Methylated cell-free DNA profiles of patients with pancreatic ductal adenocarcinoma

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science

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Johannesburg, 2017

DECLARATION

I, Mpho Mosia, declare that this dissertation is my own unaided work. It has been submitted for the degree of Master of Science in the faculty of Health Sciences in the University of the Witwatersrand, Johannesburg. It has not been submitted before any degree or examination at this or any other University.

_____

Mpho Mosia

This_____ day of _____in 20_____

DEDICATION

In memory of my father

Oupa Jonas Mosia

1964 - 2005

RESEARCH OUTPUTS

Abstract:

Mutation analysis of cell-free DNA of patients with pancreatic ductal adenocarcinoma

Mosia M J, Brand M, Duarte R and Dickens C

Presented at:

Bert Myburgh Research Forum (November, 2016) University of the Witwatersrand, Johannesburg

Poster:

Methylation profiling in cell-free DNA of patients with pancreatic ductal adenocarcinoma

Mosia M J, Brand M, Duarte R and Dickens C

Presented at:

Cancer Research Symposium (February, 2017) University of the Witwatersrand, Johannesburg

ABSTRACT

The high mortality rates of pancreatic ductal adenocarcinoma (PDAC) are largely attributed to a delayed diagnosis, of which in advanced disease, patients are unable to receive surgical resection with curative intent. Clinical presentations and genetic features shared between PDAC and other pancreatic conditions such as chronic pancreatitis (CP) are insufficient to facilitate the disease and often lead to diagnostic uncertainty at an early stage. The purpose of this study was to develop sensitive and specific non-invasive markers to aid in the detection and disease monitoring of PDAC. Here, circulating cell-free DNA (cfDNA) isolated from plasma samples of patients with PDAC (n= 155) and two control groups consisting of patients with either CP (n= 46) or critical limb ischemia (CLI) (n= 88) revealed significant differences in measured concentrations between the three patient groups (p= 0.006-Kruskal-Wallis test).When two groups were compared with each other using the Wilcoxon rank-sum test, observable differences were seen between the two pancreatic diseases: PDAC and CP (p= 0.002), and between the two controls: CP and the CLI groups (p= 0.007). A strong association was also observed in elevated cfDNA levels of CLI patients with HIV (p= 0.03), indicating a poor prognosis for patients. Results from methylation-specific PCR (MSP) in age-matched patient samples showed promoter methylation to account for the loss of *Smad4* in late-stage PDAC; with an observed association with overall increasing cfDNA levels (p= 0.03).This study indicates the potential clinical utility of cfDNA as a non-invasive tool to predict disease progression both quantitatively and qualitatively, as well as to trace epigenetic changes in tumour markers associated with PDAC. Further investigation to identify hypermethylated genes in cfDNA for the early detection of PDAC is warranted.

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LIST OF ABBREVIATIONS

5 mC	5-methylcytosine
5 hmC	5-hydroxymethylcytosine
A	Alcohol consumption
AJCC	American Joint Committee of Cancer
ATP4A	Potassium-transporting ATPase alpha chain 1
ATCC	American type cell culture
BRCA1	Breast cancer 1, early onset
BRCA2	Breast cancer 2, early onset
C	Chronic medical illness
CA	Carbohydrate antigen
Ca^2	Calcium
CANSA	Cancer association of South Africa
CEA	Carcinoembryonic antigen
CEACAM1	Carcinoembryonic antigen related cell adhesion molecule 1
CD1D	Cluster of differentiation 1
cfDNA	Cell-free DNA
CFTR	Cystic fibrosis transmembrane conductance regulator gene
CLI	Chronic limb ischemia
CNND2	c-myc and cyclin D1
CP	Chronic pancreatitis
CpG	CGI promoter
CO_2	Carbon dioxide
COX2	Cyclooxgenase-2
CT	Computed tomography
DEAD	Asp-Glu-Ala-Asp
ddPCR	Droplet digital PCR
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dUTP	Deoxyuridine Triphosphate
EGFR	Epidermal growth factor receptor
ER-beta	Estrogen receptor beta
ERCC1	Excision repair cross-complimentary molecule 1

ERCP	Endoscopic retrograde cholangiopancreatography
EUS	Endoscopic ultrasound
FNA	Fine needle aspiration
FOSB	FBJ murine osteosarcoma viral oncogene homolog B
G12A	Glycine to alanine mutation at position 12
G12C	Glycine to cysteine mutation at position 12
G12D	Glycine to aspartic acid mutation at position 12
G12R	Glycine to Arginine mutation at position 12
G12S	Glycine to serine mutation at position 12
G12V	Glycine to valine mutation at position 12
G13D	Glycine to aspartic acid mutation at position 13
GSG1	General sporulation gene 1
H	HIV status
hENT1	Human equilibrative nucleoside transporter 1
Her2/neu	Human epidermal growth factor receptor 2/protooncogene Neu
HIV	Human immunodeficiency virus
HIP/PAP	Human hepatocarcinoma-intestine-pancreas/pancreatic associated protein
HuR	Human antigen R
IL	Interleukin
IPMN	Intraductal papillary mucinous neoplasma
K-ras	V-ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LOH	Loss of heterozygosity
M	Methylated
MBD	Methyl-CpG binding domain
MCN	Mucinous cystadenoma
MGMT	O^6-methylguanine DNA methyltransferase
MIC-1	Macrophage inhibitory cytokine-1
MLH1	MutL homolog 1
MRI	Magnetic resonance imaging
MSLN	Mesothelin
MSP	Methylation-specific PCR
MUC	Mucin

N/A	Not applicable
NCR	National cancer registry
NFkB	Nuclear factor kappa beta
P16 (CDKN2A)	Cyclin dependant kinase inhibitor 2A
P53	Transformation-related protein 53
PanIN	Pancreatic intraductal neoplasms
Pap-2	Non-canonical poly (A) polymerase
PCR	Polymerase Chain Reaction
PDAC	Pancreatic ductal adenocarcinoma
PLAU	Plasminogen activator urokinase
PGK	Phosphoglycerate kinase 1
PRSS-1	Protease-serine-1 gene
Rad 51	DNA repair protein homolog 51
RAR-beta 2	Retinoic acid receptor beta 2
RASSF1	Ras association domain family member 1
RB	Retinoblastoma
RE	Regulatory edition
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
RRM1	Ribonucleotide reductase catalystic subunit M1
RSC	Maxwelle® ccfDNA plasma kit
S	Smoking
SAH	S-adenosylhomocysteine
SOCS1	Suppressor of cytokine signalling 1
SAM	S-adenosylmethionine
STAT3	Signal transducer and activator of transcription 3
Smad4 (DPC4)	Deleted in pancreatic cancer 4
SPINK1	Pancreatic secretory trypsin inhibitor 1
Ta	Annealing temperature
TET	Ten-eleven-translocation
TGFβ	Transforming growth factor beta
THBS1	Thrombospondin 1

T/M	Bisulfite-treated methylated DNA
TMEFF2	Transmembrane protein with EGF like And Two Follstatin Like Domains 2
TNFα	Tumour necrosis factor alpha
TNM	Tumour size, lymph node status, metastasis
TP53INP1	Tumour protein p53Inducible protein 1
T/U	Bisulfite-treated unmethylated DNA
U	Unmethylated
VHL	Von Hippel-Lindau

LIST OF SYMBOLS

α	Alpha
~	Approximately
bp	Base pairs
β	beta
X^2	Chi-square
°C	Degrees Celsius
µl	microlitres
ml	millilitres
min	minutes
n	number of observations
ng	nanograms
nM	nanomoles
%	Percentage
rpm	Revolutions per minute

CHAPTER ONE: LITERATURE REVIEW

1.1. Introduction: Pancreatic ductal adenocarcinoma

Pancreatic ductal adenocarcinoma (PDAC), despite 50 years of therapeutic intervention, remains the fourth leading cause of cancer-related death worldwide; and is predicted to be the second leading cause of cancer-related death in Western societies within the following decade, making it one of the most aggressive and lethal cancers (Vogelzang *et al.,* 2012; Yeo, 2015). More than 85% of pancreatic cancers are attributed to PDAC; globally it affects more than 250 000 people per year and has one of the lowest 5-year survival rates of all cancer types in both men and women, of less than 5% (Fig. 1.1) (Quaresma *et al.,* 2015; Siegel *et al*., 2015).

Amongst other factors, the risk of developing PDAC is age-dependant, affecting most individuals over the age of 45, with the average age of onset being 65. Pancreatic cancer appears to be prevalent in the African ethnic group, with epidemiological reports from different countries revealing incidences to be 50-90% higher than in other racial groups (Mittal *et al.,* 2015; Yu *et al.,* 2015). In South Africa, where its incidence is under-reported, approximately 74 new cases of pancreatic cancer are reported each year, with the highest incidences reported in the black population (NCR, 2010).

Unfortunately, effective early detection and screening methods are currently inadequate and thus the disease is often diagnosed at a late stage, frequently once metastasis has occurred. The few existing markers such as the CA 19-9 and CEA antigens are not useful in early detection of PDAC, in part because of their lack of specificity and sensitivity (Ballehaninna and Chamberlain, 2013). Hence, there lies a need to develop new diagnostic biomarkers that are efficient in the early detection of PDAC, for the sake of immediate attention and treatment in pancreatic cancer patients.

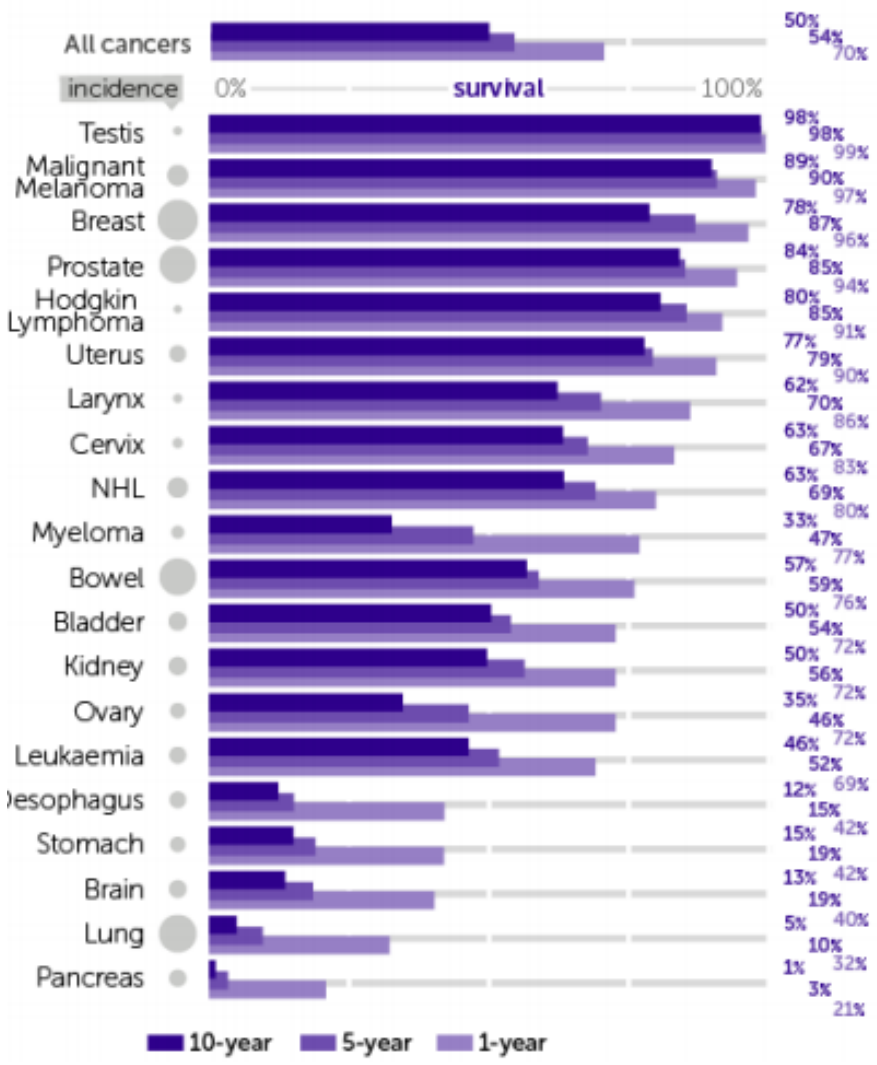

Figure 1.1 Cancer survival rates. Age-standardised one-, five- and ten-year net survival for selected cancers of adults (aged15- 99) in England and Wales between 2010 and 2011. Breast is for female only. Laryngeal is for male only (Source: cruk.org/cancerstats).

Risk factors associated with PDAC are diverse and include preventable (non-genetic) and non-preventable (genetic) factors (Fig. 1.2). A recent meta-analysis identified persons with a history of chronic pancreatitis as having the highest risk of developing PDAC, followed by those with a history of idiopathic thrombosis; moderate risk factors include tobacco smoking, diabetes mellitus, and a family history of pancreatic cancer (Maisonneuve and Lowenfels, 2015). Although there is no assured approach of preventing pancreatic cancer, the avoidance or withdrawal of alcohol and cigarette smoking has been deemed to be the best prevention method. Experimental studies have also shown antioxidant nutrients and nutraceuticals to aid in the prevention of PDAC (Li *et al*., 2015).

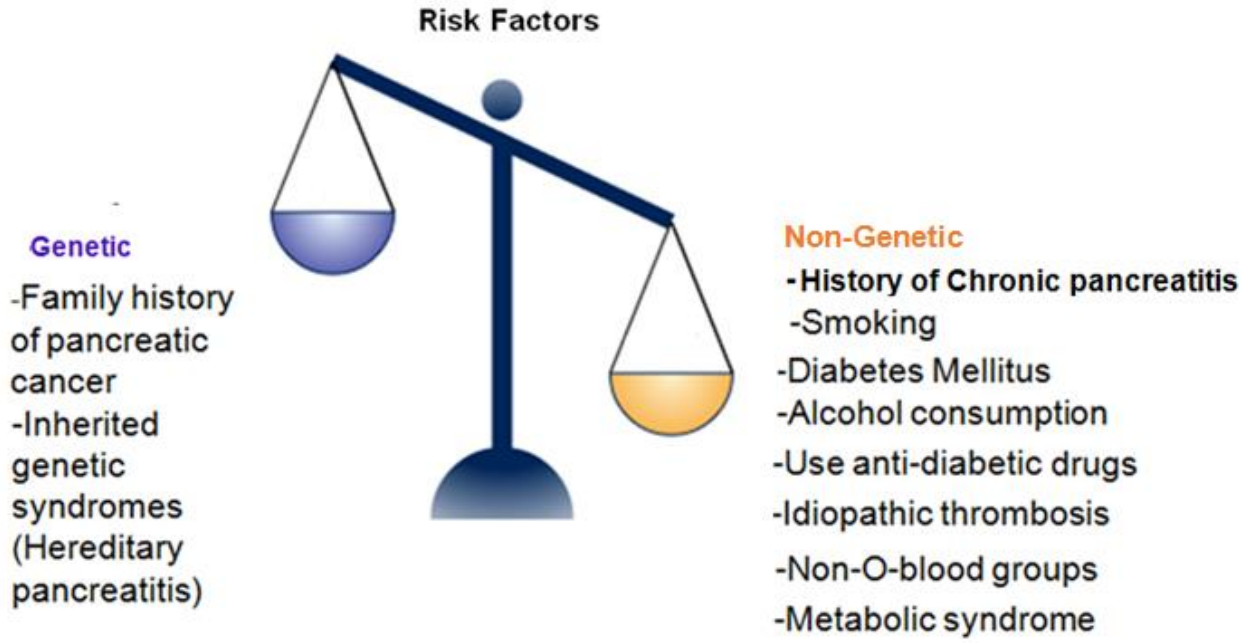

Figure 1.2 Risk factors associated with PDAC. Chronic pancreatitis is a major non-genetic risk factor for the development of PDAC.

1.1.1. Chronic pancreatitis

Chronic pancreatitis (CP), a pancreatic disease characterised by a progressive inflammation and fibrotic destruction of the pancreas, results in exocrine and endocrine function impairment. CP is hypothesised to result from repeated silent episodes of acute pancreatitis caused by a dysregulation in the secretion of pancreatic hydrolytic enzymes that aid in digestion, as well as premature acinar cell activation that may sometimes lead to malignancies. Clinical manifestations often mimic PDAC symptoms, which include weight loss, diabetes mellitus and maldigestion of dietary nutrients (Forsmark, 2013).

In men, the most commonly reported etiology is alcoholism, as opposed to idiopathic causes in women (Inui *et al.*, 2013). However, etiological factors of CP are often modified by lifestyle changes in different geographical regions. In developing countries such as South Africa for instance, where tobacco and alcohol industries are the major driving forces behind the country's economy, drinking and smoking have been inferred as the most common risks in CP patients, and have also been reported to be the strongest link for the progression of CP to PDAC (CANSA, 2010; Pinho *et al.,* 2016). Apart from the high proportion of smokers and alcohol consumers diagnosed with CP,

exposure to industrial xenobiotic compounds, particularly petrochemicals, has been linked to the high incidences of CP in the South African population (Jeppe and Smith, 2008). A large number of CP patients report being employed at industries dealing with hazardous agents and are often exposed to volatile hydrocarbons such as petrol, turpentine and diesel, known risks for CP (McNamee *et al.,* 1994). The burning of firewood and coal, which is often used in primus stoves in townships such as Soweto, as well as exposure to other solvents such as benzene, pesticides and paraffin, have also been identified as potential risks for the development of CP (Jeppe and Smith, 2006).

Genetic risk factors associated with CP include mutations in the PRSS-1 (protease-serine-1) gene, CFTR (cystic fibrosis transmembrane conductance regulator) gene and SPINK1 (pancreatic secretory trypsin inhibitor) gene (Pezzilli and Morselli-Labate, 2009) and are also associated with PDAC risk. Pancreatic duct obstructions caused by strictures, as well as hypercalcemia, commonly resulting from overactive parathyroid glands, are known contributing factors that lead to CP (Forsmark, 2013).

CP incidence rates vary geographically due to cultural differences and diagnostic criteria. In the United States, Europe and Australia, CP rates are relatively low, affecting 3.5 to 10 in 100 000 individuals (Witt *et al.,* 2010). In less developed countries, where epidemiological data is lacking, the majority of CP cases have been reported in tropical regions (Barreto and Shrikhande, 2015). Hospital admissions and discharges have previously indicated CP to be more prevalent in males as well as in the African population (Parkin *et al.,* 2002; Silverman *et al.,* 2003; Khawja *et al.,* 2015), as observed with PDAC cases (Becker *et al.,* 2014).

1.2 Disease Overview

1.2.1. PDAC Biology

PDAC is an epithelial disease originating from the pancreatic ductal and acinar cells which constitute the exocrine pancreas. Histological studies highlight three major precursor lesions that potentially give rise to the highly malignant and invasive PDAC: pancreatic intraductal neoplasms (PanIN), which likely arise from mature acinar cells or the acini-ductal epithelium junction (Miyamoto *et al.,* 2003); mucinous cystadenomas (MCN), characterised by a mucinous epithelial lining; and the intraductal papillary mucinous neoplasms (IPMN), which stem from the mucin-producing main pancreatic duct or its branches.

PanINs are the most common precursor lesions characterised by ductal epithelium with abnormally shortened telomeres (van Heek *et al.,* 2002). Lesions undergo three progressive stages that are graded depending on the degree of cytological atypia of the duct lining cells. PanIN1 lesions, readily detected in a non-diseased pancreas, present mucinous differentiation of ductal cells and minimal atypia; whereas the more advanced lesion grades (PanIN2 and PanIN3) present with marked cytologic changes that are usually detected adjacent to established adenocarcinomas or in non-tumour-bearing pancreatic cells (Hruban *et al*., 2001; Canto *et al.,* 2012). PDAC accumulates genetic alterations over a period of time, following a step-wise disease progression similar to the polyp-to-adenocarcinoma sequence proposed by Hruban *et al.,* (2000) (Fig. 1.3).

The earliest and most common gene alteration is the activation of the *K-ras* oncogene, found in nearly all PDACs as an early initiator of tumorigenesis (Löhr *et al.,* 2005). In early stage PDAC, approximately 30% of PanIN1 lesions have detectable *K-ras* mutations that accumulate during disease progression. Other notable mutations involved in progression include the activation of the *Her-2/neu* oncogene and the loss of function of three tumour suppressor genes namely, *p16* (*CDKN2A*), *p53*, and *Smad4* (*DPC4*) (Hruban *et al*., 2000; Jones *et al*., 2008). *P16*, inactivated in 90% of PDAC patients is commonly found in the PanIN2 precursor lesion (Schutte *et al*., 1997); while inactivation of *Smad4, p53* and *BRCA2* tumour suppressors associated with metastatic disease, are detected in PanIN3 lesions (Iacobuzio-Donahue *et al*., 2009).

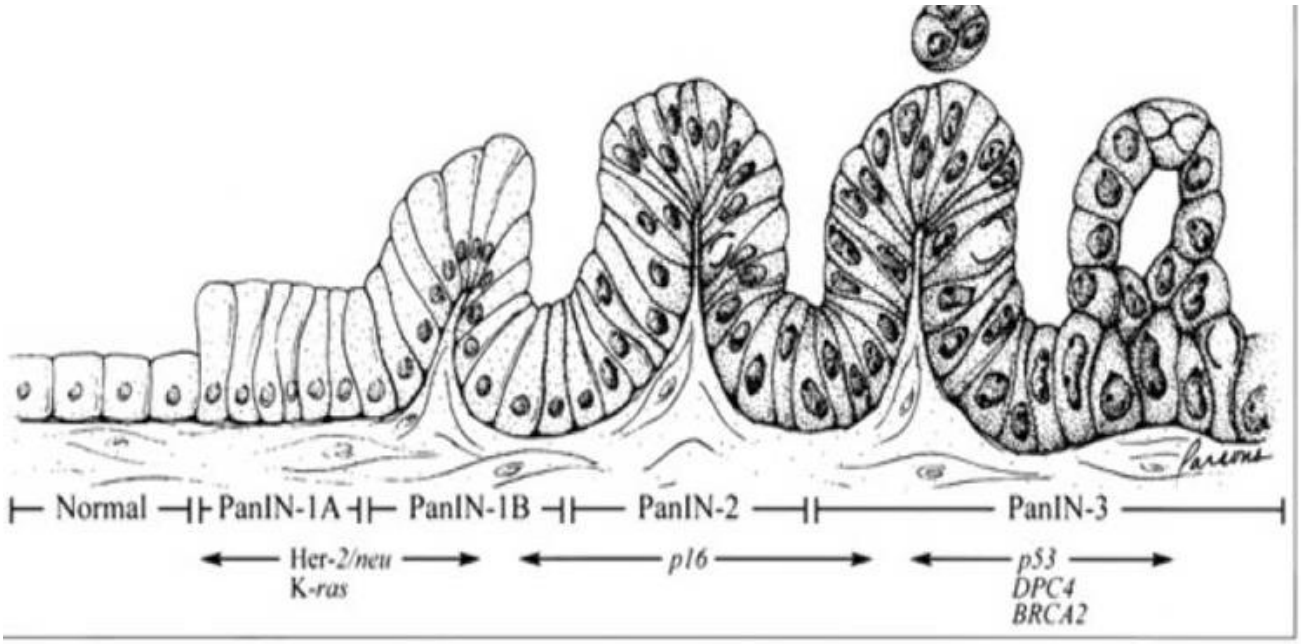

Figure 1.3 Step-wise progression model for PDAC. Disease progression is divided into five morphological stages associated with genetic alterations that accumulate over time as follows: Oncogenic activation of *K-ras* in normal pancreatic duct cells gives rise to precursor lesions. PanIN1A and PanIN1B lesions, presenting as mucinous differentiation of duct cells, accumulate *K-ras* and/or *Her-2/neu* mutations, which lead to more advanced lesions. *P16* mutations are detected in PanIN2 lesions in mid-stage disease, while mutations in *p53, DPC4* (*Smad4*) and *BRCA2* are present in PanIN3 lesions in high-grade disease (taken from Hruban *et al.,* 2000).

1.2.2. PDAC Symptoms

Approximately 60-65% of PDAC tumours are located in the head of the pancreas, with no detectable symptoms at an early stage. At a clinical level, distinguishing PDAC from other pancreatic diseases, such as CP, may be challenging as patients present with similar symptoms. As a result, 90% of PDAC patients die from malignancy due to a delayed diagnosis (Goggins, 2005). Common PDAC symptoms include abdominal pain, weight loss, jaundice, diarrhoea, nausea and vomiting (Porta *et al*., 2009). Weight loss usually results from maldigestion, anorexia and cachexia. Jaundice, the most common clinical sign of PDAC as a result of common bile duct obstruction, is also observed in CP patients. Jaundice is caused by tumours originating in the head of the pancreas through which the common bile duct travels. Tumours arising in the body/tail of the pancreas do not produce jaundice, and are thus detected at an even later stage once other symptoms such as weight loss and abdominal pain become

apparent (Stark and Eibl, 2015). Diabetes mellitus is a less common symptom associated with PDAC, occurring in approximately 25% of affected individuals while an impaired glucose tolerance, a pre-diabetic state of hyperglycemia, has been reported in approximately 40% of PDAC cases (Chari *et al.,* 2008; Pannala *et al.,* 2008).

1.2.3. Detection and Staging of PDAC Tumours

Detection methods for PDAC rely on imaging techniques such as computed tomography (CT) scans, magnetic resonance imaging (MRI), endoscopic ultrasound (EUS) and endoscopic retrograde cholangiopancreatography (ERCP). EUS and ERCP are regarded as the most sensitive tests for detecting pancreatic tumour masses (Brand *et al.,* 2000; Chang *et al.,* 1994; Canto *et al*., 2012); however, CT is regarded as the best initial diagnostic and staging test for pancreatic cancer. Although minimally non-invasive, CT has been associated with an increased risk of radiation-related cancers, and is not considered completely reliable due to previously reported false-positive diagnoses (Canto *et al.*, 2004). A recently proposed diagnostic algorithm based on Bayesian analysis, which recommends EUS and fine-needle aspiration (FNA) as follow on steps once a pancreatic mass is detected on CT, hopes to overcome the issue of misdiagnoses (Gerstenmaier and Malone, 2011). EUS, which is also minimally invasive, and FNA of pancreas masses, though better than ERCP brushings, still have a poor specificity, and a poor negative predictive value (Freeman *et al.,* 2001).

