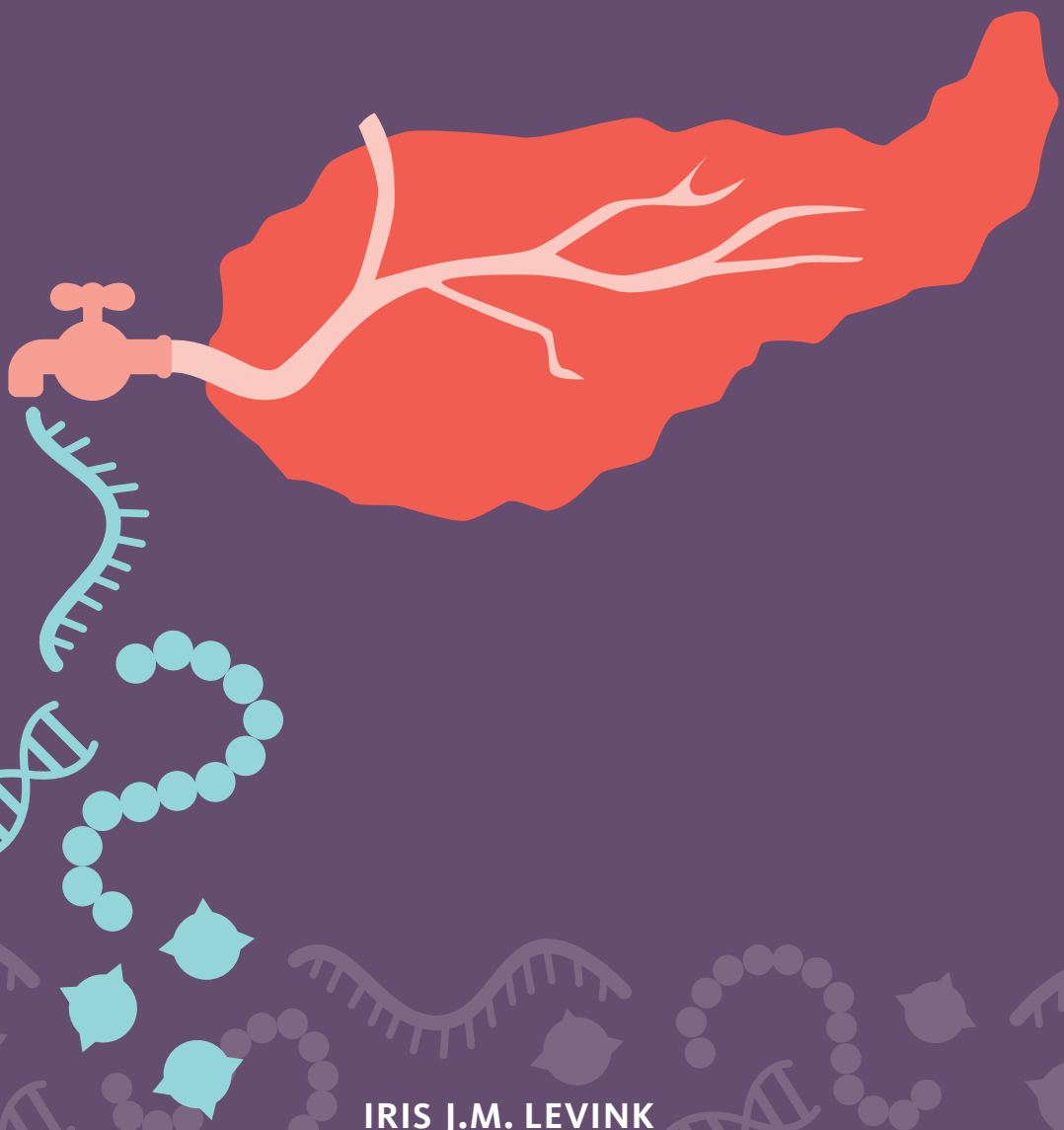
Early Detection of Pancreatic Cancer
the juicy details



IRIS J.M. LEVINK

Early Detection of Pancreatic Cancer
the juicy details

IRIS J.M. LEVINK

Early Detection of Pancreatic Cancer
the juicy details

Vroege opsporing van alvleesklierkanker
de sappige details

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof. dr. A.L. Bredenoord

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op

vrijdag 12 april 2024 om 13.00 uur

door

Iris Johanna Maria Levink
geboren te Vlijmen.

Erasmus University Rotterdam

Colofon

Early Detection of Pancreatic Cancer the juicy details

Design/Lay-out Proefschriftenbalie, Nijmegen
Print Ipskamp Printing, Nijmegen
ISBN 978-94-6473-398-3

The printing of this thesis was financially supported by: the Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center; Erasmus University Rotterdam; Nederlandse Vereniging voor Gasterenterologie; Endoss; Wassenburg Medical; Tramedico; 9Knots Solutions; Dr. Falk Pharma Benelux; Viatris; Ferring Pharmaceuticals; ABN AMRO; Chipsoft.

Promotiecommissie

Promotor
Prof. dr. M.J. Bruno

Overige leden
Prof. dr. B. Groot Koerkamp
Prof. dr. J. Mayerle
Prof. dr. J.P.H. Drenth

Copromotoren
Dr. D.L. Cahen
Dr. G.M. Fuhler

Paranimfen
Mw. F.L. Taihuttu
Dr. S.E.M. Van de Ven

Table of Contents

PART I

Introduction

CHAPTER 1

General introduction

Pancreatic cancer (PC) is a lethal disease for which incidence (3000 cases per year in The Netherlands) closely parallels mortality, due to late presentation of symptoms and lack of effective therapy for late-stage disease.[1] The classical presentation of PC is jaundice, which is caused by bile duct obstruction. For diagnosis, both an endoscopic ultrasound (EUS) and conventional tomography (CT)-scan are performed. During EUS, an endoscope containing a white-light camera and ultrasound device is orally inserted into the duodenum. This allows the gastroenterologist to search the pancreas for abnormalities and draw targeted biopsies (also known as fine-needle biopsy [FNB]) of suspicious lesions to establish a histopathological diagnosis. A CT-scan is used to evaluate the resectability; in case of >90° venous involvement, arterial involvement or distant metastases, surgical resection cannot be performed. Only 20% of patients are eligible for surgery at presentation,[2] while this provides the only chance of cure. Overall, 5% of individuals with PC survives five years after diagnosis,[3,4] whilst for individuals with (borderline) resectable disease this percentage is 15%.[5]

Premalignant lesions

PC follows from a series of genetic mutations and subsequent cellular events that together increase cell survival and inhibit cell death (**Figure 1**). This gradual process takes years from the first mutation to development of low-grade dysplasia (LGD), high-grade

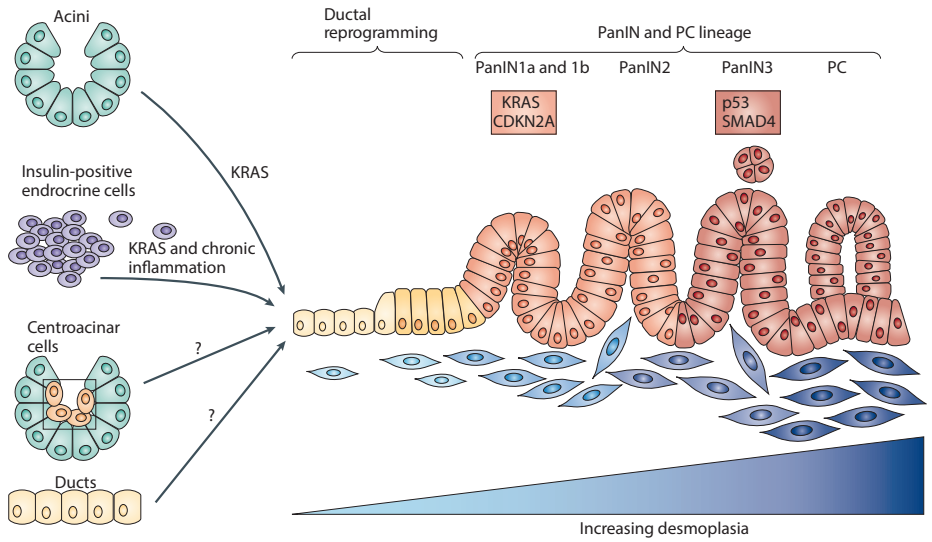


FIGURE 1 | Graphical overview of the gradual process from normal cells to ductal reprogramming (metaplasia), low-grade PanIn (also known as PanIN-1 and PanIN-2), high-grade PanIN (also known as PanIN-3) and PC with prevalent genetic mutations. A *KRAS* mutation (or *BRAF* mutation in 5% of individuals) is generally an early oncogenic event, followed by mutations in *TP53* and *SMAD4*. This image was adopted with approval from Moris *et al*. (2010). [12]

dysplasia (HGD) and, eventually, PC. Three premalignant (or 'neoplastic') lesions of PC can be identified: pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm (IPMN) and mucinous cystic neoplasm (MCN).

PanIN is a microscopic (<5mm) lesion that can occur in any part of the pancreas. It is the most common premalignant pancreatic lesion and is invisible on imaging. Features like lobular atrophy of parenchyma and chronic pancreatitis-like changes can raise suspicion for the presence of PanIN,[6,7] yet are not specific enough to warrant further diagnostic tests. For this reason, the precise prevalence in the general population is unknown.[8] An autopsy study[9] in 173 individuals without evidence of PC or IPMN (mean age 80 years) found high-grade PanIN (also known as PanIN-3) in 4% of individuals and low-grade PanIN (PanIN-1 and PanIN-2) in 77% and 28%, respectively. PanIN prevalence and its risk to become invasive is known to increase with age and the presence of diabetes mellitus.[10,11]

Intraductal papillary mucinous neoplasms (IPMN) are caused by growth of mucin-producing cells in the main or side-ducts of the pancreas, and are visible on imaging as a cystic dilation of these ducts of more than 5mm. The presence of IPMN is usually multifocal. The short-term risk of PC in individuals with IPMN is approximately 3%.[13] Three subtypes are identified, each with a distinct risk of malignancy: 1. Main-duct IPMN (MD-IPMN) carries the highest risk of malignancy, accounts for 15-21% of IPMNs, and is recognized by dilation of the main pancreatic duct; 2. Branch-duct IPMN (BD-IPMN) has the lowest risk of malignant progression, accounts for 41–64% of IPMNs and is identified by dilated side-branches; 3. Mixed-type IPMN (MT-IPMN) is a combination of the two and is seen in 22– 38% of individuals with IPMN.[14-17] Both the prevalence and risk of malignant progression of IPMNs increases with age.

MCN is a solitary cystic lesion that occurs predominantly in the pancreatic body or tail of females between 40 and 50 years old.[18] By definition, these lesions do not communicate with the pancreatic duct and contain ovarian-type stroma. At the time of resection, approximately 13% are malignant.[18] Surgical resection with negative margins is curative for non-invasive MCN. Therefore, pancreatic surgery is recommended in all individuals with MCN17. After resection, no further surveillance is needed.[17]

Pancreas surveillance

Currently, the general population is being screened for breast, colorectal and cervical cancer. This screening is effective, as these cancers have a high incidence, can be detected with a reliable non-invasive test, and curative treatment is available. This is not the case for PC, which has a low incidence, and requires either an invasive endoscopic procedure or costly MRI to detect treatable premalignant or early-malignant stages. Both modalities have a low diagnostic performance for the differentiation between no dysplasia, LGD, HGD and PC. Furthermore, treatment requires pancreatic surgery, which has an 8% morbidity and 3% mortality[19] and is not always curative due to multifocal (for PanIN or IPMN) or early spread of the disease (for PC).

In The Netherlands, the yield of pancreas surveillance is being investigated in two groups of individuals at increased risk of developing PC; those with a suspected neoplastic pancreatic cyst (IPMN and MCN), as part of the PACYFIC-registry (www.pacyfic.net), and those with a hereditary increased risk ('familial pancreatic cancer'), as part of the FPC-study (www.caps-registry.com). Pancreas surveillance aims to detect early-staged PC, or preferably HGD, in order to increase quality of life and survival. However, its (cost-) effectiveness has not yet been established. For instance, for IPMN, the number of surveillance endoscopies needed to find one individual with HGD or PC has not been determined, whereas thousands of imaging procedures are being performed yearly to find the ±3% that develops PC. Additionally, unnecessary surgery for benign lesions is common (unpublished data from PACYFIC cohort), causing harm by overtreatment.[20] Conversely, individuals often develop irresectable PC in between surveillance visits.[20]

Pancreatic cysts

For individuals with a suspected IPMN (or resected IPMN), lifelong surveillance is currently recommended by the European guidelines.[17] Surveillance is performed by EUS or MRI and focuses on the presence of absolute indications (positive cytology for HGD/PC, solid mass, jaundice, enhancing mural nodules ≥5mm, main pancreatic duct dilation ≥10mm) or relative indications for surgery (cyst diameter ≥40mm, cyst growth ≥5mm/year, enhancing mural nodules <5mm, main pancreatic duct dilation of 5-9mm, CA19.9 blood level ≥37kU/L, new-onset diabetes mellitus, acute pancreatitis). If an individual has no indications for surgery, a new visit is planned after 6 months (in the first year) to 12 months (thereafter); in case of one absolute indication or two (or more) relative indications, surgery should be considered by a multidisciplinary team.

Hereditary increased risk of pancreatic cancer

Approximately 10% of all PC cases occur in the background of familial clustering. In the Erasmus MC, Amsterdam UMC and Utrecht UMC, surveillance of individuals with an estimated ≥10% lifetime risk of developing PC ('familial pancreatic cancer' [FPC]) is performed in a research setting. This group encompasses individuals with hereditary cancer syndromes: 1. Peutz-Jeghers syndrome (*LKB1*; relative risk [RR] 76-132);[21] 2. Familial cutaneous malignant melanoma (FAMM) syndrome (*CDKN2A*; RR 15-52);[22-24] 3. Hereditary Breast and Ovarian Cancer syndrome (*BRCA1*, *BRCA2* or *PALB2*; RR 2-6); [25,26] 4. Lynch syndrome (MLH1, MSH2 and MSH6; RR 8);[27] 4. Ataxia telangiectasia syndrome (*ATM*; RR unknown); 5. Li-Fraumeni syndrome (*TP53*; RR 8).[28] Surveillance is performed yearly by EUS, starts at the age of 40 (FAMM and Peutz-Jeghers syndrome) or 50 years, and takes to the age of 75 years. Until recently, this cohort also included individuals without a detected gene mutation. However, recent results from the FPC-study[20] have

shown that the risk of PC in this group is lower than expected and does not fulfill criteria for surveillance.

Biomarkers

So far, detection of HGD and early-staged PC in individuals undergoing surveillance has been challenging, even when MRI and EUS are combined[20]. Thus, biomarkers, able to detect sub-centimeter lesions and establish their risk of malignancy, are urgently needed to complement (or replace) imaging. In addition, after diagnosis of PC, biomarkers may enable tumor characterization and treatment response prediction. During cancer development, molecules and particles – like DNA and RNA fragments, exosomes and proteins – are constantly released by cancer cells. These end up in the surrounding tissue surrounding and eventually in cyst fluid, pancreatic juice (PJ) and blood, and can therefore serve as biomarkers.

Biomarker sources

For many cancer types, tissue is the main source for characterization. While biopsy of a solid pancreatic lesion can be performed real-time during EUS with good performance for PC detection (accuracy 87%),[29] it relies on the presence of a visible lesion and is therefore not able to diagnose microscopic PC. Also, recurrent biopsies are discommended, as they are associated with complications like bleeding (0.3%) and acute pancreatitis (0.3%). [29] Furthermore, FNB risks providing information from a single cancer clone, whereas different clones (with distinct molecular changes) may be present (as cancer cells evolve over time). In addition, the low yield (cellularity) precludes complementary laboratory testing.

Blood can be collected relatively non-invasively by venipuncture. Identification of a highly specific marker would shift surveillance from repetitive imaging to blood monitoring. Also, blood sampling would likely provide information on all tumor clones. Unfortunately, detected aberrations in blood are non-specific to the pancreas and, for surveillance purposes, the concentration of informative molecules (*e.g.*, DNA fragments) scattered by PC is low, as compared to other cancers (potentially due to severe fibrosis, low cellularity and low mutational burden).

PJ may serve as an alternative biomarker source. It can be collected from the duodenal lumen during endoscopy after intravenous infusion of secretin. Secretin is a hormone that is produced by the S cells of the duodenum in response to a PH drop induced by food ingestion. This hormone stimulates the bicarbonate production from the ductal cells of the pancreas, as well as its release – together with pancreatic enzymes – into the duodenum. With the increasing availability of synthesized (human) secretin came the possibility to create a wash-out of PJ and collect it non-invasively. As compared to tissue

sampling by FNB, PJ from the duodenal lumen is less invasive, does not rely on a visible mass and is expected to consist of information on the complete range of tumor clones.[30,31] As compared to blood, biomarkers determined in PJ are expected to be more pancreas-specific. However, the use of PJ as biomarker source is challenged by the presence of enzymes (*e.g.*, trypsin, lipase, amylase, Desoxyribonuclease [DNase], ribonuclease [RNase]) that may digest any present markers.

Summary

In summary, PC is a lethal disease, for which early detection seems the only chance of cure. Surveillance of individuals at risk of developing PC due to hereditary predisposition or neoplastic cysts may increase quality of life and survival for those developing PC. So far, surveillance by imaging has not lived up to expectations. As the development of PC takes years due to an accumulation of molecular changes, it is expected that biomarkers may enable earlier detection of sub-centimeter cancer. PJ is a promising biomarker source as it is a wash-out from the ductal system from which PC originates. However, due to the current lack of research in the field, PJ collection has not (yet) been implemented into clinical practice.

References

1 Siegel RL, Miller KD and Jemal A. Cancer statistics, 2019. *CA Cancer J Clin* 2019; 69: 7-34.
2 Versteijne E, Suker M, Groothuis K, *et al*. Preoperative Chemoradiotherapy Versus Immediate Surgery for Resectable and Borderline Resectable Pancreatic Cancer: Results of the Dutch Randomized Phase III PREOPANC Trial. *J Clin Oncol* 2020; 38: 1763-1773. 2020/02/28.
3 De Angelis R, Sant M, Coleman MP, *et al*. Cancer survival in Europe 1999–2007 by country and age: results of EUROCARE-5—a population-based study. *The Lancet Oncology* 2014; 15: 23-34.
4 Nederlandse Kankerregistratie (NKR), IKNL. Verkregen via nkr-cijfers.iknl.nl op 6-8-2022.
5 Latenstein AEJ, van Roessel S, van der Geest LGM, *et al*. Conditional Survival After Resection for Pancreatic Cancer: A Population-Based Study and Prediction Model. *Annals of Surgical Oncology* 2020; 27: 2516-2524.
6 Aimoto T, Uchida E, Nakamura Y, *et al*. Multicentric pancreatic intraepithelial neoplasias (PanINs) presenting with the clinical features of chronic pancreatitis. *J Hepatobiliary Pancreat Surg* 2008; 15: 549-553. 2008/10/07.
7 Brune K, Abe T, Canto M, *et al*. Multifocal neoplastic precursor lesions associated with lobular atrophy of the pancreas in patients having a strong family history of pancreatic cancer. *Am J Surg Pathol* 2006; 30: 1067-1076. 2006/08/26.
8 Oda Y, Aishima S, Morimatsu K, *et al*. Pancreatic intraepithelial neoplasia in the background of invasive ductal carcinoma of the pancreas as a prognostic factor. *Histopathology* 2014; 65: 389-397. 2014/06/17.
9 Matsuda Y, Furukawa T, Yachida S, *et al*. The Prevalence and Clinicopathological Characteristics of High-Grade Pancreatic Intraepithelial Neoplasia: Autopsy Study Evaluating the Entire Pancreatic Parenchyma. *Pancreas* 2017; 46: 658-664. 2017/02/15.
10 Peters MLB, Eckel A, Mueller PP, *et al*. Progression to pancreatic ductal adenocarcinoma from pancreatic intraepithelial neoplasia: Results of a simulation model. *Pancreatology* 2018; 18: 928-934. 2018/08/26.
11 Matsuda Y. Age-related morphological changes in the pancreas and their association with pancreatic carcinogenesis. *Pathol Int* 2019; 69: 450-462. 2019/07/25.
12 Morris JPt, Wang SC and Hebrok M. KRAS, Hedgehog, Wnt and the twisted developmental biology of pancreatic ductal adenocarcinoma. *Nat Rev Cancer* 2010; 10: 683-695.
13 Oyama H, Tada M, Takagi K, *et al*. Long-term Risk of Malignancy in Branch-Duct Intraductal Papillary Mucinous Neoplasms. *Gastroenterology* 2020; 158: 226-237.e225.
14 Crippa S, Fernández-Del Castillo C, Salvia R, *et al*. Mucin-producing neoplasms of the pancreas: an analysis of distinguishing clinical and epidemiologic characteristics. *Clin Gastroenterol Hepatol* 2010; 8: 213-219. 2009/10/20.
15 Lee SY, Lee KT, Lee JK, *et al*. Long-term follow up results of intraductal papillary mucinous tumors of pancreas. *J Gastroenterol Hepatol* 2005; 20: 1379-1384. 2005/08/18.
16 Hwang DW, Jang JY, Lee SE, *et al*. Clinicopathologic analysis of surgically proven intraductal papillary mucinous neoplasms of the pancreas in SNUH: a 15-year experience at a single academic institution. *Langenbecks Arch Surg* 2012; 397: 93-102. 2010/07/20.

17 The European Study Group on Cystic Tumours of the P. European evidence-based guidelines on pancreatic cystic neoplasms. *Gut* 2018; 67: 789.
18 Postlewait LM, Ethun CG, McInnis MR, *et al*. Association of Preoperative Risk Factors With Malignancy in Pancreatic Mucinous Cystic Neoplasms: A Multicenter Study. *JAMA Surgery* 2017; 152: 19-25.
19 Buscail E, Cauvin T, Fernandez B, *et al*. Intraductal papillary mucinous neoplasms of the pancreas and European guidelines: importance of the surgery type in the decision-making process. *BMC Surg* 2019; 19: 115. 2019/08/24.
20 Overbeek KA, Levink IJM, Koopmann BDM, *et al*. Long-term yield of pancreatic cancer surveillance in high-risk individuals. *Gut* 2022; 71: 1152-1160. 2021/04/07.
21 Giardiello FM, Brensinger JD, Tersmette AC, *et al*. Very high risk of cancer in familial Peutz-Jeghers syndrome. *Gastroenterology* 2000; 119: 1447-1453. 2000/12/13.
22 de Snoo FA, Bishop DT, Bergman W, *et al*. Increased risk of cancer other than melanoma in CDKN2A founder mutation (p16-Leiden)-positive melanoma families. *Clin Cancer Res* 2008; 14: 7151-7157. 2008/11/05.
23 Goldstein AM, Chan M, Harland M, *et al*. High-risk melanoma susceptibility genes and pancreatic cancer, neural system tumors, and uveal melanoma across GenoMEL. *Cancer Res* 2006; 66: 9818-9828. 2006/10/19.
24 Goldstein AM, Struewing JP, Fraser MC, *et al*. Prospective risk of cancer in CDKN2A germline mutation carriers. *J Med Genet* 2004; 41: 421-424. 2004/06/03.
25 Cancer risks in BRCA2 mutation carriers. *J Natl Cancer Inst* 1999; 91: 1310-1316.
26 van Asperen CJ, Brohet RM, Meijers-Heijboer EJ, *et al*. Cancer risks in BRCA2 families: estimates for sites other than breast and ovary. *J Med Genet* 2005; 42: 711-719. 2005/09/06.
27 Kastrinos F, Mukherjee B, Tayob N, *et al*. Risk of pancreatic cancer in families with Lynch syndrome. *Jama* 2009; 302: 1790-1795. 2009/10/29.
28 Ruijs MW, Verhoef S, Rookus MA, *et al*. TP53 germline mutation testing in 180 families suspected of Li-Fraumeni syndrome: mutation detection rate and relative frequency of cancers in different familial phenotypes. *J Med Genet* 2010; 47: 421-428. 2010/06/05.
29 van Riet PA, Larghi A, Attili F, *et al*. A multicenter randomized trial comparing a 25-gauge EUS fine-needle aspiration device with a 20-gauge EUS fine-needle biopsy device. *Gastrointest Endosc* 2019; 89: 329-339. 2018/10/28.
30 Choi MH, Mejlænder-Andersen E, Manueldas S, *et al*. Mutation analysis by deep sequencing of pancreatic juice from patients with pancreatic ductal adenocarcinoma. *BMC Cancer* 2019; 19: 11.
31 Levink IJM, Nesteruk K, Visser DI, *et al*. Optimization of Pancreatic Juice Collection: A First Step Toward Biomarker Discovery and Early Detection of Pancreatic Cancer. *Am J Gastroenterol* 2020; 115: 2103-2108. 2020/10/27.

CHAPTER 2

Outline of this thesis

The aim of this thesis is to evaluate the current practice of pancreatic cyst surveillance, validate existing biomarkers for detection of pancreatic cancer (PC), and explore novel biomarkers for its earlier detection. To do so, we critically appraise current cyst surveillance guidelines and externally validate the role of two relative indications for surgery – cyst size and serum carbohydrate antigen 19.9 (CA19.9) – for the detection of PC in a pancreatic cyst surveillance cohort (**PART I**). Secondly, we explore potential serum and pancreatic juice (PJ) biomarkers (in a case-control context; **PART II**).

PART I: pancreas surveillance: the current practice

Intraductal papillary mucinous neoplasms (IPMNs) are increasingly discovered on imaging studies performed for unrelated conditions. Different consensus-based surveillance guidelines have been published (in the absence of evidence) showing distinct discrepancies in management strategies. These strategies are described and critically appraised in **Chapter 3**. One of the described guidelines is the European guidelines on pancreatic cystic neoplasms.[1] In contrast to the international Fukuoka guidelines,[2] these guidelines do not stratify surveillance frequency by size, whereas repetitive imaging of (mostly) small pancreatic cysts imposes a substantial burden on health care recourses. In **Chapter 4**, the PACYFIC dataset is used to define cyst sizes with a diminutive risk of developing high-grade dysplasia (HGD) and PC ('trivial cysts'). For such 'trivial cysts' at low risk of developing HGD or PC, a less intensive follow-up regime may suffice.

Other features may overestimate the risk of PC and cause harm by unnecessary surgery. A CA19.9 level of >37 kU/L has recently added to the European guidelines as relative indication for surgery.[1] This means that surgery should be considered in case of an increased CA19.9 value in conjunction with a second relative indication for surgery. However, this recommendation was based on retrospective surgical studies, and the role of CA19.9 monitoring in a surveillance population has not been investigated before in a prospective cohort. **Chapter 5** evaluates the performance of CA19.9 monitoring for the detection of PC in the PACYFIC cohort, and evaluates the effect on cyst management.

PART II: Potential novel biomarkers and pancreatic juice

In currently available literature, different types of biomarkers for detection of PC have mostly been investigated in blood and cyst fluid. PJ is a promising biomarker source, as it is a wash-out of the ductal system from which PC originates. In this thesis, we desired to collect PJ from our two high-risk cohorts (FPC- and PACYFIC study) and from individuals who undergo endoscopic ultrasound (EUS) for suspected PC (KRASPanc-study). At first, we aimed for non-invasive collection with the highest yield of biomarkers. In **Chapter 6**, to set up a comprehensive pipeline for PJ collection, we evaluated the optimal duration (4 *vs* 8 *vs* 15 minutes) and methodology (performing suction by the biopsy channel with *vs* without a catheter that is positioned close to the papillary orifice) by comparing the

yield of promising biomarkers (proteins, exosomal microRNA and DNA) and cell-derived organoid growth between these durations and methods.

Subsequently, the best performing methodology and duration of collection was used to collect PJ samples in order to evaluate the performance of potential biomarkers. Based on previous literature, distinct mucins and cytokines show potential as biomarkers. Mucins are heavily glycosylated proteins, that are present on the apical surface of healthy epithelial cells in the pancreatic duct. Different types of mucins are shown to be overexpressed in PC and its precursors (*e.g.*, PanIN and IPMN). Cytokines are small proteins (±20kDa) that play an important role in the interaction between cells. Cytokines, released by cancer cells, can have immune-suppressive effects (such as stimulation of anti-inflammatory cytokines, activation and maturation of inhibitory lymphocytes, among others), as well as tumor-promoting properties (such as cell proliferation, angiogenesis, metastasis). The performance of these proteins in serum and PJ for the differentiation between cases and controls are evaluated in **Chapter 7**.

One potential bloodserum-based biomarker that is currently under investigation is the glycosylation pattern of proteins. Glycosylation of proteins constitute a post-translational modification in which sugar side-chains are attached to the protein backbone. This in turn has a profound structural and functional effect on a protein. Vreeker *et al.*[3] reported that *N*-glycome analysis – the analysis of all glycosylation traits connected to the amino acid asparagine – in serum allowed differentiation of patients with PC from healthy controls. Three so-called derived glycosylation traits ('antennarity', 'sialylation', and 'fucosylation') were sufficient to identify cancer cases with an area-under-the-curve of 0.81–0.88. In **Chapter 8**, this same pipeline was used to evaluate the abundance of glycosylation traits in consecutively collected serum samples of individuals with a hereditary increased risk of PC undergoing surveillance (as part of the FPC-study). The aim of this study was to evaluate if changes in glycosylation over time are indicative of the presence of PC and predictive for future cancer development.

In **Chapter 9**, another group of markers is described: microRNAs (miRNAs). These are non-coding small RNAs with a length of ±19-24 nucleotides that play a major role in carcinogenesis by regulation of the protein expression and have previously shown promise for the detection of PC.[4] Of particular interest in PJ are those miRNAs that have been packaged in exosomes, as these are expected to protect miRNA from the enzymes (RNase) present in the PJ.[5] In this chapter, we evaluated the diagnostic performance of exosomal miRNAs in PJ. These outcomes raised suspicion that features of the transporting exosomes may differ between cases and controls. These features were further evaluated in **Chapter 10**.

One commonly investigated group of markers in PJ is based on alterations of circulating tumor DNA (ctDNA) molecules. These are short DNA fragments released into body fluids after apoptosis and necrosis of (tumor) cells. In ctDNA from plasma, increased

ctDNA levels and detection of (new) gene mutations have been associated to cancer progression. In current literature, molecular analysis ctDNA of PJ has shown promise. **Chapter 11** is a meta-analysis and systematic review of gene alterrations that have previously been investigated in PJ: gene mutations and methylation patterns. One of the most commonly mutated (onco) genes is *KRAS*, which is detected in >90% of PC cases as the driver to malignant development. A mutation in this oncogene – generally at amino acid positions G12, G13 or Q61 – result in sustained KRAS activity and persistent activation of downstream signaling pathways causing cell survival and proliferation. Gene methylation is a chemical modification of DNA, which causes epigenetic change of expression of a gene. For instance, *CDKN2A, TP53* and *SMAD4* are tumor suppressor genes that are commonly inactivated in PC, hypermethylation can cause their silencing and subsequent malignant development. PJ is expected to harbor high concentrations of ctDNA due to its close contact with the ductal system from which PC originates. ctDNA in plasma, on the other hand, may be highly diluted. **Chapter 12** compares PJ and plasma as biomarker sources for molecular analysis of these biomaterials for the detection of PC.

Lastly, chromosomal instability is a common molecular feature in human cancers and is characterized by loss of a tumor suppressor gene or amplification of an oncogene, which drive oncogenic signaling. Clinical testing for chromosomal instability during pregnancy is currently performed by noninvasive prenatal testing (NIPT), based on shallow whole-genome sequencing of maternal and fetal cell-free DNA (cfDNA). While a NIPT-test aims to diagnose chromosomal aberrations in fetal DNA, accidently, different chromosomal patterns in maternal DNA were also detected, raising suspicion of maternal malignancy.[6,7] This suggests that this technique may also be used to identify cancer in non-pregnant individuals. In **Chapter 13**, the feasibility and diagnostic performance of chromosomal instability testing for PC using this NIPT-pipeline on PJ was investigated.

References

1 The European Study Group on Cystic Tumours of the P. European evidence-based guidelines on pancreatic cystic neoplasms. *Gut* 2018;67:789.

2 Tanaka M, Fernández-del Castillo C, Kamisawa T, *et al*. Revisions of international consensus Fukuoka guidelines for the management of IPMN of the pancreas. *Pancreatology* 2017;17:738-753.

3 Vreeker GCM, Hanna-Sawires RG, Mohammed Y, *et al*. Serum *N*-Glycome analysis reveals pancreatic cancer disease signatures. *Cancer Med* 2020;9:8519-8529.

4 Szafranska AE, Davison TS, John J, *et al*. MicroRNA expression alterations are linked to tumorigenesis and non-neoplastic processes in pancreatic ductal adenocarcinoma. *Oncogene* 2007;26:4442-52.

5 Nakamura S, Sadakari Y, Ohtsuka T, *et al*. Pancreatic Juice Exosomal MicroRNAs as Biomarkers for Detection of Pancreatic Ductal Adenocarcinoma. *Ann Surg Oncol* 2019;26:2104-2111.

6 Amant F, Verheecke M, Wlodarska I, *et al*. Presymptomatic Identification of Cancers in Pregnant Women During Noninvasive Prenatal Testing. *JAMA Oncol* 2015;1:814-9.

7 Bianchi DW, Chudova D, Sehnert AJ, *et al*. Noninvasive Prenatal Testing and Incidental Detection of Occult Maternal Malignancies. *JAMA* 2015;314:162-169.

PART II

Pancreas surveillance: the current practice

CHAPTER 3

Management of Intraductal Papillary Mucinous Neoplasms: Controversies in Guidelines and Future perspectives

Authors
Iris J.M. Levink, Marco J. Bruno, Djuna L. Cahen

Current Treatment Options in Gastroenterology, 16 (2018): 316-332.

Abstract

Purpose of this review: The management of intraductal papillary mucinous neoplasm (IPMN) is currently based on consensus, in the absence of evidence-based guidelines. In recent years, several consensus guidelines have been published, with distinct management strategies. In this review, we will discuss these discrepancies, in order to guide treating physicians in clinical management.

Recent findings: The detection rate of pancreatic cysts has increased substantially with the expanded use of high-quality imaging techniques to up to 45%. Of these cysts, 24-82% are IPMNs, which harbor a malignant potential. Timely detection of high-risk lesions is therefore of great importance. Surgical management is based on the presence of clinical and morphological high-risk features, yet the majority of resected specimens appear to have a low risk of malignant progression.

Summary: International collaboration and incentive large scale prospective registries of individuals undergoing cyst surveillance are needed to accumulate unbiased data and develop evidence-based guidelines. Additionally, the development of non-invasive, accurate diagnostic tools (*e.g.*, biomarkers) is needed to differentiate between neoplastic and non-neoplastic pancreatic cysts and detect malignant transformation at an early stage (preferably at time of high-grade dysplasia).

Introduction

Intraductal papillary mucinous neoplasm (IPMN) is a pancreatic cystic lesion originating from intraductal growth of mucin producing cells. In 1980, Ohhashi *et al*.[1] were the first to describe IPMN. In 1996, it was recognized as a separate entity.[2,3] The increased detection and awareness of IPMNs led to the development of several, mainly consensus-based, periodically-revised national and international guidelines.[4-10] Wherein, notably, evidence is mainly based on surgical cohorts and information on patients that are managed conservatively is limited.

Classification of IPMN

Based on localization and extent, three subtypes can be identified; main-duct (MD-IPMN), branch-duct (BD-IPMN) and mixed-type IPMN (MT-IPMN). Every subtype exhibits a certain risk of malignancy and requires a specific therapeutic approach.

MD-IPMN can be recognized as (segmental or diffuse) dilation of the main pancreatic duct (MPD) of >5 mm. Other causes of ductal obstruction should be ruled out. It accounts for 15-21% of the IPMNs and is mostly located in the pancreatic head (64-67%).[11-13] Of the different subtypes, MD-IPMN has the highest risk to exhibit malignant disease (28-81%).[10, 12-20] Therefore, a MPD diameter ≥10 mm is considered an absolute indication for surgical resection.[10, 21]

BD-IPMN is defined as a grape-like cyst (>5 mm) that communicates with the MPD.[12, 13] It accounts for 41-64% of IPMNs and can develop multifocally throughout the pancreas, with a preference for the uncinate process.[11, 12] BD-IPMNs have the least risk of malignant progression (7-42%), yet their multifocality (40%) and high post-surgical recurrence rate (7-8%) are insidious. Interestingly, it has not been proven that multifocality increases the risk of malignancy.[10, 12-20, 22, 23] The indication for surgical resection depends on the presence of high-risk clinical and morphological features.[6, 10]

MT-IPMN meets both criteria of MD- and BD-IPMN and is seen in 22-38% of cases, of which 20-65% are malignant.[12, 13, 15, 16, 18, 19, 24-26] The therapeutic approach is the same as for MD-IPMN6.[10] Potential overlap between these subtypes should be taken into account, since 29% of patients with BD-IPMN appear to have MPD involvement after resection.[22]

IPMN is also classified according to its cellular morphology as gastric, intestinal, pancreatobiliary or oncocytic type. This classification is based on mucin expression, architecture and cytology, yet different subtypes can be seen in the same cyst. Each type exhibits a particular risk of malignancy (**Table 1**).

