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Factors which influence penetrance in Familial Pancreatic Cancer

Thesis submitted in accordance with the requirements of the University of Liverpool for the Degree of Doctor of Medicine by

Jane Victoria Butler

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Declaration

I declare that this thesis and the research upon which it is based is the result of my own work. Wherever I have incorporated the work of others it has been clearly stated. This work has not been submitted in candidature for any degree in this or any other University. Part of this work has been published in:

1. Slater EP, Langer P, Niemczyk E, Strauch K, Butler J, Habbe N, et al. PALB2 mutations in European familial pancreatic cancer families. Clinical Genetics. 2010;78:490-4.

Contribution through recruitment of families included in analyses.

2. Nicholson JA, Greenhalf W, Jackson R, Cox TF, Butler JV, Hanna T, et al. Incidence of post-ERCP pancreatitis from direct pancreatic juice collection in hereditary pancreatitis and familial pancreatic cancer before and after the introduction of prophylactic pancreatic stents and rectal diclofenac. Pancreas. 2015;44:260-5.

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3. Grocock CJ, Rebours V, Delhaye MN, Andren-Sandberg A, Weiss FU, Mountford R, et al. The variable phenotype of the p.A16V mutation of cationic trypsinogen (PRSS1) in pancreatitis families. Gut. 2010;59:357-63.

Contribution through recruitment of hereditary pancreatitis families to the EUROPAC registry.

// Abstract

Introduction

Pancreatic cancer (PDAC) confers a high mortality rate as it often presents at an advanced stage. Screening could improve early detection and survival. However, the prevalence of the disease and absence of specific screening tools mean that screening of the general population would result in many false positive tests. This may mean unnecessary overtreatment including surgery with considerable morbidity. Current screening techniques have associated risks including exposure to radiation and induction of pancreatitis. It is therefore essential to identify high risk screening populations and improve both specificity and safety of the screening modality. The European Registry of Hereditary Pancreatitis and Familial Pancreatic Cancer (EUROPAC) was established to identify individuals with a genetic predisposition for PDAC to offer secondary screening.

Thesis Aims

The aims of this thesis were to identify subpopulations of high risk families who are at the greatest risk of pancreatic cancer in order to undertake targeted screening. Furthermore, to minimise the risks and complications to individuals participating in the EUROPAC secondary screening study for early PDAC.

Materials and Methods

Familial Pancreatic Cancer (FPC) was defined as autosomal dominant predisposition for PDAC. Hereditary Pancreatitis (HP) is an autosomal dominant predisposition for pancreatitis associated with an approximately 40% life time risk of PDAC. Families with other recognised cancer syndromes conferring a predisposition to PDAC were also recruited. At recruitment, blood samples for DNA storage and cell lines were obtained for the analysis of potential candidate genes implicated in FPC. Individual and familial demographic, lifestyle and disease related data were collected to facilitate analysis of possible risk factors which could influence penetrance in these high risk groups. On confirmation of a familial predisposition, eligible individuals were invited to participate in the EUROPAC secondary screening study for early pancreatic cancer. The screening protocol comprised blood tests and imaging techniques along with analysis of molecular markers in pancreatic juice.

Results

273 potential FPC kindreds were identified and recruited; 89 had 3 or more affected family members. In FPC, age is a major risk factor but the pattern of age related risk is the same as in the general population. However, this is consistently 100 fold greater in FPC. No evidence was found for earlier age of onset in FPC contrary to other reports. Selection bias based on proband is proposed as an explanation for the apparent differences seen in the literature. Reporting bias could also explain the relationship of smoking and PDAC in FPC kindreds. No further causative mutations were found

during the preparation of this thesis but a PALB2 mutation was found in three families (possibly associated with breast cancer). A model for risk was proposed, but in this thesis only secondary screening based on previous estimates is described. The methodology involved Endoscopic Retrograde Cholangiopancreatography (ERCP) and 7 cases of pancreatitis resulted. Consequently, stenting and use of diclofenac was introduced resulting in a significant reduction in ERCP associated pancreatitis (3 of 31 screens). Use of secretin to allow sampling of pancreatic juice from the duodenum was also piloted to further improve safety. This thesis provides data to support risk stratification in FPC and an improved screening modality. A total of 212 HP kindreds were identified. A variable clinical picture and cancer risk was found in the HP families with p.R122H, p.N29I and p.A16V all conferring a significantly increased lifetime risk for the development of pancreatic cancer, in addition to the earlier age of onset of exocrine and endocrine failure. Cancer risk was shown to be increased with smoking and diabetes. Risk of PDAC increases with age equally for HP and the general population but was 28 fold higher in each age group with HP.

Conclusion

My thesis has identified specific lifestyle and disease derived risk factors which may influence risk for the development of PDAC in high risk groups. As a direct result of this thesis, amendments made to the screening protocol have improved safety and minimised harm to those individuals undertaking screening for early pancreatic cancer under the auspices of EUROPAC.

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Expansion of EUROPAC as the largest Registry of Hereditary Pancreatitis and Familial Pancreatic Cancer in Europe has been made possible through the assistance of our many collaborators in the UK and throughout Europe. I would also like to acknowledge our local clinicians in Liverpool: Professor Robert Sutton, Professor Paula Ghaneh, Mr Chris Halloran, Mr Michael Raraty, Professor Martin Lombard, Dr Howard Smart and Dr J Evans; without whose support secondary screening for early pancreatic cancer in high risk individuals would not have been possible.

List of abbreviations

µl	microliters
5-FU	5-fluorouracil
ABO	blood group derived from the ABO gene encoding for glycosyltransferase
ADE2	gene encoding for AIR-carboxylase
ADH	Aldehyde Dehydrogenase
AHH	Arylhydrocarbonhydroxylase
ALDH2	Alcohol Dehydrogenase
ARMS	Amplification Refractory Mutation System
BMI	Body Mass Index
BRCA2	Breast Cancer 2, early onset
CA19.9	Carbohydrate Antigen 19.9
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A tumour suppressor gene
CEA	Carcinoembryonic Antigen
CP	Chronic Pancreatitis
CT	Computed Tomography
CYP1A1	Cytochrome P-450 gene
DCO	Database Co-ordinator
DNA	Deoxyribonucleic Acid
DPC	Deleted in Pancreatic Carcinoma gene
EDTA	Ethylenediaminetetraacetic acid
ERCP	Endoscopic Retrograde Cholangiopancreatography
ESPAC	European Study Group for Pancreatic Cancer
EUROPAC	European Registry of Hereditary Pancreatitis and Familial Pancreatic Cancer
EUS	Endoscopic Ultrasound
FAMMM	Familial Atypical Multiple Mole Melanoma
FAMMM-PC	Familial Atypical Multiple Mole Melanoma-Pancreatic Cancer

FDR	First Degree Relative
FNA	Fine-needle Aspiration
FPC	Familial Pancreatic Cancer
GST	Glutathione S-transferase
GWAS	Genome Wide Association study
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
HP	Hereditary Pancreatitis
IPMN	Intraductal Papillary Mucinous Neoplasms
ITPN	Intraductal Tubular Papillary Neoplasms
K-RAS2	Kirsten rat sarcoma viral oncogene homolog
MCN	Mucinous Cystic Neoplasm
MDT	Multi-Disciplinary Team
mmol/l	Millimoles per litre
MMR	Mismatch Repair gene
MRCP	Magnetic Resonance Cholangiopancreatography
MRI	Magnetic Resonance Imaging
MTHFR	Methylene Tetrahydrofolate Reductase
MTR	Methionine Synthase
MTRR	Methionine Synthase Reductase
NAT	N-acetyltransferase
NFPTR	National Familial Pancreas Tumor Registry
ng/l	nanograms per litre
NHS	National Health Service
NNK	Nitrosamine 4-methylnitrosamino-1-(3-pyridyl)-1-butanone
NO	Nitric Oxide
NO-2	Nitrite group
NO-3	Nitrate group

ONOO-	Peroxynitrite
OR	Odds Ratio
PALB2	Partner and localiser of BRCA2
PANDA	Pancreatic cyst DNA Analysis study
PanGen-EU	European study into Digestive Illnesses and Genetics
PanIN	Pancreatic Intraepithelial Neoplasia
PCR	Polymerase Chain Reaction
PDAC	Pancreatic Ductal Adenocarcinoma
PRSS1	Protease Serine 1 gene
PRSS2	Protease Serine 2 gene
REC	Research Ethics Committee
RNA	Ribonucleic Acid
ROC	Receiver Operator Characteristic
RR	Relative Risk
SEER	Surveillance Epidemiology and End Results
SIR	Standardised Incidence Ratio
SMAD4	Smad family member 4/ gene encoding for a member of the Smad family of signal transduction proteins
SNP	Single Nucleotide Polymorphism
SSCP	Single Stranded Conformation Polymorphism
STK11	Serine/Threonine Kinase 11 gene
TNFα	Tumour Necrosis Factor α
TP53	Tumour Protein p53
UK	United Kingdom
US	United States of America
WHO	World Health Organisation
XRCC	X-ray Cross-Complementing proteins

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Introduction

1.1 Pancreatic ductal adenocarcinoma

1.1.1 Incidence and prevalence

Exocrine pancreatic cancer accounts for over a quarter of a million deaths worldwide each year with approximately one third arising in Europe [1]. Despite being only the thirteenth most common malignancy worldwide, it is the eighth most frequent cause of cancer mortality [2]. The lethality of this disease is further evident by the fact that mortality rates continue to approximate to incidence [2]. There has been only a marginal decline in both age-standardised incidence and mortality rates over the past 20 years [1, 3].

Pancreatic ductal adenocarcinoma (PDAC) accounts for almost 90% of all malignant neoplasms of the exocrine pancreas. This encompasses variants including mucinous non-cystic adenocarcinoma, signet-ring cell carcinoma and mixed-ductal endocrine carcinoma. They exhibit similar histological characteristics and clinical outcomes. This group of exocrine tumours is commonly collectively referred to as “pancreatic cancer”. In 2008, a total of 8,085 cases of pancreatic cancer were diagnosed in the UK [1]. This figure comprises a total of 4,001 males and 4,084 females.

1.1.2 Survival data

The overall five year survival rate in patients with PDAC remains poor at 5.6% [4]. This figure has remained relatively static for decades. The poor prognosis conferred by a diagnosis of PDAC, can be explained by the frequent late presentation of the disease at an advanced

stage. Surgical resection remains the only treatment modality to offer potential cure. Unfortunately, only up to 20% of those presenting with PDAC will have disease which is amenable to resection. Hence, potentially curative treatment cannot be offered in the majority of patients. PDAC remains largely chemoresistant, despite a gathering body of evidence to support improved survival following treatment with certain chemotherapeutic agents [5-8]. The combination of surgical resection and chemotherapy with 5-fluorouracil (5-FU) was demonstrated to be effective in early cancers, with significantly improved survival of 20-30% at five years [5]. Hence, there is an incentive to identify PDAC at an early stage to improve long term survival. A small series from Japan has shown that very early cancers (<2cm diameter, no lymph nodes involved), although rare, result in a 60% 5-year survival rate. Survival was 100% at two years [9]. Current median survival of patients with tumours that present clinically is measured in months. Early symptoms of pancreatic cancer are poorly characterised, not very specific, and are often missed by patients and their clinicians.

1.1.3 Current treatment strategies

Radical surgery for pancreatic cancer is only possible for around 20% of patients due to the frequency of vascular involvement and distant metastases at presentation [10]. Historically, the combination of significant perioperative mortality rates, coupled with overall 5-year survival rates of less than 1%, has led some clinicians to regard radical intervention in the treatment of PDAC as futile [11] [12]. This view, however, has been largely abandoned over the past 20 years, as centralisation of pancreatic surgery has resulted in dramatic improvements in perioperative management, with operative mortality rates of between 1%

- 4% now typical of most high-volume specialist centres [13-16]. Significant improvements in resectability rates, morbidity, and late postoperative survival, are also associated with greater caseloads which occur with the centralisation of services [17].

As eluded to earlier in this section, the advent of adjuvant chemotherapeutic agents has led to 5 year survival rates approaching 30% in a subgroup of patients, having undertaken surgical resection with subsequent adjuvant chemotherapy [5, 6] . However, the appropriateness of surgical intervention must be carefully considered by the Multi-Disciplinary Team (MDT), as the presence of locally advanced disease with vascular encasement, or identification of distant metastases, precludes the majority of those presenting with PDAC from attempted resection. Surgical intervention in the treatment of pancreatic cancer remains the only option for cure. Hence, in individuals with disease which is not amenable to surgery, palliative chemotherapy may be offered, pending assessment of their performance status. In some who present with advanced disease, there is a rapid clinical deterioration following diagnosis. Therefore, palliative care and symptom relief with the aim of a dignified and comfortable death may be the only treatment modality which can be offered. It is imperative that all members of the MDT work closely to deliver patient focused care at all stages of their treatment, addressing both physical and psychological concerns.

1.1.4 Surgical treatment

The majority, approximately 70%, of PDAC arise in the head of the pancreas. Hence, the standard surgical technique of partial pancreatoduodenectomy accounts for between 80-90% of all radical operations performed for this patient group [13]. Pylorus-preserving procedures have been widely adopted due to the associated preservation of gastrointestinal

function, and comparable oncological clearance when compared with historical techniques in previous randomised trials [18, 19], providing the remaining duodenal margin is viable and tumour-free. Historically, controversy has reigned regarding the role of extended lymphadenectomy in improving survival outcomes [20]. However, more established evidence from randomised controlled trials has failed to demonstrate any survival advantage associated with extended lymphadenectomy, and have indicated the potential for additional perioperative morbidity, when compared with standard surgical techniques [21, 22]. In certain cases, limited venous resection may be undertaken in those individuals in whom the tumour extends to involve the hepatic, portal or superior mesenteric vein, providing adequate macroscopic tumour clearance can be achieved [23-25]. The radiological extent of any vascular involvement is carefully evaluated by the MDT pre-operatively, as this is a key determinant in establishing resectability of the tumour.

1.1.5 Chemotherapy in the treatment of PDAC

A series of large randomised controlled trials from both Europe and the US have led to the establishment of evidence in support of a role for chemotherapy in PDAC. Initial evidence in support of chemoradiotherapy regimes conferring a survival advantage [26, 27] has been superseded by a series of large trials which reveal that, in fact, radiotherapy exerts a deleterious effect and that optimal adjuvant treatment is achieved through the use of chemotherapy in isolation [5, 6, 28]. Although initial evidence cited 5-FU as the gold standard chemotherapeutic agent in the adjuvant treatment of PDAC, more recent evidence has suggested that gemcitabine confers a longer median disease free survival coupled with a favourable toxicity profile [8]. More recently, definitive evidence from The European Study Group for Pancreatic Cancer ESPAC-3 trial did not demonstrate significant differences in progression-free survival, or quality of life scores, with the use of gemcitabine over 5-FU and folinic acid [29].

1.2 PDAC: Aetiology

1.2.1 Age, sex and ethnicity as risk factors for PDAC

Age, sex and ethnicity have all been implicated as risk factors for the development of PDAC. Population analyses have demonstrated that advancing age has an associated increased risk for development of the condition. US National Cancer Institute Surveillance and End Results (SEER) data are cross sectional analyses performed across five year age ranges. Data below pertains to the risk of developing pancreatic, colonic and breast cancer with advancing age. It can be seen that the peak lifetime risk of developing pancreatic cancer approaches 0.5%

at approximately 85 years of age. The increasing incidence of PDAC seen with advancing age in the US is reflected in worldwide data [30] [31].

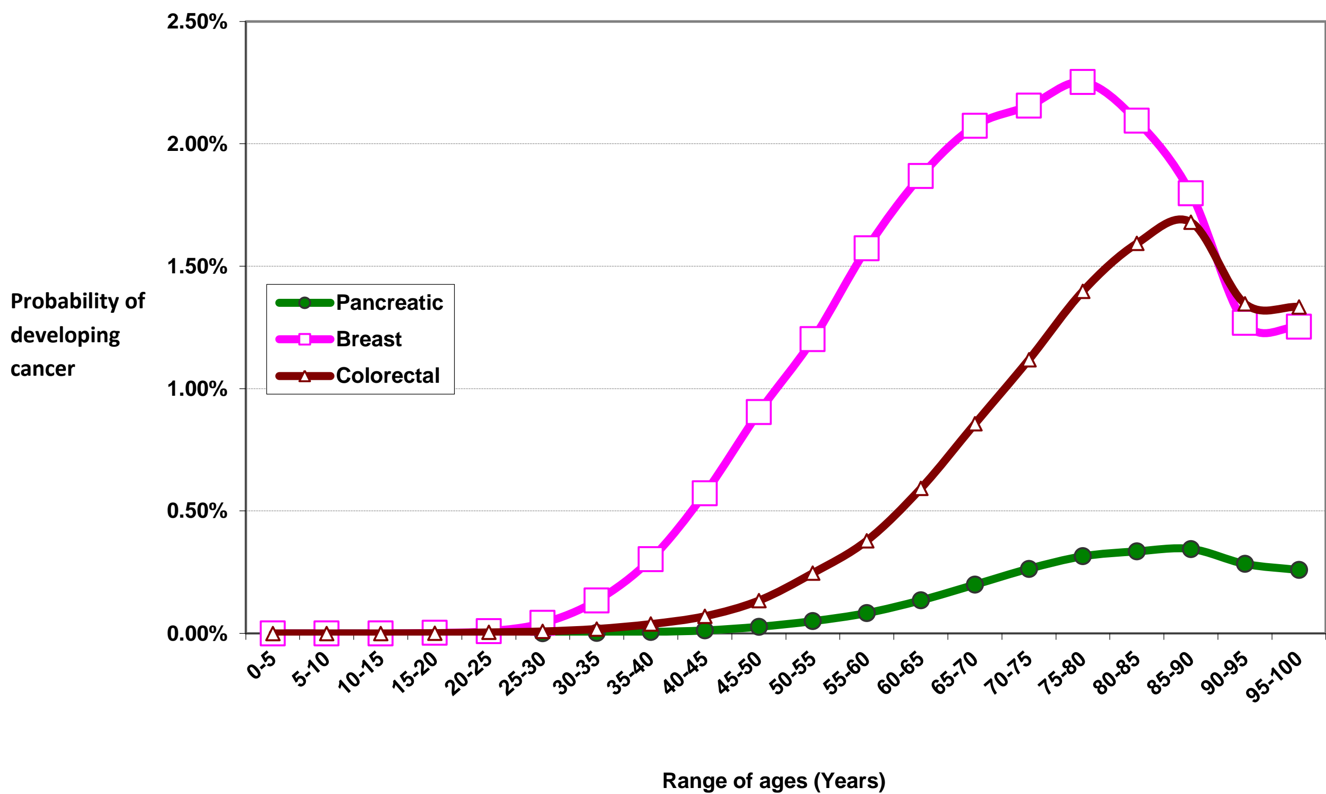


Figure 1: SEER data: cross-sectional analysis across 5 year age ranges identifying risk of development of breast, colorectal and pancreatic cancer with increasing age [4]

SEER data was obtained for the US population and shows that pancreatic cancer risk peaks in the 85-90 year age range, somewhat later than risk for breast and colorectal malignancy.

In addition to the trend of increasing incidence seen in the older population, there is established evidence to support a male preponderance to the development of PDAC. The slight female preponderance seen in UK females from 2007 data is not reflected in worldwide and historical data, where an increased incidence of PDAC has been typically recorded in males [32]. SEER data are cross sectional analyses performed across five year age ranges. SEER data recorded an incidence of 13.9 per 100,000 in US males compared to 10.7 per 100,000 in females, for the period 2000-2009. Statistics derived from worldwide

cancer registry information estimate the number of male deaths attributable to pancreatic cancer at 120,000 per annum, as compared to 107,000 female deaths [33]. Variation in risk has also been observed between those of different racial and ethnic origins. Data from SEER analysis relating to PDAC diagnoses according to race identified an increased incidence in the black population within the US. The incidence within the black male population was the highest observed among any group at 18.1 per 100,000. An apparent preponderance to the disease has been noted amongst other ethnic groups worldwide. For example, an elevated rate of pancreatic cancer is seen within the Ashkenazi Jewish population. This apparent predisposition to pancreatic carcinoma is usually associated with an inherited genetic alteration, commonly, a *BRCA2* mutation.

1.2.2 Cigarette smoking and other tobacco use as risk factors for PDAC

Many environmental and disease derived risk factors have been implicated in the development of sporadic pancreatic cancer. The most significant association is that of tobacco smoking [34-55].

A large number of cohort and case control studies have been performed examining the relationship between smoking and the development of PDAC. From this large volume of evidence, it is now widely accepted that tobacco smoking is associated with a dose dependent increased risk for the development of PDAC with an average increased risk of approximately 2 fold [34-55]. Furthermore, it is estimated that 20-30% of sporadic cases are attributable to smoking [56].

In a recent meta-analysis of 82 published papers by Iodice *et al* [56], including 42 case control, 35 cohort and 5 nested case control studies, the pooled relative risk (RR) for the

development of pancreatic cancer in current smokers was 1.74 compared to non-smokers. Very few studies have reported findings which refute this association [57-59]. In a cohort of 14,397 Taiwanese, Liaw *et al* recorded a RR of 0.3 (95% CI 0.1-0.9) for pancreatic cancer in the subgroup of male smokers versus their non-smoking counterparts despite the fact that the study did show a significant elevated risk for cancer in general for this same group of smokers. Of the 14,397 participants, 11,096 were male and amongst this group 15 pancreatic cancer deaths were recorded, compared to 110 liver cancer deaths [57]. This is a surprising ratio of pancreatic to liver cancer deaths, given that pancreatic cancer is considered to be a more likely cause of cancer death in the general male population [60]. Thus, suggesting a bias in the population or misreporting of causes of death. Two case-control studies undertaken in Japan [58] and Canada [59] have also reported a RR of <1.0 in specific study subgroups. Nishi *et al*, in a study of 141 cases and 282 controls identified a RR of 0.3 (95% CI 0.1-0.8) in a subgroup of current smokers consuming 1-2 cups of coffee daily [58]. In a Canadian study of 312 cases and 2919 controls, Hanley *et al* reported an odds ratio (OR) of 0.76 (95% CI 0.76-1.36) and 0.79 (95% CI 0.44-1.44) in a cohort of males smoking 5-15 and 15-25 pack years, respectively [59]. This reached significance, with a *p* value of 0.0059 on adjusting for age and province. However, it should be noted that individuals smoking over 25 cigarettes per day had a significantly increased risk of pancreatic cancer (1.83, 95% CI 1.23–2.72) even in this study, and the protective effect of low level smoking lost significance after multivariate analysis (p=0.49) [59].

Several authors have reported an increase in the risk of development of pancreatic cancer in females compared to their male smoker counterparts [43, 61-63] . In a Swedish cohort study of 45,906 males and 37,147 females, a reported adjusted RR of 6.90 (95% CI 1.91-24.93) was

demonstrated in the subgroup of females smoking for >40 pack years. This was compared to an adjusted RR of 4.05 (95% CI 1.81-9.05) in the corresponding male cohort [43]. However, this apparent female preponderance has not been consistently reported. Evidence from the UK Million Women Study, the largest women's health study to date, reported a RR of 2.39 (95% CI 2.10-2.73) amongst current smokers [36], which is comparable to results recorded by other large cohort studies [54, 64]. In view of this, there does not appear to be a large gender associated increased risk for the development of pancreatic cancer in smokers.

There is more consistent evidence supporting a dose-dependent relationship in cigarette smoking and pancreatic cancer. Large cohort and case control studies have consistently identified an increase in risk for the development of PDAC with increasing number of pack years [34, 36, 39-42, 46, 65].

There is limited evidence to suggest that smokers develop pancreatic cancer at earlier ages than their non-smoking counterparts. In an analysis of 29,239 histologically confirmed PDAC cases, Brand *et al* at the University of Pittsburgh, concluded that current smokers were diagnosed with pancreatic cancer 8.3 years younger than never smokers [66].

Evidence regarding former smoking demonstrates a general trend of decreasing risk with increasing number of years since stopping tobacco use, as may be expected. In their 2008 meta-analysis, Iodice *et al* reported an overall RR of 1.20 amongst former smokers from the 82 papers included [56]. Evidence across a wide range of cohort [54, 55, 62, 65] and case-control studies [39, 45, 50, 61, 63] has consistently demonstrated no increase in risk in ex-smokers 10-15 years after stopping cigarette use. The most recent evidence from a large group of 29,239 histologically confirmed pancreatic cancers, reported that past smoking was

less influential upon risk than current smoking, suggesting a key role for health education and smoking cessation in the prevention of pancreatic cancer [66].

In addition to the use of tobacco in cigarettes, tobacco is used in different forms across the world. The risk of pancreatic cancer conferred through cigar and pipe smoking has been assessed by several groups [39, 44, 61, 63, 67-70]. Although early evidence in case control studies by Wynder [67] and Howe [63] demonstrated only a marginally elevated risk associated with pipe and cigar use (RR 1.3 95% CI 0.5-3.2 and RR 1.22 95% CI 0.5-2.67 respectively), more recent studies have identified a consistent 2 to 3 fold increased risk in heavy lifetime pipe or cigar use [39, 61, 70, 71]. In a study of 526 cases versus 2153 controls, Alguacil reported a 40% increased risk for pancreatic cancer with the regular use of smokeless tobacco. These data demonstrated an apparent trend in increasing risk with increasing exposure, however, this did not reach significance [70].

Further to exposure through cigar and pipe smoking, tobacco use in water pipes, which is increasing in China, India, Pakistan and the Middle East, has only been evaluated by one case control study to date [42]. In this study of 194 cases and matched controls, a significantly increased risk for the development of pancreatic cancer was seen in sub groups of long term smokers, with additional non cigarette tobacco use. In those of greater than 30 years duration of cigarette smoking, a vastly increased risk was seen in those using non-cigarette tobacco products such as water pipes, than those who exclusively smoked cigarettes (OR 11.6 [95% CI 3.8-35.5] and OR 4.3 [95% CI 1.7-11.2] respectively) [42]. However, in isolation this relatively small case control study does not provide sufficient evidence to substantiate a link between alternative tobacco use and pancreatic cancer.

In addition to the risk posed by direct tobacco exposure, particularly through cigarette smoking, the risk conferred through indirect or passive exposure has also been examined by a number of authors [40, 42, 44, 72]. Evidence remains inconsistent regarding the role of passive smoking in the aetiology of pancreatic cancer, despite the suggestion of an increased risk in a number of case control studies [40, 42, 71, 72]. Results from a large US cohort did not demonstrate any associated increase in risk [48]. Interestingly, in a recent large US cohort of women, Bao *et al* [73] concluded that maternal smoking significantly increased the risk of pancreatic cancer, whereas paternal smoking did not exert such an effect (RR 1.42 [95% CI 1.07-1.89] vs RR 0.97 [95% CI 0.77-1.21]) [73]. This remains elevated, although not significantly so, when the analysis is limited to never smokers. From their results, Bao *et al* hypothesised as to the presence of a potential *in-utero* effect of passive smoking. Passive smoking in adult life was not associated with any increased risk in this 86,673 female cohort [73].

1.2.3 Molecular changes associated with smoking and implicated in PDAC

The influence of smoking in the mutagenesis of genes including *CDKN2A* methylation, *K-RAS2*, *TP53* and *DPC/SMAD4* which have been implicated in pancreatic carcinogenesis is, as yet, unproven. The exact mechanism of tobacco smoke derived carcinogens in the precipitation of pancreatic carcinogenesis has yet to be fully understood. There is established evidence to indicate the influence of tobacco smoking in the aetiology of pancreatic cancer, as cigarette use is implicated as the causative agent in approximately 25% of cases [33, 35, 56].

Despite this, the molecular changes which precipitate pancreatic carcinogenesis in smokers remain poorly understood and the influence of tobacco derived carcinogens upon

alterations in the main genes discussed above is still not established. When examining a potential relationship between *K-RAS2* and smoking in the aetiology of pancreatic cancer, evidence is conflicting. In an analysis of 109 incident cases of pancreatic cancer, Crous-Bou reported that the tobacco derived carcinogenic nitrosamine 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK) had the potential to exert genotoxic effects, in terms of inducing point mutations in *K-RAS2* [74]. *K-RAS2* mutations have been demonstrated to occur at a relatively early stage in the process of pancreatic carcinogenesis, as discussed above and have been seen in association with pancreatic cancer following NNK exposure in animal models [75]. However, from their series of cancer cases, Crous-Bou *et al* concluded that there was no significant association between smoking and *K-RAS2* mutations in pancreatic adenocarcinoma. In fact, those patients with *K-RAS2* mutations were less likely to be ever smokers than those with wild-type *K-RAS2*. *K-RAS2* mutations in those smoking >48 pack years was associated with an OR of 0.14 (95% CI 0.02-1.59) whereas the OR in those having smoked <28 pack years was 0.81 (95% CI 0.03-8.94) [74]. These results did not reach significance. However, they have been used to suggest an inverse association between tobacco exposure and *K-RAS2* mutations in pancreatic cancer cases.

Nevertheless, the compelling evidence seen implicating tobacco related carcinogen exposure with *K-RAS2* mutations in pancreatic cancer in animal models has been replicated by some studies in humans. Early evidence from Hruban *et al* found an increased frequency of *K-RAS2* mutations in 86% of current smokers versus only 68% of non-smokers (p=0.046) [75]. More recent evidence from a 2007 series of 83 pancreatic adenocarcinoma patients provides further evidence in support of such an association. Jiao *et al* reported a significant association between regular and heavy smoking (>36 pack years), and point mutations in

codon 12 of *K-RAS2 (p*=0.003*)* [76]*.* In this series, there was no demonstrable association between tobacco exposure and *CDKN2A* mutations or hypermethylation [76]. Further earlier series by Malats *et al* in 1997 [77] and Berger in 1999 [78] have also proposed a relationship between smoking and *K-RAS2* mutations. However, 2009 evidence from the Johns Hopkins group following sequencing of >750 million base pairs of 39 genes in 114 pancreatic cancer patients, concluded that there was no significant difference seen in the frequency of *K-RAS2* or *CDKN2A* mutations in ever smokers [35]. This group also analysed the presence of *TP53* mutations in an ever, versus never, smoking cohort. The results demonstrated the presence of *TP53* mutations in 50/64 (78%) of the ever smoking cohort compared to 44/50 (88%) never smokers, these results were not significant, so do not constitute evidence for an inverse trend [35].

Despite the most recent evidence from the Johns Hopkins group, refuting an association between smoking and the key genetic changes seen in pancreatic carcinogenesis, there were a significantly elevated number of mutations seen in the ever smoking group [35]. Smokers reportedly exhibited a mean number of 75.5 mutations per tumour, as opposed to the 56.2 mean mutations seen per tumour in non-smokers. From this, it was therefore concluded that the mechanism of tobacco derived carcinogens in pancreatic carcinogenesis is not driven by any influence upon the major genes. Indeed, when *K-RAS2*, *CDKN2A*, *SMAD/DPC4* and *TP53* were excluded from the analysis, an increased mean number of mutations were consistently demonstrated in ever smokers [35]*.* This report followed an earlier US study in 550 pancreatic cancer cases by Slebos, which did not identify any increased frequency of *K-RAS2* or *TP53* mutations in ever versus non-smokers [79].

Hence, it can be seen that the evidence supporting a role for tobacco related carcinogens in influencing the major genes involved in pancreatic carcinogenesis remains inconsistent. Therefore, in order to determine the mechanism of pancreatic carcinogenesis precipitated by tobacco derived carcinogens, research into other potential molecular changes which may influence this pathway is required. A better understanding of the type of mutation events expected will assist greatly in this; for example, the types of mutation expected to result from free radicals ingested through tobacco smoke.