Once detected, tumours are commonly staged with a triphasic CT scan, which follows the American Joint Committee on Cancer (AJCC) Pancreas Cancer Staging “TNM” (Tumour size, lymph Node status, Metastasis) format (Edge and Compton, 2010). This classification system consists of four stages: stages IA and IB, IIA and IIB, III and IV (table 1.1). Clinical assessments of tumours that follow the AJCC staging classification are currently used to predict patient survival. For example, in a small primary tumour, the presence of negative surgical margins and/or the absence of metastasis to the lymph nodes are survival predictors used for resectable disease (Rochefort *et al.,* 2013). Stages IA and IB include tumours that are limited to the pancreas, while the more advanced stages (III and IV) include tumours that extend beyond the pancreas

(Edge and Compton, 2010). Treatment options are mostly based on guidelines that distinguish between tumours that are resectable, borderline resectable, locally advanced unresectable and metastatic disease (table 1.2) (Tempero *et al.,* 2012). In addition, surgery improves patient survival in resectable and borderline resectable tumours by up to seven years, with chemotherapy improving survival up to four years in patients with unresectable tumours.

Apart from imaging tests, molecular techniques including *Smad4* immunolabelling and the plating of fibroblasts are also currently used methods to guide treatment decisions for patients with early stage tumours (Blackford *et al.,* 2009; Iacobuzio-Donahue *et al.,* 2009; Infante *et al.,* 2007).

Table 1.1 AJCC Pancreatic cancer TNM staging classification

AJCC Pancreas Cancer TNM classification

T (Tumour size)	N (lymph Node status)	M (Metastasis)
Tx-Unable to assess primary tumour	Nx-Unable to assess regional lymph nodes	M0- No distant metastasis
T0-No evidence for primary tumour	N0-No regional lymph node metastasis	M1-Distant metastasis
Tis- Carcinoma in situ	N1-Regional lymph node metastasis	
T1-Tumour limited to pancreas and <2cm in size		
T2-Tumour limited to pancreas and >2cm in size		
T3-Tumour extends beyond the pancreas		
T4-Tumour extends beyond the pancreas with the involvement of celiac axis and or superior mesenteric artery (unresectable)		

AJCC Pancreas Cancer staging

Stage	T status	N status	M status
0	Tis	N0	M0
IA	T1	N0	M0
IB	T2	N0	M0
IIA	T3	N0	M0
IIB	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
III	T4	Any N	M0
IV	Any T	Any N	M0

Classification based on Edge and Campton, 2010

Table 1.2 Patient survival prediction based on AJCC staging in pancreatic cancer

AJCC staging	Resectability of tumour	5-year survival rate (%)
Stage 0	Resectable	N/A
Stage IA	Resectable	31-39
Stage IB	Resectable	22-27
Stage IA	Resectable	16-25
Stage IIB	Resectable	8-10
Stage III	Borderline Resectable and Unresectable	0-7
Stage IV	Unresectable	0-4

Data extracted from Bilimoria *et al*., 2007; Hartwig *et al*., 2011; 2013; Isaji *et al*., 2004; Wasif *et al.,* 2010.

1.2.4. PDAC Treatment

Surgical resection remains the only option to cure PDAC, with studies showing a significant improvement in patient survival. For example, in the case of resectable disease, surgery has shown to improve the overall 5-year survival rates from 5% to up to 25%, compared to patients without it (Adham *et al.,* 2008; Conclon *et al.,* 1996; Naseef *et al.,* 2007; Schnelldorfer *et al.,* 2008; Wasif *et al.,* 2010). However, surgical resection is only performed on a minority of patients that have been diagnosed before the cancer has become inoperable. For patients receiving chemotherapy, including those with unresectable tumours, gemicitabine or 5-fluorouracil; or the two regimens in combination, are the most commonly used first-lines of treatment. More recently, FOLIFIRINOX (folinic acid, fluoruracil, irinitocen and oxiplatin), a gemcitabine-free combination therapy, as well as the nab-paclitaxel, is the preferred treatment; improving overall survival rates from 6.8 to up to 11 months compared with gemcitabine treatment alone (Conroy *et al.,* 2011; Von Hoff *et al.,* 2013).

1.2.5. PDAC Biomarkers

The use of traditional diagnostic methods such as FNA and EUS to diagnose PDAC are gradually becoming obsolete, because they lack sensitivity in early stage PDAC; as well as the potential health threats they may pose to patients. For example, the aspiration of tumour masses in PDAC when performing a fine needle aspiration, may not always give an accurate diagnosis since the needle may fail to remove enough of the tissue to yield genetic information, especially for metastasized malignancies (Eloubeidi *et al.,* 2003). Also, the increased probability of seeding cancer or pancreatitis during procedures such as FNA has previously been reported in PDAC patients (Zwirewich *et al.,* 2000).

Tumour associated glycoproteins including carbohydrate antigen CA 19-9, CEA and CA 242 are reported to play a role in the diagnosis of PDAC (Goonetilleke and Siriwardena, 2007; Ni *et al.,* 2005). One of their major disadvantages however, is that they have a limited capacity to diagnose asymptomatic patients; only showing clinical utility in the detection of recurrence after surgical resection (Kim *et al.,* 2004). Also, up to 40% of CP patients show increased levels of CA 19-9 (Rosty and Goggins, 2002; O'Brien *et al.,* 2015); suggesting that CA 19-9 may not always accurately discriminate between patients with CP and those with PDAC. Although CA 19-9 and CEA have been shown to be reliable when used in conjunction with imagining tests to make treatment decisions for PDAC patients (Haas *et al.,* 2010), other markers (table 1.3) show better sensitivity and specificity in the diagnosis, prognosis and response to therapy of PDAC patients (Fuccio *et al.,* 2013; Hu *et al.,* 2014; Sato *et al.,* 2002).

Table 1.3 Potential markers identified in liquid biopsies for PDAC

Marker	Potential clinical use	Reference
	Protein-based markers	
P53	Prognostic	Raedle *et al.,* 1996
MUC1	Diagnostic	Hamanaka *et al*., 2003
Rad51	Diagnostic	Maacke *et al.,* 2002
DEAD-box protein 48	Diagnostic	Xia *et al.,* 2005
Calreticulin	Diagnostic	Hong *et al.,* 2004
Vimentin	Diagnostic	Hong *et al.,* 2006
Osteopontin	Diagnostic	Koopmann *et al.,* 2004
MIC-1	Diagnostic	Koopmann *et al.,* 2004; 2006
PGK 1	Diagnostic	Hwang *et al.,* 2006
CEACAM1	Diagnostic	Simeone *et al.,* 2007
HIP/PAP	Diagnostic	Grønborg *et al.,* 2007
Lipocalin 2	Diagnostic	Grønborg *et al.,* 2007
PAP-2	Diagnostic	Grønborg *et al*., 2007
RNase 1	Diagnostic	Peracaula *et al.,* 2003
Hemopexin	Diagnostic	Zhao *et al.,* 2007
Kininogen-1	Diagnostic	Zhao *et al.,* 2007
Anti-thrombin-III	Diagnostic	Zhao *et al.,* 2007
Haptoglobin-related protein	Diagnostic	Zhao *et al.,* 2007
Kininogen-1	Diagnostic	Zhao *et al.,* 2007
	Gene-based markers	
MUC1	Prognostic	Winter *et al.,* 2013; Wreesman *et al*., 2013
MSLN	Prognostic	Winter *et al.,* 2013
SMAD4	Prognostic	Blackford *et al..,* 2009
FOSB	Prognostic	Wreesman *et al.,* 2004
NFκBIZ	Prognostic	Hartel *et al.,* 2008
ATP4A	Prognostic	Stratford *et al*., 2010
GSG1	Prognostic	Stratford *et al*., 2010
hENT1	Gemcitabine response	Fujita *et al.,* 2010; Nakano *et al.,* 2007
RRM1	Gemcitabine response	Fujita *et al.,* 2010
ERCC1	Platinum-based treatment response	Kamikozuru *et al.,* 2008
HuR	Adjuvant gemcitabine response	Richards *et al.,* 2010

1.3. PDAC and Chronic pancreatitis

Cancer is proposed to originate from a benign inflammatory process. In this instance, chronic diseases of the pancreas have been shown to result in the subsequent development of malignancy (Balkwill and Mantovani, 2001). The length of the lag period between the onset of a benign disease and malignant state varies for different diseases. For example, the time it takes for gastritis to develop into gastric cancer is longer (up to several decades) than for inflammatory bowel disease to develop into large bowel cancer. Approximately 5% of CP patients have been reported to develop pancreatic cancer over a period of 20 years (Raimondi *et al*., 2010), with the average age of onset for CP being mid-40s; and PDAC diagnosed at an average age of 60 in these patients. Although the exact mechanism linking CP and pancreatic cancer has not yet been established, *K-ras* activity which is present in most PDAC's (Logsdon and Ji, 2009) has shown a contribution to the development of malignant disease.

1.3.1. *K-ras* Mutations

The *K-ras* oncogene, located on the p-arm of chromosome 12, encodes for the KRAS protein, a signal transducer in the epidermal growth factor receptor (EGFR) pathway that is activated through the exchange of GDP for GTP upon growth factor-receptor binding. The oncogene is part of the RAS family of GTPases (together with *N-ras* and *H-ras*), which constitute the most frequently mutated oncogenes in human cancers (Cox *et al.,* 2014; 2015) and are characterized by single base missense mutations. In *K-ras*, 98% of the single base missense mutations are found at amino acid residues Glycine 12 (G12), Glycine 13 (G13) and Glutamic acid (Q61). Previous studies have reported *K-ras* mutations to be associated with a worse prognosis for non-resectable pancreatic tumours (Chen *et al.,* 2010); as well as shorter survival rates in tumours with positive resected tumour margins (Kim *et al.,* 2006). Out of the seven common mutation types (G12D, G12S, G12R, G12A, G13D, G12V and G12C) (Edkins *et al.,* 2006; Imamura *et al.,* 2012), four major patterns namely; G12D, G12V, G12R and G13D have been reported to account for >90% of all *K-ras* mutations in PDAC (Witkiewicz *et al.,* 2015); with G12V and G12S associated with an aggressive disease (Immervoll *et al.,* 2006).

K-ras mutations are also associated with morphological changes involving precursor lesions that lead to PDAC (Hingorani *et al.,* 2003). Carriére *et al* (2009) were able to demonstrate this in genetically engineered mouse models. In mice with a prior inflammatory insult induced by caerulein, a cholescytokinin analogue known to express *K-ras* mutations in the development of pancreatic malignancy, *K-ras* mutants were observed. Between two to six months after caerulein treatment, mice with activated oncogenic *K-ras* developed low-grade PanIN lesions as well as dedifferentiated areas of acinar cells that led to ductal metaplasia. Furthermore, acinar cells have also been proposed to be a common origin of CP linking to it to PDAC as shown in animal models where, over a period of time, these cells dedifferentiate during the induction of oxidative stress and inflammation with cholescytokinin treatment. The subsequent development of ductal pancreatic adenocarcimonas in dedifferentiated acinar cells has been shown to express transcription factors observed in PDAC (Pinho *et al.,* 2011; Rooman and Real, 2012).

1.3.2. Inflammation

The inactivation of oncogenic *K-ras* leading to malignancy is proposed to be a result of oxidative stress which, over a period of time, causes the release of inflammatory mediators accompanied by perpetual oxidative stress, ultimately leading to a benign tumour of the pancreas that progresses to an adenocarcinoma through an accumulation of mutations (Fig. 1.4). In the process of inflammation, inflammatory mediators such as tumour necrosis factor α (TNFα), IL-1β, IL-18, and chemokines are released. These have previously been reported to be up-regulated in acute pancreatitis (AP) patients (Pandol e*t al.*, 2007; Vonlaufen *et al.,* 2007), a pancreatic disorder that is believed to precede CP. Although the mechanism of tissue damage leading to inflammation remains unclear, experimental models have confirmed that transcription factors involved in secreting these inflammatory mediators are activated through Ca^{2+} and kinase dependant pathways (Awla *et al.,* 2012; Thrower *et al*., 2010; Vaquero *et al.,* 2001; Orlichenko *et al*., 2010) and are proposed to be key pathways for metastasis in pancreatic cancer (Chow *et al*., 2008).

When these inflammatory mediators are released, the process of inflammation, involving the generation of reactive oxidative species (ROS) and reactive nitrogen

species (RNS), may cause modifications of critical substrates as another way of activating the immune system (Wiseman and Halliwell, 1996). Immune cells and stromal components of the pancreas subsequently produce inflammatory cytokines and chemokines that together with ROS and RNS lead to increased cellular proliferation and damage (Chan and Leung, 2011), which progresses acute pancreatitis to CP. Secondary oxidative injury, exacerbated by perpetual inflammation, can impose additional infiltration of inflammatory mediators such as Cyclooxygenase-2 (Cox2), NF-$K\beta$ and STAT3 (Farrow and Evers, 2002; Chan and Leung, 2011) that ultimately may lead to carcinogenesis.

Genes involved in oxidative stress and inflammatory pathways that have been identified in several genetically modified mouse models include *K-ras* (Al Sati *et al.,* 2013; DeNicola *et al.,* 2011). Other than *K-ras,* the inactivated *TP53INP1* has also been observed (DeNicola *et al.,* 2011). Both *K-ras* and *TP53INP1* enhance ROS detoxification and lead to tumourigenesis. In addition, STAT3, a constitutively activated pancreatic cancer protein, has been shown to be essential in initiating cancer from CP by promoting dedifferentiation in acinar cells, which results in the cells becoming more susceptible to *K–ras* mediated transformation (Fukuda *et al.,* 2011).

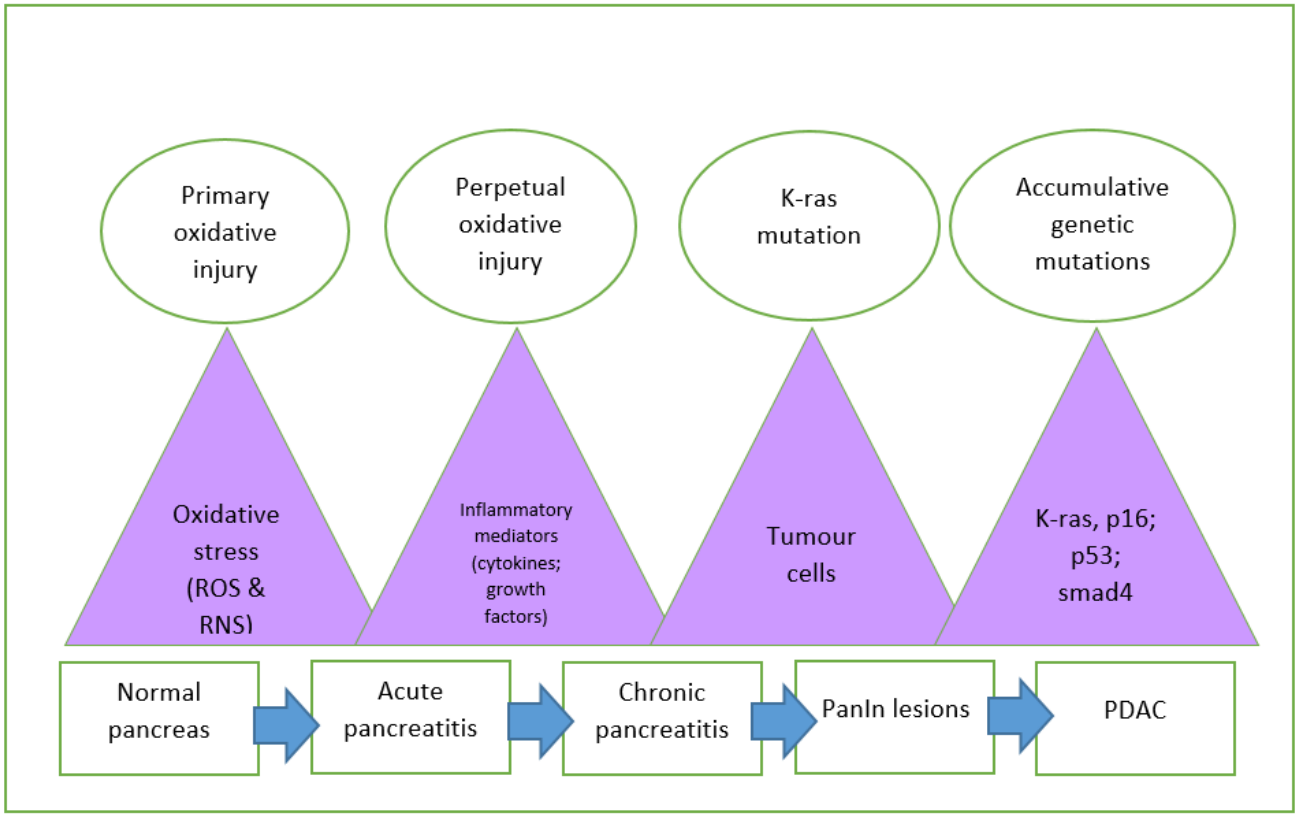

Figure 1.4 Schematic model of the development of malignancy from benign inflammatory processes in the pancreas. Malignancy is initiated by primary oxidative injury in the normal pancreas, which causes cellular damage resulting in inflammation. The release of reactive oxidative species (ROS) and reactive nitrogen species (RNS) activate the immune system leading to acute pancreatitis; which, upon perpetual oxidative injury, releases inflammatory mediators that include cytokines, chemokines and growth factors, causing chronic pancreatitis. Secondary (perpetual) oxidative injury may lead to activation of oncogenic *K-ras*, which when mutated gives rise to malignant precursor lesions. Accumulation of *K-ras* mutations, together with alterations in tumour suppressor genes including *p16, p53* and *Smad4*, aid in the progression of malignancy ultimately leading to pancreatic adenocarcinomas (PDAC).

1.4. Cell-free DNA

Advances in molecular diagnostics have shown improved clinical utility associated with the use of liquid biopsies in the discovery of tumour markers, which supersedes the traditional use of tumour biopsies to trace disease dynamics. Liquid biopsies, which are non-invasive, are able to reveal genetic alterations in PDAC (Cai *et al.,* 2014; Heitzer *et al.,* 2015) as well as other genetic disorders. Cell-free DNA (cfDNA), described as circulating DNA in the bloodstream, is detected in liquid biopsies including those of plasma, serum and urine and is mostly present as double-stranded DNA molecules, with distinctly lower molecular weights than genomic DNA (Jahr *et al.,* 2001).

Although present in both healthy and diseased patients, cfDNA in diseased patients differs significantly from healthy persons (Schwarzenbach *et al.,* 2011). The precise mechanism of the release of cfDNA into the bloodstream remains unclear; however, previous studies have reported cfDNA in healthy individuals to be derived from living cells that actively release DNA fragments, as well as apoptotic processes (Stroun *et al.,* 2001; Suzuki *et al.,* 2008; Ziegler *et al*., 2002). These DNA fragments are found to be attached to the surface of blood cells, and have low molecular weights of approximately 180-200 bp (Giacona *et al.,* 1998; Skvortsova *et al.*, 2006). In malignancies, cfDNA production mostly results from cancer cell death processes, occurring as small and much larger molecular weight fragments resulting from apoptosis and necrosis, respectively (Jahr *et al.,* 2001; Suzuki *et al.,* 2008, Wu *et al.,* 2002). Macrophages have been suggested to aid this release of DNA fragments into the bloodstream *via* phagocytosis (Gormally *et al.,* 2007).

The detection of non-tumour-derived DNA in the plasma of cancer patients is believed to originate from healthy cells adjacent to the tumour (Fig. 1.5) (García-Olmo *et al.,* 2008). This has been shown in animal experiments, where high amounts of cfDNA are released from both tumour-derived and non-tumour-derived cells. Therefore, apart from cancer cell death programmes that generate tumour-derived DNA in the blood, hypoxia, caused by deficient vascularisation (Jahr *et al.,* 2001), as well as angiogenesis (Sozzi *et al.,* 2003) may account for the non-tumour DNA present in the plasma.

The presence of cfDNA in cancer patients has also been reported to generate metastasis, a concept known as 'genometastasis' (Fig. 1.5). This was first described in an experiment involving rats with tumours, where oncogenic DNA fragments were incorporated into the genome of susceptible cells in distant organs (García-Olmo and García-Olmo, 2001). The hypothesis was later supported by cell culture experiments that showed cells cultured together with cells from a cancer patient later acquired malignancy (García-Olmo *et al.,* 2010). Methylated DNA in transformed cells has been reported to have an enhanced efficiency for penetrating into cells and thus a higher transformational potential; potentially explaining the highly elevated amounts of cfDNA in metastatic cancer (Fig. 1.5) (García-Olmo *et al.,* 2000; García-Olmo and García-Olmo, 2001; 2013; Widschwendter M and Menon, 2006; Skvortsova *et al*., 2008), often associated with a high frequency of methylation of tumour suppressor genes.

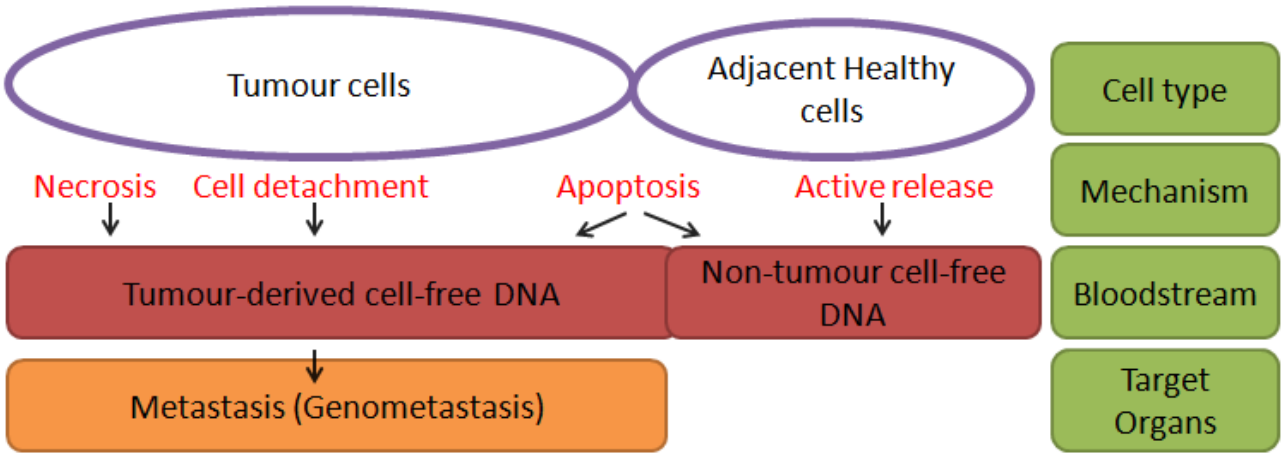

Figure 1.5 Schematic model for Genometastasis. Tumour cells shed DNA fragments into the bloodstream, known as cell-free DNA, resulting from cell-death pathways including necrosis, cell attachment and apoptosis. The high levels of cell-free DNA observed in cancer are also contributed to by cells adjacent to the tumour, which through apoptosis and active release of DNA also shed DNA fragments into the bloodstream. The enhanced efficiency of cell-free DNA resulting from tumour cells to incorporate into susceptible cells in distant (target) organs, assists in generating metastasis.

1.4.1. The clinical utility of cell-free DNA as a cancer biomarker

Several studies have identified similar specific genetic and epigenetic alterations in both tumour-derived DNA and cfDNA, demonstrating the clinical utility of using cfDNA over other invasive methods to detect cancer specific markers in patients for diagnostic and prognostic purposes. Since its first discovery in 1948, studies have reported significantly higher amounts of cfDNA in cancer patients compared with controls (Taback *et al.,* 2004; Gahan and Swaminathan 2008; Kandel, 2012; Figg and Reid, 2013; Jin *et al.,* 2012; Perkins *et al.,* 2012); as well as in patients with inflammatory disorders compared to healthy individuals (Chang *et al.,* 2003; Rhodes *et al*., 2006; Frank, 2016). In this regard, quantitative analyses of cfDNA may initially serve to distinguish diseased patients from healthy individuals.

Clinically, apart from the currently used pre-natal screening tests, BEAMing (named after its components: beads, emulsions, amplification, and magnetics), a digital assay used for detecting mutations in colorectal cancer, remains the only approved cfDNA-associated diagnostic test (Diehl *et al.,* 2005). Other techniques lack standardised protocols and appropriate controls, as highlighted in several reports (Crowley *et al.,* 2013; Fleischhacker and Schmidt, 2007; Marzese *et al.,* 2013). It is important to take into account pre-analytical and biological factors that could potentially influence quantitative and qualitative changes in cfDNA samples.

When deciding on the source of cfDNA for extraction, plasma would be recommended over serum for studies aiming to assess quantitative changes, where cfDNA concentrations would be of utmost value. Although usually detected in both plasma and serum, higher amounts of cfDNA exist in plasma as opposed to serum (Suzuki *et al.,* 2008, Holdenreider *et al.,* 2005) due to reduced DNase activity in plasma (Economidou-Karaoglou *et al.,* 1988; Funakoshi *et al.,* 1979; Cherepanova *et al.,* 2008; Tamkovich *et al.,* 2006).

However, even after having considered the source of cfDNA, inconsistent cfDNA concentrations observed across experiments suggest a lack of standardization in protocols used for extraction and quantification. cfDNA levels in cancer patients almost always vary significantly in different experiments, from a few ng/ml to thousands of ng/ml, which sometimes overlap with levels in healthy controls (Fleischhacker and Schmidt, 2007; Jung *et al.,* 2010). Moreover, cfDNA fragmentation, a prominent

feature of cfDNA derived from apoptotic cells, has also shown variation in control patients, appearing to be either higher or much lower than levels in cancer patients (Holdenrieder *et al.,* 2008; Jiang *et al.,* 2006).

These and other differences observed between studies in cfDNA are mainly attributed to variations in technical procedures from the early stages of blood sample handling (anticoagulants, collection tubes, storage conditions) and processing (centrifugation speeds), to the use of different cfDNA extraction protocols (El Messaoudi *et al.,* 2013). Furthermore, biological factors may account for variations, such as the fact that the proportion of cfDNA from tumour cells relative to the total cfDNA present in the bloodstream in cancer patients is influenced by tumour size, grade, stage and location (Diehl *et al.,* 2005; Jung *et al.,* 2010). Thus at the current moment, cfDNA quantitative analyses show better results when accompanied by the detection of qualitative changes in cancer.

1.5. DNA methylation

Previous studies have demonstrated tumour-specific epigenetic changes such as hypermethylation, in cfDNA recovered from patients with different malignancies, and the absence of these methylation markers in control subjects (Silva *et al.,* 2002; Skvortsova *et al.*, 2006; Iyer *et al.,* 2010; Liggett *et al.,* 2011). Hypermethylation, an uncommon event in normally differentiated cells, is defined as an increase in DNA methylation that results in the epigenetic silencing of tumour suppressor genes (Baylin *et al.,* 2001). The translational relevance of methylation has been demonstrated in some hypermethylated markers isolated from liquid biopsies in several malignancies for early detection (An *et al.,* 2002; Lee *et al.,* 2002; Lofton-Day *et al*., 2008), risk assessment and prognosis (Lee *et al.,* 2002; Phillip *et al.,* 2012; Li *et al*., 2012), as well as prediction to therapy response (Sharma *et al.,* 2012; Kim, *et al.,* 2010), making cfDNA a useful non-invasive tool in developing biomarkers for PDAC. Also, genes reported to be frequently methylated in cancer (table 1.4) are potential targets for cfDNA analyses for disease detection and monitoring.

