

Predicting Disease Recurrence in Pancreatic Ductal Adenocarcinoma

A thesis submitted in accordance with the requirements of
Imperial College London for the degree of MD(Res)

By

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Abstract:

Pancreatic cancer (pancreatic ductal adenocarcinoma, PDAC) is one of the most lethal tumour types worldwide. The majority of patients present late with locally advanced or metastatic disease. Therefore, despite advances in operative techniques, perioperative management and oncological treatments, the overall 5-year survival remains <5%. The reason for poor survival is due to disease recurrence even after curative surgical resection for small tumours. Determining factors that lead to disease recurrence may help in identifying those with a poor prognosis so that treatment options can be tailored to each patient. We investigated whether operative technique, clinicopathological factors or specific genetic mutations could influence disease recurrence. Further, we sought to identify a biomarker in the peripheral circulation that could be used as a prognostic marker. We confirmed that a positive medial resection margin and a high frequency of KRAS mutation in the tumour tissue result in early disease recurrence. Further, that the altered expression of five microRNAs may be useful as a blood-based prognostic predictor.

Statement of Originality

I have conducted all the experimentation presented and written in this thesis, apart from the following:

Methods The miRNA microarray was performed by Nanostring technologies, Seattle, USA

Methods Collection of blood samples for CTC analysis and CTC analysis was assisted by Mr Adam Frampton

Methods Dr Raida Ahmad (Histopathologist, Imperial College Healthcare Trust) helped to identify patient pancreatic tumour samples and perform further histological verification.

Methods Pritesh Trivedi (Histopathology technician, Imperial College Healthcare Trust) performed the transfer of the FFPE tumour samples onto slides for microdissection.

Results Statistical and bioinformatic analyses of the miRNA microarray were performed by Dr Leandro Castellano (Imperial College)

Results Analysis of the ion torrent sequencing was performed by Dr Gareth Gerrard (Imperial College)

Tamara MH Gall.

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Full manuscripts published in peer reviewed journals during MD(res) period

- 1) Pancreatic cancer: current management and treatment strategies. **Gall TM**, Tsakok M, Wasan H, Jiao LR. *Postgrad Med J*. 2015 Aug. PMID: 26243882
- 2) Pancreatic cancer: current understanding of molecular and genetic aetiologies. **Gall TM**, Wasan H, Jiao LR. *Postgrad Med J*. 2015. PMID: 26124188
- 3) microRNAs with prognostic significance in pancreatic ductal adenocarcinoma: A meta-analysis. A Frampton, J Krell, N Jamieson, **T Gall** et al. *Eur J Cancer*. 2015. PMID: 26002251
- 4) Activating mutations of GNAS and KRAS in cystic fluid can help detect intraductal papillary mucinous neoplasms of the pancreas. Frampton AE, Stebbing J, **Gall TM**, Silver B, Jiao LR, Krell J. *Expert Rev Mol Diagn*. 2015 Feb. PMID: 25656048
- 5) A case of gossypiboma mimicking intrahepatic cholangiocarcinoma. Ojha S, **Gall T**, Sodergren MH, Jiao LR. *Ann R Coll Surg Engl*. 2014 Oct;96(7):e14-6. doi: 10.1308/003588414X13946184901489. PMID: 25245716
- 6) Laparoscopic vs open approach to resection of hepatocellular carcinoma in patients with known cirrhosis: Systematic review and meta-analysis. Twaij A, Pucher PH, Sodergren MH, **Gall T**, Darzi A, Jiao LR. *World J Gastroenterol*. 2014 Jul 7;20(25):8274-81. PMID: 25009403
- 7) Radio-frequency-Assisted Liver Partition With Portal Vein Ligation (RALPP) for Liver Regeneration. **Gall TM**, Sodergren MH, Frampton AE, Fan R, Spalding DR, Habib NA, Pai M, Jackson JE, Tait P, Jiao LR. *Ann Surg*. 2014 Mar 25. PMID: 24670841
- 8) miR-15b and miR-17 Are Tumor-Derived Plasma MicroRNAs Dysregulated in Colorectal Neoplasia. Frampton AE, Krell J, **Gall TM**, Castellano L, Stebbing J, Jiao LR. *Ann Surg*. 2014 Mar 18. PMID: 24646542
- 9) Reduced Dissemination of Circulating Tumor Cells With No-Touch Isolation Surgical Technique in Patients With Pancreatic Cancer. **Gall TM**, Jacob J, Frampton AE, Krell J, Kyriakides C, Castellano L, Stebbing J, Jiao LR. *JAMA Surg*. 2014 Mar 5.

doi: 10.1001/jamasurg.2013.3643.PMID: 24599353

10) A microRNA meta-signature for pancreatic ductal adenocarcinoma. Frampton AE, Giovannetti E, Jamieson NB, Krell J, **Gall TM**, Stebbing J, Jiao LR, Castellano L. *Expert Rev Mol Diagn*. 2014 Feb 28. PMID: 24575833

11) Surgical techniques for improving outcomes in pancreatic ductal adenocarcinoma. **Gall TM**, Thompson Z, Dinneen EP, Sodergren M, Pai M, Frampton AE, Jiao LR. *Expert Rev Gastroenterol Hepatol*. 2014 Feb 4

12) Cell-free DNA for the detection of pancreatic, liver and upper gastrointestinal cancers: has progress been made? **Gall TM**, Frampton AE, Krell J, Habib NA, Castellano L, Stebbing J, Jiao LR. *Future Oncol*. 2013 Dec;9(12):1861-9. doi: 10.2217/fon.13.152. PMID: 24295416

13) Neo-adjuvant chemotherapy and primary-first approach for rectal cancer with synchronous liver metastases. **Gall TM**, Basyouny M, Frampton AE, Darzi A, Ziprin P, Dawson P, Paraskeva P, Habib NA, Spalding DR, Cleator S, Lowdell C, Jiao LR. *Colorectal Dis*. 2013 Dec 18. doi: 10.1111/codi.12534. PMID: 24344746

14) Is the detection of circulating tumor cells in locally advanced pancreatic cancer a useful prognostic marker? **Gall TM**, Frampton AE, Krell J, Jacob J, Stebbing J, Jiao LR. *Expert Rev Mol Diagn*. 2013 Oct 16. PMID: 24127751

15) Mini-probe ultrasonography for the staging of colon cancer. A systematic review and meta-analysis. **Gall TM**, Markar SR, Jackson D, Haji A, Faiz O. *Colorectal Dis*. 2013 Oct 5. doi: 10.1111/codi.12445.

16) MicroRNAs Co-operatively Inhibit a Network of Tumor Suppressor Genes to Promote Pancreatic Tumor Growth and Progression. Frampton AE, Castellano L, Colombo T, Giovannetti E, Krell J, Jacob J, Pellegrino L, Roca-Alonso L, Funel N, **Gall TM**, De Giorgio A, Pinho FG, Fulci V, Britton DJ, Ahmad R, Habib NA, Coombes RC, Harding V, Knösel T, Stebbing J, Jiao LR. *Gastroenterology*. 2013 Oct 8. doi:pii: S0016-5085(13)01431-5. 10.1053/j.gastro.2013.10.010.

- 17) Gene of the month: E-cadherin (CDH1). **Gall TM**, Frampton AE. J Clin Pathol. 2013 Aug 12. doi: 10.1136/jclinpath-2013-201768. [Epub ahead of print] No abstract available. PMID:23940132
- 18) The p53 miRNA interactome and its potential role in the cancer clinic. Krell J, Frampton AE, Colombo T, **Gall TM**, De Giorgio A, Harding V, Stebbing J, Castellano L. Epigenomics. 2013 Aug;5(4):417-28. doi: 10.2217/epi.13.41. PMID: 23895654
- 19) Circulating peripheral blood mononuclear cells exhibit altered miRNA expression patterns in pancreatic cancer. Frampton AE, Fletcher CE, **Gall TM**, Castellano L, Bevan CL, Stebbing J, Krell J. Expert Rev Mol Diagn. 2013 Jun;13(5):425-30. PMID: 23782250
- 20) Distinct miRNA profiles are associated with malignant transformation of pancreatic cystic tumors revealing potential biomarkers for clinical use. Frampton AE, **Gall TM**, Giovannetti E, Stebbing J, Castellano L, Jiao LR, Krell J. Expert Rev Mol Diagn. 2013 May;13(4):325-9. doi: 10.1586/erm.13.18. PMID: 23638815
- 21) Optimizing Unresectable Colorectal Liver Metastases for Surgery-No Limits, Any Benefits? **Gall TM**, Frampton AE, Krell J, Jiao LR. J Gastrointest Surg. 2013 Apr 17. PMID: 23592187
- 22) Blood-based miRNAs as noninvasive diagnostic and surrogate biomarkers in colorectal cancer. **Gall TM**, Frampton AE, Krell J, Castellano L, Stebbing J, Jiao LR. Expert Rev Mol Diagn. 2013 Mar;13(2):141-5. doi: 10.1586/erm.13.2. PMID: 23477554
- 23) Reactive lymphoid hyperplasia of the pancreas: a clinical conundrum. Christophides T, Frampton AE, Cohen P, **Gall TM**, Jiao LR, Habib NA, Pai M. JOP. 2013 Mar 10;14(2):207-11. doi: 10.6092/1590-8577/1327. PMID: 23474571
- 24) Circulating molecular markers in pancreatic cancer: ready for clinical use? **Gall TM**, Frampton AE, Krell J, Castellano L, Jiao LR. Future Oncol. 2013 Feb;9(2):141-4. doi: 10.2217/fon.12.187. PMID: 23414463

25) Towards a clinical use of miRNAs in pancreatic cancer biopsies. Frampton AE, **Gall TM**, Castellano L, Stebbing J, Jiao LR, Krell J. Expert Rev Mol Diagn. 2013 Jan;13(1):31-4. doi: 10.1

586/erm.12.136. PMID: 23256701

26) Is there a 'margin' for error in pancreatic cancer surgery? Frampton AE, **Gall TM**, Krell J, Ahmad R, Jiao LR. Future Oncol. 2013 Jan;9(1):31-4. doi:

10.2217/fon.12.175. PMID: 23252561

CHAPTER 1:

An Introduction To Pancreatic Cancer

1.1 DUCTAL ADENOCARCINOMA OF THE PANCREAS

Pancreatic cancer is a devastating and aggressive disease with an extremely poor prognosis despite radiological, surgical and oncological advances. The majority of cancers are exocrine (90%) arising from the ductal cells and are termed pancreatic ductal adenocarcinomas (PDAC). Other rarer types of pancreatic cancer include acinar cell carcinomas, mucinous cystadenocarcinomas, sarcomas, lymphomas, adenosquamous carcinomas, colloid carcinomas, giant cell tumours, pancreatoblastomas, signet ring cell carcinomas, undifferentiated carcinomas and neuroendocrine tumours. Evidence suggests that PDAC is a slowly developing disease, with at least fifteen years between the initiating mutations and the potential for metastatic spread¹. By the time of diagnosis, it is considered to be an invasive and progressive disease, which is highly resistant to chemotherapeutic agents. Our understanding of its tumour biology continues to rapidly improve, however 5-year survival remains at only around five per cent. Surgical resection is the only potentially curative treatment; however this is only possible in up to 25% at initial diagnosis. Those with advanced disease who are treated with palliative chemotherapy and no surgery have a median survival of just 6 months.² Even those with a curative R0 resection only have a median survival of ~29 months.³

1.2 EPIDEMIOLOGY

The GLOBOCAN project, led by the International Agency for Research on Cancer and the World Health Organisation ⁴ estimates that worldwide there are 337,872 new cases of PDAC diagnosed each year. The cumulative risk of developing the disease before the age of 75 is 0.47 for every 100 children born, which is increased in more developed regions (0.85) compared to less developed regions (0.31). Although it only accounts for 3% of new cancer cases, mortality is closely related to incidence and it is the fourth leading cause of cancer death among both males and females (National Cancer Institute, September 2010)). Age-standardised rates are higher in males compared to females and in those of White and Black ethnicity compared to Asian (Cancer Research UK, June 2014).

1.3.1 RISK FACTORS AND SCREENING

Pancreatic cancer can be divided into sporadic and familial cancer. There are four main genes affected in sporadic cancer: *KRAS* (95% of tumours); *CDKN2A* (*p16*) (90%); *p53* (75%); and *SMAD4* (55%).⁵ Multiple genetic and epigenetic mutations interlinked with environmental factors will lead to the development of PDAC. As with many cancers, the major risk factor is age, with the majority of cases occurring after the age of 60, and very few before 40. Some other identified risk factors include: Smoking; chronic pancreatitis; diabetes; obesity; other medical conditions; diet; and ABO blood type. A common feature of these risk factors is an ability to induce oxidative stress and thus DNA damage.⁶ The resultant increase in reactive oxygen species (ROS) causes damage to lipids, protein and DNA. Mechanisms exist at the cellular level to repair damaged DNA and to neutralise ROS. Activation of these repair pathways is seen in pancreatic cancer cell lines and in tumour cells.^{7, 8} Certainly, there is an increased level of direct DNA damage in patients with

pancreatic cancer.⁹ Familial pancreatic cancer is seen in patients with a first degree relative also affected and in younger patients. However, there is no standardised classification for these patients, such as with the Amsterdam criteria in familial colorectal cancer. In these cases of familial aggregation of PDAC, the *PRSS1*, *SPINK1*, *CDKN2A*, *STK11*, *ATM*, *BRCA1*, *BRCA2*, *PALB2*, *APC* and mismatch repair genes may be involved.

1.3.2 Sporadic pancreatic cancer:

Smoking: Current tobacco use is the single most important risk factor for pancreatic diseases, accountable for a quarter of cases. Results would suggest that smoking causes a 75% increase in the risk of pancreatic cancer with a higher risk in those who smoke more per day and for a longer duration. The risk persists for at least 10 years.¹⁰ Analysing 51 population based studies with nearly 3 million participants, ever smokers have a relative risk (RR) of developing PDAC of 1.60 compared to never smokers.¹¹ The relative risk is more in current smokers: RR 1.74 (95% CI 1.61-1.87) than in former smokers: RR 1.2 (95% CI 1.11-1.29)¹⁰. In addition, cigarette smokers have an increased risk compared to pipe and/or cigar smokers.¹⁰ Tobacco carcinogens may reach the pancreas via the bloodstream as with other smoking-related cancers, or directly via bile, which may explain the high incidence of head of pancreas cancers. However the pathological mechanisms behind the development of cancer is not well understood. Indeed, tobacco consumption does not appear to be associated with an increased frequency of *K-ras* mutations¹² suggesting earlier epigenetic changes.

Chronic Pancreatitis: Chronic pancreatitis (CP) is a progressive inflammatory condition with an incidence of 3.5-10 per 100,000 population.¹³ It is characterised by irreversible morphological changes resulting in loss of both exocrine and endocrine

pancreatic function. Histopathologically we see chronic inflammation, loss of acinar cells, irregular inter-lobular fibrosis, distorted ducts and patches of resolving fat necrosis.¹⁴ Mice with mutant *K-ras* expression show pancreatitis in the early stages and cerulein injections (a cholecystokinin analogue which hyperstimulates the pancreas, inducing pancreatitis) accelerates the progression to advanced cancer in these mice.¹⁵ The correlation is also seen in humans with a recent 15 year cohort study of nearly 12,000 patients with chronic pancreatitis finding an increased risk for pancreatic cancer compared to controls, with an adjusted hazard ratio of 6.9 (95% CI 5.6-8.6).¹⁶ Meta-analyses of case-control and cohort studies have also found an increased risk of pancreatic cancer in those with CP, with a RR of 5.1 (95% CI 3.5-7.3).¹⁷ The risk is more pronounced in the first 2-4 years from the diagnosis of CP¹⁶,¹⁸ and also in those with tropical pancreatitis.¹⁷ There is debate in the literature as to whether the risk is increased in those with alcoholic CP compared to those with CP of other aetiology^{16, 19}. It is possible that inflammatory insults can hyperstimulate mutated *K-ras* allowing the initiation of the sequence of carcinogenesis.²⁰ There are certainly altered cytokine levels in patients with pancreatic cancer compared to controls and these may have a causative function.²¹

Diabetes; It has long been recognised that type II diabetes may increase the risk of developing PDAC, it is seen to be both a cause and a consequence of cancer. There does not seem to be the same relationship for type I diabetics²² however, the majority of studies have not distinguished between the two types. A meta-analysis of over 9000 patients with pancreatic cancer found an 80% greater risk of pancreatic cancer in those with type II diabetes.²³ However, the RR of developing cancer appears to be increased in those with a shorter duration of disease suggesting some of the effect may be due to an early manifestation of the tumour. Although, there does remain some evidence for a causal relationship between diabetes and pancreatic cancer as a 50% increased risk still exists for those with a >10 year history of diabetes.²⁴

Furthermore, there is a dose-response pattern with an increased level of fasting glucose increasing cancer risk.^{25 26} In type II diabetes, the pancreas is exposed to high levels of insulin for many years and develops insulin resistance. It is postulated that this may be involved in the development of pancreatic cancer. Certainly, PDAC cell lines have insulin receptors and insulin increases PDAC cell proliferation in a dose-dependent manner.²⁷ This suggests that insulin may be important in tumour growth. Insulin also increases production of Insulin-like Growth Factor 1 which promotes cell growth and inhibits apoptosis in human pancreatic cancer cells.²⁸ Interestingly, diabetic medication and their pharmacological effects may also have a role in tumourigenesis with a reduced risk of PDAC seen in type II diabetics taking metformin.²⁹ Glucagon-like-peptide-1 (GLP-1) may cause proliferative effects on the exocrine pancreas³⁰ with some concerned that this increases the risk of cancer although, others have seen apoptosis of pancreatic cancer cell lines with inhibition of tumour growth following activation of the GLP-1 receptor³¹. This remains a controversial topic however most agree that the cardiovascular benefits of using GLP-1 drugs in diabetes far outweighs a potential but poorly reported risk of cancer³².

Obesity: There is increasing evidence suggesting that adiposity, measured as the body mass index (BMI) may increase the risk of developing pancreatic cancer. Two large meta-analyses analysing 3.5 million people with 8062 cases of PDAC³³ and 5 million people with 9504 cases of PDAC³⁴ have been conducted within the last decade. The RR of developing PDAC per unit increase in BMI was 1.12 (95% CI, 1.06–1.17) in the first and 1.10 (95% CI: 1.07–1.14) per 5-unit increase in the second study. A dose-response effect is seen with the highest risk in those with a BMI>30. The majority of the studies analysed controlled for the confounding factors smoking and diabetes and found no difference when taking gender into account. Adipose tissue is involved in free fatty acid metabolism and cytokine release,³⁵ resulting in an

increase of fatty acids and tumour necrosis factor- α and reduced release of adiponectin.³⁶ This leads to hyperinsulinaemia and a reduced insulin tolerance thereby stimulating cell growth as mentioned above. A Furthermore predisposing factor for cancer is pancreatic duct replication, which is increased tenfold in obese compared to lean people³⁷. In addition, increased adiposity increases lipid peroxidation³⁸ which may cause DNA damage.

Other medical conditions: There are some suggestions that other pathologies increase the risk of pancreatic cancer. For example, patients with pancreatic cancer are more likely to be *H. Pylori* positive.³⁹ Although it is not clear whether this is reverse causality, it is hypothesised that excess acidity is associated with basal stimulation of pancreatic bicarbonate production, which may increase ductal hyperplasia and increased DNA synthesis.⁴⁰ Patients with inflammatory bowel disease (IBD), both Crohn's disease and Ulcerative Colitis, have an increased life-time risk of many gastrointestinal and extra-intestinal cancers,⁴¹ including pancreatic cancer. This risk is further increased in IBD patients with associated primary sclerosing cholangitis.⁴² Furthermore, patients who have Hepatitis B have an increased risk of pancreatic cancer (odds ratio for developing pancreatic cancer in those positive for hepatitis B surface antigen (HBsAg) is 1.50 (95 % CI 1.21 to 1.87),⁴³ and those with Hepatitis C virus may also have an increased risk⁴⁴, although the association is less clear. Lastly there may be a link between periodontal disease and pancreatic cancer.⁴⁵

Diet: A meta-analysis of eleven prospective studies analysed red and processed meat consumption in 2,307,787 participants with 6643 cases of pancreatic cancer. The RR of pancreatic cancer for a 50g per day increase in processed meat consumption (about one serving) was 1.19 (95% CI 1.04-1.36), resulting in a 19% increased risk.⁴⁶ With regards to red meat, the overall RR showed no increased risk

with pancreatic cancer with an increase of 120g per day of red meat consumption. However, an association was seen in males, but not females. Importantly, not all studies controlled for the confounders obesity and diabetes and there is heterogeneity among studies in terms of meat classification and length of time for meat consumption. In theory, processed meats could increase the risk of cancer as they contain *N*-nitroso compounds, which can induce pancreatic cancer in animal models.⁴⁰ Other case-control studies have suggested a diet high in protein and unsaturated fatty acids is associated with a decreased risk of pancreatic cancer, in a dose-dependent fashion.⁴⁷ There is Furthermore limited evidence to suggest a decreased risk of cancer with a consumption of fruit and vegetables and whole grains whilst a possible increased risk exists with the consumption of sugar-sweetened soft drinks.⁴⁸ Finally, conflicting evidence is found regarding vitamin D, with some supporting a reduced risk of cancer⁴⁹ and others a higher risk,⁵⁰ with high levels of vitamin D.

ABO Blood type: There is some evidence to suggest that ABO blood type may affect risk for pancreatic cancer. In a Chinese population an increased risk of cancer was seen in those with blood group A compared to blood group O. On meta-analysis of world-wide studies, there is also an increased risk with groups B and AB compared to O.⁵¹ It is possible that there are ABO-related differences in inflammation and cytokine production but these theories are not well supported.

1.3.3 Familial pancreatic cancer:

Around 5-10% of cases of pancreatic cancer are seen to show familial clustering, with an elevated risk of pancreatic cancer in individuals with a first degree relative affected. The incidence of pancreatic cancer is estimated to rise from 9 per 100,000 per year in patients with no family history of PDAC, to 288 per 100,000 per year in

those with three or more first degree relatives with pancreatic cancer.⁵² A number of germline mutations have been identified in these familial cases of pancreatic cancer.

PRSS1 and SPINK1: Hereditary pancreatitis increases the risk of developing PDAC. The highly penetrant autosomal dominant disorder, which usually manifests by the age of 30,⁵³ is associated with mutations in *PRSS1*, which encodes for the protein trypsinogen. Acute pancreatitis is caused by premature activation of trypsinogen with recurrent attacks of acute pancreatitis leading to chronic pancreatitis and its increased cancer risk. The lifetime risk of PDAC in these patients is 35 fold by 70-75 years of age.⁵⁴ The autosomal recessive form of hereditary pancreatitis develops due to *SPINK1* mutations. This gene encodes a trypsin inhibitor and its mutations therefore lead to inappropriate trypsin activity, again resulting in pancreatitis.⁵⁵

CDKN2A: Another autosomal dominant disorder which has a 20% increased risk of pancreatic cancer by the age of 75, as well as a 60-90% risk of melanoma, is the Familial atypical mole and multiple melanoma (FAMMM) syndrome. These patients have abnormalities in *CDKN2A*. The mutant p16 proteins cannot inhibit the cyclin D1/CDK4 or cyclin D1/CDK6 complex, leading to genomic instability and uncontrolled cell division.⁵⁶

STK11 (LKB1) and ATM: Germline mutations in *STK11* are associated with Peutz-Jeghers, an autosomal dominant condition resulting in early onset, large, multiple small intestinal and colonic polyps with an increased cancer risk at these sites.⁵⁷ These patients also have a 26% increased risk of pancreatic cancer.⁵⁸ The *STK11/LKB1* gene encodes for a member of the serine/threonine kinase family, which functions as a tumour suppressor⁵⁹. The protein encoded by the *ATM* gene is also a serine/threonine kinase. It is involved in the DNA damage response signaling pathway⁶⁰, thus integral to the maintenance of genomic stability. Mutations in *ATM* result in the disease ataxia-telangiectasia characterized by cerebellar ataxia,

oculomotor apraxia, frequent infections, telangiectasias of the skin and conjunctivae and an increased rate of neoplasia, particularly haematological malignancies.⁶¹ These mutations have also been linked to pancreatic cancer.⁶²

BRCA1, BRCA2 and PALB2: These three genes encode proteins important in DNA repair pathways required to halt the development of cancer. Certain endogenous and exogenous agents may cause double-stranded DNA breaks. Protein complexes involving products coded by these genes form, enabling firstly recognition, and secondly repair of these breaks by performing exonucleolytic resection of one strand of DNA followed by replication of the single-stranded DNA.⁵⁴ People with mutations in *BRCA1* and *BRCA2* carry an 80% lifetime risk for the development of breast cancer⁶³ and germline mutations in *PALB2* are also associated with hereditary breast cancer.⁶⁴ Those with a *BRCA2* mutation also have a 3.5 fold increased risk of pancreatic cancer⁶⁵ and this germline mutation is the most common recognizable cause of familial pancreatic cancer. The risk of pancreatic cancer in *BRCA1* mutation carriers is less clear with some reporting a RR OF 2.3⁶⁶ whilst others have found no association.⁶⁷ Genome sequencing of pancreatic cancer identified *PALB2* as a mutation in 1-3% of pancreatic cancer families.⁶⁸, and carriers have a 5.9 fold increased risk of pancreatic cancer.⁶⁹

APC and Mismatch repair genes: Mutations of the *APC* gene are well known to result in familial adenomatous polyposis (FAP) and those in a number of mismatch repair genes (e.g. hMLH1, hMSH2, hMSH6, and hPMS2) predispose to hereditary non-polyposis colon cancer (HNPCC). As well as a 100% and 80% risk of developing colon cancer respectively, these patients also have a risk of extra intestinal cancers. HNPCC patients have a 3.7% risk of pancreatic cancer whilst those with FAP may have a 4 fold increased risk, although others have failed to demonstrate this.⁷⁰

1.3.4 Screening:

Considering that an earlier diagnosis of pancreatic cancer increases the likelihood of resectable disease prior to the development of metastases¹ and thus improved prognosis, there has been interest in screening for pancreatic cancer amongst the high risk groups discussed above. The International Cancer of the Pancreas Screening (CAPS) recently developed and published consortium statements regarding the management of patients at risk of familial pancreatic cancer.⁷¹ A screening programme should detect T1N0M0 PDAC and high-grade dysplastic precursor lesions to enable curative R0 resections. There was agreement that there was a role for screening with endoscopic ultrasonography (EUS) and/or magnetic resonance cholangiopancreatography (MRCP) of the pancreas in the following patients: Those with first degree relatives from a pancreatic cancer family (i.e. a family member with PDAC has at least two affected first degree relatives); and patients with Peutz-Jegher's, HNPCC, *BRCA2* mutations or *CDKN2A* mutations with at least one first degree relative affected. However, age and screening intervals were not agreed upon and Furthermore, consensus was not reached on which abnormalities should lead to surgical resection. Before a screening programme can be initiated, Furthermore advancement to improve the sensitivity and specificity of screening methods and international consensus is warranted.

1.4 PRECURSOR LESIONS

There are three types of precursor lesions in PDAC that follow a multi-step progression from neoplasia showing mild dysplasia to invasive cancer. They are: Pancreatic Intraepithelial Neoplasia (PanIN); Intraductal Papillary Mucinous Neoplasm (IPMN); and Mucinous Cystic Neoplasm (MCN). Some of these lesions may be detected on imaging, often incidentally, and either resected or kept under

surveillance to prevent the development of, or to allow early detection of, invasive carcinoma.

PanIN: PanINs are microscopic proliferative epithelial lesions in the small calibre pancreatic ducts and ductules, usually involving ducts <5mm in diameter.⁷² They are the most common precursor lesion recognised in pancreatic cancer and often found surrounding PDAC at histological analysis of surgical resections. A four-tier classification system was established in 2010⁷³ that grades the histopathological findings. PanIN-1 lesions show metaplasia and ductal hyperplasia and are subdivided into flat (PanIN-1A) and papillary (PanIN-1B) types. PanIN-2 lesions are papillary ductal lesions with atypia, low-grade or moderate dysplasia, and show nuclear abnormalities including loss of polarity, nuclear crowding, enlarged nuclei and hyperchromatism. PanIN-3 lesions show severe dysplasia, intraductal carcinoma and carcinoma-in-situ. The immunohistochemical characteristics of PanINs vary with degree of dysplasia. For example, the apomucin MUC5AC, which is not expressed in normal pancreatic ducts, is expressed in all PanINs. MUC3 and MUC4 show a progressive increase in expression with increasing dysplasia, and MUC1 is expressed almost exclusively by high grade PanIN and invasive carcinoma.⁷⁴

Carcinogenesis is thought to follow a step-wise approach from low-grade PanIN to high-grade PanIN to invasive carcinoma. With each step, certain genetic and epigenetic abnormalities have been found and the accumulation of mutations will result in PDAC (**Figure 1**). Telomere shortening is one of the earliest events in tumourigenesis and seen almost universally in PanINs, including those of low-grade.⁷⁵ Telomeres prevent fusion between the ends of the chromosomes, providing a protective effect, and their shortening occurs with age and number of cell divisions. The shortened telomeres cause chromosomal instability and promote neoplastic progression.⁷⁶ Kirsten rat sarcoma (*KRAS*) mutations are also an early event.

Mutations in this oncogene are present in 90-95% of PDAC specimens and thought to be the driver mutation for PDAC. PanINs also demonstrate alterations in tumour suppressor genes known to be associated with PDAC including: *p16/CDKN2A*, inactivated in intermediate and late lesions (PanIN-2/3); and *p53*, *SMAD4* and *BRCA2* genes inactivated in PanIN-3 lesions.⁷⁷ Epigenetic abnormalities in cancer are mostly due to methylation of the gene promoter region CpG islands, resulting in transcription silencing.⁷⁸ The hypermethylation of tumour suppressor genes is thought to be involved in carcinogenesis. Indeed, as many as 70% of PanIN-1A lesions demonstrate abnormal promoter methylation.⁷⁹

Unfortunately PanINs are not detectable by radiological imaging and thus early detection with preventative and curative resection prior to the development of carcinoma is not yet possible. Furthermore, if a PanIN is detected at intraoperative frozen section it remains unclear what the best management should be. Some would suggest further resection with PanIN-3 lesions but not with PanIN-1 and PanIN-2 lesions.⁸⁰

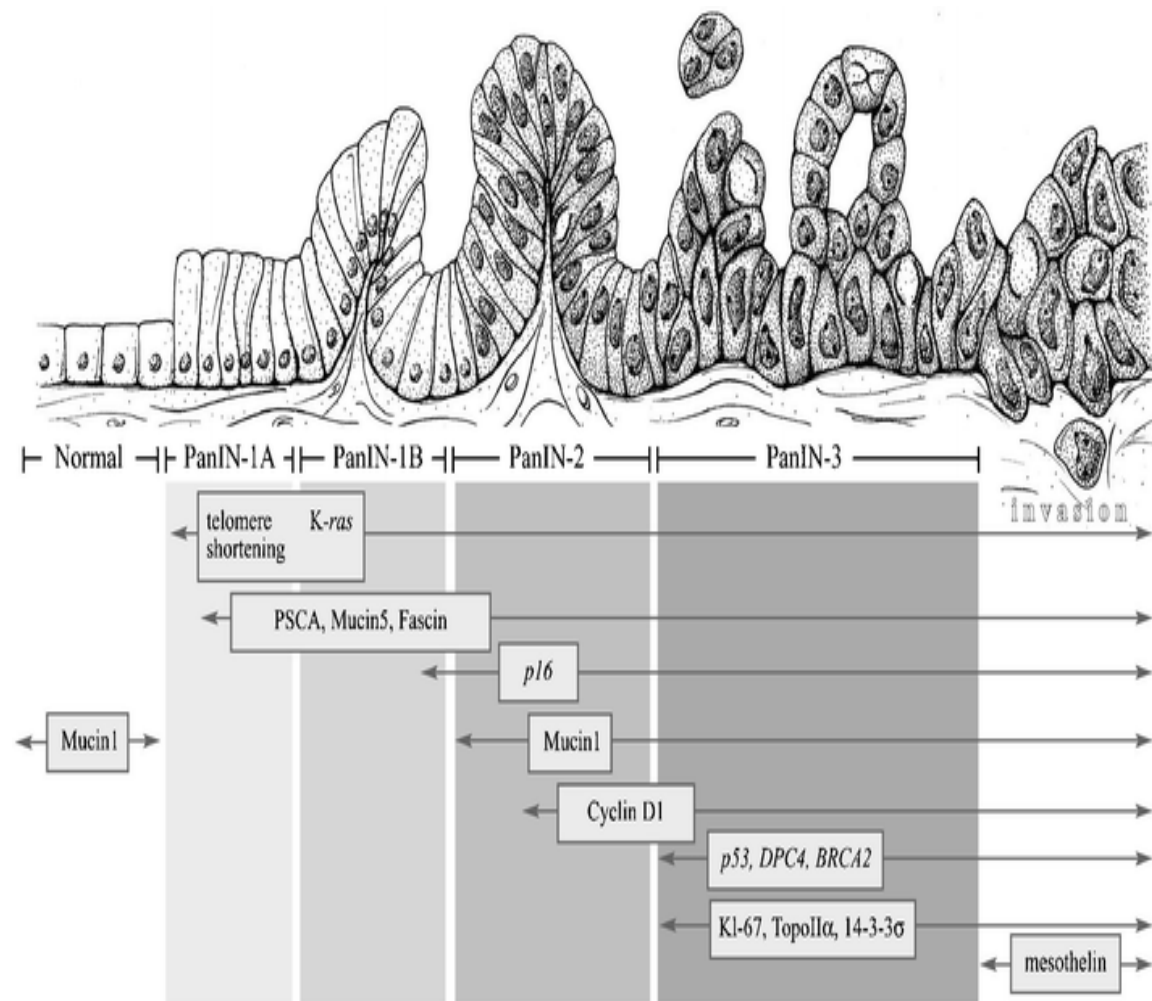


Figure 1 - Molecular changes during progression of pancreatic cancer. Normal duct epithelium progresses to infiltrating carcinoma through a series of histologically defined precursors, pancreatic intraepithelial neoplasia (PanINs). PanINs are classified into early and late lesions, starting with PanIN-1A, 1B (hyperplasia) and progressing to PanIN-2 and then to PanIN-3 or carcinoma *in situ*. The associated genetic alterations at each stage are shown below. Reproduced from⁸¹.

IPMN: IPMNs are cystic lesions arising in the main duct or branch duct epithelium of the pancreas characterised by papillary proliferation and mucin production. They are classified according to their malignant transformation from those with low-grade dysplasia, intermediate-grade dysplasia, high-grade dysplasia to those IPMNs with invasive carcinoma (IPMC). They are also divided into three types based on anatomical location: main-duct IPMN (MD-IPMN); branch duct IPMN (BD-IPMN) and mixed type IPMN (mixed IPMN) where both main and branch ducts are involved. Subtypes of IPMN based on their histological and immunohistochemical analysis of MUC expression and the intestinal marker CDX-2 are also apparent.⁸²

Genetic changes in the genes known to be involved in PDAC have been demonstrated in IPMNs, namely alterations in *KRAS*, *p16/CDKN2A*, *p53* and *SMAD4*. *Indeed >96% of IPMNs have a GNAS or KRAS mutation with more than half having both*⁸³ indicating that the activation of G-protein signalling plays an important role in IPMN. However, these changes appear to happen early in the development of IPMN and it is as yet unclear what genetic changes differentiate between low-grade dysplasia and high-grade dysplasia or IPMC.

IPMNs can be detected on computed tomography (CT) scans, magnetic resonance imaging (MRI) scans and at endoscopic ultrasound (EUS) of the pancreas. Analysis of cyst fluid CEA, taken at EUS, can further differentiate between cystic lesions. A CEA >192 ng/ml can detect mucin producing cysts (IPMNs and MCNs) with 80% accuracy.⁸⁴ The increased use and improvements in these imaging modalities has resulted in an increased detection of IPMNs as an incidental finding. Whilst we do not want to over-treat benign cysts with pancreatic resection, there is a risk of disease progression to carcinoma and management decisions must therefore be discussed at a pancreatic multidisciplinary team (MDT) meeting. The International consensus guidelines for the management of IPMNs revised in 2012,⁸⁵ recommends separate

management strategies for those with MD-IPMN / mixed IPMN and those with BD-IPMN. The risk of progression to cancer for MD-IPMN is 36-90%.^{86 87} and surgical resection is thus recommended in those with a main pancreatic duct diameter of ≥ 10 mm or for those with a main pancreatic duct diameter of 5-9mm with one of: a thickened IPMN wall; intraductal mucin; or mural nodules. For BD-IPMNs the risk of developing an invasive carcinoma has been reported to be as low as 6%⁸⁸ and therefore annual surveillance of these lesions is recommended. Resection of BD-IPMNs is indicated when there are 'worrying features' including: cyst size > 3 cm; a non-enhancing mural nodule; thickened cyst wall; and/or an abrupt change in the caliber of the main pancreatic duct.

MCN: These are mucin-producing cystic lesions of the pancreas characterised by the presence of ovarian type stroma. The epithelial lining consists of columnar cells and they are classified according to the degree of dysplasia (low-grade; intermediate-grade and high-grade). They are almost always solitary and of incidental finding in young people and located in the body or tail of the pancreas. MCNs are identified on the imaging modalities discussed above and also have a high CEA in the cyst fluid. However, they can be distinguished from IPMNs based on a lack of communication with the main pancreatic duct.⁸⁹ Although the molecular changes in MCN have not been as well researched as the other precursor lesions, due to their lower incidence, mutations have been found in *KRAS*, *p16/CKDN2A*, *p53* and *SMAD4* with an average of 16 +/- 7.6 mutations for each MCN, compared with 27 in IPMN.⁹⁰ However, unlike in IPMN, *GNAS* mutations have not been demonstrated. 3.9%- 30% of MCNs will progress to an invasive cancer, usually PDAC,^{91, 92} and thus surgical resection of MCNs is recommended.

1.5 GENETICS OF PANCREATIC CANCER

In 2008, an extensive land-mark study characterised the complex genetic network of PDAC by performing whole-exome sequencing of 24 tumours investigating 20,661 genes. This revealed that PDAC contains an average of 63 genomic alterations, mostly point mutations.⁹³ They identified 12 core signalling pathways and processes which are genetically altered in the majority of pancreatic cancers and are thought to drive PDAC tumourigenesis. These are: TGF β signaling; JNK signaling; integrin signaling; WNT/notch signaling; hedgehog signaling; control of G1/S phase transition; apoptosis; DNA damage control; small GTPase signaling; invasion; hemophilic cell adhesion; and *KRAS* signaling. Furthermore pathways have since been recognised including axon guidance and chromatin regulation.^{94 95} There are four genes which are most commonly affected in PDAC including *KRAS*, *CDKN2A*, *TP53* and *SMAD4*.