Tobacco smoke contains carcinogens such as aromatic amines and nitrosamines, which generate oxygen free radicals. Several authors have hypothesized that single-nucleotide polymorphisms (SNPs) in tobacco carcinogen-metabolising enzymes may arise through the action of oxygen free radicals [80-82]. It is proposed that this may influence pancreatic carcinogenesis, by altering the direct effect of tobacco derived carcinogens, or influence the pro-inflammatory oxidative effect which tobacco constituents exert. Hence, increasing pancreatic cancer risk [80]. An example of one such carcinogen-metabolising enzyme is cytochrome P-450. Cytochrome P-450 encodes for arylhydrocarbonhydroxylase (AHH) which is an enzyme involved in the activation of tobacco related precarcinogens, for example, polycyclic amines, aromatic amines and nitrosamines. Such activation is usually via a mechanism of *N*-hydroxylation catalyst by hepatic cytochrome P-450 [80, 81]. SNPs in the cytochrome P-450 (*CYP1A1*) gene encoding for AHH have been inconsistently associated with an increase in risk for the development of several cancers in Asian populations [82].

In a study including 309 pancreatic cancer cases, there was a non-significant increased risk for the development of pancreatic cancer seen in an African-American subgroup of 26

patients, but no increase seen in Caucasian individuals (OR 1.4 [95% CI 0.42-4.6]), in association with mutations in cytochrome P4501A1 (*CYP1A1*) [80].

Evidence from Li *et al* makes a more compelling case to support a role for cytochrome P-450 in the aetiology of smoking related pancreatic cancer. In a majority Caucasian cohort, SNPs in *CYP1A2* in ever smokers were associated with an OR of 1.6 (95% CI 1.0-2.7) compared to never smokers [83]. A significantly elevated risk was seen in female ever smokers, with a 3 fold increased risk of pancreatic cancer (95% CI 1.5-7.6) [83]. A similar apparent hormonal modulation of risk was seen in a further study by Suzuki *et al*, who recorded an elevated OR of 4.36 (95% CI 2.15-8.84) in a female subgroup who had smoked >20 pack years [81]. This has led to the hypothesis of gender related risk factors acting synergistically in influencing risk in subgroups of female heavy smokers.

In addition to the increased risk of pancreatic cancer seen in smokers with cytochrome P-450 mutations, aberrant genes encoding for enzymes which are involved in the detoxification of aromatic amines and nitroamines have also been implicated in the aetiology of smoking related pancreatic cancer. One example is that of N-acetyltransferase (NAT). There are two human NAT isoenzymes; NAT 1 and 2. NAT1 is expressed in all tissues, including the pancreas and NAT2 is specific to the liver and gastrointestinal tract [81]. There is limited evidence to suggest that SNPs in NAT1 confer an approximate 1.5 fold increased risk for the development of pancreatic cancer in heavy smokers, although no significant association has been identified for NAT2 [81, 83]. It is hypothesised that SNPs in NAT1 cause such a modification of risk in smokers via alteration of the detoxification of tobacco derived aromatic amines and nitrosamines.

SNPs in a number of other recognised carcinogen-metabolising enzymes have also been proposed in the aetiology of smoking related pancreatic carcinoma. Glutathione S-transferases (GSTs) are a group of phase II isoenzymes which are involved in protection of cells from reactive chemical intermediates and from oxidative stress as a result of exposure to carcinogens including tobacco related toxins. Their mechanism of action is as a catalyst in the addition of reduced glutathione to reactive chemicals including tobacco smoke derived carcinogens. Homozygous deletions in GST genes may be associated with lack of this enzyme and result in increased cytogenetic damage [84]. Studies aiming to determine the significance of SNPs in genes encoding for GSTs have thus far yielded inconsistent results. In a series of 309 cases versus 964 control subjects, Duell reported an association between heavy tobacco use and GST homozygous deletions in increasing the risk of pancreatic cancer [80]. In a small Asian population subgroup, this study recorded a 2 fold increased risk for the development of pancreatic cancer, independent of smoking [80]. When analysis of the large Caucasian cohort (261 cases, 890 controls) was undertaken, an apparent synergistic relationship was seen between heavy cigarette use and GST null genotypes, in certain groups. When a subgroup of Caucasian female smokers of > 40 years duration were considered, an OR (adjusted for age and sex) of 7.2 (95% CI 2.2-23.1) was recorded [80]. This evidence, therefore, supports an association between GST null phenotypes and cigarette smoking in the aetiology of pancreatic cancer. However, later data concerning this apparent relationship contradicts the earlier findings by Duell. Jiao *et al* analysed the same association in a US cohort of 352 pancreatic cancer patients, and did not identify any GST genotype-smoking interaction [84]. It is therefore too early to glean any significance from this apparent association between deletions in the GST gene and smoking. This conclusion, or lack thereof, may also be drawn in relation to other carcinogen-metabolising genes.

Inconsistent evidence also exists as to the significance of SNPs in homologous recombination repair genes. One such example is the x-ray cross-complementing group of proteins (XRCC). This group of proteins participate in homologous DNA repair in response to double-strand DNA breaks, as a result of oxygen free radical action. Impairment of homologous recombination results in genetic instability which may precipitate carcinogenesis. Limited evidence from three studies has alluded to an increased risk of pancreatic cancer in those with *XRCC1* [85], *XRCC2* [86, 87] and *XRCC3* [86, 87] mutations. It has been proposed that this effect is significantly increased by concurrent heavy tobacco use. Data from Jiao et *al* published in 2008, identified a significant trend (p=0.05) for increasing risk with increasing cigarette consumption. The OR in the subgroup of patients who had smoked <22 pack years was 1.43 (95% CI 0.59-3.48) compared to 3.42 (95% CI 1.47-7.96) in those having smoked >22 pack years [86].

Hence, it can be seen that there is limited evidence to support a role for SNPs in carcinogen-metabolising genes in the aetiology of smoking related pancreatic cancer. At present, due to the paucity of data available, no definite conclusions can be made. More work needs to be undertaken to confirm which polymorphisms may be associated with smoking related pancreatic carcinogenesis. The further understanding of such polymorphisms, and their role in cancer development, will provide an important opportunity for health education and reduction of pancreatic cancer risk.

1.2.4 Obesity and elevated body mass index as risk factors for PDAC

Over recent years, an increasing body of evidence has grown in support of a causal association between obesity and pancreatic cancer. In a recent meta-analysis of 21 studies,

encompassing 8062 pancreatic cancer cases, an increase in RR of 1.12 (95% CI 1.06-1.17) was seen with each 5 kilogram per metre^2 (kg/m^2) increase in body mass index (BMI) [88]. This positive association between increasing BMI and pancreatic cancer was demonstrated in both males and females.

Additional large cohort studies undertaken in Korea [89], Austria [90], Sweden [91] and the US [92-94] have recorded similarly elevated risk in association with increasing BMI. Estimations of risk vary between cohorts, from a modest association (RR 1.20 [95% CI 1.07-1.33] *p*=0.001) seen in white US male veterans with BMI greater than 35 [94], to a greater than 2 fold elevation in risk, recorded in an Austrian cohort of 145000 individuals with BMI of over 30 (RR 2.34 [95% CI 1.17-4.66] *p*=0.02) [90].

1.2.5 Alcohol as a risk factor for PDAC

Many authors have attempted to quantify the relationship between alcohol consumption and PDAC [34, 39, 54, 71, 88, 95-97] with varying conclusions. Currently, there is no consistent evidence supporting a causal relationship between alcohol consumption and risk. However, a small number of series have demonstrated significantly increased risk in certain subgroups of their analyses [54, 71, 96-98]. In their large cohort of 470,681, Jiao *et al* identified a significant increase in risk in those consuming more than 3 drinks, or 40g of ethanol per day (RR 1.45 [95% CI 1.17-1.80] *p*=0.002) [96]. This risk was further amplified when considering the consumption of heavy liquor (RR 1.62 [95% CI 1.24-2.10] *p*=0.001) [96]. These findings have been replicated in a number of case-control series. Most notably, in their 2010 study of 326 cases versus 652 controls, Talamini *et al* demonstrated a progressive increase in risk with rising increments of alcohol consumption. Those individuals

consuming more than 35 alcoholic beverages per week were reported to have a significantly increased risk for the development of PDAC than those consuming 7 drinks or less (OR 3.42 [95% CI 1.79-6.55] *p*=0.01) [97]. In a further case-control series, Suzuki *et al* reported a moderate increase in RR in those consuming high levels of alcohol (OR 1.90 [95% CI 1.00-3.62] *p*=0.036) [98]. Despite this failing to reach significance, Suzuki identified an apparent association between PDAC and alcohol consumption in accordance with certain SNPs. Suzuki hypothesises that SNPs in one-carbon metabolising enzymes, and their repercussions on folate metabolism, may exert some influence upon risk. It is proposed that such polymorphisms influence DNA methylation. In combination with high alcohol intake, it is suggested that these SNPs may result in alterations to protooncogenes and tumour suppressor genes [98]. In their analysis of 157 cases and 785 controls, Suzuki *et al* identified an apparent trend between SNPs in methylene tetrahydrofolate reductase (*MTHFR*), methionine synthase (*MTR*) and methionine synthase reductase (*MTRR*) and alcohol consumption in PDAC risk. The subset of individuals with *MTHFR 677 CC* genotype who consumed high levels of alcohol were found to have a significantly elevated risk of PDAC (OR 4.50 [95% CI 1.44-14.05] *p*=0.008) [98]. A trend for increased risk in association with high alcohol consumption was also reported in those with genotypes *MTR AA* and *MTRR 66 G* [98]. However, these findings have not been consistently replicated [99, 100].

In addition to the potential increased risk conferred by SNPs in *MTHFR* and *MTRR*, some studies have identified a possible association between PDAC and SNPs in the alcohol metabolising genes: alcohol dehydrogenase (*ALDH2*) and aldehyde dehydrogenase (*ADH*) [101]. In a series of 160 cases and 800 controls, Kanda *et al* reported a significant increase in risk in polymorphisms of *ADH1B*, in the subset of patients consuming greater than 30g of alcohol per day (OR 2.99 [95% CI 1.39-6.44] *p*=0.005) [101]. The group also demonstrated

non-significant associations between SNPs in *ALDH2* and *ADH1C* and heavy alcohol intake with the development of PDAC, with *p* values of 0.284 and 0.020, respectively [101]. Despite this limited body of evidence, there is no consistent data in support of a causal relationship between alcohol and PDAC.

1.2.6 Diabetes mellitus as a risk factor for PDAC

Evidence surrounding the role of diabetes in the aetiology of pancreatic adenocarcinoma remains controversial. At the time of writing, opinion remains divided as the cause or effect relationship between diabetes and pancreatic cancer. In the first large meta-analysis examining this association, Everhart *et al* concluded that individuals with a longstanding diagnosis of diabetes were at increased risk for the development of pancreatic cancer [102]. A total of 20 case control and cohort studies were included in the analysis. The key criterion for inclusion in the analysis was that study data should be derived from pancreatic cancer cases, with diagnoses of diabetes of greater than 1 year duration. Results from the meta-analysis concluded that those with diabetes of five years or greater duration had an increase in RR for pancreatic cancer (RR 2.0 [95% CI 1.6-2.8]) [102]. This evidence supports a causal association between diabetes and pancreatic cancer.

A number of large cohort studies analysing this association have been undertaken in different worldwide populations [36, 38, 64, 103-111]. The majority of these large cohort studies have reported an elevated RR of between 1.44 [107] and 2.48 [64]. Despite this, there is considerable variation in the methodology of these studies with regard to mechanism of diagnosis of diabetes. More specifically, self-reported history, versus fasting glucose or glucose tolerance testing; and in duration of follow-up. Use of fasting glucose

levels, or glucose tolerance testing, provides accurate evidence for a formal diagnosis of diabetes, and is therefore preferable; as compared to self-reporting diagnoses which may lead to considerable bias. Those cohort studies in which the author undertook fasting glucose measurement, or glucose tolerance testing, to confirm the diagnosis of diabetes [64, 103, 108-113], demonstrated a consistently elevated RR. Albeit, over variable durations of follow-up. The majority of studies, including two meta-analyses [102, 114], support a modest causal relationship between diabetes and pancreatic cancer. In the more recent meta-analysis by Huxley *et al*, conflicting conclusions are drawn following their analysis of 17 cohort and 19 case control studies. The combined OR for all included studies was 1.83 (95% CI 1.66-1.99) [114]. However, in analysis of risk variation with duration of diabetes, the group found that those with a five year or more history of diabetes had a significant 50% reduction in risk, compared to those with a shorter duration of diabetes (RR 1.5 versus 2.2 p=0.005) [114]. Their evidence, therefore, supports the theory that diabetes may be the reflection of an early manifestation of the disease, rather than any causal association. However, data from those individuals with diabetes of five or more years standing, still exhibit an increased RR of 1.5 (95% CI 1.3-1.8) [114]. This, therefore, supports a modest causal association, as a diagnosis of diabetes greater than five years prior to that of pancreatic cancer is unlikely to reflect an early manifestation of a tumour, given overall 5 year survival of approximately 5%.

Despite largely historical evidence pertaining to diabetes as a manifestation rather than cause of pancreatic cancer [115-117], evidence accumulated to date appears to support a causal association between diabetes and pancreatic cancer.

1.2.7 Chronic pancreatitis as a risk factor for PDAC

Definitive evidence for the role of chronic pancreatitis in the aetiology of PDAC was established by Lowenfels [37] *et al* in their 1993 multi-centre cohort study of 2015 individuals with chronic pancreatitis. At 10 and 20 years of follow-up, an elevated cumulative risk of 1.8% (95% CI 1.0-2.6%) and 4.0% (95% CI 2.0-5.9%), respectively, was reported in this cohort, with a standardised incidence ratio of 14.4. This association has been replicated in a number of other studies across different patient populations. In their series of 420 Chinese chronic pancreatitis patients, Wang *et al* reported a 27.2 fold increase in expected incidence of PDAC [118]. In a further Czech series of 223, Dite *et al* reported PDAC development in 4.5% of the subset of individuals with alcohol related pancreatitis [119]. However, it should be noted that both studies included relatively small cohorts. Nevertheless, chronic pancreatitis is regarded as an established risk factor for the development of PDAC.

Several mechanisms for the role of chronic pancreatic inflammation in the causation of PDAC have been proposed. Pancreatic carcinogenesis has been described as a series of specific molecular and cellular changes. Such changes are described later in this work and include alterations to certain oncogenes and tumour suppressor genes, which give rise to the development of cellular atypia. Mutations in the proto-oncogene, *K-RAS2*, are well described in pre-cursor lesions and have been consistently identified in PDAC [120]. However, work undertaken by a number of groups, including our own, have also recorded alterations in *K-RAS2* in association with chronic pancreatitis [121]. This has led to the proposal of chronic inflammation as a predisposing factor to the development of pancreatic dysplasia [122].

Explanation for the cellular atypia resulting from inflammation has been proposed by several authors, and centres on the role of free radicals. The influx of leucocytes at the site of inflammation results in increased oxygen uptake, and subsequently, the release of free oxygenated species. Examples of such products include hydroxyl radicals and nitric oxide (NO). Furthermore, NO, may react with nitrate (NO^{-}_{3}) and nitrite groups (NO^{-}_{2}) to form peroxynitrite ($ONOO^{-}$) [123, 124]. These products display oncogenic potential inducing DNA damage through impairment of cellular repair mechanisms and promotion of angiogenesis.

1.2.8 ABO group and cancer risk

ABO blood group antigen expression has long been associated with cancer development and risk. In 1953, Aird *et al* identified a positive association between blood group A and protective effect of blood group O in gastric cancer [125].

The antigenic structure of the ABO group is derived from the *ABO* gene encoding for glycosyltransferase. Glycosyltransferase catalyses transfer of different amino acids onto the H antigen. A and B alleles differ in amino acid sequence, and so will catalyse the transfer of different proteins onto the H antigen, resulting in blood groups A and B. In the case of blood group O, there is no antigenic expression due to the presence of a single base deletion. The absence of any AB antigens has been demonstrated to confer a protective effect against cancer. Early work into such an association between the AB antigen and pancreatic cancer found that there was an increased frequency of deletions in A,B and H antigens and increased incompatible expression of A and B antigens seen in association with this malignancy [126].

It has been hypothesized that expression of the different ABO groups may confer variable risk for cancer development due to an observed effect upon tumour necrosis factor α (TNFα). In a genome analysis encompassing 496,032 SNPs across 1200 individuals, polymorphisms in the *ABO* gene were found to correlate with serum TNFα levels. Those of blood group O were found to have higher serum TNFα. It was proposed that this phenomenon may explain the potential protective effect seen in blood group O. However, this association was only consistently demonstrated using one assay [127].

A genome wide association study (GWAS) in 2009 undertook genotyping of 558, 542 SNPs in 1896 individuals with pancreatic cancer and 1939 controls [128]. An association between the first intron of the *ABO* gene, located on the q arm of chromosome 9, and pancreatic cancer was reported. Analysis across different blood groups identified an increased risk for the development of pancreatic cancer associated with blood groups A and B, as compared to O [128, 129]. It is again hypothesized that this association may be attributable to the apparent relationship between this locus and circulating TNF-α level.

1.3 Molecular pathogenesis in PDAC

Carcinogenesis is a multistep process in which a number of genetic aberrations occur through damage incurred during DNA replication, or following exposure to a chemical carcinogen. Mutations in protooncogenes, oncogenes, tumour suppressor and DNA repair genes accumulate within a cell and result in uncontrolled cell proliferation [130].

1.3.1 Molecular pathogenesis – *K-RAS2* in PDAC

There is evidence to suggest that *K-RAS2* mutations occur early in the pathway of carcinogenesis. In one study from the Johns Hopkins group, mutations in the *K-RAS2*

protooncogene have been identified in 36%, 44% and 87% of pancreatic intraepithelial neoplasia (PanIN) I, II and III lesions respectively [120].

1.3.2 Molecular pathogenesis – *CDKN2A* and *CDKN2A* methylation in PDAC

CDKN2A (the gene encoding p16 protein) is a cell cycle regulatory gene which may be inactivated by mutations or deletions, or through methylation of *CDKN2A* promotor gene. Inactivation of *CDKN2A* is seen in approximately 85-90% of pancreatic cancers [131, 132], via homozygous deletion of both alleles in 40%, by intragenic mutation in a further 40%, and by hypermethylation of *CDKN2A* promoter in 15% [120]. Evidence for its inactivation at an early stage in the process of carcinogenesis, is provided in the frequency at which it is seen in PanIN precursor lesions. Loss of p16 has been identified in approximately 31% of PanIN Ia and 44% of PanIN Ib lesions [133], so suggesting that it is a relatively common and early event in the stepwise progression of pancreatic carcinogenesis [133, 134].

1.3.3 Molecular pathogenesis – *TP53* in PDAC

TP53 is a tumour suppressor gene which is mutated in 50-75% of pancreatic adenocarcinomas [133]. *TP53* mutation has been found to occur relatively late in the pancreatic carcinogenesis pathway. Evidence from the Johns Hopkins group, determining the frequency of *TP53* mutations in PanIN lesions, reported that 57% of 7 PanIN II lesions demonstrated a mutation of the *TP53* tumour suppressor, whilst none of the 48 PanIN I and II lesions included in the analysis demonstrated a *TP53* mutation [133].

1.3.4 Molecular pathogenesis – SMAD4/DPC4 in PDAC

Similarly, mutations in the *SMAD/DPC4* tumour suppressor gene and *BRCA2* DNA repair gene occur as a late event in the progression of molecular changes towards pancreatic cancer [133]. *SMAD/DPC4* is a tumour suppressor gene which is a member of the transforming-growth factor ß family. *SMAD/DPC4* becomes inactivated by homozygous deletions or through somatic mutation, with subsequent loss of heterozygosity in approximately 55% of pancreatic adenocarcinomas [133].

1.4 Precursor lesions in PDAC

A number of specific precursor lesions have been identified in the progression to pancreatic carcinogenesis, and present a similar theory to that of the adenoma-carcinoma sequence established in colorectal carcinoma [135]. Recognised premalignant lesions of the pancreas were recently formally classified by the World Health Organisation (WHO) [136]. These include established precursor entities such as PanIN, intraductal papillary mucinous neoplasms (IPMN) and mucinous cystic neoplasm (MCN) lesions [137]. In addition, the WHO classification detailed a new premalignant entity: intraductal tubular papillary neoplasms (ITPN)[137]. Attention has focused upon the recognition and treatment of these four non-invasive precursors, in an effort to prevent progression to the development of PDAC and the conferred poor prognosis.

1.4.1 Pancreatic Intraepithelial Neoplasia (PanIN)

PanIN lesions, graded I-III, are recognised as PDAC precursor lesions and increasing grade signifies progression of the cell towards carcinogenesis [138]. Evidence supporting this

theory is demonstrated by the appearance of PanINs around invasive cancers on histological reporting. In addition, the morphological changes exhibited in PanIN lesions of increasing grade are mirrored by specific molecular genetic changes, which further substantiates the role of PanINs as premalignant lesions [138]. Each grade of PanIN, and their associated cellular atypia, is associated with inactivation or activation of specific tumour suppressor genes, or oncogenes, respectively. PanIN I lesions are characterised morphologically by the presence of elongated mucin producing cells. Progression of this cellular atypia leads to the formation of PanIN II lesions. PanIN II lesions exhibit increased nuclear size and loss of polarity. The progression of these features indicates inactivation of the tumour suppressor gene *CDKN2A* [134]. PanIN III lesions are characterised by the budding off of cells into the ductal lumen and are associated with mutations in *K-RAS2*, *CDKN2A* and *TP53* genes [139].

1.4.2 Intraductal Papillary Mucinous Neoplasms (IPMN)

IPMN has been defined in the recent WHO classification as: 'an intraductal grossly visible (typically > 1.0cm) epithelial neoplasm of mucin-producing cells' [136]. The incidence of IPMN is difficult to quantify as they are frequently asymptomatic and present as an incidental finding following radiological investigation for unrelated symptoms or conditions. Alternatively, they may present with pain in association with pancreatitis, jaundice, weight loss or pancreatic insufficiency [140, 141]. IPMN predominantly affect males and occur at a mean age of 66-68 years [135, 141].

It is estimated that 70% of IPMNs occur in the head of the pancreas and account for approximately 20% of resected cystic lesions [135]. Macroscopically, IPMN are characterised by segmental dilatation of the affected ductal branch by 5mm or greater. There are 2 main

recognised subtypes; namely: main branch, which arise from the main pancreatic duct, and side branch IPMN. There may be both subtypes present indicating a ‘mixed’ type [137]. Histologically, IPMN are composed of an intraductal proliferation of mucin-producing columnar cells and can be categorised as gastric, intestinal, pancreaticobiliary or oncocytic in type [140]. IPMN are further characterised as having features of low, intermediate or high grade dysplasia. Each of which is associated with specific cellular changes. Low grade dysplasia is typified by a single layer of columnar epithelial cells, with minimal cellular atypia. Intermediate grade demonstrate nuclear aberrances including enlargement and nuclear pleomorphism and high grade dysplasia is characterised by marked atypia. This encompasses significant nuclear polymorphism, loss of cellular polarity and budding off of neoplastic cell clusters [64].

IPMN are established precursor lesions to PDAC and the prevalence of invasive malignancy varies dependent upon site. In their series of 208 resected IPMN, the Johns Hopkins centre identified that 64% of main branch IPMN were seen in association with invasive malignancy, compared to 38% of mixed and 18% of side branch lesions (p=0.001) [141]. The variable risk of invasive malignancy associated with different subtypes is reflected in the International consensus guidelines for the management of IPMN published in 2006 [142]. In their review of data, Tanaka et al reported the frequency of malignancy in main branch IPMN to range from 60-92% compared to 0-31% side branch IPMN [142]. In light of the significant increase in associated malignancy, the consensus view was that resection should be offered to individuals with main branch IPMN who are deemed fit to undergo surgical intervention. It was concluded that resection of side branch IPMN is indicated in lesions greater than 30mm

and in those who are symptomatic, as these factors were associated with an increased likelihood of malignancy [142].

The cellular atypia seen in IPMN reflects underlying molecular changes across all grades of dysplasia. The molecular changes seen in the development of PDAC have been identified in IPMN. *K-RAS2* mutations have been reported in up to 80% of IPMN, with increasing frequency in association with high grade dysplasia [137]. More specifically, in their sequence of 52 IPMN, through the application of laser capture microdissection, Chadwick *et al* reported *K-RAS2* mutations in 86% (6/7 cases) of IPMN of pancreaticobiliary type and 84% (16/19 cases) of gastric type [143]. These represented the lesions with the highest frequency of *K-RAS2* mutations. These findings have been substantiated by other groups [144]. Different subtypes of IPMN in the pancreas have distinct pathways to pancreatic cancer progression. Mutations in *CDKN2A* and *TP53* have also been described in IPMN of high grade dysplasia [137].

1.4.3 Mucinous cystic neoplasms (MCN)

The 2010 WHO classification defined MCN as 'a cyst forming epithelial neoplasm which does not communicate with the pancreatic ductal system and is composed of columnar mucin-producing epithelium associated with ovarian type subepithelial stroma' [136]. MCN account for 2-5% of pancreatic neoplasms and occur exclusively in perimenopausal women of mean age 40-50 years [145-147]. Such lesions are almost universally located in body or tail of the pancreas and typically present at a large size. In their series of 130 cases, Thompson *et al* recorded an average size of greater than 10cm at presentation [146]. The large size of MCN at presentation was further confirmed by Yamao *et al* in their later series

of 156 cases which reported an average size of 65.3mm [145]. In view of their size at presentation, the commonest presenting symptoms are of abdominal pain, weight loss, vomiting and diarrhoea [137, 146]. This symptomatology is associated with a palpable abdominal mass in 5-10% [137, 146]. Despite the large size commonly seen at presentation causing the clinical features listed; approximately 10-30% of MCN present as incidental findings on imaging undertaken for an unrelated clinical indication in the absence of any signs or symptoms [137]. MCN may be seen in association with other pancreatic or biliary conditions, such as pancreatitis; cholelithiasis; diabetes mellitus and neuroendocrine tumours [137, 145].

Macroscopically, MCN are typically characterised by multiple loculations within a thick capsule. Foci of calcification may be seen within the capsule [135, 137, 146]. One important characteristic in differentiating MCN from IPMN, is communication with the pancreatic duct. IPMN, by definition, are in communication with either the main duct or side branch. It has been proposed by some authors that the presence of such communication between cystic lesions and ductal structures is not consistent with MCN [147]. More recent series have, however, concluded that a proportion of MCN may communicate with the pancreatic duct. In their series of 156 cases, Yamao *et al* reported communication with the pancreatic duct in 18.1% of MCN assessed [145]. It has been proposed that the phenomenon of apparent communication with the pancreatic duct may occur as a result of erosion of the cyst wall into an adjacent ductal structure; thus, forming a fistula rather than a lesion of true intraductal origin [135, 137, 145].

1.4.4 Screening for precursor lesions

The role of precursor lesions in the development of pancreatic carcinogenesis is established. Specific molecular changes are recognised in association with precursor lesions as discussed above. Those individuals with recognised precursor lesions, in the presence of specific molecular changes, are at high risk for the development of PDAC. Techniques which allow diagnosis and sampling of cyst content have been utilised to identify those at high risk. Molecular analyses, in association with specific imaging modalities, have proved useful in identifying those precursor lesions at highest risk for malignant potential. The most sensitive imaging modality for the detection of pancreatic precursor lesions, which also allows cyst fluid sampling to be undertaken, is endoscopic ultrasound (EUS). EUS involves the transmission of high frequency sound waves via an endoscopically placed probe in the stomach or duodenum [148]. It is established that certain precursor lesions may be too small to be identified using cross-sectional imaging techniques such as computed tomography (CT) or magnetic resonance imaging (MRI) [149, 150]. Of 6874 individuals undergoing EUS at the Johns Hopkins centre between 2006 and 2010, 486 patients were identified as having pancreatic cysts [150]. In the diagnosis of cystic lesions of the pancreas, EUS was found to confer an increase in diagnostic yield for the prediction of neoplasia in 36% and 54% of individuals compared to CT and MRI respectively [150].

In the specific case of IPMN lesions, the International consensus guidelines on the management of IPMN as outlined by Tanaka *et al* [142] and discussed in an earlier sub-chapter of this thesis; EUS criteria of location, size and nodule involvement determine the indication for surgical intervention. More specifically, findings of a main pancreatic duct diameter greater than 10mm; branch duct diameter greater than 30mm, and the presence

of nodules, are all indicative of malignant IPMN [142]. In a series of 310 resected IPMN, Shimizu *et al* reported that branch diameter was a weaker indicator of malignancy than main duct diameter or the presence of nodules [151]. More specifically, the presence of nodules greater than 5mm in branch IPMN was found to have sensitivity and specificity of 74.3% and 72.7% respectively [151].

In addition to the improved diagnostic yield of EUS versus cross-sectional imaging in cystic precursor lesions, there is some evidence that changes seen at EUS may correlate to non-cystic, flat PanIN lesions. The recognition of PanIN lesions has been difficult using conventional imaging techniques. In a series of 24 patients undergoing surgical resection of IPMN, Maire *et al* compared the histological occurrence of PanIN lesions with pre-operative EUS findings, independent of the diagnostic features of IPMN. As opposed to IPMN and MCN, PanIN lesions are more typically characterised as flat, non-invasive lesions which are not necessarily associated with ductal dilatation. The absence of cystic change or ductal dilatation in PanIN lesions has meant that their diagnosis through imaging techniques has proven difficult. Maire *et al* reported that of 24 patients with EUS changes, independent of concurrent IPMN, 20 had histological evidence of PanIN (83%) [149]. EUS abnormalities, characterised as hyperechogenic foci and microcysts, occurred in 19 (51%) and 5 (13%) of the cohort of 24. 20 of the individuals were identified as having PanIN lesions as described above. In the remaining 4 individuals in whom PanIN lesions were not identified, chronic pancreatitis was reported following post-operative examination of the resected specimen [149]. EUS also confers the advantage of obtaining samples of cyst content for cytological and molecular analyses to further ascertain the risk for malignant potential [149, 150]. The PANDA study involving centres across the US, performed molecular analyses of cyst fluid

across a cohort of 113 individuals with confirmed malignant and benign pancreatic cysts [152]. Cyst fluid obtained via EUS fine-needle aspiration (FNA) underwent molecular analyses. Of the 113 individuals included, 25 cysts were classified as benign, non-mucinous and 88 mucinous, of which 40 were found to be malignant. On DNA analysis of cyst content aspirated at EUS, a higher number of overall mutations were identified in the mucinous group. *K-RAS2* mutations were associated with mucinous lesions with a specificity of 96% and OR of 20.9 compared to non-mucinous lesions [152]. In a smaller series of 38 mucinous lesions (35 IPMN and 3 MCN lesions), Al-Haddad *et al* defined their molecular analysis as total DNA quantity of greater or less than 40 nanograms per litre (ng/l) and the presence or absence of *K-RAS2* mutations. They reported a sensitivity of 50%, specificity of 80% and accuracy of 56.3% of molecular analysis in the diagnosis of mucinous lesions i.e. DNA quantity greater than 40 ng/l and the presence of *K-RAS2* mutation [153]. With specific regard to *K-RAS2*, mutation was absent in 22 of the 38 mucinous lesions assessed (58%).

In addition to the reported diagnostic advantage conferred by analysis of DNA quantity and *K-RAS2* mutations from cyst content aspirated at EUS, other markers have yielded promising results. In a multicentre series of 341 mucinous neoplasms, EUS imaging, plus analysis of cyst cytology and tumour markers, was undertaken by the Cooperative Pancreatic Cyst Study [154]. In the identification of mucinous versus non-mucinous cystic lesions, EUS morphology reported a sensitivity of 56.1% and specificity of 45.4% (accuracy 50.9%), and cyst cytology of 34.5% and 83.3% respectively (accuracy 58.7%). However, carcinoembryonic antigen (CEA) levels were found to have the highest accuracy of the measures evaluated with sensitivity of 75%, specificity of 83.6% and accuracy of 79.2% in the diagnosis of mucinous versus non-mucinous cystic lesions [154]. Furthermore, no combinations of the

measures assessed were found to confer any diagnostic advantage over CEA levels alone (p=< 0.0001). Further analyses of cyst CEA levels have reported similar findings. In their series of 38 mucinous lesions, Al-Haddad *et al* recorded an accuracy of 62.5% of CEA level [153]. In contrast to the Cooperative Pancreatic Cyst Study group, Al-Haddad *et al* found that the diagnostic accuracy of CEA levels was improved in combination with cytology and molecular analyses at 73% [153].

