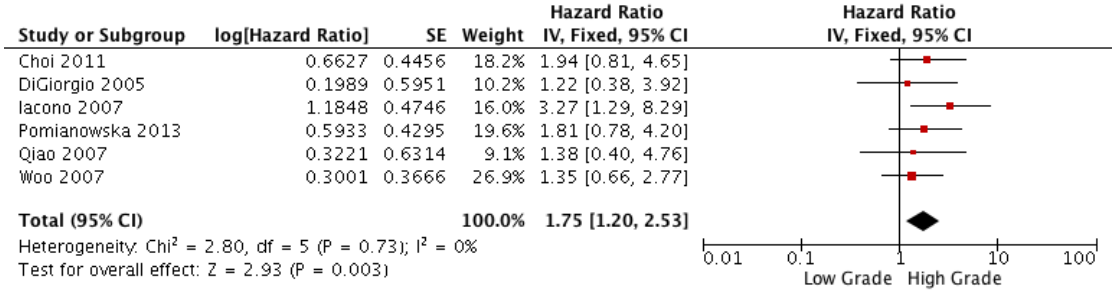
Figure 3.7 Forest plot of the relationship between UICC/AJCC stage and overall survival



3.3.3.5 Tumour Grade

Tumour grade was determined according to the degree of differentiation of the adenocarcinoma (well, moderately and poorly differentiated). The well and moderate group are then classed as low-grade tumours and the remaining lesions with poor differentiation as high grade (WHO classification). There were six studies that fully reported on grade following multivariate analysis with grade remaining independently prognostic only in the cohort presented by Iacono et al(204). The overall reported HR 1.75 (95% CI: 1.20 – 2.53; p=0.003) (Fig 3.8).

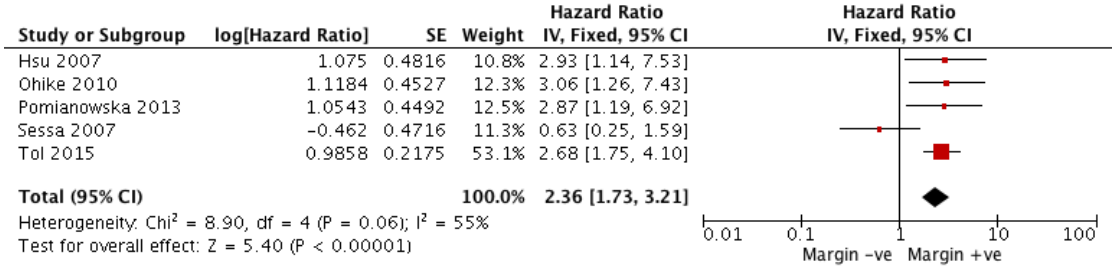
Figure 3.8 Forest plot of the relationship between tumour grade and overall survival



3.3.3.6 Resection Margin Status

Margin status was reported as R0 or R1 in all reviewed articles. There were data regarding margin status following multivariate analysis from five papers with four of those documenting almost three times the risk of death with positive microscopic resection margins. This translated to overall HR 2.36 (95% CI: 1.73 – 3.21; p<0.00001)(Fig 3.9). There was no clear consensus as to how margin was defined with two studies failing to clearly describe if they defined R1 as microscopic disease within 1mm of or at the resection margin(205, 206), two studies used the criteria of tumour at the margin(32, 182) and one study utilised within 1mm definition(175).

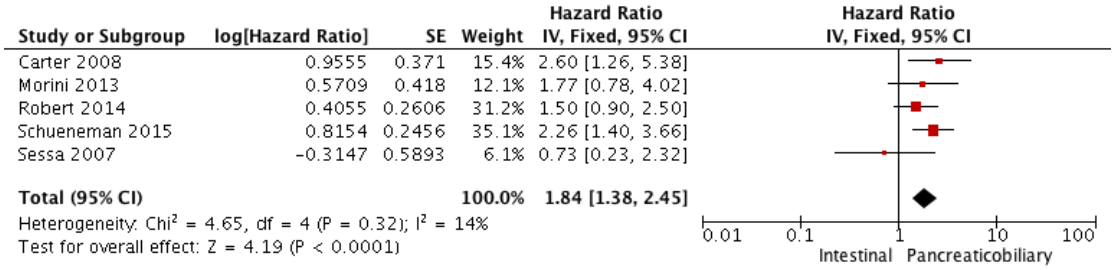
Figure 3.9 Forest plot of the relationship between margin status and overall survival



3.3.3.7 Ampullary Adenocarcinoma Subtype

Data on ampullary adenocarcinoma sub-typing according to intestinal or pancreaticobiliary phenotypes was only available in seven studies with assessment of independent prognostic impact following multivariate analysis reported in five studies. The presence of a pancreaticobiliary subtype was indicative of a poorer outcome with HR 1.84 (95%CI: 1.38 – 2.45; p<0.0001) (Fig 3.10).

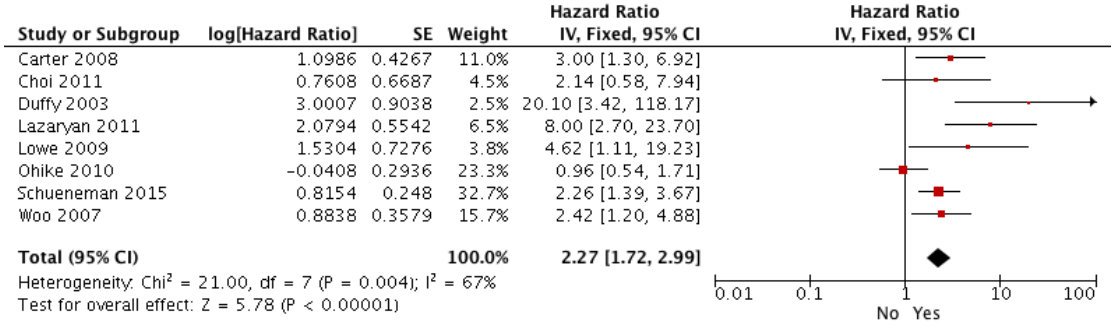
Figure 3.10 Forest plot of the relationship between subtype and overall survival



3.3.3.8 Perineural invasion

Perineural invasion was reported upon in eight papers following multivariate analysis with marked heterogeneity between analyses as demonstrated in the figure below. Overall the presence of perineural invasion was a poor prognostic features associated with significantly shortened survival. The overall HR for the combined studies was 2.27 (95%CI: 1.72 – 2.99; p<0.00001) (Fig 3.11).

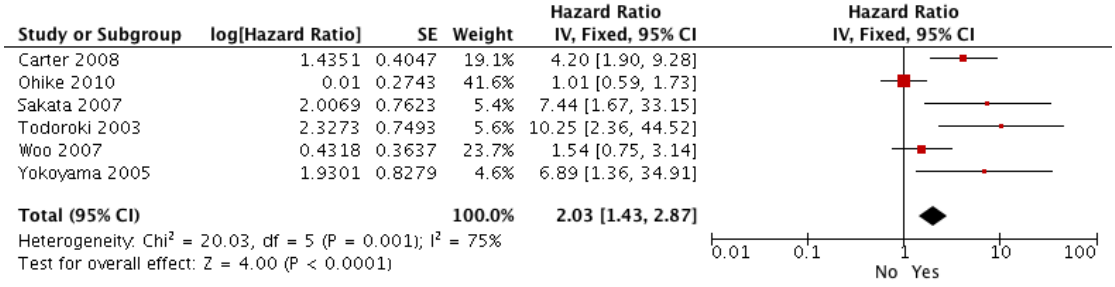
Figure 3.11 Forest plot of the relationship between perineural invasion and overall survival



3.3.3.9 Lymphatic Invasion

There were six of the 29 eligible studies that reported upon lymphatic invasion following multivariate statistical analysis with four remaining statistically significant. There was once again marked heterogeneity between studies. The overall HR was 2.03 (95%CI: 1.43 – 2.87; p<0.0001)(Fig 3.12).

Figure 3.12 Forest plot of the relationship between lymphatic invasion and overall survival



3.3.3.10 Venous invasion

A single paper identifies venous invasion as an independent prognostic factor for survival in patients with ampullary adenocarcinoma with Woo et al reporting HR 8.63 (95% CI: 2.47 – 30.2; p = 0. 001).

3.4 Discussion

There is a relative paucity of clinical data for patients with ampullary adenocarcinoma, likely due to the rarity of the disease compared to PDAC, and often limited in the most part to small patient cohorts from retrospective studies. This review of the literature, encompassing 3290 patients from 30 high quality studies, provides a comprehensive analysis of radical surgical resection by PD for ampullary adenocarcinoma and the clinicopathological prognostic factors that influence overall survival.

Radical resection by PD remains the gold standard of treatment for ampullary adenocarcinoma, owing to the high recurrence rates of 80% to 100% seen in local transduodenal resection(141, 143, 144) and the relatively unknown long-term outcomes for endoscopic ampullectomy. The marked improvement in the past decades of both surgical technique and post-operative care has reduced the operative mortality and morbidity of the procedure (213) with operative mortality between 0% and 10% readily reported(19, 33, 80, 81, 88, 141, 180, 192-194, 196, 201-204, 214). The largest study from Winter et al of 435 patients undergoing PD (for both adenoma and adenocarcinoma) reported peri-operative mortality of 2%(19). Despite these advancements the long-term survival remains poor. The median 5- year overall survival barely surpassed 50% and ranged from 33% to 68% despite the vast majority of patients undergoing an R0 resection (median 96.8%). The high negative resection margin rate suggests that it is easier to achieve R0 resections in ampullary cancer than in other periampullary adenocarcinomas with a recent systematic review of cholangiocarcinoma reporting a rate of 84%(215) whilst for PDAC, rates of 15%, 24% and 27.6%(216-218) have been reported. It also

suggests that resection margin status is not the most powerful contributing factor influencing survival as only a small proportion of patients undergo a margin positive resection however; with a HR of 2.36 with R1 disease, care must be taken to maximise potential for complete excision and utilising intra-operative frozen section to ensure microscopic clearance is advised.

The definition used to describe a R1 status in each of the manuscripts failed to demonstrate a consensus and it was not clear as to whether a standardised protocol (SP) reporting system was used which raises questions with regards to the accuracy of reporting. Research undertaken by Verbeke et al(217) looked at two comparable cohorts of patients undergoing PD resection, the first were reported using a non-standardised protocol (NSP) and the second a standardised proforma. They demonstrated that the implementation of a SP resulted in a higher R1 rate overall and that survival correlated with resection margin status in the SP cohort but not for the NSP group ($p<0.001$). They also found that there was a trend towards better median and 5-year survival after R0 resection in the SP PDAC subgroup suggesting that standardised reporting allows for more accurate specimen analysis(217).

A review of resection margin involvement and tumour origin by the same group demonstrated a wide variation in the published incidence and R1 rates of ampullary cancers from 5% to 42% and 0% to 27% respectively, with similar seen in PDAC and distal cholangiocarcinoma(219). These studies suggested that differences in pathological assessment rather than surgical practice was the main determining factor and histopathological distinction between the three cancer groups is less accurate than generally thought. They concluded that inaccurate distinction

between tumours alongside inconsistent resection margin assessment results in obfuscation of clinicopathological data and unless pathological examination becomes standardised, comparison of results between centres or multicentred trials will be of limited value(219).

From our previous work on resection margin status in PDAC using a SP reporting method for each resection specimen, we demonstrated a R1 rate of 74% that was in contrast to the majority of the previously reported literature(220). Despite this high R1 rate, the median overall survival time was comparable to those reporting much lower rates and therefore is likely a reflection on high-quality reporting rather than inadequate surgical technique(220).

An explanation for the poor prognosis attributed to ampullary adenocarcinoma despite small volume localised primary disease is the frequency of lymph node metastases (43.6%) at the time of presentation. The frequency of lymph node metastases, lymphatic invasion (47.7%), venous invasion (36%) and perineural invasion (25%) seen in resected specimens was significantly less than observed in PDAC.

Lymph node metastases has been strongly associated with poor outcome in AA, a finding reaffirmed in this review with 19 of 30 studies indicating that nodal involvement was an independent prognostic factor associated with reduced survival. Five studies further analysed lymph node involvement through assessment of number of involved lymph nodes and LNR (175, 197, 203, 207, 210) and confirmed that increasing metastatic burden significantly worsens survival and outcome.

The total number of lymph nodes harvested has also been reviewed previously. The paper from Partelli et al(221) retrospectively reviewed 127 patients undergoing PD for ampullary adenocarcinoma and concluded that removal of 12 or more lymph nodes was associated with improved survival in both N0 and N1 patients. The reason for this may be potentially related to more extensive lymphadenectomy and therefore better staging.

These data demonstrate that the pancreaticobiliary phenotype is associated with a significantly reduction in overall survival when compared to those patients with an intestinal phenotype tumour (HR 1.84, 95%CI: 1.38 – 2.45; p<0.0001). Unfortunately, subtype was only commented on in seven studies. This has potential implications for all 'periampullary cancers' (PDAC, AA, duodenal & distal cholangiocarcinoma combined) with all tumours with a pancreaticobiliary phenotype having a poorer prognosis(33, 59, 222). Potentially this histological feature determines outcome more so than anatomical tumour location, and poses the question as to whether phenotypic characterisation is a more important prognostic stratifier for pancreaticobiliary malignancies. Subsequently this may become an important component of standardized reporting for pancreaticobiliary malignancies.

There was significant heterogeneity seen in both perineural and lymphatic invasion in this meta-analysis. On further review of the papers demostating significance on multi-variate analysis there were several factors identified that may account for this. Generally, the studies consisted of small numbers with no clear or fixed histopathological process described in many cases. There were only five studies that specifically detailed the use of H&E slides for assessment(32, 42, 60, 200, 207). As

there is no recognised adjuvant therapy protocol in ampullary cancer the use of chemotherapy was discretionary throughout the studies. It was also noted that the inclusion criteria to multivariate analysis varied between some studies with p values not being uniform (<0.01 to 0.15)(32, 42, 201). The surgical technique varied slightly in two studies where a specific extending lymphadenectomy was performed with particular attention made to the superior mesenteric nodes which may result to altered outcomes(207, 212).

3.5 Strengths and Limitations

At the time of writing this thesis, to the best of knowledge, this is the only systematic review and meta-analysis of ampullary adenocarcinoma within the literature. There are a limited number of manuscripts topical to AA compared to other more common pancreaticobiliary malignancies; however, by selecting only the larger cohort studies for inclusion and limiting their publication to within the past 20 years it has been attempted to only report on the most relevant and powered studies.

A limitation of this review is the retrospective nature of all the included studies that may have allowed for the introduction of bias whilst the prognostic significance of some factors could be questioned due to the limited data available for analysis. Unfortunately this is a global problem shared amongst all the ampullary adenocarcinoma literature and is related to the rarity of this clinicopathological disease entity.

Another significant issue with the included studies is the lack of consensus regarding pathological assessment and reporting of resected specimens which must call into

question the accuracy of any subsequent survival analyses. The initial staging investigations performed in each study will not be standardised as there is no globally recognised preoperative staging criteria, again likely in part due to low incidence of AA.

As described earlier, there have been previous studies and RCTs performed to evaluate the effectiveness of adjuvant chemotherapy, however, no significant survival benefit has been determined. With this in mind, there is no standardised chemotherapy regime within the selected studies and this will result in a very heterogeneous group with regards to having received chemotherapy or not and what agent was given.

3.6 Summary

This systematic review of the clinicopathological factors influencing survival in 3290 patients with AA undergoing PD with curative intent supports that increased T stage, perineural invasion and lymph node involvement, particularly an increasing nodal metastatic burden are the strongest predictors of survival in this patient group. The influence of resection margin involvement is challenging to address as there has not been a standardized definition used throughout the literature. Clearly, the relatively small cohort numbers in some of the included studies highlights the challenges of studying patients with this disease and subsequently there is a need for future studies to be performed using a collaborative, multi-centre approach with standardization of pathological and prognostic factor reporting.

4.0 OUTCOME AND RECURRENCE FOLLOWING PANCREATICO-DUODENECTOMY: A SINGLE CENTRE EXPERIENCE

Chapter 4

Outcome and Pattern of Recurrence following Pancreaticoduodenectomy: a Single Centre Experience

4.1 Introduction

Ampullary adenocarcinoma accounts for approximately 0.2% of gastrointestinal malignancies yet represents up to 30% of resectable peri-ampullary malignancy(77). It is associated with a more favourable prognosis following surgical resection when compared with PDAC with 5-year survival times approaching 50%(19, 80, 81).

Unfortunately, a wide spectrum of outcomes exists following resection by PD. As previously evaluated in our meta-analysis of the literature (Chapter 3), pathological factors of prognostic value in AA include depth of tumour infiltration(77, 81, 192, 223), tumour differentiation(224), lymphovascular(19), perineural invasion(225) and resection margin status(194). Lymph node metastases are also an important factor(19, 32, 77, 81, 192) with both lymph node ratio (LNR)(203, 226), along with lymph node burden(210) associated with poor outcome. Adenocarcinoma of the ampulla of Vater may be classified by morphological appearance, with Kimura and co-workers initially describing intestinal and pancreaticobiliary subtypes based on morphological and IHC features(25).

Variability exists within the literature regarding the relative importance of these factors. Subsequently, the inability to predict individual outcomes for cancers affecting this anatomical location has hampered aspects of clinical decision-making, specifically the aggressiveness of therapy, choice of appropriate chemotherapeutic

strategies and clinical trial interpretation (24, 223).

Currently no standardized adjuvant therapy regimens exist for this cohort(25, 160-163). Randomized controlled trials(164-166) and institutional cohort studies(167, 168) have failed to definitively demonstrate a survival benefit for adjuvant chemotherapy. Identification of an appropriate high-risk group is therefore likely to be important for stratification in future trials. Furthermore, reporting of the site and pattern of recurrence following resection with curative intent varies significantly within the literature(88, 161), hampered by inadequate follow-up and incomplete pathological reporting.

Our review also highlighted the issue of marked variation in the pathological evaluation of tumours with no standardised histopathological reporting tool having been utilised raising questions regarding the accuracy of patient outcomes and survival being published within the literature.

4.2 Aims

In this single institution experience of a large cohort of ampullary adenocarcinoma, resected according to a standardized surgical strategy, we examined, in the context of a robust protocolised pathological method, the relative prognostic importance of pathological factors, in particular, resection margin status and morphological subtype, and furthermore evaluated the pattern and determinants of disease recurrence.

4.3 Methods

Resectional surgery was performed in the West of Scotland Pancreatic Unit, Glasgow Royal Infirmary, UK, during a twenty-four year period (January 1990 to May 2014). This analysis was limited to patients undergoing either classical (Whipple resection) or pylorus-preserving PD for ampullary adenocarcinoma, performed by the same team of surgeons. Other lesions (e.g. pancreatic, duodenal or distal bile duct adenocarcinomas, mucinous cyst-adenocarcinomas or intraductal papillary mucinous neoplasms) were excluded.

The decision to perform resection was made by a multi-disciplinary team including surgeons, oncologists, radiologists and pathologists. The criteria for resectability included: a) CT evidence of localized tumour in the peri-ampullary region; b) no evidence of superior mesenteric vein occlusion or significant tumour narrowing; c) no overt arterial involvement. (227)

4.4 Operative Procedure

The following is the standard operative approach conducted by all hepatobiliary surgeons within Glasgow Royal Infirmary. An initial roof top abdominal incision is made to enter the peritoneal cavity with subsequent retraction aided by an Omnitract retractor fitted to the operative table. The mobilisation phase of PD begins as the lesser sac is entered by freeing the greater omentum from both the transverse and ascending colon and following this plane to the right. Division of the peritoneal reflection and caudal mobilisation of the hepatic flexure of the colon exposes Gerota's fascia overlying the right kidney and the anterior aspect of the

pancreas and duodenum. This often exposes the superior mesenteric vein (SMV) at the inferior border of the pancreatic neck adjacent to the uncinate process.The peritoneum to the lateral aspect of the second part of duodenum is divided to allow extended Kocherisation to the left lateral border of the aorta to mobilise the pancreatic head which is reflected medially and exposing the vena cava.

The porta hepatis is then explored and identification of the common hepatic artery is made. The dissection is continued distally to identify the right gastric and gastroduodenal arteries. The portal vein is also sought at this point. Depending upon technique, the distal stomach or proximal duodenum is then divided followed by the proximal jejunum. The third and fourth parts of the duodenum are then fully mobilised with division of the Ligament of Treitz and then reflected underneath the superior mesenteric artery and vein exposing the pancreatic body.

The pancreas is then transected at the level of the portal vein and the pancreatic head reflected laterally allowing for separation from the SMV, portal vein and their smll tributaries. With medial retraction of the SMV and portal vein confluence, the tissue between the pancreatic head and lateral wall of the SMA is freed with division of arterial branches as required.

The resection phase requires division of the distal stomach or duodenum, jejunum, small bowel mesentery, bile duct, pancreas and mesopancreas to allow complete excision. In cases of more advanced disease there may be requirement for portal vein, SMV or SMA resection with reconstruction. The gallbladder is routinely removed and the spleen less frequently so. Transection margin frozen section analysis are routinely performed in our institution to establish the presence of

residual disease, with further pancreatic body resection undertaken until negative histopathological status is obtained. Reconstruction requires a minimum of three surgical anastomoses, namely a hepatico-jejunostomy, pancreatico-jejunostomy and gastro-jejunostomy; however a further jejuno-jejunosotomy is performed in this institution to prevent problems with bile reflux (220).

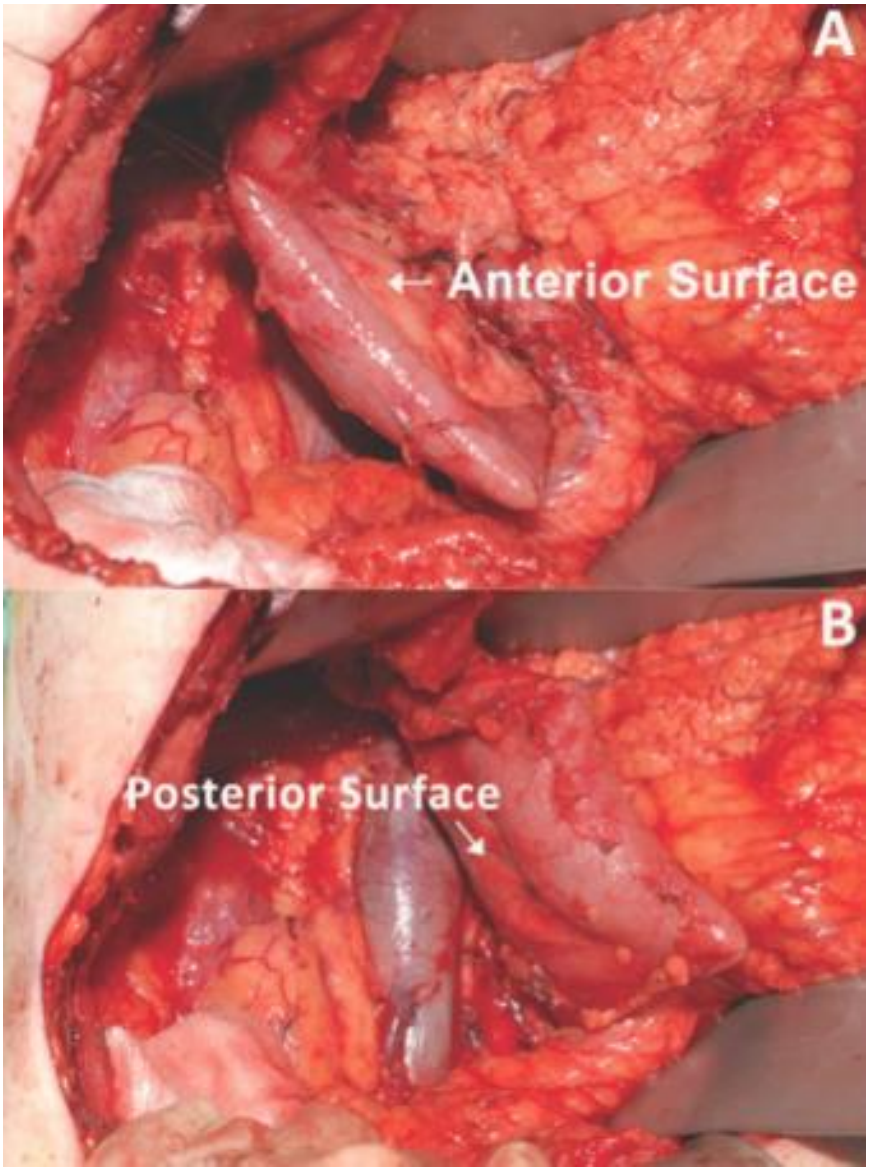


Figure 4.1a&b) Pancreatic head mobilisation

Follow-up comprised a standardized protocol of outpatient reviews. CT scans or other appropriate imaging modalities were performed whenever local–regional recurrence or distant metastatic disease was suspected; however, it is the case that our practice does not routinely perform postoperative imaging while the patient remains asymptomatic and not undergoing active treatment. Following recurrent disease, the patient was considered for chemotherapy if oncologically naïve or for rechallenge if they had received previous adjuvant chemotherapy. Detailed recurrence data was available in all except nine patients.

4.5 Pathology assessment

Ampullary tumours were defined as those unequivocally originating from the anatomical structures forming the ampulla of Vater. The pathology reports from all patients undergoing PD or PPPD during the study period for an ampullary tumour were undertaken by three senior pathologists (AKF, KO, FD) with both AFK and FD having led local standardisation and been co-authors of the British Royal College of Pathologists (RCPath) National Ampullary specimen guidelines (2002, 2019)(228). The gross and microscopic evaluation of PD resection specimens has been performed in this manner since 1996 with resection margins having not been inked prior to this time.

Clinico-pathological cohort data was collated in a prospectively maintained database including tumour stage, size, differentiation, perineural, lymphovascular invasion and resection margins status. All pathology was reviewed according to Tumour Node

Metastasis (TNM) staging in accordance with the UICC/AJCC (7th edition)(229) which corresponds to the RCPath guidelines.

Microscopic assessment and reporting included the maximum tumour diameter, the extent of local spread, tumour grade; perineural, venous and lymphatic invasion; and lymph node positivity. To my knowledge no extra or novel stains or solutions were used in this process other than those for standard reporting. Data for total number of resected lymph nodes and number of positive nodes were recorded. LNR was determined by dividing the number harboring metastasis by the total number of examined nodes. Tumour grade was categorized into high for poorly differentiated tumours and low for moderately and well-differentiated tumours.