KRAS: *KRAS* is a member of the *RAS* family of oncogenes and encodes for membrane-bound GTPases involved in regulating signal transduction.⁹⁶ The most common mutation is one amino-acid substitution in position 12 of the *KRAS* protein leading to a glycine to aspartic acid change.⁹⁷ Mutations have also been seen in codons 13 and 61. The mutations lead to constitutive activation of *RAS* hindering the ability of *RAS* to hydrolyse GTP to GDP.⁹⁸ This activation causes persistent stimulation of the downstream signalling pathways including phosphatidylinositol 3-kinase (PI3K)/AKT pathway, the RAF/MEK/ERK pathway and nuclear factor κ B,^{99, 100} resulting in increased cell proliferation, altered metabolism, apoptosis suppression, evasion of the immune response and metastasis.⁹⁷

The association of pancreatic cancer with mutated *KRAS* was established some years ago.^{101 102} More recently, exome sequencing established *KRAS* as the most

commonly mutated gene in PDAC, seen in 90-95% of cases.⁹⁵ It is seen as the driver mutation involved in the initiation or early phase of tumourigenesis.¹⁰⁵ The association between mutant *KRAS* and PDAC is supported by mice models. Mice with a mutant *KRAS* in codon 12 are born with a normal pancreas at birth but shortly develop PanINs progressing in grade and number with progression to invasive cancer in older mice.¹⁰³ Other mouse models have been developed which are defined by tissue-specific, temporally regulated and reversible expression of *KRAS*.¹⁰⁴ The induction of *KRAS* in these mice leads to precancerous lesions and its inactivation leads to regression of the lesions.¹⁰⁴ Furthermore, silencing of *KRAS* in mouse PDAC xenografts using a small interfering RNA (siRNA) reduces tumour growth and improves sensitivity to gemcitabine therapy.¹⁰⁵ Evidence that mutant *KRAS* initiates PDAC but is also required for tumour survival and chemotherapy resistance. The importance of a *KRAS* inhibitor for PDAC therapy is evident however, as yet, an effective agent to block the binding of *KRAS* to GTP has not been developed. The majority of agents developed are designed to block downstream signaling pathways such as sorafenib targeting the RAF/MEK/ERK pathway and various agents targeting the MAPK pathway at MEK, the PI3K pathway and the AKT pathway. However, clinical trials have so far shown little benefit ^{106 107 108}, perhaps due to complex feedback loops between the many pathways with deactivation of one leading to increased activation of another.

CDKN2A: The tumour suppressor gene *CDKN2A*, located on chromosome 9q21.3, encodes for the p16 protein, which is a regulator of the cell cycle at the G1/S checkpoint. It inhibits cyclin D/CDK4/6 complex, which in turn inhibits retinoblastoma protein phosphorylation and prohibits progression from G1 phase to S phase thus arresting cell proliferation.¹⁰⁹ Loss of p16 function is seen in >90% of PDACs.¹¹⁰ Although mice with a deletion of p16 do not develop PDAC,¹¹¹ mice expressing mutant *KRAS* and a p16 deletion show a highly invasive tumour with high rates of

epithelial-to-mesenchymal transition (EMT).¹¹² More recently, Furthermore evidence has shown that an expression of mutant *KRAS* and the silencing of p16 expression in a human pancreatic epithelial nestin-expressing (HPNE) cell line induces tumourigenic transformation *in vitro*.¹¹³ The transformed cells show increased cell proliferation and increased invasion with an up-regulation of EMT markers. In an orthotopic mouse model, the transformed cells exhibit increased tumour growth *in vivo*.¹¹³ This suggests that tumours with both *KRAS* mutation and p16 deletion play important roles in the tumourigenesis and invasiveness of PDAC.

TP53: The *TP53* tumour suppressor gene is located on chromosome 17p13.1 and encodes for the p53 protein. It induces cell cycle arrest or apoptosis in damaged cells but is inactivated in 50-75% of PDACs.^{114, 115} Functional loss of p53 enables cellular survival and division in the presence of DNA damage furthermore facilitating genetic mutations and instability.^{114, 116} Certainly mice with mutant *KRAS* and loss of p53 develop an invasive and metastatic PDAC, demonstrating a high degree of genetic instability.¹¹⁷ Furthermore, the combined loss of p53 and autophagy (a lysosomal recycling process essential for tissue or cell homeostasis) promotes progression from PanIN to carcinoma and alters cellular metabolism enabling rapid tumour growth.¹¹⁸

SMAD4: *SMAD4* encodes a member of the Smad family of signal transduction proteins. These are phosphorylated and activated by transmembrane serine-threonine receptor kinases in response to TGF-beta signaling. Deletion of *SMAD4* results in: increased TGF-beta signaling resulting in abnormal gene expression and transcription regulation; loss of *SMAD4*-dependent cell growth inhibition; and increased EMT.¹¹⁹ Loss of *SMAD4* is seen in 55-60% of PDACs.¹²⁰ Mice with mutant *KRAS* and loss of *SMAD4* develop a far more rapid progression to PDAC compared to mice with mutant *KRAS* alone.¹²¹ Furthermore, inactivation of *SMAD4* enhances EGFR and CD133 expression, whereas re-expression of *SMAD4* suppresses EGFR

and CD133 levels in PDAC cells. Overexpression of EGFR and CD133 has been associated with tumour malignancy, invasiveness and decreased survival.^{122 123} *SMAD4* loss of function almost always occurs in association with genetic inactivation of *TP53*, whereas *TP53* inactivation does not, indicating *SMAD4* alterations are selected for in association with *TP53* genetic alterations. This relationship also suggests that *SMAD4* inactivation occurs later than *TP53* inactivation in the genetic progression model of pancreatic carcinogenesis.¹²⁴

It is now clear that these four genes (*KRAS*, *CDKN2A*, *TP53* and *SMAD4*) contribute to the carcinogenesis of PDAC. There are also many genes seen mutated in <20% of PDACs including oncogenes such as *BRAF*, *MYB*, *AKT2* and *EGFR*; and tumour suppressors such as *MAP2K4*, *STK11*, *ACVR1B*, *ACVR2A*, *FBXW7* and *EP300*.^{76, 125-127} It is likely that a complex mix of differing sequential mutations are required for the development of PDAC enabling multiple changes in the signal transduction pathways outlined above. However, many of the exact mechanisms underlying the transformation to PDAC remain unknown. Furthermore, it is not yet clear whether certain mutations, or combination of mutations, result in a poorer prognosis, a question which this thesis will attempt to answer.

1.6 CLINICAL DIAGNOSIS OF PDAC

Around 65% of PDAC lesions are seen in the head of the pancreas (HOP) with 15% in the body and tail and the remainder diffusely involving the gland.¹²⁸ Unfortunately, wherever the anatomical location of the lesion, early pancreatic cancer is usually asymptomatic resulting in delayed detection. Symptoms tend to manifest once the tumour has invaded surrounding tissues or metastasised to other organs thus most people already have advanced disease at diagnosis.¹²⁹ The most common presenting symptoms reported are: abdominal pain, usually in the upper abdomen

which may radiate to the back, experienced by 80% of patients;¹³⁰ obstructive jaundice reported in 16-85%, in those with HOP tumours;¹³¹ and weight loss in up to 79% due to anorexia, malabsorption secondary to pancreatic ductal obstruction and cachexia. Some would argue that weight loss is often overlooked until other symptoms are apparent and suggest early upper abdominal imaging in patients with unexplained weight loss to detect any pancreatic lesions.¹³² Courvoisier's sign, defined as the presence of a non-tender palpable gallbladder and painless jaundice, implies a diagnosis of pancreatic or biliary malignancy rather than gallstones. Diabetes or impaired glucose tolerance can also occur due to damage to the pancreatic parenchyma. This is a common disease however older patients with a new diagnosis of diabetes and significant weight loss should be investigated for pancreatic cancer. Furthermore, obstruction of the pancreatic duct by the tumour may lead to acute pancreatitis. Another sign of PDAC is Trousseau's syndrome characterised by deep or superficial venous thrombosis, hypercoagulability and migratory thrombophlebitis. This syndrome is not exclusive to PDAC, seen also with lung malignancies and gliomas. Rarer symptoms reported include loss of taste, skin changes and psychological disturbances, namely depression.¹³¹

Where there is a suspicion for pancreatic cancer, Furthermore investigations must be urgently undertaken. This involves blood tests including tumour markers and radiological imaging. Routine blood tests are fairly non-specific but may reveal anaemia, hyperglycaemia or raised liver function tests either as an obstructive picture from the primary tumour obstructing the common bile duct, or secondary to liver metastases. In the clinical setting of pancreatic cancer, the most commonly used and validated tumour marker is carbohydrate antigen 19-9 (CA 19-9). CA 19-9 is expressed on the surface of cancer cells as a glycolipid and as an O-linked glycoprotein.¹³³ It is a Lewis blood group antigen of the MUC1 protein, which is commonly expressed in tumours of the upper gastrointestinal tract and may be

elevated in patients with pancreatic cancer. As CA 19-9 can only be expressed in patients belonging to the Le (α - β +) or Le (α + β -) blood groups, 5-10% of the population with the Le (α - β -) phenotype are unable to synthesise it leading to false negative results¹³³. Furthermore limitations include a considerable number of false positive results due to its elevation in other malignancies such as colorectal, liver, breast and lung cancers, as well as in benign diseases including, importantly, hyperbilirubinaemia and pancreatitis as well as in liver cirrhosis and lung disorders.¹³⁴ CA 19-9 also lacks sensitivity for early pancreatic cancers, <3cm in size,¹³⁵ and has a lower expression in poorly differentiated cancers.¹³⁵ A recent meta-analysis of 2,316 patients, found CA 19-9 to have a pooled sensitivity of 0.803 (95 % CI 0.777–0.826) and a pooled specificity of 0.802 (95 % CI 0.780–0.823).¹³⁶ Interest has been renewed however in the prognostic value of this marker following some studies demonstrating that elevated levels are an independent predictor for poor survival and low resectability rates.¹³⁷⁻¹⁴⁰

Radiological imaging of the pancreas is indicated to aid diagnosis and staging of PDAC and may include computed tomography (CT), endoscopic ultrasound (EUS) with or without fine needle aspiration (FNA), magnetic resonance imaging (MRI) and/or positron emission tomography (PET). Pancreatic cancer is staged according to The American Joint Committee on Cancer (AJCC) TNM staging system (**Table 1**). Patients are also staged clinically according to resectability, which dictates the most appropriate initial management¹⁴¹ (**Table 2**).

An ultrasound (US) scan of the pancreas may be performed as part of the initial work-up for abdominal symptoms however, this imaging modality is unable to definitively diagnose and stage patients thus a tri-phasic multi-slice pancreatic protocol CT is the gold standard investigation. This involves imaging 20-25 seconds (the arterial phase), 35-40 seconds (the pancreatic or late arterial phase) and 60-80

seconds (the portal venous phase) after administration of an intravenous contrast medium. The greatest pancreatic parenchymal enhancement is seen in the late arterial phase with maximum arterial enhancement observed in both the arterial and late arterial phase, depending on the rate of contrast administration¹⁴². Superior opacification of the superior mesenteric and portal veins is seen in the portal venous phase¹⁴². Therefore a CT with this protocol shows good images of the pancreas as well as the coeliac axis, superior mesenteric artery (SMA) and superior mesenteric and portal veins enabling accurate clinical staging and preoperative planning. Indeed, a meta-analysis involving 959 patients who had a CT performed for suspected PDAC showed that it diagnosed pancreatic cancer with a sensitivity of 91% and specificity of 85%.¹⁴³ CT is not able to detect lesions at a very early stage or distinguish between benign and pre-malignant lesions.¹⁴⁴ Furthermore, accuracy for assessing resectability is poorer with a sensitivity of 81% and specificity of 82%¹⁴³ due to undetected subtle vascular invasion and small metastases in the liver and peritoneum which only become apparent at operation.

EUS-FNA is accurate at confirming solid pancreatic lesions cytologically and has become an important part of PDAC assessment in hepatobiliary centres. High resolution images of the main pancreatic duct and surrounding parenchyma can be achieved so that structures as small as 2-3 mm can be identified. EUS has good parenchymal resolution enabling a diagnostic sensitivity of 98%.¹⁴⁵ It is superior to CT for diagnosing small tumours <3cm,¹⁴⁵ for T staging, for observing vascular invasion at the spleno-portal confluence,¹⁴⁶ and for assessing involvement of the coeliac axis, which indicates unresectable disease. Indeed, CT scanning can be falsely negative for vascular invasion in approximately 20% of PDAC patients.¹⁴⁷ EUS also has a negative predictive value approaching 100%¹⁴⁸ rendering it extremely useful for excluding PDAC in benign lesions with inconclusive findings at CT. This radiological modality is indicated in addition to CT for the following reasons: To

provide a cytological diagnosis of PDAC in unresectable cases diagnosed at CT prior to commencing oncological therapy; for differentiation between malignant and inflammatory lesions; when there are doubts as to the type of malignancy where alternative diagnoses would follow different management strategies; and for help in the characterisation of pancreatic cystic lesions.

Although CT and EUS-FNA are most frequently used for the assessment of pancreatic cancer, there may also be a role for MRI imaging of the pancreas and for PET-CT. On dynamic images, the MRI appearance of the pancreas is similar to that on contrast-enhanced CT, being hypointense to adjacent pancreatic parenchyma, but at times also appearing isointense, in which case identification of the primary tumour can be difficult.¹⁴⁹ Therefore its diagnostic sensitivity (84%) and specificity (82%) is significantly worse than CT.¹⁴³ It was thought that MRI or magnetic resonance angiography (MRA) may improve resectability assessment in PDAC with better identification of vascular structures however a meta-analysis comparing MRA to CT found no significant difference in assessing preoperative vascular invasion.¹⁵⁰ MRI may however be useful in the evaluation of pancreatic liver metastases thus avoiding unnecessary major operations without improved survival and reducing delay in starting chemotherapy. Certainly in colorectal cancer, MRI is better than CT at assessing liver metastases.¹⁵¹

A meta-analysis of 1,582 patients¹⁵² found PET-CT was able to diagnose pancreatic cancer with a sensitivity of 0.91 (95%CI: 0.88-0.93) and specificity of 0.81 (95%CI: 0.75-0.85). The sensitivity in detecting liver metastases was 0.67 (95%CI: 0.52-0.79), suggesting that the value of PET for this reason remains uncertain. However, PET-CT may be useful in determining other extra-pancreatic sites of disease e.g. lung or bone metastases, when an indeterminate lesion is seen on CT.

In addition endoscopic retrograde cholangiopancreatography (ERCP) is useful in jaundiced patients to allow placement of an endoscopic stent to relieve obstruction and can be used to obtain ductal brushings and lavage, providing tissue for diagnosis¹¹⁴.

Each patient with suspected pancreatic cancer must be discussed at a pancreatic multidisciplinary team (MDT) meeting involving pancreatic surgeons, gastroenterologists, radiologists, oncologists and nurse specialists. Investigations including blood tests, radiological imaging, FNA results and/or tissue biopsy results will be reviewed in order for a diagnosis of PDAC to be made, for staging of the disease and for management decisions.

TNM Stage	Description
T-Stage	
Tis	Carcinoma in situ
T1	Tumour <2cm and limited to pancreas
T2	Tumour >2cm and limited to pancreas
T3	Tumour extends beyond pancreas but no involvement of coeliac axis or SMA
T4	Tumour involves the coeliac axis or SMA
N-Stage	
N0	Regional lymph nodes not involved
N1	Regional lymph nodes involved
M-Stage	
M0	No distant metastases
M1	Distant metastases present

Table 1: TNM staging of pancreatic cancer according to The American Joint Committee on Cancer (AJCC) TNM staging system. T=tumour; N=nodes; M=metastases; SMA = superior mesenteric artery.

Clinical Stage	Resectability	TNM Stage	Description	Median Survival
Stage I	Resectable	T1-2 N0 M0	Tumour limited to the pancreas, no extra pancreatic disease, no lymph node involvement	17-23 months
Stage II	Resectable	T3 N0 M0 or T1-3 N1 M0	No involvement of SMA or coeliac axis, lymph nodes may be involved	17-23 months
Stage III	Borderline resectable	T4 N0-1 M0	Tumour abutment or <180° circumference of the SMA or coeliac axis	Up to 20 months
Stage III	Unresectable	T4 N0-1 M0	Tumour encasement >180° circumference of the SMA coeliac axis	8-14 months
Stage IV	Metastatic	T1-4 N0-1 M1	Metastatic disease	4-6 months

Table 2: Clinical staging of pancreatic cancer and survival. T=tumour; N=nodes; M=metastases; SMA = superior mesenteric artery. Adapted from¹²⁹

1.7 BIOMARKERS

A biological marker (biomarker), as defined by the National Institutes of Health Biomarker Working Group, is “a characteristic that is objectively measured and evaluated as an indicator of normal biological process, pathogenic processes, or pharmacological responses to a therapeutic intervention.” An ideal biomarker for pancreatic cancer would allow for early detection of disease in ‘at-risk’ patients or as part of a screening process, help to predict recurrence of disease following surgery and assess disease response to therapy. It must have a high sensitivity and specificity, a good correlation with clinical stage and be an acceptable test to the target population with few complications. In the clinical setting of pancreatic cancer, the most commonly used and validated biomarker is CA 19-9 as discussed above. However, due to its sensitivity and specificity, there is considerable interest in the scientific world to find a new biomarker for pancreatic cancer. There are numerous

reports of a large number of potential biomarkers in the literature, including various microRNAs, DJ-1, cyclophilin B, IL-8, APRIL, MIC-1, PAM4, OPN, HSP27, TPS, TPA, POA, TSGF, CAM 17.1, REG-4, haptoglobin, MUCINS, M2-PK, to name but a few ¹⁵³
¹⁵⁴. They may be derived from serum, tissue, bile, pancreatic juice, saliva or stool, however, most lack large-scale validation. Some have concentrated on gene expression in tissue and blood and on circulating tumour cells. This thesis hopes to be able to add to the discussion by assessing biomarkers as predictors of early disease recurrence namely investigating circulating tumour cells, cell-free DNA and microRNAs.

1.8.1 MANAGEMENT OF PANCREATIC CANCER

As one of the most difficult cancers to treat, pancreatic cancer remains one of the most devastating solid tumours. The tumour biology of PDAC renders the disease incredibly resistant to virtually all chemotherapy agents and the management is a worldwide challenge. The only curative management option is surgical resection however only 15-25% of patients have a surgically resectable tumour at diagnosis.¹⁵⁵ This is due to a variety of factors including the retroperitoneal location of the pancreas and the late onset of symptoms, vague abdominal and systemic symptoms and the aggressive nature of the disease in terms of tumour proliferation, invasiveness and metastatic potential. Features that preclude surgery in PDAC include distant metastatic disease and invasion of the tumour into local arteries, namely the coeliac axis or the superior mesenteric artery (SMA). Local invasion of the tumour into the portal vein (PV) or superior mesenteric vein (SMV) is seen by many as a further contraindication to surgical resection. However, more recently some specialist high volume pancreatic centres are performing PV/SMV resection routinely in patients with probable venous invasion. 57.1% of patients who have surgical resection of their pancreatic tumour have an R1 resection (tumour within

1mm of the resection margin)¹⁵⁶ and a median survival of 17.2 months. Even those with an R0 resection (no tumour within 1mm of the resection margin, a curative resection) only have a median survival of 28.7 months.¹⁵⁶ Leading many to see PDAC as a 'systemic' disease at diagnosis and that resection is simply a 'debulking' procedure that is inadequate to cure microscopic and undetectable metastatic disease. Nonetheless survival is much improved from the 8-14 months in those with unresectable locally advanced pancreatic cancer and 4-6 months in those presenting with metastatic disease.¹²⁹

1.8.2 SURGICAL MANAGEMENT

With advances in radiological techniques, PDAC is being identified at an earlier stage and more patients are deemed suitable for surgical resection than previously. The surgical management of PDAC depends on the location of the tumour. Removal of the head of the pancreas (HOP), where 65% of tumours are located,¹²⁸ involves a pancreaticoduodenectomy (PD): either a Whipples procedure or pylorus preserving pancreaticoduodenectomy (PPPD). Both operations are well described and these open procedures have become the gold-standard technique in pancreatic centres. A distal pancreatectomy and splenectomy is performed when the tumour is in the body or tail of the pancreas. Pancreatic resections can take several hours to perform requiring great surgical expertise so most countries have now centralised pancreatic surgery ensuring that only hepato-pancreato-biliary (HPB) surgeons undertake these operations in high volume centres. As such, with improvements in surgical skill and post-operative care, the morbidity and mortality for these procedures is very acceptable.

A PD involves the en-bloc removal of the distal stomach and pylorus (whipples only), the duodenum, the HOP, the common bile duct and the gallbladder. A complete

oncological operation necessitates this resection due to the anatomical proximity of these organs and shared blood supply of the HOP and proximal duodenum (the gastroduodenal artery). Three anastomoses are required at PD: Firstly, a pancreaticoenterostomy, either as a pancreaticogastrostomy or pancreaticojejunostomy, in order to re-connect the remaining pancreas and main pancreatic duct to the gastrointestinal (GI) tract; a gastrojejunostomy, to re-connect the stomach to the jejunum in a whipples procedure or a duodenojejunostomy, to re-connect the post pylorus duodenum to the jejunum in a PPPD; and a hepaticojejunostomy (choledochojejunostomy) to re-connect the common hepatic duct to the GI tract (**Figure 2**). A roux-en-Y loop is created in order to prevent gastric biliary reflux with resultant gastritis and oesophagitis. Surgeons will differ on the technique for each of these anastomoses.¹⁵⁷ Advantages of the PPPD compared to whipples may include reduced experience of dumping syndrome, reduced blood loss and shorter operating times. PPPD may be associated with a higher incidence of delayed gastric emptying and have poorer oncological outcomes.¹⁵⁸ However, no differences in terms of morbidity, mortality or survival were found in a recent Cochrane review.¹⁵⁹

Recent large observational studies evaluating PD for PDAC have been encouraging. Winter et al. looked at 1423 patients who had PD and found a median blood loss of 800mls and median length of operation of 380mins.¹⁶⁰ Morbidity was 38% and perioperative mortality only 2%. Indeed, others have shown similar morbidities of 20%-47.2%, with improvements when patients are on an enhanced recovery programme.¹⁶¹ The most common complications are delayed gastric emptying (DGE) (20-50%¹⁶²), post-operative pancreatic fistulas (POPF) (10-15%¹⁶²) and wound infections (11%¹⁶³). Others include post-operative bleeding (4-16%¹⁶²), anastomotic leaks including biliary fistulas (1-5%¹⁶²) and intestinal fistulas (3-8%¹⁶²), pancreatitis (2-3%¹⁶²), and ischaemic complications (1%¹⁶²). DGE is defined as the impossibility

of resuming oral feeding after the first postoperative week or prolonged use of a nasogastric aspiration tube.¹⁶⁴ and is graded from A to C according to its clinical impact and management. It can severely alter the nutritional state of a patient, prolonging hospital stay and has been known to last for up to 6 weeks.¹⁶⁵ It may be due to: Damage to the gastric vagus nerve system; a decrease in the plasma concentration of gastric motilin due to peripancreatic inflammation; the angulation or twisting of the gastrojejunostomy; and/or secondary to dissection of peri-pancreatic lymph nodes.¹⁶² A POPF is defined as any volume of drain fluid after postoperative day 3, with amylase content greater than 3 times serum.¹⁶⁶ They are further classified by grade according to clinical severity. Grade A fistulas are considered “transient fistulas” and have no clinical impact. Grade B fistulas require parenteral nutrition, antibiotics, or somatostatin analogues and may require re-positioning of drains. Grade C fistulas require a major change in clinical management, such as surgical exploration and often lead to an extended hospital stay.

Many are now also performing these operations laparoscopically further improving post-operative recovery for the patient. Laparoscopic pancreaticoduodenectomy (LPD) was first described by Gagner and Pomp in 1994¹⁶⁷. Technically LPD is quite a demanding operation. As such, historical opposition of LPD has focused on concerns such as the difficulty in accessing and adequately exposing the pancreas, the lack of ability to control haemorrhage from major vessels and the feasibility of performing sophisticated anatomical reconstructions and anastomoses with laparoscopic instruments. These worries have been voiced in concert with suggestions that LPD may compromise patient outcomes, by the inability to maintain oncologic surgical principles due to surgeon fatigue secondary to prolonged operative times and by the inability to palpate the tumour itself and/or surrounding vasculature.¹⁶⁸ Some concerns about the technical demands of PD laparoscopically persist because there is a lack of Level I evidence to categorically prove the safety

and benefits of LPD for these patients. To date there have been no randomised controlled trials of laparoscopic versus open PD reported in the literature. Available evidence ranges from case reports to non-randomised comparative studies with relatively small numbers, which generally fail to reach statistical significance for the majority of findings. Many of the LPD series reported in the literature exclude patients with significant co-morbidities or those likely to have difficult PDs, such as those with large tumours, vascular involvement, non-dilated ducts and extension of tumour to the uncinate process.¹⁶⁸ Furthermore, patient preference for laparoscopic or open PD introduces selection bias to potentially confound the reliability of these findings.^{169 170} Interpretation of the literature on LPD is made more difficult by the wide variety of technical practices at different centres. For instance some report an entirely laparoscopic completion of LPD, whilst others may perform the anastomoses through a small midline incision.^{170 171} Despite reservations about the quality of data available, a meta-analysis of LPD published in 2011 is cause for optimism. Of the 126 patients included in the review, none had positive tumour resection margins.¹⁶⁸ Subsequently, it has been found that in spite of longer operations, mean estimated blood loss, number of units of RBCs transfused peri-operatively, length of post-operative ICU stay and length of overall hospital stay are better after LPD than open PD (OPD), with comparable morbidity and mortality.¹⁶⁹ There is also no significant difference in tumour size, TN stage, number of positive lymph-nodes or R0 resections between either group.¹⁶⁹ A lower positive lymph-node to negative lymph-node ratio and a significantly higher total number of lymph-nodes harvested is also seen after LPD, a positive predictor of better long-term survival¹⁷¹. As alluded to above, a small number of centres have reported their experience of robotic LPD, with some promising early results. Studies have detected an increase in R0 resections,^{172 173} with one study quoting an increase from 58% to 87% with the introduction of robotic PD.¹⁷³ Similar to LPD, robotic LPD has been reported to increase the number of lymph-nodes harvested and reduce operative blood loss when compared to open

operations.^{172 173} Interestingly, robotic LPD may actually have shorter operative times than OPD.¹⁷⁴ Any consideration of the prospective benefits of robotic LPD should, however, take into account the fact that the costs of the robotic operation are estimated at more than twice that of the non-robotic LPD and appreciably more expensive than OPD.^{175 170} A randomised controlled trial comparing LPD with OPD is clearly required to assess surgical and oncologic parameters. Although the number of centres offering LPD and the number of patients suitable for LPD are both increasing, the institution of a sufficiently powered RCT comparing LPD to OPD poses sizeable logistical issues. However, the literature to date, demonstrates that LPD is feasible, safe and very promising in selected patients.

Distal pancreatectomy (DP) is performed with splenectomy for a complete oncological operation. There has been a marked improvement in outcomes following this operation in recent years and it is routinely performed both as an open and laparoscopic procedure in high volume HPB centres. The main complication following DP is POPF. POPF rates vary in the literature from 6%-32%.¹⁷⁶ Other complications relate to those of pancreatic insufficiency with endocrine failure seen in 23.4% and exocrine failure seen in 15.6%.¹⁷⁷ This operation also carries a risk of sepsis, meningitis or pneumonia, triggered mainly by *S. pneumoniae*, *N. meningitidis* and *H. influenza*, which is 35 times higher in patients following splenectomy than in the general population.¹⁷⁸ Following splenectomy, the incidence of infection is 3.2%, with a mortality of 1.4%.¹⁷⁹ There is no difference in morbidity and mortality between open and laparoscopic DP.¹⁷⁶ Five-year survival after DP for PDAC is similar to patients having surgery for HOP PDAC.¹⁵⁸ More recent techniques for DP including the radical antegrade modular pancreatectomy (RAMPS) procedure, maximise the chance of negative resection margins, with 81% achieving R0 resections.¹⁸⁰ In addition, a radical distal pancreatectomy with resection of the coeliac axis (DP-CAR), is carried out by some surgeons for pancreatic body tumours, with excellent R0

resection rates of up to 91% although with a 48%-54% morbidity^{181, 182} Median survival has been reported as 21 months by some¹⁸² but as poor as 9.25 months by others.¹⁸¹ Recently, a scoring system has been developed to predict prognosis in DP-CAR patients that may allow for greater selectivity.¹⁸³

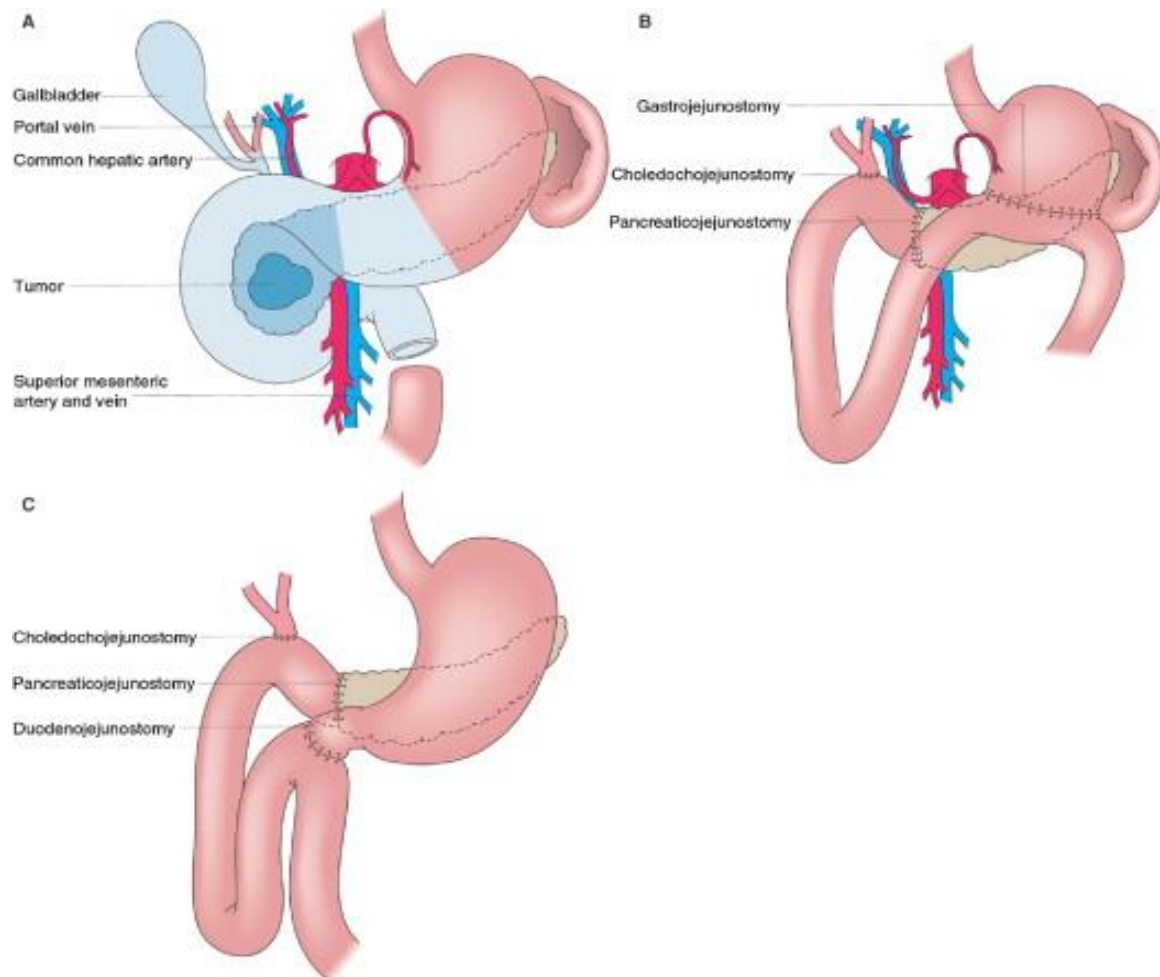


Figure 2: Pancreaticoduodenectomy. (A) The tissue to be resected in a standard pancreaticoduodenectomy. (B) Reconstruction after a standard pancreaticoduodenectomy. (C) Reconstruction after a pylorus-preserving variation.

From¹⁵⁸

1.8.3 SURGICAL MANAGEMENT TO REDUCE RECURRENCE RATES

In an effort to reduce the risk of post-operative disease recurrence and improve survival there have been a number of techniques described in the literature for pancreaticoduodenectomies including: The no-touch technique; portal vein resection; arterial resection; and newer techniques including radiofrequency ablation (RFA) and irreversible electroporation therapy (IRE)

The no-touch technique: In 1967 Turnbull reported a no-touch technique in the operative management of colon cancer.¹⁸⁴ He adopted a technique deliberately avoiding any tumour manipulation until lymphovascular ligation was complete. Using this technique on 460 patients with colorectal cancer compared to standard colectomy on 120 patients, the five-year survival improved from 34 to 50 months. It was postulated that limiting the handling of the tumour prevents the spread of cancer cells. Subsequently, it was noted that using this technique for colorectal cancer, liver metastases appeared later, and in fewer patients¹⁸⁵.

More recently, Kobayashi et al. applied the no-touch technique to PD in 10 patients with periampullary tumours¹⁸⁶. Briefly, the no-touch technique mandates that the tumour in the head of the pancreas must not be touched until complete ligation and division of the venous drainage. In the original Kobayashi paper there was no operative mortality and none of the patients developed liver metastases after a follow-up of between 5 months – 7 years. Further corroboration of the no-touch PD came from Hirota et al. who used this technique on 8 patients with pancreatic cancer and compared it to the conventional technique used with 10 patients.¹⁸⁷ The authors measured portal vein (PV) carcinoembryonic antigen (CEA) messenger RNA before and after tumour manipulation. No patients had detectable levels before resection but PV CEA messenger RNA was detected in only 13% (1/8) of those undergoing the no-

touch technique compared to 50% (5/10) of patients having the standard PD. Patients in the no-touch group had a significantly lower recurrence rate (38% compared to 90%; $p=0.043$) and longer overall survival (41.5 ± 5.6 ; 95% CI: 30.5-52.5) months compared to 21.2 ± 5.8 (95% CI: 9.9-32.5; $p=0.018$) months. The authors later published a report showing a 40% morbidity rate and no operative mortality using this technique for PD in 42 patients¹⁸⁸. For the 17 patients with PDAC, 14 (82%) had R0 resection margins and 3 (18%) had R1 resections. The two-year survival was 75%. These results are comparable to findings reported with the conventional technique and it is thus a feasible and safe operation. It is possible that handling the tumour at the time of standard surgery causes a release of circulating tumour cells (CTCs), hence contributing to future disease recurrence, an occurrence, which may therefore be improved without tumour manipulation during the no-touch technique.

There is limited data on the no-touch technique with small numbers of patients in each group. However, the technique is possible for PD, does not increase morbidity or mortality, and appears to have the possibility of improving oncological outcomes in PDAC patients who undergo PD. This thesis aims to investigate the no-touch technique and its impact on disease recurrence by using available biomarkers.

Portal vein resection: A previous contraindication to PD was local invasion of the tumour into the portal vein (PV) or superior mesenteric vein (SMV). However, more recently some specialist high volume pancreatic centres are performing PV/SMV resection routinely in patients with probable venous invasion. This element of PD remains somewhat controversial as it adds considerably to the complexity of the operation thereby increasing the possibility of bleeding, portal vein thrombosis, morbidity and mortality for the patient without conferring major improvement in long-term survival. There are currently no RCTs analysing the safety of performing PV/SMV resection in patients with PDAC. Nonetheless, from observational studies,

operative morbidity and mortality have been consistently reported as not significantly different between PD with versus without PV/SMV resection^{189 190 191 192 193 194 195 196}. Furthermore, the potential benefit of PV/SMV resection was evaluated in a recent systematic review of 1,458 patients from 28 level III evidence studies.¹⁹⁷ Median survival was 15 months (9-23 months) and the authors concluded that in high-volume centres, vascular resection has acceptable morbidity, mortality and survival outcomes and should not be seen as a contraindication to curative surgery. By pooling results from studies reporting resection margin status after PD,^{156, 198-204} a positive medial resection margin was reported in 38.2% of 701 patients. However, following PD with PV/SMV resection, the medial resection margin is only positive in 16% of patients and it is well recognised that an R0 medial resection margin is a predictor of improved survival.^{156, 199 200-204}

Of further considerable interest was a recently published paper by a group in Marseille²⁰⁵ who performed PV/SMV resection with curative intent on 34 patients with pancreatic adenocarcinoma. Histopathological examination found that 19 patients had no tumour invasion of the resected vein. These were compared to patient and tumour-matched controls i.e. patients who had PD without PV/SMV resection, all with negative resection margins. Of note median survival was 42 months in the PV/SMV resection group compared to 22 months (p=0.02). The group's finding inevitably now generates the question as to whether the PV/SMV resection should be performed routinely in PD even when there is no suspected venous tumour infiltration.

Arterial resection: Performing PD with arterial resection is more controversial. Although arterial invasion is seen as a contraindication to surgery, there is now some observational data suggesting that this operation is feasible in those with locally advanced disease and may be associated with more favourable survival compared with patients who did not undergo resection.²⁰⁶ However, a PD with arterial resection

carries an appreciable peri-operative mortality and increased morbidity thus cases must be highly selected.

Newer Techniques: There are some newer 'cutting-edge' techniques, which have been developed and may have a part to play in pancreatic cancer surgery. Radiofrequency ablation (RFA), or microwave ablation, has been successfully used in the treatment of solid tumours in the liver, lung, breast, kidney and many other tumour types. This technique has also been demonstrated to be safe and promising when performed percutaneously or endoscopically for locally advanced PDAC ^{207, 208} ^{209 210}. Increasingly, there is evidence from series around the world that open intra-operative RFA is a feasible way to provide tumour cytoreduction and partial necrosis in locally advanced pancreatic tumours, leading to good survival rates and an improvement in quality-of-life ^{211 209}. However, some have shown an increased risk of pancreatic fistula, duodenum perforation and abscess formation, and mortality related to the intervention without a clear evidence of survival benefit. Another exciting emerging modality for treatment of PDAC is Irreversible Electroporation therapy (IRE), or NanoKnife® technology. IRE is an ablation technology, which unlike RFA is not based on thermal damage to tissue, and has shown to be safe near vascular and ductal structures. IRE uses focused electrical energy to create nanoscale pores within the cell membranes of precisely targeted tissues to destroy cells within a narrow range whilst leaving neighbouring cells unaffected. The potential for use in PDAC where the tumour is adjacent or involved in key local vascular structures is clear. To date, IRE has been demonstrated to be feasible, and of value to patients as an intra-operative adjunct for open curative PD tumour margin accentuation, and in open intra-operative tumour palliative ablation ^{212 213}. However, application of this may promote tumour spread and disturbance. It remains to be seen if there is any benefit at all to patients with locally advanced pancreatic cancer.

1.8.4 ONCOLOGICAL MANAGEMENT

Adjuvant Therapy: In line with the view that pancreatic cancer is a 'systemic' disease is the opinion that all patients who have undergone resection of PDAC should be given the option of adjuvant therapy. The European Society for Medical Oncology (ESMO) and the National Comprehensive Cancer Network (NCCN) both advocate the use of adjuvant therapy, even in those with resected T1N0 disease²¹⁴. High rates of both distant and local recurrence have spurred cure efforts with systemic chemotherapy, radiotherapy and combination chemoradiotherapy. The survival benefit of adjuvant chemotherapy is clear, but the benefit of radiotherapy remains disputed. The optimal adjuvant treatment modality is thus yet to be defined. Adjuvant chemotherapy is usually initiated within four to six weeks of surgery and continued for a total of six months, however, optimal timing and duration has not been specifically addressed by randomized controlled trials.

A regimen of adjuvant chemotherapy is supported by 1A level evidence ²¹⁵, and chemotherapy alone is the therapeutic strategy most commonly practiced in the UK. Choice of chemotherapeutic agents is primarily between fluoropyrimidine-based regimens and gemcitabine^{216, 217}. Gemcitabine is usually the preferred agent given its greater tolerability in regimens involving a monthly bolus ²¹⁸. However, some clinicians argue that a weekly or infusional fluoropyrimidine schedule has an equally tolerable side effect profile and similar efficacy. Nevertheless, gemcitabine 1000mg/m² weekly for 3 weeks followed by 1 week of rest is the most commonly used regimen.