TABLE 1 | Classification of IPMN based on cellular morphology (data from surgical series).[27-29]

	Gastric type	Intestinal type	Pancreatobiliary type	Oncocytic type
Morphology	Thick finger-like papillae	Villous papillae	Complex thin branching papillae	Complex thick papillae with eosinophilic oncocytic cells
Mucin expression				
MUC 1	-	-	+	-/+
MUC 2	-	+	-	-/+
MUC 5AC	+	+	+	+
MUC 6	+	-/+ (weak)	-/+	-
Percentage of IPMNs	46-63%	18-36%	7-18%	1-8%
Location				
Head	69-72%	64-67%	63-67%	25-33%
Body or tail	28-31%	33-37%	34-37%	67-75%
Main-duct involvement	19%	63%	50%	38%
Invasive progression	10%	40%	68%	50%
Type of cancer	Tubular (79%)	Colloid > tubular	Tubular (82%)	Tubular > colloid
Mural nodules	30%	56%	57%	100%
Recurrence rate	9%	20%	46%	14%
5-year survival	85%	85%	54%	79%

IPMN = intraductal papillary mucinous neoplasm

Risk factors for malignant transformation

Both the risk of IPMN development and malignant degeneration increase with age. [12, 15, 17, 19, 20, 30] The mean age at time of IPMN detection is 65 years. There is a small male gender predisposition.[12, 19, 20] Also, lifestyle is of influence, as smoking and alcohol abuse increase the risk of having high-risk and worrisome features.[11, 31] Increased BMI and the associated presence of abdominal fat are known to play a role in the development of other pancreatic diseases (*e.g.*, type-2 diabetes mellitus [DM] and PC (PC)), due to fatty infiltration and inflammation.[32,33] Yet knowledge about the relation between abdominal fat, IPMN and malignant transformation is limited. Sturm *et al.* (2013)[34] found a relation between severe obesity (BMI≥35) and an increased risk of malignant transformation in IPMN (OR 10.1, 95% CI 1.30-78.32). [31,35] There is a causative link between IPMN and DM. 10-45% of patients with IPMN have diabetes[11-14, 16, 19, 31, 36, 37] and in case of diabetes, the risk of detecting IPMN is higher (OR 1.79; 95% CI 1.08-2.98),[35] especially in case of insulin-use (OR 6.03, 95% CI 1.74-20.84).[35] In reverse, the presence of DM is associated

with a higher risk of HGD (OR 2.02, 95% CI 1.02–4.01) and carcinoma (OR 2.05, 95% CI 1.08–3.87)[38]. Additionally, patients with chronic pancreatitis have an increased risk of IPMN (OR 10.1, 95% CI 1.30-78.32). [31,35]

Furthermore, having a family history of PC or another hereditary risk may pose a threat. Capurso *et al.* (2013)[35] compared 390 patients with IPMN with matched controls and found that 5.5% of the patients with IPMN and just 1.6% of the healthy controls had a 1st degree family member with PC (OR 2.94 95% CI 1.17-7.39 p 0.022).[31] It is unknown whether patients with a positive family history have a more rapid progression. Thus, the management (surveillance and treatment), advised by clinical guidelines, is the same as patients with sporadic IPMN.[10] The Fukuoka guideline, however, recommends surveillance at 6-months' intervals in patients with a positive family history with operated IPMN.[6]

Diagnosis

Symptoms

Most patients with IPMN are asymptomatic. Symptoms are associated with more advanced and invasive disease. Jaundice and abdominal pain are associated with invasive disease in 80 and 77% of IPMN cases, respectively. 13-32% of patients with IPMN are reported to present with secondary acute (recurrent) pancreatitis, although this incidence is based on surgical series and likely to be overestimated. Other symptoms are weight loss, new-onset diabetes, steatorrhea and back pain.[11-15, 17-20, 30, 31, 37, 39-42]

Imaging techniques

Currently, cross-sectional imaging plays a main role in lesion detection and differentiation. MRI (including MRCP) is the modality of choice, because of its superiority in the identification of MPD connectivity, mural nodules and septation,[6-8, 10] as well as cyst differentiation[43] (**Figure 1**). Additionally, the repetitive nature of cyst follow-up mandates a non-invasive modality to eliminate radiation exposure.[6,10] However, for identification of calcifications, tumor staging or surveillance of PC recurrence, addition of CT is recommended by some.[10] Secretin injection during MRCP increases the likelihood of visualizing MPD communication, yet only by 5%. More studies are needed to determine whether the addition of secretin outweighs costs and prolongation of scanning time.[44]

Endoscopic ultrasound (EUS) is a good alternative for imaging (**Figure 2**). It is mainly used to assess the presence of worrisome features and should not be performed in case of an established diagnosis or clear indication for surgery. Despite a low accuracy for differentiation between cyst types (61%-72%),[45,46] it is highly appropriate for the recognition and delineation of malignant characteristics, especially intra-cystic structures.[47-49] Addition of contrast increases the accuracy of mural nodule detection to 98%.[45]

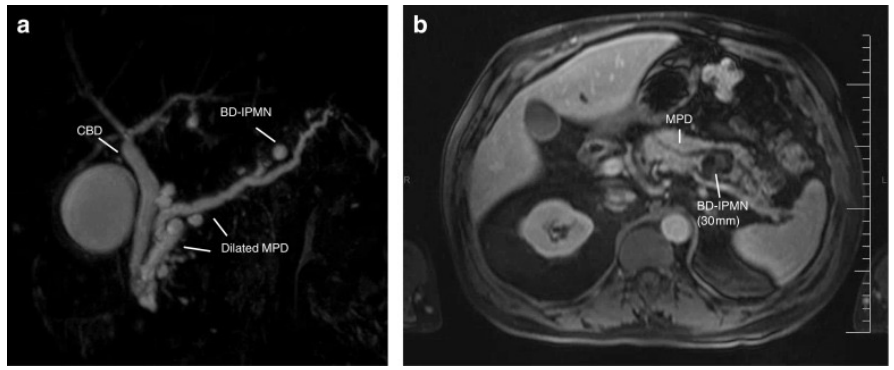


FIGURE 1 | Two examples of magnetic resonance cholangiopancreatography (MRCP; A) and magnetic resonance imaging (MRI; B) images showing intraductal papillary mucinous neoplasm (IPMN). A. MRCP shows a diluted pancreatic duct and Santorini with distal a diameter of less than 1 cm. Also, impression of a multifocal small branch-duct IPMN. **B.** MRI shows an irregular ductus pancreaticus at the level of the corpus and tail and is slightly dilated. Multiple cysteine deviations starting from the branch duct. Largest cystic lesion located in the corpus with a staining solid component. Impression of a mixed-type IPMN with solid component as sign of possible malignant transformation. The pathology result after pancreatic tail and spleen resection showed a mixed-type IPMN, both gastric and pancreatobiliary type, with moderate dysplasia; there are extensive regressive changes with mucinous extravasation and fibrosis. No high-grade dysplasia, no malignancy.

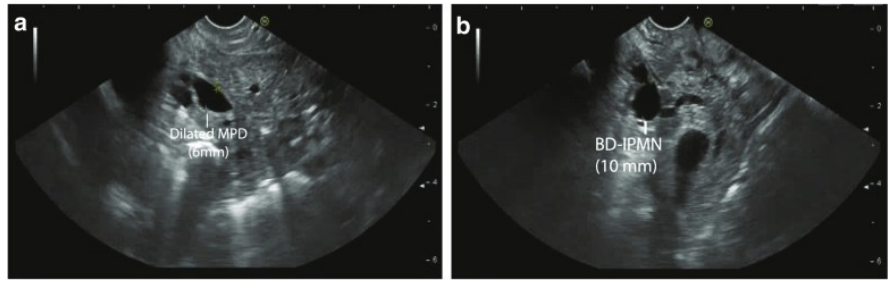


FIGURE 2 | Two screenshots of an endoscopic ultrasound (EUS) procedure. The tip of the scope is located in the D2 part of the duodenum. The PD is followed from the papilla: focalized dilation over a short trajectory with a diameter of 6 mm, slendering distally with a diameter of 2.7 mm. There is a homogeneous 10-mm cystic lesion not far from the papilla with a connection with de PD. No mural nodule or wall thickening. Conclusion: mixed-type IPMN in pancreatic head and uncinate process.

An additional benefit of EUS is that it allows for cyst fluid collection with fine-needle aspiration (FNA), which is indicated in case of indefinite imaging findings.[6,7,10] The AGA recommends EUS-FNA in patients with a cyst diameter ≥3cm, solid component or dilated MPD.[8] The Fukuoka guideline discourages FNA in case of either high-risk or worrisome features, out of fear for tumor spill.[6] Cytological cyst fluid analysis has a high specificity (91%), yet low sensitivity (65%) for differentiation between benign and malignant IPMN.[46,50-52] Sensitivity may be increased by also sampling the cyst wall and solid components.[53] The risk of complications related to cyst EUS-FNA is low (0-2.5%), although higher than for solid lesions. Potential complications are: abdominal pain, bacteremia/infection, hemorrhage and pancreatitis. Prophylactic antibiotics are recommended.[50,54-58]

Cyst fluid analysis and Biomarkers

A broad spectrum of tumor-specific (*e.g.*, DNA mutations) and tumor-associated (*e.g.*, CA19.9) markers have the potential to distinguish high- from low-risk lesions and guide decision-making (Table 2).[10] A perfect biomarker should be detectable in an early stage and specific for pancreas neoplasia. Apart from cyst fluid, other potential biomarker sources are serum and pancreatic juice.

Glycoproteins are often used as biomarkers. An increased serum level of CA19.9 (>37 U/ml) is found in 85% of the patients with PC and is used to follow the disease course.[59] For IPMN, surgical series have shown that it is an independent predictor of malignant transformation, with a (pooled) sensitivity and specificity of 40% and 89%, respectively.[60,61] An increased serum CA19.9 level is a relative indication for surgery and supplementary diagnostics are recommended.[10] Cyst fluid CA19.9 levels have limited clinical value for the identification of advanced neoplastic disease, yet low CA19.9 levels (<37 U/ml) are suggestive for a non-mucinous origin.[52] Cyst fluid CEA is mainly used for cyst differentiation. A level of <5mg/mL is highly specific (95%) for a non-mucinous cyst and a value >800ng/mL for a mucinous cyst (95%).[50] Little is known about glycoprotein detection in pancreatic juice. Hirono *et al*. (2012)[62] found a high accuracy (92%) for differentiation between benign and malignant IPMN, based on CEA levels in pancreatic juice (cutoff value > 30 ng/mL).[62]

Mutated genes are released after cell death and have high potential to serve as biomarkers. Tissue *GNAS* mutations are associated with IPMN (58-79%; OR 30, 95 % CI 7.143–127.622), IPMN-associated adenocarcinoma (36%) and mucinous carcinoma (78%).[63-66] In contrast, it is rarely detected in PC, PanIN-lesions and mucinous cystic neoplasms (MCNs). The prevalence of *GNAS* mutations differs per morphological subtype: 100% in the intestinal type, 71% in the pancreatobiliary type, 51% in the gastric type, and 0% in the oncocytic-type IPMN.[67]

KRAS is the driver mutation in most pancreatic PCs and is also detected in IPMN tissue (50%; OR 7.4, 95% CI 3.9–14.4).[66,68] However, it is less specific than *GNAS*, since *KRAS* is found in 69% of IPMN, 21% of MCN, 90% of PanIn-1 and 90% of PC patients.[66] The presence of tissue *KRAS* and *GNAS* gene mutations are not related to IPMN location (BD-IPMN *vs*. MD-IPMN).[66] In serum, Berger *et al*. (2016)[69] found in blood that total circulating cell-free DNA levels of >0.208 ng/uL distinguish between IPMN and healthy controls with 81% sensitivity and 84% specificity, and between PC and healthy controls with 83% sensitivity and 92% specificity. More specifically for *GNAS* and *KRAS*, 71% of patients with IPMN harbored cell-free circulating mutated *GNAS*. Mutated *KRAS* was not detected in patients with IPMN, although it is present in 42% of patients with PC.[69] Adding molecular testing to clinical features and morphology increases sensitivity of IPMN and MCN differentiation to 90% and 94%, respectively. However, more research is needed to distinguish whether the clinical value outweighs the high costs of these sensitive laboratory techniques.[70,71] For pancreatic juice, Suenaga *et al*. (2018)[72] found *GNAS* gene mutations in 70% of patients with IPMN. Also, *TP53* and *SMAD4* levels were found to be related to dysplasia grade, and able to distinguish IPMN from PC with a sensitivity and specificity of 32% and 100%, respectively.[72,73] A VHL gene mutation increases the probability of detecting a serous cyst Adenoma (SCA).[72,73]

Other techniques

Pancreatoscopy uses a thin scope that is introduced in the MPD during endoscopic retrograde cholangiopancreatography (ERCP) or surgery. It enables intra-ductal visualization and image-guided tissue sampling. For differentiation between benign and malignant MD-IPMN, the accuracy is relatively high (88%), yet also are the rates of post-procedural pancreatitis (7%).[74] During surgery, pancreatoscopy may be combined with intra-ductal frozen biopsies, to assess the extent of MPD involvement and guide resection.[10,75]

Needle-based confocal laser endomicroscopy (nCLE) uses a small probe (0.85 mm) that is placed in a pancreatic cyst via a 19-gauge FNA needle and provides a real-time microscopic view (width: 320 µm, resolution 3.5µm). It is able to detect a pancreatic cystic neoplasm with a sensitivity of 59-80% and a specificity of 100%. However, it is currently discouraged by the European guidelines due to high adverse event rates (7-9%). [10,76-79]

TABLE 2 | **Features suggestive for cyst-type and invasiveness.**[50,72,80-88]

Characteristic	Pseudocyst	SCA	MCN	IPMN	Malignant IPMN
Age	>40 yr	>60 yr	Young (~40 - 50yr)	>65 yr	>65 yr
Gender	F<M	F>M	F>M (>95%)	F~M	F~M
Symptoms	Regularly	Rare	Rare	Rare	Sometimes
Relation to acute pancreatitis	Mostly	No	No	Sometimes	Sometimes
Relation to chronic pancreatitis	Mostly	No	No	No	No
Calcifications	No	Sometimes (central)	Sometimes (peripheral)	No	No
Location	Not specific	Mostly distal	Mostly distal	Mostly proximal	Mostly proximal
Connection with MPD	No	No	No	Yes	Yes
Multifocality	No	Rare	No	Sometimes (BD-IPMN)	Sometimes
Serum					
Elevated CA19.9 *(>37U/mL)*	-	-	+/-	+/-	++
Mutated *KRAS*	-	-	-	-	++
Mutated *GNAS*	-	-	-	+	+/-
Cyst fluid					
Mucin	--	--	++	++	++
Amylase (*>250u/mL)*	++	--	-	+/-	+/-
CEA	--	--	+	+	++
Mutated *KRAS*	--	--	+	+	++
Mutated *GNAS*	--	--	-	++	+
Pancreatic juice					
CA19.9	--	--	-	+/-	+
CEA	--	--	-	+/-	+
Mutated *KRAS*	--	--	-	+/-	+
Mutated *GNAS*	--	--	--	++	+/-
Mutated *SMAD4* or *TP53*	--	--	--	+/-	++

CA19.9 = Cancer Antigen 19.9; CEA = Carcinoembryonic antigen; MPD = Main pancreatic duct; SCA = Serous Cyst adenoma; MCN = Mucinous cystic neoplasm; IPMN = Intraductal Papillary Mucinous Neoplasm; F = female; M = male.

Clinical strategy and surveillance

Surveillance

Nowadays, surveillance is recommended in patients with (operated) pancreatic cysts suspected for IPMN. The best utility and manner of surveillance have not been

established. At present, surveillance is based on consensus guidelines, namely the International Association of Pancreatology (IAP; 'Fukuoka guidelines'),[4-6] American College of Gastroenterology (ACG), [7] American Gastroenterological Association (AGA)[8] and European Study Group on Cystic Tumours of the Pancreas.[9,10] They all agree that the risk of malignancy should be weighed against life expectancy and co-morbidity. Confusingly, the recommended surveillance strategies differ between guidelines (**Table 3**). Large scale prospective registries of individuals undergoing cyst surveillance (*e.g.,* PACYFIC-registry; www.pacyfic.net) are needed to accumulate unbiased data and develop evidence-based guidelines.

According to all guidelines, the presence of mural nodules or solid components is most predictive for malignant disease. Mural nodules are present in 36-70% of IPMN patients with invasive disease and the size of the mural nodule is correlated with the risk of malignancy.[13,20,31,89] Additionally, a thickened cyst wall is present in ±65% of patients with invasive disease (OR 4.80; 95% CI 1.16-14.36).[13,90] In case of doubt, contrast-harmonic endoscopic ultrasound (CH-EUS) helps to differentiate between mucin and a solid component by the presence of small blood vessels in the latter.

Although cyst size is associated with invasiveness, treatment should not be determined by size alone, since small cysts do not exclude invasiveness and large cysts do not always harbor malignancy.[18,19,91-93] Both the Fukuoka and ACG guidelines base surveillance intervals on cyst size in the absence of a more practical surrogate.[6,94] The speed of cyst growth appears to be more predictive. A growth of > 2 mm/year is related to a 45% 5-year risk of developing malignancy, versus a 1.8% risk in slow growing cysts.[95-97] Due to a recorded size difference between the different imaging modalities, it is recommended not to alternate modalities between follow-up visits.[10,90,94, 98]

The mean MPD diameter is significantly larger in patients with malignant disease. Some guidelines use a 10 mm cutoff value, as absolute indication for surgery.[6,10] This is disputable, since the risk of malignancy is already increased to 59% for patients with a pancreatic duct width between 5-9 mm.[99] The AGA and ACG guidelines recommend EUS-FNA in cysts associated with a dilated MPD (ACG cutoff >5 mm, AGA non-specified).[8,17,19,94, 99-101]

According to the European, Fukuoka and ACG guidelines, the duration of surveillance should be lifelong. The AGA guideline recommends stopping surveillance in case of a stable cyst after five years. This led to publications from multiple experts showing development of PC after five years of surveillance. For instance, Kwong *et al*. (2016)[102] found an 8-fold higher mortality from non-pancreatic causes than from PC after five years of surveillance in low-risk BD-IPMN. Other studies detected high-risk features in asymptomatic BD-IPMN patients after a follow-up period of more than 5 years.[103-105] More specifically, Del Chiaro *et al*. (2017)[106] described an IPMN-related mortality of 5.8% after 10 years of follow-up in patients without high-risk features at baseline.

After resection of IPMN, lifelong surveillance is recommended, as long as the patient is able and willing to undergo surgery.[6,8,10,94] He *et al*. (2013)[107] estimated the risk of developing a new lesion after resection of non-invasive IPMN at 1.6% after one year, 14% after five years and 18% after ten years; the risk of invasive PC was ±0% after one year, 7% after five years and 38% after ten years. For invasive IPMN, post-resection surveillance is recommended solely based on symptoms, similar to PC.[6,10] However, one could argue that for patients with early-stage invasive IPMN, surveillance should restart after ±5 years of survival.

Additionally, data about the incidence of extra-pancreatic neoplasms in patients with IPMN remains controversial, since some retrospective studies show an increased risk in other cancers (*e.g*., colorectal and gastric cancer).[108-111] A large study of 1340 patients by Marchegiani *et al*. (2015)[36] did not find a higher incidence of extra-pancreatic neoplasms in patients with IPMN. Guidelines do not recommend additional imaging (*e.g*., CT) for extra-pancreatic malignancy surveillance in patients with IPMN.[6,8,10, 94]

Treatment

Guidelines recommend that surgery should be performed by an experienced surgeon in a high-volume center after consultation of - and a joint decision by - a multidisciplinary group with pancreatic expertise. Especially advanced age and the presence of co-morbidity are related to postoperative mortality of non-pancreatic cause.[112-114] On the other hand, early surgery of MCN could be considered in younger patients with no co-morbidity.[9,10]

MD-IPMN and MT-IPMN justify a more aggressive treatment approach than BD-IPMN. In general, surgery can be offered as this is justified by the high prevalence of invasive disease (MD-IPMN 11-81%; MT-IPMN 20-65%) and the high disease-specific mortality (23 per 1000 patient years; 95% CI 12-52) for untreated MD-IPMN and MT-IPMN.[115] For BD-IPMN, the guidelines are inconsistent (**Table 3**). The Fukuoka guidelines recommend surgery in case of ≥1 high-risk stigmata or ≥1 worrisome features and one of the following: Mural nodule ≥5mm, suspicious MPD, suspicious cytology.[6] The European guideline is similar, yet in case of surgical indication, age and the presence of comorbidity are advised to be taken into account.[10] ACG stress the need of decision making by a multi-disciplinary pancreatic group.[94]

In case of suspected malignancy, an oncological resection should be performed. For all IPMNs, intra-operative frozen section examination of the resection margins is recommended. For patients with MD-IPMN or MT-IPMN, intra-operative pancreatoscopy with frozen section of intraductal biopsies can be considered.[10] Patients with positive margins have a worse survival and extended resection is recommended.[15] Cysts in multifocal IPMNs should be approached autonomously due to their distinct behavior; the most suspicious lesion(s) should be removed. A total pancreatectomy is only

recommended in case of multiple worrisome features throughout the pancreas or post-surgical recurrence in the remnant pancreas. However, the majority experiences long-term symptoms related to surgery, such as severe weight loss, diarrhea (exocrine insufficiency) and/or hypoglycemic episodes in relation to brittle diabetes (endocrine insufficiency).[116,117] The survival rates one and three years after total pancreatectomy are, 80% and 65%, respectively.[117]

Pancreaticoduodenectomy (Whipple procedure), distal pancreatectomy and total pancreatectomy are performed in 42-70%, 13-47% and 3-37% of the cases.[13,15,17,31,118] These procedures are related with complications in 25% of patients, such as gastrointestinal anastomotic leakage, pancreatic fistula, intra-abdominal abscess, pancreatitis, pancreatic pseudocyst, anastomotic stenosis, cholangitis, delayed gastric emptying, ascites, diarrhea or pneumonia.[19] In-hospital morbidity is 37%, and the in-hospital and 30-day mortality 1.4% and 2.7, respectively.[15,119]

Prognosis

Recurrence after surgery

The overall recurrence rate for IPMN is ±11-20% (median 58-73 months), which increases to 65% in case of malignant IPMN.[23,94,120,121] For BD-IPMN, ±40% is multifocal, which may explain the frequent early recurrence of IPMN in the remnant pancreas (12.5%; mean follow-up 28 months).[122] Additionally, an increased age, BMI, number of resected lesions as well as an initial location in the pancreatic tail, invasiveness and a family history of PC are predictors of recurrence or disease progression.[123,124] The estimated chance to develop a new primary IPMN and related invasive PC after five years is 14% and 7%, respectively.[107,120,125] The recurrence rate for MD-IPMN is higher than for BD-IPMN. The dysplasia grade in the resection specimen is the most important predictor of the (severity of) recurrence.[23,120,126]

Survival

A large observational study by Marchegiani *et al.* (2015)[120] found a 5-year survival after resection of 77% for all IPMNs, 69% for MD-IPMN and 82% for BD-IPMN, with a median time to survival of 17, 13 and 24 months, respectively. Vanella *et al*. (2018)[115] performed a meta-analysis and found a disease specific mortality of 23 for all IPMN, 32 for MD-IPMN and 5 for BD-IPMN per 1000 patient-years. Low-grade dysplasia exhibits a similar survival as high-grade dysplasia. In case of invasive disease, the survival is significantly lower. Of all patients with IPMN-associated adenocarcinoma, 53% has lymph-node metastases, 58% peri-neural invasion and 33% vascular invasion.[120,127]

TABLE 3 | An overview of four most recent guidelines on diagnosis and management of pancreatic cystic neoplasms.[6,8,10,94]

	Revised European guideline	Revised Fukuoka guideline	ACG guideline	AGA guideline
Year publication	2018	2017	2018	2015
Diagnostic work-up	MRI: 1st choice CT: 2nd choice* EUS: supplementary FNA: Mural nodules, septations or indefinite imaging Serum CA19.9	MRI: 1st choice CT: 2nd choice* EUS: for worrisome features FNA: Indefinite imaging; discouraged in case of high-risk/worrisome features Serum CA19.9	MRI: 1st choice EUS/CT: alternative FNA: in case of indefinite imaging, high risk characteristics, cysts >2 cm (differentiation mucinous and non-mucinous) Serum CA19.9	MRI: 1st choice EUS: High-risk features FNA: ≥2 high-risk features or significant change of high-risk feature
MD-/MT-IPMN: Indications for surgery	Surgically fit patients	Surgically fit **and** ≥1 high-risk stigmata (see below)	Reference to multidisciplinary group in case of main-duct involvement	Not mentioned
BD-IPMN: High risk features/ indications surgery	**Absolute indications:** Solid mass Enhancing mural nodule ≥5 mm MPD ≥10 mm HGD/carcinoma in cytology Jaundice **Relative indications:** Cyst growth ≥5 mm/yr Cyst size ≥4cm enhancing mural nodule <5 mm MPD 5-9.9 mm Serum CA19.9 ≥37 U/ml, New-onset DM Acute pancreatitis	**High-risk stigmata:** Enhancing mural nodule >5 mm MPD >10 mm Jaundice **Worrisome features:** Growth ≥5mm/2 yrs Cyst size ≥3 cm Enhancing mural nodule <5 mm Enhancing thickened cyst wall MPD 5-9 mm PD caliber change Elevated serum CA19.9 Pancreatitis	**High-risk characteristics:** Mural nodule/solid component MPD >5 mm PD caliber change + atrophy Cyst size ≥3 mm Cyst growth >3 mm/yr HGD/carcinoma in cytology Jaundice Acute pancreatitis Elevated serum CA19.9 New-onset DM	**High-risk features:** Solid component Dilated MPD Cyst size ≥3 cm
Duration surveillance	As long as fit for surgery	As long as fit for surgery	As long as fit for surgery Individualized approach age 76-85 yr	Discontinue after 5 yrs if no significant change has occurred
Surveillance intervals	6 mo. (1st yr), then yearly	<1 cm: 6 mo., then 2-yearly 1-2 cm: 6 mo. (1st yr), yearly (2 yrs), then 2 yearly 2-3 cm: 3-6 mo. (1st yr), then yearly >3 cm: 3-6 mo.	<1 cm: 2 yrs 1-2 cm: 1 yr 2-3 cm, clear IPMN/MCN: 6-23 mo. Shorter interval for new-onset DM or cyst growth >3 mm/yr	At yrs 1, 3 and 5

TABLE 3 | Continuation.

	Revised European guideline	Revised Fukuoka guideline	ACG guideline	AGA guideline
Indication for surgery	≥1 Absolute indication ≥1 Relative indication without significant co-morbidities ≥2 Relative indications for patients with significant co-morbidities	≥1 High risk stigmata ≥1 Worrisome feature **and** ≥1 of following: Definite mural nodule, MPD involvement Suspect cytology Consider: cyst >2 cm in young and fit patient	Decided by multidisciplinary team. Referral in case of: Jaundice **or** ≥1 of following: MPD >5 mm, Cyst size ≥3 mm Caliber change MPD MPD involvement HGD/PC cytology Mural nodule	Solid component **and** dilated MPD **and/or** concerning features on EUS-FNA
Surveillance after resection	Malignancy: according to PC guidelines HGD /MD-IPMN: 6-mo (1st 2 yr), then yearly LGD: as non-operated	Malignancy: according to PC guideline 2x/yr in case of one of the following: family history of PC, surgical margin with HGD, non-intestinal type IPMN Other patients Every 6-12 mo	Malignancy: according to PC guidelines HGD: every 6-mo. Low-/intermediate grade dysplasia: every 2 yrs	Dysplasia/malignancy: every 2 yrs If not: no FU (Unless MT-IPMN or family history of PC)

ACG = American College of Gastroenterology; AGA = American Gastroenterological Association; MRI = Magnetic Resonance Imaging; CT = Computer Tomography; EUS = Endoscopic Ultrasound; FNA = Fine-needle aspiration; CH-EUS = Contrast-enhanced harmonic EUS; CA19.9 = Carbohydrate Antigen 19.9; DM = diabetes mellitus; FU = follow-up; PC = Pancreatic cancer; LGD = low-grade dysplasia; HGD = High-grade dysplasia; yr=year, mo = month.* To identify calcifications, for tumor staging or surveillance of PC recurrence.

References

1 Ohhashi K. Four cases of mucous secreting pancreatic cancer. *Prog Dis Endosc* 1982; 20: 348-351.

2 Sessa F, Solcia E, Capella C, *et al*. Intraductal papillary-mucinous tumours represent a distinct group of pancreatic neoplasms: an investigation of tumour cell differentiation and K-ras, p53 and c-erbB-2 abnormalities in 26 patients. *Virchows Arch* 1994; 425: 357-367.

3 Tulla KA and Maker AV. Can we better predict the biologic behavior of incidental IPMN? A comprehensive analysis of molecular diagnostics and biomarkers in intraductal papillary mucinous neoplasms of the pancreas. *Langenbecks Arch Surg* 2017.

4 Tanaka M, Chari S, Adsay V, *et al*. International consensus guidelines for management of intraductal papillary mucinous neoplasms and mucinous cystic neoplasms of the pancreas. *Pancreatology* 2006; 6: 17-32.

5 Tanaka M, Fernandez-del Castillo C, Adsay V, *et al*. International consensus guidelines 2012 for the management of IPMN and MCN of the pancreas. *Pancreatology* 2012; 12: 183-197.

6 Tanaka M, Fernandez-Del Castillo C, Kamisawa T, *et al*. Revisions of international consensus Fukuoka guidelines for the management of IPMN of the pancreas. *Pancreatology* 2017; 17: 738-753.

7 Elta GH, Enestvedt BK, Sauer BG, *et al*. ACG Clinical Guideline: Diagnosis and Management of Pancreatic Cysts. *Am J Gastroenterol* 2018.

8 Vege SS, Ziring B, Jain R, *et al*. American Gastroenterological Association Institute Guideline on the Diagnosis and Management of Asymptomatic Neoplastic Pancreatic Cysts. *Gastroenterology* 2015; 148: 819-822.

9 Del Chiaro M, Verbeke C, Salvia R, *et al*. European experts consensus statement on cystic tumours of the pancreas. *Dig Liver Dis* 2013; 45: 703-711.

10 The European Study Group on Cystic Tumours of the P. European evidence-based guidelines on pancreatic cystic neoplasms. *Gut* 2018;67:789.'.

11 Lee SY, Lee KT, Lee JK, *et al*. Long-term follow up results of intraductal papillary mucinous tumors of pancreas. *J Gastroenterol Hepatol* 2005; 20: 1379-1384.

12 Crippa S, Fernandez-Del Castillo C, Salvia R, *et al*. Mucin-producing neoplasms of the pancreas: an analysis of distinguishing clinical and epidemiologic characteristics. *Clin Gastroenterol Hepatol* 2010; 8: 213-219.

13 Hwang DW, Jang J-Y, Lee SE, *et al*. Clinicopathologic analysis of surgically proven intraductal papillary mucinous neoplasms of the pancreas in SNUH: a 15-year experience at a single academic institution. *Langenbeck's Archives of Surgery* 2012; 397: 93-102. journal article.

14 Salvia R, Castillo CF-d, Bassi C, *et al*. Main-Duct Intraductal Papillary Mucinous Neoplasms of the Pancreas: Clinical Predictors of Malignancy and Long-term Survival Following Resection. *Annals of Surgery* 2004; 239: 678-687.

15 Schnelldorfer T, Sarr MG, Nagorney DM, *et al*. Experience with 208 resections for intraductal papillary mucinous neoplasm of the pancreas. *Arch Surg* 2008; 143: 639-646; discussion 646.

16 Ohno E, Hirooka Y, Kawashima H, *et al*. Natural history of pancreatic cystic lesions: a multicenter prospective observational study for evaluating the risk of pancreatic cancer. *J Gastroenterol Hepatol* 2017.
17 Kim SC, Park KT, Lee YJ, *et al*. Intraductal papillary mucinous neoplasm of the pancreas: clinical characteristics and treatment outcomes of 118 consecutive patients from a single center. *J Hepatobiliary Pancreat Surg* 2008; 15: 183-188.
18 Suzuki Y, Atomi Y, Sugiyama M, *et al*. Cystic neoplasm of the pancreas: a Japanese multiinstitutional study of intraductal papillary mucinous tumor and mucinous cystic tumor. *Pancreas* 2004; 28: 241-246.
19 Nagai K, Doi R, Kida A, *et al*. Intraductal Papillary Mucinous Neoplasms of the Pancreas: Clinicopathologic Characteristics and Long-Term Follow-Up After Resection. *World Journal of Surgery* 2008; 32: 271-278.
20 Schmidt CM, White PB, Waters JA, *et al*. Intraductal papillary mucinous neoplasms: predictors of malignant and invasive pathology. *Ann Surg* 2007; 246: 644-651; discussion 651-644.
21 Yu S, Takasu N, Watanabe T, *et al*. Validation of the 2012 Fukuoka Consensus Guideline for Intraductal Papillary Mucinous Neoplasm of the Pancreas From a Single Institution Experience. *Pancreas* 2017; 46: 936-942.
22 Fritz S, Klauss M, Bergmann F, *et al*. Pancreatic main-duct involvement in branch-duct IPMNs: an underestimated risk. *Ann Surg* 2014; 260: 848-855; discussion 855-846.
23 Kang MJ, Jang JY, Lee KB, *et al*. Long-term prospective cohort study of patients undergoing pancreatectomy for intraductal papillary mucinous neoplasm of the pancreas: implications for postoperative surveillance. *Ann Surg* 2014; 260: 356-363.
24 Furukawa T, Hatori T, Fujita I, *et al*. Prognostic relevance of morphological types of intraductal papillary mucinous neoplasms of the pancreas. *Gut* 2011; 60: 509-516.
25 Shimizu Y, Yamaue H, Maguchi H, *et al*. Predictors of malignancy in intraductal papillary mucinous neoplasm of the pancreas: analysis of 310 pancreatic resection patients at multiple high-volume centers. *Pancreas* 2013; 42: 883-888.
26. Lafemina J, Katabi N, Klimstra D, *et al*. Malignant progression in IPMN: a cohort analysis of patients initially selected for resection or observation. *Ann Surg Oncol* 2013; 20: 440-447.
27. Koh YX, Zheng HL, Chok AY, *et al*. Systematic review and meta-analysis of the spectrum and outcomes of different histologic subtypes of noninvasive and invasive intraductal papillary mucinous neoplasms. *Surgery* 2015; 157: 496-509.
28 Castellano-Megias VM, Andres CI, Lopez-Alonso G, *et al*. Pathological features and diagnosis of intraductal papillary mucinous neoplasm of the pancreas. *World J Gastrointest Oncol* 2014; 6: 311-324.
29 Furukawa T, Kloppel G, Volkan Adsay N, *et al*. Classification of types of intraductal papillary-mucinous neoplasm of the pancreas: a consensus study. *Virchows Arch* 2005; 447: 794-799.
30 Ridtitid W, DeWitt JM, Schmidt CM, *et al*. Management of branch-duct intraductal papillary mucinous neoplasms: a large single-center study to assess predictors of malignancy and long-term outcomes. *Gastrointestinal Endoscopy* 2016; 84: 436-445.

31 Ridtitid W, DeWitt JM, Schmidt CM, *et al*. Management of branch-duct intraductal papillary mucinous neoplasms: a large single-center study to assess predictors of malignancy and long-term outcomes. *Gastrointest Endosc* 2016; 84: 436-445.
32 Aune D, Greenwood DC, Chan DSM, *et al*. Body mass index, abdominal fatness and pancreatic cancer risk: a systematic review and non-linear dose–response meta-analysis of prospective studies. *Annals of Oncology* 2012; 23: 843-852.
33 Smits MM and van Geenen EJ. The clinical significance of pancreatic steatosis. *Nat Rev Gastroenterol Hepatol* 2011; 8: 169-177.
34 Sturm EC, Roch AM, Shaffer KM, *et al*. Obesity increases malignant risk in patients with branch-duct intraductal papillary mucinous neoplasm. *Surgery* 2013; 154: 803-808; discussion 808-809.
35 Capurso G, Boccia S, Salvia R, *et al*. Risk Factors for Intraductal Papillary Mucinous Neoplasm (IPMN) of the Pancreas: A Multicentre Case–Control Study. *The American Journal Of Gastroenterology* 2013; 108: 1003. Pancreas and Biliary Tract.
36 Marchegiani G, Malleo G, D'Haese JG, *et al*. Association between pancreatic intraductal papillary mucinous neoplasms and extrapancreatic malignancies. *Clin Gastroenterol Hepatol* 2015; 13: 1162-1169.
37 Moris M, Raimondo M, Woodward TA, *et al*. Diagnostic Accuracy of Endoscopic Ultrasound-Guided Fine-Needle Aspiration Cytology, Carcinoembryonic Antigen, and Amylase in Intraductal Papillary Mucinous Neoplasm. *Pancreas* 2016; 45: 870-875.
38 Morales-Oyarvide V, Mino-Kenudson M, Ferrone CR, *et al*. Diabetes mellitus in intraductal papillary mucinous neoplasm of the pancreas is associated with high-grade dysplasia and invasive carcinoma. *Pancreatology* 2017; 17: 920-926.
39 Kobayashi G, Fujita N, Noda Y, *et al*. Intraductal papillary mucinous neoplasms of the pancreas showing fistula formation into other organs. *Journal of Gastroenterology* 2010; 45: 1080-1089. journal article.
40 Yamada Y, Mori H, Hijiya N, *et al*. Intraductal papillary mucinous neoplasms of the pancreas complicated with intraductal hemorrhage, perforation, and fistula formation: CT and MR imaging findings with pathologic correlation. *Abdominal Imaging* 2012; 37: 100-109. journal article.
41 Kimura W, Nagai H, Kuroda A, *et al*. Analysis of small cystic lesions of the pancreas. *Int J Pancreatol* 1995; 18: 197-206.
42 Moris M, Raimondo M, Woodward TA, *et al*. Diagnostic accuracy of endoscopic ultrasound-guided fine-needle aspiration cytology, carcinoembryonic antigen, and amylase in intraductal papillary mucinous neoplasm. *Pancreas* 2016; 45: 870-875.
43 Su Jin S, Jeong Min L, Young Jun K, *et al*. Differentiation of intraductal papillary mucinous neoplasms from other pancreatic cystic masses: Comparison of multirow-detector CT and MR imaging using ROC analysis. Journal of Magnetic Resonance Imaging 2007; 26: 86-93.
44 Rastegar N, Matteoni-Athayde LG, Eng J, *et al*. Incremental value of secretin-enhanced magnetic resonance cholangiopancreatography in detecting ductal communication in a population with high prevalence of small pancreatic cysts. *European Journal of Radiology*; 84: 575-580.