Table 1.4 Frequently hypermethylated tumour suppressor genes in cancer

Gene	**Cancer type**
MLH1	Colon, endometrial, gastric
ER-beta	Breast
RAR-beta2	Breast
BRCA1	Breast, ovarian
VHL	Kidney tumours
RB	Retinoblastoma
TMEFF2	Lung and various tumour types
DNA methyltransferase & MGMT	Various tumour types
RASSF1	Thyroid, pancreas, prostate, breast
Smad4	Gastric

1.5.1. Mechanisms of DNA methylation

Promoter methylation leads to transcription inhibition through two primary mechanisms. Firstly, the binding of transcription regulatory factors whose recognition sites contain CpG sites is impeded; and secondly, transcription factor binding is indirectly prevented by the limited access of regulatory elements known as methyl-CpG binding domains (MBDs) that bind specifically to methylated CpG sites. This gives DNA methylation an indirect regulatory role in chromatin structure and DNA accessibility for transcription factors with subsequent gene silencing (Fig. 1.6).

DNA methylation is an enzymatic reaction carried out by DNA methyltransferases (DNMTs). S-adenosylmethionine (SAM) is used as a substrate that produces S-adenosylhomocysteine (SAH) as a by-product through a base flipping reaction involving the addition of a methyl group to the 5' position of the pyrimidine ring of a cytosine producing a 5-methylcytosine (5mC). 5mC is usually reversed by a family of ten-eleven-translocation (TET) proteins converting it to 5-hydroxymethylcytosine (5hmC) through an oxidation process (Rodríguez-Dorantes *et al.,* 2004; Tahiliani *et al.,* 2009; Leung *et al*., 2013).

Several tumour suppressor genes found to be methylated in cancer regulate the cell cycle and promote apoptosis. This makes them potential targets for biomarker development, especially in the case of cfDNA, where elevated levels in cancer patients are characteristic of a high transformation potential associated with methylated DNA fragments.

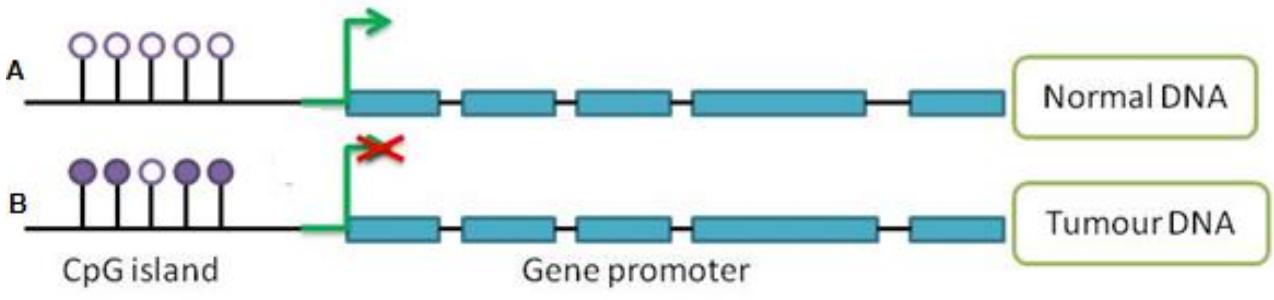

Figure 1.6 DNA methylation in gene promoters. (A) Normal DNA from healthy cells is unmethylated at the CpG island (empty lollipops) allowing for gene expression (indicated by green arrow); (B) Tumour-derived DNA from malignant cells is methylated at the CpG island of the gene promoter (filled purple lollipops) leading to transcriptional silencing (no expression) of the gene (indicated by red cross on green arrow)

1.5.2. Methylation markers in cfDNA

The detection of hypermethylated genes in plasma or serum has been shown to be useful for disease diagnosis, prognosis and predicting treatment response in several malignancies including breast, lung, colon, pancreatic and prostate cancers (Oxnard *et al.,* 2014; Stirzaker *et al*., 2015; Zhou *et al*., 2015; Haldrup *et al*., 2016); but not in PDAC. Current data suggests that differences in methylation profiles of patients with pancreatic inflammatory diseases and pancreatic malignancies calls for further investigation of hypermethylated genes in cfDNA to accurately distinguish PDAC from CP at an early stage and to monitor disease progression. Multiplexed array-mediated analyses have revealed methylation in several genes such as *CNND2, SOCS1, THBS1, PLAU*, *VHL, CD1D, MUC1 and MUC4* (Sawabu *et al.,* 2004; Liggett *et al.,* 2010; Kisiel *et al.,* 2015); as well as *p16* and *RASSF1A* in cfDNA (Fukushima *et al.,* 2003; Kisiel *et al.,* 2015; Park *et al.,* 2012). These provide a platform to profile methylation in cfDNA to develop markers for PDAC.

Apart from PDAC, promoter hypermethylation of *p16* and *RASSF1A* has been reported in several other cancers (Hu *et al*., 1997; Ohtsubo *et al*., 2003; Kawaguchi *et al.,* 2003; Gu *et al*., 2006; Tam *et al.,* 2013; Hesson *et al*., 2003; Honda *et al*., 2008; Liu *et al,* 2011); with *p16* hypermethylation being associated with a worse prognosis. However, since *p16* and *RASSF1A* hypermethylation has been reported in CP (Damman *et al*., 2003), they may be unreliable as PDAC markers, since they may lack specificity.

Amongst the other genes known to be commonly mutated in PDAC, methylation profiles of genes involved in late stage disease are poorly understood. One of these genes, *Smad4* (*DPC4*), a central mediator of TGF-β signalling, is reported to be inactivated by loss of heterzygosity (LOH) and intragenic mutations in PDAC (Hahn *et al.,* 1996; Blackford *et al.,* 2009). In other cancers including prostate cancer, Barrett's oesophagus and gastric carcinomas, hypermethylation of *Smad4* is has been observed (Onwuegbusi *et al.,* 2006; Wang *et al.,* 2007, Wang *et al*., 2007; Aitchison *et al.,* 2008; Blackford *et al.,* 2009). The inactivation of *Smad4* in cancer is associated with a high metastatic potential; and in PDAC, where its loss is observed in high-grade lesions, it has a strong prognostic potential (Dempe *et al.,* 2010; Singh *et al.,* Oshima *et al.,* 2013).

1.6. Study Rationale

Pancreatic ductal adenocarcinoma is a devastating disease. Currently mechanisms for the development of PDAC are poorly understood, making it difficult to diagnose at an early (and thus potentially curable) stage. Diagnostic uncertainty exists as other diseases, such as chronic pancreatitis, have similar presentations and genetic features and there is a lack of sensitive and specific biomarkers to assess disease stage, treatment response and overall prognosis. Hence there is a need for novel, non-invasive, sensitive and specific biomarkers to aid in the diagnosis of PDAC, as well as to predict treatment response and prognosis.

1.7. Aim and Objectives

Aim

Circulating cell-free DNA was investigated to determine cfDNA levels and whether *Smad4* inactivation is due to promoter hypermethylation in PDAC patients.

Objectives

1. To isolate and quantify cfDNA levels from patient plasma
2. To optimise droplet digital PCR for the detection of *K-ras* mutations
3. To optimise PCR conditions for *Smad4* methylation analysis

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CHAPTER TWO: METHODS AND MATERIALS

2.1. Plasma samples

Plasma samples from black patients with pancreatic ductal adenocarcinoma (PDAC), chronic pancreatitis (CP) and critical limb ischaemia (CLI) were collected in two Johannesburg hospitals (Chris Hani Baragwanath Academic Hospital and Charlotte Maxeke Johannesburg Academic Hospital) between August 2013 and April 2016 and stored at -80°C until experimentation. Ethics approval for this study was obtained under an existing study entitled *Genetic and Environmental Factors that Influence Susceptibility to Pancreas Cancer* (M130551).

The inclusion and exclusion criteria of PDAC patients and controls are summarised in table 2.1. Patients recruited for the study included persons over the age of 18, with several risk factors for PDAC. PDAC diagnosis was confirmed either by an abdominal CT scan or by histological and cytological methods. CT scans for control subjects with CP showed no evidence of pancreatic cancer, while CLI patients showed a normal pancreas. Patients that had not gone for CT or MRI scans, had unknown primary tumours, cancers or other pancreas-related diseases, were excluded from the study.

Table 2.1 Patient inclusion and exclusion criteria for the study

Inclusion Criteria	**PDAC**	**Controls**	
		Chronic pancreatitis	**Critical limb ischemia**
Age	>18 years	>18 years	>18 years
Diagnosis	Either an abdominal CT scan demonstrating PDAC and/or patients with cytologically or histologically diagnosed PDAC	Abdominal CT scan demonstrating chronic pancreatitis with no evidence of a pancreatic cancer	Abdominal CT scans do not demonstrate a malignancy, normal pancreas
Exclusion Criteria			
	PDAC	**Controls**	
Diagnosis	No abdominal CT scan or MRI scan	No abdominal CT scan.	
Other disease	Unknown primary tumours; Concurrent cancer at another organ site or a history of any type of cancer; Any other pancreas related disease	Pancreas related diseases such as acute pancreatitis	

2.2. Isolation and quantification

2.2.1 Cell-free DNA isolation

Circulating cell-free DNA (cfDNA) was extracted from plasma samples using the Maxwell® RSC ccfDNA plasma kit (RSC) (Promega) according to the manufacturer's instructions. The RSC kit is used together with the Maxwell® RSC instrument (Promega) for efficient automated cfDNA isolation from plasma volumes ranging from 0.2 - 1 ml, processing up to 16 samples in under 70 minutes. The system makes use of paramagnetic particles that provide a mobile solid phase which moves the samples through the wells of the pre-filled cartridge during the extraction process. The kit is supplied with pre-programmed purification methods for use, with disposable reagent cartridges containing 8 wells (#1: Binding buffer; #2: paramagnetic particles; #3-7: wash buffer).

Prior to DNA extraction, plasma samples were thawed at room temperature and centrifuged at 4 000 rpm for 10 min to remove any residual white blood cells. Maxwell® RSC cartridges, CSC/RSC plungers and elution tubes were placed on the deck tray as follows: After removing the seals, cartridges were placed in the tray wells with well #1 farthest away from the elution tubes; plungers were placed into well #8 of each cartridge; labelled elution tubes were placed uncapped in the elution tube positions for each cartridge; and 60 µl of elution buffer was added to the bottom of each elution tube.

Once prepared, plasma samples (Approximately 1 000 µl) were added to the binding buffer in well #1 and the RSC Instrument was set-up to run on the software system. At the end of each run, the deck tray, containing 50 µl of purified cfDNA was removed and elution tubes were immediately capped to avoid evaporation. Concentration levels were determined on the Nanodrop 2000 UV/spectrophometer (Thermo Scientific). Once cfDNA levels were measured, samples were stored at -20 °C for subsequent analyses.

2.2.2 Cell line DNA isolation

The positive control used for optimising ddPCR (droplet digital PCR) and MSP (methylation specific PCR) was the pancreatic cancer cell line, MIA PaCa-2 (ATCC) derived from the pancreas adenocarcinoma of a 65 year-old Caucasian man. The cell line was purchased and donated by Prof Geoffrey Candy's NRF Incentive funding line. Genomic DNA was extracted from cells using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. The purification procedure of the kit involves four key steps, which are carried out by QIAmp spin columns.

Cells were cultured at 37 °C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DME)/Nutrient mixture F12 (1x) (GE Healthcare Life Sciences) supplemented with 10% Foetal Calf Serum. Confluent cells (~75%) were detached from the surface of the flask by adding 2 ml trypsin (Invitrogen) and incubated for 5 min at 37 °C. Following trypsinisation, the cell suspension was aliquoted into a 1.5 ml tube and centrifuged for 5 minutes at 4000 rpm to obtain a pellet containing the cells. The pellet was resuspended in PBS (Sigma Aldrich) to a final volume of 200 µl, and 20 µl of proteinase K added to the solution to lyse the cells and release the DNA.

Cell lysis was completed by adding 200 µl of binding buffer (buffer AL) to the DNA mixture and incubating for 10 minutes at 56 °C using a heating block. The DNA mixture was subsequently precipitated by adding 200 µl of ethanol (96-100%) to the lysate and running it through the QIAamp mini spin columns to capture the DNA. DNA was washed with wash buffers (buffer AW1 and AW2) and subsequently eluted into 200 µl of elution buffer (buffer AE). Once the DNA concentration had been measured using the Nanodrop 2000 UV/spectrophometer, purified DNA was stored at -20 °C until optimisation.

2.3. *K-ras* Mutation Detection

K-ras mutations were analysed using droplet digital PCR (ddPCR). Bio-rad's ddPCR is a water-emulsion droplet-based method of performing digital PCR, where samples are prepared in a similar fashion to most standard Taqman-probe based assays. Samples are partitioned into droplets that are subsequently amplified to yield quantitative results of gene targets. A 20 µl PCR reaction mixture is compartmentalised into thousands of droplets (~20 000) prior to amplification. Results were detected fluorescently by Bio-rad's droplet reader and interpreted digitally following the binary numbers of "1" (containing target) or "0" (containing no target) (Hindson *et al.,* 2011).

To optimise the ddPCR for *K-ras* mutation screening in cfDNA, the ddPCR™ KRAS Screening Multiplex Kit (Bio-rad) was used following manufacturer's instructions. The kit allows for the detection of the 7 most common *K-ras* mutations (G12A, G12C, G12D, G12R, G12S, G12V, and G13D), using fluorophore sensitive primer-probes (FAM and HEX) that detect *K-ras* sequence variants even when present in very low amounts in a pool of wild-type background. Three randomly selected cfDNA samples from each patient group i.e. PDAC, CP and CLI, were used to optimise the three most important factors to be considered in the system workflow (Fig. 2.1) namely, cfDNA input concentration; droplet count and threshold settings.

The reaction was set up as follows: Reaction components were thawed at room temperature and briefly centrifuged; 10 µl of 2x ddPCR Supermix for probes (no dUTP), 900 nM primers, 250 nM probe (FAM and HEX) and 1 µl template DNA (50 and 25 ng/µl for cell line; 5 and 2.5 ng/µl for cfDNA) were added to a 200 µl PCR tube and adjusted to a final reaction volume of 20 µl using PCR-grade water. Droplets were generated by transferring the reaction mixture to an 8-well DG8™ cartridge (Bio-rad), followed by the addition of 70 µl of droplet oil (Bio-rad) and droplets generated using the QX200™ droplet generator (Bio-rad).

The droplets were immediately transferred to a 96-well PCR plate (Bio-rad), covered with heat-seal foil (Bio-rad), and sealed using the PX1 PCR plate sealer (Bio-rad) to avoid evaporation. Once sealed, the PCR plate was placed in the C1000 Touch™ thermal cycler (Bio-rad) and amplification carried out with the following cycling conditions: 95 °C for 10 min for enzyme activation; 40 cycles of denaturation (94 °C for 30 sec) and annealing (60 °C for 1 min). Enzyme deactivation was performed at 98

°C for 10 min and an infinite hold was set at 4 °C. Following amplification, the PCR plate was placed into the QX200™ Droplet Reader (Bio-rad) and the results analysed with the Quantasoft™ version 1.7 (RE) software (Bio-rad).

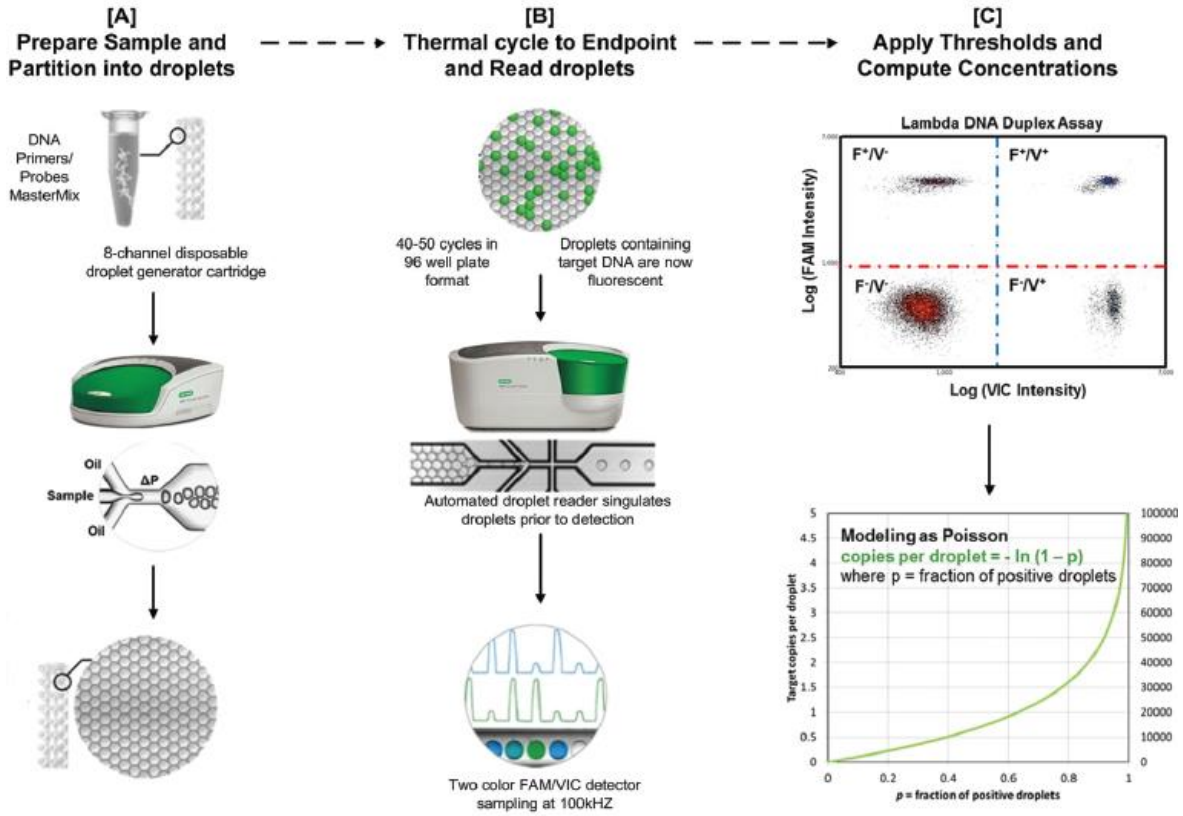


Figure 2.1 Droplet digital PCR workflow. (A) Sample preparation and droplet generation: cfDNA material and *K-ras* multiplex kit (Bio-rad), prepared in 20 µl reaction mixture is transferred into an 8-well cartridge (pictured). Droplet generation oil (~70 µl) containing emulsion stabilizing surfactant is added into adjacent cartridge wells. The cartridge is placed into a droplet generator (pictured), which generates a vacuum that creates a pressure differential which converts the aqueous sample mixture into a stable water-in-oil droplet emulsion that accumulates in the droplet collection wells of the cartridge. Droplets are then placed in a 96-well plate, foil sealed and thermal cycled. (B) After end-point thermal cycling, the plate is placed in a droplet reader (pictured) where an autosampler aspirates droplets using a microfluidic singulator, which streams them in single file past two fluorescent channels (FAM/HEX) at a rate of 100 kHz. (C) The difference in fluorescence amplitudes for droplets where amplification has occurred divides the entire droplet population into discrete clusters for FAM and VIC (HEX) assays. Four populations exist in clusters which either contain no target (F-/V-), one of the targets (F-/V+, F+/V-), or both targets (F+/V+). Setting fluorescence thresholds for each detection channel is a digital method for classifying droplets and computing the number target DNA copies based on the fraction of positive droplets by Poisson modelling (taken from Pinheiro *et al.,* 2011).

2.4 Smad4 Methylation Analysis

To determine the methylation status of *Smad4* in patient samples, methylation-specific PCR (MSP) was used. MSP, initially developed for assessing the methylation status of gene promoters in cell lines and frozen/fresh tissues (Herman *et al.,* 1996), has demonstrated its sensitivity in detecting tumour-specific hypermethylated genes in cfDNA of various malignancies (Skvortsova *et al.,* 2006; Papadopoulou *et al.,* 2006; Ellinger et al., 2008; Elshimali *et al.,* 2013; Matthaios *et al*., 2016). MSP relies on sodium bisulfite treatment of DNA to be able to distinguish methylated from unmethylated cytosines, by, under appropriate conditions, converting unmethylated cytosines to uracils, leaving methylated cytosines unmodified. Results obtained from PCR with methylation-specific primers and subsequent gel electrophoresis, can be scored as either methylated or unmethylated for the particular gene promoter without the need for further restriction and/or sequencing (Fig. 2.2).

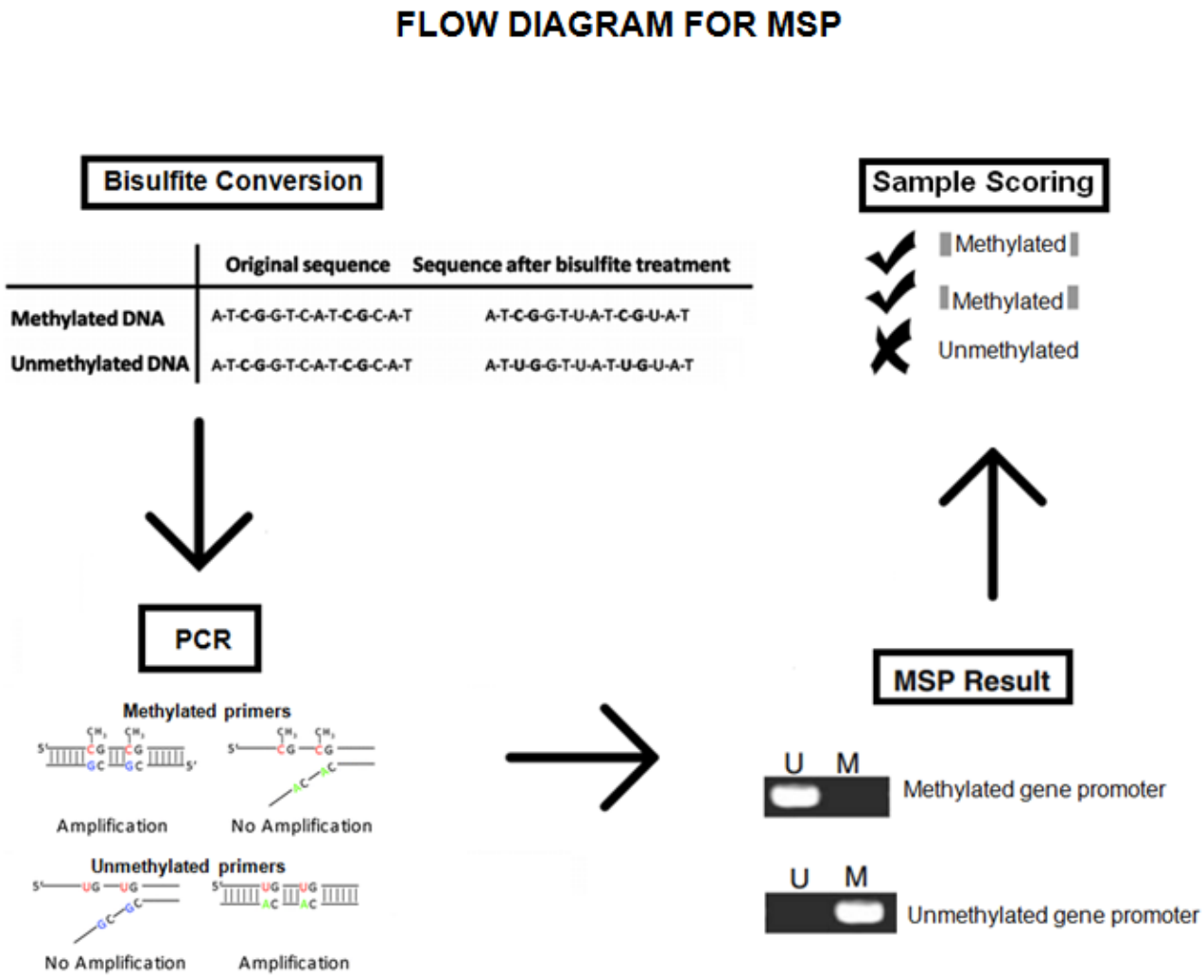


Figure 2.2 Flow Diagram for methylation-specific PCR. MSP (methylation specific PCR) begins with the bisulfite conversion of DNA samples with CpG islands, which converts unmethylated cytosines to uracils, while methylated cytosines remain unchanged. This is followed by PCR that only amplifies modified DNA using primer sets complimentary only to the formerly methylated or unmethylated alleles. PCR products with the correct molecular weight size are resolved on an agarose gel as either methylated or unmethylated; and finally scored as per sample.

2.4.1. Bisulfite Conversion

To perform bisulfite conversion on cfDNA samples, the EpiTect bisulfite kit (Qiagen) was utilised. Prior to bisulfite conversion, reagents were prepared according the manufacturer's instructions from the handbook.

For the sodium bisulfite treatment, the bisulfite mix was completely dissolved by vortexing and heated at 60 °C until the precipitate had completely dissolved. Next, a bisulfite solution consisting of 20 µl of template cfDNA (1.5 – 1.77 ng/µl), 85 µl bisulfite mix and 15 µl of DNA protect buffer was prepared in a 200 µl PCR tube, and the reaction made up to a total volume of 140 µl. After thorough mixing at room temperature, the bisulfite solution turned from green to blue indicating a correct pH for bisulfite conversion. The reaction mixture was incubated in a Bio-rad thermal cycler (MJ mini) with cycling conditions as follows: denaturation at 95 °C for 5 min; followed by 85 min incubation at 60 °C and 5 min at 95 °C; another denaturation step for 175 min at 60 °C; and an infinite hold at 20 °C.

Following bisulfite conversion, the DNA was precipitated by following the protocol for low fragmented DNA in the instruction manual. First, 310 µl of freshly prepared in buffer BL containing 10 µg/ml carrier RNA was added followed by 250 µl ethanol (96-100%). The entire reaction was transferred to a MinElute DNA spin column. Spin columns were spun down for 1 minute to collect supernatants, which were discarded each time after every step. The DNA left in the spin column was subsequently purified by performing a wash step with 500 µl buffer BW, desulphonated with 500 µl buffer BD followed by a second wash with 500 µl buffer BW. A second precipitation step was performed with 250 µl of ethanol. Purified DNA was eluted twice in 20 µl elution buffer (buffer EB) that had been pre-warmed to 60 °C, by centrifuging for 1 minute at 12 000 rpm. Eluted cfDNA was stored at 4 °C for subsequent PCR.