The rationale for chemoradiotherapy in addition to adjuvant chemotherapy is to prevent local recurrence in the pancreatic bed. 15% of patients who have surgery alone for stage I or II disease have local recurrence alone whilst 65% have both

locally recurrent and metastatic disease ²¹⁹. There is level 2B evidence supporting combined modality therapy, with infusional 5-fluorouracil regimens favoured over gemcitabine or bolus FU during the concurrent chemoradiotherapy portion²²⁰⁻²²³. Alternatively, capecitabine can be substituted for infusional FU therapy as a radiation sensitizer in patients with locally advanced pancreatic cancer as has been shown in trials for other gastrointestinal malignancies²²⁴⁻²²⁶. The regimen may consist of gemcitabine 1000mg/m² weekly for 3 weeks, with concurrent chemoradiation starting 1-2 weeks after gemcitabine. Chemoradiation would consist of 5-fluorouracil continuous IV infusion via pump during radiation and radiotherapy of 1.8Gy/day for a total of 50.4 Gy. A further 3 cycles of gemcitabine would take place 3-5 weeks after chemoradiation.

Relevant trials:

CONKO-001 trial ²¹⁷– Multinational trial in which 368 patients were randomly assigned to gemcitabine or no treatment after surgery. A 5-year survival benefit of 21% versus 10% was shown.

ESPAC-3 trial ²¹⁸– Multicentre trial in which 1088 patients were randomly assigned to six months of gemcitabine or leucovorin-modulated FU after surgery. After a median follow-up of 34 months, median survival was similar (23.6 versus 23 months respectively), but patients assigned to FU/leucovorin had more grade 3 to 4 treatment-related toxicity.

Treatment in unresectable cases: Therapeutic options for patients with unresectable PDAC include chemotherapy alone, chemoradiotherapy or radiotherapy alone. Specific methods for symptom palliation also have a significant role to provide relief of pain, obstructive jaundice, gastric outlet obstruction, and pancreatic exocrine insufficiency.

Systemic chemotherapy for patients with advanced pancreatic cancer has been shown to improve disease-related symptoms and survival compared to supportive treatment alone. FOFIRINOX, a combination chemotherapy regimen consisting of oxaliplatin, irinotecan, fluorouracil, and leucovorin, has been shown to be superior to gemcitabine in objective response rate (39% versus 11%) for patients who have a very high performance status and who can tolerate an intensive approach ²²⁷. For those who cannot tolerate this regimen, a less toxic alternative combining gemcitabine with paclitaxel or capecitabine may be trialed ²²⁸⁻²³⁰. Meanwhile, gemcitabine monotherapy provides the mainstay of treatment for the majority of patients, who cannot tolerate the side-effect profile of the above more intensive regimens and is associated with significant clinical benefit ^{231, 232}.

For patients who fail gemcitabine monotherapy or FOLFIRINOX therapy but maintain good performance status, second-line therapy should be considered. This may involve a oxaliplatin/fluoropyrimidine combination such as FOLFOX following gemcitabine monotherapy ^{233, 234}, or gemcitabine monotherapy itself following FOLFIRINOX therapy ²³⁵.

Patients with locally-advanced, unresectable cancer may also benefit from chemoradiotherapy provided they have not developed metastases during the initial phase of chemotherapy. Chemoradiotherapy with external beam RT plus concomitant low-dose infusion FU or capecitabine should be considered. The hypothesis for chemoradiotherapy's efficacy in this cohort of patients is that a period of initial disease control with chemotherapy can select patients without occult micrometastatic disease who might benefit from radiotherapy ²³⁶⁻²³⁸.

Relevant trials:

Burris trial ²³²– 126 patients with advanced symptomatic cancer were randomized to receive either gemcitabine or fluorouracil, A better clinical benefit response (24% versus 5%), improved median overall survival (5.6 months versus 4.4 months) and improved 1-year survival (18% versus 2%) were observed.

ACCORD 11 trial ²²⁷– 250 patients with chemotherapy-naïve, metastatic pancreatic cancer with an excellent performance status were randomly assigned to gemcitabine alone versus FOLFIRINOX. Patients treated with FOLFIRINOX showed significantly higher objective response rate (32% vs 9%) as well as median progression-free survival (6.4 vs 3.3 months) and overall survival (11.1 vs 6.8 months).

Neoadjuvant therapy in borderline resectable cases: Borderline resectable cases may represent patients who have focal tumour abutment of the visceral arteries or involvement of the superior mesenteric vein or portal vein that is potentially resectable and reconstructable. Operations on such cases are unlikely to achieve complete resection, thus spurring efforts to ‘downstage’ the tumour pre-operatively using chemotherapy with or without radiotherapy. This neoadjuvant therapy is highly controversial and has a poor evidence base, but the theory is that it may increase rates of margin-negative resections, provide early treatment of micrometastatic disease as well as to improve selection for patients for whom resection will not offer a survival benefit.

One proposed treatment algorithm for borderline resectable cases involves use of chemotherapy with FOLFIRINOX (or gemcitabine with or without a combination agent), followed by re-evaluation after 2 and 4 months ²³⁹. If no distant metastases have occurred, radiotherapy may take place at this point and if at 6 weeks post-treatment there is retained good performance status and no distant metastases, surgical exploration should take place. Some institutions would argue that if there is a good response to the initial neoadjuvant chemotherapy, patients should be taken

directly to surgical exploration without undergoing further radiotherapy. Given the lack of evidence for this algorithm, and that the best form of induction therapy to use is unclear, patients with borderline resectable disease should ideally be enrolled in clinical trials to further define any benefit of neoadjuvant therapy.

Recent/novel trials: There is increasing interest in molecularly-targeted therapies based on the biologic properties of pancreatic cancer. One such target is epidermal growth factor, which is frequently over-expressed in PDAC ²⁴⁰. A tyrosine kinase inhibitor of the receptor often used in lung cancer, erlotinib, has been shown to have a modest survival gain when compared to gemcitabine alone (1-year survival 23 vs 17%) ²⁴¹, however, this is unlikely to prove cost-effective. Other approaches have targeted angiogenesis and increased expression of vascular endothelial growth factor and its receptor ²⁴². Disappointingly, despite promising phase II data, a phase III trial adding bevacizumab to gemcitabine in 602 patients did not reveal any benefit ^{243, 244}.

Another attractive target has been the Ras oncogene and its encoded proteins - Ras mutations leading to constitutive activation are found in around 90% of pancreatic cancers ^{244, 245}. Early studies targeting the Ras gene using vaccines and antisense molecules have shown promise ²⁴⁵. A further therapeutic strategy has been inhibiting the post-translational addition of farnesyl group to the Ras proteins. Benzodiazepine peptidomimetic analogues blocking this process restores a normal growth pattern in Ras-transformed cells in preclinical studies ²⁴⁶, but unfortunately phase III trials of farnesyltransferase inhibitors have shown lack of clinical benefit ²⁴⁷.

1.9 AIMS AND OBJECTIVES

The hypothesis of this thesis is that currently available biomarkers for pancreatic cancer would have little impact on predicting outcomes following intervention.

At the time of starting this project there was little published research investigating differences between those with early disease recurrence following surgery for PDAC and those with no disease recurrence and whether surgical technique can alter recurrence rates. Therefore our aims were:

- 1) To review the literature regarding potential blood biomarkers in pancreatic cancer, namely cell-free DNA, circulating tumour cells and circulating microRNAs.
- 2) To determine whether the no-touch technique for pancreaticoduodenectomy (PD) reduces the number of circulating tumour cells and improves the recurrence rate in PDAC patients compared to the standard PD technique.
- 3) To identify clinicopathological factors that are significantly different between patients who develop disease recurrence within 12 months of pancreatic resection for PDAC and patients who do not develop disease recurrence for at least 2 years after surgery.
- 4) To investigate genetic and epigenetic factors, that are significantly different between patients who develop disease recurrence within 12 months of pancreatic resection for PDAC and patients who do not develop disease recurrence for at least 2 years.
 - To examine DNA changes in tumour tissue for each group.
 - To establish whether these DNA mutations can be identified in the peripheral circulation as cell-free DNA

- To determine the number of circulating tumour cells in these patients and establish whether any differences exist between each group
- To examine microRNA expression in the peripheral circulation and establish whether any differences exist between each group

CHAPTER 2:

Methods

2.1 A Literature review of potential biomarkers in pancreatic cancer:

A literature search for relevant studies using the search terms 'biomarkers' OR 'liquid biopsy' AND 'Cancer' OR 'PDAC' OR 'pancreatic cancer' OR 'pancreatic neoplasm' was conducted on PubMed, Embase, Web of Science, Cochrane Library and Google Scholar databases. The abstracts of these studies were scrutinised. Publications were categorized and further searches were conducted on the categories with the most research published including: circulating tumour cells (CTCs), cell-free DNA and micro RNAs

2.2 Surgical technique to reduce recurrence rates using CTCs as a biomarker:

2.2.1 Ethics: Approval was obtained from a UK national research ethics committee (London; 08/H0706/35) and Imperial College Healthcare NHS Trust.

2.2.2 Patients: Patients undergoing PD for likely PDAC were identified. The main inclusion criteria were those patients undergoing curative resection for PDAC and who consented for intra-operative blood sampling. A prior decision for tumour resection was made at the hepato-pancreato-biliary MDT meeting. Patients were randomised into 2 groups: Standard pancreaticoduodenectomy, ST-PD (group I) and the no-touch technique pancreaticoduodenectomy, NT-PD (group II). The primary endpoint was the increase in number of CTCs following resection. Secondary endpoints were disease-free survival and overall survival.

2.2.3 Operative technique: All operative procedures were performed by a single surgeon (LRJ). Our method for ST-PD has been previously detailed.²⁴⁸ In brief, with an assistant or the primary surgeon himself lifting and touching duodenum and the head of pancreas (HOP), the duodenum is first Kocherized to the posterior surface of pancreas at the level of left renal vein and inferior vena cava. Dissection of the liver hilum and ligation of gastroduodenal artery (GDA) is then performed close to its origin from the hepatic artery. Following tunnelling the pancreas above the superior mesenteric vein (SMV) and portal vein (PV) and division of jejunum, the surgeon places the left hand behind the HOP by holding the duodenum and head of pancreas to complete transection behind the pancreas. In comparison, the concept of the NT-PD is that the tumour must not be manipulated before the vascular and lymphatic drainage vessels are completely isolated and was performed as previously described.¹⁸⁸ Following dissection of the liver hilum to identify the common bile duct (CBD) and PV, the CBD is divided below the junction of hepatic ducts and the GDA is ligated and divided. Next, the lesser sac is entered by dividing the gastrocolic ligament with the LigaSure Vessel Sealing System (Valleylab, Boulder, CO). After division of jejunum 5-10cm from the duodenal-jejunal (DJ) flexure with a 45mm stapler, the pancreas is tunnelled at the superior mesenteric vein (SMV) and portal vein (PV) and divided with a surgical scalpel. Superior, middle and inferior pancreatic veins are identified, ligated and divided. The SMV and PV are freed completely from pancreas. All lymphatic drainage to the portal hilus is divided. Finally, Kocherization was performed and the crural tissue between the uncinate process of pancreas and superior mesenteric artery (SMA) is divided by LigaSure to complete the PD. At all times during the procedure the HOP is not grasped by the surgeon and transection of pancreatic crural is performed only after ligation and division of the venous drainage.

2.2.4 Blood sampling and analysis: Following identification and exposure of the portal vein (PV), 7.5 mL of blood was drawn directly using a 21-gauge butterfly

needle before dissection and immediately after removal of the PD specimen. Blood samples were taken at each point in a CellSave (Veridex, NJ, USA) preservative tube. The samples were then processed in our laboratory within 24 hours using the bead-based fluorescence CellSearch system (Veridex, NJ, USA) according to the manufacturer's protocol. Samples were then scanned on the CellTracks analyzer II fluorescent microscope (Veridex, NJ, USA) for analysis. The cells were evaluated for CTCs independently by 2 operators (**Figure 3**).

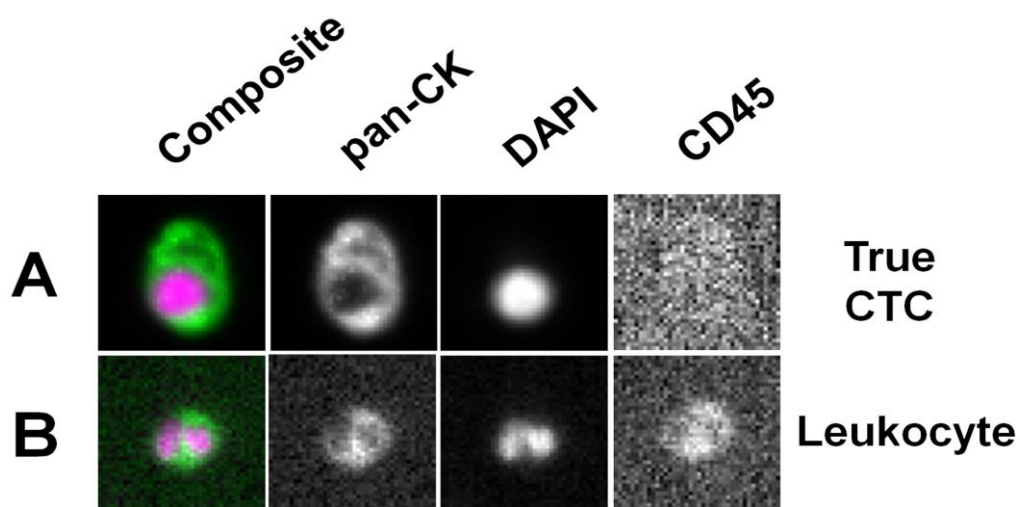


Figure 3: Identification of pancreatic circulating tumor cells (CTCs) in the portal venous circulation. (**A**) Shows a true CTC that is positive for EpCAM, pan-CK and DAPI, but negative for CD45; whereas (**B**) shows a leukocyte that is positive for CD45.

2.2.5 Follow-up: All patients had routine follow-up in the outpatients clinic at 3 monthly intervals for the first year and then six monthly intervals. Clinical examination, routine laboratory tests and a CT scan was organised at each appointment. Contrast microbubble ultrasound scan, positron emission tomography (PET) or bone scintigraphy was arranged to detect distant recurrence as necessary. Disease recurrence and date of death was determined from death certificates, clinic

letters and radiological findings. Date of disease recurrence was recorded as the date of the first CT scan to diagnose abnormal pathology. Follow-up continued until date of death or until July 2014 for surviving patients. Disease-free and overall survival was defined as the interval between date of surgery and date of death or final follow-up.

2.2.6 Statistical Analysis: The statistical analyses were performed using IBM SPSS statistics version 21 software package (IBM SPSS, Chicago, USA). Univariate analysis comparisons were made using Fisher's exact test for categorical data and the Wilcoxon signed rank test for continuous data. All tests were two-sided. Disease-free and overall survival rates were calculated by Kaplan-Meier method and compared by the log-rank test. A p-value <0.05 was considered significant.

2.2.7 Power calculation: We estimated that 85% of patients would have an increase in number of CTCs following the standard pancreaticoduodenectomy. We would consider it clinically significant if only 20% of patients had an increase in CTC numbers following the no-touch technique. We estimated that the sample size required to detect this difference at 90% power and 5% level of confidence to be 6 in each group using the following formula. Where π_1 is 85% and π_2 is 20%

$$N \text{ in each group} = f(\alpha, \beta) \times ((\pi_1 \times (100 - \pi_1) + \pi_2 \times (100 - \pi_2)) / (\pi_1 - \pi_2)^2)$$

2.3 Clinicopathological factors which may predict disease recurrence:

2.3.1 Ethics: This study was approved by our institutional review board (Hammersmith Hospital, Imperial Healthcare NHS Trust). Informed written patient consent for research was obtained before surgical treatment.

2.3.2 Patients: All patients who had a pancreatic resection from January 2001 to June 2014 were included in our retrospectively created database. Inclusion factors

for this study were: patients with histopathologically confirmed pancreatic ductal adenocarcinoma (PDAC) who had a pancreatic resection during this time period including pancreaticoduodenectomy (PD) and distal pancreatectomy (DP); and patients who developed disease recurrence (either local or distant disease) within 12 months from the operative date or who had no disease recurrence for at least 24 months after surgery. Exclusion factors included: Patients with alternative diagnoses either benign disease or malignant including ampullary carcinoma, cholangiocarcinoma, duodenal carcinoma, cystadenocarcinoma or neuroendocrine tumours; patients under 18yrs of age; patients with abandoned resections (due to locally advanced disease or metastases seen intra-operatively) or R2 resections. After preoperative evaluation with a triple phase pancreas protocol computed tomography (CT) scan and/or endoscopic ultrasound (EUS) +/- fine needle aspiration (FNA) and/or magnetic resonance imaging (MRI), a decision for surgery was made at our hepatobiliary multidisciplinary team (MDT) meeting. Surgery is offered to those who fit the criteria for pancreatic resection: absence of extrapancreatic disease; no involvement of the superior mesenteric artery (SMA) or coeliac axis; no encasement of the superior mesenteric vein (SMV) or portal vein (PV) $>180^\circ$; and fit to undergo major surgery (WHO performance status of 0-2). For patients with local venous invasion with $<180^\circ$ encasement, resection and reconstruction of the SMV or PV is performed as required. Preoperative data included age, sex, past medical history and bloods including CA 19-9, bilirubin and CRP. The cut-off value used for CA 19-9, bilirubin and CRP was 37 kunits/L, 22 $\mu\text{mol/L}$ and 20 mg/L respectively, in accordance with the literature.^{249, 250}

2.3.3 Operative procedures: All operations in our unit are performed by consultant hepatopancreatobiliary surgeons and have been previously described.²⁴⁸ Both whipples procedure and pylorus preserving pancreaticoduodenectomy (PPPD) are routinely performed as standard, without extended lymphadenectomy, Retroportal

resection extends to the tissues in the groove between the uncinate process and SMA. The pancreatico-enteric anastomosis is decided during the procedure by the surgeon and performed as either a pancreaticojejunostomy or pancreaticogastrostomy. PD is performed laparoscopically where possible. Tangential or segmental resection of the SMV or PV is performed when the pancreatic head or uncinate process can't be separated from the vein without leaving macroscopic tumour or risking venotomy. Transection margins are analysed with frozen section intraoperatively and further resection undertaken until negative margins obtained histopathologically. DP and splenectomy is performed laparoscopically where possible by standard techniques. An abdominal drain is placed during the procedure and kept until output is <30mls in 24 hours.

2.3.4 Histopathological examination: The gross and microscopic examination of specimens is performed by a hepatopancreatobiliary histopathologist according to the guidelines of the Royal College of Pathologists (available at <http://www.rcpath.org>). These guidelines recommend that the status of six discrete resection margins be documented by the reporting pathologist: the pancreatic transection margin, the medial (or superior mesenteric vessel) margin, the posterior margin, the proximal duodenal (or gastric) margin, the distal duodenal margin and the common bile duct margin. Microscopic evidence of tumour involvement within 1mm of any one of these six margins results in an R1 classification.²⁰¹ The pancreatic head is sliced at 5 mm intervals producing 10–13 slices. These slices are embedded whole in megablocks allowing extensive views of the lesion and its relation to the entire CRM and key anatomical structures.²⁰⁴ Tumour size, tumour differentiation, total number and number of involved lymph nodes, resection margin status, the presence of perineural and/or lymphovascular invasion is recorded. The lymph node ratio is the number of lymph nodes involved to the total number of lymph nodes and the cut-off used was 0.2, in accordance with the literature.²⁵¹

2.3.5 Follow-up: All patients had routine follow-up in the outpatient clinic at 3 monthly intervals for the first year and then six monthly intervals. Clinical examination, routine laboratory tests and a CT scan was organised at each appointment. Contrast microbubble ultrasound scan, positron emission tomography (PET) or bone scintigraphy was arranged to detect distant recurrence as necessary. Disease recurrence and date of death was determined from radiological findings, clinic letters, and death certificates. Date of disease recurrence was recorded as the date of the first CT scan to diagnose abnormal pathology. Follow-up continued until date of death or until July 2014 for surviving patients. Disease-free and overall survival was defined as the interval between date of surgery and date of death or final follow-up.

2.3.6 Statistical Analysis: The statistical analyses were performed using IBM SPSS statistics version 21 software package (IBM SPSS, Chicago, USA). The two groups consisted of those with disease recurrence within 12 months and those with no recurrence for at least 24 months. Univariate analysis comparisons were made using Fisher's exact test for categorical data and the Wilcoxon signed rank test for continuous data. All tests were two-sided. Variables that were significantly different ($p < 0.1$) were included in a cox regression analysis to examine the impact of these variables on early disease recurrence. Overall survival rates were calculated by Kaplan-Meier method and compared by the log-rank test.

2.4 Are disease recurrence rates affected by genetic and epigenetic changes and can these be detected in the peripheral circulation?:

2.4.1 Ethics: Approval was obtained from a UK national research ethics committee (London; 09/H0722/77) and Imperial College Healthcare NHS Trust.

2.4.2 Patients: All patients with pancreatic ductal adenocarcinoma who underwent a pancreatic resection from January 2001 until June 2014 were included on a retrospectively collected database. From this database, those who developed disease recurrence within 12 months from the date of the operation (Group I) and those that did not develop disease recurrence for at least 24 months (Group II) were identified.

2.4.3 Tumour samples: The formalin-fixed paraffin-embedded (FFPE) blocks and corresponding H&E stained slides for these patients were collected. These were reviewed with a specialist pancreatic pathologist. Areas of PDAC were then marked with a permanent marker on the glass cover-slip. FFPE blocks corresponding to each H&E slide were then cut (5-10 sections, each 10 µm thick). Microdissection of the tumour cells was then completed.

2.4.4 DNA extraction from microdissected FFPE blocks: The Qiagen QIAamp DNA FFPE kit was used to extract genomic DNA from the FFPE tissues as per the manufacturer's instructions. A volume of 160µl of deparifination solution was added to each sample in an eppendorf tube before centrifugation and incubation at 56°C. 180µl of buffer ATL (lysis buffer for purification of nucleic acid, Qiagen) and then 20µl of proteinase K was added followed by incubation at 56°C and then 90°C. The tubes were then transferred to a 2ml safe-lock microcentrifuge tube and placed into the QIAcube for DNA extraction. Finally, DNA quantification of each sample was established with the Qubit 2.0 fluorometer and the samples were stored at 4°C.

2.4.5 Ion Torrent sequencing: The ion torrent next generation sequencer (Life Technologies), a validated technique^{252 253}, was used to sequence the DNA for each sample according to the manufacturer's protocols. 10ng of DNA for each sample was used for library preparation with the Ion AmpliSeq Cancer Hotspot Panel v2 (Life Technologies). This panel gives 207 amplicons covering 2,800 COSMIC mutations from 50 oncogenes and tumour suppressor genes, including *KRAS*, *p53*, *CKDN2A* and *SMAD4*. No more than sixteen samples were processed at a time. Each library was barcoded with Ion Express Barcode Adaptors 1-16 kit and then purified with Agencourt AMPure XP reagent (Life Technologies). Following qPCR of each sample for quantification, all samples were combined in equal concentration into a single pool of 25 μ l. Template preparation was performed on the Ion PGM OneTouch system (Life Technologies) resulting in template positive ion sphere particles (ISPs). After quality control with the Ion Sphere Quality Control Kit (Life Technologies) to ensure a 10-30% template to bead ratio, the template positive ISPs were enriched on the Ion OneTouch ES system (Life Technologies). Sequencing primer and Ion PGM Sequencing 200v2 Polymerase were added to the enriched ISPs before loading onto a chip from the Ion 314 Chip Kit v2 (Life Technologies) and sequencing the samples on the Ion PGM System. Data analysis was carried out with Torrent Suite Software (Life Technologies) to generate sequence reads, trim adapter sequences and filter and remove poor signal-profile reads.

2.4.6 Power calculation: From the DNA analysis of the FFPE tissues we would consider a difference in a genetic mutation between the two groups of 60% to be clinically relevant. We estimated the sample size needed to detect a 60% difference at 90% power and 5% level of confidence to be 14 in each group with the calculation below. Where π_1 is 85%, the percentage of patients in the early recurrence group for which this mutation would be considered clinically relevant and π_2 is 40% of 85% (a 60% reduction).

$$N \text{ in each group} = f(\alpha, \beta) \times ((\pi_1 \times (100 - \pi_1) + \pi_2 \times (100 - \pi_2)) / (\pi_1 - \pi_2)^2)$$

2.4.7 Blood Samples: Patients were invited to donate blood prior to their pancreatic resection. Blood was taken and processed as follows:

2.4.7.1 For DNA sequencing: An EDTA tube of whole blood was collected and immediately processed to extract DNA using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's protocol. In brief, proteinase K and Buffer AL (Qiagen) was added to 200µl of each sample and incubated at 56°C. After the addition of 100% ethanol, the sample was applied to the QIAamp Mini spin column and centrifuged. The sample was also centrifuged after the addition of Buffer AW1, Buffer AW2 and Buffer AE (all Qiagen). DNA quantification of each sample was established with the Qubit 2.0 fluorometer and the samples were stored at -20°C. Sequencing of DNA was carried out with the ion torrent next generation sequencer as described above.

2.4.7.2 For circulating tumour cells (CTCs) analysis: 7.5mls of whole blood was collected in a CellSave (Veridex, NJ, USA) preservative tube. The samples were then processed in our laboratory within 48 hours using the bead-based fluorescence CellSearch system (Veridex, NJ, USA) according to the manufacturer's protocol. After centrifugation, the sample was placed in the CellSearch system. The plasma was aspirated and a buffer containing ferrofluid nanoparticles with antibodies to target EpCam was added. Magnets were then used to draw the ferrofluid particles to the side of the tube so that unlabelled cells were aspirated. The magnets were removed and target cells were resuspended in a buffer containing permeabilising and staining reagents. The epithelial cells were stained with antibodies labelled with phycoerythrin to target cytokeratins 8, 18 and 19. To distinguish CTCs from

leucocytes, allophycocyanin, labelled antibodies to CD45, were also used. Additionally, DAPI to stain cell nuclei and to distinguish viable cells from nonspecific debris was added. The epithelial enriched sample was then placed in a MagneSt that attracts the magnetically labelled cells. Samples were then scanned on the CellTracks analyzer II fluorescent microscope (Veridex, NJ, USA) for analysis. The cells were evaluated for CTCs independently by 2 operators.

2.4.7.3 For microRNA (miRNA) analysis – RNA extraction: Blood was collected into two EDTA tubes and immediately put on ice. Samples were processed immediately. After centrifugation, the plasma was aspirated and placed in eppendorf tubes for storage at -80°C. RNA extraction was performed at a later date using the miRNeasy serum/plasma kit (Qiagen) according to the manufacturer's protocol. For each sample, a total of 1ml of thawed plasma was used. QIAzol lysis reagent (Qiagen) and chloroform, was added to each sample. Following cold centrifugation and mixing with 100% ethanol each sample was added to an RNeasy MinElute spin column (Qiagen) and centrifuged. This step was repeated so that all 1ml of each original plasma sample was processed through the same spin column. Each spin column was washed with Buffer RWT, Buffer RPE (Qiagen) and 80% ethanol. After the addition of RNase-free water, the RNA was eluted. RNA quantification was established with the NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Scientific). RNA was stored at -80°C.

microRNA (miRNA) analysis: miRNA analysis of the extracted RNA was performed using the NanoString nCounter system (NanoString Technologies, Seattle, WA). This method enables multiplexed direct digital counting of miRNA molecules using colour-coded barcodes. The method measures a total of 800 probes including both endogenous human-associated miRNAs as well as viral miRNAs that are expressed in human cells. The hybridization method used directly interrogates target

sequences, avoiding the need for bias-prone amplification steps, even for low-abundance transcripts. Further, measurement is achieved using the digital detection of uniquely bar-coded probes, providing absolute quantification ²⁵⁴. 3µl of each sample was used following purification of the RNA with Amicon 3kD cellulose columns to remove impurities. Each RNA sample was added to the miRNA-tag ligation reaction. Following ligation, samples were diluted 1:10, and 5µl of each miRNA-tag sample (equivalent to 10ng total RNA) were added to hybridizations. Excess probes were removed using two-step magnetic bead based purification on the nCounter Prep Station (nanoString Technologies). Abundances of specific target molecules were quantified on the nCounter Digital Analyzer by counting the individual fluorescent barcodes and assessing the target molecules. For each assay, a high-density scan encompassing 600 fields of view was performed. The data was collected using the nCounter Digital Analyzer. miRNA data analysis was performed using the nSolver software analysis, (nanoString Technologies). The serum miRNA profiling data was normalized using the average signals obtained from a spike-in control (Lyophilized *C. elegans* miR-39 miRNA mimic, Qiagen), and miRNAs that gave significant hybridization signals were used for downstream analysis.

CHAPTER 3:

A Literature review of potential biomarkers in pancreatic cancer

3.1 *Introduction:*

An early diagnosis of pancreatic cancer is often delayed due to the late onset of symptoms and a limited use of current imaging modalities. Further, many clinical modalities such as EUS and FNA are invasive procedures with resultant risks to the health of a patient. Following the diagnosis of PDAC there are few options for monitoring disease progression during oncological management or for detecting disease recurrence following pancreatic resection. The gold standard being interval CT scans and blood Ca 19-9 levels. However, as discussed above these have limited sensitivities and specificities. After curative surgical resection and adjuvant chemotherapy, it is not yet possible to determine patient prognosis. Many develop disease recurrence within 12 months where as others do not develop disease recurrence for at least two years and around 5% have no evidence of PDAC at 5 years post resection. In order to improve these issues there is a need to develop a 'liquid biopsy', a blood based biomarker, able to detect PDAC and to monitor its progression with high sensitivity and specificity. Much recent work has concentrated on three potential biomarkers including circulating tumour cells (CTCs), cell-free DNA and micro RNAs.

3.2 *Circulating tumour cells (CTCs):*

In 1869, Thomas Ashworth, detected tumour cells in the blood of a patient with metastatic cancer ²⁵⁵. These cells, termed "circulating tumour cells" (CTCs), have since been of great interest in tumour biology research. CTCs are cells which circulate through the bloodstream, having been formed by tumour-induced angiogenesis ²⁵⁶. This blood-borne dissemination of tumour cells from the primary site to distant organs may lead to metastatic disease. Levels of CTCs in the

peripheral blood are low, for example only one cell per 10^5 - 10^7 with respect to mononuclear cells and therefore their detection is extremely challenging. Recently, the development of an automated detection system “CellSearch” (Veridex, NJ, USA), validated in 2007²⁵⁷ and FDA approved, has enabled us and others to focus on CTC blood levels in patients with various tumour types^{258, 259}.

CTCs are an independent predictor of progression-free survival and overall survival in metastatic breast cancer²⁶⁰⁻²⁶², colorectal cancer^{263, 264}, castrate-resistant prostate cancer²⁶⁵ and in small-cell-lung-cancer²⁶⁶. However, there are limited data on CTCs in PDAC. A literature search for relevant studies using the search terms ‘CTC’ OR ‘circulating tumour cells’ AND ‘pancreatic cancer’ was conducted on PubMed, Embase, Web of Science, Cochrane Library and Google Scholar databases. Ren et al.²⁶⁷ measured two or more CTCs (mean 16.8 ± 16.0) in 7.5mL of peripheral blood in 80.5% (33/41) of patients with advanced PDAC. No CTCs were detected in 20 healthy donors ($p < 0.01$). After seven days of the first cycle of 5-FU chemotherapy, only 29.3% (12/41) of PDAC patients had more than two CTCs in 7.5mL of peripheral blood. De Albuquerque et al.²⁶⁸ developed an immunomagnetic/RT-PCR assay and were able to detect CTCs in 47.1% (16/34) of PDAC patients, compared to none in healthy controls ($n=40$). Additionally, a shorter progression-free survival was observed for patients who had at least one CTC detected compared to those who were CTC negative. Yu et al.²⁶⁹ used CTCs from an endogenous mouse PDAC model, to identify *Wnt2* as a possible therapeutic target. A meta-analysis was conducted of nine cohort studies analysing CTCs in pancreatic cancer patients.²⁷⁰ In a total of 623 patients, 43% had positive CTCs from peripheral blood samples. These CTC-positive patients had significantly worse progression-free survival than CTC-negative patients (HR 1.89, 95% CI 1.25-4.00, $p < 0.001$) and showed worse overall survival (HR 1.23, 95% CI 0.88-2.08, $p < 0.001$). However, others have failed to correlate survival and CTCs. Indeed, in a recent study²⁷¹, only five of 20 patients

(25%) had positive CTCs in the systemic circulation and these patients did not have shorter disease-free or overall survival compared to CTC-negative patients.

Further analyses of CTCs in PDAC will provide new insights into its molecular biology, and in particular a better understanding of the metastatic process and the alterations that drive disease spread. They may well have a role in the initial diagnosis, detection of recurrent disease or aid in the selection of targeted adjuvant therapy for these patients. However, it is not clear whether CTCs will prove as beneficial biomarkers as in other cancers as small numbers are captured compared to other solid tumours. Further, to date, studies include small numbers of patients and most find CTCs in less than half of PDAC patients irrespective of disease stage.

3.3.1 *Cell-free DNA (cfDNA)*:

In 1948, Mandel and Metais published the first article to describe the occurrence of circulating nucleic acids in human plasma ²⁷². Thirty years later, in 1977, Leon et al. reported elevated levels of circulating DNA in the serum of cancer patients ²⁷³ and in 1989, plasma DNA was shown to be higher in patients with various malignancies, compared to healthy individuals ²⁷⁴. Gradually with improvements in DNA detection, the oncogenetic importance of cfDNA has been realised and several cancer-specific mutations and microsatellite alterations have been identified in plasma DNA ²⁷⁵⁻²⁷⁷. Subsequently, the widespread availability of polymerase chain reaction (PCR) techniques has allowed for a rapid increase in the ability for cfDNA detection. Emerging evidence suggests that identifying specific genetic and epigenetic mutations in cfDNA, may detect specific tumours, as well as monitor responses to treatment. Thus there is the potential for cfDNA to have significant clinical implications as a non-invasive biomarker to aid diagnosis, prognosis, monitoring and personalized therapy. A literature search for relevant studies using the search terms

'cell free DNA' OR 'cfDNA' AND 'pancreatic cancer' was conducted on PubMed, Embase, Web of Science, Cochrane Library and Google Scholar databases.

3.3.2 Origin and composition of cfDNA:

Circulating cfDNA is extracellular DNA, which occurs both in plasma and serum. The cfDNAs are mostly double-stranded molecules, consisting of small fragments of 70-200 base pairs (bp) up to larger fragments with molecular weights of 21kb^{278, 279}. It is thought that cfDNA is actively released from all living cells, as a part of normal metabolism²⁸⁰. However, 4 to 40 times greater levels are seen in patients with cancer²⁷⁹. For example, in colorectal cancer, it is estimated that up to 3.3% of the tumour DNA is fed into the circulation on a daily basis, depending on Dukes' Stage²⁸¹. cfDNA is also increased in other physiological and pathological conditions, including exercise, smoking, inflammatory disease, critical illness, sepsis and trauma²⁸²⁻²⁸⁴. Therefore there has been much postulation over the origins of circulating cfDNA. In physiologically active and pathological states, there can be a release of cfDNA from apoptosis; necrosis; direct release from viable cells and from the lysis of circulating tumour cells (CTCs). Apoptosis is programmed for a large number of cells on a daily basis. In cancer, the high cell turnover seen, leads to increased programmed cell death and thus may explain the higher concentrations of plasma cfDNA²⁷⁹. Apoptotic cell death is also characterized by fragments of around 180-720 bp, similar to a large proportion of cfDNA identified in plasma²⁸⁵. Moreover, chloroquine, is able to induce a low level of apoptosis, and its application has been shown to increase cfDNA concentration²⁸⁶. Neoplastic cells undergo rapid enlargement and start to outgrow their blood supply. This results in hypoxia and leads to necrosis. Necrotic cells are phagocytosed by macrophages and subsequently, the ingested cell components, including tumour DNA fragments, are released into the circulation²⁸¹. Normal cells close to the tumour may also become necrotic, leading to increased levels of 'normal' DNA in the circulation by the same

process. The tumour may also actively release DNA into the bloodstream. *Ex-vivo* experiments on lymphocytes have shown a release of newly-synthesised DNA that is not a result of cell death ²⁸⁷. Experiments using a rat model of colorectal cancer found increased levels of both tumour and non-tumour cfDNA in the early stages of the disease. The authors suggested that when there is minimal necrosis, there is an enhanced turnover and an increase in the active release of DNA from both cancer cells and normal adjacent tissue ²⁸⁸. The biological reasons behind these theories remain unclear. One further hypothesis for the increase of cfDNA in cancer is due to the release of DNA fragments from CTCs. CTCs are due to the blood-borne dissemination of tumour cells and are thought to be involved in the development of metastatic disease. They have been detected in a number of tumour types and their lysis may contribute to cfDNA ²⁸⁹.

Once released into the circulation, cfDNA has a short half-life of between 4 minutes to several hours ^{290, 291} and it is thought that the liver, spleen and kidneys all play a role in its clearance. DNA may exist in the blood within protein bound complexes, involving albumin, immunoglobulins, fibronectin, lactoferrin, lysozyme and serum amyloid P component ²⁹². Additionally, cfDNA can be associated with the surface of haematopoietic cells either as part of a complex or directly bound to the cell surface ²⁹³.

3.3.3 cfDNA as a biomarker for cancer:

Currently, there is debate as to whether total cfDNA levels could be used as a diagnostic marker for malignancy, as generally its sensitivity for cancer detection is low ²⁹⁴. However, identifying tumour-specific genetic and epigenetic changes in cfDNA, including gene mutations; loss of heterozygosity (LOH); methylation alterations; and microsatellite alterations may be more specific. Epigenetic changes are some of the earliest events in malignant transformation and thus their detection in

blood samples has the potential to lead to early clinical biomarkers for various cancers.

Gene mutations: Tumour-specific gene mutations, including mutations in tumour suppressor genes and oncogenes have been identified from cfDNA. Many studies have shown an agreement between mutations found in tumour tissue and those found in cfDNA. Point mutations have been found in a number of important genes including *KRAS* and *P53*.

Loss of heterozygosity: PCR-based assays have allowed for the detection of LOH. Analysing cfDNA in 388 breast cancer patients showed a significant correlation between LOH in five polymorphic markers and tumour stage, tumour size and lymph node metastasis. Others, have investigated LOH at chromosomal location 18q in colorectal cancer, a location associated with a number of tumour suppressor genes. There are as many studies showing no correlation between LOH and prognosis in tumour tissue ^{295 296 297} as those that have found a relationship ^{298 299 300}. Therefore, there is minimal research looking at LOH in peripheral blood.

Methylation alterations: DNA methylation is an important physiological process active in all cells and required for many cellular events. Methylation occurs at CpG islands, which are mainly found at the promoter areas of genes. Abnormal methylation, such as the hypermethylation of a promoter region of a tumour suppressor gene, results in gene silencing ²⁹¹. Hypomethylation leads to increased mutation rates and chromosome instability ³⁰¹. The hypermethylation of numerous genes, including APC and p16, has been identified and may be involved in the development of cancer. Hypermethylated genes found in tumour tissues, can also be detected in cfDNA ³⁰².

Microsatellite alterations: Microsatellites are short DNA sequences with lengths of 1-6 nucleotides, which are repeated up to sixty times. There are thousands of these areas in the genome. PCR techniques are able to detect alterations in these microsatellite areas, including the deletion of one of the alleles, new microsatellite areas or a LOH. Several studies have successfully identified microsatellite alterations

in cfDNA in various cancers ²⁹¹ and there is evidence to suggest that these alterations can be found at a very early stage of disease.

