The Utility of a Novel Blood Based Biomarker in the Diagnosis of Pancreatic Cancer

Lucy Ellen Nichols

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Summary

Pancreatic cancer maintains one of the worst prognoses of all malignancies. Fewer than 1% of patients survive 10-years post-diagnosis. It is an aggressive disease with as many as 80% of patients diagnosed in the most advanced stages of disease. This severely limits treatment options, contributing to the dismal prognosis.

Diagnosis remains a challenge. Often, imaging alone cannot differentiate between benign or malignant disease. Blood-based biomarker CA19-9 cannot be relied upon since it is a modified Lewis antigen so 5-10% of the population do not express it. Tissue biopsies remain the gold-standard for final confirmed diagnoses, yet collection of pancreatic biopsies is invasive, time and resource intensive and have a range of risks associated.

Blood-based biomarkers offer a less invasive, cheaper, and more accessible alternative to more traditional diagnostic techniques. Here, we explored the use of novel DNA mutation assay, the human erythrocyte PIG-A assay, as a blood-based biomarker to determine whether it had any potential in diagnosing pancreatic cancer. An elevated frequency of PIG-A mutant erythrocytes was observed within pancreatic cancer patients in comparison to controls of healthy donors and a benign pancreatic disease cohort. Furthermore, the more well-established human peripheral blood mononuclear cell cytokinesis block micronucleus assay provided a secondary measure of DNA damage. An elevation was also viewed in this assay in malignant donors. Both assays were additionally explored within an *in vitro* setting, modelling the induction of DNA damage by known risk factors for pancreatic cancer.

Given the complexity of pancreatic cancer diagnosis, a panel of biomarkers was explored, combining clinical markers of inflammation with our two DNA-based biomarkers and clinically approved CA19-9. Combination of the novel PIG-A mutation assay and CA19-9 blood-test appeared the most suitable panel of biomarkers for future exploration.

Declarations

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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STATEMENT 1

This thesis is the result of my own investigations, except where otherwise stated. Where correction services have been used, the extent and nature of the correction is clearly marked in a footnote(s).

Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

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Oh, and Tinsel the cat, whose jumping on the keyboard at the most awkward of opportunities has kept me entertained through months of writing.

Abbreviations List

5-FU – 5-Fluorouracil

ADH – Alcohol Dehydrogenase

AhR – Aryl Hydrocarbon Receptor

ALDH – Aldehyde Dehydrogenase

ANOVA – Analysis of variance

APC – Adenomatous polyposis coli gene

APC - Allophycocyanin

B[a]P – Benzo-[a]-pyrene

BMI – Body Mass Index

BPDE – Bay-region diol-epoxide

BRCA1 – Breast Cancer 1 gene

BSA – Bovine Serum Albumin

CA – Cholic Acid

CA19-9 – Carbohydrate Antigen 19-9

CBMN – Cytokinesis block micronucleus

CI – Confidence Intervals

CP – Chronic Pancreatitis

CRP – C-Reactive Protein

CT – Computerised Tomography

DCA – Deoxycholic Acid

DM – Diabetes Mellitus

DMSO – Dimethyl Sulphoxide

DNA – Deoxyribose Nucleic Acid

DQS – Dietary Quality Score

ERCP – Endoscopic Retrograde Cholangiopancreatography

EUS – Endoluminal Ultrasound

FAEE – Fatty Acid Ethyl Esters

FAP – Familial adenomatous polyposis

FBS – Foetal Bovine Serum

FDG – 18F- Fluorodeoxyglucose

FIRINOX – 5-fluorouracil, Irinotecan, Oxaplatin

FNA – Fine Needle Aspiration

FOLFIRINOX – Folinic acid, 5-fluorouracil, Irinotecan, Oxaplatin

GPA – Glycophorin A

GPI - glycosylphosphatidylinositol

HBOC – Hereditary Breast and Ovarian Cancer Syndrome

HDI – Human Development Index

HPRT – Hypoxanthine phosphoribosyltransferase

IARC – International Agency for Research on Cancer

IL-6 – Interleukin-6

IPMN – Intraductal Papillary Mucinous Neoplasm

LFT – Liver Function Tests

MCN – Mucinous Cystic Neoplasm

MN - Micronucleus

MRCP – Magnetic Resonance Cholangiopancreatography

MRI – Magnetic Resonance Imaging

NAD/H – Nicotinamide Adenine Dinucleotide

NICE – National Institute for Health and Care Excellence

NLR – Neutrophil to Lymphocyte Ratio

ONS – Office for National Statistics

PanIN – Pancreatic Intraductal Neoplasia

PBMC – Peripheral blood mononuclear cells

PBS – Phosphate Buffered Saline

PC – Pancreatic Cancer

PCR – Polymerase Chain Reaction

PDAC – Pancreatic ductal adenocarcinoma

PE – R-phycoerythrin

PET – Positron Emission Tomography

PHA - phytohemagglutinin

PIG-A – Phosphotidyl Inositol Glycan Class A (in human cells)

Pig-A – Phosphotidyl Inositol Glycan Class A (in rodent cells)

PNH – Paroxysmal Nocturnal Haemoglobinuria

RB-1 – Retinoblastoma 1 gene

REC – Research Ethics Committee

ROS – Reactive Oxygen Species

ROS – Reactive Oxygen Species

RPD – Relative Population Doubling

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1.1 Cancer

Cancer is one of the most common diseases globally, arising from the abnormal growth of cells which results in tumour formation, with exceptions found in bone marrow cancers (Roy and Saikia, 2016). Tumours initiate from a single cell which over time has acquired DNA mutations in driver genes which leads to initiation of disease (Wodarz *et al.*, 2018). Often, driver genes are either a proto-oncogene or a tumour suppressor gene. Mutation within the proto-oncogene can turn the gene into an oncogene, which then promotes the formation of cancer. Both proto-oncogenes and oncogenes encode proteins which induce cell division, inhibit cell differentiation, and inhibit cell death. Oncogenes produce these in a much higher quantity or produce a gene product with increased activity, contributing to carcinogenesis (Chial, 2008). Under normal conditions, tumour suppressor genes prevent formation of cancers through controlling cell division, initiating DNA repair mechanisms and inducing apoptosis (Hooper, 1998; American Cancer Society, 2014). Tumour suppressor genes require two mutational hits to inactivate both copies of the gene to stop tumour suppressing activities of the gene and initiate tumourigenesis (Wodarz *et al.*, 2018). Examples of tumour suppressor genes which are implicated in carcinogenesis when mutated are: *RB-1* linked with retinoblastoma and osteosarcoma, *p53* linked with a wide variety of tumours including breast and brain cancer, *BRCA1* and *2* linked with breast and ovarian cancer and *APC* linked with familial adenomatous polyposis which leads to colon carcinoma (Hooper, 1998). The result of these driver mutations is uncontrolled cell division, as seen in **Fig. 1.1A**, which forms a localised tumour. As tumours grow, requirements for nutrient supply increases and as a result, tumours attract their own blood supply to ensure survival (Arnold, 1985; Hanahan and Weinberg, 2011). Tumour cells can also harness this blood supply to enter circulation and extravasate into a novel deposit where use of proliferation, apoptosis, dormancy, and angiogenesis develop from a single cell deposit into a full-blown metastatic tumour (Steeg, 2006). Metastatic deposits signify systemic disease, which is the main cause of death in cancer patients (Steeg, 2006).
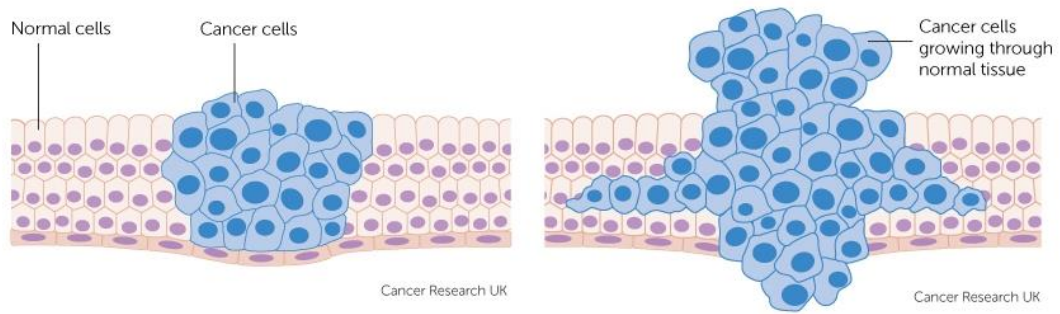
Figure 1.1: *Diagrams depicting the growth of cancer cells in blue to form **A)** a localised tumour which remains in the tissue which it originated from **B)** a locally advanced tumour which is growing into normal tissue outside of primary site of tumour* (Taken from Cancer Research UK, 2020a).

1.1.1 Cancer throughout history

Cancer is often claimed to be as old as humanity, yet evidence of cancer has been identified in prehistoric animal remains like an osteosarcoma in a hominin bone from 1.7 million years ago, before the evolution of the human race (Hajdu, 2011a; Peters and Gonzalez, 2018). The first documented written description of cancer came in approximately 3000 BC, around the same time that Stone Henge was erected. The Edwin Smith Papyrus described a bulging tumour of the breast with no curative treatment (Hajdu, 2011a). It is likely that some knowledge of tumours came before written documentation, with knowledge passed on verbally. Cancer knowledge has improved significantly over the last 5,000 years, with notable breakthroughs. Towards the 1500s, realisation came that early treatment, especially before tumours became ulcerated, resulted in better patient outcomes (Hajdu, 2011a). During the Renaissance period, many developments came as a result of physicians and surgeons uncovering more on the human anatomy and physiology through dissections (Hajdu, 2011b). Eventual replacement of gross pathology of the 18th century by microscopic pathology in the 19th century allowed for classification of tumours before treatment, and allowed for better clinical and surgical decisions to be made for the patient's benefit (Hajdu, 2012). The first half of the 1900s allowed for further tumour classification dependent on histopathological grading and an increase in oncology based research (Hajdu and Darvishian, 2013). Up to the 1970s, discovery of anti-

cancer chemotherapeutic drugs and radiation-based therapies revolutionised treatment and great advances were made with imaging modalities for visualisation of tumours, but no real improvements were made in the survival despite advances in diagnosis and treatment of disease (Hajdu and Vadmal, 2013). Most advances in cancer knowledge has come around within the last 50 years, including elucidation of the DNA damage and DNA mutations which result in tumour formation, and multidisciplinary team cancer treatments which allowed for more effective treatment and breakthroughs with patient survival (Hajdu *et al.*, 2015).

1.1.2 Incidence, mortality, and survival

Cancer is a growing concern, globally. Cancer is generally a disease of age and as other diseases are becoming more treatable, the burden of cancer is increasing. Data collected from cancer cases across the UK between the years of 1979 and 2014 in combination with ONS national population predictions were used to forecast future trends of cancer incidence and mortality between 2015 and 2035 (Smittenaar *et al.*, 2016). It was concluded that overall age standardised rate of cancer incidence would increase 0.07% per year (Smittenaar *et al.*, 2016).

A global study monitored cancer incidence and compared this with the Human Development Index (HDI). HDI was calculated using 3 aspects: life expectancy at birth, mean and expected years in education, and standard of living based on gross national income per capita, by the United Nations Development Programme (United Nations Development Programme, 2015). The study grouped nations by HDI into four categories of low, medium, high, and very high HDI and studied both cancer incidence and mortality rates, seen in **Fig. 1.2**. Cancer incidence was greater in more developed countries, but mortality is comparable between all HDI groups (Fidler *et al.*, 2018). There are various reasons which account for this imbalance between cancer incidence and national development. A westernised lifestyle made up of a high fat diet combined with alcohol intake, tobacco smoking and a more sedentary lifestyle, may contribute to this. Cancers linked with Western lifestyles include breast, prostate and colorectal cancer, which are all more prevalent in western society in comparison to developing nations (Kanavos, 2006). In contrast, lower HDI regions tend to have greater prevalence of infection-related malignancies including liver, stomach and oesophageal cancer (Fidler *et al.*, 2018). In lower HDI countries,

healthcare may not be as widespread and easily accessible. Diseases which may be easily treatable and manageable within more developed regions may not be so manageable within lower HDI areas. This may also contribute to the lower cancer incidence in lower HDI nations if life expectancy is reduced and cancer is a disease of aging. Nations with low and medium HDI have been predicted to have the biggest projected increase of 81-100% in cancer cases between 2008 and 2030 (Fidler *et al.*, 2018).
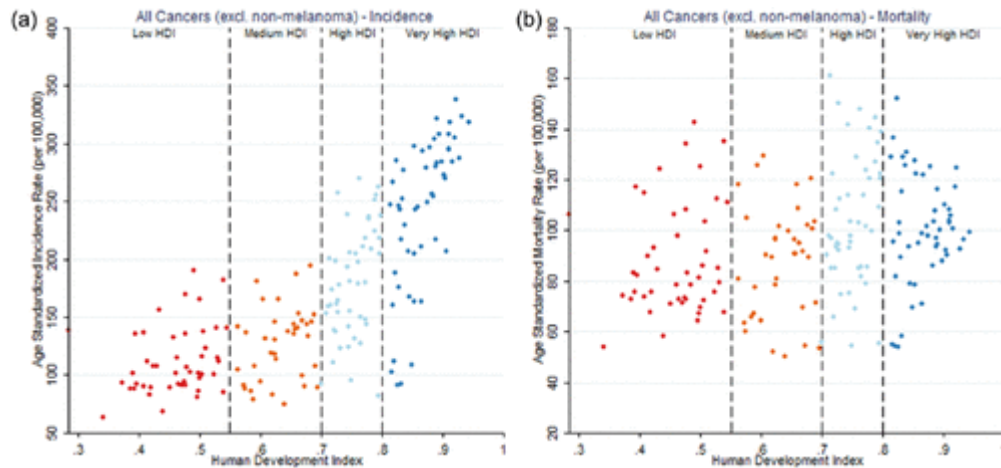
Figure 1.2: A) Cancer incidence rate (per 100,000) in countries grouped by development as measured by HDI. B) Cancer mortality rates (per 100,000) in nations grouped by development measured by HDI (Fidler *et al.*, 2018).

In a lifetime, an individual in the UK has a 50% chance of developing cancer (Cancer Research UK, 2018). There are several factors which will increase the risk of developing cancer, some factors specific for pancreatic malignancies are discussed further in **1.2.2**.

Cancer is no longer the death sentence it once was thanks to improvements in diagnosis and treatment. Now, 50 % of patients survive 10 years post cancer diagnosis which has more than doubled in the last four decades, from 24% (Cancer Research UK, 2014a). Survival is dependent on several factors including age, stage of disease at diagnosis and type of cancer since different malignancies have vastly different survival rates. Survival is greatest in patients aged 15-40, and lowest between ages of 80 and 99 (Cancer Research UK, 2016a). Patients aged over 65 account for 60% and 70% of cancer incidence and mortality, respectively (Given and Given, 2008). At the greater age, radical oncological treatments may not be tolerated

as easily, resulting from increased comorbidities and reduced functional status (Given and Given, 2008). However, there may also be some unfair barriers which block patients above a certain age from getting the treatment a younger patient may do. These include no staging of disease, no surgical options when disease may be early enough to cure through resection and the use of subclinical doses of chemotherapy and radiotherapy with no curative intent (Given and Given, 2008). Stage of disease will have a great influence on survival statistics since stage of disease will limit treatment options available. Type of malignancy plays a huge role in patient survival. Different tumour types have different characteristics which will influence how the tumour behaves, resulting in survival differences. For example, pancreatic cancer is a highly aggressive malignancy, often diagnosed once disease has spread and only surgery can cure the disease. More information on pancreatic cancer survival is found in **1.2.6**. Ten-year survival data for pancreatic cancer shows that fewer than 1% of patients survive ten years post-diagnosis, in comparison to prostate cancer which has a ten-year survival rate of 98% (Cancer Research UK, 2014a).

1.2 Pancreatic Cancer

The pancreas is a mixed gland which resides in the retroperitoneal space linked to the gastrointestinal tract, **Fig 1.3**. From an anatomical standpoint, the pancreas consists of the head, adjacent to the duodenum and the tail which is next to the spleen (Murtaugh and Keefe, 2015). The exocrine gland accounts for 80% of the pancreatic mass, comprised of acinar cells which secrete digestive enzymes into the branched pancreatic ductal network (Hezel *et al.*, 2006; Murtaugh and Keefe, 2015). In combination with bile acids stored in the gallbladder, this comprises pancreatic juice which is released into the duodenum, via the ampulla of Vater, to aid digestion, particularly of fatty foods (Murtaugh and Keefe, 2015). Endocrine islets consist of α and β cells which produce, store and secrete hormones glucagon and insulin, respectively, to maintain blood glucose homeostasis. Both endocrine and exocrine dysfunction can cause serious repercussions for the body.
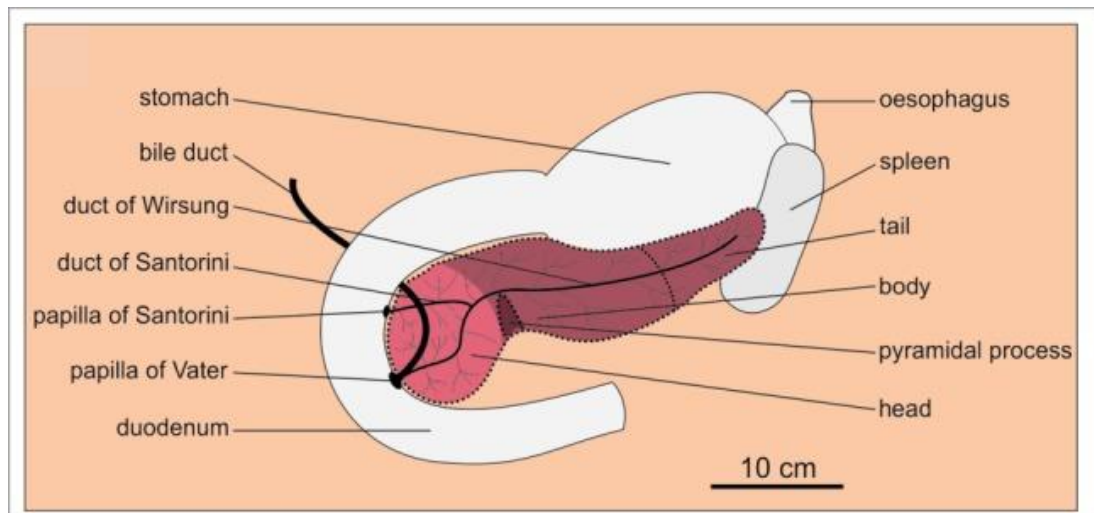
Figure 1.3: *Anatomical diagram of a human pancreas (Modified from* Dolenšek *et al.*, 2015)*.*

Cancer can arise in any region of the pancreas. Pancreatic cancer (PC) is classified dependent on the region of the gland the tumour originates from. Approximately 1-2% of PC cases arise from endocrine regions of the gland and are referred to as pancreatic neuroendocrine tumours (PNETs). They can be further classified depending on their functionality. Functional PNETs have an incidence of 0.2 cases per million and induce hormonal changes often identified through diagnostic studies (Halfdanarson *et al.*, 2008; Tadokoro *et al.*, 2016). Functional PNETs are usually named after the hormone produced in excess by the tumour, the most common form being an insulinoma (Shin *et al.*, 2010). Insulinomas are diagnosed using Whipple's triad: hypoglycaemia, low glucose and symptoms reduced after glucose administration (Miron *et al.*, 2016). The more common non-function PNET are considerably harder to identify and diagnose without altering hormonal levels (Halfdanarson *et al.*, 2008). Patients with functional PNETs have shown a 28 month increase in median survival in comparison to non-function PNET patients, indicating that earlier diagnosis and treatment are key to extending patient survival (Halfdanarson *et al.*, 2008).

Exocrine tumours account for the majority of PC cases. 85% of all PC cases are pancreatic ductal adenocarcinoma (PDAC) (Polireddy and Chen, 2016; Barone *et al.*, 2016), named after the resemblance to ductal cells (Hezel *et al.*, 2006). Most PDAC tumours arise in the head of the pancreas, allowing for infiltration into surrounding organs, namely the liver, spleen and lungs (Hezel *et al.*, 2006). PDAC has the lowest

overall survival of all solid tumours, with 1-3% survival in five years (Karandish and Mallik, 2016).

1.2.1 Epidemiology of Pancreatic Cancer

Pancreatic cancer (PC) accounts for approximately 3% of cancer cases in the UK (Cancer Research UK, 2014b). This is the equivalent to the 11th most common form of cancer in the UK (Pancreatic Cancer UK, 2020). In 2011 in the US alone, 44,030 cases of PC were diagnosed, coupled with 37,660 fatalities (Siegel *et al.*, 2011). In the UK, 10,000 cases were diagnosed coupled with 9,000 fatalities in 2016 (Pancreatic Cancer UK, 2020). Imbalances in PC incidence are seen dependent on gender. Global incidence rates in males and females were 4.9 and 3.6 per 100,000, respectively, in the year 2012 (Ilic and Ilic, 2016). As age increases, the chances of developing PC also increase, **Fig. 1.4**. Approximately 90% of PC cases occur in patients above the age of 55, with median age of diagnosis of 71 (Wood *et al.*, 2006; Gordon-Dseagu *et al.*, 2018).

Figure 1.4: *PC incidence increases at greater ages seen in England and Wales in the year 2000* (Wood *et al.*, 2006)*. Males represented by a solid line; females represented by a dashed line.*

Incidence of PC is also variable dependent on ethnicities and nationalities. A study analysed PC data collected between 1974 and 2013 within registries accounting for 30% of the US population. Ethnicity data was collected and collated with PC incidence data. Black residents were shown to have a higher incidence of PC in comparison to other ethnicities with incident rate ratio of 1.24 and 1.37 in males and

females, respectively, in comparison to the Caucasian population (Gordon-Dseagu *et al.*, 2018). In the study, the lowest incidence of PC was found in the Asian and Pacific islander population (0.78), closely followed by the native American/ native Alaskan population (0.79) and the Hispanic population (0.88) (Gordon-Dseagu *et al.*, 2018). Globally, PC incidence is not uniform. Particularly high incidence of PC is found within Armenia, Czech Republic, Slovakia, Japan and Hungary with lowest incidence in Middle Africa and Pakistan (Ilic and Ilic, 2016). The imbalance in PC incidence can be accounted for by lifestyle differences, exposure to different risk factors and different health care systems.

1.2.2 Risk Factors

Numerous risk factors have been associated with the development of PC. These have been further categorised.

1.2.2.1 Lifestyle Factors

As many as 37% of all PC cases are preventable through better lifestyle and dietary choices (Cancer Research UK, 2014b). Tobacco smoking has long been linked with lung cancer risk but additionally increases risk of PC development. As many as 29% of PC cases have been linked with smoking and additionally increases the risk of death in PC patients by 1.71-fold (Cancer Research UK, 2014b; Wang *et al.*, 2014). Obesity has been linked to increased risk of PC, likely due to inflammation and metabolic alterations. Overall, 12% of PC cases have been linked with obesity (Cancer Research UK, 2014b; Lennon *et al.*, 2016). Additionally, excessive alcohol consumption has been shown to increase PC risk by 20% (Cancer Research UK, 2014b). Many lifestyle factors are modifiable risks, with reduced risk of cancer if factors are changed, for example with losing weight, risk of PC is reduced (Xu *et al.*, 2018). More knowledge and awareness of lifestyle factors which increase the risk of cancer development may result in more lifestyle changes for the better. Lifestyle factors have been discussed further in depth in **chapter 3.1**.

1.2.2.2 Hereditary Disease

Several hereditary conditions increase the risk of developing PC. Two examples include familial adenomatous polyposis (FAP) and hereditary breast and ovarian cancer syndrome (HBOC).

FAP is autosomal dominant as a result of mutation in the adenomatous polyposis colis (APC) gene, 70% of cases are a result of inheritance and 30% are a result of a novel mutation (Lipton and Tomlinson, 2006). FAP induces an excess of colorectal polyps (>100) (Lipton and Tomlinson, 2006; Capasso *et al.*, 2018). Each individual polyp has a risk of turning malignant, greatly increasing the chances of colorectal malignancies. Patients with FAP are also at a greater risk of developing extraintestinal cancers, including cancer of the thyroid, adrenal gland, bile duct and pancreas (Giardiello *et al.*, 1993). In a study of 1,391 FAP patients, there was an increased absolute risk of developing PC with 21.4 cases per 100,000 (Giardiello *et al.*, 1993) in comparison to risk of general population, with male and female absolute risks of 5.5 and 4 per 100,000, respectively (Rawla *et al.*, 2019).

HBOC is characterised by a germline pathologic variant in *BRCA1* or *BRCA2* (Petrucelli *et al.*, 1993). It has been well established that HBOC increases the chance of developing both breast cancer in males and females, and ovarian cancer in female (Moran *et al.*, 2012). To a lesser extent, HBOC can increase the risk of developing other cancers including cancer of the prostate, pancreas, and malignant melanoma (Petrucelli *et al.*, 1993). Chances of developing PC with *BRCA1* and *BRCA2* variants are 1-3% and 2-7%, respectively, in comparison to the general risk of 0.5% (Petrucelli *et al.*, 1993). *BRCA1* and *BRCA2* both act as tumour suppressors, involved in the DNA damage response pathways. *BRCA1* is involved in checkpoint activation and DNA repair while *BRCA2* mediates homologous recombination but the relationship between the two proteins is still not fully understood (Roy *et al.*, 2012).

1.2.2.3 Pre-existing Conditions

A range of pre-existing chronic conditions will increase the likelihood of developing PC over a lifetime. Two examples are found in chronic pancreatitis and diabetes mellitus.

Chronic inflammation has been long associated with the development of cancer; in the pancreas this is no exception. Chronic pancreatitis (CP) is characterised by repeated periods of pancreatic inflammation which cause intense abdominal pain, nausea and vomiting which eventually become constant symptoms (Raphael and Willingham, 2016). Over time, the continual inflammation leads to loss of pancreatic

function leading to diabetes mellitus (DM) and maldigestion from endocrine and exocrine insufficiency, respectively (Kleeff *et al.*, 2017). CP induces a unique, complex form of diabetes described as type 3c DM. Like type 1 DM, this is insulin dependent, but there is greater risk of hypoglycaemia because of α cell involvement, reducing glucagon production (Lew *et al.*, 2017). Chronic inflammation drives NF-κB activation, which is elevated in a number of cancers, including PC (Gudkov and Komarova, 2016). Pancreatitis in all of its forms: acute, chronic, autoimmune; have all been shown to increase the risk in developing PC (Ikeura *et al.*, 2016). Chronic inflammation from CP has been shown to increase the formation of pre-malignant pancreatic lesions including pancreatic intraepithelial neoplasm, intraductal papillary mucinous neoplasm and mucinous cystic neoplasms (Krška *et al.*, 2015). CP is the strongest risk factor for PC, increasing the risk of developing PC by 13.3-fold, likely due to inflammatory changes and subsequent ROS release which initiates a cascade of DNA damage which leads to mutation (Lew *et al.*, 2017).

DM is both a risk factor for developing PC and a potential symptom of early PC so is likened to the chicken and the egg (Lishan Wang *et al.*, 2016). PC can be caused by DM in many ways. DM induces chronic inflammation in pancreatic islets, mediated by p38 MAPK. Wang *et al.* (2016) showed p38 MAPK plays a regulatory role in proliferation, apoptosis, and metastasis. All effects were only exacerbated further by hyperglycaemia. A study of 600 patients showed a significant ($p<0.0001$) increased prevalence of DM in PC patients (67%) in comparison to other malignancies (breast, lung, colorectal, prostate) and over control patients (Aggarwal *et al.*, 2013). A study of 8 autoimmune pancreatitis patients who went on to develop PC showed 65% (5) of patients had DM before PC diagnosis (Ikeura *et al.*, 2016). Any conclusions which could be drawn about DM, are complicated further given novel DM or uncontrollable blood sugar levels in longstanding DM are symptoms of PC, and they may arise before a pancreatic tumour is clinically recognisable.

Despite the complicated pattern, patients with concurrent CP and DM have a further increased risk, with a 33-fold increased risk of PC development (Lew *et al.*, 2017).

1.2.2.4 Pre-malignant Lesions

A range of pre-malignant pancreatic lesions exist which can develop into fully neoplastic disease. Three known precursors to PC are pancreatic intraepithelial

neoplasms (panIN), and then the cystic mucinous cystic neoplasms (MCN) and intraductal papillary mucinous neoplasms (IPMN) (Zamboni *et al.*, 2013). Of the three, panINs are the most abundant premalignant pancreatic lesion (Zamboni *et al.*, 2013). PanINs are typically smaller than 5mm in size and are much harder to diagnose in comparison to the larger, cystic premalignant lesions which are identifiable on radiological examinations (Yu *et al.*, 2018). As a result, panIN are often only identified through pathological examination of surgical specimens. PanINs are graded depending on their morphology, **Fig, 1.5**. Low risk panIN1 is categorised as either flat (panIN1A) and papillary (panIN1B), panIN2 are more abundant within PDAC associated pancreatic tissue and panIN3 are believed to be carcinoma *in situ* (De Wilde *et al.*, 2012).   Accumulation of specific mutations progress healthy pancreatic epithelium to advancing stages of panIN, with early stage neoplasia associated with KRAS and CDKN2A with panIN3, carcinoma *in situ*, associated with TP53 and SMAD4 (Batey, 2019). A study of surgical specimens from 95 PDAC patients who underwent a pancreatectomy discovered panIN lesions within 59% of patients, with panIN3 being the most abundant (Yu *et al.*, 2018).
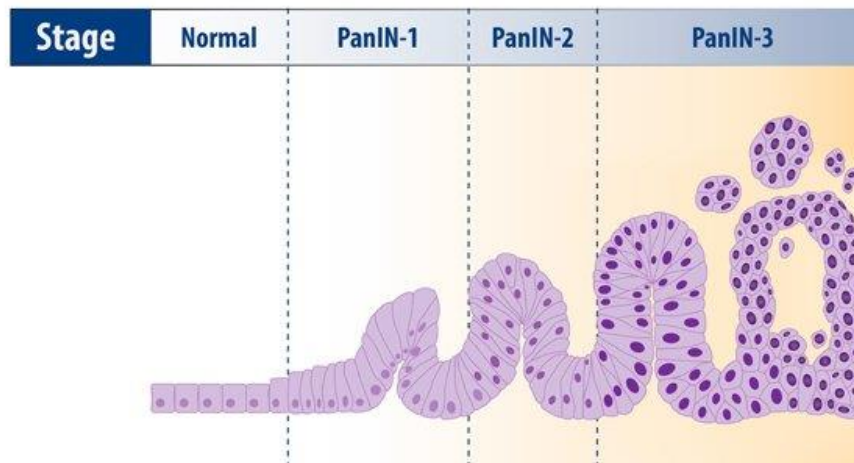
Figure 1.5*: Progression from healthy pancreatic epithelium to increasing stage of PanINs (modified from* (Batey, 2019)*.*

1.2.3 Symptoms of Pancreatic Cancer

Early stage PC is asymptomatic and so the tumours can advance undetected in the retroperitoneal space and by the time symptoms present, the disease is often too advanced for surgical intervention in around 80% of cases (Lahoud *et al.*, 2016). Common late presenting symptoms of advanced PC include upper abdominal pain, which radiates along the spine, accounting for it being one of the most painful malignancies from a clinical standpoint within its final stages (Lahoud *et al.*, 2016). Jaundice is another common symptom observed in 50% of PC cases, as a result of the obstruction of the bile duct by tumours in the head of the pancreas which are seen in 75% of all cases (Tummala *et al.*, 2011; Cancer Research UK, 2016b). Other symptoms experienced early in PC cases include weight loss, poor appetite, and new onset or poorly controlled DM. As the disease progresses, the symptoms become progressively worse (Lahoud *et al.*, 2016). The problem with the symptoms of PC is that they are unspecific, not unique to pancreatic cancer and can be ignored for some time.

1.2.4 Diagnosis

Diagnosis of PC is discussed in depth in later **chapter 4.1.1**. Diagnosis of PC is achieved through a combination of imaging, blood tests and histological confirmation since imaging and blood tests alone cannot fully differentiate between benign and malignant disease. Currently, the majority of patients are diagnosed with

advanced disease (stage III and IV), severely limiting possibility of curative treatments (National Cancer Registration and Analysis Service, 2019). Improvements in PC diagnosis are urgently needed to help change the bleak prognosis of PC patients, which have failed to improve despite therapeutic advances. Particularly, improvements in diagnosis of early stage PC where disease is still resectable, would vastly improve disease outlook.

1.2.5 Treatment

Current treatment regimens for PC are based on the tumour size, the blood vessel involvement, whether metastatic disease has been confirmed and the fitness of the patient. Under the NHS, there are three main treatment options for those with confirmed PC cases including surgery, chemotherapy, radiotherapy, or a combination thereof. To date, the only treatment with curative potential is surgery, however due to the late onset of symptoms and challenges in diagnosis, fewer than 20% of PC patients are eligible for pancreatic tumour resection (Lin *et al.*, 2015). It's estimated that 80-90% of PC patients have locally advanced or metastatic disease, which further complicates treatment (Krempien *et al.*, 2006).

Pancreatic surgery is no small undertaking. Due to complex positioning of the pancreas within the retroperitoneum and its involvement with surrounding organs and blood vessels, pancreatic surgery continues to have risks and is hailed as the most complicated general surgery (Wang *et al.*, 2016; Lemke *et al.*, 2016). Even with all the risks associated with pancreatic surgery, only 20% of patients who undergo successful surgical resection survive 5 years (Bellon *et al.*, 2016). Pancreatic surgery is categorised as open or laparoscopic. Open pancreatic surgery tends to have more associated risks and requires a lengthy hospital stay including a couple of days within intensive care. Laparoscopic or key hole surgery is used for the removal of smaller tumours, and is more commonly used for distal pancreatectomies since it has been shown to reduce blood loss, post-operative pain, and improve recovery to minimise hospital stay required in comparison to an open distal pancreatectomy (Kocaay *et al.*, 2016). Laparoscopy is still a developing technique and although has shown promises in some areas, major drawbacks still exist, and further development may bring it forward to overtake open surgery as a technique (Al-Taan *et al.*, 2010). In a study of 47,685 patients who underwent either

a liver or pancreatic resection over 10 years, minimally invasive surgical approaches were more common in patients aged 66 to 85 years (Okunrintemi *et al.*, 2016).

The aim of pancreatic surgery is complete resection of the tumour, which is the only curative option. Complete resection is referred to as R0, where no remaining cancer cells are found along the resection margins upon histopathology review. Even in surgeries where this is achieved, patient survival over 5 years is between 3 and 16% (Krempien *et al.*, 2006). Post-surgery, 92% of cases of PDAC have been shown to relapse without any ongoing therapy and this often presents around 9-15 months after the initial presentation (Krempien *et al.*, 2006; Bittoni *et al.*, 2014). Relapse is common because of the aggressive nature of PC, the disease may have progressed into regions without the surgeon's knowledge, and so remaining neoplastic tissue can develop into a tumour. There are various surgeries available which can be carried out on the pancreas depending on the disease present, the location and the vascular involvement of the rumour. Types of surgery include: the Whipple procedure, distal pancreatectomy, total pancreatectomy.

As mentioned previously, the aggressive nature of the disease, late onset of symptoms and difficulty in diagnosis results in up to 90% of patients having locally advanced or metastatic disease at presentation (Krempien *et al.*, 2006). Thus, only 20% of PC patients are candidates for surgical resection (Lin *et al.*, 2015). The remaining patients maybe suited for other therapeutic interventions such as chemotherapy and radiotherapy or may be more suited for palliative surgery. This surgery has no intention of curing the disease. Traditional palliative double bypass surgery is carried out in cases of metastatic disease to prevent gastric outlet obstruction and further jaundice to reduce future symptoms and to ease future pain (Adham and Perinel, 2019). Obstructive jaundice can also be relieved through the use of stents which support the biliary duct, allowing effective draining to relieve the symptoms of jaundice (Isayama *et al.*, 2016). Biliary stents generally come in two forms: plastic and self-expandable metal stents, each with their own benefits and risks (Moole *et al.*, 2016). In a meta-analysis, Moole *et al.* (2016) compared covered and uncovered self-expandable metal stents and found that in the thirteen studies analysed that there were no significant differences overall when comparing survival and overall adverse event rates.

Chemotherapy is one of the main treatment options available for PC patients since fewer than 20% of patients are eligible for surgery upon diagnosis (Lin *et al.*, 2015). Unlike surgery, chemotherapy is not a curative treatment option. Although there have been advances in treatment options and knowledge of PC, the outcome of therapy has failed to improve within the last 30 years (Polireddy and Chen, 2016). A range of chemotherapeutic agents have been in use against PC, yet none have had a significant impact upon survival rates. In recent years 5-fluorouracil (5-FU) was the standard chemotherapeutic treatment from discovery in the 1950s until only recently (Teague *et al.*, 2015). Its successor, gemcitabine, is a nucleoside analogue which was shown in a randomised trial of PC patients to improve disease-related pain and survival rates in comparison to patients treated with 5-FU (Burris *et al.*, 1997). This increased median survival from 4.41 months to 5.65 months and so gemcitabine became the key chemotherapeutic agent. More recently, combination therapy containing fluorouracil, oxaliplatin and irinotecan (FOLFIRINOX) outperformed gemcitabine monotherapy in a phase 2-3 trial in a number of areas including response to therapy and patient survival which was increased from 6.8 months to 11.1 months (Conroy *et al.*, 2011). Although promising, the Conroy *et al.* (2011) trials excluded patients aged over 76 so little data is available for elderly patients who make up the highest proportion of PC patients. A study was performed simply monitoring the geriatric response to FOLFIRINOX, and patients recruited were all in adequate health prior to treatment. The FOLFIRINOX therapy resulted in an overall survival of 12.51 months, which is easily comparable to the previous trials, coupled with toxicities including neutropenia and diarrhoea, which are common side effects of current chemotherapeutic regimes (Guion-Dusserre *et al.*, 2016). However, the Guion-Dusserre *et al*. (2016) study had a small sample size, only recruiting 52 patients, with only 18 having PC, with the remaining patients having colorectal cancer. As a result, further studies may be required to approve the use of FOLFIRINOX in an elderly patient cohort.

Chemotherapeutic agents can also be used in conjunction with surgical intervention to improve the chances of surgery being successful. Neo-adjuvant chemotherapy is used before surgery in the hopes of turning borderline resectable disease to resectable disease, providing a more suitable candidate for surgical resection (Mirkin *et al.*, 2016). Use of neo-adjuvant chemotherapy has increased by almost 4-fold

between 2003 and 2011 (Mirkin *et al.*, 2016). A recent study considered the efficacy of a modified FOLFIRINOX without 5-FU (FIRINOX) as a neo-adjuvant therapy. Although a small study, consisting of only 10 patients, two treatment regimens were compared (4 cycles or 8 cycles of FIRINOX). Upon comparison of histopathologic results, R0 resection rate per case was 75 and 67% with 4 cycles and 8 cycles, respectively (Okada *et al.*, 2016). Although a small sample size, Okada *et al.* (2016) demonstrated in a pilot study the efficacy of FIRINOX as a neo-adjuvant therapy, improving surgical resection rates in just 4 cycles of treatment, with toxicities reflecting commonly experienced chemotherapy side effects including leukopenia, anaemia, neutropenia, nausea and loss of appetite.

Adjuvant chemotherapy has also been shown to be advantageous to PC patients. Unlike neo-adjuvant therapy, this is administered post-surgery. A comparison of neo-adjuvant and adjuvant chemotherapy with surgery resulted in overall survivals of 20.7 months and 13.7 months respectively (Shubert *et al.*, 2016). The Shubert *et al.* (2016) study had a large sample size, following 593 patients and their treatment outcomes, and concluded that neo-adjuvant therapy resulted in an increased overall survival in comparison to adjuvant therapy. There are many reasons for neo-adjuvant therapy increasing the survival rate of PC patients in comparison to adjuvant therapy. One of the main reasons is that pancreatic surgery is no light undertaking and approximately 25% of patients who undergo surgical resection cannot undergo their expected course of adjuvant therapy due to surgical complications, comorbidities and disease recurrence (Bittoni *et al.*, 2014). Thus, fewer patients tolerate the full treatment regime and so cannot benefit from the treatment in contrast to neo-adjuvant chemotherapy patients.

Palliative treatment is an important aspect of PC management. This occurs when the disease is too advanced for surgical resection and patients are simply too weak to be able to tolerate chemotherapy or radiotherapy treatment regimes. Not only is the malignancy the most painful from a clinical standpoint, it is a complex pain consisting of visceral, somatic and neuropathic pains (Lahoud *et al.*, 2016). The traditional treatment of pain resulting from PC was opiate painkillers such as morphine. However, there are alternatives to opioid drugs. A coeliac plexus block is a temporary form of pain relief whereby an injection of anaesthetics or steroids into the celiac plexus provides long term relief of pain for a couple of months however,

celiac plexus neurolysis is a more permanent solution achieved through injection of ethanol or phenol into the coeliac plexus of nerves around the pancreas (McAninch *et al.*, 2016). In a meta-analysis comparing 27 independent studies, the coeliac plexus block was shown to temporarily reduce pain and reliance upon opioid drugs regardless of which technique was used to perform the block (Mercadante *et al.*, 2015). When coeliac plexus neurolysis is performed, its effects have been shown to last at least 6 months or up until patient death showing only minor adverse effects including diarrhoea and mild hypotension, both of which were milder than opioid side effects (Yang *et al.*, 2012).

Given the poor prognosis and current treatment options which although have advanced have not improved disease survival rates, the search for new PC treatments is ongoing. Opioid growth factors have recently shown a therapeutic potential through their interactions with the G1/S interface of the cell cycle and so have been developed into biotherapies that could be used alongside traditional chemotherapies (Zagon and McLaughlin, 2014). In their study, Zagon and McLaughlin (2014) showed that combination of opioid growth factors with chemotherapeutic drugs gemcitabine and 5-FU resulted in greater inhibition of DNA synthesis than the chemotherapeutic drugs alone. Pancreatic stellate cells are also being investigated for their potential in therapy due to the complex roles they play in the maintenance of tumour growth and proliferation (Phillips, 2012).

1.2.6 Survival

Survival in patients with PC is bleak, with PC having the worst prognosis of all gastrointestinal malignancies (Bittoni *et al.*, 2014). Despite its low incidence, it is the fourth leading cause of cancer related mortality globally and fifth within the UK (Cancer Research UK, 2014b; Aroldi *et al.*, 2016). It is predicted to become the second leading cause of cancer related mortality by 2030 (Teague *et al.*, 2015). This bleak prognosis results from most cases being too advanced for surgical resection at time of diagnosis (de Mestier *et al.*, 2016).

Median overall survival for PC patients is between 20 and 24 months (Bittoni *et al.*, 2014). One, five and ten-year survival rates for PC in the UK are 25.4, 7.3 and 5%, respectively (Cancer Research UK, 2020b). Female patients are shown to have a slight increase in survival over male PC patients, 1- and 5-year post diagnosis, but

this is not seen in the 10-year survival rates. Patient age has an influence on survival. Younger patients showed a higher 5-year survival rate in comparison to older patients. In male and female patients, 5-year survival decreases from 17% to 2% and 26% to 2% from age 15-49 to age 80-99 (Cancer Research UK, 2020b). Stage of disease also impacts upon patient survival, given more advanced disease limits treatment options available. A multinational study analysed data from 125,183 PC patients, advanced stage disease (III and IV) resulted in shorter survival in comparison to earlier disease (I and II) and surgical resection increased survival both short and long-term (Huang *et al.*, 2018).

1.3 Cancer and DNA

Cancer is a disease which starts in the DNA. DNA can be altered through mutation and DNA damage, both of which can play important roles in carcinogenesis.

1.3.1 DNA Damage

DNA is continually exposed to both endogenous and exogenous factors which can induce damage, which can in turn cause mutations as described above and hence alter health and cause disease (Chatterjee and Walker, 2017). DNA damage is defined as an alteration in the structure of DNA which could potentially cause cellular damage (Kaufmann and Paules, 1996). Depending on cell type in which the DNA damage is induced, it can have various effects. DNA damage can result in functional decline, aging and carcinogenesis (Hoeijmakers, 2009).

1.3.1.1 Quantification of DNA Damage

DNA damage quantification is a commonly used genotoxicity assessment tool. Numerous techniques can quantify the DNA damage induced in various cells and tissues following exposure to test chemicals. One of the main techniques used for this is the comet assay. First established in 1984, this assessed the migration of DNA in single cell gel electrophoresis under neutral conditions (Ostling and Johanson, 1984). Developments came in 1988, comparing DNA migration in human lymphocytes exposed to x-ray radiation and hydrogen peroxide under alkaline conditions rather than neutral conditions (Singh *et al.*, 1988). In the assay, cellular and nuclear membranes are removed through use of detergents and histones are removed from DNA using a high concentration of NaCl. DNA remains coiled within

a nucleoid region. Damaged DNA with single or double stranded breaks will not remain supercoiled without histone support. When an electrical charge is applied, the damaged, uncoiled DNA migrates towards the anode (Collins, 2015). DNA migration following exposure to genotoxic compounds is measured, quantified, and compared. During gel electrophoresis, the DNA fragments induced by DNA damage migrate from the nucleoid body to the anode to form a comet tail. The tail moment describes the DNA migration looking at both the tail length and the percentage of DNA found within the tail (Møller, 2018). The comet assay has been used in a range of cell types, assessing DNA damage in response to varying stimuli. Limitations of the assay are apparent, with wide inter-laboratory variations (Sirota *et al.*, 2014). Sources of said variations include the temperature of the alkaline solution used and the temperature the electrophoresis steps are carried out in (Sirota *et al.*, 2014).

1.3.2 DNA Mutation

Mutation results in the altering of the sequence of nucleotides in the DNA sequence, whether in the coding or non-coding regions of DNA (Mahdieh and Rabbani, 2013). DNA mutation is a heritable change in the DNA, including point mutations (base changes) and chromosome level alterations (copy number changes, translocations etc). There are three main types of point mutation, which can occur in the DNA: base substitutions, deletions, and insertions.

Substitution mutations are when nucleotides are replaced with alternative nucleotides, altering the DNA sequence. The effect that a base substitution mutation has depends on the location of the mutation within the DNA and whether it influences the protein encoded in the gene. Mutation in non-coding DNA regions may have an influence on the transcription of the downstream gene rather than altering the gene product itself. For example, the substitution mutation may increase the strength of the promoter region, resulting in greater gene expression (Mahdieh and Rabbani, 2013). Mutations in coding regions of DNA can be categorised as nonsense, missense and silent. During translation, silent mutations result in a codon which recruits the same amino acid as the wildtype sequence, missense mutations result in a codon which recruits a different amino acid, altering the resulting polypeptide's amino acid sequence and finally nonsense mutations encode a stop codon, halting translation and forming a truncated polypeptide product (Scitable,

2014). Some missense mutations are phenotypically silent, with amino acid changes not altering protein function. One of the most common mutational signatures found in majority of cancer types is a base substitution found at a CG:CG dinucleotide (Bacolla *et al.*, 2014). Commonly, a C→T mutation is induced by deamination of 5-methylcytosine to form a TG:CG. The resulting mismatched T:G causes a G→A transition during the following round of DNA replication to form TG:CA sequence (Bacolla *et al.*, 2014). Specific base substitution mutations have been linked with different cancer developments. For example, lung cancer has been linked with different base substitutions dependent on chemical cause of disease. Arsenic exposure linked lung cancer has a mutational signature of T:A→G:C in contrast to the tobacco linked lung cancer with its signature of C:G→A:T (Bacolla *et al.*, 2014).

Both deletion and insertion mutations result from a slipped template strand of DNA during DNA replication, either nucleotides can be omitted resulting in a deletion or an extra nucleotides can be added by DNA polymerase (Scitable, 2014). The resulting daughter strands of DNA with altered DNA sequence can result in a frameshift mutation. Insertion of bases of multiples of 3 (3, 6, 9 bases) will not alter frame given codons read in blocks of 3 bases. mRNA transcribed from the altered DNA acts as the template for the ribosomes for translation, but the reading frame is shifted resulting in a different amino acid sequence in the polypeptide chain (Scitable, 2014). Examples of insertion and deletion mutations are present within human cancers. Insertion within the *EGFR* exon 20 is a rare mutation. Within non-small cell lung cancer has been linked with lack of sensitivity to first line treatment, which has been suggested to be as a result of conformational changes within mutant EGFR (Byeon *et al.*, 2019). A 12 base insertion mutation has been identified in lung cancer within driver gene *HER2,* which has been identified using Sanger sequencing, next generation sequencing and a modified PCR method (Takase *et al.*, 2017). Sequencing of exons 4-10 of *p53* in samples from ovarian cancer tumours identified microdeletions within 4% of samples (Kohler *et al.*, 1993).

1.3.2.1 DNA Mutation Identification and Quantification

Various techniques have been established and utilised to identify mutations within DNA of various organisms.

1.3.2.2 PCR

The polymerase chain reaction (PCR), first carried out experimentally in 1986, is a highly useful tool used to amplify and replicate segments of DNA (Mullis *et al.*, 1986; Lorenz, 2012). It can amplify a small starting fragment of DNA 10^6-10^7 fold before additional techniques like endonuclease digestion and gel electrophoresis can be employed (Boehm, 1989). Overall, PCR has three main steps of denaturation of the DNA double strands, annealing of primers to the template DNA and elongation of the PCR product with DNA polymerase and nucleotides, repeated in a cycle up to 40 times (Lorenz, 2012). PCR as a technique can occasionally result in poor results which are a mixture of sizes seen on agarose gels, mutations can be introduced during the experiment and sometimes no product can form at all (Lorenz, 2012). Careful preparation can make the technique a powerful tool. Modifications of the technique include real-time PCR, allow the technique to be used quantitatively. PCR based techniques have been used to assess the mutation status of KRAS genes, proto-oncogenes involved in carcinogenesis of the lung, colon, rectum and pancreas (Van Mansfeld and Bos, 1992). Notably, in pancreatic cancer and lung cancer, point mutations in RAS were exclusively found within codon 12, corresponding to the active site of the GTPase (Van Mansfeld and Bos, 1992; Imamura *et al.*, 2012). Restriction fragment length polymorphism analysis on PCR products is one of the most useful PCR-based mutation detection techniques since it is quick, simple, reliable and does not rely on radiolabelled chemicals (Van Mansfeld and Bos, 1992). Allele-specific PCR has also been shown to be a valuable tool for identifying mutation and single nucleotide polymorphisms. Using highly specific primers for known alleles, any primer, which mismatches at the 3' end will stall DNA synthesis (Ugozzoli and Wallace, 1991). This has shown usefulness within a clinical setting, being used for a range of genetic and infectious disease diagnosis (Ugozzoli and Wallace, 1991).

1.3.2.3 DNA Sequencing

Despite this, PCR alone could not describe the whole DNA sequence. DNA sequencing elucidates the specific nucleotide sequence, which can then be compared against wild type DNA sequences to identify mutations. DNA sequencing was first introduced by Fred Sanger in 1977 in a ground-breaking technique, utilising dideoxy

terminator nucleotides (Sanger *et al.*, 1977; McGinn and Gut, 2013). This remained the best sequencing technique for decades. Sanger sequencing was used for the first sequencing of our own genome in the Human Genome Project, which began in 1990, at a huge time investment of multiple groups across the globe working collaboratively and a massive financial investment of approximately $3 billion (Lander *et al.*, 2001; McGinn and Gut, 2013). As novel sequencing techniques have built upon Sanger's foundations, sequencing is now a much quicker and cheaper process. Next generation sequencing techniques allow for parallel sequencing of small DNA fragments, compiled using bioinformatics and human reference genomes (Behjati and Tarpey, 2013). Next generation sequencing has huge potential to assist in a clinical setting, identifying more mutational signatures than Sanger sequencing, including larger insertions and deletions. However, drawbacks of next generation sequencing include GC rich regions, which tend to form secondary structures within the DNA, commonly found in Fragile X syndrome and Huntington's Disease and the financial sacrifice which would be required to assemble this within a clinical setting, with staff, equipment and required infrastructure. A high sample volume would be required to justify its clinical use due to the high costs (Behjati and Tarpey, 2013).

1.3.2.4 HPRT Mutation assay

The hypoxanthine phosphoribosyl transferase (*HPRT*) gene mutation assay, first established in 1981, determines the mutation status of reporter gene *HPRT* through studying resistance to toxic nucleotide analogue 6-thioguanine (Furth *et al.*, 1981; Johnson, 2012). It was first used in human lymphoblastic cell lines and more recently, it has been used in human AHH-1, MCL-5 and most commonly the Chinese hamster ovary, cell line (Furth *et al.*, 1981; Johnson, 2012). Studies have also applied the assay to primary lymphocytes isolated from humans and rodents (Lambert *et al.*, 1995; Casciano *et al.*, 1999). *HPRT* is the endogenous X-linked gene commonly mutated in the rare neurological disorder Lesch-Nyhan syndrome (Nguyen and Nyhan, 2016). The assay is split into two stages: mutant cleansing and mutant selection. Cleansing is achieved using a hypoxanthine-aminopterin-thymidine (HAT) addition into culture medium which in turn blocks endogenous and salvage pathways for nucleotide synthesis, which is fatal for HPRT$^-$ cells (and TK$^{-/-}$ HPRT$^+$). Only HPRT$^+$ cells survive the cleansing stage. Surviving cells are exposed to the test genotoxin before the second stage of mutant selection. HPRT mutants are

selected through exposure to 6-thioguanine, a toxic analogue of deoxyguanosine triphosphate. HPRT$^-$ cells survive the mutant selection phase given they are unable to incorporate toxic 6-thioguanine into their DNA through the salvage pathway (Johnson, 2012). The assay measures induction of mutation in the HPRT locus following exposure to test genotoxins, with ethyl-methanesulphonate commonly used as a positive control (Johnson, 2012). The assay has been used in a variety of *in vivo* studies, both within healthy populations and exposed workers, including those who survived nuclear events including Chernobyl and atomic bombs (Richard J. Albertini, 2001). Benefits of the assay include the simplicity of an X-linked gene, requiring one mutational hit for an altered phenotype. It is heavily relied upon technique within the genotoxicology community. However, drawbacks include both the time and labour-intensive nature of the assay (Bryce *et al.*, 2008).