45 Harima H, Kaino S, Shinoda S, *et al*. Differential diagnosis of benign and malignant branch duct intraductal papillary mucinous neoplasm using contrast-enhanced endoscopic ultrasonography. *World J Gastroenterol* 2015; 21: 6252-6260.

46 Rodriguez-D'Jesus A, Fernandez-Esparrach G, Boadas J, *et al*. Impact of endoscopic ultrasonography (EUS) and EUS-guided fine-needle aspiration on the management of pancreatic cystic lesions. *Eur J Gastroenterol Hepatol* 2016; 28: 1094-1099.

47 Kamata K, Kitano M, Kudo M, *et al*. Value of EUS in early detection of pancreatic ductal adenocarcinomas in patients with intraductal papillary mucinous neoplasms. *Endoscopy* 2014; 46: 22-29.

48 Javia S, Munigala S, Guha S, *et al*. EUS Morphology Is Reliable in Selecting Patients with Mucinous Pancreatic Cyst(s) Most Likely to Benefit from Surgical Resection. *Gastroenterol Res Pract* 2017; 2017: 9863952.

49 Lu X, Zhang S, Ma C, *et al*. The diagnostic value of EUS in pancreatic cystic neoplasms compared with CT and MRI. *Endosc Ultrasound* 2015; 4: 324-329.

50 van der Waaij LA, van Dullemen HM and Porte RJ. Cyst fluid analysis in the differential diagnosis of pancreatic cystic lesions: a pooled analysis. *Gastrointest Endosc* 2005; 62: 383-389.

51 Suzuki R, Thosani N, Annangi S, *et al*. Diagnostic yield of EUS-FNA-based cytology distinguishing malignant and benign IPMNs: a systematic review and meta-analysis. *Pancreatology* 2014; 14: 380-384.

52 Thosani N, Thosani S, Qiao W, *et al*. Role of EUS-FNA Based Cytology in Diagnosis of Mucinous Pancreatic Cystic Lesions: A Systematic Review and Meta-analysis. *Digestive diseases and sciences* 2010; 55: 2756-2766.

53 Rogart JN, Loren DE, Singu BS, *et al*. Cyst Wall Puncture and Aspiration During EUS-guided Fine Needle Aspiration May Increase the Diagnostic Yield of Mucinous Cysts of the Pancreas. *Journal of Clinical Gastroenterology* 2011; 45: 164-169.

54 Siddiqui AA, Shahid H, Shah A, *et al*. High risk of acute pancreatitis after endoscopic ultrasound-guided fine needle aspiration of side branch intraductal papillary mucinous neoplasms. *Endoscopic Ultrasound* 2015; 4: 109-114.

55 O'Toole D, Palazzo L, Arotçarena R, *et al*. Assessment of complications of EUS-guided fine-needle aspiration. *Gastrointestinal Endoscopy* 2001; 53: 470-474.

56 Hong S-KS, Loren DE, Rogart JN, *et al*. Targeted cyst wall puncture and aspiration during EUS-FNA increases the diagnostic yield of premalignant and malignant pancreatic cysts. *Gastrointestinal Endoscopy* 2012; 75: 775-782.

57 Yoon WJ and Brugge WR. The safety of endoscopic ultrasound-guided fine-needle aspiration of pancreatic cystic lesions. *Endoscopic Ultrasound* 2015; 4: 289-292.

58 Polkowski M, Larghi A, Weynand B, *et al*. Learning, techniques, and complications of endoscopic ultrasound (EUS)-guided sampling in gastroenterology: European Society of Gastrointestinal Endoscopy (ESGE) Technical Guideline. *Endoscopy* 2012; 44: 190-206.

59 Safi F, Schlosser W, Falkenreck S, *et al*. Prognostic value of CA 19.9 serum course in pancreatic cancer. *Hepatogastroenterology* 1998; 45: 253-259.

60 Wang W, Zhang L, Chen L, *et al*. Serum carcinoembryonic antigen and carbohydrate antigen 19.9 for prediction of malignancy and invasiveness in intraductal papillary mucinous neoplasms of the pancreas: A meta-analysis. *Biomed Rep* 2015; 3: 43-50.

61 Jae Ri K, Jin-Young J, Mee Joo K, *et al*. Clinical implication of serum carcinoembryonic antigen and carbohydrate antigen 19-9 for the prediction of malignancy in intraductal papillary mucinous neoplasm of pancreas. *Journal of Hepato-Biliary-Pancreatic Sciences* 2015; 22: 699-707.

62 Hirono S, Tani M, Kawai M, *et al*. The carcinoembryonic antigen level in pancreatic juice and mural nodule size are predictors of malignancy for branch duct type intraductal papillary mucinous neoplasms of the pancreas. *Ann Surg* 2012; 255: 517-522.

63 Wu J, Matthaei H, Maitra A, *et al*. Recurrent GNAS mutations define an unexpected pathway for pancreatic cyst development. *Sci Transl Med* 2011; 3: 92ra66.

64 Eliana A, Marco dal M, Andrea M, *et al*. Targeted next-generation sequencing of cancer genes dissects the molecular profiles of intraductal papillary neoplasms of the pancreas. *The Journal of Pathology* 2014; 233: 217-227.

65 Hosoda W, Sasaki E, Murakami Y, *et al*. GNAS mutation is a frequent event in pancreatic intraductal papillary mucinous neoplasms and associated adenocarcinomas. *Virchows Archiv* 2015; 466: 665-674. journal article.

66 Lee JH, Kim Y, Choi JW, *et al*. KRAS, GNAS, and RNF43 mutations in intraductal papillary mucinous neoplasm of the pancreas: a meta-analysis. *Springerplus* 2016; 5: 1172.

67 Molin MD, Matthaei H, Wu J, *et al*. Clinicopathological correlates of activating GNAS mutations in intraductal papillary mucinous neoplasm (IPMN) of the pancreas. *Ann Surg Oncol* 2013; 20: 3802-3808.

68 Kanda M, Matthaei H, Wu J, *et al*. Presence of somatic mutations in most early-stage pancreatic intraepithelial neoplasia. *Gastroenterology* 2012; 142: 730-733 e739.

69 Berger AW, Schwerdel D, Costa IG, *et al*. Detection of Hot-Spot Mutations in Circulating Cell-Free DNA From Patients With Intraductal Papillary Mucinous Neoplasms of the Pancreas. *Gastroenterology;* 151: 267-270.

70 Yang S, Che SP, Kurywchak P, *et al*. Detection of mutant KRAS and TP53 DNA in circulating exosomes from healthy individuals and patients with pancreatic cancer. *Cancer Biol Ther* 2017; 18: 158-165.

71 Springer S, Wang Y, Dal Molin M, *et al*. A combination of molecular markers and clinical features improve the classification of pancreatic cysts. *Gastroenterology* 2015; 149: 1501-1510.

72 Suenaga M, Yu J, Shindo K, *et al*. Pancreatic juice mutation concentrations can help predict the grade of dysplasia in patients undergoing pancreatic surveillance. *Clin Cancer Res* 2018.

73 Yu J, Sadakari Y, Shindo K, *et al*. Digital next-generation sequencing identifies low-abundance mutations in pancreatic juice samples collected from the duodenum of patients with pancreatic cancer and intraductal papillary mucinous neoplasms. *Gut* 2017; 66: 1677-1687.

74 Hara T, Yamaguchi T, Ishihara T, *et al*. Diagnosis and patient management of intraductal papillary-mucinous tumor of the pancreas by using peroral pancreatoscopy and intraductal ultrasonography. *Gastroenterology* 2002; 122: 34-43.

75 Navez J, Hubert C, Gigot J-F, *et al*. Impact of Intraoperative Pancreatoscopy with Intraductal Biopsies on Surgical Management of Intraductal Papillary Mucinous Neoplasm of the Pancreas. *Journal of the American College of Surgeons*; 221: 982-987.

76 Konda VJ, Meining A, Jamil LH, *et al*. A pilot study of in vivo identification of pancreatic cystic neoplasms with needle-based confocal laser endomicroscopy under endosonographic guidance. *Endoscopy* 2013; 45: 1006-1013.

77 Nakai Y, Iwashita T, Park DH, *et al*. Diagnosis of pancreatic cysts: EUS-guided, through-the-needle confocal laser-induced endomicroscopy and cystoscopy trial: DETECT study. *Gastrointest Endosc* 2015; 81: 1204-1214.

78 Le Pen C, Palazzo L and Napoleon B. A health economic evaluation of needle-based confocal laser endomicroscopy for the diagnosis of pancreatic cysts. *Endosc Int Open* 2017; 5: E987-E995.

79 Napoleon B, Lemaistre AI, Pujol B, *et al*. A novel approach to the diagnosis of pancreatic serous cystadenoma: needle-based confocal laser endomicroscopy. *Endoscopy* 2015; 47: 26-32.

80 Jian S, William RB, Christopher JD, *et al*. Molecular analysis of pancreatic cyst fluid. C*ancer Cytopathology* 2009; 117: 217-227.

81 Brugge WR, Lewandrowski K, Lee-Lewandrowski E, *et al*. Diagnosis of pancreatic cystic neoplasms: a report of the cooperative pancreatic cyst study. *Gastroenterology;* 126: 1330-1336.

82. Oh HC, Kang H and Brugge WR. Cyst fluid amylase and CEA levels in the differential diagnosis of pancreatic cysts: a single-center experience with histologically proven cysts. *Dig Dis Sci* 2014; 59: 3111-3116.

83 Springer S, Wang Y, Dal Molin M, *et al*. A Combination of Molecular Markers and Clinical Features Improve the Classification of Pancreatic Cysts. *Gastroenterology*; 149: 1501-1510.

84 Hirono S, Tani M, Kawai M, *et al*. The Carcinoembryonic Antigen Level in Pancreatic Juice and Mural Nodule Size Are Predictors of Malignancy for Branch Duct Type Intraductal Papillary Mucinous Neoplasms of the Pancreas. *Annals of Surgery* 2012; 255.

85 Rabie ME, El Hakeem I, Al Skaini MS, *et al*. Pancreatic pseudocyst or a cystic tumor of the pancreas? *Chinese Journal of Cancer* 2014; 33: 87-95.

86 Kim YH, Saini S, Sahani D, *et al*. Imaging Diagnosis of Cystic Pancreatic Lesions: Pseudocyst versus Nonpseudocyst. *RadioGraphics* 2005; 25: 671-685.

87 Costa WL, Mantoan H, Brito RH, *et al*. Pancreatic mucinous cystadenoma with serum CA 19–9 over 1,000,000 U/mL: a case report and review of the literature. *World Journal of Surgical Oncology* 2015; 13: 78.

88 Gold DV, Gaedcke J, Ghadimi BM, *et al*. PAM4 Immunoassay Alone and in Combination with CA19.9 for the Detection of Pancreatic Adenocarcinoma. *Cancer* 2013; 119: 522-528.

89 Marchegiani G, Andrianello S, Borin A, *et al*. Systematic review, meta-analysis, and a high-volume center experience supporting the new role of mural nodules proposed by the updated 2017 international guidelines on IPMN of the pancreas. *Surgery* 2018.

90 Maimone S, Agrawal D, Pollack MJ, *et al*. Variability in measurements of pancreatic cyst size among EUS, CT, and magnetic resonance imaging modalities. *Gastrointest Endosc* 2010; 71: 945-950.

91 Walsh RM, Vogt DP, Henderson JM, *et al*. Management of suspected pancreatic cystic neoplasms based on cyst size. *Surgery* 2008; 144: 677-684.

92 Jang JY, Kim SW, Lee SE, *et al*. Treatment guidelines for branch duct type intraductal papillary mucinous neoplasms of the pancreas: when can we operate or observe? *Ann Surg Oncol* 2008; 15: 199-205.

93 Weinberg BM, Spiegel BM, Tomlinson JS, *et al*. Asymptomatic pancreatic cystic neoplasms: maximizing survival and quality of life using Markov-based clinical nomograms. *Gastroenterology* 2010; 138: 531-540.

94 Elta GH, Enestvedt BK, Sauer BG, *et al*. ACG Clinical Guideline: Diagnosis and Management of Pancreatic Cysts. *Am J Gastroenterol* 2018.

95 Rautou PE, Levy P, Vullierme MP, *et al*. Morphologic changes in branch duct intraductal papillary mucinous neoplasms of the pancreas: a midterm follow-up study. *Clin Gastroenterol Hepatol* 2008; 6: 807-814.

96 Kang MJ, Jang JY, Kim SJ, *et al*. Cyst growth rate predicts malignancy in patients with branch duct intraductal papillary mucinous neoplasms. *Clin Gastroenterol Hepatol* 2011; 9: 87-93.

97 Kwong WT, Lawson RD, Hunt G, *et al*. Rapid Growth Rates of Suspected Pancreatic Cyst Branch Duct Intraductal Papillary Mucinous Neoplasms Predict Malignancy. *Dig Dis Sci* 2015; 60: 2800-2806.

98 Boos J, Brook A, Chingkoe CM, *et al*. MDCT *vs*. MRI for incidental pancreatic cysts: measurement variability and impact on clinical management. *Abdominal Radiology* 2017; 42: 521-530.

99 Abdeljawad K, Vemulapalli KC, Schmidt CM, *et al*. Prevalence of malignancy in patients with pure main duct intraductal papillary mucinous neoplasms. *Gastrointest Endosc* 2014; 79: 623-629.

100 Abdeljawad K, Vemulapalli KC, Schmidt CM, *et al*. Prevalence of malignancy in patients with pure main duct intraductal papillary mucinous neoplasms. *Gastrointestinal Endoscopy* 2014; 79: 623-629.

101 Hackert T, Fritz S, Klauss M, *et al*. Main-duct Intraductal Papillary Mucinous Neoplasm: High Cancer Risk in Duct Diameter of 5 to 9mm. *Annals of Surgery* 2015; 262: 875-881.

102 Kwong WT, Hunt GC, Fehmi SM, *et al*. Low Rates of Malignancy and Mortality in Asymptomatic Patients With Suspected Neoplastic Pancreatic Cysts Beyond 5 Years of Surveillance. *Clin Gastroenterol Hepatol* 2016; 14: 865-871.

103 Khannoussi W, Vullierme MP, Rebours V, *et al*. The long term risk of malignancy in patients with branch duct intraductal papillary mucinous neoplasms of the pancreas. *Pancreatology* 2012; 12: 198-202.

104 Tanno S, Nakano Y, Nishikawa T, *et al*. Natural history of branch duct intraductal papillary-mucinous neoplasms of the pancreas without mural nodules: long-term follow-up results. *Gut* 2008; 57: 339.

105 Farrell JJ and Fernández-del Castillo C. Pancreatic Cystic Neoplasms: Management and Unanswered Questions. *Gastroenterology*; 144: 1303-1315.

106 Del Chiaro M, Ateeb Z, Hansson MR, *et al*. Survival Analysis and Risk for Progression of Intraductal Papillary Mucinous Neoplasia of the Pancreas (IPMN) Under Surveillance: A Single-Institution Experience. *Ann Surg Oncol* 2017; 24: 1120-1126.

107 He J, Cameron JL, Ahuja N, *et al*. Is It Necessary to Follow Patients after Resection of a Benign Pancreatic Intraductal Papillary Mucinous Neoplasm? *Journal of the American College of Surgeons;* 216: 657-665.

108 Larghi A, Panic N, Capurso G, *et al*. Prevalence and risk factors of extrapancreatic malignancies in a large cohort of patients with intraductal papillary mucinous neoplasm (IPMN) of the pancreas. *Annals of Oncology* 2013; 24: 1907-1911. d

109 Kato T, Alonso S, Noda H, *et al*. Malignant, but not benign, intraductal papillary mucinous neoplasm preferentially associates with prior extrapancreatic malignancies. *Oncol Rep* 2016; 35: 3236-3240.

110 Roch AM, Rosati CM, Cioffi JL, *et al*. Intraductal papillary mucinous neoplasm of the pancreas, one manifestation of a more systemic disease? *Am J Surg* 2016; 211: 512-518.

111 Baiocchi GL, Molfino S, Frittoli B, *et al*. Increased risk of second malignancy in pancreatic intraductal papillary mucinous tumors: Review of the literature. *World J Gastroenterol* 2015; 21: 7313-7319.

112 de Wilde RF, Besselink MG, van der Tweel I, *et al*. Impact of nationwide centralization of pancreaticoduodenectomy on hospital mortality. *Br J Surg* 2012; 99: 404-410.

113 Kawakubo K, Tada M, Isayama H, *et al*. Disease-Specific Mortality Among Patients With Intraductal Papillary Mucinous Neoplasm of the Pancreas. *Clinical Gastroenterology and Hepatology*; 12: 486-491.

114 Reames BN, Ghaferi AA, Birkmeyer JD, *et al*. Hospital Volume and Operative Mortality in the Modern Era. *Annals of surgery* 2014; 260: 244-251.

115 Vanella G, Crippa S, Archibugi L, *et al*. Meta-analysis of mortality in patients with high-risk intraductal papillary mucinous neoplasms under observation. *Br J Surg* 2018; 105: 328-338.

116 Maker AV, Sheikh R and Bhagia V. Perioperative management of endocrine insufficiency after total pancreatectomy for neoplasia. *Langenbeck's Archives of Surgery* 2017; 402: 873-883.

117 Stauffer JA, Nguyen JH, Heckman MG, *et al*. Patient outcomes after total pancreatectomy: a single centre contemporary experience. *HPB : The Official Journal of the International Hepato Pancreato Biliary Association* 2009; 11: 483-492.

118 Waters JA, Schmidt CM, Pinchot JW, *et al*. CT *vs* MRCP: Optimal Classification of IPMN Type and Extent. *Journal of Gastrointestinal Surgery* 2008; 12: 101-109. journal article.

119 Gleeson EM, Shaikh MF, Shewokis PA, *et al*. WHipple-ABACUS, a simple, validated risk score for 30-day mortality after pancreaticoduodenectomy developed using the ACS-NSQIP database. *Surgery* 2016; 160: 1279-1287.

120 Marchegiani G, Mino-Kenudson M, Ferrone CR, *et al*. Patterns of Recurrence After Resection of IPMN: Who, When, and How? *Ann Surg* 2015; 262: 1108-1114.

121 Miller JR, Meyer JE, Waters JA, *et al*. Outcome of the pancreatic remnant following segmental pancreatectomy for non-invasive intraductal papillary mucinous neoplasm. *HPB : The Official Journal of the International Hepato Pancreato Biliary Association* 2011; 13: 759-766.

122 Yan L, Siddiqui AA, Laique SN, *et al*. A large multicenter study of recurrence after surgical resection of branch-duct intraductal papillary mucinous neoplasm of the pancreas. *Minerva Gastroenterol Dietol* 2017; 63: 50-54.

123 Al Efishat M, Attiyeh MA, Eaton AA, *et al*. Progression Patterns in the Remnant Pancreas after Resection of Non-Invasive or Micro-Invasive Intraductal Papillary Mucinous Neoplasms (IPMN). *Annals of Surgical Oncology* 2018.

124 Raut CP, Cleary KR, Staerkel GA, *et al*. Intraductal Papillary Mucinous Neoplasms of the Pancreas: Effect of Invasion and Pancreatic Margin Status on Recurrence and Survival. *Annals of Surgical Oncology* 2006; 13: 582-594.

125 Fujii T, Kato K, Kodera Y, *et al*. Prognostic impact of pancreatic margin status in the intraductal papillary mucinous neoplasms of the pancreas. *Surgery*; 148: 285-290.

126 Tamura K, Ohtsuka T, Ideno N, *et al*. Treatment strategy for main duct intraductal papillary mucinous neoplasms of the pancreas based on the assessment of recurrence in the remnant pancreas after resection: a retrospective review. *Ann Surg* 2014; 259: 360-368.

127 Rezaee N, Barbon C, Zaki A, *et al*. Intraductal papillary mucinous neoplasm (IPMN) with high-grade dysplasia is a risk factor for the subsequent development of pancreatic ductal adenocarcinoma. *HPB*; 18: 236-246.

CHAPTER 4

Small Cyst Size and Slow Growth Rate Are Reassuring Features in Individuals with Pancreatic Cysts Undergoing Surveillance: Results from the PACYFIC Trial

Authors

Iris J.M. Levink, Brechtje D.M. Koopmann, Sanne Jaarsma, Priscilla A. van Riet, Kasper A. Overbeek, Jihane Meziani, Marloes L.J.A. Sprij, Myrte Gorris, Jeanin E. van Hooft, Riccardo Casadei, Carlo Ingaldi, Michael B. Wallace, Sanne A. Hoogenboom, Pieter Honkoop, Marcin Polkowski, Silvia Carrara, Erik J. Schoon, Gemma Rossi, Laurens A. van der Waaij, Georg Beyer, Jilling F. Bergmann, Elizabeth Pando, Niels G. Venneman, Johanna Laukkarinen, Anne-Marie van Berkel, Frederike G.I. van Vilsteren, Matthijs P. Schwartz, Neville Azopardi, Chantal Hoge, Ellert van Soest, Marianne E. Smits, Rutger Quispel, Patrick M. Vos, Adriaan C.I.T.L. Tan, Laszlo Czacko, Ivonne Leeuwenburgh, Marco J. Bruno*, Djuna L. Cahen*, on behalf of the PACYFIC-registry work group.

*Shared authorship

Abstract

Background: Pancreatic mucinous cysts are increasingly being discovered on imaging studies performed for unrelated conditions. European guidelines do not stratify surveillance frequency by size, whereas repetitive imaging of (mostly) small cysts imposes a substantial burden on health care recourses.

Methods: The PACYFIC-registry is an international collaboration between 28 centers that investigates the yield of pancreatic cyst surveillance. We evaluated the risk of high-grade dysplasia (HGD) and pancreatic cancer (PC) for different cyst sizes and growth rates in participants who underwent at least 12 months of follow-up, underwent surgery, and/or developed HGD or PC.

Results: 1049 of 1955 PACYFIC participants met the inclusion criteria; mean age 66 years (SD 10), 63% female. Of these, 651 individuals (62%) had a baseline cyst size <20mm. During a median follow up of 26 months (IQR 25 months) and a median number of three visits (IQR 2), 46 individuals (4.4%) developed HGD/PC. The risk of HGD/PC increased with cyst size (HR 1.02 [95%CI 1.01-1.03], p=0.005; independent of other indications for surgery), and was two-fold lower in individuals with a baseline cyst <20mm, as compared to ≥20mm (HR 2.2 [95% CI 1.2-4.0], p=0.01). Individuals with a growth rate <5mm/year had a 14-fold lower risk of HGD/PC, as compared to those with faster growing lesions (HR 14 [95% CI 4.3-48], p<0.001).

Conclusion: Cysts size <20mm and growth rate <5mm/year seem 'reassuring features', associated with a diminutive risk of malignant progression. For cysts with these features – and without other indications for surgery ('trivial cysts') – a less intensive regime than currently recommended may suffice.

Introduction

Pancreatic cancer (PC) is a leading cause of cancer-related death with a mere 9% five-year survival.[1] The majority of patients has advanced disease at time of diagnosis, which hampers curative treatment. Therefore, during the last decade, intensive imaging-based surveillance programs have been propagated worldwide, aiming for earlier detection.

Intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs) are precursor lesions of PC. With increased quality (and frequency) of imaging came the detection of more and smaller lesions. Prevalence mounts with age; 76% of individuals with the age of 80 years has a pancreatic cyst with a median size of only 7mm.[2] European evidence-based guidelines on pancreatic cystic neoplasms recommend surveillance of all individuals with neoplastic or undefined cysts, as long as they are fit for surgery.[3] Choice of surgery is based on the presence of relative (RI) and absolute indications (AI) for surgery. Cyst size (≥40mm) and growth (≥5mm/year) are examples of selected RIs, based on 'strong agreement and moderate quality of evidence' (GRADE 1B). Not so much the selection of these features as RI, but their thresholds are a topic of discussion.

Current repetitive imaging of (mostly) small pancreatic cysts poses a substantial burden on health care costs and resources. Additionally, 33-72% of resected cysts prove to be benign upon histology, potentially causing unnecessary harm.[4-7] For these reasons, it is crucial to identify 'reassuring features' that indicate the presence of 'trivial cysts', defined as cysts with a diminutive risk of developing high-grade dysplasia (HGD) or PC, which can guide clinicians to perform surveillance less frequently.

We aimed to determine if small (<20mm) and slow growing pancreatic cysts, without any RI and/or AI for surgery[3] ('trivial cysts') have a lower risk of developing HGD or PC than larger cysts, and may therefore be surveilled less frequently.

Material & Methods

PACYFIC study

This is a nested cohort study of the PACYFIC-registry that has been running since 2015 and includes individuals with neoplastic and undefined pancreatic cysts – either newly or previously diagnosed or operated upon – who are being followed at the discretion of their treating physician.

In- and exclusion criteria

The current study involves participants from 14 academic and 14 community hospitals from Europe (26 centers) and the United States (2 centers). Individuals with at least 12 months of follow-up at the time of data extraction in January 2022 were included. In addition, those who developed the primary endpoint (HGD or PC) or underwent surgical resection within 12 months after inclusion were included. Excluded were individuals with PC at baseline, a history of PC and/or pancreas resection (*e.g.*, for IPMN or MCN).

Data collection

Information regarding patient and cyst characteristics, follow-up information and histological outcomes were prospectively recorded in an online case record form (www.pacyfic.net). The study was conducted according to the principles of the Declaration of Helsinki (2013), Declaration of Taipei (2016) and in accordance with the Medical Research Involving Human Subjects Act (WMO). All individuals signed informed consent prior to inclusion. All co-authors had access to the study data and have reviewed and approved the final manuscript.

Definitions

Baseline cyst size was defined as the size of the largest cyst at study entry. Definitions regarding the presence of RI and AI for surgery were based on the European evidence-based guidelines on pancreatic cystic neoplasms.[3] Solid mass, jaundice, enhancing mural nodule ≥5mm, main pancreatic duct dilation ≥10mm were considered as AI; increased levels of serum CA19.9 (≥37kU/L), main pancreatic duct (MPD) dilation 5-9mm, new-onset diabetes mellitus (DM; defined as diabetes that had developed less than two years before a visit), acute or recurrent pancreatitis in last two years and enhancing mural nodules <5mm were interpreted as RI. Cyst size and growth were based on the size of the largest cyst and were not considered as RI. Individuals with ≥2 RI were considered to have an 'AI', as the guideline recommends surgery in this case, regardless of co-morbidity. A follow-up visit was defined as a recorded visit (after the baseline visit), during which at least MRI/MRCP, EUS or CT was performed. Time-to-event was defined as the time (in months) from baseline to either surgery, development of PC or the last follow-up visit (for those who did not undergo surgery).

Statistical analysis

Patients were stratified according to size of the largest baseline cyst and divided in two groups for the main results (<20mm and ≥20mm), and five groups for the supplemental results (<10mm, 10-20mm, 21-30mm, 31-40mm and ≥40mm). Descriptive analyses of baseline characteristics were expressed as means with 95% confidence interval (CI; for normally distributed data), medians with interquartile range (IQR; for non-normally distributed data) or numbers with percentages. Differences between groups were evaluated with a Student's T-test/ANOVA for normally distributed data or a Mann–Whitney U/Kruskal-Wallis test in case of a non-normal distribution. For categorical variables, a χ^2-test was used.

To evaluate the number of participants that develop an enlarged cyst during follow-up, the prevalence of HGD/PC and AI was compared between the cyst size groups (χ^2-test) at time of surgery or second last visit (for those who did not undergo surgery). In the supplemental results, comparison between the five groups is reported, and in case of significant differences between groups (p=0.05), a post hoc analysis was performed to compare groups (significance at p=0.005, a Bonferroni correction was used to correct for multiple testing).

To evaluate the risk of HGD/PC over time according to cyst size, we performed multiple cox proportional hazards analyses. Depending on the univariable results, multivariable analyses were performed in three ways; with size as a continuous variable (corrected for significant confounding variables from the univariable analyses), as a categorical variable with two groups (corrected for significant variables from the univariable analyses), and as a categorical variable with five groups (supplemental results; without correction for confounders due to the small sample size). In case of potential overfit of the cox proportional hazards model, no analyses were performed, yet a probability curve was created to visualize the data.

Finally, cyst growth was evaluated in a cohort with ≥12 months of follow-up (excluding those individuals who underwent surgery or developed PC in the first year), and a cumulative proportional hazard curve was generated to identify a threshold for growth rate. Cox proportional hazards analysis was performed to compare two cyst growth speeds and the risk of developing HGD/PC.

Data were analyzed and graphs visualized using Statistical Package for the Social Sciences (SPSS Inc, Chicago, Illinois, version 27), R (version 4.0.2; Rstudio, version 1.3.1073, PBC, Boston, "ggplot2"), and GraphPad (GraphPad Prism version 9, La Jolla, CA). Two-sided p-values of <0.05 were considered statistically significant.

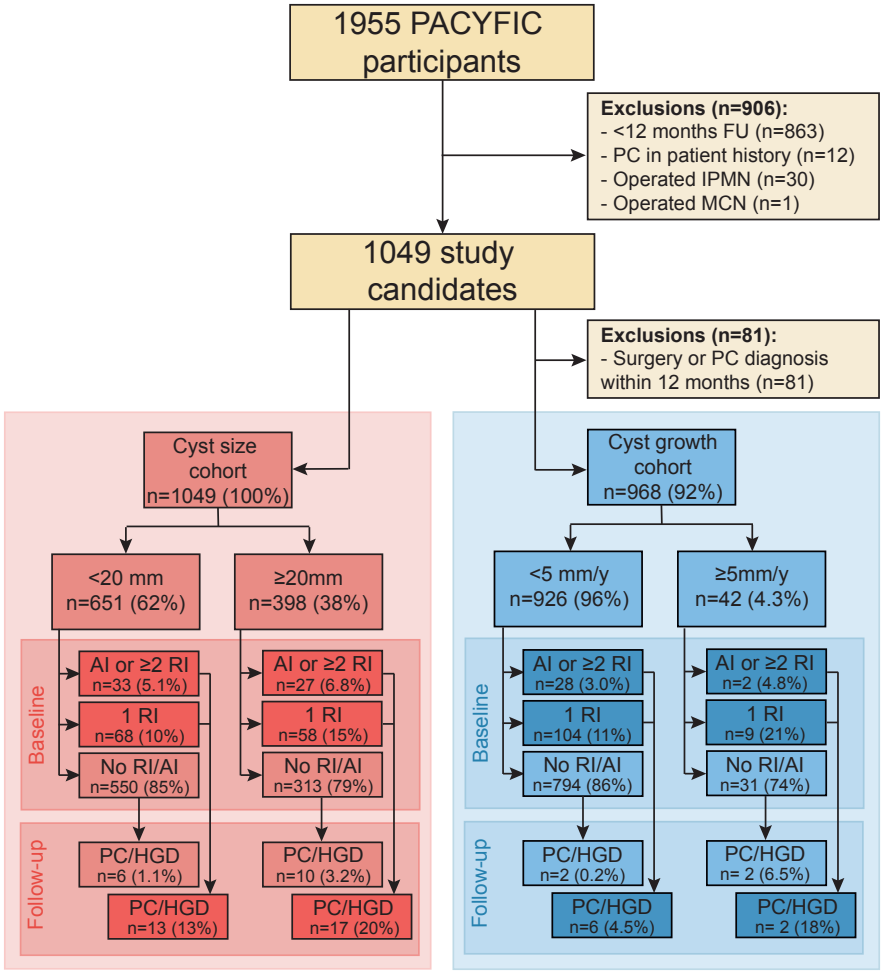


FIGURE 1 | Flowchart showing patient in- and exclusion, baseline information and follow-up information. 1049 participants were included for the cyst size cohort; this cohort was subdivided in a small cyst size group (<20mm), and larger cyst size group (≥20mm). For the cyst growth analysis, only those participants who had more than 12 months follow-up were included. Cyst size or cyst growth were not included as absolute (AI) or relative (RI) indications for surgery. PC = pancreatic cancer; HGD = high-grade dysplasia.

Results

Baseline characteristics

In total, 906 of the 1955 PACYFIC participants were excluded because of less than 12 months of follow-up (n=863), a history of PC (n=12) or previously operated IPMN (n=30)

or MCN (n=1) (**Figure 1**). Ultimately,1049 participants were included with a mean age of 66 years (SD 10) and mean BMI of 26 (SD 5). Results of the descriptive analyses are shown in **Table 1**.

The mean baseline cyst size was 19mm (SD 13) and 651 (62%) cysts measured <20mm. Participants with a cyst <20mm more often underwent MRI/MRCP without EUS than those ≥20mm (47 *vs* 38%; p=0.004), whilst for larger cysts, a combination of EUS and MRI was more often performed (29% *vs* 41%; p<0.001). Females had smaller cysts than males (mean 18mm [SD 14]) *vs* 20mm [SD 12]; p=0.007). 184 individuals (18%) experienced one or more symptoms at baseline, of which only weight loss was less common in the <20mm group (n=17 [2.6%]), as compared to the ≥20mm group (n=20 [5.0%]; p=0.04). The prevalence of 1 or more AI or RI was lower in participants with cysts <20mm (16% *vs* 21%; p=0.01). Regarding individual AI and RI, only MPD dilation was less prevalent in smaller cysts (7.4% *vs* 14%; p<0.001). In **Supplemental Table S1**, baseline characteristics of the five cyst size groups are compared.

Follow-up information

During a median of 26 months (IQR 25; range 1-74 months), the 1049 participants underwent 2643 follow-up visits. Participants with cysts <20 mm underwent a median of three visits (IQR 2; including the baseline visit) and those with larger cysts four (IQR 3; p=0.18). Also at follow-up visits, AIs or RIs were more prevalent in those with a cyst size ≥20mm than those with a cyst <20mm (p<0.001). Of these known risk factors, MPD dilation (p<0.001) and presence of an enhancing solid component (p<0.001) differed most between the two groups (**Table 2**).

Final (pathological) diagnosis

Of 1049 participants, a final diagnosis was established in 110 (9%). Of these, 16 were diagnosed with PC, yet refrained from surgery due to comorbidity or advanced disease, and 94 underwent surgery. Of these, 57 (5.4%) were diagnosed with IPMN (40 low-grade dysplasia [LGD] and 17 HGD), 13 (1.2%) with PC, 10 with MCN (1.0%, all LGD), seven with a neuro-endocrine tumor (NET) grade 1 (0.6%), one with a solid pseudopapillary neoplasm (SPN; 0.1%), one with an intraductal papillary neoplasm of the bile duct (IPNB; 0.1%) and five with non-neoplastic cysts (serous cystic adenomas [SCAs] 0.2%, pseudocyst 0.1%, lymphoepithelial cyst 0.1%, lymphangioma 0.1%). In total, 46 participants (4.4%) were diagnosed with PC (n=29 [2.7%]) or HGD (n=17 [1.6%]), after a median of 6 months (IQR 13).

TABLE 1 | Baseline characteristics for the total cohort, participants with cyst size <20mm and those with cyst size ≥ 20 mm.