3.3.4 cfDNA in PDAC:

Many cfDNA studies have investigated *KRAS* mutations from blood samples of PDAC patients (**Table 3**). Uemura et al. ³⁰³ looked at *KRAS* mutations in both tumour tissue and plasma of 28 patients with PDAC. Ninety-three percent of patients had *KRAS* mutations in pancreatic tumour tissue, as opposed to only 35% in the plasma. Other authors ³⁰⁴⁻³⁰⁷ have found plasma *KRAS* mutations in 36-73% of PDAC patients. Although these percentages vary, likely due to the different methods used, these studies also found that mutations occur more frequently in PDAC patients than those with chronic pancreatitis (CP; 0-20%). Combining the detection of *KRAS* mutations with a raised CA-19-9 accurately diagnoses PDAC with a sensitivity of 67%-98% and specificity of 77%-97% (**Table 3**). Chen et al. ³⁰⁸ found that patients with inoperable PDAC and *KRAS* mutations in plasma DNA, have significantly worse survival compared to those with wild type *KRAS* (3.9 vs. 10.2 months). Furthermore, Dabritz ³⁰⁹ correlated the presence of mutated *KRAS* in the plasma of patients with pancreatic cancer with CT findings. 15/38 patients (39%) were found to have plasma *KRAS* mutations. Only 9% of patients with complete remission of disease on CT had *KRAS* mutations. Detectable *KRAS* mutations in the plasma were clearly associated with corresponding CT scans with signs of progressive disease in 75%. When combined with CA 19-9 levels, 92% of patients with *KRAS* mutations and an elevated CA 19-9 level had progressive disease on CT as opposed to only 33.3% of those with *KRAS* wild-type and normal CA 19-9 levels. However, others have failed to correlate *KRAS* mutations with survival. ³¹⁰ Other authors have concentrated on methylation abnormalities in cfDNA. Park et al. ³¹¹ found altered methylation in 81.3% of PDAC patients, compared to 61.5% of those with CP and 3.5% of healthy controls. Although this showed limited differences between PDAC and CP, Liggett et al. ³¹²

were able to discriminate between the presence of neoplasia and inflammation with a sensitivity of 91.2% and a specificity of 90.8%. Others have used models of 5 promoter CpG sites to discriminate PDAC from healthy individuals with reasonable results (e.g. Pederson et al. ³¹³ obtained a C-statistic of 0.76 [C-statistic >0.7 indicates good predictive power of the model]; and Melnikov et al. ³¹⁴ found 76% sensitivity and 59% specificity).

Whilst all these studies have small numbers of patients and use a variety of sampling and analytical techniques for determining cfDNA changes, it is encouraging that many have found cfDNA to be clinically useful in PDAC. Although this could partly be due to publication bias, *KRAS* mutations and methylation alterations appear more frequent in the blood of patients with PDAC compared to controls. Furthermore, combining cfDNA results with other markers, such as CA 19-9, the sensitivity and specificity of detection markedly improves. Finally, cfDNA may be a good indicator of prognosis and disease progression. Despite this, few well designed trials have rigorously examined the role of cfDNA as a biomarker for cancer detection, prognosis or treatment response.

Author / Reference	Year	Patient population	DNA source	cfDNA mutation	Significant Findings
Kinugasa et al. ³⁰⁷	2015	66 PDAC	Serum	<i>KRAS</i>	<i>KRAS</i> mutations in 54.5%
Park et al. ³¹¹	2012	16 PDAC, 13 CP, 29 healthy	Plasma	Methylation	Altered methylation in PDAC compared to CP and compared to healthy controls
Dabritz ³⁰⁹	2012	38 PDAC	Plasma	<i>KRAS</i> mutations and CT findings	<i>KRAS</i> mutations in 39% of PDAC patients.. Mutations associated with signs of progressive disease.
Pederson et al. ³¹³	2011	Phase I: 132 PDAC, 60 healthy Phase II: 240 PDAC; 240 healthy	Whole blood	Methylation	Prediction model of 5 CpG sites discriminated PDAC from controls
Liggett et al. ³¹²	2010	30 PDAC, 30 CP, 30 healthy	Plasma	Methylation	91.2% sensitivity and 90.8% specificity for PDAC vs. CP differentiation
Chen et al. ³⁰⁸	2010	91 unresectable PDAC	Plasma	<i>KRAS</i> mutations	<i>KRAS</i> mutations in 33% of PDAC patients . Worse survival in those with mutations
Dabritz et al. ³⁰⁴	2009	56 PDAC, 13 CP	Plasma	<i>KRAS</i> mutations	More <i>KRAS</i> mutations in PDAC vs CP. Add CA 19-9 = 91% sensitivity for cancer diagnosis.
Melnikov et al. ³¹⁴	2009	30 PDAC, 30 healthy	Plasma	Methylation	Prediction model of 5 promoters has 76% sensitivity and 59% specificity for PDAC detection.
Uemura et al. ³⁰³	2004	28 PDAC	Tumor and	<i>KRAS</i> mutations	<i>KRAS</i> mutations in 35% of PDAC patients' plasma

			Plasma		
Dai et al. ³⁰⁵	2003	15 PDAC, 10 CP	Plasma	<i>KRAS</i> mutations	<i>KRAS</i> mutations in 73% of PDAC patients vs 20% of CP patients. <i>KRAS</i> + CA 19-9 = 66.67% sensitivity and 97% specificity for cancer detection.
Maire et al. ³⁰⁶	2002	47 PDAC, 31 CP	Serum	<i>KRAS</i> mutations	<i>KRAS</i> mutations in 47% of PDAC patients vs 13% of CP patients . <i>KRAS</i> + CA 19-9 = 98% sensitivity and 77% specificity.

Table 3: Studies detecting cfDNA in patients with PDAC. PDAC: pancreatic ductal adenocarcinoma; CP: chronic pancreatitis;

3.4 *MicroRNAs (miRNA)*:

Focussing on tiny sequences of RNA (miRNAs) in the blood may allow the discovery of more sensitive and specific cancer diagnostic/prognostic tools. miRNAs are a class of small non-coding RNAs of ~19-25 nucleotides in length, that are involved in the post-transcriptional regulation of a large number of genes and can affect many different biological pathways. In our laboratory, from pancreatic cell-lines and tumour tissue, we have previously identified 3 miRNAs (miR-21, miR-23a, and miR-27a) that act as cooperative repressors of a network of tumor suppressor genes that includes PDCD4, BTG2, and NEDD4L³¹⁵. Inhibition of these 3 miRNAs had synergistic effects in reducing proliferation of PDAC cells in culture and growth of xenograft tumors in mice. The level of inhibition was greater than that of inhibition of miR-21 alone. In 91 PDAC samples from patients, high levels of a combination of miR-21, miR-23a, and miR-27a were associated with shorter survival times after surgical resection (**Figure 4**).

A literature search for relevant studies using the search terms 'circulating micro RNAs' OR 'circulating miRNA' AND 'pancreatic cancer' was conducted on PubMed, Embase, Web of Science, Cochrane Library and Google Scholar databases. Some studies have now indicated that blood-based miRNAs are an impending clinical utility as diagnostic and/or prognostic biomarkers for PDAC (**Table 4**). Kong et al.³¹⁶ showed elevated serum miR-196a levels strongly correlated with poor survival and advanced stage disease in PDAC, and could be used to aid selection of surgical candidates. However, others have failed to find a survival correlation between PDAC patients with dysregulation of those miRNAs found to differ between PDAC and controls.³¹⁷ Bauer et al.³¹⁸ performed a large study of the PDAC miRNome in blood. They compared the differential expression of miRNAs between PDAC vs. normal pancreas / chronic pancreatitis in tissue specimens, and between PDAC vs. healthy controls / chronic pancreatitis in blood samples. The combined analysis revealed that

10 miRNAs (miR-148a, 216a, 217, 181a, 181c, 324-5p, 146a, 210, 345, 574-5p) were able to differentiate between PDAC vs. normal / inflamed pancreas ($P=0.0006$) in tissue and blood specimens. When assessing miRNAs in blood samples only, 36 miRNAs were able to distinguish PDAC vs. normal with high sensitivity and specificity (0.973 and 0.950 respectively). Therefore, combinations of miRNAs may prove more accurate at diagnosing and/or predicting outcome in PDAC patients.

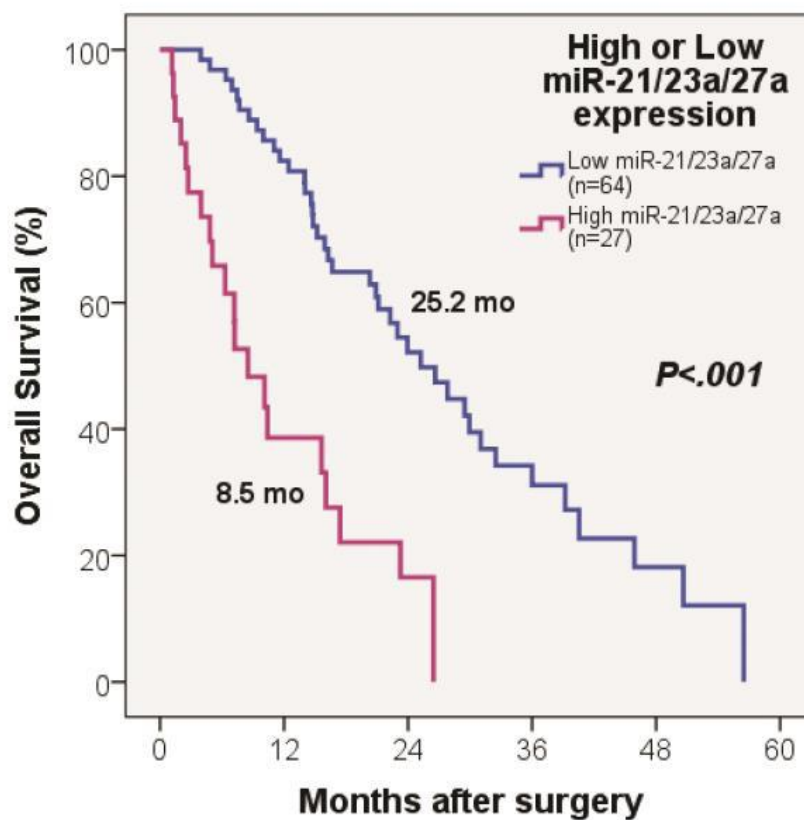


Figure 4: Overall cancer-specific survival among PDAC patients depending on tumoral miRNA expression

Author / Reference	Year	No. of Patients	Blood Type Used	miRNA expression in PDAC patients	Significance
Abue et al. ³¹⁹	2015	32 PDAC 12 IPMN 30 HC	Plasma	+miR- 483-3p +miR- 21	- Upregulated in PDAC vs HC - miR-483-3p upregulated in PDAC vs IPMN
Komatsu et al. ³²⁰	2015	71 PDAC 67 HC	Plasma	+miR-223	Upregulated in PDAC vs HC (p<0.0001)
Ganepola et al. ³²¹	2014	8 PDAC 11 HC	Plasma	+miR-642b-3p, +miR-885-5p +miR-22-3p	- All up-regulated in PDAC vs HC - Sensitivity of 91% and specificity of 91% for discriminating PDAC and HC
Carlsen et al. ³¹⁷	2014	48 PDAC 47 HC	Plasma	+miRNA-375	- Upregulated in PDAC vs HC - No correlation with survival
Wang et al. ³²²	2013	129 PDAC, 103 BPD, 60 HC	Peripheral blood mononuclear cells	+miR-27a-3p +miR-16-5p +miR-15b-5p	- All up-regulated in PDAC vs. BPD and PC vs. HC - Multivariate logistic regression model showed miR-27a-3p was able to discriminate PC from BPD and HC. - Combination of miR-27a-3p and serum CA19-9 levels provides higher diagnostic accuracy with 85.3% sensitivity and 81.6% specificity (AUC 0.886; 95% CI, 0.837–0.923%).
		80 PDAC 129 HC		+RNU2-1f	Upregulated PDAC vs HC
Bauer et al. ³¹⁸	2012	45 PDAC 38 CP	Whole Blood	87 miRNAs differentially	With statistical learning techniques, high sensitivity and specificity (AUC values 0.973 and 0.950) for discriminating PDAC or CP from HC.

		33 HC		expressed between PDAC and HC	Unable to discriminate PDAC and CP.
Liu et al. 323	2012	197 PDAC 82 CP 158 HC	Serum	+miR-20a +miR-21 +miR-24 +miR-25 +miR-99a +miR-185 +miR-191	7 miRNA-based biomarker distinguishes PDAC from HC (AUC 0.992); and PDAC from CP (AUC 0.993). - miR-21 levels associated with overall PDAC survival.
Morimura et al. 324	2011	36 PDAC 30 HC	Plasma	+miR-18a	- miR-18a significantly upregulated in PDAC (AUC 0.9369). - miR-18a levels lower in post-operative samples, than in pre-operative samples ($P=0.0077$).
Liu et al. 325	2012	140 PDAC 111 CP 68 HC	Plasma	+miR-16 +miR-21 +miR-55 +miR-181a +miR-181b +miR-196a +miR-210	miR-16, miR-196a and CA19-9 discriminate PDAC from CP and HC (AUC 0.979; sensitivity, 92.0%; specificity, 95.6%); and discriminates PDAC from CP (AUC 0.956; sensitivity, 88.4%; specificity, 96.3%).
LaConti et al. 326	2011	6 PDAC 8 HC 12 Other GI	Plasma	+miR-100a +miR-10	Panel of 9 miRNAs (miR-100a, miR- 10, miR-155, miR-199, miR- 221, miR- 21, miR- 210, miR- 223, miR- 16) distinguishes PDAC from HC and other GI cancers.

		cancers			
Li et al. ³²⁷	2010	45 PDAC 11 CP 32 HC	Serum	+miR-200a +miR-200b	• PDAC distinguished from HC with miR-200a and miR-200b (AUC 0.861 and 0.85 respectively). • No difference in miRNA expression between PDAC and CP.
Wang et al. ³²⁸	2009	49 PDAC 36 HC	Plasma	+miR-21 +miR-210 +miR-155 +miR-196a	• Combination of 4 miRNAs detects PDAC vs. HC with AUC 0.82 (95% CI, 0.70-0.94).
Kong et al. ³²⁹	2010	35 PDAC 15 CP 15 HC	Serum	+miR-21 +miR-155 +miR-196a	• miR-21 can differentiate PDAC from CP ($P<0.003$) and from HC ($P<0.001$). • miR-155 and miR-196a can differentiate PDAC and CP from HC.

Table 4: Studies detecting miRNA in patients with PDAC. PDAC, pancreatic ductal adenocarcinoma; IPMN, intraductal papillary mucinous neoplasm; CP, chronic pancreatitis; HC, healthy control; BPD, benign pancreatic disease; +, up-regulated; -, down-regulated; AUC, area under curve.

3.5 Summary:

PDAC is a lethal disease and new techniques are urgently required to enable earlier diagnosis and the identification of disease recurrence in order to improve survival. A deeper understanding of the molecular alterations in PDAC will allow the development of novel detection and therapeutic strategies³³⁰. The ideal test would be one which is non-invasive and inexpensive, which could be used clinically as diagnostic, predictive and prognostic biomarkers. Research has focused on blood based biomarkers and over the last 5 years, we have seen promising results from measuring CTCs, tumour-specific nucleic acid alterations and circulating miRNAs in PDAC patients. However, there are considerable obstacles to detecting many of these biomarkers, as they are present at extremely low concentrations in the blood and the technology required is expensive and time-consuming. Furthermore, there is no standardised validation for these tests and thus results from these early studies are variable. The numerous genetic alterations found in PDAC adds to the complexity, as we are unsure how to act on the results. Research involving these serum biomarkers needs to be consolidated in order to develop multiplex assays. This will allow the future improved detection of this disease and hopefully lead to more personalised anti-cancer therapy. Most research has investigated the use of these markers as a means of detection of PDAC compared to healthy controls or those with benign pancreatic disease with limited data on PDAC prognosis.

CHAPTER 4:

Results and Discussion

4.1 Surgical technique to reduce recurrence rates

4.1.1 HYPOTHESIS

The literature review conducted identified CTCs as a potential biomarker in PDAC, in particular, a biomarker proven to identify invasive cancers with metastatic potential in other solid tumours. As discussed in the introduction, the no-touch technique may potentially prevent the dissemination of cancer cells (or CTCs) into the circulation. A study was therefore planned in order to test whether the no-touch technique reduces PDAC recurrence, using CTCs as a biomarker.

We hypothesised that the no-touch PD technique will reduce the number of circulating tumour cells released into the circulation and reduce the disease recurrence rate in PDAC patients compared to the standard PD technique.

4.1.2 RESULTS

Based on the availability of the CellSearch system to perform the assay, and our power calculation, 12 patients with a pre-operative suspicion of PDAC were randomized into this study (ST-PD n=6 (Group I); NT-PD n=6 (Group II)). All patients had Stage II disease (potentially resectable) and none had received neo-adjuvant chemotherapy. There were no differences between the 2 groups with regards to age, operative time, length of hospital stay, complication rate, lymph-node ratio or tumor characteristics (**Table 5**). Only 1 patient (in group I) required a peri-operative blood

transfusion (2 units). For pancreatic duct reconstruction, 5 patients in each group had a longitudinal ventral pancreaticojejunostomy anastomosis (LVPA) and 1 in each group had a pancreaticogastrostomy (PG). Major complications included 1 biliary leak in group I, and 1 pancreatic leak (PG anastomosis) in group II. Both cases were treated conservatively. All tumours were confirmed histologically as PDAC and AJCC Stage IIB (pT3, N1, M0). R1 resection was defined as cancer cells within 1 mm of a circumferential or transection margin. A negative resection margin (R0) was achieved for six patients (50%) with three in each group having R1 medial resection margins.

Prior to resection of the pancreatic head, there was no difference in the number of CTCs between the 2 groups (group I, range 0-4 vs. group II, range 1-6; $P=0.306$). Following resection, an increase in the number of CTCs was seen in 5/6 (83%) patients in group I, but 0/6 in group II ($P=0.003$; **Table 6**).

The median follow-up period for the entire cohort was 14.6 months (range 10.5-27.9 months). At the end of follow-up, there were 7 deaths and 5 survivors. Three patients in the ST-PD group (50%) and two patients in the NT-PD group (33%) developed disease recurrence in the liver ($n=2$) and lung ($n=3$). There was no difference in disease-free survival (DFS) between the two groups ($p=0.421$, **Figure 5**). Median overall survival (OS) was 13.0 months (95% CI 10.1-15.9) in group I and 16.7 months (95% CI 12.6-20.8) in group II ($p=0.328$; **Figure 6**).

	Group I, n=6	Group II, n=6	p-value
Median age, years	66.3	65.6	0.92
Median operative time, hours	4	3.3	0.27
Median length of stay, days	17	16.2	0.32
Major complications	1	1	1.0 ^b
Pathological tumour stage	pT3 (100%)	pT3 (100%)	1.0
Mean tumour size, mm	35	37	0.81
Mean lymph node ratio	0.29	0.38	0.41
Positive resection margin	50% (all medial margin)	50% (all medial margin)	1.0
Perineural invasion	100%	100%	1.0
Lymphovascular invasion	100%	100%	1.0

Table 5: Clinicopathological characteristics of patients undergoing pancreaticoduodenectomy for pancreatic cancer.

Group 1 = standard pancreaticoduodenectomy. Group II = no-touch technique pancreaticoduodenectomy.

Patient Group		No. of CTCs Before Resection	No. of CTCs After Resection	Difference	Increase in CTCs after resection?
Group I	Patient 1	0	8	+8	Y
	Patient 2	1	2	+1	Y
	Patient 3	1	3	+2	Y
	Patient 4	1	1	0	N
	Patient 5	2	3	+1	Y
	Patient 6	4	6	+2	Y
Group II	Patient 1	3	2	-1	N
	Patient 2	1	1	0	N
	Patient 3	3	3	0	N
	Patient 4	1	1	0	N
	Patient 5	1	0	-1	N
	Patient 6	6	6	0	N
P Value		0.306 ^a	0.751 ^a	-	0.003^b

Table 6: Number of PV CTCs before and after resection for each patient. CTCs, circulating tumor cells; PV, portal vein; Group 1 = standard pancreaticoduodenectomy. Group II = no-touch technique pancreaticoduodenectomy;

^aWilcoxon Signed Rank test Pearson; ^bChi-Squared test, 2-tailed

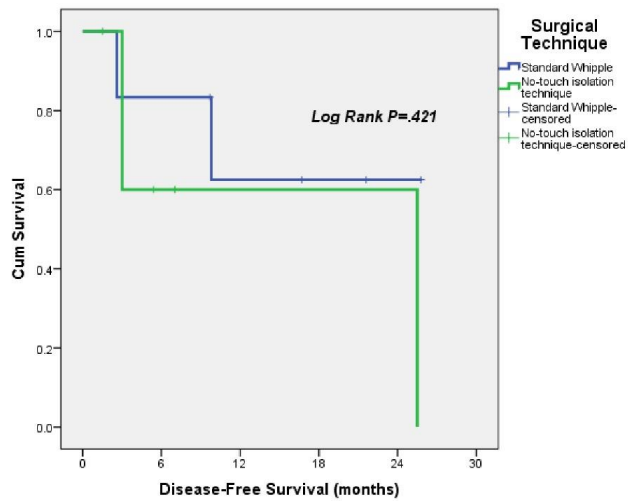


Figure 5: Kaplan-Meier curve of disease-free survival (DFS) comparing the 'no-touch' isolation pancreaticoduodenectomy to standard PD. Comparison between groups was made by log-rank test

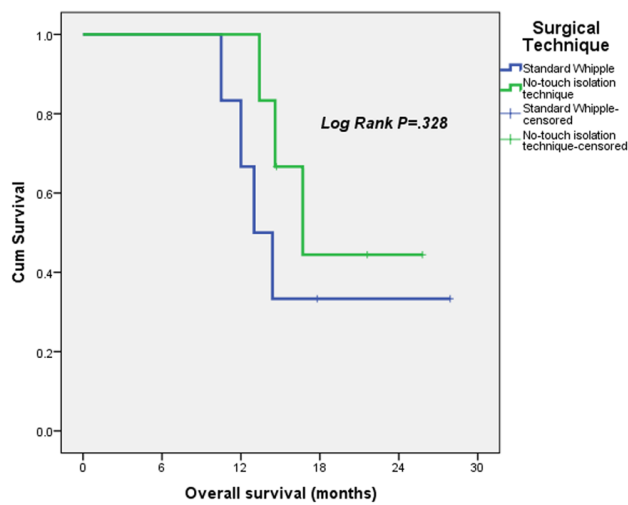


Figure 6: Kaplan-Meier curve of overall survival (OS) comparing the 'no-touch' isolation pancreaticoduodenectomy to standard PD. Comparison between groups was made by log-rank test

4.1.3 DISCUSSION

This is the first study to examine CTC numbers in the portal venous circulation during PD for PDAC. The NT-PD has since been adopted as the standard for pancreatic head resection in our patients with PDAC. There was a significant increase in the number of CTCs following a ST-PD, but not following the NT-PD method. Although the NT-PD group trended towards better DFS and OS, there was no significant difference. The log-rank test performs poorly with small sample sizes and so this may be a Type II error.³³¹ The 'no-touch' (NT) isolation technique was first described as a way of preventing the spread of cancer cells in colorectal and eye cancer by limiting the handling of the tumors.^{184, 332} In colorectal cancer (CRC), a tendency for a reduction in the number and time to liver metastases over 5 years has been seen using the NT isolation technique compared to conventional resection.¹⁸⁵ More recently, the NT isolation technique has been shown to be a safe procedure for PD^{186, 188} and distal pancreatectomy,³³³ with comparable operative morbidity and mortality. Furthermore, in a small pilot study,¹⁸⁷ carcinoembryonic antigen (CEA) messenger RNA (mRNA) was identified in PV blood samples from 50% (5/10) of patients undergoing ST-PD, compared to only 13% (1/8) having NT-PD. The latter group also had reduced disease recurrence and a longer OS. This study provided the first evidence for the biological benefit of using this technique, even though mRNA levels were not isolated from a specific cell type.

The presence of CTCs is an independent prognostic factor in metastatic breast cancer,²⁶⁰⁻²⁶² CRC,^{263, 264} castrate-resistant prostate cancer,³³⁴ and small-cell lung cancer.²⁶⁶ The detection of CTCs is extremely difficult, as they are rare and samples are often contaminated by leukocytes. However, the ability to detect, enumerate, and characterize CTCs is an important tool for the study of the metastatic cascade and

the improved clinical management of cancer patients. To date, the majority of studies have employed the CellSearch system and this is the only system with FDA approval. In recent years, other reproducible methods of CTC detection and analysis have been developed.³³⁵ However, the CellSearch system has been shown to be the most reliable tool in clinical trials using CTCs as a prognostic marker.³³⁶⁻³⁴¹ There are however some limitations to this system: The CellSearch system assumes that EpCAM-positive, panCK-positive and CD45-negative cells are CTCs.³⁴² This is a limitation of this method, and therefore our study, as CTCs which are not positive for EpCAM and panCK will go undetected. During cancer invasion and metastasis, some cancer cells undergo an epithelial-mesenchymal-transition (EMT), resulting in the downregulation of EpCAM and some cytokeratins,³⁴³ therefore these important CTCs will be missed. Certainly, newer methods such as the Epispot assay³⁴⁴ and isolation by size of epithelial tumour cells (ISET) method,³⁴⁵ which are both able to assess EpCAM-negative cells, may detect more CTCs than CellSearch. Furthermore, apoptotic cells produced by the tumor and released into the circulation could be detected as CTCs, but do not have malignant potential.³⁴⁶ Thus, it remains unclear whether the detected CTCs are functionally active and actually capable of initiating new metastases. In PDAC, CTCs that are cell surface marker CD24+ and CD44+, and also epithelial-specific antigen (ESA) positive, have been shown to demonstrate self-renewal, tumorigenicity and differentiation, whereas those negative for these markers do not.³⁴⁷ Subsequent work has also indentified CD133+,³⁴⁸ nestin,³⁴⁹ and c-MET³⁵⁰ as important markers for tumour metastasis. Indeed, CD133+ and CXCR4+ cells have been found to be essential for tumour metastasis.³⁴⁸ Recently, CTCs from a PDAC mouse model have been subjected to single-molecule RNA-sequencing.³⁵¹ This revealed an enrichment of non-canonical WNT signalling in pancreatic CTCs, which was found to increase their metastatic potential (e.g. enhanced anchorage-independent tumour-sphere formation and suppression of anoikis).³⁵¹ Quantifying the

expression of these various markers in the CTCs enumerated was beyond the scope of our study, but would be crucial in any follow-up investigations.

Peripheral CTCs have been detected using a low cut-off (≥ 1 CTC/7.5 mL) in 11% of locally advanced PDAC patients³⁵² and in 40-80.5%^{353, 354} with metastatic disease,^{8, 11, 40} compared to none in healthy individuals and those with chronic pancreatitis. Whilst we did not assess CTCs in the peripheral circulation, it appears that CTCs are easier to detect in the portal venous circulation, even in earlier stage disease, as 92% of our patients had ≥ 1 CTC/7.5 mL in the PV prior to PD. The clinical implications of detecting CTCs in PDAC are under investigation, however reports are divided regarding the association between enumeration and survival outcomes.³⁵⁵ Indeed, Kurihara et al³⁵⁴ found that in Stage III/IV disease, CTC-positive (11/26) patients had worse OS compared to those that were CTC-negative (15/26). Similarly, Bidard et al³⁵² demonstrated that in Stage III patients, CTC-positivity at any point (baseline or after 2 months chemotherapy) was associated with shorter OS (HR 2.5, 95% CI 1.2-5.4), although CTC detection did not influence DFS. Conversely, studies by Khoja et al³⁴⁵ and Negin et al³⁵⁶ failed to correlate CTC number with OS or DFS in metastatic PDAC. Plausible explanations for these findings include the small sample sizes / low statistical power of many of these studies; different detection methods and markers used and heterogeneous patient groups.^{352, 355} However, most studies do show a trend towards an association between CTC-positivity and poor survival, thus their role in metastatic development is still debated. Our power calculation was based on an estimation that 85% versus 20% of patients would have a rise in CTCs after the standard and no-touch technique respectively. It must be noted however that the number of CTCs in the portal or systemic circulation before and after operative intervention has not been previously measured in the literature and thus this estimation must be considered as somewhat arbitrary. However, in our findings

83% versus 0% of patients had a CTC rise post resection which is in line with our estimation. This was a pilot study and can now be planned on a larger scale with more patients.

Our study, albeit in a small number of patients, showed that NT-PD is comparable to ST-PD with regards to peri-operative morbidity and long-term outcome. In addition, we provide further evidence that CTCs can be detected in PDAC patients in the portal venous circulation and that the count is increased following tumour manipulation. This may affect disease recurrence and survival, but larger studies are needed for a definitive answer with regards to outcome.

4.2 Clinicopathological factors which may predict disease recurrence

4.2.1 HYPOTHESIS

In the above study on the no-touch technique, we were unable to significantly prove that surgical technique can reduce the recurrence of PDAC. It was therefore decided to plan a retrospective analysis of PDAC patients, in order to identify what clinicopathological factors may influence PDAC recurrence after curative resection.

We hypothesised that there are no clinicopathological differences between patients who develop early disease recurrence and those that develop late recurrence or no recurrence.

4.2.2 RESULTS

During the period January 2001 to June 2014, 654 patients had pancreatic resections at our hepatobiliary centre. Of these, 178 were for a histopathological diagnosis of PDAC, with 168 undergoing curative resections. The remaining 10 patients had locally advanced disease or metastatic disease evident intra-operatively and had palliative bypasses and/or biopsies. A further 16 patients had an R2 resection and were excluded from the analysis. Of the remaining 152 patients, 68 (44.74%) had evidence of disease recurrence within 12 months of surgery (Group I) and 25 (16.45%) had no evidence of disease recurrence for at least 24 months after surgery (Group II). These 93 patients were included in the analysis. 81 patients had a PD and twelve patients had a DP. Clinicopathological characteristics of the patients and tumours are shown in **table 7**. There was no 30-day mortality. Complications occurred in 30.61% (15 of 49 with available data) with major complications (Clavien-Dindo classification 3b-4)³⁵⁷ occurring in 2 patients (4.08%), one who required a second operation for wound dehiscence and one who required further ITU support following an non-ST segment elevation myocardial infarction (NSTEMI), lower respiratory tract infection (LRTI) and upper gastrointestinal bleed from a gastric ulcer.

In univariate analysis, factors associated with early recurrence compared to no or late recurrence were: resection margin status ($p=0.000$); medial resection margin status ($p=0.000$), posterior resection margin status ($p=0.032$), tumour size ($p=0.002$); T-stage ($p=0.014$); N-stage ($p=0.002$); and the presence of lymphovascular invasion ($p=0.018$). No differences between the two groups were found for: type of operation (PD or DP); age; sex; tumour differentiation; lymph node ratio; presence of perineural

invasion; pre-operative levels of CA 19-9, CRP or bilirubin; morbidity; or the use of adjuvant therapies. (**Table 7**).

Characteristic	All patients (n=93)	Group I (n=68)	Group II (n=25)	p value
Median age years (range)	66.66 (40.71-82.82)	66.18 (40.71-82.82)	67.81 (49.63-75.36)	0.697
Sex M:F (%)	52:41 (56:44)	35:33 (51:49)	17:8 (68:32)	0.168
Median preoperative CA 19-9 Kunits/L, (range) (n=54)	87.00 (1-32,876)	73.00 (1-32,876)	198.00 (1-10,195)	0.347
Preoperative CA 19-9 <37:>37 (%) (n=54)	37:17 (69:31)	28:9 (76:24)	9:8 (53:47)	0.121
Median preoperative bilirubin umol/L (range) (n=89)	18 (2-494)	18 (3-248)	17 (2-494)	0.877
Preoperative bilirubin <22:>22 (%) (n=89)	52:37 (58:42)	39:27 (59:41)	13:10 (57:43)	1.000
Median preoperative CRP mg/L (range) (n=89)	6 (1-141)	6 (1-141)	6 (1-141)	0.854
Preoperative CRP <20:>20 (n=89)	66:23 (74:26)	48:18 (73:27)	18:5 (78:22)	0.783
Type of operation PD:DP (%)	81:12 (87:13)	58:10 (85:15)	23:2 (92:8)	0.503
Morbidity no:yes (%) (n=49)	15:34 (31:69)	11:24 (31:69)	4:10 (29:71)	1.000

Differentiation poor:moderate-to- poor:moderate:well (%)	55:10:27:1 (59: 11: 29:1)	40:9:18:1 (59: 13: 26: 1)	15:1:9:0 (60: 4: 36: 0)	0.495
Resection margin status R0:R1 (%)	45: 48 (48:52)	25:43 (37: 63)	20:5 (80:20)	0.000
Medial resection margin status R0:R1 (%)	57:36 (61:39)	33:35 (49:51)	24:1 (96:4)	0.000
Anterior resection margin status R0:R1 (%)	81:12 (87:13)	59:9 (87:13)	22:3 (88:12)	1.00
Posterior resection margin status R0:R1 (%)	81:12 (87:13)	56:12 (82:18)	25:0 (100:0)	0.032
Pancreatic resection margin status R0:R1 (%)	86:7 (92:8)	65:3 (96:4)	21:4 (84:16)	0.081
Median tumour size cm (range)	2.8 (0.8-6.5)	3.0 (0.8-6.5)	2.2 (0.9-4.5)	0.002
T-stage 1:2:3:4 (%)	3:8:63:19 (3:9:68:20)	3:2:47:16 (4:3:69:24)	0:6:16:3 (0:24:64:12)	0.014
N-stage N0:N1 (%)	22:71 (24:76)	10:58 (15:85)	12:13 (48:52)	0.002
Lymph node ratio <0.2: >0.2 (%) (n=50)	21:29 (42:58)	18:24 (43:57)	3:5 (38:63)	1.000
Perineural invasion absent:present (%)	20:73 (22:78)	13:55 (19:81)	7:18 (28:72)	0.398
Lymphovascular invasion absent:present (%)	21:72 (23:77)	11:57 (16:84)	10:15 (40:60)0.018	0.018
Adjuvant chemotherapy no:yes (%)	14:79 (15:85)	12:56 (18:82)	2:23 (8:92)	0.338
Mortality no:yes (%)	14:79 (15:85)	3:65 (4:96)	11:14 (44:56)	0.000

Table 7: Clinicopathological characteristics of patients in Group I (recurrence within 12 months) and Group II (no recurrence for at least 24 months) and p-value results of univariate analysis. Comparisons were made using Fisher's exact test for categorical data and the Wilcoxon signed rank test for continuous data. All tests were two-sided.

In multivariate analysis (**Table 8**), only medial resection margin status was associated with early recurrence. (p=0.030, HR 0.031, 95%CI 0.001 – 0.717).

Mean follow-up to date of death or to final follow-up for patients still alive was 24.8 months (range 1.5 – 108.1). By definition all the early recurrence group had disease recurrence. Further, 9 patients (36%) of the no recurrence group, developed disease recurrence after 24 months. Three patients (4.41%) were alive from the early recurrence group and 11 patients (44%) were alive from the no recurrence group. Overall survival is shown in **table 9**. Overall median survival was 9.8 months (95% CI 9.0 – 10.6) for Group I which was significantly worse than the 56.1 months (95% CI 34.4 – 77.8) for Group II (p=0.000) (**Table 9** and **Figure 7**). The 1-, 3- and 5-yr survival rates of patients with early recurrence was 36%, 0% and 0% compared to 100%, 74% and 40% for patients without recurrence for at least 24 months. A cox-regression analysis showed this difference in survival was significant independent of a positive medial resection margin. P-value remained 0.000 (HR 0.018, 95% CI 0.004-0.080).

Characteristic	p-value univariate analysis	p-value multivariate analysis	HR	95% CI for HR
Resection margin status	0.000	0.347	3.787	0.237 – 60.615
Medial resection margin status	0.000	0.030	0.031	0.001-0.717

Posterior resection margin status	0.032	0.998	0.000	0.000
Pancreatic resection margin status	0.081	0.100	12.415	0.619-249.415
Tumour size	0.002	0.179	1.661	0.792-3.482
T-stage	0.014	0.666	0.808	0.308-2.124
N-stage	0.002	0.212	2.349	0.614-8.977
Lymphovascular invasion	0.018	0.178	2.732	0.632-11.808

Table 8: Multivariate analysis of clinicopathological factors that were significantly different between each group on univariate analysis. HR = Hazard ratio. CI = Confidence interval.

Group	Median Survival, months	95% CI	p-value
I (n=68)	9.8	9.0 – 10.6	0.000
II (n=25)	56.1	34.4 – 77.8	
Overall (n=93)	13.8	9.6 – 18.0	

Table 9: Survival for patients in Group I (disease recurrence within 12 months) and Group II (no disease recurrence for at least 24 months). Comparison of survival distributions made with the log rank (Mantel-Cox) significance test.

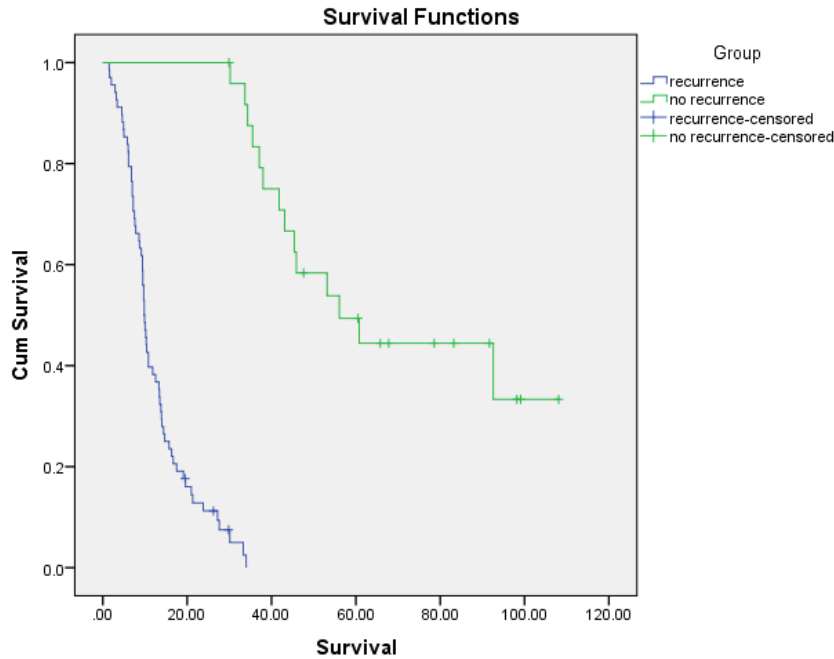


Figure 7: Kaplan Meier survival curve comparing Group I (recurrence, blue) and Group II (no recurrence, green).

4.2.3 DISCUSSION

Over a thirteen year period, our centre performed 168 curative resections for pancreatic ductal adenocarcinoma. Forty-five per cent developed disease recurrence within 12 months and 16 % did not develop disease recurrence for at least two years. It was of interest to compare these two groups to try to identify any clinicopathological factors that may predict early disease recurrence compared to no or late disease recurrence. These factors would be of prognostic value.

We had no 30 day mortality and a morbidity of 31% with major morbidity in 4%. This morbidity is comparable to the US National Surgical Quality Improvement Project data³⁵⁸. Although a number of factors were found to be associated with early recurrence on univariate analysis, only a positive medial resection margin was associated with early disease recurrence on multivariate analysis. Those who developed disease recurrence within 12 months, unsurprisingly, had a significantly

poorer overall survival with a median of 9.8 months compared to 56.1 months in those who had at least two years with no disease recurrence. Therefore, a positive medial resection margin is a prognostic indicator and predicts early disease recurrence and poor overall survival.