1.3.2.5 PIG-A Mutation Assay

1.3.2.5.1 PIG-A Assay Background

The phosphatidylinositol glycan biosynthesis class A gene (*PIG-A* in humans and *Pig-A* in rodents), found on p22.1 of the X chromosome, is also used as a reporter gene, similar to *HPRT* (Kawagoe *et al.*, 1994). *PIG-A* encodes the catalytic domain of N-acetylglucosamine transferase, used in the very first step of glycophosphatidylinositol (GPI) anchor biosynthesis (Gollapudi *et al.*, 2015). GPI anchor biosynthesis is a complex mechanism, involving 21 genes but *PIG-A* remains the only X-linked gene, so a single mutation in this gene can inactivate the enzyme and prevent GPI anchor biosynthesis (**Fig. 1.6**) (Krüger *et al*., 2015; Gollapudi *et al.*, 2015; Horibata *et al.*, 2016).
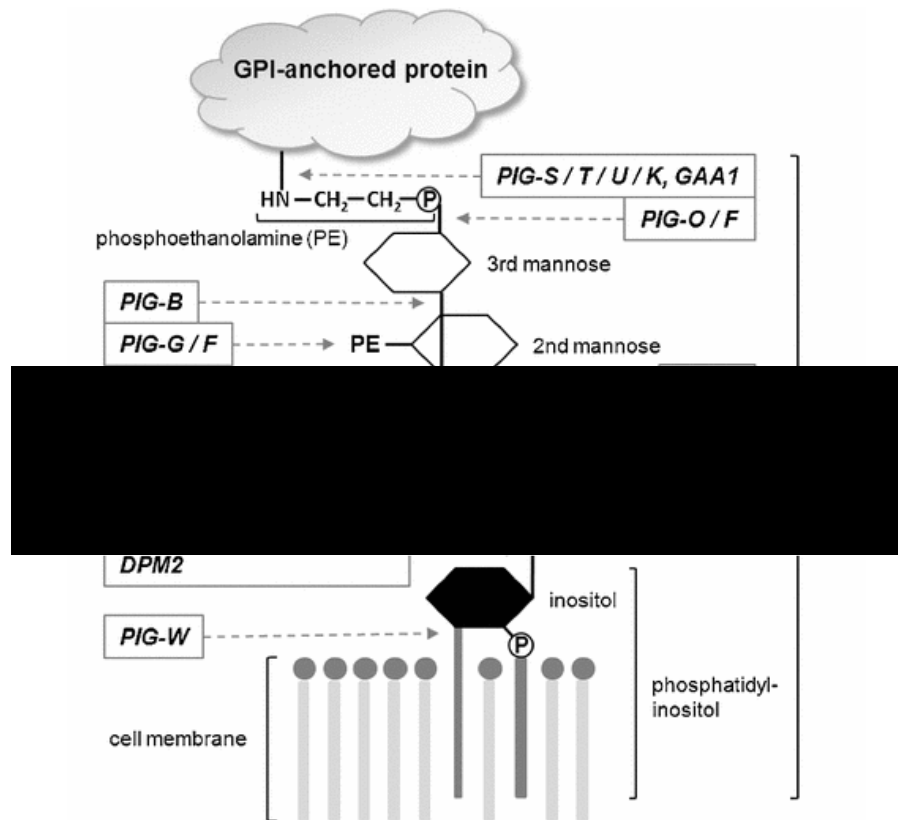
Figure 1.6: *GPI anchor structure and associated genes involved in the biosynthesis of the GPI anchor. PIG-A, highlighted, is the only X-linked gene involved* (Krüger *et al.*, 2016).

Much of the development of the assay was based on the knowledge of the rare, acquired, genetic disease paroxysmal nocturnal haemoglobinuria (PNH). An estimated incidence of the disease is around 0.1 - 0.2 person per year per 100,000 people but a definite figure is unknown (Devalet *et al.*, 2015). The most common form of PNH is due to a *de novo* somatic mutation in *PIG-A*. If this mutation arises in a pluripotent haematopoietic stem cell, all resulting blood cells (erythrocytes, leukocytes and thrombocytes) will also have this mutation in *PIG-A* (Devalet *et al.*, 2015). The result of this is significant numbers of blood cells lacking GPI anchored complement inhibitors CD-55 and CD-59 (Sahin *et al.*, 2015). CD-55, more commonly known as the decay accelerating factor, inhibits the activation of the complement system through interaction with C3 and C5 convertases (Liu *et al.*, 2005). CD-59 also inhibits the complement system through blocking the formation of the membrane attack complex through binding of C8 and C9 (Cai *et al.*, 2014). Lacking these complement inhibitors, blood cells are vulnerable to complement

attack. Notably erythrocytes are prone to intravascular haemolysis, which can lead to fatal thromboses (Chrobák, 2000). This potentially life-threatening disease can be treated through bone marrow transplantation, preventing complete bone marrow failure; and monoclonal antibody eculizumab, which prevents formation of the membrane attack complex through binding C5 (Brodsky, 2009).

Diagnosis of PNH involves flow cytometry for identifying a large population of GPI deficient cells, lacking both CD-55 and CD-59. This approach was adopted in the PIG-A mutation assay to screen for the much rarer induced PIG-A mutations through the phenotypic assessment of GPI deficient mutant blood cells using fluorescent antibodies for GPI anchored cell surface proteins (**Fig 1.7**). GPI anchor deficient cells are non-fluorescent and defined as PIG-A mutant.
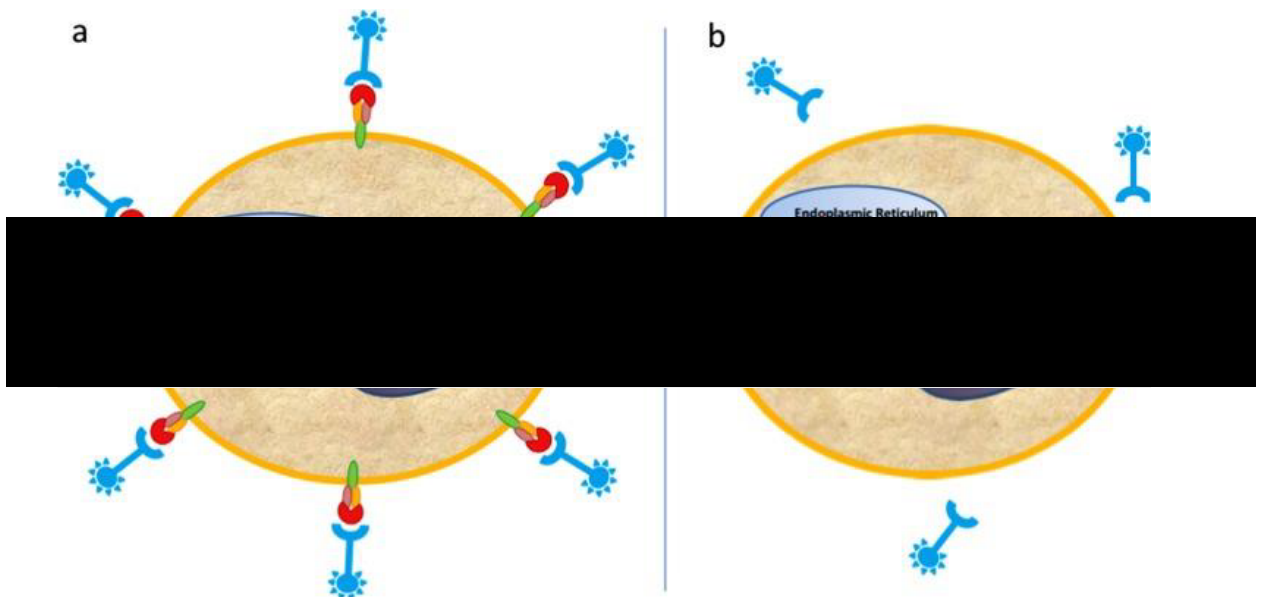
Figure 1.7: A) PIG-A wildtype cell with GPI anchor present on cell surface, GPI anchors identified with fluorescently labelled antibodies for GPI anchored proteins. B) PIG-A mutant cell deficient in GPI anchors. PIG-A mutant is non-fluorescent in flow cytometric analysis (Haboubi *et al.*, 2019).

The assay, as well as being based on knowledge of PNH and diagnostic techniques involved, aimed to mirror and build upon pre-existing genotoxicity assays. The HPRT and GPA genotoxicity assays have been previously established. The HPRT assay has been previously discussed in Chapter **1.3.2.4**. The GPA assay is an *in vivo* approach to study two erythrocyte populations using allele-specific monoclonal antibodies via flow cytometry (Grant, 2005). Loss of a single allele followed by

replication of the other allele has been linked to loss of heterozygosity in a tumour suppressor gene. As a result, this assay has been used to study genotoxicity and used more diagnostically in patients with known predispositions for developing cancer (Myers and Grant, 2014). The assay is complicated through the autosomal nature of the GPA gene, requiring test subjects to co-express the M and N allele, limiting the assay to approximately 50% of the population (Bryce *et al.*, 2008). The PIG-A assay combines the accessibility of a flow cytometry-based assay, with the simplicity of an endogenously expressed X-linked gene.

The assay has been optimised in a range of cell types both *in vitro* and *in vivo*.

1.3.2.5.2 The Human Erythrocyte PIG-A Assay

The erythrocyte PIG-A assay was first established in 2008 and since then has been extensively used in rodent genotoxicity studies, assessing induction of mutation following exposure to test chemicals (Bryce *et al.*, 2008). Following its success, the assay has been applied to a range of human blood cell populations (Rondelli *et al*., 2013). In the human erythrocyte PIG-A assay, wild type and mutant erythrocytes are distinguished using fluorescent antibodies. Various commercial kits are available for assessing the mutation status of *PIG-A* depending on the organism and cell type to be analysed. Our laboratory uses a trio of antibodies against the GPI anchored complement inhibitor proteins CD-55 and CD-59, and against the erythroid marker CD235a (Karsten *et al.*, 2010). This allows sufficient separation of the erythrocytes into wild type and PIG-A mutant populations.

Although the use of the assay in humans is in its infancy stage, it has been applied in several population studies and is showing promise. For example, a Chinese group performed a population study on a cohort of 217 healthy volunteers with ages ranging between 18 and 92 years (Cao *et al.*, 2016). The average frequency of PIG-A mutant erythrocytes in these patients was $5.25 \pm 3.6 \times 10^{-6}$. Age was shown not to be an influencing factor on the PIG-A mutant frequency by Poisson univariate analysis. Cao *et al.* (2016) also investigated whether smoking had an impact on the PIG-A mutation frequency. Smoking alone was insignificant, but cigarette pack years, defined as 20 cigarettes per day per year, was slightly associated with an increase PIG-A mutant frequency. Interestingly, in this study, there was found to be significant differences with mutant frequency in male and female donors with

mutation frequencies of 5.97 ± 4.0 and 4.19 ± 2.5 x 10^{-6} PIG-A mutant erythrocytes, respectively (Cao *et al.*, 2016). A smaller healthy population study on 52 donors found no significant difference between sex and mutant frequency, but did suggest a correlation between the age of the donor and the PIG-A mutant frequency in contrast to the Cao *et al.* (2016) study (Dertinger *et al.*, 2015). An earlier, Italian study monitored the PIG-A mutant frequency in an alternative blood cell population. Rondelli *et al* (2013) studied the peripheral granulocyte cells in a healthy population study of 110 donors, showing a mean mutant frequency of 4.9 x 10^{-6} PIG-A mutant granulocytes. This showed the assay can be applied to various blood cell populations with ease.

Not only has the assay been used in human population studies, but it has also been used in a small number of oncology studies. For example, a study of 27 cancer patients undergoing therapeutic intervention of chemotherapy, radiotherapy or a combination of the two (Horibata *et al.*, 2016). Of these, three patients (of the 27) had PC treated with gemcitabine and had blood drawn and analysed six days post-gemcitabine treatment. The conclusion of this study was that cancer patients have unremarkable levels of PIG-A mutant erythrocytes in comparison to healthy donors. However, problems with the study design lead to questioning this conclusion. A wide and inconsistent range of cancers were analysed within this study, all with differing treatment regimens, so data collected was not comparable. No baseline measures of the PIG-A mutant frequency were collected from newly diagnosed, treatment-naïve patients before their treatments were initiated. Therefore, it could not have been determined whether the mutations change over time following treatment initiation. The slow turnover of erythrocytes was not considered when choosing the timings of blood collection post-therapy. They may not have been timed best to view the phenotypic change in PIG-A mutant erythrocytes. Within our own research group, the human erythrocyte PIG-A assay has been explored in patients with oesophageal adenocarcinoma and pre-malignant Barret's metaplasia. In this study, 337 participants consisting of 137 healthy controls and 200 patients (77 gastro-oesophageal reflux, 62 non-dysplastic Barrett's metaplasia, 11 dysplastic Barrett's metaplasia, 42 oesophageal adenocarcinoma) were examined using the PIG-A assay (Haboubi *et al.*, 2019). Within this, healthy donors had a median frequency of 2.8 x 10^{-6} PIG-A mutant erythrocytes, high grade dysplastic patients had a median of 7.89

x 10^{-6} PIG-A mutant erythrocytes, and oesophageal adenocarcinoma patients had further elevated mutation frequency around 9.75 x 10^{-6} PIG-A mutant erythrocytes.

1.3.2.5.3 *In Vitro* Pig-A Assay

Given the success and ease of the erythrocyte PIG-A assay, an *in vitro* PIG-A assay has also been established. A range of cell lines commonly used within genotoxicity studies have been explored in the *in vitro* PIG-A assay, including human lymphoblastoid cell lines TK6 (Krüger *et al.*, 2015; Krüger *et al.*, 2016; Rees *et al.*, 2017; Piberger *et al.*, 2018) and metabolically capable MCL5 (Rees *et al.*, 2017) and the mouse lymphoblastoid L5178Y cell line (David *et al.*, 2018). The L5178Y cell line will be utilised within this study due to low background Pig-A mutant frequency (0.08 ± 0.06%) (David *et al*., 2018).

1.3.2.6 Micronucleus Assay

The micronucleus (MN) assay is one of the most widely used genotoxicity assays, further discussed in **chapter 3.1.6**. This well-established technique has been in use for almost 40 years (Sommer *et al.*, 2020). The MN assay monitors genomic stability through studying the induction of micronuclei, small, membrane bound vesicles containing DNA outside of the primary nucleus following exposure to genotoxins (Kisurina-Evgenieva *et al.*, 2016; Sommer *et al.*, 2020). Induction of micronuclei is achieved by a range of physical factors including radiation, as well as chemical factors which affect DNA structure (Kisurina-Evgenieva *et al.*, 2016).

MN are produced during the process of mitosis. During anaphase, chromatids migrate towards the poles of the cell as kinetochore microtubules shorten. At this stage, the MN begin to form. They can be formed through two separate processes. Disturbances with the mitotic spindle result in a whole chromosome not migrating to the cellular pole. Alternatively, unrepaired strand breaks and unrepaired DNA lesions result in an acentric chromosomal fragment not migrating far enough to be incorporated into the primary nucleus (Iarmarcovai *et al.*, 2008). Micronuclei then form, containing either the chromosomal fragment or an entire chromosome. MN containing acentric fragments are classified clastogenic events and MN containing full chromosomes are defined as aneugenic events since they result in aneuploidy (Iarmarcovai *et al.*, 2008). Different chemicals will induce either aneugenic or clastogenic MN depending on their mode of action. Example clastogenic chemicals

include mitomycin C, bleomycin and doxorubicin, and aneugenic chemicals include diethylstilbestrol, griseofulvin and vincristine sulphate (Rosefort *et al.*, 2004). Kinetochore/centromere staining of the MN post-fixation (chromosome paints or antibodies to the kinetochore) can be harnessed to determine whether chemicals act as a clastogen or an aneugen since clastogenic MN will lack kinetochores/centromeres.

The most common form of the MN assay is the cytokinesis block MN (CBMN) assay, **Fig 1.8**. Cytochalasin-B is used to block cytokinesis, discussed in **3.1.6**.
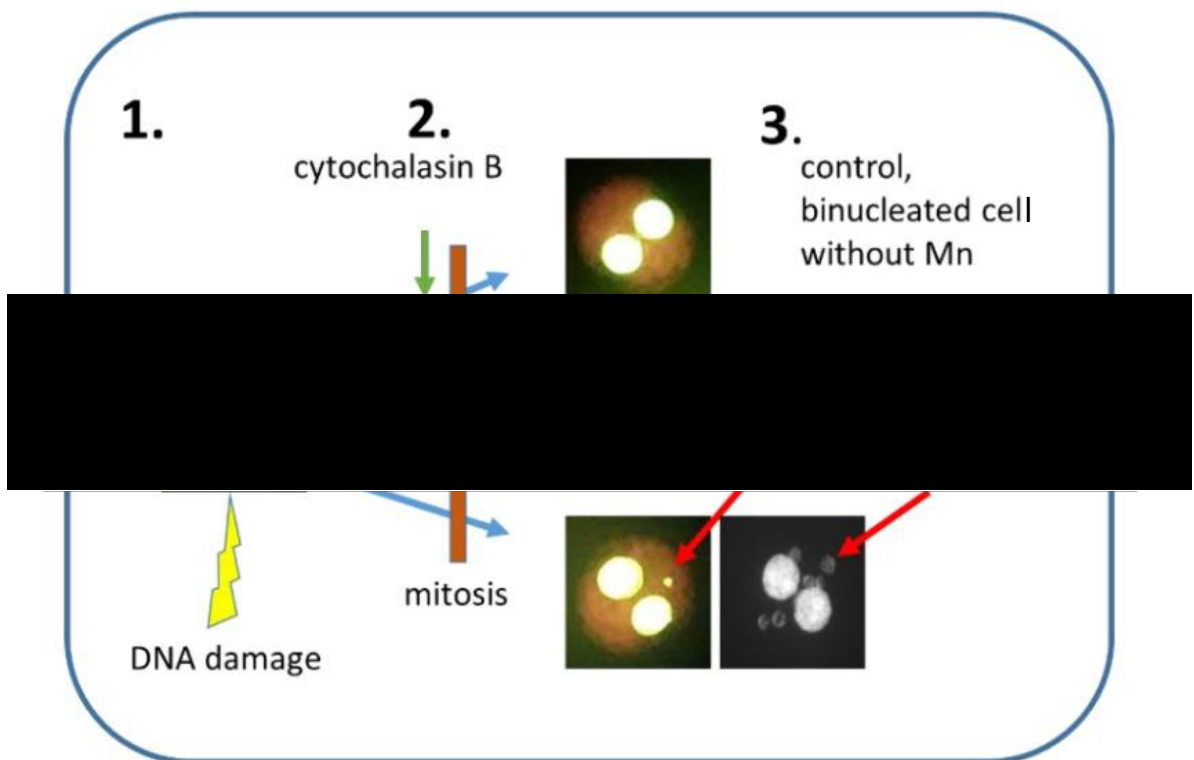
*Figure 1.8: CBMN assay **1**) Cell exposed to chemical, DNA damage is induced **2**) Cells divide in the presence of cytochalasin-B, blocking cells in the binucleate phase as cytokinesis is inhibited **3**) example binucleate cells in control, without micronuclei, and in treated cells with micronuclei, signified by red arrows* (Sommer *et al.*, 2020).

Scoring numbers of MN allows for comparison of the induction of MN by different agents or environments. Scoring criteria are shown in **Table 1.1**. These criteria were used for all scoring throughout this thesis, including in chapters **3** and **5**.

Table 1.1: Scoring criteria for MN assay according to (Fenech, 2007).

Scoring Criteria for Micronuclei
Morphologically identical to primary nuclei, but smaller
Diameter of MN between 1/16th and 1/3rd of primary nucleus
Can be readily distinguishable from staining artifacts
Not linked or connected to primary nucleus
May touch primary nuclei, but not overlap. Nuclear boundaries should be distinguishable
Staining intensity should be similar to primary nuclei, although can be brighter on occasion

There are many benefits of using the MN assay. The assay has been well established and used for decades. It is a flexible technique, established in a wide variety of cell lines, adherent or suspension, as well as primary cell cultures. This allows the assessment of DNA damage induction in a wide variety of cell and tissue types.

The *in vitro CBMN* assay has been applied to numerous cell populations isolated from human living donors. For example, blood cells have been assessed by the MN assay to monitor overall DNA damage of individuals. Within a lymphocyte population, the CBMN assay has been used a biomonitoring tool (Sommer *et al.*, 2020). The lymphocyte CBMN assay has been previously used for genotoxin exposure assessment, studying micronutrient deficiencies and excesses, assessment of genome stability, but has also been used for a measure of overall risk of development of cancer or other age-related degenerative diseases including Alzheimer's disease, Parkinson's disease and cardiovascular disease (Migliore *et al.*, 2011; Andreassi *et al.*, 2011; Bolognesi and Fenech, 2013). The human peripheral lymphocyte CBMN assay is further explored in **chapter 5.1.1**. The human buccal micronucleus cytome assay assesses the induction of DNA damage in human buccal cells, obtained through non-invasive cheek swabs (Bolognesi and Fenech, 2013). The buccal MN assay has been used to monitor induction of DNA damage induced by lifestyle and dietary factors, and also showed a correlation with cancer risk, aging

and specific neurodegenerative diseases (Sommer *et al.*, 2020). The MN assay can also be carried out in the reticulocytes lacking nuclei and less commonly in cells collected from nasal passages and derived from urine samples (Sommer *et al.*, 2020).

The *in vitro* CBMN assay is one of the best established, widely used assays for the study of induced DNA damage. It's been utilised within the field of toxicology, as well as nutritional and pharmaceutical industries (Ladeira *et al.*, 2015). It has been established in a number of cell lines including rodent cell lines CHO, V79 and L5178Y as well as human cell lines including TK6 and MCL5 (OECD, 2016).

Cytochalasin-B use results in more trustworthy results in the CBMN assay (Heddle *et al.*, 2011). Use of cytochalasin-B arrests cytokinesis and the resulting scoring of binucleated cells ensures all cells scored have undergone division following exposure to the test chemical (Heddle *et al.*, 2011). However, drawbacks of the technique include that cytochalasin-B cannot be used with all cell lines. This is seen with commonly used cell line L5178Y. Cytochalasin-B has shown poor results when used with L5178Y cell line with reports of sensitivity. Notably, cytochalasin-B has been potentially linked to poorly preserved L5178Y cells for micronucleus scoring (Oliver *et al.*, 2006; OECD, 2016).

1.4 Aims

PC diagnosis is late, resulting in late clinical intervention and poor disease prognosis. Early detection systems are urgently needed for PC. The human erythrocyte PIG-A assay has been previously explored in a patient cohort with oesophageal adenocarcinoma (Haboubi *et al.*, 2019) and various healthy population studies and shown to suggest elevated numbers of mutated cells in cancer patients. In this study, we aimed to determine whether the non-invasive, blood-based, human erythrocyte PIG-A assay has any potential in diagnosing PC. This was achieved through recruiting patients with benign and malignant disease to the study who donated whole blood and a lifestyle questionnaire. The PIG-A mutation assay was carried out on whole blood and the remainder of the blood was separated and buffy layer was harvested for the peripheral blood mononuclear blood cell (PMBC) CBMN assay to establish a second measure of DNA damage in an alternative blood cell population. Mutation induced in erythrocytes monitored by the PIG-A assay was compared with DNA damage explored in the PMBC CBMN assay, with additional

clinical parameters also assessed. I established here whether PIG-A mutation frequency was associated with pancreatic disease and if so, was it linked with disease staging. Additionally, we looked at combining biomarkers to see whether this is more predictive for PC. Alongside *in vivo* work, an *in vitro* model was utilised to study known pancreatic carcinogens which are known to increase the risk of developing PC. We aimed to monitor the DNA damage and mutation induced through high fat diets, tobacco smoking and alcohol abuse through use of the MN assay and an *in vitro* Pig-A assay. The *in vitro* model explored whether poor lifestyle choices may account for some of the mutations viewed in the human erythrocyte PIG-A assay results from patients.

Chapter 2: Materials and Methods

<u>**Chapter 2: Materials and Methods**</u>

2.1 General Materials and Methods

Specific methods are described in more detail in individual results chapters. Here, we describe general materials and methodologies used.

2.1.1 Equipment

Table 2.1: Equipment used within study and suppliers of the equipment

Equipment	Supplier
Centrifuge tubes: 15mL and 50mL	Fisherbrand
Centrifuge 5810R	Eppendorf
CO_2 air-jacketed incubators	Nuaire™
Cytospin™ 4	Thermo Scientific™
Flow cytometers:	
BD FACS Aria I™ Flow Cytometer	Becton Dickson Biosciences
Navios™ Flow Cytometer	Beckman Coulter Life Sciences
Freezer (-20°C)	Proline
Freezer (-80°C)	Proline
Fridge (4°C)	Proline
Fume hood	Clean Air Limited
Heat block	Techne Dri-Block
Microcentrifuge tubes	Eppendorf
Microscopes:	
Axio Imager Z1	Zeiss
Light microscope	Olympus BX51

Pipette tips	StarLAB
Pipettes	Gilson, Eppendorf
Pipettors	StarLAB, Fisherbrand
Shandon ™ White Filter Cards	Thermo Scientific™
Shandon™ Cytofunnel	Thermo Scientific™
Tissue Culture Flask: 25, 75, 125 cm^2	Cellstar
Tissue Culture Hood	Scanlaf Mars
SUB Aqua 18 Waterbath	Grant
Z1 Coulter Counter	Beckman Coulter Life Sciences

2.1.2 Reagents

Table 2.2: *Reagents used within the study and suppliers of the reagents*

Reagent	Supplier
Acetaldehyde Solution	Sigma-Aldrich
Acetic Acid	Fisher Scientific
Benzo-[a]-pyrene	Sigma-Aldrich
Bovine Serum Albumin (BSA)	Sigma-Aldrich
Cytochalasin B	Merck
Deoxycholic Acid	Sigma-Aldrich
Dimethyl Sulfoxide (DMSO)	Fisher Scientific
DMEM	Gibco®
DPX Mounting Medium	Fisher Scientific
Ethanol	Fisher Scientific

Foetal Bovine Serum (FBS)	Gibco®
Glutamine	Gibco®
Heat-inactivated Horse Serum	Gibco®
Histopaque®-1077	Sigma-Aldrich
Methanol	Fisher Scientific
Penicillin-Streptomycin	Gibco®
PHA	Sigma-Aldrich
Phosphate Saline Buffer (PBS)	Gibco®
Pluronic F-68	Gibco®
Potassium chloride	Sigma-Aldrich
RPMI 1640	Gibco®
RPMI Dutch Modification	Gibco®
Sodium chloride	Sigma-Aldrich
Sodium Pyruvate	Gibco®
Trypsin	Gibco®
Xylene	Sigma-Aldrich

2.1.3 Stains and Antibodies

Table 2.3: Stains and antibodies used within this study and their corresponding suppliers

Stain/Antibody	Supplier
APC anti-CD-235a	BD Pharmingen
APC anti-CD-45	Biolegend
Giemsa Stain Solution	Gurr® VWR International limited

PE anti-CD-55		BD Pharmingen
PE anti-CD-59		BD Pharmingen
PE anti-CD-90.2		Biolegend
Trypan Blue Solution (0.4%)		Sigma-Aldrich
VECTASHIELD® Antifade Mounting Medium with DAPI		Vector Laboratories
Zombie Violet™ fixable viability kit		Biolegend

2.1.4 Computer Software

Table 2.4: Computer software used in the study with associated version numbers and suppliers.

Software	Version	Supplier
Microsoft Word		Microsoft
Microsoft Excel		Microsoft
SPSS	26	IBM
BD FACS Diva	8.0	Becton Dickson Biosciences
Navios Software		Beckman Coulter Life Sciences
Metafer 4	3.9.8	Metasystems

2.2 *In Vitro* Studies

2.2.1 Cell lines

Cell lines PANC-1 and L5178Y were utilised within **chapter 3** of the thesis.

2.2.1.1 PANC-1

PANC-1 is an adherent pancreatic adenocarcinoma cell line isolated from a 56-year old Caucasian male. Cells, seen in **Fig. 2.1**, were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum, 1% glutamine and 1% penicillin-streptomycin at 37°C, 5% CO_2 humidified atmosphere. Cells were detached through trypsinisation, diluted and sub-cultured every 48 hours.
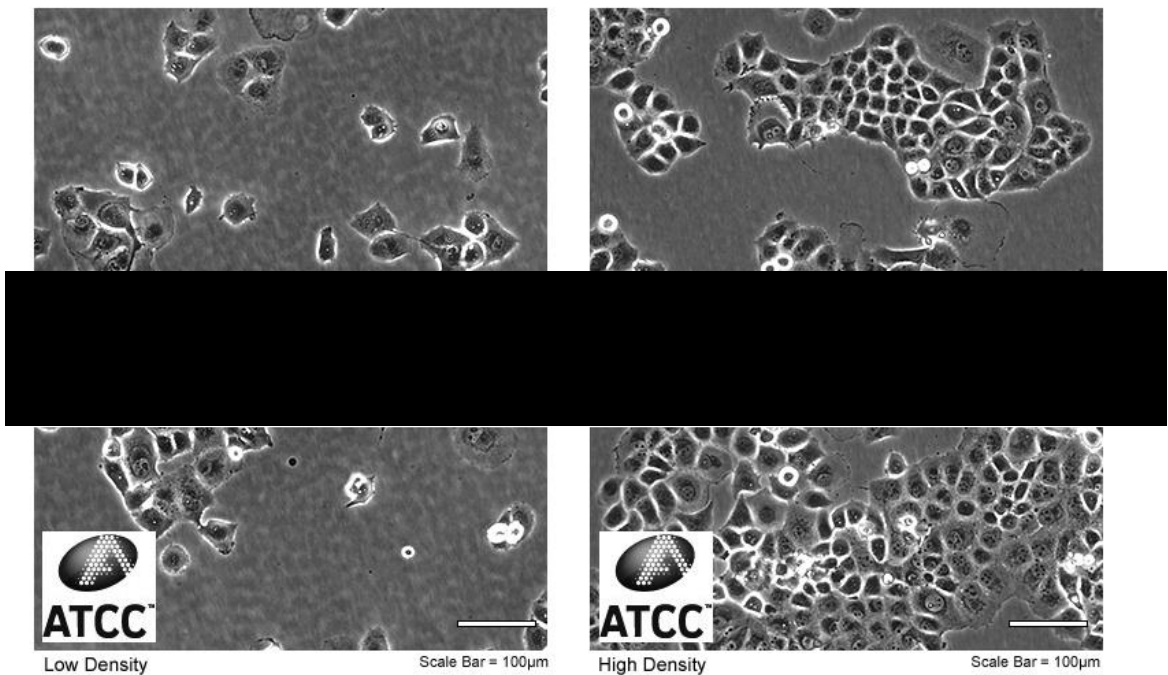
Figure 2.1: *PANC-1 cell line at both low and high-density under light microscope* (ATCC, 2016).

2.2.1.2 L5178Y

L5178Y$^{TK+/-}$ is a suspension lymphoblastoid cell line derived from a chemically induced lymphoma from *Mus musculus* (mouse) (ATCC, 2016b). Cells were maintained in RPMI Dutch modification media supplemented with 10% heat-inactivated horse serum, 1% glutamine, 1% penicillin-streptomycin, 5.5 mL sodium pyruvate and 5.5 mL pluronic F-68 at 37°C, 5 % CO_2 humidified atmosphere in the density of 3 x 10^4 and 5 x 10^5 cells/mL. L5178Y cells required sub-culturing every 24 hours.

2.2.2 Cell Culture

All cell culture was carried out in sterile conditions. This included wearing laboratory coats only worn within cell culture and gloves. All work was carried out within tissue culture hood sterilised with 70 % ethanol before and after use, with all plastic consumables sterilised through autoclaving and every item taken into the tissue culture hood was wiped down with 70 % ethanol before use. Further cell culture details are found within **chapter 3**.

2.2.3 Cell Counting

Cell counting was achieved using the automated Z1 Coulter Counter. Cell suspension was diluted with 100 µL cell suspension in 10 mL of diluent and returned and particles were counted between the sizes of 5 and 13 µm, providing a count of cells per mL. With adherent cell lines, cells were detached through trypsinisation initially so were in a solution.

Cell counting was achieved using the haemocytometer for the trypan blue exclusion assay, further detailed in **3.2.2**.

2.3 *In Vivo* Studies

2.3.1 Study Design and Patient Recruitment

Study documents were prepared, and ethical approval was sought using the integrated research application system (IRAS) (IRAS project ID: 218936). Ethical approval was granted by Plymouth and Cornwall Research Ethics Committee (REC reference: 17/SW/0012) followed by R&D approval from the Swansea Bay University Health Board.

Patients with benign and malignant pancreatic disease were recruited from October 2017 to September 2019 in the outpatient surgical clinics at Morriston Hospital, Swansea. Valid informed consent was taken, and patients completed a modified validated questionnaire and donated 9 mL of whole blood.

2.3.2 Questionnaire

Participants in the study completed a modified Extended Prostate Cancer Index Composite (EPIC) questionnaire to provide further information about dietary and lifestyle choices of the patient, **Appendix 7.1.3**.

Dietary information was used to provide a dietary quality score (DQS), calculated using guidelines in **Table 2.5** (Toft *et al.*, 2007). The DQS was first developed according to recommended guidelines for daily or weekly intake of food groups and allowed for classification of dietary habits into healthy (7-9 score), average (4-6) and poor (3) categories (Toft *et al.*, 2007).

Table 2.5*: Guide for calculating DQS from EPIC questionnaire (Toft et al., 2007).*

Food	Frequency	Score
Vegetables (cooked/raw) and/or vegetarian dishes	≥5-7 servings per week	3
	In between	2
	≤2 servings per week	1
Fruit	≥3 pieces per day	3
	≥3 pieces per week and ≤2 pieces per day	2
	≤2 pieces per week	1
Fish	≥200g per week	3
	In between	2
	No intake	1
Fat – Bread	None	3
	Vegetable margarine	2
	Butter, blended spread, or lard	1
Fat – Cooking	None, olive oil	3

	Vegetable margarine, oil	2
	Margarine, butter, blended spread, or lard	1
Fat – Summarised	6 points total	3
	3-5 point total	2
	2 points total	1

Furthermore, our questionnaire collected general health information and information regarding exercise levels for an overall snapshot of the patient's health, diet, and lifestyle.

2.3.3 Blood Collection

Whole blood was collected by venepuncture into a 9 mL sodium-heparin coated blood tube. All blood collection was carried out within the outpatient's department of Morriston Hospital, Swansea with blood taken by suitably trained staff. Blood was packaged and transported to the university in the locked boot of a car before analysis on receipt at the university.

2.4 Statistics

Statistical analysis on data collected was carried out using SPSS (v.26, IBM) computer software. Normality of data was determined using the Shapiro-Wilk test. Data determined to be normally distributed was analysed using the ANOVA and t-tests where appropriate. Non-normal data was analysed using the Mann Whitney U and Kruskal-Wallis tests where appropriate.

Chapter 3: *In Vitro* Model of Pancreatic Cancer

Chapter 3: *In Vitro* Model of Pancreatic Cancer

3.1 Introduction

3.1.1 Risk Factors for Pancreatic Cancer

Numerous risk factors have been associated with an increased risk of developing PC. As many as 37% of all cases of PC are thought to be preventable through better lifestyle and dietary choices (Cancer Research UK, 2014b). Ongoing health conditions also put patients at risk for developing PC. Diabetes mellitus increases the risk of developing PC but also poorly controlled or new onset diabetes is often a symptom of early PC (Cancer Research UK, 2016c; Lishan Wang *et al.*, 2016). In this situation, chronic inflammation in pancreatic islets, mediated by p38 MAPK, increases proliferation, apoptosis and metastasis (Lishan Wang *et al.*, 2016). Additionally, common treatment with insulin analogues have shown to increase the risk of developing PC in comparison to biguanide metformin (Amin *et al.*, 2016). Chronic inflammation of the pancreas has also been linked with PC development in patients with chronic pancreatitis. This drives NF-kB activation, and increases the development of pre-malignant pancreatic lesions including IPMN, MCN and panINs (Krška *et al.*, 2015; Gudkov and Komarova, 2016). Given the poor outcome for the disease and high proportion of avoidable cases, better education about the risks of poor lifestyle may be beneficial to reduce the 37% of avoidable causes.

3.1.2 Tobacco Smoking

Smoking continues to be a global health concern. Within the UK itself, the most recent Office for National Statistics (ONS) surveys state in 2018 that 14.7% of all adults (aged 18 and above) are current smokers (Adult smoking habits in the UK - Office for National Statistics, n.d.). When this is broken down into countries within the UK, England has the lowest incidence of smoking (14.4%) with Wales higher with 15.9% of all adults smoking. Swansea, where this study is based, had a greater than national average of 17.003% of adults smoking tobacco (Adult smoking habits in the UK - Office for National Statistics, n.d.). Increased smoking has been linked to more social deprivation, 17% of Swansea's lower super output areas, defined as small geographical regions with mean population of 1,500 (minimum population of 1,000), have been ranked in the top 10% of deprived areas in Wales in a recent study by the Welsh Government (Swansea Council, 2020). On a positive note, since 2011

there has been a 5-percentage point decrease in smokers across the UK. Much of the reduction of smoking has been attributed to awareness of health risks and alternatives including e-cigarettes which have 3.2 million users UK-wide (Adult smoking habits in the UK - Office for National Statistics, 2019).

Smoking is renowned for negative health implications whether benign or malignant. Smoking has been associated with the development of cancers of several organs. As many as 29% of PC cases have been linked with tobacco smoking (Cancer Research UK, 2014b). Smoking is often a life-long habit and as a result of each decade of smoking, there is a 16% increased risk of PC development as well as a 1.71-fold increase in risk of death in PC patients who are current smokers in comparison to non-smokers (Wang *et al.*, 2014; Barone *et al.*, 2016). Smoking has also been shown to reduce median survival time in PDAC patients from 11 to 6 months (Zhang *et al.*, 2016).

Cigarette smoke has a complex composition. Approximately 4,000 compounds are present in cigarette smoke, which could have a potential role in pancreatic carcinogenesis (Barone *et al.*, 2016). One of the key carcinogens present in cigarette smoke is benzo-[a]-pyrene (B[a]P) which is formed through incomplete organic combustion (Souza *et al.*, 2016). B[a]P exerts its genotoxic and oncogenic effects through its DNA reactive metabolites. Interestingly, B[a]P requires metabolic activation by Cytochrome P450 enzymes (e.g. CYP1A1) and can upregulate such enzymes through binding to the aryl hydrocarbon receptor (AhR), **Fig. 3.1**. AhR resides as an inactivated cytosolic complex bound to a HSP90 dimer, co-chaperone XAP2 and p23. B[a]P binding reduces the affinity of the receptor for p23 and XAP2, allowing HSP90 to translocate ligand bound AhR to the nucleus. Nuclear ligand-bound AhR dimerises with arylhydrocarbon nuclear transporter, releasing the HSP90 chaperone, binding to xenobiotic response elements inducing chromatin bending and initiating gene transcription (Wang *et al.*, 2016; Mackowiak and Wang, 2016). Initiation of gene transcription results in upregulation of numerous genes, namely xenobiotic metabolism genes in order to eliminate the environmental contaminants which enter the cell, tissue or organ including CYP1A1. A key gene targeted by the AhR activation is the AhR repressor which suppresses the transcription of AhR genes through binding the AhR and ARNT to prevent overstimulation (Larigot *et al.*, 2018). Other genes upregulated, as a result of AhR and ARNT binding to the XRE

include genes, which regulate the cell cycle and apoptosis (Procházková *et al.*, 2011; Stanford *et al.*, 2016). There have been suggestions that different ligand binding can induce different gene expression (Larigot *et al.*, 2018). AhR activation can also induce cellular changes without altering gene expression, protein interactions have also been shown to alter cell cycle progression (Barhoover *et al.*, 2010).
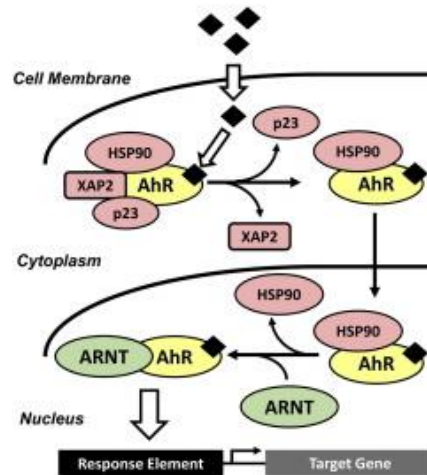
Figure 3.1: B[a]P-bound cytosolic AhR translocates to the nucleus mediated by chaperone HSP90 and binds the xenobiotic response element to induce gene transcription of various genes, including CYP1A1, responsible for B[a]P metabolism (adapted from Mackowiak and Wang, 2016).

CYP1A1 is the enzyme from the family of cytochrome P450s, which is responsible for the metabolism of B[a]P. B[a]P exerts its genotoxic and carcinogenic properties following metabolism to an active metabolite, BDPE. B[a]P metabolism to the genotoxic BDPE is dependent on aryl hydrocarbon receptor (AhR) and CYP1A1 expression, both of which have been found to be expressed in human pancreata, (Tissue expression of CYP1A1 - Summary - The Human Protein Atlas, n.d.; Yamamoto *et al.*, 2004). B[a]P is metabolically activated by CYP1A1 through sequential oxidation and hydration followed by oxidation to form the mutagenic product, bay-region diol-epoxide (BDPE), seen in **Fig 3.2** ,leads to DNA lesions through formation of DNA adducts, commonly at the N2 guanine position (Alexandrov *et al.*, 2010; Shah *et al.,* 2016). DNA adducts can lead to transversion mutations in the DNA, which increases the risk of carcinogenesis (Krais *et al.*, 2015).
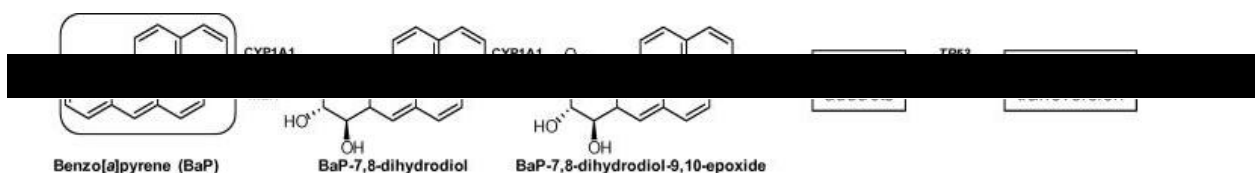
Figure 3.2: *CYP-dependent metabolism of B[a]P to its mutagenic derivative, BDPE, which forms DNA adducts (Krais et al., 2015).*

B[a]P bioavailability in the human pancreas was explored through HPLC analysis of surgical specimens from three main types of pancreatic tissue: pancreatic tumour, pancreatic tissue adjacent to tumour and healthy pancreatic tissue not adjacent to the tumour (Liu and Lun, 2012). Four main types of PAH were identified by Liu and Lun *et al.* (2012) within human pancreas tissue including B[a]P, chrysene, pyrene and 2-methylanthracene. Interestingly, concentrations of B[a]P were significantly higher in pancreatic tumour tissue and tissue adjacent to the tumour in comparison to healthy pancreatic tissue (Liu and Lun, 2012). B[a]P consumption from cigarette smoking and dietary exposure may lead to pancreatic carcinogenesis.

3.1.3 Obesity

Obesity is defined as a body mass index (BMI) above 30 kg/m^2. In England, 28.7% of adults are defined as obese with an additional 35.6% defined as overweight (BMI > 25kg/m^2) (Obesity Statistics - Commons Library briefing - UK Parliament, n.d.). Obesity is an increasing problem due to an increased caloric intake and a more sedentary lifestyle. This caloric imbalance results in excess of adipose tissue to store surplus energy. A wide variety of chronic health concerns arise from obesity including diabetes mellitus, cardiovascular disease and osteoarthritis (Cascetta *et al.*, 2018). There is also an increased risk of developing a wide range of cancers (Stone *et al.*, 2018). The World Cancer Research Fund has identified ten obesity-linked cancers including post-menopausal breast, endometrial and ovarian cancers in women, prostate cancer in men and GI cancers including oesophageal and colorectal cancers and hepatobiliary cancers including liver and gallbladder cancer and PC (Lennon *et al.*, 2016). As many as 12% of PC cases are attributed to an excess in body weight (Cancer Research UK, 2014b; Lennon *et al.*, 2016). On a positive note, obesity is a modifiable risk since weight loss has been shown to reduce the risk of cancers and may be beneficial to those at high risk of PC (Xu *et al.*, 2018).

Obesity is thought to cause cancer in a number of different ways. Inflammation and metabolic alterations are two major drivers in obesity-linked cancer development. Chronic inflammation has been linked with cancer from as early as 1863, where Verchow hypothesised inflammation combined with irritants increased cell proliferation and lead to cancer development (Coussens and Werb, 2002). The interactions between inflammation and carcinogenesis is now a more complex image than it was in 1863. Inflammation is a physiological process that has a role in wound healing and repair, usually tightly controlled by anti-inflammatory cytokines. An example of chronic inflammation leading to carcinogenesis is via the recruitment and activation of leukocytes. Leukocyte production of reactive oxygen species (ROS) induces DNA damage in proliferating cells and is designed to fight infection by killing pathogens. However, continual ROS production will also induce DNA damage in bystander host cells inducing mutation, often in p53 (Coussens and Werb, 2002). Inflammatory conditions including pancreatitis and diabetes mellitus have been previously linked with PC development.

Diets high in fat are more likely to cause obesity. Dietary fats are digested with the aid of bile. Bile is the green-coloured fluid produced by hepatocytes in the liver, stored in the gallbladder and released into the duodenum via the common bile duct. Bile has numerous roles including being the main excretory routes of fats and larger biological molecules, which are unable to be excreted via the urine including bile salts themselves and bilirubin (Boyer, 2013). Bile acids are one of the main constituents of bile besides water, which accounts for approximately 95% of bile (Boyer, 2013). Bile acids aid in the digestion of fats through emulsification of dietary fats into micelles (Chen and Cassaro, 2019). Bile acids form micelles due to their unique amphipathic structure, with a hydrophobic and a hydrophilic face (Hofmann and Hagey, 2014). Primary micelles are formed through hydrophobic interactions within the duodenum, forming a sphere where a hydrophobic core containing fat is established while polar, hydrophilic groups point externally (Pavlović *et al.*, 2018). Pancreatic lipase adsorbs to the hydrophilic surface of the micelle, allowing for enzymatic lipolysis. Bile acids as a part of the micelle increase the solubility of the digested fats, allowing for the absorption (Pavlović *et al.*, 2018).

Deoxycholic acid (DCA) is a secondary bile acid formed from the primary bile acid cholic acid (CA). Ninety percent of primary bile acids are reabsorbed and recycled,

the remaining minority are present in the large intestine and deconjugated before bacterial mediated 7α-dehydroxylation to form DCA (Narushima *et al.*, 2006). A population study showed a significant increase in serum DCA levels in overweight and obese males (BMI > 25 kg/m^2) in comparison to lean males (BMI > 23 kg/m^2) through use of UPLC-MS/MS (Xie *et al.*, 2015). In data from our research group, greater serum levels of DCA, among other bile acids, has been found in PC patients (Rees *et al.*, 2017). DCA has also shown some significance in *in vitro* genotoxicity studies. In studies of an oesophageal adenocarcinoma cell line, exposure to physiological levels of DCA (up to 300 µM) induced DNA damage, mediated by ROS production that was significantly diminished with treatment of the antioxidant vitamin C (Jenkins *et al.*, 2007).

Approximately 75% of PC patients will present with jaundice at one point during their disease journey due to extrahepatic bile duct blockage from pancreatic tumour progression (Cancer Statistics for the UK | Cancer Research UK, n.d.). This leads to an excess of bile acids in circulation, which are toxic to numerous organs. This can lead to multiple organ failure without intervention. As a result, pancreatic tissue has been shown to be particularly prone to bile acid exposure (Lu *et al.*, 2000) and this may play a role in pancreatic carcinogenesis.

3.1.4 Alcohol

Consumption of alcoholic beverages is an ingrained part of Western society. Alcohol consumption is prevalent among adults and greatest alcohol consumption is found across western, central, and eastern Europe with respective alcohol consumption per capita of 11.13, 11.64 and 11.55 litres per year (Peacock *et al.*, 2018). Globally, lowest alcohol consumption rates are found in North Africa and the Middle East, likely due to cultural and religious reasons (Peacock *et al.*, 2018). According to the 2018 NHS health survey for England 82% of adults had drank alcohol in the past year, with 49% drinking alcohol on at least a weekly basis (Health Survey for England 2018: Adult heath related behaviours, 2019). Males typically tend to drink more than females with 15.5 and 9 units consumed weekly, respectively (Health Survey for England 2018: Adult heath related behaviours, 2019). Alcohol intake has been measured in units, which are the equivalent to 10 mL or 8 g of pure alcohol, since 1987 in order to monitor and track consumption of alcohol (Alcohol units -

NHS, 2018). Unit contents of common alcoholic beverages are seen in **Table 3.1**. The NHS currently advises to not consume more than 14 units of alcohol over a week and advises to split this consumption over multiple days with alcohol free days in between (Alcohol units - NHS, 2018).

Table 3.1: *unit contents of common alcoholic beverages (Alcohol units - NHS, 2018).*

Drink	Units
Single shot of spirit (25ml, ABV 40%)	1
Alcopop (275ml, ABV 5.5%)	1.5
Small glass of wine (125ml, ABV 12%)	1.5
Bottle of lager, beer or cider (330ml, ABV 5%)	1.7
Pint of low-strength lager, beer or cider (ABV 3.6%)	2
Can of lager, beer or cider (440ml, ABV 5.5%)	2
Standard glass of wine (175ml, ABV 12%)	2.1
Pint of higher strength lager, beer or cider (ABV 5.2%)	3
Large glass of wine (250ml, ABV 12%)	3

Excessive alcohol intake can cause numerous negative health impacts from short-term or long-term alcohol abuse. Alcohol has been defined as the third largest risk factor, globally, for a range of diseases and disabilities (Rocco *et al.*, 2014). Between 2017/2018, there were over 1.2 million hospital admissions as a result of alcohol related disease or injury, which is a large burden on the NHS (Health Survey for England 2018: Adult heath related behaviours, 2019). In 2014, the UK government

stated that alcohol-related harm cost society £21 billion, incorporating costs such as NHS funding, crime and loss in productivity. Due to limitations and criticism of the methodologies for calculating the figure, including omission of certain costs and using only one study to estimate A&E costs, no further review of costs is available (Institute of Alcohol Studies, 2016). Given inflation and rising costs, this number is likely to be higher. Short term risks associated with alcohol abuse include: accidental injuries such as breaks and sprains, which require hospital treatment, increased risk of being involved in a violent event either as a victim or carrying out violence, unprotected sex, which could lead to unplanned pregnancies or sexually transmitted diseases and alcohol poisoning which requires the stomach to be pumped (Alcohol misuse - NHS, 2018). Drink driving is also a risk associated with alcohol abuse. Alcohol impairs decision making, making drink driving a real danger to society. Drink-related driving accidents caused 5,700 injuries in the UK in 2017 (Department for Transport, 2020).

Long-term alcohol dependencies can cause life-long issues, which are more complex to manage. The liver is one of the main organs responsible for the metabolism and elimination of alcohol from the body. As a result, excessive alcohol consumption can lead to both benign liver disease and liver cancer (Alcohol misuse – NHS, 2018). Chronic exposure to alcohol leads to fatty liver disease, which can sometimes develop further into cirrhosis, the final stage of liver disease (Rocco *et al.*, 2014). Ethanol is metabolised by the liver in both oxidative and non-oxidative pathways, products of which, including acetaldehyde, can be cytotoxic and genotoxic hepatotoxins inducing damage to the liver (Rocco *et al.*, 2014). Outside of the GI tract, alcohol can influence the development of heart disease and lead to an increased risk of stroke (Alcohol misuse - NHS, 2018).

The pancreas is another victim of alcohol dependency. Pancreatitis is a major, potentially life-threatening inflammation of the pancreas that often requires hospital treatment. Although pancreatitis has various causes, including gallstones, drug use and injury, alcohol is one of the main risk factors (Alcohol misuse - NHS, 2018). In the US, a third of acute pancreatitis cases are thought to be induced by alcohol (Chowdhury and Gupta, 2006). Despite this, the mechanisms involved are unclear. Pancreatitis has been thought to be stimulated by alcohol in a number of potential mechanisms including: ductal obstruction by mucoprotein plugs which inhibit cystic

fibrosis transmembrane conductance regulator (CTFR) function leading to fibrosis and scarring, alcohol increasing digestive and lysosomal enzyme contents of acinar cells increasing premature enzyme activation, and pancreatic stellate cell activation to myofibroblast-like phenotype inducing inflammation (Lankisch *et al.*, 2015; Madácsy *et al.*, 2018). Alcohol abuse alone may not be sufficient to develop pancreatitis, as fewer than 10% of heavy alcohol drinkers develop alcoholic pancreatitis, other lifestyle factors may play important roles (Vonlaufen *et al.*, 2007). In a rat model, additional inhalation of cigarette smoke acted as a contributory factor, increasing pancreatic injury in comparison to alcohol alone (Hartwig *et al.*, 2000). High fat diets may also play a contributory role in the progression of pancreatitis induced by alcohol (Chowdhury and Gupta, 2006). Alcohol abuse has also been shown to increase the chance of developing cancers around the body. Specifically, ethanol abuse has been shown to increase the risk of developing PC by 20% (Cancer Research UK, 2014b).

Ethanol is metabolised in the liver through oxidative and non-oxidative pathways as seen in **Fig 3.3** (Rocco *et al.*, 2014). In oxidative metabolism (**Fig 3.3a**), ethanol is oxidised to acetaldehyde in the liver by cytosolic alcohol dehydrogenase (ADH) in a reversible reaction, reducing NAD+ to NADH. Extrahepatic tissue metabolises ethanol to acetaldehyde using ADH, for example pancreatic tissue expresses ADH, which accounts for some of the alcohol induced injuries to the pancreas (Chiang *et al.*, 2009); non- ADH expressing tissue use catalase in peroxisomes and cytochrome P450 enzyme CYP2E1 in microsomes (Zakhari, n.d.). Acetaldehyde is further metabolised in the mitochondria to acetate using acetaldehyde dehydrogenase (ADH2) which enters the circulation. Acetate is taken in by tissues and oxidised to CO_2 in the citric acid cycle. Oxidative metabolism of ethanol eliminates 90 % of ingested alcohol (Heier *et al.*, 2016). While non-oxidative metabolism (**Fig 3.3b**) is only a minor route of metabolism, the products, which are biomolecules conjugated to ethanol including fatty acid ethyl esters (FAEE), are eliminated at a much slower rate than ethanol (Heier *et al.*, 2016). Non-oxidative metabolism of ethanol has been seen to be more prevalent in people with alcohol dependencies, often due to hepatic ADH being inhibited or deficient (Bhopale *et al.*, 2014).
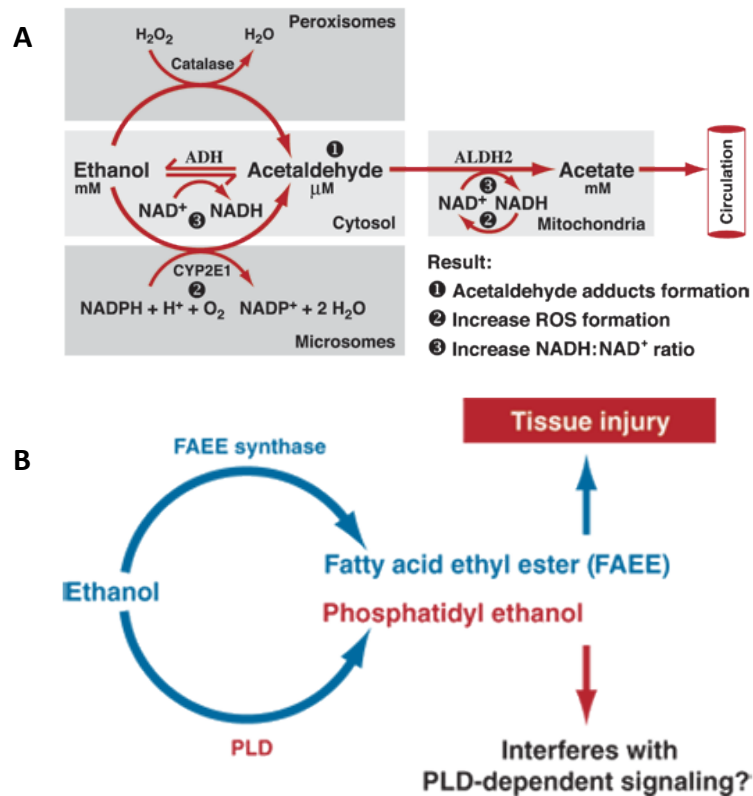
Figure 3.3: Metabolic pathways of ethanol metabolism through A) oxidative and B) non-oxidative pathways (Adapted from Zakhari, n.d.).