1.5 PDAC: rationale for screening

A diagnosis of PDAC confers a dismal prognosis. However, early detection and treatment of small asymptomatic tumours has been proven to yield five year survival rates of 60% [9]. A significant proportion of individuals diagnosed with PDAC present with metastatic disease. This is largely refractory to chemotherapy. It remains unclear as to whether the frequent advanced presentation of PDAC is attributable to early dissemination of the disease or late presentation. Yachida *et al* of the Johns Hopkins group have quantified the timing of pancreatic carcinogenesis. Through genome sequencing in seven PDAC patients, Yachida suggests an interval of at least ten years from the initiating mutation to evolution of the non-metastatic founder cell. Moreover, it is reported that a further 5 years are required for the acquisition of metastatic potential with death occurring following an additional 2 years [155]. This paper provides compelling evidence for a significant window of opportunity for the detection of pancreatic adenocarcinoma at a potentially curable stage. Therefore targeted screening, to individuals at elevated risk, has the potential to improve survival.

1.5.1 Identifying screening populations

It is established that early detection and treatment of small asymptomatic pancreatic tumours has been proven to yield five year survival rates of 60% [9]. Therefore, targeted screening to individuals at elevated risk has the potential to improve survival. In order to identify a potential screening population, we need to define those at an elevated risk for the development of pancreatic cancer. Evidence supporting the role of various environmental and disease derived risk factors has been discussed above. Table 1 demonstrates the expected sensitivity (true positive rate) and specificity (false positive rate) that screening in smokers or those with chronic pancreatitis or diabetes would yield. Screening within these populations is not feasible due to the high false positive rates seen. Major surgical resection with significant associated post-operative morbidity is to be considered in those in whom screening is positive. It is therefore imperative that benign disease, or “normal” pancreata, is recognised as such. It appears that those with a positive family history are the most feasible to screen, but further risk stratification in this group is required to ensure targeted screening with maximum yield and minimal harm.

Table 1: Relative true and false positive rates expected from undertaking screening in defined at-risk populations; assuming sensitivity and specificity of 98% of the screening modality

	Population (aged over 40)	Cases	If screening was 98% sensitive and specific	
			True +ve	False +ve
Smoking	9 million	3,000	2,940	180,000
Chronic pancreatitis	6,000	10	10	120
Type II Diabetes (late onset)	167,000	1600	1568	3340
Genetic predisposition	200,000	700	686	4,000

1.6 Genetic predisposition to PDAC

Several genetic alterations and syndromes are recognised as conferring a significantly increased risk for the development of PDAC. Hereditary Pancreatitis; Familial Atypical Multiple Mole Melanoma (FAMMM); Peutz-Jeghers; Hereditary Non-Polyposis Colorectal Cancer (HNPCC or Lynch syndrome) and Li Fraumeni syndromes all confer variable elevated risk along with certain *BRCA2* mutations. FPC is characterised by an autosomal dominant pattern of inheritance of PDAC. The underlying genetic aberrations in FPC are not yet fully understood. Attempts to further understanding of FPC have led to the identification of additional novel genes, which confer an increase in risk, but do not sufficiently explain the phenomenon fully.

1.6.1 Familial Pancreatic Cancer (FPC)

The phenomenon of autosomal dominant inheritance in pancreatic cancer was initially recorded anecdotally, prior to an analysis of 18 families (76 individuals), by Lynch *et al* in 1990. Lynch reported a family history of PDAC in 6.7% of cases compared to 0.7% of controls (p=<0.001) [156]. Further quantification of risk in those with a positive family history was undertaken in later work by Klein *et al* from the National Familial Pancreas Tumor Registry (NFPTR). In a series of 5179 individuals from 838 kindreds, a significant elevation in risk was identified with increasing numbers of affected family members [157]. Those individuals with 2 affected first degree relatives were found to have a 6.4 fold increase in risk, compared with SEER data. However, in those with 3 or more affected first degree relatives this risk increased exponentially to represent a 35 fold increase compared with expected values from SEER data [157].

1.6.2 Aetiological factors implicated in FPC

FPC demonstrates incomplete penetrance. Factors influencing cancer development in FPC have yet to be formally recognised. The phenomenon of anticipation has been described in FPC. In a series of 1223 individuals from 106 FPC kindreds with 2 or more affected individuals, McFaul *et al* concluded that anticipation for the development of pancreatic cancer between the oldest and intermediate generations was 6 years [158]. The factors which influence penetrance and anticipation in FPC are not fully understood. Limited evidence exists as to the significance of environmental and disease exposures in the onset of disease in members of FPC kindreds.

1.6.3 Smoking and FPC

Early evidence from Schenk *et al* in 2001, in a case-control study including 247 cases and 1816 of their first degree relatives (FDR), identified a greatly increased risk for the development of pancreatic cancer in those FDR who were ever smokers, with an OR of 8.23 (95% CI 2.18-31.07) [159]. When the risk factors of ever smoking and positive family history were analysed independently, results suggested that smoking may precipitate earlier age of onset of pancreatic cancer in those with an apparent inherited predisposition. These findings have been replicated in later work by a number of groups [159, 160]. However, this is potentially a result of recall bias and, in previous reports from EUROPAC, no influence of smoking was recorded [158]. Furthermore, it is notable that given autosomal dominance, previous reports indicating 100% smoking habit in affected individuals and less than 50% habit in their unaffected FDR would suggest that carriers are characterised by a predisposition for smoking, rather than a greater susceptibility for cancer if they smoke [161]. Evidence from the University of Washington group, from 251 members of 28 FPC families, suggested that unaffected members of FPC kindreds who smoke develop pancreatic cancer 10 years earlier than their non-smoking counterparts (mean age of onset 59.6 years versus 69.1 years respectively [p=0.01]) [161]. A history of ever smoking was of particular significance in the male subgroup of patients (OR 5.2 [95% CI 2.0-13.2]) compared to females (OR 2.0 [95% CI 0.8-5.2]) [161]. Furthermore, 85% of those members of FPC kindreds who developed pancreatic cancer below the age of 50 were smokers. There was a gross increase in smoking associated risk in those below the age of 50 (OR 7.6 [95% CI 1.3-43.0]). The trend for increased risk among members of FPC families who smoked was evident across all age ranges [161]. The most recent evidence, published in 2009, by the

Johns Hopkins Group concluded that smokers from FPC kindreds, who had also been exposed to environmental tobacco smoke below the age of 21, were diagnosed at a significantly younger age than non-smokers without any environmental tobacco smoke exposure (63.7 years versus 66.6 years respectively [*p*=0.05]) [162]. This trend was also apparent in an Ashkenazi Jewish FPC patient subgroup, in whom diagnoses in smokers were made 5.9 years earlier than non-smokers [162]. It can therefore be seen that, although evidence is limited, tobacco smoke may play a significant role in the onset of pancreatic cancer in members of FPC kindreds. This knowledge has important implications for health education and risk stratification in this high risk patient group.

1.6.4 Diabetes and FPC

There is no current evidence to support a causal relationship between diabetes and penetrance or anticipation in FPC kindreds. Limited evidence from the Johns Hopkins group has suggested an association between diabetes and pancreatic dysplasia in 10 cases from 4 families [161]. However, it should be noted that 5 of the 10 cases with co-existing diabetes and pancreatic dysplasia which were included for analysis, were members of the same kindred, so leading to difficulty in establishing any firm conclusions for such a link [161].

1.6.5 Hereditary Pancreatitis

Approximately 1% of all cases of pancreatitis have an underlying inherited cause. A number of genetic mutations are recognised in their association with Hereditary Pancreatitis (HP) and idiopathic pancreatitis. HP arises through mutations in the Protease Serine 1 (*PRSS1*) gene which transcribes for cationic trypsinogen. HP is characterised by the development of pancreatitis in childhood, with symptoms commonly affecting multiple generations.

Typically, symptoms of malabsorption and diabetes mellitus commence at a younger age in HP than other causes of chronic pancreatitis, and the cumulative rate of both endocrine and exocrine failure is higher [163].

In addition to the cancer syndromes which have been outlined, a significant lifetime risk of pancreatic cancer, estimated at 35-40% [163], is associated with HP. HP exhibits an autosomal dominant pattern of inheritance, with high penetrance of approximately 80% [164]. Localisation of the disease mutation in HP to the q arm of chromosome 7 was first reported by Claude Ferec and his group [164]. Later the same year, a mutation was identified in the *PRSS1* gene by Whitcomb *et al* [165]. The commonest mutation of *PRSS1* is p.R122H and constitutes a single guanine (G-) to adenine (A-) transition mutation in exon 3 of *PRSS1* which results in an Arginine (Arg) to Histidine (His) substitution at amino acid position 122 [165, 166]. This was initially described as R117H, in concordance with the chymotrypsinogen consensus numeration. The p.N29I mutation of *PRSS1* is recognised as the second most frequent alteration of the gene. This mutation is characterised by a single adenine (A-) to thymine (T-) transversion in exon 2 of *PRSS1*. This results in a change from asparagine (AAC) to isoleucine (ATC) at amino acid 29 [166]. Evidence suggests that p.N29I and p.R122H mutations of *PRSS1* are mechanistically and phenotypically similar. However, the third most frequently identified mutation in *PRSS1*, p.A16V, appears to exert a very variable phenotype. The p.A16V mutation is characterised by a missense mutation in exon 2 with replacement of cytosine (C) to thymine (T), and subsequent substitution of alanine (GCC) to valine (GTC) at amino acid position 16 [167, 168].

In addition to the recognised p.R122H, p.N29I and p.A16V mutations, there are a number of less common alterations which have been described in association with idiopathic

pancreatitis. The following mutations are not considered to confer an elevated lifetime risk for the development of pancreatic cancer. Examples include p.R122C [169], p.N29T [169], p.D22G [170] and p.K23R [171] mutations. The mechanism by which mutations in the *PRSS1* gene result in the phenotype of HP is open to debate. However, it is hypothesized that increased activation of trypsinogen, or inactivation of protective buffers, plays a key role in the pathophysiology of HP [172]. Trypsinogen, the inactive proenzyme precursor of trypsin, is synthesized by pancreatic acinar cells, prior to secretion and activation in the small intestine. It is known that intrapancreatic trypsinogen activation contributes to the pattern of increased proteolytic activity and acinar cell damage which characterises pancreatitis [172, 173]. Three trypsinogen isoforms have been identified in the human pancreas; cationic, anionic and meso-trypsinogen. These isoforms are encoded for by the *PRSS1* and *PRSS2* genes [168, 172]. It has been hypothesised that *PRSS1* mutations confer an increase in trypsinogen function and cause increased activation within the acinar cell [172, 173]. It is suggested that increased activation of trypsinogen would then trigger proteolytic activity and subsequent acinar cell damage [172, 173]. Whitcomb *et al* propose that excess trypsin activation within the normal pancreas causes hydrolysis of trypsinogen and trypsin so resulting in inactivation of trypsin [173]. This, in turn, will create a safety mechanism whereby the pancreas is protected from the effects of autodigestion. Whitcomb's group hypothesise that, in HP, this cleavage (proposed to occur at position R117H/p.R122H – corresponding to the commonest site of mutation within the gene) is prevented by a mutation at this locus, and so there is loss of the mechanism for trypsin and trypsinogen deactivation [173]. Hence, those with HP may be prone to persistent pancreatic autodigestion and recurrent pancreatitis. However, other groups refute this, Sahin-Toth has demonstrated that although rat trypsinogen, which naturally has a histidine substitution for

arginine at the presumed trypsin cleavage site (equivalent to p.R122H), was more resistant to trypsin digestion [174], the human form was not [175]. Sahin-Toth and his group went on to suggest that the human p.R122H and the p.N29I mutation were more likely to cause increased autoactivation [176]. Lerch, supported by Sahin-Toth, went on to suggest that if other autosomal dominant conditions are used as a model to hypothesize the effect of a *PRSS1* mutation; alterations within the specific causative gene usually confer a loss of function through inheritance of a defective protein, or impairment of its intracellular processing. If this were the case, it would be envisaged that a mutation in *PRSS1* would lead to a decrease in trypsinogen function, rather than the increased activation seen in pancreatitis [177]. Lerch identified that the multiple mutations in *PRSS1* which have been described, occur at distinct regions of the *PRSS1* gene, and hence, it is likely that each mutation exerts a different structural effect on trypsinogen [177]. Furthermore, Lerch and co-workers highlight evidence, using *in vivo* models, which has identified that activation of digestive proteases occurs independently of trypsin activity. These groups have in the past proposed a protective role of trypsin in defence against other, potentially more harmful, proteases [177]. As a caveat to this, Sahin-Toth has proposed a model whereby chymotrypsinogen C (caldecrin) inactivates trypsin via digestion at Leu81-Glu82, exposing the Arg122-Val123 cleavage site for tryptic digestion, and that p.R122H might therefore be of significance in reducing autodeactivation of trypsin after all [178]. Furthermore, Lerch and Sahin-Toth have convincingly shown that at least one *PRSS1* mutation (R116C) may be related to pancreatitis due to induction of the misfolded protein response, and not due to its trypsin activity at all! [179] It can therefore be seen that the specific mechanism by which *PRSS1* mutations lead to HP remain conjectural. However, regardless of how the mutations

convey their variable phenotypes, all appear to be characterised by an increased pancreatic adenocarcinoma lifetime risk in the region of 40% as highlighted above [163].

1.6.6 *BRCA2* mutations

BRCA2 is a DNA repair gene, aberrations in which are often associated with familial aggregation of pancreatic cancer cases. In a study of FPC families from the European Registry of Hereditary Pancreatitis and Familial Pancreatic Cancer, *BRCA2* mutations were identified in 5 of 26 (19%) of kindreds included in the analysis by Hahn *et al* [180]. These aberrations consisted of 3 previously reported frameshift mutations, and 2 families with novel *BRCA2* variants [180]. In a large series of 245 reportedly sporadic pancreatic cancer cases, Goggins *et al* identified 15 (27%) that had allelic loss at the 13q12 *BRCA2* locus, of which 3 were germline mutations (6174delT and 6158insT). These mutations were identified in individuals with no family history of pancreatic adenocarcinoma [181]. The frequency of *BRCA2* mutations has been found to be particularly elevated in PDAC patients of Ashkenazi Jewish origin. In their series of 145 patients, Ferrone *et al* identified *BRCA2* founder mutations in 4.4% of an Ashkenazi Jewish cohort with pancreatic cancer, as opposed to 1.1% of cancer free, non-Jewish controls. This corresponds to an OR of 3.85 (95% CI 2.1-10.8 p=0.007) [182]. It is possible that the increased risk conferred by *BRCA2* mutations may be further augmented by co-existent low penetrance polymorphisms. Such polymorphisms in isolation may not confer any increased in PDAC risk, but may result in carcinogenesis in combination with *BRCA* mutations.

1.6.7 *CDKN2A* mutations – Familial Atypical Multiple Mole Melanoma (FAMMM)

Germline mutations in the *CDKN2A* tumour suppressor gene are associated with the phenotype FAMMM. An increased risk for the development of PDAC as part of the spectrum of FAMMM was first described by Lynch and Krush in 1968 [183]. Quantitative evidence for the increased risk of PDAC in FAMMM was provided in later work by Lynch *et al* following segregation analysis in 4 kindreds [184]. This work identified an overall five-fold increase in risk for all cancers within the 4 kindreds described. In addition, an increased risk of PDAC was identified, but in too small a sample size to definitively prove an association. More substantive evidence was provided by Lynch et *al* in their analysis of 159 families, with an apparent increased risk for the development of PDAC [185]. 12% of the families analysed demonstrated a phenotype consistent with FAMMM. This led the authors to propose a new variant of the FAMMM syndrome; the FAMMM-Pancreatic Carcinoma (FAMMM-PC) syndrome. In a more recent series of 18 families, Bartsch *et al* reported a varied phenotype across proposed FAMMM-PC families. This has led to the suggestion that a number of distinct hereditary cancer syndromes may be encompassed within the umbrella of FAMMM-PC [186].

1.6.8 Peutz-Jeghers syndrome

Peutz-Jeghers syndrome is characterised by the development of hamartomatous polyps throughout the gastrointestinal tract and is associated with mutations in the *STK11* tumour suppressor gene. Such mutations confer a lifetime cancer risk of 93% [187]. More specifically, risk for the development of PDAC in Peutz-Jeghers, has been estimated at 11-32% [187]. In the case of a confirmed *STK11* mutation consistent with Peutz-Jeghers, PDAC risk has been estimated to be as high as 132-fold increase compared to expected rates

[188]. However, this risk is difficult to quantify, as such individuals may often develop carcinoma of other gastrointestinal origin prior to their presentation with a pancreatic malignancy.

There is established evidence that mutations in *STK11* can predispose to the development of IPMN lesions. In a series of 22 IPMN, Goggins *et al* of Johns Hopkins, identified *STK11* mutations in 100% (2/2) individuals with phenotypic features of Peutz-Jeghers, and in 25% (5/20) of those without [189]. Therefore, mutations in *STK11* may, in part, confer an increase in risk of PDAC through a predisposition to the development of precursor IPMN lesions.

1.6.9 Hereditary Non-Polyposis Colorectal Cancer or Lynch syndrome

The syndrome of Hereditary Non-Polyposis Colorectal Cancer (HNPCC) is associated with germline mutations in DNA mismatch repair (MMR) genes. In addition to the established risk of colorectal and endometrial carcinomas, it has been proposed that HNPCC confers an elevated risk for the development of PDAC. Some argue that this is only true of a subtype of HNPCC and as a result HNPCC is subdivided into two syndromes, Lynch Syndrome I (colorectal and endometrial only) and Lynch syndrome II which includes other cancers [190]. This risk has been difficult to quantify until recent evidence from Kastrinos *et al* [189]. In a series of 6342 individuals from 157 families with MMR mutations, an 8.6-fold increase in PDAC risk compared to the general population was reported.

1.6.10 *TP53* mutations – Li Fraumeni syndrome

Germline *TP53* mutations have been found to be associated with PDAC carcinogenesis. In a UK series of 28 Li Fraumeni families, Birch *et al* recorded a moderately elevated risk of PDAC which reached significance (p=0.007) [191]. In an earlier analysis of 475 tumours in 91 families, Kleihues *et al* recorded a frequency of PDAC of 1.3% [192].

1.6.11 Novel mutations – *PALB2*/Palladin

In the quest to identify the causative gene in FPC, a number of candidates have been identified. Most notably, mutations in Palladin and *PALB2* have been proposed as playing a significant role in FPC. However, consistent evidence definitively associating either gene with FPC remains elusive.

A significant role for Palladin in FPC was initially proposed by the University of Washington group. It is hypothesised that Palladin exerts proto-oncogene characteristics; and mutations thereof were identified in sporadic PDAC cell lines, and tissue from one FPC kindred [193]. However, such an association has not been replicated in other FPC cohorts. Following sequencing of the coding region of Palladin in 48 FPC individuals, the Johns Hopkins group refuted any association with FPC [194]. Similarly, our group, through sequencing of the locus in 74 individuals from FPC kindreds, did not identify Palladin mutations in any of those analysed [195].

More compelling evidence exists as to the role of *PALB2* in carcinogenesis in FPC. *PALB2* is a binding partner for *BRCA2* and has been implicated in breast cancer families. Its association with FPC was initially proposed following the identification of truncating mutations in 3 of 96 individuals from US FPC kindreds [196]. During my work towards this thesis, families I

recruited were included in the analysis of a European series of kindreds, furthering our understanding of *PALB2* as a potential candidate gene. In our series of 81 European FPC families, sequencing of the 13 exons of *PALB2* also identified 3 truncating mutations (3.7%). Significantly, all 3 mutations were identified in kindreds with a history of breast cancer, proposing a causative role for *PALB2* in this particular subset of families [197]. A number of smaller studies in sporadic and familial pancreatic cancer individuals have not demonstrated any role for *PALB2* [198, 199]. Despite this, *PALB2* is gaining more widespread acceptance for its role in pancreatic carcinogenesis in a certain subset of FPC families.

1.7 Secondary screening for early pancreatic cancer in high risk groups

1.7.1 Screening centres and recruitment strategies

Strategies for screening for PDAC in high risk groups have been established by a number of worldwide groups. Two of the first groups to offer screening in the US and Europe were the NFPTR of the Johns Hopkins Medical Institute, and the European Registry of Familial Pancreatic Cancer and Hereditary Pancreatitis (EUROPAC). In this section of my thesis, I shall outline the different screening techniques employed by different worldwide groups and the underlying rationale behind the differing strategies. Identification and recruitment of participants is a key determinant in ensuring that only those deemed at highest risk for the development of PDAC will be exposed to screening techniques, and the potential complications thereof. The potential risks and complications of different screening modalities will be discussed in detail later in this thesis. The NFPTR and EUROPAC vary in their recruitment approaches. The NFPTR of the Johns Hopkins Medical Institute recruit

families following an individual's diagnosis of PDAC; this individual is the proband for that family. Inclusion on the registry requires recognition of a positive family history; family history in this sense meaning a FDR with pancreatic cancer. This approach is cross-sectional, i.e. the probands must have cancer during the very small window of the registry recruitment. Previous reports from the NFPTR show a highly elevated risk of prospective pancreatic cancer in such families [200]. However, this risk was overwhelmingly due to a very small number of families with a very strong family history. Only one out of 98 families with 2 existing cases of pancreatic cancer had a prospective cancer, in contrast one out of just 4 families with 5 or more cases had a prospective cancer. Clearly, family classification is dependent on the observer, if 2 out of 5 cases of cancer are known originally, and 3 new cases are subsequently identified, there has not been any change to actual cancer risk, merely a change to assumed cancer risk. If we accept that there are families with equivalent biologically defined risk in the group with 2 cases, and the group of families with 5 cases, this would strongly suggest that most families are random clusters, but there are fewer random clusters in the families with more cases [200]. EUROPAC tries to reduce the number of random clusters by adopting a longitudinal approach to recruiting families. This is based mostly on concerned healthy individuals with multiple (two or more) pancreatic cancer cases in their family approaching the registry. The rationale being that, recruitment on the basis of an affected proband can only include families where a cancer case is current. This will represent only a very small proportion of genuine families in any catchment zone, given the unfortunately short life expectancy of people diagnosed with PDAC. Clearly, if the proband is defined as being a pancreatic cancer patient, at least one case is already guaranteed in that family. The proband will know the cause of death of a certain number of individuals in their family (n). A conservative estimate of the probability of a second cancer,

assuming the null hypothesis of no genetic predisposition, will be the probability of dying from pancreatic cancer (in the general population) multiplied by n. In fact it will be slightly higher than this, bearing in mind that the proband will be highly motivated and sensitised to cancer compared to other causes of death. In contrast, concerned healthy individuals reporting to EUROPAC will be describing cancer cases dating back over a potentially extensive period. We believe this helps to reduce random clusters and extend the proportion of true FPC pedigrees that will be sampled.

Since the advent of screening for early pancreatic cancer in high risk individuals, further groups have adopted differing recruitment and surveillance strategies, with variable yields in cancer diagnosis [201-207]. Most registries recruit participants following referral by Clinical Genetics departments upon confirmation of their status as high risk, or self-referral of concerned individuals in the knowledge of their affected family members. The recruitment strategy of referral, or self-presentation, of high risk families is adopted by most groups, rather than enrolling affected probands [201, 203-207].

1.8 Screening strategies in high risk groups

Once a screening population has been defined and identified, strategies as to how to perform screening for early PDAC need to be developed. Screening centres worldwide adopt different protocols to optimise potential for detection of early lesions, and minimise potential adverse effects of any screening modality employed. Various biochemical parameters and imaging modalities are used in combination, in an attempt to maximise diagnostic accuracy. However, such investigations will only identify detectable lesions, and do not confer any benefit in risk stratification, or recognition of specific individual molecular markers which are recognised in the pathway of carcinogenesis. Hence, more invasive

surveillance techniques are advocated by some groups to allow for more targeted stratification of individual risk. The biochemical and imaging modalities, and their relative sensitivity and specificity, will be considered in more detail. The International Cancer of the Pancreas Screening Consortium recently published data summarising screening programmes across a number of groups [208]. The imaging modalities utilised by each group, along with their diagnostic yield were summarised. A total of 12 programmes were outlined across the US and Europe [201-207, 209, 210]. All except two surveillance protocols have adopted EUS, alongside either CT or MRI, as their primary imaging modality. These two groups advocate the role of MRI screening as an initial imaging modality, with a view to further imaging in the event of a detected abnormality [206, 207]. More definitive risk stratification can be offered by ERCP, with the collection of pancreatic juice for analysis of molecular markers. This technique is offered to high risk screening cohorts by our group, and through the surveillance regime adopted by the University of Washington [201]. In this context, ERCP is undertaken with the specific aim of analysis of molecular markers to achieve more targeted risk stratification on an individual basis. Other worldwide groups offer ERCP, but only in the event of an abnormality detected at EUS [202, 207]. In this eventuality, ERCP or EUS is undertaken with a view to confirmation of cytological diagnosis, rather than risk stratification through molecular analyses.

Variable yields of PDAC and precursor lesion diagnoses have been reported across differing worldwide surveillance cohorts and protocols. A technique of annual MRI is adopted by two groups; in a cohort of FPC individuals, with a yield of 3 PDAC and 15 cystic lesions after screening in 262 individuals [207]; and in 79 FAMMM patients, yielding 7 (9%) PDAC diagnoses and detection of 9 (11%) precursor lesions [206]. In those groups advocating EUS

as the first-line imaging modality [202-205], the yield of cancer diagnoses has been reported as 7/38 in a cohort of FPC and Peutz-Jeghers individuals [202], and 6/51 in a further US FPC screening group [205]. In their series of 76 FPC individuals, a German EUROPAC collaborating group reported the detection of abnormalities at EUS of 33% [204].

The technical considerations and potential complications of the biochemical, radiological and more invasive molecular screening modalities, used across the surveillance protocols reviewed above will be discussed in the following sub-chapters.

1.8.1 CA19.9

Carbohydrate antigen (CA) CA19.9 is a sialylated Lewis blood group carbohydrate antigen, which is elevated in over 80% of pancreatic cancers [211]. However, if used as a screening tool in isolation, CA19.9 lacks specificity [212, 213], and is not produced by those who are Lewis *a* and *b* negative [214, 215].

1.8.2 Fasting glucose

The measurement of serum glucose is utilised in the early detection of pancreatic cancer. A number of different techniques may be performed. In a case-control study analysing the prevalence of pancreatic cancer associated diabetes mellitus, Pannala et al defined 5.5 millimoles per litre (mmol/l) as the upper limit of the normal reference range [216].

Alternative screening tools for the detection of diabetes mellitus have been used by other groups. Two large US cohort studies identified a trend in increased development of pancreatic cancer in those with an elevated post prandial, or post load, glucose [64, 106]. Post prandial glucose measurement involves the administration of a glucose load, for

example, 50g oral glucose load, and drawing of blood at a specified interval following this challenge. This may be one or two hours in most cases. The resultant plasma glucose level is measured in mmol/l. A result of greater than 11.1 mmol/l is generally considered as diagnostic of diabetes mellitus [64, 106].

1.8.3 Imaging modalities utilised in screening for PDAC

Much evidence exists as to the sensitivity and specificity of various imaging modalities in the detection of pancreatic precursor, or early malignant, lesions. A discussion of the comparative diagnostic yield of precursor lesions when utilising EUS versus CT and MRI has been undertaken earlier in this thesis. In this subchapter, the imaging modalities of CT, MRI and EUS, and their role in the diagnosis of early PDAC in varying clinical contexts, will be reviewed.

CT as a screening modality

CT scanning involves the projection of x-rays from several angles in order to achieve cross sectional images. The specificity of CT in reliably distinguishing between malignant and benign pancreatic disease has been estimated at 59-93% [206-209]. With specific regard to screening, the use of CT has recently been evaluated by the International Cancer of the Pancreas Screening Consortium [208]. In a series of 216 high risk individuals screened across 4 US centres, the group evaluated the use of CT in the detection of asymptomatic pancreatic lesions [217]. A total of 283 solid and cystic pancreatic lesions were identified in the cohort of 216 individuals using three imaging modalities i.e. CT, MRI and EUS. CT detected 39 (13.8%) of the lesions. With specific regard to potential precursor cystic lesions, CT visualised fewer cysts greater than 1cm than MRI and EUS. Of those lesions less than 1cm, the CT detection rate fell to 11% [217].

MRI as a screening modality

MRI involves the application of an oscillating magnetic field at a resonant frequency, and subsequent detection of the resultant specific frequency radio waves emitted from hydrogen atoms. This technique confers an advantage in the avoidance of exposure to ionising radiation. Several series have evaluated the use of MRI techniques in the screening of high risk groups [205, 206, 218]. In a series of 51 patients from 43 kindreds, Verna *et al* undertook MRI and EUS screening over a period of 3 years. A total of 2 pancreatic masses and 3 cystic lesions were detected by both modalities. In a larger series of 109 high risk individuals, Ludwig *et al* employed a single cycle screening technique of magnetic resonance cholangiopancreatography (MRCP). The overall diagnostic yield of MRCP was reported at 8.3% (9/109 patients) and was concluded to be a safe screening technique. Following identification of an abnormality at MRCP, individuals underwent further imaging with EUS [218]. Further evidence for the diagnostic yield of MRI was reported by Vasen *et al* in their Dutch series of 79 FAMMM patients [206]. Annual screening MRI scans, undertaken over a median follow-up period of 4 years, detected PDAC in 7(9%) individuals [206]. The use of MRI as an effective screening tool was also evaluated, alongside CT and EUS, in the larger US series by Canto *et al* [217]. In their analysis of 283 lesions in 216 high risk individuals, MRI visualised 218 (77%) of the detected abnormalities [217]. With specific regard to cystic precursor lesions, MRI detected 33% of sub-1cm abnormalities, and 90% greater than 1cm in size [217].

EUS as a screening modality

Evidence pertaining to the role of EUS in the detection of precursor lesions of the pancreas has been discussed earlier in this thesis. The International Cancer of the Pancreas Screening

Consortium has advocated EUS, and/or MRI, in the initial screening of high risk individuals [208]. This followed work by Canto, which has been summarised in the above subchapters, discussing CT and MRI techniques [217]. The effectiveness of EUS as a screening technique was assessed in the cohort of 216 high risk individuals. EUS conferred the highest detection rate of 229 (79%) of the total 283 visualised lesions [217]. In the detection of precursor cystic lesions, EUS detected 100% of cysts greater than 1cm in size, a significantly higher rate than CT (62.5%) and MRI (90%) *[p=*0.007*]*[217]. Further support for the use of EUS in screening of unaffected members of FPC kindreds, was provided in earlier work by Poley *et al* in 2009. This group reported results from first-time EUS screening in a series of 44 high risk individuals, from families with a variety of cancer syndromes [218]. The high risk cohort was recruited prospectively and undertook screening between April 2005 and October 2007. In this period, 3 asymptomatic pancreatic mass lesions which were subsequently demonstrated to be ductal adenocarcinomas were identified. In addition, 7 (15.9%) cystic abnormalities suggestive of IPMN were diagnosed [218]. This evidence would appear to suggest a high pick up of asymptomatic cancers in high risk individuals via EUS. However, evidence from a different established screening group refutes this finding. In their 5 year follow-up of 76 high risk individuals, undertaking screening including EUS and MRI, Langer et al concluded that, although screening led to the identification of precursor lesions in 25 (33%) of high risk individuals via EUS alone, undertaking long term surveillance in all high risk individuals delivers a relatively low yield [219] . This evidence from the Marburg group suggests that risk stratification, even in those deemed at high risk for the development of pancreatic cancer, is required in order to target secondary screening to improve the yield of abnormalities.