The medial resection margin faces the superior mesenteric vein (SMV) and has a shallow groove-like shape and a fairly smooth surface. To the left is a relatively small area of rougher texture that faces the superior mesenteric artery (SMA) and joins by an acute angle the slightly fibrous but smooth posterior surface of the pancreatic head, which overlies the aortocaval groove ³⁵⁹ (**Figure 8**). Several retrospective trials have previously identified resection margin status as an independent prognostic factor in PDAC patients ^{160, 360, 361 362 363 364}. In the European Study Group for Pancreatic Cancer-1 (ESPAC-1) study, which involved 541 PDAC patients and is the largest randomized trial in pancreatic cancer patients, those with an R1 resection status had a median survival of 10.9 months versus 16.9 months for patients with R0 margins ³⁶⁵. Although, resection margin status was only a significant independent prognostic factor when tumour grade and lymph node involvement were both absent from the model. Further, other retrospective studies have failed to identify resection margin status as a prognostic factor³⁶⁶ including an American study of 360 consecutive pancreatic cancer patients ³⁶⁷. However, few authors have clarified which margins were R0 or R1. Whilst we found overall resection margin status to be significant on univariate analysis, this association dissipated on a multivariate regression analysis when taking into account the four separate resection margins. This is in agreement with Zhang et al. who demonstrated that an R0 medial margin was an independent prognostic factor ($P = 0.002$, $HR = 0.381$; 95% CI 0.207-0.701)

A positive resection margin may predict a more biologically aggressive tumour. Despite macroscopic tumour clearance, a microscopic invasion of the tumour to within 1mm of the resection margin implies that some tumour cells have remained at the resection bed in the patient. As the medial resection margin is in close proximity to the SMV and SMA, our results may suggest that these microscopic tumour cells may enter the systemic circulation to enable metastatic development.

The results of this study would suggest that by improving medial resection margin status, we can prolong disease-free and overall survival in PDAC patients undergoing curative resections. By performing portal venous resection and/or SMV resection at the time of pancreaticoduodenectomy, it may be possible to reduce R1 resection rates. Indeed, in a recent study of 359 PDAC patients, the 131 patients who had additional venous resection had significantly more R0 resections ($p=0.02$)³⁶⁸. By pooling results from studies reporting resection margin status after PD,^{156, 198-204} a positive medial resection margin was reported in 38.2% of 701 patients (**Table 10**). A positive medial resection margin varied in these studies from 22.4% - 51.4%. Surgical technique and patients selection likely explains this heterogeneity. However, following PD with PV/SMV resection, the medial resection margin is only positive in 16% of patients²⁰⁵. Others have suggested completely excising the mesopancreas during pancreaticoduodenectomy. The mesopancreas is defined as the soft tissue between the pancreatic head and SMA³⁶⁹. It includes nerves, vessels and lymphatics. A total mesopancreas excision leads to a significantly increased rate of R0 resections³⁷⁰ and is facilitated by using the posterior or artery-first approach³⁷¹.

This study was limited by its retrospective nature. Further, it looks at differences between two distinct groups of patients. We can therefore only conclude that those

with a positive medial resection margin are more likely to develop disease recurrence within 12 months than to have a disease-free survival of at least two years.

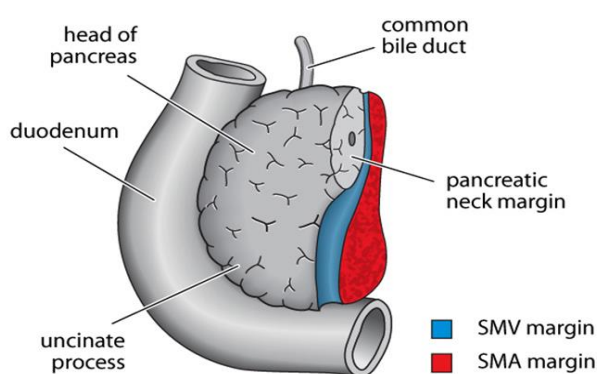


Figure 8: A head of pancreas resection with the superior mesenteric vein (SMV) and superior mesenteric artery (SMA) margins shown. These margins contribute to the medial resection margin. From The Royal College of Pathologists of Australasia (<http://www.rcpa.edu.au>).

Author, year	Total no. of positive (R1/R2) resections	Positive pancreatic transection margin	Positive anterior transection margin	Positive posterior transection margin	Positive medial transection margin
John, 2013 ¹⁹⁸	52/70 (74.3%)	2/70 (2.9%)	21/70 (30%)	32/70 (45.7%)	33/70 (47.1%)
Zhang, 2012 ¹⁵⁶	48/84 (57.1%)	3/84 (3.6%)	26/84 (31.0%)	36/84 (42.9%)	25/84 (29.8%)
Jamieson, 2010 ¹⁹⁹	109/148 (73.6%)	15/148 (10.1%)	40/148 (27.0%)	48/148 (32.4%)	50/148 (33.8%)
Menon, 2009 ²⁰⁰	22/27 (81.5%)	3/27 (11.1%)	3/27 (11.1%)	13/27 (48.1%)	12/27 (44.4%)
Campbell, 2009 ²⁰¹	128/168 (76.2%)	38/168 (22.6%)	6/168 (3.6%)	69/168 (41.1%)	64/168 (38.1%)
Fusai, 2008 ²⁰²	40/67 (59.7%)	4/67 (6.0%)	7/67 (10.4%)	18/67 (26.9%)	15/67 (22.4%)

Esposito, 2008 ²⁰³	84/111 (75.7%)	3/111 (2.7%)	8/111 (7.2%)	39/111 (35.1%)	57/111 (51.4%)
Verbeke, 2006 ²⁰⁴	22/26 (84.6%)	2/26 (7.7%)	4/26 (15.4%)	14/26 (53.8%)	12/26 (46.2%)
TOTAL	505/701 (72.0%)	70/701 (10.0%)	115/701 (16.4%)	269/701 (38.4%)	268/701 (38.2%)

Table 10: Studies reporting R1 status for each resection margin after pancreaticoduodenectomy and pooled results.

4.3 Are disease recurrence rates affected by genetic and epigenetic changes and can these be detected in the peripheral circulation?

4.3.1 HYPOTHESES

When analysing clinicopathological factors, which affect disease recurrence in PDAC, we only identified a positive medial resection margin as being an independent predictor of recurrence. We therefore thought it was of interest to investigate if other tumour factors, such as genetic mutational differences could also be responsible for PDAC recurrence. Further, with the results of our literature review suggesting that biomarkers may also influence survival, we planned an investigation to identify these biomarkers as potential indicators of recurrence.

We hypothesised that:

1) DNA mutations can be detected in pancreatic tumour tissue and differences can be seen between patients with short disease-free survival and those with long disease free survival.

2) These DNA mutations can also be detected in the peripheral circulation

3) Other circulating biomarkers including circulating tumour cells and circulating miRNA can be used as prognostic markers

4.3.2 RESULTS OF ION TORRENT SEQUENCING ON TUMOUR TISSUE

Ion Torrent sequencing:

As discussed above, 152 patients underwent curative resections for PDAC from January 2001 to June 2014. 68 (44.74%) had evidence of disease recurrence within 12 months of surgery (Group I) and 25 (16.45%) had no evidence of disease recurrence for at least 24 months after surgery (Group II). The FFPE blocks identifying the tumour and the corresponding H&E slides were retrieved for 34 patients (21 in Group I and 13 in Group II). Following microdissection of the cut FFPE blocks, a mean of 18.3 +/- 14.7 ng/µl of DNA was extracted from each tumour sample.

Technical performance of the Ion Ampliseq Cancer Hotspot Panel v2: DNA sequencing was carried out using the ion torrent next generation sequencer (Life Technologies) as detailed in Chapter 2. All samples were processed once with an 81% success rate. Samples with poor sequence reads were processed a second time. Successful results were generated in 18 patients from Group I and 11 patients from Group II. Mutations in 34 loci of 12 oncogenes and tumour suppressor genes were identified as well as 382 single nucleotide polymorphisms (SNPs). The mean read length was 103bp with a mean of 197,452 reads per sample.

Summary of gene mutations: All samples identified at least 1 mutated oncogene or tumour suppressor gene. 11 samples had one mutation, 7 had two mutations, 5 had

three mutations, 2 had 4 mutations and one sample had 5 identified mutations.

Table 11 summarises the mutations found in each sample.

KRAS mutations: 17/18 (94%) of group I samples contained a KRAS point mutation. Of these: 15/17 (88%) were a missense substitution at location 25398284 on chromosome 12 substituting guanine for adenine in 12 samples (corresponding to a COSMIC mutation ID of COSM521) and guanine for thymine in 5 samples (corresponding to a COSMIC mutation ID of COSM520); one mutation was at location 25398285 substituting guanine for cytosine (corresponding to a COSMIC mutation ID of COSM518); and one mutation was at location 25380275 substituting adenine for thymine. The mean frequency of the mutation found in each sample was 16.93% (SD 11.04). This compares to 11/11 (100%) of group II samples, which contained a KRAS point mutation ($p=0.13$). These were on chromosome 12, with a mutation ID of COSM521 in 5 samples ($p=0.4513$), COSM520 in 3 samples ($p=0.6784$) and COSM518 in 3 samples ($p=0.535$). The mean frequency of the mutation found in each sample was 7.55% (SD 5.76) $p = 0.0078$. See **Table 12** and **Figure 9**.

TP53 mutations: In Group I, 8/18 (44%) had TP53 mutations in 7 different locations of chromosome 17, all missense substitutions. In Group II, 3/11 (27%) had TP53 mutations ($p=0.86$) in 3 different locations on chromosome 17. The mean frequency of the mutation found in each sample was 14.3% (SD 9.81) in Group I and 9.12% (SD 10.94) in Group II ($p=0.23$). **Table 12** and **Figure 9**.

CDKN2A mutations: In group I, 2/18 (11%) had CDKN2A mutations compared to 0/11 (0%) in Group II ($p=0.25$). Both mutations were missense substitutions of thymine for cytosine on chromosome 12. **Table 12**.

SMAD4 mutations: In group I, 2/18 (11%) had SMAD4 mutations compared to 0/11 (0%) in Group II ($p=0.25$). Both mutations were missense substitutions on chromosome 18. **Table 12.**

Multiple mutations: Two or more mutations were seen in 11/18 (61%) of samples from Group I with 3/18 (17%) having three or more. In Group II, 5/11 (48%) ($p=0.41$) and 1/11 (9%) ($p=0.57$) had two or more and three or more mutations respectively. Interestingly, 11/18 (61%) patients in group I, compared to only 3/11 (27%) patients in group II ($p=0.12$) had a *KRAS* mutation and a mutation in either *p53*, *CDKN2A* or *SMAD4*. **Table 12.**

These results show that patients with a higher frequency of *KRAS* mutation in the tumour sample, i.e., more of the tumour DNA contains the mutated *KRAS* gene, are significantly more likely to develop disease recurrence within 12 months than have at least 2 years without disease recurrence. On regression analysis, taking into consideration a positive medial resection margin, also found to significantly differ between the two groups, both factors retain their significance (high frequency of *KRAS* mutation: $p=0.002$ and positive medial resection margin: $p=0.022$).

Group	Sample	Mutated Gene	Chromosome	Position	Frequency of mutation (%)
1	1	KRAS TP53	12 17	25398284 7578275	8.8 9.7
1	2	KRAS CDH1 TP53	12 16 17	25398284 68846147 7578263	7.1 50.4 11
1	3	KRAS	12	25398284	6.9
	4	JAK2 CDKN2A KRAS STK11	9 9 12 19	5073770 21971028 25398284 1223125	7.9 37 12 53.9
1	5	CDKN2A KRAS TP53	9 12 17	21971186 25398284 7578535	10.1 5.6 8
1	6	KRAS TP53	12 17	25398284 7577548	16 9.8
1	7	KRAS TP53 SMAD4	12 17 18	25398284 7573996 48591896	10.5 5.2 4.8
1	8	KRAS TP53	12 17	25398284 7578555	16.1 13.6
1	9	KRAS TP53	12 17	25398284 7578263	43.2 35.3
1	10	ERBB2	17	37880994	13.1

1	11	KRAS	12	25398284	7.3
1	12	PIK3CA CDKN2A CDKN2A KRAS SMAD4	3 9 9 12 18	178936093 21971120 21971142 25380275 48591904	30.2 40 34.6 37.6 61.9
1	13	PTEN PTEN KRAS	10 10 12	89692883 89720862 25398284	13.5 12.5 23.1
1	14	KRAS	12	25398284	17.5
1	15	KRAS	12	25398284	20.5
1	16	KRAS	12	25398284	7.1
1	17	KRAS	12	25398285	27.3
1	18	PTEN KRAS TP53 SMAD4	10 12 17 18	89692905 25398284 7577542 48591918	14.8 21.1 21.9 20.3
2	1	TP53 KRAS	17 12	7577121 25398285	2.6 1.4
2	2	KRAS	12	25398284	13.2
2	3	KRAS TP53	12 17	25398284 7578208	13.3 21.8
2	4	KRAS GNAS	12 20	25398285 57484420	5.5 4.7
2	5	APC	5	112175240	3.6

		KRAS	12		3.1
		TP53	17	25398284	13.9
				7577558	
2	6	KRAS	12	25398284	13.9
2	7	KRAS	12	25398284	17.8
2	8	KRAS	12	25398284	3.3
2	9	KRAS	12	25398284	3.4
2	10	KRAS	12	25398284	4.3
		STK11	19	1223125	37.5
2	11	KRAS	12	25398284	3.3

Table 11: Mutations found in each sample.

	KRAS mutation (%)	Mean frequency of KRAS mutation, %	TP53 mutation (%)	Mean frequency of TP53 mutation, %	CDKN2A mutation (%)	SMAD4 mutation (%)	Two or more mutations	Three or more mutations
Group I (n=18)	17 (94)	16.93	8 (44)	14.30	2 (11)	2 (11)	11 (61)	3 (17)
Group II (n=11)	10 (91)	7.55	3 (27)	9.12	0 (0)	0 (0)	5 (48)	1 (9)
p- value	0.13	0.0078	0.86	0.23	0.25	0.25	0.41	0.57

Table 12: The number of samples with KRAS, TP53, CDKN2A, SMAD4 and multiple mutations in each group. The mean frequency of KRAS and TP53 mutations per sample is also shown for each group. P-values calculated using a one-tailed student's t-test for continuous data and chi squared test for categorical data.

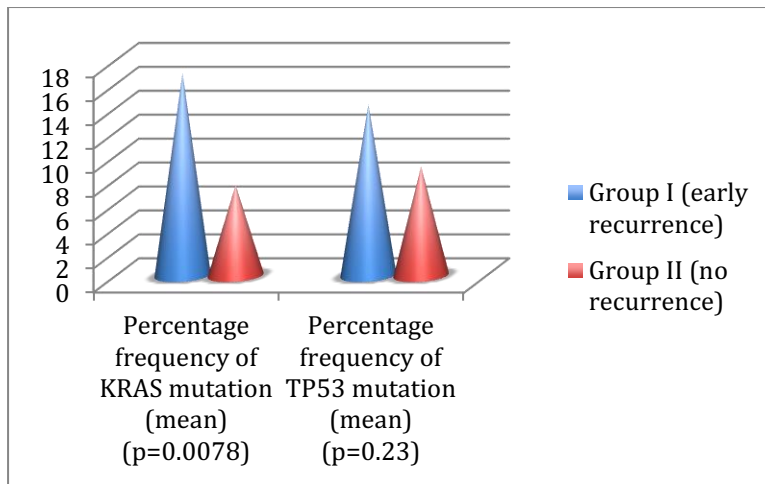


Figure 9: Graph showing the mean percentage frequency of KRAS and TP53 mutations in each group.

4.3.3 DISCUSSION OF ION TORRENT SEQUENCING ON TUMOUR TISSUE

4.3.3.1 Our Results:

We performed high-throughput DNA sequencing using the Ion Torrent multigene next generation sequencer on FFPE tumour samples from 18 patients with a short disease-free survival and 11 patients with a long disease-free survival. Our goal was to identify genetic differences between these two groups in order to identify a prognostic indicator. We were able to perform the analysis with sufficient full-length reads and a mean read length of 103bp using 16 samples per chip with a starting DNA volume range of 1.65 to 76.5 ng/μl. The success rate of this technique was 81%. We identified that tumour samples from those with a short disease-free survival had a higher frequency of *KRAS* mutation than tumour samples from those with long disease-free survivals.

4.3.3.2 Pancreatic cancer sequencing:

Cancer is the result of an accumulation of genetic damage in key genes and signalling pathways resulting in an uncontrolled growth of mutated cells. Each

mutation, whether a single point mutation, or a structural rearrangement of chromosomes can lead to a change in protein formation and affect a signalling pathway. Combinations of mutations and signalling pathway disruptions may cause a functional change with suppression of tumour suppressor genes and/or activation of oncogenes and subsequent growth of the carcinoma. The first whole exome sequencing of pancreatic cancer was published by Jones et al. in 2008⁹³. The authors designed primers for 219,229 amplicons covering the sequences of protein-coding exons from 20,735 genes and analysed 24 advanced PDAC tumour samples. An average of 48 somatic mutations were identified in each sample. Further analyses found 69 gene sets that were genetically altered in the majority of the tumour samples. 31 of which involved 12 core signalling pathways and processes that were each altered in 67 to 100% of each tumour examined. These pathways and the cancer-altering genes mutated to affect these pathways and processes are summarised in **Figure 10**. Biankin et al.⁹⁵ later performed further exome sequencing of PDAC, analysing 142 early cases of sporadic PDAC tumour samples from patients suitable for curative operations. They supported the high prevalence of genetic mutations known to be important in PDAC, observing mutations in 38 of the 79 genes reported as mutated in more than one sample in the study by Jones et al. They defined novel mutations in a total of 1,456 genes, all-be-it commonly in low frequencies, confirming the complexity of PDAC tumour biology. Further, they added to the previous knowledge of signalling pathways and processes affected in PDAC by identifying gene sets also involved in chromatin modification (*EPC1* and *ARID2*) and axon guidance (*SLIT2*, *ROBO2*, *ROBO1*), implicated in cancer cell growth, survival, invasion and angiogenesis³⁷². The role of DNA damage repair in the pathogenesis of PDAC was highlighted as mutations in *ATM* were identified in 8% of the samples. By evaluating the consequences of these mutated genes and altered

pathways, four genes were identified as being of functional significance in PDAC including: *KRAS*; *TP53*; *CDKN2A* and *SMAD4*.

These two landmark studies were paramount in identifying the genetic mutations, driver genes and targeted signalling pathways that are involved in PDAC. Although the genes mutated with high prevalence and shown to be functionally important (*KRAS*, *TP53*, *CDKN2A* and *SMAD4*) were confirmed in both studies, there were multiple varying mutations found in each. Further, each tumour sample analysed had different mutations in differing frequencies. It is likely that multiple gene mutations can affect the same pathway and multiple aberrant pathways can lead to the initiation and progression of PDAC. One clear difference between the two studies was the use of advanced PDAC samples in the first and early PDAC samples in the second. This may therefore suggest that each tumour can develop new mutations during its development and growth. Certainly, during the progression of normal pancreatic tissue to PanIN-1a, PanIN-1b, PanIN-2, PanIN-3 and finally PDAC, varying genetic alterations are seen^{144, 373 72, 374 375 75 77}. Early PanIN-1 lesions show point mutations in *KRAS*, *GNAS* and telomere shortening³⁷⁶. Later lesions, PanIN-2 and PanIN-3, exhibit mutations in *CDKN2A* with *TP53* and *SMAD4* inactivating mutations occurring in PanIN-3 lesions. Although PanIN2 lesions often contain as many mutations as PanIN3 and invasive tumor samples³⁷⁴, suggesting that the effects of the mutations are also time dependent.

Survival analyses were not conducted in the reports of genetic sequencing of PDAC and thus we were interested to perform a genetic analysis of our patients with short and long disease-free survivals to establish whether any differences exist between the two groups.

choice due to its reliability and an automated machine was developed as the first generation of DNA sequencers. Over time, improved efficiency led to lower costs and mass production of the Sanger method, which was used to complete the Human Genome Project in April 2003, sequencing 90% of the human genome, with less than 1 bp error per 100,000 bp of the finished sequence³⁸³. However, the first generation techniques have high costs and require a significant amount of time and large DNA quantities to assess multiple molecular alterations. Thus in the last decade there has been the development of next-generation sequencing. These are technologies that use multiplex assays to enable the simultaneous analysis of millions of sequences, resulting in lower costs in a shorter time period. Various next-generation sequencers have been developed with the most accurate results obtained from the Ion Semiconductor technique, pyrosequencing, sequencing by synthesis, sequencing by ligation and chain termination (the Sanger technique)³⁸⁴. The Ion Semiconductor sequencer (Ion Torrent sequencing, Life Technologies) was released by Ion Torrent at the end of 2010. It is based on the detection of hydrogen ions that are released during the polymerization of DNA. The template DNA strand to be sequenced is exposed to each nucleotide in turn. If the template nucleotide is incorporated into the DNA molecules by the polymerase, a hydrogen ion is released. The change in pH is detected by a hypersensitive ion sensor. The template DNA base pairs are sequentially flooded with each synthetic nucleotide. If it is not the correct nucleotide then no voltage will be detected and if two nucleotides are added a double voltage is detected. It is the first commercial sequencing machine that does not require fluorescence and camera scanning leading to higher speeds, low costs and a smaller instrument size³⁸⁴. Further, only 10ng of DNA is required for accurate analysis. The technique has been validated for many solid tumour types with a similar level of accuracy to polymerase chain reaction (PCR) and Sanger sequencing^{252, 385, 386}. At our centre, the Ion Torrent semiconductor is established as a next-generation

sequencer for DNA from solid tumours and is already used clinically to target oncological treatment of small-cell lung cancer, colorectal cancer and lymphoma. We therefore decided to use this method of gene sequencing for this study.

4.3.3.4 *KRAS* mutations and effects:

The *KRAS* oncogene encodes a small GTPase protein (RAS) that cycles between GTP-bound active and GDP-bound inactive states. GTPase-activating proteins induce hydrolysis of GTP to the inactive form. The mutations we have identified stop the interaction between the GTPase-activating proteins and *KRAS* leading to its persistent activation. Activation of *KRAS* stimulates a number of downstream signalling pathways and these in turn have a role in cell proliferation, altered metabolism, apoptosis suppression, evasion of the immune response and metastasis⁹⁷. The effector pathways of the activated RAS protein are known to include the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, the RAF/MEK/ERK pathway and nuclear factor κ B (NF- κ B) pathway.

(PI3K)/AKT pathway (**Figure 11**): The PI3Ks are a kinase family which phosphorylate the 3-hydroxyl group of phosphoinositides. Tyrosine kinases, including: epidermal growth factor receptors (EGFRs); platelet derived growth factor receptors (PDGF-Rs); and insulin-like growth factor1 receptors (IGF-1), initiate the activation of PI3K. One way of initialising this activation is by phosphorylating RAS to its active form. When activated, these kinases convert their substrates phosphatidylinositol 4,5-bisphosphate (PIP₂) into PIP₃. PTEN is able to dephosphorylate PIP₃ back to PIP₂. PIP₃ in turn enables pyruvate dehydrogenase lipoamide kinase isozyme 1 (PDK1) to phosphorylate protein kinase B (Akt), which becomes activated. The mammalian target of rapamycin (mTOR) kinase protein also phosphorylates Akt. This combined activation of Akt activates and inhibits a number

of downstream proteins with a resultant effect of: increased protein synthesis and growth; increased cell survival; and increased proliferation³⁸⁷.

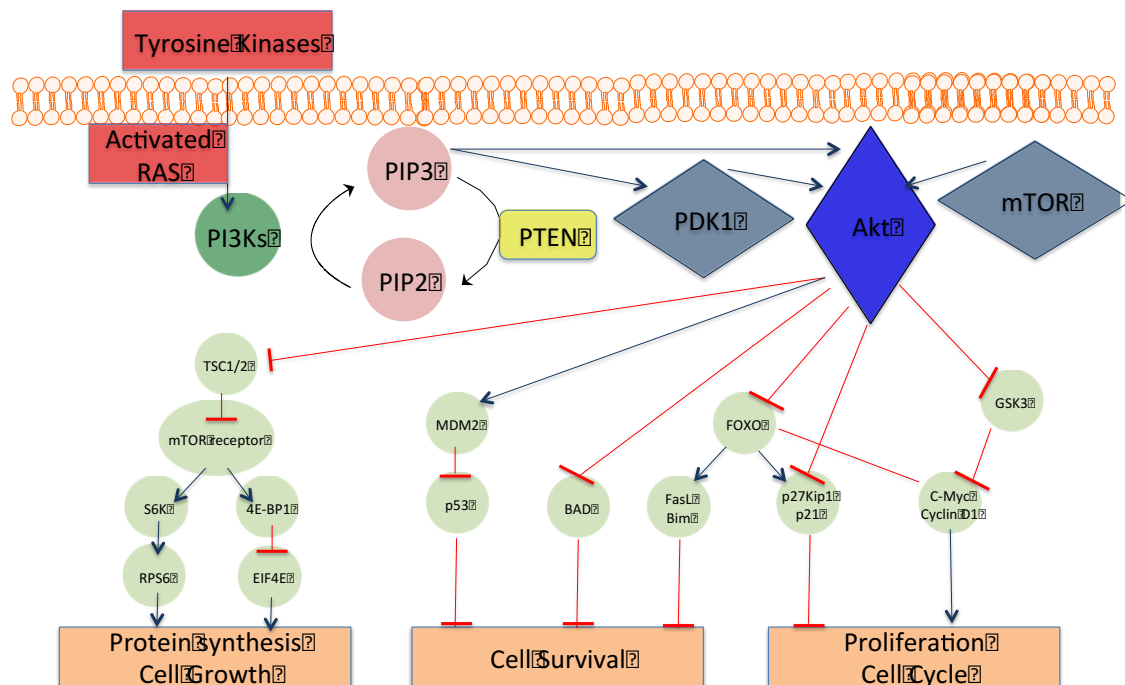


Figure 11: The PI3K/Akt/mTOR signaling pathway and associated inhibitors. Tyrosine kinases activate PI3K via activation of RAS. The active kinase generates PIP3 at the lipid membrane. PIP3 facilitates the phosphorylation of Akt by PDK1, while the mTOR-ricor complex contributes a second phosphate residue to Akt. As the central effector of the PI3K pathway, Akt transmits signal to a host of downstream substrates, thus influencing a variety of key cellular functions. Adapted from ref ³⁸⁷.

RAF/MEK/ERK pathway (**Figure 12**): Raf is a serine/threonine kinase and is activated by the active form of RAS which binds to Raf-1 leading to its recruitment to the cell membrane. This enables Raf-1 to be phosphorylated and activated by other kinases. Raf-1 then binds ATP and phosphorylates mitogen-activated protein kinase (MEK) which in turn phosphorylates extracellular-signal-regulated kinase (ERK). ERK has a number of downstream effects leading to the activation of transcription factor targets including NF- κ B, CREB, Ets-1, AP-1 and c-Myc. These induce the

expression of genes important for cell cycle progression and prevention of apoptosis³⁸⁸.

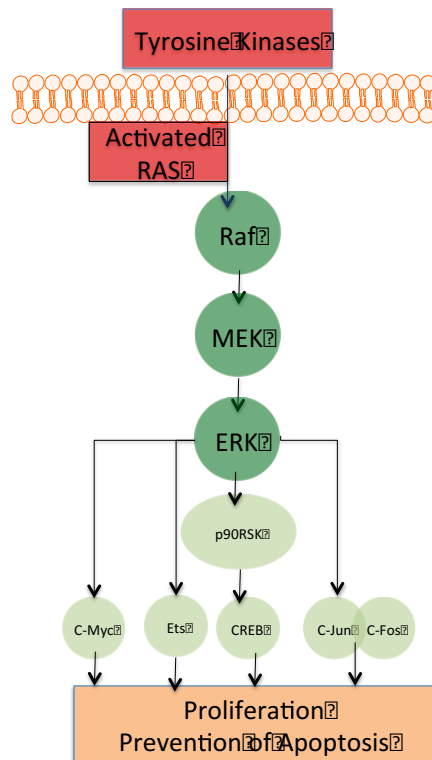


Figure 12: The RAF/MEK/ERK pathway. Activated RAS and other kinases phosphorylate Raf which in turn activates MEK and ERK. This results in the activation of transcription factors which cause the expression of genes resulting in cell proliferation and the prevention of apoptosis. Adapted from ref ³⁸⁸.

NF-κB pathway: NF-κB is a transcription factor controls over 150 genes and several biological responses including immune signalling, inflammation, proliferation, apoptosis and development. The five members of this family are usually inactive in the cytoplasm by the binding of their inhibitor proteins (IκBs), which prevent NF-κB from entering the nucleus. Activation of the pathway requires phosphorylation of IκB by an IκB kinase (IKK), which marks it for ubiquitination and proteasomal degradation, thus releasing NF-κB, which translocates to the nucleus ³⁸⁹. It is widely believed that activated RAS can contribute to the activation of this complex pathway.

Considering *KRAS* is the most frequently mutated gene in PDAC and is also one of the earliest genetic changes found in low-grade PanINs, it is thought to be an early driver mutation for the development of PDAC. Mice models with endogenous overexpression of mutant *KRAS* on chromosome 12 have been created. These *KRAS*^{G12D} mice developed early and late stage PanINs. As the mice aged, high grade PanINs were observed with increased frequency. This substantiates *KRAS* mutation as an important initialising event in pancreatic cancer. Indeed, 7% of these mice also developed metastatic PDAC at 6 and 8 months ¹⁰³. The low frequency of progression of PanIN lesions to PDAC suggests that further genetic mutations are required for disease progression. When the *KRAS*^{G12D} mice models are created with inactivating mutations for tumour suppressor genes, including p53, CDKN2A or SMAD4, they develop accelerated PanINs and PDAC ^{112 117 121}. The inactivation of different tumour suppressor genes is found to completely alter the type of precursor lesion that develops and the state of the malignant disease ³⁹⁰. Collins et al. generated mouse models for inducible *KRAS*^{G12D} allowing for inducible and reversible expression of this mutated gene. During early tumorigenesis, precancerous lesions developed with the induction of the mutant *KRAS*. Interestingly, the inactivation of *KRAS*^{G12D} led to regression of the lesions to barely detectable or undetectable on MRI scanning in just 3 weeks ³⁹¹. With re-expression of *KRAS* they saw rapid recurrence of the primary tumour mass and metastatic development. This is in agreement with studies on human pancreatic cell lines that require oncogenic *KRAS* for sustained proliferation and survival. So, continuous oncogenic *KRAS* signalling is essential for tumour progression and maintenance as well as initiation ³⁹².

We detected *KRAS* mutations in 94% of the tumour samples from Group I (short disease-free survival) and 100% of tumour samples from Group II (long disease-free

survival). This is in agreement with other studies and repeatedly reported as the most commonly mutated gene in PDAC. Indeed, the COSMIC database (<http://www.sanger.ac.uk/genetics/CGP/cosmic/>) reports that 82% of the total number of PDAC samples in the published literature have a *KRAS* mutation. This includes various studies and method heterogeneity may explain our higher percentage. In Group I (early recurrence) 71% of samples corresponded to a COSM521 mutation, 29% corresponded to a COSM520 mutation and 6% had a COSM518 mutation. Similarly in Group II (late recurrence), 45% had a COSM521 mutation, 27% had a COSM520 mutation and 27% had a COSM518 mutation. These 3 COSMIC mutations are also those most frequently reported in the literature with the COSMIC database stating the COSM521 mutation in 42% of *KRAS* mutated PDAC samples, the COSM520 mutation in 40% of samples and the COSM518 mutation in 15% of samples. Notably, the long disease-free survivors had fewer patients with a COSM521 mutation and more patients with a COSM518 although this did not reach significance. We also identified that samples containing a higher frequency of *KRAS* mutation were observed in the short disease-free survival group compared to the long disease-free survival group.

KRAS mutational status has been associated with shorter disease-free and overall survival in patients following pancreatic resection in some studies.^{393, 394} However, in agreement with others³⁹⁵, we did not find that *KRAS* mutations were more common in those with a short, compared to a long, disease-free survival likely due to the high percentage of patients with *KRAS* mutations in all PDAC samples and the reported necessity for *KRAS* in tumour development. A higher frequency of oncogenic *KRAS* was seen in the short disease-free survival group which suggests that more aggressive tumours have more DNA with the oncogenic *KRAS*, which we know is

required for the maintenance of PDAC and for the development of metastatic disease³⁹¹.

Although mouse models have not compared specific mutational statuses with tumour progression, there is some published literature suggesting that the type of *KRAS* mutation may play a role in disease recurrence and progression. An analysis of 27 PDAC tumour samples suggested that those with a COSM521 mutation had worse overall survivals than those with other *KRAS* mutations³⁹⁶. Although this result did not reach significance it is a similar finding to our results and hints towards different *KRAS* mutations having different effects on tumourigenesis and the signalling pathways affected. Indeed, others have reported that overall survival is reduced in those with a COSM521 mutation (HR=2.42; 95%CI 1.14–2.67; $P=0.03$)³⁹⁷.

4.3.3.5 *TP53* mutations and effects:

Tumour protein 53 (*p53*) is a nuclear phosphoprotein and functions as a transcription factor. It is widely regarded as a tumour suppressor gene. It activates the transcription of several genes that carry out the *p53* dependent functions in a cell. *P53* plays a central role in cell cycle regulation, regulation of apoptosis, and DNA repair³⁹⁸. When an upstream event causes DNA damage from a stressor such as: ultraviolet light; irradiation; chemical agents; oxidative stress; osmotic shock; ribonucleotide depletion; or dysregulated oncogene expression, there is a rapid increase in the level of *p53* in the cell by prolonging the *p53* half life, and an increase in its activation as a transcription factor. The increase is proportional to the extent of DNA damage. The N-terminal domain of *p53* contains a large number of phosphorylation sites so it is believed that these are targets for protein kinases transducing stress signals. One of the downstream targets of *p53* is transcription of *p21*. *P21* binds to cyclin complexes thus inhibiting kinase activity. Cyclin complexes

have an important role in the p16–cyclin D₁–cdk4–Rb pathway, which is central to the regulation of the G1-to-S phase transition during cell growth. Thus *p53* has a role in G1 arrest via the transcription of *p21*³⁹⁸. *P53* is also able to initiate apoptosis by gene transcription and by direct protein signalling. Interestingly, *p53*^{-/-} mice are unable to produce the normally observed apoptosis of thymocytes after exposure to DNA damaging stimuli³⁹⁹.

In cancer, mutations in *p53* result in a loss of normal function allowing the cells to escape DNA damage control checkpoints and programmed G1 arrest or cell death, thus contributing to genomic instability¹¹⁴. In pancreatic cancer, the mutated gene is seen in late PanINs and therefore, although not an initializing event, likely contributes to the development and progression of PDAC. Indeed, loss of *p53* or mutated *p53* permits the growth of KRAS-mutated cells in mice, promoting the progression of premalignant lesions to PDAC and driving metastatic development⁴⁰⁰.

We identified *p53* mutations in 44% of tumour samples from the short disease-free survivors and 27% of those from the long disease-free survivors. This is in concordance with the literature where *p53* is repeatedly shown to be the second most commonly mutated gene in PDAC. Out of our eleven patients with *p53* mutations, 10 of these were at different locations on chromosome 17. Again, this is in agreement with the COSMIC database where 202 different *p53* mutations have been identified in 395 samples. Although the difference in mutational status between our two groups did not reach significance in our small sample set, it indicates a trend that patients with *p53* mutations have worse survival. As discussed above, they may be unable to arrest cell growth in this micrometastatic disease, resulting in earlier recurrence of detectable lesions. Certainly, *P53* has been shown to be one of the top three upstream regulators of the proteins associated with short-term survival in

PDAC⁴⁰¹. Further, mutant *p53* increases chemoresistance to gemcitabine⁴⁰² which would therefore shorten survival.

4.3.3.6 *CDKN2A* mutations and effects:

Cyclin-dependent kinase Inhibitor 2A (*CDKN2A*) is a tumour suppressor gene which codes for several proteins including p16, INK4A and ARF. P16 is a key regulator of the G1-S transition of the cell cycle by inhibiting cyclin dependent kinases such as CDK4 and CDK6. The kinases phosphorylate retinoblastoma protein (pRB) which results in progression from G1 phase to S phase¹¹⁴. Thus its mutation and loss of function inhibits cell growth. *CDKN2A* mutations usually occur in the later stages of the pancreatic tumour progression model⁴⁰³. Mice with a knock-in KRAS mutation and a p16 knock-out show rapid progression of PanIN lesions to PDAC^{112, 115}. Our results identified a *CDKN2A* mutation in 11% of those with short disease-free survivals and in 0% of those with long disease-free survivals although this was not a significant finding. The COSMIC database reports that 23% of all PDAC samples reported in the literature have a *CDKN2A* mutation. Our results are in agreement with previous studies finding a poorer survival in those with a *CDKN2A* mutation but without reaching significance³⁹⁷.

4.3.3.7 *SMAD4* mutations and effects:

Mothers against decapentaplegic homolog 4 (*SMAD4*) is a tumour suppressor protein involved in cell signaling. It is essential in the SMAD pathway, altering the transforming growth factor TGF- β pathway. In the TGF- β pathway, TGF- β dimers bind to and activate transmembrane type II receptors which phosphorylate and activate a type I cell surface receptor. This leads to the activation of SMADS which bind to SMAD4. The SMAD complex can then translocate to the nucleus where it interacts with transcriptional activators to regulate gene expression. Many TGF- β

ligands use this pathway and thus SMAD4 is involved in biological functions including, of note, apoptosis and the cell cycle. SMAD4 deficiency inhibits the TGF- β -induced cell cycle arrest and migration, contributing to tumour formation ⁴⁰⁴. In our study, 11% of those with short disease-free survivals and 0% of those with long disease-free survivals had a *SMAD4* mutation, suggesting a trend for better survival in those without this mutation. The COSMIC database reports *SMAD4* mutations in 21% of PDAC samples. Previous work is in agreement with our results finding that a loss or mutation of *SMAD4*, in tumours from those who had died of PDAC, correlated with distant metastases ²¹⁹. Further, analysis of *SMAD4* on 174 tumour specimens found *SMAD4* status to be significantly correlated with portal vein invasion, lymph vessel invasion, and perineural invasion and was an independent prognostic factor ⁴⁰⁵.

4.3.3.8 Multiple mutations:

Considering each driver mutation in PDAC acts through differing pathways, we would expect multiple mutations to worsen tumourigenesis and link to survival. Particularly when the mutated *KRAS*, acting as an oncogene, is in combination with a tumour suppressor gene. Certainly, when mouse models with mutated *KRAS* are combined with loss-of-function alleles of *p53* and/or *CDKN2A*, an acceleration of PDAC growth, more genetic instability and a tendency for poorly differentiated tumours is seen ³⁹⁰.

Although we found no significant differences between our two groups in terms of number of diver mutations identified, there was a trend towards more mutations in the short disease-free survival group. Indeed, 61% of short disease-free survival patients compared to only 27% of long disease-free survival patients had a *KRAS* mutation and a mutation in either *p53*, *CDKN2A* or *SMAD4*. This is in agreement with others who have found poor survivals in patients with concomitant *KRAS* and

CDKN2A mutations³⁹⁷, and the number of driver gene alterations has been found to be an independent prognostic factor for overall survival²¹⁹.

4.3.3.9 *Limitations:*

This study is greatly limited by its size. We had a total of 18 patients in group I and 11 patients in group II, which falls short of our power calculation target of 14 in each group, and puts our data at risk of a type II error. Further, due to difficulty in obtaining the FFPEs and their corresponding slides we were only able to extract DNA from one part of the tumour in each sample. It is well known that PDACs exhibit tumour mutational heterogeneity and thus we may have found different results if each sample had come from a different part of the PDAC specimen.