	Total cohort (n=1049)	Cyst size <20 mm (n=651)	Cyst size ≥20 mm (n=398)	P-value
Age, mean (SD)	66 (10)	66 (10)	67 (10)	0.38
Female sex, n (%)	660 (63)	442 (68)	218 (55)	<0.001
BMI, mean (SD)[a]	26 (5.4)	26 (4.7)	27 (6.5)	0.43
Symptoms[b], n (%)	184 (18)	100 (15)	84 (21)	0.03
Jaundice	4 (0.4)	1 (0.2)	3 (0.8)	0.13
Acute pancreatitis	31 (3.0)	18 (2.8)	13 (3.3)	0.64
Steatorrhea	9 (0.9)	3 (0.5)	6 (1.5)	0.07
Abdominal pain	103 (10)	61 (9.4)	42 (11)	0.53
Weight loss	37 (3.5)	17 (2.6)	20 (5.0)	0.04
Diabetes mellitus, n (%)	159 (15)	87 (13)	72 (18)	0.10
Diabetes de novo[c]	13 (1.2)	7 (1.1)	6 (1.5)	0.52
MPD dilation, n (%)	105 (10)	48 (7.4)	57 (14)	<0.001
MPD 5-9mm	81 (7.7)	35 (5.4)	46 (12)	<0.001
MPD ≥10mm	24 (2.3)	13 (2.0)	11 (2.8)	0.42
Solid component, n (%)	30 (2.9)	15 (2.3)	15 (0.4)	0.12
Mural nodule <5mm	9 (0.8)	4 (0.6)	5 (1.3)	0.27
Mural nodule ≥5mm	4 (0.4)	2 (0.3)	2 (0.5)	0.62
Enhancing solid mass	17 (1.6)	9 (1.4)	8 (2.0)	0.44
Elevated serum CA19.9, n (% of determined)[d]	56 (5.3)	31 (4.8)	25 (6.3)	0.32
AI or RI at baseline, n (%)	186 (18)	101 (16)	85 (21)	0.01
AI and/or ≥2 RI	60 (5.7)	33 (5.1)	27 (6.7)	0.05
1 RI	126 (12)	68 (10)	58 (15)	0.02
Working diagnosis, n (%)				
Unspecified cyst	115 (11)	87 (13)	28 (7.0)	0.001
BD-IPMN	825 (79)	515 (79)	310 (78)	<0.001
MT-IPMN or MD-IPMN	87 (8.3)	43 (6.7)	44 (11)	0.001
MCN	22 (2.1)	6 (0.9)	16 (4.0)	<0.001

[a] Body mass index (BMI): Missing value in 647 (62%) participants; [b] Any symptom of jaundice, acute (recent or recurrent) pancreatitis, steatorrhea, abdominal pain and/or weight loss; [c] Diagnosis of diabetes mellitus for 2 years or less; [d] Serum CA19.9 was unknown at 480 baseline visits (46%; 301 [46%] participants with cysts <20mm; 179 [45%] with cysts ≥20mm) participants. AI = absolute indication of surgery; BD-IPMN = branch-duct IPMN; CT = computed tomography; EUS = endoscopic ultrasound; MD-IPMN = Main-duct IPMN; MPD = main pancreatic duct; MRCP = magnetic resonance cholangio-pancreatography; MRI = magnetic resonance imaging; MT-IPMN = mixed-type IPMN; PC = pancreatic cancer; RI = relative indication for surgery.

TABLE 2 | Patient and cyst characteristics per follow-up visit. A follow-up visit is defined as a visit where at least MRI/MRCP, EUS or CT is performed. Baseline visits were excluded from these analyses.

	All follow-up visits (n=2643)	Follow-up visits with cyst size <20 mm (n=1501)	Follow-up visits with cyst size ≥20mm (n=1142)	P-value
Symptoms[a], n (%)	261 (10)	117 (7.8)	144 (13)	<0.001
Jaundice, n (%)	4 (0.2)	2 (0.1)	2 (0.2)	1.00
Steatorrhea, n (%)	22 (0.8)	4 (0.3)	18 (1.6)	<0.001
Abdominal pain, n (%)	166 (13)	75 (5.0)	91 (8.0)	0.002
Weight loss, n (%)	66 (2.5)	36 (2.4)	33 (2.9)	0.43
MPD dilation, n (%)	297 (11)	137 (9.1)	160 (14)	<0.001
MPD 5-9mm	223 (8.4)	93 (6.2)	130 (11)	<0.001
MPD ≥10mm	74 (2.8)	44 (2.9)	30 (2.6)	0.64
Solid component, n (%)	45 (1.7)	13 (0.9)	32 (2.8)	<0.001
Mural nodule <5mm	16 (0.6)	6 (0.4)	10 (0.9)	0.12
Mural nodule ≥5mm	4 (0.2)	2 (0.1)	2 (0.2)	0.78
Enhancing solid mass	25 (0.9)	5 (0.3)	20 (1.8)	<0.001
Elevated serum CA19.9, n (% of determined)[b]	146 (9.7)	84 (9.6)	62 (10)	0.81
AI or RI at visit, n (%)	548 (21)	266 (18)	282 (25)	<0.001
AI and/or ≥2 RI	130 (4.9)	67 (4.5)	63 (5.5)	0.01
1 RI	418 (16)	199 (13)	219 (19)	<0.001
Modality, n (%)				
MRI/MRCP only (+/- CT)	1832 (69)	1067 (71)	765 (67)	0.01
EUS only (+/- CT)	643 (24)	341 (23)	302 (26)	0.04
MRI + EUS (+/- CT)	55 (2.1)	26 (1.7)	29 (2.5)	0.15
CT only	113 (4.3)	67 (4.5)	46 (4.0)	0.61

[a] Any symptom of jaundice, steatorrhea, abdominal pain and/or weight loss; DM de novo and pancreatitis as these were not included out of risk of duplication; as these are noted for a period of two years; [b] Serum CA19.9: Missing value for 1145 follow-up visits (43%; 41% of follow-up visits with cysts <20mm; 45% of those with cysts ≥20mm). AI = absolute indication of surgery; CT = computed tomography; EUS = endoscopic ultrasound; MPD = main pancreatic duct; MRCP = magnetic resonance cholangio-pancreatography; MRI = magnetic resonance imaging; PC = pancreatic cancer; RI = relative indication for surgery.

TABLE 3 | Univariable and multivariable analysis of predictors for high-grade dysplasia (HGD) and pancreatic cancer (PC). MRI = magnetic resonance imaging; EUS = endoscopic ultrasound; MPD = main pancreatic duct.

	Univariable analysis		Multivariable analysis	
	HR (95% CI)	p-value	HR (95% CI)	p-value
Age, years	(0.97-1.03)	0.91		
<65 years	0.88 (0.49-1.59)	0.67		
Male gender	0.60 (0.34-1.08)	0.09		
Diabetes mellitus	1.30 (0.75-2.25)	0.36		
Imaging modality				
MRI	0.58 (0.29-1.14)	0.11		
EUS	0.88 (0.12-6.43)	0.88		
AI or 2 RI at baseline	26.2 (14.6-46.9)	<0.001	24.0 (13.3-43.2)[a]	<0.001
MPD ≥10mm	9.61 (4.3-21.6)	<0.001		
Jaundice	21.5 (5.19-89.3)	<0.001		
Mural nodule ≥5mm	39.5 (12.0-130.7)	<0.001		
Solid mass	30.5 (15.0-62.0)	<0.001		
1 RI at baseline	3.03 (1.62-5.69)	<0.001	2.34 (1.24-4.43)[b]	0.009
MPD 5-9 mm	9.02 (4.98-16.3)	<0.001		
CA19.9 ≥37kU/L*	2.50 (1.02-6.14)	0.05		
Mural nodule <5mm	30.7 (12.7-74.2)	<0.001		
Acute pancreatitis	5.95 (2.52-14.1)	<0.001		
Cyst size (mm)	1.02 (1.01-1.03)	<0.001	1.02 (1.01-1.03)[c]	0.005

*at baseline 480 missing variables. [a] adjusted for presence of 1RI and cyst size; [b] adjusted for the presence of AI and cyst size; [c] adjusted for the presence of RI and AI at baseline.

Predictive value of cyst size

A 1mm larger initial cyst size increased the risk of developing HGD/PC within 26 months by 2% (HR 1.02 [95% CI 1.01-1.03]; p=0.005; **Table 3**). Participants with an initial cyst ≥20mm had a 2-fold higher risk of developing HGD/PC, as compared to those with cysts <20mm (HR 2.2 [95% CI 1.2-4.0], p=0.01; **Figure 2**). Similarly, initial cysts ≥15mm harbored an increased risk of developing HGD/PC within 26 months, as compared to cysts <15 mm (HR 2.1 [95% CI 1.1-4.1], p=0.03). A cutoff of ≥30mm was associated with a nearly 3-fold higher risk of developing HGD/PC (HR 2.8 [95% CI 1.5-5.3], p<0.001); a cyst size ≥40mm was not associated with an increased risk of developing HGD or PC (**Supplemental Figure S2**).

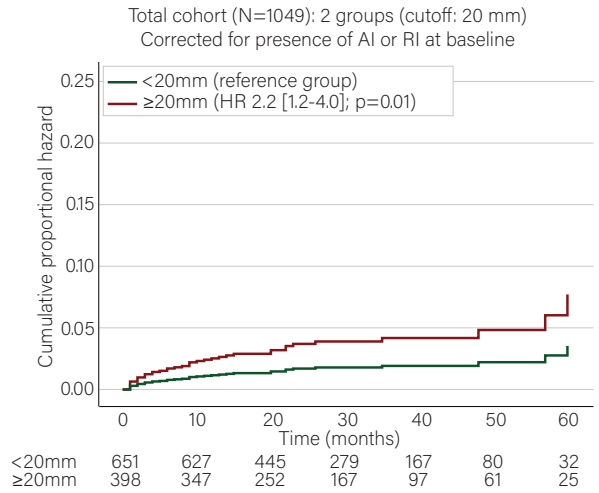


FIGURE 2 | Cumulative hazard plot showing a 2-fold increased risk of developing high-grade dysplasia (HGD) or pancreatic cancer (PC) in individuals with cyst size ≥ 20mm, independent of presence of relative indications for surgery (RIs) or absolute indications for surgery (AIs) at baseline. Cox proportional hazards analysis was performed; HR = hazard ratio, intervals are 95% confidence intervals (CIs); p<0.05 for statistical significance.

Risk of HGD and PC at follow-up

During follow-up, participants whose cysts grew to (or remained) ≥20mm during follow-up (n=420 [40%]) were more often diagnosed with HGD/PC (7.1% *vs* 2.5%; p<0.001) and AI (8.8% *vs* 5.1%; p=0.02) than those whose cysts remained smaller (**Figure 3A-C**). Even in the absence of other AI (n=980), HGD/PC was more prevalent if cysts became ≥20mm (2.6%) than if not (0.7%; p=0.01). HGD/PC was diagnosed at age <65, 65-74 and ≥75 years in 16 (4.7%), 17 (4.6%) and 11 (4.2%; p=0.96) individuals, respectively. **Supplemental Figure S1** shows the comparison of these characteristics between the five cyst size groups.

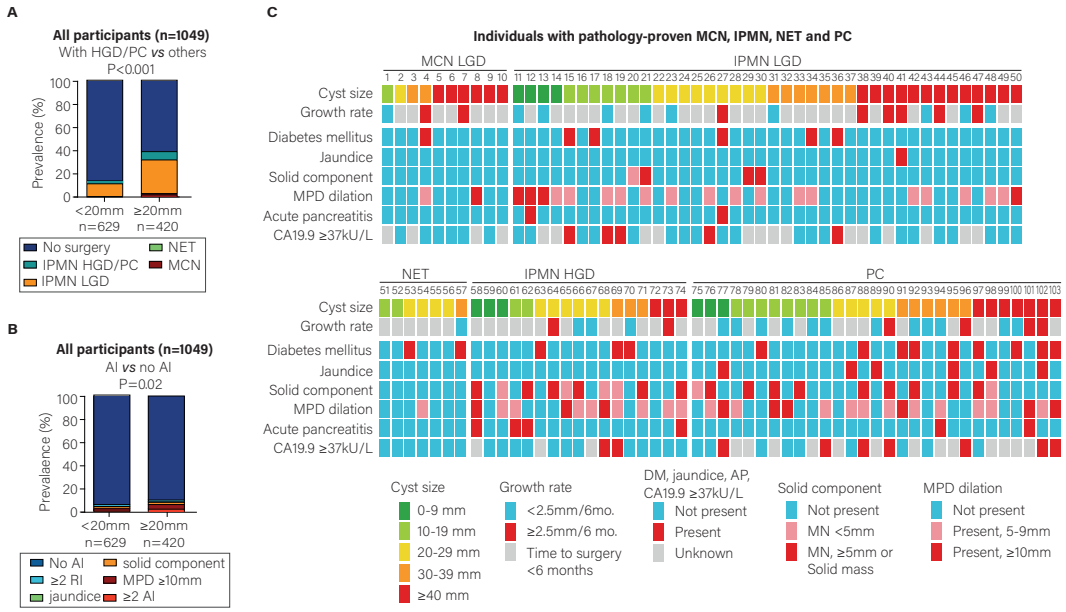


FIGURE 3 | **Results of cross-sectional analysis for two cyst size groups. A.** Prevalence of high-grade dysplasia (HGD) or pancreatic cancer (PC) was higher in participants with a cyst size ≥20mm than for <20mm. **B.** In participants with a cyst size ≥20mm, AIs were more prevalent than those with a cyst size of <20mm. **C.** Overview of presence of relative indications for surgery (RI) and AI in association with cyst size (and growth) and pathology results for individuals with pathology-proven mucinous cystic neoplasm (MCN, n=10), intraductal papillary mucinous neoplasm (IPMN, n=57), neuroendocrine tumor (NET, n=7) or pancreatic cancer (PC, n=29). Seven participants with solid pseudopapillary neoplasm (SPN, n=1; cyst size 70 mm), intraductal papillary neoplasm of the bile duct (IPNB, n=1; 49 mm), serous cystic adenoma (SCA, n=2; 35-36mm), pseudocyst (n=1; 25mm), lymphoepithelial cyst (n=1; 20mm), lymphangioma (n=1; 16mm) were not included in this figure due to low patient numbers. **A-B.** Percentages were compared using χ^2-test (significance level p<0.05). LGD = low-grade dysplasia; AP = acute pancreatitis; DM = diabetes mellitus.

Predictive value of cyst growth

Of 1049 study participants, 968 with at least 12 months follow-up were included in the growth analysis. Cyst size increased in 247 (26%) individuals, of which 42 (4.4%) showed growth ≥5mm; 19 (3.0%) with baseline cysts <20mm and 23 (6.7%) with cysts ≥20mm at baseline (p=0.008; **Figure 4A**). In this fast-growing group, four individuals developed HGD or PC (9%), whereas only eight (0.9%) in the <5mm/year group developed malignancy. In analogy, univariable analysis showed that 1mm/year faster cyst growth harbored a 12% higher risk of HGD/PC (HR 1.12 [1.04-1.21], p=0.002). Growth ≥5mm/year was associated with a 14-fold higher risk of HGD/PC as compared to growth <5mm (HR 14.43 [4.32-48.23], p=<0.001; Figure 4B-C).

Figure 4D shows growth speed in relation to the final pathological diagnosis (n=70) in this subgroup. Interestingly, of the eight individuals with a growth rate of ≥5mm/year (3 LGD, 2 HGD, 3 PC), none with LGD had additional RI or AI at time of resection, while all individuals with HGD and PC did (**Figure 4E**). Of the controls, 37 (3.9%) demonstrated growth >5mm/year.

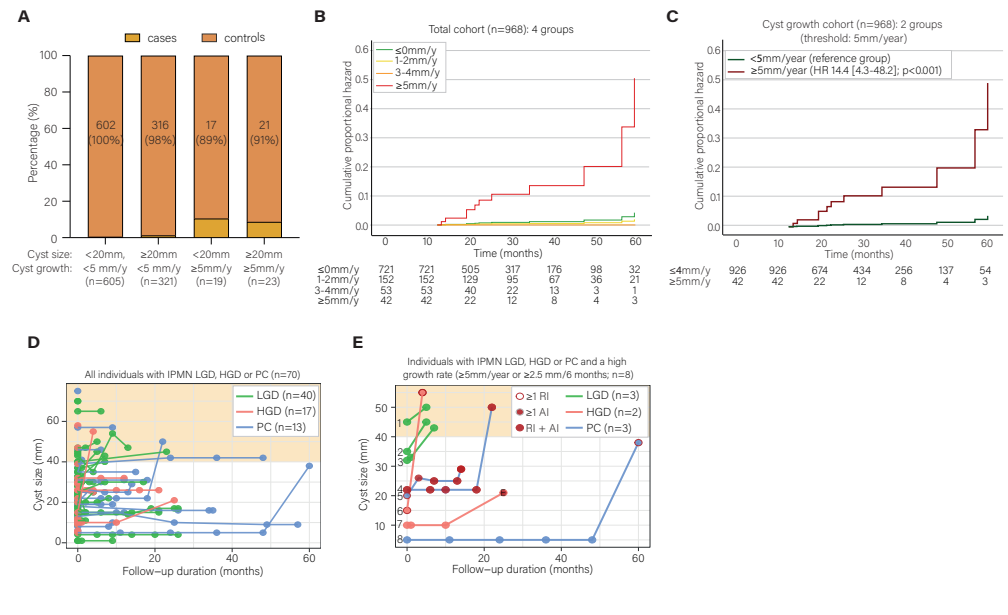


FIGURE 4 | **Cyst growth analysis showed an increased risk of high-grade dysplasia (HGD) or pancreatic cancer (PC) in individuals with a growth rate of ≥5mm/year. A.** The risk of HGD/PC in case of a growth rate ≥5mm/year seems independent of cyst size. However no statistical test was possible due to the low number of events. **B.** Visualization of risk curves shows an increased cumulative risk of HGD or PC in case of an annual cyst growth of 5mm, as compared to the three other groups (size decrease or no growth, 1-2mm/year and 3-4mm/year). No statistical test was performed due to low number of events. **C.** The cox proportional hazards model showed that the risk of HGD/PC is 14-fold higher in individuals with a cyst growth ≥5mm/year than those with a lower growth rate. **D.** Overview of the course of cyst size for surgery cases developing low-grade dysplasia (LGD), HGD and PC. **E.** Detailed overview of surgery cases with a growth rate of ≥5mm/year. HR = hazard ratio; RI = relative indication or surgery; AI = absolute indication for surgery; described intervals are 95% confidence intervals; p-values <0.05 were considered significant.

Discussion

This multicenter prospective cohort study shows that – during the first 26 months of follow-up – a 1mm larger baseline cyst is associated with a 2% higher risk of developing HGD/PC, independent of the presence of other AI or RI. The risk of HGD/PC was 2-fold lower in individuals with cysts <20mm, as compared to those ≥20mm. Cyst growth of <5mm/year was associated with a 14-fold lower risk of developing HGD/PC and seems an independent and even more important risk factor than cyst size.

Pancreatic cysts are identified more frequently than ever, and current intensive surveillance poses a high burden on patients and health care resources. Thus, identification of 'reassuring features' associated with a diminutive risk of development to PC is crucial. A small/stable cyst size may be such a feature. Studies focusing on the malignancy risk of small cysts are scarce. In a retrospective study on 49 individuals with ≤20mm cysts, none developed PC after >5 years of follow-up.[8] Ciprani *et al.*[9] evaluated the risk of malignant transformation in 806 individuals with cysts <15mm (median follow-up 58 months). 14 (1.7%) developed HGD/PC, and growth ≥ 2.5mm/year was the strongest predictor of malignancy (HR=29.54, P <0.001). Other studies investigated surrogate endpoints (risk of worrisome features [WF] and high-risk stigmata [HRS]). Capurso *et al.*[10] (n=540, median follow-up 52 months) showed a 2-fold increased risk of WF/HRS for BD-IPMN >15mm. Similar results were found by Lee *et al.*[11] (n=982, median follow-up 96 months), who showed a two-fold increased risk (HR 1.87, p=0.005) of WF/HRS in individuals with a cyst >15 mm. More recently, Marchegiani *et al.* (2023)[12] stressed that surveillance can even be discontinued at a certain age and cyst size (age 65 years at size <15mm; age 75 at size <30mm) after five years of surveillance without developing RI or AI. In analogy with these results, we now observed that cysts <20mm harbor a two-fold lower risk of developing HGD/PC, independent of other RI and AI. Together, these findings endorse using cyst size <20mm as a 'reassuring feature', provided that no RI or AI are present.

Whilst not the main objective of the study, we also evaluated the risk of HGD and PC in larger cyst sizes and showed that cysts ≥30mm have an increased risk of developing HGD or PC, as compared to those <30mm. The European pancreatic cyst guidelines[3] consider cyst size ≥40mm to be a RI. However, this recommendation was based on predominantly surgical studies investigating the 30 mm threshold.[13-17] The study by Masica *et al.*[18] (2017) selected a ≥40mm cyst size as 'top composite marker' (but without comprehensive evaluation of this threshold), after evaluating 584 individuals with surgically resected IPMNs. They found a median size of 26mm for low- and intermediate-grade IPMNs versus 33mm for HGD and invasive IPMNs. The cox proportional hazards model of this study shows the risk of developing HGD/PC to be 3-fold higher for the 30mm threshold and 2-fold for the 40mm threshold, which may endorse using a cyst size of 30mm as threshold. Based on our case-control analysis, however, a threshold of 40mm may suffice, as HGD/PC and AI were more prevalent in cysts ≥40mm than smaller cysts (**Figure 2**). Future studies are needed to define this threshold.

The current study shows that a 1mm larger initial cyst size is associated with a 2% increased risk of HGD/PC. However, this should be interpreted in light of the likely low background risk of neoplastic pancreatic cysts under surveillance. For instance, an assumed 3% risk would increase to 3.06% by 1 mm growth.

With regard to cyst growth speed, we found a 14-fold increased risk of HGD/PC in individuals with a cyst growth ≥5mm/year, which endorses the growth speed threshold as recommended by the European cyst guidelines. Similar findings on the predictive value of cyst growth speed were reported by others. In 2019, Marchegiani *et al.*[19] showed that growth ≥2.5mm/year was associated with an increased 10-years cumulative incidence of PC, as compared to growth <2.5mm/year (16.7% *vs* 1.8%, p=0.029). Other studies looked at surrogate endpoints. Tsai *et al.*[20] (2015; 135 individuals, ≥1 year follow-up)[20] showed that those with cyst growth >1mm/year more often developed morphological changes. Yamazaki *et al.*[21] (2021; 283 individuals, median 56 months follow-up) showed that cyst growth (yet not initial cyst size) was associated with the development of HRS. Our sample size did not allow for appraisal of the most appropriate cutoff (or age) to reduce surveillance frequency or even discontinue it, but **Figure 4A** indicates that cyst growth of 5mm/year may be a better cutoff than 2.5mm/year.

Based on our results, a less intensive follow-up regime may suffice for smaller cysts. The Fukuoka guidelines (2017)[22] already propagate a size-based approach. After six-month of stable follow-up, they recommend two-yearly follow-up for cysts <10mm, yearly for 10-20mm cysts and every 3-6 months for larger cysts, with possible lengthening of intervals when cysts remain stable. Conversely, in the European guidelines (2018) the management does not depend on cyst size subgroups, yet marks cyst size ≥40mm as RI requiring intensified 6-monthly surveillance or surgery in case of a second RI or AI (depending on patient fitness). For future European guidelines[3], we would opt for a similar cyst-size based approach with lengthening of surveillance intervals after a period (*e.g.*, three years) of a stable cyst size <20mm without RI and AI. Discontinuation of surveillance could even be considered at some point for low-risk cysts and addition of other reassuring features (*e.g.*, older age or lack of multifocality[23,24]) may aid decision-making. However, in contrast to the Fukuoka guidelines,[22] we would not encourage surgery based on cyst size alone, as this may lead to unnecessary harm related to surgery of lesions without HGD or PC (upon post-surgical pathology).

As compared to current literature, the PACYFIC-study is a unique international multi-center registry that prospectively records data of a representative population under pancreatic cyst surveillance in general clinical practice. The relatively large sample size allowed us to use HGD/PC as an endpoint and not AI as a surrogate. The study also has limitations. First, cyst management was determined by the treating physician and not formally based on guideline recommendations. For example, EUS was used more often for cysts ≥20mm, and MRI more often for smaller cysts, perhaps driven by a higher prevalence of RI or AI in larger cysts. This may have influenced results, as Huynh (2020)[25]

observed EUS to slightly underestimate cyst size by 1-4 mm, whereas MRI overestimates by 3mm. This also emphasizes the importance of performing surveillance with the same imaging modality, to allow for reliable growth estimation.

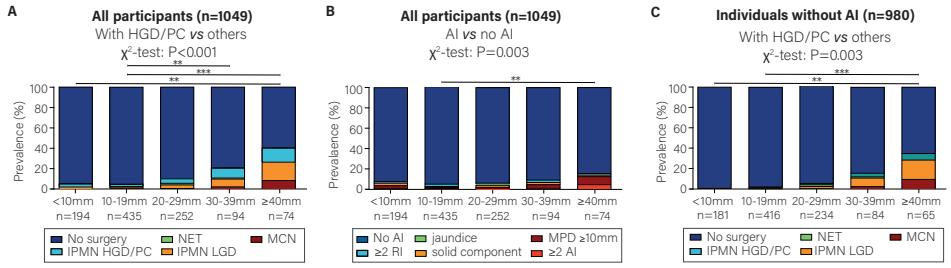
A second limitation is that our study contains missing values, largely because at initiation, certain risk factors had not yet been identified. For instance, an elevated serum CA19.9 had not yet been identified as a RI at the start of our study, and thus determination was initially often omitted. The same holds true for cyst growth. Referral bias of higher risk cysts may be another limitation, as 14 of 28 participating centers were performing tertiary care. This would also explain why smaller cysts (<10mm) still had a relatively high prevalence of AI in this cohort. Whilst this trial aims to recruit individuals with neoplastic cysts, other cystic lesions (*e.g.*, SCAs, lymphoepithelial cysts, pseudocysts) often show similar morphological changes on imaging and may have been misdiagnosed as IPMN. Furthermore, this study has a limited follow-up time of 26 months. Hence, we are unable to draw conclusions on the HGD/PC risk on the longer term (especially considering that only 12 HGD/PC were diagnosed past the first year of follow-up). Finally, the number of malignant cases is low (especially in the cyst growth cohort). Therefore, future analyses (with more cases) are required to evaluate the long-term risk.

In conclusion, this study shows that cyst size and growth rate are independent predictors of the development of HGD/PC. Cysts smaller than 20mm and an annual growth of less than 5mm appear to be 'reassuring features'. Such 'trivial cysts' may require less frequent monitoring, provided that no other RI or AI are present. Low-risk cysts should be a focal point of attention for future studies, to alleviate the burden of intensive follow-up regimes on patients and health care resources. However, clinical studies with extended follow-up and more cases are required to draw definite conclusions for the longer term.

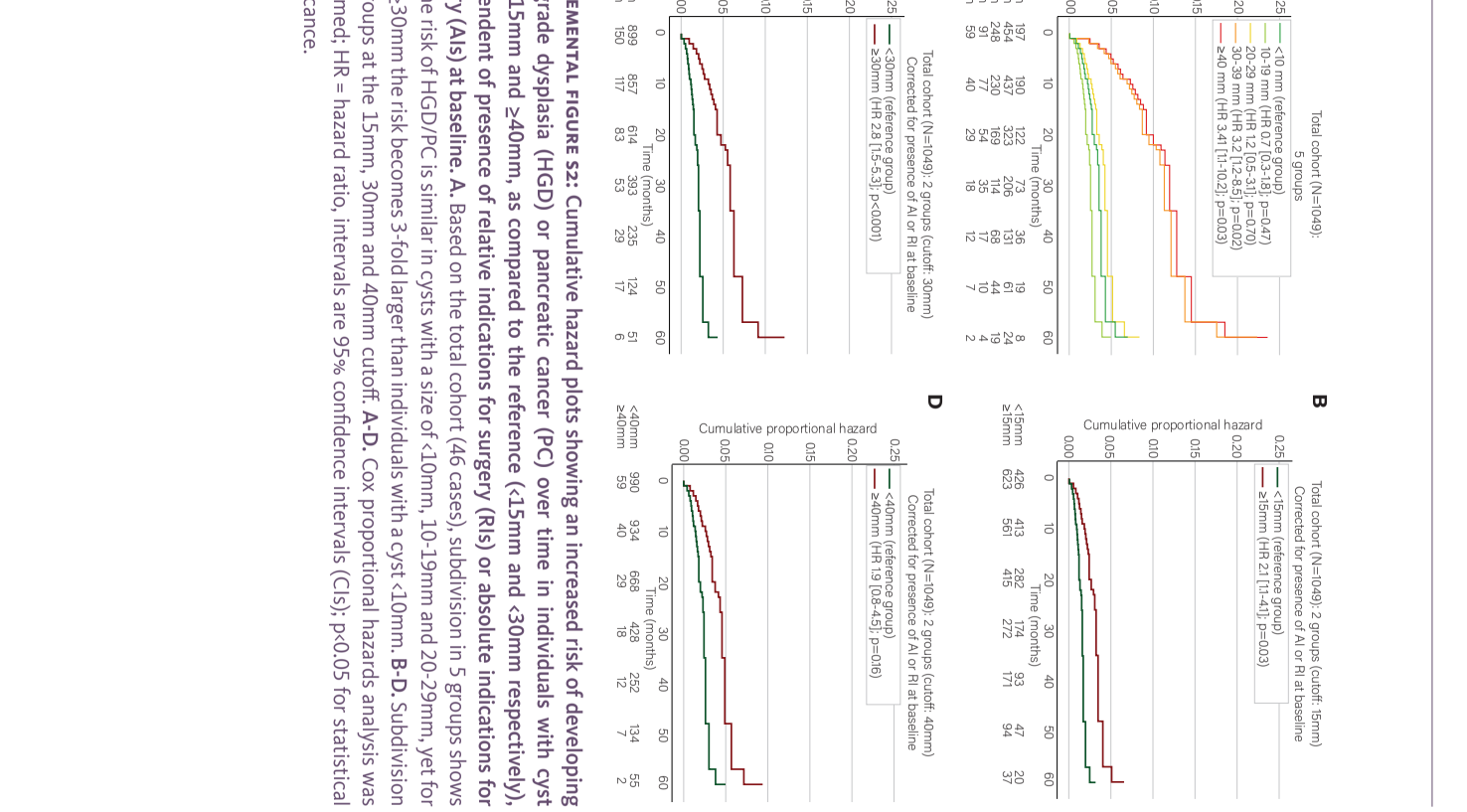
References

1 Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. *CA Cancer J Clin* 2020;70:7-30.
2 Kromrey M-L, Bülow R, Hübner J, *et al*. Prospective study on the incidence, prevalence and 5-year pancreatic-related mortality of pancreatic cysts in a population-based study. *Gut* 2018;67:138.
3 The European Study Group on Cystic Tumours of the P. European evidence-based guidelines on pancreatic cystic neoplasms. *Gut* 2018;67:789.
4 Konings ICAW, Canto MI, Almario JA, *et al*. Surveillance for pancreatic cancer in high-risk individuals. *BJS open* 2019;3:656-665.
5 Zelga P, Hernandez-Barco YG, Qadan M, *et al*. Number of Worrisome Features and Risk of Malignancy in *Intraductal Papillary Mucinous Neoplasm*. Journal of the American College of Surgeons 2022;234.
6 Marchegiani G, Crippa S, Perri G, *et al*. Surgery for Intraductal Papillary Mucinous Neoplasms of the Pancreas: Preoperative Factors Tipping the Scale of Decision-Making. *Annals of surgical oncology* 2022;29:3206-3214.
7 Poruk KE, Shahrokni A, Brennan MF. Surgical resection for intraductal papillary mucinous neoplasm in the older population. *Eur J Surg Oncol* 2022;48:1293-1299.
8 Handrich SJ, Hough DM, Fletcher JG, *et al*. The natural history of the incidentally discovered small simple pancreatic cyst: long-term follow-up and clinical implications. *AJR Am J Roentgenol* 2005;184:20-3.
9 Ciprani D, Weniger M, Qadan M, *et al*. Risk of malignancy in small pancreatic cysts decreases over time. *Pancreatology* 2020;20:1213-1217.
10 Capurso G, Crippa S, Vanella G, *et al*. Factors Associated With the Risk of Progression of Low-Risk Branch-Duct Intraductal Papillary Mucinous Neoplasms. *JAMA Netw Open* 2020;3:e2022933.
11 Lee BS, Nguyen AK, Tekeste TF, *et al*. Long-term follow-up of branch-duct intraductal papillary mucinous neoplasms with No change in first 5 Years of diagnosis. *Pancreatology* 2021;21:144-154.
12 Marchegiani G, Pollini T, Burelli A, *et al*. Surveillance for Presumed BD-IPMN of the Pancreas: Stability, Size, and Age Identify Targets for Discontinuation. *Gastroenterology* 2023 Oct;165(4):1016-1024.e5.
13 Woo SM, Ryu JK, Lee SH, *et al*. Branch duct intraductal papillary mucinous neoplasms in a retrospective series of 190 patients. *Br J Surg* 2009;96:405-11.
14 Sadakari Y, Ienaga J, Kobayashi K, *et al*. Cyst size indicates malignant transformation in branch duct intraductal papillary mucinous neoplasm of the pancreas without mural nodules. *Pancreas* 2010;39:232-6.
15 Hirono S, Tani M, Kawai M, *et al*. The carcinoembryonic antigen level in pancreatic juice and mural nodule size are predictors of malignancy for branch duct type intraductal papillary mucinous neoplasms of the pancreas. *Ann Surg* 2012;255:517-22.
16 Sahora K, Mino-Kenudson M, Brugge W, *et al*. Branch duct intraductal papillary mucinous neoplasms: does cyst size change the tip of the scale? A critical analysis of the revised international consensus guidelines in a large single-institutional series. *Ann Surg* 2013;258:466-75.

17 Ohtsuka T, Kono H, Tanabe R, *et al*. Follow-up study after resection of intraductal papillary mucinous neoplasm of the pancreas; special references to the multifocal lesions and development of ductal carcinoma in the remnant pancreas. *Am J Surg* 2012;204:44-8.
18 Masica DL, Dal Molin M, Wolfgang CL, *et al*. A novel approach for selecting combination clinical markers of pathology applied to a large retrospective cohort of surgically resected pancreatic cysts. *Journal of the American Medical Informatics Association* 2016;24:145-152.
19 Marchegiani G, Andrianello S, Pollini T, *et al*. "Trivial" Cysts Redefine the Risk of Cancer in Presumed Branch-Duct Intraductal Papillary Mucinous Neoplasms of the Pancreas: A Potential Target for Follow-Up Discontinuation? *Am J Gastroenterol* 2019;114:1678-1684.
20 Tsai H-M, Chuang C-H, Shan Y-S, *et al*. Features associated with progression of small pancreatic cystic lesions: A retrospective study. *World journal of gastroenterology* 2015;21:13309-13315.
21 Yamazaki T, Tomoda T, Kato H, *et al*. Risk Factors for the Development of High-risk Stigmata in Branch-duct Intraductal Papillary Mucinous Neoplasms. *Intern Med* 2021;60:3205-3211.
22 Tanaka M, Fernández-del Castillo C, Kamisawa T, *et al*. Revisions of international consensus Fukuoka guidelines for the management of IPMN of the pancreas. *Pancreatology* 2017;17:738-753.
23 Marchegiani G, Pollini T, Burelli A, *et al*. Surveillance for Presumed BD-IPMN of the Pancreas: Stability, Size, and Age Identify Targets for Discontinuation. *Gastroenterology* 2023.
24 Overbeek KA, van Leeuwen N, Tacelli M, *et al*. International external validation of a stratification tool to identify branch-duct intraductal papillary mucinous neoplasms at lowest risk of progression. *United European Gastroenterology Journal* 2022;10:169-178.
25 Huynh T, Ali K, Vyas S, *et al*. Comparison of imaging modalities for measuring the diameter of intraductal papillary mucinous neoplasms of the pancreas. *Pancreatology* 2020;20:448-453.



SUPPLEMENTAL FIGURE S1: Results of case-control analysis for five cyst size groups. A. Prevalence of high-grade dysplasia (HGD) or pancreatic cancer (PC) was higher in individuals with a cyst size ≥40mm than for <20mm (and 30-39mm higher than 10-19mm). **B.** In individuals with a cyst size ≥40mm, absolute indications for surgery (AIs) were more prevalent than those with a cyst size of 10-19mm. The prevalence of main pancreatic duct dilation (MPD) was notably high in individuals with a cyst <10mm ('main-duct IPMN'). **C.** For individuals without absolute indications for surgery (AI; n=980), those with a cyst size ≥40mm more often harbored HGD or PC than those <20mm. **A-C.** Percentages were compared using χ^2-test (significance level p<0.05). In case of significance, all groups were directly compared (significance level <0.005 due to Bonferroni correction for multiple testing), *** <0.001, ** <0.005.



SUPPLEMENTAL FIGURE S2: Cumulative hazard plots showing an increased risk of developing high-grade dysplasia (HGD) or pancreatic cancer (PC) over time in individuals with cyst size ≥15mm and ≥40mm, as compared to the reference (<15mm and <30mm respectively), independent of presence of relative indications for surgery (RIs) or absolute indications for surgery (AIs) at baseline. A. Based on the total cohort (46 cases), subdivision in 5 groups shows that the risk of HGD/PC is similar in cysts with a size of <10mm, 10-19mm and 20-29mm, yet for cysts ≥30mm the risk becomes 3-fold larger than individuals with a cyst <10mm. **B-D.** Subdivision in 2 groups at the 15mm, 30mm and 40mm cutoff. **A-D.** Cox proportional hazards analysis was performed; HR = hazard ratio, intervals are 95% confidence intervals (CIs); p<0.05 for statistical significance.