Resection margin involvement (R1) was defined according to the RCPath guidelines as the presence of tumour at or in an involved lymph node within 1 mm of a margin when assessed by microscopy of haematoxylin and eosin (H&E) stained slides(228). On receipt of the specimen, this margin status analysis requires inking of the four pancreatic resection margins (pancreatic transection, medial, posterior and anterior surface) as described previously(220, 230). The medial margin is regarded as the tissue plane running alongside the lateral borders of the mesenteric vessels and the posterior margin comprises the smooth pancreatic surface lying behind the medial margin(220, 230) **Figure 4.2a&b**. This method of reporting was applied uniformly to ampullary resections in our institution throughout the study period under the supervision of the aforementioned pancreatic pathologists (AKF, FD, KO).

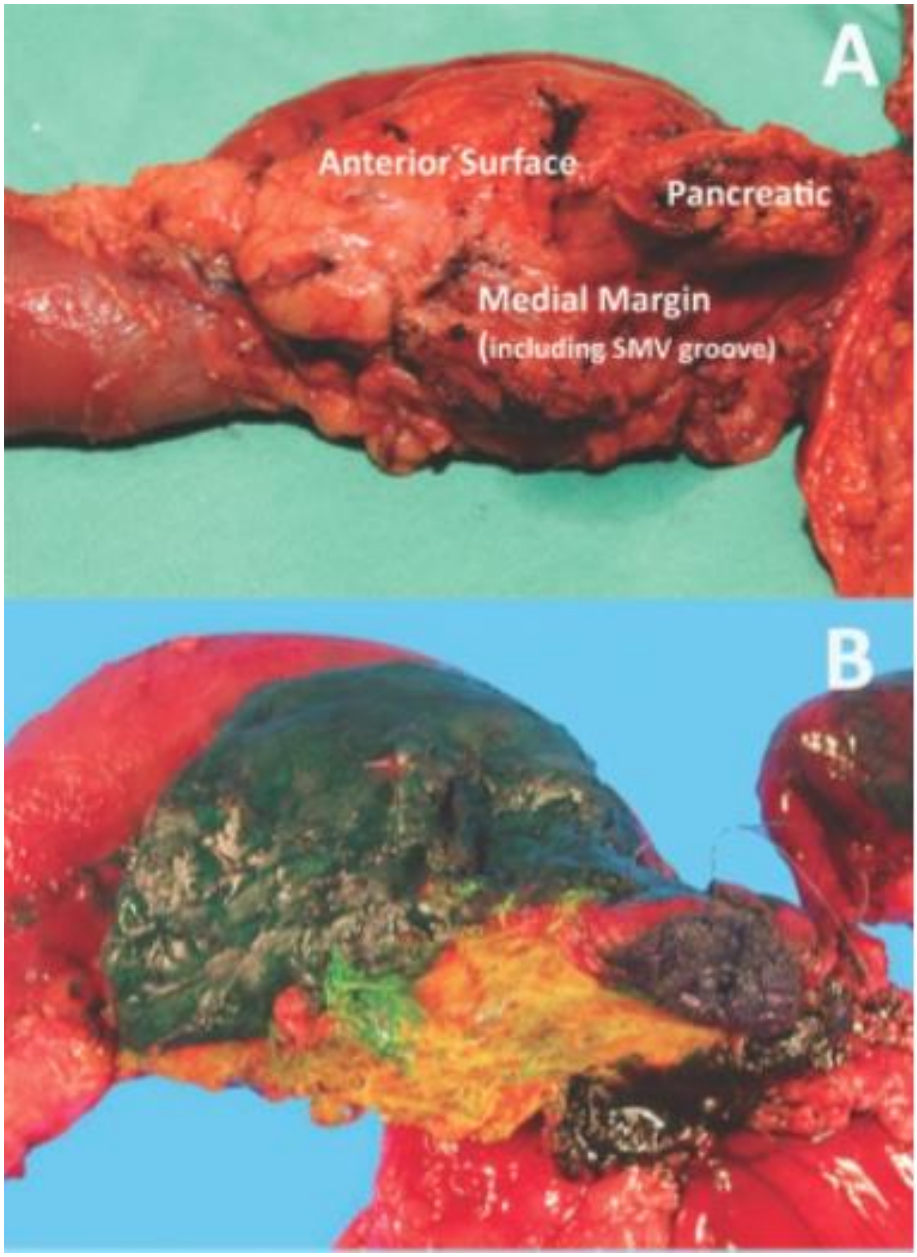


Figure 4.2a) Pancreatic resection specimen **b)** with margin inking

Regarding morphological subtyping a minimum of 4 H&E slides were reviewed for each case. All tumours were categorized according to the original criteria(25), later revised by Albores-Saavedra and co-workers(231). Tumours of the intestinal subtype had a more cribiform appearance macroscopically with tall/elongated pseudo-stratified columnar epithelium with elongated nuclei often found at the cytoplasmic

border of the nucleus. Pancreaticobiliary tumours had branching glands macroscopically with a simple cuboidal epithelium with no stratification and a single-layer of nuclei of differing sizes. Tumours found to have elements of both morphology were classified on the predominant features.

Patients were divided into four groups based on LNR, including those with no lymph node metastases (LNR = 0) and three groups with positive lymph nodes as follows: LNR = 0.01-0.2; LNR ≥ 0.2-0.4; and LNR > 0.4(23). Lymph node positive patients were additionally evaluated according to the number of involved nodes identified (1 - 3 and ≥ 4 nodes).

4.6 Statistics

Categorical variables were compared using the Mantel –Haenszel (χ^2) test. The Mann-Whitney U test was used to compare continuous variables following confirmation of a normal distribution of values. The principal outcome measure was length of survival as measured from the time of the original surgery. Length of survival following surgery and cause of death were obtained from our prospectively maintained database and validated using the NHS Scotland Information Services Department (http://www.isdscotland.org). Kaplan-Meier survival analysis was used to analyze the overall survival from the time of surgery. Patients alive at the time of follow-up were censored. The last follow-up period for patients still alive was June 2014. To compare the length of survival between curves, a log-rank test was performed. A Cox proportional hazards model was used for multivariate analysis to adjust for competing risk factors, and the hazard ratio (HR) with 95% confidence

intervals (CIs) was reported as an estimate of the risk of disease-specific death. Variables that were found to be significant on univariate analysis at $P < 0.10$ were included in multivariate analysis in a backwards stepwise fashion. The statistical significance for a test was set at a $P \leq 0.05$. All statistical analyses were performed using SPSS version 22.0 (IBM Corporation, NY, USA).

4.7 Results

4.7.1 Patient characteristics

From a total of 564 PDs performed, 127 patients were identified as having had a PD for ampullary adenocarcinoma with full clinicopathological follow up available. The 90-day mortality was 3.9% (5 patients) with these survival data being censored from analysis.

Patient demographics and tumour characteristics are summarized in **Table 1.** The median age was 64.0 years (range 35-80). There were 77 men (60.6%) and 50 women (39.4%). No vascular resections were required. 39 patients received adjuvant chemotherapy (30.7%). At a median follow up of 100.0 months, the median overall survival was 35 months (95% CI: 17.8-52.2). At the time of follow-up 51 patients (40.2%) remained alive following resection. 62 patients (48.8%) had died of recurrent disease. The deaths of 14 (11.0%) patients were not attributable to ampullary adenocarcinoma.

Table 4.1: Clinicopathological factors and outcome for patients with ampullary adenocarcinoma (n = 127)

	Total Cohort			Histological Intestinal Subtype			Histological Pancreaticobiliary Subtype		
	n = 127			n = 69			n = 58		
Variables	**No. (%)**	**Median OS**	***P* value Log-**	**No. (%)**	**Median OS**	***P* value Log-rank**	**No. (%)**	**Median OS**	***P* value Log-rank**
Sex									
Male	77 (60.6)	35.0		40 (58.0)	92.0		37 (63.8)	21.0	
Female	50 (39.4)	32.0	0.927	29 (42.0)	121.0	0.919	21 (36.2)	26.0	0.622
Age (years)									
Mean	63.1			61.9			64.5		
Median	64.0			62.0			66.5		
Range	35.0 – 80.0			35.0 – 80.0			43.0 – 77.0		
Outcome									
Follow-up (months)	0.3 – 289.0			0.3 – 289.0			0.4 – 164.0		
Median follow-up	100.0			104.0			90.5		
Death AC	62 (48.8)			28 (40.6)			34 (58.6)		
Death other	14 (11.0)			6 (8.6)			8 (13.7)		
Death unknown	0 (0)			0 (0.0)			0 (0.0)		
Alive	51 (40.2)			35 (50.7)			16 (27.6)		
Stage									
I	28 (22.1)	NR		18 (26.1)	NR		10 (17.2)	NR	
II	77 (60.6)	29.0		42 (60.9)	50.0		35 (60.3)	20.0	
III	22 (17.3)	21.0	<0.0001	9 (13.0)	27.0	0.001	13 (22.4)	12.0	0.049
T Stage									
T1	10 (7.9)			8 (11.6)			2 (3.4)		
T2	37 (29.1)	NR		22 (31.9)	NR		15 (25.9)	26.0	
T3	58 (45.7)			30 (43.5)			28 (48.3)		
T4	22 (17.3)	27.0	0.001[a]	9 (13.0)	35.0	0.009[a]	13 (22.4)	21.0	0.269[a]
N Stage									
N0	56 (44.1)	NR		35 (50.7)	NR		21 (36.2)	NR	
N1	71 (55.9)	20.0	<0.0001	34 (50.3)	27.0	0.003	37 (63.8)	14.0	<0.0001
LNR									
0	55 (43.3)	NR		34 (49.3)	NR		21 (36.2)	NR	
0.01 - 0.2	45 (35.4)	27.0		25 (36.2)	92.0		20 (34.5)	15.0	
≥ 0.2 - 0.4	20 (15.7)	14.0		7 (10.1)	26.0		13 (22.4)	14.0	
≥ 0.4	7 (5.5)	12.0	<0.0001	3 (4.3)	13.0	<0.0001	4 (6.9)	6.0	<0.0001
Lymph node burden									
0	52 (43.3)	NR		34 (49.3)	NR		22 (37.9)	NR	
1 – 3	38 (31.7)	25.0		21 (30.4)	92.0		19 (32.8)	15.0	
> 4	30 (25.0)	15.0	<0.0001	14 (20.3)	26.0	<0.0001	17 (29.3)	12.0	<0.0001
Differentiation									
Well	5 (3.9)			3 (4.3)			2 (3.4)		
Moderate	87 (68.5)	67.0		54 (78.3)	121.0		33 (56.9)	30.0	
Poor	35 (27.6)	19.0	0.001[b]	12 (17.4)	48.0	0.793[b]	23 (39.7)	12.0	0.001[b]
Tumour size									
≤ 20mm	72 (56.7)	48.0		38 (55.1)	NR		34 (58.6)	30.0	
> 20mm	55 (43.3)	29.0	0.107	31 (44.9)	55.0	0.380	24 (41.4)	14.0	0.026
Margins (≤ 1 mm)									
R0	87 (68.5)	55.0		54 (78.3)	NR		33 (56.9)	24.0	
R1	40 (31.5)	27.0	0.013	15 (21.7)	32.0	0.025	25 (43.1)	15.0	0.724
Subtype									
Intestinal	65 (51.2)	121.0							
Mixed	4 (3.1)								
Pancreaticobiliary	58 (45.7)	21.0	<0.0001[c]						
Perineural Invasion									
Negative	90 (70.9)	67.0		53 (76.8)	NR		37 (63.8)	24.0	
Positive	37 (29.1)	22.0	0.002	16 (23.2)	32.0	0.001	21 (36.2)	21.0	0.855

Venous Invasion									
Negative	90 (70.9)	69.0		51 (73.9)	NR		39 (67.2)	29.0	
Positive	37 (29.1)	17.6	<0.0001	18 (26.1)	32.0	0.018	19 (32.8)	12.0	0.002
Lymphatic invasion									
Negative	82 (64.6)	92.0		47 (68.1)	NR		35 (60.3)	30.0	
Positive	45 (35.4)	24.0	0.003	22 (31.9)	32.0	0.078	23 (39.7)	15.0	0.033
Chemotherapy									
Adjuvant	39 (30.7)	31.0		20 (29.0)	NR		19 (32.8)	30.0	
No Adjuvant	88 (69.3)	44.0	0.597	49 (71.0)	121.0	0.765	39 (67.2)	14.0	0.119

a) T1/2 *Vs* T3/4 for survival analyses based on AJCC TNM Staging System: Ampullary Cancer 7th Edition, 2009. b) Differentiation: Well/ Moderate (low grade) *Vs* Poor (high grade) for survival analyses. c) Intestinal and mixed subtype *Vs* pancreaticobiliary subtype for survival analyses. Comparison between intestinal and pancreaticobiliary phenotype (χ^2 test) # $P < 0.05$, ## $P < 0.005$. NR - Median survival time not reached.

4.7.2 Pathological factors

The median tumour size was 20 mm (range 5-110 mm). Most tumours were low grade (72.4%), T stage 3 or 4 (63.0%), node-positive (N1) (55.9%), with negative resection margins (68.5%). Perineural, venous and lymphatic invasion was present in 29.1%, 29.1% and 35.4% respectively. According to tumour morphology: 69 (54.3%) were of intestinal and 58 (45.7%) of pancreaticobiliary phenotype. The former were more likely to be lower grade ($P = 0.005$) and have R0 status ($P = 0.005$) according to χ^2 test analysis (**Table 4.1).** Four tumours had a mixed phenotype.

The overall median number of positive lymph nodes was 1 (range 1-16), while the median number in the N1 group was 3 (range 1-16). The median number of nodes evaluated was similar over the study period (1990-2002, 17 [Interquartile range (IQR) 12-21], *vs.* 2003-2013, 21 [IQR 17-26], $P < 0.0001$). A high LNR was associated with adverse pathological tumour characteristics including higher stage, higher grade, with perineural, venous and lymphatic invasion along with R1 status ($P < 0.05$). In contrast, patients with T1 disease had no evidence of lymph node metastases, perineural, venous, lymphatic invasion and were all R0 resections ($P < 0.0001$).

TABLE 4.2. Demographic, operative, pathologic and treatment characteristics by resection margin status in 127 patients undergoing resection for ampullary adenocarcinoma

	No. (%) patients		
	R0 Resection[a]	**R1 Resection[a]**	***P* value**
Total No. of patients	87 (68.5)	40 (31.5)	
Gender			
Female	34 (39.1)	16 (40.0)	0.922
Male	53 (60.9)	24 (60.0)	
Age (yrs)†			
Median	62.0	68.0	0.015
Mean	61.6	66.4	
Range	35.0-78.0	49.0-80.0	
Tumour stage			
T1	10 (11.5)	0 (0)	<0.0001
T2	31 (35.6)	6 (15.0)	
T3	38 (43.7)	20 (50.0)	
T4	8 (9.2)	14 (35.0)	
Lymph node status			
N0	48 (55.2)	8 (20.0)	<0.0001
N1	39 (44.8)	32 (80.0)	
Tumour size (mm)[b]			
Median	18.0	25.0	0.003
Mean	20.9	27.2	
Range	5-55	10-110	
Tumour grade			
Low	62 (71.3)	30 (75.0)	0.662
High	25 (28.7)	10 (25.0)	
Perineural invasion			
No	69 (79.3)	21 (52.5)	0.002
Yes	18 (20.7)	19 (47.5)	
Venous invasion			
No	70 (80.5)	20 (50.0)	<0.0001
Yes	17 (19.5)	20 (50.0)	
Lymphatic invasion			
No	60 (69.0)	22 (55.0)	0.126
Yes	27 (31.0)	18 (45.0)	
Morphological subtype			
Intestinal	54 (62.1)	15 (37.5)	0.010
Pancreaticobiliary	33 (37.9)	25 (62.5)	
Adjuvant chemotherapy			
No	65 (74.7)	23 (57.5)	0.051
Yes	22 (25.3)	17 (42.5)	

a) Resection margin positive (R1) if microscopic evidence of tumour ≤ 1 mm from any pancreatic margin. b) Mann-Whitney U test used.

Clinicopathological and treatment characteristics were evaluated according to resection margin status in **Table 4.2.** Patients with R1 tumours (31.5%) in addition to more frequently demonstrating a pancreaticobiliary phenotype were larger (P = 0.003), with higher T stage ($P < 0.0001$), more likely to be lymph node positive ($P <$ 0.0001), with more frequent perineural ($P = 0.002$) and venous invasion ($P <$ 0.0001).

4.7.3 Clinicopathological variables associated with survival

For the entire cohort of 127 patients according to univariate analysis, standard clinicopathological variables associated with reduced overall survival included higher AJCC stage ($P < 0.0001$), higher T stage ($P = 0.001$), lymph node status ($P < 0.0001$), high grade ($P = 0.001$), R1 status ($P = 0.013$), perineural ($P = 0.002$), venous ($P <$ 0.0001), lymphatic invasion ($P = 0.003$), pancreaticobiliary phenotype ($P <$ 0.0001)(**Table 3.1**). Survival at 3yrs, 5yrs and 10 yrs was 49%, 42% and 36% respectively.

Patients with N0 disease had a significantly prolonged survival compared to N1 patients (median survival not reached [NR] versus 20.0 months, 95%CI: 14.1-29.9, $P < 0.0001$). The N1 group was stratified with those patients with 1-3 positive nodes having a prolonged overall survival compared to those with ≥ 4 nodes involved (25.0 months, 95%CI: 15.5-34.5 versus 15.0 months 95%CI: 8.1-21.9, $P < 0.0001$). LNR also successfully stratified outcome as patients with an LNR of ≥ 0.4 or ≥ 0.2-0.4 had a reduced overall survival (12.0 months, 95%CI: 3.6-20.4 versus 14.0 months, 95%CI: 6.8-21.2 months respectively), compared to patients with an LNR of 0.01-0.2 (27 months, 95%CI: 18.5-35.5 months $P < 0.0001$). In the R0 cohort (68.5%), those with

no lymph node metastases had a 5 yr overall survival of 66%. While resection margin involvement had a negative impact on survival for the T1/T2 stage tumours (R0 [NR] *vs.* R1 [14.0 months, $P < 0.0001$]) this effect was not evident in higher stage T3/T4 tumours ($P = 0.739$).

According to morphological phenotype the median overall survival following resection for patients with an intestinal phenotype tumour was 121.0 months in comparison to 21.0 months (95%CI: 11.0-31.0) for the pancreaticobiliary subtype ($P < 0.0001$) (**Table 4.1**). For the intestinal phenotype: AJCC stage, T stage, lymph node status, LNR, lymph node burden, resection margin status, perineural, venous and lymphatic invasion were significant predictors of survival (**Table 4.1**, **Fig. 4.3).** Survival at 3 years, 5 years and 10 years was 63%, 54% and 46% respectively. For the pancreaticobiliary phenotype: AJCC stage, lymph node status, LNR, lymph node burden, tumour grade, tumour size, venous invasion and lymphatic invasion were prognostic factors on univariate analysis **(Table 4.1, Fig. 4.4).** Survival at 3 years, 5 years and 10 years was 29%, 26% and 22% respectively.

Figure 4.3 Kaplan Meier graphs demonstrating clinicopathological factors influencing survival stratified by intestinal phenotype

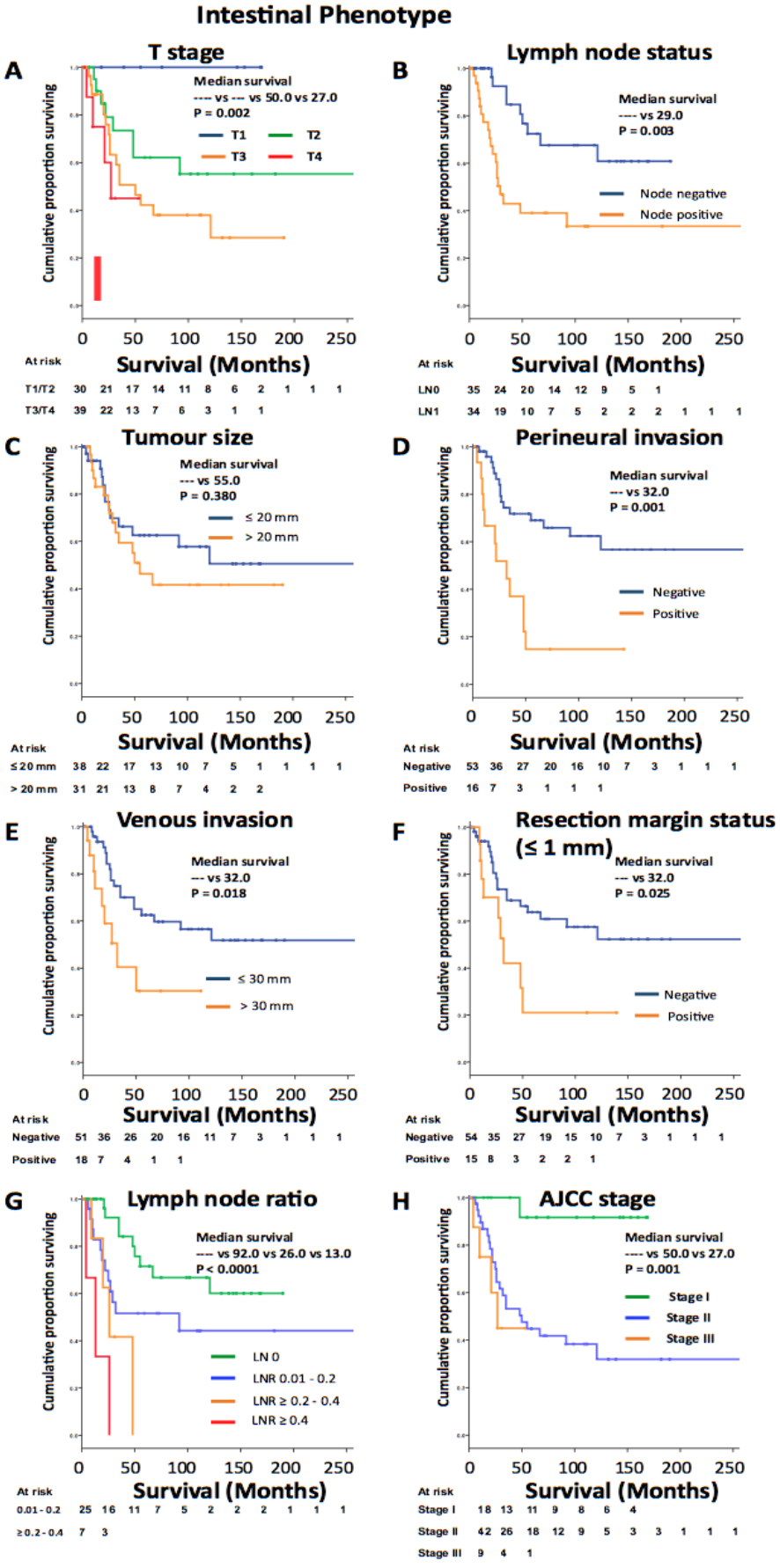
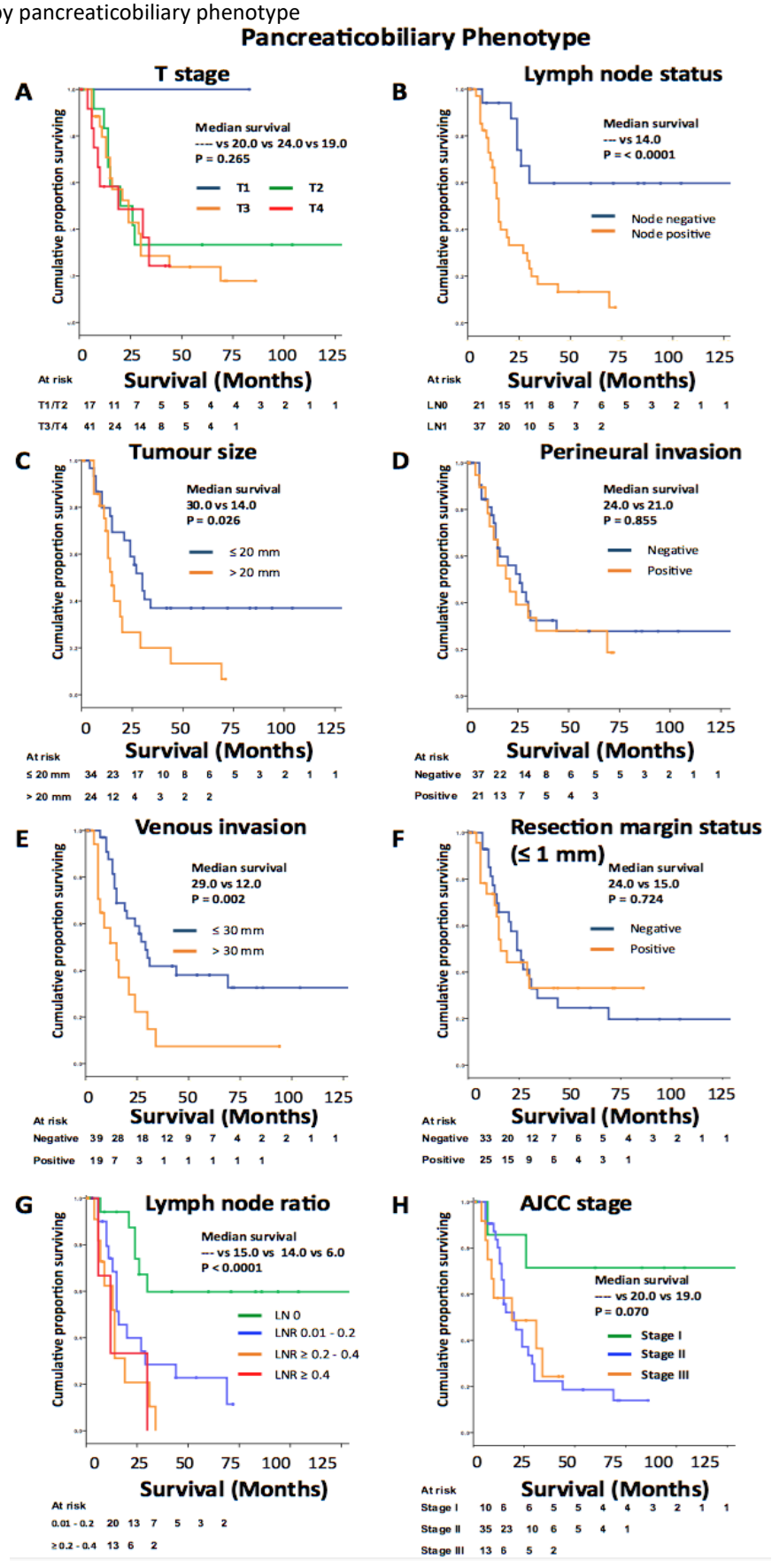


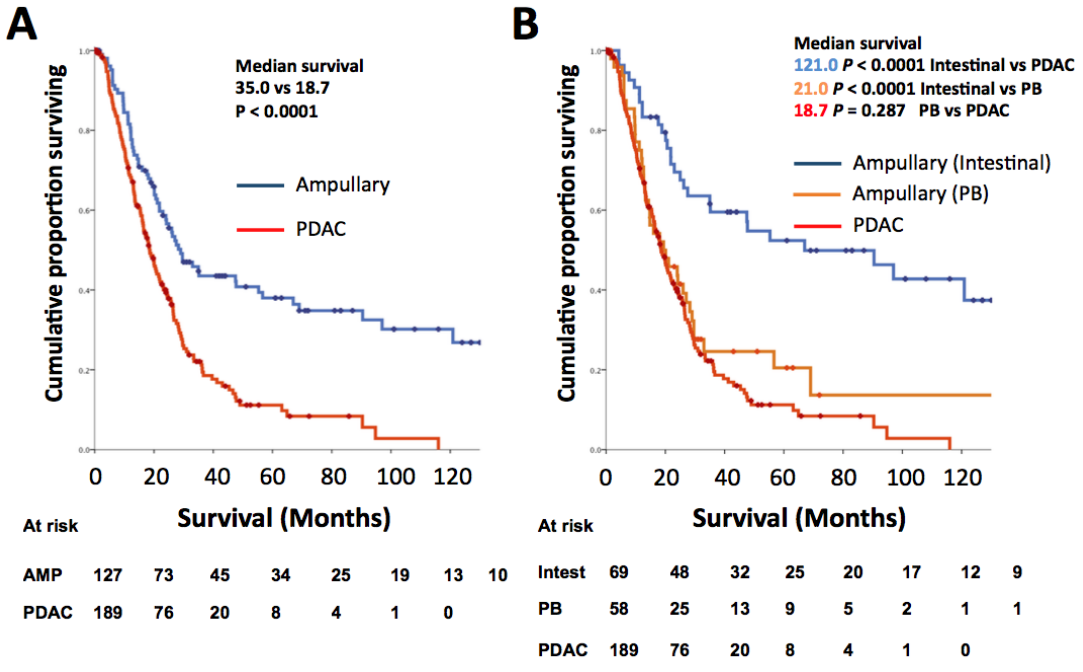
Figure 4.4 Kaplan Meier graphs demonstrating clinicopathological factors influencing survival stratified by pancreaticobiliary phenotype



While adjuvant chemotherapy failed to impact on survival for the overall cohort, for the lymph node-positive patients, chemotherapy (n = 26) improved outcome compared to those who did not receive adjuvant therapy (n = 45) (29.0 months, 95%CI: 16.0-42.0 *vs.* 14.0 months, 95%CI: 10.0-17.9, *P* = 0.015). Adjuvant chemotherapy failed to impact significantly on survival in either intestinal (*P* = 0.765) or pancreaticobiliary (*P* = 0.119) subgroups. Limited cohort size prevented evaluation of different adjuvant chemotherapy regimens (Gemcitabine *vs.* 5FU) according to morphological subtype.