2.4.2. Polymerase Chain Reaction (PCR)

PCR on converted DNA was performed on bisulfite converted DNA and products analysed using gel electrophoresis. For MSP optimisation, *RASSF1A* MSP primers (Dammann *et al.*, 1999) and *Smad4* primers (Wei *et al.*, 2016) (table 2.4) together with the KAPA2G Fast ReadyMix PCR Mix (KAPA Biosystems) were used to prepare the PCR reaction. This kit makes use of the KAPA Fast DNA polymerase designed to improve sensitivity and product yield, compared to conventional *Taq* polymerase. To optimise primer specificity, the EpiTect PCR Control DNA set (Qiagen), consisting of 10 ng/µl of human untreated genomic DNA, bisulphite-treated methylated and bisulfite-treated unmethylated DNA was used.

Table 2.2 MSP primer information

Gene	Primer Sequence	Annealing temperature	Expected size (bp)
RASSF1A M	5'-CGAGAGCGCCTTTAGTTTCGTT'-3 (sense) 5'-CGATTAAACCCGTACTTCGCTAA-3' (antisense)	58	192
RASSF1A U	5'-GGGGGTTTTGTGAGAGTGTGTTT -3' (sense) 5'-CCCAATTAAACCCATACTTCACTAA-3' (antisense)	58	204
SMAD4 M	5'-GTAACGAGATGTTAATTTTTTCGGC-3' (sense) 5'-ACTTATCGAAAAACCACTAAACATACG-3' (antisense)	58	274
SMAD4 U	5'-TTTGTAATGAGATGTTAATTTTTTTGGT-3' (sense) 5'-CAACTTATCAAAAAACCACTAAACATACA-3' (antisense)	58	269

Reaction mixtures were set-up according to the manufacturer's instruction with the exception of the 2x Epitect Master Mix being replaced with 10 µl KAPA2G Fast ReadyMix (2x). Components added to the 20 µl reaction mixture prepared on ice were as follows: 10 µl KAPA2G Fast ReadyMix (2x), 1.25 µl sense primer (1µM), 1.25 µl reverse primer (1 µM), 5 ng template DNA (bisulfite converted) and RNase-free water to make it up to a 20 µl volume. Next, PCR tubes were transferred to the MJ mini

thermal cycler and DNA was amplified with the following conditions: initial denaturation at 95 °C for 3 min; 40 cycles of denaturation at 95 °C for 15 sec, annealing according to table 2.4 and extension at 72 °C for 30 sec; a final extension at 72 °C for 1 min; and an infinite hold at 4 °C. PCR products were loaded onto a 2% agarose gel and electrophoresis at 100 V was run. Gels were visualised under Bio-rad's Gel doc and analysed using the Image Lab software version 5.2 (Bio-rad).

2.4.3. Statistical Analyses

Statistical evaluation was performed using Stata version 13.1 (StataCorporation, College Station, Texas) with p-values of < 0.05 considered statistically significant. To test for the normality of the data, the Shapiro-Wilk test was performed with p-value >0.05 considered to represent a normal distribution. Median differences in cfDNA concentration levels were compared between PDAC and controls using the Kruskal-Wallis rank test for non-parametric variables, which was also used to determine the associations between cfDNA concentrations and individual clinical characteristics (chronic medical illness, HIV, gender, cigarette smoking and alcohol consumption), as well as the length of plasma storage at -80 °C. For categorical variables, Pearson's χ^2-test was used to determine the association between *Smad4* methylation and individual clinical characteristics (chronic medical illness, HIV, gender, cigarette smoking and alcohol consumption). Predictable outcomes of *Smad4* methylation based on patient characteristics were determined using logistic regression.

CHAPTER THREE: QUANTIFICATION ANALYSIS OF CELL-FREE DNA

Cell-free DNA (cfDNA) is detectible in patients suffering from cancer and other destructive diseases; with higher cfDNA levels having been reported in cancer patients compared to control subjects (Van der Vaart and Pretorius, 2010; Schwarzenbach *et al.,* 2011). Quantitative changes in cfDNA are known to be influenced by pre-analytical factors such as blood-processing, cfDNA isolation and storage conditions, as well as biological factors such as tumour stage and size in the case of cancer, inflammation and other associated diseases; however, no variable has consistently been shown to be associated with cfDNA levels (Jung *et al.,* 2010).

3.1. Patient demographics

In this study, cfDNA concentration levels were compared between PDAC and two control patient groups that either suffered from chronic pancreatitis (CP) or critical limb ischemia (CLI), to determine whether or not concentration levels differed between the three groups. Patient demographics and concentrations, presented in table 3.1 (also see Appendix A), were used to assess associations related to increased cfDNA levels in patients. The mean ages of patients ranged between 50 and 58 years; with at least 50% of all patient groups diagnosed with a chronic medical illness, and having smoked cigarettes at least once in their lives. Most smokers (89%) and alcohol consumers (96%) were observed in the CP control group, with less smokers (62%) and drinkers (68%) observed in the PDAC group.

Approximately 1/3 of patients had been diagnosed with HIV, of which minimal differences were observed between patient groups ($p=0.954$). The same applied to chronic medical illness ($p=0.640$). However, statistical analyses pointed out notable differences between the ages of patients in each sample group ($p<0.001$), with PDAC patients being oldest (58.3±11.7 years) and CP patients being the youngest (50.4±9.4 years). Significant variations were also seen in patients that consumed alcohol and cigarettes ($p<0.001$ for both).

Table 3.1 Patient demographics for cfDNA quantitative analysis

	PDAC	CP	CLI	p-value*
Number of patients	155	46	88	
Mean age	58.3±11.7	50.4±9.4	52.2±15.9	<0.001**
Chronic medical illness	63.0% (97/154)	60.9% (28/46)	56.8% (50/88)	0.640
Smoked cigarettes	61.6% (93/151)	89.1% (41/46)	50.0% (44/88)	<0.001
Consume alcohol	67.4% (99/147)	95.7% (44/46)	42.5% (37/87)	<0.001
HIV status	38.4% (33/86)	36.4% (12/33)	36.1% (22/61)	0.954

* p-values calculated using a χ^2 test
**p-value calculated using an ANOVA test

3.2. Cell-free DNA levels in patients

Plasma-derived cfDNA concentrations were quantified spectrophometrically in patients with PDAC (n= 155), CP (n= 46) and a control group (n=88). cfDNA levels were the highest among patients with PDAC, followed by CP patients, whilst patients with CLI, with a healthy pancreas, showed to have the least (Fig. 3.1). Due to the wide dispersion of cfDNA concentrations within the patient groups, which did not follow a normal distribution ($p < 0.05$; Shapiro-Wilk W test), statistical inferences could only be made using median concentrations (See Appendix B for Stata outputs).

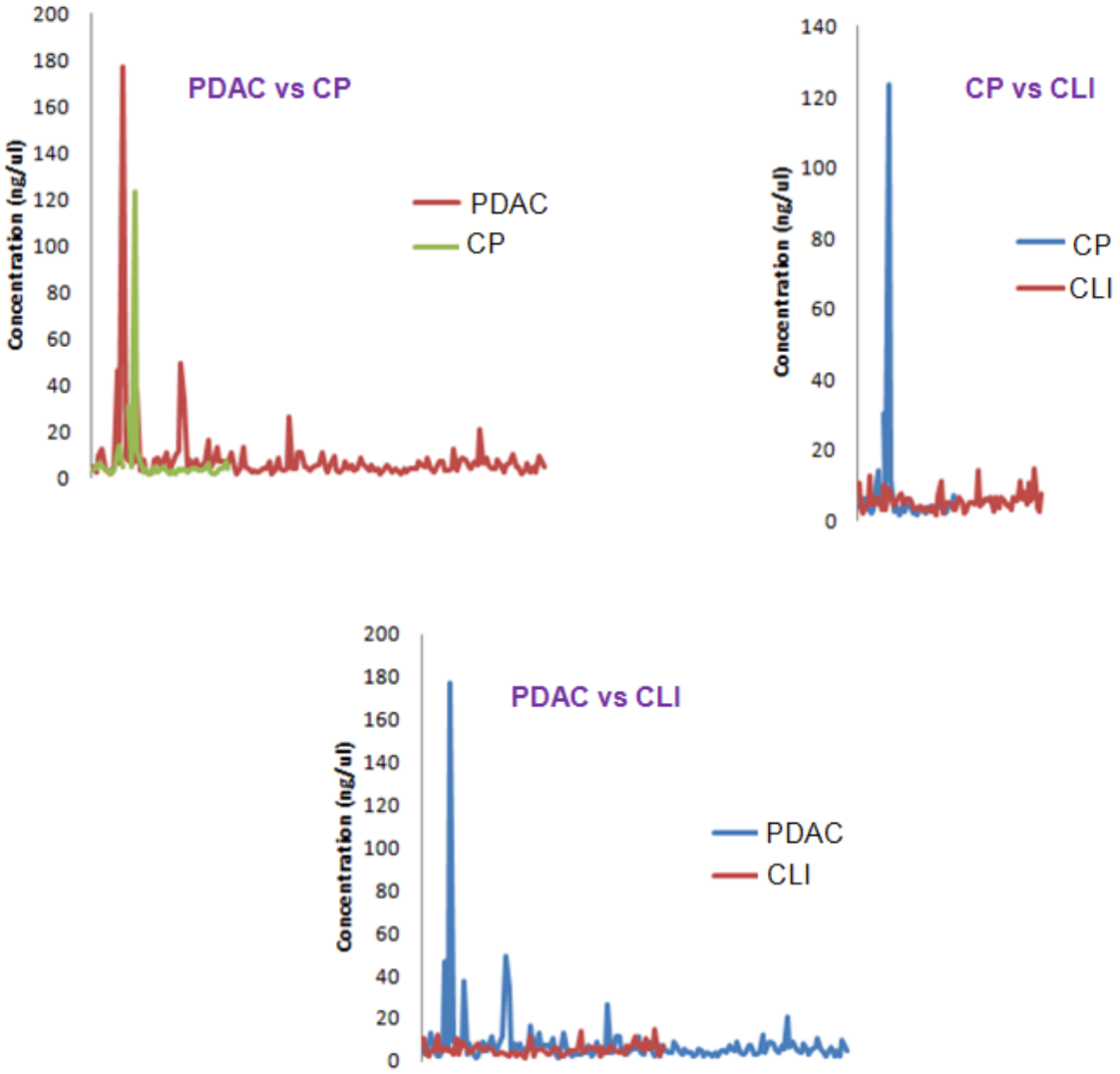


Figure 3.1 Comparisons of cfDNA concentration levels between patient groups

The Kruskal-Wallis H test was performed to make statistical inferences from the data, which appeared to be widely dispersed according to the calculated median concentrations amongst the groups (see Appendix B). Results from the test revealed significant differences in concentration between the three patient groups, with medians being 5.10 ng/ul for PDAC; 3.85 ng/ul for CP and 5.05 ng/ul for the CLI control groups ($p= 0.006$). Furthermore, when the Wilcoxon rank-sum test was performed to compare statistical significance between two groups, major differences were observed between the PDAC and CP groups ($p= 0.002$), as well as the CP and the CLI control groups ($p= 0.007$) (Table 3.2).

Table 3.2 Statistical summary of measured cfDNA levels

		Median (range) cfDNA (ng/ul) and significance of differences between groups		
Patient group	**N**	**PDAC**	**CP**	**CLI**
PDAC	155	**5.1** **(3.9-7.6)**	p= 0.002	P=0.433
CP	46	p= 0.002	**3.85** **(3-5.2)**	P= 0.007
Control group	88	p= 0.433	p= 0.007	**5.05** **(3.85-6.6)**

3.3. Effects of Clinical factors on cfDNA levels

The Kruskal-Wallis rank test was performed to determine whether there were any associations between cfDNA levels and clinical characteristics (table 3.3). Results indicated no relationship between overall cfDNA levels in patients and clinical risk factors associated with PDAC and CP (smoking, alcohol consumptions and chronic illness) ($p > 0.05$). Similarly, no associations were observed between PDAC cfDNA levels and its risk factors. HIV, though not a risk for PDAC, revealed a strong relationship with cfDNA levels in CLI patients ($p = 0.034$). A strong positive association was also observed between cfDNA levels and the length at which plasma was stored between 0 and 3 years at -80°C ($p < 0.001$).

These results demonstrated the use of cfDNA levels in distinguishing patients with pancreatic disorders from those with a healthy pancreas. Associations between cfDNA

levels and clinical factors could only be established for CLI patients diagnosed with HIV (based on median comparisons); as well as the duration at which plasma was stored prior to cfDNA isolation. An association was seen between length of storage and cfDNA concentration (Fig 3.2). However, since the length of storage may have affected cfDNA yield, further assessment needed to be carried out to determine whether or not common genetic alterations could be detected in cfDNA; as well as to assess the DNA methylation, with the expectancy of identifying biomarkers that discriminate PDAC from CP.

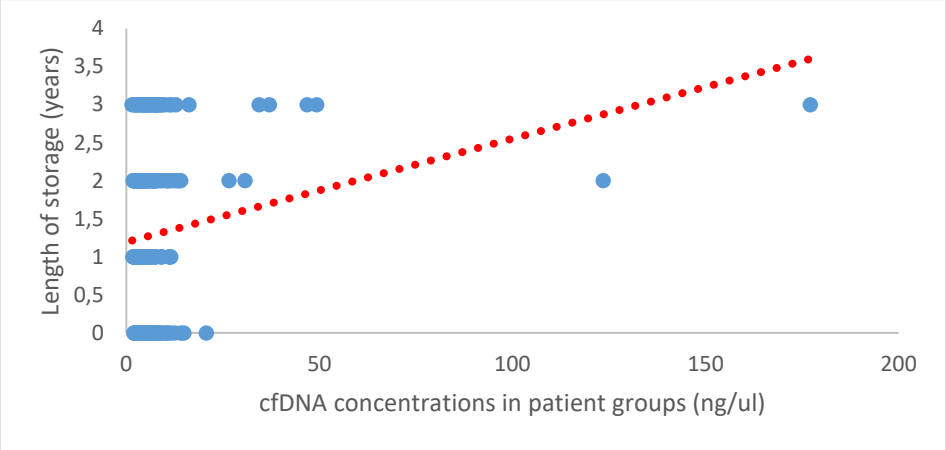


Figure 3.2. A chart showing a correlation between cfDNA concentrations with length of storage. The red-dotted regression line indicates increase in concentration with an increase in storage time prior to cfDNA isolation.

Table 3.3 Associations between cfDNA levels and clinical factors

Patient Characteristics		Observations	**cfDNA concentration (ng/ul)**		**p-value***
Chronic medical illness			**Yes**	**No**	
	Overall	**174/278**	**4.9 (3.5 - 6.9)**	**5.0 (3.9 - 7.1)**	**0.415**
	PDAC	**97/154**	**5.1 (3.7 - 7.4)**	**5.7 (4.1 - 7.9)**	**0.226**
	CP	**27/45**	**3.4 (2.9 - 5.3)**	**4.3 (3.0 - 5.2)**	**0.308**
	CLI	**50/88**	**5.1 (4.1 - 6.8)**	**5.0 (3.7 - 6.4)**	**0.536**
HIV status			**HIV+**	**HIV-**	
	Overall	**67/179**	**4.6 (3.2 - 6.3)**	**4.6 (3.6 - 6.3)**	**0.453**
	PDAC	**33/86**	**5.0 (3.7 - 7.4)**	**4.3 (3.7 - 5.7)**	**0.558**
	CP	**12/32**	**3.8 (2.8 - 5.2)**	**3.4 (2.9 - 4.6)**	**0.969**
	CLI	**22/61**	**4.4 (3.2 - 5.3)**	**5.3 (4.3 - 6.9)**	**0.034**
Smoking			**Yes**	**No**	
	Overall	**177/284**	**4.6 (3.6 - 7.4)**	**5.1 (3.6 - 6.8)**	**0.872**
	PDAC	**93/151**	**5.1 (3.7 - 8.0)**	**5.4 (4.0 - 7.4)**	**0.982**
	CP	**40/45**	**4.1 (3.1 - 5.3)**	**2.8 (2.8 - 3.4)**	**0.187**
	CLI	**44/88**	**5.2 (3.9 - 6.6)**	**5.0 (3.6 - 6.5)**	**0.570**
Alcohol consumption			**Yes**	**No**	
	Overall	**179/279**	**4.7 (3.7 - 6.6)**	**5.3 (3.5 - 7.3)**	**0.736**
	PDAC	**99/147**	**5.1 (3.7 - 7.6)**	**5.7 (3.8 - 7.7)**	**0.811**
	CP	**43/45**	**3.9 (2.9 - 5.3)**	**3.2 (3.0 - 3.4)**	**0.408**
	CLI	**37/87**	**5.1 (4.6 - 6.5)**	**5.2 (3.5 - 6.6)**	**0.492**
Length of storage (years)					
	Overall	**289/289**	**4.9 (3.6 - 7.0)**		**<0.001**
	0-1	**79/289**	**5.5 (4.1 - 6.9)**		
	1-2	**91/289**	**4.1 (3.2 - 5.3)**		
	2-3	**75/289**	**4.9 (3.5 - 7.4)**		
	>3	**44/289**	**6.4 (4.4 - 9.1)**		

* p-values calculated using a Wilcoxon rank-sum (Mann-Whitney) test

CHAPTER FOUR: GENE ANALYSIS IN CELL-FREE DNA

4.1. K-ras mutation analysis in cfDNA

The observed concordance rate of mutations between tumour- and plasma- derived DNA in previous studies promotes the use of liquid biopsies over traditional methods to monitor cancer genetics. Since results from quantification analyses indicated that the duration of plasma storage prior to extraction, a pre-analytical factor in cfDNA isolation, could have affected the reliability of cfDNA samples, mutational analysis was performed to determine whether or not cfDNA was useful in detecting genetic alterations that occur in PDAC.

One of the most common cancer-identifiers, *K-ras,* has shown to be the gene of choice in initiating experimental studies involving cfDNA (Schwarzenbach *et al.,* 2011; Taly *et al*., 2013; Thierry *et al*., 2014; Bettegowda *et al*., 2014). *K-ras* mutations, present in nearly all PDACs as early initiators (Löhr *et al.,* 2005), have also been identified in cfDNA of PDAC patients; but not yet in CP patients, where the presence of mutations are suggested to select for patients with a high risk for developing PDAC (Bournet *et al.* 2009; Shi *et al.,* 2008; Talar-Wojnarowska *et al*., 2004). For this reason, the detection of *K-ras* mutations in cfDNA samples from PDAC, CP and CLI patients was assessed using droplet digital PCR (ddPCR), a recently commercialized technology that enables the precise quantification and detection of target DNA sequences in a sample.

In cfDNA, the use of ddPCR to detect *K-ras* mutations demonstrates its ability to screen for mutations with sufficient sensitivity in DNA obtained by non-invasive blood collection (Taly *et al.,* 2013; Oxnard *et al.,* 2014; Zhu *et al.,* 2015). However, because of the highly fragmented nature of cfDNA, factors affecting the end-signal detection from sample preparation to data analysis (Fig. 2.1) such as the number of droplets measured per PCR reaction, as well as input DNA concentration (Pinheiro *et al.,* 2011; Strain *et al*., 2013) had to be assessed to optimise ddPCR for *K-ras* analysis in patient samples.

4.1.1. Droplet digital PCR optimisation

4.1.1.1 Input cfDNA concentrations

Previous studies have made use of low input cfDNA amounts, ranging from picograms to nanograms to either quantify DNA targets or detect mutations using ddPCR (Combaret *et al.,* 2015; Kinugasa *et al*., 2015; Guttery *et al.,* 2015; Sanmamed *et al*., 2015; Hyman *et al.,* 2015; Tsao *et al*., 2015; Guibert *et al*.; 2016; Taly *et al*., 2013; Janku *et al*., 2016) (table 4.1). The most commonly used input concentration appeared to be 10 ng/µl. However, due to the limited amounts of cfDNA available for this *K-ras* study, ddPCR was optimised to determine the lowest amplifiable concentration of cfDNA that could be used to detect mutations.

For the assessment of the cfDNA, two concentrations (5 and 2.5 ng/µl) were investigated to determine if the droplet reader could detect positive droplets from low input amounts of fragmented DNA after thermal cycling. Mutation-positive cluster positions were predicted using positive (cell line) and negative (CLI cfDNA patient) controls, which were chosen according to a known health status. In addition, the discrimination between positive (containing target) and negative (containing no target) droplets was based on thresholds that were manually set above the NTC (6000 for FAM channel and 2000 for HEX channel).

When high input concentrations were used (50 ng/µl for the positive control and 5 ng/µl for cfDNA samples), target DNA concentrations in positive droplets read by QuantaSoft were higher than at low input concentrations (25 ng/µl for the positive control and 2.5 ng/µl for cfDNA samples), meaning that the droplet reader quantified half of the input DNA (Fig. 4.1). Furthermore, higher mutant to wild type ratios were observed in cfDNA inputs of 5 ng/µl compared to 2.5 ng/µl, similar to the results published by Taly *et al.* (2013), with the exception of the PDAC cfDNA samples where both concentrations yielded ratio's in the same range 0.3 and 0.31.

These results demonstrated the potential clinical utility of ddPCR's ability to detect rare mutations in cfDNA by a multiplexing approach, which not only increases the number of targets measured in a single reaction, but also scales down the amount of clinical material required to analyse multiple mutations, with only as little as 2.5 ng/µl needed to amplify cfDNA.

Table 4.1 Summary of ddPCR input cfDNA concentrations in the literature

Cancer type	**Gene Biomarker**	**DNA concentration (ng/µl) in 20 µl reaction volume**	**Reference**
Neuroblastoma (NB)	ALK	10	Combaret *et al.,* 2015
Pancreatic cancer	K-ras	Up to 130	Kinugasa *et al*., 2015
Breast cancer	ESR1, PIK3CA, TP53	5	Guttery *et al.,* 2015
Melanoma	BRAF	*1.6	Sanmamed *et al*., 2015
Langerhans cell histiocytosis (LCH) and Erdheim–Chester disease (ECD)	BRAF	Up to 60	Hyman *et al.,* 2015
Melanoma	BRAF , N-ras	10	Tsao *et al*., 2015
Gastric cancer	HER2	10	Kinugasa *et al*., 2015
NSCLC	K-ras	10	Guibert *et al*.; 2016
CRC	K-ras	0.3-283	Taly *et al*., 2013
Metastatic cancers	K-ras	16	Janku *et al*., 2016

*Concentration in picograms (pg/µl)

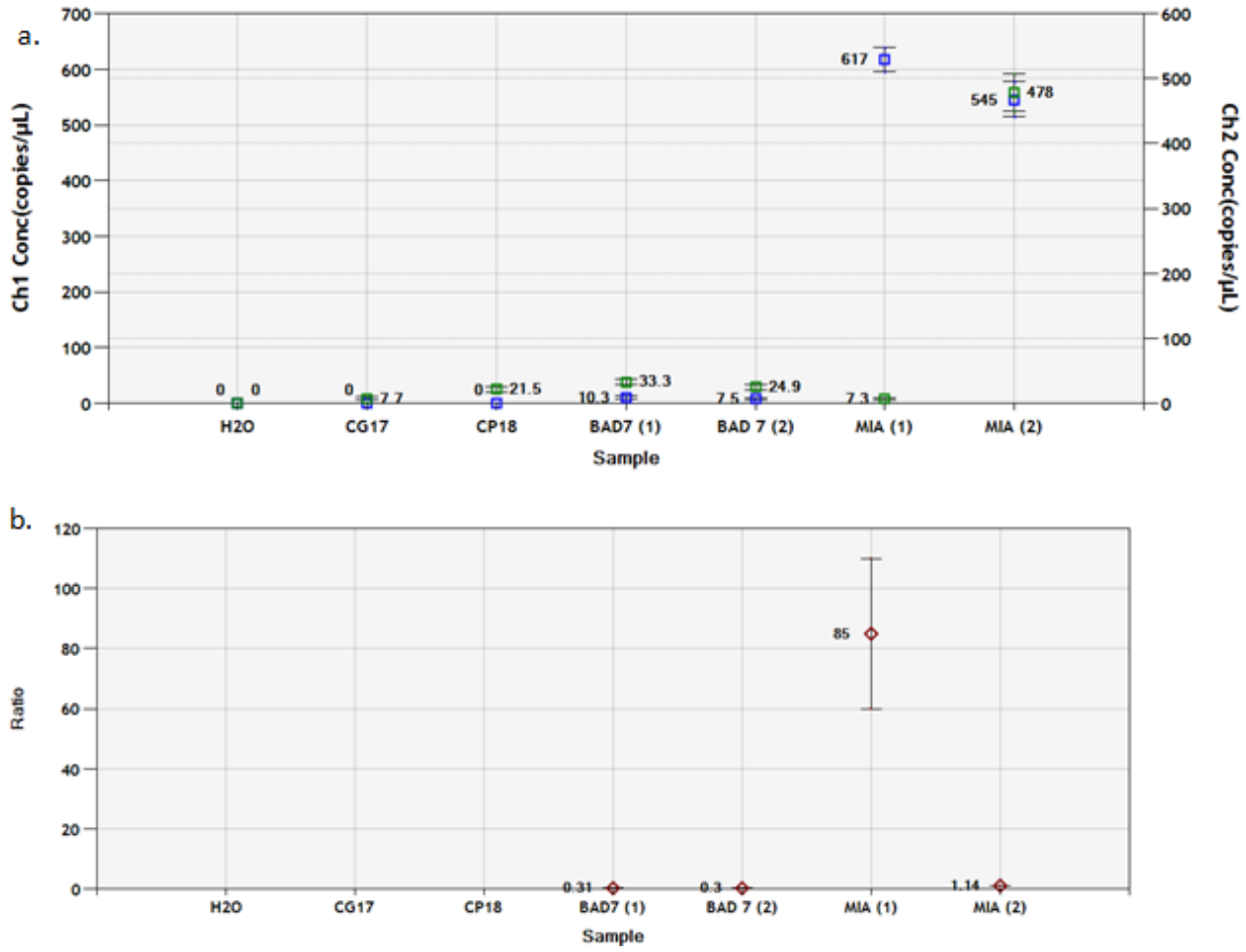


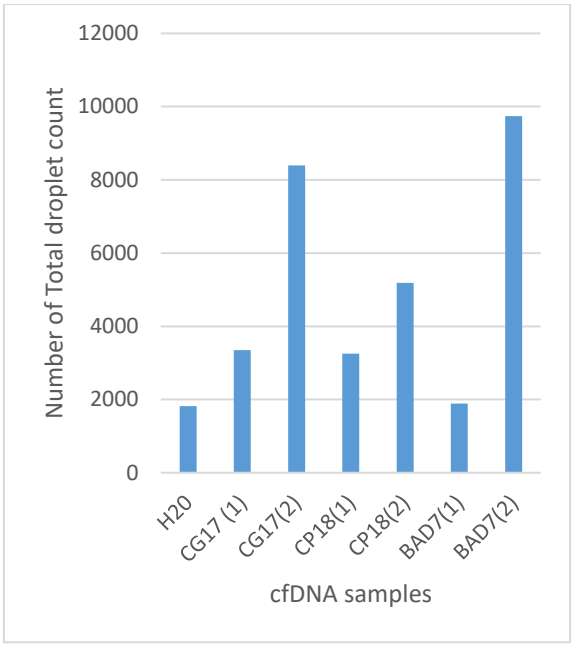
Figure 4.1 Screenshot of positive droplets detected by droplet reader from QuantaSoft. (a) Concentrations quantified by QuantaSoft using Poisson distribution of: wildtype (green box) and mutant (blue box) *K-ras*. (b) *K-ras* mutant ratios. Thresholds were set at 6000 for FAM channel and 2000 for HEX channel. Duplicate concentrations were run on each sample, with numbers in brackets (1) and (2) indicating 2.5 and 5 ng/µl, respectively. Sample IDs abbreviated as H2O: non-template control; CG17: CLI control sample 17; CP18: Chronic pancreatitis control sample 18; BAD7: PDAC sample 7; and MIA: pancreatic cancer cell line. Points represent percentage of positive (mutant and wildtype) alleles detected in the sample; error bars represent Poisson distribution error bars.