1.8.4 CT versus EUS in the detection of PDAC

The main imaging modalities undertaken by EUROPAC in screening for early pancreatic cancer are CT and EUS. These techniques are summarised earlier in this thesis. Defining sensitivity of these imaging techniques depends upon the stage of the cancer, and definition of specificity depends on the control group used. Therefore, these estimates vary greatly in the literature. CT and EUS have been found to exhibit a sensitivity of 69-90% [219-222] and >90% respectively [223, 224]. EUS has not been demonstrated to be as specific in the presence of pancreatitis, with poor specificity of 64%. However, in the absence of pancreatitis, the specificity of EUS has been demonstrated to be 96% [225]. Hence, EUS is primarily utilised in the screening of eligible unaffected members of FPC kindreds with expected 'normal' pancreata, whereas CT is used as the follow-up imaging modality of choice in those with HP.

2 Thesis aims and objectives

2.1 Thesis Aims

In this thesis I aimed to identify subpopulations of high risk families who are at the greatest risk of pancreatic cancer in order to target them for screening.

2.2 Thesis Objectives

To achieve the aims of my thesis I had several objectives, notably:

1. Registering families with multiple cases of pancreatic cancer and/or pancreatitis and obtaining full epidemiological data stratified by kindred. I achieved this objective through recruitment of individuals and families eligible as high risk kindreds either through self-referral or via a collaborating clinician. Confirmation of the family as having a genuine predisposition to PDAC was obtained and familial and individual demographic, lifestyle and disease related data was obtained at the time of registration through completion of questionnaires. Completion of corroborating medical questionnaires was also sought from the referring clinician.
2. Comparison of age of onset in sporadic pancreatic cancer with age of onset of cancer in high risk kindreds. This analysis was achieved through data collection and Kaplan Meier survival analysis in high risk family groups following their registration.
3. Comparing lifestyle characteristics and other demographics in cancer patients on the registry with sporadic pancreatic cancer patients (prospectively identified and as recorded in the literature). This thesis objective was achieved through collection of individual and familial lifestyle and disease related data at registration and Kaplan Meier survival analyses.

4. Investigation of genetic factors that could be used in stratification of risk within kindreds and in sporadic pancreatic cancer. This objective was achieved through registration of high risk families, in the confirmation of diagnoses and construction of pedigrees and in the collection of DNA and cell line blood samples for storage and analysis.
5. Parallel pilot screening of high risk individuals and comparing outcomes with predictions based on the previous objectives. Achieved through recruitment of high risk individuals to the EUROPAC secondary screening study. Through collection of pancreatic juice samples for the analysis of molecular markers to stratify risk based on previous work by our group and discussed further in the later Materials and Methods section of this thesis.
6. Evaluating the risks of screening compared to outcome measures, and optimising the screening process. This objective was achieved through the recording of complications and morbidity encountered as a result of the EUROPAC secondary screening study and in the implementation of techniques to minimise harm, with particular reference to amendment of the screening protocol for the collection pancreatic juice.

3 Materials and Methods

3.1 Introduction to the European Registry of Familial Pancreatic Cancer and Hereditary Pancreatitis (EUROPAC) - Background to registry, eligibility criteria and recruitment of families.

EUROPAC has been working on the genetic and clinical characterisation of Hereditary Pancreatic Diseases since it was established in 1997. EUROPAC is the largest European

registry of FPC and HP kindreds, both of which are associated with an increased risk for the development of pancreatic cancer as discussed above. A major part of this thesis has involved the expansion of this registry, as will be described later.

Registration with EUROPAC as either an FPC or HP kindred, was achieved either through self-referral following consultation of our internet website, or via referral from a clinician or Clinical Geneticist. Registration with EUROPAC as an FPC kindred requires confirmation of multiple cases of pancreatic cancer affecting multiple generations. A minimum of two cases suggesting an autosomal dominant mode of inheritance, was the minimum criterion for registration. My key role was in the recruitment of individuals either through self-referral or via a collaborating clinician or geneticist. I provided information regarding the registry and eligibility for participation in the secondary screening study to interested individuals. I was responsible for collecting and collating individual and family data using questionnaires at the time of registration. I undertook consent and collection of blood samples for DNA storage and research as part of the recruitment process. The specific parameters of data collected and mode of storage are discussed in more detail later in this chapter.

The probability of a family carrying a genetic predisposition for the development of pancreatic cancer is increased with increasing numbers of affected first degree relatives. This phenomenon was established by research undertaken by Klein *et al* from the US NFPTR and has been discussed earlier in this thesis [157]. Those individuals with 2 affected first degree relatives were found to have a 6.4 fold increase in risk compared to SEER data. However, in those with 3 or more affected first degree relatives this risk increased exponentially to represent a 35 fold increase compared with expected values from SEER data[157].

The FPC registry also includes families of additional cancer syndromes which confer an increased risk for the development of pancreatic cancer. Such syndromes include Peutz-Jeghers, Breast-Ovarian, FAMMM, Neurofibromatosis, Li Fraumeni and HNPCC syndromes. Many Breast-Ovarian families carry *BRCA2* mutations. *BRCA2* has a very variable phenotype which may include a preponderance for pancreatic adenocarcinoma. Although a number of candidate genes have been identified [195, 197], the gene loci which is responsible for FPC has yet to be formally described. Through familial based association testing, EUROPAC has identified a haplotype and is continuing to sequence candidates in this region. I was involved in the recruitment of such families and in the collection of blood samples from affected and unaffected individuals from high risk families to work towards achieving this aim.

3.1.1 Research Ethics Committee approvals

The EUROPAC registries of FPC and HP were founded in 1997 following Research Ethics Committee (REC) approval for the collection and analysis of data and blood samples from these high risk groups. Ethical approval for the FPC registry was obtained via consultation with the North West Multi-Centre Research Ethics Committee (REC reference: 03/8/069). Similar approval was sought for the HP registry from the Scotland A Research Ethics Committee (REC reference: 04/0/010). The favourable ethical approval obtained for the FPC and HP registries encompasses collection and collation of data for research purposes in these high risk groups. Our ethical approval also encompasses the collection and storage of blood for research purposes and DNA storage from consenting individuals.

Screening ethics

EUROPAC has ethical approval to undertake screening on a research basis in those individuals who fulfil criteria as high risk (REC refs: 07/H1008/153 Central Manchester REC; 07/H1211 Warwickshire REC). A key part of my work towards this thesis was in the authorship of a significant amendment to the screening protocol and associated ethical application and approval. I was responsible for counselling and consenting eligible individuals wishing to participate in the secondary screening study as well as co-ordinating the investigations which comprise the screening protocol.

3.1.2 Identification and referral of participants

Families which may be suitable for recruitment to the EUROPAC FPC and HP registries were identified through self-referral by an individual, or referral by a collaborating clinician. In the case of the former, individuals may be alerted to the possibility of an underlying inherited increased risk for the development of pancreatic cancer within their family and seek further information and advice. EUROPAC operates and maintains a website (www.europac-org.eu), which individuals concerned about their risk may refer to. The website contains information regarding the FPC and HP registries, and contact information. We receive enquiries via email and telephone from individuals concerned about their pancreatic cancer risk seeking advice.

EUROPAC works closely with Clinical Genetics Departments and Hepatobiliary physicians and surgeons across the UK and Europe. Individuals seeking advice regarding the possibility of a familial predisposition to pancreatic cancer may often be referred to a local Clinical Geneticist for advice by their primary care physician. The Clinical Geneticist may then refer the individual to EUROPAC for further advice, and possible inclusion on the FPC or HP

registries. In this case, it was usual to receive a letter of referral from the clinician involved which indicates that the individual has consented to receive the relevant registry participant information sheet. This was then duly sent to the potential participant for consideration prior to completion of the registry questionnaires and consent.

EUROPAC maintains links with Pancreatic Cancer UK and the Pancreatic Cancer Research Fund and attends scientific meetings across the UK and Europe. This provides education and awareness of the registries to both patient, and professional, groups.

Individuals with pancreatitis or pancreatic cancer suitable for inclusion on the HP or FPC registries were regularly reviewed in the regional Hepatopancreatobiliary specialist clinic. The clinicians who form the Professorial Hepatopancreatobiliary unit were all involved in recruitment to EUROPAC in the identification and education of participants. I was responsible for liaising with interested eligible individuals and orchestrated their clinic attendance and consent prior to registration.

In addition to the active and passive method of recruitment discussed, EUROPAC works alongside further studies in the recruitment of eligible individuals and families. This process is reciprocal and applies to a number of studies currently being undertaken within the Professorial Hepatopancreatobiliary unit at the Royal Liverpool University Hospital National Health Service (NHS) Trust. I worked in liaison with other members of the research team in this recruitment process.

3.1.3 Data acquisition

The EUROPAC HP and FPC registries acquire information from registering eligible individuals via the use of ethically approved personal and family history questionnaires. The “EUROPAC

Study Questionnaire of Familial Pancreatic Cancer (FPC)/ Hereditary Pancreatitis (HP)" contains questions relating to personal identifiable information, such as full name, address and contact details. This initial demographics section also includes questions about GP contact details, occupation, brief educational background, ethnicity, religious origin, height and weight. The questionnaire also comprises questions relating to exposure to specific risk factors which may be implicated in risk stratification in FPC and HP. The "Smoking" section of the questionnaire asks for the participant to indicate whether they are a current, ex or never smoker and to provide quantitative information regarding their exposure. Similar information was sought for alcohol intake. Dietary factors were quantified to some degree by the inclusion of questions relating to the average weekly consumption of meat, fish, fresh fruit, greasy or fried food and dairy products.

The registering individual was also asked to provide information regarding any history of specific medical conditions which may be relevant; such as cystic fibrosis, diabetes, pancreatitis and other specified intra-abdominal conditions. A further section of the questionnaire asked participants to provide information regarding their past surgical history. The information received from the questionnaires was entered into the high risk database under the corresponding fields described above. I was responsible for issuing the appropriate questionnaires prior to registration and in the collection and entry of data into the databases described below.

Confirmation of the pancreatic, and significant other, cancer diagnoses within each potential FPC family being considered for addition to the registry was sought via a variety of methods. Histological reports concerning each of the affected family members were sought, where possible, through liaison with clinicians and hospital trusts involved in the provision

of their care. In the absence of histological confirmation, information regarding an affected family member's diagnosis was sought from the regional Cancer Registry organisation. This requires consent to be provided by the registering individual. This consent was indicated on a specific form which requests details of the deceased family member, in order for us to approach the relevant Cancer Registry for information. Finally, in the event that such information was not available, or the date of death of an affected relative predates the registration of all known cancer deaths, then a copy of their death certificate was sought from the registering individual.

In addition to the personal and family history questionnaires detailed above, each participant was required to complete a consent form which contains clauses relating to the holding and use of their data for research purposes; access to relevant medical records by the research team; storage of DNA and blood for research purposes and molecular analysis of such samples. Participants indicated their consent for each of these clauses and contributed a voluntary blood sample at their discretion. The provision of a blood sample for research purposes was not a prerequisite for inclusion on the respective registry. Working alongside the EUROPAC database co-ordinator, I was responsible for ensuring appropriate informed consent was obtained and that diagnoses of affected individuals within the kindred was confirmed prior to inclusion on the EUROPAC registry.

In the event of a participant consenting to provide a blood sample for research purposes, this sample was processed for two purposes. An EDTA sample was processed for purposes of DNA storage within the Regional Molecular Genetics Laboratory based at the Liverpool Women's Hospital. A second sample obtained within a sodium-heparin vial underwent lymphocyte preparation for cell line storage in liquid nitrogen at the Division of Surgery and

Oncology at the University of Liverpool. I was responsible for obtaining informed consent from individuals wishing to provide a blood sample for research purposes and involved in the collection and processing of such samples for storage. I worked in liaison with other members of the research team who were principally involved in the preparation and analysis of blood samples from participating individuals.

The EUROPAC registries work in collaboration with, and handle data from, The European study into Digestive Illnesses and Genetics (PanGen-EU). PanGen-EU is a large, European wide epidemiological study recruiting individuals with a diagnosis of pancreatic adenocarcinoma and matched controls. The study involved a structured questionnaire which was completed by a research interviewer by directly questioning eligible participants. The questions within the questionnaire related to epidemiological factors which may be implicated in the development of pancreatic adenocarcinoma. The questionnaire was structured and featured questions pertaining to different dietary intakes; smoking; alcohol; water consumption; exercise habits and occupations over a participant's lifetime. Samples for DNA extraction and SNP analysis were taken in parallel to the questionnaire. Buccal (salivary), urine and blood samples were collected from consenting participants. I worked in liaison with other members of the research team in the identification of individuals who were eligible for recruitment to PenGen-EU and other studies undertaken within our unit.

Data obtained from patient notes

It can be necessary to obtain specific clinical data from a participant's clinical notes and the EUROPAC registry consent includes a specific clause to this effect. Only researchers directly involved with EUROPAC, who hold either a full or honorary NHS Trust contract, were entitled to have access to such participant information.

Participation in the EUROPAC secondary screening study requires each individual's details to be entered onto the NHS trust computer system for recording of patient results and records. The NHS Trust databases involved were only accessible by individuals who held full or honorary NHS contracts, following training by the NHS Trust Computer Services department who issued access usernames and passwords to eligible researchers.

Access to the NHS Trust computerised records provided data regarding the results of screening investigations undertaken, as well as access to demographic data if required. Screening reports were included in the research records of study participants and stored within a secure locked filing cabinet within the NHS Trust building.

Data obtained as part of the screening program

Data obtained from the NHS Trust computerised records and patient notes were accessed by researchers involved in co-ordination of the secondary screening study, as detailed in the participant consent. Such data may be entered into the high risk database to the relevant fields outlined previously. It was necessary to include clinical data on the high risk database in order to track progress with each participant's involvement with the study. Clinical data, such as results from screening investigations, were held on the high risk database to allow for data analysis of the screening study.

3.2 EUROPAC Databases

Three databases have been established and are maintained as part of the EUROPAC registry. The databases each fulfil a different role in the storage of specific information regarding

family data and samples collected. These three databases will be described in detail and are referred to as:

1. The high risk database
2. The anonymous tissue database
3. The names database

In the construction and maintenance of the databases, our group apply specific principles which will be discussed in more detail. In work undertaken towards this thesis I was involved in the input of data and maintenance of the databases described below.

3.2.1 Guiding principle

It is imperative that the storage of personal and demographic data referring to individual study participants is held on secure systems to maintain confidentiality. Furthermore, in order to minimise researcher bias, it is good practice for research data to be held separately from identifiable information. Thus researchers can access a bank of research data for analysis, without unnecessary exposure to participant identifiable information.

In addition to this principle, a further key aim when considering the storage of participant information is that the systems in place for the storage of such information should be secure to ensure that access by an unauthorised individual, by accident or intent, is virtually impossible. The systems in place should act to ensure that clinical and research data cannot be linked by unauthorised individuals.

3.2.2 Definitions:

Coded:

‘Coded’ refers to data which may be traced to a specific study participant. This may include follow-up data for which referral to medical case notes may be required.

Anonymous:

‘Anonymous’ refers to data which cannot be traced to an individual study participant. One example is in the event that multiple samples were obtained with data that cannot be traced to the specific study participant and the samples were then randomised prior to storage of data. e.g. if multiple samples were obtained with data that cannot be traced back to the participant, and the samples were then randomised before storage of data.

Participant:

This refers to any individual donating a sample or information for research having given informed consent.

3.2.3 The high risk database

Information regarding those high risk individuals registered with EUROPAC was held on a Progeny database. Progeny is a family tree based database with a spreadsheet format for individual participants (individual spreadsheet), and a spreadsheet format for pedigrees (pedigree spreadsheet). The database was created to have 316 fields in the individual spreadsheet, and 30 fields in the pedigree spreadsheet.

As discussed in our guiding principles regarding the maintenance of databases; it is preferable to maintain databases as two separate entities; one containing demographic information that could be linked to a participant and the other confidential information. However, it was impractical to keep two databases of family based information

synchronised and so, under this exceptional circumstance, it was considered acceptable to have a single database with separate hierarchical password access for demographic information and clinical or research data.

Researcher access to fields was in accordance with a user hierarchy which was set at 3 levels: “Co-ordinator” (DCO), “Geneticist” (G) and “Molecular” (M). DCO is the only level able to fully access information regarding participant contact data, such as telephone number and home address. The DCO user password does not allow access to confidential research data on participants or risk estimates. G gives access to data related to familial information and clinically relevant data (risk estimates, genetic tests etc.) but, in contrast to the DCO user password, level G access does not provide any of the information which would be required to contact the specific participant. Username M gives access to all research data, but in a coded format.

The security levels necessitated some redundancy of field, in most cases redundant fields were populated from other fields (e.g. “Year of Birth” can be populated automatically from “Date of Birth”). The reason for the hierarchy was to preserve anonymity of participants from unauthorised access, and multiple passwords were not routinely held by any one individual. The level of appropriate access was determined by each research team member’s role in the addition of data. Necessary members of the research team were given access during separate sessions to different levels of the hierarchy.

Most nominal data were entered via dropdown options. Where appropriate, calculations were automatically carried out on the basis of entered data to populate other fields. One example is “BMI” which was calculated from height and weight entry, where weight in kilograms was calculated automatically if weight was entered in stones. Additional comment

fields were also available to add further information in the form of free text where appropriate.

Data entry onto the high risk Progeny database was usually via forms linked to individuals on a family tree presentation, although direct entry into spreadsheets was also possible.

Individual/Global spreadsheet fields

The entry of data into the Individual spreadsheet fields was governed by user access. As discussed above, use of the G username password allowed access to specific data which differs from that accessed via the DCO username and accompanying password. Use of the ‘All’ username allowed access to all fields encompassing the DCO, G and M passwords.

System fields

These fields were populated on the basis of the family tree and were accessible to all users. They are summarised as follows:

“Pedigree name” - this is a coded number referring to the specific family tree; “UPN” or “Unique Personal Number” – refers to a specific individual within the family tree; “Adopted” – refers to adoptive status of a registered individual; “Deceased status”; “Degree of relation” – refers to that how that specific individual was related to the proband; “Father ID”; “Folder name” – which refers to groupings of particular families with common mutations, this field does not specify the family diagnosis; “G:P” or “Generation: Position” – refers to that generation and position of the individual relative to the proband; “Gender”; “Gender Unknown”; “Global ID”; “Individual name” – refers to a code allocated to that specific family member; “Marked By”; “Mother ID”; “No Issue”; “Proband status” – referring

to whether the proband was affected or not; “SAB” or spontaneous abortion; “Twin status”; “Twin relationship”. These data fields were accessible by all users.

Personal Details

These fields were entered directly or populated on the basis of other fields, an example being the calculation of computed age from the entry of dates of birth.

All user passwords included access to information within the fields:

“Date registered; Computed Age; Age of Death; Age at last contact; Affected; Cause of death; Nationality; Other diagnosis; Individual Number; Study Number; User global ID; Year of Birth, Year of Death”.

Patient identifiable information, such as held in the forename and surname fields, was accessible only via the DCO password. Similarly, the fields entitled “Date of Birth; Date of Death; Hospital Unit Number; NHS Number” and “Tissue Database code”, which refers to the location of stored participant samples, were also accessible via the DCO access level.

Other fields such as the “age difference of affected parent; average age of affected; family number” and free text comments fields were accessible via the G and M user names.

Address

Participant identifiable contact information held within the fields of “E-mail; Telephone Number; Postcode; Street; Town” were accessible via the DCO password only.

All levels of access include more general information regarding the “City; Country; County; Family UK region; UK Region” of study participants. These specific data fields contain generic

information concerning the region of origin of a participant and their family with no inclusion of information which may result in the identification of the particular individual.

Clinician Details

Information regarding the Clinical Genetics Department involved in each family was accessed via the DCO and G user name passwords. These data include “Clinical Geneticist E-mail; Clinical Geneticist Forename; Clinical Geneticist Hospital; Clinical Geneticist Surname; Clinical Geneticist Telephone”. In certain cases, the Clinical Geneticist was also included in the Referrer fields if the family were recruited to EUROPAC as a result of their referral for inclusion on the registry.

Additional clinician details including “GP Address City; GP Address Country; GP Address County; GP Address Postcode; GP Address Street; GP Address Town; GP Surname; Ordered Referrer Surname; Referral; Referrer Address City; Referrer Address Country; Referrer Address County; Referrer Address Part 1; Referrer Address Part 2; Referrer Address Part 3; Referrer Address Post Code; Referrer Address Town; Referrer E-mail; Referrer Surname; GP Address Telephone; Month referred; Referrer Address Telephone” were accessible via the DCO user name.

Lifestyle

Information regarding risk factors such as “Alcohol; Smoking; Age started drinking (alcohol); Age started smoking; Age stopped drinking (alcohol); Age stopped smoking; Age variable for smoking; Alcohol amount; Estimated smoking amount; Estimated Stop Smoking; Pack Years; Smoking amount; Years Smoking; Lifestyle notes” (free text) were accessible by those using the G user name.

Linkage

Further characterisation of the genetic mutations associated with FPC is obviously a key area of our on-going work. In view of this, the linkage folder includes an extensive number of fields pertaining to specific genetic regions of interest, as well as mutations in *BRCA2* and other genes relating to established familial cancer syndromes. These fields were accessible to those with M level of entry to the database and are entitled: “1539 Standardised-a1; 1539 Standardised-a2; 1596 Standardised-a1; 1596 Standardised-a2; 1597 Standardised-a1; 1597 Standardised-a2; 1603 Standardised-a1; 1603 Standardised-a2; 1617 Standardised-a1; 1617 Standardised-a2; 2952 Standardised-a1; 2952 Standardised-a2; 393 Standardised-a1; 393 Standardised-a2; 413 Standardised-a1; 413 Standardised-a2; 415 Standardised-a1; 415 Standardised-a2; 1539 allele1; 1539 allele2; 1596 allele1; 1596 allele2; 1597 allele1; 1597 allele2; 1603 allele1; 1603 allele2; 1617 allele1; 1617 allele2; 2952 allele1; 2952 allele2; 393 allele1; 393 allele2; 413 allele1; 413 allele2; 415 allele1; 415 allele2; Chance of exclusion; Disease Allele; Disease Allele?; Exclusion criteria; Standardisation 377/ABI-a1; Standardisation 377/ABI-a2; Used on array; Consistent with Linkage; Hi risk allele; PPID Polymorphism; Palladin Mutation; Palladin SNP; ; 4q Class; D3S1277-a1; D3S1277-a2; D3S1289-a1; D3S1289-a2; D3S1297-a1; D3S1297-a2; D3S1304-a1; D3S1304-a2; D4S1539-a1; D4S1539-a2; D4S1596-a1; D4S1596-a2; D4S1597-a1; D4S1597-a2; D4S1603-a1; D4S1603-a2; D4S1617-a1; D4S1617-a2; D4S2952-a1; D4S2952-a2; D4S393-a1; D4S393-a2; D4S413-a1; D4S413-a2; D4S415-a1; D4S415-a2; D5S424-a1; D5S424-a2; D5S641-a1; D5S641-a2; D8S260-a1; D8S260-a2; D8S505-a1; D8S505-a2; DXS1001-a1; DXS1001-a2; DXS1047-a1; DXS1047-a2; Li's Codes; Linkage analysis; Number used in 4q; Pseudo” and “sequencer type”. In addition to the listed association loci of interest, information regarding the

presence or absence of a familial *BRCA2* Mutation, or *CDKN2A* Mutation, were also entered via the G and M levels of access.

Risk calculation

In the absence of a recognised underlying attributable mutation in FPC, the risk to individuals from each specific kindred was approximated from the following fields, which relate to risk calculation. The combination of these factors will help to determine the probability of the individual being a carrier of the familial mutation. These data fields are entitled: “Affected code; Affected Siblings; Calculate affected 1; Calculated affected 2; Difference in age with affected parent; Difference in age with sibling; High adjusted risk; High Risk; LH1 probability; LH2 probability; Low adjusted risk; Low risk; Medium risk probability; Penetrance High risk Calc; Penetrance low calc; Penetrance Not calculated; Probability sib; Probability based on cohort; Probability based on parent unadjusted; Probability by parent; Probability of being a carrier; Probability on age; Probability on age unadjusted; Probability risk adjusted; Probability sibling unadjusted; Sum of Cohort 1 adjusted risk; Unaffected Siblings”. Risk calculation in each participant was the key in determining which individuals were at highest risk and would benefit from targeted secondary screening. Information regarding these risk calculation parameters were read and entered by researchers with G user name access to the database.

1901 Census

The folder entitled 1901 census is self-explanatory, and contains the fields: “Age in 1901; City 1901; Civil Parish 1901; County 1901”. Such information could be accessed by all user names. More detailed information localising an individual to a specific location or street was

accessible only by the DCO. Such fields comprise: “1901 Ident; Town 1901 and Street in 1901”.

Details of cancer

The DCO may enter specific data regarding an individual’s specific “Cancer Registry Number” and “Date of diagnosis of cancer”. Fields detailing “Histological confirmation; Year of diagnosis” and specific “Histological Reports” were available to all levels of access.

EUROPAC screening

After appropriate risk estimation, the decision that screening was indicated for a particular individual was made. Information regarding the process of instigating, and continued participation, in the screening study was maintained on the database. All levels of access entered data in the fields of “Interested in Screening; Referred for screening; Followed up and Date of last screen”. More specific information regarding results of screening investigations and molecular analyses was entered by the M user name under the fields listed: “Notes on screening; ERCP complication; ERCP complications Amylase; ERCP CRP Level; *K-RAS2*; *K-RAS2* in tumour; methylation (in pancreatic juice*)*; methylation in tissue; Molecular Test of Tissue; Somatic mutation results; Nature of *BRCA2* test; *CDKN2A* Mutation; *CDKN2A* Test; *TP53*; *TP53* in tumour; Peutz-Jegher Test; Type of screening; CA19-9; Date of CA19-9; Molecular Screen; Year of Latest CT; Year of Latest ERCP; Year of Latest EUS; CT; ERCP; EUS; Latest CT Report; Latest EUS report; MRCP; Hospital; Summary of Scans; Transabdominal Ultrasound”.

Non-clinical details regarding the screening clinicians involved in delivering a secondary screening study participant's care i.e. "Radiologist Forename; Radiologist Surname" were entered by the DCO.

Family history

All levels of access to the Progeny high risk database allowed the fields of "Age of affected parent; Age of oldest affected sibling; Age of Parent; Age of parent 2" and "Transmission" to be read and entered. Access to clinical information and further risk stratification information within the fields "Known causes of death; Anticipation; Bias in transmission; Calculate affected; Cohort Individual; Cohort Parent; Generations; Li Hsu Generation; New Average age; Sum of cohort based probability; Sum of maternal; Sum of paternal; Total FPC; Coeliac Disease; Crohns Disease; Total Diabetes; Total gastric; Total other cancers; Total pancreas related; Year of diabetes diagnosed; Parents with data; Penetrance; Siblings with data; Family Diagnosis; Proband affected" and "Risk Category" was withheld from those with DCO and M levels of access and was only accessible via the G user name.

Samples

As part of the registration process, as detailed above; each registering individual was asked to provide a voluntary blood sample for research purposes and DNA storage. These data were entered into the appropriate fields on the Progeny website via the M user name. The specific fields comprise: "DNA Stored; Location of additional Sample; Location of Blood; Location of Blood DNA; Location of Cell Line; Location of Tissue Block; Type of tissue; Any other samples; Blood DNA; Blood Sample; Cell Line; Date when sample was taken; DNA from Tissue; Number of samples" and "Tissue Block". This enabled the location all of the samples

taken from a study participant and stored in the University of Liverpool Division of Surgery and Oncology freezer and liquid nitrogen facilities to be accurately recorded, and hence obtained readily for linkage and association analyses where appropriate.

Weight and Height

Clinical information regarding a study participant's weight and height were recorded in the fields of "BMI; Computed Kg; Computed Metres; Feet; Height in m; Inches; Pounds; Stones; Weight in Kg; Obesity" and "Record of weight changes". As these data are clinical and may relate to risk they were accessible via the G user password.

Actions required

The fields detailing specific actions to be undertaken relate to the logistical process of recruiting an individual who has expressed an interest in the registry and was suitable for inclusion. In view of this, the DCO entered information under the fields "Date action required; Date (registration) pack sent; Description of action; DNA needed" and "Pack details", as all of these parameters do not contain any clinical information but provided an overview of the participant's progress in the registration process.

Health

The clinical data fields relating to a study participant's relevant personal medical history were entered by the G user name. The following parameters were included and represent medical conditions which may affect risk for cancer development, specifically: "Age diabetes diagnosed; Date diabetes diagnosed; Diabetes; General Health Notes; Neurofibromatosis; Other Cancers; Pancreas related cancer; Pancreatitis; Type of Diabetes; Operation".

Participant consent

Recording of each study participant's informed consent for their data to be included on the EUROPAC registry was obviously of great importance. In view of this, data regarding appropriate consent was included in the fields "Consent for access to Information; Consent for blood sample; Consent for Deceased Relatives Notes and Consent for Deceased Relatives Tissue", which was accessible by all users.

Pedigree spreadsheet fields

System fields

In addition to the coded data relating to each individual from a registered family, the system fields folder within the pedigree spreadsheet fields details the coded name, or Pedigree Number, given to the pedigree, as well as the grouping to which the family belong. This grouping is referred to as the “Folder” to which the family belongs and did not provide specific details of a family’s diagnosis.

Statistical analysis

Within the fields included in the statistical analysis folder, data concerning risk stratification in a family was held. For example, the phenomenon of anticipation has been described in FPC and “Average Anticipation; Average age of onset; Consistent with anticipation” were specific fields accessible via the G user name along with “Transmission characteristic” and “Descriptive Statistics”. All of these fields were utilised in risk calculation when considering screening in an unaffected FPC family member.

Family details

The G user name allowed access to specific details regarding the familial diagnosis, namely, “Family Diagnosis; Family Number; Nationality; UK region; Other Cancers; Number of affected; Total Diabetes; Total other cancer types; Total Pancreatic related cancers; Notes” (which allows the addition of free text). In their primary role in the construction of the

family pedigrees, the DCO had access to indicate the status of the proband of each family via the Proband field.

Linkage fields

As described in the Individual/ Global spreadsheet fields, any association of linkage of the family to a particular loci was added in the following fields “4q32-34; Chance of Distribution (linked); Chance of Distribution (Unlinked); Chr 3 1297-1304; Chr 5 424-641; Chr 8 505-260; Chr3 1277-1289; Notes on linkage; Probability of Elimination; X Chr 1001-1047; Eliminated”. These fields were accessed via the M user name.

Clinician

Similar to the Individual/ Global spreadsheet, a specific referrer sometimes alerted a number of family members to the EUROPAC registry, and so represented the referring clinician for the family. This data was registered by the DCO under the field “Referring Clinician”.

3.2.4 The anonymous tissue database

The anonymous tissue database is a Microsoft Access database which stores pseudonymised study participant data via a series of linked tables, which refer to the location of stored samples from study participants within the Division of Surgery and Oncology, plus relevant clinical and research data. No participant identifiable data was held on this database. All study participants were pseudonymised using a specific code.