Patients with ampullary adenocarcinomas had a prolonged overall survival compared to a similarly managed PDAC cohort (35.0 *vs.* 18.6 months, *P* < 0.0001) (**Fig. 4.5A**) (230). However, the overall survival of pancreaticobiliary phenotype patients does not significantly differ from the PDAC cohort (21.0 *vs.* 18.7 months, *P* = 0.278) (**Fig. 4.5B**).

Figure 4.5 A) Kaplan Meier graph demonstrating AA survival compared to PDAC and **B)** comparison of survival to PDAC when AA stratified by intestinal and pancreaticobiliary phenotypes



4.7.4 Multivariate analysis

Multivariate Cox proportional hazards models were created including univariate prognostic factors. For the overall cohort (n = 127) factors independently associated with poor outcome included: lymph node involvement (HR = 4.13, 95%CI: 2.30-7.43, *P* < 0.0001), venous invasion (HR = 2.97, 95%CI: 1.78-4.96, *P* = 0.002), and pancreaticobiliary phenotype (HR = 2.47, 95%CI: 1.50-4.08, *P* = 0.001) (**Table 4.3**).

Table 4.3 Predictors of survival following pancreaticoduodenectomy using multivariate Cox regression analysis for 127 patients with ampullary adenocarcinoma

Multivariate		**Overall survival**	
		HR (95% CI)	***P* value**
Patient Related Factors			
Lymph node status	N0/ N1	4.13 (2.30-7.43)	< 0.0001
Morphological Subtype	Intestinal/ Pancreaticobiliary	2.47 (1.50-4.08)	0.001
Venous invasion	Absent/ Present	2.97 (1.78-4.96)	0.002
Tumour stage	T1/T2 *Vs* T3/T4	2.49 (1.40-4.40)	0.203
Lymphatic invasion	Absent/ Present	2.13 (1.30 3.49)	0.297
Tumour grade	Low/ High	0.44 (0.26-0.72)	0.306
Margin status (1 mm)	R0/ R1	1.91 (1.15-3.17)	0.526
Perineural invasion	Absent/ Present	2.15 (1.30-3.56)	0.952

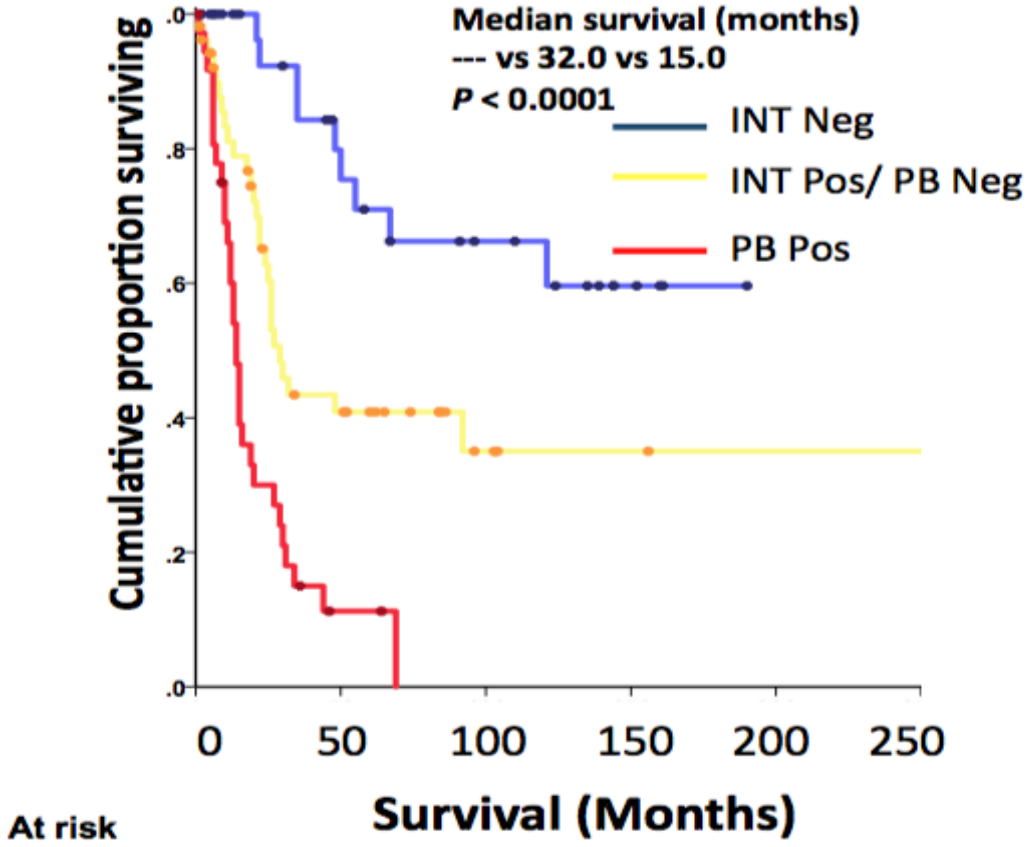
Following recalculation of univariate prognostic factors according to histological phenotype, for the intestinal phenotype tumours, independent predictors of poor overall survival were: lymph node involvement (HR = 3.23, 95%CI: 1.45-7.22, *P* = 0.003); high tumour stage (HR = 2.83, 95%CI: 1.24-6.50, *P* = 0.028); and perineural invasion (HR = 3.87, 95%CI: 1.80-8.28, *P* = 0.002) **(Table 4.4).** For the pancreaticobiliary phenotype tumours, the independent prognostic predictors of poor outcome were: lymph node involvement (HR = 4.52, 95%CI: 1.86-10.98, *P* < 0.0001) and venous invasion (HR = 2.95, 95%CI: 1.51-5.75, *P* = 0.005) (**Table 4.4**).

Table 4.4. Predictors of survival following pancreaticoduodenectomy using multivariate Cox regression analysis for A) intestinal and B) pancreaticobiliary ampullary adenocarcinoma cohorts

Multivariate		**Overall survival**	
Intestinal phenotype (n = 65)		**HR (95% CI)**	***P* value**
Perineural invasion	Absent/ Present	3.87 (1.80-8.28)	0.002
Lymph node status	N0/ N1	3.23 (1.45-7.22)	0.003
Tumour stage	T1/ T2 *Vs* T3/ T4	2.83 (1.24-6.50)	0.028
Venous invasion	Absent/ Present	2.67 (1.18-6.02)	0.386
Lymphatic invasion	Absent/ Present	1.96 (0.93-4.17)	0.603
Margin status (1 mm)	R0/ R1	2.50 (1.12-5.59)	0.645
Pancreaticobiliary phenotype (n = 55)			
Lymph node status	N0/ N1	4.52 (1.86-10.98)	<0.0001
Venous invasion	Absent/ Present	2.95 (1.51-5.75)	0.005
Tumour size (mm)	< 20/ ≥ 20	2.15 (1.10-4.20)	0.122
Lymphatic invasion	Absent/ Present	2.06 (1.06-3.99)	0.166
Tumour grade	Low/ High	0.32 (0.17-0.63)	0.218

A combination of histological phenotype along with the most powerful independent prognostic factor, lymph node status, enabled stratification of the cohort into three prognostic groups. First, those patients with an intestinal phenotype (n = 35) and no lymph node involvement had an excellent prognosis, with a 5-year survival of 72.0% (median survival not yet reached). Second, the group of patients with a pancreaticobiliary phenotype and lymph node metastases (n = 37) had a poor prognosis, with a 5-year survival of 11.0% and median survival of 15.0 months (95%CI: 12.9-17.1 months). Third, the remaining intestinal phenotype patients with lymph node metastases or pancreaticobiliary phenotype with no lymph node involvement [n = 55]) had an intermediate prognosis, with a 5-year survival of 46.0% and median survival of 32.0 months (95%CI: 5.9-66.1 months) **(Figure 4.6).**

Figure 4.6 Kaplan Meier graph comparing survival in the intestinal LN negative group, intestinal LN positive & pancreaticobiliary LN negative group and the pancreaticobiliary LN positive group



At risk											
INT Neg	35	24	20	14	12	9	5	1	0		
INT Pos/ PB Neg	55	30	17	12	7	3	3	2	1	1	1
PB Pos	37	10	3	0							

4.7.5 Time variable calculation

As data was collected over a 24 year period a time variable calculation was performed to ensure there was no effect on survival over time. The cohort was dichotomised with early patients operated on from 1990 to 2006 and the later patients from 2007 to 2014.

On univariate analysis for patients with an intestinal phenotype there was no significant relationship between the time of resection (early v late) and survival (P=0.891). Therefore, time of resection was not added into the multivariate analysis for further assessment.

On univariate analysis for patients with a pancreaticobiliary phenotype there was a minimally significant relationship between time of resection and survival (P=0. 075). This was therefore included in the multivariate analysis from **Table 4.4** but was found not to be an independent predictor of overall survival and did not affect lymph node status or venous invasion as being independently significant.

4.7.6 Pattern of Recurrence

From the 62 (48.8%) cancer specific deaths, full disease recurrence data was available for 53 patients. A single site of initial recurrence was seen in 36 (67.9%) patients with multiple sites affected in the remaining 17 (32.1%) **(Table 4.5).** Metastatic disease involved various sites including 32 (60.4%) patients with any liver metastases, 20 (37.7%) liver metastases as the only site, 18 (34%) locoregional recurrences, with 12 (22.6%) lung, 5 (9.4%) bone, 4 (7.5%) peritoneal, 2 (3.8%) brain

and 5 (9.4%) metastases at other sites including skin and adrenals. 5 (9.4%) patients developed liver and locoregional recurrence simultaneously.

We analyzed the pathological factors that potentially influenced site of recurrence (**Table 4.5).** Liver metastases were associated with the presence of venous ($P < 0.05$) and lymphatic invasion ($P < 0.05$). Local recurrence was associated with lymph node involvement ($P = 0.008$). Furthermore local recurrence was more likely as lymph node burden ($P = 0.023$) or LNR increased ($P = 0.027$). Although bone metastases were rare (9.4%), all occurred following a resection for pancreaticobiliary subtype tumour ($P = 0.03$). Resection margin status did not correlate with any pattern of recurrence. It was noted that T1 tumours demonstrated no metastatic potential with no recurrence seen within this subgroup.

During the follow-up period 10 (7.9%) patients developed a secondary malignancy including 4 (3.1%) breast cancers, 2 (1.6%) NSCLCs, 2 (1.6%) prostate cancers, 1 (0.8%) follicular lymphoma and 1 (0.8%) oesophageal.

TABLE 4.5 Demographic, operative, pathologic and treatment characteristics by disease recurrence in 53 patients with metastatic disease following resection for ampullary adenocarcinoma

	Metastatic Disease Location n (%)									
	Local Recurrence		**Liver Metastases**		**Lung Metastases**		**Bone Metastases**		**Peritoneal Disease**	
Total No. of patients	18 (34.0)		32 (60.4)		12 (22.6)		5 (9.4)		4 (7.5)	
Tumour stage										
T1	0 (0.0)	*0.372*	0 (0.0)	*0.645*	0 (0.0)	*0.072*	0 (0.0)	*0.451*	0 (0.0)	*0.190*
T2	5 (27.8)		9 (28.1)		2 (16.6)		2 (40.0)		0 (0.0)	
T3	8 (44.4)		18 (56.3)		5 (41.7)		3 (60.0)		4 (100)	
T4	5 (27.8)		5 (15.6)		5 (41.7)		0 (0.0)		0 (0.0)	
Lymph node status										
N0	0 (0.0)	***0.008***	6 (18.8)	*0.657*	2 (16.6)	*0.691*	1 (20.0)	*0.965*	2 (50.0)	*0.134*
N1	18 (100)		26 (81.2)		10 (83.4)		4 (80.0)		2 (50.0)	
Tumour size (mm)										
<20	8 (44.4)	*0.630*	17 (53.1)	*0.465*	6 (50.0)	*0.941*	5 (100)	***0.017***	0 (0.0)	***0.041***
>20	10 (55.6)		15 (46.9)		6 (50.0)		0 (0.0)		4 (100)	
Tumour grade										
Low	10 (55.6)	*0.912*	17 (53.1)	*0.528*	9 (75.0)	*0.144*	2 (40.0)	*0.431*	4 (100)	*0.069*
High	8 (44.4)		15 (46.9)		3 (25.0)		3 (60.0)		0 (0.0)	
Perineural invasion										
No	10 (55.6)	*0.776*	18 (56.3)	*0.538*	6 (50.0)	*0.823*	3 (60.0)	*0.736*	1 (25.0)	*0.246*
Yes	8 (44.4)		14 (43.7)		6 (50.0)		2 (40.0)		3 (75.0)	
Venous invasion										
No	8 (44.4)	*0.089*	16 (50.0)	***0.050***	4 (33.3)	***0.029***	3 (60.0)	*0.986*	2 (50.0)	*0.659*
Yes	10 (55.6)		16 (50.0)		8 (66.7)		2 (40.0)		2 (50.0)	
Lymphatic invasion										
No	9 (50.0)	*0.621*	14 (43.7)	*0.048*	5 (41.7)	*0.302*	3 (60.0)	*0.803*	2 (50.0)	*0.844*
Yes	9 (50.0)		18 (56.3)		7 (58.3)		2 (40.0)		2 (50.0)	
Morphological subtype										
Intestinal	6 (33.3)	*0.210*	15 (46.9)	*0.774*	7 (58.3)	*0.302*	**0 (0.0)**	***0.033***	2 (50.0)	*0.844*
Pancreaticobiliary	12 (66.7)		17 (53.1)		5 (41.7)		5 (100)		2 (50.0)	
Adjuvant chemotherapy										
No	12 (66.7)	*0.888*	20 (62.5)	*0.296*	7 (58.3)	*0.418*	3 (60.0)	*0.690*	4 (100)	*0.153*
Yes	6 (33.3)		12 (37.5)		5 (41.7)		2 (40.0)		0 (0.0)	

4.8 Discussion

Understanding the prognostic influence of clinicopathological factors in a disease with a broad range of outcomes such as adenocarcinoma of the ampulla of Vater is of considerable importance. Enabling patients to be better selected for surgery, justify the allocation of significant adjuvant therapy if aggressive pathology findings are present or enable the expectation of good prognosis if sinister features are absent is possible.

We have focused upon the survival of a cohort of 127 patients with resected primary ampullary adenocarcinoma treated over three decades at a single institution. A relatively consistent surgical approach was accompanied by careful pathological review with uniform tumour classification according to the AJCC staging system (7th edition)(229) along with a detailed standardized specimen preparation protocol with focus on resection margin status, using evidence of microscopic disease within 1mm of the resection margin as an R1 specimen.

The results from our single centre cohort are comparable to those seen from our meta-analysis of the reviewed literature. Our data demonstrated statistically significant survival differences in T stage, N stage, margin status, morphological subtype, perineural, lymphatic and venous invasion which is in keeping with other studies. When further interrogated using cox regression survival analysis all the aforementioned HRs were similar except lymph node positivity had a stronger effect on survival in our cohort (HR 4.13 versus HR 1.75). This is also true for morphological subtype (HR 2.47 versus HR 1.84). An R1 resection margin demonstrated an increased risk in the meta-analysis (HR 1.91 versus HR 2.36) and may be attributable

to the heterogenic method of pathological reporting of margin status in the literature. Overall, our results can be validated from an original meta-analysis with large patient numbers.

The current study validates the current AJCC staging system for ampullary adenocarcinoma demonstrating sufficient survival discrimination between tumour stages. Yet traditional staging criteria do not adequately stratify all patients, as up to 60% of patients will eventually succumb to recurrent disease, with some subgroups identified as having particularly poor prognosis(81, 192). In the present study, perineural invasion was not an independent prognostic factor as has been proposed previously(225) which is consistent with our meta-analysis as marked heterogeneity was apparent following evaluation. Nodal involvement however, significantly reduced overall survival from a 5yr survival of 66% to 22%, one of the largest survival differences reported and approximate a large population based study(24). Due to the impact of lymph node involvement, some have advocated extended lymphadenectomy for PDAC and ampullary adenocarcinoma to improve pathological staging and survival, however such extended resections have failed in subsequent randomized trials(132).

As in PDAC(232), N1 patients are not homogenous with regard to outcome therefore other prognostic refinements must be considered. We did not identify any evidence of lymph node spread in patients with T1 disease regardless of morphological subtype. This has important implications for endoscopic ampullectomy, as nodal spread may be less common than previously reported. This contrasts with a recent single institution assessment of 350 ampullary adenocarcinoma resections that

identified a 28% N1 rate in these early stage patients(19). This difference may simply be explained by the low frequency of T1 lesions (n = 10) in the present study.

In the node-positive patients, survival could be stratified by both lymph node burden and LNR, with high LNR cases associated with a more aggressive pathological phenotype. Importantly, when analysed separately both remained independent prognostic factors. The findings from our cohort are in keeping with those from the meta-analysis which found LN positivity to be a factor of poor prognosis; however, a LN burden of >3 or a high LNR showed much stronger correlation to poor survival, both possessing HRs of 8.18 $p<0.00001$.

Recently, the SEER database of 678 resected ampullary cancers was used to determine the most appropriate lymph node cut-offs(233). 54% were N0 with a 5-year survival of 77%, compared to 43% for those with 1-3 nodes and 7% for > 3 nodes involved. The authors concluded that the total number of positive nodes and not LNR was the most powerful prognostic nodal variable(229). Conversely Falconi and co-workers concluded that LNR independently predicted survival(210). The median number of retrieved lymph nodes in our study (n = 19) was greater than in the previous studies(203, 210) suggesting high quality lymphadenectomy at our institution. It is likely that within future pathological reporting datasets lymph node burden along with minimal adequate total lymph node retrieval standards will serve to enhance stratification.

Although IHC evaluation of caudal-related homeobox 2 (CDX2), cytokeratin and apomucin expression variation can differentiate intestinal and pancreaticobiliary phenotypes and has yielded prognostic information(31, 35, 39, 234, 235) simple

histological evaluation is often overlooked(236). While initial analysis of subtype morphology revealed no significant variation in pathological features(200) an increased frequency of lymphatic, vascular and perineural invasion was demonstrated recently(237). We identified intestinal type tumours were of lower grade and significantly less likely to be R1 resections. Perineural invasion had a similar frequency between subtypes, although had prognostic impact only in the intestinal subtype. Despite these associations morphological subtyping provided strong independent prognostic information and therefore further biological explanation is required to account for the variation in outcome.

Although not all studies have identified histological subtype as prognostic, many were under-powered(29, 32, 34, 238) with most recent studies including our meta-analysis supporting our data(25, 33, 195, 200, 239). Considering the histological subtypes separately, multivariate analysis yielded differing results, confirming divergent clinicopathological behaviour. In particular T stage, lymph node spread and perineural invasion were independent predictors of poor outcome in the intestinal subtype compared to lymph node spread and venous invasion in the pancreaticobiliary subtype. Our results indicate that more valuable prognostic information is obtained from morphological subtyping than provided by tumour stage or tumour grade. Certainly standardized reporting of these pathological criteria should be incorporated within future standard (UICC/AJCC) staging classification.

The overall survival of intestinal and pancreaticobiliary subtypes shows marked heterogeneity (**Fig 4.5b**) with median overall survival of 121 months versus 21

months ($p<0.0001$). The pancreaticobiliary subtype shows a marked similarity to PDAC in the survival curve with no significance in survival seen (21 months versus 18.7 months $p=0.287$). This raises the question as to whether ampullary cancer is in fact two separate disease processes and as pancreaticobiliary subtypes show great similarity to PDAC they should be treated as such and considered for neoadjuvant chemotherapy regimes such as FOLFIRONOX (combination folinic acid, 5-fluorouracil, irinotecan and oxaliplatin regarded as first line in PDAC). Again, the marked variance in survival also questions whether subtype and not origin is a more important classification tool for biliary cancers.

These data suggest a high (31.5%) R1 rate, in excess of that previously reported throughout the literature (0-25%)(19, 23, 32, 84, 200, 203, 206, 239) and from our meta-analysis (median 3.2%, 0-25%). We believe this reflects our use of a SP alongside high-quality pathological specimen examination in accordance with the British RCPath guidelines rather than inadequate operative resection with representative survival figures seen within this cohort similar to our previous findings for PDAC(220). A prospective evaluation of resection margin involvement which employed a similar ≤ 1 mm R1 clearance definition, margin inking and standardized specimen examination technique identified a comparable figure of R1 involvement (27%)(217).

While patients with R1 resections had tumours with detrimental pathological features including lymph node positivity, perineural and vascular invasion, it would appear that resection margin involvement is not as powerful a prognostic factor in the overall ampullary adenocarcinoma cohort as in PDAC(218). This may be

explained by the anterior and posterior circumferential margins being the most commonly involved margins owing to the location of ampullary tumours at the anatomical crevice between the duodenal wall and the posterior and anterior aspect of the pancreatic head. We have previously reported that involvement at these 'mobilization' margins has lesser prognostic significance in PDAC(220). Nevertheless, the negative prognostic impact of anterior margin involvement in this cohort underlines the importance of rigorous pathological assessment at all circumferential and transection margins. Interestingly, in the intestinal subtype, R1 involvement did compromise survival although was not an independent prognostic factor. However, R1 status was not a prognostic factor in the pancreaticobiliary subtype cohort.

At present there is no proven role for adjuvant therapy in ampullary adenocarcinoma and as such our cohort did not receive a standardized adjuvant regimen nor did morphological subtype alter adjuvant strategy. The ESPAC-3 adjuvant therapy trial for periampullary cancer revealed a negative result(169). The potential benefit of adjuvant chemotherapy was only demonstrated in multivariate analysis when adjusted for other prognostic variables, suggesting that poor-prognosis tumours were potentially associated with responsiveness. The studies included in our meta-analysis demonstrated a wide variation in use of adjuvant chemotherapy between 6.3% and 67% with no clear consensus in use. Again, there was no significant outcomes in any of the included studies.

The evidence from single-institutional studies supports this, with a recent chemoradiation evaluation demonstrating that in N1 patients, survival was prolonged(168). Only 31% received adjuvant chemotherapy in the current study, but

as shown previously(168) we found adjuvant chemotherapy provided a small but significant benefit for the N1 group only, although this may simply reflect selection bias. While adjuvant chemotherapy had limited impact according to morphological subtype, potentially gemcitabine regimens may be more effective in the pancreaticobiliary subtypes with 5FU beneficial to those patients with intestinal subtypes.

This potential heterogenicity in response may make it difficult to detect a statistically significant difference in clinical trials of unselected patients. Certainly posthoc analysis based on histological subtype alone in the ESPAC3 study did not identify differential treatment responsiveness. Unfortunately cohort size did not allow stratification by chemotherapy regimen in the present study.