4.3.3.10 *Conclusions:*

From next-generation gene sequencing of 29 PDAC tumours we found that a high frequency of *KRAS* mutation was more likely to occur in those with short, rather than long, disease-free survival and this was an independent prognostic marker. Further, a trend towards worse survival was seen in those with a COSM521 mutation of *KRAS* and a trend towards better survival was seen in those with a COSM518 mutation of *KRAS*. Short disease-free survivors also trended towards having multiple mutations, particularly of *p53*, *CDKN2A*, and *SMAD4*. Genetic mutational analysis could therefore direct personalised therapy and be used to stratify patients for more aggressive local or systemic treatment following surgical resection.

4.3.4 RESULTS FROM BLOOD SAMPLES:

4.3.4.1 cfDNA sequencing

Considering patients with a high frequency of KRAS mutation are more likely to develop disease recurrence within 12 months than to have at least two years free from disease, it is of interest to sequence cell-free DNA from blood samples of these patients as a potential prognostic biomarker. Blood samples from the peripheral circulation were collected from a total of sixteen patients (8 in Group I and 8 in Group II). A mean of 63.67 +/- 24.37 ng/μl of DNA was extracted from each sample. A total of 256 SNPs were identified from the total of 16 samples. A genetic mutation was identified in two samples: an APC gene mutation and an STK11 mutation, the location of which corresponded to the tumour sample mutations seen in these patients. No other mutations, specifically no KRAS mutations were identified in any samples.

4.3.4.2 Circulating tumour cells (CTCs) analysis: As cell-free DNA was not identified as a biomarker in this study, it was decided to look at alternative prognostic biomarkers. CTC analysis was undertaken from the peripheral circulation blood samples in the same sixteen patients (8 in Group I and 8 in Group II). A median of 0 CTCs per sample (range 0-2) were found, with a median of 0.5 in Group I and 0 in Group II (p=0.33). **Figure 13.**

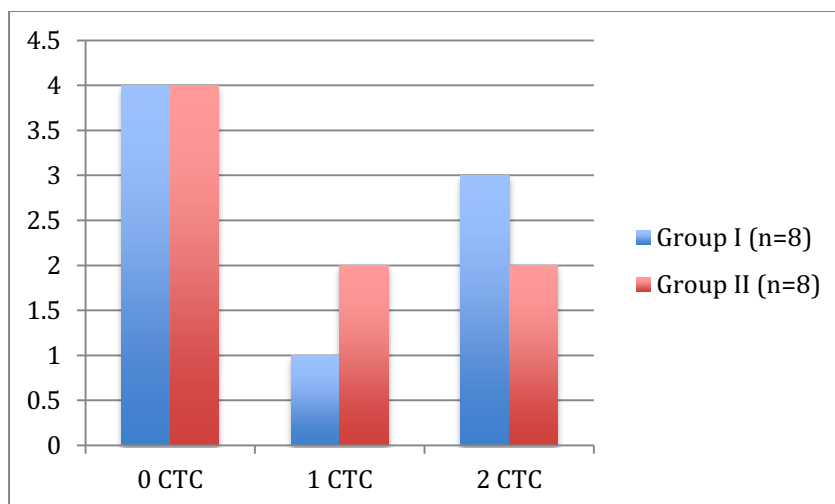


Figure 13: CTC analysis for Group I (recurrence within 12 months) and Group II (no recurrence for 24 months). No significant difference found between each group ($p=0.33$).

4.3.4.3 microRNA (miRNA) analysis: Our group has previously found that a combination of miR-21, miR-23a and miR-27a from PDAC tumour samples is associated with shorter survival after surgical resection⁴⁰⁶. Therefore, we hypothesised that circulating miRNAs may distinguish between our two groups of patients and be of potential use as prognostic biomarkers.

Blood from the sixteen patients used above was also analysed for miRNAs using the NanoString nCounter system expression assay. Using a significance value of <0.01 , five miRNAs were differentially expressed between patients with short disease-free survival and those with long disease-free survival: hsa-miR-548ah-5p; hsa-miR-550b-3p; hsa-miR-223-3p; hsa-let-7b-5p and hsa-let-7c. Three were downregulated and two upregulated in Group I compared to Group II (**Table 13**). These miRNAs were entered into the MiRTarBase database (May 2015). Only one of these differentially expressed miRNAs has been found to target genes identified as mutated in the genetic sequencing of our PDAC samples, namely hsa-let-7b-5p

(target KRAS). None of these miRNAs have been previously identified as prognostic indicators in pancreatic cancer.

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miRNA	Base Mean	Log2 Fold Change	Adjusted p-value	Upregulated/ Downregulated in Group I compared to Group II	PDAC Gene Target
hsa-miR-548ah-5p	42.63822508	-0.712787648	0.001728	Down-regulated	Nil known
hsa-miR-550b-3p	46.53592309	-0.61247248	0.245326	Down-regulated	Nil known
hsa-miR-223-3p	155.6722423	0.603150078	0.080409	Up-regulated	Nil known
hsa-let-7b-5p	60.40780961	0.607372968	0.085134	Up-regulated	KRAS ⁴⁰⁷
hsa-let-7c	41.9846227	-0.346058076	0.085134	Down-regulated	Nil known

Table 13: The five most differentially expressed circulating microRNAs between Group I and Group II.

4.3.5 DISCUSSION OF BLOOD RESULTS

4.3.5.1 Gene sequencing of cell-free DNA (cfDNA):

Genetic sequencing of blood DNA did not reveal any mutations in the four driver genes associated with PDAC and seen in the sequencing of the patients' tumour samples. The mutational analysis of cf-DNA is therefore not a useful prognostic marker in patients with resectable PDAC. The Ion Torrent™ PGM sequencer has been used by others to detect mutations in cfDNA of patients with HCC ⁴⁰⁸, methylmalonic acidemia ⁴⁰⁹, chronic lymphocytic leukemia ²⁵³ and breast cancer ⁴¹⁰. Whilst some concentrated on DNA extraction from plasma, others used whole-blood samples, such as in our study. We had high volumes of DNA extracted from our samples and sufficient full-length reads were obtained. Further, multiple SNPs were detected as well as two gene mutations. Interestingly these gene mutations were in a germline *STK11* variant and in *APC*, and both mutations were seen in the corresponding tumour analysis. This suggests that the Ion Torrent performed technically well and was able to analyse the DNA mutational status appropriately. However, we can report that the genetic mutations identified in the tumour were not seen in peripheral blood DNA. Indeed, both *STK11* and *APC* mutations are known to be a familial cause of PDAC suggesting that these two patients had familial circulating DNA with these mutation defects, which may have led to the cause of the PDAC rather than be resultant from it. Other studies which have detected mutation defects in cfDNA (Table 3) only detected *KRAS* mutations in the blood of 33-54.5% of PDAC patients compared to the 82% of PDAC tumour samples known to show *KRAS* mutations. Further, the majority of the studies took blood samples from those with unresectable disease and thus at a later stage than our patient cohort. With this in mind it is therefore likely that patients who have resectable pancreatic cancer have

not yet shed tumour cells with DNA containing the mutational defect into the systemic circulation and cfDNA cannot be used as a prognostic marker in these patients.

4.3.5.2 Analysis of circulating tumour cells (CTCs):

There were no differences seen in the number of CTCs detected in the peripheral circulation between the short- and long- disease-free survival patients. Our finding of CTCs in 50% of the total number of patients concurs with the literature where at least one CTC has previously been detected in the blood of 43-80% of PDAC patients.^{267, 268, 270} Although a shorter progression-free survival has previously been correlated with the detection of CTCs, all these studies were conducted in patients with unresectable progressive disease undergoing oncological treatment. In the only other study conducted on patients with resectable PDAC, only 25% had detectable CTCs and, in agreement with our study, these patients did not have shorter disease-free or overall survival compared to the CTC-negative patients²⁷¹. The detection of CTCs however has been linked to survival in other cancers, including metastatic breast cancer²⁶⁰⁻²⁶², colorectal cancer^{263, 264}, castrate-resistant prostate cancer²⁶⁵ and in small-cell-lung-cancer²⁶⁶. In each of these cases, which differs from all reports of CTCs in PDAC, large numbers of CTCs are detected. Indeed, in breast cancer, a cut-off of at least 5 CTCs/7.5ml blood is required to enumerate CTC reported differences in median progression-free or overall survival,⁴¹¹ but we only detected a maximum of 2 CTCs/7.5ml blood in our patients. Although the CellSearch system is the most commonly used for CTC detection and the only one with FDA approval, it may be that PDAC cells have specific characteristics which means they are not detected by this method. Indeed, the CellSearch system assumes that cancer cells have epithelial tissue by relying on the detection of cytokeratins 8- 18- and 19-, proteins found in the intracytoplasmic cytoskeleton of epithelial tissue. However, some cancer cells undergo an epithelial-mesenchymal-transition (EMT),

resulting in the downregulation of EpCAM and some cytokeratins³⁴³. EMT signifies an aggressive cancer and facilitates cancer invasion, so the known aggressive nature of PDAC would suggest that many of its cells undergo an early EMT even in those with resectable disease. An analysis of E-cadherin expression (which is expressed in epithelial cells) confirms this theory as 43% of PDAC tumours from those with resectable disease had partial or complete loss of E-cadherin⁴¹².

Pancreatic cancer cell lines harboring *KRAS* mutations do tend to exhibit EMT induction, and the EGFR/Ras pathway correlates with the genesis and promotion of EMT-induced tumor-initiating cells⁴¹³. Moreover, EMT status is associated with poor prognosis and adverse clinical outcomes in PDAC⁴¹², and cancer cells with EMT possess features that represent resistance to chemotherapy⁴¹⁴.

Ultimately, we may be missing the detection of many pancreatic tumour cells, which have undergone EMT in keeping with the invasive nature of the disease. Newer technologies for CTC detection in PDAC may be on the horizon, but until these have been validated CTCs are unlikely to be of use as prognostic biomarkers in pancreatic cancer.

4.3.5.3.1 Analysis of circulating miRNAs:

miRNAs are small endogenous non-coding RNA molecules around 22 nucleotides in length. This compares to messenger RNAs (mRNA) which are approximately 2000-5000 base pairs in length. They function in post transcriptional regulation of gene expression and RNA silencing by targeting mRNA for either direct cleavage, translational repression or transcript destabilisation⁴¹⁵. The total expression of miRNA directly affects the degree to which mRNA targets are altered. Following transcription of DNA, RNA polymerase copies the DNA to form one-stranded mRNA. The mRNA is transported from the nucleus to the cytoplasm where it can then be

translated by ribosomes and specifies the amino acid sequence of the protein products of gene expression. Considering miRNAs can affect mRNA and resultant gene expression, numerous mechanisms of miRNA biogenesis and behaviours have come under scrutiny. The miRNA pathways are extremely complex with thousands of miRNAs established and each one thought to target around 400 genes⁴¹⁶.

Disruption of these processes can disrupt the homeostasis of miRNAs, causing them to act pathologically, or, pathologically abnormal gene expression may result in differential expression of miRNAs. Compared to RNA, miRNAs are remarkably stable and protected from degradation due to their small size⁴¹⁷. Techniques to measure their expression have thus been developed and their role in human disease has been extensively investigated. Almost all developmental and physiological processes are linked to miRNAs and their dysregulation has been linked to many diseases including cancer⁴¹⁸. They have been shown to act as both oncogenes and tumour suppressors and expression profiling of miRNAs has associated specific individual or groups of miRNAs with certain cancers. The PDAC miRNome has been profiled and distinctive miRNA signatures have been shown to be useful in detecting pancreatic cancer from FFPE tissue⁴¹⁹, blood³¹⁸, EUS-FNA samples⁴²⁰ and stool samples. Expression profiling has found that pancreas-specific miRNAs are dysregulated in PDAC compared to the normal pancreas, chronic pancreatitis and other tumours. Each report in the literature has identified aberrant miRNA expression in numerous miRNAs and the majority report unique but differing signatures of both up-regulated and down-regulated miRNAs⁴²¹. Ma et al.⁴²² conducted a robust meta-review of the profiled miRNome and identified a diagnostic miRNA signature comprising up-regulated miR-21, miR-23a, miR-31, miR-100, miR-143, miR-155 and miR-221; and down-regulated miR-148a, miR-217 and miR-375. This meta-review found that miR-21 is one of the most frequently up-regulated miRNAs in PDAC although it is also dysregulated in many other malignancies.

4.3.5.3.2 MiRNAs with prognostic significance:

A recent meta-analysis⁴²³ pooled results of 20 studies analysing 1525 patients with PDAC and correlated the tumour miRNA results with survival. They identified a prognostic significance for up-regulated tumoural miR-10b; miR-21; miR-155; miR-196a/b; miR-203 and miR-222; and down-regulated miR-34a/b. These were independently prognostic for overall survival in PDAC. miR-21 was the strongest predictor of overall survival even when adjusting for other prognostic factors (HR: 2.66) and when adjusting for adjuvant therapy (HR: 2.72). However, some of the publications pooled contained a mixture of resectable and locally advanced PDACs. As those with increasing stage of PDAC have shorter survivals, those patients with locally advanced disease may have been driving the miR-21 result.

4.3.5.3.3 Our Findings:

We identified five circulating miRNAs that were differentially expressed between patients with short- and those with long- disease free survivals: hsa-miR-548ah-5p; hsa-miR-550b-3p; hsa-miR-223-3p; hsa-let-7b-5p and hsa-let-7c. Although none of these miRNAs have been previously reported as being of prognostic significance in PDAC, the let-7 family is known to target *KRAS*.

4.3.5.3.4 The let-7 family:

Lethal-7 (let-7) was first identified as a heterochronic gene in *C. elegans* in 2000. The let-7 mutants failed to exit the cell cycle and the larval cells died⁴²⁴. The 3'UTR of *RAS* was later identified as a top-scoring candidate containing let-7 complementary sites. They found that let-7 was able to regulate *RAS* expression⁴²⁵. Similar let-7 complementary sequences were also found in the 3'UTR of human *RAS* mRNAs. There are 12 different members of the let-7 family (let-7a-1, 7a-2, 7a-3, 7b,

7c, 7d, 7e, 7f-1, 7f-2, 7g, 7i, and mir-98) expressed in humans. These all have a highly conserved region of nucleotides, important for target recognition and suggestive that the members share some targets and functions⁴²⁶. In human cancer, members of the let-7 family are widely reported as tumour suppressor miRNAs with down-regulation observed in many cancer types. In prostate cancer, almost all members were down-regulated although in ovarian cancer, only levels of let-7d and let-7f were reduced⁴²⁷. Reduced let-7 expression corresponds to elevated *RAS* expression in small-cell lung cancer⁴²⁸ and increased let-7 expression suppresses *RAS* and abolishes tumorigenesis in non-small-cell lung cancer⁴²⁹ and cell growth in a mouse model of breast cancer⁴³⁰. The mechanism for let-7 down-regulation in *KRAS* driven cancers is thought to be because *KRAS* up-regulation leads to the induction of LIN-28 (a negative regulator of let-7) which thus reduces let-7 expression⁴³¹.

In addition, let-7 suppresses growth and cell cycle progression in cancer by repressing cell cycle regulators such as cyclins A, D1, D3 and CDK4 and by suppressing MYC expression, abolishing MYC-induced cell growth in Burkitt-lymphoma cells⁴²⁶. In addition, there are examples of let-7 showing oncogenic behavior with the up-regulation of some members seen in high grade transformation in lymphoma⁴³² and let-7a targets caspase-3mRNA, an essential protease activated during apoptosis in squamous cell carcinoma and hepatocellular carcinoma cell lines⁴³³.

In PDAC, let-7 has been seen to be up-regulated in normal ductal cells compared to PDAC cell-lines and in patient samples, has been shown to be expressed in normal pancreatic cells but not in cancerous cells⁴³⁴. In PDAC cells with mutated *KRAS*, down-regulation of *KRAS* and MAPK and a strong inhibition of cell proliferation was

seen with the introduction of let-7a. However, mice injected with *KRAS*-mutant PDAC cells over-expressing let-7a failed to exhibit stunted tumour growth⁴³⁴. In human patients, Ali *et al.*⁴³⁵, found that let-c and d were down-regulated in PDAC FNA samples compared to samples from the normal pancreas. Further, significantly shorter survival after potentially curative resection was seen in lung cancer patients with reduced *let-7* expression ($P = 0.0003$)⁴²⁸. This supports our finding of down-regulated let-7c in the short disease-free survival group and a tumour suppressor role of let-7c in PDAC. In contrast, Henry *et al.*⁴³⁶ found let-7b to be up-regulated in the aspirate of cancerous cystic tumours, which supports our finding of up-regulated let-7b in the short disease-free survival group and an oncogenic role of let-7b in PDAC.

4.3.5.3.5 miR-548:

miR-548 is from a larger human gene family located on almost all human chromosomes, with 69 members documented⁴³⁷. There is little published research regarding its influence on cancer, which may reflect its low expression levels compared to other miRNAs and thus difficulty in detecting expression with some miRNA expression profiling methods available. However, there are more than 3,500 hsa-mir-548 target genes and analysis of their expression profiles and functional affinities suggests that they have cancer-related regulatory roles⁴³⁸. In fact, some members of this family have been shown to be important as tumour suppressors. Indeed, one member has been identified in the fourth intron of the human *FHIT* gene on a xenograft mouse model injected with tumour cells and on human tissue analysis of cervical, brain, colon, melanoma and lung cancers. Increased expression of this miRNA was able to inhibit tumour cell growth⁴³⁹. Although *FHIT* was not analysed by the ion torrent package used in our gene sequencing study, previous work has identified that the tumour suppressor gene *FHIT* is expressed in pancreatic ductal

cells and aberrant in PDAC ^{440, 441 442 443}. The nanostring technique for miRNA analysis is particularly advantageous in identifying miRNAs with low expression profiles. We found that a member of the miR-548 family was down-regulated in the short disease-free survival patients and this may be due to its regulatory influence on *FHIT*, which is aberrantly expressed in PDAC.

4.3.5.3.6 miR-550:

According to miRBase, hsa-miR-550 is located in chromosomal region 7p14, and two members of the hsa-miR-550 family have been identified: hsa-miR-550a and hsa-miR-550b. miR-550 has been shown to regulate *CPEB4* ⁴⁴⁴. In PDAC, the expression of *CPEB4* is up-regulated, driving the growth and invasion of cancer cells ⁴⁴⁵. miR-550 has previously been shown to be dysregulated in non-small-cell-lung cancers ⁴⁴⁶, MALT lymphomas ⁴⁴⁷ and hepatocellular carcinoma (HCC) ⁴⁴⁴. Further, in childhood acute lymphoblastic leukaemia, miR-550 was down-regulated in patients with early relapse ⁴⁴⁸ and in HCC, miR-550a markedly promoted cell migration and invasion. More specifically, miR-550b-3p, which was differentially expressed in our study, has been found to be dysregulated in cervical cancer ⁴⁴⁹. Whilst not previously reported as dysregulated in PDAC, these studies confirm an important role for miR-550 in cancer and as a potential prognostic indicator, which may be via its regulation of *CPEB4*.

4.3.5.3.7 miR-223:

miR-223 comprises a family of miRNAs which are known to regulate a number of biological processes with roles in granulopoiesis, haematopoietic differentiation, immune cell function and activation, inflammatory disorders and infection. As well as these, it is well known to be dysregulated in cancer, particularly haematological malignancies⁴⁵⁰. There is also increasing evidence for miR-223 in solid cancers.

Certainly HeLa cells injected into mice show suppressed proliferation and tumour growth when the cells are overexpressed with miR-223⁴⁵¹. This was caused by reduced expression of the miR-223 target IGF1R and downstream Akt/mTOR/p70S6K signaling. miR-223 may also influence cell proliferation by targeting the transcription factor FOXO1⁴⁵². In HCC, down-regulation of miR-223 causes up-regulation of STMN1, a regulatory protein frequently overexpressed in cancer⁴⁵³. Of note, an elevated STMN1 expression has been associated with tumour progression and poor prognosis in PDAC ⁴⁵⁴. Further, a p53 mutation associated with poor prognosis represses miR-223 in breast and colon cancer cell lines⁴⁵⁵. Interestingly, there is also evidence of its up-regulation, as seen in our results, potentiating metastatic disease. It is over expressed in metastatic gastric cancer cells⁴⁵⁶ and in recurrent ovarian cancer, compared to primary tumours suggesting a metastatic signature⁴⁵⁷. In PDAC, miR-223 has been identified as strongly up-regulated in PDAC tissue compared to normal pancreas^{419, 458, 459}. Therefore, up-regulation of miR-223 is seen in PDAC and our results show this may be of prognostic significance. However, the mechanisms of this action remain poorly understood.

4.3.5.4 *Limitations:*

The blood samples for the cfDNA, CTCs and circulating miRNAs were obtained from 8 short disease-free survival patients and 8 long disease-free survival patients. This was therefore a limited study in terms of sample size and subject to the effect small sample sizes have on extreme outcomes. Furthermore, we did not validate our miRNA results using alternative methods of miRNA detection (e.g. Quantitative Real Time Reverse Transcription PCR). In addition, independent groups of patients would be required to fully validate these findings, including a large cohort of pre-operative

PDAC patients. In the near future, we intend to assess the prognostic ability of these miRNAs in a prospective manner.

4.3.5.5 Conclusions:

From these results we can conclude that neither CTCs, detected using the CellSearch System, nor gene mutational analysis of cfDNA are likely to be of benefit as prognostic biomarkers in PDAC. We have identified five circulating miRNAs (hsa-miR-548ah-5p; hsa-miR-550b-3p; hsa-miR-223-3p; hsa-let-7b-5p and hsa-let-7c) that may be of prognostic value. Prognostic miRNA signatures for PDAC have differed with each publication thus it is clear that large scale multi-centred prospective clinical studies should be carried out to determine the most effective combination of miRNAs for predicting survival outcomes in PDAC. There is a lack of blood-based miRNA research so until larger analyses are undertaken, we cannot reliably predict prognosis in PDAC and we are far from using circulating miRNAs as a biomarker. Once we have these large scale results, it is possible that a miRNA signature could be used as a PDAC prognostic biomarker thus impacting therapeutic aggressiveness and allowing both clinician-selection and patient-choice as to the benefit of surgical resection and chemotherapy regimes.

CHAPTER 5:

Summary of findings and future perspective

Pancreatic ductal adenocarcinoma (PDAC) has one of the poorest survivals of any solid cancer. Despite major advances in genetics; our knowledge of tumourigenesis; oncological treatment and surgical technique, the 5-year survival rate has shown almost no improvement over the last two decades. This is in contrast to almost all other cancers of the human body. Most pancreatic cancers will not be operable at initial diagnosis and the treatment is for prolonging life by a few months and/or for palliation of symptoms. Those with an early diagnosis of a small operable tumour who undergo pancreatic resection have a significantly improved overall survival. However, even 95% of these patients eventually develop disease recurrence and die within 5 years. It is not currently possible to predict prognosis in these patients in order to identify short and long-term survivors, which could lead to a more tailored and personalised management approach.

This thesis investigated potential predictors of disease recurrence in patients post pancreatic resection for PDAC. A literature review identified three promising prospective biomarkers: circulating tumour cells (CTCs); cell-free DNA and circulating microRNAs. We used CTCs to determine whether different surgical approaches could influence disease recurrence rates, specifically comparing the standard pancreaticoduodenectomy (PD) and the no-touch PD technique. Although the number of CTCs in the portal vein was significantly more following the standard PD compared to the no-touch PD, the difference in disease-free and overall survival did not reach significance. This suggested that an increase in CTCs does not necessarily lead to disease recurrence and changing surgical technique may not influence prognostic outcome. We therefore aimed to find clinicopathological factors

which could predict disease recurrence in PDAC patients. Two groups of patients were compared: short disease-free survivors (recurrence <12 months post-operatively) and long disease-free survivors (no recurrence for >24 months). Multivariate analysis identified a positive medial resection margin as the only independent prognostic indicator in patients following curative surgical resection. Although survival is much better for these patients, those with R0 resection are still likely to develop disease recurrence. We therefore hypothesised that there may be genetic differences between these two groups of patients and that these genetic alterations could predict early disease recurrence. From genetic sequencing of tumour tissue we were able to identify mutations in the key driver genes known to play a role in the pathogenesis of PDAC. Similar mutations were seen in the short- and long- disease free survivors however, the early recurrence group had significantly more DNA with mutant KRAS, that is, a higher frequency of KRAS mutation. The early recurrence group also trended towards having a COSM521 mutation of *KRAS* and multiple mutations, particularly of *p53*, *CKDN2A*, and *SMAD4*. The late recurrence group trended towards having a COSM518 mutation of *KRAS*. These genetic differences between the two groups could be analysed for each patient to predict prognosis. We also wanted to identify whether the same mutations could be seen from the sequencing of cell-free DNA from peripheral blood samples, which could lead to a prognostic biomarker. However, the mutations seen in the tumour tissue could not be identified in the peripheral circulation. This led us to look for other prognostic biomarkers in the peripheral circulation including CTCs and microRNAs. Although the numbers of CTCs did not appear to differ between the two groups, we did find five microRNAs whose expression was significantly different between the two groups: hsa-miR-548ah-5p; hsa-miR-550b-3p; hsa-miR-223-3p; hsa-let-7b-5p and hsa-let-7c.

In summary, our research has highlighted the importance of medial resection margin status; frequency of KRAS mutation; and the expression of five circulating microRNAs, in predicting disease recurrence in PDAC. However, there were limitations to all the research conducted, particularly in view of the retrospective nature of patient identification and of small sample sizes. The next stage of this project should involve prospective validation of our findings. All PDAC patients suitable for resection should have blood collected and stored for the analysis of these five microRNAs and their expression should be confirmed by at least two methods. The histology will report on medial resection margin status and the tumour tissue DNA should be analysed for KRAS frequency. Should these findings be reproduced in this prospective manner then a multi-centred approach should be taken in order to further validate our work. If our results can be validated and replicated then we may find these factors are reliable predictors of disease recurrence. This could lead to personalised treatment such that those with poorer disease-free survival prediction may undergo more aggressive oncological treatment.

REFERENCES

1. Yachida S, Jones S, Bozic I, et al. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. *Nature* 2010; 467(7319):1114-7.
2. Poplin E, Wasan H, Rolfe L, et al. Randomized, multicenter, phase II study of CO-101 versus gemcitabine in patients with metastatic pancreatic ductal adenocarcinoma: including a prospective evaluation of the role of hENT1 in gemcitabine or CO-101 sensitivity. *J Clin Oncol* 2013; 31(35):4453-61.
3. Gall TM, Thompson Z, Dinneen EP, et al. Surgical techniques for improving outcomes in pancreatic ductal adenocarcinoma. *Expert Rev Gastroenterol Hepatol* 2014; 8(3):241-6.
4. Ferlay J, Shin HR, Bray F, et al. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010; 127(12):2893-917.
5. Iacobuzio-Donahue CA, Velculescu VE, Wolfgang CL, et al. Genetic basis of pancreas cancer development and progression: insights from whole-exome and whole-genome sequencing. *Clin Cancer Res* 2012; 18(16):4257-65.
6. Mena S, Ortega A, Estrela JM. Oxidative stress in environmental-induced carcinogenesis. *Mutat Res* 2009; 674(1-2):36-44.
7. Koorstra JB, Hong SM, Shi C, et al. Widespread activation of the DNA damage response in human pancreatic intraepithelial neoplasia. *Mod Pathol* 2009; 22(11):1439-45.
8. Lister A, Nedjadi T, Kitteringham NR, et al. Nrf2 is overexpressed in pancreatic cancer: implications for cell proliferation and therapy. *Mol Cancer* 2011; 10:37.
9. Hocevar BA, Kamendulis LM, Pu X, et al. Contribution of environment and genetics to pancreatic cancer susceptibility. *PLoS One* 2014; 9(3):e90052.
10. Iodice S, Gandini S, Maisonneuve P, et al. Tobacco and the risk of pancreatic cancer: a review and meta-analysis. *Langenbecks Arch Surg* 2008; 393(4):535-45.
11. Alsamarrai A, Das SL, Windsor JA, et al. Factors That Affect Risk for Pancreatic Disease in the General Population: A Systematic Review and Meta-analysis of Prospective Cohort Studies. *Clin Gastroenterol Hepatol* 2014.
12. Porta M, Crous-Bou M, Wark PA, et al. Cigarette smoking and K-ras mutations in pancreas, lung and colorectal adenocarcinomas: etiopathogenic similarities, differences and paradoxes. *Mutat Res* 2009; 682(2-3):83-93.
13. Witt H, Apte MV, Keim V, et al. Chronic pancreatitis: challenges and advances in pathogenesis, genetics, diagnosis, and therapy. *Gastroenterology* 2007; 132(4):1557-73.
14. Kloppel G, Maillet B. Pathology of acute and chronic pancreatitis. *Pancreas* 1993; 8(6):659-70.
15. Carriere C, Young AL, Gunn JR, et al. Acute pancreatitis markedly accelerates pancreatic cancer progression in mice expressing oncogenic Kras. *Biochem Biophys Res Commun* 2009; 382(3):561-5.
16. Bang UC, Benfield T, Hyldstrup L, et al. Mortality, cancer, and comorbidities associated with chronic pancreatitis: a Danish nationwide matched-cohort study. *Gastroenterology* 2014; 146(4):989-94.

17. Raimondi S, Lowenfels AB, Morselli-Labate AM, et al. Pancreatic cancer in chronic pancreatitis; aetiology, incidence, and early detection. *Best Pract Res Clin Gastroenterol* 2010; 24(3):349-58.
18. Ekblom A, McLaughlin JK, Karlsson BM, et al. Pancreatitis and pancreatic cancer: a population-based study. *J Natl Cancer Inst* 1994; 86(8):625-7.
19. Anderson MA, Zolotarevsky E, Cooper KL, et al. Alcohol and tobacco lower the age of presentation in sporadic pancreatic cancer in a dose-dependent manner: a multicenter study. *Am J Gastroenterol* 2012; 107(11):1730-9.
20. Zambirinis CP, Pushalkar S, Saxena D, et al. Pancreatic cancer, inflammation, and microbiome. *Cancer J* 2014; 20(3):195-202.
21. Blogowski W, Deskur A, Budkowska M, et al. Selected cytokines in patients with pancreatic cancer: a preliminary report. *PLoS One* 2014; 9(5):e97613.
22. Zendejdel K, Nyren O, Ostenson CG, et al. Cancer incidence in patients with type 1 diabetes mellitus: a population-based cohort study in Sweden. *J Natl Cancer Inst* 2003; 95(23):1797-800.
23. Huxley R, Ansary-Moghaddam A, Berrington de Gonzalez A, et al. Type-II diabetes and pancreatic cancer: a meta-analysis of 36 studies. *Br J Cancer* 2005; 92(11):2076-83.
24. Silverman DT, Schiffman M, Everhart J, et al. Diabetes mellitus, other medical conditions and familial history of cancer as risk factors for pancreatic cancer. *Br J Cancer* 1999; 80(11):1830-7.
25. Jee SH, Ohrr H, Sull JW, et al. Fasting serum glucose level and cancer risk in Korean men and women. *JAMA* 2005; 293(2):194-202.
26. Batty GD, Shipley MJ, Marmot M, et al. Diabetes status and post-load plasma glucose concentration in relation to site-specific cancer mortality: findings from the original Whitehall study. *Cancer Causes Control* 2004; 15(9):873-81.
27. Fisher WE, Boros LG, Schirmer WJ. Insulin promotes pancreatic cancer: evidence for endocrine influence on exocrine pancreatic tumors. *J Surg Res* 1996; 63(1):310-3.
28. Ohmura E, Okada M, Onoda N, et al. Insulin-like growth factor I and transforming growth factor alpha as autocrine growth factors in human pancreatic cancer cell growth. *Cancer Res* 1990; 50(1):103-7.
29. Wang Z, Lai ST, Xie L, et al. Metformin is associated with reduced risk of pancreatic cancer in patients with type 2 diabetes mellitus: A systematic review and meta-analysis. *Diabetes Res Clin Pract* 2014.
30. Butler AE, Campbell-Thompson M, Gurlo T, et al. Marked expansion of exocrine and endocrine pancreas with incretin therapy in humans with increased exocrine pancreas dysplasia and the potential for glucagon-producing neuroendocrine tumors. *Diabetes* 2013; 62(7):2595-604.
31. Zhao H, Wei R, Wang L, et al. Activation of glucagon-like peptide-1 receptor inhibits growth and promotes apoptosis of human pancreatic cancer cells in a cAMP-dependent manner. *Am J Physiol Endocrinol Metab* 2014.
32. Ryder RE. The potential risks of pancreatitis and pancreatic cancer with GLP-1-based therapies are far outweighed by the proven and potential (cardiovascular) benefits. *Diabet Med* 2013; 30(10):1148-55.
33. Larsson SC, Orsini N, Wolk A. Body mass index and pancreatic cancer risk: A meta-analysis of prospective studies. *Int J Cancer* 2007; 120(9):1993-8.
34. Aune D, Greenwood DC, Chan DS, et al. Body mass index, abdominal fatness and pancreatic cancer risk: a systematic review and non-linear dose-response meta-analysis of prospective studies. *Ann Oncol* 2012; 23(4):843-52.

35. Rajala MW, Scherer PE. Minireview: The adipocyte--at the crossroads of energy homeostasis, inflammation, and atherosclerosis. *Endocrinology* 2003; 144(9):3765-73.
36. Preziosi G, Oben JA, Fusai G. Obesity and pancreatic cancer. *Surg Oncol* 2014.
37. Butler AE, Galasso R, Matveyenko A, et al. Pancreatic duct replication is increased with obesity and type 2 diabetes in humans. *Diabetologia* 2010; 53(1):21-6.
38. Olusi SO. Obesity is an independent risk factor for plasma lipid peroxidation and depletion of erythrocyte cytoprotective enzymes in humans. *Int J Obes Relat Metab Disord* 2002; 26(9):1159-64.
39. Risch HA, Lu L, Kidd MS, et al. Helicobacter pylori seropositivities and risk of pancreatic carcinoma. *Cancer Epidemiol Biomarkers Prev* 2014; 23(1):172-8.
40. Risch HA. Etiology of pancreatic cancer, with a hypothesis concerning the role of N-nitroso compounds and excess gastric acidity. *J Natl Cancer Inst* 2003; 95(13):948-60.
41. Kappelman MD, Farkas DK, Long MD, et al. Risk of cancer in patients with inflammatory bowel diseases: a nationwide population-based cohort study with 30 years of follow-up evaluation. *Clin Gastroenterol Hepatol* 2014; 12(2):265-73 e1.
42. Ananthakrishnan AN, Cagan A, Gainer VS, et al. Mortality and extraintestinal cancers in patients with primary sclerosing cholangitis and inflammatory bowel disease. *J Crohns Colitis* 2014.
43. Majumder S, Bockorny B, Baker WL, et al. Association Between HBsAg Positivity and Pancreatic Cancer: a Meta-Analysis. *J Gastrointest Cancer* 2014.
44. Huang J, Magnusson M, Torner A, et al. Risk of pancreatic cancer among individuals with hepatitis C or hepatitis B virus infection: a nationwide study in Sweden. *Br J Cancer* 2013; 109(11):2917-23.
45. Fitzpatrick SG, Katz J. The association between periodontal disease and cancer: a review of the literature. *J Dent* 2010; 38(2):83-95.
46. Larsson SC, Wolk A. Red and processed meat consumption and risk of pancreatic cancer: meta-analysis of prospective studies. *Br J Cancer* 2012; 106(3):603-7.
47. Jansen RJ, Robinson DP, Frank RD, et al. Fatty acids found in dairy, protein and unsaturated fatty acids are associated with risk of pancreatic cancer in a case-control study. *Int J Cancer* 2014; 134(8):1935-46.
48. Pericleous M, Rossi RE, Mandair D, et al. Nutrition and pancreatic cancer. *Anticancer Res* 2014; 34(1):9-21.
49. Bao Y, Ng K, Wolpin BM, et al. Predicted vitamin D status and pancreatic cancer risk in two prospective cohort studies. *Br J Cancer* 2010; 102(9):1422-7.
50. Zablotska LB, Gong Z, Wang F, et al. Vitamin D, calcium, and retinol intake, and pancreatic cancer in a population-based case-control study in the San Francisco Bay area. *Cancer Causes Control* 2011; 22(1):91-100.
51. Risch HA, Lu L, Wang J, et al. ABO blood group and risk of pancreatic cancer: a study in Shanghai and meta-analysis. *Am J Epidemiol* 2013; 177(12):1326-37.

52. Klein AP, Brune KA, Petersen GM, et al. Prospective risk of pancreatic cancer in familial pancreatic cancer kindreds. *Cancer Res* 2004; 64(7):2634-8.
53. Whitcomb DC. Genetic risk factors for pancreatic disorders. *Gastroenterology* 2013; 144(6):1292-302.
54. Rustgi AK. Familial pancreatic cancer: genetic advances. *Genes Dev* 2014; 28(1):1-7.
55. Chen JM, Ferec C. Genetics and pathogenesis of chronic pancreatitis: the 2012 update. *Clin Res Hepatol Gastroenterol* 2012; 36(4):334-40.
56. Campbell PJ, Yachida S, Mudie LJ, et al. The patterns and dynamics of genomic instability in metastatic pancreatic cancer. *Nature* 2010; 467(7319):1109-13.
57. Rustgi AK. The genetics of hereditary colon cancer. *Genes Dev* 2007; 21(20):2525-38.
58. Korsse SE, Harinck F, van Lier MG, et al. Pancreatic cancer risk in Peutz-Jeghers syndrome patients: a large cohort study and implications for surveillance. *J Med Genet* 2013; 50(1):59-64.
59. Ollila S, Makela TP. The tumor suppressor kinase LKB1: lessons from mouse models. *J Mol Cell Biol* 2011; 3(6):330-40.
60. Marechal A, Zou L. DNA damage sensing by the ATM and ATR kinases. *Cold Spring Harb Perspect Biol* 2013; 5(9).
61. Jayadev S, Bird TD. Hereditary ataxias: overview. *Genet Med* 2013; 15(9):673-83.
62. Roberts NJ, Jiao Y, Yu J, et al. ATM mutations in patients with hereditary pancreatic cancer. *Cancer Discov* 2012; 2(1):41-6.
63. Domchek SM, Weber BL. Clinical management of BRCA1 and BRCA2 mutation carriers. *Oncogene* 2006; 25(43):5825-31.
64. Rahman N, Seal S, Thompson D, et al. PALB2, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene. *Nat Genet* 2007; 39(2):165-7.
65. Cancer risks in BRCA2 mutation carriers. The Breast Cancer Linkage Consortium. *J Natl Cancer Inst* 1999; 91(15):1310-6.
66. Thompson D, Easton DF, Breast Cancer Linkage C. Cancer Incidence in BRCA1 mutation carriers. *J Natl Cancer Inst* 2002; 94(18):1358-65.
67. Axilbund JE, Argani P, Kamiyama M, et al. Absence of germline BRCA1 mutations in familial pancreatic cancer patients. *Cancer Biol Ther* 2009; 8(2):131-5.
68. Jones S, Hruban RH, Kamiyama M, et al. Exomic sequencing identifies PALB2 as a pancreatic cancer susceptibility gene. *Science* 2009; 324(5924):217.
69. Casadei S, Norquist BM, Walsh T, et al. Contribution of inherited mutations in the BRCA2-interacting protein PALB2 to familial breast cancer. *Cancer Res* 2011; 71(6):2222-9.
70. Roberts NJ, Klein AP. Genome-wide sequencing to identify the cause of hereditary cancer syndromes: with examples from familial pancreatic cancer. *Cancer Lett* 2013; 340(2):227-33.
71. Canto MI, Harinck F, Hruban RH, et al. International Cancer of the Pancreas Screening (CAPS) Consortium summit on the management of patients with increased risk for familial pancreatic cancer. *Gut* 2013; 62(3):339-47.