Clinical fields which were of relevance to the study are included in this database and the fields comprise: “P Number” which is the pseudonymised code allocated to each study participant; “P Link” refers to the linking “Unique Personal Number” or “UPN” field of the high risk database thus allowing participant information to be linked across the two databases; “Author” of the database entry; “Paired” includes details of the type of samples stored for a participant; more specifically, blood, pancreatic juice or tissue; furthermore the time and date of the taking and processing of the samples was also recorded. Participant identifiable information was entered into fields entitled “Hospital Number”; “Hospital (name)”; “Gender”; “Age”; “Date of birth”; “Year of birth”; as well as the date and year of entry of the participant onto the database. Details regarding the vital status were also included under the field headings “Alive/ Deceased”; “Date of Death”; “Year of death”. Last contact and event time data were also entered under similar parameters. Clinical information was entered within the fields “Diagnosis” and “Aetiology”. Furthermore, details regarding the diagnosis of specific relevant conditions, notably diabetes, malabsorption and pancreatitis were entered into fields including “Date of Diagnosis”; “Year of Diagnosis” and “Treatment” (specific to the relevant condition). Exposure to risk factors which are implicated in pancreatic disease were entered under the following field headings “Smoking

Y/N/Ex"; "Alcohol Y/N/Ex", along with quantitative data fields detailing lifetime exposure i.e. "Age started/ stopped"; "Light/Moderate/Severe" intake/ exposure. Information regarding the surgical history of each participant, where relevant, was also entered onto the anonymous database. Clinical parameters including blood test results for pancreatic and other tumour markers, in addition to other specified tests were also entered under the corresponding fields where appropriate. In addition to blood test results, different imaging modalities were recorded on the anonymous database, under the appropriate field headings "Transabdominal ultrasound"; "Laparoscopic ultrasound"; "Magnetic Resonance Imaging"; "Endoscopic ultrasound"; "Endoscopic retrograde cholangiopancreatography (ERCP)" along with the corresponding dates of each investigation. In the event of a participant involved in a specific cancer related or chemotherapy trial being added to the database, fields such as "Chemotherapy" and specific trial information was recorded along with any histological reports and prognostic information following surgical intervention.

One of the most important aspects of the anonymous database was in the recording of data relating to the location of participant samples within the Division of Surgery and Oncology at the University of Liverpool. The "P number" allocated to each participant on the "Names" database was applied to all samples derived from that individual. The appropriate location within the freezer room or liquid nitrogen storage facilities was entered for the corresponding "P Number" under the fields "Urine"; "Serum"; "Plasma"; "Cell Line"; "Pancreatic juice"; "Duodenal juice"; "Bile". Similarly, any tissue samples obtained from resected specimens, for example, normal pancreas, pancreatic tumour, duodenum, common bile duct etc had their locations entered. In certain cases, the method of collection of samples was recorded. In the case of pancreatic juice, the method of collection i.e. via

ERCP or per operatively, holds significance, as the presence of radiological contrast or blood within the sample influenced subsequent DNA extraction or analysis. The dates of all samples collected were entered accordingly.

3.2.5 The names database

The "Names" database is a separate Microsoft Access database, which is comprised of a single table, which records the allocated pseudonymised code with identifiable participant information. In view of the sensitive nature of the data held, the "Names" database is held on a secure, password protected disk which is stored in a locked fireproof safe. The fields incorporated in the "Names" database comprise "Surname"; "Forename"; "Hospital Number"; "Gender";"Date of birth"; "Year of birth"; "Age"; "Date of registration", "Year of registration"; "Last contact"; "Referring clinician"; "Consent taken by" to include the name of the clinician who consented the patient for inclusion on the database and taking of any samples for storage; "Code for consent form" refers to the version code, number and date of the consent form completed by the participant and finally; "P Link" refers to whether an individual included on the database has a registration code for the high risk Progeny database, more specifically, this refers to the "Unique Personal Number" or "UPN" entered in the "System Fields" folder of the Individual/ Global spreadsheet on the high risk Progeny database discussed earlier.

3.2.6 Statistical analysis

Data from our databases, follow-up questionnaires and clinical tests were analysed using a variety of statistical methods. Descriptive statistics, including graphical methods, were used

to determine the distribution of the data. From this information, summary measures of the data can be determined for use in further analyses. Continuous and categorical data were collected as part of the registry and secondary screening studies and were analysed using parametric tests, for example, t-test and paired t-test, or, in the case of data which was not normally distributed, non-parametric tests such as chi-squared (χ^2) or Fisher's exact test.

Regression describes the relationship between two random variables. Linear regression was undertaken to analyse the relationship between one continuous response and one explanatory variable. Linear regression can only be applied to data which appears to follow a linear trend. In the case of there being multiple explanatory variables for a response, multiple linear regression techniques may be employed after identification of the most significant explanatory variables using simple linear regression methods.

A logistic regression method of analysis is used more commonly in survival analysis as it pertains to the (log) odds or probability of an event (response variable), commonly, death, occurring. Survival analysis, or 'time-to-event' analysis, methods were used in the analysis of follow-up data. In this type of analysis, the response variable was the time from a specific trigger to subsequent event. For example, time interval between exposure versus non-exposure to a specific risk factor, and subsequent death. A Kaplan Meier curve was used to give an estimate of the proportion of individuals still alive at different points in the study.

In order to gain a better understanding of the relationship between multiple specific variables of interest upon survival, Cox's proportional hazards model was used. This model can adjust for the confounding effect of extrinsic variables upon the explanatory variables included in the analysis.

In the analysis of sensitivity and specificity of, for example, a specific screening method, receiver operator characteristic (ROC) curves were used. ROC curves plot sensitivity i.e. number of true positives (x axis) versus 1-specificity i.e. false positives (y axis). This allows for the calculation of the area under the curve (AUC) which can be used as a comparator between different diagnostic tests.

3.2.7 Sample collection and processing

In addition to the acquisition of data from participants at the time of registration via the questionnaires discussed above; EUROPAC requests the voluntary provision of blood for the purposes of DNA storage and analysis. This was undertaken in conjunction with the Regional Genetics Service at the Liverpool Women's Hospital where storage of participant samples was undertaken on an anonymised basis. I consented individuals prior to research samples being obtained and was involved in taking, preparing and storing such samples. Upon completion of this process, samples taken were recorded on the databases described above.

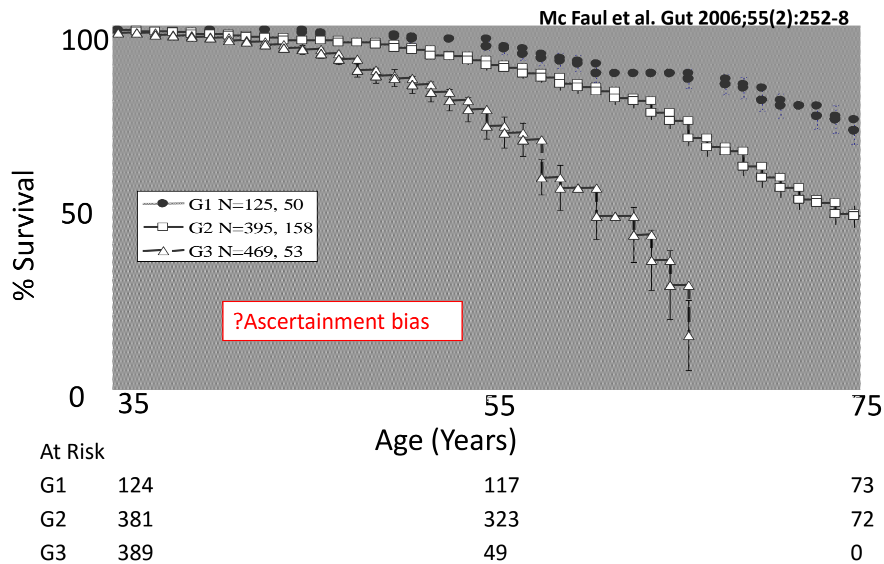
3.3 EUROPAC secondary screening study for early pancreatic cancer in FPC and HP

Upon registration with EUROPAC, eligible participants were offered information regarding participation in the secondary screening study for the detection of PDAC. I counselled and consented all individuals prior to their registration. Participation in the secondary screening study was offered to those who fulfilled criteria as high risk, following registration with EUROPAC as outlined above. Those individuals who wished to be considered for screening were invited to attend our specialist Hepatobiliary clinic to discuss the protocol in detail prior to participation. Secondary screening was offered from the age of 40 in most circumstances. However, previous work by EUROPAC has established the phenomenon of

anticipation in FPC i.e. the development of PDAC at progressively younger ages in successive generations [158]. Hence, eligibility for participation in the secondary screening study was considered below the age of 40 in kindreds demonstrating a strong trend of anticipation. I arranged attendance at Hepatobiliary clinic for counselling of all individuals prior to obtaining informed consent for participation in the secondary screening study. Following an individual's inclusion in the study I then instigated their relevant screening investigations according to the EUROPAC secondary screening protocol. The protocol is outlined later in this work.

3.3.1 Phenomenon of anticipation in FPC

Evidence from EUROPAC data has found that the onset of disease occurs a median of 10 years earlier in successive generations [158]. On initial inspection, it may appear that such a phenomenon could be attributable to ascertainment bias. However, statistical correction of such bias has been undertaken via the equation outlined in Figure 2. This equation decrees that no anticipation can predict cancer death in second and third generation FPC kindreds from survival data in the first generation. Figure 2 demonstrates low risk calculation regression analysis of uncensored Kaplan Meier survival in first generation of FPC kindreds and risk in successive second (G2) and third generations (G3) [158].



Equations used to calculate Estimate (E)	G1 80* Families			G2 106* Families			G3 101* Families		
	O	E	*p*	O	E	*p*	O	E	*p*
Low Risk $E = \sum Pr. (1 - ((Age \times 0.03) - 1.2)$ (For Age=55 to 90) + $\sum Pr$ (for over 90)	0.63	0.65	0.374	1.52	0.82	$<10^{-6}$	0.5	0.09	$<10^{-6}$
High Risk $E = \sum Pr. (1 - e^{\ln(Age)x5.35 - 23})$ (For Age = 40 to 75) + $\sum Pr$ (for over 75)	0.63	1.14	0.003	1.52	1.32	0.417	0.5	0.13	0.028

Results of iterative simulation to predict cancer deaths in G1, G2 and G3 individuals

Figure 2: The phenomenon of anticipation in FPC

Kaplan Meier survival analysis of cohorts of EUROPAC FPC individuals from different generations and dates of birth. The numbers below the graph show the number of family members from each group at each time point who were considered in the survival analysis. It can be seen that age of death of individuals was significantly different between generations G1, G2 and G3 (989 at risk, 261 affected; $p>0.001$) [158]. The Low Risk equation is calculated from survival data in G1. Application of the Low Risk equation to G2 and G3 demonstrates a significant increase in the observed rate of PDAC versus the expected according to the survival seen in G1 ($p<10^{-6}$). The High Risk equation is derived from survival data as seen in G3. Application of this equation to individuals in G1 demonstrates a significant reduction in observed versus expected survival ($p=0.003$) which is the reverse of the trend seen in G3.

3.3.2 Positive family history risk and false positive rate – need for further stratification of risk.

EUROPAC currently offers screening on a research basis to those high risk individuals with a defined genetic predisposition who meet certain eligibility criteria. Those who may be

eligible to participate include unaffected members of FPC kindreds, those from families with recognised high risk cancer syndromes and affected HP patients with p.R122H/C, p.N29I/T and p.A16V mutations of their *PRSS1* gene, or those with an apparent autosomal dominant mode of inheritance of idiopathic pancreatitis, even in the absence of a known *PRSS1* mutation. Secondary screening for pancreatic cancer is offered on a research basis to high risk individuals by a number of groups across Europe and the US. Within these populations, defined as having a genetic predisposition to pancreatic cancer, further risk stratification is required. FPC individuals are at a significantly elevated risk for the development of pancreatic adenocarcinoma as demonstrated by the earlier comparison with SEER data, and so provide a potentially attractive screening group. However, further stratification of risk is required before consideration of screening. If all those individuals in the UK with one affected FDR were to be screened, then this would identify a cohort numbering approximately 200,000 (Table 1). This figure was derived from the probability of having a FDR who has died from pancreatic cancer multiplied by the number of people within the UK population aged over 40 (approximately 30 million). The probability of having a FDR who has died from pancreatic cancer can be estimated by multiplying the average number of deceased FDR an individual in the UK has, by the proportion of deaths attributable to pancreatic cancer. This calculation was used to determine the 200,000 cohort. If a 98% sensitive and specific screening test were to be applied to this group, although 686 of the 700 expected cases would be positively identified, there would also be a high number of false positives wrongly detected, approximately 4000. In a worked example assuming a 0.1% risk of pancreatic cancer within a population, application of a screening test with 98% sensitivity and specificity to a cohort of 1000 participants would identify one true positive cancer case. However, in assuming a 0.1% risk in this population and applying a 98% specific

test, although 980 true negatives would be correctly excluded, 20 false positives would be wrongly identified as having cancer. It would therefore be unethical to apply such screening in a population with such low risk, as in the detection of one true cancer case, 20 people would be wrongly diagnosed and potentially harmed through unnecessary treatment. In a second example considering a population of 1000 participants at 10% risk for the development of cancer, assuming a 98% sensitive and specific test, 98 individuals would be correctly diagnosed with cancer. In the same test, one would expect to positively exclude 882 individuals with a false positive result in 18. This equates to 98 individuals being correctly diagnosed with 18 participants being wrongly diagnosed as having cancer. Although the risk of 10% in this theoretical screening population is elevated compared to that seen in our screening cohort, the anticipated risk of pancreatic cancer development falls between these two worked examples, and such risk should be stratified with the aim of yielding maximal benefit to those at highest risk whilst minimising harm to those who are at lowest risk for the development of pancreatic cancer. In view of this, the current screening criteria for participation in the EUROPAC secondary screening study are individuals over the age of 40 from FPC kindreds with confirmation of two or more affected relatives. Despite this, in the absence of any genetic testing for the, as yet unknown mutation responsible for FPC, any screening programme may expect to identify a number of false positives, particularly when only 50% of individuals will be expected to carry the mutation. In order to gain higher specificity rates from screening, further aetiological factors which may influence the penetrance of pancreatic cancer in genetically predisposed individuals should be identified and incorporated into the risk stratification process.

3.3.3 Risk stratification model and clinical interface

We can use the phenomenon of anticipation and other risk factors in a model for risk stratification in FPC kindreds. My thesis centred on determining factors which influence the age dependent risk. The combination of this risk which was determined by age, anticipation and various environmental and disease exposures can be combined with estimation that the family was a true FPC family, and that the individual was a carrier of the mutation concerned, to determine the probability that the individual will develop cancer. Obviously this approach to screening will change in the advent of further characterisation of the underlying genetic alteration. Previous work undertaken by our group formulated the risk stratification interface as outlined in Figure 3. Risk stratification through determination of the probability of the family as a true FPC kindred and the individual as a carrier, along with the age dependent risk, allowing for the phenomenon of anticipation, will enable clinicians to target screening to those at highest risk. The principle of the risk stratification model is outlined in Figure 3. Work undertaken towards this thesis has specifically aimed to quantify age dependent risk, according to lifestyle factors, which influence penetrance in FPC, so contributing to the risk model and targeted screening of those at highest risk.

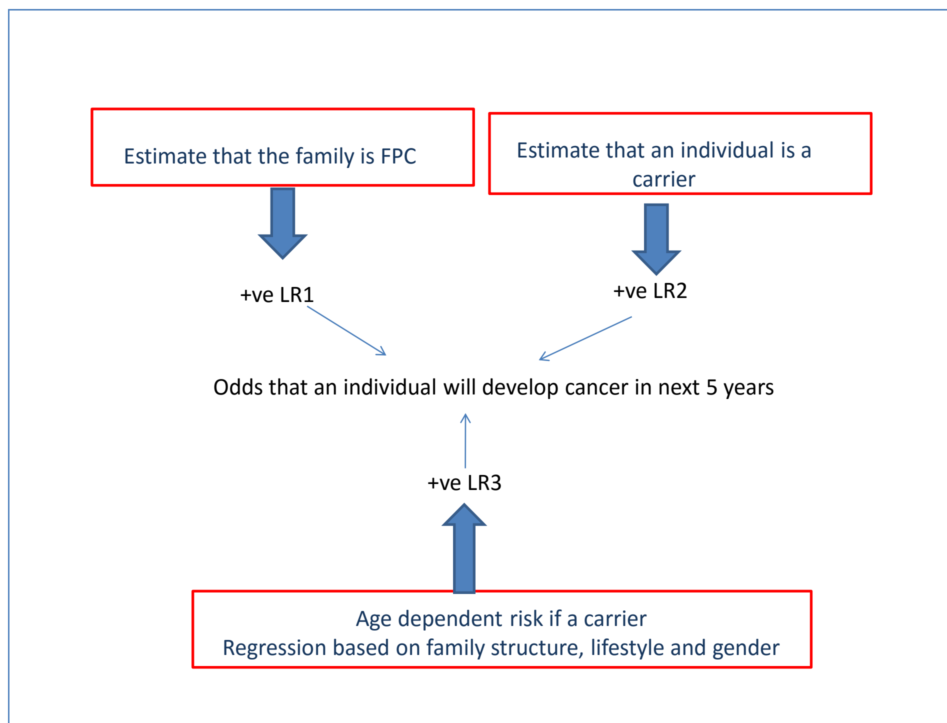


Figure 3 EUROPAC risk stratification model
The model assumes that individuals can be put into groups with a mean time to event determined by a selection of critical co-variates. The co-variates examined included gender, smoking, age and parental history of cancer.

3.3.4 Overview of EUROPAC screening protocol

EUROPAC, along with other registries worldwide have developed multi-modality screening protocols. The screening protocols adopted by other registries have been summarised earlier in this thesis. Throughout the period of this work, the screening programme evolved in various ways, partly as a direct result of the data presented later, and partly due to evaluation of data from other centres. The secondary screening protocol, as employed in 2010 is outlined in Figure 4. A chief part of the work towards this thesis was centred upon the recruitment of individuals to the secondary screening study and orchestration of screening investigations according to our protocol. Data collected from our screening cohort will be presented later. A significant part of this work was in the amendment of the EUROPAC screening protocol with the aim of improved participant safety as stated in my thesis aims and objectives. The protocol comprises a combination of blood tests, imaging

modalities and molecular analyses. Initial baseline fasting glucose and CA19.9 were measured and repeated on an annual basis. A 5 millilitre (ml) serum sample was obtained from our screening participants for measurement of CA19.9. In the EUROPAC secondary screening study for early pancreatic cancer, plasma fasting glucose, measured in mmol/l, was used as a screening tool for diabetes mellitus. Plasma analysis of fasting glucose required the patient to have fasted for a minimum of 8 hours. The sample was taken using a EDTA tube and 3ml volume was required for the analysis. The reference range, as stated by the Royal Liverpool University Hospital Trust Department of Biochemistry, was 3.5-6.0 mmol/l. This reference range may vary between groups. Alternative plasma glucose measurements were utilised by other screening centres as previously discussed.

Baseline imaging of the pancreas was sought via a single CT scan upon entry into the screening program. In addition, EUS was undertaken at baseline and on a repeated basis.

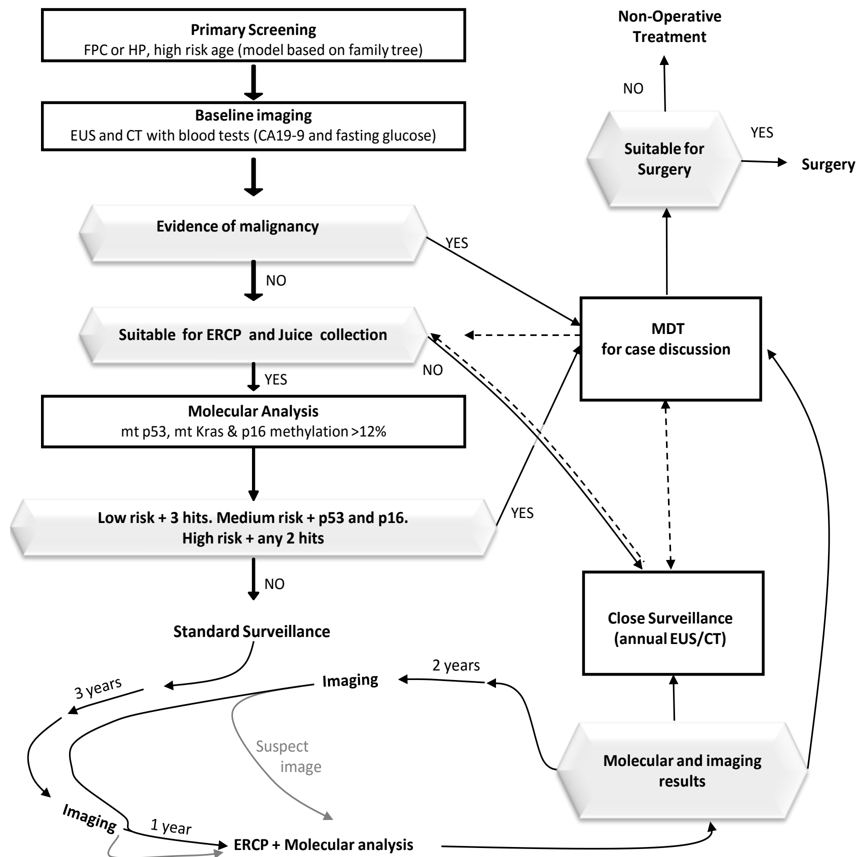


Figure 4: EUROPAC secondary screening protocol

Schematic representation of the EUROPAC secondary screening protocol incorporating primary screening, baseline imaging, pancreatic juice collection for molecular analysis and resultant risk-dependent screening schedule.

3.3.5 ERCP and pancreatic juice collection

In addition to the blood tests and imaging modalities described, EUROPAC undertook ERCP and pancreatic juice collection for analysis of molecular markers. These molecular markers, namely, *K-RAS2* mutations, *CDKN2A* methylation and *TP53* mutations aid in risk stratification and identification of pre-cancerous, or early malignant, lesions. The role of *K-RAS2*, *CDKN2A* and *TP53* in pancreatic carcinogenesis has been described earlier. I was responsible for collection, preparation and storage of juice samples for further analysis by another member of our team.

ERCP procedures were undertaken on a day case basis for high risk individuals participating in the EUROPAC secondary screening study. All procedures were undertaken by a Consultant Endoscopist under sedation with 1-5mg intravenous Midazolam plus an opiate analgesic, Fentanyl 50-100mg or Pethidine 50mg, dependent upon endoscopist preference, and an anti-peristaltic agent. Selective cannulation of the pancreatic duct, in order to aspirate pancreatic juice for analysis, was confirmed by fluoroscopy prior to aspiration. Pancreatic juice aspiration was then undertaken following the administration of intravenous secretin, this aspect of the screening study will be discussed in more detail in a subsequent sub-chapter. A maximum of 10 ml of pancreatic juice was then aspirated for analysis. Upon completion of the procedure, the endoscopist deployed a 5 French gauge stent. The stent utilised was plastic and self-expelling. Follow-up abdominal x-ray was undertaken at 6 weeks to ensure expulsion of the stent. In addition to the deployment of prophylactic pancreatic stents at ERCP, participants also received 50mg of the anti-inflammatory Diclofenac per rectally within 30 minutes of completion of the procedure. The use of prophylactic stents and per rectal Diclofenac was instigated following an amendment to the EUROPAC screening protocol undertaken by me with the aim of improved participant safety.

Molecular analysis of pancreatic juice

The use of molecular techniques for the detection of key mutations in pancreatic carcinogenesis is an attractive strategy for screening patients at risk of pancreatic cancer. Following my collection, preparation and storage of pancreatic juice samples, another member of the research team undertook analyses according to the standard operating procedure described later in this chapter. Point mutations of *K-RAS2* occur in around 80% of

tissue samples taken from sporadic PDAC [226]. *K-RAS2* oncogene activation appears to occur in preinvasive pancreatic cancer [227] and is also mutated in various types of ductal hyperplasia [226-228]. *K-RAS2* mutations have been detected in pancreatic juice samples of patients with pancreatic cancer with a sensitivity of between 67%-100% [229, 230]. They have also been detected in patients with chronic pancreatitis, and in patients with normal pancreata [231-234]. This suggests that *K-RAS2* is a sensitive marker for the presence of malignancy, but lacks specificity, particularly in the presence of chronic pancreatitis.

The tumour suppressor gene *CDKN2A* is deleted or mutated in 85% of tissue samples from PDAC [131, 235]. A subset of FAMMM kindreds that carry *CDKN2A* mutations, have a significantly increased risk of pancreatic cancer [236]. Aberrant methylation of cytosine residues in the promoter region of *CDKN2A* is an important mechanism of gene inactivation in the molecular pathogenesis of pancreatic cancer [237, 238]. Detection of *CDKN2A* promoter CpG island methylation has been examined as a screening modality for PDAC [239-241] but only on the basis of detection or non-detection. Reported sensitivity was low (<20% to 40%), and although specificity was reported to be high in pancreatic juice, this could have been because of the low sensitivity of the test, and the authors confirm the presence of methylated sequences in normal tissue. In the analysis of *CDKN2A* methylation utilised in the EUROPAC secondary screening study, we used a sensitive polymerase chain reaction (PCR) modality combined with quantification to improve specificity.

TP53 gene mutations occur in approximately 50% of pancreatic cancers [242] and mutations have also been detected in pancreatic juice using a technique called single stranded conformation polymorphism (SSCP). Of 26 patients with PDAC, 11 (42%) carried a *TP53* mutation, whilst none of the 16 patients with chronic pancreatitis had a detectable *TP53* mutation [243]. It is believed that *TP53* mutations are more likely to arise relatively late

during pancreatic carcinogenesis and are rare in chronic pancreatitis [243, 244]. The presence of mutant *TP53* in combination with *K-RAS2* mutations in pancreatic juice has also been described. In their analysis, Wang *et al* recorded *TP53* and *K-RAS2* mutations in pancreatic juice in 42.9% (9 of 21) and 81.0% (17 of 21) of cancer cases respectively. No *TP53* mutations were seen in 25 patients with chronic pancreatitis, whilst *K-RAS2* mutations were detected at an incidence of 28% (7 of 25)[245].

The specificity (percentage of chronic pancreatitis controls or benign biliary disease controls correctly identified by each of the three screening tests) and sensitivity (percentage of pancreatic cancer correctly identified by the screening tests) of the molecular tests have already been evaluated. Mutations of *TP53* were detected in 20 of 48 cases (42%) of PDAC, none in 49 biliary controls and 2 (4%) in 49 patients with chronic pancreatitis. *K-RAS2* mutations were seen in 31/57 (54%) cancer patients, but also in 13/61 (21%) biliary controls, and 23/67 (34%) with chronic pancreatitis. 26 out of 42 (62%) cancer patients had *CDKN2A* promoter methylation levels >12% compared to 1/22 (5%) biliary controls and 2/20 (10%) individuals with chronic pancreatitis. The specificities and sensitivities based on these results are given in Table 2 below. Combination of *TP53* results with *CDKN2A* promoter methylation increased the discrimination between patients with malignant and benign disease of the pancreas: 27 (79%) out of 34 cancer patients had a *TP53* mutation or high level *CDKN2A* promoter methylation compared to 1/18 (6%) controls and (28%) 5/18 with chronic pancreatitis. The analysis of molecular markers in pancreatic juice, as undertaken

by our group, is summarised by the Bayesian approach depicted below [121]

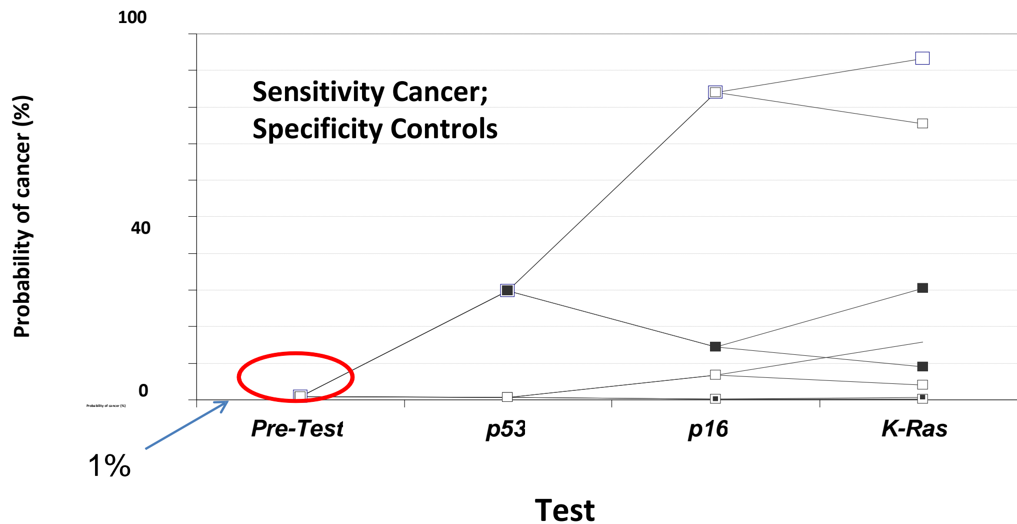


Figure 5: Bayesian model for molecular screening of pancreatic juice

Molecular analyses (p16 methylation, K-Ras mutation and p53 mutation) were carried out on pancreatic juice DNA from patients recruited with PDAC and benign biliary disease. This allowed a calculation of sensitivity and specificity for each marker (for detection of PDAC). Each marker was tested in each group for independence and on the basis of independence a Bayesian approach was adopted. Briefly, odds and likelihood ratios of having cancer were calculated on the basis of the specificities and sensitivities for each marker and the likelihood ratios used sequentially to calculate probability of cancer (the combination obviously having the same value in whichever order the markers are used). The data is also presented in table 2 below [121].

Table 2: Sensitivity and specificity of analysis of TP53, K-RAS2 and CDKN2A methylation in pancreatic juice [121]

	TP53	***K-RAS2***	***CDKN2A* methylation <12%**
Specificity vs pancreatitis (%)	96 (86-99)	66 (54-76)	90 (70-97)
Specificity vs controls (%)	100 (93-100)	79 (67-87)	95 (78-99)
Sensitivity (%)	42 (28-56)	54 (42-68)	62 (47-75)

3.3.6 Preparation of pancreatic juice aspirate samples taken at ERCP prior to DNA extraction

A maximum of 10ml of pancreatic juice was taken during cannulation of the pancreatic duct at ERCP. Within two hours of collection, the chilled juice was transferred to a sterile 1.5ml Eppendorf tube and spun at 4000g for ten minutes. The supernatant was aspirated and immediately stored at –80ºC. I undertook this process of collection, preparation and storage of pancreatic juice samples prior to further analyses by another member of our research team according to the standard operating procedure outlined below.

Pancreatic juice aspirate DNA extraction

To 200 microlitres (µl) of pancreatic juice aspirate, equal volumes of sterile water and Phenol: Chloroform: Isoamyl Alcohol 25:24:1 were added. The aqueous and organic phases were separated by centrifugation. DNA was precipitated overnight at 4ºC using 2.5 volumes of 100% ethanol and 20µl 3M sodium acetate pH 6.8. The DNA was dissolved in 200µl of molecular grade water.

Functional assay of TP53

The *TP53* assay is based on the principles of a yeast functional assay system designed by Flaman et al. [246]. The most important modification was the use of genomic DNA, rather than RNA. Exons five to eight, which contain the mutation hotspots 42, were chosen, as these exons code for the DNA binding domain of the *TP53* protein.

Polymerase Chain Reaction (PCR) Amplification and linkage of TP53 exons 5,6 and 7

Genomic DNA was amplified by PCR; the conditions were 95ºC for 12 minutes, followed by 40 cycles of 94ºC for 30 seconds, 60ºC for 30 seconds and 72ºC for 1 minute. The PCR mixture contained 0.25 units of pfu DNA polymerase, 1x pfu buffer (Promega) with 2mM Magnesium Sulphate, 20 µmol of each primer and 0.2 mm dntps. Primers were designed to allow PCR linkage of exons 5-6 and exons 7-8 and then to link 6 to 7, where possible the overlapping primers were used to amplify the exons directly from genomic DNA, otherwise an external (intronic) primer was used to give product followed by a second nested PCR.

Strains, vector, media and yeast functional assay

The reporter yeast strain yig-397 was co-transformed with the PCR product of *TP53* exons 5 to 8 and a circular expression vector carrying full length *TP53* (pls76). The vector *TP53* sequences were replaced by PCR amplified sequence in vivo by homologous recombination. Wild type *TP53* allows expression of ADE2 and consequently white yeast colonies, whilst mutant *TP53* sequences result in no expression of ADE2 and so red colonies. White and red transformants appear following 48 hours incubation on selective plates lacking leucine and with a low adenine concentration (10mg/l). The uncut circular vector was used rather than gap repair. Although this dramatically decreases the ratio of red to white colonies, it also decreases the number of false positive colonies. Previous studies [246, 247] have shown that the *TP53* functional assay of tissue containing wild-type *TP53* gave 5-10 % red colonies with the gap repair vector. The background resulted from PCR-induced error, alternative splicing of intron 44 and self-ligated vector with gap repair 41 (this latter problem was avoided by the use of undigested vector). To identify mutations, plasmids were rescued

from three red yeast colonies by alkaline lysis and transformed into E coli DH5α and sequenced. If two sequences from different yeast carried the same *TP53* mutation the sample was classified as mutant.