The predictors for pattern of recurrence vary greatly in the ampullary cancer literature. An R1 resection did not affect the pattern of first recurrence. Specifically, margin positivity did not result in greater frequency of local or regional recurrences. Consistent with the literature, the most common metastatic sites were the liver followed by locoregional and lung. Indeed, more than a half of patients with recurrence died with the presence of liver metastases. We encountered a higher level of lung metastases than reported previously. Lymph node metastases were strongly associated with local recurrence while distant metastases, particularly liver and lung, were associated with venous and lymphatic invasion as identified previously(88). Further emphasizing the importance of morphological subtyping, we noted for the first time, patients succumbing to bone metastases all had pancreaticobiliary subtype tumours. These data suggest potential for modification

of follow-up regimens to account for variation in pattern of recurrence according to histological subtype as well as ensuring adequate thoracic imaging to identify frequent pulmonary recurrence.

In view of the recently documented PDAC intestinal variant(33, 231) potentially the challenge of differentiating the precise anatomical origin of a periampullary lesion may be less important than classification according to histological phenotype for the purpose of prognosis and management(219).

An important result of the present study is the favorable overall outcome associated with resected ampullary adenocarcinoma. For N0 patients with clear resection margins, 5-year survival was 66%. The absence of lymph metastases and intestinal phenotype identifies patients with an overall survival approaching 70% at 10 years suggesting long-term survival may only be possible if an intestinal phenotype is identified. These figures are encouraging and highlight the importance of early identification of periampullary malignancy, accurate morphological phenotyping along with the minimization of morbidity in the post-operative period. The favorable prognosis of intestinal tumours may have implications for more aggressive endoscopic management of this subgroup. Furthermore, preoperative MRI scanning has potential to differentiate morphological phenotype(240). Chung et al analysed 50 ampullary carcinomas with 15 of intestinal phenotype on immunohistochemistry. Of these 15, all were nodular, 87% were isointense and 73% demonstrated an oval filling defect on standard MR and MRCP imaging. Unfortunately MRI was not routinely available for our cohort.

Although limited to 127 PD resections, this represents a large cohort of true ampullary adenocarcinoma with complete follow-up data. More comprehensive analysis will only be achieved via multi-centre studies(239). The quality of pathological assessment is highlighted by the high R1 rate (32%) supported by our previous PDAC assessment (74%)(220). While morphological subtype classification requires subjective assessment, the frequency matches the prior literature. Molecular marker evaluation may yet improve classification reproducibility, however, high quality morphological assessment along with careful lymph node examination can yield excellent prognostic discrimination. In the future, genomic sequence profiling will undoubtedly provide further molecular insight into these subtypes.

4.9 Conclusions

In summary, this validated single centre analysis of resected ampullary adenocarcinoma demonstrates that long-term survival can be expected for patients with intestinal phenotype and lymph node negative tumours. It questions whether pancreaticobiliary subtype tumours should be treated in a similar manner to PDAC due to their similar characteristics and survival. While R1 status is more common than previously reported, standard pathological factors must be considered in the context of morphological phenotype. Notably, site of recurrence was related to the tumour phenotype. These findings lend support to the routine integration of phenotype classification in pathological reporting, appropriate stratification of future

adjuvant therapy trials and increasingly detailed molecular characterization of ampullary adenocarcinoma.

5.0 ROLE OF ENDOSCOPIC AMPULLECTOMY IN THE DIAGNOSIS AND MANAGEMENT OF AMPULLARY LESIONS

Chapter 5

The role of endoscopic ampullectomy in the diagnosis and management of ampullary lesions

5.1 Introduction

Ampullary lesions have historically been diagnosed by histological examination of biopsy specimens to assess the cellular origin of the lesion and the extent of dysplasia. Before the availability of endoscopic ampullectomy as treatment option, the majority of patients diagnosed with an ampullary adenoma underwent surgical resection as it was regarded as the sole intervention option without a significant recurrence risk.

Such major surgery was undertaken as it has been demonstrated that ampullary adenomas have malignant potential and adenoma to carcinoma progression exists within these lesions, similar to that seen in the colon(14). The transformation of an adenoma to in-situ or invasive carcinoma has been reported in the region of 25-85%(17) Confounding this, the accuracy of diagnostic biopsy specimens have been questioned with previous studies having shown false-negative rates of 17% to 40% for forceps biopsy in the detection of infiltrating carcinomas(153-155). For these reasons complete removal is mandatory for curative therapy. The only exception to this is the presence of low-grade dysplasia (LGD) within an adenoma in FAP patients, where in the absence of symptoms, regular surveillance is often adequate(78).

Endoscopic ampullectomy with curative intent was first described in 1993 by Binmoeller et al(146). It is regarded as an important diagnostic tool in the initial investigation of patients with an ampullary lesion as it provides larger pathology specimens for analysis, therefore improving diagnostic accuracy and refining diagnosis. More recently there has been an increasing role for endoscopic resection and it is now preferred over surgical resection for dysplastic adenomata given it is recognized as a safe and reliable treatment modality(183) with much-reduced morbidity(145).

The management approach to invasive ampullary adenocarcinoma remains controversial with some reports having suggested endoscopic treatment is a viable alternative in select ampullary cancer patients(103), particularly if patient comorbidity precludes formal surgical exploration and resection which is associated with significant morbidity burden. However, other authors deem the presence of an adenocarcinoma to be a criterion for exclusion from endoscopic management(146, 186, 241). Despite this, endoscopic ampullectomy is now being considered as curative therapy, in place of extensive surgery, for patients with high-grade dysplasia (HGD) adenomas, carcinoma in situ or even focal T1 tumours although the literature to support such management is lacking and often anecdotal(242).

Definitive therapeutic indications for use of endoscopic ampullectomy have yet to be defined. This relates to the previously described difficulty in accurately determining disease grade within an adenomatous lesion; both from low diagnostic accuracy in forceps biopsy specimens and understaged lesions on CT(89, 90, 93, 94).

The aim of the current study was to evaluate the use of endoscopic ampullectomy in dysplastic and malignant ampullary lesions over a fifteen-year period whilst considering management pathways, outcomes, complications and overall long-term survival in a retrospective cohort of patients at a single centre in an effort to develop a a comprehensive treatment algorithm.

5.2 Methods

A retrospective search of patient records was undertaken and a database of all patients referred and treated endoscopically for an ampullary lesion over a 15-year period from 1st January 1999 to 1st April 2014 inclusive was collated. All patients were staged and treated either primarily by endoscopic or surgical management or a combination of both by the same team of surgeons. This analysis was limited to patients undergoing management for pathologically confirmed ampullary adenoma and adenocarcinoma with other lesions (e.g. pancreatic, duodenal or distal bile duct adenocarcinomas, mucinous cystadenocarcinomas or intraductal papillary mucinous neoplasms (IPMN), and adenosquamous tumours) being excluded.

A multi-disciplinary team consisting of surgeons, oncologists, radiologists and pathologists decided upon which treatment option each patient should receive based on initial biopsy findings when available, cross sectional imaging for local and metastatic disease, patient fitness and preference. Assessment of fitness was made objectively by reviewing each patient's co-morbidities.

Clinicopathological data regarding the cohort of patients was held within the created database that was prospectively maintained. Patients were identified from both the hepatobiliary multidisciplinary meeting, nurse specialist patient files and GRIs endoscopic reporting database where coded for an ampullary lesion. Ampullary lesions were defined as those unequivocally arising from the duodenal anatomical structures that form the ampulla of Vater. The lesions were classified as LGD, HGD or adenocarcinoma and treatment groups were categorised in a hierarchical manner from most definitive to least; PD, surgical bypass, ampullectomy and biopsy +/- ablative therapy including biliary stenting procedures. Individuals were then allocated to the highest treatment category as per their recorded individual treatment pathway. Pre-procedural, procedural and post-procedural data was retrieved from both case notes and intranet based patient records in each case. The endoscopic diagnosis, final histology, lesion size, treatment pathway, number of endoscopic sessions, surgical intervention if any, complications, follow-up and survival for each patient was reviewed and recorded.

Patients whose lesion was amenable to endoscopic ampullectomy and who did not already have a formal pathological diagnosis of invasive adenocarcinoma from simple biopsy underwent a “staging” ampullectomy to improve diagnostic yield. The set criteria for ampullectomy Included: 1) that the lesion must be localised, 2) it must be exophytic with no ulceration, 3) the base of which could be entirely encompassed within a snare. Ablative therapies were utilised in sessile lesions unsuitable for ampullectomy or surgical intervention. Biliary stenting was times necessary but did

not precluded the subsequent utilization of ablative therapies. A single surgeon performed all endoscopic procedures.

5.3 Endoscopic Procedure

5.3.1 General Considerations

The procedure itself is an advanced endoscopic therapeutic technique that requires an endoscopist with extensive training and experience to perform well. The aim of the procedure should be to perform a complete en-bloc excision of the ampullary lesion in question with larger lesions with extra-ampullary extension removed in as few pieces as possible. The depth of excision should be flush to the duodenal wall to minimize recurrence from minor ductal extensive within the ampulla. The ability to perform en-bloc excision has the advantage of allowing more accurate histological assessment and reduced recurrence(101). The importance of adequate initial excision is paramount as repeat procedures for partially resected lesions encounter problems with submucosal fibrosis, both increasing the difficulty and risk of complications.

5.3.2 Excision Technique

Prior to commencing the procedure written consent is obtained following a detailed discussion regarding the procedure itself, its associated complications and alternatives. An intravenous cannula is placed preferentially in the right hand for ease of access and the patient is then placed in the left lateral position. The patient

then receives a dose of intravenous midazolam (3 to 5mg) with or without fentanyl (2.5 to 5 mcg) as sedation. A side viewing endoscope is then inserted via the oropharynx into the oesophagus and onwards until the duodenum is reached. The ampulla is then located within the second part and the lesion assessed for resectability for which the criteria are described above.

Once the lesion is deemed suitable for excision a stable endoscope position should be obtained with the lesion in direct view. A thin wire snare should then be selected to maximize current through the tissue and then opened to encompass the ampulla with the snare tip anchored at the ampullary apex. The snare is then closed maximally whilst maintaining contact at the apex to achieve the correct tissue plane. The ampulla itself should be relatively mobile compared to the adjacent duodenal wall and absence of this raises concerns regarding deep invasion or duodenal inclusion within the snare.

The diathermy current is then applied through the snare to excise the lesion. If there is extra-ampullary extension of the lesion, submucosal injection of saline or gelofusine with methylene blue (with or without adrenaline) into the duodenum can be used to elevate this component from deeper layers for excision. Multiple base biopsies are then obtained from the site of excision in an effort to identify evidence of residual malignant disease. The patient is then recovered and returned to the ward for observation.

5.4 Statistical Analysis

Comparison of categorical variables was done using the χ^2 test. The Mann-Whitney U test was used to compare continuous variables following confirmation of a normal distribution of values. The principal outcome measure was length of survival as measured from the time of the original intervention. Length of survival following intervention and cause of death were obtained from our prospectively maintained database and validated using the NHS Scotland Information Services Department (http://www.isdscotland.org). Kaplan-Meier survival analysis was used to analyze the overall survival from the time of intervention. Patients alive at the time of follow-up were censored. The last follow-up period for patients still alive was April 2015. To compare the length of survival between curves, a log-rank test was performed. Statistical significance was set at a *P* value of ≤ 0.05. All statistical analyses were performed using SPSS version 22.0 (IBM Corporation, NY, USA).

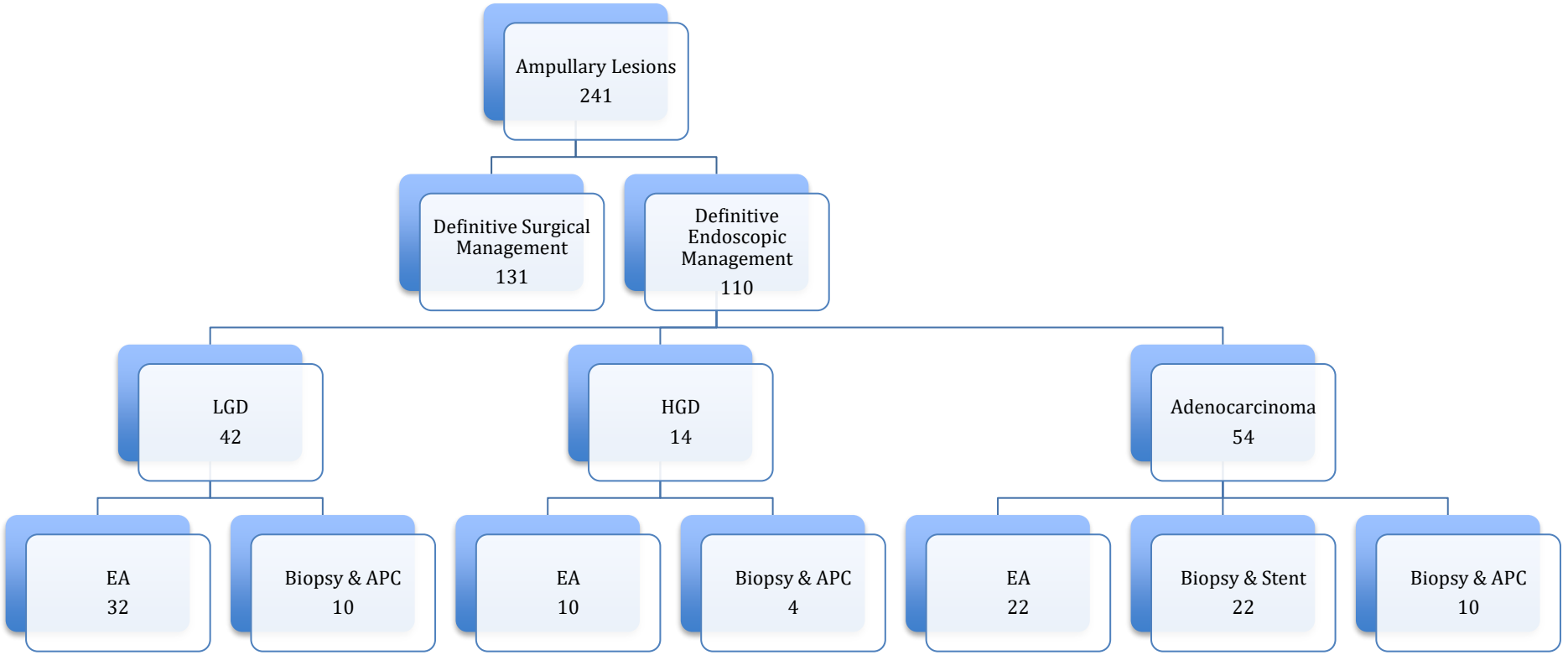
5.5 Results

5.5.1 Patient Characteristics

A total of 241 patients were identified as having been referred to the West of Scotland Pancreatic Unit at Glasgow Royal Infirmary for management of an ampullary lesion from January 1999 to April 2014 of which 76 underwent endoscopic ampullectomy as part of a diagnostic and management algorithm. The 90-day mortality was 1.3% with a minimum follow-up of 12 months.

The patient demographics, lesion characteristics, procedure specifics and definitive management are summarized in **Table 5.1** and **Figure 5.1**. The median age for this cohort was 71.0 years (range 38-87 years). There were 42 men (55.3%) and 34 women (44.7%). There were 59 patients (77.6%) who had undergone prior endoscopic biopsy with the remaining 17 (22.4%) progressing straight to endoscopic ampullectomy. At a median follow up of 68.5 months, the median overall survival was 83 months (95% CI: 44.6 - 121.4). At the time of follow-up 41 patients (53.9%) remained alive following endoscopic ampullectomy. 28 patients (36.8%) had died of recurrent ampullary disease. The deaths of 7 (9.2%) patients were attributable to other medical co-morbidities.

Figure 5.1 Flow chart of definitive endoscopic management outcome for all patients diagnosed with an ampullary lesion between 1999 and 2014



LGD = low grade dysplasia; HGD = high grade dysplasia; EA = endoscopic ampullectomy; APC = argon plasma coagulation

Table 5.1 Clinicopathological factors of patients undergoing endoscopic ampullectomy (n = 76)

	Total Cohort			Adenocarcinoma Cohort		
	n = 76			n = 34		
Variables	**No. (%)**	**Median OS**	***P* value Log-rank**	**No. (%)**	**Median OS (Months)**	**P value Log-rank**
Sex						
Male	42 (55.3)	89		18 (52.9)	-	
Female	34 (44.7)	-	P=0.290	16 (47.1)	34	P=0.537
Age (years)						
Mean	68.6			71.8		
Median	71.0			74.0		
Range	38.0 – 87.0			51.0 – 87.0		
Outcome						
Follow-up (months)	12.0 – 186.0			12.0 – 185.0		
Median follow-up	68.5			51.5		
Death AC	28 (36.8)			15 (44.1)		
Death other	7 (9.2)			4 (11.8)		
Death unknown	0 (0)			0 (0)		
Alive	41 (53.9)			15 (44.1)		
Previous Biopsy						
Yes	59 (77.6)	35		19 (55.9)	34	
No	17 (22.4)	-	P=0.069	15 (44.1)	34	P=0.944
Grade						
LGD	32 (42.1)	NR				
HGD	10 (13.2)	58				
Adenocarcinoma	34 (44.7)	34	p<0.0001			
FAP						
Yes	5 (6.6)	-		0 (0)	-	
No	71 (93.4)	-	P=0.251	34 (100)	34	-
Tumour Size						
≤ 20mm	43 (58.1)	-		16 (50.0)	35	
> 20mm	31 (41.9)	58	P=0.020	16 (50.0)	28	P=0.466
Diagnostic Change						
Upgraded	17 (28.8)	-		13 (68.4)	-	
Downgraded	3 (5.1)	20		0 (0)		
Same	39 (66.1)	-	P=0.009	6 (31.6)	13	P=0.187
Resection Margin -ve						
LGD	29 (90.6%)					
HGD	9 (90%)					
Adenocarcinoma	25 (73.5%)					
Combined	63 (82.9%)					
Definitive Treatment						
Endoscopic	64 (84.2)	-		22 (64.7)	30	
Surgical Bypass	3 (8.8)	17		3 (8.8)	17	
Whipple	9 (26.5)	-	P=0.042	9 (26.5)	-	P=0.046
Complications						
Nil	54 (71.1)	-		29 (85.3)	35	
Pancreatitis	10 (13.2)	-		0 (0)	-	
Bleeding	8 (10.5)	-		4 (11.8)	14	
Cholangitis	3 (3.9)	-		1 (2.9)	34	
AKI	1 (1.3)	-	P=0.287	0 (0.0)	-	P=0.257

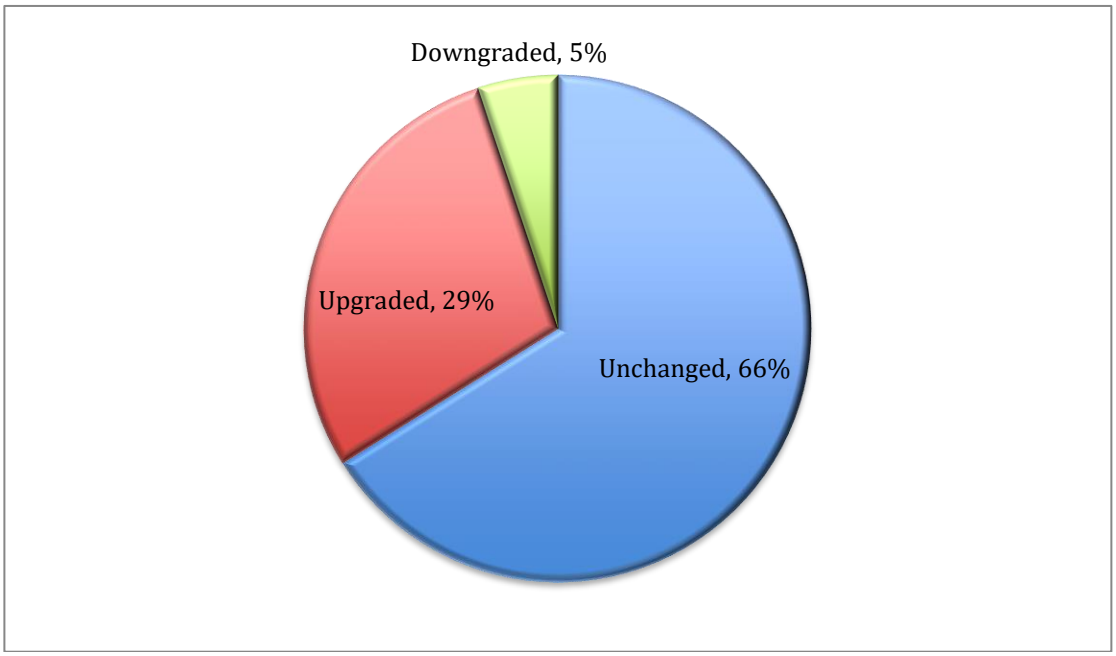
5.5.2 Pathological Factors

Of the 76 patients to have undergone endoscopic ampullectomy, a final histological diagnosis of LGD was seen in 32 patients (42.1%), HGD in 10 patients (13.2%) and invasive adenocarcinoma in 34 patients (44.7%). Five patients (6.6%) had a history of Familial Adenomatous Polyposis (FAP).

5.5.2.1 Diagnostic Accuracy

Endoscopic ampullectomy were performed on biopsy proven adenomas or adenocarcinomas in 59 patients (77.6%) with the remaining 17 (22.4%) proceeding directly to ampullectomy. The mean tumour size was 17 mm (range 5 – 50 mm). According to the original biopsy histopathological diagnosis of LGD or HGD, the diagnosis was upgraded in 17 cases (28.8%) and downgraded in three tumours (5.1%). When considering the upgraded cases, 7 (41.2%) were from LGD to adenocarcinoma, 6 (35.3%) from HGD to invasive disease and 4 (23.5%) from LGD to HGD.

Figure 5.2 Pie chart of diagnostic accuracy of forceps biopsy following EA



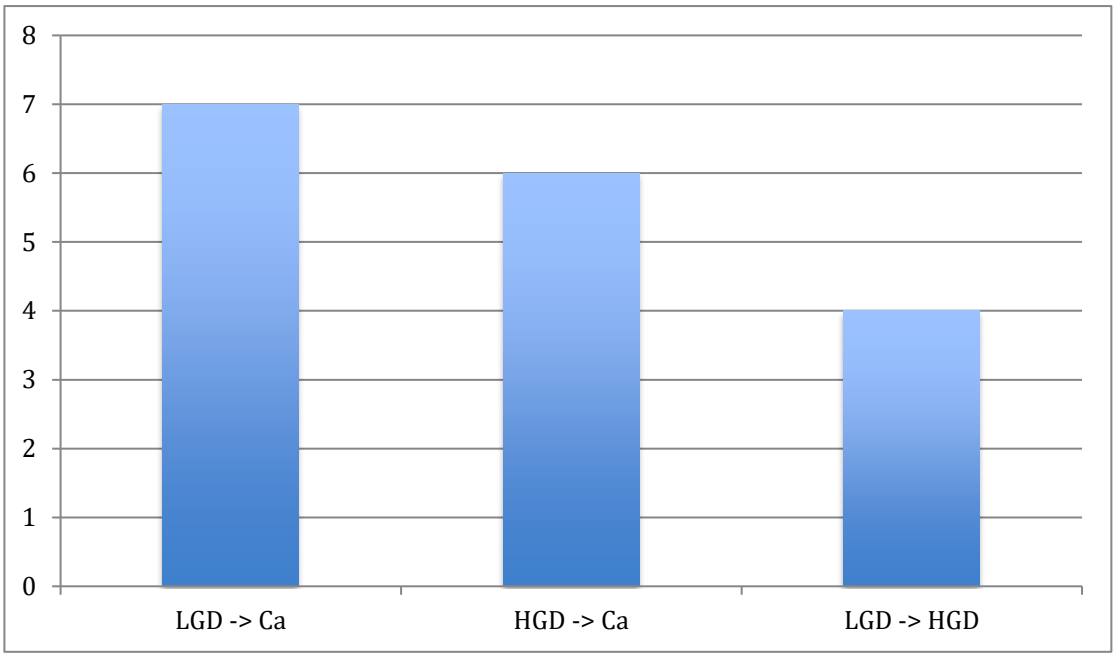
No patient underwent repeat ampullectomy for residual lesions as the snare was only attempted when the lesion was encompassed in its entirety on first sitting. There were 13 (17.1%) of the 76 patients whose histology demonstrated incomplete resection with the remaining 63 (82.9%) patients having no evidence of residual disease at the diathermy margin.

5.5.2.2 LGD & HGD Group

An EA procedure was regarded as definitive management in 64 patients (84.2%). All the patients with a final diagnosis of either LGD or HGD were treated definitively by endoscopic ampullectomy +/- APC and surveillance. No patients within the HGD group proceeded to formal surgical resection as 7 patients were unfit for this and the remaining 3 elected for endoscopic management all of which demonstrated good results with histological improvement in each case. There was a single case of

disease progression to adenocarcinoma following incomplete initial resection after 27 months in the medically unfit group indicating a single endoscopic treatment failure.

Figure 5.3 Chart demonstrating breakdown of diagnostic change from biopsy following EA



LGD = low grade dysplasia; HGD = high grade dysplasia; Ca = invasive malignancy

5.5.2.3 Adenocarcinoma Group

Within the adenocarcinoma group (N = 34), a total of nine patients (26.5%) underwent surgical treatment by pancreaticoduodenectomy with a further three patients (8.8%) requiring surgical bypass. The remaining 22 patients (64.7%) were managed by endoscopic ampullectomy and subsequent surveillance due to significant co-morbidity precluding surgery in 13 cases (38.2%), patient preference in three (8.8%), complete excision and subsequent normal biopsies in three (8.8%), locally advanced disease in two (5.9%) and a single patient (2.9%) who had previously undergone an oesophagectomy precluding further duodenal resection.

There were six of the 22 endoscopically managed patients with adenocarcinoma still alive at the time of follow-up with three demonstrating no evidence of tumour recurrence. Of the deaths within this group, four (25%) were not related to ampullary adenocarcinoma and therefore of unrelated cause.

5.5.3 Complications

Post-endoscopy complications were mostly mild in nature and seen in 22 patients (28.9%) with acute pancreatitis in 10 (13.2%), bleeding in 8 (10.5%), cholangitis in 3 (3.9%) and acute kidney injury (AKI) in a single patient (1.3%). A single patient (1.3%) died following EA from a post-procedural myocardial infarction having suffered a significant haemorrhage.