Acetaldehyde is a toxic intermediate produced through the oxidation of ethanol. Under normal physiological conditions, it is produced in small quantities and is usually metabolised rapidly using a delicate balance of ADH to ALDH. However, in chronic alcoholism there is decreased elimination by oxidation with either reduced ALDH function or by mitochondrial dysfunction which leads to an accumulation of acetaldehyde (Cederbaum, 2012). Pharmaceutical interventions for alcoholism include disulfiram, an ALDH2 inhibitor. By blocking acetaldehyde oxidation, an excess of acetaldehyde accumulates in the blood, serum, liver and pancreas following treatment with ethanol and disulfiram (Petersen, 1992; He *et al.*, 2001). This acts as a deterrent for alcohol consumption as a result of negative side effects include nausea, sweating and an increased heart rate (Cederbaum, 2012).

Acetaldehyde has been classified by the IARC as "possibly carcinogenic to humans" in group 2B (IARC, 1987). Studies demonstrated carcinogenicity in animals but there was inadequate evidence to prove this in humans. Despite this, acetaldehyde

has been shown to be genotoxic. It induces single and double-stranded DNA breaks in primary human lymphocytes from low concentrations of acetaldehyde, although double stranded breaks were only induced at high concentrations (100mM) (Singh and Khan, 1995). DNA damage induction was also studied using the *in vitro* cytokinesis block micronucleus (CBMN) assay in the MCL-5 cell line showed an induction of clastogenic micronuclei in response to acetaldehyde, contrasting the aneugenic micronuclei induced following ethanol exposure (Kayani and Parry, 2010). One of the main routes by which acetaldehyde exerts its genotoxic effects is through formation of DNA adducts. Adducts are formed through reaction of acetaldehyde with the exocyclic amino group on deoxyguanosine (dG) (Mizumoto *et al.*, 2017a). Adducts formed include the more abundant N^2-ethylidene-2'-deoxyguanosine (N^2-ethylidene-2'-dG), and the less abundant N^2-ethyl-2'-deoxyguanosine (N^2-Et-dG), and α-S and α-R-methyl-γ-hydroxy-1, N^2-propano-2'-deoxyguanosine (CrPdG). N^2-ethylidene-2'-dG has shown to have a half-life around 35h and is highly unstable (Hori *et al.*, 2012). Increasing concentrations of acetaldehyde adducts increase the risk of a transversion mutations, which in turn increases the risk of developing cancers (Heymann *et al.*, 2018). Acetaldehyde has also been found to form protein adducts with proteins including cytochrome C oxidase, impairing the electron transport chain; haemoglobin, putting red blood cells at risk of haemolysis and CYP2E1, which may have an effect of limiting the accumulation of acetaldehyde itself (Heymann *et al.*, 2018).
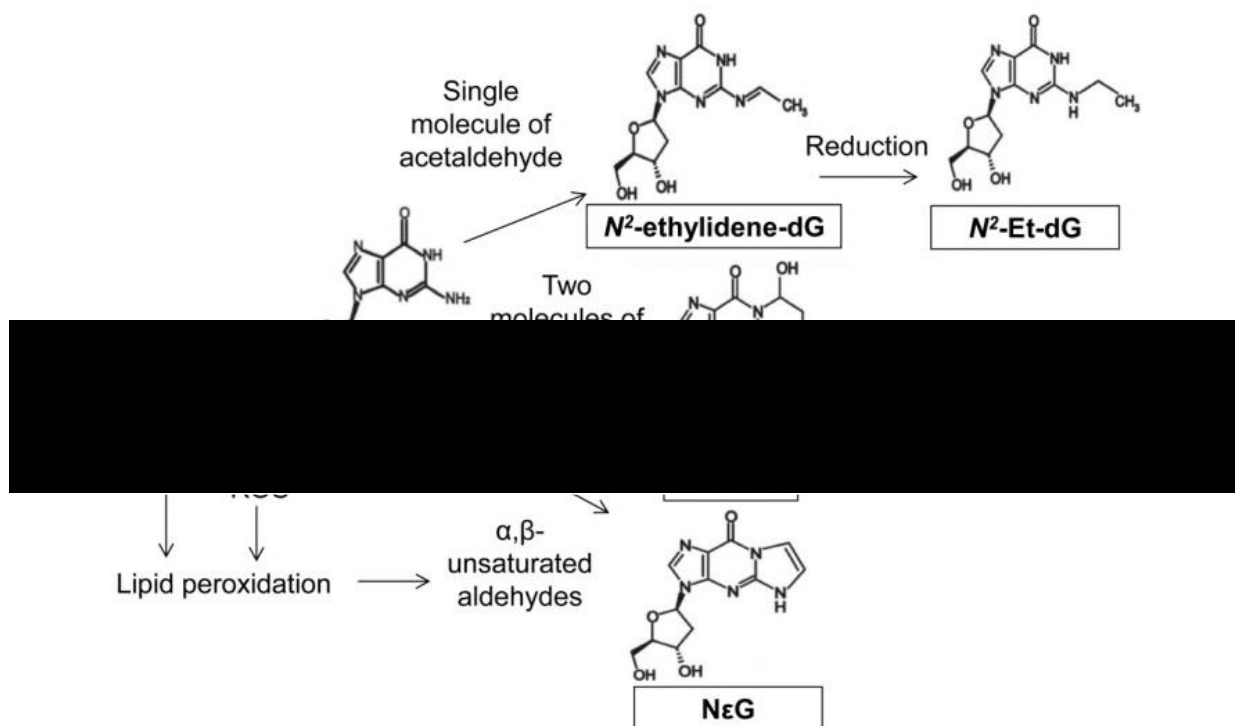
Figure 3.4: *formation of acetaldehyde induced DNA adducts deoxyguanosine (dG)* (Mizumoto *et al.*, 2017).

3.1.5 Measuring DNA damage induced by lifestyle factors

DNA damage induced by exogenous factors, including the lifestyle factors discussed prior, can be quantified, and compared. A range of techniques exist for the quantification of how much the DNA has been damaged, which can look at numerous cell types from various organisms.

The CBMN assay, discussed in **1.3.2**, is one of the most well established and commonly used genotoxicity assays. In this, the induction of small, membrane bound, DNA containing vesicles, called MN, outside of the primary nucleus is studied in response to DNA damage (Kisurina-Evgenieva *et al.*, 2016). MN are produced in response to genomic damage and the contents can vary from acentric chromosomal fragments, induced by clastogenic DNA lesions, up to full chromosomes, due to mitotic disturbances leading to aneuploidy (Iarmarcovai *et al.*, 2008). The type of MN can be identified using kinetochore staining. The CBMN assay is one of the most common *in vitro* techniques for quantifying chromosomal damage (Fenech, 2007; Rossnerova *et al.*, 2016). Cytochalasin-B blocks cells in the multinucleate stage of mitosis through inhibiting cytokinesis (Ridler and Smith,

1968). Cytokinesis is blocked by preventing actin polymerisation so no actin ring is formed to mediate cytoplasmic division (Heddle *et al.*, 2011). Cells being in the binucleate stage also ensures cells have undergone division following exposure to the test chemical. The frequency of binucleated cells with MN provide an overall view on the stability of the chromosomes, and whether this is altered following chemical exposure.
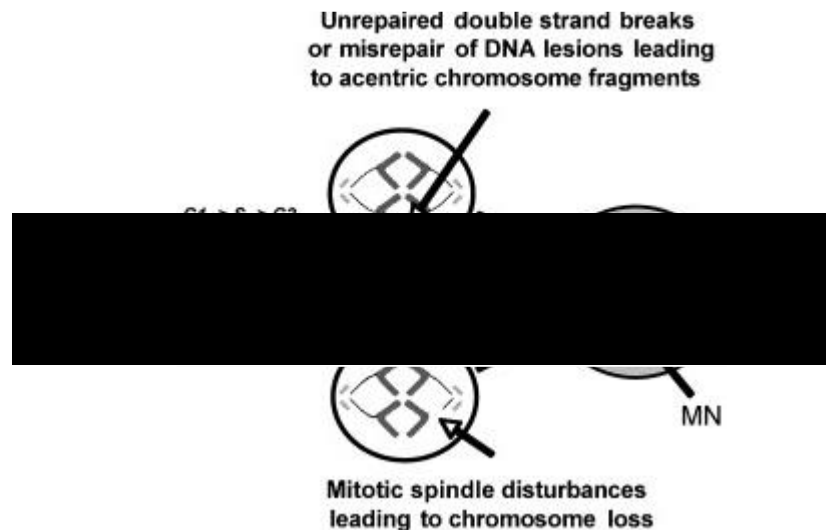
Figure 3.5: production of MN from cell undergoing mitosis in the presence of cytochalasin B in the CBMN assay, producing a binucleate cell with a MN. MN can be formed in two mechanisms(Iarmarcovai et al., 2008).

As well as assessing chromosomal stability in the CBMN assay, induction of point mutations will be studied in the *in vitro* Pig-A assay, described in chapter **1.3.2.5.3**..

3.1.6 Aims

Since lifestyle factors have a such a significant influence on the development of PC, studying them and their mechanisms is important to understand more about pancreatic carcinogenesis. Here, we aim to study the impact of smoking, a high fat diet and alcohol abuse modelled using B[a]P, DCA and acetaldehyde in the PDAC cell line PANC-1 in an *in vitro* model of PC. Cytotoxicity will be studied at increasing doses using both relative population doubling (RPD) values and the trypan blue exclusion assay. Chromosomal stability will be monitored using the *in vitro* CBMN assay in the PANC-1 cell line and a modified mononucleate MN assay will be carried out in the cytochalasin-B sensitive L5178Y cell line. Additionally, the *in vitro* Pig-A assay in the L5178Y cell line will be used to assess the induction of

point mutation following exposure to the trio of chemicals linked with lifestyle factors. Here, we will employ the same techniques we will be using in our *ex vivo* experiments on patient samples. This will aid in the analysis of patient data, since many of the patients recruited may also be influenced by these lifestyle factors explored in this chapter.

3.2 Methods

3.2.1 Cell culture

3.2.1.1 PANC-1

Pancreatic adenocarcinoma (PANC-1) cells were maintained in DMEM media (Gibco) supplemented with 10% foetal bovine serum, 1% glutamine and 1% penicillin streptomycin at 37°C, 5% CO_2 humidified atmosphere. Cells were subcultured through media removal, washing cell monolayer twice in PBS (Gibco) followed by trypsinisation. Detached cells were neutralised through the addition of media and trypsin-media mix removed through centrifugation at 30 g for 5 minutes. Supernatant was discarded and pellet was resuspended in fresh complete media and diluted further before incubation. PANC-1 required subculturing every 48 hours.

3.2.1.2 L5178Y

Mouse lymphoma (L5178Y$^{TK+/-}$) cells were maintained in RPMI Dutch modification (Gibco) supplemented with 10 % heat-inactivated horse serum, 1% glutamine, 1% penicillin streptomycin, 5.5 mL sodium pyruvate (Gibco) and 5.5 mL pluronic F-68 (Gibco). Cells were maintained at 37°C, 5 % CO_2 humidified atmosphere in the density of 3 x 10^4 and 5 x 10^5 cells/mL. L5178Y required subculturing every 24 hours.

3.2.2 Cytotoxicity Measure in PANC-1

PANC-1 cells between passages 18 and 24 were seeded at 1x10^5 cells/mL in T25 flasks in complete PANC-1 media as described previously. Cells were returned to an incubator and left to adhere for 24 hours. An hour before exposure to chemicals, cells were put into suspension through trypsinisation to be counted by Beckman Coulter Counter. 100 µL of cell suspension was added to 10 mL Coulter Counter diluent (Beckman) and cells were counted between sizes of 5 and 17µm. Cells were left to adhere to the flask once again for an hour in the incubator before exposure to

test chemicals: B[a]P, DCA and Acetaldehyde. B[a]P was made up in a solution of DMSO to make final concentrations ranging from 0-50 µM, a solvent control of DMSO was used. DCA was diluted in ethanol with concentrations ranging from 0-300 µM with ethanol alone as a solvent control. Acetaldehyde solution (Sigma) was diluted further with media in concentrations of 0-2.5 µM. Following 24 hour exposure to the test chemicals, media was removed, and the cell monolayer was washed twice in PBS before 10 mL fresh media was added. Cells were incubated for further 48 hours to recover before cytotoxicity analysis.

Cells were removed from flasks through trypsinisation and resuspended in 10 mL media. Cells were counted again with the Beckman Coulter Counter and cell counts were recorded. The relative population doubling (RPD) was calculated using the following formulae:

$$Population\ Doubling\ (PD)$$
$$= \frac{\log\ (Cell\ count\ after\ exposure\ \div Cell\ count\ before\ exposure)}{\log(2)}$$

$$Relative\ Population\ Doubling\ (RPD) = (PD \div Control\ PD) \times 100$$

The viability of treated cells was also determined through the trypan blue exclusion assay. 10 µL of the harvested cell suspension was diluted in 1:1 ratio with 0.4% trypan blue solution (Sigma) and mixed through gentle pipetting. 10 µL of this mix was loaded onto a haemocytometer for analysis under a light microscope. The four outer squares of the haemocytometer were counted, **Fig. 3.6**. Live cells are impermeable to the stain and remain white whereas dead cells are permeable to the stain and so dead cells appeared blue under the microscope.
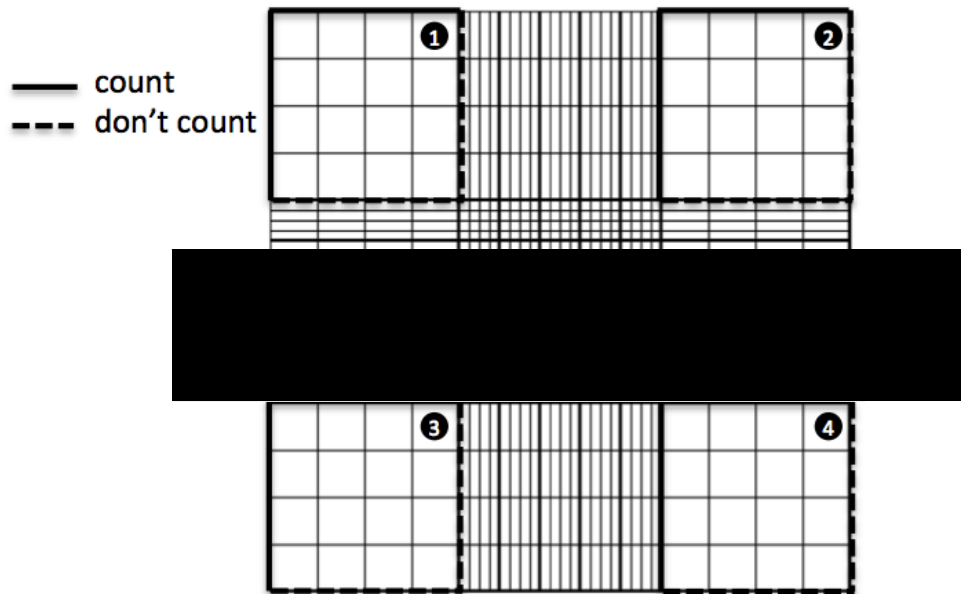
Figure 3.6: *How a haemocytometer would look under a light microscope. The four outer large squares labelled 1, 2, 3 and 4 are counted with cells touching top and far left edges (solid line) are counted whereas cells touching the right hand side and lower edge (dashed line) would not be counted (Fuentes, n.d.).*

The percentage of viable cells was then determined using the following formula:

$$Cell\ viability\ (\%) = (1 - (No.of\ blue\ cells \div total\ number\ of\ cells)) \times 100$$

3.2.3 Cytokinesis Block Micronucleus Assay in PANC-1 cell line

PANC-1 cells were seeded at the density of 2 x 10^5 cells/mL in a T25 flask in supplemented media. Cells were returned to an incubator to adhere for 24 hours. An hour before dosing with test chemicals, satellite flasks were removed, cells were detached and counted as described above. Cells were then left to adhere for an hour. Cells were dosed as described above with B[a]P, DCA and acetaldehyde. Following 24 hour exposure, media was removed, and cells were washed twice with PBS. 10 mL fresh media was added to all flasks along with 30 µL cytochalasin B (0.6 µg/mL) to all test flasks excluding satellite flasks. Cells were left for 1 cell cycle (48 hours) to recover before cells were harvested and satellite flasks were counted for RPD calculations.

Harvested cells were fixed according to two protocols by Seager *et al.* (2014): one for the semi-automated Metafer scoring and one for the more traditional manual scoring.

3.2.3.1 Semi-automated Metafer Scoring

Harvested cells were washed in PBS and suspended in 0.56% hypertonic KCl solution. Cells were centrifuged at 14 g for 10 minutes, supernatant was discarded. The pellet was resuspended in fixative 1 (5 parts methanol: 6 parts 0.9% NaCl solution: 1-part acetic acid) and incubated for 10 minutes before centrifugation. Supernatant was removed and pellet was resuspended in fixative 2 (5 parts methanol: 1-part acetic acid) and incubated for 10 minutes before centrifugation. Cells were washed twice more in fixative 2 before storage overnight at 4°C. Microscope slides were stored in fixative 2 were transferred into ddH$_2$O before being wiped with slide tissue. Cells were centrifuged and resuspended in 1 mL fixative 2. 100 µL cell suspension was spread over the slide, checked under a light microscope to check if cell density suitable and then left to airdry for 24 hours. On day of analysis, the slide was stained with 30 µL Vectashield Mounting Medium, a coverslip was added, and slides were left to incubate for 10 minutes protected from light.

Stained slides with coverslips were scanned using the Zeiss Imager.Z2 system with Metafer 4 software version 3.9.8 using classifier "MicronucFL-10-BN0sjmodif". Slides originally scanned at 10 x magnification but 63 x objective and oil used to confirm or deny the presence of MN following guidelines outlined by Fenech (2007), explored in **Table 1.1**. 2000 binucleated cells were scored per slide, with 3 scored per dose and per biological repeat (n=3).
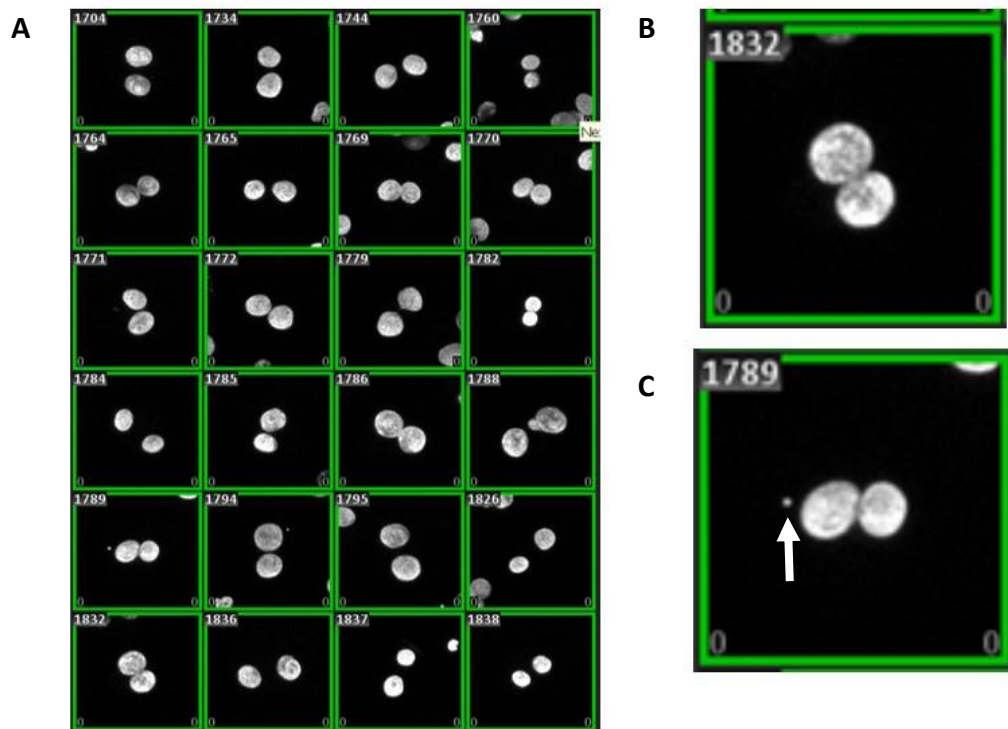
Figure 3.7: Screenshots from Metafer 4 software showing A) a gallery of binucleate PANC-1 cells which the software has identified B) a closer image showing the binucleate PANC-1 cell with no identifiable objects in black surrounding nuclei C) image of binucleate PANC-1 cell with a potential micronuclei, highlighted by an arrow. If this was seen during scoring, the eyepiece and 63 x objective and oil would be used to either confirm or deny the presence of a micronuclei. All images shown at 10 x magnification.

3.2.3.2 Manual Scoring

Harvested cells were washed in PBS and resuspended in 1 mL PBS. 100 µL of cell suspension was adhered to microscope slides using a Cytospin at 1200 rpm (162.58 g) for 5 minutes. Slides were left to airdry before fixing in 90% ice cold methanol for 10 minutes. Once dry from fixing step, cells were stained with an 20% Giemsa solution for 8 minutes before washing and being left to airdry. Once dry, slides were dipped in xylene solution for 10 seconds and coverslips were applied using DPX mounting medium before slides were left to dry in a fume hood.

Prepared and stained slides for manual scoring were scored under a standard light microscope at 100 x objective using oil. Care was taken to assess all cells on the slide, following left to right hand side of the cells in the dot on the slide before dropping down and following right to left and so on until entire slide examined. 1000 binucleated cells were examined per slide and of these, the number of cells with micronuclei as outlined by Fenech *et al*. (2007), **Table 1.1**, were quantified. 3 slides were examined per dose, only one biological repeat was quantified.
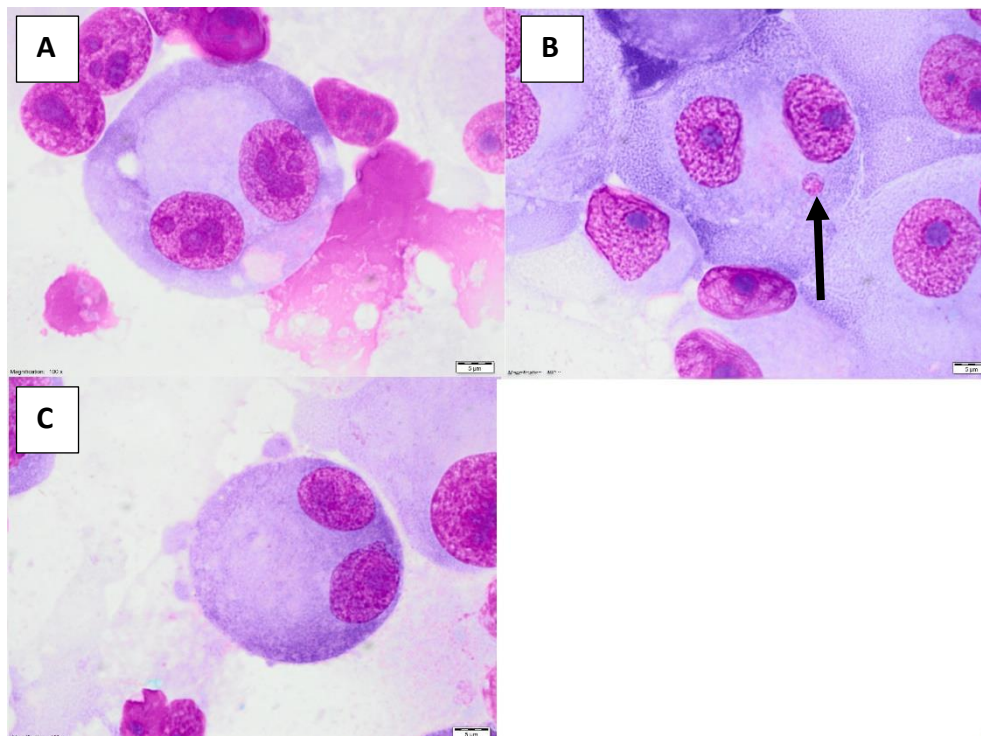
Figure 3.8: *Examples of bicnucleated PANC-1 cells scored by manual scoring with Giemsa stain under 100 x magnification on a light microscope. A) A binucleate cell B) A binucleate cell with a micronuclei denoted by an arrow C) a binucleate with a small amount of debris in the cytoplasm, which is stained a pink colour.*

3.2.4 Mononucleate Micronucleus Assay in L5178Y cell line

L5178Y cells were seeded at the density of 2 x 10^5 cells/mL in T25 flasks in complete media. Cells were returned to the incubator for 24 hours. Cells were counted for cytotoxicity calculations as seen above, before exposure to chemicals. Cells were exposed to test chemicals (DCA and acetaldehyde) while in a low serum media. DCA solutions made up with ethanol in the range of 0-300 µM with ethanol as a solvent control. Acetaldehyde solution was diluted further with media in the range of 0-150 µM. Low serum media was made up the same as the complete media as seen above but with only 5% of heat inactivated horse serum. Following a 4 hour exposure period, cells were washed twice in a wash media made up of blank RPMI 1640 with added 1% penicillin streptomycin before cell pellets were resuspended in complete media and returned to the incubator for 24 hour. After 24 hour incubation, cells were harvested following the steps in **3.2.3.1** up to the step of scoring, since no cytochalasin B was used this is the mononucleate micronuclei assay and so "MONOMicronucFL-10-BN0sjmodif" classifier was used instead. However, micronuclei were still identified using the details outlined by Fenech (2007), defined in **Table 1.1**.

3.2.5 *In Vitro* Pig-A Assay in L5178Y cell line

3.2.5.1 Cell dosing

L5178Y cells were revived from liquid nitrogen storage and grown in complete media for 6 days ensuring that the cells were always in their logarithmic growth phase. On the first day of the Pig-A assay, 6 x 10^6 cells were seeded in 10 mL of 5% serum media with the cells counted once seeded. Cells were exposed to test chemicals for 4 hours: DCA diluted in ethanol at doses of 20 and 150 µM with vehicle control of ethanol and acetaldehyde solution diluted in media at doses of 0.25 and 1 µM with media as a vehicle control. Following 4 hours exposure, cells were washed twice in wash media and then were resuspended in complete media and returned to the incubator. The experiment was carried out in triplicate.

3.2.5.2 *In Vitro* Pig-A Assay Preparation

In vitro Pig-A assay was carried out on days 1, 5, 9, 12, 15, 18 and 21 following test chemical exposure. On Pig-A analysis days, 2 x 10^6 cells were added to centrifuge

tubes. 1 per replicate of doses and 1 live cell control, 1 dead cell control, 1 mutant tube and 3 untreated L5178Y cells. Cells were washed once in wash media at 250 g for 5 minutes. A dead cell control was heated to 90°C for 3 minutes on a heating block to ensure cell death. Cell pellets were resuspended in 200 µL Zombie Violet Fixable Viability dye (Biolegend) (1:200 dilution in PBS) and incubated in the dark for 30 minutes at room temperature. Cells were washed once in antibody wash buffer (0.2% BSA in PBS) before resuspending pellet in 300 µL antibody solution (0.18 µg of antibody:1 µL of APC-CD-45 and PE-CD-90.2 (Biolegend) to 1110 µL antibody dilution buffer). Tubes were incubated protected from light at 4°C on a rocker for 30 minutes followed by 10 minutes at room temperature. Cells were washed once in antibody wash buffer (0.2% BSA in PBS) and resuspended in 1 mL antibody wash buffer. Samples were then analysed immediately by flow cytometry.

3.2.5.3 *In Vitro* Pig-A Assay Flow Cytometry Analysis

Stained samples were immediately analysed via flow cytometry. Samples were analysed using the Beckman Coulter Navios and Navios software. 250,000 events were analysed per sample on a low flow rate to try and capture the rare Pig-A mutation.

Initially, live and dead cells were discriminated between using the zombie violet viability dye. Live cells are impermeable to the dye, leaving dead cells positively stained. **Fig 3.9** shows an example cytogram from both a live and dead cell control which was ran at the beginning of each experiment. The live cells in **Fig 3.9A** remain unstained by the zombie violet dye, unlike the heated dead cell control in **Fig 3.9B**. Live cells were therefore put into gate C. In this example, the live cell control had 95.56% of cells in gate C (238,902 cells) in comparison to just 4.28% (5,288) in the dead control.
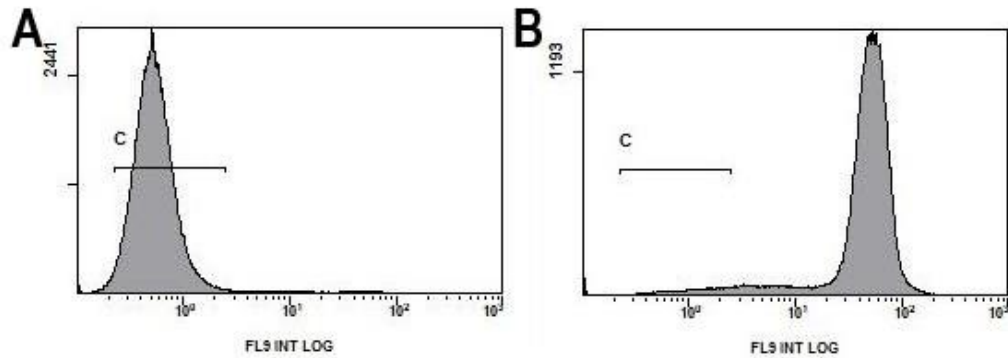
Figure 3.9: *Zombie violet viability dye discrimination seen in **A**) a live cell control and **B**) a dead cell control which had been heated at 90°C for 3 minutes to induce cell death. Live cells are identified in gate C.*

All live cells from gate C were then gated further dependent on APC fluorescence. APC-CD-45 antibody ensured L5178Y cells were being analysed, not debris or other contaminants. Cells which stained positive for the APC-CD-45 antibody were categorised into gate D. Cells in gate D were then analysed by a final cytogram with APC fluorescence for the APC-CD-45 antibody on the X-axis and PE fluorescence on the Y-axis for the PE-CD-90.2 antibody, which identified GPI anchored CD-90.2. Gates were drawn in this cytogram based on a positive control of fluorescently activated cell sorted L5178Y Pig-A mutant cells. Mutant cells were identified as those with positive APC fluorescence but PE fluorescence beneath the threshold set using the mutant control. In the example shown in **Fig 3.10**, 0.04% of cells are defined as mutant.
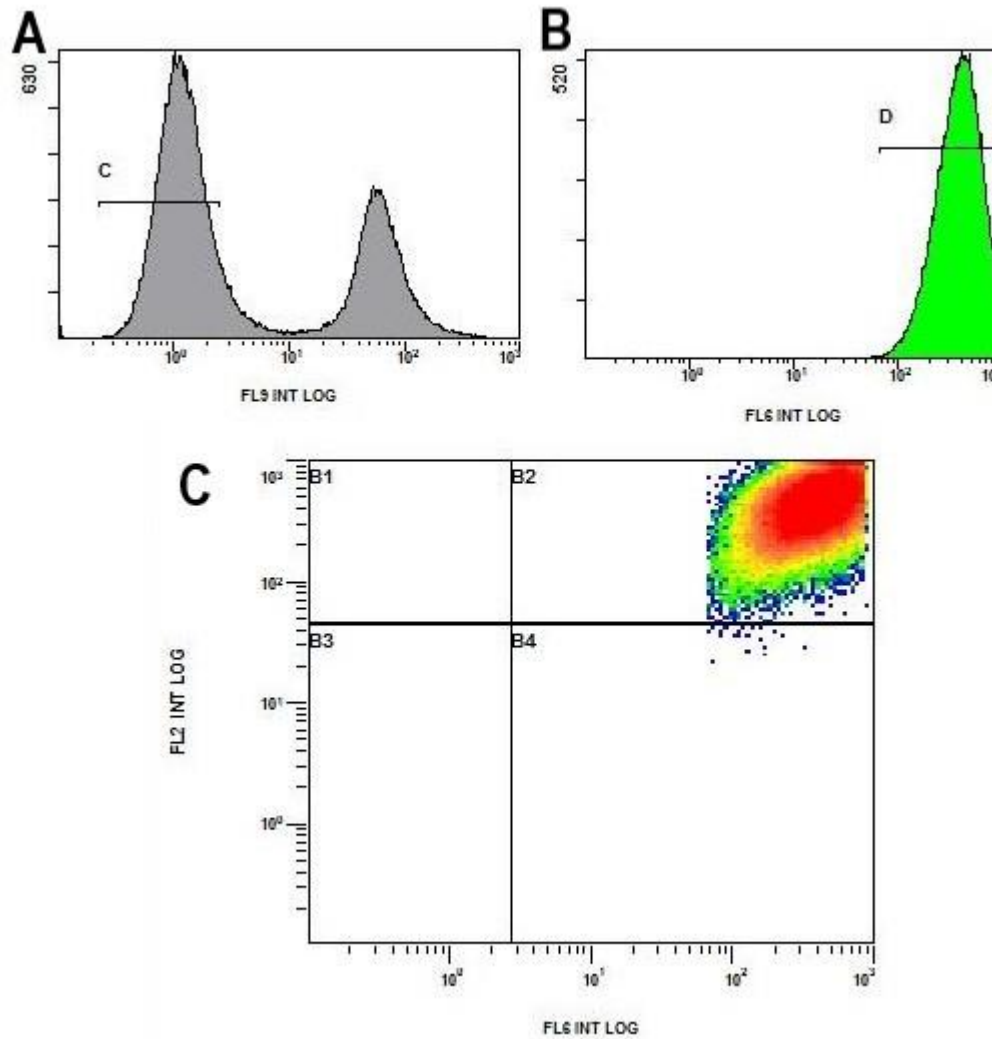
Figure 3.10: Example graphs from flow cytometry analysis of stained sample during in vitro Pig-A assay A) Live-dead discrimination using the zombie violet viability dye. Population in gate C are live. 60.02% of cells are live. B) Selection of CD-45 positive cell population. 93.29% of live cells from gate C are CD-45 positive and are grouped in gate D. C) Cytogram of all cells in gate D (live, CD-45 positive population) with APC fluorescence on X-axis and PE fluorescence on the Y-axis. Solid line gate drawn based on positive control mutant population. Cells below this line in quadrant B4 are mutant. In this example, 0.04% of live, CD-45 positive cells are CD-90.2 deficient and are defined as mutant.

3.3 Results

3.3.1 *In Vitro* Cytotoxicity Assessment and CBMN Assay in PANC-1 cell line

The pancreatic adenocarcinoma cell line PANC-1 was used to model PC. Literature searches for the risk factors of PC identified chemicals of B[a]P, DCA and acetaldehyde, which modelled smoking, a high fat diet and alcohol abuse.

Initial cytotoxicity experiments established the sensitivity of the PANC-1 cell line to 24 hr exposures to the chemicals to determine suitable dose ranges for subsequent experiments. Cytotoxicity was determined through two methods, RPD and the trypan blue exclusion assay. The cytotoxicity of B[a]P is seen in **Fig. 3.11**. A dose dependent decrease in both RPD and cell viability determined by the trypan blue exclusion assay was seen. A significant decrease in viability was seen at 3µM using RPD (p= 0.045) but this was not reflected in the trypan blue assay. Viability determined by trypan blue exclusion decreases became significant from 10 µM (p= 0.01) and the top dose of 50 µM (p= 0.003).
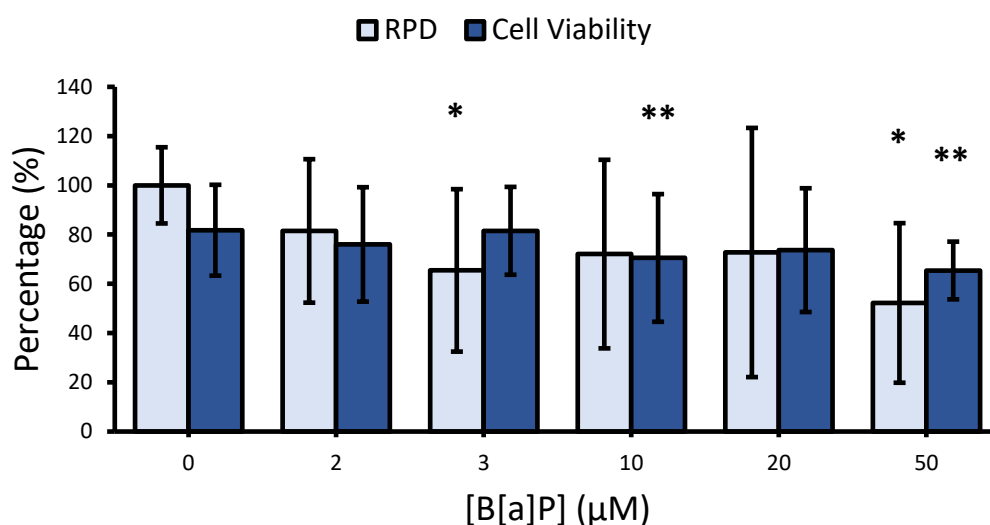
Figure 3.11: *Cytotoxicity determined through RPD and the trypan blue exclusion assay in the PANC-1 cell line exposed to increasing concentrations of B[a]P over 24 hours. Bars represent the mean (n=3) and error bars show standard deviation. (* show p<0.05, ** show p<0.01).*

The cytotoxicity assessment of DCA, **Fig. 3.12,** did not show a dose-dependent decrease in the levels found at normal physiological levels (up to 300 µM). A decrease in RPD was seen around 300 µM, although this is non-significant and not

reflected in the trypan blue assay (p= 0.093 trypan blue, p= 0.481 RPD). An expanded range of doses were also assessed, **Appendix 7.2** which showed cytotoxicity beyond 50 % above 300 µM DCA.
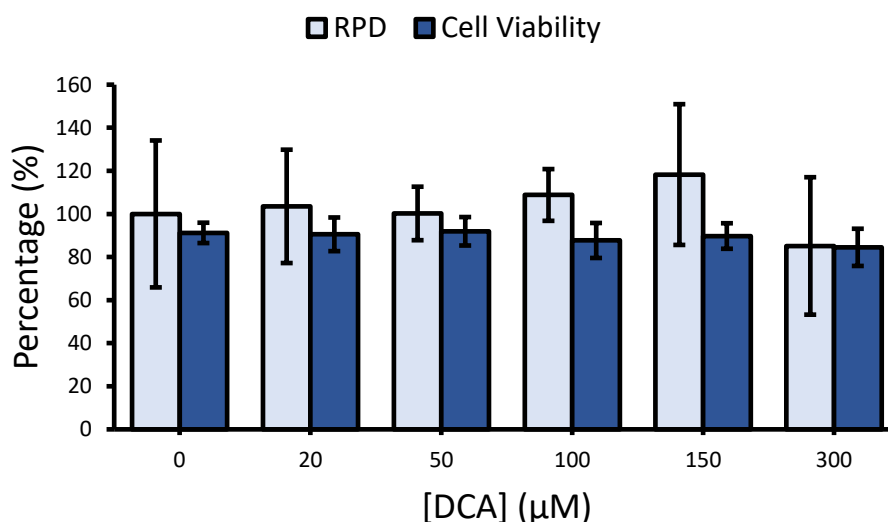
Figure 3.12: Cytotoxicity determined by RPD and the trypan blue exclusion assay in PANC-1 cell line exposed to increasing concentrations of DCA over 24 hours. Bars represent the mean value (n=3) and error bars show the standard deviation.

Acetaldehyde exposure significantly reduced the RPD of PANC-1 cells from 0.75 µM upwards but this was not reflected in the trypan blue exclusion viability assay, **Fig 3.13** (p=0.006 at 0.75 µM**,** p=0.011 at 1µM, p=0.004 at 2.5 µM RPD, p= 0.535 trypan blue). Comparing the three test chemicals and the dose ranges selected, B[a]P reduced the RPD and the viability the most, but acetaldehyde is potentially the most potent, inducing significant decreases at lower doses.
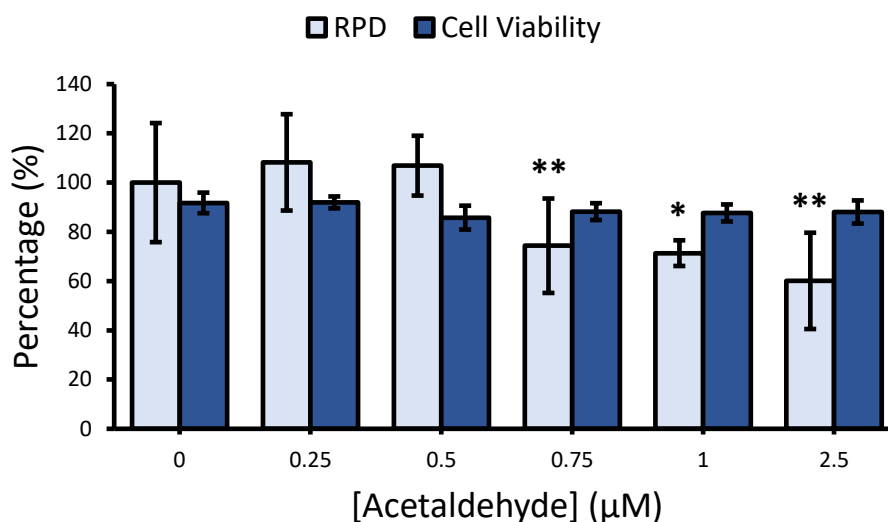
Figure 3.13: *Cytotoxicity determined through RPD and the trypan blue exclusion assay in PANC-1 cell line exposed to increasing concentrations of acetaldehyde for 24 hours. Bars represent the mean (n=3) with error bars representing the standard deviation. (* shows p<0.05, ** shows p<0.01)*

Following the initial cytotoxicity experiments, suitable doses were selected to use for the *in vitro* CBMN assay. The methods of scoring of the *in vitro* CBMN assay are divided into two methods the traditional manual scoring or semi-automated scoring method using the Metafer system. Traditional manual slide preparation and scoring detailed in **3.2.3.2** examines 1000 binucleated cells per slide under 100 x magnification on a light microscope and the binucleated cells with MN are quantified by the user. In comparison, the semi-automated Metafer system, detailed in **3.2.3.1**, examines 2000 binucleated cells per slide and the user confirms or denies the presence of MN using the eyepiece and 62 x magnification and oil. The variation between the techniques may result in differing results if quantified through the different techniques. Both methods were compared using a replicate of the *in vitro* CBMN assay with a control and the top dose of B[a]P, **Fig 3.14**. Similar results were observed with both techniques but due to the ease and greater statistical power of the semi-automated system, this was used for the remainder of the experiment.
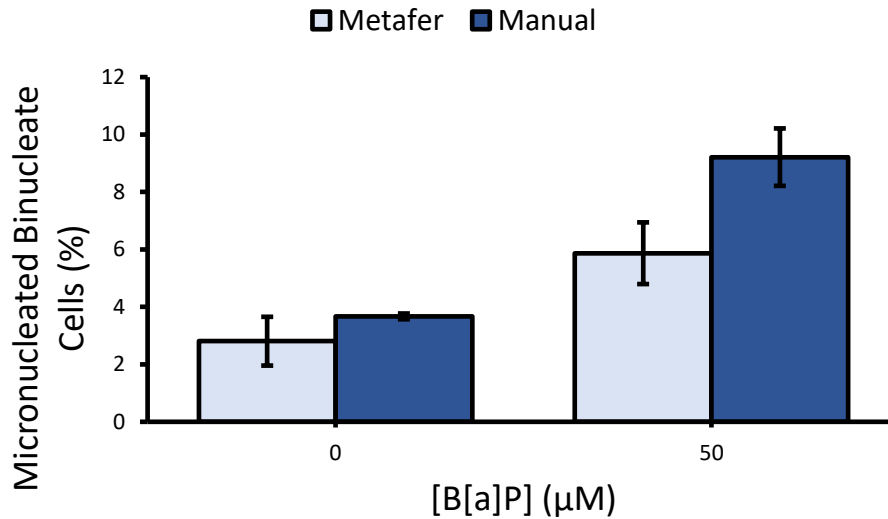
Figure 3.14: *Comparison of manual and semi-automated Metafer scoring for the CBMN assay in the PANC-1 cell line. 3 slides were scored per dose, per method (n=1). Bars represent the mean and the error bars show the standard deviation.*

The quantity of binucleated PANC-1 cells with additional micronuclei were scored according to Fenech (2007) rules with the semi-automated Metafer system using a fluorescent microscope. Two thousand binucleated cells were scored in triplicate per biological repeat (n=3) repeated three times in total with cells of increasing passages to score 18,000 cells per dose. Cells were exposed to B[a]P, DCA and acetaldehyde in separate experiments.

A dose dependent induction of micronuclei was seen in PANC-1 cells following 24 hour exposure to B[a]P in the *in vitro* CBMN assay, **Fig. 3.15**. Induction of micronuclei was seen at the two highest doses of B[a]P analysed with micronuclei frequencies of 2.301 ± 2.301 % and 4.905 ± 4.905 % at 20 and 50 µM, respectively (p= 0.04, p=0.01). Below 20 µM of B[a]P, there is no statistical difference from vehicle control cells.
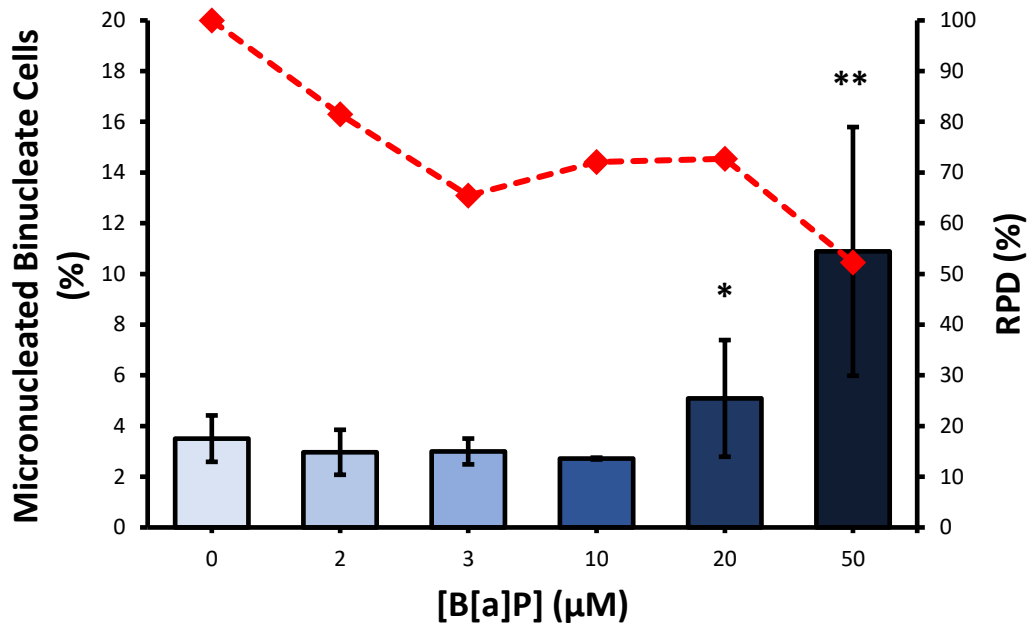
Figure 3.15: *Percentage of binucleated PANC-1 cells with micronuclei following 24 hour exposure to B[a]P (n=3). There's a dose dependent induction of micronuclei with significance above 20μM. Bars represent the mean and error bars represent the standard deviation. Red dashed line indicates the RPD. (* shows p<0.05, ** shows p<0.01).*

DCA didn't show a dose-dependent induction of MN, **Fig. 3.16**. Despite this, elevated MN levels at 50 and 300 µM of $3.79 \pm 0.551\%$ and $4.059 \pm 1.332\%$, respectively, showed a significant increase over the vehicle control (p =0.05 and 0.011 respectively).
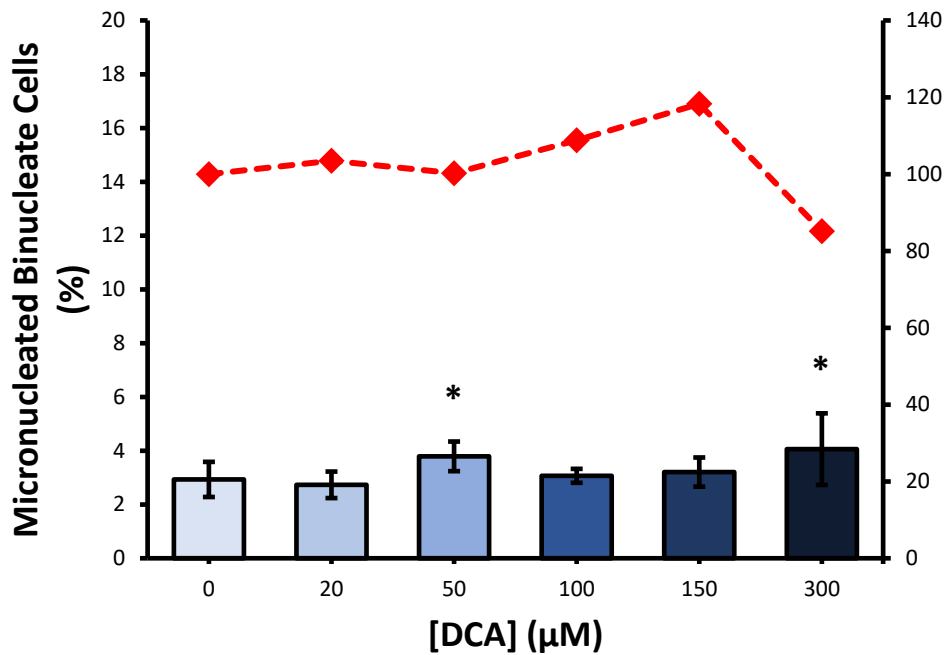
Figure 3.16: *Percentage of binucleated PANC-1 cells with micronuclei following 24 hour exposure to DCA. No clear pattern is shown, even if significant increases at 50 and 300 µM. Bars represent the mean and the error bars represent the standard deviation. Red line shows the RPD. (* shows p<0.05)*

The induction of micronuclei in binucleated PANC-1 cells was studied in the *in vitro* CBMN assay following 24h exposure to acetaldehyde, **Fig. 3.17**. Micronuclei frequency started 5.273% in the vehicle control. From 0.55 µM to 1 µM there was an elevated frequency of micronucleated binucleate PANC-1 cells ranging from 7.438 ± 4.727% to 8.267 ± 2.510%. This was close to significance following a t-test at 0.5 µM (p = 0.08). The greatest induction of micronuclei was at 2.5 µM acetaldehyde with 15.667 ± 10.941 with a p-value of 0.006.
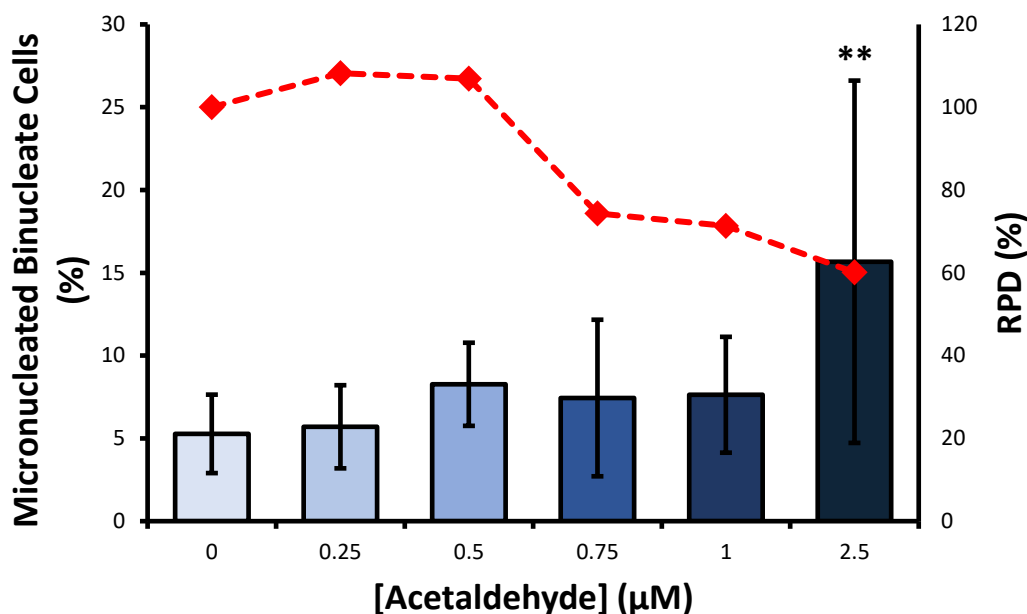
Figure 3.17: *Percentage of binucleated PANC-1 cells with micronuclei following 24 hour exposure to acetaldehyde. A dose dependent increase in MN induction is seen at 0.5 and 2.5 μM of acetaldehyde. Bars represent the mean (n=3) and the error bars represent the standard deviation. Red line shows RPD. (* shows p<0.05, ** shows p<0.01)*

3.3.2 *In Vitro* mononucleate micronucleus assay with L5178Y cell line

Since the *in vitro* Pig-A assay has been optimised for a couple of suspension cell lines, including L5178Y, mutational modelling by Pig-A had to be performed in this cell line. We therefore chose to compare MN response in comparison to the PANC-1 cell line explored earlier in the chapter. The L5178Y cell line is not metabolically competent, so assays following exposure to B[a]P were not performed. Additionally, the mononucleate MN assay had to be performed using the L1578Y cell line due to the sensitivity of the cell line to cytochalasin-B. Comparison of the MN results allowed selection of appropriate doses for the in vitro Pig-A assay in the L1578Y cells.

Results of the *in vitro* MN assay in the L5178Y cell line are found in **Appendix 7.3**. Initial cytotoxicity experiments determined the suitable maximum doses of DCA and acetaldehyde, following 4 hour exposure, which did not reduce RPD below 50 %.

RPD in the L5178Y cell line was reduced by 150 µM DCA and 1 µM acetaldehyde to 71.67% and 74.34%, respectively. Doses above these concentrations reduced RPD to below the 50 %, threshold outlined in the OECD guidelines for the *in vitro* micronucleus assay (OECD, 2016). Both chemicals showed induction of MN at top doses, but only acetaldehyde showed significant induction (p=0.003).

3.3.3 *In Vitro* Pig-a assay in L5178Y Cell Line

Following on from initial cytotoxicity experiments and the mononucleate MN assay, the *in vitro* Pig-A assay was carried out to determine the frequency of GPI-deficient L5178Y cells over a 21-day period following exposure to the chemical of interest or control substance. Previous *in vitro* studies have shown a peak in Pig-A mutant cells around day 10 post chemical exposure (David *et al.*, 2018). Monitoring the frequency of Pig-A mutant L5178Y cells over a 21-day period was carried out to determine the peak expression of the mutant phenotype.