4.1.1.2 Total droplet counts

The accepted events for the analysis of ddPCR data are total droplet counts greater than 10 000. However, if events are lower than this number, careful evaluation of the data is required. Since, for technical reasons, only approximately 30 µl of droplets could be transferred to the PCR microplates, instead of the recommended 40 µl after droplet generation, the total event number of generated droplets was much lower in most instances than the recommended total.

To determine the lowest total droplet count for ddPCR for which target sequences could be reliably detected, droplets with differing amounts of cfDNA were run in duplicate (5 and 2.5 ng) and analysed using the QuantaSoft program. Results revealed varying total droplet counts across all samples (Fig. 4.2a) even though the input volume, approximately 30 μl, was similar. Positive droplets, containing either wild-type, mutant or both target sequences were detected in most cfDNA samples, including samples that had the lowest total droplet counts i.e. 1886 events (Fig. 4.2b). The absence of positive droplets in one of the control groups (total events =3345), was not attributed to a low total droplet count, since samples that had even lesser total droplet counts could still reveal positive droplets; more so, *K-ras* mutants (Fig. 4.2b). Thus, other factors contributing to droplet stability such as emulsion degradation during thermal cycling could have occurred. In this regard, a droplet count of 1886 was to be considered a cut-off value for subsequent analyses.

a.



b.

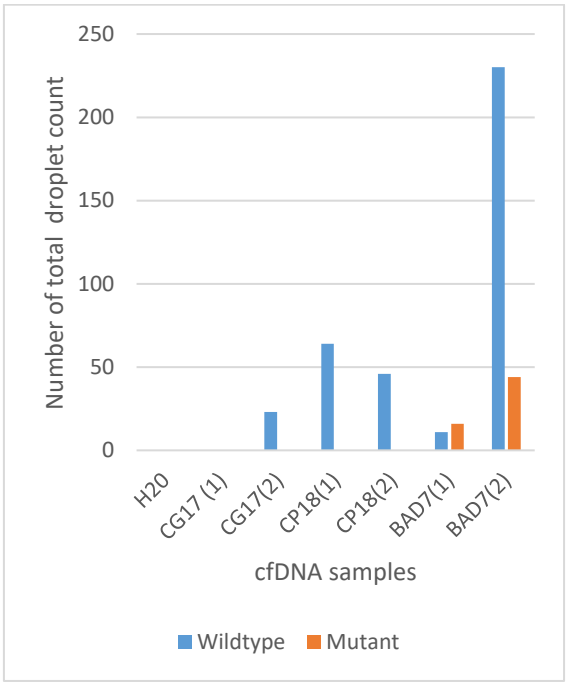


Figure 4.2 Droplet counts detected by droplet reader in ddPCR. (a) Total droplet counts. Blue-bars define total events containing both positive and negative droplets. (b) Positive droplet counts. Blue bars define wild-type *K-ras*; red bars define mutant *K-ras*. Duplicate concentrations were run on each sample, with numbers in brackets (1) and (2) indicating 2.5 and 5 ng/µl, respectively. Sample IDs abbreviated as H2O: non-template control; CG17: CLI control sample 17; CP18: Chronic pancreatitis control sample 18; BAD7: PDAC sample 7; and MIA: pancreatic cancer cell line.

4.1.2. K-ras mutation analysis in patient cfDNA

To detect *K-ras* mutations in cfDNA samples, the ddPCR's rare mutation detection assay (RMD) was performed on samples matched according to age. Patient demographics (table 4.2) represent a total of 42 matched patient groups (n=126) ranging between 23- 70 years of age (mean age: 50). Approximately half of the patients in each group had been diagnosed with a chronic medical illness. CP patients had the highest proportion of smokers (88%) and drinkers (96%), while CLI patients had the least. For the 25 PDAC, 30 CP and 28 CLI patients with a known HIV status, the highest proportion of patients diagnosed with HIV came from PDAC patients (52%), followed by the CLI group (46%), and CP having the least (40%). Statistical analyses showed little or no difference in patient group ages (p=1.00), chronic medical illness (p=0.681) and a positive HIV status (p=0.671). However differences were observed in patients that smoked cigarettes (p=0.003) and consumed alcohol (p<0.001); again, pointing to chronic pancreatitis patients as being the major alcohol and cigarette consumer group.

Table 4.2 Patient demographics for genetic screening

	PDAC	**CP**	**CLI**	**p-values***
Mean age	50.1 ± 10.2	50.0 ± 9.4	50.1 ± 10.1	1.000**
Chronic medical illness	54.8% (23/42)	59.5% (25/42)	50.0% (21/42)	0.681
Smoked cigarettes	60.0% (24/40)	88.1% (37/42)	57.1% (24/42)	0.003
Consume alcohol	74.1% (29/39)	95.2% (40/42)	53.7% (22/41)	<0.001
HIV status	52.0% (13/25)	40% (12/30)	46.4% (13/28)	0.671

* p-values calculated using a χ^2 test
**p-value calculated using an ANOVA test

Ten matched samples (n=30) were subject to ddPCR in each run, with a total of five runs. Results analysed on Quantasoft showed variability in total droplet events (Fig. 4.3) ranging from 737 to 12369 events. Samples with droplet counts lower than the set cut-off value of 1886 were excluded from further analysis. Positive and negative droplets were distinguished based on FAM and HEX thresholds set in the above optimisation experiments. However, due to the significant variability in fluorescence amplitudes observed in run 2-5 (Fig. 4.4), which showed rain droplet events or "noise" i.e. droplets with intermediate fluorescence that could have most likely been caused by emulsion degradation during thermal cycling, thresholds could not be set for approximately ¾ of cfDNA samples. The droplet generation oil used here had passed its expiry date by a few weeks; thus results here demonstrate the effect of droplet emulsion degradation on end point signals in the droplet reader.

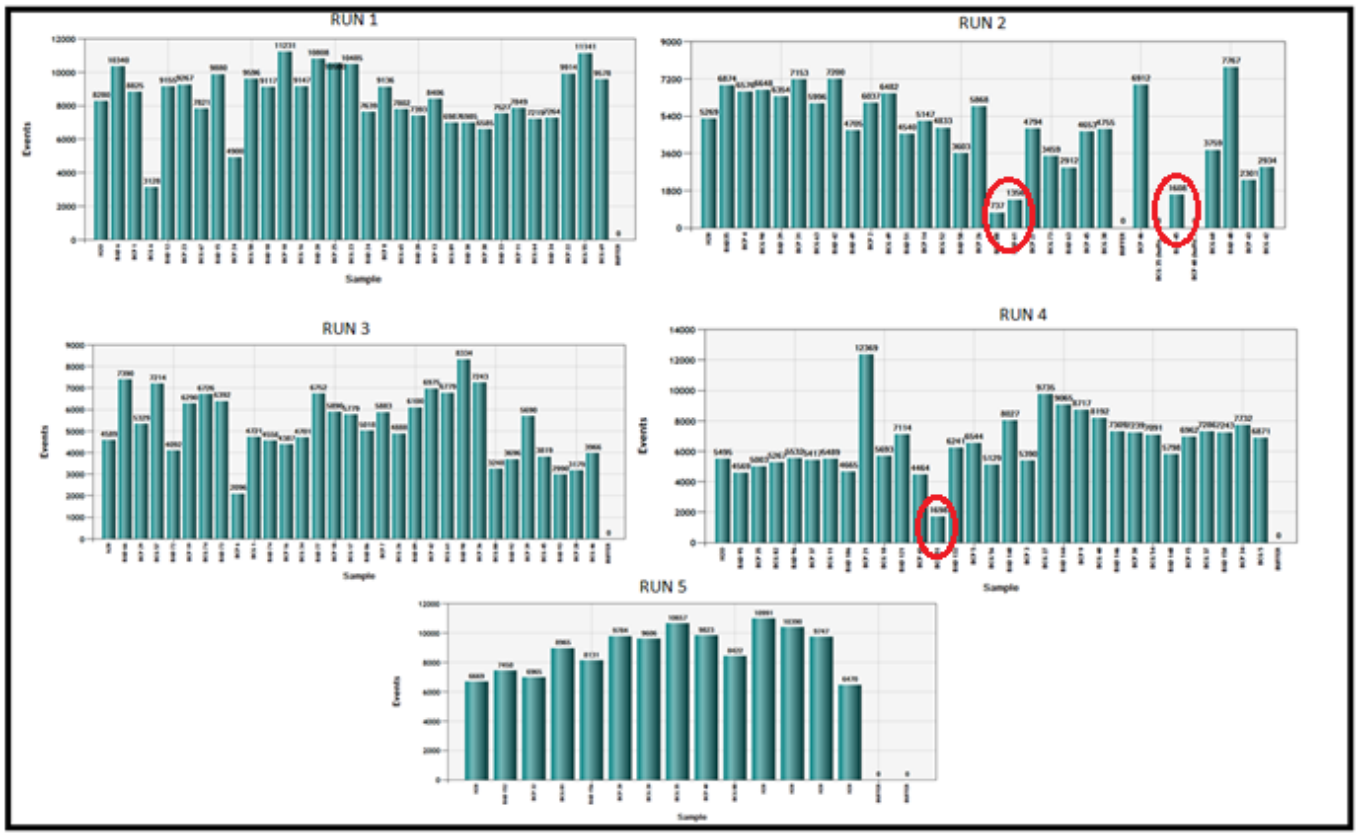


Figure 4.3. Screenshots taken from QuantaSoft representative of bar graphs of total events in ddPCR of matched cfDNA sample groups. Total droplet counts (positive + negative droplets) ranged between 737 and 12 369. Zero droplets were detected in the buffer controls (shown by a green dot in the last two columns of run 5 and last columns of run 1, 3 and 4 *buffer in run 2 was contaminated-indicated by a green bar). Bars encircled with red represent droplet counts that were less than the cut-off value of 1886. These were excluded out of the analysis. "Event" on the y-axis represents the number of droplets detected by ddPCR.

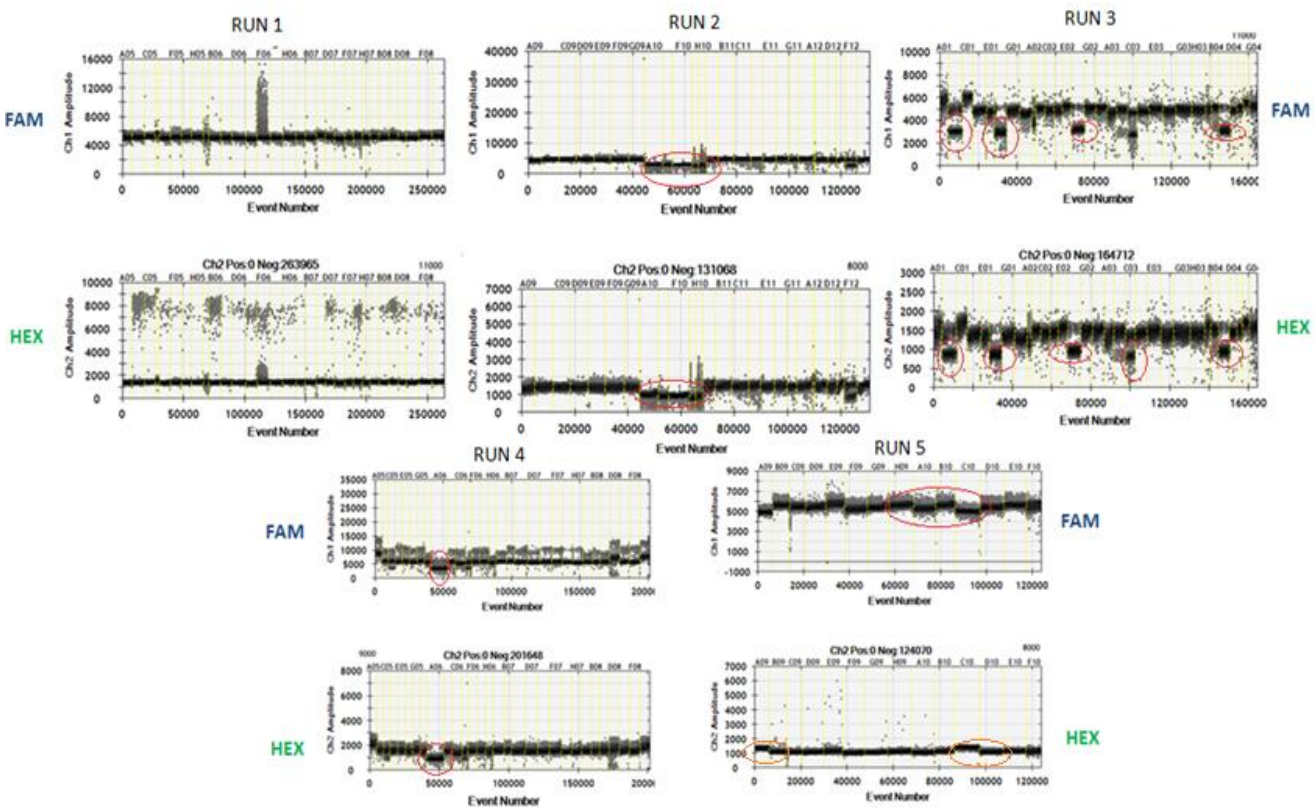


Figure 4.4 Screenshots taken from QuantaSoft representative of 1-dimensional plots for matched cfDNA sample runs. FAM and HEX channels on y-axis show uniform fluorescence amplitudes across all samples in run 1; whereas runs 2, 3, 4 and 5 showed variability in amplitudes (indicated by orange circles). "Event number on x-axis represent negative detected alleles (grey); and positive alleles that cannot be clearly seen on this picture, but appear as orange for mutants and blue for wildtypes in QuantaSoft.

Since most of the samples showed intermediate fluorescence, setting different thresholds of such droplets came with the risk of making false-positive calls. Therefore, only samples in the first ddPCR (run 1), were considered for further analysis. Two-dimensional plots revealed a clear distinction between negative droplets and wild-type *K-ras* (Fig. 4.5). However, clear cluster separation between negative droplets and mutant *K-ras* was not observed. This was due to one of the PDAC samples that showed a very high fluorescence, which in turn, compromised the mutant clustering plot. Nonetheless, positive counts that distinguish mutants from wild-types could be established, revealing *K-ras* mutants in cfDNA of patients with PDAC (4/10), CP (5/10) and CLI (6/10). A Wilcoxon Mann-Whitney test revealed no association between *K-ras* mutations and cfDNA concentration levels (table 4.3; Appendix B). However, due to a small sample size of results obtained in *K-ras* mutation analysis, there is

insufficient statistical power to make any inferences concerning *K-ras* mutations and the cfDNA levels of patients in this study.

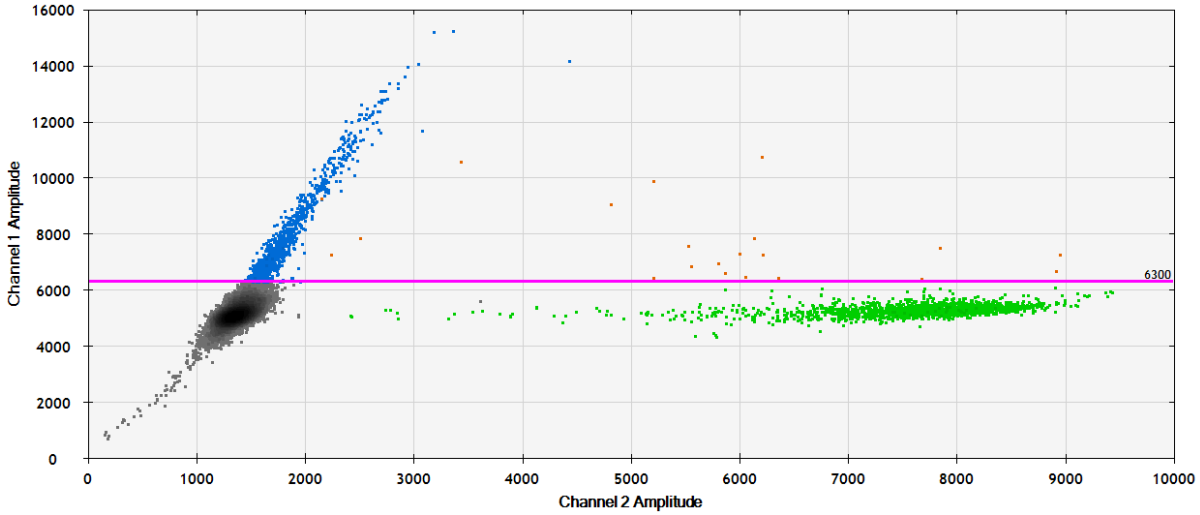


Figure 4.5 Two-dimensional plot showing cluster separation. Cluster colours are represented as Blue: mutant KRAS alleles; Green: KRAS wildtype alleles; Orange: *K-ras* mutant and wildtype; Grey: Negative droplets containing no *K-ras* alleles. FAM and HEX thresholds were linear throughout all samples. Plot represents the first ddPCR run.

Table 4.3 Associations between cfDNA levels and *K-ras* mutations

		cfDNA concentration (ng/ul)		
	Observations	**G12 mutation**	**WT only**	**p-value***
Overall	15/30	5.7 (4.2 - 11.1)	6.1 (4.6 - 30.7)	0.430
PDAC	4/10	9.5 (7.9 - 12.0)	21.2 (6.1 - 37)	0.829
CP	5/10	4.2 (3.4 - 4.2)	5.1 (4.6 - 14.1)	0.141
CLI	6/10	6.0 (4.8 - 12.6)	4.6 (3.3 - 6.5)	0.286

* p-values calculated using a Wilcoxon rank-sum (Mann-Whitney) test

4.2 Methylation profiling in cfDNA

In PDAC, the progression of PanIN lesions to an invasive ductal adenocarcinoma is driven by the loss or inactivation of common tumour suppressor genes including *p16* (*CDKN1A*), *p53, Smad4* (*DPC4*) and *BRCA 2*, once activating *K-ras* mutations have occurred (Hruban *et al.,* 2000). *K-ras* and *p16* mutations are observed in both low- and high-grade PanIN lesions (Moskaluk *et al.,* 1997), whereas *Smad4* mutations are present only in high-grade lesions that harbour *K-ras* mutations (Wilentz *et al.,* 2000). *RASSF1A* is also another gene frequently inactivated in PDAC (Dammann *et al.*, 2003), but has not been clearly associated with *K-ras* activating mutations; and the point at which *RASSF1A* methylation occurs during disease progression is also unknown. Aberrant methylation has been reported to be associated with *p16* and *RASSF1A* inactivation in PDAC (Fukushima *et al.,* 2002; Dammann *et al.,* 2002). However, loss of *Smad4* expression in PDAC has only been associated with mechanisms other than methylation (LOH and intragenic mutations) (Hahn *et al.,* 1996; Iacobuzio-Donahue *et al.,* 2000). Thus, the methylation status of *Smad4* was assessed in patient cfDNA samples using methylation specific PCR (MSP), a technique that facilitates the detection of promoter hypermethylation at CpG islands of DNA.

4.2.1 MSP Optimisation

Successful amplification of a PCR product, indicative of the presence of either methylated (M) or unmethylated (U) alleles may often be influenced by several technical factors including DNA yield, primer specificity and PCR conditions i.e. annealing temperature. For this reason MSP conditions were optimised. Methylated (M) and unmethylated (U) primers for the *RASSF1A* gene promoter were used as control primer sets to determine the lowest input cfDNA concentration needed for successful amplification after bisulfite treatment. In addition, the Epitect control DNA set (Qiagen), which consisted of completely methylated, completely unmethylated bisulfite converted and unconverted DNAs; as well as the MIA PaCa-2 cell line, were used to validate the binding specificity of *RASSF1A* MSP primers and to optimise thermal cycling conditions.

4.2.1.1 Bisulfite conversion cfDNA yields

Following the “sodium bisulfite conversion of unmethylated cytosines in small amounts of fragmented DNA” protocol of the Epitect® bisulfite handbook, to determine the efficiency of bisulfite treatment, randomly selected cfDNA samples (n= 13) from the PDAC, CP and CLI groups, as well as the cell line, gave concentrations ranging between 2.1 ng/µl to 10.4 ng/µl (table 4.4). The high cfDNA concentrations post bisulfite treatment were however not a true reflection of cfDNA yield due the presence of carrier RNA during the bisulfite conversion procedure. Therefore, to eliminate these false-positive values, “true” cfDNA yields were calculated (Eq. 1). Converted cfDNA samples amplified with *RASSF1A* M and U primers (Bachman *et al.,* 1999) showed picogram amounts of bisulfite treated DNA (ranges 70 pg/µl -1.25 ng/µl) to be detectable on the electrophoresis gel after MSP amplification (Fig. 4.6), thus validating the bisulfite conversion.

Furthermore, samples (table 4.4) amplified with *RASSF1A* control primers revealed unmethylated *RASSF1A* in all cfDNA samples*,* whereas a slight ambiguity was observed in the methylation status of *RASSF1A* in the cell line, indicated by a strong band in the M primer amplification and a faint one in the U primer. Although the stronger (merhylated) band appeared at the correct molecular weight region (93 bp), the weaker (unmethylated) one appeared just under 200 bp, raising concerns about the binding specificity of the *RASSF1A* M primers.

$$[DNAf] = \frac{[DNAi]}{EV}$$

Equation 1

Where:

$[DNAf]$ = Final DNA concentration after bisulfite treatment

$[DNAi]$ = Initial DNA concentration before bisulfite treatment

EV = Volume of elution buffer that converted DNA was eluted into

Table 4.4 cfDNA yields from bisulfite treatment

cfDNA		Concentration (ng/µl)		
SAMPLE ID	**DNA sample**	**Initial**	**After bisulfite treatment**	**Actual cfDNA yield**
1	Cell line	50	14	1.25
2	BAD 00003	8.4	15	0.21
3	BAD 00004	10.4	15	0.26
4	BAD 00006	5.8	14	0.15
5	BCP 00002	5.0	12	0.13
6	BCP 00005	4.3	16	0.11
7	BCP 00008	5.7	12	0.14
8	BCP 000010	4.6	17	0.12
9	BCP 00007	3.4	14	0.09
10	BCG 000014	2.1	15	0.05
11	BCG 000017	2.8	14	0.07
12	BCG 000023	2.8	16	0.07
13	BCG 000029	2.8	20	0.07
14	BCG 000030	3.9	14	0.10

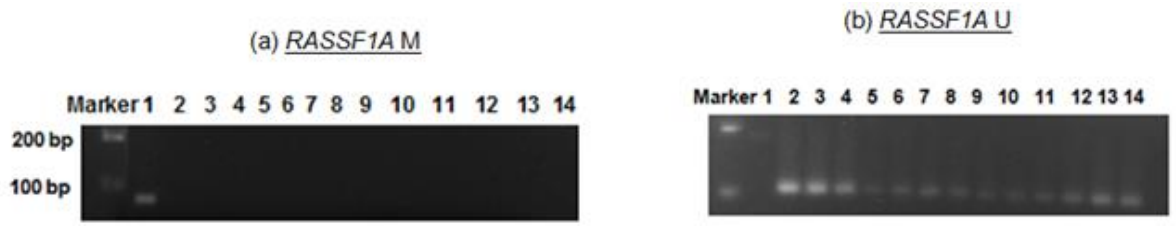


Figure 4.6 *RASSF1A* methylation profile on small amounts of bisulfite-treated cfDNA. Bands indicate PCR products from table 4.4 amplified with *RASSF1A* M and U primers resolved on a 2% agarose gel (a) Amplification with *RASSF1A* M primers showing the cell line to amplify the correct region (93 bp) (b) Amplification with *RASSF1A* U primers showing cfDNA samples to amplify the correct region (106 bp), and a faint band in the cell line just below the 200 bp region. Gel lanes labelled according to DNA samples in table 4.4.

4.2.1.2 Primer specificity

PCR amplification with M and U *RASSF1A* primers using the Epitect control DNA set further revealed the lack of binding specificity of these MSP primers. Products resolved on an electrophoresis gel showed binding of *RASSF1A* M primers in all control DNAs, including the cell line; binding of the *RASSF1A* U primers was also observed in all control DNAs, with faint bands in the cell line DNA (Fig. 4.7). Since *RASSF1A* MSP primers from Bachman's group (1999) failed to bind specifically to control DNA, even at higher annealing temperatures, new primers from Dammann's group (2000) were used to validate MSP primer specificity for this gene on the control DNA set.

M primers bound with high specificity to bisulfite-treated methylated control DNA (Fig. 4.8). In addition, *RASSF1A* showed to be methylated in the MIA PaCa-2 cell line, supporting previous results from Damman's group (2003). U primers also bound to bisulfite-treated unmethylated DNA with high specificity. Results from amplification of *RASSF1A* MSP primers on control DNA sets were thus showed the use of the control DNA set to determine primer binding specificity.