5.5.4 Overall Survival following Endoscopic Ampullectomy

It was not possible to obtain sufficient information and data regarding disease free survival to perform meaningful statistical analysis in relation to the EA cohort and have therefore used overall survival which is a limitation to the following findings.

Patients whose disease graded histologically as LGD had significantly prolonged overall survival compared to those with HGD and invasive adenocarcinoma respectively (median survival not reached [NR] *vs.* 58 months, 95%CI: 0.0 – 129.9 *vs.* 34 months, 95%CI: 26.1–41.9 $p < 0.001$) **(Figure 5.4)**. When considering the ampullary adenocarcinoma group alone, endoscopic ampullectomy as definitive management achieved similar overall survival when compared to the cohort of patients who

underwent PD (n = 127, chapter 4) up to 36 months (49% versus 54% respectively) and there was no significant statistical difference in median survival (30 months, 95%CI: 22.6 – 37.4 *vs.* 35 months, 95%CI: 17.8 – 52.2 *p=0.152*) **(Figure 5.5)**.

Figure 5.4 Kaplan Meier of overall survival following EA stratified by histological disease grade

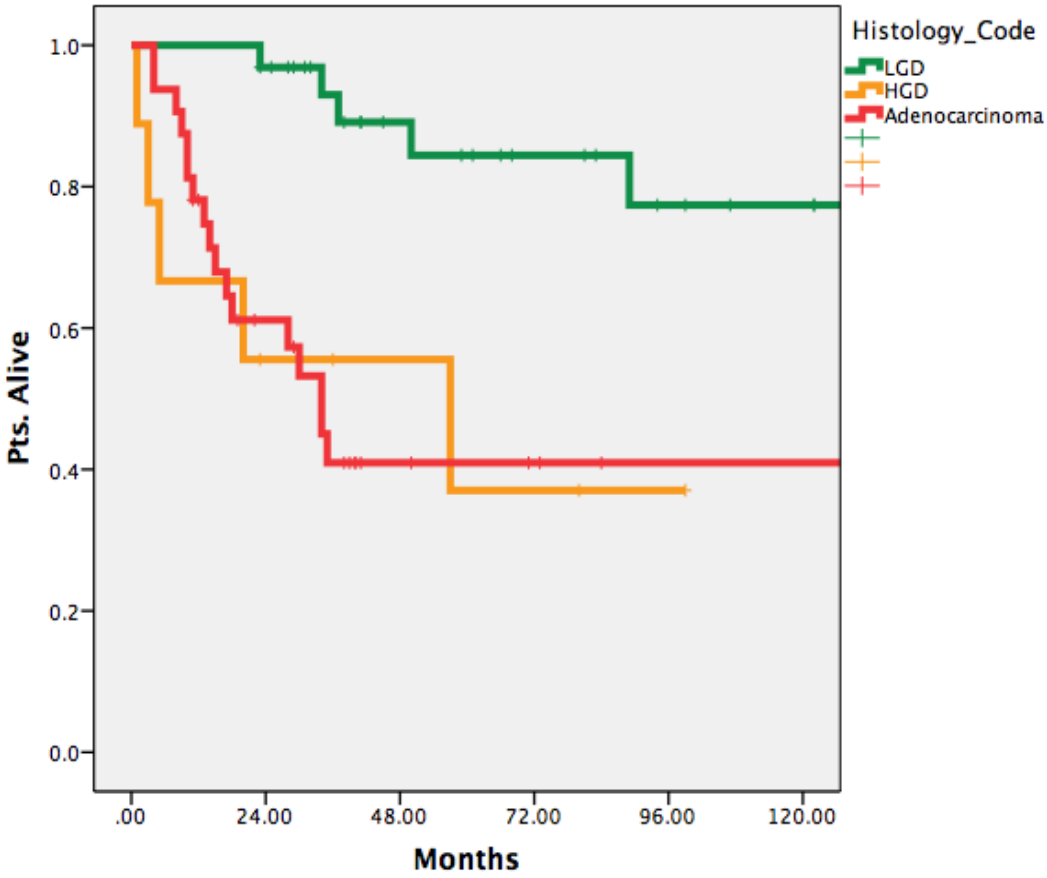
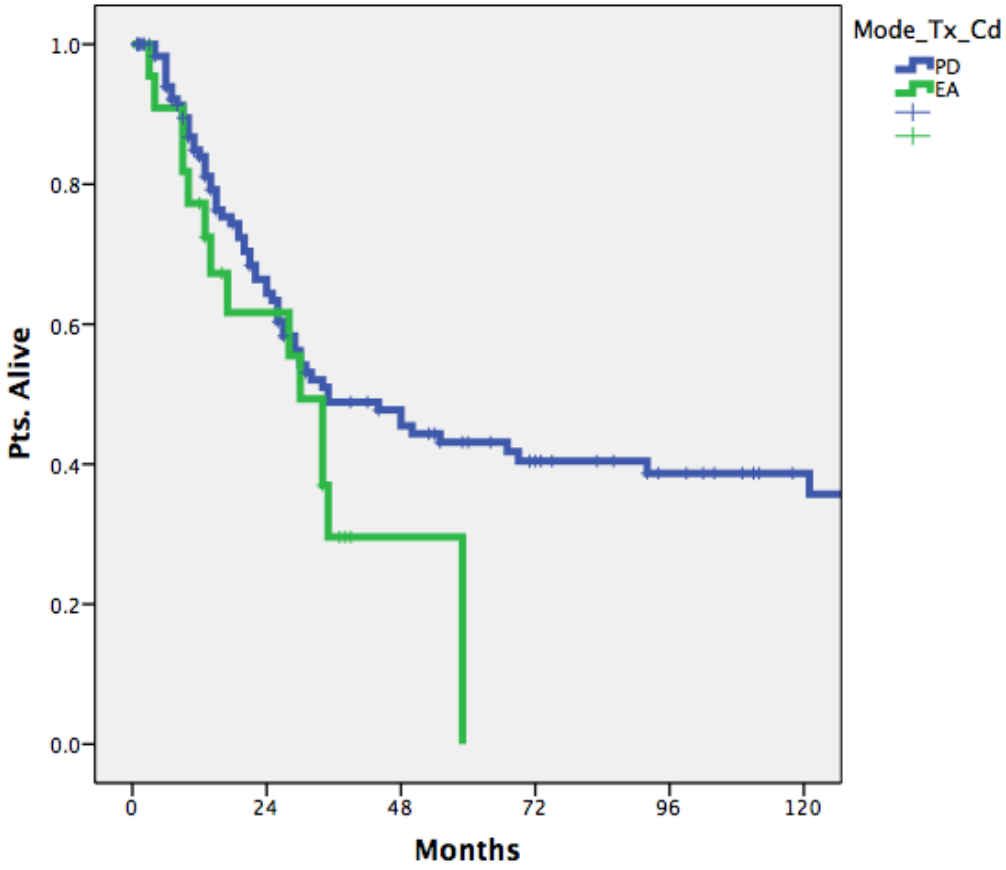


Figure 5.5 Kaplan Meier of overall survival in patients undergoing EA v PD for AA



5.6 Discussion

Complete removal of ampullary lesions is mandatory due to their malignant potential. There is no debate that EA has been established as a safe and effective alternative therapeutic modality to surgical resection in the management of non-cancerous lesions of the ampulla with these data reaffirming this stance.

Due to the focal nature of dysplastic change or malignant transformation it does not come as a surprise that random ampullary biopsies result in potentially high false-negative rates of 11% to 60%(141, 153, 154, 156, 243). The data from our series further confirms the inaccuracy of forceps biopsy with a change in histological diagnosis made in more than a third of patients and 28.8% of patients having an upstaged lesion. An upgraded disease stage, particularly to invasive malignancy, will obviously have a major influence on management strategy. Therefore these data support the concept EA must be performed in all patients with endoscopically resectable lesions containing as a crucial staging tool to permit a larger specimen to refine diagnosis and guide the most appropriate definitive management.

EUS will also play a role in as a staging tool in assessing the lesion and determining the potential depth of invasion or the presence of intraductal extension. The method has an excellent tumour detection rate in excess of 90%(87) and can accurately identify and sample locoregional lymph nodes(102) for evidence of metastatic disease making this modality particularly complementary to EA.

The complication rate following EA in our series was 28.1% with a mortality rate of 1.3%. These figures are higher than those recognised for conventional ERCP although were comparable to those from other published series (9.7% to 35%)(146, 147, 183,

186, 241, 244-246) and from a literature review by Laleman et al that reported a mean complication rate of 27.9%(152). The increase in complications, most notably acute pancreatitis and bleeding, compared to ERCP is likely to be related to the relative increase in tissue trauma required to excise the ampulla.

LGD lesions of the ampulla are adequately managed with EA and surveillance with excellent outcomes, as evidenced from our cohort where no patients disease progressed. The only deviation from this plan is the presence of a lesion with a large intraductal component that is incompletely excised. In this rare situation it is difficult to be certain no focal malignancy exists and a PD may be warranted.

The optimal treatment strategy of HGD lesions remains heterogeneous with no clear consensus. Kim et al reported a high recurrence rate in patients with HGD and therefore should all undergo radical surgical resection unless their general fitness precluded this(247); however this data showed a trend to question this as disease progression was only seen in a single patient with positive EA margins in our small cohort of 10 patients. The remaining patients were successfully managed with surveillance endoscopy with no recurrence.

Therefore, complete resection of an HGD lesion by EA is an acceptable curative management alternative to major surgical resection according to these data. The literature further supports this view as several studies report EA as a credible curative option(17, 248-250) furthermore Lee et al found that resected HGD surgical specimens demonstrated no lymph node or lymphovascular invasion(243).

Our cohort of 10 patients with resected T1 tumours following PD all demonstrated no lymph node involvement; perineural, lymphatic or venous invasion. Despite previous reports of T1 tumours possessing lymphovascular invasion in 11% to 56%(242, 243) our data disputes this and raises the possibility of EA being a suitable alternative to PD in early tumours that have been completely resected endoscopically, particularly in those with borderline fitness for PD, although further studies with larger patient numbers are needed to clarify this hypothesis . When considering our endoscopically managed adenocarcinoma group of 22 patients further, 3 patients had normal surveillance biopsies and no evidence of recurrence at 35, 40 and 41 months respectively which highlights that carefully selected patients with early invasive disease can have excellent outcomes from endoscopic management.

When considering adenocarcinoma, at 36 months following initiation of definitive management both unmatched EA and PD cohorts had similar survival with 49% and 54% of patients respectively remaining alive. However, there was a significant worsening of outcome from this point onward for the EA group compared to the surgically managed cohort **(Figure 5.5)**. When considering median survival, again there was no significant statistical difference with 30 months in the EA group and 35 in the PD group. This displays that short to medium term outcomes are comparable for both treatment modalities and although EA is perhaps less likely to achieve long-term cure, it is possible to achieve excellent survival with regular endoscopic therapy. This makes EA an attractive option for elderly or frail patients and those

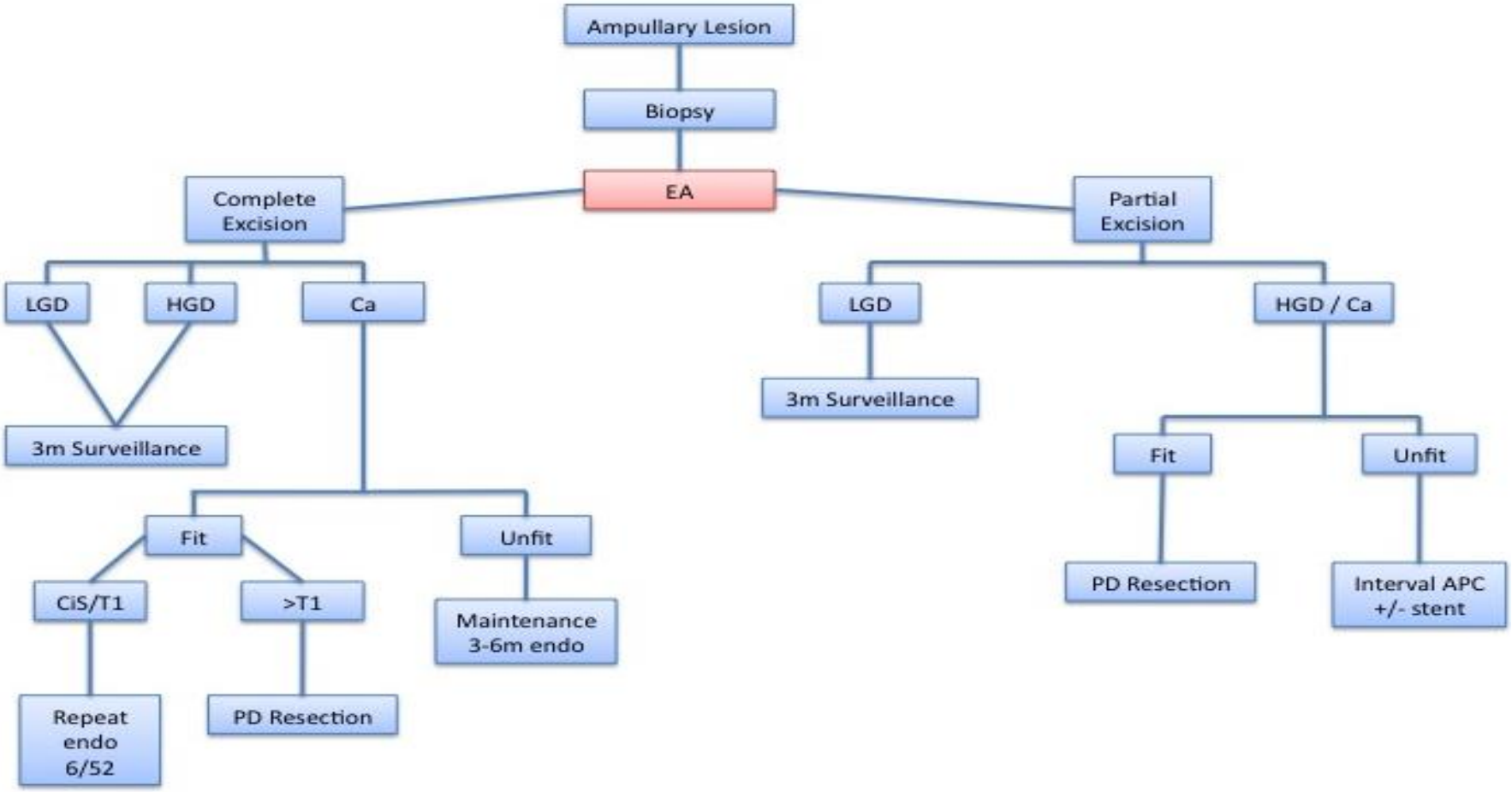
who have significant comorbidity and surgical characteristics that would make PD particularly high risk.

The integration of comorbidity scoring systems and tools that evaluate a patients overall wellbeing to aid in this decision making process prior to the implementation of management may identify those patients who are likely to die with their ampullary disease rather than from it. These include the ASA physical status classification system that assesses fitness prior to surgery and Charleson index which predicts one-year mortality for a patient with a range of comorbidities.

From this data analysis and experience an algorithm for the management of all ampullary lesions has been developed as a proposed guideline for clinical use **(Figure 5.6)**. Completely excised dysplastic lesions and partially excised LGD should undergo initial 3 monthly surveillance and then at 6-monthly intervals for a minimum of 3 years. There have been reports of late recurrence even after 5 years(148, 251) and therefore long-term and even indefinite surveillance is warranted in select patients.

For those select patients undergoing endoscopic management of HGD or T1 tumours with clear resection margins an early first follow-up endoscopy should be performed after 6 weeks to verify complete resection and no residual disease is present. This is key as residual disease in certain cases would change management to the surgical pathway immediately and initial complete resection has seen to be the most significant factor affecting endoscopic success(252).

Figure 5.6 Proposed algorithm for the management of ampullary lesions



EA = endoscopic ampullectomy; LGD = low grade dysplasia; HGD = high grade dysplasia; Ca = invasive malignancy; CiS = carcinoma in situ; PD = pancreaticoduodenectomy; APC = argon plasma coagulation

5.7 Conclusion

EA is a safe and effective method of curative resection for dysplastic adenomata when complete excision is achieved whilst also being an important staging tool in the initial assessment of ampullary lesions as it allows for more meticulous histological analysis. In this regard, all patients with an endoscopically resectable lesion should undergo EA. Patients with incomplete resection of HGD or invasive disease should still undergo PD as definitive management where possible. The same is true of completely excised adenocarcinomas although our data suggests that a select cohort of T1 tumours may be adequately treated endoscopically, avoiding the risks of major surgical intervention. However, in view of the limited data available, a large prospective trial is needed to confirm outcomes following EA and produce quality evidence for the development of global guidelines.

6.0 TISSUE BIOMARKERS INFLUENCING SURVIVAL IN AMPULLARY ADENOCARCINOMA

Chapter 6

Immunohistochemical markers influencing survival and prognosis in Ampullary Adenocarcinoma

6.1 Introduction

As discussed in the previous chapters, the clinicopathological factors of resected specimens following PD help to characterise the behaviour of ampullary adenocarcinoma and predict prognosis for patients with the disease(19, 33, 83, 140, 177, 211). A detailed review of these factors are reported on within the meta-analysis presented in **section 3.3.3**. Unfortunately, these routinely assessed histological factors do not entirely explain the marked variability in outcome regularly observed in patients with AA patients following surgical resection. As a result, there is a requirement for superior prognostic markers to be identified to potentially enhance management of AA and provide insight into the underlying biology.

In recent years there have been new factors identified that are implemented in prognostication which are not routinely included with the AJCC 7th edition TNM classification of ampullary cancers. These include the ability to categorise ampullary tumours into histological subtypes that allows for identification of two heterogeneous groups with marked variation in survival as demonstrated within our 127 patient cohort (121 months for Intestinal vs. 21 months for pancreaticobiliary). This development has been augmented by the identification of cytokeratin and

mucin molecular markers, namely CK7, CK20, MUC1, MUC2 and CDX2 that have been implemented in enhancing the characterization of the subtypes and survival outcomes (34, 59, 61, 63). With these markers being closely correlated to subtype the validity of their independent impact on survival must be carefully considered.

There have been a number of key molecular aspects involved in the progression of PDAC identified over recent years with subsequent targeted pharmacological treatments in evolution; however, this area has mostly remained unexplored in AA. It is of interest to ascertain how the expression of different protein markers vary in AA particularly in the context of intestinal and pancreaticobiliary subtype tumours. As previously described, both subtypes have marked heterogeneous behavioural traits and so is of interest to establish how the underlying biomarker pathways that produce overall clinical and survival differences were affected.

The aim of this chapter was to further elucidate the prognostic ability of molecular markers involved in signaling pathways found within AA with a view to implementation in the clinical setting. Each investigated marker was grouped according to its biological function to aid in presentation; senescence, apoptosis, angiogenesis, invasion and metastases, insensitivity to growth inhibition and self-sufficiency to growth signals.

The primary aim was to evaluate each candidate biomarker and assess its relationship to clinicopathological factors and overall survival within a large post radical surgical resection AA cohort using IHC.

6.2 Methods

6.2.1 Patient cohort

All patients who were included in the immunohistochemical analysis underwent surgical resection with curative intent by either classical PD or PPPD in the West of Scotland Pancreatic Unit, GRI, over a 22-year period. The surgical procedure was performed by one of four surgeons and all were for an ampullary adenocarcinoma subsequently confirmed by histopathological analysis. A detailed description of the operative procedure pathological assessment has been detailed in sections **4.4** and **4.5**. No other biliary tract malignancies or surgical interventions were included in the study. The decision to perform radical surgical resection with curative intent was made at the multi-disciplinary team meeting. There was no standardised adjuvant therapy following PD, in part due to the negative studies and RCTs evaluating chemotherapy benefit post-operatively(47, 164, 166, 168, 169, 253). Follow-up comprised of a standardised protocol of regular out-patient review and CT imaging was only performed when recurrence or complications were suspected. From the 127 patients reviewed earlier in chapter 4, it was able to perform IHC marker staining on the specimens for 81 resections.

6.2.2 Statistical Analysis of Data

Categorical variables were compared using the Mantel –Haenszel (χ^2) test. The Mann-Whitney U test was used to compare continuous variables following confirmation of a normal distribution of values. The principal outcome measure was

length of survival as measured from the time of the original surgery. Length of survival following surgery and cause of death were obtained from our prospectively maintained database and validated using the NHS Scotland Information Services Department (http://www.isdscotland.org). Kaplan-Meier survival analysis was used to analyze the overall survival from the time of surgery. Patients alive at the time of follow-up were censored. The last follow-up period for patients still alive was June 2014. To compare the length of survival between curves, a log-rank test was performed. A Cox proportional hazards model was used for multivariate analysis to adjust for competing risk factors, and the hazard ratio (HR) with 95% confidence intervals (CIs) was reported as an estimate of the risk of disease-specific death. Variables that were found to be significant on univariate analysis at $P < 0.10$ were included in multivariate analysis in a backwards stepwise fashion. The statistical significance for a test was set at a $P \leq 0.05$. All statistical analyses were performed using SPSS version 22.0 (IBM Corporation, NY, USA).

6.2.3 TMA construction

Previously, more conventional IHC required the use of whole tissue sections and was therefore highly reagent and sample intensive. This technology has evolved over the years to create a platform enabled for high volume assessment of DNA, RNA and protein expression allowing gene validation and correlation of targets with the tumours themselves and clinicopathological data. Creation of the TMA's for this IHC analysis was carried out by highly trained scientists within the pathology laboratory to ensure high quality specimens for analysis.

The selected H&E stained sections were reviewed and then multiple defined areas of invasive disease as well as normal ampullary tissue, pancreas, duodenum and bile duct were marked on the slides. It was decided six cores should be obtained to gain a true representation of the tumour due to the heterogeneous nature of AA. The criteria for selection was as follows:

1) High tumour content (minimal contaminants e.g. stromal tissue)

2) Definitive invasive ampullary adenocarcinoma (not HGD)

3) Tumour size (volume enough for 18 x 0.6mm cores (leaving residual tissue for further work should it be required)

6.2.4 Array design and construction

Conventionally, it is standard practice to construct array outlines in an asymmetrical format to allow easy sample identification and cores should be randomised throughout the block. The array was designed to include 6 tumour cores, 2 normal cores and control tissue with the ability to differentiate between each TMA and was constructed in a block of Paraplast using a manual tissue arrayer. A wax core was removed at least 5.7mm from the recipient block edges using a hollow needle and then a core of tumour was removed from the first donor block using the matching marked H&E slide as a reference. The specimen core obtained was then placed into the recipient block. The position of the recipient block was changed each time and the process was repeated, positioning, a total of 1290 cores (6 x 0.6mm core x 215) across 7 different arrays. There was a minimum distance of 0.8mm kept between each core. The control tissues were arrayed in duplicate in each array and each

array with specimen tissue was incubated at 40°c for 15 minutes and then allowed to cool prior to sectioning. A prior test TMA was created with a number of practice tissue core lines for antibody optimization.

6.2.4.1 Sectioning

The tissue blocks were first cooled to -10°c (Tissue Tec© cooler) and then sectioned using a tape-transfer system and a Leitz 1512 microtome. A window of adhesive tape was applied to the array surface and the microtome blade was positioned under one edge to allow cutting of 5µm sections. The section was then fixed to an adhesive slide by 35 seconds of UV curing, TPC solvent released the adherent tape window and the slides were subsequently baked for 5 minutes at 80°c and then stored at 4°c.

6.2.4.2 Haematoxylin & Eosin (H&E) staining

The wax from the sections was removed in Histoclear and then washed in graded alcohol. The sections were then immersed in haematoxylin for 4 minutes, rinsed under water, briefly immersed in acid alcohol and placed in Scott's tap water for 1 minute. The sections were rinsed once more then placed in eosin Y for 30 seconds. A period of section dehydration was then undertaken using graded alcohol and Histoclear.

6.2.5 Immunohistochemisty (IHC)

For the IHC protocol FFPE sections were dewaxed in xylene for 20 minutes and rehydrated through intermediate alcohols. Protocols were first optimised using FFPE sections then applied to the test TMAs and then the final ampullary TMA sections. To prepare for IHC evaluation the sections were stained using a DAKO Autostainer. This process began by incubating the sections in goat serum blocker for an initial 20 minutes and then the primary antibody for a further 30 minutes. They were then rinsed in TBS, submerged in 0.3% hydrogen peroxidase for 5 minutes to block endogenous peroxidases and then again washed with TBS. Envision ChemMate was used for antibody detection and 3,3-diaminobenzidine for visualization with copper sulphate used as an enhancing agent. An antibody diluent was used on the negative controls.

A combination of 0.1% trypsin and 0.1% calcium chloride within a Tris buffer was used when pretreatment enzyme digestion was required. This was undertaken for 25 minutes at a temperature of 37°c. Similarly, when microwave pressure cooker treatment was required slides were submerged in dH_2O containing 0.55g EDTA and 0.87% Tri base and then heated at full pressure for 5 minutes.

6.2.5.1 Antibodies

Twelve antibodies were used in this study. There were:

1. B-catenin	(Monoclonal Mouse Anti-Human; clone βcatenin)	Dako Ltd (Ely, UK)
2. P53	(Monoclonal Mouse Anti-Human; clone DO-7)	Dako Ltd (Ely, UK)
3. P21	(Monoclonal Mouse Anti-Human; clone SX118)	Dako Ltd (Ely, UK)
4. SMAD4	(Monoclonal Mouse Anti-Human; clone B-8)	Santa Cruz (Ca, USA)
5. CDX2	(Polyclonal Rabbit Anti-Human)	Santa Cruz (Ca, USA)
6. LkB1	(Polyclonal Rabbit Anti-Human; clone ab58786)	Abcam (UK)
7. mTOR	(Polyclonal Rabbit Anti-Human; 2971)	Cell Signalling (Ma, USA)
8. GSK3β	(Polyclonal Rabbit Anti-Human; 9331)	Cell Signalling (Ma, USA)
9. Cyclin D1	(Monoclonal Rabbit Anti-Human; clone SP4)	Lab-Vision Neomarkers (Ca, USA)
10. SRC	(Monoclonal Rabbit Anti-Human; 2109)	Cell Signalling (Ma, USA)
11. MUC1	(Monoclonal Rabbit Anti-Human; 14161)	Cell Signalling (Ma, USA)
12. CDX2	(Monoclonal Rabbit Anti-Human; 12306	Cell Signalling (Ma, USA)

The selection of these antibodies was made for several reasons. Following an extensive review of the current literature of IHC markers in ampullary cancer **(Table 1.1)** there were few candidate biomarker proteins identified for use in this cohort due to the paucity of published data. The markers MUC1 and CDX2 have previously been extensively investigated in their role in the assessment of histological phenotyping of pancreaticobiliary and intestinal subtypes(35, 42, 254) and using these would allow validation within our cohort and confirm previous findings.