72. Hruban RH, Takaori K, Klimstra DS, et al. An illustrated consensus on the classification of pancreatic intraepithelial neoplasia and intraductal papillary mucinous neoplasms. *Am J Surg Pathol* 2004; 28(8):977-87.
73. Hruban RH, Adsay NV, Albores-Saavedra J, et al. Pancreatic intraepithelial neoplasia: a new nomenclature and classification system for pancreatic duct lesions. *Am J Surg Pathol* 2001; 25(5):579-86.
74. Nagata K, Horinouchi M, Saitou M, et al. Mucin expression profile in pancreatic cancer and the precursor lesions. *J Hepatobiliary Pancreat Surg* 2007; 14(3):243-54.
75. van Heek NT, Meeker AK, Kern SE, et al. Telomere Shortening Is Nearly Universal in Pancreatic Intraepithelial Neoplasia. *Am J Pathol* 2002; 161(5):1541-1547.
76. Maitra A, Kern SE, Hruban RH. Molecular pathogenesis of pancreatic cancer. *Best Pract Res Clin Gastroenterol* 2006; 20(2):211-26.
77. Hustinx SR, Leoni LM, Yeo CJ, et al. Concordant loss of MTAP and p16//CDKN2A expression in pancreatic intraepithelial neoplasia: evidence of homozygous deletion in a noninvasive precursor lesion. *Mod Pathol* 2005; 18(7):959-963.
78. Clark SJ. Action at a distance: epigenetic silencing of large chromosomal regions in carcinogenesis. *Hum Mol Genet* 2007; 16 Spec No 1:R88-95.
79. Sato N, Fukushima N, Hruban RH, et al. CpG island methylation profile of pancreatic intraepithelial neoplasia. *Mod Pathol* 2008; 21(3):238-44.
80. Distler M, Aust D, Weitz J, et al. Precursor Lesions for Sporadic Pancreatic Cancer: PanIN, IPMN, and MCN. *Biomed Res Int* 2014; 2014:474905.
81. Koorstra JB, Feldmann G, Habbe N, et al. Morphogenesis of pancreatic cancer: role of pancreatic intraepithelial neoplasia (PanINs). *Langenbecks Arch Surg* 2008; 393(4):561-70.
82. Furukawa T, Kloppel G, Volkan Adsay N, et al. Classification of types of intraductal papillary-mucinous neoplasm of the pancreas: a consensus study. *Virchows Arch* 2005; 447(5):794-9.
83. Furukawa T, Kuboki Y, Tanji E, et al. Whole-exome sequencing uncovers frequent GNAS mutations in intraductal papillary mucinous neoplasms of the pancreas. *Sci Rep* 2011; 1:161.
84. Brugge WR, Lewandrowski K, Lee-Lewandrowski E, et al. Diagnosis of pancreatic cystic neoplasms: a report of the cooperative pancreatic cyst study. *Gastroenterology* 2004; 126(5):1330-6.
85. Tanaka M, Fernandez-del Castillo C, Adsay V, et al. International consensus guidelines 2012 for the management of IPMN and MCN of the pancreas. *Pancreatology* 2012; 12(3):183-97.
86. Salvia R, Fernandez-del Castillo C, Bassi C, et al. Main-duct intraductal papillary mucinous neoplasms of the pancreas: clinical predictors of malignancy and long-term survival following resection. *Ann Surg* 2004; 239(5):678-85; discussion 685-7.
87. Schmidt CM, White PB, Waters JA, et al. Intraductal papillary mucinous neoplasms: predictors of malignant and invasive pathology. *Ann Surg* 2007; 246(4):644-51; discussion 651-4.
88. Grutzmann R, Post S, Saeger HD, et al. Intraductal papillary mucinous neoplasia (IPMN) of the pancreas: its diagnosis, treatment, and prognosis. *Dtsch Arztebl Int* 2011; 108(46):788-94.

89. Matthaei H, Schulick RD, Hruban RH, et al. Cystic precursors to invasive pancreatic cancer. *Nat Rev Gastroenterol Hepatol* 2011; 8(3):141-50.
90. Wu J, Jiao Y, Dal Molin M, et al. Whole-exome sequencing of neoplastic cysts of the pancreas reveals recurrent mutations in components of ubiquitin-dependent pathways. *Proc Natl Acad Sci U S A* 2011; 108(52):21188-93.
91. Yamao K, Yanagisawa A, Takahashi K, et al. Clinicopathological features and prognosis of mucinous cystic neoplasm with ovarian-type stroma: a multi-institutional study of the Japan pancreas society. *Pancreas* 2011; 40(1):67-71.
92. Baker ML, Seeley ES, Pai R, et al. Invasive mucinous cystic neoplasms of the pancreas. *Exp Mol Pathol* 2012; 93(3):345-9.
93. Jones S, Zhang X, Parsons DW, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science (New York, N.Y.)* 2008; 321(5897):1801-1806.
94. Shain AH, Giacomini CP, Matsukuma K, et al. Convergent structural alterations define SWItch/Sucrose NonFermentable (SWI/SNF) chromatin remodeler as a central tumor suppressive complex in pancreatic cancer. *Proc Natl Acad Sci U S A* 2012; 109(5):E252-9.
95. Biankin AV, Waddell N, Kassahn KS, et al. Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. *Nature* 2012; 491(7424):399-405.
96. Hall A. The cellular functions of small GTP-binding proteins. *Science* 1990; 249(4969):635-40.
97. Pylayeva-Gupta Y, Grabocka E, Bar-Sagi D. RAS oncogenes: weaving a tumorigenic web. *Nat Rev Cancer* 2011; 11(11):761-74.
98. Bryant KL, Mancias JD, Kimmelman AC, et al. KRAS: feeding pancreatic cancer proliferation. *Trends Biochem Sci* 2014; 39(2):91-100.
99. Shields JM, Pruitt K, McFall A, et al. Understanding Ras: 'it ain't over 'til it's over'. *Trends Cell Biol* 2000; 10(4):147-54.
100. Downward J. Targeting RAS signalling pathways in cancer therapy. *Nat Rev Cancer* 2003; 3(1):11-22.
101. Almoguera C, Shibata D, Forrester K, et al. Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. *Cell* 1988; 53(4):549-54.
102. Smit VT, Boot AJ, Smits AM, et al. KRAS codon 12 mutations occur very frequently in pancreatic adenocarcinomas. *Nucleic Acids Res* 1988; 16(16):7773-82.
103. Hingorani SR, Petricoin EF, Maitra A, et al. Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. *Cancer Cell* 2003; 4(6):437-50.
104. Collins MA, Bednar F, Zhang Y, et al. Oncogenic Kras is required for both the initiation and maintenance of pancreatic cancer in mice. *J Clin Invest* 2012; 122(2):639-53.
105. Réjiba S, Wack S, Aprahamian M, et al. K-ras oncogene silencing strategy reduces tumor growth and enhances gemcitabine chemotherapy efficacy for pancreatic cancer treatment. *Cancer Sci* 2007; 98(7):1128-1136.
106. Kindler HL, Wroblewski K, Wallace JA, et al. Gemcitabine plus sorafenib in patients with advanced pancreatic cancer: a phase II trial of the University of Chicago Phase II Consortium. *Invest New Drugs* 2012; 30(1):382-6.
107. Rinehart J, Adjei AA, Lorusso PM, et al. Multicenter phase II study of the oral MEK inhibitor, CI-1040, in patients with advanced non-small-cell lung, breast, colon, and pancreatic cancer. *J Clin Oncol* 2004; 22(22):4456-62.

108. Bodoky G, Timcheva C, Spigel DR, et al. A phase II open-label randomized study to assess the efficacy and safety of selumetinib (AZD6244 [ARRY-142886]) versus capecitabine in patients with advanced or metastatic pancreatic cancer who have failed first-line gemcitabine therapy. *Invest New Drugs* 2012; 30(3):1216-23.
109. Rayess H, Wang MB, Srivatsan ES. Cellular senescence and tumor suppressor gene p16. *Int J Cancer* 2012; 130(8):1715-25.
110. Iacobuzio-Donahue CA, Velculescu VE, Wolfgang CL, et al. Genetic Basis of Pancreas Cancer Development and Progression: Insights from Whole-Exome and Whole-Genome Sequencing. *Clinical Cancer Research* 2012; 18(16):4257-4265.
111. Sharpless NE, Bardeesy N, Lee KH, et al. Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis. *Nature* 2001; 413(6851):86-91.
112. Aguirre AJ, Bardeesy N, Sinha M, et al. Activated Kras and Ink4a/Arf deficiency cooperate to produce metastatic pancreatic ductal adenocarcinoma. *Genes Dev* 2003; 17(24):3112-26.
113. Chang Z, Ju H, Ling J, et al. Cooperativity of Oncogenic K-Ras and Downregulated p16/INK4A in Human Pancreatic Tumorigenesis. *PLoS One* 2014; 9(7):e101452.
114. Hidalgo M. Pancreatic Cancer. *New England Journal of Medicine* 2010; 362(17):1605-1617.
115. Pérez-Mancera PA, Guerra C, Barbacid M, et al. What We Have Learned About Pancreatic Cancer From Mouse Models. *Gastroenterology* 2012; 142(5):1079-1092.
116. Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. *Nat Med* 2004; 10(8):789-99.
117. Hingorani SR, Wang L, Multani AS, et al. Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell* 2005; 7(5):469-83.
118. Jonckheere N, Vincent A, Van Seuning I. Of autophagy and in vivo pancreatic carcinogenesis: The p53 status matters! *Clin Res Hepatol Gastroenterol* 2014.
119. Frampton AE, Gall TM, Giovannetti E, et al. Distinct miRNA profiles are associated with malignant transformation of pancreatic cystic tumors revealing potential biomarkers for clinical use. *Expert Rev Mol Diagn* 2013; 13(4):325-9.
120. Perez-Mancera PA, Guerra C, Barbacid M, et al. What we have learned about pancreatic cancer from mouse models. *Gastroenterology* 2012; 142(5):1079-92.
121. Bardeesy N, Cheng KH, Berger JH, et al. Smad4 is dispensable for normal pancreas development yet critical in progression and tumor biology of pancreas cancer. *Genes Dev* 2006; 20(22):3130-46.
122. Maeda S, Shintani H, Kurahara H, et al. CD133 expression is correlated with lymph node metastasis and vascular endothelial growth factor-C expression in pancreatic cancer. *Br J Cancer* 2008; 98(8):1389-97.
123. Tobita K, Kijima H, Dowaki S, et al. Epidermal growth factor receptor expression in human pancreatic cancer: Significance for liver metastasis. *Int J Mol Med* 2003; 11(3):305-9.
124. Yachida S, Iacobuzio-Donahue CA. Evolution and dynamics of pancreatic cancer progression. *Oncogene* 2013; 32(45):5253-60.

125. Vincent A, Herman J, Schulick R, et al. Pancreatic cancer. *The Lancet* 2011; 378(9791):607-620.
126. Schonleben F, Qiu W, Remotti HE, et al. PIK3CA, KRAS, and BRAF mutations in intraductal papillary mucinous neoplasm/carcinoma (IPMN/C) of the pancreas. *Langenbeck's archives of surgery / Deutsche Gesellschaft fur Chirurgie* 2008; 393(3):289-96.
127. Sohn TA, Yeo CJ. The molecular genetics of pancreatic ductal carcinoma: a review. *Surg Oncol* 2000; 9(3):95-101.
128. Artinyan A, Soriano PA, Prendergast C, et al. The anatomic location of pancreatic cancer is a prognostic factor for survival. *HPB (Oxford)* 2008; 10(5):371-6.
129. Vincent A, Herman J, Schulick R, et al. Pancreatic cancer. *Lancet* 2011; 378(9791):607-20.
130. Takhar AS, Palaniappan P, Dhingsa R, et al. Recent developments in diagnosis of pancreatic cancer. *BMJ* 2004; 329(7467):668-73.
131. Stapley S, Peters TJ, Neal RD, et al. The risk of pancreatic cancer in symptomatic patients in primary care: a large case-control study using electronic records. *Br J Cancer* 2012; 106(12):1940-4.
132. Elli M, Piazza E, Franzone PC, et al. Considerations on early diagnosis of carcinoma of the pancreas. *Hepatogastroenterology* 2003; 50(54):2205-7.
133. Kannagi R. Carbohydrate antigen sialyl Lewis a--its pathophysiological significance and induction mechanism in cancer progression. *Chang Gung Med J* 2007; 30(3):189-209.
134. Wu E, Zhou S, Bhat K, et al. CA 19-9 and pancreatic cancer. *Clin Adv Hematol Oncol* 2013; 11(1):53-5.
135. Steinberg W. The clinical utility of the CA 19-9 tumor-associated antigen. *Am J Gastroenterol* 1990; 85(4):350-5.
136. Gui JC, Yan WL, Liu XD. CA19-9 and CA242 as tumor markers for the diagnosis of pancreatic cancer: a meta-analysis. *Clin Exp Med* 2014; 14(2):225-33.
137. Distler M, Pilarsky E, Kersting S, et al. Preoperative CEA and CA 19-9 are prognostic markers for survival after curative resection for ductal adenocarcinoma of the pancreas - A retrospective tumor marker prognostic study. *Int J Surg* 2013.
138. Barton JG, Bois JP, Sarr MG, et al. Predictive and prognostic value of CA 19-9 in resected pancreatic adenocarcinoma. *J Gastrointest Surg* 2009; 13(11):2050-8.
139. Hata S, Sakamoto Y, Yamamoto Y, et al. Prognostic impact of postoperative serum CA 19-9 levels in patients with resectable pancreatic cancer. *Ann Surg Oncol* 2012; 19(2):636-41.
140. Hartwig W, Strobel O, Hinz U, et al. CA19-9 in potentially resectable pancreatic cancer: perspective to adjust surgical and perioperative therapy. *Ann Surg Oncol* 2013; 20(7):2188-96.
141. Varadhachary GR, Tamm EP, Abbruzzese JL, et al. Borderline resectable pancreatic cancer: definitions, management, and role of preoperative therapy. *Ann Surg Oncol* 2006; 13(8):1035-46.
142. McNulty NJ, Francis IR, Platt JF, et al. Multi--detector row helical CT of the pancreas: effect of contrast-enhanced multiphasic imaging on enhancement of the pancreas, peripancreatic vasculature, and pancreatic adenocarcinoma. *Radiology* 2001; 220(1):97-102.

143. Bipat S, Phoa SS, van Delden OM, et al. Ultrasonography, computed tomography and magnetic resonance imaging for diagnosis and determining resectability of pancreatic adenocarcinoma: a meta-analysis. *J Comput Assist Tomogr* 2005; 29(4):438-45.
144. Ghaneh P, Costello E, Neoptolemos JP. Biology and management of pancreatic cancer. *Gut* 2007; 56(8):1134-52.
145. De Angelis C, Brizzi RF, Pellicano R. Endoscopic ultrasonography for pancreatic cancer: current and future perspectives. *J Gastrointest Oncol* 2013; 4(2):220-30.
146. Dewitt J, Devereaux BM, Lehman GA, et al. Comparison of endoscopic ultrasound and computed tomography for the preoperative evaluation of pancreatic cancer: a systematic review. *Clin Gastroenterol Hepatol* 2006; 4(6):717-25; quiz 664.
147. Snady H. EUS criteria for vascular invasion: analyzing the meta-analysis. *Gastrointest Endosc* 2007; 65(6):798-807.
148. Klapman JB, Chang KJ, Lee JG, et al. Negative predictive value of endoscopic ultrasound in a large series of patients with a clinical suspicion of pancreatic cancer. *Am J Gastroenterol* 2005; 100(12):2658-61.
149. Tamm EP, Balachandran A, Bhosale PR, et al. Imaging of pancreatic adenocarcinoma: update on staging/resectability. *Radiol Clin North Am* 2012; 50(3):407-28.
150. Zhang Y, Huang J, Chen M, et al. Preoperative vascular evaluation with computed tomography and magnetic resonance imaging for pancreatic cancer: a meta-analysis. *Pancreatol* 2012; 12(3):227-33.
151. Niekel MC, Bipat S, Stoker J. Diagnostic imaging of colorectal liver metastases with CT, MR imaging, FDG PET, and/or FDG PET/CT: a meta-analysis of prospective studies including patients who have not previously undergone treatment. *Radiology* 2010; 257(3):674-84.
152. Wang Z, Chen JQ, Liu JL, et al. FDG-PET in diagnosis, staging and prognosis of pancreatic carcinoma: a meta-analysis. *World J Gastroenterol* 2013; 19(29):4808-17.
153. Ballehaninna UK, Chamberlain RS. Biomarkers for pancreatic cancer: promising new markers and options beyond CA 19-9. *Tumour Biol* 2013.
154. Gall TM, Frampton AE, Krell J, et al. Circulating molecular markers in pancreatic cancer: ready for clinical use? *Future Oncol* 2013; 9(2):141-4.
155. Goodman MD, Saif MW. Adjuvant therapy for pancreatic cancer. *JOP* 2014; 15(2):87-90.
156. Zhang Y, Frampton AE, Cohen P, et al. Tumor infiltration in the medial resection margin predicts survival after pancreaticoduodenectomy for pancreatic ductal adenocarcinoma. *J Gastrointest Surg* 2012; 16(10):1875-82.
157. Chen YJ, Lai EC, Lau WY, et al. Enteric reconstruction of pancreatic stump following pancreaticoduodenectomy: a review of the literature. *Int J Surg* 2014.
158. Matsuoka L, Selby R, Genyk Y. The surgical management of pancreatic cancer. *Gastroenterol Clin North Am* 2012; 41(1):211-21.
159. Diener MK, Fitzmaurice C, Schwarzer G, et al. Pylorus-preserving pancreaticoduodenectomy (pp Whipple) versus pancreaticoduodenectomy (classic Whipple) for surgical treatment of periampullary and pancreatic carcinoma. *Cochrane Database Syst Rev* 2011(5):CD006053.

160. Winter JM, Cameron JL, Campbell KA, et al. 1423 pancreaticoduodenectomies for pancreatic cancer: A single-institution experience. *J Gastrointest Surg* 2006; 10(9):1199-210; discussion 1210-1.
161. Coolsen MM, van Dam RM, van der Wilt AA, et al. Systematic review and meta-analysis of enhanced recovery after pancreatic surgery with particular emphasis on pancreaticoduodenectomies. *World J Surg* 2013; 37(8):1909-18.
162. Lermite E, Sommacale D, Piardi T, et al. Complications after pancreatic resection: diagnosis, prevention and management. *Clin Res Hepatol Gastroenterol* 2013; 37(3):230-9.
163. Grobmyer SR, Pieracci FM, Allen PJ, et al. Defining morbidity after pancreaticoduodenectomy: use of a prospective complication grading system. *J Am Coll Surg* 2007; 204(3):356-64.
164. Wente MN, Bassi C, Dervenis C, et al. Delayed gastric emptying (DGE) after pancreatic surgery: a suggested definition by the International Study Group of Pancreatic Surgery (ISGPS). *Surgery* 2007; 142(5):761-8.
165. Tani M, Terasawa H, Kawai M, et al. Improvement of delayed gastric emptying in pylorus-preserving pancreaticoduodenectomy: results of a prospective, randomized, controlled trial. *Ann Surg* 2006; 243(3):316-20.
166. Bassi C, Dervenis C, Butturini G, et al. Postoperative pancreatic fistula: an international study group (ISGPF) definition. *Surgery* 2005; 138(1):8-13.
167. Gagner M, Pomp A. Laparoscopic pylorus-preserving pancreatoduodenectomy. *Surg Endosc* 1994; 8(5):408-10.
168. Ammori BJ, Ayiomamitis GD. Laparoscopic pancreaticoduodenectomy and distal pancreatectomy: a UK experience and a systematic review of the literature. *Surg Endosc* 2011; 25(7):2084-99.
169. Asbun HJ, Stauffer JA. Laparoscopic vs open pancreaticoduodenectomy: overall outcomes and severity of complications using the Accordion Severity Grading System. *J Am Coll Surg* 2012; 215(6):810-9.
170. Cho A, Yamamoto H, Nagata M, et al. Comparison of laparoscopy-assisted and open pylorus-preserving pancreaticoduodenectomy for periampullary disease. *Am J Surg* 2009; 198(3):445-9.
171. Dulucq JL, Wintringer P, Mahajna A. Laparoscopic pancreaticoduodenectomy for benign and malignant diseases. *Surg Endosc* 2006; 20(7):1045-50.
172. Chalikhonda S, Aguilar-Saavedra JR, Walsh RM. Laparoscopic robotic-assisted pancreaticoduodenectomy: a case-matched comparison with open resection. *Surg Endosc* 2012; 26(9):2397-402.
173. Zeh HJ, Zureikat AH, Secrest A, et al. Outcomes after robot-assisted pancreaticoduodenectomy for periampullary lesions. *Ann Surg Oncol* 2012; 19(3):864-70.
174. Buchs NC, Addeo P, Bianco FM, et al. Robotic versus open pancreaticoduodenectomy: a comparative study at a single institution. *World J Surg* 2011; 35(12):2739-46.
175. Kang CM, Kim DH, Lee WJ, et al. Conventional laparoscopic and robot-assisted spleen-preserving pancreatectomy: does da Vinci have clinical advantages? *Surg Endosc* 2011; 25(6):2004-9.
176. Pericleous S, Middleton N, McKay SC, et al. Systematic review and meta-analysis of case-matched studies comparing open and laparoscopic distal pancreatectomy: is it a safe procedure? *Pancreas* 2012; 41(7):993-1000.

177. Iacono C, Verlato G, Ruzzenente A, et al. Systematic review of central pancreatectomy and meta-analysis of central versus distal pancreatectomy. *Br J Surg* 2013; 100(7):873-85.
178. Hansen K, Singer DB. Asplenic-hyposplenic overwhelming sepsis: postsplenectomy sepsis revisited. *Pediatr Dev Pathol* 2001; 4(2):105-21.
179. Bisharat N, Omari H, Lavi I, et al. Risk of infection and death among post-splenectomy patients. *J Infect* 2001; 43(3):182-6.
180. Strasberg SM, Fields R. Left-sided pancreatic cancer: distal pancreatectomy and its variants: radical antegrade modular pancreatosplenectomy and distal pancreatectomy with celiac axis resection. *Cancer J* 2012; 18(6):562-70.
181. Jing W, Zhu G, Hu X, et al. Distal pancreatectomy with en bloc celiac axis resection for the treatment of locally advanced pancreatic body and tail cancer. *Hepatogastroenterology* 2013; 60(121):187-90.
182. Hirano S, Kondo S, Hara T, et al. Distal pancreatectomy with en bloc celiac axis resection for locally advanced pancreatic body cancer: long-term results. *Ann Surg* 2007; 246(1):46-51.
183. Miura T, Hirano S, Nakamura T, et al. A new preoperative prognostic scoring system to predict prognosis in patients with locally advanced pancreatic body cancer who undergo distal pancreatectomy with en bloc celiac axis resection: a retrospective cohort study. *Surgery* 2014; 155(3):457-67.
184. Turnbull RB, Jr., Kyle K, Watson FR, et al. Cancer of the colon: the influence of the no-touch isolation technic on survival rates. *Ann Surg* 1967; 166(3):420-7.
185. Wiggers T, Jeekel J, Arends JW, et al. No-touch isolation technique in colon cancer: a controlled prospective trial. *Br J Surg* 1988; 75(5):409-15.
186. Kobayashi S, Asano T, Ochiai T. A proposal of no-touch isolation technique in pancreatoduodenectomy for perampullary carcinomas. *Hepatogastroenterology* 2001; 48(38):372-4.
187. Hirota M, Shimada S, Yamamoto K, et al. Pancreatectomy using the no-touch isolation technique followed by extensive intraoperative peritoneal lavage to prevent cancer cell dissemination: a pilot study. *JOP* 2005; 6(2):143-51.
188. Hirota M, Kanemitsu K, Takamori H, et al. Pancreatoduodenectomy using a no-touch isolation technique. *Am J Surg* 2010; 199(5):e65-8.
189. Chakravarty KD, Hsu JT, Liu KH, et al. Prognosis and feasibility of en-bloc vascular resection in stage II pancreatic adenocarcinoma. *World J Gastroenterol* 2010; 16(8):997-1002.
190. Murakami Y, Uemura K, Sudo T, et al. Benefit of portal or superior mesenteric vein resection with adjuvant chemotherapy for patients with pancreatic head carcinoma. *J Surg Oncol* 2013; 107(4):414-21.
191. Moldovan SC, Moldovan AM, Dumitraescu T, et al. The advantages of retropancreatic vascular dissection for pancreatic head cancer with portal/superior mesenteric vein invasion: posterior approach pancreaticoduodenectomy technique and the mesopancreas theory. *Chirurgia (Bucur)* 2012; 107(5):571-8.
192. Muller SA, Hartel M, Mehrabi A, et al. Vascular resection in pancreatic cancer surgery: survival determinants. *J Gastrointest Surg* 2009; 13(4):784-92.
193. Kaneoka Y, Yamaguchi A, Isogai M. Portal or superior mesenteric vein resection for pancreatic head adenocarcinoma: prognostic value of the length of venous resection. *Surgery* 2009; 145(4):417-25.

194. van Geenen RC, ten Kate FJ, de Wit LT, et al. Segmental resection and wedge excision of the portal or superior mesenteric vein during pancreatoduodenectomy. *Surgery* 2001; 129(2):158-63.
195. Riediger H, Makowiec F, Fischer E, et al. Postoperative morbidity and long-term survival after pancreaticoduodenectomy with superior mesenterico-portal vein resection. *J Gastrointest Surg* 2006; 10(8):1106-15.
196. Toomey P, Hernandez J, Morton C, et al. Resection of portovenous structures to obtain microscopically negative margins during pancreaticoduodenectomy for pancreatic adenocarcinoma is worthwhile. *Am Surg* 2009; 75(9):804-9; discussion 809-10.
197. Chua TC, Saxena A. Extended pancreaticoduodenectomy with vascular resection for pancreatic cancer: a systematic review. *J Gastrointest Surg* 2010; 14(9):1442-52.
198. John BJ, Naik P, Ironside A, et al. Redefining the R1 resection for pancreatic ductal adenocarcinoma: tumour lymph nodal burden and lymph node ratio are the only prognostic factors associated with survival. *HPB (Oxford)* 2013; 15(9):674-80.
199. Jamieson NB, Foulis AK, Oien KA, et al. Positive mobilization margins alone do not influence survival following pancreatoduodenectomy for pancreatic ductal adenocarcinoma. *Ann Surg* 2010; 251(6):1003-10.
200. Menon KV, Gomez D, Smith AM, et al. Impact of margin status on survival following pancreatoduodenectomy for cancer: the Leeds Pathology Protocol (LEEPP). *HPB (Oxford)* 2009; 11(1):18-24.
201. Campbell F, Smith RA, Whelan P, et al. Classification of R1 resections for pancreatic cancer: the prognostic relevance of tumour involvement within 1 mm of a resection margin. *Histopathology* 2009; 55(3):277-83.
202. Fusai G, Warnaar N, Sabin CA, et al. Outcome of R1 resection in patients undergoing pancreatoduodenectomy for pancreatic cancer. *Eur J Surg Oncol* 2008; 34(12):1309-15.
203. Esposito I, Kleeff J, Bergmann F, et al. Most pancreatic cancer resections are R1 resections. *Ann Surg Oncol* 2008; 15(6):1651-60.
204. Verbeke CS, Leitch D, Menon KV, et al. Redefining the R1 resection in pancreatic cancer. *Br J Surg* 2006; 93(10):1232-7.
205. Turrini O, Ewald J, Barbier L, et al. Should the portal vein be routinely resected during pancreaticoduodenectomy for adenocarcinoma? *Ann Surg* 2013; 257(4):726-30.
206. Mollberg N, Rahbari NN, Koch M, et al. Arterial resection during pancreatectomy for pancreatic cancer: a systematic review and meta-analysis. *Ann Surg* 2011; 254(6):882-93.
207. Pai M, Valek V, Tomas A, et al. Percutaneous Intraductal Radiofrequency Ablation for Clearance of Occluded Metal Stent in Malignant Biliary Obstruction: Feasibility and Early Results. *Cardiovasc Intervent Radiol* 2013.
208. Varshney S, Sewkani A, Sharma S, et al. Radiofrequency ablation of unresectable pancreatic carcinoma: feasibility, efficacy and safety. *JOP* 2006; 7(1):74-8.
209. Carrafiello G, Ierardi AM, Fontana F, et al. Microwave ablation of pancreatic head cancer: safety and efficacy. *J Vasc Interv Radiol* 2013; 24(10):1513-20.
210. Steel AW, Postgate AJ, Khorsandi S, et al. Endoscopically applied radiofrequency ablation appears to be safe in the treatment of malignant biliary obstruction. *Gastrointest Endosc* 2011; 73(1):149-53.

211. Lygidakis NJ, Sharma SK, Papastratis P, et al. Microwave ablation in locally advanced pancreatic carcinoma--a new look. *Hepatogastroenterology* 2007; 54(77):1305-10.
212. Martin RC, 2nd, McFarland K, Ellis S, et al. Irreversible electroporation therapy in the management of locally advanced pancreatic adenocarcinoma. *J Am Coll Surg* 2012; 215(3):361-9.
213. Narayanan G, Hosein PJ, Arora G, et al. Percutaneous irreversible electroporation for downstaging and control of unresectable pancreatic adenocarcinoma. *J Vasc Interv Radiol* 2012; 23(12):1613-21.
214. Seufferlein T. Pancreatic adenocarcinoma: ESMO - ESDO Clinical Practice Guidelines for diagnosis, treatment and follow-up (vol 23, pg 33, 2012). *Annals of Oncology* 2013; 24:171-171.
215. Ryan DP. Adjuvant therapy for resected exocrine pancreatic cancer. In: Post TW, ed. UpToDate. Waltham, MA: UpToDate; 2014.
216. Neoptolemos JP, Dunn JA, Stocken DD, et al. Adjuvant chemoradiotherapy and chemotherapy in resectable pancreatic cancer: a randomised controlled trial. *Lancet* 2001; 358(9293):1576-85.
217. Oettle H, Post S, Neuhaus P, et al. Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing curative-intent resection of pancreatic cancer: a randomized controlled trial. *JAMA* 2007; 297(3):267-77.
218. Neoptolemos JP, Moore MJ, Cox TF, et al. Effect of adjuvant chemotherapy with fluorouracil plus folinic acid or gemcitabine vs observation on survival in patients with resected periampullary adenocarcinoma: the ESPAC-3 periampullary cancer randomized trial. *JAMA* 2012; 308(2):147-56.
219. Iacobuzio-Donahue CA, Fu B, Yachida S, et al. DPC4 gene status of the primary carcinoma correlates with patterns of failure in patients with pancreatic cancer. *J Clin Oncol* 2009; 27(11):1806-13.
220. Kooby DA, Gillespie TW, Liu Y, et al. Impact of adjuvant radiotherapy on survival after pancreatic cancer resection: an appraisal of data from the national cancer data base. *Ann Surg Oncol* 2013; 20(11):3634-42.
221. Forgensen J. Resected adenocarcinoma of the pancreas--616 patients: results, outcomes, and prognostic indicators. *J Gastrointest Surg* 2001; 5(6):681; author reply 681.
222. Hsu CC, Herman JM, Corsini MM, et al. Adjuvant chemoradiation for pancreatic adenocarcinoma: the Johns Hopkins Hospital-Mayo Clinic collaborative study. *Ann Surg Oncol* 2010; 17(4):981-90.
223. Sohn TA, Yeo CJ, Cameron JL, et al. Resected adenocarcinoma of the pancreas-616 patients: results, outcomes, and prognostic indicators. *J Gastrointest Surg* 2000; 4(6):567-79.
224. Schneider BJ, Ben-Josef E, McGinn CJ, et al. Capecitabine and radiation therapy preceded and followed by combination chemotherapy in advanced pancreatic cancer. *Int J Radiat Oncol Biol Phys* 2005; 63(5):1325-30.
225. Stokes JB, Nolan NJ, Stelow EB, et al. Preoperative capecitabine and concurrent radiation for borderline resectable pancreatic cancer. *Ann Surg Oncol* 2011; 18(3):619-27.
226. Mukherjee S, Hurt CN, Bridgewater J, et al. Gemcitabine-based or capecitabine-based chemoradiotherapy for locally advanced pancreatic cancer (SCALOP): a multicentre, randomised, phase 2 trial. *Lancet Oncol* 2013; 14(4):317-26.

227. Conroy T, Desseigne F, Ychou M, et al. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *N Engl J Med* 2011; 364(19):1817-25.
228. Vaccaro V, Sperduti I, Milella M. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *N Engl J Med* 2011; 365(8):768-9; author reply 769.
229. Herrmann R, Bodoky G, Ruhstaller T, et al. Gemcitabine plus capecitabine compared with gemcitabine alone in advanced pancreatic cancer: a randomized, multicenter, phase III trial of the Swiss Group for Clinical Cancer Research and the Central European Cooperative Oncology Group. *J Clin Oncol* 2007; 25(16):2212-7.
230. Hoff V. Results of a randomized phase III trial (MPACT) of weekly nab-paclitaxel plus gemcitabine versus gemcitabine alone for patients with metastatic adenocarcinoma of the pancreas with PET and CA19-9 correlates. *J Clin Oncol* 2013; 31.
231. Rothenberg ML, Moore MJ, Cripps MC, et al. A phase II trial of gemcitabine in patients with 5-FU-refractory pancreas cancer. *Ann Oncol* 1996; 7(4):347-53.
232. Burris HA, 3rd, Moore MJ, Andersen J, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol* 1997; 15(6):2403-13.
233. Yoo C, Hwang JY, Kim JE, et al. A randomised phase II study of modified FOLFIRI.3 vs modified FOLFOX as second-line therapy in patients with gemcitabine-refractory advanced pancreatic cancer. *Br J Cancer* 2009; 101(10):1658-63.
234. Demols A, Peeters M, Polus M, et al. Gemcitabine and oxaliplatin (GEMOX) in gemcitabine refractory advanced pancreatic adenocarcinoma: a phase II study. *Br J Cancer* 2006; 94(4):481-5.
235. Walker EJ, Ko AH. Beyond first-line chemotherapy for advanced pancreatic cancer: an expanding array of therapeutic options? *World J Gastroenterol* 2014; 20(9):2224-36.
236. Krishnan S, Rana V, Janjan NA, et al. Induction chemotherapy selects patients with locally advanced, unresectable pancreatic cancer for optimal benefit from consolidative chemoradiation therapy. *Cancer* 2007; 110(1):47-55.
237. Arvold ND, Ryan DP, Niemierko A, et al. Long-term outcomes of neoadjuvant chemotherapy before chemoradiation for locally advanced pancreatic cancer. *Cancer* 2012; 118(12):3026-35.
238. Huguet F, Andre T, Hammel P, et al. Impact of chemoradiotherapy after disease control with chemotherapy in locally advanced pancreatic adenocarcinoma in GERCOR phase II and III studies. *J Clin Oncol* 2007; 25(3):326-31.
239. Ryan D. Initial chemotherapy and radiation for nonmetastatic locally advanced unresectable, borderline resectable, and potentially resectable exocrine pancreatic cancer. Waltham, MA: UpToDate, 2014.
240. Bruns CJ, Solorzano CC, Harbison MT, et al. Blockade of the epidermal growth factor receptor signaling by a novel tyrosine kinase inhibitor leads to apoptosis of endothelial cells and therapy of human pancreatic carcinoma. *Cancer Res* 2000; 60(11):2926-35.
241. Moore MJ, Goldstein D, Hamm J, et al. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase

- III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol* 2007; 25(15):1960-6.
242. Itakura J, Ishiwata T, Shen B, et al. Concomitant over-expression of vascular endothelial growth factor and its receptors in pancreatic cancer. *Int J Cancer* 2000; 85(1):27-34.
 243. Van Cutsem E, Vervenne WL, Bennouna J, et al. Phase III trial of bevacizumab in combination with gemcitabine and erlotinib in patients with metastatic pancreatic cancer. *J Clin Oncol* 2009; 27(13):2231-7.
 244. Bos JL. ras oncogenes in human cancer: a review. *Cancer Res* 1989; 49(17):4682-9.
 245. Alberts SR, Schroeder M, Erlichman C, et al. Gemcitabine and ISIS-2503 for patients with locally advanced or metastatic pancreatic adenocarcinoma: a North Central Cancer Treatment Group phase II trial. *J Clin Oncol* 2004; 22(24):4944-50.
 246. James GL, Goldstein JL, Brown MS, et al. Benzodiazepine peptidomimetics: potent inhibitors of Ras farnesylation in animal cells. *Science* 1993; 260(5116):1937-42.
 247. Van Cutsem E, van de Velde H, Karasek P, et al. Phase III trial of gemcitabine plus tipifarnib compared with gemcitabine plus placebo in advanced pancreatic cancer. *J Clin Oncol* 2004; 22(8):1430-8.
 248. Sommerville CA, Limongelli P, Pai M, et al. Survival analysis after pancreatic resection for ampullary and pancreatic head carcinoma: an analysis of clinicopathological factors. *J Surg Oncol* 2009; 100(8):651-6.
 249. Ballehaninna UK, Chamberlain RS. Serum CA 19-9 as a Biomarker for Pancreatic Cancer-A Comprehensive Review. *Indian J Surg Oncol* 2011; 2(2):88-100.
 250. Mahmoud FA, Rivera NI. The role of C-reactive protein as a prognostic indicator in advanced cancer. *Curr Oncol Rep* 2002; 4(3):250-5.
 251. Slidell MB, Chang DC, Cameron JL, et al. Impact of total lymph node count and lymph node ratio on staging and survival after pancreatectomy for pancreatic adenocarcinoma: a large, population-based analysis. *Ann Surg Oncol* 2008; 15(1):165-74.
 252. Belardinilli F, Capalbo C, Buffone A, et al. Validation of the Ion Torrent PGM sequencing for the prospective routine molecular diagnostic of colorectal cancer. *Clin Biochem* 2015.
 253. McClure R, Mai M, McClure S. High-throughput sequencing using the Ion Torrent personal genome machine for clinical evaluation of somatic hypermutation status in chronic lymphocytic leukemia. *J Mol Diagn* 2015; 17(2):145-54.
 254. Waggott D, Chu K, Yin S, et al. NanoStringNorm: an extensible R package for the pre-processing of NanoString mRNA and miRNA data. *Bioinformatics* 2012; 28(11):1546-8.
 255. Ashworth T. A case of cancer in which cells similar to those in the tumours were seen in the blood after death. *Aust Med J* 1869; 14(3):146-149.
 256. Cen P, Ni X, Yang J, et al. Circulating tumor cells in the diagnosis and management of pancreatic cancer. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* 2012; 1826(2):350-356.
 257. Riethdorf S, Fritsche H, Müller V, et al. Detection of Circulating Tumor Cells in Peripheral Blood of Patients with Metastatic Breast Cancer: A Validation

- Study of the CellSearch System. *Clinical Cancer Research* 2007; 13(3):920-928.
258. Jiao LR, Apostolopoulos C, Jacob J, et al. Unique localization of circulating tumor cells in patients with hepatic metastases. *J Clin Oncol* 2009; 27(36):6160-5.
 259. Shaw JA, Brown J, Coombes RC, et al. Circulating tumor cells and plasma DNA analysis in patients with indeterminate early or metastatic breast cancer. *Biomark Med* 2011; 5(1):87-91.
 260. Jacob J, Krell J, Castellano L, et al. Determination of cut-offs for circulating tumor cell measurement in metastatic cancer. *Expert Rev Anticancer Ther* 2011; 11(9):1345-50.
 261. Cristofanilli M, Hayes DF, Budd GT, et al. Circulating Tumor Cells: A Novel Prognostic Factor for Newly Diagnosed Metastatic Breast Cancer. *Journal of Clinical Oncology* 2005; 23(7):1420-1430.
 262. Rack B, Schindlbeck C, Schneeweiss A, et al. Prognostic relevance of circulating tumor cells (CTCs) in peripheral blood of breast cancer patients before and after adjuvant chemotherapy: The German SUCCESS-Trial. *J Clin Oncol* 2008; 26(15S):503.
 263. Rahbari NN, Aigner M, Thorlund K, et al. Meta-analysis Shows That Detection of Circulating Tumor Cells Indicates Poor Prognosis in Patients With Colorectal Cancer. *Gastroenterology* 2010; 138(5):1714-1726.e13.
 264. Cohen SJ, Punt CJA, Iannotti N, et al. Relationship of Circulating Tumor Cells to Tumor Response, Progression-Free Survival, and Overall Survival in Patients With Metastatic Colorectal Cancer. *Journal of Clinical Oncology* 2008; 26(19):3213-3221.
 265. de Bono JS, Scher HI, Montgomery RB, et al. Circulating Tumor Cells Predict Survival Benefit from Treatment in Metastatic Castration-Resistant Prostate Cancer. *Clinical Cancer Research* 2008; 14(19):6302-6309.
 266. Hou JM, Greystoke A, Lancashire L, et al. Evaluation of circulating tumor cells and serological cell death biomarkers in small cell lung cancer patients undergoing chemotherapy. *Am J Pathol* 2009; 175(2):808.
 267. Ren C, Han C, Zhang J, et al. Detection of apoptotic circulating tumor cells in advanced pancreatic cancer following 5-fluorouracil chemotherapy. *Cancer Biol Ther* 2011; 12(8):700-706.
 268. de Albuquerque A, Kubisch I, Breier G, et al. Multimarker Gene Analysis of Circulating Tumor Cells in Pancreatic Cancer Patients: A Feasibility Study. *Oncology* 2012; 82(1):3-10.
 269. Yu M, Ting DT, Stott SL, et al. RNA sequencing of pancreatic circulating tumour cells implicates WNT signalling in metastasis. *Nature* 2012; 487(7408):510-3.
 270. Han L, Chen W, Zhao Q. Prognostic value of circulating tumor cells in patients with pancreatic cancer: a meta-analysis. *Tumour Biol* 2014; 35(3):2473-80.
 271. Bissolati M, Sandri MT, Burtulo G, et al. Portal vein-circulating tumor cells predict liver metastases in patients with resectable pancreatic cancer. *Tumour Biol* 2015; 36(2):991-6.
 272. Mandel P, Metais P. [Not Available]. *C R Seances Soc Biol Fil* 1948; 142(3-4):241-3.
 273. Leon SA, Shapiro B, Sklaroff DM, et al. Free DNA in the serum of cancer patients and the effect of therapy. *Cancer Res* 1977; 37(3):646-50.