SUPPLEMENTAL TABLE S1: Baseline characteristics five cyst size groups

	Total cohort (n=1049)	Cyst size **<10 mm** (n=197)	Cyst size **10-19 mm** (n=454)	Cyst size **20-29 mm** (n=248)	Cyst size **30-39 mm** (n=91)	Cyst size **≥40mm** (n=59)	P-value
Age, mean (SD)	66 (10)	65 (10)	67 (10)	67 (9)	66 (10)	68 (12)	0.03
Female sex, n (%)	660 (63)	136 (69)	306 (67)	145 (58)	43 (47)	30 (51)	<0.001
BMI, mean (SD)[a]	26 (5.4)	26.5 (4.7)	26.3 (4.8)	26.4 (6.1)	28.2 (8.6)	26.4 (4.0)	0.75
Symptoms[b]**, n (%)**	184 (18)	35 (18)	65 (14)	47 (19)	21 (23)	16 (27)	0.16
Jaundice, n (%)	4 (0.4)	-	1 (0.2)	2 (0.8)	1 (1.1)	-	0.45
Acute pancreatitis, n (%)	31 (3.0)	7 (3.6)	11 (2.4)	4 (1.6)	3 (3.3)	6 (10)	0.21
Steatorrhea, n (%)	9 (0.9)	2 (1.0)	1 (0.2)	3 (1.2)	2 (2.2)	1 (1.7)	0.28
Abdominal pain, n (%)	103 (10)	20 (10)	41 (9.0)	29 (12)	10 (11)	3 (5.1)	0.67
Weight loss, n (%)	37 (3.5)	6 (3.0)	11 (2.4)	9 (3.6)	5 (5.5)	6 (10)	0.03
Diabetes mellitus, n (%)	159 (15)	21 (11)	66 (14)	35 (14)	27 (30)	10 (17)	0.007
Diabetes de novo[c], n (%)	13 (1.2)	3 (1.5)	4 (0.9)	4 (1.6)	2 (2.2)	-	0.5
MPD dilation, n (%)	105 (10)	24 (12)	24 (5.3)	27 (11)	14 (15)	16 (27)	<0.001
MPD 5-9mm, n (%)	81 (7.7)	19 (9.6)	16 (3.5)	23 (9.3)	11 (12)	12 (20)	<0.001
MPD ≥10mm, n (%)	24 (2.3)	5 (2.5)	8 (1.8)	4 (1.6)	3 (3.3)	4 (6.8)	0.12
Solid component, n (%)	30 (2.9)	8 (4.0)	7 (1.5)	8 (3.2)	5 (5.5)	2 (3.4)	0.68
Mural nodule <5mm, n (%)	9 (0.8)	2 (1.0)	2 (0.4)	2 (0.8)	3 (3.3)	-	-
Mural nodule ≥5mm, n (%)	4 (0.4)	1 (0.5)	1 (0.2)	-	1 (1.1)	1 (1.7)	-
Enhancing solid mass, n (%)	17 (1.6)	5 (2.5)	4 (0.9)	6 (2.4)	1 (1.1)	1 (1.7)	-
Elevated serum CA19.9, n (% of determined)[d]	56 (5.3)	11 (5.6)	20 (4.4)	16 (6.5)	4 (4.4)	5 (8.5)	0.65
AI or RI at baseline, n (%)	186 (18)	44 (23)	57 (13)	49 (20)	21 (23)	15 (25)	0.05
AI and/or ≥2 RI, n (%)	60 (5.7)	13 (6.6)	20 (4.4)	12 (4.9)	8 (8.8)	7 (12)	0.08
1 RI, n (%)	126 (12)	31 (16)	37 (8.1)	37 (15)	13 (14)	8 (14)	0.002
Working diagnosis							
Unspecified cyst, n (%)	115 (11)	39 (20)	48 (11)	15 (6.0)	8 (8.8)	5 (8.5)	<0.001
BD-IPMN, n (%)	825 (79)	134 (68)	381 (84)	205 (82)	71 (78)	34 (58)	<0.001
MT-IPMN or MD-IPMN, n (%)	87 (8.3)	23 (12)	20 (4.4)	22 (8.9)	10 (11)	12 (20)	<0.001
MCN, n (%)	22 (2.1)	1 (0.5)	5 (1.1)	6 (2.4)	2 (2.2)	8 (14)	<0.001
Modality, n (%)							
MRI/MRCP (+/- CT)	459 (44)	107 (54)	200 (44)	101 (41)	33 (36)	18 (31)	0.003
EUS (+/- CT)	169 (16)	29 (15)	73 (16)	40 (16)	13 (14)	14 (23)	0.55
MRI + EUS (+/- CT)	348 (33)	44 (22)	142 (31)	97 (39)	41 (45)	24 (41)	<0.001
CT only	73 (7.0)	17 (8.6)	39 (8.6)	10 (4.0)	4 (4.4)	3 (5.1)	0.12

[a] BMI: Missing value in 647 (62%) participants; [b] Any symptom of jaundice, acute (recent or recurrent) pancreatitis, steatorrhea, abdominal pain and/or weight loss; [c] Diagnosis of diabetes mellitus for 2 years or less; [d] Serum CA19.9: Missing value for 480 (46%) participants. AI = absolute indication of surgery; BD-IPMN = branch-duct IPMN; CT = computed tomography; EUS = endoscopic ultrasound; MD-IPMN = Main-duct IPMN; MPD = main pancreatic duct; MRCP = magnetic resonance cholangio-pancreatography; MRI = magnetic resonance imaging; MT-IPMN = mixed-type IPMN; PC = pancreatic cancer; RI = relative indication for surgery.

CHAPTER 5

The Additive Value of CA19.9 Monitoring in a Pancreatic Cyst Surveillance Program

Authors

Iris J.M. Levink, Sanne C. Jaarsma, Brechtje D.M. Koopmann, Priscilla A. van Riet, Kasper A. Overbeek, Jihane Meziani, Marloes L.J.A. Sprij, Riccardo Casadei, Carlo Ingaldi, Marcin Polkowski, Megan M.L. Engels, Laurens A. van der Waaij, Silvia Carrara, Elizabeth Pando, Marlies Vornhülz, Pieter Honkoop, Erik J. Schoon, Johanna Laukkarinen, Jilling F. Bergmann, Rossi Gemma, Frederike G.I. van Vilsteren, Anne-Marie van Berkel, Trevor Tabone, Matthijs P. Schwartz, Adriaan C.I.T.L. Tan, Jeanin E. van Hooft, Rutger Quispel, Ellert van Soest, Laszlo Czacko, Marco J. Bruno, Djuna L. Cahen, on behalf of the PACYFIC-registry work group.

United European Gastroenterol J., 2023 Sep;11(7):601-611.

Abstract

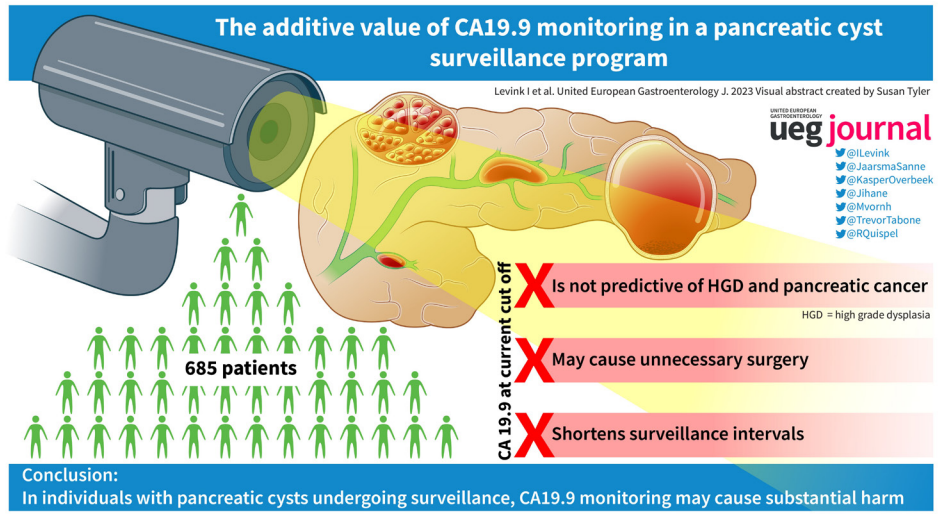
Background: Surveillance of pancreatic cysts focuses on the detection of (mostly morphologic) features warranting surgery. European guidelines consider elevated CA19.9 as a relative indication for surgery. We aimed to evaluate the role of CA19.9 monitoring for early detection and management in a cyst surveillance population.

Methods: The PACYFIC-registry is a prospective collaboration that investigates the yield of pancreatic cyst surveillance performed at the discretion of the treating physician. We included participants for whom at least one serum CA19.9 value was determined with a minimum follow-up of 12 months.

Results: Of 1865 PACYFIC participants, 685 met the inclusion criteria for this study (mean age 67 years, SD 10; 61% female). During a median follow-up of 25 months (IQR 24, 1966 visits), 29 participants developed high-grade dysplasia (HGD) or pancreatic cancer (PC).
At baseline, CA19.9 ranged from 1 to 591kU/L (median 10kU/L [IQR 14]), and was elevated (≥37kU/L) in 64 participants (9%). During 191 of 1966 visits (10%), an elevated CA19.9 was detected, and these visits more often led to intensified follow-up (42%) than those without an elevated CA19.9 (27%; p<0.001). An elevated CA19.9 was the sole reason for surgery in five participants with benign disease (10%).
The baseline CA19.9 value was (as continuous or dichotomous variable at the 37kU/L threshold) not independently associated with HGD or PC development, whilst a CA19.9 of ≥133kU/L was (HR 3.8, 95% CI 1.1-13, p=0.03).

Conclusion: In this pancreatic cyst surveillance cohort, CA19.9 monitoring caused substantial harm by shortening of surveillance intervals (and performance of unnecessary surgery). The current CA19.9 cutoff was not predictive for HGD and PC, whereas a higher cutoff may decrease false-positive values. The role of CA19.9 monitoring should be critically appraised prior to implementation in surveillance programs and guidelines.

Graphical abstract



Introduction

Pancreatic cancer (PC) is a leading cause of cancer-related death, with a mere five-year survival of 9%.[1] Timely detection is expected to increase the chance of curative surgery and prolonged survival.[2,3] Intraductal Papillary Mucinous Neoplasms (IPMNs) of the pancreas are neoplastic cystic lesions with a potential for malignant progression. They are increasingly being detected in asymptomatic individuals who undergo cross-sectional imaging for unrelated reasons. As precursor lesions of PC, surveillance is recommended by seeking (mostly) morphological changes (so called relative [RI] and absolute [AI] indications for surgery).[4]

Serum carbohydrate antigen 19.9 (CA19.9) is a tumor marker that is used to monitor the disease course in patients with PC. In the latest update of the European guidelines on cystic neoplasms,[4] an elevated CA19.9 level was added as a RI, meaning that surgery should be considered in the presence of a second RI. This recommendation was based on retrospective surgical studies,[4-7] but the yield of CA19.9 monitoring in a surveillance population has not yet been investigated. Conversely, CA19.9-related harm, due to unnecessary shortening of surveillance intervals and surgery, is unclear.

The aim of this study is to evaluate the role of serum CA19.9 monitoring in individuals undergoing pancreatic cyst surveillance within the PACYFIC-registry, by assessing: 1. The range and variability of CA19.9 levels at baseline and during follow-up; 2. The clinical impact of an elevated value; 3. The diagnostic performance of CA19.9 for the detection of HGD or PC; 4. The risk of developing HGD or PC over time for different CA19.9 cutoffs.

Material and Methods

The PACYFIC registry

The PACYFIC-registry is an ongoing prospective multicenter cohort study (http://www.pacyfic.net) that follows individuals who undergo pancreatic cyst surveillance at the discretion of their treating physician. The study has been running since 2015 and includes individuals with a pancreatic cyst (either newly or previously diagnosed, or previously operated upon) for whom cyst surveillance is warranted according to the treating physician. Exclusion criteria are a history of chronic pancreatitis, suspected pseudocyst or walled-off necrosis, suspected serous cystadenoma, Von Hippel-Lindau disease and limited life expectancy (<2 years). It was designed to investigate the long-term yield of this surveillance. The surveillance period ends if the cyst appears to be benign (due to new insights) or is no longer present, or if the participant is no longer fit for surgery, is lost to follow-up, withdraws participation, or has passed away. A total of 23 academic and community hospitals from Europe (n=22) and The United States (n=1) contributed to this study.

The PACYFIC registry was approved by the ethical review board of Erasmus University Medical Center in 2014 (MEC-2014-021). Written informed consent was obtained from each participant prior to inclusion. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

PACYFIC data are prospectively collected at each follow-up visit and stored in a digital case record form. Registered patient information includes sex, age, body mass index (BMI), presence of symptoms or diabetes mellitus (DM). Cyst characteristics include the working diagnosis, size, presence of AI or RI and histological outcomes (of fine-needle aspiration or biopsy or surgery). Per study participant, each CA19.9 value was tested at the same center (using the same laboratory technique).

In- and exclusion criteria for the current study

All PACYFIC-participants were considered for inclusion in the current study. As surveillance within the PACYFIC-registry is performed upon the discretion of the treating physician, CA19.9 is not always determined. Participants without a recorded CA19.9 value were excluded, as well as those with less than 12 months of follow-up. However, participants – for which a definitive dysplasia grade was established within 12 months after the first recorded CA19.9 value – were included. Individuals with a history of PC or jaundice at baseline were also excluded, as these conditions may influence CA19.9 levels.[8]

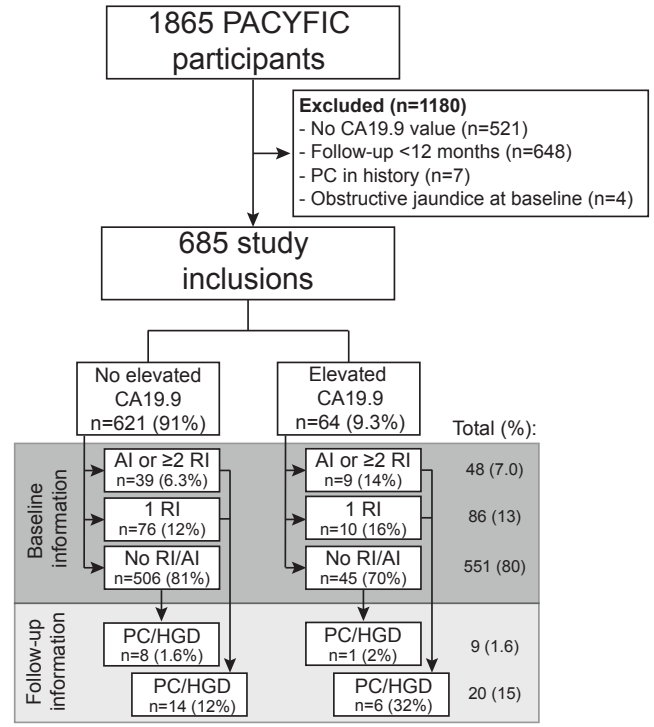


FIGURE 1 | Flowchart of patient in- and exclusion, baseline information and follow-up information. All PACYFIC participants were considered for inclusion in the current study. Participants without a recorded CA19.9 value, as well as those with less than 12 months of follow-up (with the exception of those participants for which a pathological cyst diagnosis was established within the first 12 months after the first recorded CA19.9 value), were excluded. Additionally, individuals with a history of PC or jaundice at baseline were excluded, as these conditions may influence CA19.9 levels. CA19.9 values and (baseline) cyst growth were not included as absolute (AI) or relative (RI) indications for surgery[4]; HGD = high-grade dysplasia; PC = pancreatic cancer.

Definitions

Definitions regarding the presence of RI and AI were based on the European evidence-based guidelines on pancreatic cystic neoplasms.[4] The presence of a solid mass, (tumor-related) jaundice, enhancing mural nodules ≥5 mm or main pancreatic duct (MPD) dilation >10 mm as considered as 'AI'; RI was defined as the presence of one of the following: MPD dilation 5-9 mm, new-onset DM (DM developed within two years prior to visit), acute pancreatitis, enhancing mural nodules <5 mm and a cyst size ≥40 mm (based on the largest cyst). Cyst growth ≥5 mm/year was not included as RI at baseline, as growth is not assessable at that time. Individuals with ≥2 RIs were considered to have an 'AI', as the guideline recommends surgery in this case, regardless of co-morbidity.

'Baseline' is defined as the first visit during which CA19.9 was recorded, whilst follow-up is the period from 'baseline' until time of analysis or end of the study. 'Cases' were defined as individuals who developed pathology-proven HGD or PC and 'controls' as those who did not. Time-to-event was defined as the time (in months) from the first CA19.9 value determination to development of HGD/PC or the last follow-up visit.

Statistical analysis

Patients were stratified according to baseline CA19.9 values (threshold 37 kU/L). Results were expressed as mean with standard deviation (SD; for normally distributed data), median with interquartile range (IQR; for non-normally distributed data) or numbers with percentages. Differences between groups were assessed with a student's t-test/ANOVA for normally distributed data or a Mann–Whitney-U/Kruskal-Wallis-test for non-normally distributed data. For categorical variables, a χ^2-test was used. A correlation between variables was performed using Spearman.

To evaluate the impact of an elevated value on cyst management (regarding surveillance intervals or interventions) were described as proportions (of visits), and compared by a χ^2-test. General surveillance intervals' were based on recommendations by the European guidelines[4] (6 months' interval during the first year and 12 months' thereafter).

Median CA19.9 values were compared with the Mann–Whitney U test. A Receiver operating characteristic (ROC) curve was generated for different cutoffs; besides 37kU/L, two other cutoffs were selected upon visualization of clear angles on the ROC curve (aiming for high specificity). Subsequently, the diagnostic performance (sensitivity, specificity, positive predictive value [PPV], negative predictive value [NPV], accuracy) for these thresholds were calculated; 95% confidence intrevals (CIs) are "exact" Clopper-Pearson CIs. For individuals with a pathological diagnosis, CA19.9 levels from the last visit before this diagnosis were used. Otherwise, levels from 12 months prior were used to minimize the risk of occult PC.

To evaluate the risk of HGD or PC related to baseline CA19.9 values, multiple (univariable and multivariable) proportional hazard's models were generated, with CA19.9 as a continuous and dichotomous variable (at thresholds selected by performed ROC analyses).

Two-sided P-values of less than 0.05 were considered statistically significant. Data were analyzed and graphs visualized using Statistical Package for the Social Sciences (SPSS Inc, Chicago, Illinois, version 27) and GraphPad (GraphPad Prism version 9, La Jolla, CA).

Results

Characteristics of study participants

Of the 1865 PACYFIC participants, 1180 were excluded; 521 for lack of CA19.9 determination, 648 for insufficient follow-up duration, seven for a history of PC and four for jaundice at baseline (**Figure 1**). The mean age of the 685 included individuals was 67 years (SD 10) and 61% were female. BD-IPMN was the most common working diagnosis (77%), followed by unspecified cyst (9.5%) and mixed-type (MT-IPMN) or main-duct IPMN (MD-IPMN; 9.3%; **Table 1**).

During follow-up, a pathological diagnosis was established in 77 individuals (11%); by surgery in 64 (9.3%) and by fine-needle aspiration (FNA) in 13 (1.9%). Of these, 15 (2.2%) had PC (7 resected, 8 irresectable), 14 (2.0%) HGD IPMN (all resected), 27 (4.0%) LGD IPMN (23 resected), six (0.9%) LGD MCN (all resected), and 15 (2.2%) other diagnoses: lympho-epithelial cyst (n=1), lymphangioma (n=1), pseudocyst (n=2), neuroendocrine tumor grade 1 (NET; n=6, 5 resected), serous cystadenoma (SCA; n=4), solid pseudopapillary neoplasm (SPN; n=1).

CA19.9 results

At baseline, CA19.9 values ranged from 1 to 591 kU/L (median 10 kU/L; IQR 14) and were elevated in 64 participants (9.3%). Those with an elevated CA19.9 at baseline were older (p=0.05), had a larger cyst size (p=0.03) and more often an AI or RI (p=0.03), ≥1AI (p=0.004), weight loss (p=0.04), a solid component (p=0.03) or diabetes mellitus (DM; p=0.01; **Table 1**).

During a median follow-up of 25 months (IQR 24; 0-72 months), a median of three surveillance visits took place (IQR 2; range: 1-11 visits), in which a median of three CA19.9 values were determined (IQR 2; range 1-11). Serum CA19.9 ranged from 1 to 1470 kU/L (median 10, IQR 14) and levels were elevated at 191 of 1966 visits (9.7%) in 96 individuals (14%).

The clinical impact of an elevated value

Figure 2 depicts the impact of elevated CA19.9 levels on cyst management. A shortened interval was chosen more often during visits with an elevated value (42%), as compared to those without (27%, p<0.001). Ten of 96 participants with an elevated value underwent surgery (10%). Of these, four had HGD or PC (40%, **Figure 3A**) and six benign disease (60%; **Figure 3B**). In the latter group, an elevated value was the sole reason for surgery in five of 48 participants. Thus, omission of CA19.9 testing may have prevented unnecessary surgery for this group.

TABLE 1 | Baseline characteristics

	Total cohort (n=685)	CA19.9 <37kU/L (n=621)	CA19.9 ≥37kU/L (n=64)	p-value
Age, mean (SD)	67 (10)	67 (10)	69 (8)	**0.05**
Female sex, n (%)	416 (61)	379 (61)	37 (58)	0.62
BMI, mean (SD)[a]	26 (5.1)	26 (5.0)	27 (6.2)	0.89
Previously operated cyst, n (%)	18 (2.6)	18 (2.9)	-	0.17
AI or RI, n (%)[b,c]	134 (20)	115 (19)	19 (30)	**0.03**
≥1 AI, n (%)[b]	48 (7.0)	39 (6.3)	9 (14)	**0.004**
1 RI, n (%)[c]	86 (13)	76 (12)	10 (16)	0.10
Symptoms				
Acute pancreatitis, n (%)	20 (2.9)	19 (3.1)	1 (1.6)	0.50
Steatorrhea, n (%)	4 (0.6)	4 (0.6)	-	0.52
Abdominal pain, n (%)	70 (10)	66 (11)	4 (6.3)	0.25
Weight loss, n (%)	23 (3.4)	18 (2.9)	5 (7.8)	**0.04**
Size largest cyst, median mm (IQR)	16 (13)	16 (13)	18 (13)	**0.03**
Cyst size ≥40 mm, n (%)	42 (6.1)	37 (6.0)	5 (7.8)	0.53
Diabetes mellitus, n (%)	122 (18)	103 (17)	19 (30)	**0.01**
New-onset diabetes mellitus, n (%)	8 (1.2)	7 (1.1)	1 (1.6)	081
MPD dilation, n (%)	87 (13)	75 (12)	12 (19)	0.14
MPD 5-9 mm, n (%)	60 (8.8)	52 (8.4)	8 (13)	0.29
MPD ≥ 10 mm, n (%)	18 (2.6)	14 (2.3)	4 (6.3)	0.06
Dilated, unknown PD diameter n (%)	9 (1.3)	9 (1.4)	-	0.33
Solid component, n (%)	22 (3.2)	17 (2.7)	5 (7.8)	**0.03**
Mural nodule <5 mm, n (%)	8 (1.2)	5 (0.8)	3 (4.7)	**0.006**
Mural nodule ≥5 mm, n (%)	4 (0.6)	4 (0.6)	-	0.52
Enhancing solid mass (other), n (%)	10 (1.5)	8 (1.3)	2 (3.1)	0.31
Working diagnosis, n (%)[d]				
Unspecified cyst	65 (9.5)	56 (9.0)	9 (14)	0.20
BD-IPMN	525 (77)	481 (78)	44 (69)	0.15
MT-IPMN or MD-IPMN	64 (9.3)	57 (9.2)	7 (11)	0.19
MCN	12 (1.8)	12 (1.9)	-	0.26
NET	3 (0.4)	3 (0.5)	-	-
PC	2 (0.3)	1 (0.2)	1 (1.6)	-
No visible cyst (previous surgery)	8 (1.2)	8 (1.3)	-	0.38
Unknown	6 (0.9)	3 (0.5)	3 (4.7)	-

[a] Body mass index (BMI) was a missing value for 349 individuals; [b] Absolute indications for surgery (AI): solid mass, enhancing mural nodule (≥5 mm), main pancreatic duct dilation ≥10 mm or ≥2 relative indications for surgery (RI); Patients with baseline jaundice were excluded[4]; [c] RI: main pancreatic duct (MPD) 5-9 mm, cyst diameter ≥40 mm, new-onset diabetes mellitus, (recent or recurrent) acute pancreatitis, mural nodule <5 mm. Growth-rate (as a rate cannot be calculated from one single time point) and serum CA19.9 value were not included as RIs; [d] Not pathology-proven, upon imaging interpretation of the radiologist or endosonographer. SD = standard deviation, IQR = interquartile range, BD-IPMN = branch-duct IPMN, MT-IPMN = mixed-type IPMN, MD-IPMN = main-duct IPMN, MCN = mucinous cystic neoplasm, NET = neuroendocrine tumor, PC = pancreatic cancer.

Seven (5 PC, 2 HGD) of 29 individuals with HGD or PC had an elevated CA19.9 value (24%; **Figure 3A**). Of these, five had at least one AI (4 resectable, 1 irresectable), and two had 1 RI (#1 and #8; both were unresectable at time of diagnosis). Thus, in this study, no malignancy was detected in a resectable stage due to an elevated CA19.9 value (alone or in addition to 1 RI). Of 542 controls with at least two CA19.9 values, 79 (14%) had at least one elevated CA19.9 value during follow-up. **Figure 3C** depicts the variation in these values.

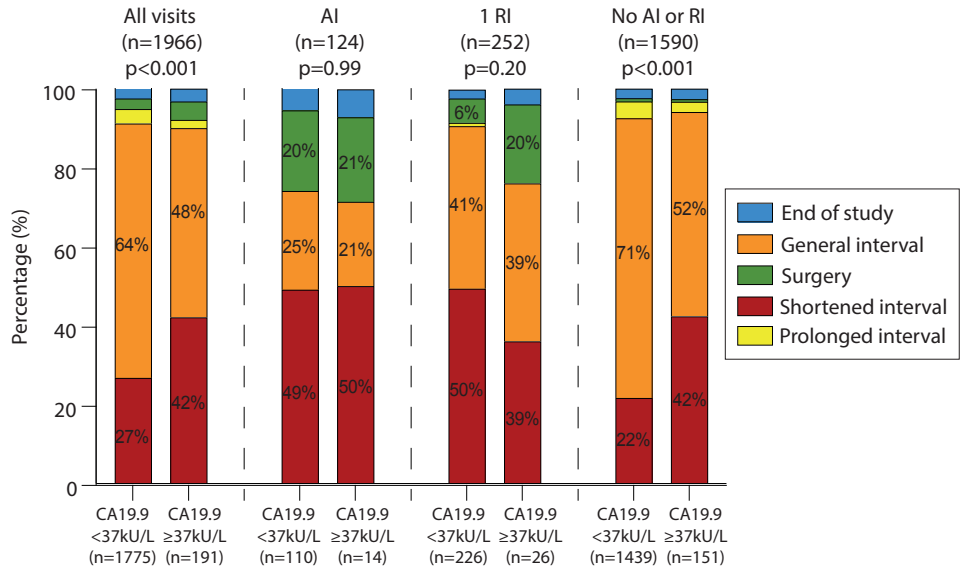


FIGURE 2 | **The clinical consequences of an elevated value at all visits, as well as for those visits with and without detected absolute (AI) or relative (RI) indications for surgery.** For Individuals without AI or RI, those with an elevated CA19.9 value more often underwent a shortened surveillance interval, as compared to those without an elevated value (χ^2-test). Management was based on the decision of the physician after imaging. A general surveillance interval was a 6 months' interval during the first year of surveillance and a 12 months' interval during follow-up afterwards (as based on the recommendations in the European Guidelines.[4]

The diagnostic performance of CA19.9

The 29 individuals with HGD or PC (n=29) did not have a higher median CA19.9 level (11 kU/L [IQR 24], range 1-1470) than the benign group (n=656; 10 kU/L [IQR 12], range 1-605; p=0.18). However, those with HGD or PC more often had a CA19.9 value ≥37 kU/L (24% *vs* 8%; p=0.002; **Figure 4A**). Subgroup analysis showed no difference in median CA19.9 levels between dysplasia grades (p=0.58, **Figure 4B**) and the presence or absence of AI and/or RI (**Figure 4C**).

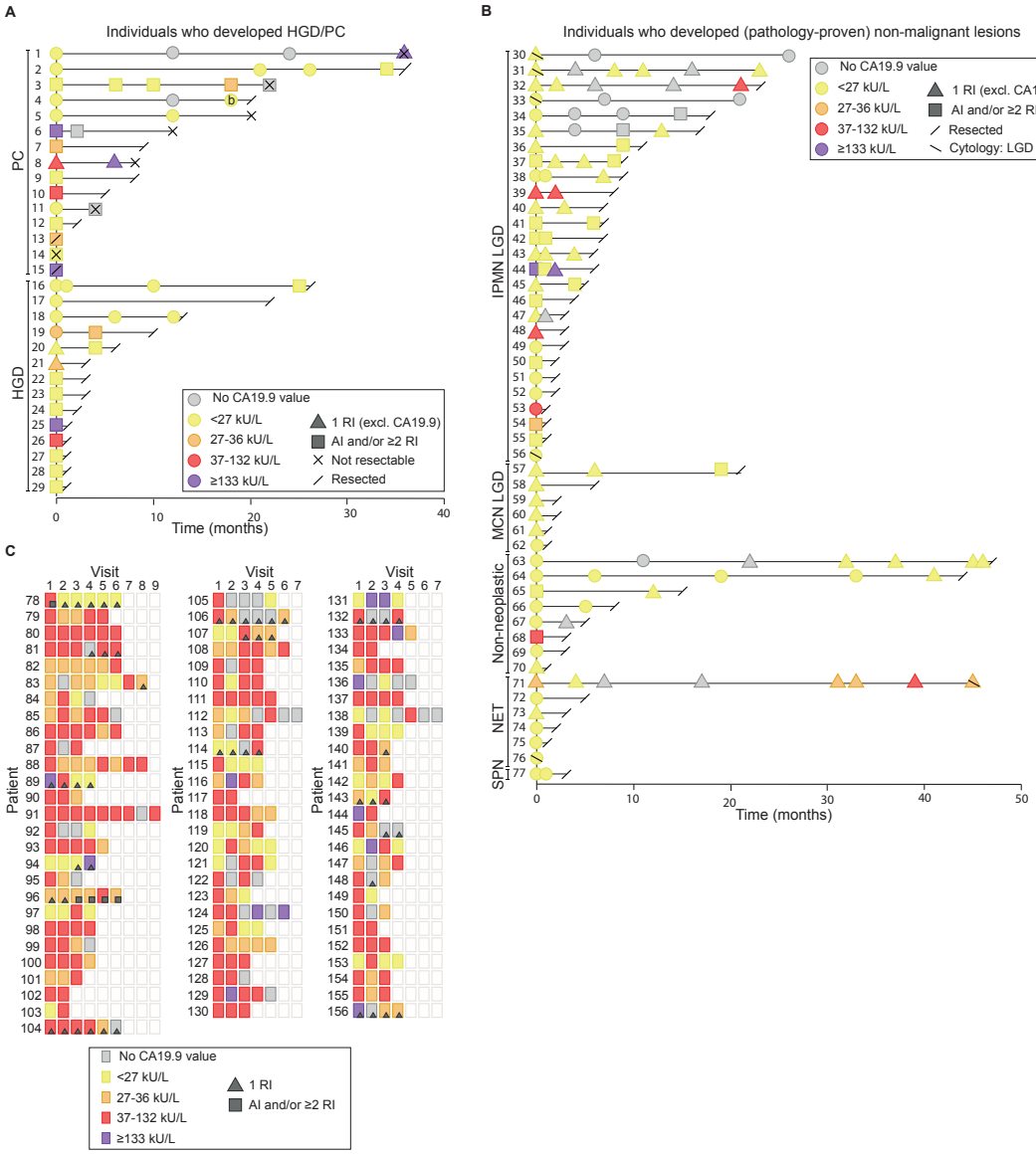
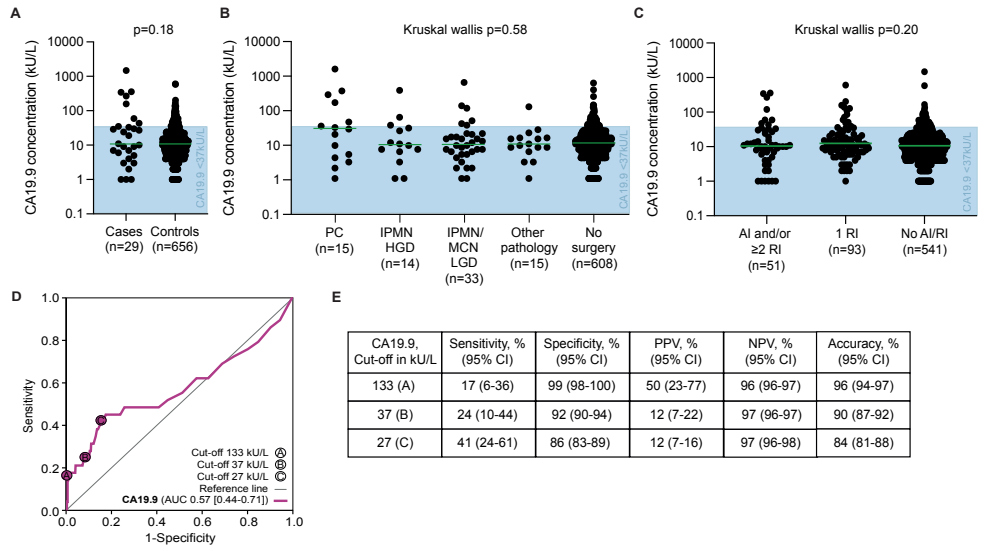


FIGURE 3 | The role of CA19.9 monitoring on treatment management. A-B. Presence of relative indications for surgery other than CA19.9 elevation (RI), absolute indications for surgery (AI)[4] and elevated CA19.9 values over time for individuals who develop high-grade dysplasia (HGD) or pancreatic cancer (PC; A) and other pathology-proven lesions (B), the FNA prior to surgery in patient 4 ('b') showed no dysplasia; **C.** Overview of participants with two or more available CA19.9 values who did not undergo surgery, showing the high frequency of elevated values. IPMN = intraductal papillary mucinous neoplasm; MCN = mucinous cystic neoplasm; PA-proven = pathology proven; NET = neuro-endocrine tumor; SPN = solid pseudopapillary neoplasm.

To determine the most reliable cutoff for PC detection, a ROC-curve was created (AUC 0.57 [95% CI 0.44-0.71]; **Figure 4D**). The cutoff of ≥37kU/L was able to differentiate between HGD/PC and controls with a sensitivity of 24% (95% CI 10-44%), specificity of 92% (95% CI 90-94%) and accuracy of 90% (95% CI 87-92%; **Figure 4E**).

Two alternative higher and lower cutoff values were chosen based on this ROC curve. A higher cutoff (A; 133 kU/L) significantly increased specificity (99% [95% CI 98-100]) and PPV (50% [95% CI 23-77]), without changing the NPV (96% [95% CI 96-97%]). In contrast, a lower cutoff of 27 kU/L increased sensitivity to 41% (95% CI 24-61%), yet decreased specificity (86% [95% CI 83-89]) and accuracy (84% [95% CI 81-88]), without changing the PPV and NPV **Figure 4E**).



CA19.9, Cut-off in kU/L	Sensitivity, % (95% CI)	Specificity, % (95% CI)	PPV, % (95% CI)	NPV, % (95% CI)	Accuracy, % (95% CI)
133 (A)	17 (6-36)	99 (98-100)	50 (23-77)	96 (96-97)	96 (94-97)
37 (B)	24 (10-44)	92 (90-94)	12 (7-22)	97 (96-97)	90 (87-92)
27 (C)	41 (24-61)	86 (83-89)	12 (7-16)	97 (96-98)	84 (81-88)

FIGURE 3 | Subgroup analyses do not show differences in median CA19.9 values. However, CA19.9 is able to differentiate cases from controls with high specificity, yet low sensitivity. **A.** The median CA19.9 value is not higher in cases with high-grade dysplasia (HGD) or pancreatic cancer (PC) than controls; **B.** Individuals with PC, HGD, low-grade dysplasia (LGD), other pathology (heterogeneous group of SPN, NET, SCA, lympho-epithelial cyst, lymphangioma and pseudocyst); **C.** The median CA19.9 level was not higher in individuals with absolute (AI) or relative (RI) indications for surgery[26], as compared to those without; **D.** Receiver operator characteristic (ROC) curve of serum CA19.9 with three cutoffs (37 kU/L) and two selected based on visualized angles in the curve); **E.** Diagnostic performance at the three cutoffs. Described data do not have equal variances, therefore nonparametric tests were used (A-C). 95% confidence intervals (CIs) are "exact" Clopper-Pearson confidence intervals (D-E). AUC = area under the curve; NPV= negative predictive value.

The risk of future development of HGD or PC for different CA19.9 cutoffs

The univariable model showed a 1% higher risk of HGD or PC for each 1kU/L increase in CA19.9 (HR 1.01, 95% CI 1.00-1.01; p<0.001). When evaluating CA19.9 as a binary parameter, the HRs were 2.8 (95% CI 1.3-6.0; p=0.009) for the 27 kU/L threshold, 2.6 (95% CI 1.0-6.3, p=0.04) for 37 kU/L, and 11 (95% CI 3.4-37, p<0.001) for the 133 kU/L threshold (**Supplemental Table 1**). Multivariable analysis showed that the 37 kU/L threshold, continuous or dichotomous, was not independently (of the presence of AI or RI) associated with HGD/PC, whereas the 27 kU/L (HR 2.3; 95% CI 1.1-5.0, p=0.04) and 133 kU/L thresholds were (HR 3.8; 95% CI 1.1-13, p=0.03; **Figure 5A-C, Supplemental Table S1**).