3.3.3.1 DCA

The Pig-A mutant frequency was monitored in L5178Y cells exposed to DCA over a 21-day period, **Fig. 3.18.** Pig-A mutant frequencies in untreated L5178Y cells peaked around day 9 with 0.074 % of the cells showing a spontaneous mutant phenotype. The remaining treated cell types showed an unclear pattern across the 21-day monitoring period. Despite this, a peak was seen around day 9 and a decrease at day 15.
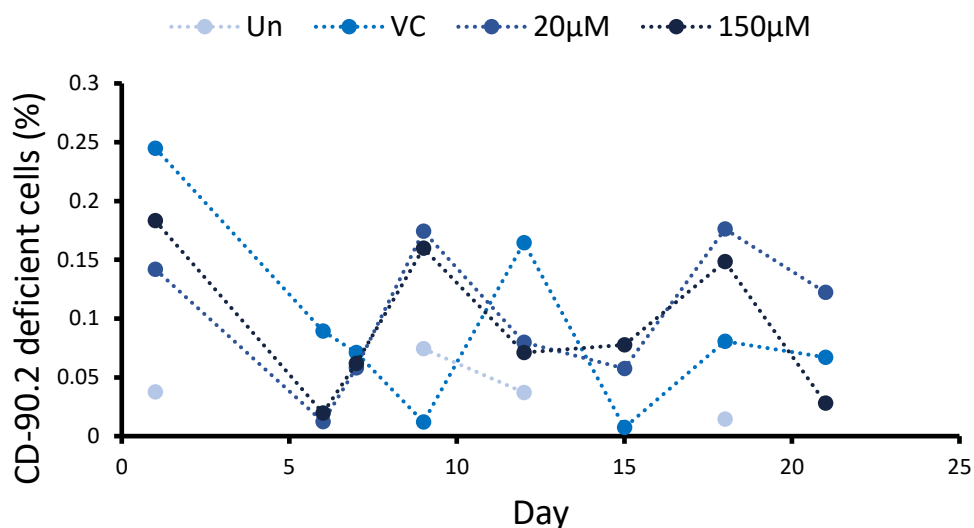
Figure 3.18: *Frequency of CD-90.2 deficient live L5178Y cells measured across a 21-day period following 4 hour exposure to 20 or 150 μM of DCA, vehicle control (VC) ethanol or untreated (Un). (n=3)*

Although a complex pattern, the Pig-A mutant frequencies peaked on day 9 following exposure to DCA and the vehicle control. Day 9 was chosen as a suitable time point to compare the mutations induced, **Fig. 3.19**. Both doses of DCA induced Pig-A mutant frequencies of 0.174 and 0.160% at 20 and 150 µM, respectively. Although there was an increase, this was not statistically significant in comparison to either the untreated L5178Y cells or the cells exposed to the vehicle control (p=0.360).
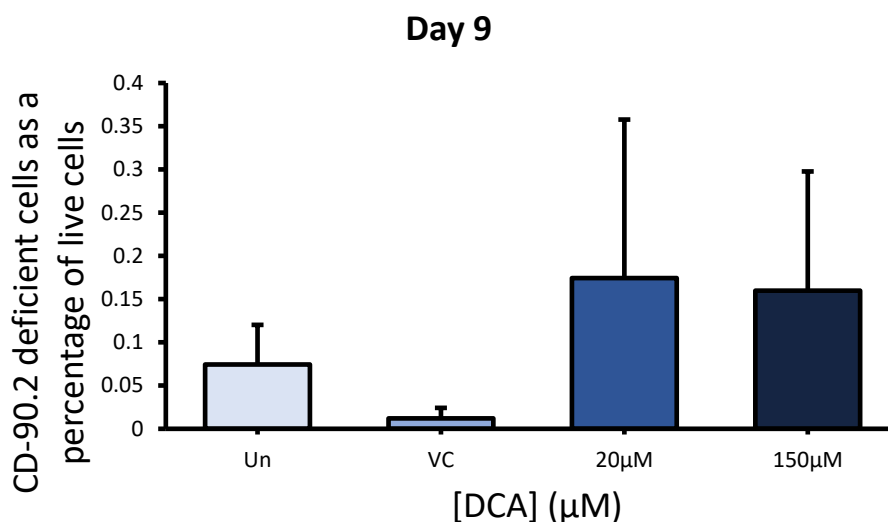
Figure 3.19: *Percentage of CD-90.2 deficient live L5178Y cells on day 9 following a 4 hour exposure to DCA. Bars represent the mean value of CD90.2 deficient cells (n=3) and error bars show the standard deviation.*

3.3.3.2 Acetaldehyde

The induction of Pig-A mutations following 4 hour exposure to acetaldehyde was monitored in the L5178Y cell line over 21-days, **Fig. 3.20**. In comparison to the pattern with DCA, **Fig. 3.18**, there is a clearer induction of mutation in the exposed populations around day 9 and these return to baseline mutation frequencies over the remainder of the experiment. **Fig. 3.21** depicts the Pig-A mutant frequencies at the peak of phenotypic expression day 9. At both doses of acetaldehyde there is an induction of Pig-A mutant cells in comparison to the vehicle control. This is not dose-dependent, as Pig-A mutant frequencies are very similar with $0.233 \pm 0.151\%$ and $0.243 \pm 0.068\%$, respectively for 0.25 and 1 µM of acetaldehyde. However, despite this, statistical significance was only seen at 1 µM following a t-test which provided a p-value of 0.023.
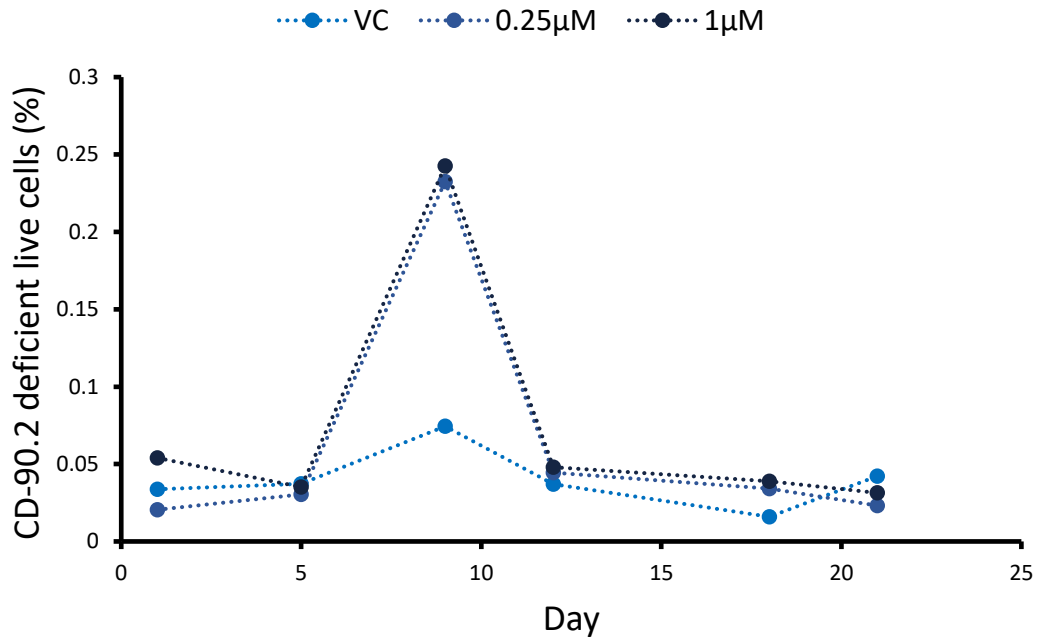
Figure 3.20: *Percentage of CD-90.2 deficient cells as a percentage of live L5178Y cells across a 21-day period following 4 hour exposure to acetaldehyde. Mutant frequency peaks around day 9, with treated cells having more mutations induced in comparison to the vehicle control (VC) which was exposed to media alone (n=3).*
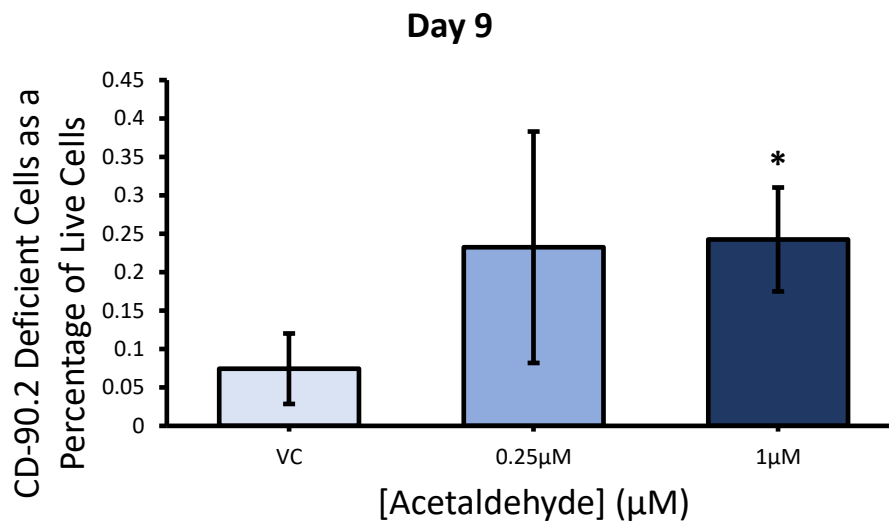
Figure 3.21: *Induction of CD-90.2 deficient live L5178Y cells on day 9 as a result of 4 hour exposure to acetaldehyde at 0.25 or 1 µM or the vehicle control (VC) of media. Bars show the mean value and error bars show the standard deviation. (* show $p < 0.05$).*

3.4 Discussion

Here, we have explored the induction of DNA damage induced by three chemicals to model known risk factors for PC. This includes B[a]P, DCA and acetaldehyde to model cigarette smoking, a high fat diet and alcohol abuse, respectively. Experiments utilised included the *in vitro* CBMN assay in the PANC-1 cell line and mononucleate MN assay in the L5178Y cell line. This was followed up by the *in vitro* Pig-A assay in the L5178Y cell line. Both mutation methods will be employed with human samples in future chapters.

Table 3.2: *Table change in RPD(%), induction of MN (%) following either the CBMN in PANC-1 cell line or mononucleate MN assay in L5178Y, and induction of Pig-A mutants (%) in the L5178Y on day 9 (peak in mutant phenotype). Results shown in PANC-1 cell line (grey boxes) and L5178Y cell line (white boxes). PANC-1 cells were exposed to chemicals for 24 hours and L5178Y cells were exposed for 4 hours. (* p<0.05, ** p<0.01, NS non-significant).*

Chemical	RPD (%)	MN (%)	Pig-A Mutant (%)
B[a]P	50 µM reduction 52.24 ± 32.40 (*)	20 µM induction 5.09 ± 2.30 (*) 50 µM induction 10.89 ± 4.91 (**)	-
	-	-	-
DCA	300 µM reduction 85.14 ± 31.91 (NS)	50 µM induction 3.79 ± 0.55 (*) 300 µM induction	-

		4.06 ± 1.33 (*)	
	150 µM reduction 71.67 ± 26.52 (NS)	150 µM induction 0.26 ± 0.10 (NS)	20 µM induction 0.17 ± 0.18 (NS) 150 µM induction 0.16 ± 0.14 (NS)
Acetaldehyde	0.75 µM reduction 74.35(**) 1 µM reduction 71.34(*) 2.5 µM reduction 60.09(**)	2.5 µM induction 15.67 ± 10.94 (**)	-
	1 µM reduction 74.34 ± 16.97 (*)	1 µM induction 0.37 ± 0.08 (**)	1 µM induction 0.24 ± 0.07 (*)

Table 3.2 compares induction of DNA damage by the 3 chemicals associated with risk factors for PC. All three chemicals in turn reduced the RPD in both the PANC-1 cell line and induced DNA damage in the CBMN assay. We also assessed the cytotoxicity in the L5178Y cell line followed by the mononucleate MN assay and in vitro Pig-A assay following exposure to only DCA and acetaldehyde. The L5178Y cell line was chosen since the *in vitro* Pig-A assay has been optimised in this cell

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line, but drawbacks of the cell line include its sensitivity to cytochalasin B, which results in the mononucleate MN assay being employed; and the lack of metabolic competency, so B[a]P wasn't explored due to the need of S9 mix to ensure metabolism to the active metabolite BDPE.

Cytotoxicity was assessed using both RPD and the trypan blue exclusion assay. RPD alone only provides a measure of how a treated cell population doubles in comparison to a control cell population. Population doubling alone cannot give a full picture of cytotoxicity as we do not know further information including whether cells quantified are alive or viable, introducing possible error. The trypan blue exclusion assay differentiates live cells from dead since the azo dye is impermeable to intact plasma membranes. Under microscopic examination, this leaves live, viable cells white whereas non-viable cells with compromised membranes are blue. This technique is vulnerable to false positive non-viable cells with intact membranes and false negatives, where cells are able to repair their membranes to become viable once more (Strober, 2015). Haemocytometer counting also provides counting errors around 10% (Aslantürk, 2018). Haemocytometer counting can also be time consuming with numerous samples. A combination of RPD and the trypan blue assay provides a more accurate overview of cytotoxicity.

From **Table 3.2,** acetaldehyde appears to be the most potent cytotoxic agent, reducing RPD significantly from the lowest concentrations of 0.75 µM. Acetaldehyde has been previously shown to induce pancreatitis-like injuries *in vitro* in isolated pancreas models, but this hasn't been able to be reproduced *in vivo* (He *et al.*, 2001). It is also seen that L5178Y cell line is more sensitive to DCA than the PANC-1 cell line, showing greater reduction in RPD at a lower dose. PANC-1 may be less sensitive since pancreatic ductal epithelial cells would be naturally exposed to more bile acids than a lymphoblastoid cell line. PANC-1 cell growth inhibition has previously been achieved through exposure to crude bile in the media (Lu *et al.*, 2000). A longer, 48 hour exposure achieved greater inhibition, so perhaps the 24 hour exposure used in our PANC-1 cells was not long enough to see significant RPD decrease. In contrast, a 4 hour exposure in the L5178Y cell line reduced RPD further, this is attributed to greater sensitivity and a considerably shorter cell cycle than PANC-1 cells.

The *in vitro* MN assay is one of the most used techniques in *in vitro* genotoxicity studies. The CBMN has a benefit over the mononucleate variety since cytochalasin B ensures cells scored have undergone cell division following chemical exposure. The CBMN was harnessed in the PANC-1 cell line but L5178Y cells have been shown to be negatively impacted by cytochalasin B, but the rapid cell cycle of L5178Y cells ensures that division has occurred following chemical exposure thus the mononucleate MN assay was used (Lorge *et al.*, 2016). Background MN frequency observed in PANC-1 cultures exposed to vehicle controls ranged between 3 and 5%. Since 3 separate solvents were used to dissolve test chemicals, they could not be combined. DMSO, ethanol and media were used to dissolve and dilute B[a]P, DCA and acetaldehyde, respectively. Natural variation in background MN frequency is expected.

Fig. 3.14 compares manual and semi-automated MN scoring techniques. Semi-automated scoring was used for this study due to ease of technique, user friendly software, clear imaging of scored cells and more cells scored per slide (2,000 in comparison to 1,000). The software also allowed for quicker observation of cells in comparison to the more time-intensive manual scoring. Despite the improvements, the semi-automated scoring still required human input to be able to confirm or deny presence of MN. Some studies have used flow-cytometry based techniques in order to score MN in both the L5178Y and TK6 cell lines (Bryce *et al.*, 2007). Although scoring thousands of cells within minutes is quicker, semi-automated scoring allowed for visualisation of individual nuclei, making the Metafer approach better suited to the study.

Similarly to RPD, acetaldehyde appears to be the most potent genotoxin to induce MN at lower concentrations than DCA and B[a]P. Acetaldehyde significantly (p < 0.01) induces MN at 1 µM and 2.5 µM in the L5178Y and PANC-1 cell line, respectively. This supports the idea that the L5178Y cell line is more sensitive than PANC-1. Lymphoblastoid cell line, MCL5, has also shown an increase MN frequency following acetaldehyde exposure (Kayani and Parry, 2010). Acetaldehyde has been shown to induce DNA damage through forming DNA adducts at the N^2 position of guanine rings (Mizumoto *et al.*, 2017). This is the same residue, which is targeted by the genotoxic B[a]P metabolite, BDPE during adduct formation (Alexandrov *et al.*, 2010; Shah *et al.*, 2016). MN induction is well studied with

B[a]P, given its status as a model clastogen. HepG2 studies have shown induction of MN from around 25 µM, given B[a]P is often used as a positive control at 25 or 50 µM following 24 hour exposure, similar to results seen in our PANC-1 cells (Wu *et al.*, 2005; Wei *et al.*, 2009). Previous studies in lymphoblastoid lines have shown induction of MN in the TK6 cell line (Takeiri *et al.*, 2019) and the L5178Y cell line (Kirsch-Volders *et al.*, 2003), but both required S9 mix since both cell lines lack metabolic capabilities. Kirsch-Volders *et al.* (2003) showed significant induction of MN in the L5178Y cell line from 8 µM with the use of S9 mix. Here, PANC-1 have required more than double that dose to induce significant MN. DCA appears to be the least potent genotoxic agent explored within this study. No significant results were seen with the L5178Y cell line, but some significant increases were seen in the PANC-1 cell line, but this was not dose dependent. The unclear results seen as a result of DCA exposure may be linked to the ROS based mechanism in which DCA induces DNA damage (Jenkins *et al.*, 2007). ROS play an important role in PC at high levels whereby they damage macromolecules in the cells, but low levels of ROS have also shown an important role as secondary messengers (Durand and Storz, 2017). A delicate balance of ROS levels is used in PC development to maintain activation of oncogenic pathways while being low enough to evade cell senescence (Durand and Storz, 2017).

We also explored the induction of DNA damage using the *in vitro* Pig-A assay, which studies mutation induction rather than chromosomal stability as seen in the MN assay. Recently, studies have focused on development of the assay *in vitro*, although without much success as the *in vivo* counterpart. Many cell lines have been investigated for their use in the assay including TK6 and metabolically capable MCL5 cell lines (Rees *et al.*, 2017). The TK6 cell line is well established in genotoxicity studies, often utilised for the HPRT and CBMN assays. Various groups established the assay in this cell line (Krüger *et al.*, 2015; Krüger *et al.*, 2016; Rees *et al.*, 2017; Piberger *et al.*, 2018). Various problems limited this development. TK6 cells have a high background frequency of GPI-deficient cells which require a pre-cleansing before assay initiation (David *et al.*, 2018). Next generation sequencing identified a heterozygous deletion in the Pig-L locus on chromosome 17, which also resulted in a GPI anchor deficient phenotype alongside a mutation in Pig-A (Krüger *et al.*, 2015). This results in the TK6 cell line being unsuitable for the flow cytometry

based Pig-A assay since surface markers alone couldn't distinguish between Pig-A or Pig-L mutation (Krüger *et al.*, 2015). The L5178Y is superior in comparison, having two copies of Pig-L, a lower Pig-A mutant frequency which remains stable (0.08 ± 0.06%) and no pre-cleansing steps are required before the assay (David *et al.*, 2018).

Here, we monitored the percentage of GPI deficient live L5178Y cells over a period of 21-days post DCA and acetaldehyde exposure. Expression of the Pig-A mutant phenotype peaked around day 9, similar to Pig-A mutant peaks reported on days 8 and 7 post-exposure (David *et al.*, 2018). This expression period allows for the recirculation of cell surface anchors, which are in a constant state of flux and for the production and trafficking of freshly synthesised GPI anchors to the extracellular surface membrane, or not in the case of GPI-anchor deficient cells.

DCA showed an unclear pattern across the 21-day period, **Fig. 3.18**. Despite this, a peak in Pig-A mutant frequency was found on day 9. This unclear pattern could be attributed to the ROS-based mechanism by which DCA exerts its genotoxic effects. David *et al*. (2018) assessed the *in vitro* Pig-A assay in chemicals with known modes of action. Two of those studied, mitomycin C and 4-nitroquinoline-N-oxide, partially exert their effects through ROS production. Mitomycin C did not induce Pig-A mutant cells, but 4-nitroquinoline-N-oxide did at the day 8 mark. No data was available for the 21-day monitoring period and they may have potentially shown an unclear pattern as seen here with DCA.

Acetaldehyde, in contrast, showed a clearer pattern over the 21-day expression monitoring period and induced more Pig-A mutants on day 9 than DCA even at lower concentrations. This again strengthens the idea that acetaldehyde is a more potent genotoxin than DCA, we cannot draw conclusions about B[a]P since we did not assess it in the L5178Y cell line. Cells treated with acetaldehyde showed similar pattern in mutation across the 21-day period as seen with EMS, an adduct forming genotoxin which targets N^7 of the guanine ring as opposed to N^2, which acetaldehyde favours (David *et al*. 2018). A peak in mutant frequency was seen around day 9 followed by a steady return to baseline level of mutant cells, which may signify DNA repair mechanisms at work to repair damage induced by acetaldehyde or may show mutant cells dying (Seitz and Homann, 2007).

Although the *in vitro* Pig-A assay has shown potential in a few studies, and in this study with acetaldehyde, more papers exist which are more critical than supportive of the method. The assay is in its infancy, with several issues, which need to be overcome before the assay is as useful as its rodent erythrocyte counterpart. Problems arise with long experiment time. Cells are revived and maintained for 28 days (6 days exponential growth, 1-day exposure, 21 days monitoring Pig-A mutation status). This increases contamination risk, which occurred during this study a few times, which required fresh vials of cells revived, starting over again. Another overall issue is found in the fragility of the GPI anchor on the surface of test cell line, whether that is L5178Y, MCL-5 or TK6. Care was taken to resuspend pellets carefully with gentle flicking and no mixing with pipettes as found in other staining protocols. Excessive roughness during processing resulted in false high mutant frequencies due to mechanical shearing of GPI anchors from the extracellular surface. One group in fact showed an increase in GPI-deficient cells through increased flicking of samples (unpublished). Additionally, staining was an issue, perhaps due to insufficient mixing, while taking care not to shear GPI anchors. Occasionally following the four-hour processing and staining stages seen in **3.2.5**, cells were insufficiently stained, and no results were able to be collected. More reliable and less time-intensive techniques may have been more suitable to determine the quantity of GPI anchor deficient cells, but the flow cytometry based Pig-A assay in blood cells has been such a reliable and quick technique to quantify GPI anchor deficient blood cells, it doesn't seem to have translated as easily into an *in vitro* setting. This is a shame, since a reliable *in vitro* Pig-A assay may be suitable for use as a control to calibrate flow cytometers and as a method to standardise results collected on different machines.

3.5 Conclusion

To conclude, in this chapter we have investigated an *in vitro* model in response to three chemicals linked to three known risk factors for PC. We have compared decrease in viability by RPD and induction of MN in the PANC-1 and L5178Y cell lines, followed by the *in vitro* Pig-A assay in the L5178Y cell line. Comparing all three chemicals assessed, acetaldehyde has shown to be the most potent genotoxin, inducing significant DNA damage, quantified through the MN assay and the *in vitro* Pig-A assay, at lower doses than the other chemicals assessed. B[a]P would be the

second most potent, given PANC-1 results but this chemical was not explored in the L5178Y cell line due to lack of metabolic capacity. Other studies carried out using S9 mix would support this conclusion. DCA would be the least potent chemical assessed. Additionally, the L5178Y cell line appeared to be more sensitive than the PANC-1 cell line. We have shown induction of DNA damage, assessed in two methods, following exposure to chemicals linked with poor diet, alcohol abuse and smoking. This is important preparatory work for a future chapter, where the MN assay and PIG-A assay will be utilised *ex vivo*. The work carried out in this chapter may account for some of the mutation we will see in donors, who may be patients affected by the lifestyle factors we have explored.

Chapter 4: The Human Erythrocyte PIG-A Assay as a diagnostic test for Pancreatic Cancer

Chapter 4: The Human Erythrocyte PIG-A Assay as a diagnostic test for Pancreatic Cancer

4.1 Introduction

4.1.1 PC Diagnosis

PC is a fairly rare form of cancer, only accounting for 3 % of cancer cases in the UK which is equivalent to the 10th most common form of cancer (Cancer Research UK, 2020c). In 2017, there were 10,377 new cases of PC diagnosed with an even gender distribution with males accounting for 51 % of cases (Cancer Research UK, 2020c). Most PC cases are unfortunately diagnosed at advanced stages. Between the years of 2012 and 2017, 52.57 % of PC patients were diagnosed with stage IV disease, more than the total of patients with either lower staged disease (I-III) or disease of an undetermined stage (National Cancer Registration and Analysis Service, 2019).

There are numerous routes to a PC diagnosis. Currently, 46 % of cases are diagnosed via emergency presentation, which accounts for A&E attendance, emergency admission from GP, or emergency inpatient or outpatient referral (Cancer Research UK, 2016d). More PC patients present through emergency presentation (46 %) than general cancers (20 %) (Murchie *et al.*, 2017). A number of factors have also been associated with increased chance of emergency presentation of general cancers including no prior GP appointment (OR = 3.89; CI 95 % 2.14 - 7.09), upper GI cancer, including PC (OR = 18.97; CI 95 % 6.08 - 59.23) and also ethnicity (OR = 2.78; CI 95 %) (Murchie *et al.*, 2017). Emergency presentation of cancer has been associated with high short-term mortality, contributing to the poor prognosis of PC given the high proportion of emergency PC admissions (Mcphail *et al.*, 2013). Reducing the emergency presentation of PC has been predicted to increase the number of patients who would survive over one year (Pancreatic Cancer UK, 2013). Not only does the emergency route contribute to poorer disease outcome, it further burdens the NHS financially. Each individual emergency admission with PC costs approximately £5,000, triple the cost of other emergency admissions. This accumulates to costing the NHS approximately £14,650,000 per annum (Pancreatic Cancer UK, 2013). The high proportion of emergency PC presentation highlights the lack of early diagnosis. Earlier diagnosis would allow for more patients to be

diagnosed with potentially curable disease and improve the poor survival statistics for PC patients.

Only 17 % of PC patients in England were diagnosed through the NHS's "two week wait" referral route (Cancer Research UK, 2016d). Through this, patients with suspected cancer symptoms are referred for an urgent appointment with a specialist clinician within a fortnight. These emergency referrals have increased from 903,000 in 2009 - 10 to more than 1.5 million in 2014 - 15 (Bankhead, 2017). No formal "two week wait" pathway exists in Wales. Urgent referrals are available for suspected cancer cases to be seen urgently by suitable clinicians. NICE have set out guidelines in their 2015 report "Suspected cancer: recognition and referral" specifying patients over the age of 40 displaying jaundice should have a specialist appointment within a fortnight from initial GP presentation, and patients over the age of 60 displaying two or more symptoms need an urgent computer tomography (CT) within a fortnight (NICE, 2015). The formal "two week wait" system in England has only been in place a short period of time and it is too soon to tell whether it has made any benefit to patient survival (Bankhead, 2017).

In Wales alone, two rapid diagnostic clinics have been established to help speed up cancer diagnoses to allow for better patient outcomes. Early detection of cancer is key for better treatment options and, in turn, longer survival. Particularly with PC, there is a desperate need for advances in early diagnosis, given the current bleak outlook for PC patients. In time, with more rapid diagnostic clinics and better diagnostic techniques available for PC, more patients would be diagnosed with early stage disease and have more treatment regimes available to them. In turn, this would increase the patient survival and improve on bleak outlooks, which have failed to improve over the last four decades.

4.1.1.1 PC Diagnosis Methods

4.1.1.1.1 Blood based biomarkers for pancreatic disease

To date, there is only one blood-based biomarker in use for PC. The serum carbohydrate antigen 19-9 (CA19-9) is normally found in healthy patients at physiological levels below 37 kU/L (Thaker *et al.*, 2014). It is elevated in PC, but can also be elevated in a number of benign conditions (Howaizi *et al.*, 2003). With the standard cut off at 37 kU/L, the test has a sensitivity of 81% and a specificity of

90% (Thaker *et al.*, 2014). PC patients can have levels of CA19-9 as high as 1,000 kUmL and, at this level, the test shows an increased specificity at 99.8%, but at a compromise with 4% sensitivity (Thaker *et al.*, 2014). Despite this being the only biomarker in use for PC, the assay isn't without its problems. Elevated CA19-9 levels are non-specific and are often raised in benign conditions including diabetes mellitus, jaundice, pancreatitis and chronic liver disease (Howaizi *et al.*, 2003). More issues are raised since CA19-9 is a modified Lewis antigen and secretion of the marker are reliant on allele expression (Tempero *et al.*, 1987). As a result, 5 – 10% of the population have the Lewis negative phenotype and the test will not be clinically relevant (Ballehaninna and Chamberlain, 2011).

Often, the CA19-9 blood test is used in a wide panel of test results in order to narrow down the source of the patient's symptoms. Another test result, which is helpful, is the c-reactive protein (CRP) level. CRP is produced in hepatocytes in response to inflammatory IL-6 (Agalianos and Dervenis, 2017). CRP indicates systemic inflammation and has been shown to be elevated in a wide range of cancers (Inoue *et al.*, 2015). Many cancers including PC have an inflammatory origin (pancreatitis) and so the detection of an inflammatory biomarker is relevant. Normal physiological levels of CRP are below 5 mg/L (NHS, 2020). Elevation of CRP has been identified in PC patients, notably in patients with metastatic disease (Basso *et al.*, 1988). Additionally, PC impacts upon the function of the liver and so monitoring the liver function tests (LFTs) is beneficial for diagnosing pancreatic disease. Elevated LFTs, especially bilirubin, can signify bile duct obstruction, which would require an intervention to relieve (Kim and Ahuja, 2015).

4.1.1.1.2 Imaging techniques

Imaging of the pancreas and surrounding organs is imperative when there is a suspected pancreatic pathology. The first port of call after the first presentation of symptoms is an abdominal ultrasound (Ciaravino and D'Onofrio, 2019). The ultrasound gives an image of the pancreas, gallbladder, liver and surrounding structures, allowing the identification of any abnormalities. PDAC, the most common solid pancreatic tumour, is identifiable by a solid, hyper-echoic mass and duct dilatation on ultrasound imaging (Lee and Lee, 2014; Ciaravino and D'Onofrio, 2019). Challenges are associated with pancreatic ultrasound, with careful

preparation and positioning of the ultrasound wand for diagnostic imaging. Patient preparation includes 7 - 8 hour fasting, as food consumption may occasionally result in false impression of pancreatic tumours, and 500 - 700 mL of non-carbonated fluid should be consumed 10 – 15 minutes prior to ensure the stomach is full of liquid and not gas, which can obstruct the view of the pancreas and result in non-diagnostic imaging (Sirli and Sporea, 2010). If abnormalities are shown from an ultrasound, or imaging is insufficient, but symptoms and blood test results suggest pancreatic disease, further imaging techniques will be employed to further elucidate the issue and determine any involvement with surrounding organs and structures.

Further imaging techniques include CT, magnetic resonance imaging (MRI) and positron emission tomography (PET) scans. CT scans of the abdomen and thorax are used to assess both local disease and any potential metastatic deposits. It is the most valuable imaging modality for the assessment of the resectability of PC, given the clear imaging of the associated vascular structures, but is limited due to often missing smaller peritoneal metastatic deposits (Lee and Lee, 2014). MRI imaging is preferred to CT as it has greater contrast when assessing soft tissue (Lee and Lee, 2014). The adapted technique magnetic resonance cholangiopancreatography (MRCP) can be used to visualise the ductal system in the pancreas and can identify strictures and abnormalities often caused by tumours. PET scanning is less common for PC, but it is sometimes used in combination with CT scanning to distinguish between benign lesions and secondary tumours. Tumour cells have an increased glycolytic rate, commonly known as the Warburg Effect. This is exploited in PET scanning. The label, 18F-fluorodeoxyglucose (FDG) is a radioactive glucose analogue, which is taken up into cells but cannot be metabolised through glycolysis (Yeh *et al.*, 2018). FDG enters the cells via glucose transporters, is phosphorylated by hexokinase, but cannot be metabolised further, unlike glucose (Rahman *et al.*, 2019). Tumour cells, with their increased glucose uptake accumulate more of the radiolabelled FDG and so emit more radiation than healthy tissues, which is captured through PET scanning, **Fig. 4.1B**. Problems arise as a result of glucose intolerance in PDAC, increased serum glucose decreased FDG uptake in pancreatic tumours for false negative results, and normal physiological processed result in the uptake of FDG in some tissues which can cause false positives (Lee and Lee, 2014; Yeh *et al.*, 2018). As a result, PET scans are used in limited scenarios where the nature of a

potential metastatic deposit is queried and is not clarified further through alternative scans and a biopsy is unsuitable. CT and MRI scans are the first port of call for visualising the pancreas and surrounding organs, with PET scanning used in special circumstances, **Fig. 4.1**.
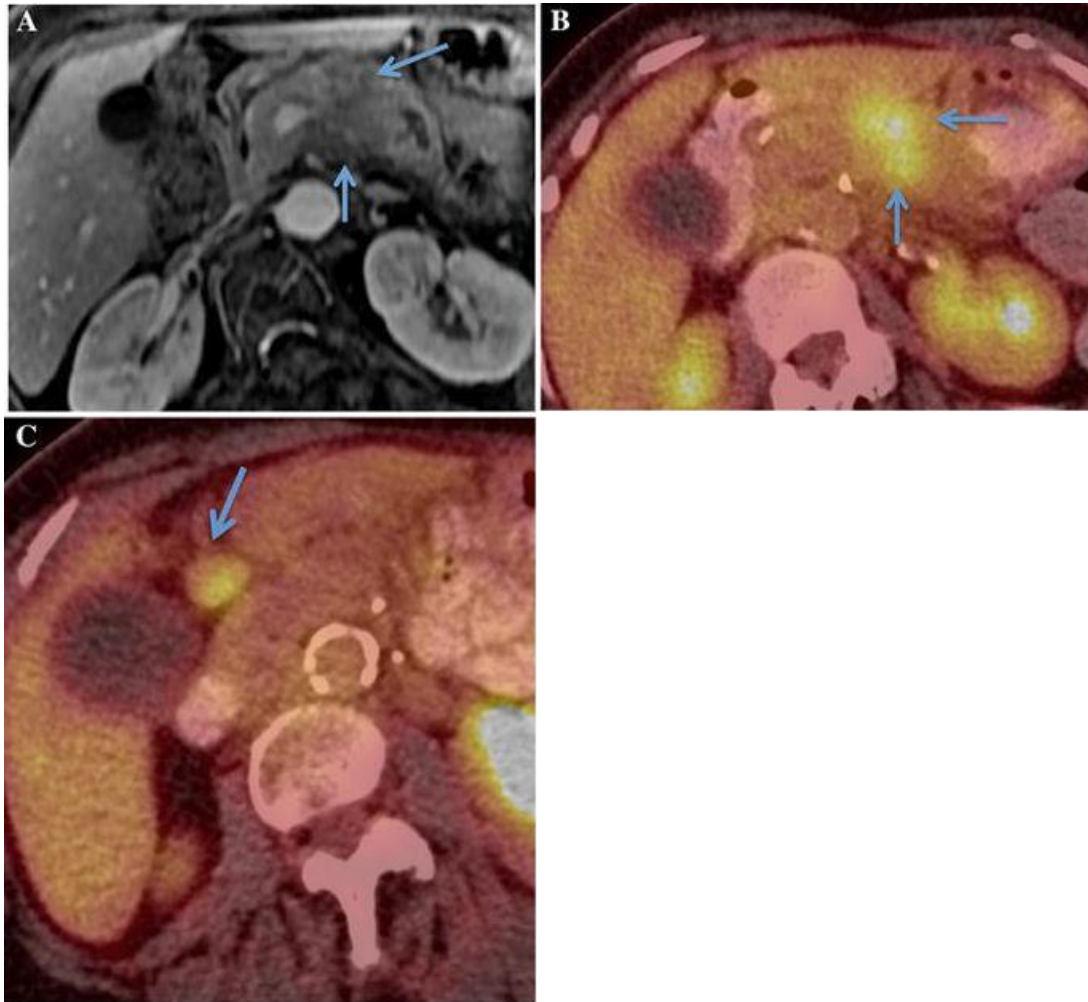
Figure 4.1: *Initial staging scans of an 80-year-old female diagnosed with PDAC. **A)** MRI scan identified hypoenhancing mass in the body of the pancreas, identified by arrow **B)** PET/CT showing avid FDG uptake in primary mass, corresponding with MRI mass, identified by arrows **C)** An avid FDG peripancreatic lymph node identified by PET/CT (Modified from Yeh et al., 2018).*

4.1.1.1.3 Biopsy

Often in cancer, the only definitive proof of malignancy, rather than benign tissue, or inflammatory disease, is a histological confirmation given how ambiguous blood test and imaging results can be. Biopsies and cell brushings are methods to collect sufficient tissue for a diagnosis. There are several techniques available to do this. Endoscopic techniques include endoscopic ultrasound (EUS) and endoscopic retrograde cholangio-pancreatography (ERCP) are used to further image the pancreas and simultaneously collect tissue biopsies or cytology for histopathological analysis. To date, endoluminal ultrasonography (EUS) is one of the most sensitive imaging techniques used for the visualisation of the pancreas and is often used in combination with biopsy-collecting techniques such as fine needle aspiration (FNA) (Harris and Buscaglia, 2010). EUS combines the endoscope's ability to reach the organs along the gastrointestinal tract with an ultrasound probe which can visualise smaller structures more easily for elucidation (The National Pancreas Foundation, 2014). Benefits include that its high resolution has been shown to identify small tumours between 2 and 3 mm (Helmstaedter and Riemann, 2008). Additionally, combination with FNA allows for the aspiration of a biopsy sample for immediate histological assessment and in combination with EUS, has shown to have an accuracy ranging from 71 – 90 % (Harris and Buscaglia, 2010). Although highly accurate, this technique isn't without its limitations. EUS can only be used to identify local metastases, but when combined with larger scale imaging such as a CT or MRI it paints a fuller picture. Questions have also been raised due to the extensive training required for the effective use of this technique and for the resulting images to be meaningful (Handgraaf *et al.*, 2014). Similarly, this technique is invasive and does not come without risks. Complications that have been attributed to EUS-FNA include bleeding, iatrogenic pancreatitis, and perforation with a 3% cumulative prevalence (Majumder *et al.*, 2012). Predictions state the use of core needle biopsies in the future instead of FNA to collect a larger sample of tissue whereby other aspects including the stroma could be analysed (Vera *et al.*, 2016). ERCP also allows the endoscopist to collect biopsies through similar techniques as EUS and carries the associated risks.

Suspected metastatic deposits, which are unable to be confirmed to be malignant from scans alone, and where the primary has been identified but not confirmed by

histology, would require biopsies before treatment could initiate. If the metastatic deposit is seen on CT scans, it is possible for biopsy to be taken through ultrasound guided biopsy by a trained consultant radiologist. If no metastatic disease is found on imaging, but it is suspected due to very elevated CA19-9 blood test results, a staging laparoscopy may be carried out (keyhole surgery). The minimally invasive exploratory surgery examines the inside of the abdomen to decide whether the PC is resectable in the future. Use of a staging laparoscopy has identified occult metastases, which were not present on imaging studies, in 15 – 51% of PC patients at high risk for unresectable disease (De Rosa *et al.*, 2016).

All biopsies and brushings are analysed by a trained pathologist to confirm or deny the presence of cancer. Often, cancer is not confirmed until after resection or surgical biopsy. Pancreatic surgeries are now focused in regional hepato-pancreato-biliary centres, where a specialised team of consultant surgeons, radiologists, and pathologists along with dedicated nursing staff allow for better specialised care for the complex pancreatic needs. Pathologists with special interest in pancreatic pathology assess tumours both macroscopically and microscopically to report back official diagnoses.

4.1.1.2 PC Diagnosis Challenges

There are a variety of problems when it comes to diagnosing PC. Currently, 80% of patients are diagnosed once their disease is either locally advanced or metastatic, which is too late for surgical intervention (Lahoud *et al.*, 2016). Almost 50% of patients are diagnosed with stage IV disease (Public Health England and Cancer Research UK, 2018). As a result, only around 10% of patients diagnosed with PC had surgery as part of their treatment journey, whether alone or in combination with therapy (Public Health England and Cancer Research UK, 2018). Currently, radical surgical resection is the only curative treatment option for PC, so delays to diagnosis resulting in more advanced disease at diagnosis is detrimental and will contribute to the poor disease prognosis. Problems have been highlighted by NICE in their 2015 report "Suspected cancer: recognition and referral" (NICE, 2015). This report provides guidelines for clinicians to aim for earlier diagnosis of PC to ensure the disease is still treatable. While most patients are diagnosed late, there is no screening system in place for early detection of PC. With low incidence of PC and presentation

with vague, non-specific symptoms; it is not a cost-efficient method. Other malignancies, like oesophageal cancer, have ongoing endoscopic screening (Lakatos *et al.*, 2016). Questions have been raised to whether it would be worthwhile following up patients predisposed to developing PC such as those with familial links of PDAC.

One of the many factors, which contributes to the late diagnosis of PC is the lacking symptoms, or only having minor, vague symptoms which can be easily brushed away. Early stage disease is often asymptomatic and so the disease can progress undetected within the retroperitoneal space. Once symptoms present, the disease has often spread into surrounding organs and tissues. A late-presenting symptom is jaundice, observed in 50% of PC cases, caused by the head of pancreas tumour expanding, causing common bile duct obstruction (Tummala *et al.*, 2011; Cancer Research UK, 2016b). Some earlier, vague symptoms which can arise include fatigue, unexpected weight loss and poor appetite. They can start off unnoticeable and progressively get worse as the disease advances (Lahoud *et al.*, 2016). Often, these symptoms can just be brushed aside and ignored as just a part of the aging process. This results in patients presenting through emergency admission for pain or jaundice.

Questions have also been raised about the invasive nature of diagnostic techniques used for visualisation of the pancreas. This is particularly seen with endoscopic based approaches including EUS and ERCP mentioned above. There are risks of putting patients already in considerable pain through procedures which could result in side effects. Highly trained and skilled medical staff are also required to perform these procedures, which may not be available at local hospitals, hence travel to regional centres may be required.

More problems arise with diagnosing PC from differentiating benign and malignant disease. The blood-based approach, using the CA19-9 serum marker, which has been shown to be elevated in benign conditions lead to false positive results. Additionally, the risk of serum negative patients means the test could also give false negatives and not identify PC at all. This was seen in a case by Muratore *et al.* (2016) of a patient who underwent a total pancreatectomy and islet autotransplantation for a suspected case of chronic pancreatitis, who later went on to develop PDAC within the

transplanted regions of the liver 10 months post-transplantation. At the time of the operation, serum analysis showed CA 19-9 levels were insignificant at 7 kU/L, yet retrospective serum analysis on the same sample showed elevated levels of microRNAs associated with early stage PDAC (Muratore *et al.*, 2016). Imaging modalities also have problems differentiating between benign and malignant disease. Definitive proof of malignancy is often from histopathological examination of biopsies and cytology samples from the suspected tumour. However, sometimes tissue sampling is non-diagnostic. A study of 276 EUS patients found that 130 also had FNA to biopsy the suspected tumour. Of these, 67 patients had a positive confirmation of cancer, whereas 63 were negative for malignancy. Follow-up of the negative FNA patients found 17 with a final diagnosis of PC despite lacking pathology confirmation (Spier *et al.*, 2009). Lack of a tissue confirmation cannot rule out a diagnosis of PC. Often, final diagnoses are made following pancreatic resection.

4.1.2 Blood-Based Biomarkers

Blood-based biomarkers are small biological markers which circulate in the blood, which can be identified and analysed to provide information about the health of the patient. Biomarkers exist for several different physiological processes and diseases. There are several benefits of blood-based biomarkers. In comparison to a lot of other diagnostic and biomonitoring techniques, it is a much cheaper alternative with blood tests ranging in cost from 24p to £13.28 depending on service provider (Robinson, 2013). Blood tests also have a benefit of being a comparatively non-invasive method, especially considering endoscopic and surgical techniques. They are also an expected part of the healthcare system; regular blood tests are carried out during diagnostic investigation. Therefore, adding an additional biomarker into the battery of markers and metabolites already quantified and analysed would not be any extra commitment for the patient, but would require a little extra work from the haematology department. Blood collection is a common technique and can be carried out in local GP surgeries and health centres, which is more accessible for patients than some of the more complex investigations. Some blood-based biomarkers, such as the CA19-9 marker discussed prior, may not be suited for a biomarker alone but in combination with imaging, histology and other blood test results could help contribute to a full picture of disease. Alone, CA19-9 may be helpful as a tool to

stratify patients to determine those who are more in need of the more invasive and complicated procedures. This can be seen in patients with resectable PC from imaging but with CA19-9 elevated above 150 kU/L. These patients are selected to undergo staging laparoscopy to ensure there is no metastatic disease which would prevent a full pancreatic resection in the coming weeks (De Rosa *et al.*, 2016).

4.1.3 PIG-A Assay

The human erythrocyte PIG-A assay was discussed previously in chapters **1.3.2.5** and **1.3.2.5.1**. The assay monitors the mutation status of X-linked *PIG-A* through flow cytometric detection of GPI anchored proteins CD-55 and CD-59 on the erythrocyte surface.

4.1.3.1 Flow Cytometry

The mutation status of *PIG-A* is determined indirectly by flow cytometry. Flow cytometry as a technique has been in use since the 1970s and has matured over the time to become a sensitive and sophisticated technique (Büscher, 2019). It is a commonly used analytical technique, often associated with blood analyses and immunological studies, making it an ideal technique for analysis of surface proteins on erythrocytes. Thousands of cells can be analysed per second, allowing for millions of cells to be analysed in a short period of time. This is invaluable when assessing a rare event, such as the PIG-A mutation.

Flow cytometers are made up of several parts. From a basic perspective there is a light source to excite fluorophores, a flow cell, where particles are focused into a single file stream for analysis through hydrodynamic focusing, and a detector to record the fluorescence emitted by fluorophores (**Fig. 4.2**) (Büscher, 2019). In modern, complex machines, multiple lasers and detectors are used for more complex analyses.
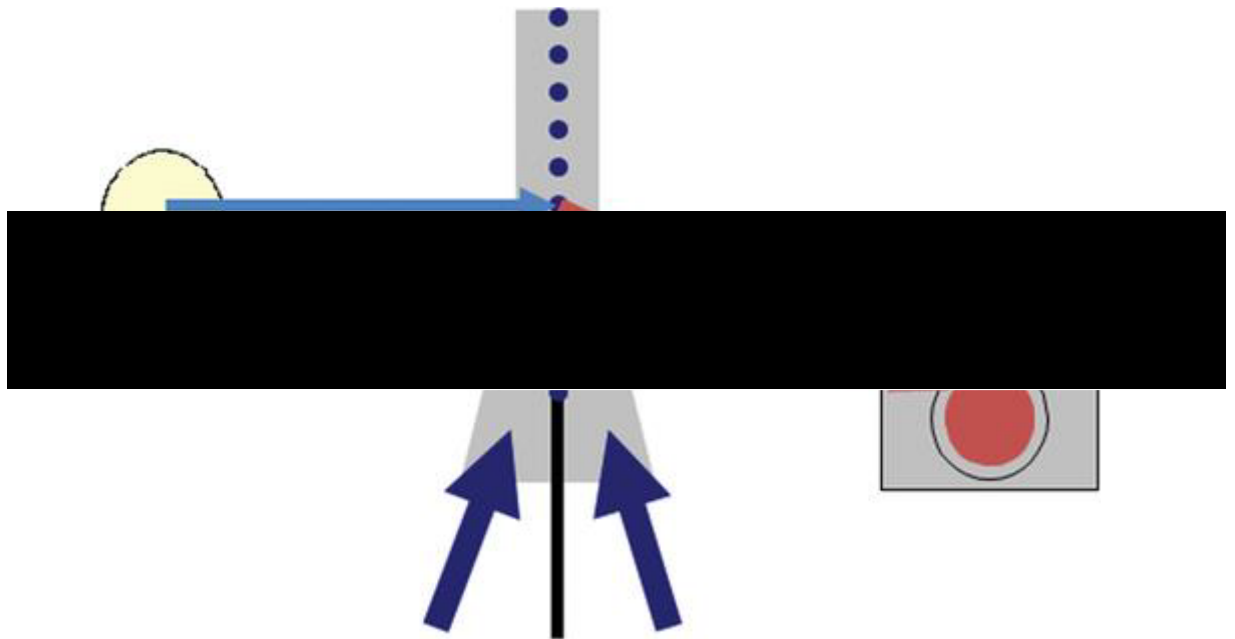
Figure 4.23: *Diagram depicting interior workings of a flow cytometer. A light source excites fluorophores in a single stream of focused particles, any fluorescence and light scattering is detected by the detector and sent to a computer for analysis* (Büscher, 2019)*.*

In this study, two fluorophores are used, conjugated to antibodies specific for CD235a, the erythroid marker, and CD55 and CD59, the GPI anchored proteins of interest. The two fluorophores of choice for this assay are allophycocyanin (APC) and R-phycoerythrin (PE), with the excitation and emission spectra found in **Fig. 4.3**. Both PE and APC are bright fluorophores, which allows for sufficient fluorescence intensity even with less expressed markers (Maecker *et al.*, 2004). Between the two, PE is the brighter fluorophore and was used in the PIG-A assay to identify the GPI anchored proteins CD-55 and CD-59 (Biolegend, 2020). When using multiple fluorophores in an experiment, it is important to ensure the excitation and emission spectra do not overlap to avoid complication and need for additional compensation to remove the fluorescence overlapping into alternative channels (Maecker *et al.*, 2004). The excitation and emission spectra of PE and APC are shown in **Fig. 4.3**. PE is excited at 495 nm and emits at 578 nm, around the yellow-orange range of visible light. In comparison, APC is excited at 650 nm and emits light at 660 nm, which is in the red wavelength of visible light. There is sufficient

distance between the two spectra to collect clear results.
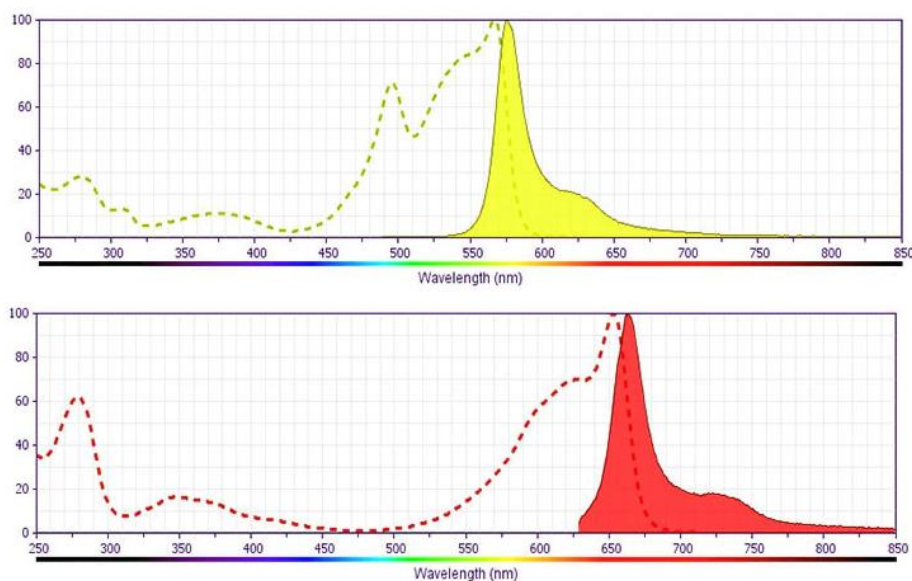
Figure 4.3: *Excitation and emission spectra of the two fluorophores used in this study.* ***A)*** *PE is excited at 495 nm and emits at 578 nm* ***B)*** *APC is excited at 650 nm and emits at 660 nm* (Absorption and Emission Spectra | BD Biosciences-US, 2020).

4.1.4 Aims

In this study, we aim to use the human erythrocyte PIG-A mutation assay in a cohort of patients with PC and benign pancreatic disease, not previously explored in detail in the literature. PC diagnosis remains a real clinical challenge and the search for novel blood-based biomarkers continues. The flow cytometry-based erythrocyte PIG-A assay quantifies GPI anchor deficient red blood cells by detecting GPI anchored CD-55 and CD-59 on the surface of red blood cells. GPI anchor deficient cells have been previously shown to have a mutation in the PIG-A gene. Quantifying GPI deficient erythrocytes provides a measure of circulating DNA damage, which has been used extensively in rodent genotoxicity studies and in a few healthy human population studies. We aim to determine whether patients with PC have an elevated level of circulating mutant red blood cells in comparison to healthy and benign control donors, and whether this has any potential in diagnosing PC.

4.2 Methods

4.2.1 Ethics and Permissions

The study was planned, and documents were prepared for submission for research ethics committee (REC) approval. A protocol, patient informed consent form, patient information sheet and modified validated questionnaire (**Appendix 7.1.3**) were submitted via the integrated research application system (IRAS) (IRAS project ID:218936) to the Plymouth and Cornwall REC (REC reference: 17/SW/0012). Favourable opinion was granted on the 25th January 2017.

Valid informed consent was granted from patients with benign pancreatic conditions including pancreatitis and pre-malignant conditions, and patients with malignant pancreatic disease. Patients donated a completed lifestyle questionnaire and 10mL whole blood in a sodium-heparin coated blood tube by venepuncture. Samples were returned to the university for analysis.

4.2.2 The Human Erythrocyte PIG-A Assay

4.2.2.1 Blood Preparation

10 mL of venous whole blood was collected in sodium-heparin coated Vacutainer (Becton Dickson) tubes from consenting blood donors. Each tube was gently inverted 5 times. 10 µL whole blood pipetted into bottom of 15 mL centrifuge tube: once for an unstained instrumental calibration sample (ICS), and in triplicate for stained samples. Care was taken to ensure no blood was caught up the sides of the centrifuge tube to ensure thorough staining. Stained triplicates were stained with 5 µL APC anti-CD235a (diluted 1:10) (BD Pharmingen) (0.1 µL/10^6 cells), 20 µL PE anti-CD59 and 20 µL PE anti-CD55 (both BD Pharmingen) (0.4 µL/10^6 cells). Samples were mixed through gentle pipetting. Mixtures were incubated at room temperature, protected from light for 30 minutes.

Following 30 minute incubation, samples centrifuged at 500 g at room temperature for 5 minutes with full breaks and acceleration. Supernatants removed, with care taken not to disturb cell pellets. Pellets were washed twice in 2 mL antibody wash buffer (0.2 % BSA (Sigma) in PBS (Gibco)) using same centrifuge settings. Cell pellets were suspended in 1 mL antibody wash buffer (0.2 % BSA (Sigma) in PBS

(Gibco)) and transferred to flow cytometry tubes before immediate flow cytometry analysis.

4.2.2.2 Flow Cytometry

Initially, PIG-A blood samples were run on two flow cytometers: the FACSAriaI and the Beckman Coulter Navios. Unfortunately, over the course of the study, the FACSAriaI broke beyond repair and so all remaining samples ran solely on the Navios system.

4.2.2.2.1 FACS Aria

The BD FACSAria I (BD Biosciences) collected 100,000 events for the unstained ICS and 1,000,000 events positive for APC and PE for stained samples. A low flow rate and higher number of events used for stained samples since the PIG-A mutant phenotype is rare event. Data was collected and analysed using BD FACSDiva software, version 8.0 (BD Biosciences). Gates were drawn dependent on ICS. Gated population in the stained sample had positive staining for both APC and PE were determined to be wild type erythrocyte population. APC positive and PE negative population were concluded to be PIG-A mutant erythrocyte population and were quantified per million of wildtype erythrocytes.

4.2.2.2.2 Navios

The Navios (Beckman Coulter) flow cytometer analysed samples in a similar way to FACSAria seen in **4.2.2.1**. Analysis was carried out using the Navios software (Beckman Coulter). Gating techniques remained the same with gates on stained samples drawn in respect to the unstained ICS. APC and PE positive population determined to be wildtype erythrocytes and APC positive, PE negative population determined to be PIG-A mutant erythrocytes, **Fig 4.4**. Mutant erythrocytes quantified per million wildtype erythrocytes. The only difference is all samples were analysed for 12 minutes, including the ICS, rather than for 1,000,000 events. More than 1,000,000 events were captured per sample.
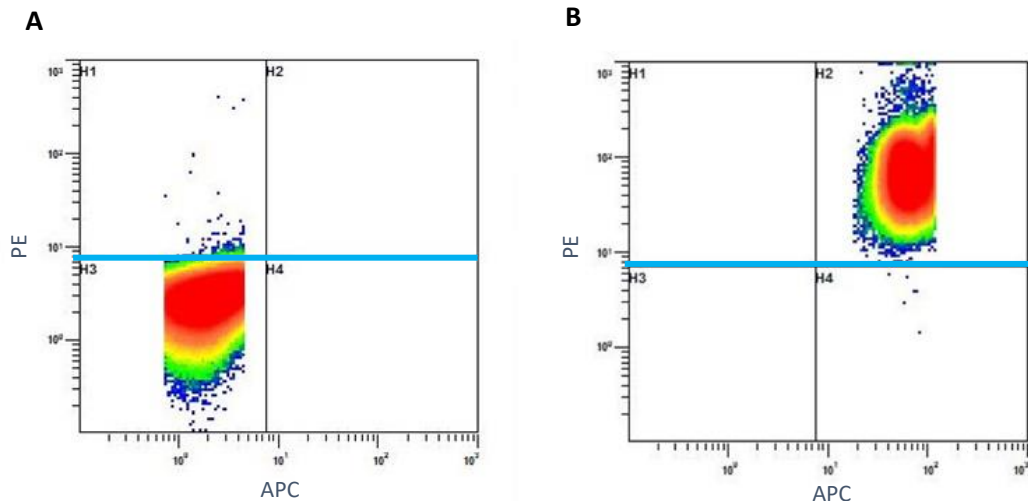
Figure 4.4: Example cytograms from a healthy donor A) Unstained ICS which gates(blue line) are drawn by B) stained sample, showing wildtype red blood cells in H2 quadrant, mutant cell population in quadrant H4. In this example, 7 mutant red blood cells per 1.4 million wild-type red blood cells, or 5 x 10^{-6} PIG-A mutant erythrocytes.