274. Stroun M, Anker P, Maurice P, et al. Neoplastic characteristics of the DNA found in the plasma of cancer patients. *Oncology* 1989; 46(5):318-22.
275. Sorenson GD, Pribish DM, Valone FH, et al. Soluble normal and mutated DNA sequences from single-copy genes in human blood. *Cancer Epidemiol Biomarkers Prev* 1994; 3(1):67-71.
276. Vasioukhin V, Anker P, Maurice P, et al. Point mutations of the N-ras gene in the blood plasma DNA of patients with myelodysplastic syndrome or acute myelogenous leukaemia. *Br J Haematol* 1994; 86(4):774-9.
277. Nawroz H, Koch W, Anker P, et al. Microsatellite alterations in serum DNA of head and neck cancer patients. *Nat Med* 1996; 2(9):1035-7.
278. Stroun M, Anker P, Lyautey J, et al. Isolation and characterization of DNA from the plasma of cancer patients. *Eur J Cancer Clin Oncol* 1987; 23(6):707-12.
279. Jahr S, Hentze H, Englisch S, et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Res* 2001; 61(4):1659-65.
280. Jung K, Fleischhacker M, Rabien A. Cell-free DNA in the blood as a solid tumor biomarker—A critical appraisal of the literature. *Clinica Chimica Acta* 2010; 411(21–22):1611-1624.
281. Diehl F, Li M, Dressman D, et al. Detection and quantification of mutations in the plasma of patients with colorectal tumors. *Proc Natl Acad Sci U S A* 2005; 102(45):16368-73.
282. Atamaniuk J, Vidotto C, Tschan H, et al. Increased concentrations of cell-free plasma DNA after exhaustive exercise. *Clin Chem* 2004; 50(9):1668-70.
283. Zhang R, Shao F, Wu X, et al. Value of quantitative analysis of circulating cell free DNA as a screening tool for lung cancer: a meta-analysis. *Lung Cancer* 2010; 69(2):225-31.
284. Rainer TH, Lam NY. Circulating nucleic acids and critical illness. *Ann N Y Acad Sci* 2006; 1075:271-7.
285. Suzuki N, Kamataki A, Yamaki J, et al. Characterization of circulating DNA in healthy human plasma. *Clin Chim Acta* 2008; 387(1-2):55-8.
286. Morozkin ES, Babochkina TI, Vlassov VV, et al. The effect of protein transport inhibitors on the production of extracellular DNA. *Ann N Y Acad Sci* 2008; 1137:31-5.
287. Stroun M, Maurice P, Vasioukhin V, et al. The origin and mechanism of circulating DNA. *Ann N Y Acad Sci* 2000; 906:161-8.
288. Garcia-Olmo DC, Samos J, Picazo MG, et al. Release of cell-free DNA into the bloodstream leads to high levels of non-tumor plasma DNA during tumor progression in rats. *Cancer Lett* 2008; 272(1):133-40.
289. Schwarzenbach H, Alix-Panabieres C, Muller I, et al. Cell-free tumor DNA in blood plasma as a marker for circulating tumor cells in prostate cancer. *Clin Cancer Res* 2009; 15(3):1032-8.
290. Lo YM, Zhang J, Leung TN, et al. Rapid clearance of fetal DNA from maternal plasma. *Am J Hum Genet* 1999; 64(1):218-24.
291. Fleischhacker M, Schmidt B. Circulating nucleic acids (CNAs) and cancer--a survey. *Biochim Biophys Acta* 2007; 1775(1):181-232.
292. Chelobanov BP, Laktionov PP, Vlasov VV. Proteins involved in binding and cellular uptake of nucleic acids. *Biochemistry (Mosc)* 2006; 71(6):583-96.

293. Rykova EY, Morozkin ES, Ponomaryova AA, et al. Cell-free and cell-bound circulating nucleic acid complexes: mechanisms of generation, concentration and content. *Expert Opin Biol Ther* 2012; 12 Suppl 1:S141-53.
294. Jung K, Fleischhacker M, Rabien A. Cell-free DNA in the blood as a solid tumor biomarker--a critical appraisal of the literature. *Clin Chim Acta* 2010; 411(21-22):1611-24.
295. Zauber NP, Wang C, Lee PS, et al. Ki-ras gene mutations, LOH of the APC and DCC genes, and microsatellite instability in primary colorectal carcinoma are not associated with micrometastases in pericolic lymph nodes or with patients' survival. *J Clin Pathol* 2004; 57(9):938-42.
296. Ogino S, Nosho K, Irahara N, et al. Prognostic significance and molecular associations of 18q loss of heterozygosity: a cohort study of microsatellite stable colorectal cancers. *J Clin Oncol* 2009; 27(27):4591-8.
297. Bertagnolli MM, Redston M, Compton CC, et al. Microsatellite instability and loss of heterozygosity at chromosomal location 18q: prospective evaluation of biomarkers for stages II and III colon cancer--a study of CALGB 9581 and 89803. *J Clin Oncol* 2011; 29(23):3153-62.
298. Watanabe T, Wu TT, Catalano PJ, et al. Molecular predictors of survival after adjuvant chemotherapy for colon cancer. *N Engl J Med* 2001; 344(16):1196-206.
299. Choi SW, Lee KJ, Bae YA, et al. Genetic classification of colorectal cancer based on chromosomal loss and microsatellite instability predicts survival. *Clin Cancer Res* 2002; 8(7):2311-22.
300. Alazzouzi H, Alhopuro P, Salovaara R, et al. SMAD4 as a prognostic marker in colorectal cancer. *Clin Cancer Res* 2005; 11(7):2606-11.
301. Feinberg AP, Cui H, Ohlsson R. DNA methylation and genomic imprinting: insights from cancer into epigenetic mechanisms. *Semin Cancer Biol* 2002; 12(5):389-98.
302. Board RE, Knight L, Greystoke A, et al. DNA methylation in circulating tumour DNA as a biomarker for cancer. *Biomark Insights* 2008; 2:307-19.
303. Uemura T, Hibi K, Kaneko T, et al. Detection of K-ras mutations in the plasma DNA of pancreatic cancer patients. *J Gastroenterol* 2004; 39(1):56-60.
304. Dabritz J, Preston R, Hanfler J, et al. Follow-up study of K-ras mutations in the plasma of patients with pancreatic cancer: correlation with clinical features and carbohydrate antigen 19-9. *Pancreas* 2009; 38(5):534-41.
305. Dai MH, Zhao YP, Cai LX, et al. [Combined detection of K-ras mutation and CA 19-9 level in plasma of patients with pancreatic cancer]. *Zhonghua Wai Ke Za Zhi* 2003; 41(5):332-5.
306. Maire F, Micard S, Hammel P, et al. Differential diagnosis between chronic pancreatitis and pancreatic cancer: value of the detection of KRAS2 mutations in circulating DNA. *Br J Cancer* 2002; 87(5):551-4.
307. Kinugasa H, Nouse K, Miyahara K, et al. Detection of K-ras gene mutation by liquid biopsy in patients with pancreatic cancer. *Cancer* 2015.
308. Chen H, Tu H, Meng ZQ, et al. K-ras mutational status predicts poor prognosis in unresectable pancreatic cancer. *Eur J Surg Oncol* 2010; 36(7):657-62.
309. Dabritz J, Preston R, Hanfler J, et al. K-ras mutations in the plasma correspond to computed tomographic findings in patients with pancreatic cancer. *Pancreas* 2012; 41(2):323-5.

310. Singh N, Gupta S, Pandey RM, et al. High levels of cell-free circulating nucleic acids in pancreatic cancer are associated with vascular encasement, metastasis and poor survival. *Cancer Invest* 2015; 33(3):78-85.
311. Park JW, Baek IH, Kim YT. Preliminary study analyzing the methylated genes in the plasma of patients with pancreatic cancer. *Scand J Surg* 2012; 101(1):38-44.
312. Liggett T, Melnikov A, Yi QL, et al. Differential methylation of cell-free circulating DNA among patients with pancreatic cancer versus chronic pancreatitis. *Cancer* 2010; 116(7):1674-80.
313. Pedersen KS, Bamlet WR, Oberg AL, et al. Leukocyte DNA methylation signature differentiates pancreatic cancer patients from healthy controls. *PLoS One* 2011; 6(3):e18223.
314. Melnikov AA, Scholtens D, Talamonti MS, et al. Methylation profile of circulating plasma DNA in patients with pancreatic cancer. *J Surg Oncol* 2009; 99(2):119-22.
315. Frampton AE, Castellano L, Colombo T, et al. MicroRNAs Co-operatively Inhibit a Network of Tumor Suppressor Genes to Promote Pancreatic Tumor Growth and Progression. *Gastroenterology* 2013.
316. Kong X, Du Y, Wang G, et al. Detection of Differentially Expressed microRNAs in Serum of Pancreatic Ductal Adenocarcinoma Patients: miR-196a Could Be a Potential Marker for Poor Prognosis. *Dig Dis Sci* 2010.
317. Carlsen AL, Joergensen MT, Knudsen S, et al. Cell-free plasma microRNA in pancreatic ductal adenocarcinoma and disease controls. *Pancreas* 2013; 42(7):1107-13.
318. Bauer AS, Keller A, Costello E, et al. Diagnosis of pancreatic ductal adenocarcinoma and chronic pancreatitis by measurement of microRNA abundance in blood and tissue. *PLoS One* 2012; 7(4):e34151.
319. Abue M, Yokoyama M, Shibuya R, et al. Circulating miR-483-3p and miR-21 is highly expressed in plasma of pancreatic cancer. *Int J Oncol* 2015; 46(2):539-47.
320. Komatsu S, Ichikawa D, Miyamae M, et al. Malignant potential in pancreatic neoplasm; new insights provided by circulating miR-223 in plasma. *Expert Opin Biol Ther* 2015; 15(6):773-85.
321. Ganepola GA, Rutledge JR, Suman P, et al. Novel blood-based microRNA biomarker panel for early diagnosis of pancreatic cancer. *World J Gastrointest Oncol* 2014; 6(1):22-33.
322. Wang WS, Liu LX, Li GP, et al. Combined Serum CA19-9 and miR-27a-3p in Peripheral Blood Mononuclear Cells to Diagnose Pancreatic Cancer. *Cancer Prev Res (Phila)* 2013.
323. Liu R, Chen X, Du Y, et al. Serum microRNA expression profile as a biomarker in the diagnosis and prognosis of pancreatic cancer. *Clin Chem* 2012; 58(3):610-8.
324. Morimura R, Komatsu S, Ichikawa D, et al. Novel diagnostic value of circulating miR-18a in plasma of patients with pancreatic cancer. *Br J Cancer* 2011; 105(11):1733-40.
325. Liu J, Gao J, Du Y, et al. Combination of plasma microRNAs with serum CA19-9 for early detection of pancreatic cancer. *Int J Cancer* 2012; 131(3):683-91.

326. LaConti JJ, Shivapurkar N, Preet A, et al. Tissue and serum microRNAs in the Kras(G12D) transgenic animal model and in patients with pancreatic cancer. *PLoS One* 2011; 6(6):e20687.
327. Li A, Omura N, Hong SM, et al. Pancreatic cancers epigenetically silence SIP1 and hypomethylate and overexpress miR-200a/200b in association with elevated circulating miR-200a and miR-200b levels. *Cancer Res* 2010; 70(13):5226-37.
328. Wang J, Chen J, Chang P, et al. MicroRNAs in plasma of pancreatic ductal adenocarcinoma patients as novel blood-based biomarkers of disease. *Cancer Prev Res (Phila)* 2009; 2(9):807-13.
329. Kong X, Du Y, Wang G, et al. Detection of differentially expressed microRNAs in serum of pancreatic ductal adenocarcinoma patients: miR-196a could be a potential marker for poor prognosis. *Dig Dis Sci* 2011; 56(2):602-9.
330. Biankin AV, Waddell N, Kassahn KS, et al. Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. *Nature* 2012.
331. Wang R, Lagakos SW, Gray RJ. Testing and interval estimation for two-sample survival comparisons with small sample sizes and unequal censoring. *Biostatistics* 2010; 11(4):676-92.
332. Barnes JP. Physiologic resection of the right colon. *Surg Gynecol Obstet* 1952; 94(6):722-6.
333. Hirota M, Hashimoto D, Ishiko T, et al. Distal pancreatectomy using a no-touch isolation technique. *Scand J Surg* 2012; 101(3):156-9.
334. de Bono JS, Scher HI, Montgomery RB, et al. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. *Clin Cancer Res* 2008; 14(19):6302-9.
335. O'Flaherty JD, Gray S, Richard D, et al. Circulating tumour cells, their role in metastasis and their clinical utility in lung cancer. *Lung Cancer* 2012; 76(1):19-25.
336. Onstenk W, Gratama JW, Foekens JA, et al. Towards a personalized breast cancer treatment approach guided by circulating tumor cell (CTC) characteristics. *Cancer Treat Rev* 2013.
337. Schindlbeck C, Andergassen U, Hofmann S, et al. Comparison of circulating tumor cells (CTC) in peripheral blood and disseminated tumor cells in the bone marrow (DTC-BM) of breast cancer patients. *J Cancer Res Clin Oncol* 2013; 139(6):1055-62.
338. Munzone E, Botteri E, Sandri MT, et al. Prognostic value of circulating tumor cells according to immunohistochemically defined molecular subtypes in advanced breast cancer. *Clin Breast Cancer* 2012; 12(5):340-6.
339. Gazzaniga P, Gradilone A, de Berardinis E, et al. Prognostic value of circulating tumor cells in nonmuscle invasive bladder cancer: a CellSearch analysis. *Ann Oncol* 2012; 23(9):2352-6.
340. Naito T, Tanaka F, Ono A, et al. Prognostic impact of circulating tumor cells in patients with small cell lung cancer. *J Thorac Oncol* 2012; 7(3):512-9.
341. Shamash J, Jacob J, Agrawal S, et al. Whole blood stem cell reinfusion and escalated dose melphalan in castration-resistant prostate cancer: a phase 1 study. *Clin Cancer Res* 2012; 18(8):2352-9.
342. Attard G, Crespo M, Lim AC, et al. Reporting the capture efficiency of a filter-based microdevice: a CTC is not a CTC unless it is CD45 negative--letter. *Clin Cancer Res* 2011; 17(9):3048-9; author reply 3050.

343. Muller V, Alix-Panabieres C, Pantel K. Insights into minimal residual disease in cancer patients: implications for anti-cancer therapies. *Eur J Cancer* 2010; 46(7):1189-97.
344. Deneve E, Riethdorf S, Ramos J, et al. Capture of Viable Circulating Tumor Cells in the Liver of Colorectal Cancer Patients. *Clin Chem* 2013.
345. Khoja L, Backen A, Sloane R, et al. A pilot study to explore circulating tumour cells in pancreatic cancer as a novel biomarker. *Br J Cancer* 2012; 106(3):508-16.
346. Rossi E, Basso U, Celadin R, et al. M30 neoepitope expression in epithelial cancer: quantification of apoptosis in circulating tumor cells by CellSearch analysis. *Clin Cancer Res* 2010; 16(21):5233-43.
347. Li C, Heidt DG, Dalerba P, et al. Identification of pancreatic cancer stem cells. *Cancer Res* 2007; 67(3):1030-7.
348. Hermann PC, Huber SL, Herrler T, et al. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell Stem Cell* 2007; 1(3):313-23.
349. Matsuda Y, Naito Z, Kawahara K, et al. Nestin is a novel target for suppressing pancreatic cancer cell migration, invasion and metastasis. *Cancer Biol Ther* 2011; 11(5):512-23.
350. Herreros-Villanueva M, Zubia-Olascoaga A, Bujanda L. c-Met in pancreatic cancer stem cells: therapeutic implications. *World J Gastroenterol* 2012; 18(38):5321-3.
351. Yu M, Ting DT, Stott SL, et al. RNA sequencing of pancreatic circulating tumour cells implicates WNT signalling in metastasis. *Nature* 2012; advance online publication.
352. Bidard FC, Huguet F, Louvet C, et al. Circulating tumor cells in locally advanced pancreatic adenocarcinoma: the ancillary CirCe 07 study to the LAP 07 trial. *Ann Oncol* 2013.
353. Ren C, Han C, Zhang J, et al. Detection of apoptotic circulating tumor cells in advanced pancreatic cancer following 5-fluorouracil chemotherapy. *Cancer Biol Ther* 2011; 12(8):700-6.
354. Kurihara T, Itoi T, Sofuni A, et al. Detection of circulating tumor cells in patients with pancreatic cancer: a preliminary result. *J Hepatobiliary Pancreat Surg* 2008; 15(2):189-95.
355. Tjensvoll K, Nordgard O, Smaaland R. Circulating tumor cells in pancreatic cancer patients: Methods of detection and clinical implications. *Int J Cancer* 2013.
356. Negin B, Meropol N, Alpaugh R, et al. Characterization and prognostic significance of circulating tumor cells in the peripheral blood of patients with metastatic pancreatic cancer. ASCO Meeting Abstr, Vol. 28, 2010. pp. 4127.
357. Dindo D, Demartines N, Clavien PA. Classification of surgical complications: a new proposal with evaluation in a cohort of 6336 patients and results of a survey. *Ann Surg* 2004; 240(2):205-13.
358. Bhayani NH, Miller JL, Ortenzi G, et al. Perioperative outcomes of pancreaticoduodenectomy compared to total pancreatectomy for neoplasia. *J Gastrointest Surg* 2014; 18(3):549-54.
359. Verbeke CS, Menon KV. Redefining resection margin status in pancreatic cancer. *HPB (Oxford)* 2009; 11(4):282-9.

360. Jamieson NB, Chan NI, Foulis AK, et al. The prognostic influence of resection margin clearance following pancreaticoduodenectomy for pancreatic ductal adenocarcinoma. *J Gastrointest Surg* 2013; 17(3):511-21.
361. Trede M, Schwall G, Saeger HD. Survival after pancreatoduodenectomy. 118 consecutive resections without an operative mortality. *Ann Surg* 1990; 211(4):447-58.
362. Gall FP, Kessler H, Hermanek P. Surgical treatment of ductal pancreatic carcinoma. *Eur J Surg Oncol* 1991; 17(2):173-81.
363. Millikan KW, Deziel DJ, Silverstein JC, et al. Prognostic factors associated with resectable adenocarcinoma of the head of the pancreas. *Am Surg* 1999; 65(7):618-23; discussion 623-4.
364. Wenger FA, Peter F, Zieren J, et al. Prognosis factors in carcinoma of the head of the pancreas. *Dig Surg* 2000; 17(1):29-35.
365. Neoptolemos JP, Stocken DD, Dunn JA, et al. Influence of resection margins on survival for patients with pancreatic cancer treated by adjuvant chemoradiation and/or chemotherapy in the ESPAC-1 randomized controlled trial. *Ann Surg* 2001; 234(6):758-68.
366. Geer RJ, Brennan MF. Prognostic indicators for survival after resection of pancreatic adenocarcinoma. *Am J Surg* 1993; 165(1):68-72; discussion 72-3.
367. Raut CP, Tseng JF, Sun CC, et al. Impact of resection status on pattern of failure and survival after pancreaticoduodenectomy for pancreatic adenocarcinoma. *Ann Surg* 2007; 246(1):52-60.
368. Kulemann B, Hoepfner J, Wittel U, et al. Perioperative and long-term outcome after standard pancreaticoduodenectomy, additional portal vein and multivisceral resection for pancreatic head cancer. *J Gastrointest Surg* 2015; 19(3):438-44.
369. Popescu I, Dumitrascu T. Total meso-pancreas excision: key point of resection in pancreatic head adenocarcinoma. *Hepatogastroenterology* 2011; 58(105):202-7.
370. Kawabata Y, Tanaka T, Nishi T, et al. Appraisal of a total meso-pancreatoduodenum excision with pancreaticoduodenectomy for pancreatic head carcinoma. *Eur J Surg Oncol* 2012; 38(7):574-9.
371. Adham M, Singhirunnusorn J. Surgical technique and results of total mesopancreas excision (TMpE) in pancreatic tumors. *Eur J Surg Oncol* 2012; 38(4):340-5.
372. Mehlen P, Delloye-Bourgeois C, Chedotal A. Novel roles for Slits and netrins: axon guidance cues as anticancer targets? *Nat Rev Cancer* 2011; 11(3):188-97.
373. Hruban RH, Goggins M, Parsons J, et al. Progression Model for Pancreatic Cancer. *Clinical Cancer Research* 2000; 6(8):2969-2972.
374. Murphy SJ, Hart SN, Lima JF, et al. Genetic alterations associated with progression from pancreatic intraepithelial neoplasia to invasive pancreatic tumor. *Gastroenterology* 2013; 145(5):1098-1109 e1.
375. Griffin CA, Hruban RH, Morsberger LA, et al. Consistent chromosome abnormalities in adenocarcinoma of the pancreas. *Cancer Res* 1995; 55(11):2394-9.
376. Amato E, Molin MD, Mafficini A, et al. Targeted next-generation sequencing of cancer genes dissects the molecular profiles of intraductal papillary neoplasms of the pancreas. *J Pathol* 2014; 233(3):217-27.

377. Jones S, Zhang X, Parsons DW, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science* 2008; 321(5897):1801-6.
378. Wu R. Nucleotide sequence analysis of DNA. I. Partial sequence of the cohesive ends of bacteriophage lambda and 186 DNA. *J Mol Biol* 1970; 51(3):501-21.
379. Sanger F, Air GM, Barrell BG, et al. Nucleotide sequence of bacteriophage phi X174 DNA. *Nature* 1977; 265(5596):687-95.
380. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* 1977; 74(12):5463-7.
381. Prober JM, Trainor GL, Dam RJ, et al. A system for rapid DNA sequencing with fluorescent chain-terminating dideoxynucleotides. *Science* 1987; 238(4825):336-41.
382. Feldmann H, Aigle M, Aljinovic G, et al. Complete DNA sequence of yeast chromosome II. *EMBO J* 1994; 13(24):5795-809.
383. Schmutz J, Wheeler J, Grimwood J, et al. Quality assessment of the human genome sequence. *Nature* 2004; 429(6990):365-8.
384. Liu L, Li Y, Li S, et al. Comparison of next-generation sequencing systems. *J Biomed Biotechnol* 2012; 2012:251364.
385. Fujita S, Masago K, Takeshita J, et al. Validation of an Ion Torrent Sequencing Platform for the Detection of Gene Mutations in Biopsy Specimens from Patients with Non-Small-Cell Lung Cancer. *PLoS One* 2015; 10(6):e0130219.
386. Lin MT, Mosier SL, Thiess M, et al. Clinical validation of KRAS, BRAF, and EGFR mutation detection using next-generation sequencing. *Am J Clin Pathol* 2014; 141(6):856-66.
387. Markman B, Dienstmann R, Tabernero J. Targeting the PI3K/Akt/mTOR pathway--beyond rapalogs. *Oncotarget* 2010; 1(7):530-43.
388. Chang F, Steelman LS, Lee JT, et al. Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention. *Leukemia* 2003; 17(7):1263-93.
389. Verstrepen L, Beyaert R. Receptor proximal kinases in NF-kappaB signaling as potential therapeutic targets in cancer and inflammation. *Biochem Pharmacol* 2014; 92(4):519-29.
390. Morris JPt, Wang SC, Hebrok M. KRAS, Hedgehog, Wnt and the twisted developmental biology of pancreatic ductal adenocarcinoma. *Nat Rev Cancer* 2010; 10(10):683-95.
391. Collins MA, Brisset JC, Zhang Y, et al. Metastatic pancreatic cancer is dependent on oncogenic Kras in mice. *PLoS One* 2012; 7(12):e49707.
392. Zimmermann G, Papke B, Ismail S, et al. Small molecule inhibition of the KRAS-PDEdelta interaction impairs oncogenic KRAS signalling. *Nature* 2013; 497(7451):638-42.
393. Schultz NA, Roslind A, Christensen IJ, et al. Frequencies and prognostic role of KRAS and BRAF mutations in patients with localized pancreatic and ampullary adenocarcinomas. *Pancreas* 2012; 41(5):759-66.
394. Sinn BV, Striefler JK, Rudl MA, et al. KRAS mutations in codon 12 or 13 are associated with worse prognosis in pancreatic ductal adenocarcinoma. *Pancreas* 2014; 43(4):578-83.
395. Hruban RH, van Mansfeld AD, Offerhaus GJ, et al. K-ras oncogene activation in adenocarcinoma of the human pancreas. A study of 82 carcinomas using a

- combination of mutant-enriched polymerase chain reaction analysis and allele-specific oligonucleotide hybridization. *Am J Pathol* 1993; 143(2):545-54.
396. Miglio U, Oldani A, Mezzapelle R, et al. KRAS mutational analysis in ductal adenocarcinoma of the pancreas and its clinical significance. *Pathol Res Pract* 2014; 210(5):307-11.
 397. Rachakonda PS, Bauer AS, Xie H, et al. Somatic mutations in exocrine pancreatic tumors: association with patient survival. *PLoS One* 2013; 8(4):e60870.
 398. Levine AJ. p53, the cellular gatekeeper for growth and division. *Cell* 1997; 88(3):323-31.
 399. Lowe SW, Schmitt EM, Smith SW, et al. p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* 1993; 362(6423):847-9.
 400. Morton JP, Timpson P, Karim SA, et al. Mutant p53 drives metastasis and overcomes growth arrest/senescence in pancreatic cancer. *Proc Natl Acad Sci U S A* 2010; 107(1):246-51.
 401. Chen R, Dawson DW, Pan S, et al. Proteins associated with pancreatic cancer survival in patients with resectable pancreatic ductal adenocarcinoma. *Lab Invest* 2015; 95(1):43-55.
 402. Fiorini C, Cordani M, Padroni C, et al. Mutant p53 stimulates chemoresistance of pancreatic adenocarcinoma cells to gemcitabine. *Biochim Biophys Acta* 2015; 1853(1):89-100.
 403. Wilentz RE, Geradts J, Maynard R, et al. Inactivation of the p16 (INK4A) tumor-suppressor gene in pancreatic duct lesions: loss of intranuclear expression. *Cancer Res* 1998; 58(20):4740-4.
 404. Izeradjene K, Combs C, Best M, et al. Kras(G12D) and Smad4/Dpc4 haploinsufficiency cooperate to induce mucinous cystic neoplasms and invasive adenocarcinoma of the pancreas. *Cancer Cell* 2007; 11(3):229-43.
 405. Yamada S, Fujii T, Shimoyama Y, et al. SMAD4 expression predicts local spread and treatment failure in resected pancreatic cancer. *Pancreas* 2015; 44(4):660-4.
 406. Frampton AE, Castellano L, Colombo T, et al. MicroRNAs cooperatively inhibit a network of tumor suppressor genes to promote pancreatic tumor growth and progression. *Gastroenterology* 2014; 146(1):268-77 e18.
 407. Chin LJ, Ratner E, Leng S, et al. A SNP in a let-7 microRNA complementary site in the KRAS 3' untranslated region increases non-small cell lung cancer risk. *Cancer Res* 2008; 68(20):8535-40.
 408. Vaca-Paniagua F, Oliver J, Nogueira da Costa A, et al. Targeted deep DNA methylation analysis of circulating cell-free DNA in plasma using massively parallel semiconductor sequencing. *Epigenomics* 2015; 7(3):353-62.
 409. Sun Y, Jiang T, Ma D, et al. [Detection of pathogenic mutations for methylmalonic acidemia using new-generation semiconductor targeted sequencing]. *Zhonghua Yi Xue Yi Chuan Xue Za Zhi* 2015; 32(1):56-9.
 410. Rothe F, Laes JF, Lambrechts D, et al. Plasma circulating tumor DNA as an alternative to metastatic biopsies for mutational analysis in breast cancer. *Ann Oncol* 2014; 25(10):1959-65.
 411. Moussavi-Harami SF, Wisinski KB, Beebe DJ. Circulating Tumor Cells in Metastatic Breast Cancer: A Prognostic and Predictive Marker. *J Patient Cent Res Rev* 2014; 1(2):85-92.

412. Hong SM, Li A, Olin K, et al. Loss of E-cadherin expression and outcome among patients with resectable pancreatic adenocarcinomas. *Mod Pathol* 2011; 24(9):1237-47.
413. Jiang JH, Liu C, Cheng H, et al. Epithelial-mesenchymal transition in pancreatic cancer: Is it a clinically significant factor? *Biochim Biophys Acta* 2015; 1855(1):43-49.
414. Li Y, Kong D, Ahmad A, et al. Pancreatic cancer stem cells: emerging target for designing novel therapy. *Cancer Lett* 2013; 338(1):94-100.
415. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004; 116(2):281-97.
416. Friedman RC, Farh KK, Burge CB, et al. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* 2009; 19(1):92-105.
417. Kosaka N, Iguchi H, Ochiya T. Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. *Cancer Sci* 2010; 101(10):2087-92.
418. Siomi H, Siomi MC. Posttranscriptional regulation of microRNA biogenesis in animals. *Mol Cell* 2010; 38(3):323-32.
419. Bloomston M, Frankel WL, Petrocca F, et al. MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. *JAMA : the journal of the American Medical Association* 2007; 297(17):1901-1908.
420. Frampton AE, Gall TM, Castellano L, et al. Towards a clinical use of miRNAs in pancreatic cancer biopsies. *Expert Rev Mol Diagn* 2013; 13(1):31-4.
421. Wan C, Shen Y, Yang T, et al. Diagnostic value of microRNA for pancreatic cancer: a meta-analysis. *Arch Med Sci* 2012; 8(5):749-55.
422. Ma MZ, Kong X, Weng MZ, et al. Candidate microRNA biomarkers of pancreatic ductal adenocarcinoma: meta-analysis, experimental validation and clinical significance. *J Exp Clin Cancer Res* 2013; 32:71.
423. Frampton AE, Krell J, Jamieson NB, et al. microRNAs with prognostic significance in pancreatic ductal adenocarcinoma: A meta-analysis. *Eur J Cancer* 2015; 51(11):1389-1404.
424. Reinhart BJ, Slack FJ, Basson M, et al. The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* 2000; 403(6772):901-6.
425. Johnson SM, Grosshans H, Shingara J, et al. RAS is regulated by the let-7 microRNA family. *Cell* 2005; 120(5):635-47.
426. Su JL, Chen PS, Johansson G, et al. Function and regulation of let-7 family microRNAs. *Microrna* 2012; 1(1):34-9.
427. Bussing I, Slack FJ, Grosshans H. let-7 microRNAs in development, stem cells and cancer. *Trends Mol Med* 2008; 14(9):400-9.
428. Takamizawa J, Konishi H, Yanagisawa K, et al. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer Res* 2004; 64(11):3753-6.
429. Kumar MS, Erkeland SJ, Pester RE, et al. Suppression of non-small cell lung tumor development by the let-7 microRNA family. *Proc Natl Acad Sci U S A* 2008; 105(10):3903-8.
430. Yu F, Yao H, Zhu P, et al. let-7 regulates self renewal and tumorigenicity of breast cancer cells. *Cell* 2007; 131(6):1109-23.

431. Paranjape T, Heneghan H, Lindner R, et al. A 3'-untranslated region KRAS variant and triple-negative breast cancer: a case-control and genetic analysis. *Lancet Oncol* 2011; 12(4):377-86.
432. Lawrie CH, Chi J, Taylor S, et al. Expression of microRNAs in diffuse large B cell lymphoma is associated with immunophenotype, survival and transformation from follicular lymphoma. *J Cell Mol Med* 2009; 13(7):1248-60.
433. Tsang WP, Kwok TT. Let-7a microRNA suppresses therapeutics-induced cancer cell death by targeting caspase-3. *Apoptosis* 2008; 13(10):1215-22.
434. Torrisani J, Bournet B, du Rieu MC, et al. let-7 MicroRNA transfer in pancreatic cancer-derived cells inhibits in vitro cell proliferation but fails to alter tumor progression. *Human Gene Therapy* 2009; 20(8):831-844.
435. Ali S, Saleh H, Sethi S, et al. MicroRNA profiling of diagnostic needle aspirates from patients with pancreatic cancer. *Br J Cancer* 2012; 107(8):1354-60.
436. Henry J, Bassi C, Giovinazzo F, et al. MicroRNA from Pancreatic Duct Aspirate Differentiates Cystic Lesions of the Pancreas. *Annals of Surgical Oncology* 2013; 20(3):661-666.
437. Liang T, Guo L, Liu C. Genome-wide analysis of mir-548 gene family reveals evolutionary and functional implications. *J Biomed Biotechnol* 2012; 2012:679563.
438. Piriyaopongsa J, Jordan IK. A family of human microRNA genes from miniature inverted-repeat transposable elements. *PLoS One* 2007; 2(2):e203.
439. Hu B, Ying X, Wang J, et al. Identification of a tumor-suppressive human-specific microRNA within the FHIT tumor-suppressor gene. *Cancer Res* 2014; 74(8):2283-94.
440. Sorio C, Baron A, Orlandini S, et al. The FHIT gene is expressed in pancreatic ductular cells and is altered in pancreatic cancers. *Cancer Res* 1999; 59(6):1308-14.
441. Birnbaum DJ, Adelaide J, Mamessier E, et al. Genome profiling of pancreatic adenocarcinoma. *Genes Chromosomes Cancer* 2011; 50(6):456-65.
442. Bloomston M, Kneile J, Butterfield M, et al. Coordinate loss of fragile gene expression in pancreatobiliary cancers: correlations among markers and clinical features. *Ann Surg Oncol* 2009; 16(8):2331-8.
443. Tsujiuchi T, Sasaki Y, Kubozoe T, et al. Alterations in the Fhit gene in pancreatic duct adenocarcinomas induced by N-nitrosobis(2-oxopropyl)amine in hamsters. *Mol Carcinog* 2003; 36(2):60-6.
444. Tian Q, Liang L, Ding J, et al. MicroRNA-550a acts as a pro-metastatic gene and directly targets cytoplasmic polyadenylation element-binding protein 4 in hepatocellular carcinoma. *PLoS One* 2012; 7(11):e48958.
445. Ortiz-Zapater E, Pineda D, Martinez-Bosch N, et al. Key contribution of CPEB4-mediated translational control to cancer progression. *Nat Med* 2012; 18(1):83-90.
446. Rani S, Gately K, Crown J, et al. Global analysis of serum microRNAs as potential biomarkers for lung adenocarcinoma. *Cancer Biol Ther* 2013; 14(12):1104-12.
447. Thorns C, Kuba J, Bernard V, et al. Deregulation of a distinct set of microRNAs is associated with transformation of gastritis into MALT lymphoma. *Virchows Arch* 2012; 460(4):371-7.

448. Xu L, Liang YN, Luo XQ, et al. [Association of miRNAs expression profiles with prognosis and relapse in childhood acute lymphoblastic leukemia]. *Zhonghua Xue Ye Xue Za Zhi* 2011; 32(3):178-81.
449. Witten D, Tibshirani R, Gu SG, et al. Ultra-high throughput sequencing-based small RNA discovery and discrete statistical biomarker analysis in a collection of cervical tumours and matched controls. *BMC Biol* 2010; 8:58.
450. Haneklaus M, Gerlic M, O'Neill LA, et al. miR-223: infection, inflammation and cancer. *J Intern Med* 2013; 274(3):215-26.
451. Jia CY, Li HH, Zhu XC, et al. MiR-223 suppresses cell proliferation by targeting IGF-1R. *PLoS One* 2011; 6(11):e27008.
452. Wu L, Li H, Jia CY, et al. MicroRNA-223 regulates FOXO1 expression and cell proliferation. *FEBS Lett* 2012; 586(7):1038-43.
453. Wong QW, Lung RW, Law PT, et al. MicroRNA-223 is commonly repressed in hepatocellular carcinoma and potentiates expression of Stathmin1. *Gastroenterology* 2008; 135(1):257-69.
454. Li J, Hu G, Kong F, et al. Elevated STMN1 Expression Correlates with Poor Prognosis in Patients with Pancreatic Ductal Adenocarcinoma. *Pathol Oncol Res* 2015.
455. Masciarelli S, Fontemaggi G, Di Agostino S, et al. Gain-of-function mutant p53 downregulates miR-223 contributing to chemoresistance of cultured tumor cells. *Oncogene* 2014; 33(12):1601-8.
456. Li X, Zhang Y, Zhang H, et al. miRNA-223 promotes gastric cancer invasion and metastasis by targeting tumor suppressor EPB41L3. *Mol Cancer Res* 2011; 9(7):824-33.
457. Laios A, O'Toole S, Flavin R, et al. Potential role of miR-9 and miR-223 in recurrent ovarian cancer. *Mol Cancer* 2008; 7:35.
458. Szafranska AE, Davison TS, John J, et al. MicroRNA expression alterations are linked to tumorigenesis and non-neoplastic processes in pancreatic ductal adenocarcinoma. *Oncogene* 2007; 26(30):4442-52.
459. Jamieson NB, Morran DC, Morton JP, et al. MicroRNA molecular profiles associated with diagnosis, clinicopathologic criteria, and overall survival in patients with resectable pancreatic ductal adenocarcinoma. *Clin Cancer Res* 2012; 18(2):534-45.