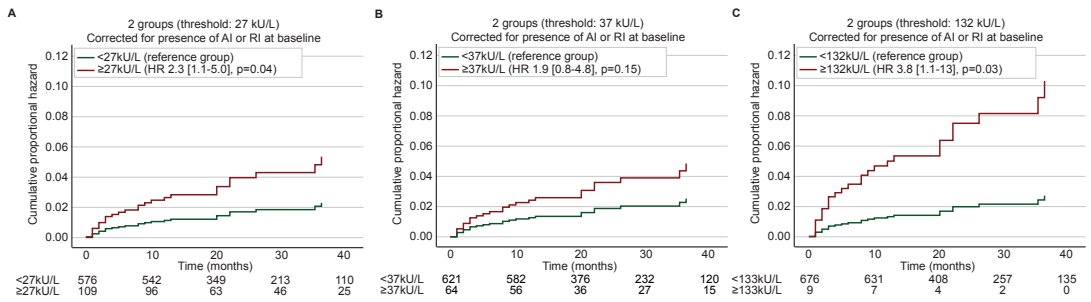


FIGURE 5 | Cox proportional hazard's model. A-C. The risk of developing high-grade dysplasia (HGD) and pancreatic cancer (PC) for three different thresholds (A, B, C; corrected for the presence of absolute indications for surgery (AI) and relative indications for surgery (RI)[4] at baseline). Intervals are 95% confidence intervals.

Discussion

Surveillance of (presumed neoplastic) pancreatic cysts by seeking morphologic changes on imaging remains a challenge. The current study aimed to evaluate the additive value of CA19.9 monitoring and its potential harm using data extracted from the PACYFIC registry. It shows that – for individuals under pancreatic cyst surveillance – serum CA19.9 (at a cutoff level of 37 kU/L or as a continuous variable) is not independently associated with the development of HGD or PC. Moreover, CA19.9 monitoring led to substantial unnecessary shortening of surveillance internals and even surgery. Without CA19.9 determination, surgery might have been prevented in six of 37 individuals with IPMN harboring LGD. An alternative threshold of 133 kU/L was associated with a 4-fold increased risk of developing HGD or PC (independent of presence of baseline AI or other RI), with a high specificity but low sensitivity.

Our observed CA19.9 values were in accordance with previous literature.[5,7,9,10] CA19.9 is a marker for advanced disease and seems less suitable for early detection. Data by Ciprani *et al*. (2020)[9] support this hypothesis. In their study, individuals who underwent surgery for IPMN (n=594) did not show a higher proportion of HGD (relative to LGD) in the elevated CA19.9 group (n=128). However, it did show a higher proportion of T3-tumors (relative to T1) and CA19.9 was independently associated with advanced disease (perineural invasion and lymphogenic metastases). The majority (72%) of their HGD/PDAC cases had normal CA19.9 levels. This percentage was lower in our cohort (53%), perhaps because of the smaller number of participants with advanced disease. Our percentage is similar to the 51% observed by Oyama *et al*. (2020),[11] who performed surveillance in 1404 individuals with BD-IPMN. Of 22 cases with resectable disease, none had an elevated CA19.9 value without having ≥1 AI and/or ≥2 RI (similar to the current study). Manen *et al*. (2020)[10] also showed higher CA19.9 levels in patients with (locally) advanced (n=224), as compared to resectable (n=151) PC. Interestingly, Fahrmann *et al*. (2020),[12] evaluated the predictive value of CA19.9 determination at 6-months' intervals in healthy individuals. In this study, 175 patients developed PC and were compared to 875 controls with prostate, lung, colorectal or ovarian cancer. CA19.9 was able to catch localized (early) PC in 50% of cases, as long as it was measured frequently (every 0-6 months). Thus, based on these studies, CA19.9 may be a marker for late-stage disease requiring short interval determination to be of value for the detection of early-stage disease.

A surveillance protocol ideally involves diagnostic tests with a low number of false positives (high specificity), which is not the case for current CA19.9 testing. Elevated CA19.9 values are not only seen in PC, yet also in obstructive jaundice, as well as hepatic (cirrhosis and hepatitis), gastrointestinal cancer, pulmonary, gynecological and endocrine (DM or hypothyroidism) diseases.[13-15] Thus, false positive outcomes are common, as our result show. Also, we established that this often leads to an intensified follow-up regimen, which could cause harm due to unnecessary diagnostic procedures (such as EUS/FNA) and psychological distress.[16-18] Moreover, in the current study, 45% of operated individuals underwent surgery for benign disease, while we know that pancreatic surgery is associated with substantial grade III-IV (Clavien-Dindo) morbidity (4-31%) and mortality (2-6%).[19-23]

We found CA19.9 levels >37 kU/L not to be associated with the development of HGD or PC. The first cutoff for PC detection was set by Pleskow *et al*. at 70 kU/L. Recently, the current cutoff was debated by our group, Levink *et al* (2022),[4] as well as Ciprani *et al*. (2020)[9] and others. Ciprani *et al*.[9] proposed 100 kU/L as new cutoff, which would alter the sensitivity and specificity for PC detection from 41% and 85% (cutoff 37kU/L), to 23% and 97%. Fahrmann *et al*. (2020)[12] observed a sensitivity of 27% and specificity of 99% for invasive IPMN at a cutoff of 97 kU/L. These results are similar to our cross-sectional analysis, showing that individuals with a CA19.9 value ≥133 kU/L had a higher risk of developing HGD or PC.

Based on these results, we propose using this higher threshold to reduce false positive results. Future research (in a larger cohort with more cases) is needed to evaluate if the

threshold of 133 kU/L is indeed optimal to detect early-stage PC. Additionally, as CA19.9 value <27kU/L had a lower risk of HGD or PC development, one may argue that a value below this threshold may be a soothing feature. However, we believe this is unlikely, as CA19.9 is not generated by all pancreatic cells and 6-22% of the Caucasian population is not able to produce CA19.9.[25]

This study has limitations. Whilst the PACYFIC cohort is unique in its size, it is still too small to draw definite conclusions on CA19.9-related risk of HGD or PC. Also, the low number of malignant cases does not allow us to correct for confounding variables. The follow-up duration is short (especially for those with HGD or PC) and solely allows short-term predictions. Thus, other multicentric efforts with longer follow-up durations are needed to validate our results. In addition, the choice to determine CA19.9 was at the discretion of the treating physician. This may have caused selection of individuals at higher risk and overestimation of the role of CA19.9 in a surveillance population. Moreover, two individuals already had a suspicion of PC at time of first CA19.9 value.

A general limitation related to prospective cyst surveillance studies is their mixed population. Other lesions (*e.g.*, SCAs, lymphoepithelial cysts, pseudocysts) often show similar morphological changes on imaging and are therefore misdiagnosed as IPMN. Thus, these results cannot be extrapolated to IPMN, yet can be extrapolated to other neoplastic cyst populations. Critical appraisal of the differential diagnosis by the treating physician is required for each patient undergoing pancreas surveillance. As all controls had at least 12 months of follow-up, the presence of PC is unlikely within this group.

In conclusion, CA19.9 monitoring in its current form does not contribute to early-stage PC detection and causes harm by unnecessary shortening of surveillance intervals and surgery. This calls for critical appraisal of current recommendations and may lead to either omitting CA19.9 monitoring altogether, or exploring the potential of higher cutoff values, aiming for a higher specificity in combination with the highest achievable sensitivity.

References

1 Siegel RL, Miller KD and Jemal A. Cancer statistics, 2020. CA: *A Cancer Journal for Clinicians* 2020; 70: 7-30.

2 Alexakis N, Gomatos IP, Sbarounis S, *et al*. High serum CA 19.9 but not tumor size should select patients for staging laparoscopy in radiological resectable pancreas head and peri-ampullary cancer. *European Journal of Surgical Oncology (EJSO)* 2015; 41: 265-269.

3 Canto MI, Harinck F, Hruban RH, *et al*. International Cancer of the Pancreas Screening (CAPS) Consortium summit on the management of patients with increased risk for familial pancreatic cancer. *Gut* 2013; 62: 339-347. 2012/11/09.

4 European evidence-based guidelines on pancreatic cystic neoplasms. *Gut* 2018; 67: 789-804. 2018/03/27.

5 Kim JR, Jang J-Y, Kang MJ, *et al*. Clinical implication of serum carcinoembryonic antigen and carbohydrate antigen 19-9 for the prediction of malignancy in intraductal papillary mucinous neoplasm of pancreas. *Journal of Hepato-Biliary-Pancreatic Sciences* 2015; 22: 699-707.

6 Wang W, Zhang L, Chen L, *et al*. Serum carcinoembryonic antigen and carbohydrate antigen 19-9 for prediction of malignancy and invasiveness in intraductal papillary mucinous neoplasms of the pancreas: A meta-analysis. *Biomed Rep* 2015; 3: 43-50.

7 Jang J-Y, Park T, Lee S, *et al*. Proposed Nomogram Predicting the Individual Risk of Malignancy in the Patients With Branch Duct Type Intraductal Papillary Mucinous Neoplasms of the Pancreas. *Annals of Surgery* 2017; 266.

8 Zhao B, Cheng Q, Cao H, *et al*. Dynamic change of serum CA19-9 levels in benign and malignant patients with obstructive jaundice after biliary drainage and new correction formulas. *BMC Cancer* 2021; 21: 517. 2021/05/09.

9 Ciprani D, Morales-Oyarvide V, Qadan M, *et al*. An elevated CA 19.9 is associated with invasive cancer and worse survival in IPMN. *Pancreatology* 2020; 20: 729-735.

10 van Manen L, Groen JV, Putter H, *et al*. Elevated CEA and CA19-9 serum levels independently predict advanced pancreatic cancer at diagnosis. *Biomarkers* 2020; 25: 186-193. Article.

11 Oyama H, Tada M, Takagi K, *et al*. Long-term Risk of Malignancy in Branch-Duct Intraductal Papillary Mucinous Neoplasms. *Gastroenterology* 2020; 158: 226-237.e225. 2019/09/02.

12 Fahrmann JF, Schmidt CM, Mao X, *et al*. Lead-Time Trajectory of CA19-9 as an Anchor Marker for Pancreatic Cancer Early Detection. *Gastroenterology* 2021; 160: 1373-1383. e1376. 2020/12/14.

13 Kim S, Park BK, Seo JH, *et al*. Carbohydrate antigen 19-9 elevation without evidence of malignant or pancreatobiliary diseases. *Scientific Reports* 2020; 10: 8820.

14 Pavai S and Yap SF. The clinical significance of elevated levels of serum CA 19.9. *Med J Malaysia* 2003; 58: 667-672. 2004/06/12.

15 Marrelli D, Caruso S, Pedrazzani C, *et al*. CA19-9 serum levels in obstructive jaundice: clinical value in benign and malignant conditions. *The American Journal of Surgery* 2009; 198: 333-339.

16 Overbeek KA, Cahen DL, Kamps A, *et al*. Patient-reported burden of intensified surveillance and surgery in high-risk individuals under pancreatic cancer surveillance. *Fam Cancer* 2020; 19: 247-258.
17 Mizuide M, Ryozawa S, Fujita A, *et al*. Complications of Endoscopic Ultrasound-Guided Fine Needle Aspiration: A Narrative Review. *Diagnostics (Basel)* 2020; 10: 964.
18 Marinelli V, Secchettin E, Andrianello S, *et al*. Psychological distress in patients under surveillance for intraductal papillary mucinous neoplasms of the pancreas: The "Sword of Damocles" effect calls for an integrated medical and psychological approach a prospective analysis. *Pancreatology* 2020; 20: 505-510. 2020/01/18.
19 Wundsam HV, Rösch CS, Kirchweger P, *et al*. Long-Term Quality of Life after Pancreatic Surgery for Intraductal Papillary Mucinous Neoplasm. *European Surgical Research* 2021; 62: 80-87.
20 Buscail E, Cauvin T, Fernandez B, *et al*. Intraductal papillary mucinous neoplasms of the pancreas and European guidelines: importance of the surgery type in the decision-making process. *BMC Surg* 2019; 19: 115. 2019/08/24.
21. Poruk KE, Shahrokni A and Brennan MF. Surgical resection for intraductal papillary mucinous neoplasm in the older population. *Eur J Surg Oncol* 2022; 48: 1293-1299. 2021/12/11.
22 Palmeri M, Di Franco G, Bianchini M, *et al*. Prognostic impact of conservative surgery for pancreatic IPMNs. *Surg Oncol* 2021; 38: 101582. 2021/04/24.
23 Mintziras I, Wächter S, Manoharan J, *et al*. Postoperative morbidity following pancreatic cancer surgery is significantly associated with worse overall patient survival; systematic review and meta-analysis. *Surg Oncol* 2021; 38: 101573. 2021/04/16.
24 Levink IJM, Visser IJ, Koopmann BDM, *et al*. Protein Biomarkers in Pancreatic Juice and Serum for Identification of Pancreatic Cancer. *Gastrointestinal Endoscopy* 2022.
25 Lee T, Teng TZJ and Shelat VG. Carbohydrate antigen 19-9 - tumor marker: Past, present, and future. *World J Gastrointest Surg* 2020; 12: 468-490.

SUPPLEMENTAL TABLE 1 | Univariable and multivariable analysis of predictors for high-grade dysplasia (HGD) and pancreatic cancer (PC) development.

	Univariable analysis		Multivariable analysis	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**
Age, years	0.98 (0.95-1.02)	0.28		
<65 years	0.7 (0.3-1.4)	0.31		
Male gender	0.8 (0.4-1.7)	0.57		
DM, yes	1.5 (0.6-3.5)	0.34		
CA19.9, kU/L	(1.00-1.01)	<0.001	1.00 (1.00-1.01)[a]	0.11
Elevated ≥27 kU/L	2.8 (1.3-6.0)	0.009	2.3 (1.1-5.0)[a]	0.04
Elevated ≥37 kU/L	2.6 (1.0-6.3)	0.04	1.9 (0.8-4.8)[a]	0.15
Elevated ≥133 kU/L	11 (3.4-37)	<0.001	3.8 (1.1-13)[a]	0.03
≥1 AI or ≥2 RI at baseline, present	28 (13-58)	<0.001	22 (10-47)[b]	<0.001
MPD ≥10 mm	9.2 (3.5-24)	<0.001		
Mural nodule ≥5mm	46 (13-160)	<0.001		
Solid mass	26 (10-64)	<0.001		
≥2 RI	14 (5.8-36)	<0.001		
1 RI at baseline, present	4.6 (2.2-9.7)	<0.001	3.1 (1.4-6.6)[c]	0.004
MPD 5-9 mm	7.7 (3.6-16)	<0.001		
Mural nodule <5 mm	47 (18-119)	<0.001		
Acute pancreatitis	6.1 (2.1-18)	0.006		
Cyst size ≥40 mm	2.9 (1.03-8.5)	0.05		

[a] Corrected for both ≥1 AI and/or ≥2 RI and 1 RI at baseline. [b] Corrected for CA19.9 (as continuous variable) and the presence of 1 RI at baseline; [c] Corrected for CA19.9 (as continuous variable) and the presence of ≥1 AI and/or ≥2 RI at baseline. HR = Hazard ratio, CI = confidence interval, DM = diabetes mellitus, AI = absolute indication for surgery, RI = relative indication for surgery, MPD = main pancreatic duct.

PART III

Potential biomarkers and pancreatic juice

CHAPTER 6

Methodology of Pancreatic Juice Collection from the Duodenum for Biomarker Discovery and Early detection of Pancreatic Cancer

Authors
Iris J.M. Levink*, Kateryna Nesteruk*, Dido I. Visser, Anieta M. Sieuwerts†, Célio J.C. Fernandes, Maurice P.M.H. Jansen, Lydi M.J.W. van Driel, Jan-Werner Poley, Maikel P. Peppelenbosch, Djuna L. Cahen, Gwenny M. Fuhler, Marco J. Bruno

*These Authors share first authorship
† Deceased

Adopted from: *Am J Gastroenterol*. 2020 Dec;115(12):2103-2108.

Abstract

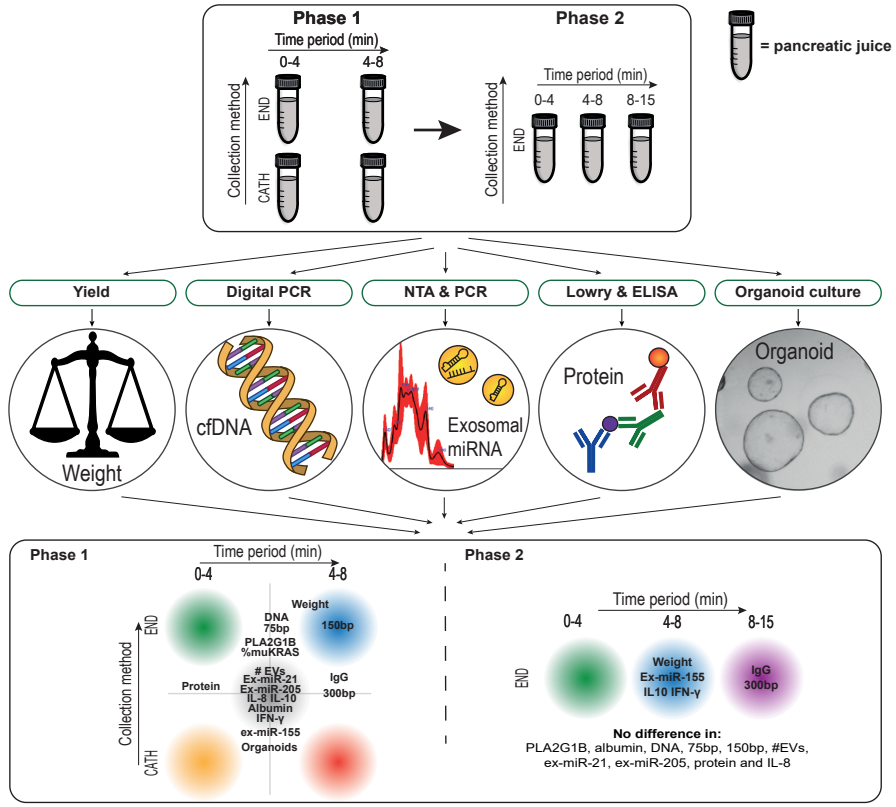
Background: Earlier detection of pancreatic cancer (PC) may improve treatment opportunities and increase survival rates. Pancreatic juice (PJ), being in close contact with pancreatic ductal cells, is a potential promising biomarker source. However, the optimal collection method remains unclear. We aimed to establish the most effective PJ collection method for detection of a variety of biomarkers.

Methods: In individuals with PC or a hereditary predisposition for PC (FPC), secretin-stimulated pancreatic juice (PJ) was collected from the duodenal lumen during endoscopic ultrasound (EUS). The yield of biomarkers and organoids was compared for two collection techniques (endoscope-suction-channel [END] *vs* catheter [CATH] based) and three time periods (0-4 vs 4-8 vs 8-15 minutes).

Results: 23 FPC and 18 PC patients were evaluated. Given the volume of PJ (p=0.0005) and PLA2G1B (p=0.09) concentration, collection with END was superior to CATH. Although collection during 4-8 minutes led to increased blood contamination (based on IgG; p=0.04), this did not decrease pancreatic content (based on PLA2G1B; p=0.41). Collection beyond 8 minutes (t=8-15 min) resulted in increased blood contamination and lower biomarker levels (based on EV-miR-155 and cytokines). Culturing of PJ-derived organoids was feasible for all collection methods and gene expression analysis showed a potential pancreatic origin.

Conclusion: This study provides the first step towards standardization of PJ collection for biomarker detection. Collection of PJ with END for up to 8 minutes is the most effective method for detection of diverse potential biomarkers. Future studies are needed to compare these biomarkers between patients with different pancreatic conditions.

Graphical abstract



Introduction

Comprehensive surveillance programs in individuals with hereditary predisposition for pancreatic cancer (PC) or pancreatic cysts have shown that early detection of PC by imaging is often challenging; by the time it becomes detectable, many patients may have already developed advanced disease.[1-5]

Recent research has shown that pancreatic juice (PJ) is a promising source of biomarkers for the detection of pancreatic dysplasia and cancer.[6-9] A wash-out of PJ from the pancreatic ductal system can be provoked by intravenous secretin infusion during endoscopic ultrasound (EUS) and enables non-invasive collection from the duodenal lumen. PJ is – by virtue of its origin – in close contact with ductal cells (the location where PC develops) and therefore potentially rich in diagnostic biomarkers.

Historically, PJ was collected after direct cannulation of the pancreatic duct during endoscopic retrograde cholangiopancreatography (ERCP). Although this technique ensures PJ purity, it poses a risk of pancreatitis, reported to be as high as 25%.[10] This is of a particular concern in high-risk individuals undergoing surveillance in whom PJ collection is performed repetitively. In contrast, development of pancreatitis due to juice collection from the duodenal lumen after secretin stimulation has not been described thus far.

One of the reasons that secretin-stimulated PJ collection has not yet been implemented in routine surveillance programs is the lack of a standardized protocol. Optimization of the methodology and duration of collection is needed to maximize the yield of biomarkers. For instance, a through-the-scope catheter may decrease (duodenal) contamination by precise positioning of the catheter close to the ampullary orifice, while performing suction directly with the endoscope suction channel may yield higher volumes (**Figure 1**). Additionally, the first flush after secretin injection may be stagnant remnants of earlier ejections, and unsuitable for biomarker detection. Alternatively, the first wash-out may be more concentrated, while prolonged collection may dilute the biomarkers of interest.

In this study, we investigated the feasibility of analyzing potential biomarkers in PJ, including cell-free DNA (cfDNA), exosomal microRNA (EV-miR) and cytokines, by comparing two collection techniques and three time periods. Furthermore, we explored the potential of culturing organoids from the cellular content of PJ, as organoids may serve as an unlimited diagnostic source for targeted personalized medicine.

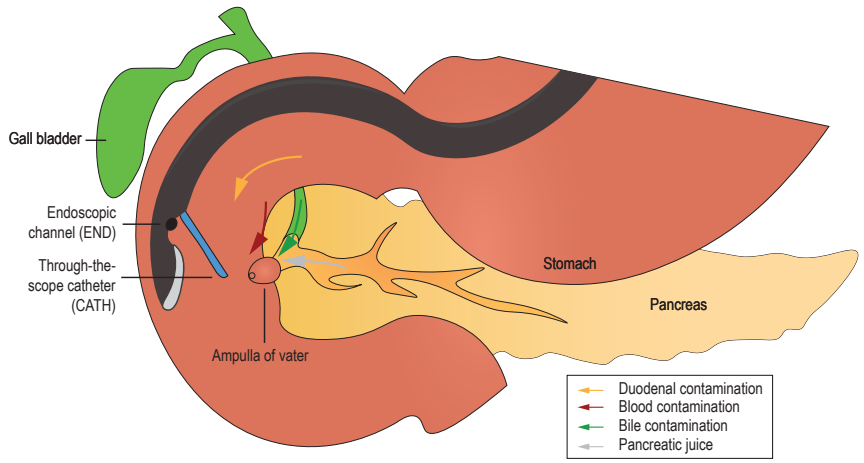


FIGURE 1 | Graphical representation of the positions of the two collection methods: performing suction using a trough-the-scope-catheter (CATH) or with the endoscope suction channel (END).

Material and Methods

Patient inclusion

This prospective study performed at the Erasmus University Medical Center in Rotterdam involves patients who underwent EUS for suspected (sporadic) PC (phase 1 and 2) and high-risk individuals under surveillance for either a hereditary predisposition for PC (FPC; phase 1 and 2) or pancreatic cysts (phase 2) between August 2018 and November 2019. The Erasmus Medical Center ethical review board approved the study, and the included individuals gave written informed consent before enrolment. The study was carried out according to the ethical principles for medical research involving human subjects from the World Medical Association Declaration of Helsinki.

Study design

The study comprised of two phases (**Figure 1**). During the first phase, we compared two collection techniques (utilization of a through-the-scope catheter [CATH] *vs* performing suction through the endoscopic channel [END]) and two collection timeframes (0-4 minutes *vs* 4-8 minutes), by assessing concentrations (or expression) of selected biomarkers. The collection timeframes were based on agreement by three experienced endoscopists, who judged an 8-minute collection as logistically feasible when performing multiple juice collections per half-day EUS program. During this first phase, we performed additional assessments; 1. Phospholipase A2 group1B (PLA2G1B; representing true pancreas-derived material), total IgG and albumin concentrations (representing blood and bile contamination); 2. Reproducibility between endoscopists; 3. The effect of adding a protease inhibitor on the biomarker concentration; 4. The optimal way to isolate DNA from PJ (Nucleospin Kit *vs*. Maxwell Kit); and 5. The ability to grow organoids from PJ and development of an organoid culture protocol.

After determination of the optimal collection and analysis techniques in the first phase, in the second phase, we investigated whether biomarker detection improved with even longer collection. Taking into account the results from Suenaga *et al*.,[11] we extended the collection duration to 15 minutes, and performed a paired-wise comparison – based on the same biomarkers as in phase 1 – between juice collected during the three timeframes (0-4, 4-8 and 8-15 minutes).

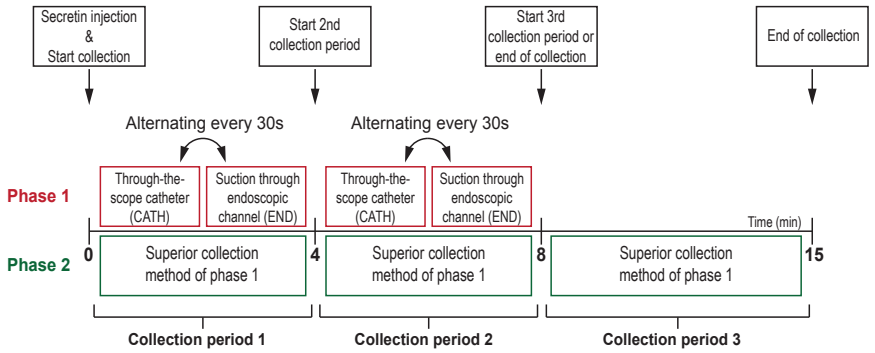


FIGURE 2 | Graphical overview of pancreatic juice collection during endoscopic ultrasound. Collection starts immediately after secretin injection. For phase 1, collection takes two times four minutes with alternating collection techniques. For phase 2, collection is prolonged to 15 minutes. The superior collection technique is used in this phase.

Pancreatic Juice Collection

PJ collection was performed by three experienced endoscopists. To reduce duodenal contamination, duodenal fluid was aspirated prior to juice collection. Next, a wash-out of PJ was stimulated by intravenous administration of human synthetic secretin (ChriRhoStim, Burtonsville, MD, 16µg/patient). Collection started immediately after injection and took eight (phase 1) or 15 minutes (phase 2). To enable comparison of the different timeframes, after each collection timeframe (at t=4 for phase 1, at t=4 and t=8 for phase 2), both the mucus extractor (END) and syringe (CATH) were replaced. The collection techniques were alternated every 30 seconds (**Figure 2**).

Collection of PJ in the first technique (END) was performed by applying suction with the endoscope (Pentax Medical, Tokyo, Japan). For this, a mucus extractor (Pennine Healthcare, Derby, United Kingdom, 15 mL) was attached to the proximal end of the endoscopic channel.

For the second technique (CATH), a catheter (Huijbregtse, 7 Fr, Cook Medical, Bloomington, IN) was passed through the scope and positioned close to the ampullary orifice (without cannulation; **Figure 1**). A three-way stopcock syringe was attached to the proximal end, to prevent efflux of juice back to the duodenum, through which suction was applied by the assisting nurse.

After collection, PJ for organoid culture was kept on ice and processed within two hours. For the other tests, PJ was aliquoted into 1.5 mL tubes and Protease Inhibitor (Thermo Fisher Scientific, Waltham, MA, USA, #78430) was added in 50% of the subjects (randomly). PJ was snap-frozen within 10 minutes after collection. Samples were weighed as a proxy for the yield of collected PJ and stored at -80°C until further use.

Pancreatic juice contamination

Samples were weighed as a proxy for the yield of collected PJ. Juice contamination was evaluated by documenting juice color, assuming that pure PJ is transparent, while contamination with blood or bile colors it more red or green. Contamination was further evaluated by assessment of concentrations of PLA2G1B, albumin and IgG by enzyme linked immunosorbent assays (ELISA; see 'ELISA' in **Supplementary Material and Methods**).

cfDNA quantification and qualification

To investigate the optimal technique for cfDNA isolation, two extraction kits were used according to manufacturer's instructions: (1) the silica membrane-based NucleoSpin DNA kit (Bioké, Leiden, The Netherlands, #6181527); and (2) the automatic bead-based Maxwell RSC cfDNA Plasma kit (Promega, Fitchburg, WI, AX1115), and KRAS mutational load as well as length of isolated DNA were determined (**Supplementary Material and Methods**)

Extracellular vesicle isolation and miRNA analysis

Extracellular vesicles (EVs) were isolated from 400 µL of PJ and analyzed by Nanoparticle Tracking Analysis (NTA), for details see Supplementary Material and Methods. miRNA (miR) was isolated from 200 uL EV preparation and miRNA-specific cDNA was prepared (miR-16, miR-21, miR-205, miR-155) as described before.[12,13] MiRNA expression changes were calculated relative to miR-16 as a reference gene using the $2^{-\Delta\Delta Ct}$ method,[14] as reported before.[15-18] Additionally, we explored the possibilities of normalization to EV-concentration (ΔCT) in this analysis.

ELISA

The total protein concentration in PJ was assessed by Lowry protein assay (Bio-Rad, Hercules, CA).[19] Interleukin-8 (IL-8), interleukin-10 (IL-10), and interferon-γ (IFN-γ) were measured by ELISA according to manufacturer's protocol of the used kits (Thermo Fisher Scientific, Waltham, MA, #88-8086, #88-7106, #88-7316). For details see **Supplementary Material and Methods**.

Organoid growth

Organoid culture was based on a protocol for culture of tissue-derived pancreatic organoids described by Broutier *et al.*[20] Analysis of pancreas-related genes was performed by *qPCR* for CK19, CK7, SOX9, Axin2 and CDX1 as described.[21] For details see **Supplementary Material and Methods**.

Statistical analysis

For paired analyses, either Wilcoxon Matched Pairs test (2 groups) or Friedman test (>2 groups) was performed. Alternatively, for unpaired comparisons we performed either a Mann-Whitney U (2 groups) test or a Wilcoxon Signed Rank test (>2 groups). P-values of subgroup analyses were only displayed when significant ($p \leq 0.05$). Correlations were made using Spearman's correlation.

Results

Patients

For phase 1, juice was collected for up to eight minutes from 41 patients (FPC N=23; PC N=18; see **Table 1** for patient details). Juice collections were not always sufficient to allow matched comparison for all biomarkers. While all measured samples are represented in results below, statistical analysis was performed using paired tests if the experimental set up allowed this. For phase 2, juice was collected for up to 15 minutes in 10 patients (see **Supplementary Table S1** for patient details).

TABLE 1 | **Characteristics of patients included in phase 1 of this study.**

	FPC (N=23)	PC (N=18)
Age (years), median (IQR)	60 (48-67)	68 (63-72)
Gender, n female (%)	17 (74)	9 (50)
Indication		
Surveillance, n (%)	23 (100)	-
Suspected PC, n (%)	-	13 (72)
Fiducials placement, n (%)	-	5 (28)
Mutation carrier		
BRCA1, n (%)	1 (4)	-
BRCA2, n (%)	3 (13)	-
CDKN2A, n (%)	6 (26)	-
Peutz-Jeghers, n (%)	1 (4)	-
Pancreatic cyst		
Undefined cyst, n (%)	7 (30)	-
SB-IPMN, n (%)	4 (17)	-
If cyst: Size in mm, median (IQR)	5 (2-9)	-
If cyst: absolute or relative indications for surgery,[22] n (%)	0 (0)	-

PC = Pancreatic cancer; FPC = Familial pancreatic cancer; SB-IPMN = Side-branch intraductal papillary mucinous neoplasm; IQR = interquartile range.

Phase 1: The yield of pancreatic juice & contamination

Overall, the mean quantity of juice per 8-min collection was 10.4g (95% CI: 7.2-13.5). Suction with the endoscopic channel collected three times more juice (mean 7.8g; 95% CI: 5.1-10.4), as compared to the catheter (mean 2.6g; 95% CI: 1.7-3.6; p=0.0005; **Figure 3A**). Regarding timing, the first 4 minutes provided a mean of 4.6g juice (95% CI: 2.8-6.5) and the second timeframe 5.7g (95% CI: 4.1-7.4; p=0.06; **Figure 3B**). Overall, the volume of collected juice did not differ between endoscopists (p=0.39; **Supplemental Figure S1**).

To evaluate the purity of PJ, we assessed juice sample concentrations of PLA2G1B, a pancreas-specific phospholipase. In our tested samples, PLA2G1B was detected in 123/130 samples. Of the 7 samples that did not contain PLA2G1B, 5 were collected with the catheter and 2 with the endoscopic channel. PLA2G1B concentrations were higher in PJ collected with the endoscopic channel (p=0.09), as compared to through-the scope-catheter (**Figure 3C**). These results did not only show that collection with the endoscopic channel provides more fluid, but also that its content is more likely to be of pancreatic origin. PLA2G1B concentrations were constant over time (P=0.41; **Figure 3D**). Hence, the higher yield of ejected juice at later time points is not a result of dilution with other sources of fluid.

We noticed evident differences in PJ color between individual donors, ranging from bright green to dark red, suggesting different levels of contamination with bile or blood (for examples see **Figure 3E**). Color of the samples did not affect PLA2G1B concentration (**Figure 3F**), suggesting that blood or bile contamination does not affect the concentration of pancreas-derived fluid. Total IgG was detectable in 95% of samples, with the highest concentration measured in dark red samples (p=0.01; **Figure 3G**), suggesting that IgG presents an objective measure of blood contamination. Indeed, all samples without detectable IgG were either transparent or green. While total IgG concentration did not depend on the performed collection method (p=0.14; **Figure 3I**), it was higher in samples collected in the 4-8 min timeframe (p=0.04; Figure 3J), suggesting increased blood contamination during the course of collection. Albumin, as a measure of both bile and blood contamination, was detectable in all evaluated samples. Although transparent samples appeared to contain less albumin, differences were not significant between sample color groups (p=0.19; **Figure 3H**). Furthermore, albumin levels did not differ according to the collection techniques or timeframes (**Figure 3K, L**).

In toto, these data suggest that collection with endoscopic channel results in the highest yield of PJ. Although collection during the second timeframe leads to more blood contamination, this did not decrease the concentration of the pancreatic component.

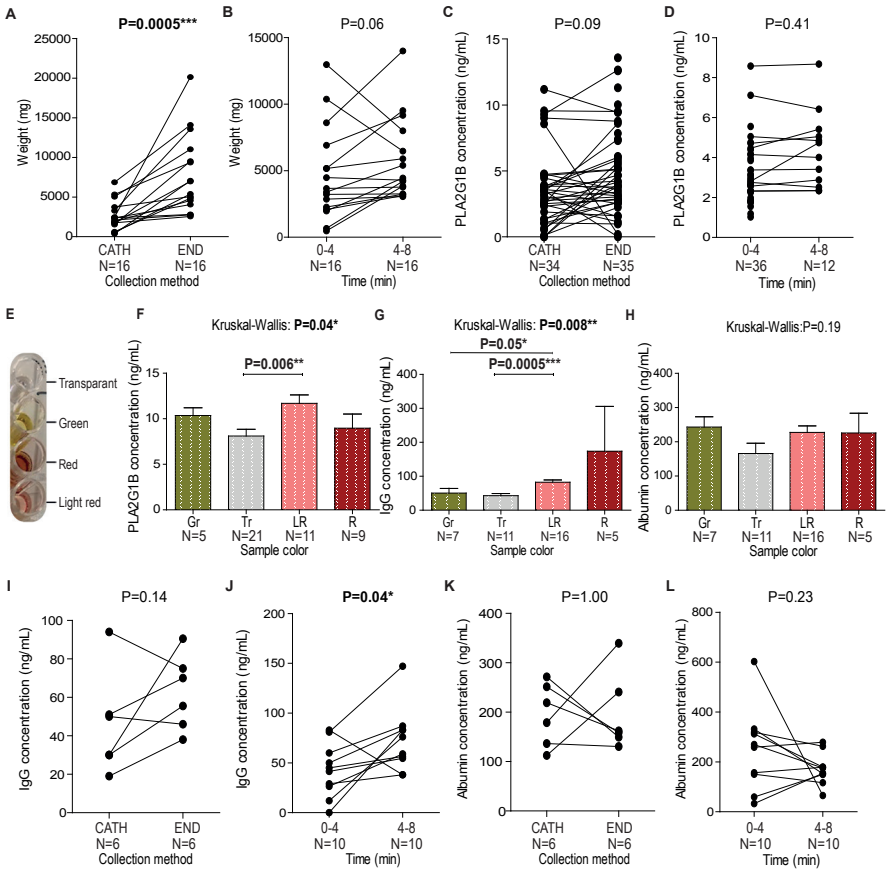


FIGURE 3 | **Comparison of the volume of PJ collection and juice contamination.** (A, B) The juice volume was determined by weighing the tubes containing all collected PJ and compared for two collection methods (A) and time periods (B); collection with endoscopic channel (END) resulted in a higher volume as compared to catheter (CATH). (C, D) The presence of the pancreatic marker PLA2G1B was compared for the two collection methods (C) and time frames (D). (E-H) Different colors of collected juice were observed, examples ranging from green (Gr) to transparent (Tr), light red (LR) and red (R) are shown (E). PLA2G1B, IgG and albumin concentrations in PJ samples stratified according to color score (F-H). (I-L) The level of contamination was evaluated by determination of IgG (I, J) and albumin concentrations (K, L) for the two collection methods and time periods. The second collection period resulted in more contamination with IgG (J).