4.3 Results

4.3.1 Healthy, Benign and Malignant PIG-A

In total, six healthy donors, 10 donors with benign pancreatic disease and 30 donors with pancreatic cancer provided blood for the human erythrocyte PIG-A mutation assay (**Fig 4.5**). The median PIG-A mutant frequencies for healthy donors were 4.55 (95% CI 1.48-6.51) mutant red blood cells per million, similar to reported values from healthy population studies in the literature. Donors with benign pancreatic disease showed a median PIG-A mutant frequency of 2.64 (95% CI 2.11 - 4.16) per million red blood cells. The benign conditions of these donors included pancreatitis, pancreatic cysts, IPMN and PanINs. Comparison of healthy and benign donors using a non-parametric Mann Whitney U test determined there was no statistical difference between the two groups (U = 27, p = 0.745). The median mutant frequency for PC patients is 5.81 (95% CI 6.25 - 10.86) PIG-A mutant red blood cells per million. A non-parametric Kruskal-Wallis test showed a statistical difference between the groups of blood donors (p = 0.021) with mean ranks of 16.67, 15.10 and 27.03 in the groups. A significant increase in mutant red blood cells is seen between patients with

benign and malignant pancreatic disease (p = 0.013). Although an increase between the mean values in the healthy and malignant group, the comparatively closer median values and overlapping confidence intervals in the two groups results in a non-significant increase (p= 0.078). With a bigger population of donors (particularly the healthy volunteers), perhaps this increase would be significant.
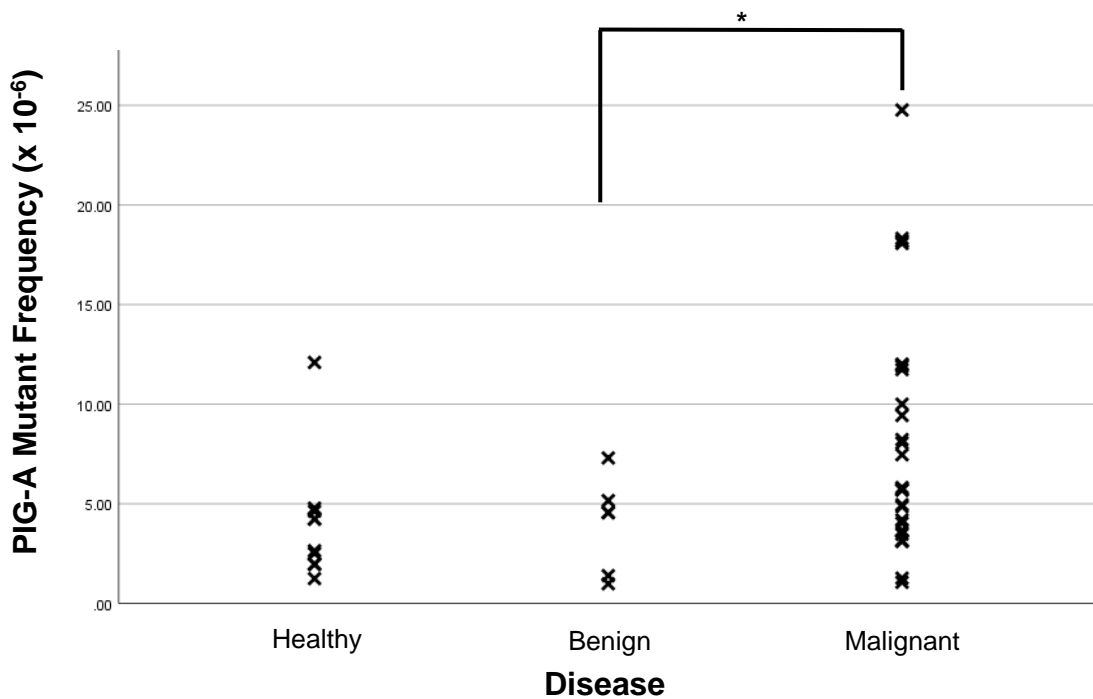
Figure 4.5: *Dot plot comparing PIG-A mutant erythrocytes frequencies in donors who are healthy (n=6), with benign (n=10) or malignant pancreatic disease (n=30). Donors with cancer of the pancreas have a higher frequency of mutant erythrocytes. (p<0.05 signified with *)*

4.3.2 TNM Tumour Staging

TNM tumour staging is used to define the size of the tumour, lymph node involvement and whether the disease has metastasised, providing a more detailed view of the disease. The TNM staging currently in use is the AJCC/UICC 8th edition, which is defined in **Appendix 7.4**.

T-stage defines the primary tumour size. T-staging is further defined in **Appendix 7.4**. As T-stage increases the tumour size does. **Fig. 4.6** explores the PIG-A mutant frequency in PC donors with increasing T-stage. No clear pattern is seen as tumour size increases. An increase is seen at T4 with an PIG-A mutant frequency of 11.9 x

10^{-6}, but only one donor had T4 staged tumour so more donors would be needed to confirm this increase. A Kruskal-Wallis test showed there was no statistical significance and that T-stage of disease did not increase the amount of mutant red blood cells (p=0.472).
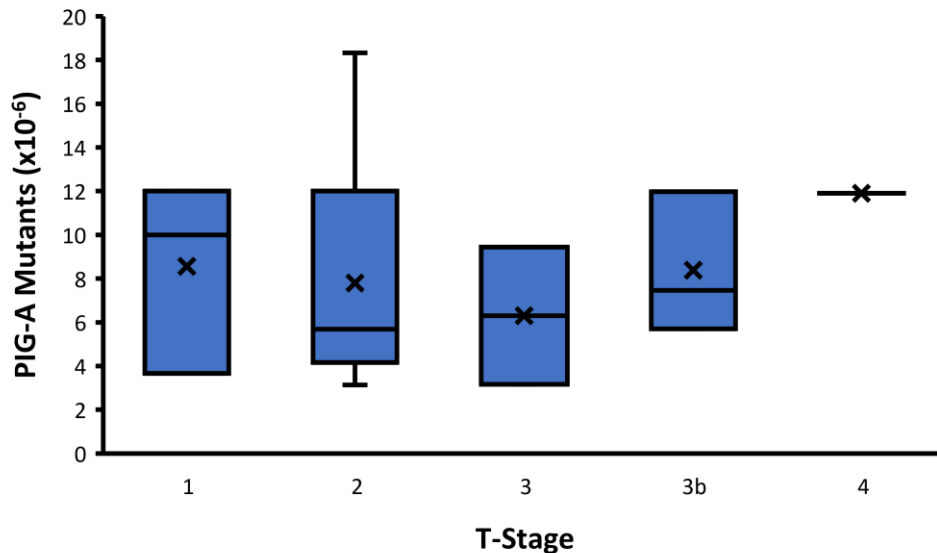
Figure 4.6: *PIG-A mutation frequency in PC patient donors with increasing tumour size (T-stage) (n = 3 T1, n = 9 T2, n = 2 T3, n = 3 T3b, n = 1 T4). Crosses show the mean values.*

Lymph node involvement in disease increases as the cancer becomes more advanced and begins to spread. Definitions of N-staging is found in **Appendix 7.4**. No correlation is seen with the lymph node involvement of the tumour and the mean PIG-A mutant frequency, **Fig. 4.7**. The median values start lower for N0 disease with 5.69 x 10^{-6}, it is then higher and comparable for N1 and N2 disease with medians of 7.46 and 7.58 PIG-A mutant per million erythrocytes, respectively. A Kruskal-Wallis statistical test showed non-significance overall (p = 0.7), with no clear correlation between nodal involvement and the frequency of PIG-A mutant red blood cells.
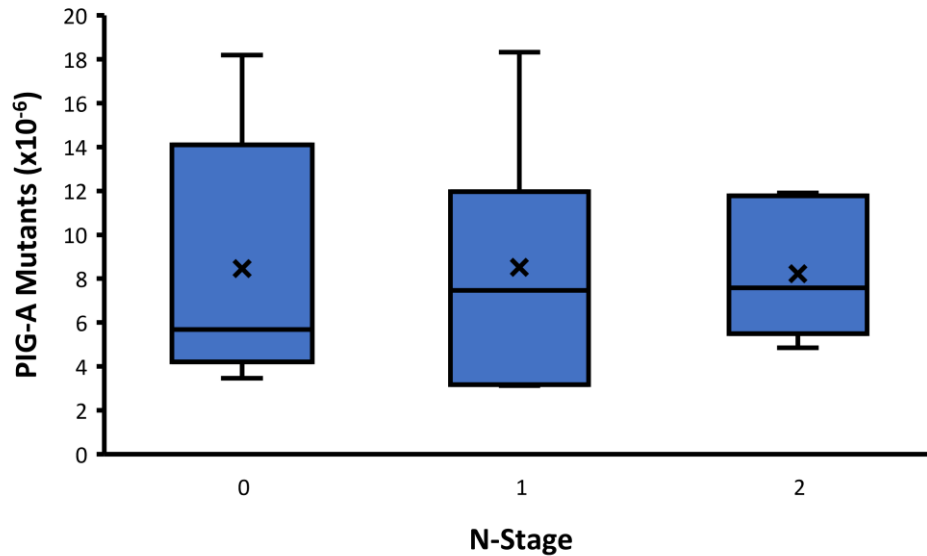
Figure 4.7: *PIG-A mutation frequency in donors with PC with increasing lymph node involvement (n = 5 N0, n = 7 N1, n = 6 N2). Crosses represent the mean.*

Metastasis has been shown to increase the mean frequency of PIG-A mutant erythrocytes in comparison to patients with no metastatic disease, **Fig. 4.8**. Donors with localised disease had median of 6.60 (95% CI 6.01 - 10.81) mutant red blood cells per million analysed in comparison to 8.22 (95% CI 5.20 - 19.69) mutant red blood cells per million in donors with metastatic disease. A Mann Whitney U statistical test showed this increase in PIG-A mutant erythrocytes was non-significant (p = 0.226).
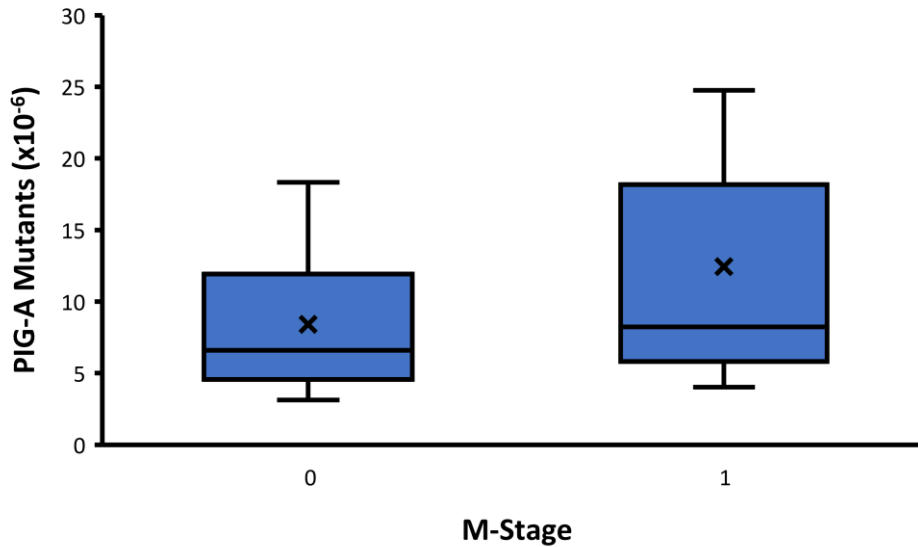
Figure 4.84: *PIG-A mutant frequency in donors with PC with patients with localised disease (0) or metastatic disease (1) (n = 18 M0, n = 7 M1). Crosses represent the mean.*

From individual TNM staging of donors, numerical tumour stages were determined, **Appendix 7.4**. Of the patients with staging data available, donors were categorised as follows: 2 patients as stage IA, 4 were stage IB, 6 were IIB, 6 in stage III and 7 in stage IV. No donors were found to be stage IIA. Donors in stages IB, IIB and III had very similar levels of mean mutant red blood cells with 8.073 ± 6.804, 7.954 ± 6.137 and 8.228 ± 3.200 x 10^{-6} PIG-A mutant erythrocytes, respectively. Donors in stage IA had an elevated mean mutant frequency in comparison to higher stages, with 11.004 ± 1.421 x 10^{-6}. However, it should be noted that only two donors had the earliest stage disease, and a greater number of donors may alter this result. Median values of PIG-A mutant erythrocytes are high for stage IA, perhaps because only two donors had stage IA disease. Values for stages IB and IIB were more similar with 5.32 (95% CI 2.75 - 18.90) and 5.57 (95% CI 1.51 - 14.39), with a slight elevation at the more advanced stages III and IV with 7.58 (95% CI 4.87 - 11.59) and 8.22 (95% CI 5.20 - 19.69), respectively. Despite this elevation at more advanced stages, this was determined to be non-significant through the Kruskal-Wallis statistical test (p = 0.501).
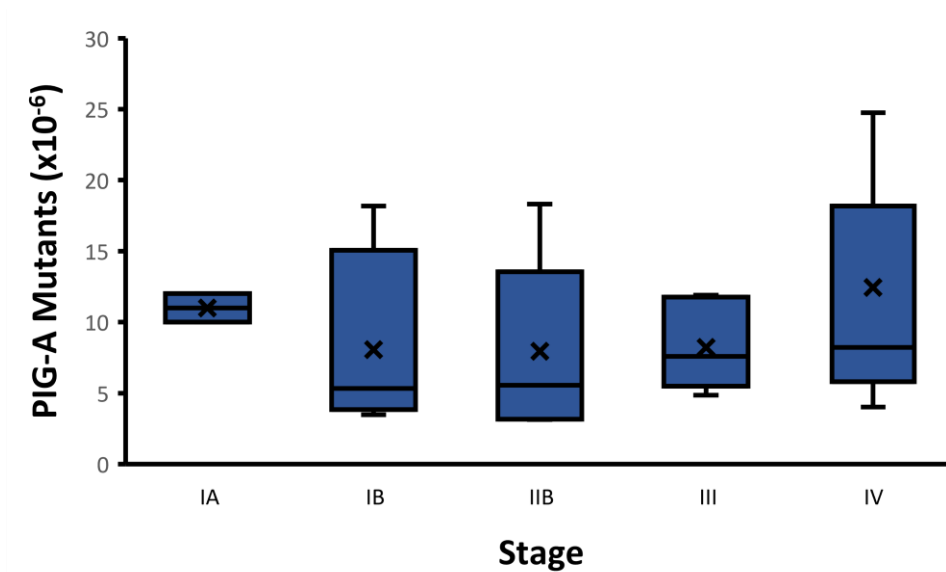
Figure 4.9*: Box and whisker plot of PIG-A mutant erythrocyte frequency as stage of PC increases (n = 2 IA, n = 4 IB, n = 6 IIB, n = 6 III, n = 7 IV).*

For the PC patients who underwent pancreatic resection as part of their disease journey, resection completeness was assessed. Five patients had complete resection with clear resection margins, but six had incomplete surgical resections where tumour was present on the resection margins microscopically. No difference was seen between the mutant PIG-A frequency and the completeness of the resection, **Fig. 4.10**.
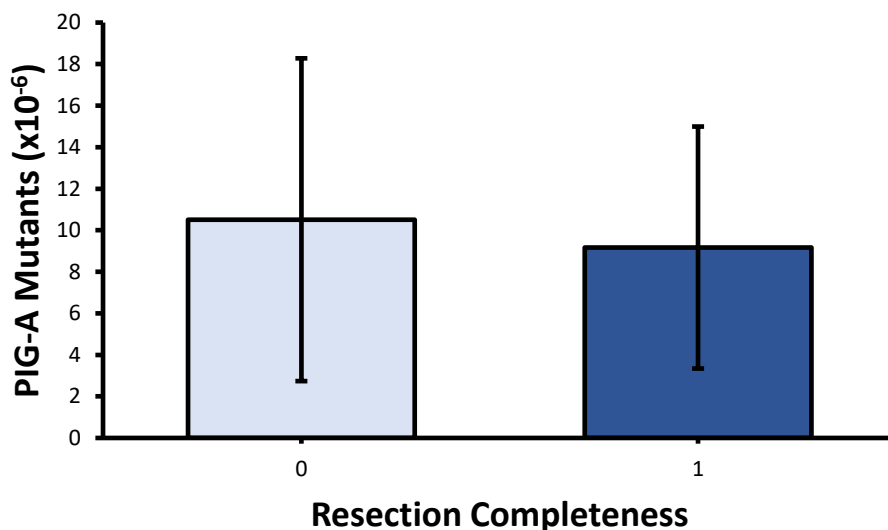
Figure 4.105*: Resection completeness of the PC patients who underwent pancreatic resection as part of their treatment. Completeness rated as 0, complete (n = 5), or 1,*

where microscopic tumour was present along resection margins (n = 6). Bars represent the mean and the error bars represent the standard deviation.

4.4 Discussion

In this study, we have explored the human erythrocyte PIG-A mutation assay in a novel population, i.e. patients with benign and malignant pancreatic disease, to determine whether there is an elevated number of circulating mutated red blood cells in PC patients. The human erythrocyte PIG-A mutation assay is in its infancy relatively, having previously been used extensively in rodent genotoxicity studies. The assay is straightforward, requiring only a trio of antibodies so the staining protocol is simple and quick, only requiring a 30-minute incubation step. Samples can go from arrival at the university laboratories to samples being run on the flow cytometer within an hour and a half. It is a high throughput method, analysing over 3,000,000 erythrocytes per donor over a 40-minute period. Batches of samples can also be run together to make it even more high throughput. It is also a cheaper alternative to other diagnostic techniques, with estimates from our laboratory that this test only costs £30 per patient, mainly due to fluorescent antibody costs. As well as being cheap it only requires 40 µL of blood in total per patient, considerably less than other tests require. In previous studies, clinical laboratories have been shown to receive 8.5 times more blood than is needed for analysis for full blood counts, with 12 times more blood received for electrolyte panels (Dale and Ruby, 2003). This resulted in average blood volumes being discarded of 2.8 mL/tube and 2.0 mL/tube for full blood counts and electrolyte analysis, respectively. Our blood test, which only uses a tiny volume of 40 µL could easily be added into the battery of tests run on a single blood sample used in a patient with suspected PC, reducing wasted blood samples while potentially providing further information which could be beneficial to the patient.

One drawback of the assay is that the determination of the mutation in *PIG-A* is solely down to the phenotypic lack of GPI anchored CD55 and CD59, rather than through studying the DNA directly. DNA cannot be studied in the mature erythrocyte since the nucleus and organelles are expelled during terminal erythrocyte maturation within the bone marrow (Moras *et al.*, 2017). Previous studies have shown in alternative nucleated blood cell populations that mutation in the PIG-A

gene has resulted in a GPI deficient phenotype (Dobrovolsky, *et al.*, 2008). The use of antibodies against two different GPI anchored proteins, CD55 and CD59, also ensures the lack of GPI-linked proteins is due a mutation involved in GPI anchor biosynthesis rather than in a random mutation in the gene for the markers themselves. For novel biomarkers, the necessary equipment should ideally be present in a clinical setting, especially if the equipment is not cheap to purchase or maintain. However, flow cytometry is now found within most large hospitals around the United Kingdom. For example, the Cardiff and Vale University Health Board have flow cytometry and use it for the processing of immunological samples, primarily for exploration of immunodeficiencies (CVUHB, 2016) and Swansea Bay University Health Board also uses flow cytometry. Haematology based flow cytometry is also used for the detection of PNH, the potentially fatal disease caused by a somatic PIG-A mutation, resulting in GPI deficient blood cells, which become lysed at the hands of the complement system. It is also diagnosed by flow cytometric detection of red blood cells lacking in CD-55 and CD-59, or by the use of fluorescently labelled aerolysin which binds GPI anchors to induce haemolysis (Besa, 2019). Since PNH diagnosis is currently available within the clinical setting using a near-identical set-up to our erythrocyte PIG-A assay, there is potential that this test could be rolled out into a clinical setting if proven to be suitable, although this is admittedly a long way off.

Through using the human erythrocyte PIG-A assay, the mutant frequency was established in healthy donors and donors with benign and malignant pancreatic disease. Healthy donors were found to have a median PIG-A mutant frequency of 4.55×10^{-6} (95 % CI 1.48 - 6.51) and patients with benign pancreatic disease were shown to have a very similar mutant frequency of 2.64×10^{-6} (95 % CI 2.11 - 4.16), no statistical difference was found between the two groups, **Fig. 4.5**. These mutant frequencies as well as being comparable to each other, were similar to reported mutant frequencies from healthy population studies including means of 2.9×10^{-6} and 5.25×10^{-6} and median value of 2.8×10^{-6} (Dertinger *et al.*, 2015; Cao *et al.*, 2016; Haboubi *et al.*, 2019). This suggests that patients with benign pancreatic disease have not accumulated significant DNA damage and mutation in their blood cells over the healthy population. In a rodent study, acute necrotizing pancreatitis was induced in a Wistar-albino rat population and DNA damage was assessed in

tissues using the comet assay. Peripheral lymphocytes were assessed and showed no significant differences in comparison to lymphocytes from healthy rats measured by tail intensity, moment or migration (Şahin *et al.*, 2007). Although this is in a separate blood cell population and a rodent model, it supports results seen in our donors with benign pancreatic disease. In contrast, a human study performed the comet assay on lymphocytes from acute pancreatitis patients and showed a significant induction of DNA damage in comparison to controls, which was also positively correlated with the severity of disease calculated by the Balthazar scoring system (Dur *et al.*, 2016). Conflicting evidence is available within the literature, this may come as a result of using alternative techniques for identifying DNA damage. The comet assay is a quantitative technique which studies single and double strand breaks within the DNA which has been in use for over 25 years. It is sensitive enough to detect even low level DNA damage in small samples (Tsai *et al.*, 2017). However, the comet assay may also detect transient breaks in the DNA induced by DNA repair processes. This normal physiological response may not ultimately lead to fixed DNA mutations. It may be sensitive enough to identify DNA damage in benign inflammatory conditions like pancreatitis, as well as in fully malignant disease. The PIG-A mutation assay may be less sensitive, since it studies mutation rather than damage, and only show an induction of mutation in malignant disease. More work on the PIG-A erythrocyte assay would have to be performed to establish this, especially given this is only a pilot study.

Patients with malignant disease have shown to have a significantly elevated PIG-A mutant frequency, median of 5.81 (95 % CI 6.25 - 10.86) PIG-A mutant red blood cells per million, over patients with benign pancreatic disease (p = 0.013), but not quite over healthy donors (p = 0.078), **Fig. 4.5**. An increase in PIG-A mutant frequency was also seen in oesophageal adenocarcinoma patients, who were shown to have a three-fold increase in mutant frequency in comparison to healthy and gastroesophageal reflux disease patients (Haboubi *et al.*, 2019). This is more than the 2.075-fold increase viewed in PC patients in comparison to benign patients. This increase in erythrocyte PIG-A mutant frequency viewed in this study is due to an accumulation of mutation and DNA damage which occurs over several years before tumour formation. Computer modelling has predicted the time frame from tumour initiation into a founding cell of the tumour to be an average of 11.7 years, although

most tumours aren't diagnosed until subclones with metastatic capabilities have already disseminated and formed metastatic deposits, a further 5 - 7 years later (Yachida *et al.*, 2010). This allows a window for diagnosis of 5 - 7 years when PC is present, and potentially treatable.

Questions have been raised about the sources of the PIG-A mutations seen in the erythrocytes of the population of PC patients studied. Mature erythrocytes lack nuclei since nuclei and other organelles are expelled from the cell during terminal erythrocyte maturation within bone marrow (Moras *et al.*, 2017). This suggests mutation in PIG-A would have occurred before the shedding of the nuclei before maturation. Prior studies have established a Pig-A assay in rodent bone marrow erythroid cell populations following treatment with known mutagens 7,12-dimethyly-benz[a]anthracene and N-ethyl-N-nitrosourea, both of which induced Pig-A mutant erythrocytes (Revollo *et al.*, 2018; Revollo *et al.*, 2019). The increase in both Pig-A mutant immature bone marrow erythroid cells and circulating mature erythrocytes suggests the mutation occurs within the bone marrow before erythrocyte maturation. If this is the case, some form of tumour-secreted factor may be responsible for mutations induced in the bone marrow erythroid precursors.

A study monitored proteins overexpressed within the secretome of PDAC cell line PANC-1 in comparison to control healthy pancreatic cell line HPDE cell line. Stable isotope labelling with amino acids in cell culture in tandem with liquid chromatography and DNA microarrays allowed for identification of 68 proteins expressed more than 1.5-fold more in the PDAC cell secretome than that of the healthy pancreatic epithelium (Grønborg *et al.*, 2006). Several upregulated proteins, with a wide variety of roles, had not been associated with PC previously (Grønborg *et al.*, 2006).

In particular, upregulated proteins cathepsin D and TNF-α were of significant interest in the Grømborg *et al*. (2006) study. Aspartatyl protease cathepsin D is found upregulated in several cancers, notably breast and colorectal cancers, and in a range of neurological diseases (Zaidi *et al.*, 2008). Secretome analysis of PC cell line PANC-1 revealed a 7-fold increase in cathepsin D expression in comparison to non-neoplastic pancreatic HDPE cell line (Grønborg *et al.*, 2006). A similar study explored the oesophageal squamous cell carcinoma secretome and identified a larger

population of 120 upregulated proteins in malignancy (Kashyap *et al.*, 2010). Cathepsin D was also observed to be upregulated in oesophageal squamous cell carcinoma, but with a lower 4.88-fold change in comparison to the 7-fold change viewed in PC (Grønborg *et al.*, 2006; Kashyap *et al.*, 2010). Cathepsin D's role in malignancy is complicated due to the protein acting as both a mitogen and a protease (Mahajan *et al.*, 2020). Mitogenic activity of cathepsin D is viewed in pancreatic and gastric cancers, where cancer cell proliferation and dissemination is caused by post-translational induction of cathepsin D by anterior gradient 2 (Chen *et al.*, 2017). Poorer prognosis was associated with higher cathepsin D expression within the pancreas in a study of 403 PC patients (Mahajan *et al.*, 2020). This was partially attributed to mitogenic activity of cathepsin D but also due to resistance to gemcitabine through cathepsin D's proteolytic activity and its complex regulation by acid sphingomyelinase through breakdown of the cathepsin D activator ceramide (Mahajan *et al.*, 2020).

Pro-inflammatory cytokine TNF-α was shown to be overexpressed in PC cell lines and levels are further elevated in cells resistant to chemotherapy (Zhao *et al.*, 2016). In circulation, TNF-α will encounter numerous cell types, including haematopoetic stem cells within the bone marrow. TNF-α plays a key pro-survival role in haematopoetic stem cells, yet this is not without risks (Yamashita and Passegué, 2019). TNF-α has shown to be mutagenic in the *in vitro* chromosomal aberration and CBMN studies through ROS mediated mechanisms (Yan *et al.*, 2006). Endogenous mitogens and mutagens, exemplified here with cathepsin D and TNF-α, upregulated in PC may circulate and induce PIG-A mutations within haematopoetic stem cells. Haematopoetic stem cell survival, expansion and erythropoiesis may lead to the circulation of fully mature PIG-A mutant erythrocytes. This may account for some of the PIG-A mutant erythrocytes viewed within our PC patient cohort within this study.

Within the cohort of cancer patients studied, patients with metastatic PC were shown to have an elevated number of PIG-A mutant red blood cells in comparison to patients with no distant metastases (**Fig. 4.8**). Despite an increase being seen, this was non-significant following Kruskal-Wallis statistical analysis (p = 0.472). This may become significant with a bigger patient population with metastatic disease. Patient recruitment, following ethical approval, was based within pancreatic surgery

clinics at a tertiary referral centre. Care was taken not to approach patients during their initial appointments where the diagnosis of PC may have been new. As a result, fewer patients with metastatic disease were recruited since following initial appointments, those with obvious metastatic disease from imaging were referred to local oncology departments for chemo/radiotherapy and they did not undergo surgery. All the patients here with metastatic disease were patients who we found to have metastatic deposits histologically confirmed during operation (staging or resection), which ultimately resulted in an abandoned resection and alternative palliative treatment options pursued. In further studies, the potential to recruit palliative PC patients from oncology departments before starting therapy would be beneficial to capture as many patients as possible to get a bigger population of patients with metastatic PC patients to get results which may become significant.

Cancer is a disease of mutation and accumulating DNA damage. PC is no exception. In this study, we have shown an elevated frequency of PIG-A mutant peripheral erythrocytes in comparison to healthy donors and donors with benign pancreatic disease. Circulating markers of DNA damage are hoped to reflect the state of the disease and may show some potential as diagnostic or staging tools. One study examined both the nuclear and mitochondrial DNA from lymphocytes from 48 cancer patients in comparison to 48 controls using quantitative PCR. Damage to the genome was detected through reduced amplification, indicative of lesions blocking the action of DNA polymerase (Jansen *et al.*, 2015). PDAC patients were shown to have significant reduction in mitochondrial DNA damage (p = 0.03) and borderline significant reduction in damage in nuclear DNA (p = 0.08) (Jansen *et al.*, 2015). It was suggested that the increased oxidative stress in cancer in turn increased the DNA repair capacity of the lymphocytes. This reduction of DNA damage contrasts our findings of an increased DNA mutation in peripheral red blood cells. Not only does cellular DNA mutation show potential in diagnosing PC, cell-free DNA has been gaining interest. Analysis of cell-free DNA in 112 patients with pancreatic lesions (including 70 PDAC patients) showed hot spot mutations in KRAS, which were only present in malignant disease or pre-malignant patients not in healthy controls (Xiaoyu Liu *et al.*, 2019). Additionally, cell-free DNA has shown the ability to diagnose early stage PC, with cell-free DNA reflecting mutations seen within the primary tumour. Interestingly, the Liu *et al.* (2019) study also showed that cell-free

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DNA concentrations did not reflect the CA19-9 levels or overall disease staging but was correlated with the size of the tumour in those with localised disease. Blood-based biomarkers have shown potential for use in pancreatic disease, further research is needed to identify new markers and to investigate their usefulness.

Given the limitations of current widely used CA19-9 as a biomarker for PC, previously discussed, it cannot be fully relied upon alone for PC diagnosis. When used in conjunction with additional biomarkers for other clinical features including systemic inflammation, it may perform better than the single biomarkers alone to provide further information and provide a more rounded picture of the patient's disease (Brand *et al.*, 2011). One study established a panel of biomarkers for PC consisting of the trio of biomarkers CA19-9, ICAM-1 and osteoprotegerin. Within a training dataset, PC patients were differentiated from healthy controls and benign patients with sensitivity and specificity of 88% and 90% for healthy donors and 76% and 90% for benign donors, respectively (Brand *et al.*, 2011). Furthermore, a validation set showed great specificity for PC over breast, lung and colon cancer with specificities of 100%, 97% and 97%, respectively (Brand *et al.*, 2011). This is superior over CA19-9 as a single biomarker, with 81% sensitivity and 90% specificity with the cut-off value of 37 kU/L (Thaker *et al.*, 2014). We aim to explore a novel biomarker panel in the next chapter to determine whether the combination of CA19-9 with the novel biomarker PIG-A, explored within this chapter, shows any improvement upon single biomarker use. Additionally, we aim to add in c-reactive protein and the neutrophil-to-lymphocyte ratio to the biomarker panel. As markers of clinical and sub-clinical inflammation, it may be beneficial due to the strong inflammatory links in PC development.

4.5 Conclusion

PC diagnosis continues to be a challenge to clinicians due to the late presentation, vague symptoms, and aggressive nature of the disease. The erythrocyte PIG-A assay has been used extensively in rodent genotoxicity studies and less frequently in human population studies. More recently, the assay has been used in two studies monitoring cancer cohorts. In this study, we aimed to examine a population of patients with pancreatic disease, both benign and malignant, to determine whether the assay has any potential in diagnosing PC. In this study, 46 donors: 6 healthy

volunteers, 10 patients with benign pancreatic disease and 30 PC patients; donated heparinised whole blood which was analysed through the PIG-A mutation assay. Healthy donors and patients with benign disease had comparable median mutation frequencies of 4.55 (95 % CI 1.48 - 6.51) and 2.64 (95 % CI 2.11 - 4.16) mutated red blood cells per million, in keeping with reported values from population studies in the literature. PC patients were shown to have a significantly elevated frequency of mutated red blood cells (5.81 (95 % CI 6.25 - 10.86)) over patients with benign disease (p = 0.013). Staging data on the tumours allowed the examination of any correlations with tumour size, nodal involvement, metastasis, overall staging, and resection completeness of those who underwent surgery. No significant differences were found trends were seen with PC patients with metastatic disease, and those with stages III and IV disease had a slight elevation. With larger patient cohorts, these results may have become significant. Regardless, the PIG-A assay has shown some potential for detecting those patients with pancreatic disease.

Chapter 5: Peripheral Blood Mononuclear Cell Cytokinesis Block Micronucleus Assay and Additional Biomarkers for Pancreatic Cancer

Chapter 5: Peripheral Blood Mononuclear Cell Cytokinesis Block Micronucleus Assay and Additional Biomarkers for Pancreatic Cancer

5.1 Introduction

5.1.1 The Cytokinesis Block Micronucleus Assay in Peripheral Blood Mononuclear Cells

The CBMN assay is a well-established technique for assessing chromosomal stability in a range of cell lines and primary cell cultures, as discussed previously in **1.3.1.6** and **3.1.5**. The induction of small, membrane bound vesicles containing fragmented DNA is studied following exposure to stimuli to determine whether the stimuli is genotoxic. As well as being established in numerous cell lines, primary human cells have also been explored using this assay. *Ex vivo* applications of the assay include monitoring induction of DNA damage induced through lifestyle and dietary factors in lymphocytes and buccal cells from cheek swabs (Bolognesi and Fenech, 2013; Sommer *et al.*, 2020). Human reticulocytes are also used in the CBMN assay and less commonly urine and nasal swab derived cells have been explored with the assay (Sommer *et al.*, 2020).

The CBMN assay has also been established in human peripheral blood mononuclear cells (PBMCs). PBMCs consist of lymphocytes and monocytes and are isolated from the buffy layer. Lymphocytes are white blood cells, which circulate through the lymphatic system and the blood (Blum and Pabst, 2007), consisting of B-cells, T-cells and natural killer cells. Only 2% of lymphocytes are present in blood, with the remainder present in organs and the lymphatic system (Blum and Pabst, 2007). Healthy adults should have lymphocytes within the range of 1.0 to 4.0 x 10^9 cells/L (Gloucester Hospitals NHS Foundation Trust, 2020). Deviations from this can signify infection or disease. Lymphocytosis, the elevation of lymphocyte counts, can be caused by infection, chronic inflammation of autoimmune disorders and blood or lymphatic malignancies (NCH Healthcare System, 2019). In contrast, low lymphocyte counts, lymphocytopenia, can be as a result of malnutrition, autoimmune disorders, chronic infections like AIDS and also blood and lymphatic cancers (MSD, 2020).

Through studying the induction of MN in circulating blood cells, genomic damage can be quantified whether the damage comes as a result of exposure to genotoxic

compounds, through disease states or simply from increasing age (Kisurina-Evgenieva *et al.*, 2016). A healthy population study in a middle aged Korean population monitored the MN induction in a study of 150 male donors and 150 female donors with insignificant medical history (Cho *et al.*, 2017). An increase in MN was seen with increasing age across both male and female donors, with female donors having an elevated frequency of MN above male donors with $2.69 \pm 1.18\%$ in comparison to $1.86 \pm 0.71\%$ ($p < 0.001$) (Cho *et al.*, 2017). A Croatian population study also determined women to have an elevated frequency of MN in comparison to male donors with frequencies of $0.516 \pm 0.298\%$ and 0.479 ± 0.344 %, respectively (Gajski *et al.*, 2018). Not only did age and gender show an influence on MN frequency, Gajski *et al.* (2018) also determined that the sampling period and sampling season influenced the frequency of MN. Male donors were found to have highest MN frequency during the summer, whereas female donors had peak MN frequency in Spring time (Gajski *et al.*, 2018). Additionally, Gajski *et al.* (2018) saw that nuclear buds (defined as MN-like protrusions from the main nuclei which haven't fully detached) were highest frequency in summer and winter for male and female donors, respectively. Radiation exposure was also observed to increase the MN frequency in the Croatian population study (Gajski *et al.*, 2018).

As well as monitoring genotoxicity in healthy populations, the assay has been used as a biomonitoring tool following exposure to occupational hazards. Industrial risks often involve exposure to potentially harmful chemicals or radiation over extended periods. A study of industrial radiographers who study the safety of materials through use of X-ray machines or gamma radiation emitting sources were assessed with the lymphocyte CBMN assay using 60 exposed workers and 40 non-exposed controls (Shakeri *et al.*, 2017). Not only did exposed workers show an elevated frequency of MN in comparison to control workers, a dose dependent increase in MN was seen with workers exposed to increasing radiation doses (Shakeri *et al.*, 2017). Further studies have explored the lymphocyte CBMN assay in areas with naturally occurring high background radiation including Kerala in southern India, in both adult (Karuppasamy *et al.*, 2016) and new-born populations (Das and Karuppasamy, 2009); as well as west Sulawesi, Indonesia (Syaifudin *et al.*, 2018). Industrial chemical exposure has been explored using the lymphocyte CBMN assay. Coke oven workers have also been shown to have an increased MN frequency in

comparison to control workers in a population in Northeast China with frequencies of 0.74 ± 0.43% and 0.30 ± 0.30%, respectively (Cheng *et al.*, 2009). Exposure to styrene, polycyclic aromatic hydrocarbons and common pesticides have also been explored (Bolognesi and Holland, 2016; Costa *et al.*, 2016; Sram *et al.*, 2016).

Accumulation of DNA damage, as quantified in the lymphocyte CBMN assay, can lead to the development of disease. This has been explored in several benign and malignant diseases. Cardiovascular disease is a prime example of disease explored using the assay, given established links between atherosclerotic plaque development and genomic instability from oxidative stress (Andreassi *et al.*, 2011). Both coronary artery disease and ischaemic heart disease were shown to have elevated frequency of micronuclei in lymphocytes in comparison to healthy donors (1.19 ± 0.17 %, 0.59 ± 0.12 % and 0.36 ± 0.07 %, respectively) (Botto *et al.*, 2001). An additional study of patients with dilated cardiomyopathy, confirmed through ultrasonography, were shown to have an elevated frequency of micronuclei in comparison to an age and sex matched control population ($p < 0.001$) (Sitaraman *et al.*, 2014). Neurodegenerative diseases have also been explored using the lymphocyte CBMN assay, since genome instability has been shown to increase the risk of neurodegeneration (Lee *et al.*, 2015). A study compared the circulating DNA damage in patients with pre-disease mild cognitive impairment and fully developed Alzheimer's disease to establish whether genome stability gets progressively worse as disease severity increases (Lee *et al.*, 2015). Although no significant overall difference was seen between patients with mild impairment and Alzheimer's disease with MN frequency, a significant decrease was seen when mental state was examined, suggesting a link with declining cognitive ability (Lee *et al.*, 2015).

Malignant disease has also been explored, given cancer is a disease resulting from an accumulation of DNA damage. A study examined 44 Serbian patients with breast, uterine or pharyngeal cancers in comparison to a benign healthy donor cohort. Cancer patients were shown to have an elevated frequency of MN with 1.518 ± 0.505% in comparison to healthy donors with 0.645 ± 0.275% ($p < 0.001$) (Milosevic-Djordjevic *et al.*, 2010). Interestingly, no differences were viewed when examining gender, primary tumour site or smoking status of donor (Milosevic-Djordjevic *et al.*, 2010). Elevated MN frequency has been linked with increased risk of developing cancer, due to an increased burden of DNA damage. Studies have

explored the elevated MN frequency, showing increased risks of developing bladder, thyroid and colorectal malignancies (Bayram *et al.*, 2014; Ionescu *et al.*, 2015; Pardini *et al.*, 2017). Furthermore, a large international study, collating results from the HUMN studies, established that an elevated frequency of MN was linked with an increased risk of cancer development (Bonassi *et al.*, 2007). However, when specifically observing pancreatic and hepatobiliary cancers, Bonassi *et al* (2007) saw a reduced risk of cancer development with increasing MN frequency, although this was non-significant (p = 0.27). Studies have also monitored the MN response to oncological therapies. A study following patients with metastatic colorectal cancer through their systemic therapy journeys and recorded the frequency of MN in peripheral lymphocytes before, during and after therapy over a six-month period (Nikolouzakis *et al.*, 2019). Patients who responded in similar manners to the therapy were shown to have a similar pattern in MN frequency across the six-month period, often with a dip in the middle and increase at final reading, making a v-shaped pattern. Patients with shallower 'V' trends having better response to therapy than non-responders, who had the deepest 'V'(Nikolouzakis *et al.*, 2019). This allows for potential prediction of therapeutic response from the assay as well as radiological imaging. Radiotherapy and chemoradiotherapy have also been explored in patients with head and neck cancers, with patients treated with chemoradiotherapy showed an elevated frequency of MN in binucleated lymphocytes, whereas radiotherapy alone did not show an elevation in comparison to healthy controls (Unal *et al.*, 2016). However, limitations of this study, including the sampling time post-therapy which ranges from 60 to 239 months after therapy, may limit the reliability of data (Unal *et al.*, 2016). PC has also been examined using the CBMN assay, with PC displaying elevated frequencies of peripheral lymphocytes with MN (Chang *et al.*, 2004; Chang *et al.*, 2011).

5.1.2 Other Biomarkers for PC

A range of biomarkers are currently in use for PC, although their uses are limited. Given the diagnostic challenges and poor prognosis, the search for novel PC biomarkers continues in the hope to improve the dire outlook for PC patients. A panel of biomarkers, using multiple in combination, may improve diagnostic capabilities in comparison to lone biomarkers.

5.1.2.1 CA19-9

CA19-9 is a clinically used serum biomarker for PC, further discussed in **4.1.1.1**. Elevated CA19-9 levels can be as a result of PC, but also due to a number of benign conditions including diabetes mellitus, jaundice and pancreatitis (Howaizi *et al.*, 2003).

5.1.2.2 Neutrophil to Lymphocyte Ratio

From routine full blood counts the neutrophil to lymphocyte ratio (NLR) can be calculated by division of number of neutrophils by number of lymphocytes (Gürağaç and Demirer, 2016). This provides a measure of sub-clinical inflammation within the patient (Gürağaç and Demirer, 2016; Faria *et al.*, 2016; Forget *et al.*, 2017). A healthy population study of 413 non-geriatric donors resulted in a mean NLR of 1.65 ± 1.96 with a range of 0.78 to 3.53 (Forget *et al.*, 2017). NLR values above this signifies increased inflammatory stress in the patient (Farkas, 2019).

Patients with inflammatory conditions, cancers and systemic infections have been shown to have elevated NLR (Gürağaç and Demirer, 2016). Autoimmune inflammatory conditions including rheumatoid arthritis and Behçet's disease were shown to have increased NLR, associated with increased disease severity (Chandrashekara *et al.*, 2017; Hammad *et al.*, 2018). Additionally, NLR has been shown to be a predictor for metabolic syndrome (Liu *et al.*, 2019).

NLR has been shown to be elevated in a range of malignancies (Gürağaç and Demirer, 2016). The immune system plays a complex role in carcinogenesis. Neutrophils play an active role in a tumour microenvironment, displaying both pro- and anti-tumour properties (Wu *et al.*, 2019). Tumour promoting activities of neutrophils include the production of ROS, chemokines, cytokines and enzymes which increase the chance of developing cancer (Wu *et al.*, 2019). In PC, neutrophils secrete the cytokine TGF-β, which in turn increases collagen production, to contribute to the high levels of desmoplasia displayed in pancreatic malignancies (Aoyagi *et al.*, 2004). In contrast, lymphocyte infiltration in primary tumours has shown some prognostic value in some solid tumours (Hendry *et al.*, 2017). NLR has shown potential when studying malignancies of the breast, kidney, colon, ovary and the pancreas (Faria *et al.*, 2016).

Both neutrophils and lymphocytes have been implicated in previous PC studies. Immunohistochemical studies of PC established high neutrophil concentration within the tumour microenvironment was linked with poorer disease prognosis in comparison to those with high levels of lymphocyte infiltration (Hendry *et al.*, 2017). Various studies have evaluated the NLR's use in PC. In a study of 206 PC patients, NLR was found to be significantly associated with overall survival of patients through both univariate and multivariate analysis (Piciucchi *et al.*, 2017). Additionally, within all patients and patients with metastatic PC, Piciucchi *et al.* (2017) determined that patients with NLR below 5 had increased survival in comparison to patients with greater NLR values. A meta-analysis determined that NLR is a useful prognostic tool within both metastatic and localised PC patients, but usefulness can be limited due to both false positives and negatives (Mowbray *et al.*, 2018). In contrast, a recent study combined the use of NLR with CA19-9 results within PC to improve upon NLR as a lone prognostic indicator (Sakamoto *et al.*, 2018).

5.1.2.3 CRP

CRP, like NLR, is another biomarker of clinical inflammation. CRP was first discovered in 1930 from serum of patients infected with pneumococci, which reacted with 'fraction C' containing a carbohydrate antigen of the capsule, hence the name C-reactive protein (Tillett and Francis, 1930). CRP is produced and released from hepatocytes in the liver in response to inflammatory IL-6 (Agalianos and Dervenis, 2017). CRP transcription is induced by IL-6, primarily through activation of transcription factors STAT3 and C/EBPβ (Voleti and Agrawal, 2005). It is an acute phase protein, produced during inflammation or infection where concentrations rise and fall rapidly dependent on presence of inflammatory stimulus (Nehring *et al.*, 2020). It has been shown to increase in concentration as much as 3000-fold in response to stimuli (Ansar and Ghosh, 2013). Elevated CRP levels therefore indicate systemic inflammation (Inoue *et al.*, 2015). CRP plays roles in activation of the complement system through binding to its ligands of both phosphocholine, a common component in cell wall antigens of infectious agents such as pneumococci; and autoantigens including apoptotic cells, chromatin and histones (Szalai, 2004).

A healthy Chinese population study evaluated the CRP concentration in 6060 healthy volunteers, with the average baseline CRP value to be 1.18 (range 0.6 - 2.47) mg/L (Tang *et al.*, 2018). It was established that CRP levels increased with age of the donor, particularly above age 45 and CRP levels were found to be greatest in male donors (Tang *et al.*, 2018). Elevated CRP values are found in patients with a range of conditions. CRP has been well established as a marker for cardiovascular disease (Ansar and Ghosh, 2013). Atherosclerotic plaque formation in early cardiovascular disease is linked with inflammation and so observing inflammatory markers allows for monitoring of disease (Diederichsen *et al.*, 2018). In a large study of 1179 participants with no cardiac history, patients who went on to experience cardiovascular events (including stroke, myocardial infarction) were proven to have elevated CRP in comparison to patients who did not go onto develop cardiovascular disease (2.7 and 1.4 mg/L) (Diederichsen *et al.*, 2018).

Acute pancreatitis is a complex, potentially fatal inflammatory condition, which requires careful management and treatment. In response to acute pancreatitis, CRP levels raise and peak after 72 - 96 hours (Schütte and Malfertheiner, 2008). Greatly elevated CRP is the gold standard for pancreatitis, with values greater than 150 mg/dL, have been linked with necrotising pancreatitis with a specificity and accuracy over 80 and 86%, respectively (Schütte and Malfertheiner, 2008; Staubli *et al.*, 2015). PC is a malignancy linked with inflammation and prior attacks of pancreatitis increase the risk of developing PC. A comprehensive systematic review examined the prognostic capabilities of CRP in 485 total PC patients with resectable disease, 48 % of patients were determined to have high CRP, linked with poorer disease prognosis in 4 of the 6 studies (Stevens *et al.*, 2015). However, the conclusions were limited with studies analysed having varying definitions of high CRP ranging from 3 to 10 mg/L (Stevens *et al.*, 2015). A study combined the use of CRP and platelet levels to separate PC patients by median survival time (Miyamoto *et al.*, 2017).

5.1.2.4 Bilirubin

Bilirubin concentrations are determined from liver function tests, commonly carried out in patients with ongoing pancreatic disease. Haemoglobin is broken down within the spleen in two stage catalytic degradation producing the haem group and the yellow coloured pigment bilirubin (Kalakonda *et al.*, 2018). Under normal

physiological conditions, healthy adults should have bilirubin levels below 21 µmol/L (North Bristol NHS Trust, 2020). Excess bilirubin within the serum causes jaundice, signifying disease within the liver, bile ducts or pancreas (Kalakonda *et al.*, 2018). Obstructive jaundice caused by compression of the bile duct can occur in both benign and malignant disease processes, including PC. A study of 1026 patients with obstructive jaundice, 546 patients (53.22%) presented with malignant disease consisting of PDAC, hilar or distal cholangiocarcinoma, ampulla and ductal adenocarcinoma (Garcea *et al.*, 2011). Garcea *et al.* (2011) were able to distinguish between benign and malignant obstructive jaundice with optimal sensitivity and specificity of 71.9% and 86.9%, respectively, with bilirubin levels above 100 µmol/L. Not only can bilirubin levels reflect malignant obstructive jaundice; it has also been used as a predictor for disease outcome. A study of 113 patients with pancreatic head tumours identified pre-operative bilirubin above 4.6 mg/dL to be a useful marker to predict recurrence of tumour post-surgery (Yang *et al.*, 2016).

5.1.2.5 Novel Biomarkers

Due to the limitations in the current blood-based system with CA19-9, many teams are currently searching for novel biomarkers for PC, which may offer a more suitable alternative. For example, PAM4 is showing promise. PAM4 (clivatuzumab) is a murine monoclonal antibody, which has demonstrated a high specificity for mucin MUC5AC, which is expressed in early pancreatic neoplasia (David V Gold *et al.*, 2013; Liu *et al.*, 2015). The Gold *et al.* (2013) study demonstrated the diagnostic capabilities of PAM4 and found it able to identify two thirds of stage I PDAC due to elevated levels of the MUC5AC. As a result, this shows promise for earlier diagnosis of PC. A phase I clinical trial using PAM4 also showed that it is not only limited to diagnosis and can also be used as a therapeutic intervention. Ocean *et al.* (2012) gave 38 untreated patients ytrium-90-labelled humanised PAM4 in combination with a low-dose of chemotherapeutic gemcitabine. 16% of patients showed partial responses to the treatment and 42% were shown to have disease stabilisation as determined by computer tomography. Although promising, 28 of the 38 patients developed grade 3/4 myelosuppression following the first cycle of treatment (Ocean *et al.*, 2012).

Additionally, intercellular adhesion molecule-1 (ICAM-1) is showing potential for diagnosis. ICAM-1 plays a role in cell-cell adhesion in normal conditions, under inflammatory stress and also in malignancy (Tempia-Caliera *et al.*, 2002). Comparison of healthy and malignant pancreatic tissue showed a 5.4-fold increase in ICAM-1 expression and so is being explored for diagnostic potential (Tempia-Caliera *et al.*, 2002). A recent study by Mohamed *et al.* (2016) analysed whether serum ICAM-1 values would have a better chance than CA19-9 of differentiating between benign pancreatic conditions such as pancreatitis from malignant PC. It was determined that ICAM-1 had a greater specificity and sensitivity than CA-19-9 with differentiating between benign and malignant conditions yet performed equally poorly when staging PC (Mohamed *et al.*, 2016).

Several blood-based biomarkers are being explored with the aim of earlier diagnosis of PC. A blood-based system is beneficial in several manners. To start, a blood-based system is comparatively cheaper and more accessible than scanning techniques discussed above. This would allow for blood samples to be taken on a more local basis, such as their local GP surgery or outpatient referral unit should pancreatic disease be a potential cause of the patient's visit. Additionally, the results from the blood-test would either confirm or deny the need of visualisation of the pancreas and so reduces unnecessary appointments in already busy radiology departments. A high level of biomarkers would also allow for fast-tracking of patients to scans and screenings sooner to catch the disease in an earlier, more treatable stage.

Blood-tests are also a lot less invasive than the endoscopic techniques used for visual confirmation of the tumour. Given the painful nature of late disease, additional unnecessary pain and discomfort is not ideal for the patient. EUS and ERCP, although highly accurate do risk considerable side effects including iatrogenic pancreatitis, perforation and bleeding which are not to be taken lightly (Majumder *et al.*, 2012). Additionally, blood-based systems allow for repeated measurements to be taken over a period of time and can be used as a screening system, given none is currently in place for PC, such as those present for oesophageal cancer (Lakatos *et al.*, 2016). Patients deemed at risk of developing PC, such as those with a family history of PC, those with diabetes, chronic pancreatitis, and genetic predisposition for developing cancer would be ideal targets of continual screening with a blood-based system. The monitoring would allow identification of early changes and so

would allow the disease to be caught at a stage where surgical resection could be possible.

5.1.3 Aims

In this chapter, we aim to explore the human PBMC CBMN assay in patients with benign and malignant pancreatic disease. As elevated frequencies of binucleated PBMCs with MN have been seen in other malignancies, we aim to determine whether the assay has potential in PC for both diagnosis and staging. Additionally, we will be exploring additional clinical parameters CA19-9, CRP and NLR both alone and in combination with the PBMC CBMN and erythrocyte PIG-A assay results we have seen in chapter 4. Given the complexity of PC diagnosis and drawbacks of individual biomarkers, a combination of biomarkers may offer a more suitable approach for PC diagnosis and staging to improve patient outcome.

5.2 Methods

5.2.1 Ethics and Permissions

As seen in chapter **4.2.1**.