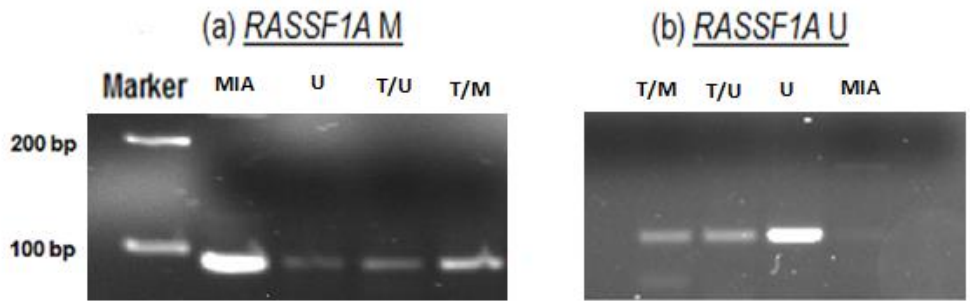


Figure 4.7 Non-specific binding of RASSF1A M and U primers on control DNA. Bands indicate PCR products of control DNA amplified with (a) RASSF1A M (93 bp region) and (b) RASSF1A U (106 bp region) primers resolved on a 2% agarose gel. DNA abbreviated as T/M for bisulfite-treated methylated; T/U for bisulfite-treated unmethylated; U for untreated DNA; and MIA for the MIA PaCa-2 cell line.

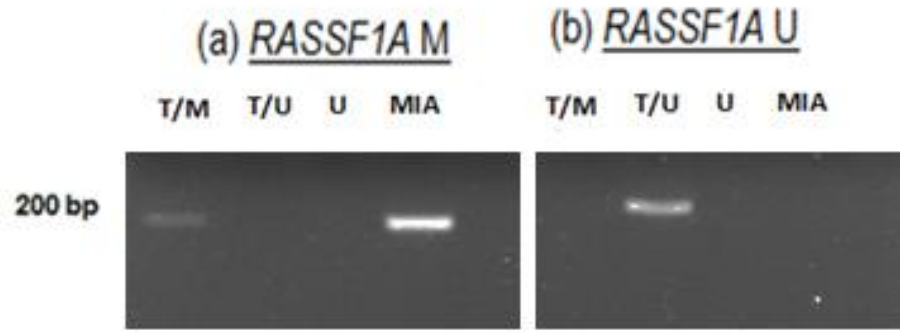


Figure 4.8 Binding specificity of *RASSF1A* MSP primers on control DNA. Bands indicate PCR products of control DNA amplified with (a) RASSF1A M (192 bp region) and (b) RASSF1A U (204 bp region) primers resolved on a 2% agarose gel. DNA abbreviated as T/M for bisulfite-treated methylated; T/U for bisulfite-treated unmethylated; U for untreated DNA; and MIA for the MIA PaCa-2 cell line.

To validate the specificity of *Smad4* MSP primers designed by Wei's group (2016) to be used in this study, the control DNA set was once again used. Initially, amplification performed at annealing temperatures (Ta) of 50 °C and 54 °C for and M and U primers, respectively, showed a lack of binding specificity for M primers on bisulfite-treated methylated DNA, indicated by a faint band at an incorrect molecular weight region (approximately 150 bp) (Fig. 4.9), although binding specificity was observed for U primers on bisulfite-treated unmethylated DNA. *Smad4* appeared to be unmethylated in the cell line, indicated by a weak band (Fig. 4.9b) showing that *Smad4* is most likely methylated in mid- or late-stage disease, since MIA PaCa-2 is associated with early

stage pancreatic cancer. The annealing temperature was increased to 58 °C for both primer sets to improve binding specificity, which showed a significant improvement, indicated by stronger bands at the correct binding region (Fig. 4.10).

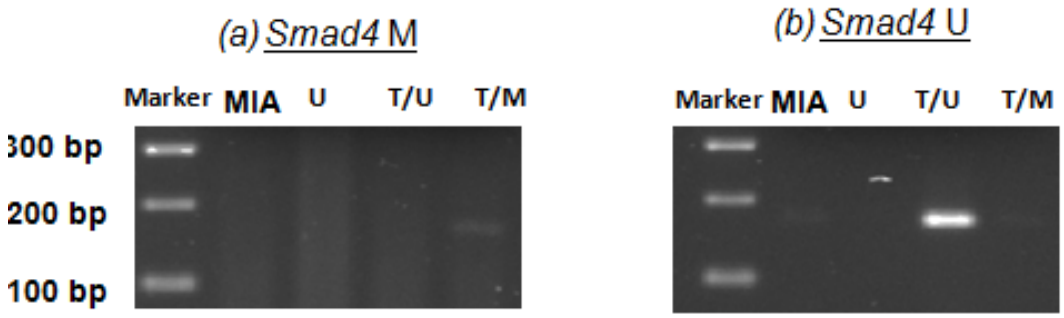


Figure 4.9 Binding specificity of *Smad4* MSP primers at low annealing temperatures on control DNA. Bands indicate PCR products of control DNA amplified with (a) *Smad4* M primers, showing no band at 274 bp region and (b) *Smad4* U primers (269 bp region) resolved on a 2% agarose gel. DNA abbreviated as T/M for bisulfite-treated methylated; T/U for bisulfite-treated unmethylated; U for untreated DNA; and MIA for the MIA PaCa-2 cell line.

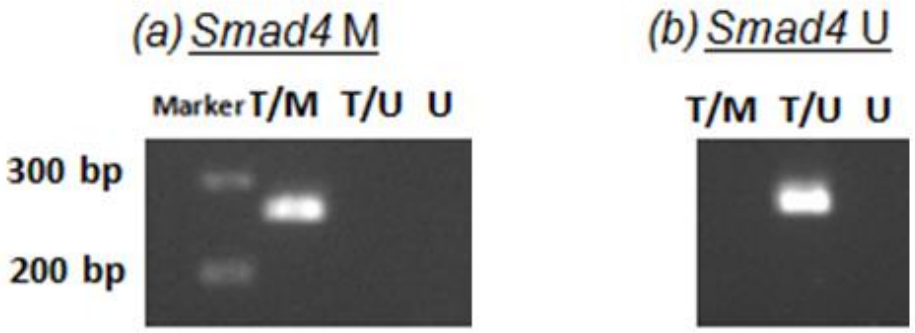


Figure 4.10 Binding specificity of *Smad4* MSP primers with optimised PCR conditions. Bands indicate PCR products of control DNA amplified at Ta = 58 °C instead of 50 °C and 54 °C for M and U primers, respectively. Bands show PCR products of (a) *Smad4* M (274 bp region) and (b) *Smad4* U (269 bp region) primers resolved on a 2% agarose gel. DNA abbreviated as T/M for bisulfite-treated methylated; T/U for bisulfite-treated unmethylated; U for untreated DNA.

4.2.2. Methylation of *Smad4* in patient cfDNA

To investigate *Smad4* promoter methylation in PDAC, matched cfDNA samples (table 4.2) were amplified with *Smad4* M and U primers under previously optimised PCR conditions (section 4.2.1). Samples were scored as either having “methylated” or “unmethylated” *Smad4* alleles based on banding patterns determined above (Fig. 4.10). Results revealed *Smad4* methylation in all patient groups (table 4.5), with PDAC patients having the highest *Smad4* methylation frequency (20%) although the differences between the groups were not statistically significant (p=0.107). Statistical analyses showed no association between *Smad4* methylation and risk factors associated with PDAC and CP (smoking, alcohol consumption and chronic illnesses); for HIV there were more HIV positive PDAC patients with methylation than CP or CLI patients (p =0.04) (table 4.6; see Appendix B for STATA outputs). A significant association was observed between cfDNA levels overall and *Smad4* methylation (p= 0.04); further revealing a significant probability of methylation with elevated cfDNA levels (95% CI, 1.00-1.14, p = 0.055; linear regression).

Logistic regression revealed the likelihood of *Smad4* methylation to increase with smokers (OR= 2.40, 95% CI, 0.27-21.37, p= 0.433) and alcohol consumers (OR =2.45, 95% CI, 0.27-21.86, p= 0.421), though less significantly in PDAC smokers (OR= 1.85, 95% CI, 0.17-20.26, p= 0.616) and alcohol consumers (OR= 1.43, 95% CI, 0.13-16.03, p= 0.772) compared with the CLI control group. However, these associated probabilities were not statistically significant.

Table 4.5 Summary of *Smad4* methylation profiles in patient groups

	Smad4 methylation	
Patient group	Methylated	Unmethylated
PDAC	20% (5/25)	80% (20/25)
CP	4.3% (1/23)	95.7% (22/23)
Control group	4.3 % (1/23)	95.7% (22/23)

Table 4.6 Associations and risks between clinical factors and *Smad4* methylation

Patient characteristics		**Methylated *Smad4***				**Logistic regression**		
		Methylated	**Unmethylated**	**p-value***		**Odds ratio**	**p-value**	**95% CI**
Chronic medical illness	Overall	2/40 (5.0%)	38/40 (95.0%)	0.530		0.27	0.138	0.05-1.52
	PDAC	1/13 (7.7%)	12/13 (92.3%)			0.17	0.138	0.02-1.78
	CP	0/15 (0%)	15/15 (100%)			-†	-	-
	CLI	1/12 (8.3%)	11/12 (91.7%)			-†	-	-
Smoking	Overall	6/51 (11.8%)	45/51 (88.2%)	0.177		2.40	0.433	0.27-21.37
	PDAC	4/17 (23.5%)	13/17 (76.5%)			1.85	0.616	0.17-20.26
	CP	1/21 (4.8%)	20/21 (95.2%)			-†	-	-
	CLI	1/13 (7.7%)	12/13 (92.3%)			-†	-	-
Alcohol consumption	Overall	6/50 (12.0%)	44/50 (88.0%)	0.233		2.45	0.421	0.28-21.86
	PDAC	4/18 (22.2%)	14/18 (77.8%)			1.43	0.772	0.13-16.03
	CP	1/21 (4.8%)	20/21 (95.2%)			-†	-	-
	CLI	1/11 (9.1%)	10/11 (90.9%)			-†	-	-

HIV	Overall	3/20 (15.0%)	17/20 (85%)	0.038		0.42	0.368	0.06-2.78
	PDAC	3/7 (42.9%)	4/7 (57.1%)			0.19	0.207	0.01-2.50
	CP	0/7 (0%)	7/7 (100%)			-†	-	-
	CLI	0/6 (0%)	6/6 (100%)			-†	-	-
CfDNA concentration (ng/ul)	Overall	7.9 (5.2 - 26.6)	5.0 (3.8 - 6.8)	0.037**		1.07	0.055	1.00-1.14
	PDAC	12.8 (7.9 - 26.6)	7.1 (4.6 - 12.3)	0.118**		1.04	0.252	0.97-1.12
	CP	3.8 (3.8 - 3.8)	4.0 (3.2 - 5.1)	0.880**		0.84	0.801	0.21-3.36
	CLI	5.2 (5.2 - 5.2)	5.2 (3.9 - 6.6)	1.000**		0.92	0.817	0.44-1.90

† Insufficient values to perform logistic regression analysis

* p-values calculated using a χ^2 test

** p-values calculated using a Wilcoxon rank-sum (Mann-Whitney) test

CHAPTER FIVE: DISCUSSION

The development of early detection markers remains a major focus in PDAC, a disease characterized by the worst survival rates when compared to other cancer types, especially for the overwhelming majority of patients who present with unresectable disease and are thus unable to receive curative treatment (Bilimoria *et al*., 2007). To resolve the high death rates in PDAC patients, usually caused by a delayed diagnosis, specific biomarkers need to be established. Making use of a liquid biopsy as a cancer marker in clinical medicine for the purpose of early detection, prognosis and the monitoring of treatment response has shown to be a significant improvement in the field. In the case of PDAC, where patients normally present symptoms indistinguishable from those of chronic pancreatitis (CP), a pancreatic condition that often mimics the aetiology of PDAC, liquid biopsies derived from plasma DNA could potentially serve as early markers to discriminate between the two diseases.

Cell-free DNA (cfDNA), isolated from blood plasma is easily accessible, reliable and represents a promising non-invasive approach to PDAC diagnosis (Elshimali *et al.,* 2013). Here, quantified levels of cfDNA from patients suffering from PDAC, CP or CLI, revealed detectable cfDNA amounts (nanograms) in all samples, consistent with previous findings showing the presence of cfDNA in cancer patients, as well as patients with pathologies other than cancer (Schwarzenbach *et al*., 2011; Jin *et al.,* 2012; Perkins *et al.,* 2012; Figg and Reid, 2013). Earlier studies that focussed on quantifying plasma DNA levels made use of detection methods that lacked analytical sensitivity, which consequently led to the erroneous assumption that cfDNA could only be detected in malignancies. However, recent developments have shown the presence of cfDNA in almost all individuals; whether burdened with diseases such as cancer or inflammatory disorders; or healthy.

In the present study, the extraction method chosen had to allow for the purification of amplifiable cfDNA from patient plasma samples. For this, the Maxwell® RSC ccfDNA Plasma Kit (RSC) was chosen based on a recent study by Sorber's group (2012), which demonstrated RSC's extraction efficiency over other commonly used kits such as the QIAamp® circulating nucleic acid kit (Qiagen). RSC showed consensus with previous results in that isolation was achievable with a fully automated protocol that

yielded quantifiable amounts. Furthermore, since cfDNA may sometimes pose challenges in the subsequent evaluation of genes due to its innate characteristics, such as the high rate of fragmentation and its low abundance as compared to genomic DNA (Jahr *et al*., 2001; Suzuki *et al.,* 2008), the fact that in this study downstream applications i.e. *Smad4* methylation analysis could be performed, further validated the isolation efficiency of the RSC system.

Major sources of cfDNA shedding into the bloodstream are thought to be *via* apoptosis and necrosis. In apoptotic cells, DNA fragment lengths of ~180 bp are often seen (Jahr *et al.,* 2001). The cfDNA fragment lengths of 170-180 bp support the hypothesis of apoptosis being a source of cfDNA. In cancer patients, total cfDNA is generally increased due to tumour cells having a high cellular turnover (Stroun *et al.,* 2000; Li *et al.,* 2003). The same could be concluded for PDAC patients in this study; which had the highest cfDNA levels compared to the control groups. Furthermore, though significantly lower than PDAC levels, cfDNA levels in patients with CP were significantly elevated compared to patients suffering from critical limb ischemia (CLI) patients, all of whom had a healthy pancreas. These high plasma DNA levels in inflammatory disease have previously been associated with dead and dying cells (Jiang and Pisetsky, 2004; 2005); with the hypothesis suggesting increased cfDNA levels may be associated with a greater level of inflammation due to a high rate of cell death (Mittra *et al*., 2012); which in the case of CP, may be attributed to an elevated apoptotic index (Bateman *et al.,* 2002).

Based on epidemiological studies, CP is one of the major clinical risk factors used to narrow down populations for screening for PDAC (Urayama, 2015; Raimondi *et al.,* 2010). Ideally, comparing cfDNA levels between PDAC and CP could serve as a useful tool in the initial diagnosis of PDAC. However, due to the wide range of concentrations quantified within the three groups, establishing reference limits or cut-off values is a major challenge; one that has previously been encountered in several studies (Jung *et al.,* 2010). Also, cfDNA concentration levels are likely to vary between studies due to a number of factors ranging from biological causes (disease stage, risk factors and other medical conditions) to technical methods (blood processing and storage). For instance, in this study the length of plasma storage prior to cfDNA isolation was found to be strongly associated with cfDNA concentration levels, a factor previously reported

to have a significant impact on cfDNA yield (table 3.1) (Sozzi *et al.,* 2005). Such pre-analytical factors should be of great consideration prior to quantitative analyses.

Here, environmental risks associated with PDAC and CP were evaluated for their effects on cfDNA levels in patients. Alcohol consumption, a common cause of CP (Coté *et al.*, 2011); as well as smoking, an established risk factor of PDAC and CP (Secretan *et al.,* 2009; Talamini *et al.,* 2010) showed no influence on cfDNA concentration levels. The two risk factors, often found to coincide (Yadav *et al.,* 2009), also had no association on cfDNA levels in CP patients, which had of the highest proportion of smokers and drinkers (table 3.1).

Chronic illness, although not specific to either PDAC or CP, was also another factor considered to potentially affect cfDNA levels in patients, since high cfDNA levels have previously been reported in patients with pathologies other than cancer such as myocardial infarction, trauma and stroke (Chang *et al.,* 2003; Rhodes *et al*., 2006; Frank, 2016). Data compiled for this study did not specify the nature of the chronic disease. In the case of PDAC, conditions such as diabetes mellitus, hepatitis B or *helicobacter pylori* infections would be known risks that could potentially affect cfDNA levels in PDAC patients (Chari *et al.,* 2005; Ben *et al.,* 2012). Nonetheless, in our patients having a chronic illness showed no effect on cfDNA concentration levels.

HIV on the other hand, was found to be associated with increased cfDNA levels in CLI patients. CLI is a disease characterised by a reduced blood flow and atypical leg pain resulting in major limb loss if left untreated; as well as a high incidence of cardiovascular comorbidities including stroke and myocardial infarction (Davies, 2012). Since increased plasma DNA levels in patients with the latter comorbidities have previously been reported (Chang *et al.,* 2003; Rainer *et al.,* 2003; Rainer and Lam, 2006), results here suggest that HIV, which is associated with a higher risk for CLI in patients with a marked increase in carotid Intima-Media Thickness (Brand *et al.,* 2012), could potentially be a comorbidity associated with a worse prognosis in CLI patients. In this regard, quantifying cfDNA levels in CLI patients could be a promising predictive tool, which could aid in treatment decision-making in affected patients.

Limited information could be obtained from solely quantifying cfDNA levels, with the added uncertainty of potential effects posed on data quality by pre-analytical factors i.e. duration of plasma storage, comparison of cfDNA levels in patients would have to be accompanied with the detection of known genetic markers to better differentiate PDAC patients from controls.

Array-based studies revealing highly discriminant methylated DNA markers in PDAC show epigenetic-based screening strategies to be a promising approach to identify potential diagnostic and prognostic markers (Omura *et al.,* 2008; Vincent *et al.,* 2011; Nones *et al.,* 2014; Kisiel *et al.*, 2015). In cancer, where different mechanisms such as loss of heterozygosity (LOH) and gene mutations contribute to the inactivation of tumour suppressors (Chan *et al.,* 2001), it is essential to investigate other pathways leading to the gene repression, especially if the gene has been proposed as a prominent cancer marker. Here, DNA methylation of *Smad4*, a gene previously reported to be inactivated through genetic events other than hypermethylation (Blackford *et al.,* 2009; Wood and Hruban, 2015), was assessed; first, by pre-screening of cfDNA samples for *K-ras* mutations, since *Smad4* inactivation is known to occur only in the presence of mutated *K-ras* (Bardeesy *et al.,* 2006; Kojima *et al*., 2007); and secondly to confirm that cfDNA was sufficiently intact to analyse *Smad4* methylation.

Droplet digital PCR (ddPCR) was used to analyse *K-ras* mutations in cfDNA samples. This system shows a significant advantage over other PCR-based methods (quantitative or real-time PCR) in that it quantifies absolute target concentrations with a simplified workflow for experimentation (Yung *et al.,* 2009; Huggett and Whale, 2013; Strain *et al*., 2013; Dreo *et al*., 2014), thus eliminating the need to perform calibrations. ddPCR assays have been developed for screening multiple mutations, particularly in cfDNA (Taly *et al*., 2013), making it possible to detect all seven *K-ras* mutations associated with PDAC.

In the present study, optimisation experiments revealed that mutation detection was possible with low cfDNA amounts (2.5ng). Experiments also showed that a low droplet count (<10 000) did not have any effect on detecting mutants. Unfortunately, when comparing the matched samples, due to technical challenges, mutations could only be detected in a minority of cfDNA samples. Thus, the results failed to validate the

optimised protocol used for *K-ras* mutations in cfDNA. The high variability observed in the fluorescence amplitudes may be due to droplet emulsion degradation as a consequence of the expired droplet oil that was used during droplet generation. Also, since a positive control was not included in these runs, it was difficult to define cluster thresholds, which could have potentially led to false-positive results for cfDNA samples analysed in the first run; a possible explanation for the fact that the CLI patients showed the highest proportion of *K-ras* mutations. Since there have been no reports suggesting mutations of *K-ras* in CLI patients, these results most likely indicate an error in data analysis. However, this did not hinder the analysis of *Smad4* methylation, since low levels of fragmented DNA were shown to be amplifiable.

The loss of *Smad4* is more specific and sensitive to PDAC than other cancers (van Heek *et al.,* 2002. It is correlated with a poor prognosis, mainly due the presence of metastasis (Blackford *et al.,* 2009; Iacobuzio-Donahue *et al.,* 2009), and has previously been associated with two genetic alterations; namely homozygous deletions (or LOH) and intragenic mutations (Singh *et al.,* 2012; Chen *et al*., 2014). In this study, hypermethylation, an epigenetic mechanism commonly associated with the loss of *p16* in PDAC (Fukushima *et al.,* 2002) was shown to be another mechanism leading to *Smad4'*s reduced expression in higher-grade PDAC. Using a method first developed by Herman's group's to show differential methylation of *p16* (Herman *et al.,* 1996), cfDNA proved to be a reliable source to demonstrate gene hypermethylation.

Herman's protocol for methylation specific PCR (MSP) was optimised for low cfDNA amounts. "True" DNA yields calculated for bisulfite converted DNA, though very low (from 72 pg/μl), produced detectable PCR products when optimised with *RASSF1A* MSP primers. Results from MSP optimisation also revealed MIA PaCa-2, a pancreatic cancer cell line associated with early-stage PDAC (Dammann *et al*., 2003), to have methylated *RASSF1A;* but not *Smad4*. In this regard, methylation analyses of both *RASSF1A* and *Smad4* show prognostic potential in early- and late- stage PDAC, respectively, since alterations in *Smad4* have previously been implicated in high-grade disease (Blackford *et al.,* 2009; Dempe *et al.,* 2010; Singh *et al.,* Oshima *et al.,* 2013).

For the matched samples, *K-ras* mutation analysis was deemed inconclusive. However, *Smad4* analysis could still be performed without prior knowledge of *K-ras* status, since studies have consistently emphasized the dependence of *Smad4*

inactivation preceding *K-ras* mutations in the development of PDAC (Fig. 1.3) (Blackford *et al.,* 2009; Iacobuzio-Donahue *et al.,* 2009; Singh *et al.,* 2012). This has also been shown in *p16*, where activating mutations of *K-ras* always occur before *p16* hypermethylation in mid-stage PDAC (Mazur and Siveke, 2011, Singh *et al.,* 2012; Chang *et al.,* 2014).

Results showed an association between *Smad4* methylation and increasing cfDNA levels. In this regard, elevated cfDNA levels appear to be characteristic of a high apoptotic index of which, in the case of *Smad4*, a metastatic PDAC tumour marker, correspond to a highly malignant disease (Wang *et al.,* 2013). The fact that *Smad4* inactivation is reported in metastatic PDAC, further supports the theory of "Genometastasis" (Fig. 1.6), initially hypothesized by García-Olmo's group (1999), which suggests the high transformation potential associated with methylated DNA fragments, leading to the invasion of susceptible cells in distant organs, to be reflected by elevated cfDNA levels in cancer patients (Skvortsova *et al*., 2008). Therefore, the observed cfDNA levels in patients with hypermethylated *Smad4* could be indicative of patients with metastatic PDAC.

In the case of the two control patients showing hypermethylated *Smad4*, logistic regression models revealed minor odds of *Smad4* being methylated in these control groups; as well as in smokers and alcohol consumers. Since there have been no reports suggesting *Smad4* inactivation in either CP or CLI, the likelihood of *Smad4* hypermethylation in these patients could be compared to that of drinking and smoking; with no evidence supporting the underlying cause of this observed probability.

Our study shows that, apart from intagenic mutations and LOH, DNA methylation was a contributing mechanism leading to the inactivation of Smad4 in PDAC patients, an established prognostic marker in PDAC. Results also indicate that high cfDNA levels may not only aid in differentiating patients with pancreatic disorders from those with a healthy pancreas, but may also serve as a predictive tool to assess metastasis in PDAC in patients with methylated Smad4. Also, hospitalised patients that smoke, consume alcohol and/or diagnosed with HIV have a slightly high probabability of Smad4 transcriptional silencing due to promoter hypermethylation.

Conclusion

PDAC is a disease characterised by various aetiologies, some of which are attributed to CP, a disease that often times leads to a delayed diagnosis of PDAC in patients. This study shows the potential clinical utility of cfDNA as a prognosis tool in patients with PDAC. Epigenetic changes that affect the expression of *Smad4* by promoter methylation in cfDNA provide a platform for prognosticating the disease non-invasively. However, since *Smad4* promoter methylation could only be detected in only a small subset of PDAC patient samples (~5-fold higher than in control groups), the data and results presented here would seem far too preliminary to conclude that promoter methylation of the *Smad4* gene to be a definite platform for disease prognosis. Therefore, further investigation to identify early detection markers, as well as prognostic epigenetic markers for PDAC remains warranted.