The antibodies used were also readily available in our lab for use in IHC evaluation and had previously been used on TMA's for our PDAC cohort with interesting findings; in particular the LKB1-p21 axis where LKB1 suppression may serve as an

alternative to p53 suppression and drive pancreatic cancer evolution(189). Similarly, the markers COX2, β-catenin and GSK3β have been shown to be associated with clinicopathologcal status and overall survival following resection in PDAC(255) and SMAD4 inactivation has been associated with a poorer prognosis(256, 257).

The progression from a histologically normal epithelium to LGD to HGD to invasive carcinoma appears to be associated with the build up multiple genetic mutations with each of these protein markers being involved in different steps of the cell cycle pathway including senescence, invasion and metastases; angiogenesis, self sufficiency and growth; and insensitivity to growth inhbition.

6.2.5.2 Tissue microarray and image capture archiving

The slides were digitally captures, stored and archived by CO once staining of the TMA sections was completed. The Slidepath Digital Image Hub (http://ld.dih.slidepath.com/login.php) was used as a digital microscope to allow visualization and scoring of the TMAs. These scores were linked to the tumour specimen with a unique identifier to allow integration of into the patient cohort database with the SPSS program.

6.2.5.3 TMA assay quantification – modified histoscore

This is the routine scoring method used in this insititution and has been used in this manner several times before(255, 258). It is a semi-quantitative ordinal scoring

system and is the default method pathologists are familiar with in the analysis of TMAs. This method considers three key factors:

1) Percentage of positive stained cells

2) Intensity of positive staining

3) Percentage of tumour within core specimen

The main issue with this method of visual analysis is its semi-quantitative nature which reduces data output from absolute values to an ordinal scale. Each of the markers within this thesis were scored using a weighted histoscore. When scoring each core, the proportion of cells staining was multiplied by the intensity of staining to achieve an overall score out of 300. This was done for each cell compartment – nucleus, cytoplasm and membrane. The digital microscope allowed for accurate scoring at multiple magnifications.

The quantity of cells staining was given as a percentage and the intensity of staining was characterised in a 4 tier system; nil, weak, moderate and strong staining with a score of 0 to 3 given to each respectively. The overall score out of 300 was achieved by calculating the % cells with weak staining (x1) + % cells with moderate staining (x2) + % cells with strong staining (x3).

For example, in a slide with 25% weak staining, 25% moderate staining and 50% strong staining the histoscore would be: (25 x 1) + (25 x 2) + (50 x 3) = 225

All of the proposed prognostic markers were scored by CRF and double scored by a consultant pathologist alongside other trained members within the research team (NBJ). If there was any marked variance in overall histoscore between team members then reassessment of the core was undertaken simultaneously and a score agreed upon.

6.2.5.4 IHC evaluation

A semi-quantitative analysis of protein expression for the panel of proposed prognostic markers was undertaken using the Histoscore method. The association between combinations of the studied markers; including CDX2, MUC1, B-catenin, Cyclin D1, LKB1, p53, SRC, GSK3β, mTOR, SMAD4, P21 and COX were evaluated using Spearman's Correlation Coefficient analysis. The identification of correlation between the putative markers and clinicopathological factors were obtained.

6.2.5.5 Cutoff point determination for survival analysis

For each proposed prognostic marker the cohort was dichotomised into low and high groups according to the histoscore obtained. The point of separation was determined at the median point of scores and when this would split equal scores the two groups were created at the closest score that was not the median, either higher or lower. This subsequently allowed for Kaplan Meier survival analysis and Cox proportional hazards modeling to be performed on the cohorts.

6.3 Results

From the 127 patients who underwent a PD for ampullary cancer between January 1990 and May 2013, it was able to perform IHC marker staining on the specimens of for 81 cases, all of which were included in the study. The following markers were analysed: MUC1, CDX2, B-catenin, Cyclin D1, LKB1, p53, SRC, GSK3β, SMAD4, mTOR, p21 and COX2. The weighted histoscore method was used as discussed in **section 6.2.5.2**. The histoscore median and range for each proposed marker used to determine cut-off values for high and low scoring used for subsequent survival analysis are displayed in **Table 6.1**.

The number of specimens for each protein marker was variable and ranged from 47 to 81. This was because an additional array of 30 patients allowed for a total of 81 specimens for some markers but only 50 patients had the broader range of markers evaluated.

Table 6.1 Marker median histoscores and ranges

Protein	No. of specimens	Median Histoscore	Interquartile Range	Histoscore Range
CDX2	81	5.00	87.5	0.0 – 300
MUC1	81	0.00	1.00	0.0 – 4.00
B-Catenin	52	0.00	15	0.0 – 225
Cyclin D1	52	78.75	105.6	3.0 – 237.5
Cyclin D1 Nuclear	52	2.00	1.5	0.0 – 3.00
LKB1	52	165.00	133.75	0.0 – 300
p53	52	28.75	165	0.0 – 300
p53 Nuclear	52	1.00	2.5	0.0 – 3.00
SRC	49	100.00	100	0.0 – 300
GSK3β	47	0.00	15	0.0 – 225
SMAD4 Nuclear	52	20.00	80	0.0 – 270
SMAD4 Cytoplasmic	52	60.00	143.75	0.0 – 300
mTOR	39	230.00	100	100 – 300
P21	52	25.00	51.8	0.0 – 162.5
P21 Nuclear	52	1.00	1.00	0.0 – 3.00
COX2 Nuclear	50	70.00	86.38	10 – 220
COX2 Cytoplasmic	50	195.00	93.13	17.5 – 300
COX2 Membranous	50	28.75	65	0.0- 300

6.3.1 Protein Expression and Survival

To assess whether levels of protein expression were associated with poorer survival in ampullary cancer, survival analysis was undertaken and graphs created for patients expressing low levels (below cut off) and high levels (above cut off) of molecular proteins. The survival data for protein expression was compared using the log rank test with **Table 6.2** demonstrating overall survival for each marker dependant on level of staining.

From initial analysis, 6 protein markers demonstrated strong statistical significance all with $p < 0.05$, namely CDX2, MUC1, SRC, LKB1, COX2 and P21. While expression of B-catenin, Cyclin D1, p53, GSK3β, SMAD4 and mTOR all failed to correlate with

outcome. **Table 6.2** and **Figure 6.1** demonstrates Kaplan Meier graphs of the statistically significant markers.

Figure 6.1 Kaplan Meier graphs demonstrating biomarkers that influence survival

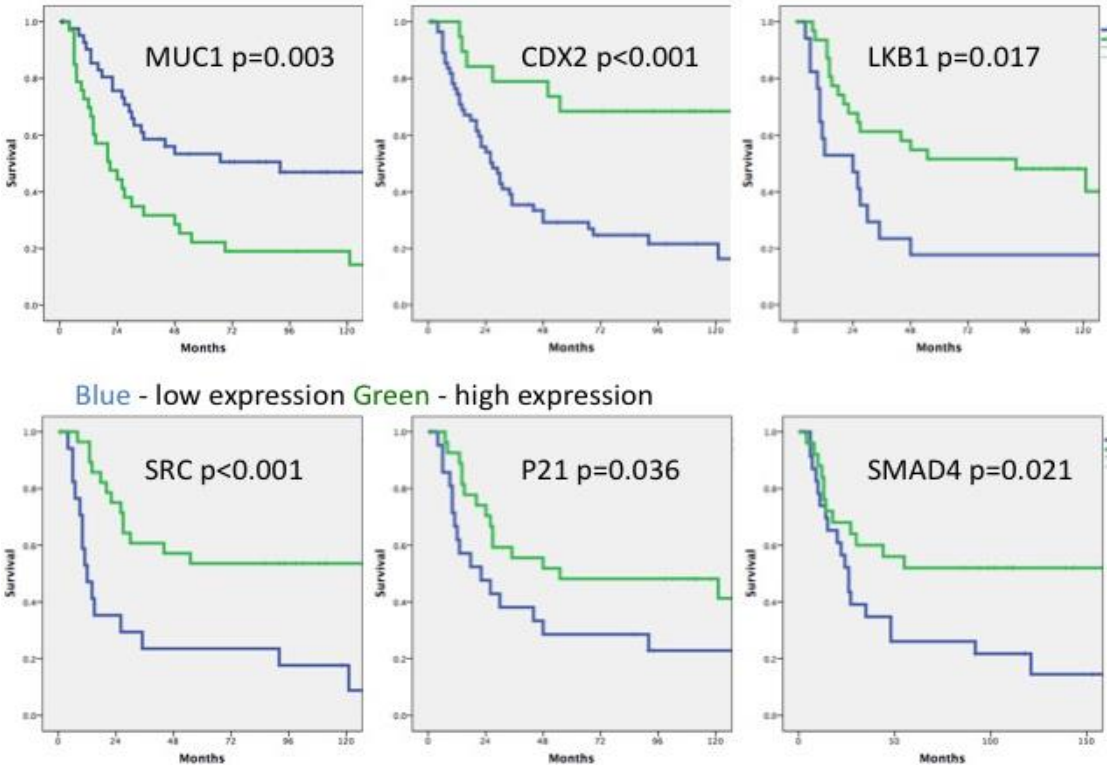


Table 6.2 Survival following resection for Ampullary adenocarcinoma stratified by protein expression

Marker	Number of patients (%)	Mean OS (months)	Median OS (months)	*P* value (Logrank)
CDX-2				
Low	41 (50.6)	47.8	29	
High	40 (49.4)	136.4	55	**0.001**
MUC-1				
Low	46 (56.7)	99.1	92.0	
High	35 (43.3)	43.0	21.0	**0.003**
B-Catenin				
Low	30 (57.7)	70.1	26.0	
High	22 (42.3)	83.8	55.0	0.271
Cyclin D1				
Low	26 (50.0)	56.4	24.0	
High	26 (50.0)	93.4	48.0	0.113
Cyclin D1 Nuclear				
Low	22 (42.3)	69.6	30.0	
High	30 (57.7)	84.5	27.0	0.616
LKB-1				
Low	26 (50.0)	49.5	26.0	
High	26 (50.0)	107.1	121.0	**0.017**
P53				
Low	27 (51.9)	61.7	26.0	
High	25 (48.1)	90.4	48.0	0.336
P53 Nuclear				
Low	31 (59.6)	72.9	30.0	
High	21 (40.4)	77.7	27.0	0.828
SRC				
Low	17 (34.7)	39.2	12.0	
High	32 (65.3)	109.3	----	**0.001**
GSK3B				
Low	26 (55.3)	70.2	20.0	
High	21 (44.7)	87.8	55.0	0.192
SMAD4 Nuclear				
Low	27 (51.9)	56.7	24.0	
High	25 (48.1)	99.7	48.0	0.109
SMAD4 Cytoplasmic				
Low	26 (50.0)	49.0	24.0	
High	26 (50.0)	108.7	----	**0.021**
P21				
Low	25 (48.1%)	50.0	17.6	
High	27 (51.9%)	99.1	55.0	**0.036**
P21 Nuclear				
Low	33 (63.5%)	60.4	26.0	
High	19 (36.5%)	103.1	55.0	0.121
COX2 Nuclear				
Low	24 (48%)	61.0	20.0	
High	26 (52%)	96.1	55.0	0.194
COX2 Cytoplasmic				
Low	25 (50%)	54.6	26.0	
High	25 (50%)	102.8	121.0	0.090
COX2 Membranous				
Low	25 (50%)	60.3	24.0	
High	25 (50%)	101.9	55.0	0.147

6.3.2 Senescence markers prognostic influence

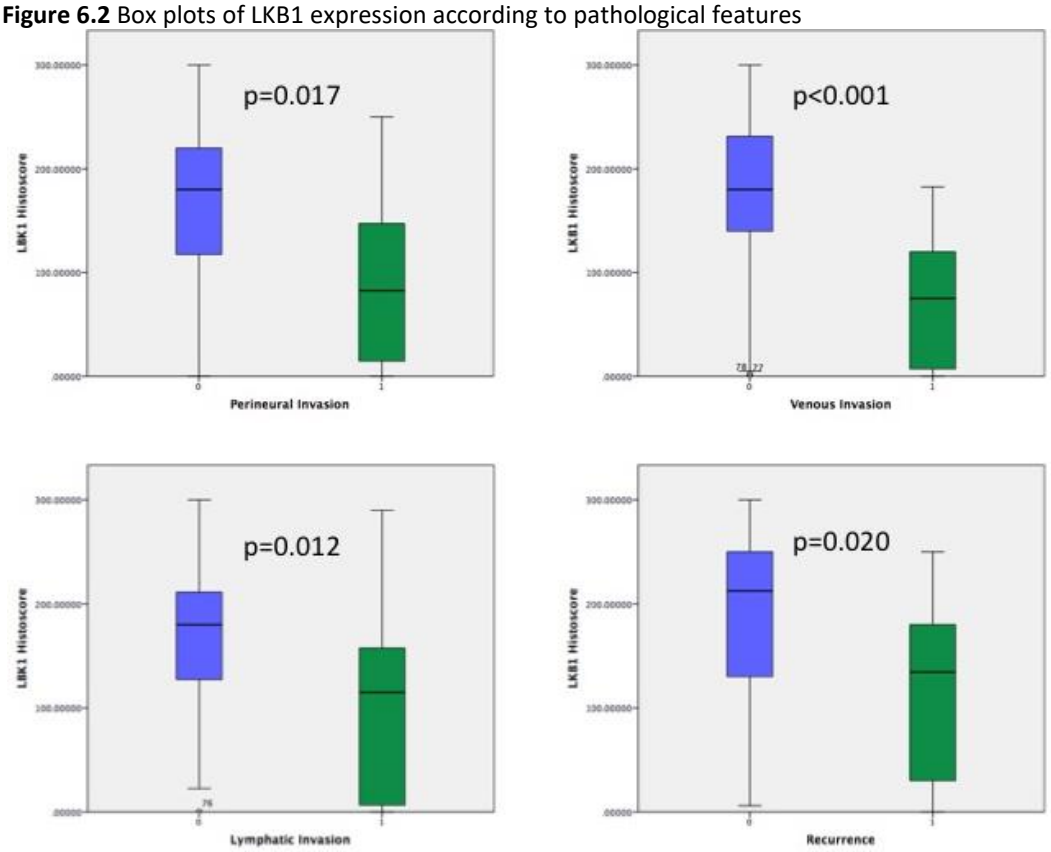
There is evidence to suggest that aberrant expression of p21 and p53 can provide prognostic information in patients with PDAC partly due to their well-established roles in pathogenesis. Unfortunately, the same cannot be said for AA with three studies from the past 15 years failing to demonstrate any correlation with survival (29, 55, 57). To date there has been no investigation of the role of LKB1 in ampullary cancer although low expression in PDAC has been associated with reduced overall survival (Morton et al 200x).

6.3.2.1 LKB1 correlation with clinicopathological Features

The expression of LKB1 was not associated with T stage, lymph node status or ratio, grade or tumour size. However; perineural, lymphatic and venous invasion were all significantly associated with lower median LKB1 expression (all $p < 0.02$) as was positive resection margin status ($p = 0.046$)(**Figure 6.2**).

In univariate analysis, low LKB1 expression was associated with significantly reduced overall median survival compared with high expression following resection (26 months versus 121 months, $p = 0.017$)(**Figure 6.1, Table 6.2**).

Figure 6.2 Box plots of LKB1 expression according to pathological features

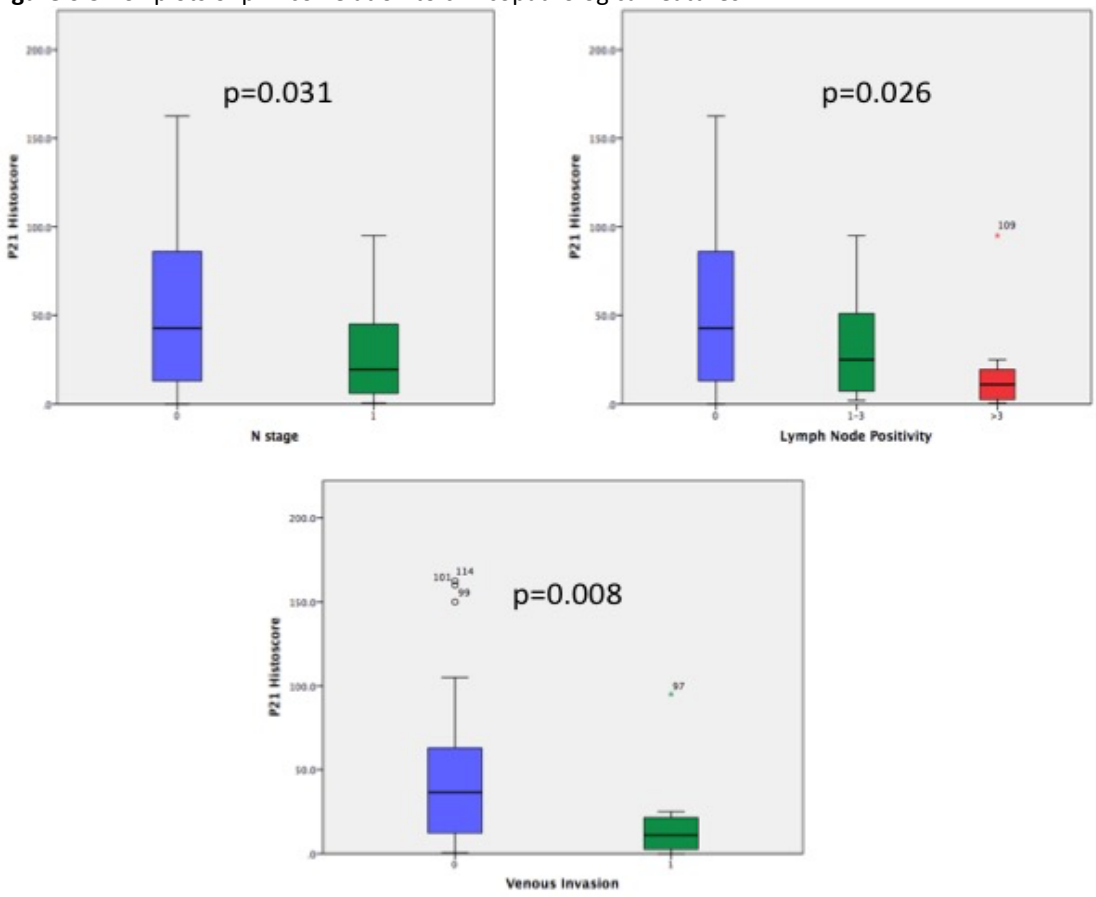


6.3.2.2 p21 correlation with clinicopathological Features

The expression of p21 did not show any correlation with T stage, grade or tumour size, resection margin status, histological subtype, perineural invasion, lymphatic invasion or the lymph node ratio. Interestingly, low p21 expression did show a significant association with N stage (p=0.031), number of positive nodes (p=0.026) and venous invasion (p=0.008) (**Figure 6.3**).

In univariate survival analysis, low p21 expression was associated with significantly reduced overall median survival compared with high expression following resection (17.6 months versus 55 months, p = 0.036)(**Figure 6.1, Table 6.2**).

Figure 6.3 Box plots of p21 correlation to clinicopathological features



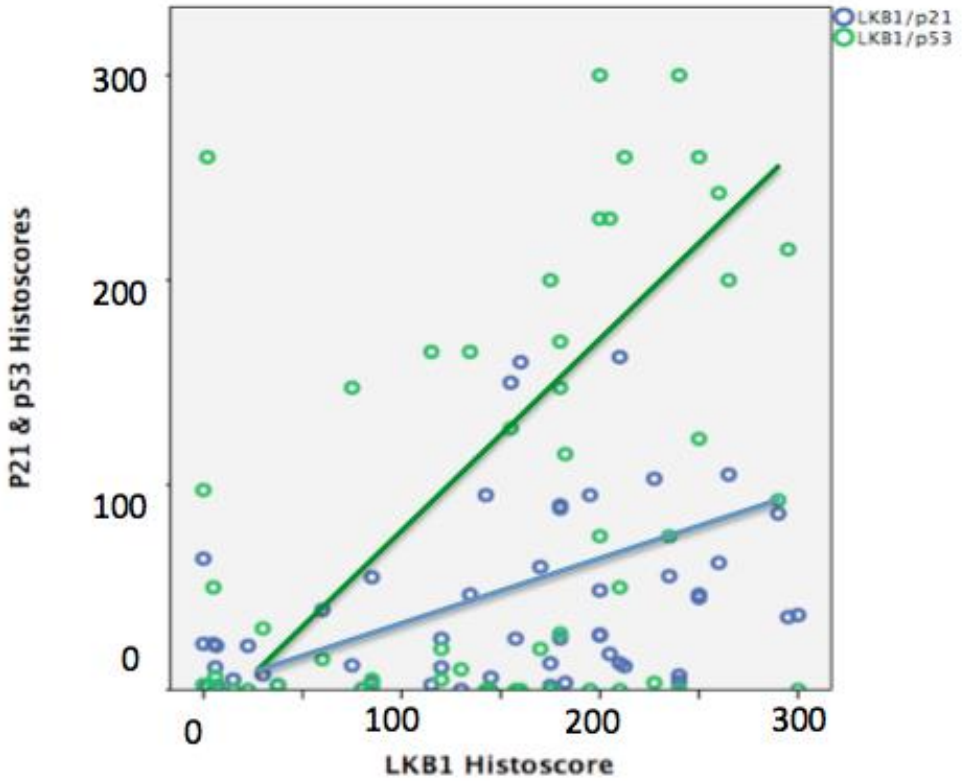
6.3.2.3 p53 correlation with clinicopathological Features

There were no significant correlation between p53 and any clinicopathological factors evaluated including survival following resection.

6.3.2.4 Relationship between LKB1, p21 and p53

Within the AA specimens, LKB1 showed a significant direct correlation to both p21 (Spearman's rho correlation coefficient = 0.40; p = 0.003) and p53 (Spearman's rho correlation coefficient = 0.35; p = 0.012); however, no association was evident between p21 and p53 (**Figure 6.4**).

Figure 6.4 Scatter plot demonstrating correlation between LKB1 and both p21 & p53



6.3.3 Invasion & metastases markers prognostic influence

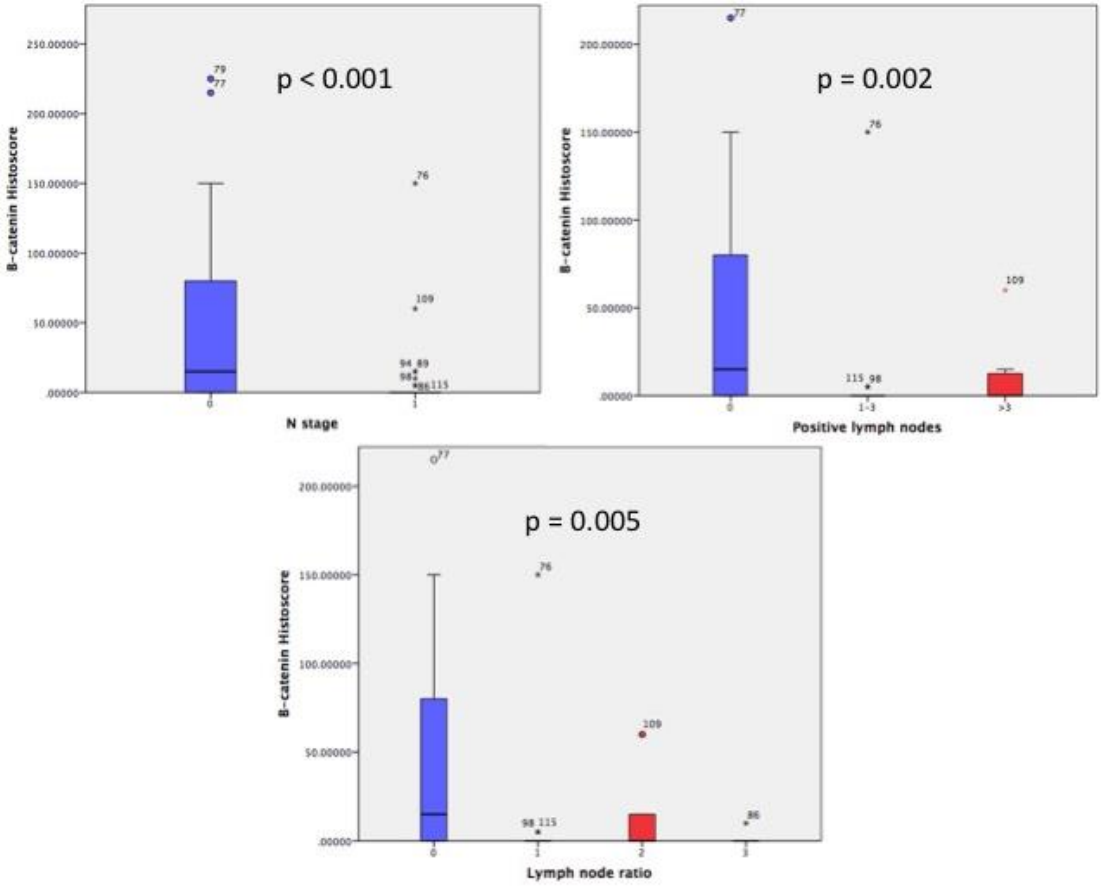
B-catenin and GSK3β have both been implicated in PDAC as markers that are both strongly linked to overall outcome and survival. A single paper by Sung et al(58) identified in the earlier protein marker review proposed over expression of B-catenin correlates to cell grade, increased TNM stage and both lymphatic and perineural invasion. There were no papers identified on the investigation of GSK3β and therefore both markers were reviewed on the AA TMAs.

6.3.3.1 B-Catenin correlation with clinicopathological features

The expression of B-catenin did not show any correlation towards T stage, tumour grade or tumour size; positive resection margins, histological subtype, perineural invasion, lymphatic invasion, venous invasion or recurrence. There was a significant association with nodal disease, in particular a significant correlation between increased N stage ($p < 0.001$), number of positive nodes ($p = 0.002$) and the LNR ($p = 0.005$) and reduced expression of B-catenin(**Figure 6.5**).