Amplification Refractory Mutation System (ARMS) analysis for K-RAS2 mutations

ARMS is a mutation specific PCR assay controlled with amplification of an overlapping sequence with mutation independent primers. Real-time PCR was performed using the Roche lightcycler (Roche Diagnostics) and detection was via SYBR Green I. In practice, there was normally a degree of amplification of the wild type sequence even with mutation specific primers, hence control primers were used for comparison, that amplify wild type and mutant sequences with equal efficiency. Threshold cycles with mutant specific primers were plotted against threshold cycles using the control primers. The assay had been calibrated using varying concentrations of normal blood DNA, at least 100 pairs of PCR reactions for each mutation specific primer. This allowed 98% confidence intervals to be produced on a linear regression curve. Samples were analysed in triplicate for each *K-RAS2* mutation, and threshold cycles plotted. If all three points were below the 98% confidence limit for the regression the sample was considered to contain mutant sequences, otherwise it was classified as wild type.

Reaction conditions for ARMS

Primers specific for the six recognised point mutations at codon 12 were used (aspartate, arginine, valine, serine, alanine and cysteine). Real time PCR was performed with an initial

denaturation step of 10 minutes at 95ºc. This was followed by 60 cycles of: 2 seconds at 95ºc, 20 seconds at the annealing temperature of 61ºc, 20 seconds at 72ºc and 10 seconds at 81ºc. This was followed by a melting curve ramp from 72 to 95ºc over 10 minutes. Quantification analysis was performed on each sample using the lightcycler software (Version 5.32). Melting curve analysis was performed to verify that pure PCR products were produced.

Real-time PCR measurement of CDKN2A promoter methylation in pancreatic juice aspirate

DNA from pancreatic juice aspirate was subjected to sodium bisulfite modification as described previously [248]. Real-time PCR amplification was carried out in a 20 µl of volume with 2 µl Light Cycle DNA Mastermix, with a primer concentration of 1µm. PCR reactions were carried out on the lightcycler amplification and detection system. Pre-incubation was performed for 10 minutes at 95 ºC. DNA was amplified for 60 cycles of 20 seconds at 95ºC, 10 seconds at 67ºC for methylated primer (65ºC for unmethylated primer), and 10 seconds at 72ºC. Data were analyzed by the quantification program software. The methylation index (%) in a sample was calculated using the following equation: Methylation index = [M/(M+U)] x 100%, where 'M' is the quantity of methylated *CDKN2A* sequences measured by MSP following bisulfite conversion, and 'U' is the quantity of unmethylated *CDKN2A* sequences measured by real-time MSP following bisulfite conversion [249].

3.3.7 Reduction of ERCP pancreatitis in FPC screening using PR Diclofenac and temporary self-expelling pancreatic stents

Despite the advantages in risk stratification achieved through the collection and analysis of pancreatic juice via ERCP; cannulation of the pancreatic duct poses a significant risk of pancreatitis. The procedure associated risk of pancreatitis following ERCP varies greatly from 1% to in excess of 30% [250, 251] [252]. This risk varies in accordance with exposure to certain risk factors, which may be technical or patient derived. Young age has been consistently demonstrated to confer a significantly increased risk of the development of post-ERCP pancreatitis [251-253]. This is a risk factor which is of particular concern, as those unaffected family members belonging to confirmed FPC kindreds were invited to commence screening from the age of 40, and so fall into this category of elevated risk. In order to minimise the risk of pancreatitis in the EUROPAC screening cohort, an amendment to the protocol was undertaken as part of my work towards this thesis. The advent of the anti-inflammatory agent Diclofenac, administered per rectally, and the deployment of temporary self-expelling pancreatic stents resulted in significantly reduced ERCP pancreatitis in our screening cohort.

3.4 The use of intravenous Secretin for duodenal juice collection for analysis of molecular markers

A mechanism of collection of genetic material derived from pancreatic juice for stratification via molecular analysis; whilst avoiding pancreatic duct cannulation, and the resultant risk of pancreatitis, would be preferable to minimise risk exposure in the screening population. In view of this, the technique of juice collection has been modified with the

objective of improving safety, and reducing procedure related risks, in our screening cohort. The modification involves hormonal stimulation of secretion of pancreatic juice and collection within the duodenum (first part of the small bowel). This technique has been used successfully in the analysis of biochemical markers in chronic pancreatitis [254] and in two previous studies similar to that which was proposed i.e. analysing molecular markers for pancreatic cancer [250, 251]. This method involves the use of a synthetic gut hormone, secretin, which is administered intravenously and stimulates production and secretion of pancreatic juice. The stimulated juice is then collected within the duodenum, with the ultimate aim of avoiding cannulation of the pancreatic duct, and hence the associated risk of pancreatitis.

The techniques of ERCP and EUS are employed in those with suspected malignant or benign disease for a variety of diagnostic and therapeutic interventions. ERCP may be undertaken in patients with obstruction of the biliary tree secondary to a mass lesion in the head of the pancreas, or due to intraductal stones. In the event of a probable malignant mass affecting the head of the pancreas, stent deployment is undertaken via ERCP in patients pre-operatively or as a palliative procedure. ERCP and EUS are also undertaken to obtain samples for cytological or histological analysis of a suspected pancreatic adenocarcinoma. We recruited patients with benign or malignant disease attending for either ERCP or EUS for a prescribed clinical indication.

We have previously demonstrated that the analysis of molecular markers in pancreatic juice can enable discrimination between malignant and benign disease [121]. We have ethical approval to perform the technique of pancreatic juice collection via ERCP for molecular analyses of *K-RAS2* and *TP53* mutations plus *CDKN2A* methylation in order to stratify risk in a number of defined high risk screening populations (REC refs: 07/H1008/153 Central

Manchester REC; 07/H1211 Warwickshire REC). Similar studies have been performed analysing the sensitivity and specificity of duodenal fluid for the detection of molecular markers which may indicate early pancreatic adenocarcinoma. These studies have been undertaken in small sample groups and have yielded inconsistent results [239, 255, 256]. We aimed to evaluate the sensitivity and specificity of molecular markers within duodenal fluid as an indicator of pancreatic carcinogenesis by comparing analyses from malignant and benign patient groups. It was envisaged that the results of such analyses could be interpreted as a surrogate for the presence of molecular markers in pancreatic juice in high risk screening patients. A significant part of my work towards this thesis was in the ethical approval for a prospective cohort study for the collection and analysis of duodenal juice in the screening of high risk individuals for pancreatic cancer (REC Reference: 10/H1010/19 NRES Committee North West Haydock).

3.4.1 Historical use of Secretin for duodenal juice sampling and rational for use in screening for early pancreatic cancer

It is hypothesised that the sensitivity and specificity of molecular markers in pancreatic juice will be replicated in similar analyses of duodenal aspirate. The collection of duodenal fluid does not require cannulation of the pancreatic duct, which is the event that precipitates procedure related pancreatitis during ERCP. Thus, this technique is likely to be safer in our cohort of screening patients, provided that the sensitivity and specificity are comparable to that seen in pancreatic juice.

Post-ERCP pancreatitis may affect 2-15% of those undertaking the procedure depending upon technical and patient derived risk factors. Of particular relevance to the EUROPAC cohort of screening patients is the increased risk which has been consistently identified in

patients below the age of 50 [251, 253, 257, 258]. In a study of 2444 procedures, a large multicentre trial performed in Italy recorded a significantly increased risk of ERCP related pancreatitis in patients below the age of 60 (OR 1.53 [95% CI 1.06-2.20] p=<0.001)[258]. The reported severity of post-ERCP pancreatitis varies across different cohorts and may be classified as mild, moderate or severe. The majority of post-ERCP pancreatitis is classed as mild and characterised by the onset of abdominal pain within 24 hours following the procedure. This is associated with a threefold elevation in serum amylase; a human enzyme which exhibits grossly elevated levels in the presence of pancreatic inflammation i.e.in acute pancreatitis. In a large multicentre US trial examining outcomes in 11,497 ERCP procedures, 75% of the 304 reported cases of post procedure pancreatitis were mild in severity. There was an associated mortality rate of 0.06% with ERCP induced pancreatitis in this large cohort [250]. This demonstrates that, although post-ERCP pancreatitis is mild in the majority of cases, it may be a cause of significant morbidity, and in severe cases, with the associated onset of multi-organ failure, may be fatal. A number of studies have examined the influence of certain procedure and patient related risk factors in the development of post-ERCP pancreatitis. Among the factors most consistently demonstrated as conferring an increased risk of post-procedure pancreatitis is age. A number of large cohort studies have identified a significantly increased risk in individuals undertaking ERCP below the age of 50-60 years [253, 257, 258]. As EUROPAC commences screening in eligible individuals from the age of 40, and they may undertake ERCP for juice collection at a maximum of every 18 months, the evidence suggesting an increase in risk of ERCP-pancreatitis within this age group is an important consideration. It should be noted that, although the highest risk of ERCP pancreatitis is conferred through therapeutic procedures including sphincterotomy and multiple contrast injections into the pancreatic duct [250, 251, 257, 258], certain other

patient related factors bear significance when considering the risk to a screening cohort. The majority of patients involved in the EUROPAC secondary screening study for early pancreatic cancer have not undertaken any ERCP intervention prior to their participation in the screening programme. One study has identified an increased complication rate in such individuals who underwent ERCP with cannulation via a native papilla i.e. in those who have not undertaken any previous therapeutic ERCP [259]. In order to reduce the risk of procedure related pancreatitis in our screening cohort we administered a synthetic gut hormone, secretin, to enable collection of pancreatic juice within duodenal aspirate, so avoiding cannulation of the pancreatic duct and the inherent risk of procedure related pancreatitis. Secretin is a 27 amino-acid polypeptide hormone secreted in an inactive form, prosecretin, by the S cells of the duodenal mucosa in response to acidity (low pH) within the duodenal lumen. The acidic conditions which stimulate the release of prosecretin occur through the presence of gastric contents entering the duodenum during the digestive process. Secretin exerts a number of effects within the digestive process. It targets the pancreas where it stimulates the secretion of bicarbonate rich pancreatic juice from pancreatic acinar cells. The alkaline bicarbonate rich juice is secreted into the duodenum so increasing the pH within the duodenal lumen. This process serves as an aid to digestion and protective mechanism. Lowering of the intraluminal pH within the duodenum enables activation of other digestive enzymes, and buffering the acidity of the gastric contents prevents damage to the small bowel mucosa. Secretin also relaxes the sphincter of Oddi and may facilitate pancreatic duct cannulation at ERCP, as a result of widening of the orifice [260]. Secretin has other functions, in stimulating release of bile from the liver and inhibiting pepsin production from G cells within the gastric antrum, so further increasing the pH within the duodenum by reducing acid production within the stomach. Secrelux® is a purely

synthetically derived porcine secretin which is available for investigational clinical use to aid diagnosis at MRCP and ERCP in a number of clinical conditions including sphincter of Oddi dysfunction and chronic pancreatitis [254, 261]. Biologically derived porcine secretin was originally extracted by Jorpes and Mutt in 1961 [262] and has been used for clinical diagnostic purposes in the US since 1981 [260]. Research into the pharmacological efficacy of synthetically derived porcine secretin compared to a biological derivative has demonstrated identical effects [263]. The active ingredient within synthetic porcine secretin is secretin pentachloride, and the manufacturer's (Sanochemia Diagnostics International) guidelines recommend intravenous administration at a dose of 1 CU (international unit) per kilogram over a period of 1-2 minutes for juice collection in the diagnosis of exocrine pancreatic function. Previous pharmacokinetic analysis in 15 volunteers has recorded a half-life of porcine secretin of 2.84 ($\pm$ 0.62) minutes in humans [264]. From the manufacturer's data, reported undesired effects include occasional diarrhoea resulting from a frequently observed increase in secretion of pancreatic amylases, lipases and trypsin. This may be associated with abdominal pain and nausea or vomiting. Rapid infusion may result in flushing or faintness. Other reported effects include urinary urgency and a transient fall in blood glucose, which may be observed in diabetics. Synthetic secretin is licensed for patient use in a variety of clinical scenarios. Secretin stimulated EUS and MRCP are performed in individuals with recurrent pancreatitis of uncertain aetiology [265]. There is limited evidence to suggest that secretin stimulated EUS and MRCP achieve a higher diagnostic yield than ERCP in this patient group [265]. The administration of synthetic secretin at MRCP has been found to improve pancreatic duct delineation [265, 266]. This effect occurs as a result of stimulation of the exocrine pancreas to secrete bicarbonate rich fluid which causes transient dilatation of the pancreatic duct [261]. In the context of screening for early PDAC, recent

evidence has highlighted an important potential advantage in the use of intravenous secretin. A recent randomised, double-blind, placebo-controlled trail by Jowell *et al* reported a significant reduction in post-ERCP pancreatitis following the use of intravenous secretin. In their series of 889 participants, the incidence of post-ERCP pancreatitis in the secretin cohort was 8.7%, versus 15.1% in the placebo group (p=0.004).

In addition to its use in various imaging modalities, secretin is also administered to stimulate pancreatic juice secretion for collection for biochemical analyses, including bicarbonate measurement [267-269]. There is a limited body of evidence regarding molecular analysis of duodenal fluid as a surrogate marker for pancreatic juice, and its potential role in the detection of early pancreatic cancer [255, 256].

3.4.2 Molecular analysis of secretin stimulated duodenal aspirate

Secretin stimulated duodenal aspirate was taken at screening ERCP, the process of which has been described earlier in this work. Prior to cannulation of the ampulla and pancreatic duct, the endoscopist undertook suction aspiration of existing gastric debris in order to mimimise contamination of the collected duodenal sample with junk DNA. The researcher then administered secretin in accordance with the manufacturer's guidance as outlined above. The endoscopist then employed continuous aspiration of the resultant ampullary secretions for a maximal 10 minute collection period, or at such time that 10ml of aspirate was obtained. Larger volumes of duodenal aspirate were collected, compared to that of pancreatic juice, in order to maximise the diagnostic yield in subsequent molecular analyses. The endoscopist then proceeded on to cannulate the ampulla and pancreatic duct to obtain pancreatic juice samples for comparative analyses. The ERCP and recovery were then managed as previously detailed.

3.4.3 Duodenal juice analysis protocol

EUROPAC has ethical approval for the collection of pancreatic juice for the analysis of molecular markers. The collection, analysis and storage of duodenal aspirate was undertaken according to this protocol. My role was in authorship of the protocol for ethical approval and in recruitment and sample collection. Collection and analysis of *K-RAS2*, *CDKN2A* methylation and *TP53* mutation was performed in accordance with the techniques previously validated by our group and discussed earlier in this work [121].

Sample collection was undertaken in individuals attending for clinically indicated ERCP or EUS. No procedure was undertaken in the absence of clinical need for the sole objective of sample collection. The comparative sensitivity and specificity of molecular markers in duodenal aspirate versus pancreatic juice, from individuals with pancreatic cancer and chronic pancreatitis, plus a control cohort with benign biliary disease, determined the feasibility of this technique in effective screening of high risk groups. It was proposed that the collection of duodenal aspirate as a surrogate marker for pancreatic juice may enable risk stratification, while minimising the morbidity of ERCP-associated pancreatitis. The results of this analysis will be discussed later in this thesis.

4. RESULTS: Registries

4.1 FPC registry: Demographics

The EUROPAC FPC and HP registries were established in 1997 and, at the time of writing, we had recruited 1000 families who appear to exhibit an underlying genetic predisposition to the development of pancreatic adenocarcinoma. This included 401 families who were registered with the EUROPAC FPC registry. Of these, there were 273 kindreds who displayed an autosomal dominant pattern of inheritance consistent with FPC. This encompassed 16 families in whom there appeared to be an inherited predisposition to gastric cancer in conjunction with an elevated pancreatic cancer risk. Of those families demonstrating a clear autosomal pattern of inheritance, 184 kindreds had 2 affected cases and 89 had 3 or more affected family members. Table 3 provides an overview of the FPC registry during the period of work towards this thesis:

Table 3: Summary of FPC database

Table 3a: Summary of FPC database

Country	Total number of families	Family diagnosis		Family diagnosis FPC/FPC?	
				Number of affected at registration	
		FPC/FPC?	Other inc. *BRCA2*	2 affected	>2 affected
UK	327	216	111	146	70
France	22	14	8	11	3
Germany	12	11	1	9	2
Greece	6	6	0	4	2
Italy	6	5	1	2	3
Latvia	6	6	0	5	1
Hungary	4	4	0	3	1
Eire	4	1	3	0	1
Sweden	3	1	2	0	1
Belgium	3	3	0	2	1
Denmark	2	2	0	0	2
Spain	2	2	0	1	1
Netherlands	1	1	0	1	0
Portugal	1	0	1	0	0
Switzerland	1	0	1	0	0
Japan	1	1	0	0	1
TOTAL	401	273	128	184	89

Table 3b: Summary of UK FPC families
Histological confirmation includes cases where pathology reports and/or cancer registry and/or good medical records confirm the diagnosis of PDAC.

No families registered	Total no. individuals registered	Pancreatic cancer cases	Histologically confirmed cases	DNA stored		Cell line	
				Affected	Unaffected	Affected	Unaffected
390	11737	857	442	111	509	48	240

The EUROPAC FPC registry also encompasses those families with hereditary cancer syndromes which confer an increased risk for the development of pancreatic adenocarcinoma. Examples include kindreds with mutations in *BRCA2*, Peutz-Jeghers, Breast-Ovarian, FAMMM and HNPCC syndromes. A summary of families registered with specific cancer syndromes and an associated increased risk for the development of pancreatic cancer is given in Table 4.

Table 4: Summary of specific cancer syndrome families registered with EUROPAC

Encompassing families with the following diagnoses: *BRCA2* Breast Ovarian; *BRCA2* FPC; HNPCC; FAMMM; Breast Ovarian; Peutz-Jeghers; Neurofibromatosis.

No. of Families	Diagnosis
8	*BRCA2* BOv
5	*BRCA2* FPC
4	HNPCC
5	FAMMM
24	BOv
3	PJS
2	NF

4.2 HP registry – updated figures and breakdown/ demographics

At the time of completion of the work towards this thesis, the EUROPAC HP registry had 540 registered families, which represents the largest series across Europe. As described earlier, the criteria for registration as an HP kindred are either an apparent autosomal dominant pattern in inheritance for pancreatitis of early onset in the absence of other causative factors; or a confirmed mutation of the *PRSS1* gene. Table 5a summarises kindreds registered with the EUROPAC HP registry from across Europe during the time of writing of this thesis.

Table 5: Summary of HP database

Table 5a: Summary of HP database

Country	p.R122H families	p.N29I families	A16V families	NEG ALL HP families	Copy number variance	Other mutation	Familial Idiopathic Pancreatitis (FIP)	Possible HP	CFTR families	SPORADIC	TOTAL
UK	30	24	3	19	1	1	28	45	15	139	305
France	29	9	1	3	5	10	6	4	0	1	68
Germany	19	4	0	5	0	1	0	1	0	3	33
Eire	5	2	0	1	0	0	2	4	1	12	27
Denmark	2	0	3	1	0	0	4	1	1	12	24
Belgium	2	1	1	1	0	0	2	1	0	6	14
Italy	3	1	0	4	1	1	2	1	1	0	14
Netherlands	2	1	0	1	0	0	1	0	0	0	5
Finland	1	0	0	0	0	0	0	0	0	1	2
Greece	0	0	0	0	0	0	0	0	0	1	1
Sweden	2	0	0	0	0	0	0	3	0	8	13
Switzerland	3	0	1	2	0	0	0	1	0	1	8
Hungary	2	0	0	1	0	0	0	0	0	4	7
Czech	0	2	0	1	0	0	3	0	0	0	6
Spain	0	0	0	0	0	0	0	1	1	1	3
Turkey	0	1	0	0	0	1	1	0	0	0	3
Norway	0	0	1	0	0	0	0	0	0	0	1
Poland	2	0	0	0	0	0	0	0	0	0	2
Portugal	0	0	0	0	0	0	0	0	0	1	1
Sri Lanka	0	0	0	0	0	0	0	0	0	1	1
USA	0	0	1	0	0	1	0	0	0	0	2
TOTAL	102	45	11	39	7	15	49	62	19	191	540

A summary of the mutations present within those families registered with EUROPAC HP registry is presented in Table 5b.

Table 5b: Summary of *PRSS1* mutations identified in individuals registered with the EUROPAC HP registry.

Mutation	Families	Affected individuals	Pancreatic cancer cases (in affected individuals)
True HP	212	839	49(46)
p.R122H	102	462	25 (22)
p.N29I	45	185	11 (11)
p.A16V	11	25	1 (1)
Neg All	39	145	11 (11)
Other mutation	15	22	1(1)

4.3 RESULTS: Epidemiological factors implicated in cancer development in HP

In previous work undertaken by EUROPAC, in the largest series of HP individuals in Europe to date, Howes *et al* identified cumulative risk for pancreatic cancer development with age. In a cohort of 233 individuals, cumulative cancer risk increased with advancing age from 0.5% (95% confidence interval [CI]: 0.0 – 1.3%) at 40 years to 33.3% (95% CI: 19.0 - 47.5%) at 80 years of age [163]. When considering cumulative risk from onset of symptoms, at 70 years from onset, the risk for cancer development was 44% (95% CI: 8.0 – 80%)[158].

4.3.1 Risk according to *PRSS1* mutation

The risk of pancreatic cancer conferred by the p.A16V mutation of *PRSS1* is difficult to quantify. In the largest series of p.A16V individuals to date, previous work by Grocock *et al* [167] using data from the EUROPAC HP registry, has revealed that p.A16V exerts a very variable phenotype. The cancer risk associated with the p.A16V genotype is difficult to characterise [167], the cumulative lifetime risk of pancreatic adenocarcinoma conferred with p.R122H and p.N29I mutations is widely accepted. In the largest series of p.A16V families to date, our group characterised the phenotypic variation seen in p.A16V [167]. The phenotypic and clinical features of the p.A16V families described in this series are outlined in Table 6 [167].

Table 6: Summary of EUROPAC p.A16V families

Family	Classification	Number testing positive for p.A16V* (with pancreatitis)	Median age of onset (IQR) All patients testing positive for p.A16V*	Median age of onset (IQR) Patients with pancreatitis	Endocrine pancreatic failure (with pancreatitis)	Exocrine pancreatic failure (with pancreatitis)	Pancreatic cancer cases (with pancreatitis)
A	HP	7(3)	18 years (7-53)	10 years (5-21)	4(0)†	1(1)	0
B	HP	2(2)			2(1)	2(2)	0
C	HP	2(2)			0	0	0
D	HP	4(4)			0	0	0
E	HP	5(2)			0	0	1(0)
F	HP	3(2)			2(2)	2(2)	1(0)
G	Idiopathic	3(1)	Not reached (5-Not reached)	2 (1-5)	1(0)†	0	0
H	Idiopathic	3(1)			1(1)	1(1)	0
I	Idiopathic	4(1)			0	0	0
J	Single Generation	4(4)	27 (25-28)	27 (25-28)	0	2(2)	1(1)
Totals		37(22)	26 (7-54)	10 (5-26)	10(4)	8(8)	3(1)

In light of this evidence, it was concluded that cancer risk should be considered within the context of each kindred. In the event of a single case of idiopathic disease, then risk for the development of cancer should be considered as per any individual with idiopathic pancreatitis. However, in those families which exhibit a pattern of inheritance consistent with true HP, then the cancer risk should be considered as that of true HP. As summarised above, a number of *PRSS1* mutations have been described and are associated with a variable clinical picture and cancer risk. p.R122H, p.N29I and p.A16V have been found to confer a significantly increased lifetime risk for the development of pancreatic cancer, in addition to the earlier age of onset of exocrine and endocrine failure. Other mutations in *PRSS1* have been described in association with idiopathic disease and have been summarised earlier. For the purposes of discussion of those mutations which confer an elevated pancreatic cancer risk, these mutations will not be mentioned further.

4.3.2 Age – SEER data and EUROPAC HP data demonstrating 28 fold increase in risk

Data pertaining to the lifetime risk for development of pancreatic adenocarcinoma in certain *PRSS1* mutations associated with HP, have identified a constant increase in risk with progressive age. Comparison of EUROPAC HP survival data with SEER statistics demonstrated an approximate 28-fold increased risk in the HP cohort when comparison to US SEER data is made. This trend appears constant with increasing age as detailed in Figure 6.

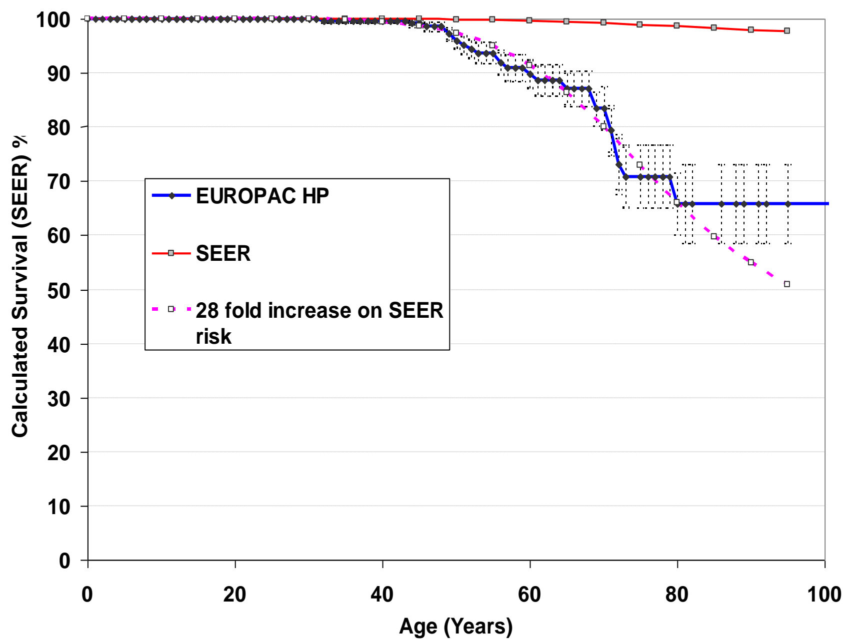


Figure 6: Kaplan-Meier curve demonstrating EUROPAC HP risk compared to 28 fold increase on SEER data.
SEER data (from the US) provides cross-sectional analyses of cancer incidence and survival across 5 year age ranges. I used SEER data to extrapolate survival by utilising the incidence (/10,000 population) to determine the percentage (%) risk of PDAC within each 5 year age range (red continuous line). Kaplan Meier analysis was applied to HP individuals to give the continuous blue line. Comparison of HP risk in age brackets (from Kaplan Meier) to the equivalent SEER data gave an average ratio of 28 (EUROPAC:SEER). The SEER risks were multiplied by 28 and applied as above to produce a pseudo Kaplan Meier curve (discontinuous pink line).

4.3.3 Cancer risk in HP according to smoking and diabetes status

From EUROPAC HP data, we have analysed the influence of smoking status and diabetes mellitus on cancer risk in HP. In Figures 7-10 the relationship between diabetes onset, smoking status and cancer development can be seen. The data reveal that although there is evidence that diabetes influences age of death from cancer in HP; that this effect is overwhelmed by the effect of smoking.

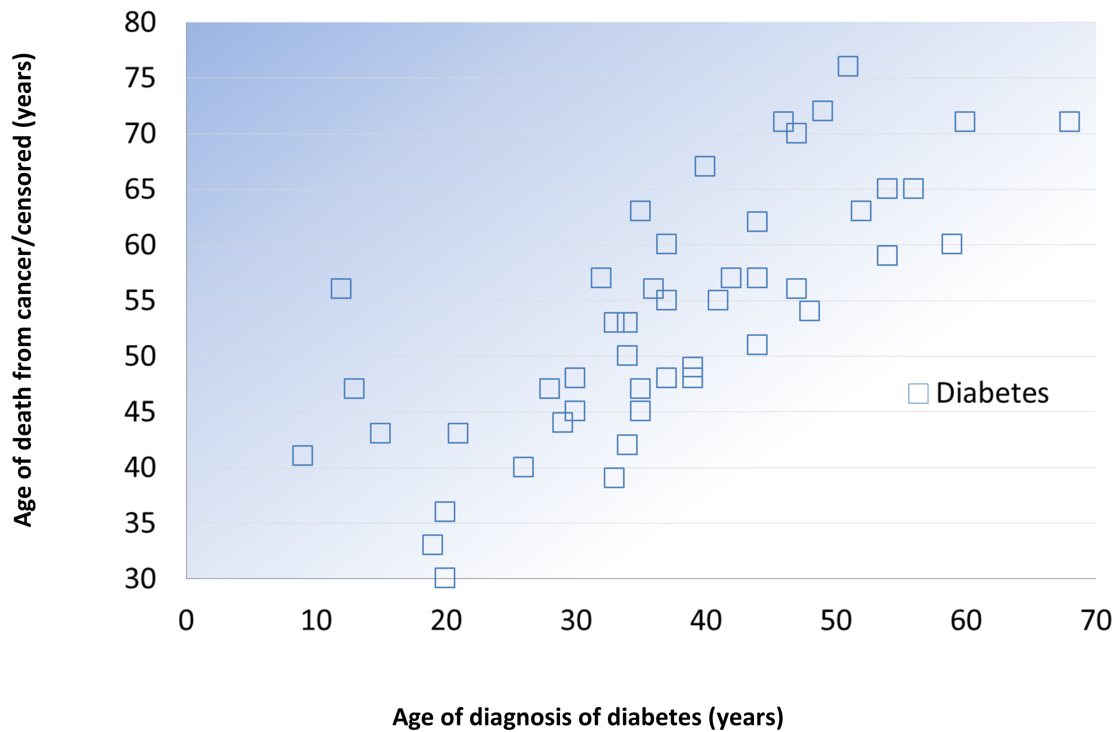


Figure 7: Age of onset of diabetes and age of cancer death in the EUROPAC HP cohort (just non-smokers)
Date of death denotes age of death in years from a confirmed diagnosis of PDAC or the age of last contact, which is denoted as the point at which the data for that individual is 'censored' and analysed. The blue squares are all patients with diabetes.

Figure 7 demonstrates the age of onset of diabetes and cancer death in our HP cohort. The following figure demonstrates age of onset of diabetes in a cohort of non-smoking HP individuals. It can be seen that diagnosis of diabetes demonstrates a proportional relationship to age of death from cancer as evidenced by the clustering of cases at the trend-line.