CHAPTER SIX: REFERENCES

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APPENDIX A-Table of patient parameter proportions

	Patient Group																	
SN	**PDAC**						**CP (Control Group 1)**						**CLI (Control Group 2)**					
	Conc (ng/µl)	**L**	**H**	**C**	**A**	**S**	**Conc (ng/µl)**	**L**	**H**	**C**	**A**	**S**	**Conc (ng/µl)**	**L**	**H**	**C**	**A**	**S**
1	**5,3**	3		1	1	0	**4,2**	3		0	1	1	10,5	2	2	0	1	1
2	**2,8**	3		0	1	1	4,5	3		0	1	1	**3,9**	2	2	1	0	1
3	**9,2**	3		0	1	1	6,2	3		1	1	0	1,9	2	2	1	1	1
4	**12,8**	3		0	1	1	6	2		1	1	1	4,9	2	2	1	1	0
5	**4,4**	3	1	1	1	1	3	2	1	0	1	1	4,9	2	1	1	1	1
6	**2,3**	3		1	1	1	3	2	2	1	0	1	12,6	2	2	1	0	0
7	**2,1**	3		1	1	1	2	2		0	1	0	4,7	2	2	1	0	1
8	**5,4**	3		1	1	1	3,4	2	2	1	0	0	4,9	2	2	1	1	0
9	46,8	3	2	1	0	1	11,1	2	2	0	1	1	6,5	2	2	1	1	0
10	**6,1**	3	2	0	1	0	14,1	2	2	1	1	1	4,7	2	2	1	1	1
11	177,1	3	2	1	1	1	5,3	2	1	1	1	1	5,2	2	2	1	1	1
12	8,9	3		1	0	1	30,7	2	2	1	1	1	3,4	2	3	1	0	1
13	7,9	3		0	0	0	5,2	2	2	0	1	1	10,4	2	2	1	0	1
14	6,6	3		1	0	0	123,5	2	2	0	1	1	3,2	2	1	1	1	1
15	7,1	3		0	1	1	11,6	2	1	0	1	1	9,3	2	2	0	1	1
16	37	3	1	0	1	1	7,4	2	1	1	1	1	7,9	2	2	1	0	1
17	3,2	3		0	1	1	2,8	2	2	1	1	1	5,5	2		1	1	1
18	7,9	3	1	0	1	1	4,3	2	2	1	1	1	4,3	2	2	1	1	1
19	3,6	3		1	1	0	1,8	2	2	0	1	1	5	2	2	1	0	0
20	1,5	3		1	0	1	2,8	2	2	0	1	1	7	2	2	0	1	1
21	2,3	3		1	0	0	5,1	2	1	0	1	0	7,9	2	2	1	0	0
22	7,9	3		0	0	0	2,9	2	2	0	1	1	5,8	2	2	1	0	1
23	8,9	3	1	1	0	0	4,2	1	2	1	1	1	4,8	2	2	1	1	0
24	4,5	3		0	1	1	4,6	1	2	1	1	1	6,3	2	2	1	0	0
25	7,1	3	1	0	0	0	**3,2**	1	1	1	1	1	6,4	2	2	0	0	0
26	11,1	3		0	1	1	**1,9**	1	1	1	1	1	5,3	2	2	0	0	0
27	2,9	3	2	0	1	1	**3,3**	1	2	1	1	1	3,5	2	2	1	0	0
28	5,7	3	2	1	0	0	**1,8**	1		1	1	1	3	2	1	0	0	0
29	9,8	3		0	1	1	**3,9**	1	1	1	1	1	3,8	2	2	0	1	1
30	11,7	3		1	1	1	**3,2**	1	2	0	1	1	4,3	2	2	0	0	1
31	49,3	3		0	0	1	**3,8**	1	1	1	1	1	**3**	1	1	0	0	0
32	34,4	3		1	1	1	**2,4**	1	2	1	1	1	**2,6**	1	1	0	0	0
33	4,3	3		0	1	0	**3,6**	1	2	1	1	1	**3,6**	1	1	1	0	0
34	7,9	3		0	0	0	**4,2**	1		1	1	1	**3,7**	1	1	0	1	1
35	5,7	3		0	1	1	**3,8**	1	2	0	1	1	**2,5**	1		1	0	0
36	7,9	3		1	0	0	**3,3**	1	2	1	1	1	**4,1**	1	1	0	0	0
37	5,5	3		1		0	**3,4**	0		1	1	1	**2,9**	1	1	0	0	0
38	4,7	3		1		0	**3,8**	0	1	1	1	1	**1,7**	1	1	1	0	0
39	7,2	3		1	1	0	**6,2**	0		0	1	1	**7**	1	2	1	0	0

40	16,2	3		1	1	0	**2,6**	0	1	0	1	1	**11,5**	1		1	1	1
41	4,2	3	2	1	1	1	**2**	0	1	1	1	1	**2,1**	1		0	0	1
42	7,4	2	1	1	0	0	**2,8**	0	2	1	1	1	**4,8**	1	1	0	0	0
43	13,4	2	2	1	1	1	**4,5**	0		0	1	0	**5,3**	1	2	1	1	1
44	7,1	2	3	1	0	0	**4,4**	0		0	1	1	**5,3**	1	2	1	0	0
45	7,4	2	1	1	0	0	**7,1**	0		1	1	1	**4,6**	1	2	1	1	1
46	7,4	2	1	0	1	1	**4,3**	0		1	1		**3,3**	1	1	0	0	0
47	6,5	2		0	3	18							**3**	1		1	1	1
48	10,7	2		0	2								**5,4**	1	2	0	0	1
49	**4**	2		1	2	12							**6,6**	1	1	0	0	0
50	1,8	2	1	1	0	1							**5,3**	1	1	1	0	0
51	4,2	2	1	1	1	1							**2**	1		1	0	0
52	13,5	2		1	1	1							**2,6**	1		0		1
53	4,6	2	1	1	1	1							**4,3**	1	2	1	0	1
54	4,1	2	2	1	0	0							**5,1**	0	1	0	1	1
55	2,4	2	2	0	1	1							**5,1**	0		0	1	0
56	3,5	2	2	1	1	1							**4,7**	0		0	1	0
57	2,7	2	1	1	1	1							**5,7**	0		1	1	1
58	3,2	2	1	1	1	1							**14,1**	0	1	0	0	1
59	4,5	2	2	0	1	1							**4,2**	0		0	0	0
60	**4,2**	2	2	1	1	1							**5,3**	0	2	1	1	0
61	**6,9**	2	2	0	1	1							**6**	0		0	0	1
62	**2,1**	2		1	0	0							**6,3**	0	2	1	1	1
63	**3,3**	2	2	1	1	1							**5,7**	0	1	0	0	1
64	**8,7**	2	1	1	1	1							**6,5**	0		0	1	1
65	**3,9**	2	1	1	1	1							**2,6**	0	2	0	0	0
66	**3,3**	2	1	1	1	1							**6,8**	0	2	1	0	0
67	**4,5**	2	2	1	0	0							**4**	0		0	1	1
68	**26,6**	2	2	0	0	0							**3,6**	0		0	0	0
69	**3,9**	1		1	1	1							**6,9**	0	2	1	0	0
70	**4,3**	1	2	0	1	1							**5,5**	0		0	0	0
71	**11,2**	1	2	0	0	0							**4,8**	0	1	1	1	0
72	**11,1**	1		1	1	1							**4,1**	0		1	0	0
73	**4,6**	1	2	1	1	1							**4,8**	0		1	0	0
74	**5,1**	1	2	0	1	1							**3,3**	0	2	1	1	1
75	**3,5**	1	2	0	0	1							**6,9**	0		1	0	0
76	**5,1**	1	1	1	1	1							**5,6**	0		1	0	0
77	**5,7**	1	2	0	1	1							**7,4**	0		1	1	0
78	**6**	1		0	1	1							**11,3**	0	2	1	0	1
79	**11,4**	1	2	0	1	1							**6,1**	0		0	1	1
80	**3,9**	1	2	0	0	0							**8,3**	0		0	1	1
81	**2,9**	1	2	1	1	1							**4,6**	0	1	0	1	1
82	**6,1**	1	1	1	0	0							**10,7**	0		0	0	0
83	**9,2**	1		0	1	1							**5,5**	0		0	0	0
84	**3,6**	1	2	1	0	0							**6,6**	0	1	0	1	1
85	**2,5**	1	3	0	1	1							**14,9**	0	1	1	1	1

86	**4**	1	2	1	0	0							**3,9**	0	1	0	1	1
87	**7,5**	1		1	1	1							**2,5**	0	2	1	0	1
88	**4,5**	1	2	1	0	0							**7,5**	0		1	0	0
89	**5,6**	1	1	1	0	1												
90	**3,7**	1	2	0	1	1												
91	**4**	1		1	1	0												
92	**9,1**	1	2	1	1	0												
93	**6,2**	1		0	1	1												
94	**5**	1	1	0	1	0												
95	**3,1**	1	2	1	1	1												
96	**5,5**	1	2	1	0	0												
97	**3,7**	1	1	1	1	1												
98	**4,1**	1		0	1	1												
99	**2,2**	1	2	0	1	1												
100	**3,2**	1	1	1	1	0												
101	**5,8**	1		0	1	1												
102	**5**	1	1	1	1	1												
103	**2,5**	1	2	0	0	0												
104	**4,3**	1		1	0	0												
105	**3,5**	1	2	1	0	0												
106	**2**	1		1	1	1												
107	**4**	1	2	0	1	1												
108	**2,5**	1	1	1	1	0												
109	**4,5**	1	1	0	0	0												
110	**4,4**	1		0	0	1												
111	**3,9**	1	1	1	1	1												
112	**7,4**	1	2	1	0	0												
113	**5,7**	1	2	1	1	1												
114	**5,1**	1		1		1												
115	**8,9**	1		1	1	1												
116	**4,1**	1	2	1	1	1												
117	**2,8**	1	2	0	1	0												
118	**4,1**	1	2	0	1	1												
119	**7,4**	1	1	1	0	0												
120	**7,4**	1		1	0	1												
121	**3,2**	1	2	0	0	0												
122	**3,2**	1	1	1	0	1												
123	**3,9**	0		0	1	1												
124	**12,5**	0		0	1	1												
125	**3,7**	0	2	1	1	1												
126	**7,7**	0		1	1	1												
127	**9**	0	2	1	1	1												
128	**8**	0	2	0	1	1												
129	**5,3**	0		1	1	0												
130	**4,2**	0	2	0	0	0												
131	**7,6**	0	1	1	1	1												

132	**5,7**	0	2	1	0	0													
133	**20,7**	0		0	1	1													
134	**6,3**	0	1	1	1	1													
135	**8,9**	0		1	0	0													
136	**5,9**	0		1	1	0													
137	**4,5**	0	2	1	1	0													
138	**4,3**	0	2	0	1	1													
139	**8,1**	0		1	1	0													
140	**5,9**	0	2	0	1	1													
141	**2,7**	0		1	0	0													
142	**5,7**	0		1	1	1													
143	**6,6**	0		1	1	0													
144	**10,3**	0		1	0	1													
145	**5,2**	0	1	1	1	0													
146	**4,9**	0		1	0	0													
147	**2**	0		1	0	0													
148	**3,7**	0	2	1	1	1													
149	**6,1**	0	1	1	1	0													
150	**2,5**	0																	
151	**4,6**	0	2	0	1	1													
152	**2,6**	0	1	1	1	1													
153	**9,8**	0		1		1													
154	**6,2**	0		1	0	0													
155	**5,1**	0	2	1	1	1													

*Highlights indicate sample number cut-offs for each patient group i.e. Yellow = PDAC (n=155); Blue = CP (n=46); Green = CLI (n= 88). Abbreviations: SN = Sample number; Conc = Concentration measured with UV Spec; L = Length of storage (years); H = HIV status; C = Chronic medical illness; A = Alcohol consumption; S = Smoking. The number “1” in columns indicates “yes”; and “0“indicates “No”; except for HIV status where “2” indicates positive; and “1“indicates a negative HIV status; and LOS where numbers indicate years of storage. Black boxes indicate information was not available regarding that sample’s parameter.

Appendix B - Stata commands and outputs

```
. summ age if disease==1

    Variable |       Obs        Mean    Std. Dev.       Min        Max
-------------+--------------------------------------------------------
         age |       155    58.30839    11.71243       23.8       92.8

. summ age if disease==2

    Variable |       Obs        Mean    Std. Dev.       Min        Max
-------------+--------------------------------------------------------
         age |        46    50.43478    9.361035        27         70

. summ age if disease==3

    Variable |       Obs        Mean    Std. Dev.       Min        Max
-------------+--------------------------------------------------------
         age |        88    52.23182    15.93414        25       89.3

. anova  age disease if age!=.

                           Number of obs =     289     R-squared     =  0.0657
                           Root MSE      = 12.8409     Adj R-squared =  0.0592

                  Source |  Partial SS    df       MS           F     Prob > F
              -----------+----------------------------------------------------
                   Model |  3317.43886     2  1658.71943      10.06     0.0001
                         |
                 disease |  3317.43886     2  1658.71943      10.06     0.0001
                         |
                Residual |  47158.1744   286  164.888722
              -----------+----------------------------------------------------
                   Total |  50475.6132   288  175.262546

.
. tab disease chr_med_ill, row chi2

+----------------+
| Key            |
|----------------|
|   frequency    |
| row percentage |
+----------------+

           |      chr_med_ill
   disease |         0          1 |     Total
-----------+----------------------+----------
      PDAC |        57         97 |       154
           |     37.01      62.99 |    100.00
-----------+----------------------+----------
        CP |        18         28 |        46
           |     39.13      60.87 |    100.00
-----------+----------------------+----------
   Control |        38         50 |        88
           |     43.18      56.82 |    100.00
-----------+----------------------+----------
     Total |       113        175 |       288
           |     39.24      60.76 |    100.00

          Pearson chi2(2) =   0.8941   Pr = 0.640

.
. tab disease smoke , row chi2
```

```
+----------------+
| Key            |
|----------------|
|   frequency    |
| row percentage |
+----------------+

           |         smoke
   disease |         0          1 |     Total
-----------+----------------------+----------
      PDAC |        58         93 |       151
           |     38.41      61.59 |    100.00
-----------+----------------------+----------
        CP |         5         41 |        46
           |     10.87      89.13 |    100.00
-----------+----------------------+----------
   Control |        44         44 |        88
           |     50.00      50.00 |    100.00
-----------+----------------------+----------
     Total |       107        178 |       285
           |     37.54      62.46 |    100.00

          Pearson chi2(2) =  19.8294   Pr = 0.000

.
. tab disease alc , row chi2

+----------------+
| Key            |
|----------------|
|   frequency    |
| row percentage |
+----------------+

           |          alc
   disease |         0          1 |     Total
-----------+----------------------+----------
      PDAC |        48         99 |       147
           |     32.65      67.35 |    100.00
-----------+----------------------+----------
        CP |         2         44 |        46
           |      4.35      95.65 |    100.00
-----------+----------------------+----------
   Control |        50         37 |        87
           |     57.47      42.53 |    100.00
-----------+----------------------+----------
     Total |       100        180 |       280
           |     35.71      64.29 |    100.00

          Pearson chi2(2) =  38.2495   Pr = 0.000

.
. tab disease hiv, row chi2

+----------------+
| Key            |
|----------------|
|   frequency    |
| row percentage |
+----------------+

           |          HIV
   disease |  Positive   Negative |     Total
-----------+----------------------+----------
      PDAC |        33         53 |        86
           |     38.37      61.63 |    100.00
-----------+----------------------+----------
```

```
        CP |        12         21 |        33
           |     36.36      63.64 |    100.00
-----------+----------------------+----------
   Control |        22         39 |        61
           |     36.07      63.93 |    100.00
-----------+----------------------+----------
     Total |        67        113 |       180
           |     37.22      62.78 |    100.00

          Pearson chi2(2) =   0.0940   Pr = 0.954

.
. kwallis concentration, by( disease)

Kruskal-Wallis equality-of-populations rank test

  +---------------------------+
  | disease | Obs | Rank Sum |
  |---------+-----+----------|
  |    PDAC | 155 | 23944.00 |
  |      CP |  46 |  5036.50 |
  | Control |  88 | 12924.50 |
  +---------------------------+

chi-squared =    10.343 with 2 d.f.
probability =     0.0057

chi-squared with ties =    10.346 with 2 d.f.
probability =     0.0057

. tabstat concentration, stat( p50) by( disease)

Summary for variables: concentration
     by categories of: disease (disease)

disease |       p50
--------+----------
   PDAC |       5.1
     CP |      3.85
Control |      5.05
--------+----------
  Total |       4.9
--------------------

. ranksum concentration if disease!=3, by( disease)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

     disease |      obs    rank sum    expected
-------------+---------------------------------
        PDAC |      155     16711.5       15655
          CP |       46      3589.5        4646
-------------+---------------------------------
    combined |      201       20301       20301

unadjusted variance   120021.67
adjustment for ties      -50.37
                     ----------
adjusted variance     119971.30

Ho: concen~n(disease==PDAC) = concen~n(disease==CP)
             z =   3.050
    Prob > |z| =   0.0023

. ranksum concentration if disease!=1, by( disease)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test
```

```
     disease |      obs    rank sum    expected
-------------+--------------------------------
          CP |       46        2528        3105
     Control |       88        6517        5940
-------------+--------------------------------
    combined |      134        9045        9045

unadjusted variance    45540.00
adjustment for ties      -21.24
                       ----------
adjusted variance      45518.76

Ho: concen~n(disease==CP) = concen~n(disease==Control)
             z =  -2.704
    Prob > |z| =   0.0068

. ranksum concentration if disease!=2, by( disease)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

     disease |      obs    rank sum    expected
-------------+--------------------------------
        PDAC |      155     19322.5       18910
     Control |       88     10323.5       10736
-------------+--------------------------------
    combined |      243       29646       29646

unadjusted variance   277346.67
adjustment for ties      -91.85
                       ----------
adjusted variance     277254.81

Ho: concen~n(disease==PDAC) = concen~n(disease==Control)
             z =   0.783
    Prob > |z| =   0.4334

.
. tabstat concentration if chr_med_ill==1, stat(n p50 p25 p75) by(disease)

Summary for variables: concentration
     by categories of: disease (disease)

disease |         N       p50       p25       p75
--------+--------------------------------------------
   PDAC |        97       5.1       3.7       7.4
     CP |        27       3.4       2.9       5.3
Control |        50       5.1       4.1       6.8
--------+--------------------------------------------
  Total |       174       4.9       3.5       6.9
------------------------------------------------------

. tabstat concentration if chr_med_ill==0, stat(n p50 p25 p75) by(disease)

Summary for variables: concentration
     by categories of: disease (disease)

disease |         N       p50       p25       p75
--------+--------------------------------------------
   PDAC |        57       5.7       4.1       7.9
     CP |        18      4.25         3       5.2
Control |        38      4.95       3.7       6.4
--------+--------------------------------------------
  Total |       113         5       3.9       7.1
------------------------------------------------------

. ranksum concentration, by(chr_med_ill)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test
```

```
 chr_med_ill |      obs    rank sum    expected
-------------+---------------------------------
           0 |      113     16831.5       16272
           1 |      174     24496.5       25056
-------------+---------------------------------
    combined |      287       41328       41328

unadjusted variance   471888.00
adjustment for ties     -148.16
                     ----------
adjusted variance     471739.84

Ho: concen~n(chr_me~l==0) = concen~n(chr_me~l==1)
             z =   0.815
    Prob > |z| =   0.4153

. ranksum concentration if disease==1, by(chr_med_ill)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

 chr_med_ill |      obs    rank sum    expected
-------------+---------------------------------
           0 |       57        4741      4417.5
           1 |       97        7194      7517.5
-------------+---------------------------------
    combined |      154       11935       11935

unadjusted variance    71416.25
adjustment for ties      -32.03
                     ----------
adjusted variance      71384.22

Ho: concen~n(chr_me~l==0) = concen~n(chr_me~l==1)
             z =   1.211
    Prob > |z| =   0.2260

. ranksum concentration if disease==2, by(chr_med_ill)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

 chr_med_ill |      obs    rank sum    expected
-------------+---------------------------------
           0 |       18         458         414
           1 |       27         577         621
-------------+---------------------------------
    combined |       45        1035        1035

unadjusted variance     1863.00
adjustment for ties       -2.45
                     ----------
adjusted variance       1860.55

Ho: concen~n(chr_me~l==0) = concen~n(chr_me~l==1)
             z =   1.020
    Prob > |z| =   0.3077

. ranksum concentration if disease==3, by(chr_med_ill)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

 chr_med_ill |      obs    rank sum    expected
-------------+---------------------------------
           0 |       38      1617.5        1691
           1 |       50      2298.5        2225
-------------+---------------------------------
    combined |       88        3916        3916
```

```
unadjusted variance    14091.67
adjustment for ties       -8.44
                     ----------
adjusted variance      14083.23

Ho: concen~n(chr_me~l==0) = concen~n(chr_me~l==1)
             z =  -0.619
    Prob > |z| =   0.5357

.
. tabstat concentration if hiv==1, stat(n p50 p25 p75) by(disease)

Summary for variables: concentration
     by categories of: disease (disease)

disease |         N       p50       p25       p75
--------+--------------------------------------------
   PDAC |        33         5       3.7       7.4
     CP |        12       3.3       2.9      5.75
Control |        22      4.35       3.2       5.3
--------+--------------------------------------------
  Total |        67       4.6       3.2       6.3
-------------------------------------------------------

. tabstat concentration if hiv==2, stat(n p50 p25 p75) by(disease)

Summary for variables: concentration
     by categories of: disease (disease)

disease |         N       p50       p25       p75
--------+--------------------------------------------
   PDAC |        53       4.3       3.7       5.7
     CP |        20      3.85      2.85      5.15
Control |        39       5.3       4.3       6.9
--------+--------------------------------------------
  Total |       112       4.6       3.7      6.35
-------------------------------------------------------

. ranksum concentration, by(hiv)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

         hiv |      obs    rank sum    expected
-------------+---------------------------------
    Positive |       67        5778        6030
    Negative |      112       10332       10080
-------------+---------------------------------
    combined |      179       16110       16110

unadjusted variance   112560.00
adjustment for ties      -54.17
                     ----------
adjusted variance     112505.83

Ho: concen~n(hiv==Positive) = concen~n(hiv==Negative)
             z =  -0.751
    Prob > |z| =   0.4525

. ranksum concentration if disease==1, by(hiv)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

         hiv |      obs    rank sum    expected
-------------+---------------------------------
    Positive |       33      1501.5      1435.5
    Negative |       53      2239.5      2305.5
-------------+---------------------------------
    combined |       86        3741        3741
```

```
unadjusted variance    12680.25
adjustment for ties      -13.16
                     ----------
adjusted variance      12667.09

Ho: concen~n(hiv==Positive) = concen~n(hiv==Negative)
             z =   0.586
    Prob > |z| =   0.5576

. ranksum concentration if disease==2, by(hiv)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

         hiv |      obs    rank sum    expected
-------------+---------------------------------
    Positive |       12         199         198
    Negative |       20         329         330
-------------+---------------------------------
    combined |       32         528         528

unadjusted variance      660.00
adjustment for ties       -0.85
                     ----------
adjusted variance        659.15

Ho: concen~n(hiv==Positive) = concen~n(hiv==Negative)
             z =   0.039
    Prob > |z| =   0.9689

. ranksum concentration if disease==3, by(hiv)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

         hiv |      obs    rank sum    expected
-------------+---------------------------------
    Positive |       22         541         682
    Negative |       39        1350        1209
-------------+---------------------------------
    combined |       61        1891        1891

unadjusted variance     4433.00
adjustment for ties       -4.92
                     ----------
adjusted variance       4428.08

Ho: concen~n(hiv==Positive) = concen~n(hiv==Negative)
             z =  -2.119
    Prob > |z| =   0.0341

.
. tabstat concentration if smoke==1, stat(n p50 p25 p75) by(disease)

Summary for variables: concentration
     by categories of: disease (disease)

disease |         N       p50       p25       p75
--------+--------------------------------------------
   PDAC |        93       5.1       3.7         8
     CP |        40      4.05       3.1      5.25
Control |        44      5.15       3.9      6.55
--------+--------------------------------------------
  Total |       177       4.6       3.6       7.4
------------------------------------------------------

. tabstat concentration if smoke==0, stat(n p50 p25 p75) by(disease)

Summary for variables: concentration
```

```
     by categories of: disease (disease)

disease |         N       p50       p25       p75
--------+-------------------------------------------
   PDAC |        58       5.4         4       7.4
     CP |         5       2.8       2.8       3.4
Control |        44      4.95       3.6      6.45
--------+-------------------------------------------
  Total |       107       5.1       3.6       6.8
-------------------------------------------------

. ranksum concentration, by(smoke)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

       smoke |      obs    rank sum    expected
-------------+---------------------------------
           0 |      107     15355.5     15247.5
           1 |      177     25114.5     25222.5
-------------+---------------------------------
    combined |      284       40470       40470

unadjusted variance   449801.25
adjustment for ties     -144.09
                     ----------
adjusted variance     449657.16

Ho: concen~n(smoke==0) = concen~n(smoke==1)
             z =   0.161
    Prob > |z| =   0.8720

. ranksum concentration if disease==1, by(smoke)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

       smoke |      obs    rank sum    expected
-------------+---------------------------------
           0 |       58        4414        4408
           1 |       93        7062        7068
-------------+---------------------------------
    combined |      151       11476       11476

unadjusted variance    68324.00
adjustment for ties      -31.79
                     ----------
adjusted variance      68292.21

Ho: concen~n(smoke==0) = concen~n(smoke==1)
             z =   0.023
    Prob > |z| =   0.9817

. ranksum concentration if disease==2, by(smoke)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

       smoke |      obs    rank sum    expected
-------------+---------------------------------
           0 |        5        78.5         115
           1 |       40       956.5         920
-------------+---------------------------------
    combined |       45        1035        1035

unadjusted variance      766.67
adjustment for ties       -1.01
                     ----------
adjusted variance        765.66

Ho: concen~n(smoke==0) = concen~n(smoke==1)
```

```
            z =  -1.319
    Prob > |z| =   0.1871

. ranksum concentration if disease==3, by(smoke)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

       smoke |      obs    rank sum    expected
-------------+---------------------------------
           0 |       44        1890        1958
           1 |       44        2026        1958
-------------+---------------------------------
    combined |       88        3916        3916

unadjusted variance    14358.67
adjustment for ties       -8.60
                     ----------
adjusted variance      14350.07

Ho: concen~n(smoke==0) = concen~n(smoke==1)
            z =  -0.568
    Prob > |z| =   0.5703

.
. tabstat concentration if alc==1, stat(n p50 p25 p75) by(disease)

Summary for variables: concentration
     by categories of: disease (disease)

disease |         N       p50       p25       p75
--------+--------------------------------------------
   PDAC |        99       5.1       3.7       7.6
     CP |        43       3.9       2.9       5.3
Control |        37       5.1       4.6       6.5
--------+--------------------------------------------
  Total |       179       4.7       3.7       6.6
------------------------------------------------------

. tabstat concentration if alc==0, stat(n p50 p25 p75) by(disease)

Summary for variables: concentration
     by categories of: disease (disease)

disease |         N       p50       p25       p75
--------+--------------------------------------------
   PDAC |        48      5.65      3.75      7.65
     CP |         2       3.2         3       3.4
Control |        50      5.15       3.5       6.6
--------+--------------------------------------------
  Total |       100       5.3       3.5      7.25
------------------------------------------------------

. ranksum concentration, by(alc)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

         alc |      obs    rank sum    expected
-------------+---------------------------------
           0 |      100       14218       14000
           1 |      179       24842       25060
-------------+---------------------------------
    combined |      279       39060       39060

unadjusted variance   417666.67
adjustment for ties     -135.59
                     ----------
adjusted variance     417531.08
```

```
Ho: concen~n(alc==0) = concen~n(alc==1)
             z =   0.337
    Prob > |z| =   0.7358

. ranksum concentration if disease==1, by(alc)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

         alc |      obs    rank sum    expected
-------------+---------------------------------
           0 |       48        3610        3552
           1 |       99        7268        7326
-------------+---------------------------------
    combined |      147       10878       10878

unadjusted variance    58608.00
adjustment for ties      -28.67
                     ----------
adjusted variance      58579.33

Ho: concen~n(alc==0) = concen~n(alc==1)
             z =   0.240
    Prob > |z| =   0.8106

. ranksum concentration if disease==2, by(alc)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

         alc |      obs    rank sum    expected
-------------+---------------------------------
           0 |        2          31          46
           1 |       43        1004         989
-------------+---------------------------------
    combined |       45        1035        1035

unadjusted variance      329.67
adjustment for ties       -0.43
                     ----------
adjusted variance        329.23

Ho: concen~n(alc==0) = concen~n(alc==1)
             z =  -0.827
    Prob > |z| =   0.4084

. ranksum concentration if disease==3, by(alc)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

         alc |      obs    rank sum    expected
-------------+---------------------------------
           0 |       50        2120        2200
           1 |       37        1708        1628
-------------+---------------------------------
    combined |       87        3828        3828

unadjusted variance    13566.67
adjustment for ties       -8.04
                     ----------
adjusted variance      13558.63

Ho: concen~n(alc==0) = concen~n(alc==1)
             z =  -0.687
    Prob > |z| =   0.4921

.
. tabstat concentration, stat(n p50 p25 p75) by(losyrs)

Summary for variables: concentration
```

```
     by categories of: losyrs (LOS (yrs))

  losyrs |         N       p50       p25       p75
---------+-------------------------------------------
       0 |        79       5.5       4.1       6.9
       1 |        91       4.1       3.2       5.3
       2 |        75       4.9       3.5       7.4
       3 |        44       6.4      4.35      9.05
---------+-------------------------------------------
   Total |       289       4.9       3.6         7
-------------------------------------------------------

. kwallis concentration, by( losyrs)

Kruskal-Wallis equality-of-populations rank test

  +-------------------------+
  | losyrs | Obs | Rank Sum |
  |--------+-----+----------|
  |      0 |  79 | 12518.50 |
  |      1 |  91 | 10248.00 |
  |      2 |  75 | 11180.50 |
  |      3 |  44 |  7958.00 |
  +-------------------------+

chi-squared =    23.996 with 3 d.f.
probability =     0.0001

chi-squared with ties =    24.004 with 3 d.f.
probability =     0.0001

.
. summ age if disease==1 & ddpcrrun==1

    Variable |       Obs        Mean    Std. Dev.       Min        Max
-------------+--------------------------------------------------------
         age |        42     50.0619    10.22004      23.8       70.6

. summ age if disease==2 & ddpcrrun==1

    Variable |       Obs        Mean    Std. Dev.       Min        Max
-------------+--------------------------------------------------------
         age |        42    50.02381     9.43912         27         70

. summ age if disease==3 & ddpcrrun==1

    Variable |       Obs        Mean    Std. Dev.       Min        Max
-------------+--------------------------------------------------------
         age |        42       50.05    10.10403         25         70

. anova  age disease if age!=. & ddpcrrun==1

                           Number of obs =     126     R-squared     =  0.0000
                           Root MSE      = 9.92703     Adj R-squared = -0.0163

                  Source |  Partial SS    df       MS           F     Prob > F
              -----------+----------------------------------------------------
                   Model |  .031904762     2  .015952381       0.00     0.9998
                         |
                 disease |  .031904762     2  .015952381       0.00     0.9998
                         |
                Residual |  12121.1402   123  98.5458556
              -----------+----------------------------------------------------
                   Total |  12121.1721   125  96.9693771

.
. tab disease chr_med_ill if ddpcrrun==1, row chi2
```

```
+----------------+
| Key            |
|----------------|
|   frequency    |
| row percentage |
+----------------+

           |      chr_med_ill
   disease |         0          1 |     Total
-----------+----------------------+----------
      PDAC |        19         23 |        42
           |     45.24      54.76 |    100.00
-----------+----------------------+----------
        CP |        17         25 |        42
           |     40.48      59.52 |    100.00
-----------+----------------------+----------
   Control |        21         21 |        42
           |     50.00      50.00 |    100.00
-----------+----------------------+----------
     Total |        57         69 |       126
           |     45.24      54.76 |    100.00

          Pearson chi2(2) =   0.7689   Pr = 0.681

.
. tab disease smoke if ddpcrrun==1, row chi2

+----------------+
| Key            |
|----------------|
|   frequency    |
| row percentage |
+----------------+

           |         smoke
   disease |         0          1 |     Total
-----------+----------------------+----------
      PDAC |        16         24 |        40
           |     40.00      60.00 |    100.00
-----------+----------------------+----------
        CP |         5         37 |        42
           |     11.90      88.10 |    100.00
-----------+----------------------+----------
   Control |        18         24 |        42
           |     42.86      57.14 |    100.00
-----------+----------------------+----------
     Total |        39         85 |       124
           |     31.45      68.55 |    100.00

          Pearson chi2(2) =  11.3332   Pr = 0.003

.
. tab disease alc if ddpcrrun==1, row chi2

+----------------+
| Key            |
|----------------|
|   frequency    |
| row percentage |
+----------------+

           |          alc
   disease |         0          1 |     Total
-----------+----------------------+----------
      PDAC |        10         29 |        39
           |     25.64      74.36 |    100.00
-----------+----------------------+----------
        CP |         2         40 |        42
```

```
           |      4.76      95.24 |    100.00
-----------+----------------------+----------
   Control |        19         22 |        41
           |     46.34      53.66 |    100.00
-----------+----------------------+----------
     Total |        31         91 |       122
           |     25.41      74.59 |    100.00

          Pearson chi2(2) =  18.9264   Pr = 0.000

.
. tab disease hiv if ddpcrrun==1, row chi2

+----------------+
| Key            |
|----------------|
|   frequency    |
| row percentage |
+----------------+

           |          HIV
   disease |  Positive   Negative |     Total
-----------+----------------------+----------
      PDAC |        13         12 |        25
           |     52.00      48.00 |    100.00
-----------+----------------------+----------
        CP |        12         18 |        30
           |     40.00      60.00 |    100.00
-----------+----------------------+----------
   Control |        13         15 |        28
           |     46.43      53.57 |    100.00
-----------+----------------------+----------
     Total |        38         45 |        83
           |     45.78      54.22 |    100.00

          Pearson chi2(2) =   0.7982   Pr = 0.671
```