In univariate survival analysis, while the overall median survival was reduced for those patients with reduced B-catenin expression seen within the AA TMA cohort this difference was not significant (26 months low expression versus 55 months high expression, $p = 0.271$)(**Table 6.2**).

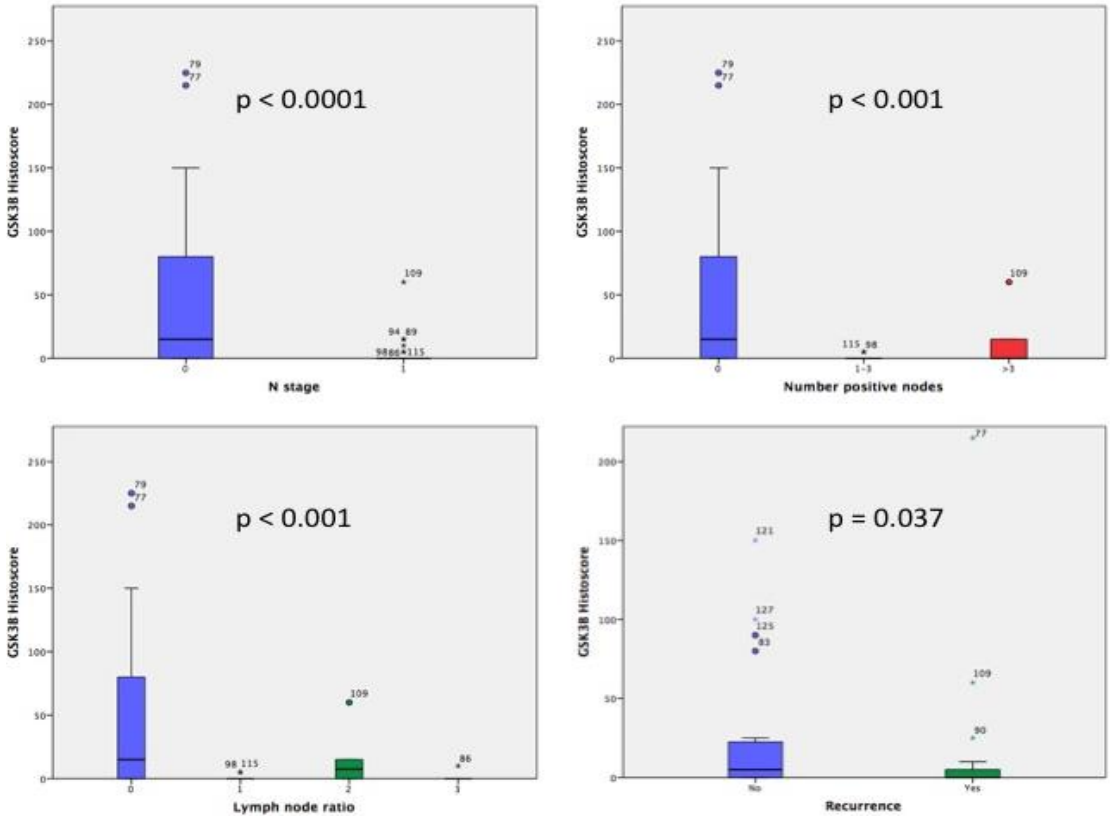
Figure 6.5 Box plots of B-catenin correlation to clinicopathological features



6.3.3.2 GSK3β correlation with clinicopathological features

The expression of GSK3β showed an unsurprising similarity to B-catenin in terms of correlation to clinicopathological features. Its loss showed the same affinity for N stage ($p < 0.0001$), number of positive nodes ($p < 0.001$) and the LNR ($p < 0.001$)(**Figure 6.6**). However, there was weak correlation with resection margin involvement ($p = 0.048$); however, did not show any correlation towards T stage, tumour grade or tumour size, subtype, perineural invasion, lymphatic invasion or venous invasion. While there was a trend towards reduced survival in patients who had low expression, this did not translate into a significant association with overall median survival seen within the cohort (20 months low expression versus 55 months high expression, $p = 0.192$)(**Table 6.2**).

Figure 6.6 Box plots of GSK3β correlation to clinicopathological factors



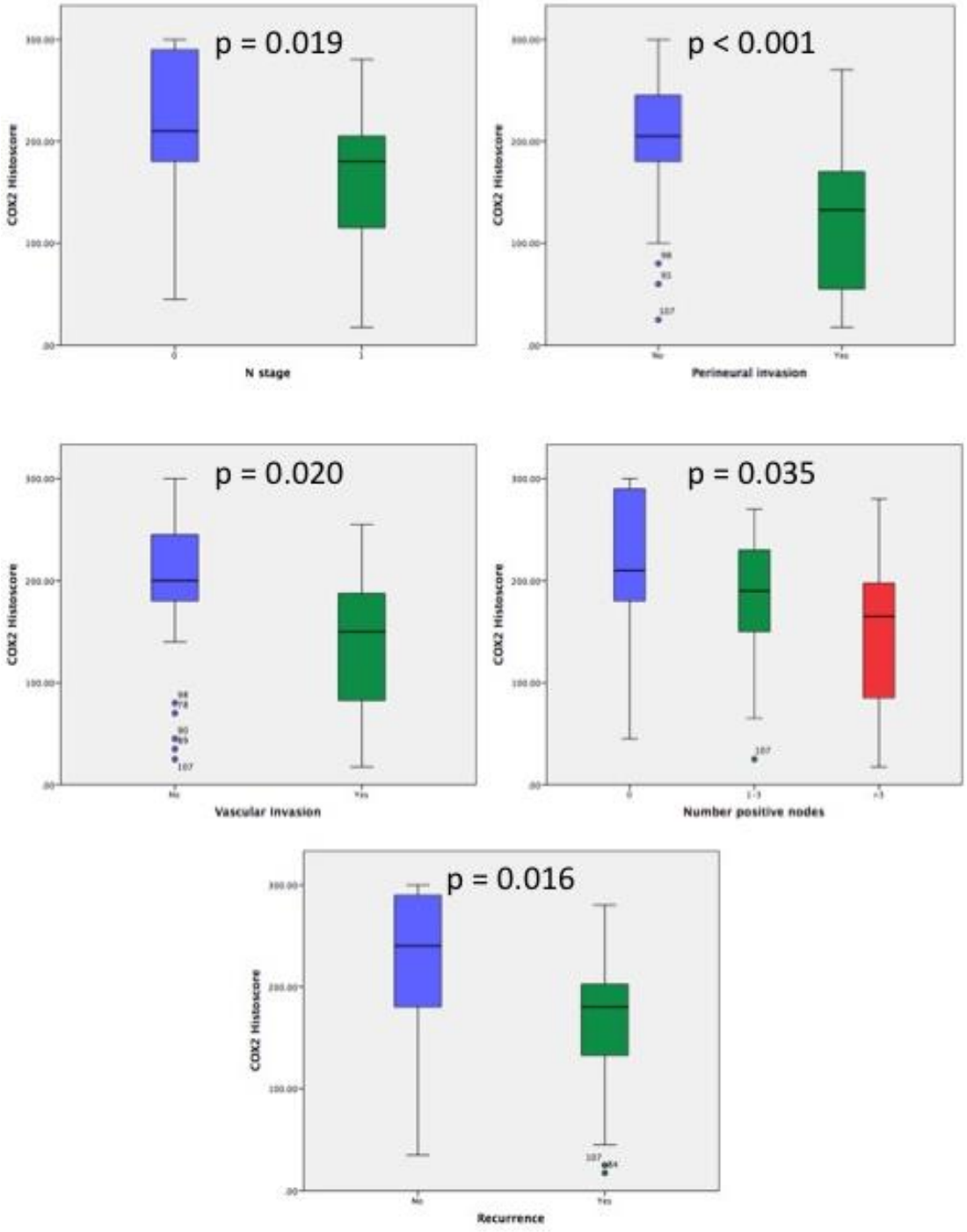
6.3.4 Angiogenesis markers prognostic influence

There were two identified papers from the performed literature search that reported on COX2 expression and its effects in ampullary cancer, predominantly improved survival with over expression. Having said this, there is strong laboratory and experimental evidence to suggest that COX2 plays a role in the evolution of gastrointestinal malignancies, having been identified as a prognostic marker in PDAC(259). We chose to further interrogate the prognostic role of COX2 in AA within the TMA cohort.

6.3.4.1 COX2 correlation with clinicopathological factors

COX2 staining was predominantly seen within the cytoplasm of epithelial cells rather than the nucleus or membrane (median histoscore 195 versus 70 versus 28.75). The expression of COX2 did not show any correlation towards T stage, tumour grade or tumour size; positive resection margins, subtype, lymphatic invasion and lymph node ratio. It did show a significant correlation with N stage ($p = 0.019$), perineural invasion ($p < 0.001$), venous invasion ($p = 0.020$), and number of positive nodes ($p = 0.035$)(**Figure 6.7**). There was a marked difference in median overall survival with low and high expression of COX2 (26 months versus 121 months); however, this did not translate into a statistical overall median survival advantage ($p = 0.090$)(**Table 6.2**).

Figure 6.7 Box plots of COX2 correlation to clinicopathological features



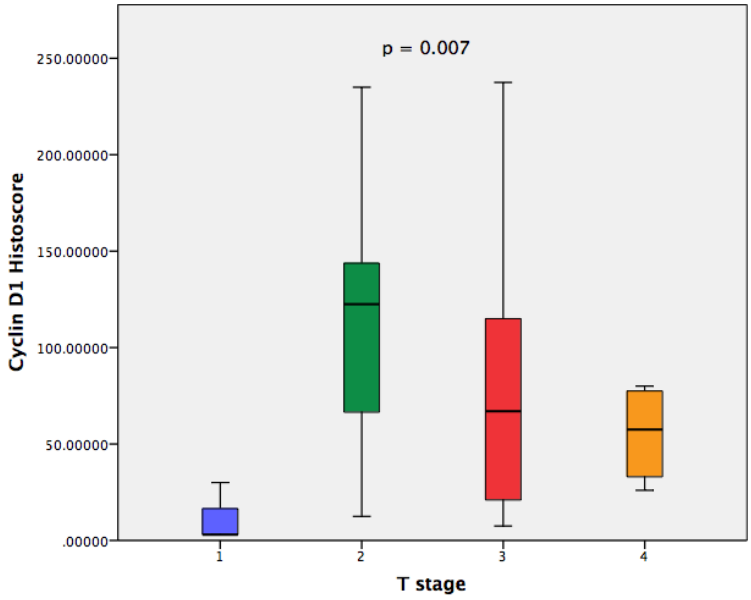
6.3.5 Markers of self-sufficiency for growth prognostic influence

The prognostic value of the protein markers SRC and cyclin D1 were evaluated in this section. There was one previous report on the prognostic value of cyclin D1 in the literature by Guo et al(57) that found that low expression correlated with overall survival.

6.3.5.1 Cyclin D1 correlation with clinicopathological factors

The expression of cyclin D1 demonstrated affinity towards T stage only (p = 0.007) (**Figure 6.8**). The histoscore was low in T1 disease (3-30), maximum expression was seen in T2 and then lost with advancing T stage thereafter. There was no correlation towards tumour grade or size; positive resection margins, subtype, lymphatic invasion, perineural invasion, venous invasion, recurrence or any LN evaluation. There was also no significant difference in median overall survival with expression loss of cyclin D1 (24 months versus 48 months; p = 0.113)(**Table 6.2**).

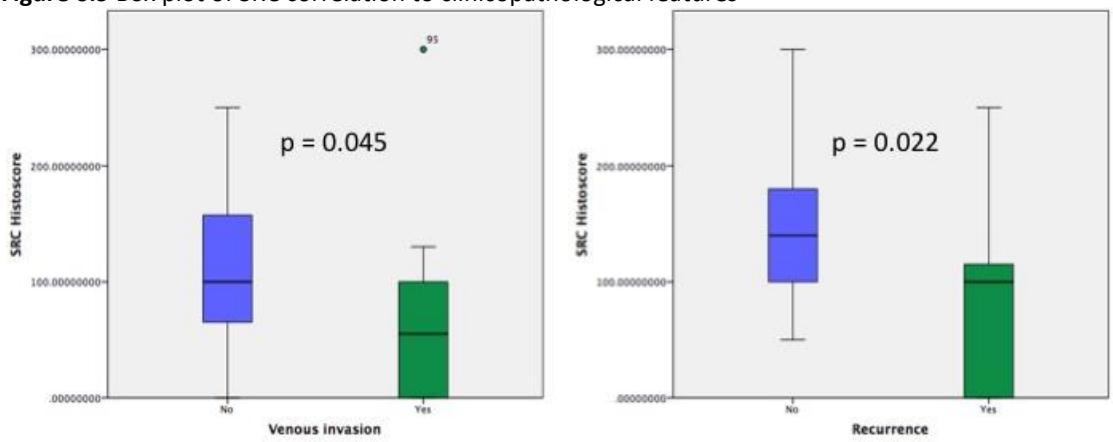
Figure 6.8 Box plot of cyclin D1 correlation to T stage



6.3.5.2 SRC correlation with clinicopathological factors

SRC staining was only seen within the membrane of epithelial cells rather than the nucleus or cytoplasm. The expression of SRC only showed strong correlation with venous invasion (p = 0.045) and tumour recurrence (p = 0.022) with the former only just reaching statistical significance (**Figure 6.9**). All of the other clinicopathological features reviewed showed no relationship to the SRC protein. Perhaps surprisingly, in view of the limited associated to clinicopathology there was a significant effect towards a poorer overall survival with low expression of SRC (12 months versus median survival not reached [NR] months; p < 0.001 (**Table 6.2, Figure 6.1**).

Figure 6.9 Box plot of SRC correlation to clinicopathological features



6.3.5.3 Relationship between cyclin D1 and SRC

From the AA specimens included in the TMAs, there was no direct correlation between cyclin D1 and SRC proteins (Spearman's rho correlation coefficient = 0.096; p = 0.510).

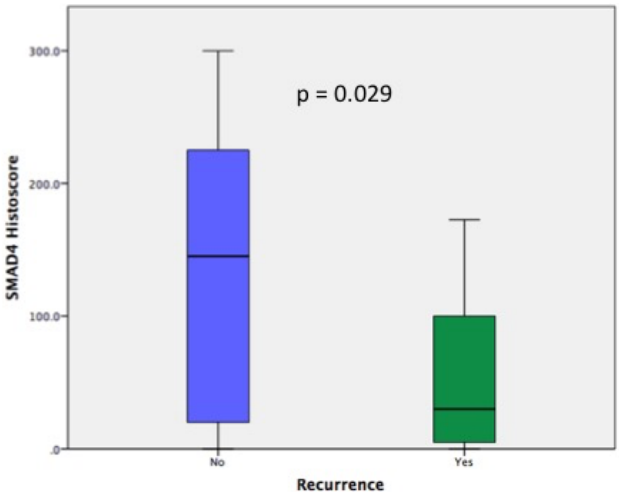
6.3.6 Insensitivity to growth inhibition markers prognostic influence

SMAD4, which is part of the TGFβ pathway, is inactivated in approximately 50% of pancreatic cancers, either by allele mutation or by homozygous loss of both alleles. When the PDAC cohort was evaluated there was a significant reduction in overall survival associated with loss of SMAD4 in the cytoplasm (DATA NOT SHOWN). The prognostic influence of this protein was therefore assessed in the AA TMA cohort.

6.3.6.1 SMAD4 correlation with clinicopathological factors

SMAD4 expression was seen within both the cytoplasm and nucleus of the ampullary tumour epithelium however loss was not correlated with clinicopathological features(**Figure 6.10**). While there was no survival impact associated with loss of nuclear SMAD4 expression, there was a significant overall poorer survival associated with low expression within the cytoplasm of 24 months compared to median survival not reached [NR](p = 0.021)(**Table 6.2, Figure 6.1**).

Figure 6.10 SMAD4 correlation to tumour recurrence



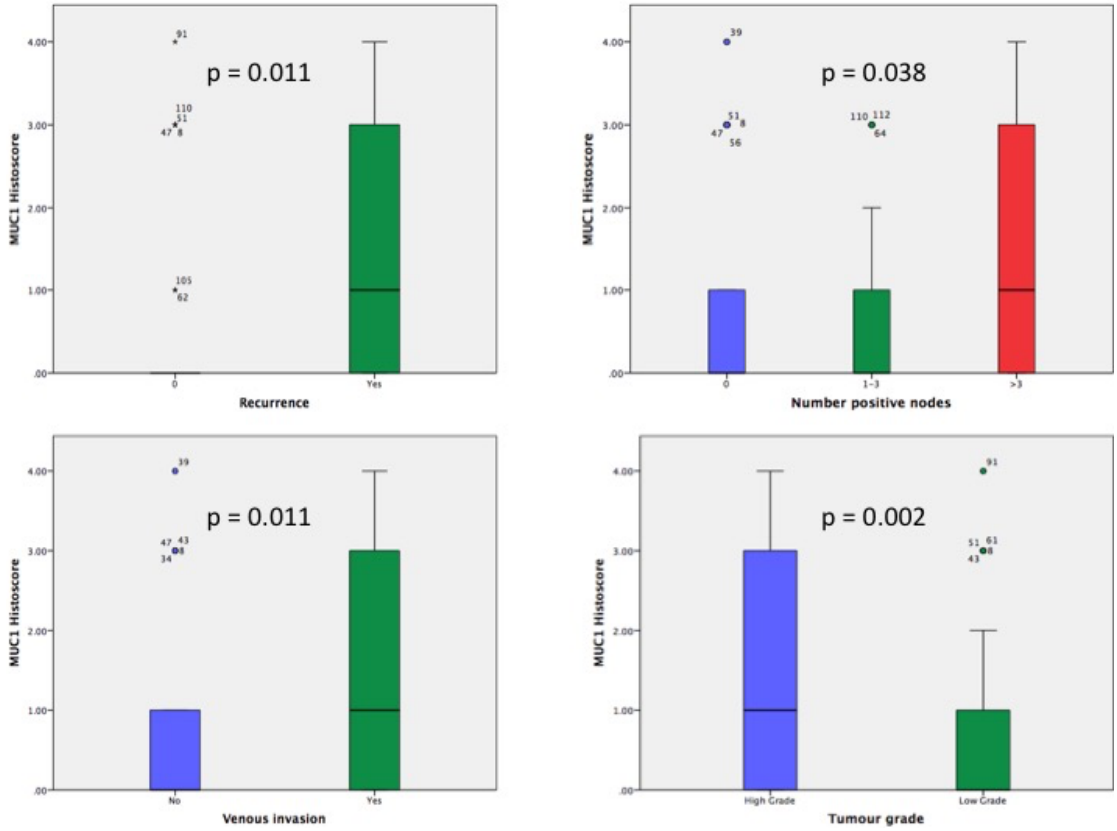
6.3.7 MUC1 and CDX2 markers prognostic influence

MUC1 and CDX2 have been the most extensively researched markers in the context of ampullary cancer with the majority of studies reporting that they are key markers in the delineation of histological subtype (high MUC1 and low CDX2) in pancreaticobiliary subtype and the converse seen in the intestinal subtype tumours) with subsequent survival implications as a result(31, 34, 59, 63). These markers were evaluated in the TMAs to revalidate this data within our cohort.

6.3.7.1 MUC1 correlation with clinicopathological factors

MUC1 expression was seen to correlate with clinicopathological features, namely tumour grade (p = 0.002), venous invasion (p = 0.011), number of positive nodes (p = 0.038) and tumour recurrence (p = 0.011)(**Figure 6.11**). There was no association with any other evaluated factors and surprisingly, although there was a trend towards subtype it did not reach statistical significance (p = 0.095). There was a significant overall survival benefit with low expression of MUC1 (67 months versus 20 months; p = 0.003)(**Table 6.2, Figure 6.1**).

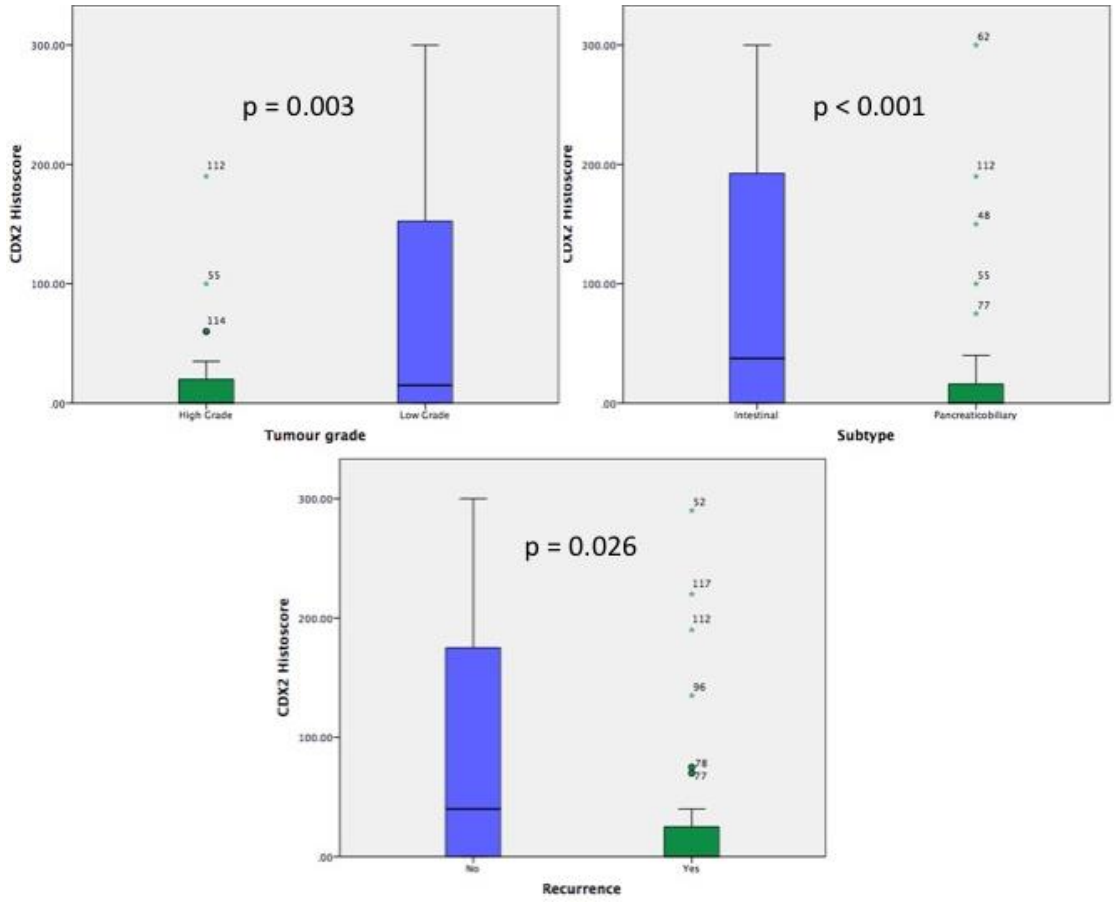
Figure 6.11 Box plots of MUC1 correlation to clinicopathological features



6.3.7.2 CDX2 correlation with clinicopathological factors

CDX2 expression was seen to have a strong association with tumour subtype ($p < 0.001$), and grade ($p = 0.003$)(**Figure 6.12**). There was a no relationship between the other clinicopathological features. High CDX2 expression was associated with a prolonged overall median survival when compared to tumours with low CDX2 expression (26 months versus median survival not reached [NR]($p < 0.001$)(**Table 6.2, Figure 6.1**).

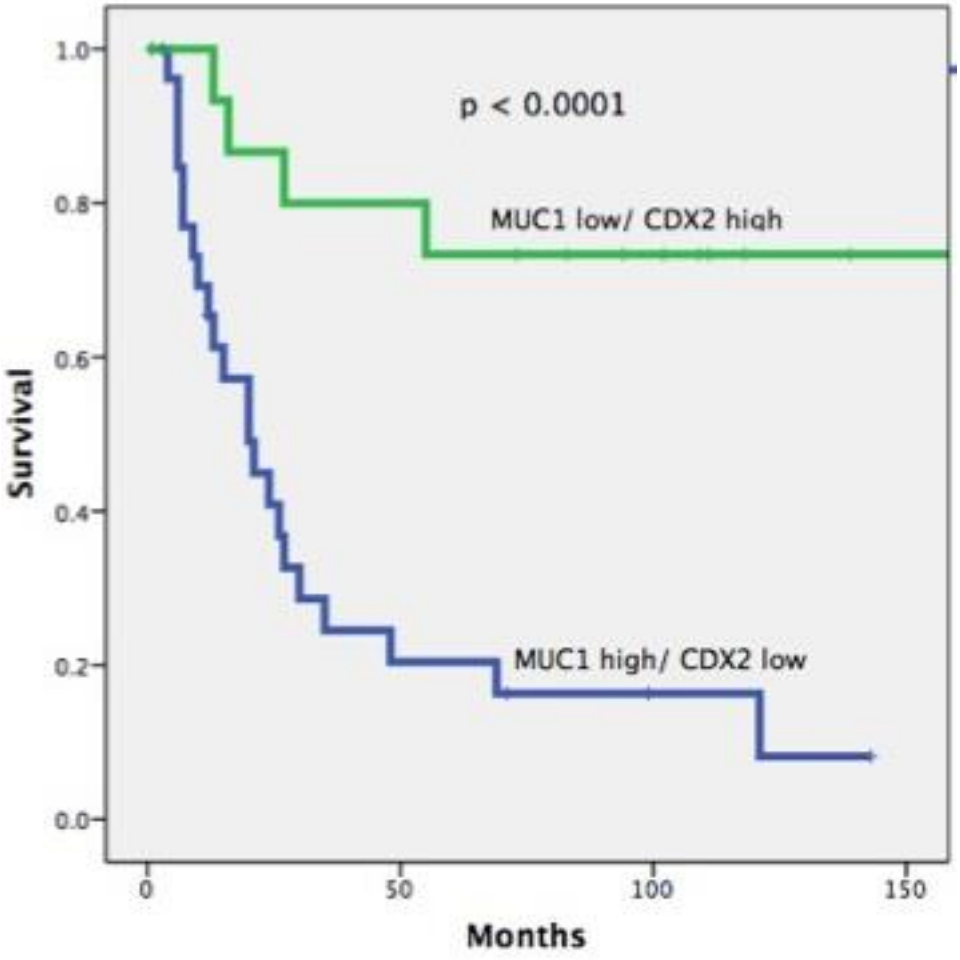
Figure 6.12 Box plots of CDX2 correlation to clinicopathological features



6.3.7.3 Relationship between MUC1 and CDX2

There was no direct correlation between the MUC1 and CDX2 markers; however, when patients were stratified into those who had either a tumour with high MUC1 expression and low CDX2 (pancreaticobiliary phenotype) or one with low MUC1 and high CDX2 expression (intestinal phenotype) marked heterogeneity was seen in overall survival (20 months 95%CI: 10.4-29.5, versus median survival not reached [NR]; $p < 0.0001$) implying the presence of two different disease and prognosis (**Figure 6.13**).