5.2.2 The PBMC CBMN Assay

5.2.2.1 PBMC Isolation

10 mL of donated heparinised whole blood was inverted 5 times gently. 4.5 mL of heparinised whole blood was gently layered on top of an equal volume of Histopaque$^®$-1077 (Sigma) in a 15 mL centrifuge tube. This was carried out twice. The layered blood and Histopaque were centrifuged at 104 g for 30 minutes with the lowest break and acceleration settings available. Following centrifugation, the plasma was removed and stored for future analysis, and the white buffy layer was removed through gentle swirling action with Pasteur pipette (**Fig. 5.1**). Buffy layer was washed three times in 10 mL pre-warmed PBS before the cell pellet was resuspended in 10 mL complete PBMC media (RPMI 1640 (Gibco) supplemented with 10% FBS, 1% glutamine and 1% penicillin-streptomycin). Buffy layer cells were suspended in media and returned to incubator for 24 hour incubation.
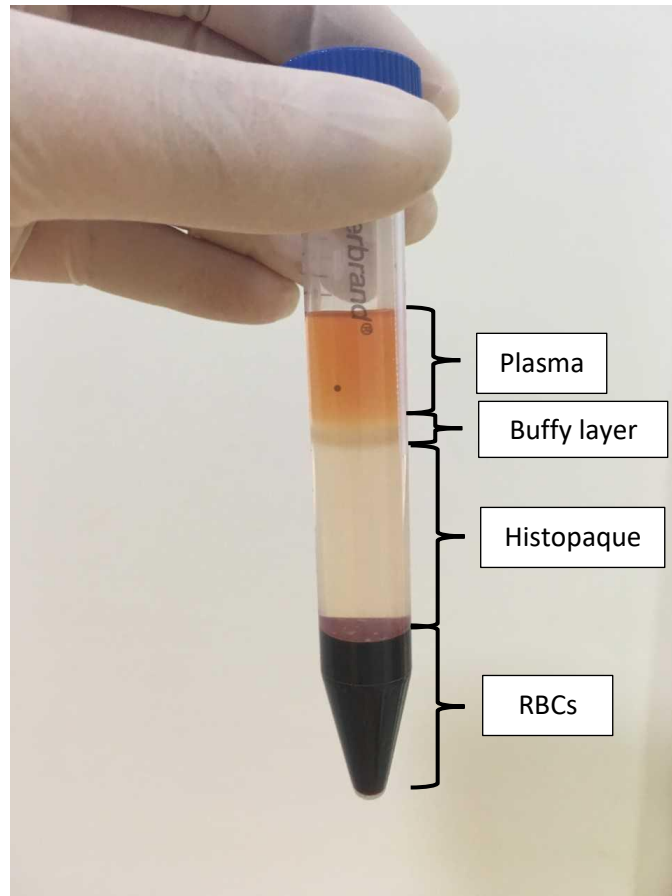
Figure 5.1: *Blood separation following centrifugation with Histopaque®-1077 into (top to bottom): plasma, buffy layer, histopaque, red blood cells.*

5.2.2.2 PBMC Micronucleus Assay

Following 24 hour incubation, the PBMCs were centrifuged (21 g, 5 minutes) and resuspended in plain RPMI 1640 (Gibco) supplemented with 1% penicillin-streptomycin. Ten microlitres of PHA were added to induce cell division in the lymphocytes and cells were returned to the incubator for 48 hours. After incubation, 30 µL (0.6 µg/mL) cytochalasin B was added, cells returned to incubator for further 48 hour incubation. Cells were harvested after final incubation and washed three times in PBS (Gibco).

5.2.2.3 Slide Preparation

Freshly washed, harvested cells were resuspended in 1 mL PBS, with care taken to resuspend the cell pellet by gentle flicking to prevent cell damage and lysis. 100 µL cell suspension was adhered to microscope slides using a Cytospin centrifuge at 30 g for 5 minutes. Initially, one slide was made and assessed under a light microscope to

determine whether the cell density suitable before remaining slides. If suitable, the remaining slides were centrifuged. If not, the cell density was adjusted through addition or removal of PBS from cell suspension and remainder of slides prepared. Slides were airdried and fixed in 90 % ice-cold methanol for 10 minutes. Slides were airdried again before staining in 20 % Giemsa (Gurr® VWR International Limited) for eight minutes. Slides were washed and airdried. Slides were dipped in xylene for 10 seconds within a fume hood and the coverslip applied to cytodot using DPX mounting medium and air dried before scoring.

5.2.2.4 Slide Scoring

Prepared and stained slides were manually scored under a light microscope at 100 x objective and oil. Care was taken to assess all cells on the slide. One thousand binucleated lymphocytes were examined per slide, with those with micronuclei as defined in **Table 1.1** (Fenech, 2007) were quantified. Five slides were prepared per donor with three scored.

5.2.3 Human Erythrocyte PIG-A Assay

As described in **4.2.2**.

5.3 Results

5.3.1 PBMC CBMN Assay

The human PBMC CBMN assay was performed on PBMCs isolated from peripheral blood from consenting donors with benign and malignant pancreatic disease and healthy donors (**Fig. 5.2**). Healthy CBMN data was kindly provided from another study within this laboratory. Healthy donors and benign patients had similar frequencies of PBMCs with MN with $0.397 \pm 0.214\%$ and $0.427 \pm 0.243\%$ in healthy and benign donors, respectively. As a result, healthy and benign donors were were combined. Healthy donors and patients with benign pancreatic disease, such cysts and pancreatitis, have a lower frequency of PBMC with micronuclei in comparison to PC patients with mean frequencies of $0.438 \pm 0.235\%$ and $1.619 \pm 1.001\%$, respectively. A parametric t-test showed this increase seen in cancer patients is significant ($p<0.000$).
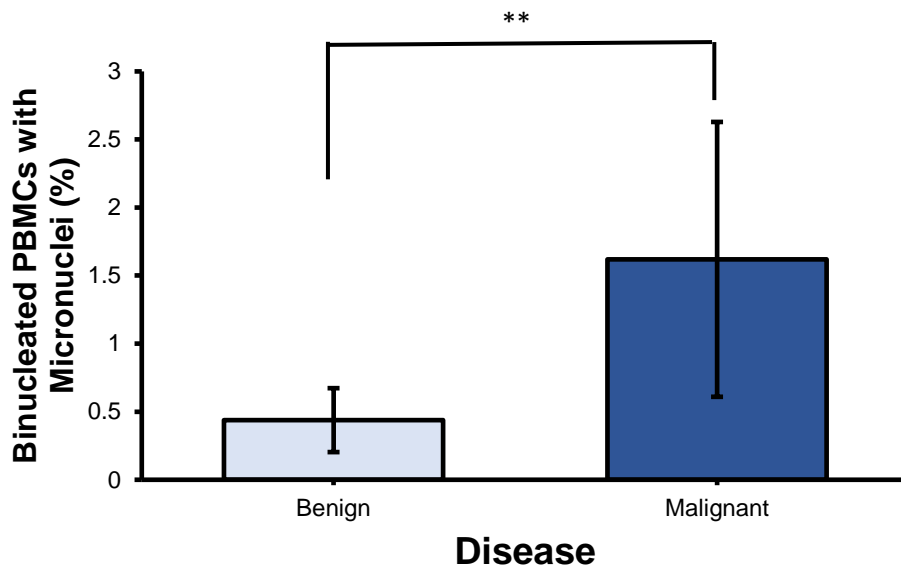
Figure 5.2: *Frequency of micronuclei within binucleated PBMCs from patients with benign and healthy (n=12) and malignant (n=18) pancreatic disease. Bars represent the mean and error bars show standard deviation. (** signifies p<0.01)*

Given the increase between benign and malignant donors, PC patient PBMC CBMN results were explored further to explore the relationship. TNM tumour staging allowed further comparison, further described in **Tables 7.1 and 7.2** in **Appendix 7.4**. T-stage (**Fig. 5.3**) represents the size of the tumour. As tumour size increases, the frequency of binucleated PBMC also increases up to T3b disease. A one-way ANOVA determined this increase to be non-significant (p=0.742). No post-hoc analyses could be performed since only one donor was sized T3.
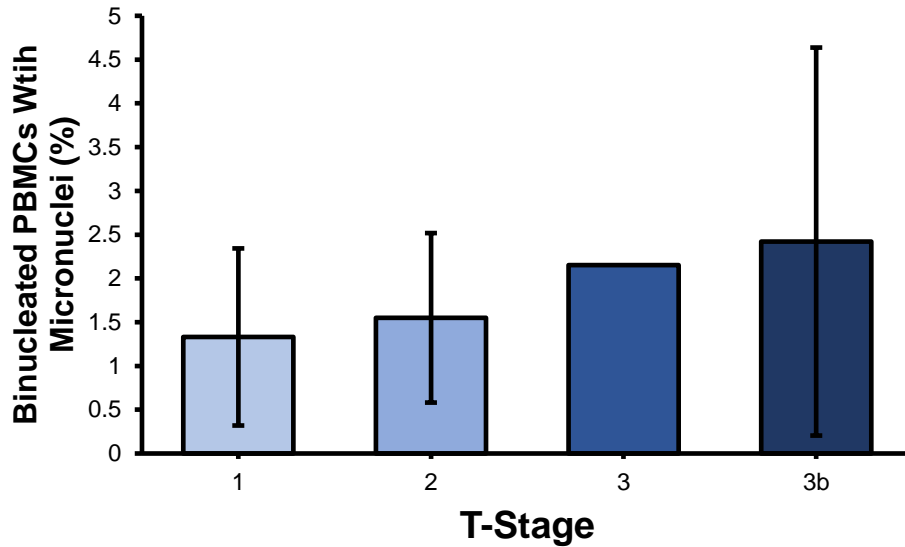
Figure 5.3: *Frequency of micronucleated binucleate PBMCs of PC patients as tumour size, represented by T-staging increases (n=4 T1, n=6 T2, n=2 T3, n=1 T3b). Bars represent the mean and the error bars represent the standard deviation.*

Lymph node involvement, described by N-stage, was also associated with an increase in micronuclei in peripheral PMBCs of PC patients (**Fig. 5.4**). A one-way ANOVA determined the increase to be non-significant (p=0.590).
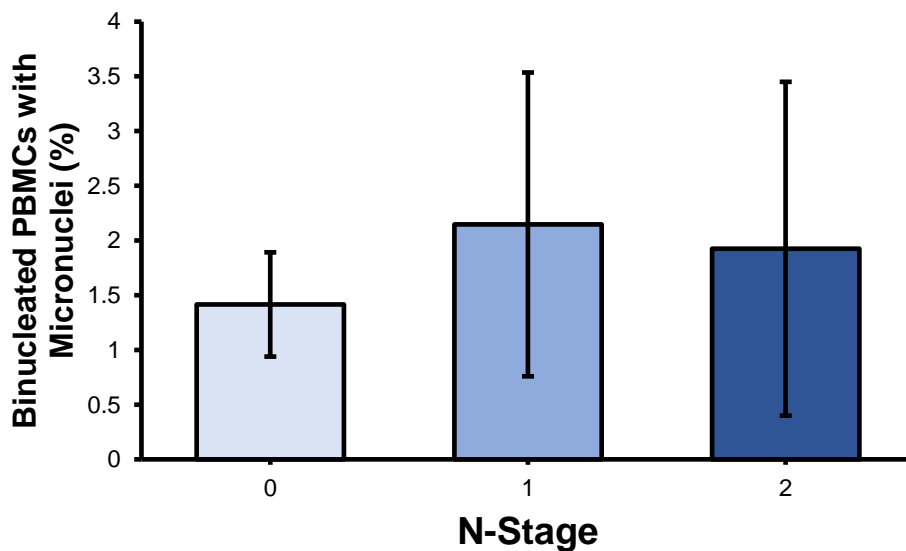
Figure 5.4: Frequency of binucleated PBMCs with micronuclei in PC patients as nodal involvement of disease, signified by N-stage, increases (n=5 N0, n=5 N1, n=2 N2). Bars represent the mean and error bars show the standard deviation.

Unlike with tumour size and lymph node involvement, the presence of metastatic disease does not result in an induction of micronuclei in human PBMCs in PC patients (**Fig. 5.5**). The decrease was determined non-significant through a t-test (p=0.581).
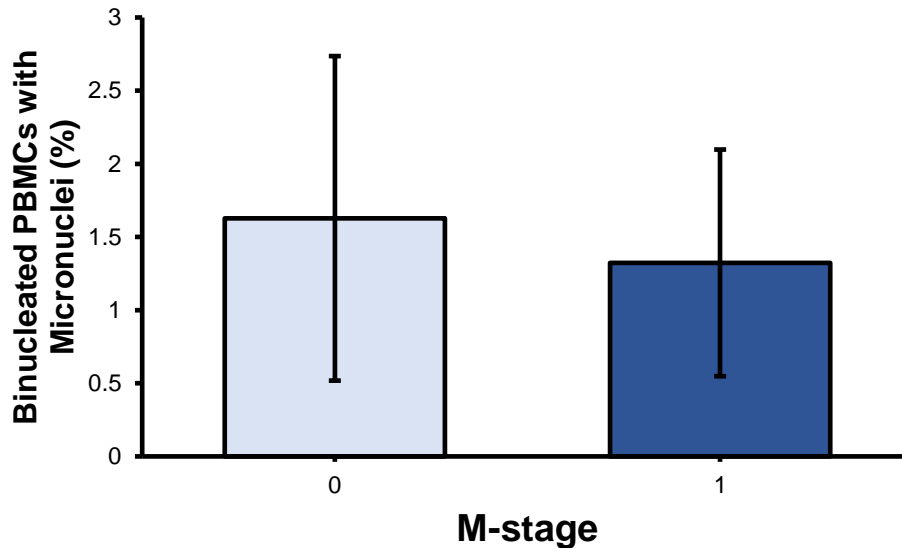
Figure 5.5*: Frequency of binucleated PBMCs with micronuclei from PC patients with either non-metastatic disease (M0) or distant metastatic disease (M1) (n=15 M0, n=3 M1). Bars represent the mean and error bars the standard deviation.*

TNM staging was used to determine overall staging of disease in PC patients, seen in **Fig. 5.6**. The frequency of PBMCs with micronuclei increased from stage IA to stage IIB, with a decrease to stage IV. No significant increases were observed through a one-way ANOVA (p=0.257). Stage IA (0.794±0.559%) and stage IIB (2.147±01.388%) disease had the largest difference, but this increase was non-significant (p=0.597).
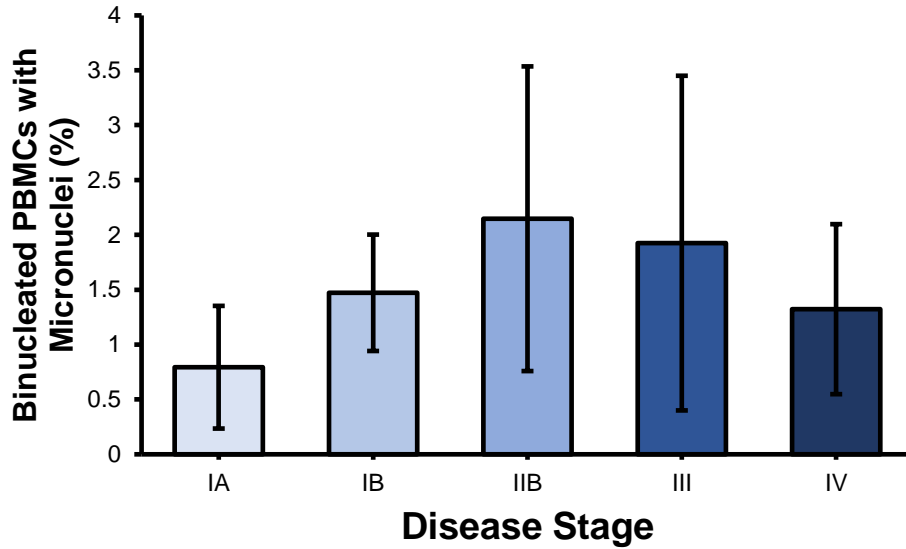
Figure 5.6: *Frequency of binucleated PBMCs with micronuclei from peripheral blood of PC patients as overall stage of disease increases (n=2 IA, n=4 IB, n=4 IIB, n=3 III, n=3 IV). Bar shows the mean and error bars represent the standard deviation.*

Since both the human erythrocyte PIG-A assay and the human PBMC CBMN assay assess DNA damage in two different forms in two alternative blood cell populations, their results were compared (**Fig. 5.7**). Overall, a slight negative correlation was viewed in donors suggesting that there was little evidence of correlation between these two DNA mutational endpoints in patient blood cells.
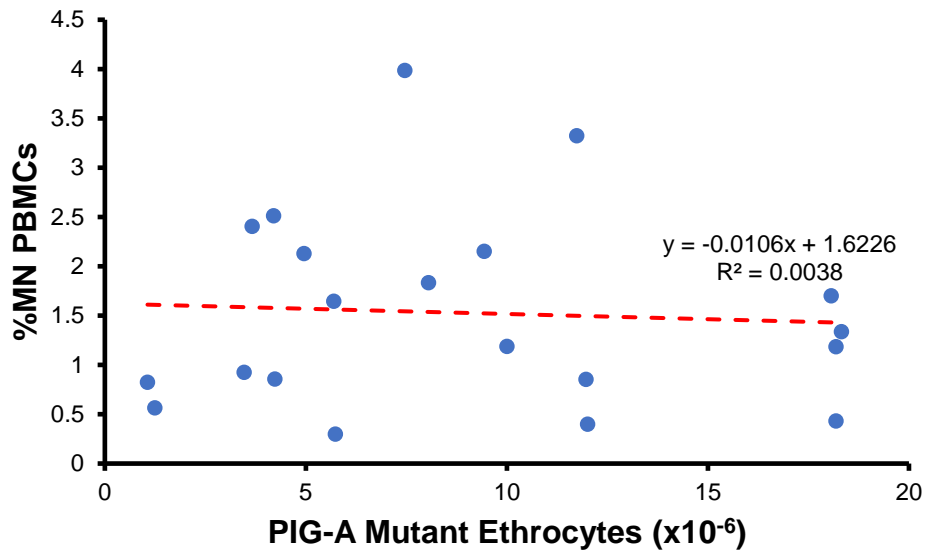
Figure 5.7: *Correlation between the human erythrocyte PIG-A assay and human peripheral PBMC CBMN assay in patients with pancreatic disease (n=20).*

5.3.2 Lone Biomarker Studies

Here, we show the differences between benign and malignant pancreatic patients and PC of increasing stages for CA19-9, NLR and CRP independently.

5.3.2.1 CA19-9

CA19-9, the currently in use biomarker for PC, levels were examined within donors with benign and malignant disease. Corresponding CA19-9 values were only available for 27 donors. Elevated CA19-9 serum concentrations were viewed in some donors with malignant disease, but this was shown to be non-significant through a Mann Whitney U test (p=0.530). It is noteworthy that of the 22 donors with PC, 27.273% of donors (6) were shown to have insignificant CA19-9 levels ranging from 2 to 33 kU/L. All benign donors in the study were shown to have CA19-9 levels below the cut-off of 37 kU/L. There is a 237.93-fold increase between serum CA19-9 concentrations in malignant pancreatic donors in comparison to those with benign disease.
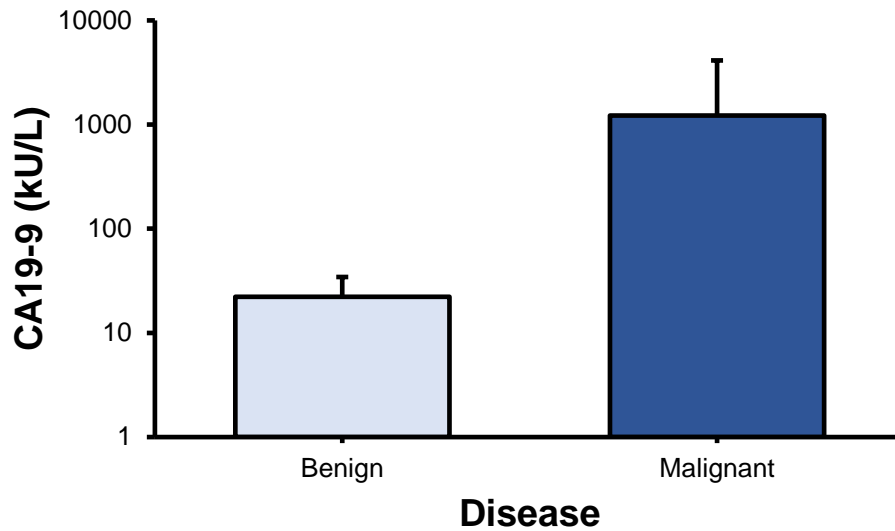
Figure 5.8: *Mean serum CA19-9 concentrations in patients with benign and malignant pancreatic disease (n=5 benign, n=22 malignant). Bars represent the mean and error bars show the standard deviation.*

Further examination of PC patients' CA19-9 levels determined an increase from stage IA disease to later stages, although this difference was determined to be non-significant using a Kruskal-Wallis test (p=0.454). Furthermore, given we only have CA19-9 data from one donor with stage IA PC, this cannot be fully relied upon.
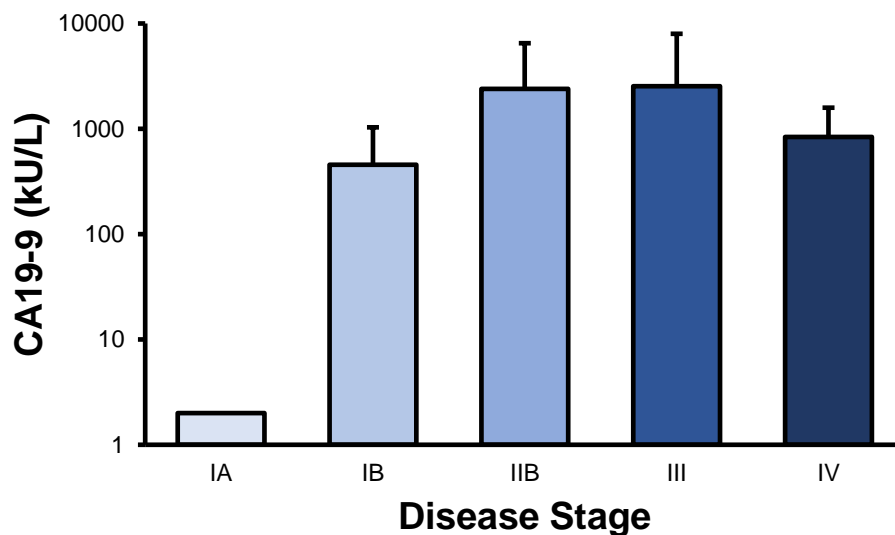
Figure 5.9: *Mean concentrations of serum CA19-9 in PC patients with increasing staged disease (n=1 IA, n=4 IB, n=3 IIB, n=5 III, n=6 IV).*

5.3.2.2 NLR

The NLR, calculated from full blood counts, is a measure of sub-clinical inflammation. NLR values were established for 28 donors with both benign and malignant pancreatic disease. An elevated NLR was seen in PC patients, with a 1.61-fold increase seen over patients with benign disease, although this was non-significant through a Mann Whitney U test (p=0.104).
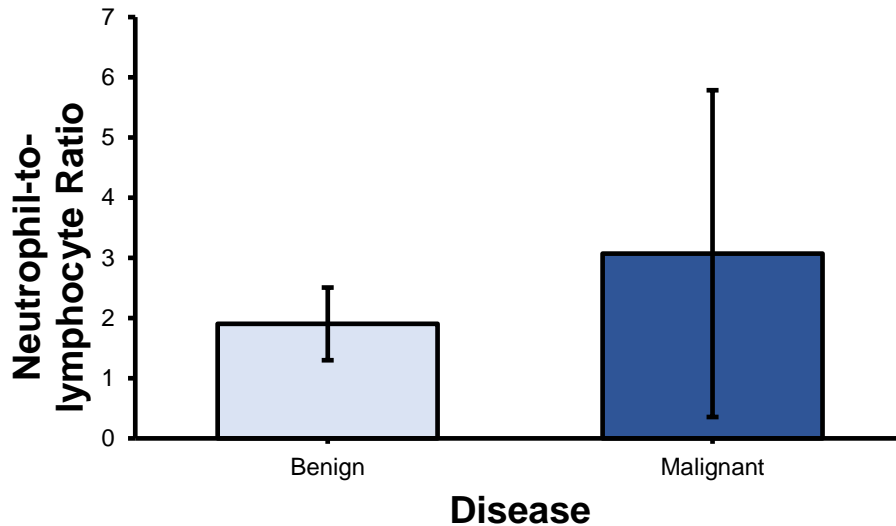
Figure 5.10: *Mean NLR values in patients with benign and malignant pancreatic disease (n=6 benign, n=22 malignant).*

Further examination of PC patients with respect to overall staging of disease showed an unclear pattern. Statistically significant differences were identified between the groups (p=0.000) but this is likely due to the one donor with staged IA and their high NLR value. Given one donor within this group, no post-hoc analyses could be carried out.
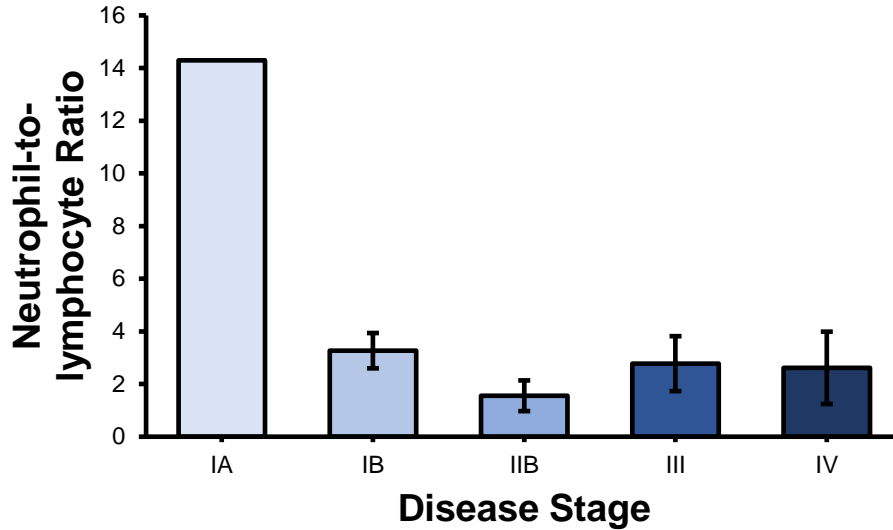
Figure 5.11: Mean NLR in PC patients with increasing tumour staging (n=1 IA, n=4 IB, n=3 IIB, n=5 III, n=6 IV).

5.3.2.3 CRP

CRP concentrations signify clinical inflammation. Overall, PC patients were shown to have elevated CRP levels, with a 2.727-fold increase seen over patients with benign pancreatic disease, but given the large standard deviation in the malignant donor group, this was determined non-significant (p=0.605). CRP values below 5 are normal, 45.455% (10) of the PC donors were shown to have CRP levels elevated above this.
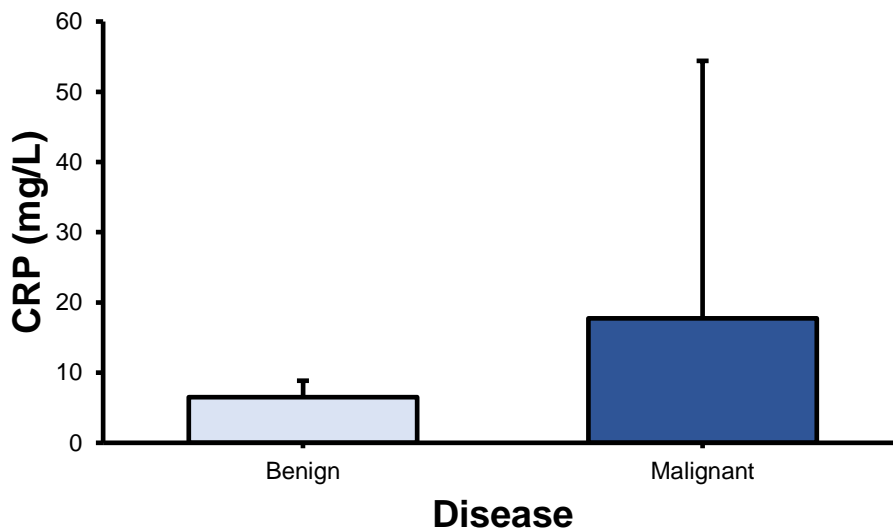
Figure 5.12: Mean CRP values in patients with benign and malignant pancreatic disease (n=6 benign, n=22 malignant).

Further examination of PC patients showed an unclear pattern with CRP values as stage of PC increases. Without stage IB disease, a steady stepwise increase in CRP concentration is seen as disease becomes more advanced. However, IB disease was shown to have a highly elevated mean CRP of 47.25 ± 84.5 mg/L, due to one of the four donors having a greatly elevated CRP of 174. No results were statistically significant.
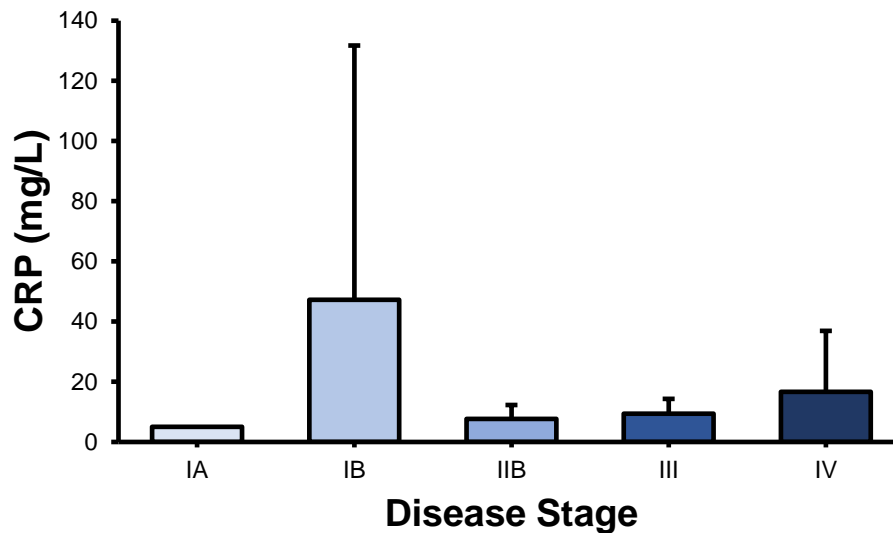
Figure 5.13: Mean CRP concentrations in PC patients with increasing disease stage (n=1 IA, n=4 IB, n=3 IIB, n=4 III, n=6 IV).

5.3.3 Combination of Biomarkers

Combining the human erythrocyte PIG-A mutant frequency with the PBMC CBMN results and other clinical parameters from patients, we aimed to further separate patients and determine whether this had any potential for the diagnosis of pancreatic disease. Different parameters were combined through simple multiplication to provide an overall arbitrary factorial score, with each biomarker having equal weighting.

5.3.3.1 PIG-A and CA19-9

Multiplication of PIG-A mutant frequencies and CA19-9 values resulted in further separation of benign and malignant donors. Benign donors had a mean score of 83.067 ± 54.013 in comparison to malignant donors with scores of 12707.143 ± 29877 (**Fig. 5.14**). The increase was shown to be close to significance through a Mann Whitney U test (p=0.053).
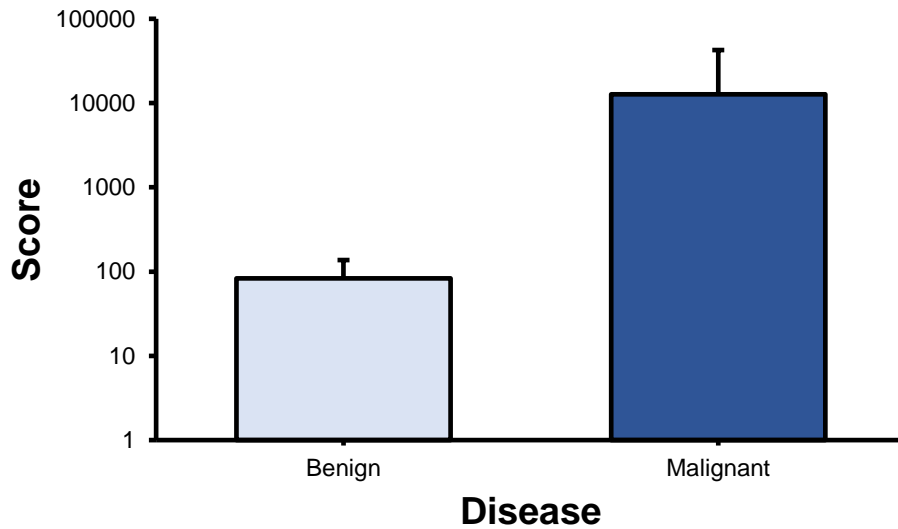
Figure 5.14: *Score from combining PIG-A and CA19-9 blood test results in patients with benign and malignant pancreatic disease (n=6 benign, n=20 malignant).*

The resulting score was also explored for overall disease staging (**Fig. 5.15**). Early stage disease (IA) was shown to have a lower score in comparison to later stages, which were much more comparable. This may result from only one donor analysed with stage IA disease, but also may signify an early stage disease marker but more work would need to be done to determine this. No statistical differences between the groups were determined through a Kruskal-Wallis test (p=0.428).
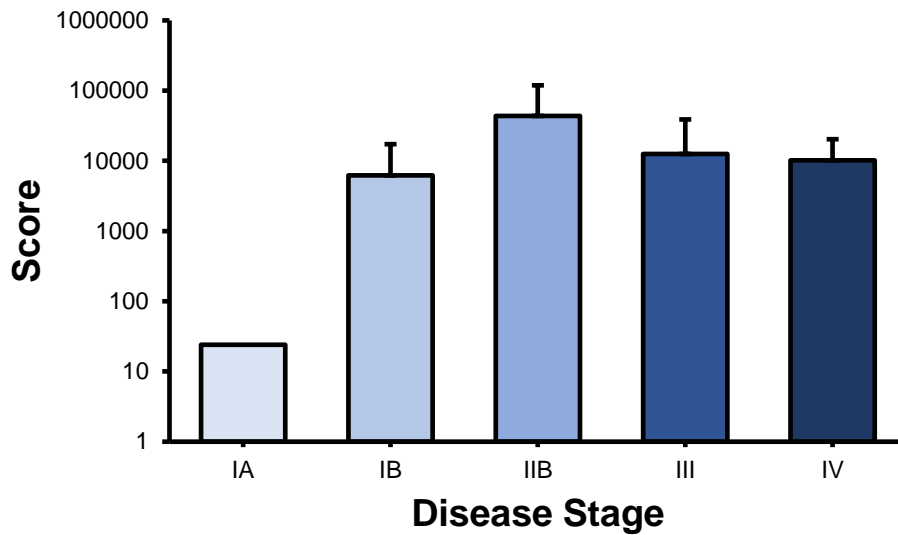
Figure 5.15: *Score from combination of PIG-A and CA19-9 across PC patients with increasing stage of disease (n=1 IA, n=3 IB, n=3 IIB, n=5 III, n=6 IV).*

5.3.3.2 PIG-A and Neutrophil-to-Lymphocyte Ratio

The NLR and PIG-A results were combined through multiplication. Differences between benign and malignant donors were seen in **Fig. 5.16**. There is a five-fold increase between the score of benign and malignant donors, which came close to significance (p=0.059) following a Mann Whitney U test.
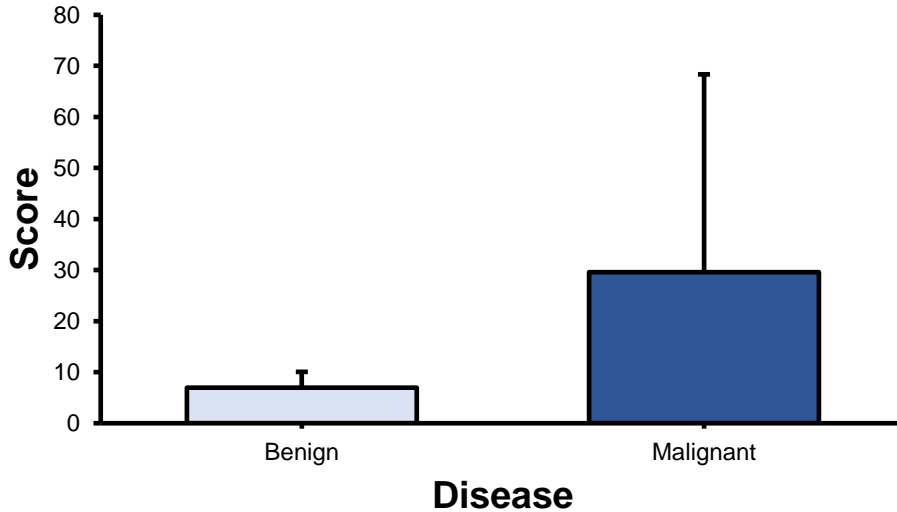
Figure 5.16: Score from NLR and PIG-A results in donors with benign and malignant disease (n=6 benign, n=22).

TNM staging of PC patients was used to establish whether there is a link between score and stage of disease, **Fig. 5.17**. Stage IA disease was shown to have a higher score than higher stage disease, but this could be explained by one donor with stage IA disease. This high value seen in the one donor with IA disease resulted in a significant difference between groups determined through a one-way ANOVA (p=0.001).
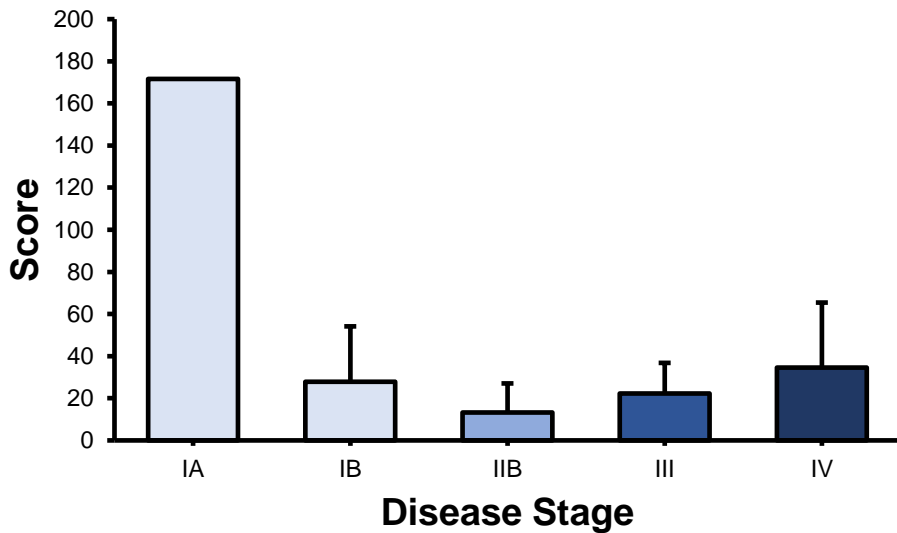
Figure 5.17: Score calculated with PIG-A and NLR values as PC stage increases (n=1 IA, n=3 IB, n=3 IIB, n=5 III, n=6 IV).

5.3.3.3 PIG-A and C-Reactive Protein

PIG-A mutant frequency and biomarker for clinical inflammation, CRP, were combined through multiplication to produce a score. A 4.775-fold increase in score was seen between benign and malignant donors (**Fig. 5.18**). The increase in score seen in malignant donors was established as a significant increase by the Mann Whitney U test (p=0.033).
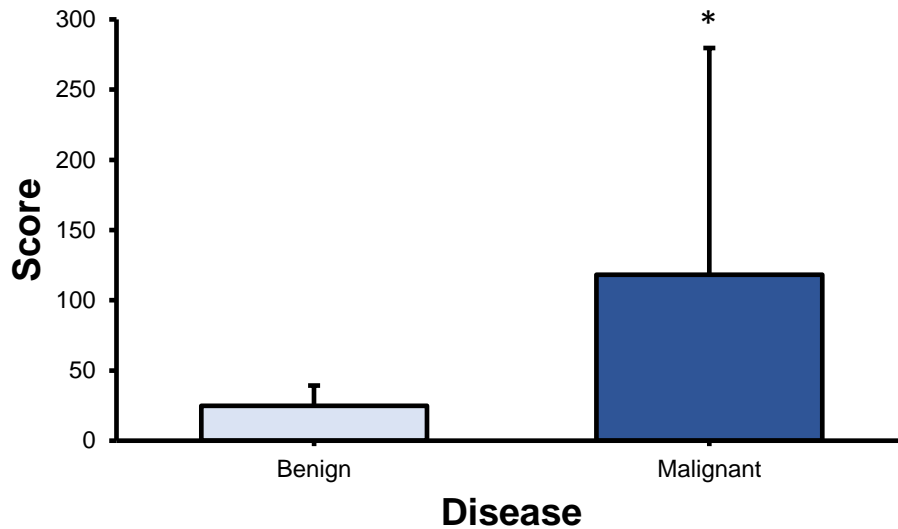
Figure 5.18: *Score generated with CRP and PIG-A values in patients with benign and malignant pancreatic disease (n=6 benign, n=22 malignant).(* shows p<0.05).*

TNM staging of donors with PC allowed further comparison of score generated with CRP values and PIG-A mutant frequencies. Stage IB and stage IV disease was shown to have scores four-times that of stage IA disease, **Fig. 5. 19**. But overall pattern was unclear, and no statistically significant differences were viewed between groups using a one-way ANOVA (p=0.671).
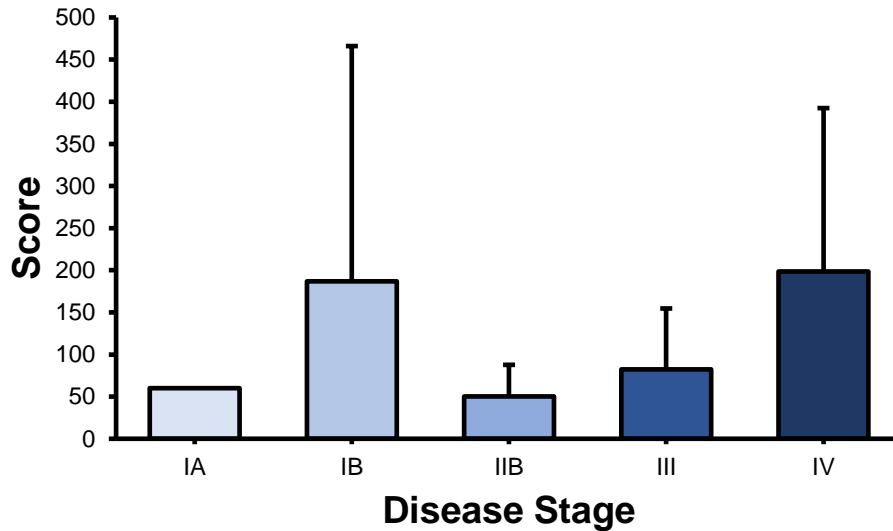
Figure 5.19: Score generated with CRP and PIG-A values as disease stage increases in donors with PC (n=1 IA, n=4 IB, n=3 IIB, n=4 III, n=6 IV).

5.3.3.4 PIG-A and PBMC CBMN

Combining the PIG-A mutant frequency with frequency of PMBC with micronuclei through multiplication provided a score which was explored. Donors with malignant disease had 6.751-times greater score than donors with benign pancreatic disease (**Fig. 5.20**) although this increase was deemed non-significant through a t-test (p=0.137).
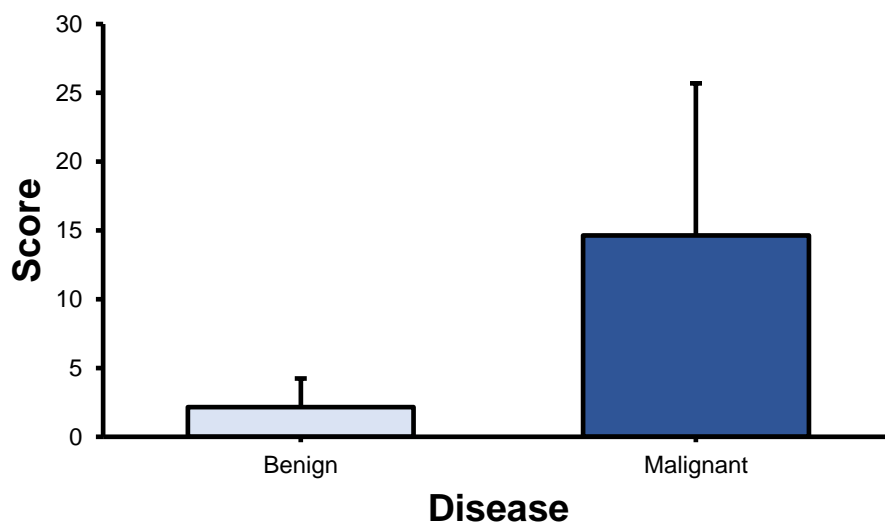
Figure 5.20: Score generated with PIG-A and PBMC CBMN assay in patients with benign and malignant pancreatic disease (n=3 benign, n=17 malignant).

TNM staging provided overall staging of disease. An elevation in score is seen as disease becomes more advanced, with a peak seen in stage III disease (**Fig. 5.21**). No statistically significant differences were seen in the score as staging of disease increases (p=0.696).
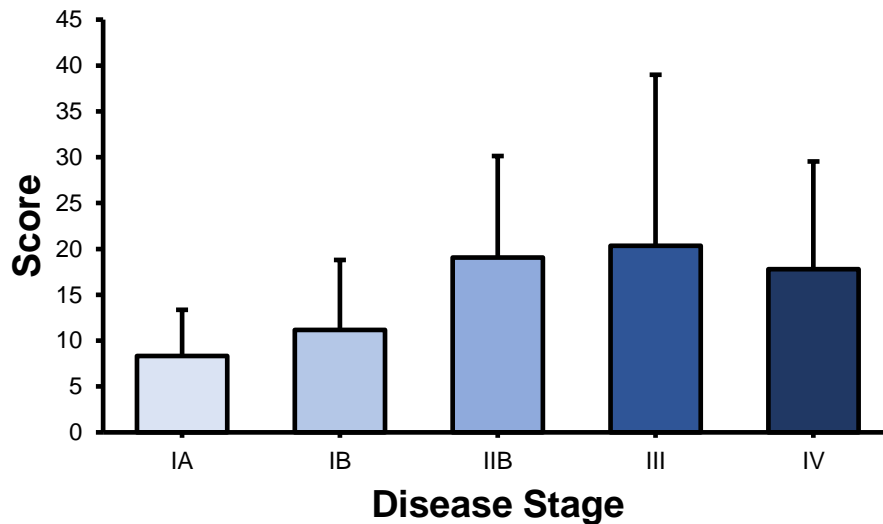
Figure 5.21: Score calculated from PIG-A and PBMC CBMN results as disease stage increases in PC patients (n=2 IA, n=4 IB, n=4 IIB, n=3 III, n=3 IV).

5.3.3.5 PIG-A, CA19-9, NLR, CRP and PBMC CBMN

A combination of all biomarkers assessed may be more likely to further separate patients. Data for all parameters was not available for all patients and so only results for a maximum of 13 patients could be analysed. Of the thirteen, no patients had benign disease and so patients with malignant disease were only able to be analysed. TNM staging was explored to determine whether there was a discernible difference between scores generated with the staging of disease. Firstly, size or T-stage of disease was examined (**Fig. 5.22**). Although patients with T1 disease showed a lower score, 669.917 in comparison to 468483 and 173856, this was deemed non-significant through a one-way ANOVA (p=0.631) and no further post-hoc tests could be carried out due to small sample sizes.
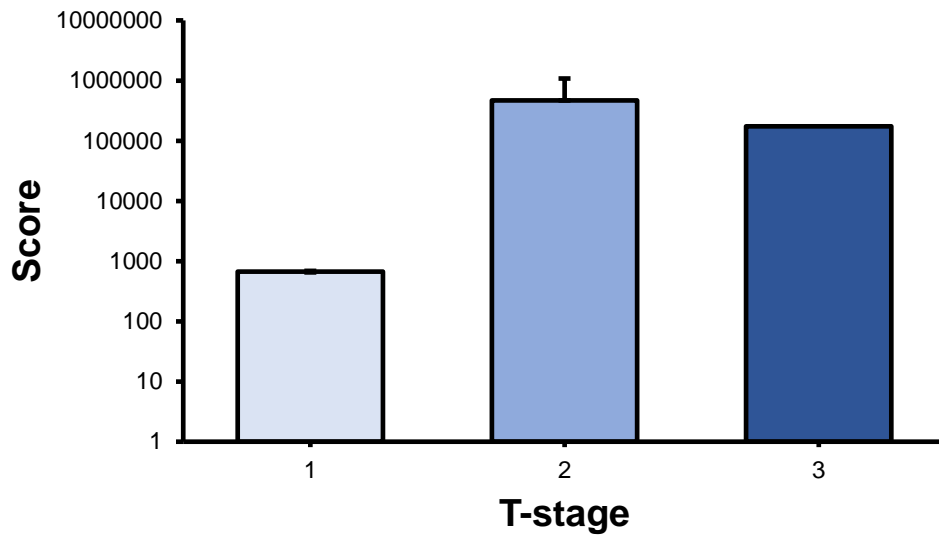
Figure 5.22: *Comparison of score by T-stage of disease from PC patients (n=2 T1, n=5 T2, n=1 T3). Bars show the mean and error bars show the standard deviation.*

Nodal involvement in patients with PC was explored (**Fig. 5.23**). Fewer patients had available N-staging, accompanied with other clinical parameters. There was an unclear pattern overall with an increase between N0 and N1, followed by N2 disease showing a lower score than both N0 and N1 disease. A one-way ANOVA determined no statistically significant differences between the groups (p=0.495), even with post-hoc analyses.
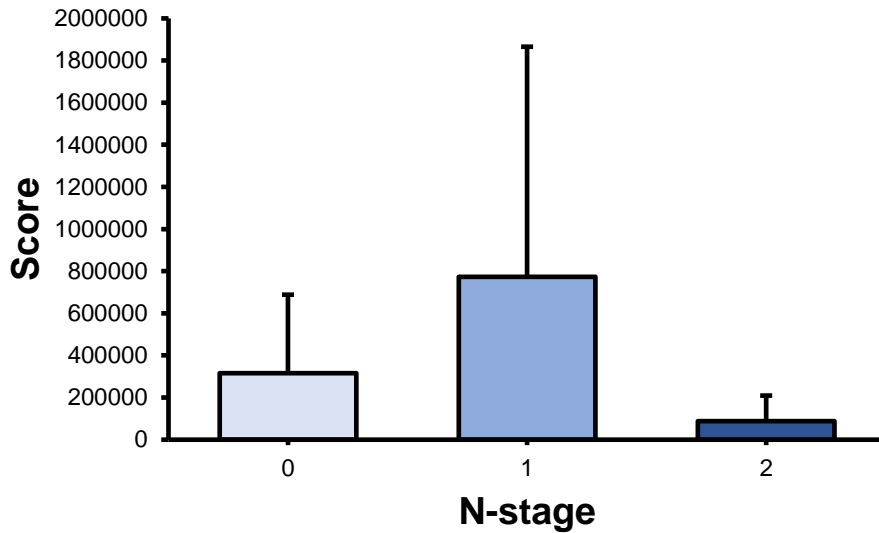
Figure 5.23: Biomarker score with increasing nodal involvement of disease in PC patients (n=4 N0, n=2 N1, n=2 N2).

Patients with metastatic disease (**Fig. 5.24**) showed an increase in score over patients with localised PC, 698,523 in comparison to 298,902. This was determined to be non-significant through a T-test (p=0.140).
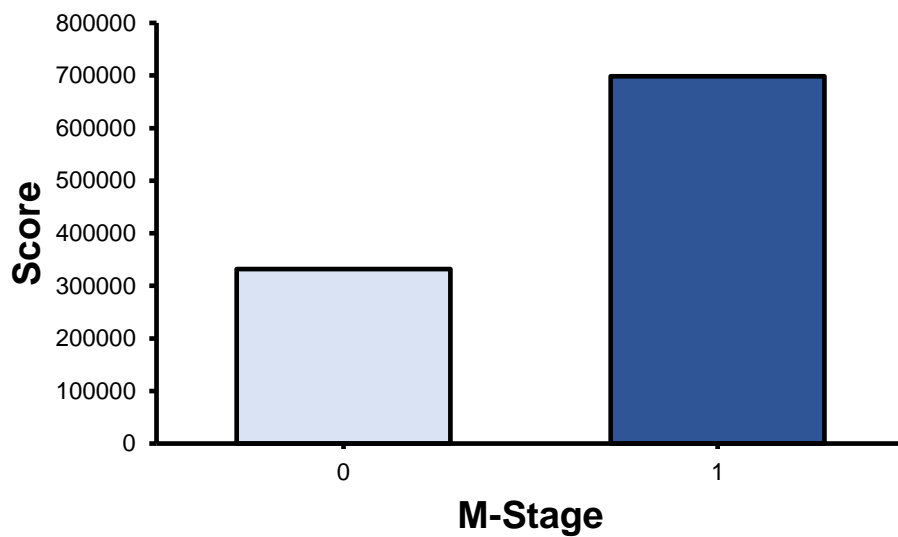
Figure 5.24: Biomarker score in patients with localised PC (M0) and metastatic PC (M1) (n=10 M0, n=3 M1).

Finally, overall staging of PC was compared with the biomarker score calculated through multiplying biomarker values together (**Fig. 5.25**). Stage IA disease had a lower score of 683.521 in comparison to more advanced disease which was determined to be non-significant through a one-way ANOVA (p=0.645). This may be because of small sample size, which resulted in only donor with complete parameter data having stage IA disease.
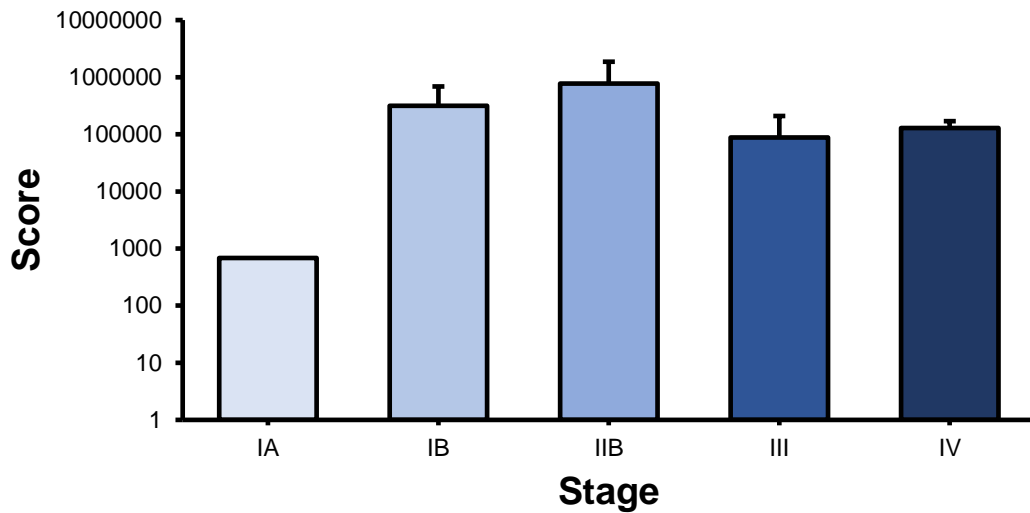
Figure 5.25: *Biomarker score in PC patients with increasing stage of PC (n=13).*

5.3.4 PIG-A: High or Low

Here, PIG-A mutant erythrocyte frequency of all donors was analysed. **Fig. 5.26** shows the frequency distribution of the PIG-A mutant frequency of all donors.
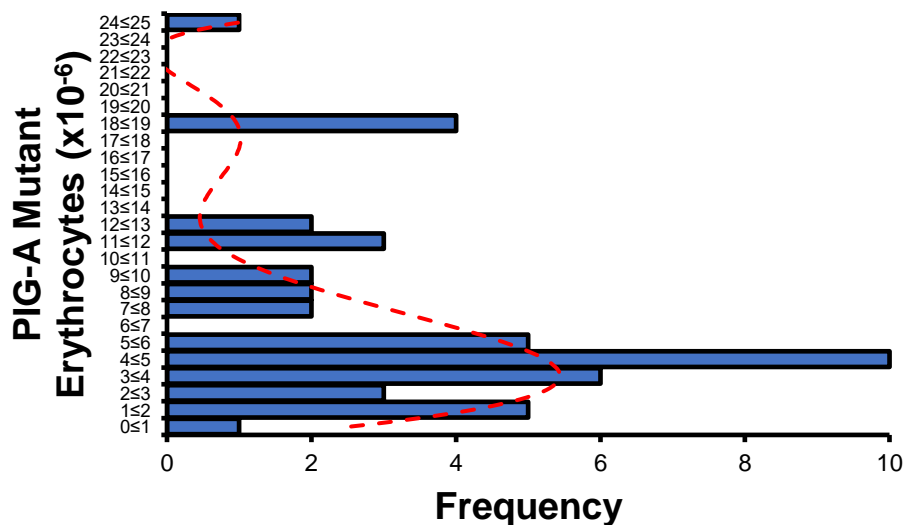
Figure 5.26: *Frequency distribution of PIG-A mutation frequencies from all donors in the study (n=45). Red line shows line of best fit.*

The median of all PIG-A mutant erythrocyte frequencies from all donors was determined to be 4.860×10^{-6}. Any PIG-A mutant frequencies below this value were defined as low PIG-A values and any equal to or above the median were defined as high PIG-A values. Proportions of donors with high or low PIG-A values within each donor group is seen in **Fig. 5.27.** Within groups of donors, healthy and benign donors had only 33.33% and 10% of donors with high PIG-A values. In comparison, 68.97% of PC patients displayed high PIG-A values.
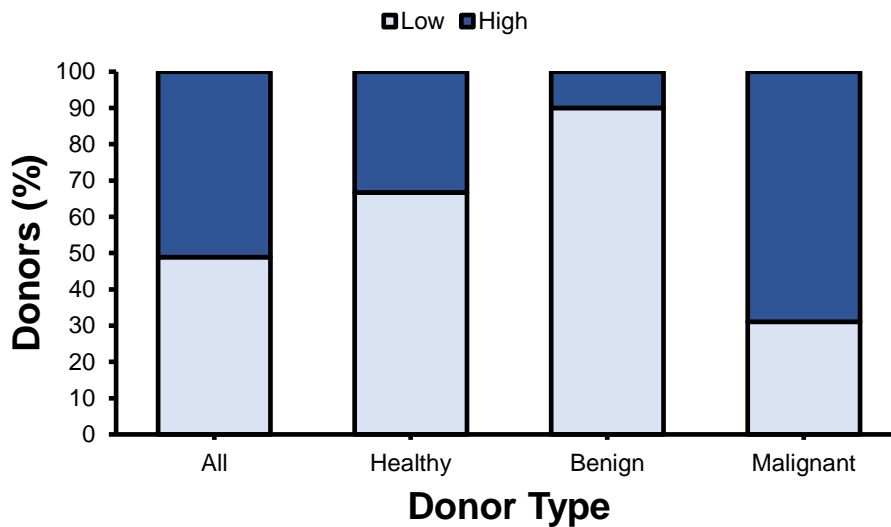
Figure 5.27: *Percentage of donors with PIG-A mutant frequencies below and above 4.8×10^{-6}, defined as low and high PIG-A values, within different donor groups (n=6 healthy, n= 10 benign, n=29 malignant).*

TNM staging data was also explored for patients with malignant disease. Tumour size, classified by T-stage, is explored in **Fig. 5.28**. In the highest stages, T3b and T4, all donors have high PIG-A values in contrast to the smaller sized tumours, which have 50% and below of donors with low PIG-A values.
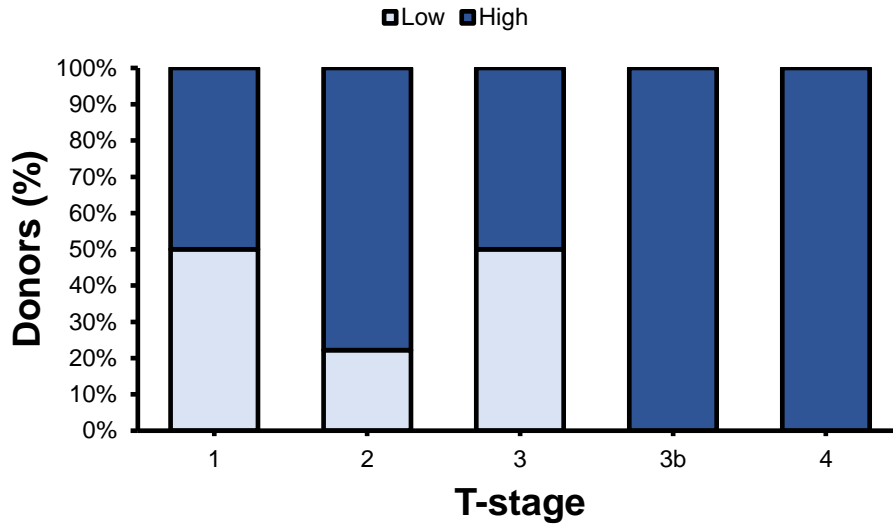
Figure 5.28: Percentage of donors with high and low PIG-A values across increasing T-stage in donors with PC (n=2 T1, n=9 T2, n=2 T3, n=3 3b, n=1 T4).