KRAS analysis

```
.
. tabstat concentration, stat(n p50 p25 p75) by(g12mutation)

Summary for variables: concentration
     by categories of: g12mutation (G12 Mutation)

g12mutation |         N       p50       p25       p75
------------+--------------------------------------------
          0 |        15       6.1       4.6      30.7
          1 |        15       5.7       4.2      11.1
------------+--------------------------------------------
      Total |        30       5.9       4.2      12.6
-------------------------------------------------------

. tabstat concentration if disease==1, stat(n p50 p25 p75) by(g12mutation)

Summary for variables: concentration
     by categories of: g12mutation (G12 Mutation)

g12mutation |         N       p50       p25       p75
------------+--------------------------------------------
          0 |         6     21.15       6.1        37
          1 |         4       9.5       7.9     11.95
------------+--------------------------------------------
      Total |        10       9.5       7.9      34.4
-------------------------------------------------------
```

```
. tabstat concentration if disease==2, stat(n p50 p25 p75) by(g12mutation)

Summary for variables: concentration
     by categories of: g12mutation (G12 Mutation)

g12mutation |         N       p50       p25       p75
------------+------------------------------------------
          0 |         5       5.1       4.6      14.1
          1 |         5       4.2       3.4       4.2
------------+------------------------------------------
      Total |        10       4.4       3.4       5.3
--------------------------------------------------------

. tabstat concentration if disease==3, stat(n p50 p25 p75) by(g12mutation)

Summary for variables: concentration
     by categories of: g12mutation (G12 Mutation)

g12mutation |         N       p50       p25       p75
------------+------------------------------------------
          0 |         4      4.55       3.3       6.5
          1 |         6         6       4.8      12.6
------------+------------------------------------------
      Total |        10       5.4         4       7.9
--------------------------------------------------------

. ranksum concentration, by(g12mutation)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

 g12mutation |      obs    rank sum    expected
-------------+---------------------------------
           0 |       15       251.5       232.5
           1 |       15       213.5       232.5
-------------+---------------------------------
    combined |       30         465         465

unadjusted variance      581.25
adjustment for ties       -1.94
                     ----------
adjusted variance        579.31

Ho: concen~n(g12mut~n==0) = concen~n(g12mut~n==1)
             z =   0.789
    Prob > |z| =   0.4299

. ranksum concentration if disease==1, by(g12mutation)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

 g12mutation |      obs    rank sum    expected
-------------+---------------------------------
           0 |        6          34          33
           1 |        4          21          22
-------------+---------------------------------
    combined |       10          55          55

unadjusted variance       22.00
adjustment for ties       -0.53
                     ----------
adjusted variance         21.47

Ho: concen~n(g12mut~n==0) = concen~n(g12mut~n==1)
             z =   0.216
    Prob > |z| =   0.8291

. ranksum concentration if disease==2, by(g12mutation)
```

```
Two-sample Wilcoxon rank-sum (Mann-Whitney) test

 g12mutation |      obs    rank sum    expected
-------------+---------------------------------
           0 |        5        34.5        27.5
           1 |        5        20.5        27.5
-------------+---------------------------------
    combined |       10          55          55

unadjusted variance       22.92
adjustment for ties       -0.28
                          ----------
adjusted variance         22.64

Ho: concen~n(g12mut~n==0) = concen~n(g12mut~n==1)
             z =   1.471
    Prob > |z| =   0.1412

. ranksum concentration if disease==3, by(g12mutation)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

 g12mutation |      obs    rank sum    expected
-------------+---------------------------------
           0 |        4          17          22
           1 |        6          38          33
-------------+---------------------------------
    combined |       10          55          55

unadjusted variance       22.00
adjustment for ties        0.00
                          ----------
adjusted variance         22.00

Ho: concen~n(g12mut~n==0) = concen~n(g12mut~n==1)
             z =  -1.066
    Prob > |z| =   0.2864
```

SMAD4 methylation analysis

```
.
. tab disease smad4_meth, row chi2

+----------------+
| Key            |
|----------------|
|   frequency    |
| row percentage |
+----------------+

           |      SMAD4_Meth
   disease |         0          1 |     Total
-----------+----------------------+----------
      PDAC |        20          5 |        25
           |     80.00      20.00 |    100.00
-----------+----------------------+----------
        CP |        22          1 |        23
           |     95.65       4.35 |    100.00
-----------+----------------------+----------
```

```
   Control |        22          1 |        23
           |     95.65       4.35 |    100.00
-----------+----------------------+----------
     Total |        64          7 |        71
           |     90.14       9.86 |    100.00

          Pearson chi2(2) =   4.4651   Pr = 0.107

.
. tab disease smad4_meth if chr_med_ill==1, row chi2

+----------------+
| Key            |
|----------------|
|   frequency    |
| row percentage |
+----------------+

           |      SMAD4_Meth
   disease |         0          1 |     Total
-----------+----------------------+----------
      PDAC |        12          1 |        13
           |     92.31       7.69 |    100.00
-----------+----------------------+----------
        CP |        15          0 |        15
           |    100.00       0.00 |    100.00
-----------+----------------------+----------
   Control |        11          1 |        12
           |     91.67       8.33 |    100.00
-----------+----------------------+----------
     Total |        38          2 |        40
           |     95.00       5.00 |    100.00

          Pearson chi2(2) =   1.2686   Pr = 0.530

. tab disease smad4_meth if smoke==1, row chi2

+----------------+
| Key            |
|----------------|
|   frequency    |
| row percentage |
+----------------+

           |      SMAD4_Meth
   disease |         0          1 |     Total
-----------+----------------------+----------
      PDAC |        13          4 |        17
           |     76.47      23.53 |    100.00
-----------+----------------------+----------
        CP |        20          1 |        21
           |     95.24       4.76 |    100.00
-----------+----------------------+----------
   Control |        12          1 |        13
           |     92.31       7.69 |    100.00
-----------+----------------------+----------
     Total |        45          6 |        51
           |     88.24      11.76 |    100.00

          Pearson chi2(2) =   3.4664   Pr = 0.177

. tab disease smad4_meth if alc==1, row chi2

+----------------+
| Key            |
|----------------|
|   frequency    |
| row percentage |
```

```
+----------------+

           |      SMAD4_Meth
   disease |         0          1 |     Total
-----------+----------------------+----------
      PDAC |        14          4 |        18
           |     77.78      22.22 |    100.00
-----------+----------------------+----------
        CP |        20          1 |        21
           |     95.24       4.76 |    100.00
-----------+----------------------+----------
   Control |        10          1 |        11
           |     90.91       9.09 |    100.00
-----------+----------------------+----------
     Total |        44          6 |        50
           |     88.00      12.00 |    100.00

          Pearson chi2(2) =   2.9111   Pr = 0.233

. tab disease smad4_meth if hiv==1, row chi2

+----------------+
| Key            |
|----------------|
|   frequency    |
| row percentage |
+----------------+

           |      SMAD4_Meth
   disease |         0          1 |     Total
-----------+----------------------+----------
      PDAC |         4          3 |         7
           |     57.14      42.86 |    100.00
-----------+----------------------+----------
        CP |         7          0 |         7
           |    100.00       0.00 |    100.00
-----------+----------------------+----------
   Control |         6          0 |         6
           |    100.00       0.00 |    100.00
-----------+----------------------+----------
     Total |        17          3 |        20
           |     85.00      15.00 |    100.00

          Pearson chi2(2) =   6.5546   Pr = 0.038

.
. tabstat concentration, stat(n p50 p25 p75) by(smad4_meth)

Summary for variables: concentration
     by categories of: smad4_meth (SMAD4_Meth)

smad4_meth |         N       p50       p25       p75
-----------+--------------------------------------------
         0 |        64      4.95       3.8       6.8
         1 |         7       7.9       5.2      26.6
-----------+--------------------------------------------
     Total |        71       5.1       3.8       7.9
----------------------------------------------------------

. tabstat concentration if disease==1, stat(n p50 p25 p75) by(smad4_meth)

Summary for variables: concentration
     by categories of: smad4_meth (SMAD4_Meth)

smad4_meth |         N       p50       p25       p75
-----------+--------------------------------------------
         0 |        20      7.05       4.6     12.25
         1 |         5      12.8       7.9      26.6
```

```
-----------+--------------------------------------------
     Total |        25       7.9       5.1      13.4
-------------------------------------------------------

. tabstat concentration if disease==2, stat(n p50 p25 p75) by(smad4_meth)

Summary for variables: concentration
     by categories of: smad4_meth (SMAD4_Meth)

smad4_meth |         N       p50       p25       p75
-----------+--------------------------------------------
         0 |        22         4       3.2       5.1
         1 |         1       3.8       3.8       3.8
-----------+--------------------------------------------
     Total |        23       3.8       3.2       5.1
-------------------------------------------------------

. tabstat concentration if disease==3, stat(n p50 p25 p75) by(smad4_meth)

Summary for variables: concentration
     by categories of: smad4_meth (SMAD4_Meth)

smad4_meth |         N       p50       p25       p75
-----------+--------------------------------------------
         0 |        22       5.2       3.9       6.6
         1 |         1       5.2       5.2       5.2
-----------+--------------------------------------------
     Total |        23       5.2       3.9       6.6
-------------------------------------------------------

. ranksum concentration, by(smad4_meth)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

  smad4_meth |      obs    rank sum    expected
-------------+---------------------------------
           0 |       64        2196        2304
           1 |        7         360         252
-------------+---------------------------------
    combined |       71        2556        2556

unadjusted variance     2688.00
adjustment for ties       -1.76
                     ----------
adjusted variance       2686.24

Ho: concen~n(smad4_~h==0) = concen~n(smad4_~h==1)
             z =  -2.084
    Prob > |z| =   0.0372

. ranksum concentration if disease==1, by(smad4_meth)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

  smad4_meth |      obs    rank sum    expected
-------------+---------------------------------
           0 |       20         237         260
           1 |        5          88          65
-------------+---------------------------------
    combined |       25         325         325

unadjusted variance      216.67
adjustment for ties       -0.42
                     ----------
adjusted variance        216.25

Ho: concen~n(smad4_~h==0) = concen~n(smad4_~h==1)
             z =  -1.564
```

```
    Prob > |z| =   0.1178

. ranksum concentration if disease==2, by(smad4_meth)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

  smad4_meth |      obs    rank sum    expected
-------------+---------------------------------
           0 |       22         265         264
           1 |        1          11          12
-------------+---------------------------------
    combined |       23         276         276

unadjusted variance       44.00
adjustment for ties       -0.13
                     ----------
adjusted variance         43.87

Ho: concen~n(smad4_~h==0) = concen~n(smad4_~h==1)
             z =   0.151
    Prob > |z| =   0.8800

. ranksum concentration if disease==3, by(smad4_meth)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

  smad4_meth |      obs    rank sum    expected
-------------+---------------------------------
           0 |       22         264         264
           1 |        1          12          12
-------------+---------------------------------
    combined |       23         276         276

unadjusted variance       44.00
adjustment for ties       -0.04
                     ----------
adjusted variance         43.96

Ho: concen~n(smad4_~h==0) = concen~n(smad4_~h==1)
             z =   0.000
    Prob > |z| =   1.0000

.
. logistic smad4_meth chr_med_ill

Logistic regression                               Number of obs   =         71
                                                  LR chi2(1)      =       2.45
                                                  Prob > chi2     =     0.1177
Log likelihood = -21.636514                       Pseudo R2       =     0.0535

------------------------------------------------------------------------------
  smad4_meth | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
 chr_med_ill |   .2736842   .2393411    -1.48   0.138     .0493016    1.519283
       _cons |   .1923077   .0939087    -3.38   0.001     .0738471    .5007948
------------------------------------------------------------------------------

. logistic smad4_meth chr_med_ill if disease==1

Logistic regression                               Number of obs   =         25
                                                  LR chi2(1)      =       2.69
                                                  Prob > chi2     =     0.1008
Log likelihood = -11.163632                       Pseudo R2       =     0.1076

------------------------------------------------------------------------------
  smad4_meth | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
 chr_med_ill |   .1666667   .2012691    -1.48   0.138     .0156288    1.777347
```

```
       _cons |         .5   .3061862    -1.13   0.258     .1505628    1.660437
------------------------------------------------------------------------------

. logistic smad4_meth chr_med_ill if disease==2
note: chr_med_ill != 0 predicts failure perfectly
      chr_med_ill dropped and 15 obs not used


Logistic regression                               Number of obs   =          8
                                                  LR chi2(0)      =      -0.00
                                                  Prob > chi2     =          .
Log likelihood = -3.0141613                       Pseudo R2       =    -0.0000

------------------------------------------------------------------------------
  smad4_meth | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
 chr_med_ill |          1  (omitted)
       _cons |   .1428571   .1527207    -1.82   0.069     .0175764    1.161114
------------------------------------------------------------------------------

. logistic smad4_meth chr_med_ill if disease==3
note: chr_med_ill != 1 predicts failure perfectly
      chr_med_ill dropped and 11 obs not used


Logistic regression                               Number of obs   =         12
                                                  LR chi2(0)      =      -0.00
                                                  Prob > chi2     =          .
Log likelihood = -3.4420318                       Pseudo R2       =    -0.0000

------------------------------------------------------------------------------
  smad4_meth | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
 chr_med_ill |          1  (omitted)
       _cons |   .0909091   .0949514    -2.30   0.022      .011737    .7041389
------------------------------------------------------------------------------

.
. logistic smad4_meth smoke

Logistic regression                               Number of obs   =         70
                                                  LR chi2(1)      =       0.73
                                                  Prob > chi2     =     0.3926
Log likelihood = -22.390387                       Pseudo R2       =     0.0161

------------------------------------------------------------------------------
  smad4_meth | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
       smoke |        2.4   2.677312     0.78   0.433     .2695604    21.36812
       _cons |   .0555556   .0570779    -2.81   0.005     .0074165    .4161539
------------------------------------------------------------------------------

. logistic smad4_meth smoke if disease==1

Logistic regression                               Number of obs   =         24
                                                  LR chi2(1)      =       0.27
                                                  Prob > chi2     =     0.6022
Log likelihood = -12.145922                       Pseudo R2       =     0.0111

------------------------------------------------------------------------------
  smad4_meth | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
       smoke |   1.846154   2.256231     0.50   0.616     .1682647    20.25549
       _cons |   .1666667   .1800206    -1.66   0.097     .0200653    1.384368
------------------------------------------------------------------------------

. logistic smad4_meth smoke if disease==2
note: smoke != 1 predicts failure perfectly
```

```
      smoke dropped and 2 obs not used


Logistic regression                                     Number of obs   =         21
                                                        LR chi2(0)      =       0.00
                                                        Prob > chi2     =          .
Log likelihood = -4.0203257                             Pseudo R2       =     0.0000

------------------------------------------------------------------------------
  smad4_meth | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
       smoke |          1  (omitted)
       _cons |        .05   .0512348    -2.92   0.003     .0067104    .3725564
------------------------------------------------------------------------------

. logistic smad4_meth smoke if disease==3
note: smoke != 1 predicts failure perfectly
      smoke dropped and 10 obs not used


Logistic regression                                     Number of obs   =         13
                                                        LR chi2(0)      =       0.00
                                                        Prob > chi2     =          .
Log likelihood = -3.5254618                             Pseudo R2       =     0.0000

------------------------------------------------------------------------------
  smad4_meth | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
       smoke |          1  (omitted)
       _cons |   .0833333   .0867361    -2.39   0.017     .0108358     .640881
------------------------------------------------------------------------------

.
. logistic smad4_meth alc

Logistic regression                                     Number of obs   =         69
                                                        LR chi2(1)      =       0.77
                                                        Prob > chi2     =     0.3798
Log likelihood = -22.263899                             Pseudo R2       =     0.0170

------------------------------------------------------------------------------
  smad4_meth | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         alc |   2.454545   2.738716     0.80   0.421     .2755645    21.86346
       _cons |   .0555556   .0570779    -2.81   0.005     .0074165    .4161539
------------------------------------------------------------------------------

. logistic smad4_meth alc if disease==1

Logistic regression                                     Number of obs   =         24
                                                        LR chi2(1)      =       0.09
                                                        Prob > chi2     =     0.7676
Log likelihood = -12.238079                             Pseudo R2       =     0.0036

------------------------------------------------------------------------------
  smad4_meth | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         alc |   1.428571   1.762089     0.29   0.772     .1273436    16.02606
       _cons |         .2    .219089    -1.47   0.142     .0233661    1.711885
------------------------------------------------------------------------------

. logistic smad4_meth alc if disease==2
note: alc != 1 predicts failure perfectly
      alc dropped and 2 obs not used


Logistic regression                                     Number of obs   =         21
                                                        LR chi2(0)      =       0.00
```

```
                                                Prob > chi2     =          .
Log likelihood = -4.0203257                     Pseudo R2       =     0.0000

------------------------------------------------------------------------------
  smad4_meth | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         alc |          1  (omitted)
       _cons |        .05   .0512348    -2.92   0.003     .0067104    .3725564
------------------------------------------------------------------------------

. logistic smad4_meth alc if disease==3
note: alc != 1 predicts failure perfectly
      alc dropped and 11 obs not used


Logistic regression                             Number of obs   =         11
                                                LR chi2(0)      =       0.00
                                                Prob > chi2     =          .
Log likelihood = -3.3509971                     Pseudo R2       =     0.0000

------------------------------------------------------------------------------
  smad4_meth | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         alc |          1  (omitted)
       _cons |         .1   .1048809    -2.20   0.028     .0128012    .7811739
------------------------------------------------------------------------------

.
. logistic smad4_meth hiv

Logistic regression                             Number of obs   =         49
                                                LR chi2(1)      =       0.83
                                                Prob > chi2     =     0.3618
Log likelihood = -15.731871                     Pseudo R2       =     0.0257

------------------------------------------------------------------------------
  smad4_meth | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         hiv |   .4197531   .4046197    -0.90   0.368     .0634572     2.77656
       _cons |   .4204152   .6100607    -0.60   0.550     .0244629    7.225188
------------------------------------------------------------------------------

. logistic smad4_meth hiv if disease==1

Logistic regression                             Number of obs   =         15
                                                LR chi2(1)      =       1.81
                                                Prob > chi2     =     0.1787
Log likelihood =  -7.794518                     Pseudo R2       =     0.1039

------------------------------------------------------------------------------
  smad4_meth | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         hiv |   .1904762   .2502563    -1.26   0.207     .0145042    2.501429
       _cons |     3.9375   7.341289     0.74   0.462     .1019047    152.1413
------------------------------------------------------------------------------

. *logistic smad4_meth hiv if disease==2
. logistic smad4_meth hiv if disease==3
note: hiv != 2 predicts failure perfectly
      hiv dropped and 6 obs not used


Logistic regression                             Number of obs   =         10
                                                LR chi2(0)      =       0.00
                                                Prob > chi2     =          .
Log likelihood = -3.2508297                     Pseudo R2       =     0.0000

------------------------------------------------------------------------------
```

```
  smad4_meth | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         hiv |          1  (omitted)
       _cons |   .1111111   .1171214    -2.08   0.037     .0140771    .8770063
------------------------------------------------------------------------------

.
. logistic smad4_meth concentration

Logistic regression                               Number of obs   =         71
                                                  LR chi2(1)      =       3.36
                                                  Prob > chi2     =     0.0668
Log likelihood = -21.180926                       Pseudo R2       =     0.0735

------------------------------------------------------------------------------
   smad4_meth | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
--------------+---------------------------------------------------------------
concentration |   1.067604     .03638     1.92   0.055     .9986299    1.141343
        _cons |   .0574127   .0329264    -4.98   0.000     .0186569    .1766753
------------------------------------------------------------------------------

. logistic smad4_meth concentration if disease==1

Logistic regression                               Number of obs   =         25
                                                  LR chi2(1)      =       1.28
                                                  Prob > chi2     =     0.2582
Log likelihood = -11.870978                       Pseudo R2       =     0.0511

------------------------------------------------------------------------------
   smad4_meth | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
--------------+---------------------------------------------------------------
concentration |    1.04425    .039467     1.15   0.252     .9696914    1.124541
        _cons |   .1342002    .105772    -2.55   0.011     .0286329    .6289866
------------------------------------------------------------------------------

. logistic smad4_meth concentration if disease==2

Logistic regression                               Number of obs   =         23
                                                  LR chi2(1)      =       0.09
                                                  Prob > chi2     =     0.7683
Log likelihood = -4.0700548                       Pseudo R2       =     0.0105

------------------------------------------------------------------------------
   smad4_meth | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
--------------+---------------------------------------------------------------
concentration |   .8367266   .5928833    -0.25   0.801     .2086621    3.355239
        _cons |   .0936072   .2704908    -0.82   0.412     .0003248    26.97573
------------------------------------------------------------------------------

. logistic smad4_meth concentration if disease==3

Logistic regression                               Number of obs   =         23
                                                  LR chi2(1)      =       0.06
                                                  Prob > chi2     =     0.8029
Log likelihood = -4.0822982                       Pseudo R2       =     0.0076

------------------------------------------------------------------------------
   smad4_meth | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
--------------+---------------------------------------------------------------
concentration |   .9175248   .3410409    -0.23   0.817     .4428219    1.901107
        _cons |   .0734575   .1612506    -1.19   0.234     .0009943    5.426935
------------------------------------------------------------------------------
```

APPENDIX C – Ethics clearance certificate