Figure 6.13 Kaplan Meier of effects of MUC1 & CDX2 on survival

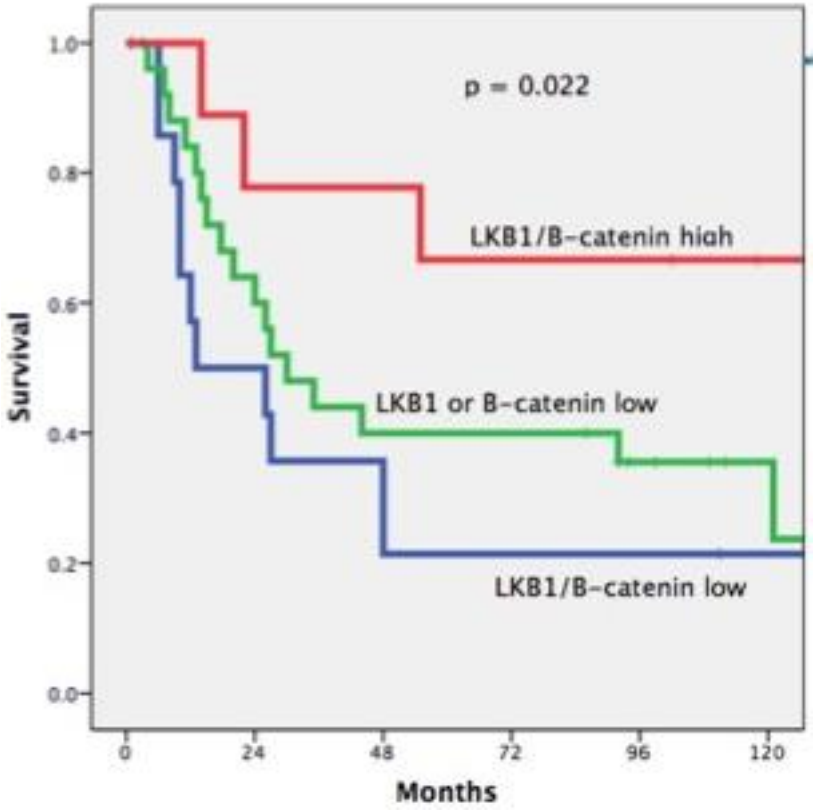


6.3.8 Metastatic phenotype

From the evaluated protein markers, B-catenin and GSK3β were the two most strongly correlated ($p < 0.001$) with the presence of lymph node metastases followed by p21 ($p = 0.031$). When considering venous and perineural invasion, LKB1 was identified as having the strongest correlation (all $p < 0.02$). The relationship of these markers together with regards to local invasion was addressed to establish their prognostic value and potential to reveal a metastatic phenotype. As GSK3β and B-catenin are implicated in the same biological process only the latter was included in the analysis.

There were 11 of 52 (21.2%) patients who were found to have high expression of LKB1 and B-catenin and 15 of 52 (28.8%) patients had low expression of both. The remaining 26 patients (50%) were in a group where one marked demonstrated high expression and the other low. In those with high expression 7 patients (63.6%) had no evidence of LN or microscopic metastases. There were 2 patients with N1 disease, 1 patient with perineural invasion and 1 with lymphatic invasion. This contrasts to the low expression group where 13 of 15 (86.7%) patients had evidence of nodal disease and microscopic invasion. Of the 12 patients with N1 status 11 (91.7%) had simultaneous microscopic invasion and a single patient had microscopic invasion only. 8 of 12 patients (66%) had invasion at more than a one site with venous being most common (8/12; 66%) followed by lymphatic and perineural (both 5/12; 41.6%). The median overall survival of patients stratified by LKB1 and B-catenin expression showed significantly poorer survival with loss of expression of both markers compared to the mixed and high expression groups (13 months, 95%CI: 0.0-38.0 versus 30 months, 95%CI: 15.0-44.2 versus median survival not reached [NR]; p = 0.022)(**Figure 6.14**).

Figure 6.14 Kaplan Meier graph of effect of LKB1/B-catenin expression on survival

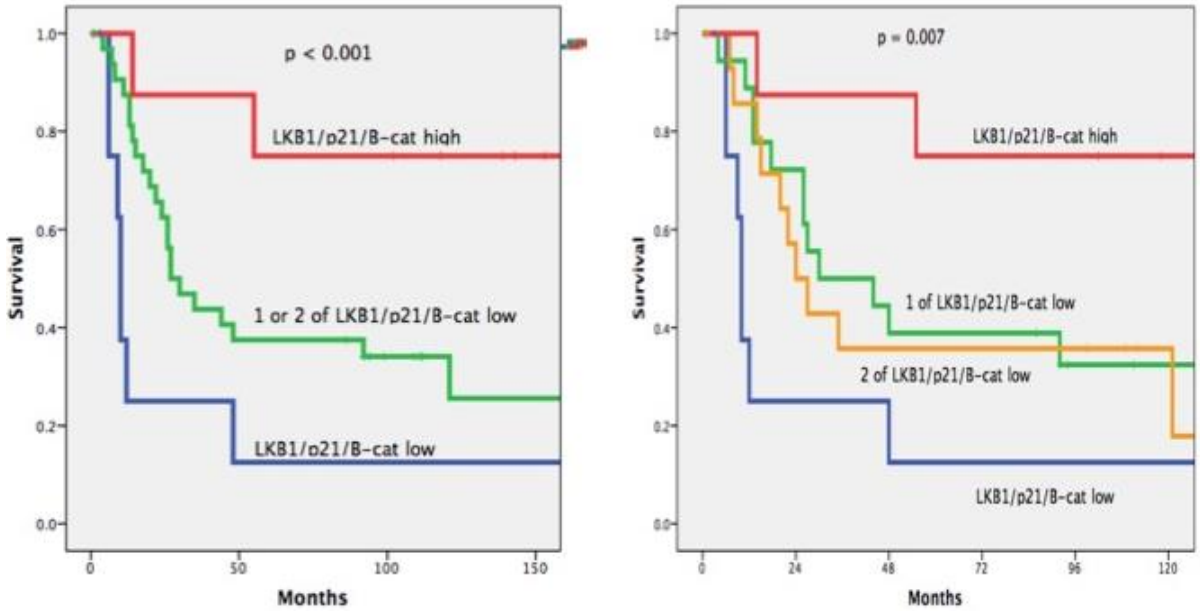


With the addition of p21 to the combination of LKB1 and B-catenin the markers were re-evaluated in an effort to obtain greater prognostic power. There were 8 of 52 (15.4%) patients who were found to have high expression of all 3-protein markers and a further 9 of 52 (17.3%) with low expression. The remaining 35 (67.3%) had a mixture of low and high expression of the three markers. In the high expression group 5 patients (62.5%) had no nodal or microscopic invasive disease and the remaining three each had one of N1 disease, lymphatic invasion or perineural invasion. Of the 9 patients in the low expression group, 8 (88.9%) patients had evidence of nodal metastases and 7 (77.8%) had signs of microscopic invasion, all of which were at multiple sites (3/7, 42.9% perineural and 6/7, 85.7% both venous and

lymphatic). In the patients with N1 staged disease, 87.5% had evidence of concurrent microscopic invasive disease.

Following survival analysis there was a significant overall median survival disadvantage with the loss of all three markers compared to the mixed group and the high expression group (10 months, 95%CI: 8.6-11.0 versus 27 months, 95%CI: 14.5-39.5 versus median survival not reached [NR]; p = 0.001). When the mixed group was further stratified into single and double marker loss the overall survival difference remained statistically significant (10 months, 95%CI: 8.6-11.0 versus 30 months, 95%CI: 0.0-65.3 versus 24 months, 95%CI: 14.8-33.1 versus median survival not reached [NR]; p = 0.007)(**Figure 6.15**).

Figure 6.15 Kaplan Meier graphs of effect of LKB1/B-catenin/P21 on survival



6.4 Discussion

IHC remains at the forefront of biological molecular assessment of malignant tumours. It is multi-faceted in the information it provides; allowing description of protein marker expression in relation to the histopathological appearance of the tumour(260) whilst also allowing determination of the location and intensity of expression. There are multiple roles for IHC in laboratory diagnostics today and is routinely employed for breast cancer (oestrogen/progesterone receptor staining), cytokeratin staining for tumour delineation and the identification of lymphoma to name but a few. In AA, IHC has been used to evaluate cytokeratins, namely CK7 and CK20, to aide accurate determination of subtype(34, 59, 61, 62) and more recently Chang et al were able to demonstrate the use MUC1 and CDX2 in three distinct cohorts to further classify AA into histomolecular phenotypes(31). As there is wide variation seen in survival of patients following PD for AA, often greatly prolonged compared to PDAC, the identification of patients with molecularly identified aggressive disease can impact management.

An issue with the markers mentioned above is their limited clinical value other than subtype identification. From the comprehensive review of IHC prognostic markers following AA resection presented in **Table 1.1** little headway has been made in identifying any relevant markers that can be applied to the clinical setting with the majority of data coming from single studies. When considering ampullectomy specimens, the identification of a molecular marker that possesses a strong correlation to lymph node or lymphovascular invasion, effectively predicting a metastatic phenotype, would potentially have great clinical impact in terms or

management strategy by providing prognostic information at an earlier point in time.

The data included in the current chapter is the first time that semi-quantitative assessment of multiple prognostic protein markers has been performed in AA. The mirrored PDAC cohort from the same institution has underwent the same TMA evaluation, with the results from that cohort being consistent with the published literature(259).

The evaluation of LKB1 expression in AA is particularly interesting. While there has been previous work evaluating the role of LKB1 in PDAC this present work remains the first of its type in AA. These data have demonstrated that LKB1 deficiency correlates most strongly with microscopic invasion. It also correlates with loss of p21 expression with a significant overall poorer median survival, and that LKB1 deficiency may act as an alternative to p53 mutation in ampullary adenocarcinoma tumour evolution. These results support the theory that LKB1 may act as a tumour suppressor gene in the periampullary region and that may function, at least in part, by prohibiting p21 expression. As mentioned, low levels of p21 and LKB1 were correlated, data that is consistent with the previous findings from PDAC studies that loss of LKB1 expression prevents culture induced cellular senescence(261) and allows BRAF mutant melanoma cells to proliferate(262).

p21 is a CDK inhibitor and prevents progression through the cell cycle mainly at S phase. The literature review identified a single paper that had previously assessed its value in ampullary cancer with no prognostic value having been identified(57). These data found low p21 expression to correlate with N1 disease, increased lymph

node positivity and venous invasion that translated to an overall reduction of median overall survival suggesting that it does indeed have prognostic value. There were however several tumours in this cohort that were found to have low p21 with high LKB1 expression suggesting that LKB1 is not the only factor implemented in this process and is likely multi-factorial.

p53 is a tumour suppressor protein that acts to promote apoptosis or arrest the cell cycle in the presence of DNA damage and abnormal cells, and is frequently mutated in the development of ampullary adenomas to adenocarcinoma(263). The fact that p53 is mutated, rather than deleted, suggests that mutant p53 provides some tumour cell growth advantage. Despite over 40 studies assessing the prognostic impact of p53in PDAC(259) and a further 3 in AA(29, 55, 57) no significant correlation to clinicopathological factors or survival impact has been seen. This is also true of the AA cohort reported in this thesis. Interestingly, p53 correlated with LKB1 expression, which suggests this may play a role in the initial dysregulation of LKB1.

B-catenin and the associated E-cadherin are important proteins involved in the maintenance of cell to cell adhesion and in contributing to B-catenin activation(264). B-catenin also functions as a transcription factor(58) with dysfunction possibly contributing to the development of ampullary cancer(265). This study demonstrated that loss of membrane B-catenin was not associated with a statistically significant reduction in median overall survival; however, did show a marked correlation towards the presence of lymph node metastases. As a result, the prognostic relevance of loss of B-catenin expression highlights a cohort of patients at risk of

early local spread and the necessity for further investigation into early B-catenin targeted anti-metastatic therapies.

The expression of nuclear B-catenin was not thought to be reproducible and therefore not formally assessed in this cohort and is not a common feature of ampullary cancers(266). Certainly, nuclear localisation is regarded as a method by which B-catenin can alter gene expression and therefore future work is required to resolve the prognostic relevance of this. The work provides the first evaluation of GSK3β expression related to AA and overall survival. These data suggest that reduced expression of GSK3β was also strongly associated with lymph node involvement and tumour recurrence following resection. This unfortunately does not support the theory that GSK3β plays a role in the inactivation of B-catenin. Therefore further interrogation of the interaction between GSK3β and B-catenin is required.

There were two studies within the literature detailing the role COX-2 expression has in the prognosis of ampullary cancer, in particular the increased frequency of intestinal phenotype and improved survival with over expression. The latter was also true from our cohort although statistical significance was not met (121 months versus 26 months; p=0.090). The converse is true from previous evaluation in other GI malignancies, as COX-2 expression was associated with worse overall survival independent of stage and tumour histological grade in PDAC(267) and with poor prognosis, larger tumour size and nodal disease in colorectal cancer(268). Loss of COX-2 expression had significant correlation to nodal involvement, perineural invasion and venous invasion which is in keeping with previously described COX-2 expression having an established relationship with angiogenesis(269). Due to the

paucity of published data on COX-2 in the context of ampullary cancer it is difficult to ascertain whether it is a plausible target for therapy compared to the multitude of evidence for COX-2 inhibition preventing colorectal cancer. A previous study has reported that high COX-2 influences promoted Lkb1 expression in polyps of Peutz-Jeghers syndrome patients(270) which is also true within the AA patients within this study (Spearman's rho correlation coefficient = 0.60; $p < 0.001$). This finding is in keeping with previous studies that suggest COX-2 may inactivate tumour suppressor genes including LKB1. Having said this, as the relationship possesses a positive correlation within the AA cohort, the effect on LKB1 must be indirect in nature.

This data confirms the previously published work by Chang et al on the utility of MUC1 and CDX2 by Chang et al(31) who state that these markers allow for the most accurate classification of AA into two distinct histomolecular phenotypes. Despite, only CDX2 having a statistically significant correlation to subtype in this cohort when both markers were combined two distinct homogenous groups were identified with marked variability in median overall survival between the two. This has clinical significance as high MUC1/low CDX2 pancreaticobiliary subtype tumours behave in a similar manner to true PDACs and suggests they should possibly be treated as such with consideration of neoadjuvant therapies prior to definitive surgery.

The work within this chapter was the first of its kind for the majority of the markers included. A potential subsequent step is investigating the prognostic utility of these markers in preoperatively collected EA specimens. A completely novel aspect of this thesis has been the attempt to identify a metastatic phenotype in ampullary cancer by grouping protein markers by the strength of their correlation to lymph node

metastases and microscopic invasion. It was possible to stratify patients into subgroups where when p21, LKB1 and B-catenin were all lost the likelihood of having local disease spread out with primary tumour was nearly 90%. This has clinical significance as if this were applied to EA specimens, patients prospectively planned for definitive endoscopic management due to borderline fitness would ultimately be better managed by high-risk radical surgical intervention with this marker profile. Clearly, due to the small number of patient data, further, larger studies in a validation group are required before definite conclusions can be made in this regard. However, this work has provided evidence that such protein markers and their expression can be utilised as adjuncts to traditional tumour staging and to influence management strategies by redefining outcomes.

6.5 Limitations

The value of IHC protein marker evaluation has increased with the introduction of TMA technology by providing the ability to perform assessment of large numbers of specimens simultaneously(271) making test conditions standardised and hence reducing multi-test error. Having said this, variations will still exist in the experimental procedures themselves such as antigen retrieval, scoring inconsistency and marker cut-off point selection variability all of which can influence the prognostic value of the proposed protein marker in question. The introduction of automated scoring techniques may allow for standardisation of these areas and overall time reduction. Furthermore tumour heterogeneity also limits the

applicability of results generated from TMA analysis highlighting the importance of multicentre validation cohorts as well as full section histological analysis.

This current chapter included 47 to 81 patients depending on which protein marker being evaluated. TMA analysis requires multiple numbers of cores and tumour samples to provide meaningful output due to its semi-quantitative nature and therefore further larger cohorts are required to test this profiling method. Despite use of a semi- quantitative scoring method, which provided a continuous score for protein marker expression, there was still the potential for bias associated with the subjective categorical assessment undertaken by the examiner. The proposed prognostic protein markers evaluated in this chapter are not an inclusive set and systematic review of the literature suggests that markers such as S100A4, hENT and EGFR may possess prognostic capability. To increase the strength of these data findings, a complete data set of protein markers could be achieved by undertaking further IHC work and creating extra TMAs so that data is available on all 127 patients recorded. A further validation cohort would also like to have been tested to assess the validity of the findings current chapter of work.

7.0 CONCLUSIONS

Chapter 7 Conclusions

7.1 Thesis Summary

This thesis evaluates several different methods for the improvement of the prediction of survival and outcome in patients undergoing management of Ampullary adenocarcinoma. Initially, the conventional clinicopathological features reported in the literature were interrogated and tested in chapter 3 to obtain a general understanding of the prognostic value of traditional clinicopathological features. Prior to the assessment of biomarker for prognostication, a large cohort of patients undergoing resection at a single institution with detailed and complete clinicopathological data available were assessed and compared with the literature in chapter 4. This focused on subtype variability and the prognostic role of lymph node involvement and notably highlighted a particularly high rate of margin involvement in AA compared to other documented series. The first novel conclusion made was that pancreaticobiliary subtype tumours behave in a remarkably similar way to true PDAC tumours and should perhaps be treated as such with consideration of neoadjuvant therapy regimens that are now being increasingly utilized in the management of PDAC. Chapter 5 then focused on the evolving role of endoscopic ampullectomy in the management of ampullary tumours. Certainly these data suggest that ampullectomy will upgrade lesions from LGD to HGD or HGD to adenocarcinoma in 28.8%. Furthermore long-term survival can be achieved with endoscopic resection alone in those patients for who are unsuitable for PD due to frailty or significant comorbidity. IHC molecular evaluation of proposed prognostic protein biomarkers were performed in chapter 6 with interesting outcomes seen for

a number of candidate markers. From this, an attempt to develop a metastatic phenotype was undertaken by combining the markers with prognostic correlation towards metastatic disease. Potentially the implementation of IHC assessment of a selection of markers in EA specimens could provide the clinician with a more detailed information into the biology of tumour and enable a more precise management algorithm particularly for those patients who possess a tumour with favourable prognosis according to histological and molecular characterisation and should be subjected to a highly morbid PD.

7.2 Overall Conclusions

Ampullary adenocarcinoma is a rare disease with the incidence having been estimated to be 6 per million persons per year(272) The gold standard of therapy remains PD and as patients typically manifest symptoms earlier in the course of the disease, a relatively high resection rate of up to 80% is seen within the literature(193). While overall prognosis is generally better than for PDAC, there is significant heterogeneity as to the clinical behaviour of ampullary adenocarcinoma following PD, with 5-year survival rates ranging between 37% to 68%(19, 140, 211). Therefore, it is important that we attempt to establish the clinicopathological and molecular features of AA that can help more precisely predict outcome and guide therapeutic strategies most effectively. Particularly, as PD remains a high-risk operative procedure with a 30-day mortality of up to 5% and morbidity seen in up to 50% of patients(112) and therefore caution must be exercised when selecting operative candidates.

When considering conventional cancer reporting using the AJCC cancer staging 7th edition TNM criteria, the clinicopathological factors influencing survival in AA from the literature were in keeping with other biliary and GI tract malignancies. This system has adapted very little over the years and while it accurately predicts prognosis for some patients with what is an extremely heterogonous disease it fails to discriminate between two distinct histological subtypes(173).

Chapter 3 highlighted the variability in pathological reporting within the literature. Employing a meta-analysis, that is the first of its kind in ampullary adenocarcinoma it was possible to capture the features indicative of a poorer prognosis in ampullary cancer following radical surgical resection following. The vast majority of patients achieved an R0 resection (median 96.8%) which raises two points; if accurate, as so few patients undergo a margin positive resection, margin status is not a major contributing factor influencing survival; it questions the adequacy of pathological reporting in the literature. Our resected AA R1 rate was much higher than seen in the literature at 31.5% using a SP reporting method although median overall survival figures for the entire cohorts were comparable. This was also true within the PDAC cohort(220)and therefore is thought to reflect high-quality reporting rather than poor surgical technique compared to other centres. The presence of lymph node involvement was an independent predictor of outcome in 19 of the 30 included studies and increasing burden demonstrated the strongest association with survival. A pancreaticobiliary subtype tumour is associated with a significantly reduced overall survival mortality rate when compared to the intestinal subtype. This appears to be true in all other periampullary adenocarcinomas(33, 59, 222) and questions whether

'periampullary cancers' should be classified by subtype over anatomical location whilst emphasises the importance of this features inclusion in standardised histopathological reporting guidelines(173). Our patient cohort data indicate that more valuable prognostic information is obtained from morphological subtyping than provided by tumour stage or tumour grade.

Related to the reviewed clinicopathological features in the literature, chapter 4 allowed analysis and comparison of the AA patient cohort undergoing resection within this institution to previously published work. Nodal involvement once again significantly reduced overall survival from a 5yr survival of 66% to 22% although did not stratify patients in a homogenous manner. No T1 tumours displayed evidence of lymph node or microscopic invasion, which has important implications for EA and raises the question to it being a valid alternative treatment option in early stage disease. Intestinal and pancreaticobiliary subtypes shows marked heterogenicity with marked variance in prognosis. The AA pancreaticobiliary subtype behaved similarity to PDAC with no significant difference in survival observed. This questions whether AA is in fact two distinct disease processes and as the pancreaticobiliary subtype mirrors PDAC raises the possibility of whether it should be treated as such and potentially considered for neoadjuvant chemotherapy as is currently being considered in PDAC(273). Clearly considerable further clinical and molecular research will be required before this strategy could be sanctioned. However, potentially this strategy could appropriately avoid resection in those patients who experience early recurrence as a result of micro-metastatic disease. Again, the

marked variance in survival also questions whether subtype and not origin is a more important classification tool for pancreaticobiliary cancers.

With the relatively recent introduction of EA, chapter 5 assessed its increasing role in the diagnosis and management of patients with an ampullary lesion. These data support the need for mandatory EA in all patients with an ampullary adenoma in an effort to more accurately determine nature of the lesion (LGD versus HGD). The frequency of upstaging suggests that endoscopic biopsy alone is perhaps inadequate prior to decision making for the management of patients with ampullary lesions particularly. For patients with adenocarcinoma resected by EA, survival was seen to mirror the surgical resection cohort up to 30 months demonstrating that EA provides excellent medium term outcomes and, although perhaps not curative, makes definitive endoscopic management an attractive option for elderly patients and those with significant comorbidity that make PD high risk. A proposed treatment guideline has been developed from our early experience and evaluated data which will require prospective validation.

A review of the literature pertaining to prognostic protein markers in AA was performed prior to chapter 6 with a paucity of data available compared to that seen in PDAC(259). The lack of useful markers may be related to the rarity of the disease process resulting in small numbers for evaluation. A number of protein markers prognostic value was assessed in the TMA cohort, individually, based on functional groups and then by correlations with clinicopathological features. The cohort demonstrated that low expression of SMAD4, p21, SRC and LKB1 were all significantly associated with poor overall survival. A novel undertaking was the

attempt to identify a metastatic phenotype; markers with the strongest correlation to nodal disease or microscopic invasion were grouped with LKB1, p21 and B-catenin being associated with a very poor survival. This has implications to the clinical setting as evaluation of these markers in EA specimens would identify patients with a highly invasive tumour, reinforcing the need for the patient to undergo PD as the only curative option even in the face of borderline fitness.

The implementation of these evaluated biomarkers alongside the previous work by Chang et al(31), who demonstrated that MUC1 and CDX2 expression were determinates of differing histomolecular phenotypes in AA, will allow for the further stratification of the disease, in both ampullectomy and resection specimens, and assessment of biomarker specific therapeutic responsiveness with the potential for use of focused chemotherapy regimes.

As previously discussed, recent genomic research has further interrogated ampullary tumour evolution and has identified that some ampullary tumours display characteristics of intestinal tumours such as microsatellite instability, inactivation of the tumour suppressor gene ELF3 by a high frequency of mutations and disruption of the WNT signaling pathway(43, 274). This was not homogenous for tumour type or subtype; however with the implementation of further subtyping by extensive biomarker assessment it may be possible to identify which tumours exhibit these characteristics and therefore adopt a custom treatment plans and pave the way for genomic WNT targeted therapies alongside tailored chemoradiotherapy regimes.

In conclusion, this work consists of one of the largest ampullary adenocarcinoma cohorts studied to date which has demonstrated marked heterogeneity in outcome when considering standard clinicopathological factors and has went some way to suggest ways of improving tumour classification by identifying new biochemical markers that allow for a much more homogenous analysis of outcomes. These markers have also allowed for the identification of 'high risk' tumours, particularly those at significant risk of metastatic dissemination, and when applied to current practice with further work will enable the clinician to identify tumours that are appropriate for less aggressive intervention compared to current standards(21).

Leading on from this piece of work, the next step would be to obtain further specimen samples and perform more immunohistochemistry to first obtain a patient cohort with a complete protein marker dataset. This will allow for further evaluation in greater numbers, improving the results and providing more robust findings. It would also be appropriate to seek a similar cohort of patients from another institution and apply the same experiements we have here to validate our findings.

As we are aware, pancreatic cancer research has progressed greatly in the past five to ten years with the introduction of genomic profiling to identify specific gene alterations within PDACs that may make tumour cells susceptible to specific chemotherapy regimes with some progress having been made in this field from an ampullary perspective as mentioned earlier(43). It would seem that the next step in the interrogation of ampullary carcinoma from this work is to adopt precision medicine and look to identify markers both as predictors of survival (similar to the

metastatic phenotype identified in this work) and those targetable for patient tailored adjuvant and neoadjuvant therapies.

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