A similar pattern was viewed when exploring nodal involvement (**Fig. 5.29**). More advanced disease, N2, had 100% of donors with high PIG-A values with lower proportions seen in N0 and N1 disease.
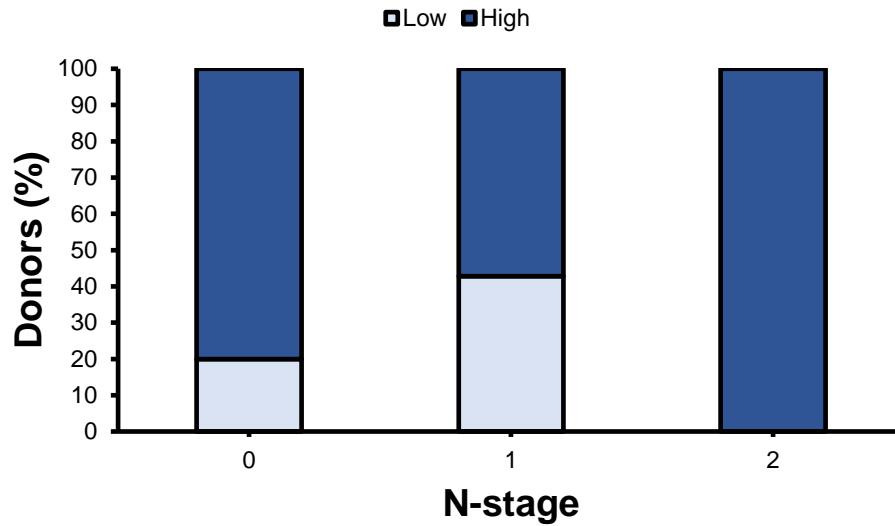
Figure 5.29: Percentage of donors with high and low PIG-A values with increasing lymph node involvement in donors with PC (n=5 N0, n=7 N1, n=5 N2).

Additionally, M-staging (**Fig. 5.30**) also showed an increased percentage of donors with high PIG-A values with metastatic disease in comparison to non-metastatic disease (77.78% and 85.71%).
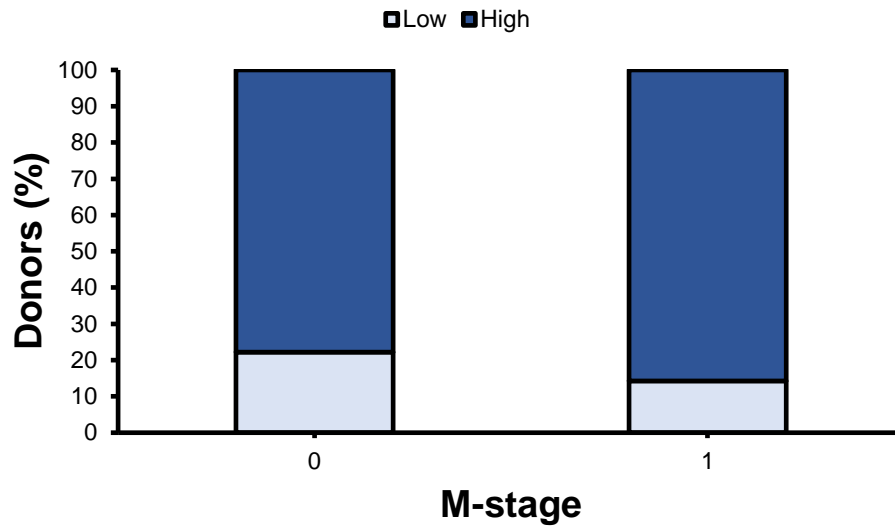
Figure 5.30: Percentage of donors with high or low PIG-A values with localised PC or metastatic PC (n=18 M0, n=7 M1).

The pattern seen in overall staging of PC is less clear (**Fig. 5.31**). This may be because of the higher percentage of PC patients having high PIG-A values.
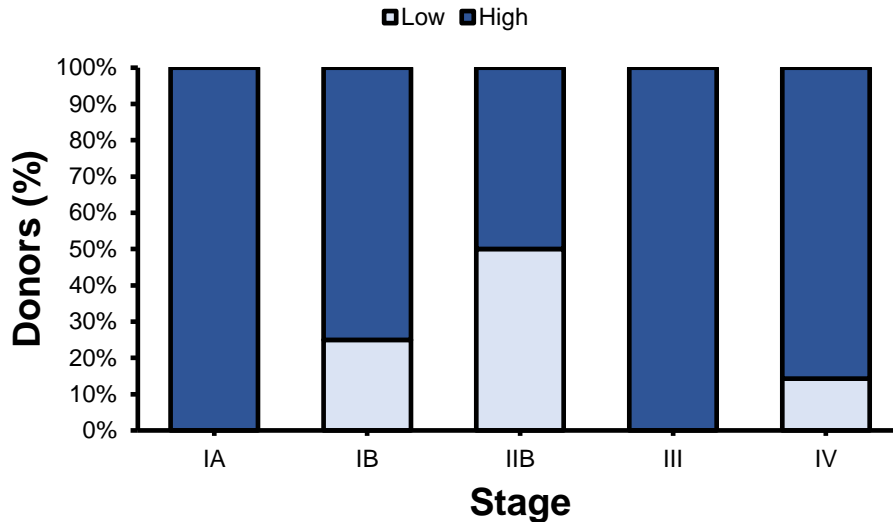
Figure 5.31: *Percentage of donors with high and low PIG-A values as stage of PC increases (n=2 IA, n=4 IB, n=6 IIB, n=6 III, n=7 IV).*

5.3.5 Questionnaire Data

Questionnaire responses were minimal, with only 8 donors fully completing and returning questionnaires. Within this small cohort, dietary quality score (DQS) was evaluated in benign and malignant donors. Donors with malignant disease were shown to have a mildly elevated DQS, signifying a diet which is nutritionally better, but this increase was non-significant (p=0.172). Furthermore, no link was observed between DQS, dietary fat content and alcohol consumption with PIG-A alone. Interestingly, a slight decrease in average PIG-A mutant frequency was seen in past-smokers in comparison to non-smokers (p=0.176). An interesting finding showed an increased PIG-A mutant frequency as self-reported health declined; however, this was also determined non-significant (p=0.180). Further questionnaire responses are explored further in depth within **Appendix 7.5**.

5.3.6 Survival

Given the abysmal survival rates for PC, the survival of patients was explored. Of the PC patients we have survival data for, 20% (5) of PC patients have unfortunately passed away since donating to the study.

Staging of PC has an influence over survival. Early stage disease shows greater survival (100%) in comparison to stage IIB disease (83.33%) and IV disease (42.86%) (**Fig. 5.32**).
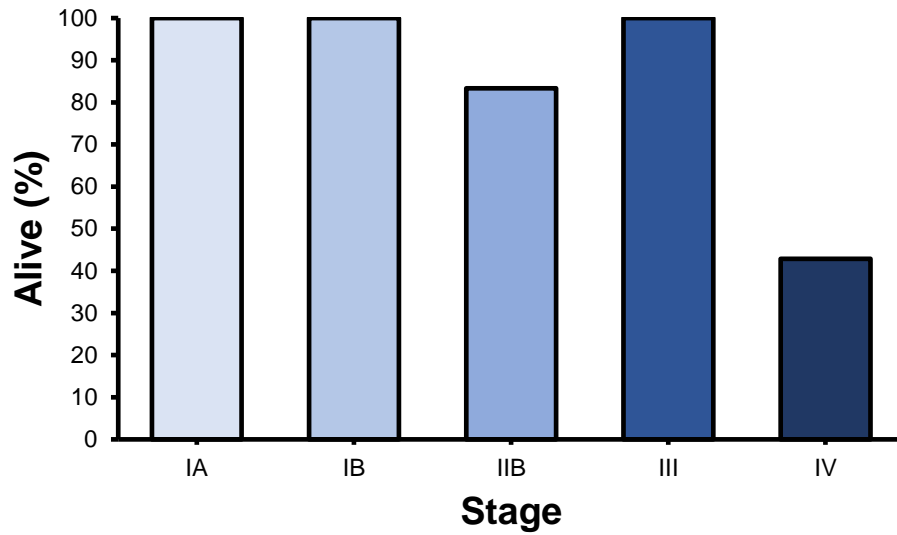
Figure 5.32: *Percentage of live donors with PC at increasing stages of disease (n=2 IA, n=4 IB, n=6 IIB, n=6 III, n=7 IV).*

We aimed to explore whether there were differences in PIG-A mutant frequency in patients who survived in comparison to patients who did not. Patients who survived were shown to have a lower PIG-A mutant frequency in comparison to patients who did not survive with mean PIG-A mutant frequencies of 9.143 ± 5.852 and $11.132 \pm 6.702 \times 10^{-6}$, respectively. This difference was determined to be non-significant through a Mann Whitney U test (p=0.684).
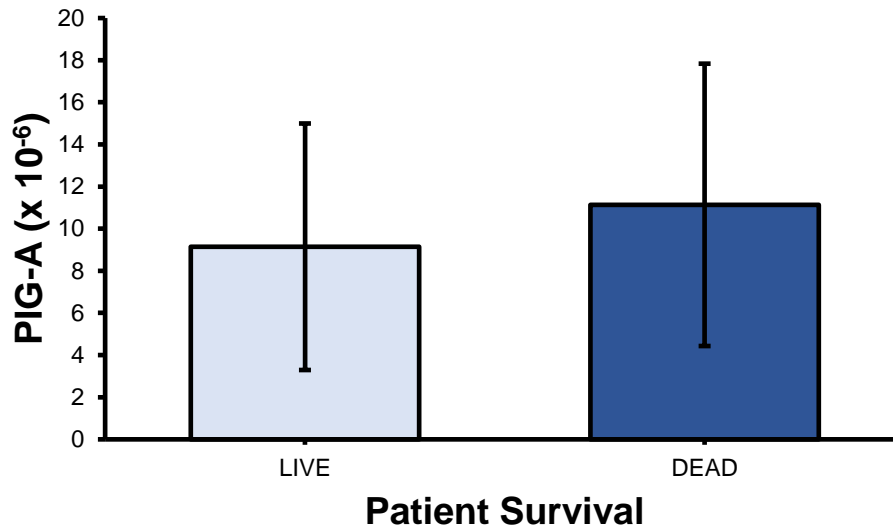
Figure 5.33*: Mean PIG-A mutant frequencies in PC patients who did and did not survive (n=20 live, n=5 dead).*

Furthermore, we aimed to see whether the results from the human PBMC CBMN assay correlated with survival. A decrease in micronuclei frequency was observed within PBMCs of PC patients who died, although this was deemed non-significant through a Mann Whitney U test (p=0.638).
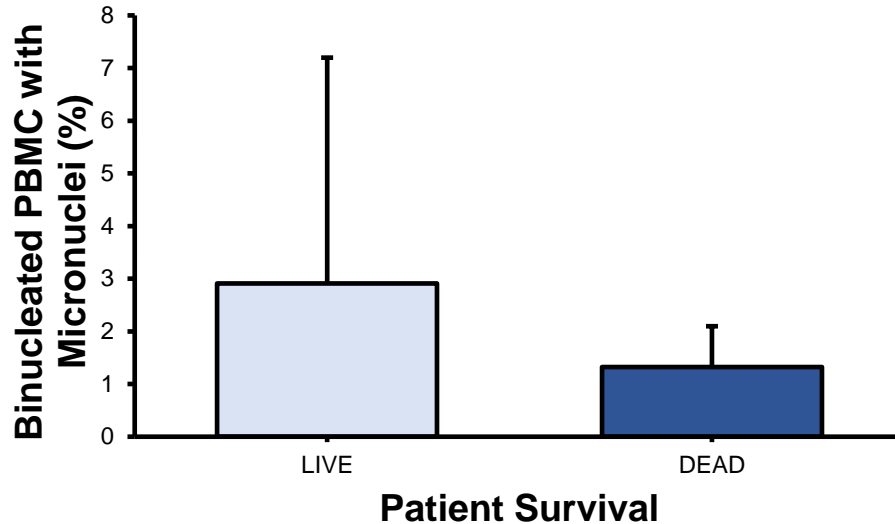
Figure 5.34*: Induction of micronuclei using the human PBMC CBMN assay in PC patients who did and did not survive (n=12 live, n=3 dead).*

5.4 Discussion

In this chapter, the PBMC CBMN assay was harnessed to assess genome stability within donors with benign and malignant pancreatic disease, which is also loosely correlated with cancer staging. Further clinical parameters have also been examined, and through combining of multiple biomarkers, we aimed to improve the use of them in comparison to lone biomarkers.

5.4.1 PBMC CBMN

Within this study, we have shown a significantly elevated frequency of micronuclei within PBMCs of donors with malignant pancreatic disease in comparison to benign donors. This correlated with the data in Chapter 4 showing increased PIG-A mutant erythrocytes in PC. Previous studies have also explored the PBMC CBMN assay within PC patient populations, with results supporting the results seen in our study. An elevated MN frequency was seen in a population of PC patients in comparison to controls with $1.39 \pm 0.43\%$ and $1.11 \pm 0.43\%$ respectively (Chang *et al.*, 2004). Lymphocytes from PC patients in the Chang *et al*. (2004) study were also shown to induce greater MN in response to H_2O_2, indicative of BRCA1 mutations. A larger case-control study monitored the MN frequency in peripheral lymphocytes from 346 newly diagnosed, histopathologically confirmed PC patients and 446 healthy

controls also showed an increased frequency of 15.3 ± 0.3 in PC patients in comparison to 10.7 ± 0.3 (Chang *et al.*, 2011).

Exploring PC patients further, it is seen that the percentage of PMBC with micronuclei differs depending on TNM staging of disease. Tumour size, nodal involvement and overall staging tend to increase the DNA damage viewed in PMBCs, although this has not been significant in this study (p= 0.742, 0.590 and 0.581, respectively). In contrast, as disease becomes metastatic, there appears to be less chromosomal damage than in patients with localised disease. In the large published PC study, no significant association was discovered between the stage of disease and the frequency of MN (Chang *et al.*, 2011). This is supporting our results, where no significance was determined. Although a larger population may have made the increases seen in tumour size, nodal involvement, and overall staging statistically significant. Previous studies across other malignancies show contrasting results. A study of breast cancer within an Iranian population showed highest micronuclei frequency within donors with metastatic disease, and lymph node involvement didn't show an increase in micronuclei (Salimi and Eskandari, 2018). A study of bladder cancer showed a higher frequency of MN in donors with disease not invading the muscular lining of the bladder in contrast to those with more advanced disease, whose disease was infiltrating the muscular wall of the bladder, although differences were non-significant (Pardini *et al.*, 2017).

Correlation between the PBMC CBMN assay and PIG-A results in donors with pancreatic disease was slightly negative. This is likely since the PIG-A assay measuring the frequency of specific point mutations within *PIG-A* rather than monitoring overall genome stability, seen in the CBMN assay and so results from both may not reflect each other. For example, a murine study explored diet-induced obesity using both the blood cell CBMN assay and Pig-A assay. Mice fed on a diet of 60% fat were shown to have 3-4 fold increased frequency of Pig-A mutant cells but no change was shown in MN frequency, suggesting diet induced obesity is mutagenic (Wickliffe *et al.*, 2016). Individual patients may be exposed to unique combinations of lifestyle factors and environmental factors, which will in turn affect DNA in different manners. For example, tobacco smoking is mutagenic so a donor who had smoked all their life may show higher PIG-A mutations and less in the way of micronuclei in PBMCs in contrast to a donor who has lived a healthy life but is

geriatric and DNA stability may have decreased, leading to cancer formation who may have higher percentage of MN in PMBCs but insignificant mutagenicity measured through PIG-A.

The *ex vivo* CBMN assay has been well established and used for a long time. Once one is used to the assay, it was a simple and convenient assay with easily determined endpoints. Drawbacks of the assay included the difficulty of PBMC separation. PBMC isolation, achieved through density gradient separation using histopaque, required gentle layering of whole blood onto histopaque. Several samples were ruined through incorrect layering of the blood on top of the histopaque, breaking the surface and mixing the two layers. This resulted in incomplete separation of the layers, seen in **Fig. 5.1**, resulting in red stained plasma coupled with no observable buffy layer. As a result, PBMC CBMN results were not available for all donors in this study. Additionally, the protocol followed for the assay required optimisation, with initial attempts having too few binucleated PBMCs for feasible scoring, requiring a doubling of the time exposed to Cytochalasin-B from 24 to 48h. Slide preparation was also a challenge initially due to the fragility of primary PBMCs. Suspension of cell pellets had to be achieved through gentle flicking of the tube otherwise microscope slides would be prepared, stained, and observed under a light microscope to find that PBMCs would be lysed. Additionally, in comparison to established cell lines, slide quality was considerably poorer in the primary PBMCs, with additional time required for scoring slides in comparison to established cell lines. With advances in semi-automated scoring systems such as the Metafer system used within **Chapter 3** and the novel flow cytometry based scoring of the PBMC CBMN assay (Rodrigues *et al.*, 2018), slide preparation and scoring issues may no longer be relevant in the future.

5.4.2 Combining Biomarkers

Given the current challenges associated with diagnosis of PC, combining multiple biomarkers to form a battery of tests is likely to outperform individual biomarkers alone. Here, we combined biomarkers through simple multiplication of individual values to give a score. Multiplication of values to provide a score gives all biomarkers explored within the study equal weighting.

Table 5.1: *Average scores generated through combining different biomarkers in donors with benign and malignant pancreatic disease.*

Combination of Biomarkers	Benign	Malignant	Fold Change
PIG-A Alone	4.028±3.101	8.557±6.060	2.124
PIG-A and CA19-9	83.067±54.013	12707.143±29877	152.97
PIG-A and NLR	6.966±3.100	29.592±38.764	4.248
PIG-A and CRP	24.851±14.419	118.18±161.46	4.755
PIG-A and CBMN	2.167±2.070	14.633±11.057	6.751
All the above	N/A	391122±540552	N/A

Combination of CA19-9 with PIG-A resulted in the largest fold-change between benign and malignant donors of 159.97, which is 72.016 times greater than the fold change when using just PIG-A alone. Differentiation between benign and malignant pancreatic disease is a common diagnostic problem. Challenges arise between differentiation of benign and malignant disease both from imaging studies and blood test results, often with malignancy only confirmed through histopathological examination of surgical specimens. Issues have been raised previously with CA19-9 alone, given its elevation in benign conditions (Howaizi *et al.*, 2003) and cases of insignificant levels in malignant disease (Muratore *et al.*, 2016). In this study, CA19-9 levels alone give a 67.33-fold increase between benign and malignant disease, where combination with PIG-A increases the difference to 159.97-fold. This suggests use in combination with PIG-A may result in better differentiation between benign and malignant disease.

Table 5.2: *Average scores generated by combining biomarkers in donors with PC with increasing stage disease, and associated fold-changes in score from earliest disease to most advanced (IA to IV).*

Combination of Biomarkers	IA	IB	IIB	III	IV	Fold change (IA – IV)
PIG-A Alone	11.004	8.073	7.954	8.228	12.447	1.131
PIG-A and CA19-9	24.018	6209.3	43612	12533	10163	423.155
PIG-A and NLR	171.606	27.842	13.249	22.266	34.572	0.201
PIG-A and CRP	60.044	186.83	50.234	82.343	198.54	3.307
PIG-A and CBMN	8.337	11.168	19.060	20.342	17.791	2.134
All of the above	683.52	315751	773170	88033	128954	188.661

Table 5.2 explores scores of combined biomarkers for PC patients. Combining CA19-9 and PIG-A again provides the largest fold-change between the earliest stage disease and most advanced disease. A combination of the two gives greater separation than even the combination of all biomarkers together. Previous studies have combined novel biomarkers with current biomarker CA19-9 to improve CA19-9 performance within PC (Kaur *et al.*, 2017; Murali Manohar *et al.*, 2017; Jahan *et al.*, 2019). Additionally, combination of CA19-9 with current biomarker CA-125 in ovarian cancer has been used to differentiate between serous and epithelial type cancers (Zhang *et al.*, 2018). Continual use of the CA19-9 blood test may continue to be beneficial in patients while in combination with other novel biomarkers yet care

should still be taken given that 5-10% of the population remain Lewis antigen negative (Ballehaninna and Chamberlain, 2011).

Other than PIG-A alone, the poorest performing biomarker panel appeared to be PIG-A in combination with NLR. Previous studies had shown the usefulness of NLR when in used in combination with current PC biomarker, CA19-9, as a prognostic marker in 66 patients with recurring PC (Sakamoto *et al.*, 2018). However, NLR as a biomarker has been shown to be non-specific, having been explored in a range of disease processes including systemic infection (Gürağaç and Demirer, 2016), metabolic syndrome (Chuan Chuan Liu *et al.*, 2019), various autoimmune disorders (Chandrashekara *et al.*, 2017; Hammad *et al.*, 2018) and a range of malignancies (Gürağaç and Demirer, 2016). General inflammatory markers including CRP and NLR, although have prognostic capabilities within PC, may not be the most suitable tools for diagnostic and staging.

5.4.3 High or Low PIG-A

Through a simple binary high-low system, with a cut-off point established using the median value of all PIG-A values from all donors, we have explored the usefulness of PIG-A as a biomarker for pancreatic disease. It was established that more donors with malignant disease had PIG-A values which fell into the high category than in comparison to healthy and benign donors. This is an important hurdle for biomarkers to pass, however tweaking of the cut-off point may differentiate benign and malignant donors further to minimise the risk of both false-positives and false-negatives, both which can be detrimental to the patient's journey.

5.4.4 Questionnaire

Questionnaire data collected from patients provided insight into lifestyle and dietary choices from patients with both benign and malignant pancreatic disease. Dietary quality, quantitated using the DQS outlined in **Table 2.5**, was shown to be non-significantly elevated in patients with PC (p=0.172). This tends to contrast literature sources, which have previously linked PC development with poor dietary choices. As much as 30-50% of PC cases have been linked to nutritional intake, with grilled meats and fried foods increasing PC risk (Midha *et al.*, 2016). Furthermore, in our questionnaire data, we have seen a decreased frequency of circulating PIG-A mutant erythrocytes in past smokers and frequent consumers of alcoholic beverages in

comparison to controls. This is unexpected given both smoking and alcohol consumption give rise to known mutagens including B[a]P and acetaldehyde, renowned for their DNA adduct formation as seen in the *in vitro* data in **Chapter 3.**

There are limitations with our questionnaire data given the poor patient response, resulting in only eight fully, correctly completed questionnaires being received. Questionnaires were provided to donors to complete in their own time and return using a stamped, addressed envelope. It is understandable given the amount of information and leaflets provided to the patients from both the clinical and research staff that it may be forgotten and be the least important document provided. In hindsight, perhaps the patients should have completed the questionnaires within surgical clinics, however these were often tight for time with the high volume of patients to see in a limited time frame. The resulting data, although interesting, cannot be fully relied upon because of the small population. This may account for some of the deviations from trends seen within the literature.

5.4.5 Survival

Our survival data shows an increased survival in comparison to reported values within the literature and discussed in **1.2.6**. Median overall survival of PC patients has been estimated between 20 and 24 months (Bittoni *et al.*, 2014). Currently, within the UK, 25.4% of patients survive 1 year post-diagnosis (Cancer Research UK, 2020b). This is close to our 20% patient survival but given the time frame of patient recruitment between October 2017 and September 2019, we would expect the survival to be lower. However, with longer patient follow-up this may reveal closer results to reported values. Additionally, recruitment within surgical clinics may be skewing our data, since patients undergoing surgery have better overall survival both in the short and long-term (Huang *et al.*, 2018).

5.5 Conclusion

Here, we explored the peripheral PBMC CBMN assay for its use diagnosing and staging PC. Exploring the PBMCs CBMN in a population of PC patients, we discovered an elevated frequency of MN in PBMCs of PC patients in comparison to patients with benign pancreatic disease (p<0.000), supporting elevations also viewed within the literature. Within PC donors, increases in MN frequency were also viewed

as TNM staging and overall staging of disease increased, although this was non-significant.

Combinations of biomarkers were also explored, to determine whether a whole panel of clinical parameters in combination with our PBMC MN and PIG-A results would be useful in a diagnostic or staging setting. Of all assessed, combination of CA19-9 and PIG-A appeared to show the most promise, with the greatest fold changes when differentiating between benign and malignant pancreatic disease and when examining staging of PC.

Furthermore, overall survival data and questionnaire data was analysed. Survival data requires longer follow-up to determine whether values are in line with literature. Poor questionnaire involvement with patients limits the usefulness of data collected from questionnaires.

Chapter 6: General Discussion

<u>**Chapter 6: General Discussion**</u>

6.1 General Discussion

Despite PC being one of the rarer malignancies, only accounting for 3% of all cancer cases in the UK (Cancer Research UK, 2020c), it remains the fourth leading cause of cancer related mortality in the UK (Cancer Research UK, 2014b). With median overall survival between 20 and 24 months, PC has one of the worst prognoses of all solid malignancies (Bittoni *et al.*, 2014). PC is expected to become the second leading cause of cancer related mortality by 2030 (Bittoni *et al.*, 2014). The bleak prognosis arises from a combination of vague, late presenting symptoms, late diagnosis, limited treatment options and an aggressive disease state often believed to be systemic at diagnosis. PC diagnosis remains a challenge, with blood-based biomarker CA19-9 not being fully relied upon and difficulties differentiating between benign and malignant disease from imaging studies alone. Often, a definitive diagnosis is only achieved following histopathological examination of biopsies or surgical specimens. Advances in PC diagnosis are needed urgently to improve patient outcomes, increase survival, and save lives.

Blood-based biomarkers are a cheap, non-invasive, and accessible methods of determining information about a patient's health from a small blood sample. Current blood-based biomarkers for PC are discussed in **5.1** including CA19-9, CRP, bilirubin, and the neutrophil to lymphocyte ratio. Novel biomarkers are continually being investigated for their promise in PC diagnosis, with ICAM-1 and MUCA5 showing great potential (Tempia-Caliera *et al.*, 2002; David V. Gold *et al.*, 2013).

6.2 Presented Work

Here, we have examined the use of two blood-based assays for their potential use in PC diagnosis. Both assays quantify DNA mutation within separate blood cell populations. Given that cancer is a disease initiated within the DNA, an increased mutational load may be indicative of malignant disease and even the staging of the malignancy.

6.2.1 Human Erythrocyte PIG-A Assay

The human erythrocyte PIG-A assay was used to examine a novel population of patients with benign and malignant pancreatic disease alongside healthy donors.

Healthy donors were found to have a median mutant frequency of 4.55 x 10^{-6} (95% CI 1.48-6.51), comparable to reported healthy donor mutant frequencies from population studies including means of 2.9 x 10^{-6} and 5.25 x 10^{-6} and median value of 2.8 x 10^{-6} (Dertinger *et al.*, 2015; Cao *et al.*, 2016; Haboubi *et al.*, 2019). Patients with benign pancreatic disease showed a comparable mutant frequency in comparison to healthy donors, with a median of 2.64 x 10^{-6} (95% CI 2.11-4.16).

PC patients were found to have an elevated mutant frequency over benign pancreatic donors with a median of 5.81 (95% CI 6.25-10.86) (p=0.013) but not over healthy donors (p=0.078). A previous study within our research group has shown an elevated PIG-A mutant frequency in oesophageal adenocarcinoma patients, with a 3-fold increase shown between oesophageal adenocarcinoma patients in comparison to healthy donors and gastroesophageal reflux disease patients (Haboubi *et al.*, 2019). This is greater than our 2.075-fold increase seen in our study between benign and malignant pancreatic donors, but this does not diminish the potential shown here within PC. Other DNA based assays have also shown an increased circulating DNA mutation in PC donors, including within the lymphocyte CBMN assay (Chang *et al.*, 2004).

Furthermore, our results showed a slight increase in PIG-A mutant frequency in patients with metastatic disease, although this was determined non-significant. Further study and a larger population of donors with metastatic disease may examine whether this increase becomes significant and if so, whether this could be exploited for use as a staging tool. Given the invasive nature of a lot of diagnostic techniques for PC, stratification of patients could be carried out based upon PIG-A mutant frequency and reduce unnecessary invasive procedures and fast-track patients with greatly elevated PIG-A for quicker access to the right procedures and clinicians.

6.2.2 Human PBMC Cytokinesis Block Micronucleus Assay

The human PBMC CBMN assay was used to explore a cohort of patients with both benign and malignant pancreatic disease. An elevated frequency of PBMCs with micronuclei was viewed in PC patients (p<0.000). This elevation within the malignant pancreatic cohort has also been viewed within a PC population in a previous study of PC patients (Chang *et al.*, 2004). In a later experiment, the group further classified BRCA1 mutant patients through a challenge assay and exposure to

H_2O_2 (Chang *et al.*, 2004). A further, larger population study carried out by the same group also established an elevated MN frequency within PC patients, although it was unclear whether this cohort was treatment naïve (Chang *et al.*, 2011). Previous studies have also explored the PBMN CBMN assay in other malignancies. A study of colorectal cancer patients throughout therapeutic interventions took pre-treatment measures and compared their results to control, non-cancer patients. The treatment naïve colorectal cancer patients were shown to have a 3.45-fold increased frequency of MN over healthy controls (Nikolouzakis *et al.*, 2019). This is a similar to the 3.696-fold increase viewed here in PC patients over benign and healthy donors. This fold-increase is also larger than those seen in previous PC studies of 1.577 and 1.252 (Chang *et al.*, 2004; Chang *et al.*, 2011). Furthermore, a study of papillary thyroid cancer showed a 2.67-fold increase over an age and gender matched control population (Gerić *et al.*, 2015). Furthermore, a study of a range of cancers including breast, uterine and pharyngeal cancers identified a 2.35-fold increase in MN frequency in cancer patients in comparison to benign donors. This places our MN induction differences within the same fold-change as seen within the literature, but greater than previous PC specific studies.

The large PC population study determined no changes in MN frequency as staging of PC increases (Chang *et al.*, 2011). In contrast, our study has seen slight elevation in MN frequency as tumour size and nodal involvement increases and a decrease in MN frequency as disease becomes metastatic, although these results remain non-significant. A larger population within our study may have led to significant results, with greater statistical power behind the results.

6.2.3 Biomarker Panel

Currently, the only biomarker clinically in use for PC is CA19-9. However, its use is limited since it's a modified Lewis antigen (Tempero *et al.*, 1987). As a result, 5-10% of the population has a Lewis negative phenotype and so CA19-9 test results may not be clinically relevant (Ballehaninna and Chamberlain, 2011). For example, in our study 27.27% of donors with a clinically confirmed PC diagnosis were shown to have insignificant CA19-9 levels below the 37 kU/L cut-off value (Thaker *et al.*, 2014). The CA19-9 levels within PC patients ranged from as low as 2 kU/L to 33

kU/L (mean 9.333±11.793). As a result, CA19-9 alone would not be a reliable blood-based biomarker for PC.

Given the multifactorial complexity of PC, a panel of biomarkers with a wide range of sources is likely to provide a better option for diagnosing PC from a blood-based perspective. A biomarker panel of a tumour marker in combination with biomarkers for other features like systemic inflammation can perform better than a tumour marker alone and provide further clinical information (Brand *et al.*, 2011). Building upon this, we aimed to study a panel of biomarkers including the PC tumour marker CA19-9, two DNA mutation assays: the novel PIG-A assay and PBMC CBMN assay, and two inflammatory markers: CRP for clinical and NLR for subclinical inflammation, given the strong links with inflammation in pancreatic carcinogenesis. In this pilot study, we simply multiplied the values from each of the five lone biomarkers to provide an arbitrary factorial score. Multiplication gave each biomarker equal weighting and was simple enough for our pilot study with a relatively small population. Other larger studies have combined biomarkers through more complicated methods, including regression analysis, receiving operating characteristic curves and the area under the curve (Balasenthil *et al.*, 2011; Fahrmann *et al.*, 2019). These techniques could be used on future, larger studies using our biomarker panel in the future, if this panel shows promise.

We studied a biomarker panel of all the previously mentioned markers, alongside smaller panels of a duo of PIG-A and each marker individually. Since no donors with all five biomarkers recorded had benign disease, a fold change between benign and malignant donors could not be established for the full panel. However, when overall staging of PC there was a 188.661-fold increase in biomarker score from stage IA disease to stage IV, but this difference was determined non-significant through a one-way ANOVA (p=0.645). The largest increases were viewed in the combination of PIG-A and CA19-9 of 152.97 and 423.155-fold increases when disease and disease stage were compared, respectively. The increase between benign and malignant donors almost reached significance (p=0.053) whereas the increase viewed in overall disease staging was non-significant (p=0.428).

Combination of CA19-9 and PIG-A appeared to the most suitable combination of biomarkers assessed in this study for both PC diagnosis and staging. Although care

should be taken due to the Lewis antigen negative population (Ballehaninna and Chamberlain, 2011). Combination of CA19-9, which is not expressed in 5-10% of the population (Ballehaninna and Chamberlain, 2011), with the ubiquitously expressed PIG-A gene may improve upon the use of CA19-9 alone. For example, our combination of PIG-A and CA19-9 has increased the fold-change viewed in overall staging than that of just CA19-9 alone. Furthermore, previous studies have also combined novel biomarkers with CA19-9 to improve the performance of CA19-9 in PC patients (Kaur *et al.*, 2017; Murali Manohar *et al.*, 2017; Jahan *et al.*, 2019).

6.2.4 *In Vitro* Model of Pancreatic Cancer

In an *in vitro* model of PC, we aimed to determine the sources of some of the mutations viewed in the *in vivo* studies and to further explore the role that dietary and lifestyle choices play in pancreatic carcinogenesis. PC development can be heavily influenced by lifestyle factors, with as many as 37% PC cases thought to be avoidable by making healthier life choices (Cancer Research UK, 2014b). We modelled smoking, a high fat diet and alcohol abuse using associated chemicals B[a]P, DCA and acetaldehyde in both the PDAC cell line PANC-1 and the L5178Y cell line, widely used in genotoxicity assessments.

Smoking has been shown to be linked with 29% of all PC cases (Cancer Research UK, 2014b) and is implicated in reducing median survival time in PDAC by almost half (Zhang *et al.*, 2016). Here, smoking modelled by B[a]P was only explored within the metabolically competent PANC-1 cell line. An induction of MN in the CBMN assay was seen at both 20 and 50μM. B[a]P has a known carcinogenic mechanism, undergoing sequential oxidation, hydration and further oxidation to form ultimate mutagenic product BPDE, which forms adducts namely at N2 guanine position (Alexandrov *et al.*, 2010; Shah *et al.*, 2016). B[a]P is only one of over 4,000 chemicals in the complex mixture of compounds present in cigarette smoke which could play a role in pancreatic carcinogenesis (Barone *et al.*, 2016). Due to this complex composition of cigarette smoke and clear induction of DNA mutation, viewed by the CBMN assay in PANC-1, we expected to view an elevated PIG-A mutant frequency in erythrocytes of smokers. Questionnaire data from study participants showed a small snapshot of lifestyle and dietary choices from eight donors. Within this, we had two groups of never smokers and past smokers, with no

current smokers. Interestingly, our past smokers showed a slight reduction in PIG-A mutant frequency in comparison to the non-smoking group, but this was non-significant. This does not reflect the *in vitro* work, but this could come as a result of a small population of donors who completed the questionnaire not being truly representative, an alternative measure of mutation in the PIG-A assay and CBMN assay and additionally may show past smokers having a higher activity of DNA repair mechanisms following on from years of smoking.

Obesity is an increasing concern globally. Several malignancies have been linked with obesity, including PC. Specifically, 12% of PC cases have been attributed to obesity (Cancer Research UK, 2014b). High-fat diets are likely to cause obesity. Dietary fats are digested with the aid of bile with bile acids emulsifying fats into micelles which can then be absorbed (Boyer, 2013; Pavlović *et al.*, 2018; Chen and Cassaro, 2019). Increased serum concentrations of bile acid DCA has been observed in obese donors (Xie *et al.*, 2015). Following DCA treatment, induction of MN was viewed in the PANC-1 cell line at 50 and 300μM (significantly) and at 150μM in the L5178Y cell line (non-significant). Previous studies have also shown induction of DNA damage by DCA in an oesophageal adenocarcinoma cell line, diminished by vitamin C treatment highlighting the ROS-based genotoxic mechanism (G. J.S. Jenkins *et al.*, 2007). Furthermore, an induction of Pig-A mutants were viewed in the *in vitro* Pig-A assay in L5178Y, but this was non-significant. Questionnaire data also showed an increased PIG-A mutant frequency in donors who had poorer DQS values with regards to dietary fats, but this was also non-significant. Induction of PIG-A mutants viewed both *in vivo* and *in vitro*, although non-significant may signify the mutagenic potential of obesity. A larger donor population would further explore this. Additionally, studying further bile acids involved in obesity and inflammatory markers associated with obesity-driven inflammation may provide further insight.

Consumption of alcoholic beverages is ingrained in western society, but overindulgence and abuse of alcohol can lead to a range of long-term health problems. Alcohol abuse has increased the risk of PC development by 20% (Cancer Research UK, 2014b). Alcohol is one of the most common inducers of pancreatitis, with up to a third of acute pancreatitis cases in the US coming as a result of it (Chowdhury and Gupta, 2006). Ethanol is metabolised within the body to toxic intermediate acetaldehyde, which was used to model alcohol abuse *in vitro*. Of the

three chemicals tested, acetaldehyde was the most potent, inducing higher proportions of DNA damage at lower doses than either DCA or B[a]P. Significant induction of MN were viewed in both the PANC-1 and L5178Y cell lines at both 2.5 and 1µM, respectively. Following on from this, Pig-A mutations were induced in a dose dependent manner following acetaldehyde exposure, with significance at 1µM. Similar to B[a]P, acetaldehyde forms DNA adducts, primarily at the N2 guanine position (Mizumoto *et al.*, 2017b). Both questionnaire and human *in vivo* PIG-A data did not reflect *in vitro* work. In fact, we saw a negative correlation between erythrocyte mutant PIG-A frequency and alcohol units consumed per week. Equally important to consider is the small population of completed questionnaires and this data was skewed by one donor with high alcohol consumption. A larger population would allow further exploration of this and to determine whether mutations seen *in vitro* may reflect some of those seen in the *in vivo* PIG-A mutation assay.

6.3 Future Work

Although promising results have been seen in this thesis, this is still only a preliminary pilot study. With only 30 patients diagnosed with PC as donors to the study, it is sufficient for preliminary results within our pilot study. Future work on a larger scale would be beneficial to give further statistical power behind the results collected. A particularly interesting patient cohort, which would be good to recruit to future studies would be PC patients from oncology departments before initiating chemo or radiotherapy. These patients would have disease too advanced for surgery and are a patient group we may have missed since ethical approval granted permission from recruitment within surgical departments. Having more patients with higher staged disease would be good to examine, allowing better analysis of the PIG-A results with regards to staging of disease. For example, only one donor in this study had T4 disease and so very little information could have been elucidated from this.

An interesting further study would be a fully blinded study, where diagnoses can be predicted using the cut-off value determined in **chapter 5.3.3**, 4.860×10^{-6}, with mutation frequencies above this value would be predicted to be cancer. In our study, 68.97% of all PC patients were defined to have a high PIG-A in comparison to just 10% of benign pancreatic donors. While taking into consideration the small

population studied in this pilot study, a greater patient population in a future study may result in fewer than 10% of benign donors with high PIG-A, and a greater proportion of PC patients with high PIG-A. Further work could then be carried out once official diagnoses are revealed by modifying the cut-off value to improve its diagnostic value by improving accuracy, sensitivity, and specificity.

Questions have been raised about the specificity of PIG-A as a biomarker for pancreatic malignancy due to the elevation viewed within oesophageal adenocarcinoma. Currently these are the two malignancies explored within our research group, given both diseases have dismal prognoses. Although a wider range of malignancies were explored by Horibata *et al.* (2016) during therapeutic interventions, no pre-treatment bloods were taken, so we cannot determine whether there are elevated PIG-A mutant frequencies in other malignancies. Further studies to establish the PIG-A mutant frequencies across a variety of malignancies would be beneficial. If elevated PIG-A mutant frequencies are common across all cancers, the human erythrocyte PIG-A assay could be incorporated into a battery of tests used in suspected cancer cases to help differentiate between benign and malignant disease. Suspected blood-borne malignancies would be especially interesting to examine, since bone marrow is the source of blood cell precursors. Furthermore, exploration of other malignancies would determine whether PIG-A mutant elevation is non-specific or purely limited to gastrointestinal malignancies and will ultimately guide the future use of the assay.

The human PBMC CBMN assay is well established and has been used extensively. Beneficial future work which would improve upon the current PBMC CBMN assay would include automation of the scoring of the assay. Semi-automated Metafer scoring of the CBMN assay carried out in the PANC-1 and the L5178Y cell lines within **chapter 3**. Primary human PBMCs were too small to be identified using the current classifiers. Producing and testing a new classifier on the Metafer system, which could identify primary human PBMCs would be better in the future. Although manual scoring remains the gold standard for the CBMN assay, the semi-automated Metafer system has its advantages over the manual system. Semi-automated scoring allows for quicker examination of cells, with an entire slide being able to be scored within 30 minutes, in comparison to a minimum of an hour for manual CBMN scoring. Additionally, the Metafer system requires the scoring of 2,000 binucleated

cells per slide, in comparison to 1,000 cells in manual scoring. This provides a greater statistical power for results collected through this manner. Semi-automated scoring would also bypass a lot of the problems associated with slide preparation discussed within **5.4.1**.

6.4 Conclusion

Challenges in diagnosing PC result in the majority of patients diagnosed with advanced PC, too late for curative intervention. This helps to maintain the dismal prognosis faced by PC patients. Improvements in PC diagnosis are needed urgently to help improve the bleak outlook.

Here, we have assessed the use of two blood-based biomarkers and explored their use within a biomarker panel for PC diagnosis. The human erythrocyte PIG-A assay monitors the mutation status of the X-linked PIG-A gene through flow cytometric detection of GPI anchored proteins on the erythrocyte surface. We have viewed an increased frequency of PIG-A mutant erythrocytes within donors with PC in comparison to both benign pancreatic and healthy donors. Furthermore, when PC staging was examined, an elevated PIG-A frequency was observed in patients with metastatic disease in comparison to localised disease. The assay has potential for use in diagnosis and staging of PC. It may also show potential for patient stratification for more invasive investigative procedures for PC diagnosis such as EUS or ERCP. An elevated frequency of MN in PBMCs was also observed within PC patients, but did not show much potential with staging disease. A range of biomarker panels were assessed, with a combination of CA19-9 and PIG-A showing most potential for diagnosing and staging PC, improving upon use of the single biomarkers alone.

A further *in vitro* study was also used to identify lifestyle and dietary choices which may account for PIG-A mutants seen within the *in vivo* study.

Chapter 7: Appendices

7.1 Study Documentation

The following documents were prepared and applied for REC committee approval via IRAS. Approval granted by Cornwall and Plymouth REC and ABMU R&D.

Dept. of Surgery

Morriston Hospital

Swansea

SA6 6NL
01792 487418

Patient Information Sheet– 25th January 2017, Version 4.0

Information Sheet for Research Participants

Study title:

Blood cell mutation analysis in pancreatic disease

Chief Investigator: Miss Lucy Nichols, Co-investigator: Professor Gareth Jenkins, Clinical lead: Professor Bilal Al-Sarireh.

You are being invited to take part in a research study which makes part of a PhD. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and members of hospital staff. Ask us if there is anything that is not clear or if you would like more information. Please take time to decide whether or not you wish to take part in the study.

If you do decide to take part, please let us know beforehand if you have been involved in any other study during the last year. If you decide not to take part or to withdraw at any other time without explanation, your future care will not be affected by your decision. Thank you for reading this.

What is the purpose of the study?

You are undergoing investigations for pancreatic disease. We would like to develop blood-based tests so that in the future we can detect pancreatic disease more simply. In order to do so we require donated blood samples from patients with a range of pancreatic diseases. We will compare the results of our test in patients with different types of disease. We then can see if our newly developed test can (in the future) detect early forms of pancreatic disease.

We plan to investigate this by:

a) Asking you to fill out a questionnaire about your lifestyle.
b) Taking a blood test from the intravenous line which will be inserted as a matter of routine. This will be approximately 10mls (2 teaspoons).
c) We may later analyse tissue taken during biopsy and/or surgery for routine purposes. You will not have to provide extra tissue samples, we will use "left-over" tissue. We will obtain this from the pathology dept.
d) We may carry out genetic analysis on the blood sample donated.
e) Samples will be disposed of at the end of the study.

Why have I been chosen?

You are being investigated for the presence of pancreatic disease.

Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part, you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive in the future.

What do I have to do?

You will be approached and informed about the study while attending the Department for your follow-up clinic appointment, OR while attending your follow-up outpatient appointment in the main outpatients department. The researcher will:

- Ask you about your health in general, and any previous operations

- Explain the purpose of this study in further details and answer your questions

Taking part in this study will not affect the standard treatment that you receive.

You can opt out of the study at any stage.

What will happen to me if I take part?

If you decide to take part in this study you will be asked to sign the necessary consent form, then you will have:

1) A questionnaire which will take no longer than 10 minutes to complete, which will ask you about your diet, lifestyle and medications. It will also ask you to give your weight and height measurements.

2) A blood test will also be offered to you, which you can accept or refuse. We will take this blood using a standard blood test.

3) We may (in some cases) obtain some "left-over" biopsy tissue to study some cellular details to compare to the blood data. You will not have to provide any additional biopsies for this.

What are the possible disadvantages and risks of taking part?

There are no known risks associated with taking part. The blood test will be taken by trained staff, although some minimal discomfort may be experienced, as per all blood tests.

What are the possible benefits of taking part?

There will not be immediate benefits from taking part in this study. The study will help develop a novel blood test that may be beneficial to patients in the future.

Will my taking part in this study be kept confidential?

All information that is collected about you during the course of the research will be kept strictly confidential. Your name and address will be removed from the information when it is shown to other medical staff outside the study.

Who is organising the research?

This study is being organised by the staff of the surgery department of ABM University Health Board and Swansea University.

Who has reviewed the study?

The Research Ethics Committee of Cornwall and Plymouth has reviewed this study.

What if something goes wrong?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect, or about the way you have been treated during the course of this study then you should immediately inform Professor Bilal Al-Sarireh (Dept Surgery, Morriston hospital, 01792 487418). The normal National Health Service complaint complaints mechanisms are also available to you. If you are still not satisfied with the response, you may contact the Mr Raymond Cibrowski, Registrar & Chief Operating Officer (Email: researchgovernance@swansea.ac.uk or misconduct@swansea.ac.uk).

Contact for Further Information

If you want more information, before or after you return your form, you can phone (9am-5pm Mon-Fri)

Professor Gareth Jenkins on

Miss Lucy Nichols on

Professor Bilal Al-Sarireh

The Cornwall and Plymouth Research Ethics Committee has reviewed and approved the above statement.

7.1.2 Informed Consent Form

Dept of Surgery

Morriston Hospital

Swansea

SA66NL

Title of research "Blood cell mutation analysis in pancreatic disease"

CI: Prof Gareth Jenkins

The participant should complete the whole of this sheet himself/herself.

(Please initial each statement if it applies to you)

I have read the Information Sheet for Patients dated November 2016

I have received enough information about the study, have been given

the opportunity to ask questions and discuss the project and have received

satisfactory answers to all my questions.

I understand that relevant sections of my medical notes may be accessed by

members of the research team, the

Health Board or from regulatory authorities for the purposes of monitoring or auditing

The study, including the risks and benefits, has been explained to me by

a member of the clinical research team

I consent to having a blood sample taken for analysis by the research team

I agree to completing the questionnaire associated with this study

I understand that researchers may access "left-over" biopsy material for further analysis ☐

I understand that I am free to withdraw from
the study at any time, without having to give a reason for
withdrawing and without affecting my future medical care. ☐

Signed.. Date................................

(NAME IN BLOCK CAPITALS)...…

Investigator's signature......................................…… Date: ....

(NAME IN BLOCK CAPITALS)...…

1 copy for patient, 1 copy for Principal Investigator, 1 copy for hospital notes

7.1.3 Questionnaires

The REC requested separate questionnaires for male and female participants to avoid confusion around final questions about male or female specific health concerns.

7.1.3.1 Questionnaire – Male

Pancreatic Disease Questionnaire. V2. 20/01/2017

DIET.

We are interested in the type of foods you eat and how frequently you eat them.

Questions about your diet

1. Do you eat meat?   **Yes ☐   No ☐**

If **Yes**, how many times a week do you eat meat?

 _____times a week

If **No**, how old were you when you last ate meat?

_____years old

2. Do you eat any fish? **Yes** ☐ **No** ☐

If **Yes**, how many times a month do you eat the following?

(put '0' if less than once a month)

Fatty fish _____times a month Other fish _____times a month

(including sardines, (including Cod, tuna, haddock)

salmon, mackerel, herring)

If **No**, how old were you when you last ate fish?

_____years old

3. Do you eat any dairy products? **Yes** ☐ **No** ☐

(including milk, cheese, butter, yoghurt)

If **No**, how old were you when you last ate dairy products? _____years old

4. **Do you eat any eggs?** **Yes** ☐ **No** ☐

(including eggs in cakes and other baked foods)

If **Yes**, how many eggs do you eat each week?

_____eggs each week

Put "0" if eaten less than once a week

If **No**, how old were you when you last ate eggs?

_____years old

5. What type of milk do you use most often?

Full cream ☐ Soya milk fortified with calcium ☐

Semi-skimmed ☐   Soya milk not fortified with calcium ☐

Skimmed/fat free ☐   Other   ☐

None   ☐

How much milk do you drink each day, including milk with tea, coffee, cereals, etc.

Less than a quarter of a pint (<150ml) ☐ Three quarters of a pint ☐

                                                   (450ml)

Quarter of a pint (150ml)   ☐ One pint (600ml)   ☐

Half a pint (300ml)   ☐ More than one pint   ☐

                                                   (>600ml)

6. What type of spread do you use most often on bread, crispbreads, vegetables, etc.?

Butter   ☐ Hard margarine   ☐

                                                 (in wrapper not tub)

Dairy spread e.g. Clover   ☐ Soya margarine or   ☐

                                               other milk free margarine

Low or reduced fat spread   ☐ Cholesterol lowering   ☐

                                             spread e.g.benecol/Flora pro active

Olive based spread e.g. Olivio ☐ Other margarine   ☐

Polyunsaturated margarine   ☐ None   ☐

e.g. Flora

How thickly do you spread it?

Thick ☐   Medium ☐   Thin ☐

Do you add it to potatoes?   **Yes ☐ No ☐**

Do you add it to other vegetables?   **Yes ☐ No ☐**

7. What type of fat do you use most often for cooking?

Butter	☐ Lard	☐
Soft margarine	☐ Olive oil	☐
Hard margarine	☐ Other vegetable oil	☐
Solid vegetable fat e.g. White flora	☐ None	☐

8. Do you eat organic food?

Never ☐

Sometimes ☐

Usually ☐

Always ☐

9. How much bread, crispbread, etc. do you normally eat each day?

(Put '0' if none)   (Put '0' if none)

White bread _____   Crispbread _____

(slices per day)   (biscuits per day)

Brown bread _____   Sweet biscuits _____

(slices per day)   (biscuits per day)

Wholemeal bread _____

(slices per day)

10. What type of beakfast cereal do you eat most often?

Bran cereal	☐	Muesli, oat clusters, etc	☐
e.g. Branflakes			
Wholemeal cereal	☐	Other	☐
e.g. Weetabix		e.g. cornflakes, Rice krispies	
Porridge,	☐	None	☐
hot oat cereal			

How many bowls of cereal do you eat each week?