Phase 1: Cell-free DNA-analysis

As cfDNA has been suggested as a potential source for early cancer detection, we first aimed to establish the optimal cfDNA isolation procedure for PJ. The concentration of isolated cfDNA in PJ ranged from 42-14563 ng/mL, which is substantially higher than found in blood of PC patients.[23,24] When comparing the yield between Nucleospin and Maxwell isolation methods, the overall concentration of cfDNA was higher with

Nucleospin (p=0.03; **Figure 4A**). cfDNA fragment length and mutational burden are two biomarkers currently explored for detection of cancer.[25-28] The yield of 75bp, 150bp, and 300bp fragments of cfDNA did not differ between the two isolation kits (**Figure 4B, C**). However, for mutational analysis, we observed that both the percentage of *KRAS* that was mutated (%mu*KRAS*; p=0.0006) and reproducibility (p=0.06) of mutant *KRAS* detection in cfDNA isolated with the Maxwell kit was superior to that isolated by the Nucleospin kit (**Figure S2E, F**). Thus, depending on the analysis to be performed, different cfDNA isolation kits may be preferred. Subsequent analyses reported in this manuscript were performed on results generated after cfDNA isolation with the Maxwell kit.

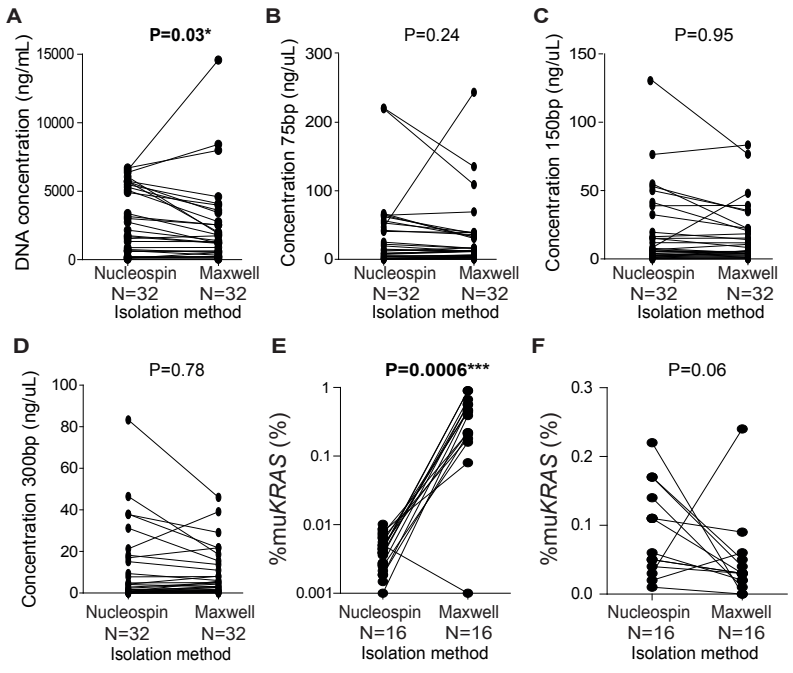


FIGURE 4 | **Comparison of total DNA concentration and mutational component for two DNA isolation kits.** The cfDNA was isolated Maxwell and Nucleospin cfDNA isolation kits, and the concentration of DNA and the percentage mutated KRAS (%muKRAS) were determined. (A) The total concentration of DNA was higher when using the Nucleospin kit. (B-C) The concentration of isolated 75bp, 150bp and 300bp fragments did not differ between the two cfDNA isolation methods. (E) A significantly higher %muKRAS was detected in cfDNA isolated by Maxwell kit. (F) KRAS mutation rate was determined by digital PCR at least two times for individual samples, and error was indicated by a standard deviation. The standard deviation was lower (indicating higher reproducibility) when measurements were performed after isolation with the Maxwell kit.

cfDNA isolation and analyses may be influenced by dilution or contamination with duodenal fluid, bile or blood. Neither total cfDNA concentration, yield of different length fragments, nor percentage mutated *KRAS* (%mu*KRAS*) was correlated with

PLA2G1B concentration. However, we did detect a correlation between total IgG and cfDNA concentrations (r=0.53; P=0.01), as well as 300bp fragments (r=0.47; P=0.03; **Supplementary Figures S2A-C**), suggesting that blood contamination may potentially result in the presence of contaminating genomic DNA fragments.

Next, we investigated cfDNA yield for the different PJ collection methods. The yield of cfDNA isolated from PJ collected with the endoscopic channel was significantly higher than from juice collected with the catheter (p=0.008; **Figure 5A**), but was not affected by the timeframe of collection (p=0.11; **Figure 5B**). Similarly, concentrations of 75bp (p=0.04) and 150bp (p=0.04) fragments were higher in juice collected with the endoscopic channel (**Figure 5C-E**). 150bp and 300bp fragment concentrations were higher in juice collected during the 4-8 min timeframe, as compared to the 0-4 min timeframe (p=0.02 and 0.008, respectively, **Figure 5G, H**), yet the concentration of 75bp fragments did not differ between timeframes (**Figure 5F**). This may be due to blood contamination, in accordance with our findings that the second timeframe harbors higher concentrations of IgG (**Figure 3J**), and the fact that these IgG concentrations are correlated with longer DNA fragments (**Supplementary Figure S2C**). The percentage of mutated *KRAS* showed a trend in favor of collection with the endoscopic channel (p=0.13; **Figure 5I**), yet did not differ for the different timeframes (**Figure 5J**).

Thus, cfDNA isolation and measurement is possible in PJ samples, yet the optimal method depends on the eventual cfDNA analysis. The concentration of shorter DNA fragments is higher when using the endoscopic channel. Longer collection seems to result in a higher concentration of longer DNA fragments due to blood contamination, however this does not affect the detection of mutated *KRAS*.

Phase 1: miRNA analysis

Tumor cells release extracellular vesicles (EVs), which are detectable in blood and may contain tumor-specific markers. EVs are of particular interest in PJ, since these are thought to protect microRNAs (miRs) against RNA-degrading enzymes.[15] We selected three miRs isolated from EVs (EV-miR-21, EV-miR205 and EV-miR155)[7,15,29] to investigate the feasibility of isolating EVs from PJ in a subset of samples. EVs were present in all tested samples. Their number ranged from $3.5*10^7$ to $56.6*10^{12}$ per mL and was not associated with either the PJ collection method (**Figure 6A**) or timeframe (**Figure 6B**). EVs size ranged from 82 to 245 nm, which is in line with the reported size of exosomes,[30] and was also not affected by method or timing of PJ collection (**Figure 6C, D**).

We subsequently determined the expression of selected EV-miRs using two different normalization approaches. First, we normalized against the number of EVs present in the samples, effectively giving a measure of the expression level of the relevant EV-miR per EV. While there was no difference for EV-miR-21 (**Supplementary Figure S3A**), expression of EV-miR-205 and EV-miR-155 were higher in PJ collected by the catheter as compared

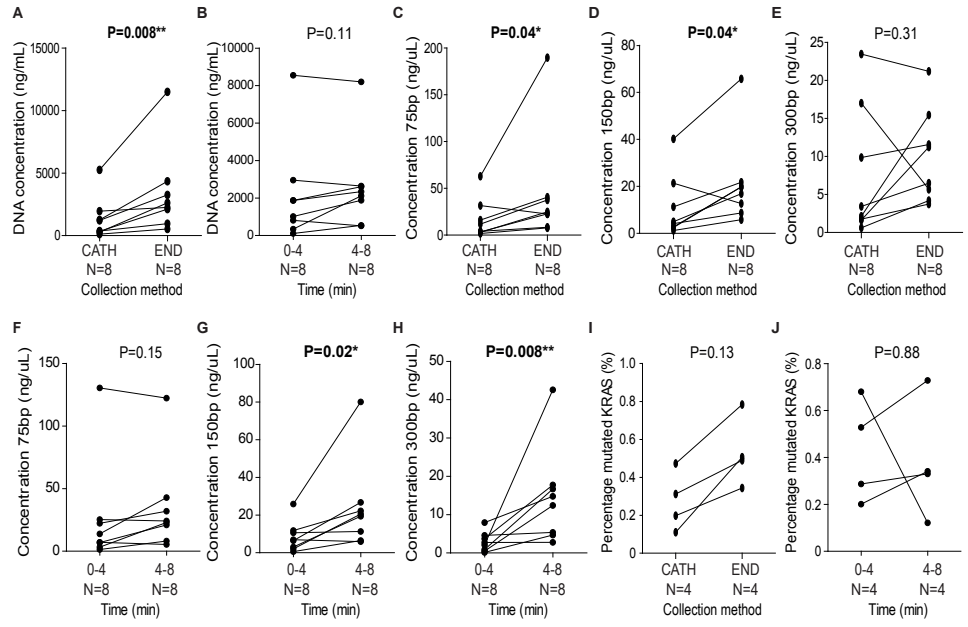


FIGURE 5 | **Comparison of the yield of (short-fragment) DNA for the different collection methods and time periods.** cfDNA isolation was performed using the Maxwell kit. (A) The total yield of DNA was higher for collection with the endoscopic channel (END), as compared to the catheter (CATH). (B) Total yield of DNA was not affected by the timeframe of PJ collection. (C-E) Concentration of 75bp, 150 bp, but not 300 bp DNA fragments was higher in PJ collected by the endoscopic channel. (F-H) Concentration of longer DNA-fragments (150 bp, 300 bp, but not 75bp) was higher in PJ collected during the 4-8 min timeframe. (I, J) Detection of mutated KRAS showed a trend in favor of PJ collected with the endoscopic channel (I), yet was not affected by timeframe of PJ collection (J).

to by performing suction directly with the endoscope (**Supplementary Figure S3B, C**; p=0.06 and p=0.03, respectively). When studying the different timeframes of collection, no differences were observed for any of the miRs (**Supplementary Figure S3D-F**).

As both blood and bile contain EVs, contamination of PJ with these fluids may bias results when normalizing towards the number of EVs present in these juices. While the number of EVs was not associated with PLA2BG and IgG levels (**Supplementary Figure S2D, E**), we did find an association with the color of juice (**Supplementary Figure S2F**). We therefore performed a second normalization procedure using EV-miR16 as an internal control, as this miR has been described to be pancreas specific.[31,32] After normalization with EV-miR16, a correlation between PLA2G1B and EV-miR-21, and EV-miR-155 expression was observed (**Supplementary Figure S6G-J**), suggesting that normalization against EV-miR16 indeed improves pancreatic-specific measurements. PJ collection technique or timeframe did not affect the levels of EV-miR-21, EV-miR-205 or EV-miR155 (**Figure 6E-J**).

Thus, meaningful miR measurements can be performed in PJ, irrespective of collection method or time frame of collection, but normalization approaches to miR data should be carefully considered.

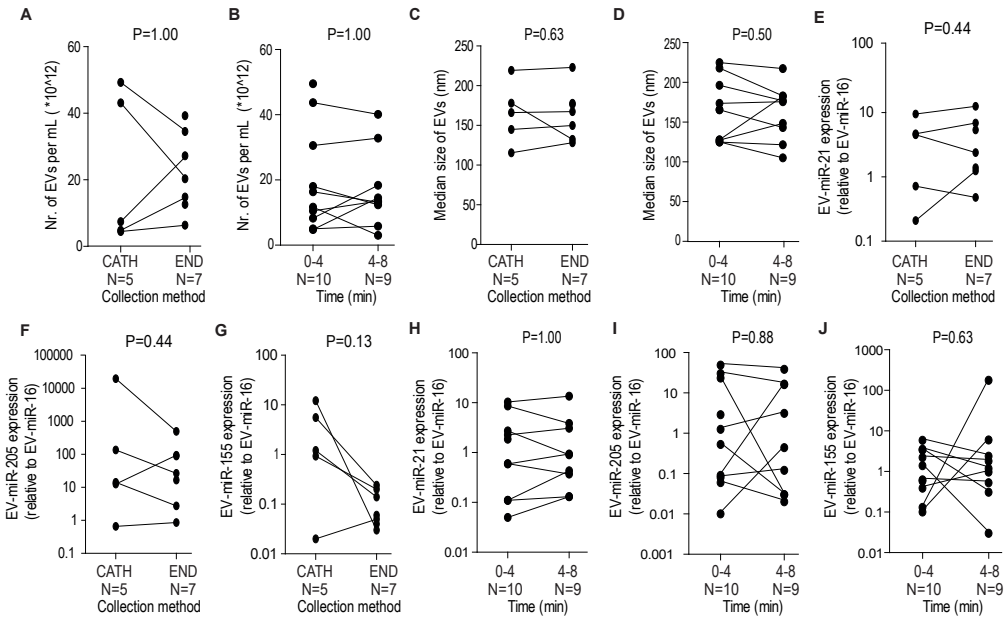


FIGURE 6 | The concentration of extracellular vesicles (EVs) and expression of selected miRs does not differ in PJ collected with different collection methods and time periods. The yield of extracellular vesicles isolated from PJ and their size were determined by nanoparticle tracking analysis. No differences were observed for yield of EVs and their size between PJ collected with either the catheter (CATH) or the endoscopic channel (END) (A, C) or the different time periods (B, D). The expression of EV-miR-21, EV-miR-205 and EV-miR-155 (relative to EV-miR-16) for the collection methods (E-G) and time periods (H-J).

Phase 1: Protein analysis

Next, we investigated the presence of protein-based biomarkers in PJ. The mean protein concentration of the tested samples was 8.99 µg/µl (95% CI: 8.31-9.67). The overall protein concentration in PJ was not affected by the different collection methods (**Figure 7A**). However, PJ collected in the first timeframe (0-4 min) contained significantly higher protein concentrations as compared to juice collected during the second timeframe (p=0.06, **Figure 7E**).

Several pro-and anti-inflammatory cytokines have been suggested as biomarkers for PC. We investigated a panel of those (*i.e.*, IL-6, IL-8, IL-10 IL-13, TNF-α and IFN-γ). In a pilot of 32 samples, IL-6 and IL-13 were undetectable and TNF-α was only detectable in a few samples. Therefore, these were not studied further. In contrast, IL-8, IL-10 and

IFN-γ were detectable in 86.9%, 41.7% and 85.8% of tested samples, respectively. When comparing concentrations of these cytokines in PJ collected with the different collection methods and timeframes, no significant differences were observed (**Figure 7B-D, F-H**). Concentrations of IL-10 (r=0.31; p=0.008) and IFN-γ (r=0.24; p=0.04) were positively correlated with PLA2G1B, while IL-8 was not (r=0.20; p=0.10; **Supplementary Figure S2M-O**). The fact that these cytokines are correlated with PLA2G1B, yet not with albumin or IgG (not shown), may implicate that these cytokines are valuable as pancreas-specific biomarker. Thus, meaningful cytokine analyses can be performed in PJ. We have no evidence that blood contamination affects cytokine concentrations.

As enzymes present in PJ may affect the stability of biomarkers, we compared protein levels for samples that were stored in the presence of protease inhibitors. No indication for an improvement of biomarker detection in the presence of either of these inhibitors was observed at selected storage conditions (**Supplementary Figure S4**).

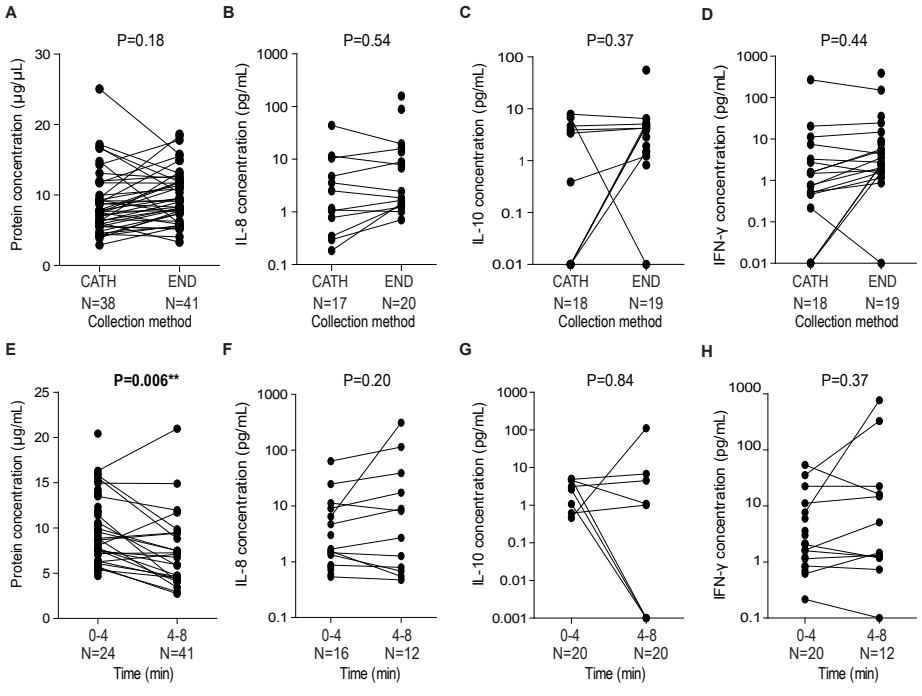


FIGURE 7 | Comparison of total protein and cytokine concentrations in pancreatic juice collected with different methods. (A-D) The protein and cytokine concentration in pancreatic juice collected with the catheter (CATH) or the endoscopic channel (END) were compared. (E-G) Total protein and cytokine concentrations in pancreatic juice collected during two different timeframes were compared. Total protein concentration was higher in juice collected during the first four minutes.

Phase 1: Establishment of organoid cultures from pancreatic juice

To analyse the cellular component of PJ, the cell pellet of concentrated PJ was seeded into 3D cultures. Organoid growth was evaluated after 2 weeks of culture. First, we set up and improved the culturing conditions, seeding 30 PJ samples from 30 patients (**Figure 8A;** Protocol A). We established the use of Matrigel as a matrix (BME also worked), pre-treatment of the cell pellet with collagenase, and application of a cell strainer prior to seeding, as standard protocol. However, a high level of infection was observed (43% of cases). For this reason, we adjusted the protocol (**Figure 8A**; Protocol B) by washing the cell pellet with 100 µg/mL of vancomycin and adding vancomycin to the culturing medium (10 µg/mL), in addition to penicillin-streptomycin and primocin (n=29 PJ). Nonetheless, this did not improve contamination rates (42%). Culture-based analysis of the infected samples revealed that the majority of these samples was contaminated with fungus and yeast, which appeared to be present in the PJ prior to seeding. Hence, in a third protocol (**Figure 8A**; Protocol C), another antibiotic regimen – antibiotic-antimycotic – was used, which resulted in a decrease of the infection rate to 15% (6/40). Additionally, the combination of organoid passaging using TrypLE with mechanical disruption by pipet resulted in higher numbers of organoids after passaging as compared to mechanical disruption alone. The rate of cultures yielding uninfected organoids was 23% (7/30), 24% (7/29) and 35% (14/40) for the three protocols, respectively, and the median number of organoids per seeded sample was 3 (range 1-25). While efficient passaging of organoids was dependent on the number of organoids that initially grew out, established lines tolerated freeze/thaw cycles and a minimum of 5 passages. **Figure 8B and C** show representative images of organoids from PJ and from pancreatic tissue, respectively.

Subsequently, we compared the effect of PJ collection methods on organoid growth (performed using protocol C). As seen from **Figure 8D**, PJ obtained by the catheter resulted in better organoid growth, with a success rate of 53.3% (8 of 15 uninfected samples). Unpaired analysis showed that timeframes did not influence the rate of organoid growth when the catheter and endoscopic channel specimens were assessed together (**Figure 8E**). For seven patients, juice was seeded for organoid growth from both the endoscopic channel and catheter (same time frames of collection). For one of these patients, organoids grew from PJ derived by the endoscopic channel but not from the catheter, and for two of them, organoids grew from juice collected with the catheter but not with the endoscopic channel. For these 7 patients, PJ collected during the 4-8 min timeframe showed more efficient organoid growth, although this did not reach statistical significance (p=0.08). Together these data suggest that organoid growth is most efficient in PJ collected by catheter during the 4-8-minute timeframe.

The ability to culture organoids from PJ was not associated with the concentration of PLA2G1B in that juice (p=0.84), suggesting that other cell sources may contribute to organoid growth. To investigate the cellular origin of cultured organoids, expression of several pancreatic markers (*CK19, CK7, AXIN2, SOX9*) and an intestinal (*CDX1*) marker

were assessed.[33] *CK19, CK7, AXIN2, SOX9* were expressed in all organoid lines from PJ (**Supplementary figure S5A-E**). Interestingly, organoids derived from a PC patient showed higher levels of *CK19* and *CK7*. In addition, *CDX1*, which may be a marker of intestinal metaplasia was highest in organoids derived from IPMN patients. These data show that culturing of organoids from PJ is feasible, and patient-specific (**Figure 8F**).

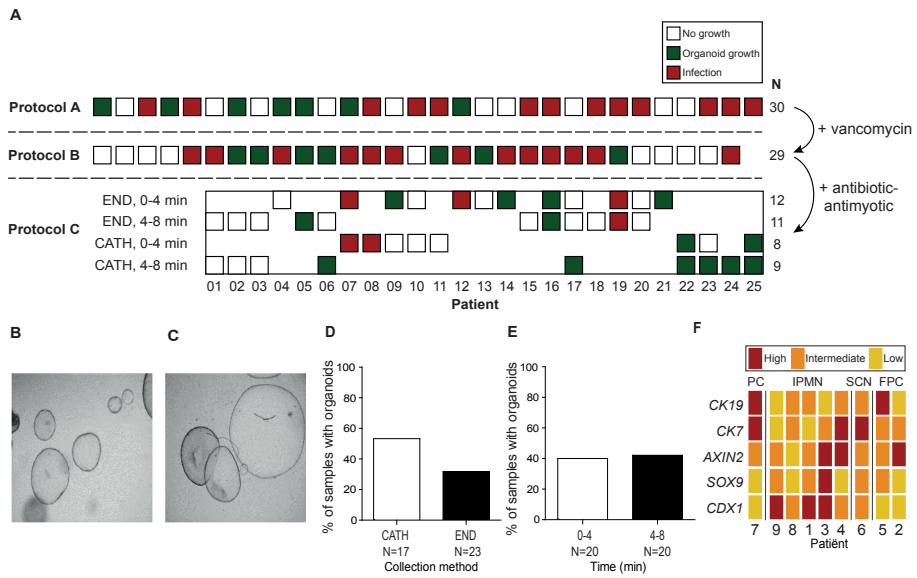


FIGURE 8 | Workflow and yield of organoids during the sequential steps of organoid culture development. (A) First, we established the use of Matrigel as a matrix (BME also worked), pre-treatment of the cell pellet with collagenase, and application of a cell strainer prior to seeding, as standard protocol (protocol A). Due to a high level of infection (43% of cases), we added vancomycin to the culturing medium (protocol B). Culture-based analysis of infected samples revealed contamination with fungus and yeast, after addition of antiobiotic-antimyotic the infection rate decreased (Protocol C); (B-C) Representative picture of organoid culture from pancreatic tissue (B)and PJ (C); (D) PJ collection with catheter (CATH) results in a higher yield of organoids as compared to collection with endoscope suction channel (END); (E) Timeframe of PJ collection does not affect organoid growth; (F) Heatmap showing genes expressed in organoids cultured from PJ of patients with differential diagnoses (PC=pancreatic ductal adenocarcinoma, IPMN = intraductal papillary mucinous neoplasm, SCN = serous cystic neoplasm, FPC=individual with hereditary risk of developing PC without morphologic abnormalities).

Phase 2: 15-minute collections

Having shown the feasibility of measuring DNA, EV-miR and cytokine levels in PJ and demonstrating that collection method and timeframe influence the total volume of juice, concentration of (short-segment) DNA and organoid growth, we next investigated whether even longer duration of PJ collection would further affect biomarker detection.

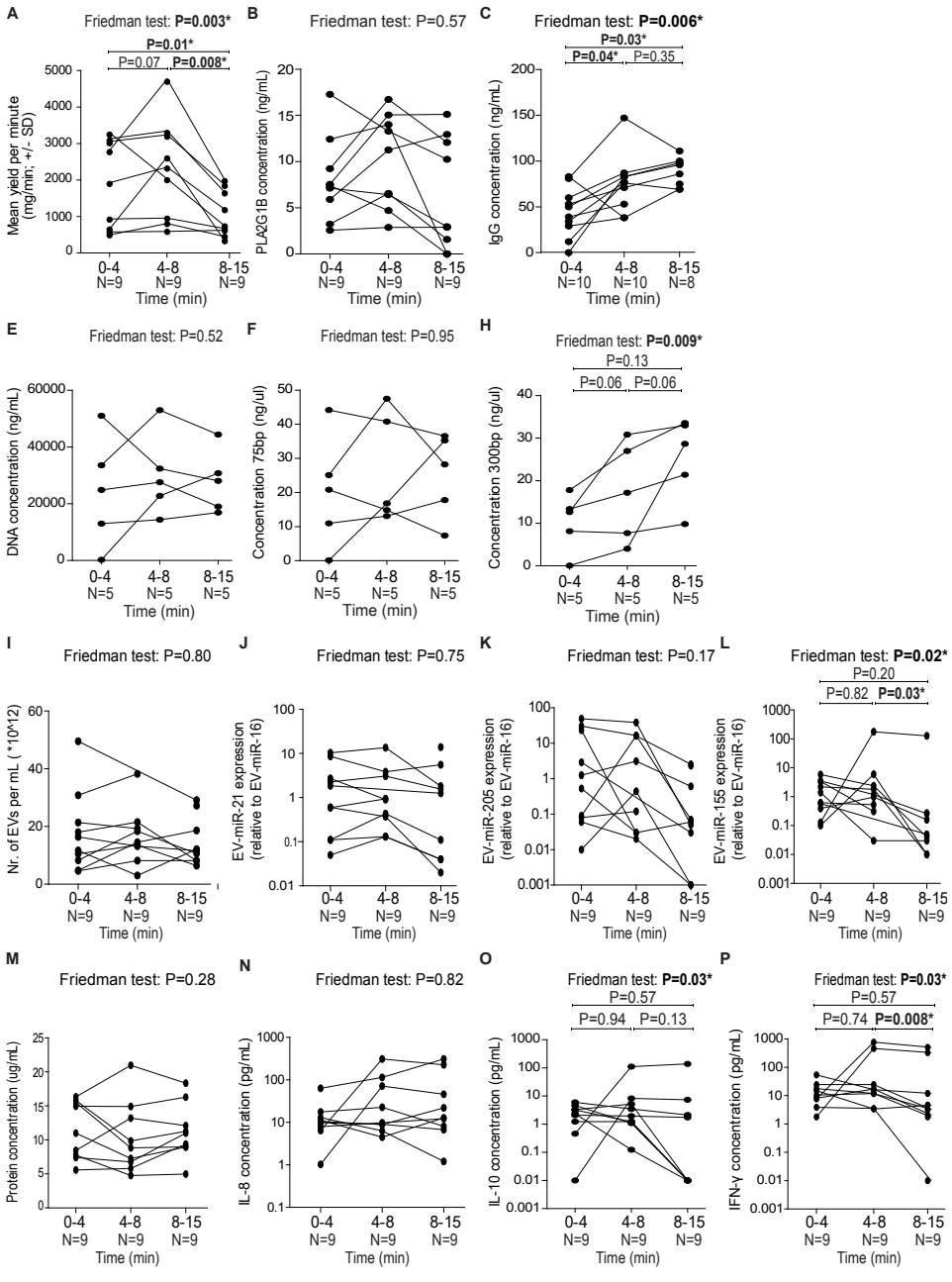


FIGURE 9 | The yield of pancreatic juice and biomarkers for longer collection time. The yield of pancreatic juice, IL10 and IFN-γ and the expression of EV-miR-155 decreases after 8 minutes of collection. In contrast, the concentration of IgG and long-segment DNA (300bp) increases after 8 minutes. The change of yield of PJ (per minute; A), contamination (B-D), DNA (E-H), extracellular vesicles (I) proteins (K-N), and EV-miR (J-L) over time is shown.

We extended the duration of collection to 15 minutes, using only the endoscopic channel. Juice was collected from 10 patients (see **Supplementary Table S1** for patient details), with the aim to perform side-by-side comparison of three different timeframes of PJ collection (0-4, 4-8 and 8-15 minutes). While this was feasible for most patients, the 8-15 timeframe did not yield sufficient material for all patients to perform all analyses.

During the last phase of collection (8-15 min), the volume of juice collected per minute was significantly lower as compared to the first two collection timeframes (p=0.003; **Figure 9A**) and harbored a higher concentration of IgG, while PLA2G1B and albumin were unaffected (**Figure 9B-D**). A longer collection duration did not affect the total or shorter fragment DNA concentration, although in line with a higher level of blood contamination, a higher concentration of longer fragments of DNA (300 bp) was found (**Figure 9E-H**). Neither the yield of collected EVs nor the expression of EV-miR-21 changed over time (**Figure 9I, J**). However, a decrease in the level of EV-miR-205 was seen in PJ collected after the second timeframe, which was even stronger for EV-mir-155 (**Figure 9K, L**). The overall protein concentration and IL-8 levels did not vary per timeframe of collection (**Figure 9M, N**). However, the concentrations of IL-10 and IFN-γ were reduced in PJ collected during the 8-15 min timeframe in all but one patient (**Figure 9O, P**). Thus, we have no evidence that extending PJ collection beyond 8 minutes improves detection of biomarkers in PJ, while longer collection times may result in increased blood contamination.

Discussion

Only recently, PJ has become a focus of attention as a promising biomarker source for PC. The yield of biomarker determination is likely influenced by the collection and processing methods, but this has not been studied so far. Therefore, this study evaluated the best methodology and duration of PJ collection for detection of potential PC biomarkers, including cfDNA, extracellular vesicles, ex-miR and cytokines. In addition, we showed feasibility of establishing organoid cultures from PJ for future personalized medicine studies.

Juice can be collected from the duodenum by direct suction through the endoscopic channel after having positioned the tip of the endoscope close to the ampulla of Vater. To decrease duodenal contamination (duodenal/gastric juice, bile), Suenaga *et al.*[34] described the use of an endoscopic distal cap, which, when positioned on the tip of a forward-viewing scope, shows a modest increase in cfDNA mutation detection rate in collected samples. However, since simultaneous suction and occlusion of the ampulla and renewed introduction of a forward-viewing scope poses an additional risk of complications (*e.g.*, pancreatitis and perforation, respectively), we tested an alternative method by performing suction utilizing a catheter passed through a linear echoendoscope of which the distal tip is positioned close to the ampullary orifice (without touching it). Nevertheless, our results indicate that for total volume of juice, concentration of cfDNA,

and the degree of pancreatic content (based on PLA2G1B) in juice, collection with the endoscopic channel outperforms collection using a dedicated catheter. This is potentially because of the wider diameter of the endoscope channel better facilitates aspiration of PJ when it is more viscous.

Regarding the duration of collection, Suenaga *et al.*[11] have recommended to perform PJ collection for 15 minutes, based on the percentage mutated *KRAS* recovered from PJ samples. However, our results indicate that most cellular constituents measured (*i.e.*, DNA, mRNA, diverse proteins and cells) showed optimal detection ranges within the 0-4- or 4-8-minute timeframes and declined in juice collected beyond eight minutes after secretin injection. Thus, longer collection timeframes may result in the actual dilution of biomarkers, such as cytokines, which cannot be concentrated. Moreover, as a panel of biomarkers may eventually be required, a collection method allowing optimal detection of all these markers combined is preferable. Our data – suggesting that collection of juice for up to 8 minutes (instead of 15 minutes) is optimal – has the additional benefit of putting less strain on a busy endoscopy schedule, in particular when performing multiple juice collections per day.

The detection of tumor-specific mutations in cfDNA, overexpressed EV-miR and increased protein concentrations in PJ may provide valuable biomarkers.[6,7,15,35-37] However, laboratory-based choices should be made with caution. In the current study, we confirm that cfDNA isolation from PJ is feasible with both kits tested. However, detection of mutated genes appears to be more sensitive with DNA isolated by the Maxwell kit, potentially due to less contamination with genomic DNA. For miRNA, normalization approaches should be carefully considered. As compared with EV-count, a pancreas-specific EV-miR (*e.g.*, EV-miR-16) as internal control may provide more solid information on EV-miR expression. As confirmed in the current study, EV-miR expression (relative to EV-miR-16) is correlated with PLA2G1B and EVs in PJ are originated from both the pancreas and blood (or bile). The same holds true for cytokine measurements; detected cytokines may be originated from contaminating fluids, in particular blood. However, the correlation of cytokine levels with PLA2G1B, but not IgG, suggests pancreatic origin of these cytokines rather than blood. Nevertheless, normalization with either PLA2G1B and/or IgG could be considered in future studies.

PJ-based cytology has been investigated intensively. Tanaka *et al.*[9] recently published a meta-analysis on papers (N=193) that investigated biomarkers from PJ, cyst fluid, or serum of patients with malignant IPMN and determined that cytology in PJ has the highest AUC and sensitivity values (AUC 0.84, sensitivity 54%, specificity 91%). Since the concentration of cells may be low in case of secretin stimulation, exploration of ways to increase cellular content is needed. In theory, organoids yield an unlimited source of cells for diagnosis (DNA analysis, staining, immunohistochemistry) and baseline treatment response prediction (DNA, *in vitro* sensitivity testing). Here we show that establishment of organoid cultures from PJ is feasible. Gene expression analysis has shown that

organoids are of pancreatic origin and may have patient-specific characteristics. While further analysis and characterization of these organoids is required, these results do open up the tantalising possibility of capturing premalignant lesions in early stages and open up avenues for personalised treatment modalities.

In conclusion, for secretin-stimulated PJ collection with an EUS-scope, we established that the most effective method is by collection directly through the suction channel of the endoscope for no longer than eight minutes. This resulted in the most optimal detection of a variety of potential biomarkers based on their yield, concentration or expression, and the ability to culture organoids. This study provides a first step towards standardization of PJ collection for evaluation of a variety of biomarkers. Future studies are needed to compare these biomarkers between patients with different pancreatic conditions to determine their diagnostic accuracy for PC detection.

References

1 Konings IC, Harinck F, Poley JW, *et al*. Prevalence and Progression of Pancreatic Cystic Precursor Lesions Differ Between Groups at High Risk of Developing Pancreatic Cancer. *Pancreas* 2017;46:28-34.

2 Konings I, Canto MI, Almario JA, *et al*. Surveillance for pancreatic cancer in high-risk individuals. *BJS Open* 2019;3:656-665.

3 Goggins M, Overbeek KA, Brand R, *et al*. Management of patients with increased risk for familial pancreatic cancer: updated recommendations from the International Cancer of the Pancreas Screening (CAPS) Consortium. *Gut* 2020;69:7-17.

4 Canto MI, Almario JA, Schulick RD, *et al*. Risk of Neoplastic Progression in Individuals at High Risk for Pancreatic Cancer Undergoing Long-term Surveillance. *Gastroenterology* 2018;155:740-751 e2.

5 Vasen H, Ibrahim I, Ponce CG, *et al*. Benefit of Surveillance for Pancreatic Cancer in High-Risk Individuals: Outcome of Long-Term Prospective Follow-Up Studies From Three European Expert Centers. *J Clin Oncol* 2016;34:2010-9.

6 Suenaga M, Yu J, Shindo K, *et al*. Pancreatic Juice Mutation Concentrations Can Help Predict the Grade of Dysplasia in Patients Undergoing Pancreatic Surveillance. *Clin Cancer Res* 2018;24:2963-2974.

7 Wang J, Raimondo M, Guha S, *et al*. Circulating microRNAs in Pancreatic Juice as Candidate Biomarkers of Pancreatic Cancer. *J Cancer* 2014;5:696-705.

8 Noh KW, Pungpapong S, Wallace MB, *et al*. Do cytokine concentrations in pancreatic juice predict the presence of pancreatic diseases? *Clin Gastroenterol Hepatol* 2006;4:782-9.

9 Tanaka M, Heckler M, Liu B, *et al*. Cytologic Analysis of Pancreatic Juice Increases Specificity of Detection of Malignant IPMN - A Systematic Review. *Clin Gastroenterol Hepatol* 2019.

10 Yamakawa K, Masuda A, Nakagawa T, *et al*. Evaluation of efficacy of pancreatic juice cytology for risk classification according to international consensus guidelines in patients with intraductal papillary mucinous neoplasm; a retrospective study. *Pancreatology* 2019;19:424-428.

11 Suenaga M, Dudley B, Karloski E, *et al*. The Effect of Pancreatic Juice Collection Time on the Detection of KRAS Mutations. *Pancreas* 2018;47:35-39.