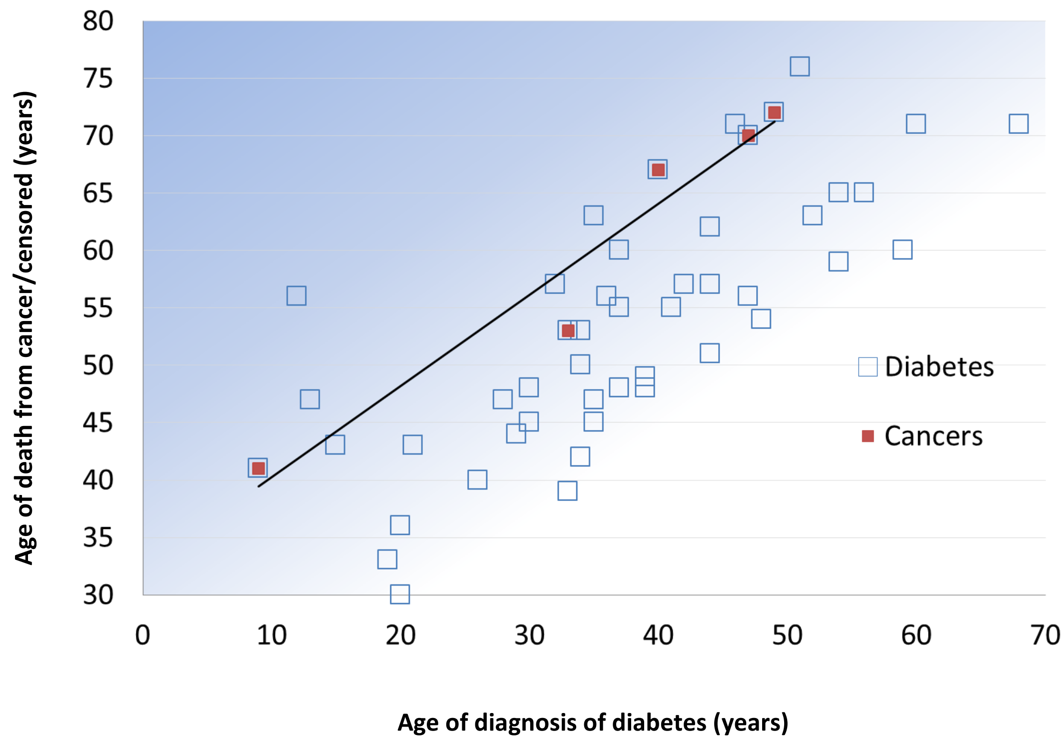


Figure 8: Age of onset of diabetes and age of death from cancer in HP non-smokers
Date of death denotes age of death in years from a confirmed diagnosis of PDAC or the age of last contact, which is denoted as the point at which the data for that individual is 'censored' and analysed. Cancers are marked on in red the line indicates a linear regression for the cancer patients.

Evidence from the EUROPAC HP registry therefore appears to suggest a trend in age of onset of diabetes and age of death from cancer. However, Figure 9 details the same data in a subgroup of HP smokers. The effect of age of onset of diabetes has been superseded by the influence of smoking on cancer risk.

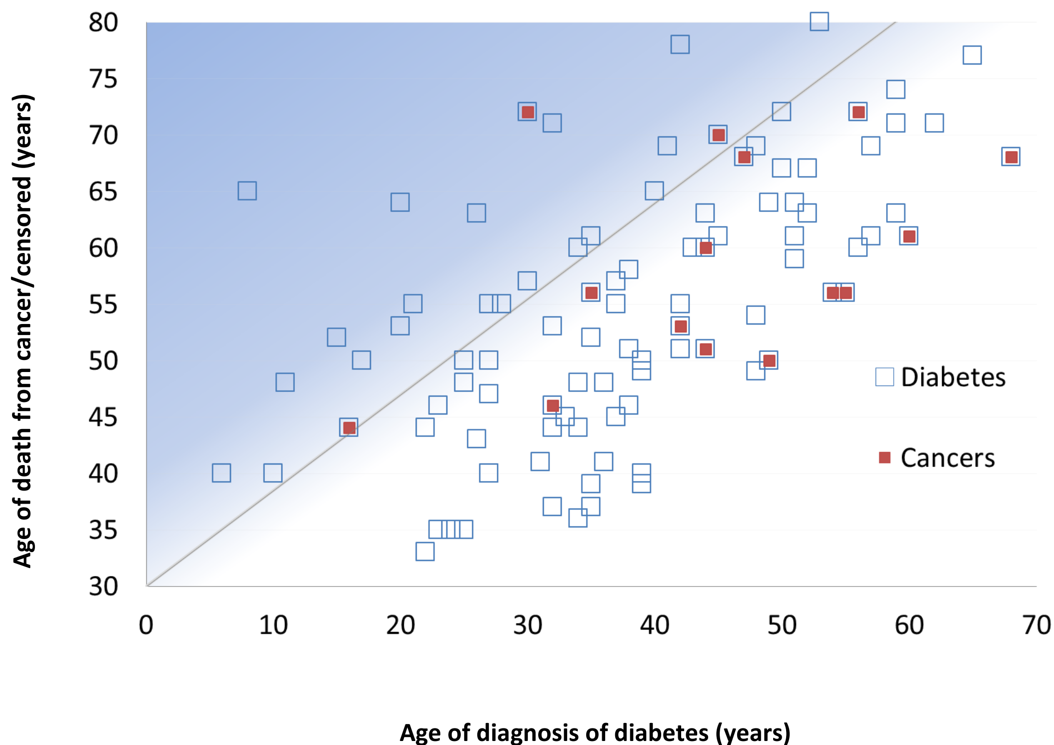


Figure 9: Plot of age of onset of diabetes and age of cancer death in a cohort of HP smokers

The trend in aggregation of cancer cases seen in the non-smoker HP cohort is not replicated in the smoking group. The assumption made is that of smoking causing cancer. The trend seen in the EUROPAC HP cohort is that of smoking as causative of both diabetes and cancer. The line represents a proposed screening age of 20 years after diabetes diagnosis, this misses most cancers in smokers but would have missed none in the non-smoking cohort.

It can be seen that the relationship between age of onset of diabetes and death from cancer which is apparent in non-smoking HP individuals, has been confounded by the influence of smoking on cancer risk in HP. The effect of smoking upon cancer risk in the EUROPAC HP cohort is further evident when ex-smokers are considered (Figure 10).

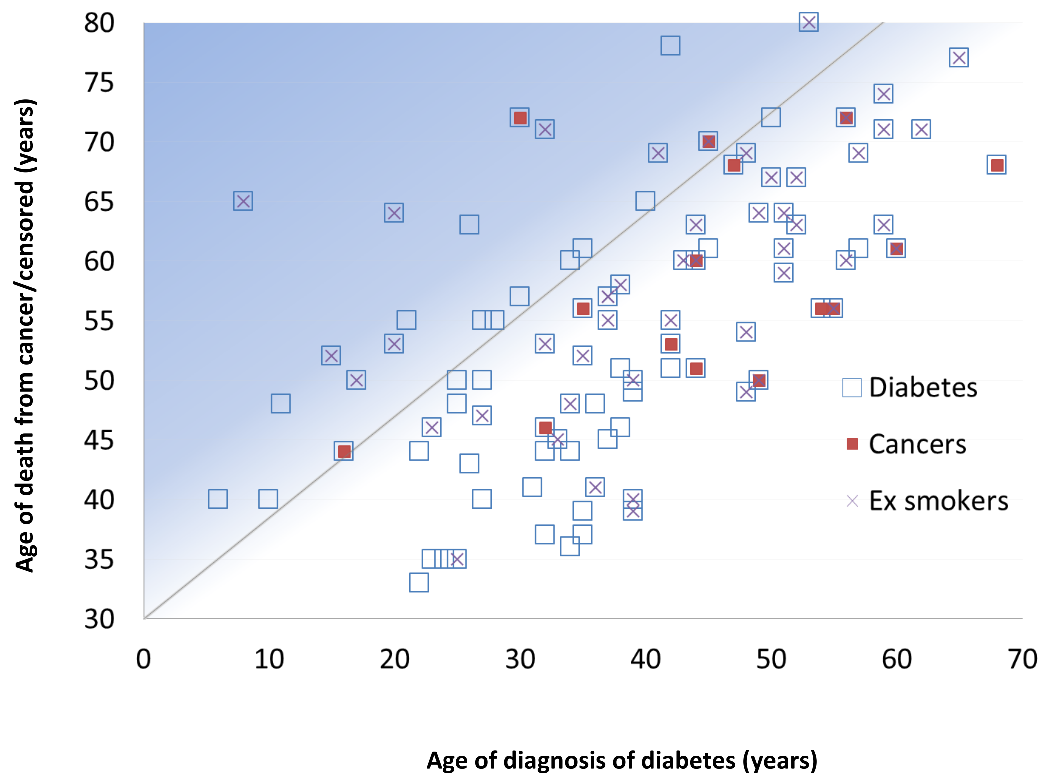


Figure 10: Plot of age of onset of diabetes and age of death from cancer in HP ex-smokers

In analysis incorporating ex-smokers, it can be seen that the proposed screening age at 20 years post onset of diabetes would be too late to identify the majority of cancers in ex-smokers with HP.

4.4 RESULTS: Epidemiological factors implicated in cancer development in FPC

4.4.1 Influence of age of onset in FPC

Previous analysis of survival data from the EUROPAC FPC Registry has identified a 100-fold increased risk when compared to SEER data [32] at progressive age ranges (Figure 11); this will be further addressed in this thesis.

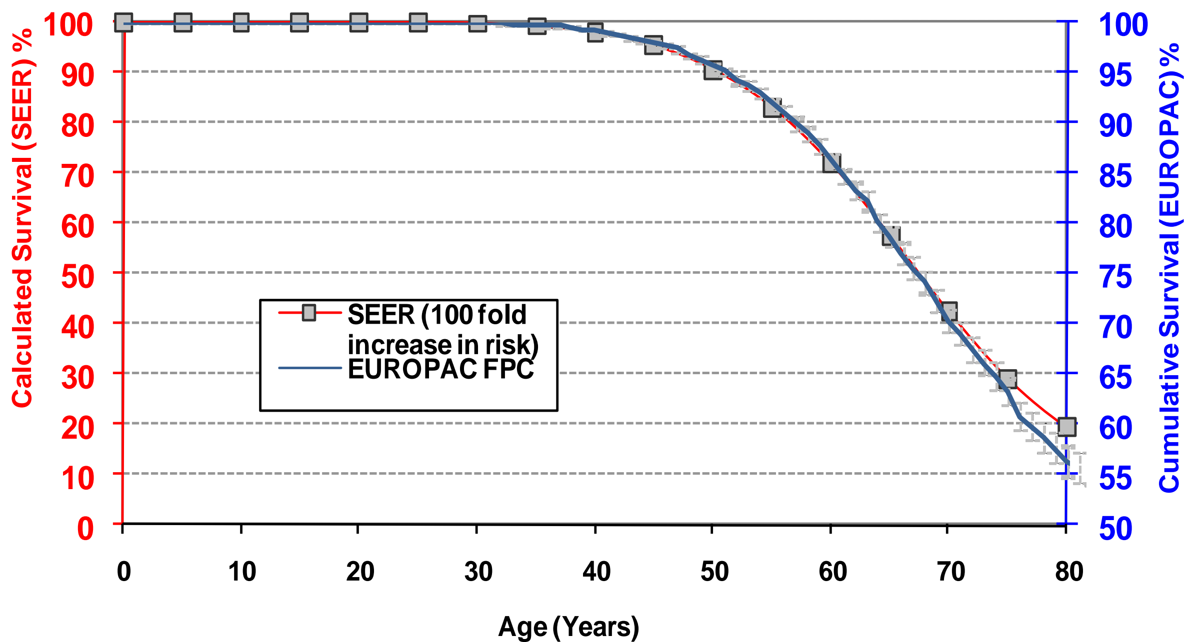


Figure 11: Survival data from EUROPAC FPC individuals and 100-fold increase in risk from US SEER data
SEER data was used as a basis for calculation of % risk and applied to the EUROPAC FPC cohort as described in Figure 6

4.4.2 Age of onset in FPC

It has been proposed that families with an autosomal dominant predisposition for pancreatic cancer also have an earlier age of onset than individuals with no clear genetic predisposition. This is consistent with observations in other cancer syndromes and was systematically addressed by Brune [270] *et al* based on families recruited by the NFPTR. In their paper entitled "Importance of Age of Onset in Familial Pancreatic Cancer kindreds", Brune *et al* reported a significantly elevated risk in kindreds with younger age of onset for existing affected individuals. This was based on a comparison of families where affected relatives were above or below 50 years of age. Being a member of a younger onset family conferred a standardised incidence ratio (SIR) of 9.31 (95% CI 3.42-20.28, p=<0.001) compared to an SIR of just 6.34 (95% CI 4.02-9.51, p =<0.001) in the older onset families

[270]. However, in this thesis I propose that this apparent trend is attributable to ascertainment bias. Previous reports from the NFPTR show a highly elevated risk of prospective pancreatic cancer in such families [157]. However, this risk was overwhelmingly due to a very small number of families with a very strong family history. Only one out of 98 families with 2 existing cases of pancreatic cancer had a prospective cancer, in contrast one out of just 4 families with 5 or more cases had a prospective cancer. Clearly, family classification is dependent on the observer, if 2 out of 5 cases of cancer are known originally and 3 new cases are subsequently identified there has not been any change to actual cancer risk, merely a change to assumed cancer risk. If we accept that there are families with equivalent biologically defined risk in the group with 2 cases and the group of families with 5 cases, this would strongly suggest that most families are random clusters, but there are fewer random clusters in the families with more cases [157]. EUROPAC tries to reduce the number of random clusters by adopting a longitudinal approach to recruiting families as explained earlier in this work. To ascertain whether bias through variation in recruitment strategy could explain the young age of onset reported by Brune *et al*, we have compared the characteristics of families recruited to our registry on the basis of an affected versus an unaffected proband, including the estimated cancer risk in FDR. For the purposes of this analysis, the total number of affected individuals within a family included probands in addition to FDR. It is surmised that the greater the number of PDAC cases confirmed within a kindred, the greater the likelihood of a genuine familial predisposition to PDAC. Brune *et al* proposed that there is a lower age of onset in FPC versus sporadic PDAC, therefore the age of onset should be lower in kindreds with greater numbers of affected relatives. Kindreds included for analysis were divided depending upon the number of confirmed PDAC cases in the family i.e. 2 and 3 or more (Figure 12). Families were recruited via probands

who, following informed consent, completed family and personal health questionnaires. Where appropriate, clinicians also completed parallel questionnaires, which were used for confirmation of clinical details and hospital and pathology notes were also obtained. Clinics were arranged and family members were invited to attend via the proband. Individuals were classified as affected based on histological confirmation of PDAC where available. Where histology was not available good quality medical notes or reliable cancer registry information were used. FPC was defined as families with two or more affected individuals not fulfilling criteria for any other familial cancer syndrome. We assessed 289 families (706 affected) of which 91 families had 3 or more affected cases (310 in total). As was reported by Brune et al, there was a trend for earlier onset in families with greater number of affected individuals but despite large numbers of individuals included this does not reach significance (Figure 12).

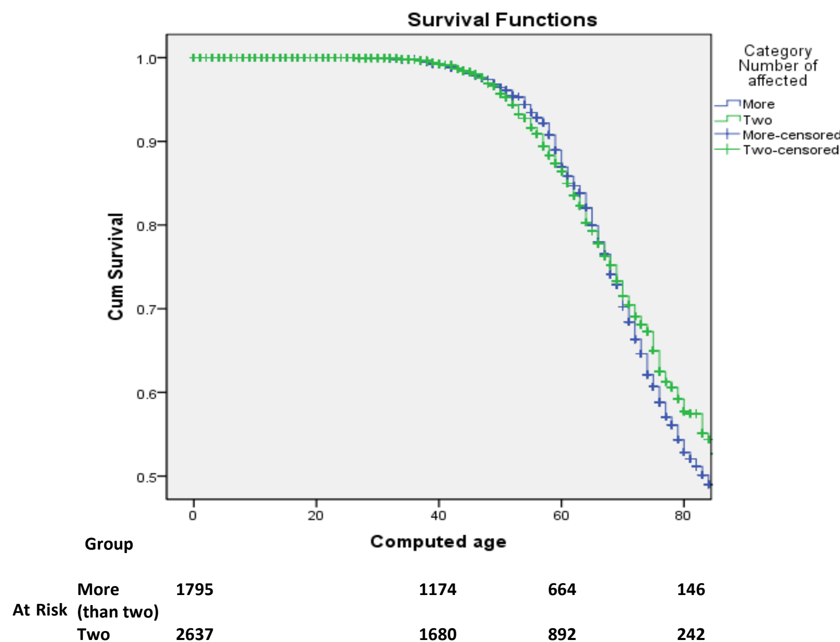


Figure 12: Cumulative survival for all on kindred individuals including probands
Kaplan-Meier curve demonstrating survival in on-kindred FPC in families with 2 versus 3 or more affected individuals. There is a non-significant trend of earlier age of onset in those families with 3 or more cancer cases i.e. those more likely to reflect 'true' FPC kindreds with a genuine predisposition to PDAC (p=0.24).

Figure 12 highlights a trend for earlier onset in families with greater number of cancers but this did not reach significance (Mantel Cox Log rank, p=0.24). Furthermore, removal of probands from the analysis reverses the apparent trend for apparent earlier onset in families most likely to have an autosomal dominant predisposition to PDAC.

If the family is identified because of an affected individual appearing in clinic, there is a tendency for younger cases to be followed up more rigorously. This will be equally true if the individual chosen actually comes from a genuine family, or if they come from a random cluster. Therefore, there may be a tendency to identify a younger age of onset in registered families because of bias, rather than in any genuine younger age of onset in legitimate FPC. To address ascertainment bias, we excluded all probands (whether affected or not), and

only included the FDR of these probands. As seen in Figure 13, this gave a small but significant apparent survival benefit for families with more cancer cases; thus not demonstrating earlier onset for those families with more than 2 affected individuals, but the opposite. Clearly this is in direct contradiction to the data obtained by Brune *et al.* This cannot be explained merely by the form of analysis and must reflect some fundamental difference between the EUROPAC and NFPTR cohorts.

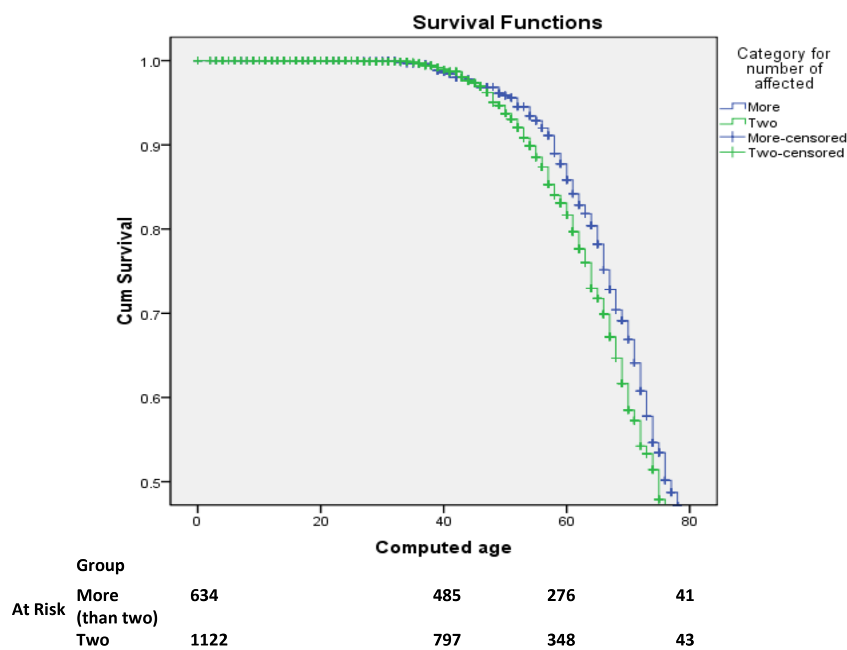


Figure 13: Cumulative survival for first degree relatives of probands

Exclusion of probands from survival analysis (FDR only) demonstrates an apparent significant improvement in survival in those FDR from families with 3 or more affected individuals versus only 2, contrary to the trend expected in the presence of a genuine autosomal dominant predisposition (p=0.021).

Figure 13 demonstrates a significant survival advantage for those families with 3 or more affected individuals (Mantel Cox Log Rank p=0.021).

Ascertainment bias in registries ensures that registered individuals will have at least one case of pancreatic cancer in an FDR, regardless of whether cancer is genetically predisposed or not. Consider the family in Figure 14, which illustrates a typical registered family where

two generations are affected. Consider two hypothetical cases with identical family structures: Where the proband is in generation 2 (G2, individual A), or where the proband is in G3 (individual B). By definition, proband B will have FDR who are comparatively young i.e. offspring will be in G4 rather than G3. Ascertainment bias will ensure that there will be at least one affected FDR individual, regardless of which generation the proband is in. In calculating SIR, the numerator is the number of affected individuals and the denominator is mainly unaffected individuals. In Kaplan-Meier survival analysis, any individual who has not reached an outcome event is said to be censored. Whichever way age of onset is considered there is a bias defined by the nature of the proband. Neither the numerator in SIR or the event times in a Kaplan-Meier analysis will be unchanged (as the number of affected FDR is fundamental to family selection), but the denominator in calculating an SIR and the age of censorship in a Kaplan-Meier analysis will both be younger if the proband is in G3 as compared to G2.

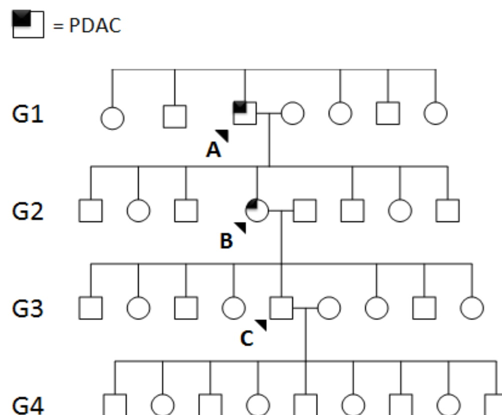


Figure 14: Typical FPC pedigree demonstrating 2 affected individuals in successive generations. 3 possible probands are marked, A and B affected and C not affected with PDAC.

This figure provides an illustration of the variable age in potential onset of PDAC in an FPC kindred dependent upon the proband. The NFPTR recruit families by presentation of an affected proband while EUROPAC mainly recruits from probands who are not affected. The families must have at least two affected individuals. If A is an affected proband then, for recruitment B must have also developed PDAC; and as B is a descendant of A she must have developed the cancer earlier than A. In the event of B presenting to the registry as an affected proband, both A + B must be affected, but A may have developed PDAC at a younger age than B. C cannot be recruited under the NFPTR system as a proband but would be a typical proband for EUROPAC. If C is the proband, then A + B must have PDAC and age of onset may be earlier or later than the present age of C. Therefore, a bias in age of onset can be introduced dependent upon which generation is recruited as proband.

If the family is selected on the basis of a non-affected individual reporting two cases of cancer in their family, then the report will almost certainly come from an individual in G3 or G4 (such as C in Figure 14), simply because by the time an individual in G3 develops cancer, most individuals in G1 and G2 will be over 60. There can also be a considerable delay between an individual in G2 developing cancer and the family being recruited.

To further investigate this, our group undertook survival analyses for families with affected and unaffected probands. The Kaplan-Meier curve in Figure 15 compares event time in FDR of affected probands with the FDR of unaffected probands. A significant earlier onset was seen with the unaffected probands. This can be explained by ascertainment bias which dictates that the “proband affected” cohort of individuals will contain a greater number of older censored FDRs as compared to the “proband unaffected” group. Hence, the appearance of an apparent earlier age of onset.

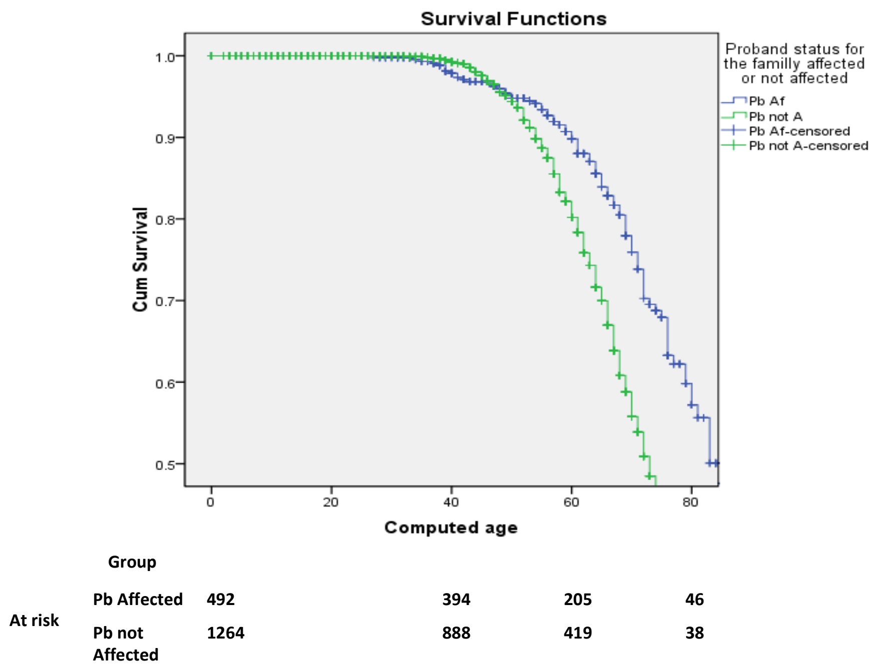


Figure 15: Cumulative survival for all FDR in proband (Pb) affected (Af) and not affected (not A) groups.
Referring to the example in Figure 14, the Kaplan Meier curve above compares FDR with proband affected (proband A when B is affected and vice versa; and proband C (unaffected) when A + B are affected). There appears to be a significant earlier age of onset in FDR whose probands were unaffected as compared to affected.

This figure demonstrates an apparent earlier age of onset in FDR of unaffected probands (Mantel Cox Log Rank p=<0.0001). However, in the analysis of a more stringent selection of families, this effect is confounded (Figure 16). Our group propose that the apparent significant influence of proband status on age of onset is due to the phenomenon of ascertainment bias. If the effect seen in Figure 15 is due to ascertainment bias, then it should be reduced or eliminated if the proportion of random clusters is minimised. The Kaplan-Meier curve in Figure 16 compares survival, dependent upon proband affected status, in FDR of families with 3 or more cases i.e. those families more likely to represent ‘true’ FPC, rather than an effect of random clustering.

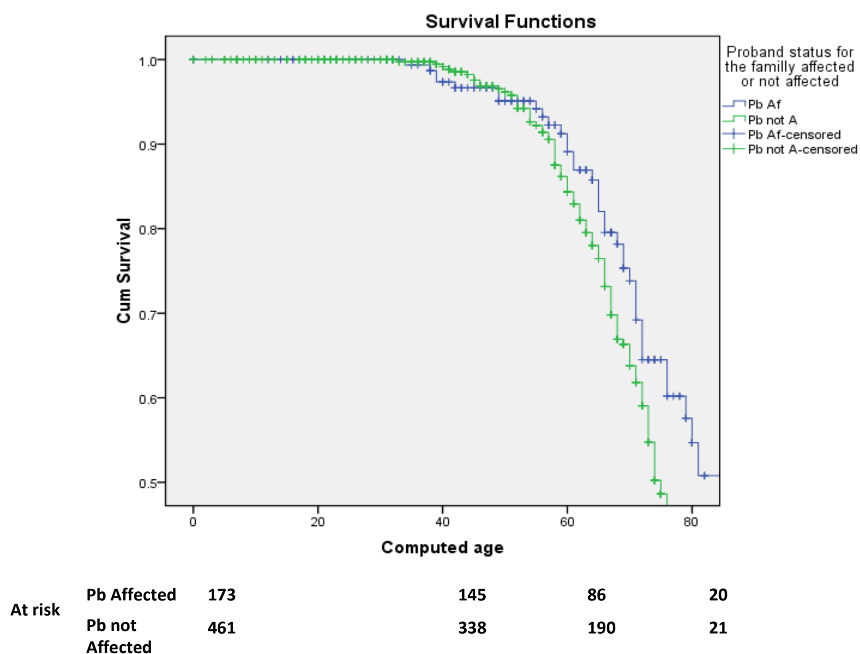


Figure 16: Cumulative survival for FDR of families with 3 or more affected individuals according to proband status. Survival in more 'genuine' FPC families with 3 or more affected family members where proband is affected versus unaffected demonstrating a trend for improved survival in those with an affected proband. This reflects the NFPTR recruitment policy.

In analysis of individuals from perceived "genuine" FPC families, with three or more cancer cases, according to affected proband status; there is a trend for improved survival in those FDR from families where the proband is affected, but this is less marked (Mantel Cox Log Rank p=0.05). In contrast, the evidence for ascertainment bias on the basis of the nature of the proband is accentuated in families with a less stringent selection i.e. two cases or less (see Figure 17).

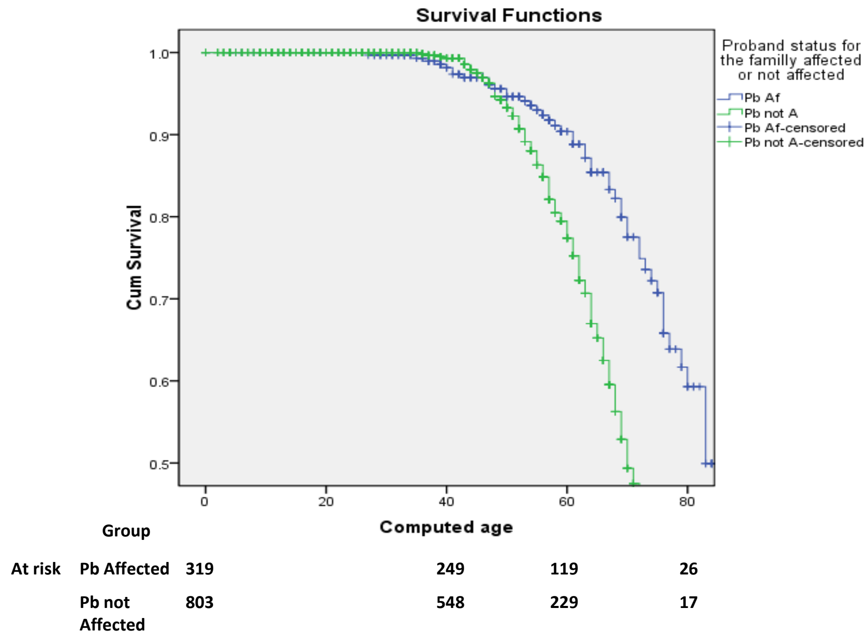


Figure 17: Survival in FDR of families with 2 or less affected individuals

The above Kaplan-Meier curve is the same analysis as in figure 16, but applied to those families with 2 affected family members, thus representing those less likely to have a genuine genetic predisposition to FPC. There is an apparent significant trend in survival in FDR where the proband is affected.

The families included in the survival analysis demonstrated in Figure 17 are more likely to represent random clusters rather than a genuine predisposition to PDAC. A significant survival advantage is seen in those individuals from families with an affected proband (Mantel-Cox Log Rank p=<0.0001). It can therefore be seen that ascertainment bias can change apparent age of onset. If the results from Brune *et al.* are, indeed, due to ascertainment bias then it should be possible to generate a similar effect with EUROPAC families simply by restricting family selection to kindreds with an affected proband. This is demonstrated in Figure 18.

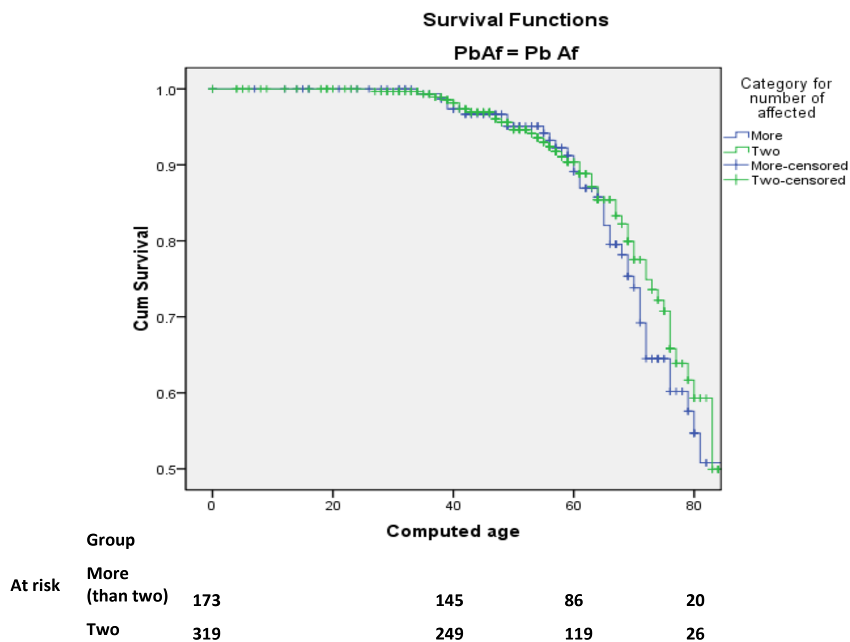


Figure 18: Kaplan-Meier plot for all families in whom the proband was affected at the time of registration split by the total number of confirmed PDAC cases i.e. less than 3 versus 3 or more.