_____bowls a week

Put '0' if none

11. How much of the following do you drink each day?

Put '0' if none

Tea	____ cups daily	Pure fruit juice	____ glasses daily
Herb tea	____ cups daily	fruit drinks, squash	____ glasses daily
Coffee	____ cups daily	"Diet" fizzy soft drinks	____ glasses/cans daily
Water	____ glasses daily	Fizzy soft drinks	____ glasses/cans daily

12. How many teaspoons of sugar, in total, do you add to your tea, coffee, cereal, fruit, etc. each day

_____teaspoons each day

Put '0' if none

13. At present, about how many alcoholic drinks do you have each week?

Put '0' if none

Beer, lager or cider _____pints each week   Sherry or fortified wine _____glasses each week

Red wine _____glasses each week   Spirits – whisky, gin,

brandy _____glasses each week

White wine _____glasses each week

13. How often do you eat the following?

Please cross one box for each item

	Never	Seldom	Once a week	2-4 times a week	5-6 times a week	Once or more a day

Fresh fruit					
Dried fruit (raw)					
Stewed fruit, tinned fruit					
Pasta, e.g. spaghetti					
Rice					
Pizza					
Chips					
Other potatoes					
Peas					
Baked beans					
Lentils, dried beans					
Tomatoes					
Carrots					
Green vegetables					
Other cooked vegetables					
Salad/raw vegetables					
Tofu					
Soya meat, burgers, TVP					

	Never	Seldom	Once a week	2-4 times a week	5-6 times a week	Once or more a day
Other veggie burgers						
Cheese						
Cottage cheese						
Soya cheese						
Yoghurt, dairy						

desserts						
Soya, yoghurt, soya desserts						
Cream, ice cream						
Soya cream, ice cream						
Cakes, puddings, pies, buns, etc						
Chocolate, any type						
Other sweets, e.g. boiled sweets						
Crisps, Hula hoops, etc						
Peanut butter, salted nuts						
Other nuts and seeds not in muesli						
Jam, marmalade						
Yeast extract, Marmite						

LIFESTYLE.

We are interested in the type of activities you undertake.

Questions about your lifestyle

1. Have you ever smoked cigarettes   **Yes** ☐   **No** ☐

If you have stopped smoking cigarettes, how old were you when you gave up? _____years old

If you smoke now, how many cigarettes, do you usually smoke each day? _____cigarettes a day

2. Do you smoke cigars?   Yes ☐ No ☐

3. Do you smoke a pipe?   Yes ☐ No ☐

4. Do you have a paid job at present?

Yes, full-time ☐   Yes, part-time ☐   No ☐

If **Yes**, we would like to know the type and amount of physical activity involved in your work.

Please put a cross in the appropriate box.

Sedentary occupation   ☐

You spend most of your time sitting (such as in an office)

Standing occupation   ☐

You spend most of your time standing or walking, but your work does not require intense physical effort (e.g. shop assistant, hairdresser, guard)

Manual work   ☐

This involves some physical effort including handling of heavy objects and use of tools (e.g. plumber, electrician, carpenter)

Heavy manual work   ☐

This involves very vigorous physical activity including handling very heavy objects (e.g. docker, miner, bricklayer, construction worker)

5. In a typical week during the past year, how many hours did you spend per week on the following activities? Put '0' if none

                        In Summer   In Winter

Walking, including to

work, shopping and   _____hours per week   _____hours per week

leisure time

Cycling, including

cycling to work,   ____hours per week   ____hours per week

and during leisure time

Gardening   ____hours per week   ____hours per week

Do-It-Yourself   ____hours per week   ____hours per week

<u>In Summer</u>   <u>In Winter</u>

Physical exercise such   ____hours per week   ____hours per week

as keep-fit/aerobics,

swimming, jogging, tennis, etc.

Housework, such as   ____hours per week   ____hours per week

cleaning, washing,

cooking and childcare

6. In a typical week during the past 12 months, did you practice any of these activities vigorously enough to cause sweating or a faster heartbeat?

Yes ☐ No ☐

If **Yes**, for how many hours per week in total did you practice such vigorus activity? _____hours per week

7. What is your weight?

Stones_____pounds____ OR ____.__ Kg

8. Please indicate your marital status

Single   ☐   Separated   ☐

Married or living as married ☐   Divorced   ☐

Widowed   ☐

HEALTH.

We are interested in your past medical history and your current state of health.

Questions about your health

1. In the past six years, have you had any broken/fractured bones?

Yes ☐ No ☐

If **Yes**, please give details: _____(bone) _____(year)

_____(bone) _____(year)

_____(bone) _____(year)

2. In the last six years, has your doctor told you that you that you had any of the following?

	Yes	Year first diagnosed	No
Cancer			
Type of cancer:			
Polyps in large intestine			
Enlarged prostate (men only)			
High blood pressure			
High blood cholesterol			
Angina			
Stroke			

	Yes	Year first diagnosed	No
Heart Attack			
Cardiac arrhythmias/ palpatations/ irregular heartbeat			
Blood clot in leg			
Blood clot in lung or elsewhere			
Diabetes			
Thyroid disease			
Cataract in eye			
Stomach ulcer			
Duodenal ulcer			
Gallstones			
Have you had your gallbladder removed?			
Crohn's disease			
Ulcerative colitis			
Asthma			
Bronchitis/emphysema			
Rheumatoid Arthritis			
	Yes	Year first diagnosed	No
Osteoarthritis			
Depression requiring treatment			
Other significant Illnesses or operations, please give details			

3. Do you regularly take any vitamins, minerals or other supplements?

Yes ☐   No ☐

If **Yes**, do you take: (you can cross more than one box)

Multivitamins (with minerals)   ☐ Vitamin A ☐

Multivitamins (without minerals) ☐ Vitamin B ☐

Fish oil (including cod liver oil)   ☐ Vitamin C ☐

Evening primrose oil   ☐ Vitamin D ☐

Garlic   ☐ Vitamin E ☐

Iron   ☐ Other   ☐ _____

Zinc   ☐   (name and brand)

Calcium   ☐

4. Have you taken any medications for most of the last 4 weeks?

Yes ☐ No ☐

If **Yes**, please list them below: (you can name more than one box)

_____
_____
_____
_____
_____

5. About how many bowel movements do you have each week?

____a week

How often do you take laxatives? (Put '0' if never)

____times a month

6. How would you describe your health now?

Excellent ☐   Good ☐   Fair ☐   Poor ☐

QUESTION FOR MEN ONLY

7. Have you had a vasectomy?   Yes ☐ No ☐

If **Yes**, at what age? _____years old

Thank you for taking the time to complete this questionnaire.

Please return to Miss Lucy Nichols

Institute of Life Science, Swansea University SA28PP

OR

Bring this questionnaire with you on the day of your appointment

7.1.3.2 Questionnaire – Female

Pancreatic Disease Questionnaire. V2. 20/01/2017

DIET.

We are interested in the type of foods you eat and how frequently you eat them.

Questions about your diet

1. Do you eat meat?   **Yes ☐   No ☐**

If **Yes**, how many times a week do you eat meat?

_____times a week

If **No**, how old were you when you last ate meat?

_____years old

2. Do you eat any fish?   **Yes ☐   No ☐**

If **Yes**, how many times a month do you eat the following?

(put '0' if less than once a month)

Fatty fish _____times a month   Other fish _____times a month

(including sardines,   (including Cod, tuna, haddock)

salmon, mackerel, herring)

If **No**, how old were you when you last ate fish?

_____years old

3. Do you eat any dairy products?   **Yes ☐   No ☐**

(including milk, cheese, butter, yoghurt)

If **No**, how old were you when you last ate dairy products? _____years old

4. **Do you eat any eggs?**   **Yes ☐ No ☐**

(including eggs in cakes and other baked foods)

If **Yes**, how many eggs do you eat each week?

_____eggs each week

Put "0" if eaten less than once a week

If **No**, how old were you when you last ate eggs?

_____years old

5. What type of milk do you use most often?

Full cream   ☐   Soya milk fortified with calcium   ☐

Semi-skimmed   ☐   Soya milk not fortified with calcium ☐

Skimmed/fat free ☐   Other   ☐

None   ☐

How much milk do you drink each day, including milk with tea, coffee, cereals, etc.

Less than a quarter of a pint (<150ml) ☐ Three quarters of a pint ☐

(450ml)

Quarter of a pint (150ml)   □ One pint (600ml)   □

Half a pint (300ml)   □ More than one pint   □

(>600ml)

6. What type of spread do you use most often on bread, crispbreads, vegetables, etc.?

Butter   □ Hard margarine   □

(in wrapper not tub)

Dairy spread e.g. Clover   □ Soya margarine or   □

other milk free margarine

Low or reduced fat spread   □ Cholesterol lowering   □

spread e.g.benecol/Flora pro active

Olive based spread e.g. Olivio □ Other margarine   □

Polyunsaturated margarine   □ None   □

e.g. Flora

How thickly do you spread it?

Thick □   Medium □   Thin □

Do you add it to potatoes?   Yes □ No □

Do you add it to other vegetables? Yes □ No □

7. What type of fat do you use most often for cooking?

Butter   □ Lard   □

Soft margarine   □ Olive oil   □

Hard margarine   □ Other vegetable oil   □

Solid vegetable fat e.g. White flora □ None   □

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8. Do you eat organic food?

Never ☐

Sometimes ☐

Usually ☐

Always ☐

9. How much bread, crispbread, etc. do you normally eat each day?

(Put '0' if none)   (Put '0' if none)

White bread ____   Crispbread ____

(slices per day)   (biscuits per day)

Brown bread ____   Sweet biscuits ____

(slices per day)   (biscuits per day)

Wholemeal bread ____

(slices per day)

10. What type of beakfast cereal do you eat most often?

Bran cereal ☐   Muesli, oat clusters, etc ☐

e.g. Branflakes

Wholemeal cereal ☐   Other ☐

e.g. Weetabix   e.g. cornflakes, Rice krispies

Porridge, ☐   None ☐

hot oat cereal

How many bowls of cereal do you eat each week?

____bowls a week

Put '0' if none

11. How much of the following do you drink each day?

Put '0' if none

Tea	_____ cups daily	Pure fruit juice	_____ glasses daily
Herb tea	_____ cups daily	fruit drinks, squash	_____ glasses daily
Coffee	_____ cups daily	"Diet" fizzy soft drinks	_____ glasses/cans daily
Water	_____ glasses daily	Fizzy soft drinks	_____ glasses/cans daily

12. How many teaspoons of sugar, in total, do you add to your tea, coffee, cereal, fruit, etc. each day

_____teaspoons each day

Put '0' if none

13. At present, about how many alcoholic drinks do you have each week?

Put '0' if none

Beer, lager or cider _____pints each week   Sherry or fortified wine _____glasses each week

Red wine _____glasses each week   Spirits – whisky, gin, brandy _____glasses each week

White wine _____glasses each week

13. How often do you eat the following?

Please cross one box for each item

	Never	Seldom	Once a week	2-4 times a week	5-6 times a week	Once or more a day
Fresh fruit						
Dried fruit (raw)						
Stewed fruit, tinned fruit						
Pasta, e.g. spaghetti						
Rice						

Pizza						
Chips						
Other potatoes						
Peas						
Baked beans						
Lentils, dried beans						
Tomatoes						
Carrots						
Green vegetables						
Other cooked vegetables						
Salad/raw vegetables						
Tofu						
Soya meat, burgers, TVP						
	Never	Seldom	Once a week	2-4 times a week	5-6 times a week	Once or more a day
Other veggie burgers						
Cheese						
Cottage cheese						
Soya cheese						
Yoghurt, dairy desserts						
Soya, yoghurt, soya desserts						
Cream, ice cream						
Soya cream, ice cream						

Cakes, puddings, pies, buns, etc						
Chocolate, any type						
Other sweets, e.g. boiled sweets						
Crisps, Hula hoops, etc						
Peanut butter, salted nuts						
Other nuts and seeds not in muesli						
Jam, marmalade						
Yeast extract, Marmite						

LIFESTYLE.

We are interested in the type of activities you undertake.

Questions about your lifestyle

1. Have you ever smoked cigarettes   **Yes** ☐   **No** ☐

If you have stopped smoking cigarettes, how old were you when you gave up? _____years old

If you smoke now, how many cigarettes, do you usually smoke each day?   _____cigarettes a day

2. Do you smoke cigars?   **Yes** ☐ **No** ☐

3. Do you smoke a pipe?   **Yes ☐ No ☐**

4. Do you have a paid job at present?

Yes, full-time ☐   Yes, part-time ☐   No ☐

If **Yes**, we would like to know the type and amount of physical activity involved in your work.

Please put a cross in the appropriate box.

Sedentary occupation   ☐

You spend most of your time sitting (such as in an office)

Standing occupation   ☐

You spend most of your time standing or walking, but your work does not require intense physical effort (e.g. shop assistant, hairdresser, guard)

Manual work   ☐

This involves some physical effort including handling of heavy objects and use of tools (e.g. plumber, electrician, carpenter)

Heavy manual work   ☐

This involves very vigorous physical activity including handling very heavy objects (e.g. docker, miner, bricklayer, construction worker)

5. In a typical week during the past year, how many hours did you spend per week on the following activities? Put '0' if none

	In Summer	In Winter
Walking, including to work, shopping and leisure time	_____hours per week	_____hours per week
Cycling, including cycling to work, and during leisure time	_____hours per week	_____hours per week

Gardening   _____hours per week   _____hours per week

Do-It-Yourself   _____hours per week   _____hours per week

<u>In Summer</u>   <u>In Winter</u>

Physical exercise such   _____hours per week   _____hours per week

as keep-fit/aerobics,

swimming, jogging, tennis, etc.

Housework, such as   _____hours per week   _____hours per week

cleaning, washing,

cooking and childcare

6. In a typical week during the past 12 months, did you practice any of these activities vigorously enough to cause sweating or a faster heartbeat?

Yes ☐ No ☐

If **Yes**, for how many hours per week in total did you practice such vigorus activity? _____hours per week

7. What is your weight?

Stones_____pounds_____ OR ____.__ Kg

8. Please indicate your marital status

Single   ☐   Separated   ☐

Married or living as married ☐   Divorced   ☐

Widowed   ☐

HEALTH.

We are interested in your past medical history and your current state of health.

Questions about your health

1. In the past six years, have you had any broken/fractured bones?

Yes ☐ No ☐

If **Yes**, please give details: _____(bone) _____(year)

_____(bone) _____(year)

_____(bone) _____(year)

2. In the last six years, has your doctor told you that you that you had any of the following?

	Yes	Year first diagnosed	No
Cancer			
Type of cancer:			
Polyps in large intestine			
Enlarged prostate (men only)			
High blood pressure			
High blood cholesterol			
Angina			
Stroke			
Heart Attack			
Cardiac arrhythmias/ palpatations/ irregular heartbeat			
Blood clot in leg			
Blood clot in lung or elsewhere			
Diabetes			

	Yes	Year first diagnosed	No
Thyroid disease			
Cataract in eye			
Stomach ulcer			
Duodenal ulcer			
Gallstones			
Have you had your gallbladder removed?			
Crohn's disease			
Ulcerative colitis			
Asthma			
Bronchitis/emphysema			
Rheumatoid Arthritis			
	Yes	Year first diagnosed	No
Osteoarthritis			
Depression requiring treatment			
Other significant Illnesses or operations, please give details			

3. Do you regularly take any vitamins, minerals or other supplements?

Yes ☐ No ☐

If **Yes**, do you take: (you can cross more than one box)

Multivitamins (with minerals)   ☐ Vitamin A ☐

Multivitamins (without minerals) ☐ Vitamin B ☐

Fish oil (including cod liver oil)   ☐ Vitamin C ☐

Evening primrose oil   ☐ Vitamin D ☐

Garlic   ☐ Vitamin E ☐

Iron   ☐ Other   ☐ _____

Zinc   ☐   (name and brand)

Calcium   □

4. Have you taken any medications for most of the last 4 weeks?

 Yes ☐ No ☐

If **Yes**, please list them below: (you can name more than one box)

_____
_____
_____
_____
_____

5. About how many bowel movements do you have each week?

____a week

How often do you take laxatives? (Put '0' if never)

____times a month

6. How would you describe your health now?

Excellent ☐   Good ☐   Fair ☐   Poor ☐

QUESTIONS FOR WOMEN ONLY

8. Have you had your menopause (stopped having periods)?

Yes ☐   No ☐   Not sure ☐

                    (because taking HRT, irregular periods, etc)

If **Yes**, how old were you when you stopped having periods?

_____years old

9. How many periods have you had in the last 12 months?

_____periods (Put '0' if no periods)

10. Have you ever taken the contraceptive pill?

Yes ☐ **No** ☐

If **Yes**, for how long altogether have you used the pill? _____years

(Put '0' if less than one year)

Are you currently taking the contraceptive pill?　　**Yes** ☐ **No** ☐

If **No**, at what age did you stop? _____years old

11. Have you ever taken Hormone Replacement Therapy (HRT)?
Yes ☐ **No** ☐

If **Yes**, for how long altogether have you used HRT?　　_____years

(Put '0' if less than one year)

Are you currently taking HRT?　　　　　　　　**Yes** ☐ **No** ☐

If **No**, at what age did you stop?　　　　　　_____years old

12. During the last six years, have you had any children?

Yes ☐ **No** ☐

If **Yes**, please enter the years of birth and sex below:

　1.　_____(YEAR)　Boy ☐ Girl ☐
　2.　_____(YEAR)　Boy ☐ Girl ☐
　3.　_____(YEAR)　Boy ☐ Girl ☐

13. Have you ever had a hysterectomy (womb removed)?

Yes ☐ **No** ☐

If Yes, at what age _____years old

14. Have you ever had an operation to remove one or both ovaries?　　**Yes** ☐ **No** ☐ **Don't know** ☐

If **Yes**, were one or both ovaries removed?

One ☐   Both ☐   Don't know ☐

At what age? _____years old

15. Have you ever had breast screening by mammography (x-ray)?   **Yes ☐**
No ☐

If **Yes**, how many times in the last ten years? _____times

When did you last have a breast screen?   _____*YEAR*

16. Have you ever had a cervical smear test?

   Yes ☐ No ☐

If **Yes**, how many times in the last ten years?   _____times

When did you last have a cervical smear?   _____*YEAR*

Thank you for taking the time to complete this questionnaire.

Please return to Miss Lucy Nichols

Institute of Life Science, Swansea University SA28PP

OR

Bring this questionnaire with you on the day of your appointment

7.2 PANC-1 Expanded Dose Range Viability

Outside of the dose ranges of DCA tested in the PANC-1 cell line in **chapter 3.3.1**, other doses were assessed but not used further.

DCA doses beyond 300μM were shown to be too cytotoxic to PANC-1 cells through severe reduction of RPD. RPD was reduced beyond the point of usefulness within genotoxicity assessment, **Fig. 7.1**.
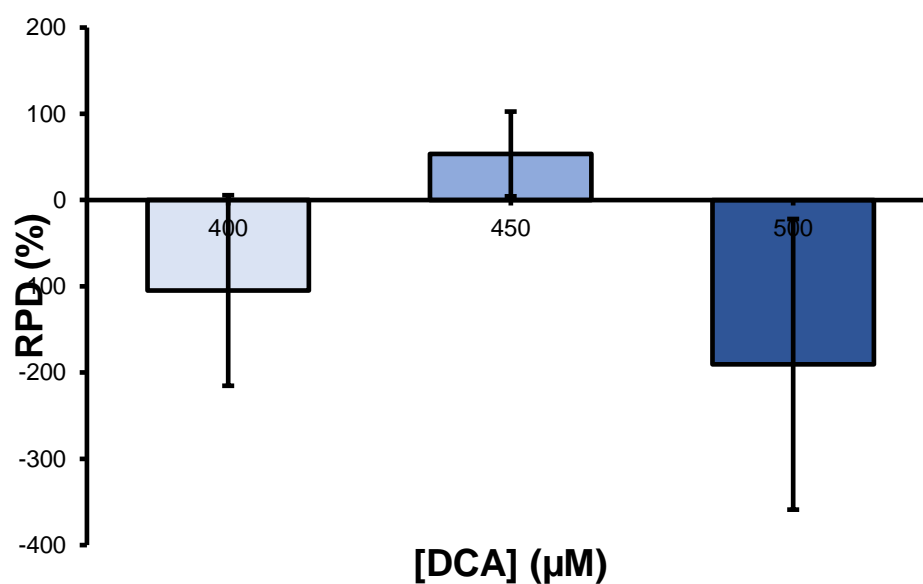
Figure 7.1: *Cytotoxicity in PANC-1 determined by RPD in increasing concentrations of DCA. Bars represent means and error bars show the standard deviation.*

7.3 L5178Y Mononucleate MN Assay
7.3.1 DCA

Initial cytotoxicity experiments determined a suitable dose range of DCA in the L5178Y cell line, which was more sensitive in comparison to the PANC-1 cell line. From the vehicle control, there was an increase to an RPD of 116.00 ±27.50% at 20μM and then a decrease at 150μM of 71.67±26.52%. No statistical significance was determined via the Mann Whitney U assay, although at top dose showed a p-value of 0.088. Above this dose, results were inconsistent and below 50±5% RPD guidelines suitable for the MN assay.
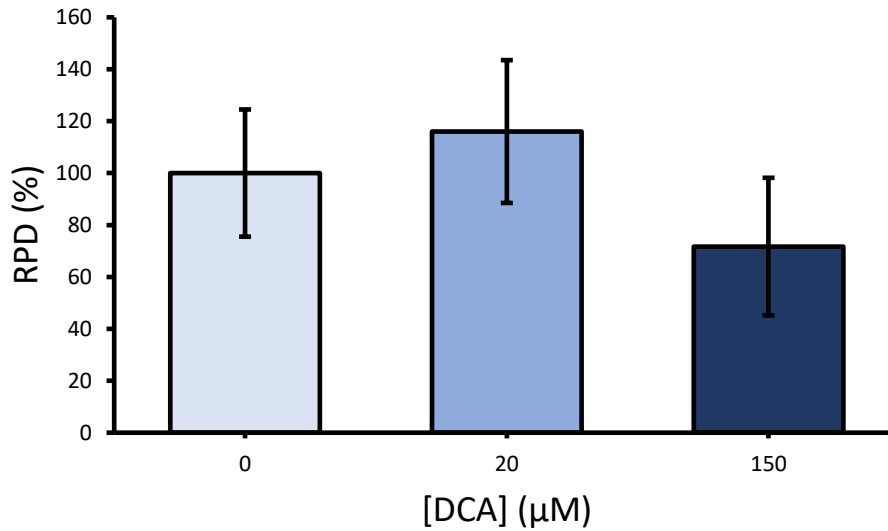
Figure 7.2*: Cytotoxicity induced by increasing DCA concentrations in L5178Y cell line determined by RPD calculations. Bars represent the mean (n=3) and the error bars show the standard deviation (* show p<0.05, ** show p<0.01).*

The mononucleate MN assay in the L5178Y cell line showed no statistically significant increase in micronuclei at both concentrations of DCA used, **Fig 7.3**. The results from the vehicle control and 20µM DCA are comparable with 0.23±0.11% and 0.22±0.02% cells with micronuclei, respectively. 150µM DCA induced 0.26±0.10% micronucleated cells, which is not significantly higher than the control in a t-test (p=0.823).
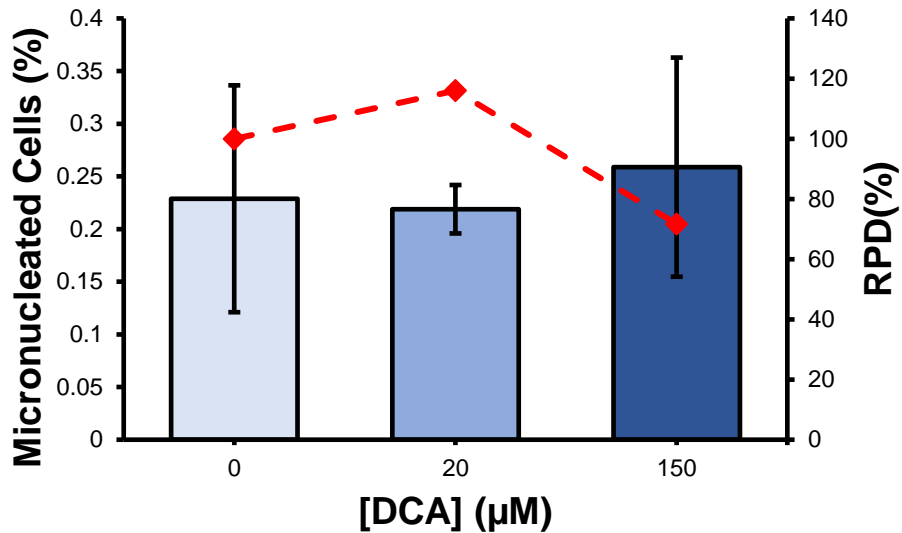
Figure 7.3: *Induction of micronuclei in the L5178Y cell line following 4h exposure to increasing DCA concentrations for 4h in the mononucleate MN assay. Bars represent the mean (n=3) and the error bars show the standard deviation. Red dashed line represents the RPD.*

7.3.2 Acetaldehyde

Initial dose-range finding experiments determined a suitable dose range of acetaldehyde in the L5178Y cell line since these cells were more sensitive than the PANC-1 cell line. Cytotoxicity was determined through RPD calculations, **Fig 7.4**. A dose-dependent decrease in RPD was observed which was significant at 1µM (p=0.021) in the Mann Whitney U statistical test.
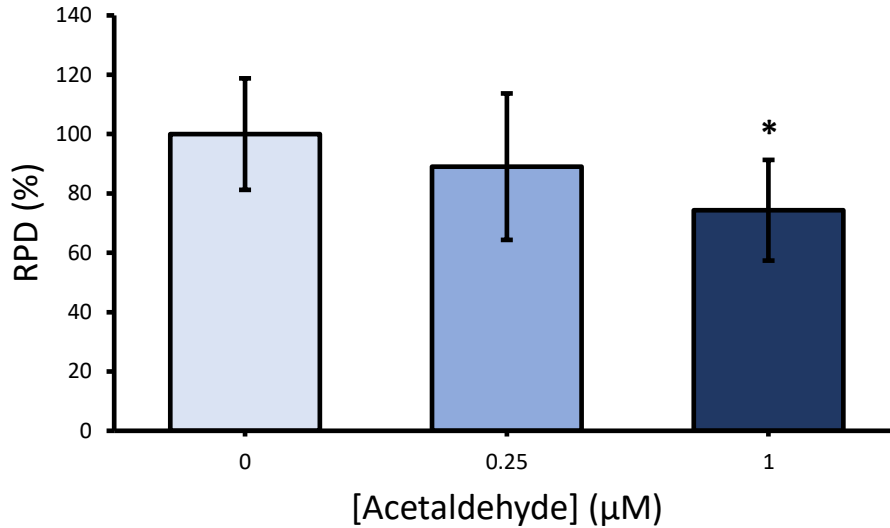
Figure 7.4: *Cytotoxicity induced by 4h exposure to increasing concentrations of acetaldehyde in the L5178Y cell line. There is a dose-dependent decrease in RPD as acetaldehyde dose increases. Bars represent the mean (n=3) and error bars show the standard deviation. (* show p<0.05)*

The induction of micronuclei was studied in the mononucleate micronucleus assay following acetaldehyde exposure, **Fig. 7.5**. Similarly to DCA, micronuclei induction by the vehicle control and the lowest dose, 0.25µM, was comparable with 0.17±0.03% and 0.16±0.11%, respectively. Significant induction of micronuclei was seen at 1µM acetaldehyde, increasing to 0.37±0.08%. A t-test provided a p-value of
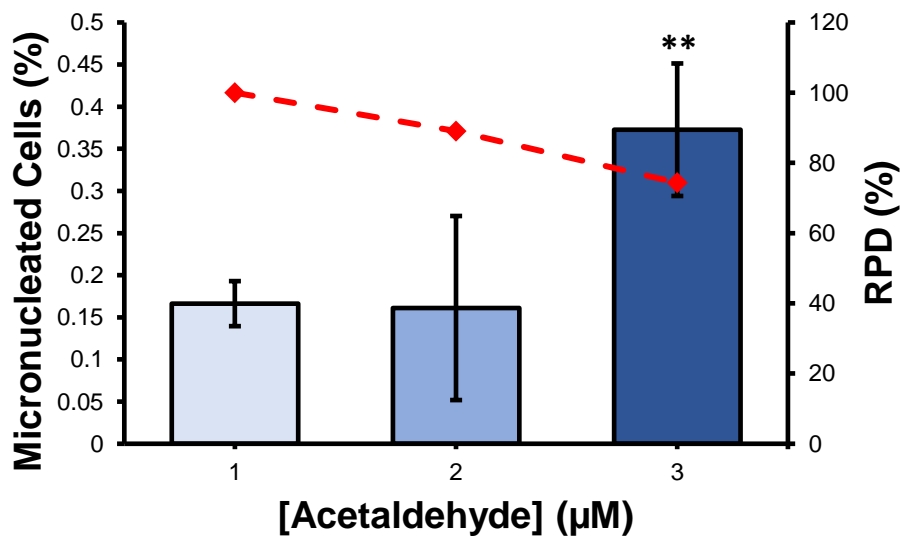
0.003.

Figure 7.5: Induction of micronuclei in L5178Y cell line following 4h exposure to acetaldehyde in the mononucleate micronucleus assay. A significant induction was seen at 1µM actealdehyde. Bars represent the mean and the error bars represent the standard deviation. Red dashed line represents the RPD (shows p<0.05, ** shows p<0.01).*

7.4 TNM Staging Definitions

The most up to date staging for pancreatic tumours is the AJCC 8th edition, further described below. This provides information about the size, lymph node involvement and metastasis of the disease to provide a better all-around image of disease.

Table 7.1: definitions of TNM staging for pancreatic tumours according to the AJCC/UICC 8th edition (Cong *et al.*, 2018)

Stage	Definition
T1	Maximum tumour diameter ≤2cm
T2	Maximum tumour diameter >2, ≤4cm
T3	Maximum tumour diameter >4cm
T4	Tumour involves coeliac axis, common hepatic artery, or superior mesenteric artery
N0	No regional lymph node metastasis
N1	Metastasis in 1-3 regional lymph nodes
N2	Metastasis in ≥4 lymph nodes
M0	No distant metastasis
M1	Distant metastasis

Table 7.2: *Definitions of staging dependent on pancreatic tumour TNM staging from the AJCC/UICC 8th edition* (Cong *et al.*, 2018).

Stage	T	N	M
IA	T1	N0	M0
IB	T2	N0	M0
IIA	T3	N0	M0
IIB	T1-T3	N1	M0
III	T4 (or any T)	Any N (N2)	M0
IV	Any T	Any N	M1

7.5 Questionnaire Data

Questionnaire responses for donors in the study was poor. Only 8 donors returned completed questionnaires with correctly filled out questions. Of 8 donors, 7 donors had PC and 1 had benign pancreatic disease. Dietary Quality Score values were calculated from examining the dietary portion of the questionnaire, discussed in **2.3.2** and **Table 2.5**. DQS values were similar, with a range of 8-10. Patients with PC were shown to have a marginally elevated DQS values in comparison to benign donors with mean scores of 9.143±0.69 and 8, respectively. This was determined non-significant through a t-test (p=0.172). A lower DQS signifies a poorer diet, so this suggests a better diet within this small population of PC patients. Only one donor has benign disease, so may not be truly representative.
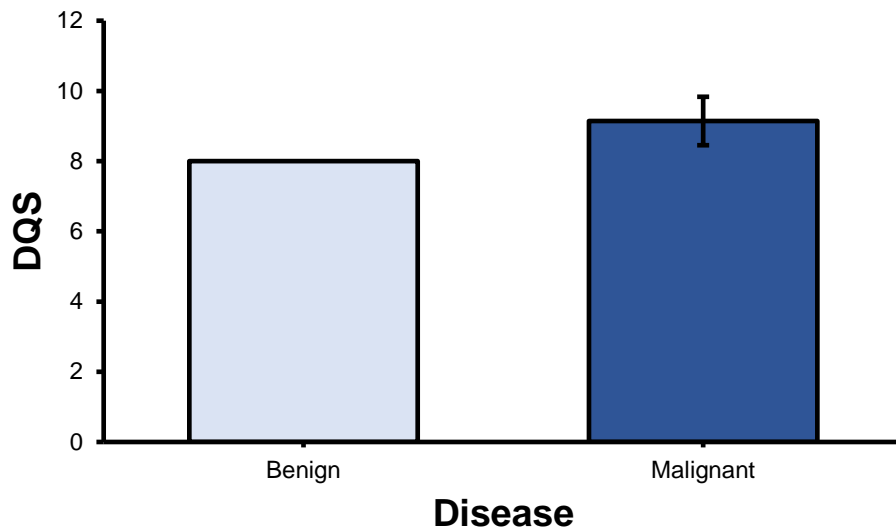
Figure 7.6: *Mean DQS scores in malignant and benign pancreatic donors (n=8).*

We examined whether there was a link between PIG-A mutant frequencies and DQS in donors with both benign and malignant disease. Only 5 of the 8 donors with completed questionnaires had full PIG-A results. No overall link was seen between the two.
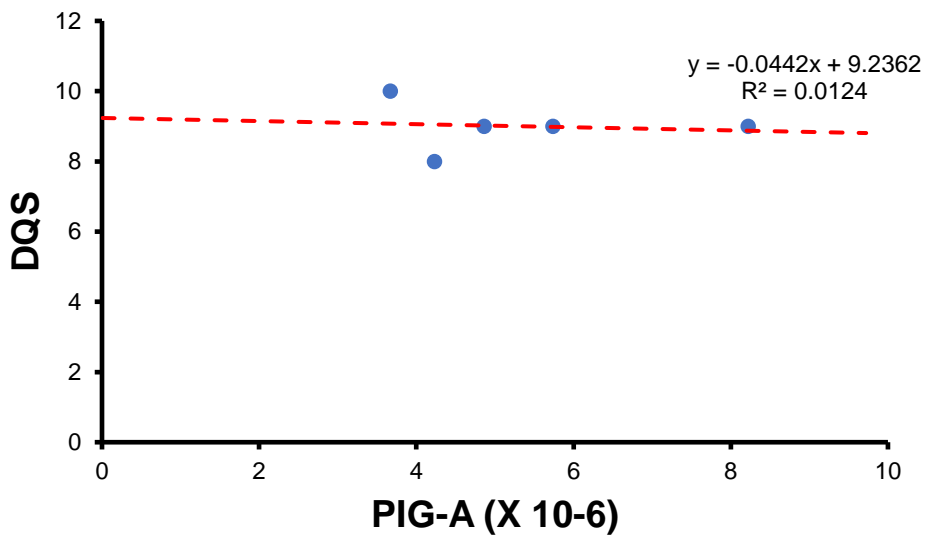
Figure 7.7: *Correlation between PIG-A mutant frequencies and DQS in donors with benign and malignant pancreatic disease (n=5).*

Further in-depth analysis explored dietary fats and whether that had any impact on the PIG-A mutant frequency. When examining fats used on bread, a greater PIG-A mutant frequency was seen with a lower score, with no significance (p=0.176). However, with cooking fats and overall fats the changes are not as obvious.
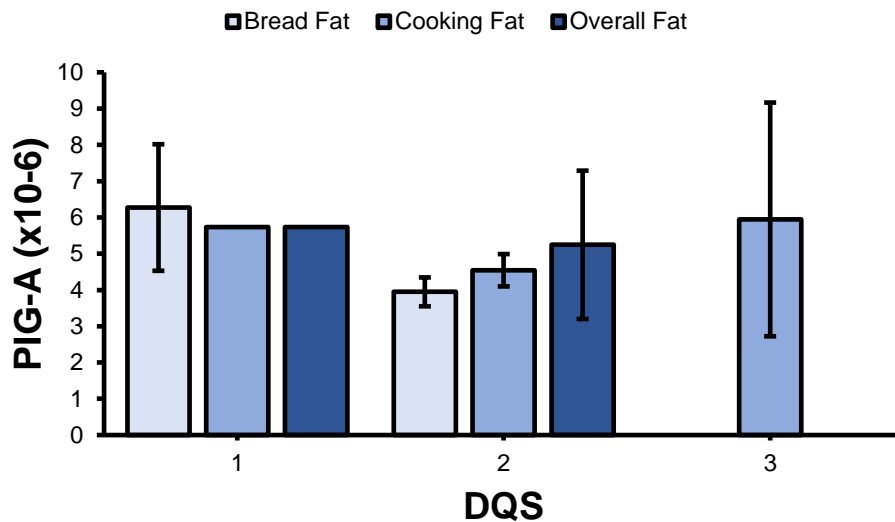
Figure 7.8: *Mean PIG-A in donors with increasing DQS values for fat used in bread and cooking, and overall fat use (n=5)*

Smoking status of the donors was assessed. 50 % of our donors with completed questionnaires were past smokers with remaining 50% have never smoked. We further examined whether the smoking status of the donor influenced PIG-A mutant frequency. Patients who had never smoked (n=3) were shown to have an elevated PIG-A mutant frequency over the previous smokers (n=2) although this was non-significant through a t-test (p=0.176). The small population may contribute to this.
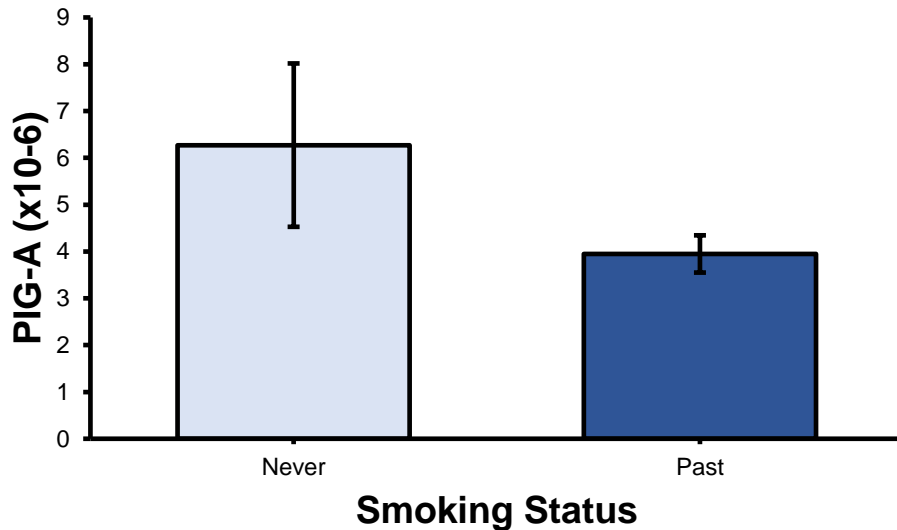
Figure 7.9: *Smoking status in donors and corresponding mean PIG-A mutant frequencies (n=5).*

Alcohol consumption was also examined. The lone benign donor did not consume any alcohol. In contrast, across the 7 malignant donors there was an average weekly alcohol consumption of 7.514±12.961 units, ranging from 0 to 33.3 units. A comparison of the PIG-A mutant frequency and the units of alcohol consumed per week, **Fig. 7.10**, did not show much of a correlation and was skewed from one donor who consumed 33.3 units per week.

$$y = -4.3129x + 30.164$$
$$R^2 = 0.2752$$
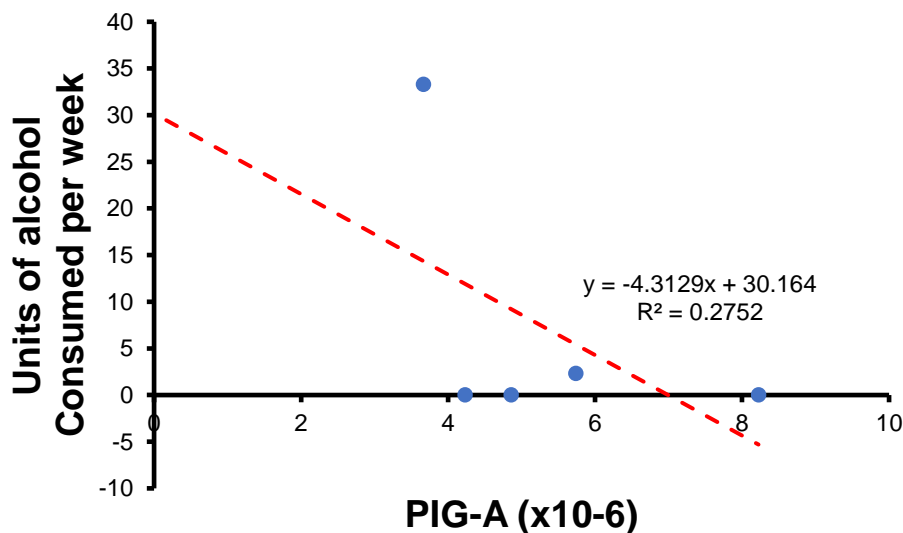
Figure 7.10: *Relationship between alcohol consumption in units per week and PIG-A mutant frequency (n=5).*

Further information collected from the questionnaire allowed further insight into other health conditions experienced by the patients within our study. Diabetes mellitus was the most common comorbidity, with 62.5% (5) of our patients recording it as an ongoing health concern. Previously, diabetes mellitus has been described as both a symptom of early PC and a cause of PC. High cholesterol was the next most common condition experienced, besides pancreatic pathologies, being experienced by 37.5% (3) patients. Interestingly, the third most common other condition was cataracts, seen in 25% (2) of our patients. Self-reported health was a question found in the questionnaire. Interestingly, those with worse self-reported health tended to have an elevated PIG-A mutant frequency, although this was deemed non-significant through a one-way ANOVA (p=0.180).
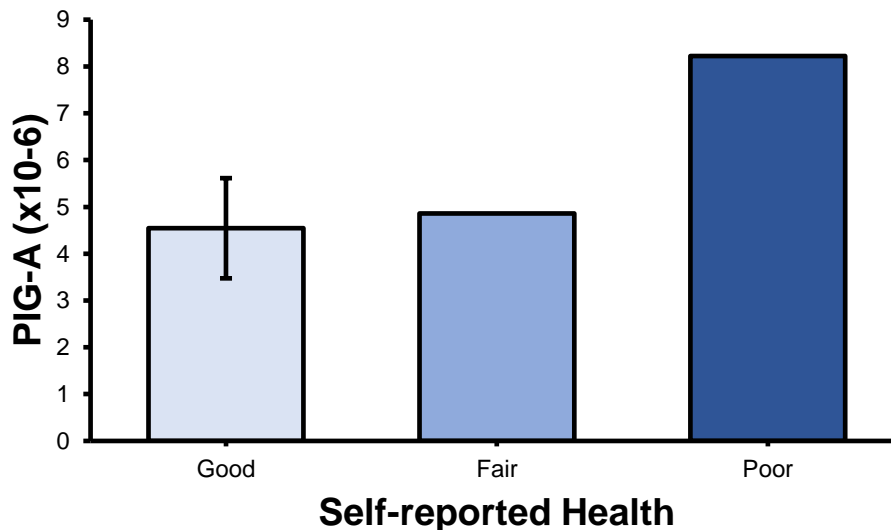
Figure 7.11: Mean PIG-A mutant frequencies in decreasing self-reported health (n=5).

7.6 Conference Attendance

Poster – April 2017 – Swansea University Medical School Postgraduate Research Conference

Although survival rates for cancers have improved, survival for pancreatic cancer patients has failed to improve within the last four decades. Most cases are diagnosed at an advanced stage where patients are not eligible for surgical intervention. As a result, there is a push towards non-invasive techniques for earlier diagnosis of pancreatic cancer, which could extend patient survival.

The Pig-A assay has been used extensively in rodent models to monitor genotoxic responses to stimuli and more recently has been applied to human population studies. Particularly, cancer patients have been shown to have a higher mutation frequency than healthy individuals. X-linked *Pig-A* is ubiquitously expressed and encodes the catalytic domain of N-acetyl glucosamine transferase responsible for the first step of GPI anchor biosynthesis. The mutation status of peripheral erythrocytes was determined using a trio of antibodies against GPI anchored proteins and flow cytometry. In parallel with the micronucleus assay, a more complete view of *in vivo* mutations is given. Initial consenting patients donated 10ml of peripheral blood from which a Pig-A mutation frequency was measured to determine whether the assay has potential in differentiating between benign and malignant pancreatic conditions.

The micronucleus assay was also used to assess chromosomal damage in an *in vitro* model of pancreatic cancer induced by poor diet and lifestyle. Approximately 37% of cases of pancreatic cancer can be avoided through better dietary and lifestyle choices. Benzo-[a]-pyrene, a common contaminant of cigarette smoke, was shown to induce micronuclei formation in the panc-1 cell line following 24-hour exposure.

Flash Presentation – May 2018 – Swansea University Medical School Postgraduate Research Conference

<u>**Can the Novel PIG-A Assay Diagnose Pancreatic Cancer?**</u>

Fewer than 5% of pancreatic cancer patients survive 5 years post-diagnosis since the majority of patients are diagnosed with advanced disease. Advances in earlier diagnosis of pancreatic cancer would improve survival rates of pancreatic cancer patients.

A novel blood test determines the mutation status of the X-linked PIG-A gene through flow cytometric detection of GPI anchored proteins on the surface of red blood cells. Previous studies have shown higher levels of PIG-A mutant erythrocytes in cancer patients and so the test is being explored within a pancreatic cancer cohort to determine its potential in diagnosis. 10µL whole blood stained with a triplicate of antibodies against erythrocyte specific CD-235a and GPI-anchored CD-55 and CD-59. A GPI deficient population was identified and quantified as PIG-A mutants. To date, 13 patient samples resulted in mean mutant frequencies of $8.47\pm6.70 \text{ X}10^{-6}$ in

malignant pancreatic patients and 2.15 ± 0.32 $X10^{-6}$ in benign patients. Additionally, primary lymphocytes were harvested from whole blood using histopaque, cultured for 48h in presence of phytohemagglutinin and with cytokinesis block cytochalasin B for an additional 48h. Giemsa stained slides were scored for the frequency of micronuclei in 1000 binucleated lymphocytes. This provided a measure of chromosomal stability and a secondary measure of *in vivo* DNA damage.

An *in vitro* approach was also used to assess DNA damage induced by known risk factors because 39% of pancreatic cancer cases are linked to avoidable causes. Micronuclei induction was studied in the pancreatic ductal adenocarcinoma cell line PANC-1 in response to a trio of chemicals, benzo-[a]-pyrene, bile acid deoxycholic acid and acetaldehyde, to mimic smoking, poor diet and excess alcohol consumption. An increase in DNA damage was seen following exposure to the three chemicals independently. Additional endpoints will be assessed and this will also be correlated with *in vivo* PIG-A data collected from patients.

Poster – September 2018 – UKEMS Annual Meeting, Magdalen College, Oxford

<u>PIG-A Assay: Potential Pancreatic Cancer Diagnostic Tool?</u>

Pancreatic cancer remains one of the greatest challenges of modern medicine. It is an aggressive disease with consistently low survival rates, with little improvement over the past four decades. Eighty percent of patients are diagnosed with metastatic disease, limiting treatment to palliative chemo and radiotherapy. Earlier diagnosis would allow more patients to have potentially curative surgery.

 The human erythrocyte PIG-A assay has been investigated to determine its use as a new diagnostic tool for pancreatic cancer. X-linked PIG-A gene encodes a catalytic subunit involved in GPI anchor biosynthesis. A single mutation is enough to result in GPI deficient cells. Mutation in the PIG-A gene is identified through flow cytometric detection of GPI anchored CD-55 and CD-59 on the surface of erythrocytes. Patients were recruited from surgical clinics at a tertiary referral centre for pancreatic cancer, at Morriston Hospital, Swansea. To date, 24 patients have been recruited. PIG-A mutation frequencies in a healthy, benign pancreatic and malignant pancreatic cohort are 3.71 ± 2.03 (n=3), 3.04 ± 1.40 (n=4) and 8.12 ± 6.60 (n=13), respectively. Pancreatic cancer patients show a higher mean PIG-A mutant frequency and a wider range of

mutant frequencies, potentially linked to stage of disease. The lymphocyte micronucleus assay was used to provide a secondary measure of DNA damage. An *in vitro* model of pancreatic cancer was also explored to determine sources of the DNA mutations reflected in the *in vivo* tests. Specifically, alcohol consumption, smoking and obesity were modelled using acetaldehyde (0-2.5µM), B[a]P (0-50µM) and deoxycholic acid (0-500µM) exposure in the PANC-1 cell line. Significant micronuclei induction was seen using B[a]P above 20µM and acetaldehyde at 2.5µM but the pattern was less clear with deoxycholic acid.

A larger patient cohort is needed to fully elucidate whether the PIG-A assay will be beneficial as either a diagnostic or staging tool alongside imaging studies.

Oral Presentation – April 2019 - UKEMS/BTS Joint Meeting – Robinson College, Cambridge

In *vitro* and in *vivo* study of the PIG-A assay in pancreatic cancer.

Pancreatic cancer is a complex disease. Approximately 40% of cases are attributed to avoidable lifestyle and dietary causes. It is difficult to diagnose due to a late onset of non-specific symptoms. The PIG-A assay has been used extensively in rodent toxicology studies and more recently in humans and *in vitro*. Through flow cytometric detection of fluorescently labelled GPI-anchored proteins, the mutation status of the x-linked PIG-A gene is determined.

The human erythrocyte PIG-A assay has been applied to samples from donors with benign and malignant pancreatic conditions, with the lymphocyte micronucleus assay used as a secondary measure of DNA damage. Reported PIG-A mutation frequencies for healthy volunteers are comparable to values found for healthy patients and benign pancreatic patients (4.59±2.11 and 3.37±1.21 respectively). Cancer patients have an increased level of mutated erythrocytes (8.33±6.16). As the TNM stage of the tumour increased there was an increase in the number of mutated erythrocytes, but this was significant as disease became metastatic. An increase in micronucleated lymphocytes was seen in cancer patients (1.80%) in comparison to benign patients (1.29%).

The *In vitro* Pig-A assay and micronucleus assay have been used to monitor mutations induced by pancreatic carcinogens deoxycholic acid, benzo-[a]-pyrene and

acetaldehyde to model a high fat diet, smoking and alcohol abuse respectively. Mutations seen in the *in vitro* assay may reflect some mutations seen *in vivo*. This increase in mutations quantified through both the PIG-A and micronucleus assay may be useful as a diagnostic or staging tool for patients with pancreatic disease.

Poster – May 2019 – Pancreatic Symposium, Cancer Research UK Cambridge Institute, Cambridge

<u>**PIG-A Assay: potential novel diagnostic tool for pancreatic cancer**</u>

As much as 80% of all pancreatic cancer patients are diagnosed with locally advanced or metastatic disease, limiting treatment options and causing a poor prognosis. Blood-based biomarkers are a cheap, easy and accessible method of diagnosis and monitoring disease progression. Currently used CA19-9 although helpful cannot be fully relied upon so the search for more blood-based biomarkers for pancreatic cancer is of high importance.

The human erythrocyte PIG-A assay is being explored to determine whether it has any potential in diagnosing pancreatic cancer. The simple blood-based assay monitors the mutation status of the x-linked PIG-A gene through detecting GPI anchored proteins on the erythrocyte surface by flow cytometry. PIG-A mutant erythrocytes lack GPI anchors, in turn lacking the GPI anchored proteins and so are non-fluorescent during analysis. The mutant erythrocytes are quantified per million red blood cells analysed. Healthy donors in population studies have approximately 5 mutant erythrocytes per million analysed. Studies within our research group have shown elevated level of mutation in peripheral erythrocytes of gastrointestinal cancer patients and so this is being investigated in pancreatic cancer, where improvements in diagnosis are mostly needed.

To date, 45 consenting donors have provided 10ml heparinised blood and a lifestyle questionnaire. Blood was analysed in parallel with the human erythrocyte PIG-A assay and the lymphocyte cytokinesis block micronucleus assay to provided two measures of DNA damage in two separate blood cell populations. Stated PIG-A mutation frequencies in population studies are comparable to the PIG-A mutation frequencies found in healthy and benign pancreatic patients (4.59±2.11 and 3.37±1.21 respectively). Elevated mutated erythrocytes are found in cancer patients (8.67±6.33). Comparison of TNM tumour staging showed a significant increase in

mutated erythrocytes in metastatic disease. An increase in micronucleated lymphocytes were also seen in cancer patients in comparison to benign patients with frequencies of 1.80% and 1.29%, respectively. No significance was found when comparing TNM staging with results from the lymphocyte cytokinesis block micronucleus assay.

In this pilot study, an increase has been viewed in the frequency of mutated erythrocytes in cancer patients in comparison to patients with benign disease. There was also an increase in mutated erythrocytes in patients with metastatic pancreatic cancer. The PIG-A assay has shown potential for use either as a diagnostic tool or a staging tool for those with pancreatic disease, although greater patient numbers are required to fully explore this.

Oral Presentation – May 2019 – Swansea University Medical School Postgraduate Research Conference

<u>An investigation of the PIG-A assay in pancreatic cancer</u>

Within the last 4 decades, cancer survival rates have improved in most malignancies. Pancreatic cancer, however, has bucked the trend with a consistently low five-year survival rate of 7%. It's an aggressive disease with 40% of cases caused by lifestyle and dietary causes. With vague, non-specific symptoms, 80% of patients are diagnosed with advanced disease. Improvement in diagnosis would allow for earlier intervention and may improve patient outcome.

The PIG-A assay is being explored for its use in pancreatic disease. The assay monitors the mutation status of the x-linked PIG-A gene through flow cytometric detection of GPI-anchored proteins on the surface of cells. It has been used extensively in rodent genotoxicology studies but more recently in human blood cell populations and *in vitro* studies.

The human erythrocyte PIG-A assay has been carried out on heparinised blood samples from consenting donors with benign and malignant pancreatic disease. To date, 44 patients have donated samples. PIG-A mutation frequencies in healthy and benign donors (4.59 ± 2.11 and 3.37 ± 1.21 respectively) are comparable to reported mutation frequencies in population studies. An elevated level of mutations are seen in cancer patients (8.33 ± 6.16). Clinical parameters have been correlated with the

mutation frequency, with metastatic disease having a significant increase in mutated erythrocytes in comparison to localised disease. The lymphocyte micronucleus assay was used as a secondary measure of DNA damage in an alternative cell population. An increase in micronucleated lymphocytes was seen in cancer patients.

An *in vitro* model of pancreatic cancer using PANC-1 cell line modelled exposure to known risk factors for pancreatic carcinogenesis including a high fat diet (deoxycholic acid), smoking (benzo-[a]-pyrene) and alcohol abuse (acetaldehyde). Induction of DNA damage was studied using the micronucleus assay and the *in vitro* Pig-A. Mutations seen in the *in vitro* study may reflect some of those seen *in vivo*.

Poster – November 2019 – ACEMS/JEMS Joint Meeting – Tokyo

<u>A novel blood based biomarker for pancreatic cancer</u>

Diagnosing pancreatic malignancies remains a challenge due to late presentation and vague symptoms. Approximately 80% of patients present too late for potentially curative surgery. Earlier diagnosis would increase likelihood of patients being eligible for potentially curative resection that may improve patient outcomes.

The PIG-A assay is being explored for its use in pancreatic disease. The mutation status of X-linked *PIG-A* is determined via flow cytometric detection of GPI anchored proteins on the surface of erythrocytes. Mutation in *PIG-A* has previously been shown to result in GPI anchor deficient cells that are quantified. To date, 51 consenting patients with pancreatic disease including 23 benign patients and 28 treatment naïve cancer patients have donated blood samples. Whole blood was stained with antibodies against erythrocyte specific CD-235a and GPI anchored CD-55 and CD-59 before flow cytometry analysis. Cancer patients have shown an elevated frequency of mutated erythrocytes (8.67 ± 6.33) in comparison to benign and healthy donors (4.59 ± 2.11 and 3.37 ± 1.21).As tumour staging becomes more advanced, the PIG-A mutant frequency increases which is significant when disease is metastatic. Chromosomal stability was monitored in the lymphocytes from the donors using the micronucleus assay. An increase in micronucleated lymphocytes was seen in cancer patients and there is a positive correlation when compared to PIG-A mutation frequency.

An increase of DNA damage assessed by two methods is seen in pancreatic cancer patients that may be potentially useful in a clinical setting.

Chapter 8:

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