Survival analysis of EUROPAC families limited to those with proband affected. There is an apparent survival advantage seen in those with 2 affected family members compared to those with 3 or more ('genuine' FPC) which reflects the observation of the NFPTR and reflects the recruitment policy of the NFPTR.

Analysis of FDR from families with an affected proband demonstrates an apparently significant increased survival in those individuals from families with less than 3 cases (Mantel-Cox Log Rank p=0.007). By limiting survival analysis to those families with an affected proband, in accordance with the selection criteria applied by the NFPTR; an apparent trend for decreased survival with increasing number of affected family members can be seen (Figure 18). Similarly, if the effect is due to bias then it should be minimised or reversed in families with unaffected probands, as seen in Figure 19 (Mantel-Cox Log Rank p=0.007).

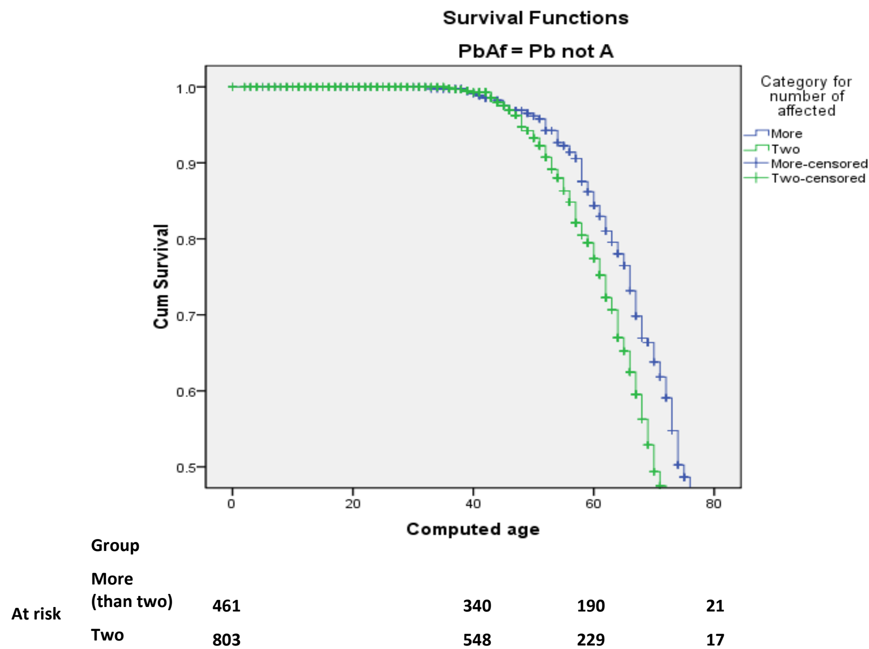


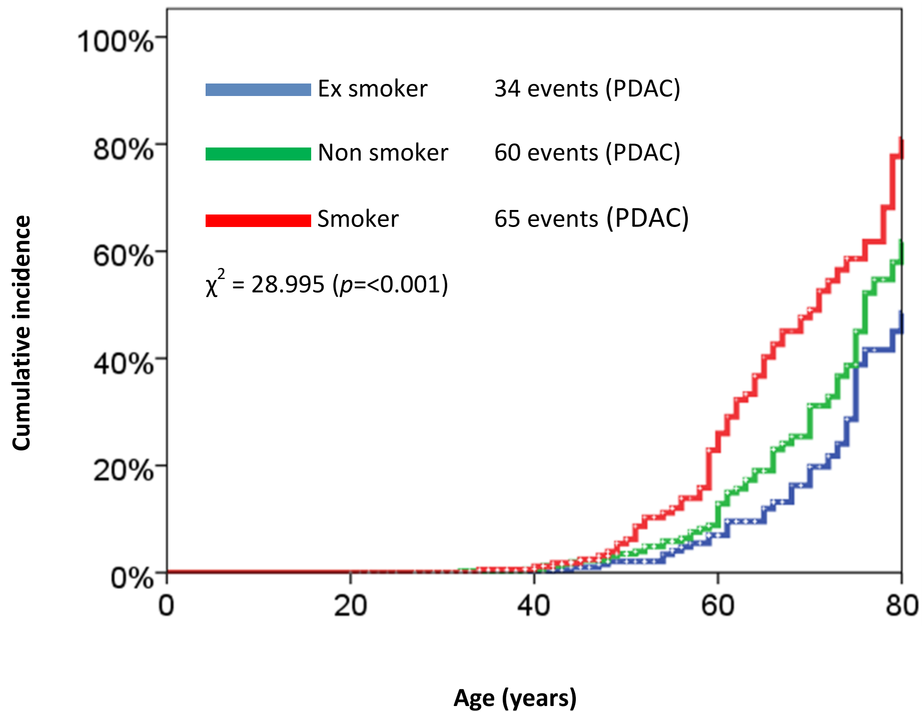
Figure 19: Cumulative survival in FDR of families with unaffected probands.
In survival analysis in EUROPAC families with unaffected probands, the reverse of the trend demonstrated in Figure 18 can be seen (*p*=0.007). Thus the NFPTR observation is dependent on their recruitment policy.

The recruitment policies of the NFPTR and EUROPAC both produce biases which can lead to an impression of younger age of onset in particular families. In the case of EUROPAC, this can give the impression of younger onset in families with fewer affected family members. While recruitment by the NFPTR, gives the impression of younger onset with more affected family members. The observations from the NFPTR published by Brune *et al* can be duplicated in the EUROPAC cohort, by restricting family inclusion to only those families where the proband is affected. As families reported by unaffected individuals are at least as legitimate as families encountered on the basis of affected individuals recruited in clinic, and arguably will include less falsely identified families; it is not reasonable to assume an earlier age of onset for familial pancreatic cancer (in comparison with sporadic disease). This is in contrast to reports from other cancer syndromes and may appear counterintuitive, particularly as we have previously argued that anticipation is a characteristic of FPC.

However, nothing we or others have published previously excludes late onset in FPC individuals, indeed, that is exactly the feature we previously described for earlier generations.

4.5 Smoking and risk in FPC

Evidence discussed earlier in this work has demonstrated a causative role for smoking in the onset of sporadic PDAC. Analysis of our data has identified an apparent phenomenon of recall bias with no definitive evidence of smoking as significantly influencing PDAC risk. Figure 20 demonstrates survival in the EUROPAC FPC cohort according to smoking status as reported by the proband, whether affected or unaffected. The data suggest an apparent trend in poorer survival associated with smoking; a trend which has been reported in sporadic cases and has been discussed earlier in this work.



At Risk				
Ex	**238**	**212**	**115**	**16**
Non	**367**	**311**	**136**	**11**
Smoker	**206**	**163**	**74**	**7**

Figure 20: Survival in EUROPAC FPC cohort according to smoking status
Survival analyses in the EUROPAC cohort dependent upon status as a current, ex or never smoker. This data suggests an increase in incidence in the smoking group. However, there appears to be an increase in non-smokers versus ex-smokers which conflicts with established data for sporadic disease.

Smoking data taken at the time of a participant's registration with EUROPAC is taken for the registering individual and affected family members, through use of the individual and family history questionnaires. Accuracy of the data recorded by the registering individual is subject to recall bias. An individual reporting their own smoking status will recall with accuracy whether they are a current or ex-smoker. On recalling the smoking status of affected relatives, particularly in the context being sensitised to their family cancer risk, an individual is more likely to report a relative as a smoker or non-, rather than ex-. This introduction of bias can be seen in the survival analysis of ever (encompassing current and ex-smokers) versus non-smokers. It can be seen from the following survival analysis, that there is no

significant relationship between smoking and cancer risk in the EUROPAC FPC cohort. This is explained by the over-reporting of smoking status in affected family members by sensitised unaffected probands.

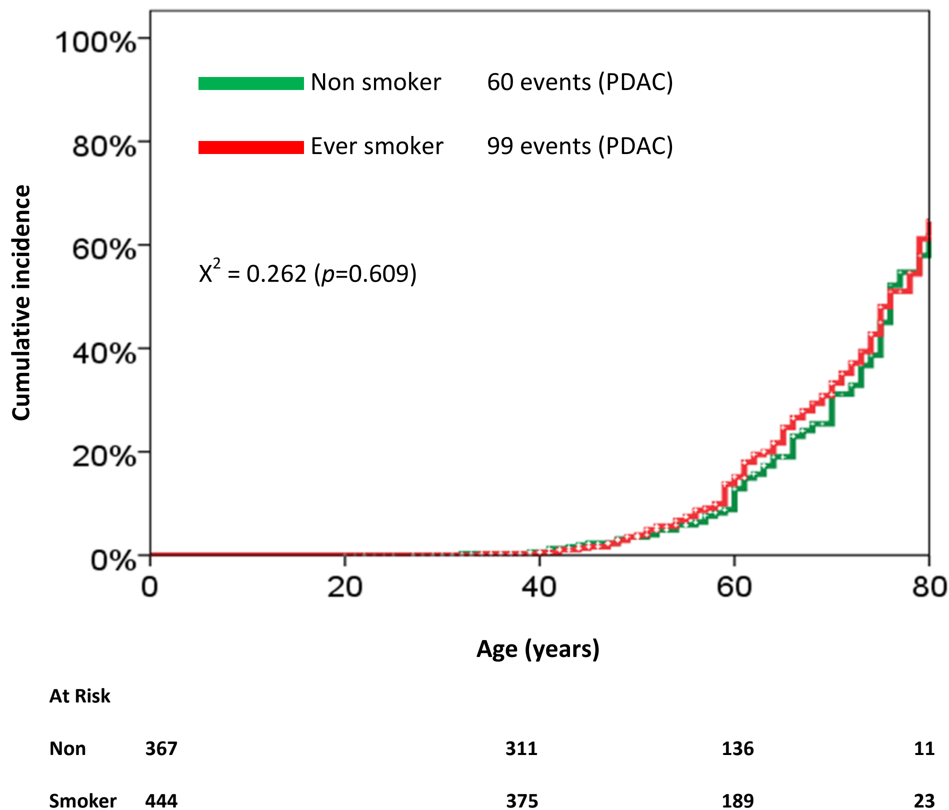


Figure 21: Survival data in the EUROPAC FPC cohort according to ever versus non-smoking status
Upon classification of smoking history as ever versus non, there is no significant difference in survival, the original difference in survival could be explained by recall bias.

4.6 Obesity and cancer risk in FPC

Research into the effect of obesity on the development of sporadic pancreatic cancer has suggested a trend in cancer risk with increasing BMI [88-94]. BMI data are poorly reported in the EUROPAC FPC cohort. In addition, BMI is a dynamic measurement with considerable variation in an individual over time. Hence, an isolated BMI taken at the time of an individual's registration is unlikely to yield any benefit in stratification of risk.

4.7 Diabetes and cancer development in FPC

Evidence surrounding the influence of diabetes on PDAC risk in sporadic cases remains conjectural, with conflict continuing as to whether the relationship is one of affect or effect. In the EUROPAC cohort of FPC families, only one family demonstrated an apparent relationship between affected family members and a diagnosis of diabetes. This evidence is insufficient to utilise diagnosis of diabetes as a rationale for risk stratification in the screening of FPC kindreds.

4.8 Genetic modifiers

4.8.1 ABO group and cancer risk

Genome wide analysis work into potential genetic modifiers which may be key in the development of PDAC have highlighted a possible influential role of the ABO gene. It is widely established that Group A individuals have an increased lifetime risk for the development of gastric cancer. It is proposed that such a link may also be of significance in PDAC. Data collected from sporadic PDAC individuals by our group is largely drawn from the North West of England and North Wales. Historical data concerning distribution of ABO groups across the UK reveals an increased prevalence of group O within this region, with the suggestion that group O has a frequency of 28.5% higher in North Wales and 16.6% higher in North West England than group A. It should be noted that this distribution is based on data from 1970 [125]. Although there is likely to have been an element of population shift in this time, this data suggests that one would expect to see an increased prevalence of pancreatic cancer cases in blood group O, due to the elevated frequency compared to A.

Sporadic cancer and ABO group – analysis of tissue database data

ABO group data from a series of 232 cases of sporadic pancreatic adenocarcinoma treated in our centre revealed 107 (47%) individuals were of blood group A; 86 (37%) ABO group O; 32 (14%) group B and just 7 (3%) AB. With regard to Rhesus status, only 39 of the 232 total patients were Rhesus negative, with 16 of the 107 group A patients included within this figure. In order to determine whether this risk factor is of any significance in pancreatic cancer development, greater numbers are required and the geographical context of the cohort taken into account due to the heavily population dependent nature of ABO grouping.

4.9 Novel mutations

4.9.1 *PALB2*

A number of novel mutations have been proposed as candidate genes in the causation of FPC. *PALB2* (partner and localiser of *BRCA2*) is a DNA repair gene localised to chromosome 16. Families recruited as part of my work towards this thesis were included in the analysis of a series of 81 European kindreds [197]. In total, 3 *PALB2* mutations were identified across the 81 families. One specific example is the recognition of a frameshift mutation in exon 11 of the *PALB2* gene, which is demonstrated in Figure 22 below. Analysis across 81 European kindreds was undertaken by members of the EUROPAC team in co-operation with our German collaborators Slater *et al* [197]. My role was in the recruitment of families included in the analysis.

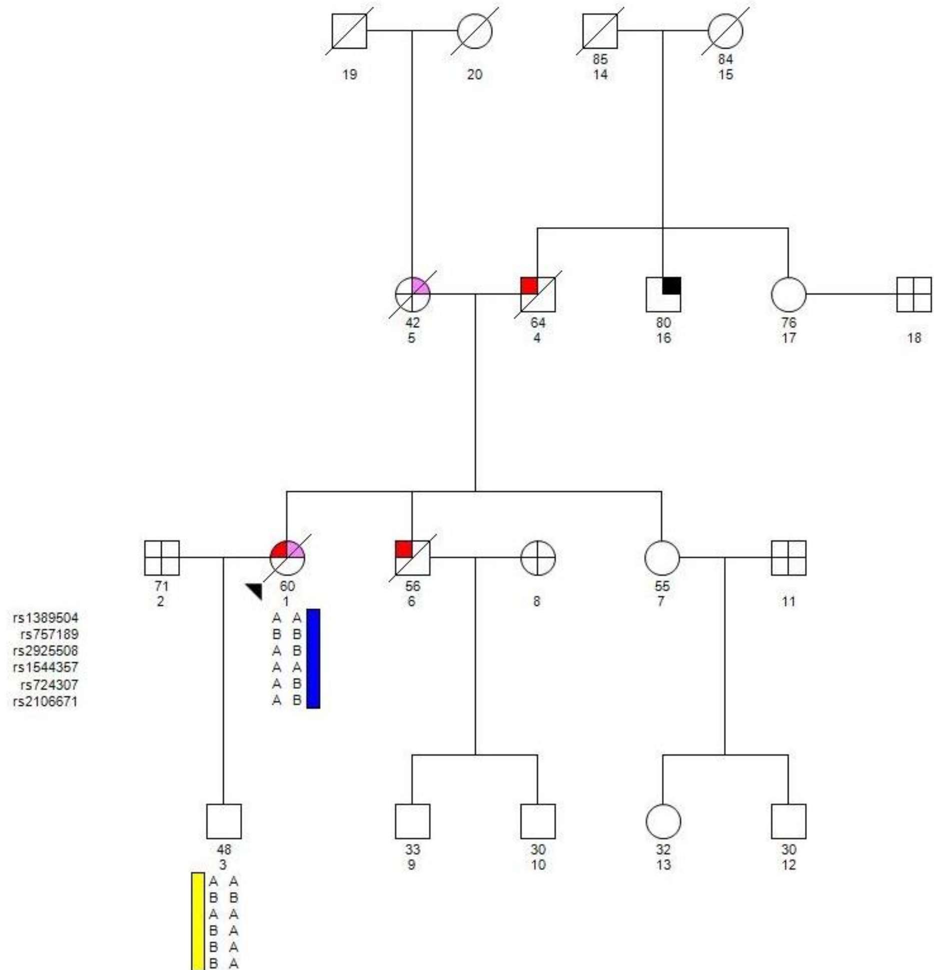


Figure 22: Frameshift mutation in exon 11 of PALB2.

Demonstrating SNPs relating to *PALB2* in affected individuals within one EUROPAC FPC kindred. A total of 81 European kindreds were included in the analysis which was undertaken by members of the EUROPAC team and our collaborators in Germany [197].

4.10 RESULTS: Secondary screening study

4.10.1 Total numbers currently recruited to secondary screening (UK figures) and total number of screening procedures

Table 7 records the number of individuals participating in the EUROPAC secondary screening study across our UK collaborating centre at the time of writing of this thesis.

Table 7: Screening centres and numbers recruited to the EUROPAC secondary screening study

Screening Centre	FPC	HP
Liverpool	47	22
London	17	6
Nottingham	5	0
Leeds	3	0
Southampton	1	2
Glasgow	2	1
Leicester	1	0
Newcastle	1	1
Edinburgh	0	3
Other	5	3
TOTAL	**82**	**38**

4.10.2 Experience with ERCP for juice collection and associated complications

HP

A total of 23 ERCP procedures were undertaken in HP individuals under the auspices of the EUROPAC secondary screening study. No occurrences of pancreatitis were reported in this patient cohort at the time of completion of this thesis. The definition of post ERCP pancreatitis in our group being: greater than three times elevation of normal serum amylase. It is surmised that the apparent low risk of post-ERCP pancreatitis in the EUROPAC HP cohort may be attributable to the abnormal anatomy, and more specifically, presence of pancreatic ductal dilatation seen in the HP screening sub-group, as compared to the normal anatomy seen in FPC.

FPC

The EUROPAC secondary screening protocol has been outlined earlier in this work. As part of their participation in the study, participants are offered ERCP for pancreatic juice collection for the analysis of molecular markers. This technique and analysis has been detailed in an earlier sub-chapter. Participants in the secondary screening study were invited to undergo ERCP following an informed consent process and explanation of risk. There is a procedure associated risk of pancreatitis associated with ERCP. In the earlier phase of screening, 7 cases of ERCP pancreatitis were recorded during 16 procedures between 2003 and 2007. In order to minimise this risk, the measures of temporary pancreatic stenting and the administration of an anti-inflammatory medication (Diclofenac) per rectally post-procedure were introduced during my work towards this thesis. The

technique of ERCP with self-expelling pancreatic stent deployment and administration of Diclofenac has been described in the materials and methods section.

4.10.3 Diclofenac and pancreatic stents in the reduction of ERCP pancreatitis

The early experience of EUROPAC in the use of ERCP for pancreatic juice collection for molecular analysis between 2003-2007 yielded a concerning rate of ERCP associated pancreatitis of 7/16 (43.8%) in the FPC cohort. In order to minimise this risk, the measures of temporary self-expelling stents and administration of per rectal Diclofenac were implemented in an amendment to the EUROPAC secondary screening protocol. These measures resulted in a significant reduction in the rate of ERCP pancreatitis in the EUROPAC FPC screening cohort. In the subsequent period following instigation of self-expelling pancreatic stents and Diclofenac, a total of 31 ERCP procedures were undertaken in FPC individuals. Of these 31 procedures, a diagnosis of acute pancreatitis, as defined by the biochemical parameter described above, was recorded in 3 individuals. Hence, a significant reduction in ERCP associated pancreatitis to 9.7% from 43.8% (p=0.020). The current overall rate of ERCP pancreatitis from 2003 is 10/47 procedures (21.3%).

4.11 Analysis of secretin stimulated duodenal aspirate as a surrogate for pancreatic juice

The risk of ERCP associated acute pancreatitis in the EUROPAC FPC cohort poses a significant threat of morbidity in this, usually otherwise well, group [271]. A fundamental requirement of any feasible screening test is to do no harm. In view of this, our group has undertaken work to evaluate the diagnostic merit of analysis of molecular markers in surrogate

pancreatic juice. Collection of secretin stimulated pancreatic juice within the duodenum may yield similar diagnostic value in the determination of FPC risk, while avoiding invasive cannulation of the pancreatic duct, and the associated morbidity of acute pancreatitis. EUROPAC has ethical approval for the collection and analysis of pancreatic juice for molecular markers using the methodology described earlier in this thesis. The same technique for analysis of duodenal juice as a surrogate marker following secretin stimulation has received ethical approval as described above (Figure 6). As part of the work towards this thesis, a prospective cohort study of molecular markers in duodenal aspirate and pancreatic juice from individuals with pancreatic cancer, chronic pancreatitis and benign biliary disease controls was designed and approved.

4.12 Secretin stimulated duodenal juice sampling: provisional results from cohort of PDAC, CP and biliary controls

Recruitment of individuals from three cohorts: pancreatic cancer, chronic pancreatitis (CP) and benign biliary disease was undertaken. Secretin stimulated duodenal aspirate and pancreatic juice samples were collected from a total of 173 patients. The numbers recruited in each of the subgroups described is outlined in Table 8 below.

Table 8: Numbers recruited and median age

COHORT	TOTAL	MEDIAN AGE (Years)/ IQR	
Cancer	38	68	(62-75)
CP	23	57	(41-67)
Control	112	56	(57-78)

A total of 173 patients underwent sample collection and analysis. Analysis of duodenal aspirate and pancreatic juice for *TP53* and *K-RAS2* mutation and *CDKN2A* methylation yielded results as recorded in Table 9 below.

Table 9: Results of analysis of *TP53* mutation, *CDKN2A* methylation and *K-RAS2* mutation in pancreatic cancer, chronic pancreatitis and benign biliary controls

COHORT	NUMBER	*TP53* mutation (%)	*CDKN2A* methylation >12% (%)	*K-RAS2* mutation (%)
CANCER	38	2 (6%)	17 (50%)	14 (38%)
CP	23	0 (0%)	12 (52%)	10 (43%)
CONTROL	112	5 (4%)	48 (43%)	69 (62%)

Table 10: Sensitivity and specificity of molecular markers in duodenal aspirate in cancer and benign disease

	NUMBER	*TP53* mutation (%) (n = 162)	*CDKN2A* methylation >12% (%) (n = 164)	*K-RAS2* mutation (%) (n = 172)
MALIGNANT	38	2	17	14
BENIGN	135	5	60	79
***p* VALUE**	N/A	0.517	0.426	**0.049**
SENSITIVITY (95% CI)		0.06 (0.01-0.23)	0.41 (0.25-0.59)	0.40 (0.24-0.58)
SPECIFICITY (95% CI)		0.96 (0.91-0.99)	0.66 (0.57-0.74)	0.42 (0.34-0.51)

The results, as outlined in Table 10, demonstrate that the specificity and sensitivity of the detection of molecular markers in duodenal aspirate do not correlate with that of pancreatic juice collected by cannulation of the pancreatic duct at ERCP. Only analysis of wild type *K-RAS2* demonstrated an apparent trend in correlation nearing significance. On current evidence, secretin stimulated collection of duodenal aspirate is not sufficiently sensitive or specific to be utilised as a surrogate marker for risk stratification in high risk individuals. It is hypothesised that this may be due to the comparative high volume of junk DNA present within the duodenum, despite aspiration prior to collection. This effect may be exacerbated as a result of pH change within the duodenum compared to the comparatively alkaline pancreatic duct. Work by our group continues in the exploration of factors which may aid detection of early pancreatic cancer in high risk individuals. The aim of EUROPAC is in optimising the safety and efficacy of screening for pancreatic cancer in those at highest risk, whilst continuing efforts in the identification of candidate genes to definitively delineate FPC.

5. DISCUSSION

The aims of this thesis were in the identification of subpopulations of high risk kindreds at greatest risk, to offer targeted screening for early pancreatic cancer; and to ensure the safety of those individuals undergoing screening. I was able to achieve this aim through completion of a number of objectives outlined earlier in this work. My first objective was in the registration of families with an apparent or recognised autosomal dominant predisposition for the development of PDAC and obtaining full epidemiological data. A total of 1000 families have been recruited to the FPC and HP registries at the time of writing. The numbers of individuals and kindreds recruited during the period of work towards this thesis are demonstrated in Table 3 and Table 5. Data obtained through individual, family history and collaborating clinician questionnaires at the time of registration enabled me to achieve my second objective in the comparison of age of onset in sporadic pancreatic cancer with age of onset of cancer in high risk kindreds. In my analysis of the age of onset in affected family members I have shown that the previously proposed phenomenon of increased familial risk associated with early age of onset; as reported by Brune et al [270], may be artefactual. In Figures 12-19 of section 4.4.2, in the analysis of EUROPAC FPC kindreds, I have demonstrated that the apparent phenomenon of early age of onset affecting risk can be explained by ascertainment bias. The recognition of this is important in ensuring that screening is not offered inappropriately merely on the basis of young age of onset within kindreds or at an unduly young age. Thus, minimising exposure to the potential risks associated with screening for early pancreatic cancer. There does appear to be a caveat to this, and that is in the recognition of those families which display the phenomenon of anticipation. Previous work in the analysis of EUROPAC FPC kindreds has demonstrated that

onset can occur at increasingly earlier age in certain families, with a clear autosomal dominant pattern affecting successive generations [158]. This phenomenon of anticipation, i.e. progressively earlier onset across multiple successive generations, and a higher total number of affected family members, are both associated with increased familial risk. Therefore, the number affected, and age of onset in successive generations should be elicited in the stratification of risk. However, the presence of few, or isolated, early age of onset affected family members, does not confer any increase in risk; according to the findings from my work towards this thesis. In addition to risk stratification according to age, my analysis of data pertaining to lifestyle characteristics and other demographics in familial and sporadic PDAC fulfilled my third objective as stated earlier in this work. The influence of specific lifestyle factors on the development of sporadic PDAC has been extensively reported. My work towards risk stratification in FPC and HP kindreds included analysis of the influence of environmental risk factors which have been implicated in pancreatic carcinogenesis. There is established evidence, across many populations, to support a relationship between cigarette smoking and PDAC. Figures 20 and 21 of section 4.5 illustrate survival analysis according to smoking status in the EUROPAC FPC cohort. I determined that recall bias in the reporting of smoking status by sensitised, unaffected individuals creates an apparent trend of poorer survival within the smoking sub-group. This can be shown to be as a result of the over-reporting of smoking status as demonstrated in Figures 20 and 21. Through the collection and analysis of epidemiological data, I have been able to evaluate the influence of specific lifestyle factors upon cancer risk in both EUROPAC FPC and HP cohorts. In a cohort of EUROPAC HP patients, I analysed the effect of diabetes and smoking upon age of cancer development. In HP, the age of onset of diabetes has been demonstrated to exhibit an effect upon age of death from cancer. However, further analysis in sub-groups,

according to smoking status, reveal that any effect of diabetes is confounded by the overwhelming effect of smoking upon cancer risk in HP. These findings are highlighted in the survival analyses in Figures 7-10 in subchapter 4.3.3.

Evidence from the analysis of sporadic PDAC has consistently proposed an increase in risk with cigarette smoking. Analysis of other lifestyle factors in the implication of pancreatic carcinogenesis has failed to yield such conclusive evidence. Other proposed environmental risk factors for pancreatic carcinogenesis include obesity. With regard to the EUROPAC FPC cohort, obesity was not found to confer any significant influence upon risk as discussed in section 4.6. It is proposed that the recording of BMI over time can exhibit significant variation. Therefore, an isolated BMI measurement; reported at the time of registration as an FPC kindred is unlikely to be useful in the stratification of risk. Similarly, when considering diabetes as an aid to risk stratification; analysis of the FPC cohort only demonstrates a consistent relationship between diabetes and PDAC in one family. This is discussed more fully in section 4.7. Controversy has existed regarding the role of diabetes as cause or effect in sporadic pancreatic carcinogenesis, and its concomitant diagnosis does not appear to be a reliable risk modifier in FPC.

Through my recruitment of families to the EUROPAC registry I was able to contribute to fulfilling another of my thesis objectives in the investigation of genetic factors that could be used in stratification of risk within kindreds and in sporadic pancreatic cancer. Through individual and family registration, our group compiled epidemiological data to aid risk stratification and collected DNA and cell line samples. In association with their registration, our group has obtained DNA from 111 affected and 509 unaffected individuals, and cell line samples from 48 and 240 affected and unaffected individuals, following recruitment to the

FPC registry. The collection and storage of DNA and cell lines from kindreds with an apparent autosomal dominant predisposition to PDAC allows for ongoing work into potential candidate genes. The identification of novel mutations which may be implicated in FPC continues to be a key focus of the work undertaken by EUROPAC. During work towards this thesis, our group reported an association with a novel mutation in PALB2. In a series of 81 families, 3 PALB2 mutations were identified. This analysis is discussed in section 4.9.1 and Figure 22.

In genome wide association studies, an association between PDAC and the ABO gene locus has been reported [129]. In data collected from 232 sporadic cases treated in our unit, 101/232 (47%) of individuals were blood group A. However, it is difficult to draw significant conclusions from this due to the population and geographical dependence of ABO group distribution. Further work on larger Europe wide populations would be required to ascertain meaningful results. Work contributing towards the recognition of PALB2 and potential role of ABO fulfil my thesis objective of the investigation of genetic factors in risk stratification in FPC kindreds.

My final thesis objectives pertained to secondary screening for early pancreatic cancer in high risk groups. At the time of writing a total of 82 FPC and 38 HP individuals had been recruited to the respective EUROPAC screening studies for early PDAC as denoted in Table 7. The paramount aim of any screening strategy must first be to do no harm. Prior to my work in revising the EUROPAC screening protocol, a significant number of participants had suffered from post-ERCP pancreatitis with an associated morbidity and potential mortality. As part of this work, I helped to introduce a novel approach to the collection of pancreatic juice with the intention of reducing risks from screening. The use of Diclofenac and

pancreatic stenting reduced the incidence of ERCP-associated pancreatitis in our screening cohort (from 43.8% to 9.7% [*p*=0.020]). The results are discussed in section 4.10.3. The improvement of participant safety in our screening cohort was an important aim of this thesis; the significant reduction in procedure-associated morbidity demonstrates that I have achieved this objective. In further work towards the aim of improved participant safety, I also established a protocol for the collection and analysis of duodenal aspirate, as a proposed surrogate marker for pancreatic juice. The technique is discussed earlier in this thesis and results are highlighted in Tables 8-10 in section 4.12. It minimised the risk of ERCP related pancreatitis by negating the need for pancreatic duct cannulation. Although, results from the subset analysis of benign and malignant cases do not yet support a role for duodenal juice in the analysis of molecular markers in early pancreatic cancer; this thesis stands as a basis for expanding this study and as such achieves the aim of furthering patient safety and increasing screening yield.

In achieving my thesis objectives, we are now better able to understand the factors which influence penetrance in FPC. This has implications for risk stratification in FPC and hence, in the targeted screening of those at highest risk. In parallel, refinement of techniques utilised in the EUROPAC secondary screening study for early pancreatic cancer, reduce the exposure of asymptomatic individuals to procedure associated morbidity. The recruitment of high risk kindreds undertaken as part of this thesis has facilitated the collection of DNA and cell lines in affected and unaffected individuals with an apparent familial predisposition to PDAC. Work towards the identification of potential candidate genes continues in parallel to screening in high risk groups. Until the advent of the discovery of a causative gene for FPC, work continues into the stratification of risk and optimisation of EUROPAC secondary

screening modalities to ensure targeted screening of those at highest risk whilst ensuring minimal harm. This thesis has contributed to fulfilling this aim through the demonstration of demographic and lifestyle factors which influence risk and appropriate advent of screening. The identification of potential bias in age related risk in FPC is an important concept in ensuring that screening and any associated risks are not undertaken in young individuals in the absence of a genuine predisposition. Future work continues into further stratification of risk with the potential for the development of a risk stratification clinical interface and in the exploration of duodenal aspirate as a surrogate marker for pancreatic juice to minimise ERCP related morbidity and improve participant safety in secondary screening for early PDAC.

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