12 Verhoeven CJ, Farid WRR, de Ruiter PE, *et al*. MicroRNA profiles in graft preservation solution are predictive of ischemic-type biliary lesions after liver transplantation. *Journal of Hepatology* 2013;59:1231-1238.

13 Farid WRR, Pan Q, van der Meer AJP, *et al*. Hepatocyte-derived microRNAs as serum biomarkers of hepatic injury and rejection after liver transplantation. *Liver Transplantation* 2012;18:290-297.

14 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402-8.

15 Nakamura S, Sadakari Y, Ohtsuka T, *et al*. Pancreatic Juice Exosomal MicroRNAs as Biomarkers for Detection of Pancreatic Ductal Adenocarcinoma. *Annals of Surgical Oncology* 2019.

16 Xu J, Cao Z, Liu W, *et al*. Plasma miRNAs Effectively Distinguish Patients With Pancreatic Cancer From Controls: A Multicenter Study. *Ann Surg* 2016;263:1173-9.

17 Abue M, Yokoyama M, Shibuya R, *et al*. Circulating miR-483-3p and miR-21 is highly expressed in plasma of pancreatic cancer. *Int J Oncol* 2015;46:539-47.

18 Wang J, Chen J, Chang P, *et al*. MicroRNAs in plasma of pancreatic ductal adenocarcinoma patients as novel blood-based biomarkers of disease. *Cancer Prev Res (Phila)* 2009;2:807-13.

19 Lowry OH, Rosebrough NJ, Farr AL, *et al*. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265-75.

20 Broutier L, Andersson-Rolf A, Hindley CJ, *et al*. Culture and establishment of self-renewing human and mouse adult liver and pancreas 3D organoids and their genetic manipulation. *Nat Protoc* 2016;11:1724-43.

21 Janmaat VT, Liu H, da Silva RA, *et al*. HOXA9 mediates and marks premalignant compartment size expansion in colonic adenomas. *Carcinogenesis* 2019;40:1514-1524.

22 The European Study Group on Cystic Tumours of the P. European evidence-based guidelines on pancreatic cystic neoplasms. *Gut* 2018;67:789.

23 Sorber L, Zwaenepoel K, Deschoolmeester V, *et al*. A Comparison of Cell-Free DNA Isolation Kits: Isolation and Quantification of Cell-Free DNA in Plasma. *The Journal of Molecular Diagnostics* 2017;19:162-168.

24 Pietrasz D, Pécuchet N, Garlan F, *et al*. Plasma Circulating Tumor DNA in Pancreatic Cancer Patients Is a Prognostic Marker. *Clinical Cancer Research* 2017;23:116.

25 Underhill HR, Kitzman JO, Hellwig S, *et al*. Fragment Length of Circulating Tumor DNA. *PLoS Genet* 2016;12:e1006162.

26 van Dessel LF, Vitale SR, Helmijr JCA, *et al*. High-throughput isolation of circulating tumor DNA: a comparison of automated platforms. *Mol Oncol* 2019;13:392-402.

27 Mouliere F, Robert B, Arnau Peyrotte E, *et al*. High fragmentation characterizes tumour-derived circulating DNA. *PLoS One* 2011;6:e23418.

28 Mouliere F, Chandrananda D, Piskorz AM, *et al*. Enhanced detection of circulating tumor DNA by fragment size analysis. *Sci Transl Med* 2018;10.

29 Sadakari Y, Ohtsuka T, Ohuchida K, *et al*. MicroRNA expression analyses in preoperative pancreatic juice samples of pancreatic ductal adenocarcinoma. *JOP : Journal of the pancreas* 2010;11:587-592.

30 Kowal J, Arras G, Colombo M, *et al*. Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. *Proceedings of the National Academy of Sciences* 2016;113:E968-E977.

31 Wang X, Zhang X, Yuan J, *et al*. Evaluation of the performance of serum miRNAs as normalizers in microRNA studies focused on cardiovascular disease. *Journal of thoracic disease* 2018;10:2599-2607.

32 Lange T, Stracke S, Rettig R, *et al*. Identification of miR-16 as an endogenous reference gene for the normalization of urinary exosomal miRNA expression data from CKD patients. *PloS one* 2017;12:e0183435-e0183435.

33 Boj Sylvia F, Hwang C-I, Baker Lindsey A, *et al*. Organoid Models of Human and Mouse Ductal Pancreatic Cancer. *Cell* 2015;160:324-338.

34 Suenaga M, Sadakari Y, Almario JA, *et al*. Using an endoscopic distal cap to collect pancreatic fluid from the ampulla (with video). *Gastrointestinal Endoscopy* 2017;86:1152-1156.e2.
35 Choi MH, Mejlænder-Andersen E, Manueldas S, *et al*. Mutation analysis by deep sequencing of pancreatic juice from patients with pancreatic ductal adenocarcinoma. *BMC Cancer* 2019;19:11.
36 Yu J, Sadakari Y, Shindo K, *et al*. Digital next-generation sequencing identifies low-abundance mutations in pancreatic juice samples collected from the duodenum of patients with pancreatic cancer and intraductal papillary mucinous neoplasms. *Gut* 2017;66:1677-1687.
37 Sadakari Y, Ohtsuka T, Ohuchida K, *et al*. MicroRNA expression analyses in preoperative pancreatic juice samples of pancreatic ductal adenocarcinoma. *Jop* 2010;11:587-92.
38 Vitale SR, Sieuwerts AM, Beije N, *et al*. An Optimized Workflow to Evaluate Estrogen Receptor Gene Mutations in Small Amounts of Cell-Free DNA. *J Mol Diagn* 2019;21:123-137.

Supplementary Material and Methods

cfDNA quantification and qualification

To investigate the optimal technique for cfDNA isolation, two extraction kits were used according to manufacturer's instructions: (1) the silica membrane-based NucleoSpin DNA kit (Bioké, Leiden, The Netherlands, #6181527); and (2) the automatic bead-based Maxwell RSC cfDNA Plasma kit (Promega, Fitchburg, WI, AX1115). To quantify the concentration of total (double stranded) DNA, the Quant-iT dsDNA High-Sensitivity (HS) Assay Kit was employed, according to manufacturer's instructions. To quantify 75 base-pair (bp), 150bp and 300bp DNA fragments, the ProNex® DNA QC Assay (Promega, Fitchburg, WI) a human-specific, multiplexed probe-based quantitative polymerase chain reaction (qPCR) was used, according to the manufacturer's protocol. This assay also includes an internal positive control to test for false-negative results that may occur in the presence of PCR inhibitors.

KRAS mutational load determination

To generate sufficient copies of DNA, cfDNA was pre-amplified with the Taqman PreAmp master mix (Thermo Fisher Scientific, Waltham, MA, #4488593). For this, the 20x primer-probe *KRAS* Screening assay (Bio-Rad, Hercules, CA, #186-3506) was diluted 100 times in LoTe (3 mM Tris-HCL (pH 8.0)/0.2 mM EDTA (pH 8.0)). A PreAmp reaction mix consisting of Taqman, Pre-Amp master mix (4 µL), 100-fold diluted KRAS Screening assay (2 µL) and DNA (0.1-4.0 ng in 2 µL) was prepared and PCR was performed under the following conditions: cycle at 95°C for 10 minutes, 15 cycles at 95°C for 15 seconds and 60°C for 4 minutes, followed by a cool-down to 4°C. Finally, 8 µL of pre-amplified product was diluted 10-fold in LoTe. *KRAS* copies present in the pre-amplified product were quantified in a regular quantitative PCR (primer sequences and PCR amplification program). For this, 2 µL of the 10-fold diluted pre-amplified sample was added to 2.5 µL PCR mastermix (GCBiotech, Waddinxveen, The Netherlands, #BIO-84020), 4.5 µL H_2O and 0.5 µL 20x KRAS-Screening assay. PCR conditions were as follows: 1 cycle at 95°C for 3 minutes, followed by 45 cycles at 92°C for 10 seconds and 60°C for 1 minute. After this quantification, 10 to 30 ng amplification product from the first round of PCR was used for digital polymerase chain reaction (dPCR, Thermo Fisher Scientific, Waltham, MA, quant3D studio). In each sample, *KRAS* hotspot mutations were assessed with the *KRAS* Screening Multiplex Kit (Bio-Rad, Hercules, CA, #186-3506). Primers (final concentration, 900 nM) and probes (final concentration 250 nM) present in this kit were designed to detect mutated G12S, G12D, G12R, G12V, G13D and wild-type (WT) *KRAS*. The fluorescent label FAM was used to quantify the number of mutated copies and 5'-Hexachloro-Fluorescein-CE Phosphoramidite (HEX) was used to determine the number of WT copies. The final cycle quantification values of HEX were used to calculate the optimal volume of sample to load into the dPCR chip, as described before.[38] dPCRs were performed with the QuantStudio 3D Digital PCR System (Thermo Fisher Scientific,

Waltham, MA), according to the manufacturer's protocol. For this, each pre-amplified diluted DNA sample was portioned into 20.000 wells of a QuantStudio 3D Digital PCR v2 Chip and run on a ProFlex 2x Flat PCR System (Thermo Fisher Scientific, Waltham, MA). The target-specific optimized PCR program was as follows: 10 minutes at 96°C, followed by 40 cycles of 30 seconds incubation at 98°C, and 2 minutes at 52°C, and a final pause at 10°C. Chips were read in a QuantStudio 3D dPCR instrument, and analyzed with web-based QuantStudio 3D dPCR Analysis Software version 3.4.1 (Thermo Fisher Scientific, Waltham, MA).

Extracellular vesicle isolation and analysis

400 µL of PJ was centrifuged for 10 min at 4000 RPM 4°C to remove debris. Then, 100 µL of Total Extracellular vesicle (EV) Isolation Reagent (Thermo Fisher Scientific, Waltham, MA, #4478359) was added to 200 µL of supernatant and kept on a rollerbank at 4°C overnight. After this, samples were centrifuged for 1h at 14000 RPM and the pellet was resuspended in 400 µL of PBS (filtered with 0.2µM filter). For Nanoparticle Tracking Analysis (NTA), samples were diluted 1:1000 in PBS. The size and concentration of the extracellular vesicles (EV) were detected by NanoSight NS300 (NTA 3.4 Build 3.4.003 software). Concentrated EVs were stored at -80°C until further analysis.

miRNA analysis

miRNA (miR) was isolated from 200 uL EV preparation with QIAzol Lysis Reagent (Qiagen, Hilden, Germany, #79306) and miRNeasy Mini kit (Qiagen, Hilden, Germany, #217004) according to manufacturer's recommendations. miRNA-specific complementary DNA (cDNA) was prepared using the Taqman microRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, #217004; miR-16, miR-21, miR-205, miR-155), as described before.[12,13] In a modified protocol, every cDNA reaction consisted of 0.4 µL dNTP mix, 1.35 µL Multiscribe RT enzyme (500U/µL), 2.0 µL 10x RT Buffer, 0.25 µL RNase inhibitor, 1.0 µL of each RT primer, and 5 µL of diluted template RNA. The total reaction volume was adjusted to 20 µL with nuclease-free water. All cDNA and qPCR reactions were performed according to the manufacturer's instructions and carried out in duplicate. Each qPCR reaction consisted of 6 µL TaqMan Universal PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, #4324018), 0.5 µL microRNA-specific PCR primer and 5.0 µL of the previously 1:5 diluted cDNA. The final volume of every PCR reaction was adjusted to 12 µL with nuclease-free water. MiRNA expression changes were calculated relative to miR-16 as a reference gene using the $2^{-\Delta\Delta Ct}$ method,[14] as reported before.[15-18] Additionally, we explored the possibilities of normalization to EV-concentration (ΔCT) in this analysis.

ELISA

The total protein concentration in PJ was assessed by Lowry protein assay (Bio-Rad, Hercules, CA).[19] Interleukin-8 (IL-8), interleukin-10 (IL-10), and interferon-γ (IFN-γ) were

measured by Enzyme-Linked Immuno Sorbent Assay (ELISA) according to manufacturer's protocol of the used kits (Thermo Fisher Scientific, Waltham, MA, #88-8086, #88-7106, #88-7316). Briefly, Immunosorb plates (Nunc, Hardenberg, The Netherlands) were coated with cytokine-specific capture antibody overnight at 4 °C and plates were blocked with ELISA diluent for 1 hour at room temperature. PJ (75 µL per well) was incubated at 4 °C overnight, after which biotin conjugated detection antibody was added at room temperature for 1 hour. Following incubation of avidin-HRP for 30 minutes at room temperature, tetramethylbenzidine (TMB) substrate was added. Reactions were stopped by addition of sulfiric and absorbance was read at 450 nm (Tecan Infinite200 pro plate reader). Assessment of the concentrations of PLA2G1B (pancreatic marker, MyBiosource, San Diego, #MBS703283) and IgG (Thermo Fisher Scientific, Waltham, MA, #BMS2091) were performed similarly, using pre-coated, pre-blocked plates according to the manufacturer's protocol. Albumin levels were detected using an in-house designed protocol.

Organoid growth

Organoid culture was based on a protocol for culture of tissue-derived pancreatic organoids described by Broutier et al.[20] 2-4 mL of PJ was collected in a 15 mL tube containing 5 mL of basal medium supplemented with 100 µg/mL of vancomycin and 1x of Antibiotic-Antimycotic (100X) (Thermo Fisher Scientific, Waltham, MA, #15240062) and kept on ice until processing. PJ was incubated with collagenase II (0.1 mg/mL; Sigma-Aldrich, St. Louis, MO, #C9891-100MG) for 20 min on a shaker at 37°C to dissociate tissue clumps. To remove collagenase, samples were centrifuged at 1350 RPM for 5 min and pellet was washed with 5 mL of wash medium, which is DMEM (Thermo Fisher Scientific, Waltham, MA, #41965039) supplemented with 1% UltraGlutamine I (Alanyl-L-Glutamine, Westburg BV, Leusden, NL, #BE17-605E/U1), 1% sodium pyruvate (Life Technologies, Carlsbad, CA, #11360070), 1% FBS (Sigma-Aldrich, St. Louis, MO, #F7524-500ML), 100 µgmL of vancomycin, and 1x of Antibiotic-Antimycotic (100X). Next, cells were passed through a 70 µM cell strainer to remove clumps or debris and washed once with 10 mL of wash medium and once with 5 mL of basal medium (Advanced DMEM/F-12 [Invitrogen, Carlsbad, CA, #12634-028], with 1% GlutaMAX [Invitrogen, Carlsbad, CA, #35050-079] and 10mM HEPES [Invitrogen, Carlsbad, CA, #15630-056]). The pellet was seeded in a pre-warmed 24-well plate for cell suspension (Corning, Corning, NY) in a 50 µL drop of matrigel (BD bioscience, Franklin Lanes, New Jersey, USA #356231) or BME (Bio-Techne, Minneapolis, MN, #3533-010-02). Droplets were incubated at 37°C until solidified. 500 µL of pancreatic organoid isolation and expansion medium was added to each well: Advanced DMEM/F-12, 1% penicillin/streptomycin, 1% GlutaMAX, HEPES 10 mM, 1:50 B27 supplement (without vitamin A) (Invitrogen, Carlsbad, CA, #17504-044), 1:100 N2 supplement (Invitrogen, Carlsbad, CA, #17502-048), 1 mM N-acetylcysteine (Sigma-Aldrich, St. Louis, MO, #A9165-5G), 30% (v/v) Wnt3a-conditioned medium, 5% (v/v) Rspo1-conditioned medium, 10 mM nicotinamide (Sigma-Aldrich, St. Louis, MO, #N0636), 10 nM recombinant human [Leu15]-gastrin I (Sigma-Aldrich, St. Louis, MO,

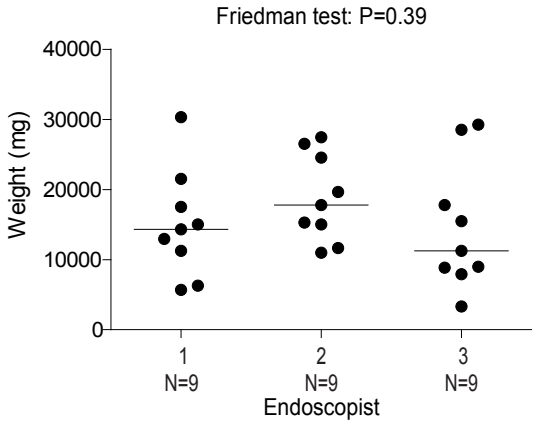
#G9145), 50 ng/mL recombinant human EGF (Peprotech EL Ltd Princeton, NJ, #AF10015), 100 ng/mL recombinant human FGF10 (PeproTech EC Ltd, Princeton, NJ, #100-26), 5% (v/v) Noggin-conditioned medium, 5 µM A83-01 (Tocris, Abingdon, UK, #2939), and 3 µM PGE-2 (Tocris, Abingdon, UK, #2296). 10 µg/mL vancomycin, 10 µM RhoK inhibitors (Y-27632; R&D Systems Europe, #1254/10) and 1X Antibiotic-Antimycotic (100X) and 10 µG/mL vancomycin were added only during the first 2-3 days post-seeding. Plates were kept under standard tissue culture conditions (37 °C, 5% CO2) for at least 2 weeks and checked for organoid growth. Medium was replaced 3 times per week. Organoids were passaged by mechanical disruption with a pipette or in combination with TrypLE™ Express Enzyme (1X) (Thermo Fisher Scientific, Waltham, MA, #12604013). All variations of the protocol are indicated in the result section.

The *qPCR* for CK19, CK7, SOX9, Axin2 and CDX1 was performed as described.[21] In short, total RNA from organoids was isolated for cDNA preparation. Primers used (first forward, then reverse: CK19 (CTACAGCCACTACTACACGAC, CAGAGCCTGTTCCGTCTCAAA), CK7 (GGGGACGACCTCCGGAATAC, CTTGGCACGCTGGTTCTTGA), SOX9 (GGAAGTCGGTGAAGAACGGG, TGTTGGAGATGACGTCGCTG), Axin2 (TATCCAGTGATGCGCTGAC, TTACTGCCCACACGATAAGG), and CDX1 (GTGGCAGCGGTAAGACTC, GTTCACTTTGCGCTCCTTTGC). Data are calculated based on $2^{-\Delta\Delta Ct}$ method[14] and presented as relative expression to RP2 (forward AAGCTGAGGATGCTCAAAG, reverse CCCATTAAACTCCAAGGCAA).

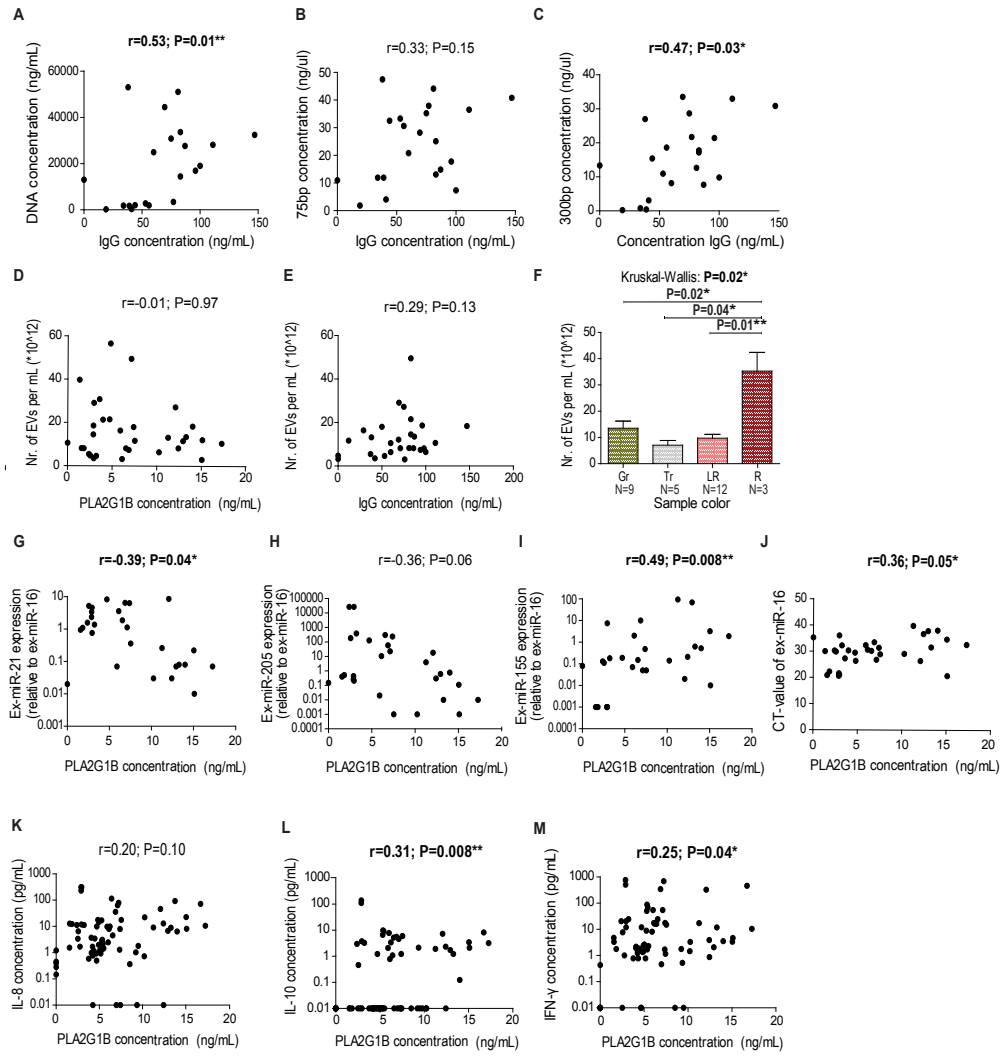
SUPPLEMENTAL TABLE S1 | Characteristics of patients included in phase 2 of the study.

	Age (years)	Gender	Mutation carrier	EUS indication	Morphology pancreas	Pathology proven (yes/no)
FPC1	53	F	No	Surveillance	No abnormalities	No
FPC2	71	M	BRCA2	Surveillance	Multifocal SB-IPMN, largest cyst 14 mm, no worrisome features.	No
FPC3	63	F	No	Surveillance	Multifocal SB-IPMN, largest cyst 9 mm, no worrisome features	No
FPC4	66	F	No	Surveillance	No abnormalities	No
FPC5	40	F	No	Surveillance	No abnormalities	No
PC1	73	F	NA	Fiducial placement	LAPC	Yes
PC2	72	F	NA	Diagnosis	LAPC	Yes
Cyst1	53	F	NA	Surveillance	Multifocal SB-IPMN, largest cyst 12 mm, no worrisome features	No
Cyst2	48	F	NA	Surveillance	SB-IPMN, 24 mm, non-enhanced (thickened) septation.	No
Cyst3	65	F	NA	Surveillance	MT-IPMN, PD 5mm, cyst 20mm, enhancing mural nodule.	No

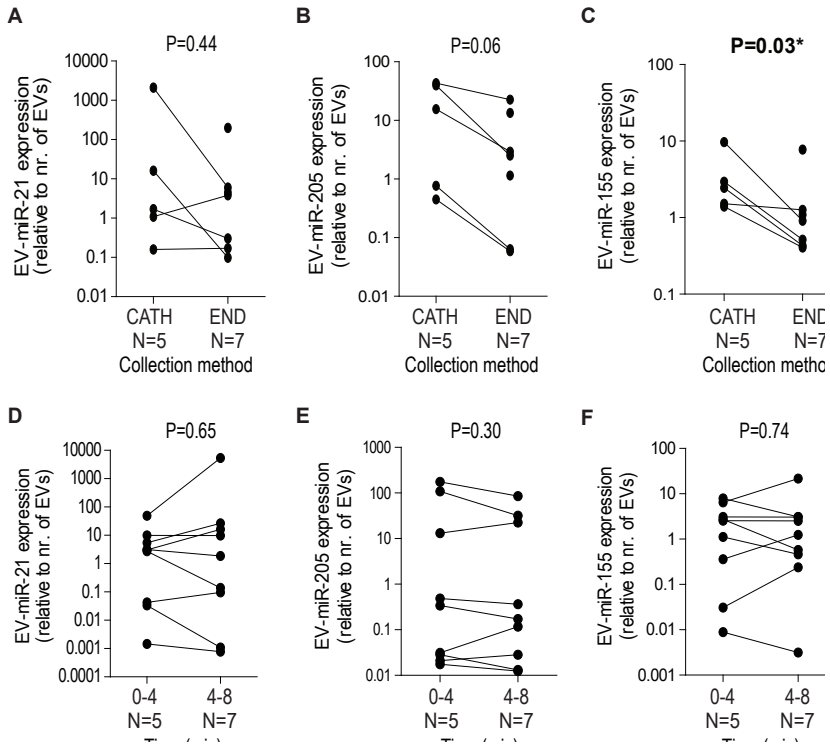
NA = not applicable; FPC = familial pancreatic cancer; PC = pancreatic cancer; LAPC = locally advanced pancreatic cancer; SB-IPMN = Side-branch intraductal papillary mucinous neoplasm; MT = mixed-type intraductal papillary mucinous neoplasm; EUS = endoscopic ultrasound.



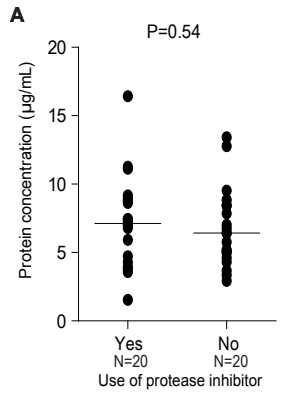
SUPPLEMENTAL FIGURE S1 | The collected volume of pancreatic juice did not differ between the endoscopists. Groups were matched based on study cohort (FPC N=6; PC N=3).



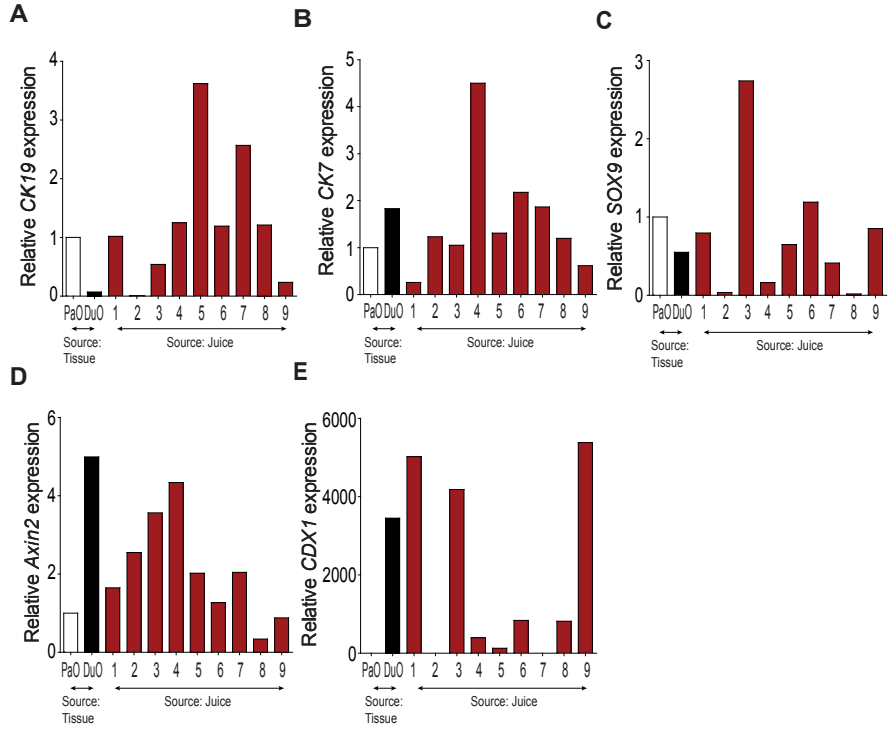
SUPPLEMENTAL FIGURE S2 | Color and biomarker concentration in relation to PLA2G1B and IgG concentration. (A-C) The total and 300bp DNA concentration is correlated to the IgG concentration (A, C), while 75bp concentration is not (B). The number of EVs was not associated with PLA2G1B concentration (not shown). (D, E) The number of extracellular vesicles is correlated to the concentration of PLA2G1B (D) and IgG4 (E). (F) The number of extracellular vesicles (EVs) in PJ stratified by color. Examples of PJ colors ranging from green (Gr) to transparent (Tr), light red (LR) and dark red (R). (G-J) The tested EV-miRs were correlated with PLA2G1B. EV-miR-21 and EV-miR-205 were, surprisingly, negatively correlated to PLA2G1B (G, H), while EV-miR-155 was positively correlated (I). (J) The CT-value of EV-miR-16, used in this study as internal control was correlated with PLA2G1B. (K-M) IL-8 was not, yet IL-10 and IFN-γ were correlated with PLA2G1B concentration. Although cytokines are known to be present in blood, cytokine concentrations were not associated with IgG or sample color (not shown).



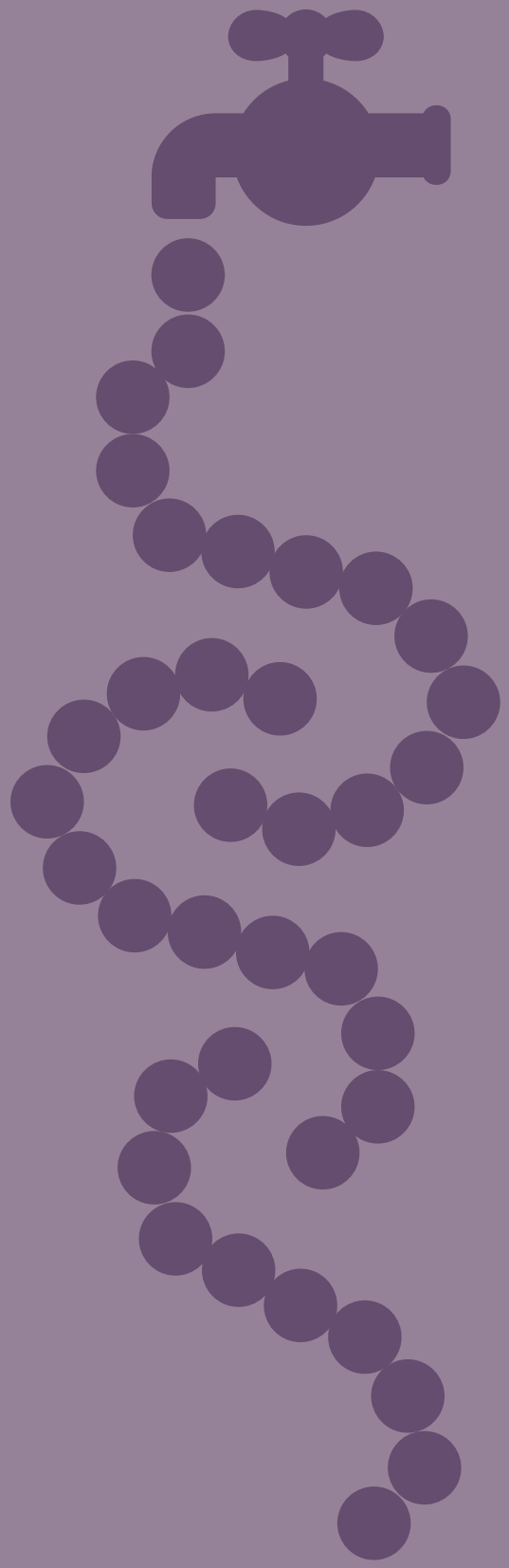
SUPPLEMENTAL FIGURE S3 | **Extracellular vesicle microRNA-155 (EV-miR-155) levels (relative to the number of EVs) are higher in PJ collected through suction with the catheter.** The expression of the selected EV-miRs was compared for the collection methods (A-C) and time periods (D-F). However, as both blood and bile contain EVs, contamination of PJ with these fluids may bias results when normalizing towards the number of EVs present in these juices.



SUPPLEMENTAL FIGURE S4 | **The addition of protease inhibitor does not affect protein concentration.**



SUPPLEMENTAL FIGURE S5 | Barcharts showing expression of *CK19*, *CK7*, *SOX9* and *AXIN2* (as reported for organoids cultured from pancreatic tissue; A-D) and *CDX1* (intestinal marker; E).



CHAPTER 7

Protein Biomarkers in Pancreatic Juice and Serum for Identification of Pancreatic Cancer

Authors
Iris J.M. Levink, Isis J. Visser, Brechtje D.M. Koopmann, Lydi M.J.W. van Driel, Jan Werner Poley, Djuna L. Cahen, Marco J. Bruno*, Gwenny M. Fuhler*

*Authors contributed equally

Gastrointest Endosc. 2022 Nov;96(5):801-813.e2.

Abstract

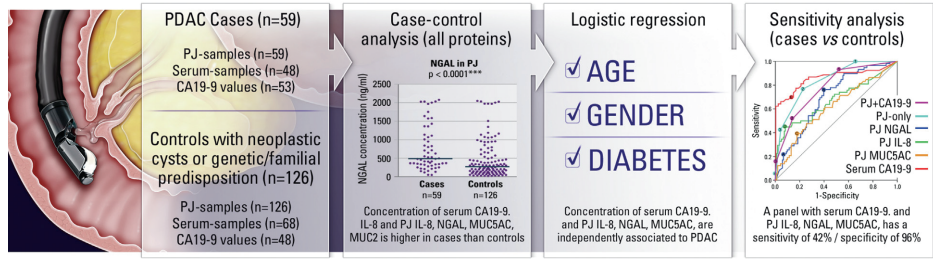
Background and aims: To date, surveillance of high-risk individuals for pancreatic cancer (PC) has not lived up to expectations, as identification of curable stages through imaging remains challenging. Biomarkers are therefore needed. Pancreatic juice (PJ) may be a promising source, being in direct contact with the ductal epithelial lining from which PC arises. We aimed to develop a panel of biomarkers from serum and PJ to detect PC for future surveillance purposes.

Methods: All patients who underwent PJ collection upon secretin stimulation at the Erasmus MC were included. Both PJ and serum were evaluated. Protein levels were determined by Lowry assay. Potential biomarkers (IL-8, IFN-γ, NGAL, MUC5AC, MUC2, PLA2G1B) were selected based on previously reported outcomes and assessed with ELISA. Serum CA19.9 values were determined by electrochemiluminescence immunoassay.

Results: This study included 59 cases and 126 surveilled controls (who underwent PJ collection), of whom 71 with a hereditary predisposition (35 genetic, 36 familial) and 55 with (suspected neoplastic) pancreatic cysts. CA19.9 values were available for 53 cases and 48 controls.
Serum CA19.9, as well as PJ IL-8, NGAL and MUC5AC were associated with PC independent of age, gender and presence of diabetes mellitus. Serum CA19.9 had a significantly higher AUC (0.86 [95% CI 0.79-0.94]) than individual PJ markers (AUC 0.62 to 0.70). A combination of PJ markers and serum CA 19.9 (panel 2: sensitivity 42% [95% CI 29-57], specificity 96% [95% CI 86-100]) did not improve diagnostic performance compared to CA 19.9 alone (sensitivity 70% [95% CI 56-82], specificity 85% [95% CI 72-94]).

Conclusions: High levels of serum CA19.9 and PJ-derived proteins are associated with PC. Prospective surveillance studies including individuals at risk of developing PC are required to validate these findings.

Graphical abstract



Pancreatic juice (PJ) is collected from the duodenum after stimulation by secretin; PDAC = pancreatic ductal adenocarcinoma; PJ+CA19.9 = PJ NGAL, PJ IL-8, PJ MUC5AC and serum CA19.9; PJ-only = PJ NGAL, PJ IL-8 and PJ MUC5AC

Introduction

Pancreatic cancer (PC) is a lethal disease for which the incidence closely parallels mortality, due to late presentation of symptoms and lack of effective therapy for late-stage disease.[1] Surveillance programs in individuals with a hereditary predisposition have shown that early detection based on imaging is highly challenging, even when imaging modalities are combined. By the time neoplasia becomes detectable, many patients already have advanced disease.[2-4] Additionally, in surveillance programs, resection of abnormalities which prove to be benign upon histological assessment are no rarity, causing unnecessary harm.[5] Likely, the best way to improve PC survival is by earlier detection. Biomarkers hold promise, as structural cellular changes are expected to occur months to years before a lesion becomes apparent on imaging. Thus, they may complement imaging in ruling out malignant disease.[6]

To date, carbohydrate antigen 19.9 (CA19.9) is the only PC serum marker that has been implemented in clinical practice to assess treatment response and detect recurrence. Despite guideline recommendations,[7] use for surveillance purposes is controversial, as higher values (>37kU/L) are regularly observed in patients with no or low-grade dysplasia (limited specificity),[8] and low values do not rule out malignant progression (limited sensitivity). While several other blood-based biomarkers have been proposed, thus far, none have lived up to expectations or been implimented.[9,10] For instance, Zhang *et al.* (2015)[11] reported in a meta-analysis that CEA, as compared to CA19.9, has a lower sensitivity of 40% [95% CI 37-42] and similar specificity of 81% [95% CI 79-83]) and is therefore not useful in clinical practice. With a specificity of 83% (95% CI 81-85; sensitivity 68% [95% CI 66-70]) CA-242 has a similar diagnostic performance to that of CA19.9 (sensitivity of 75% [95% CI 73-77]; specificity 78% [95% CI 75-80]), and is also not clinically implemented.

Pancreatic juice (PJ) may serve as an alternative biomarker source. Biomarkers determined in juice are expected to be more pancreas-specific, as this fluid constitutes

a wash-out of the pancreatic ductal system and has been in close contact with the ductal cells from which PC originates. As compared to either PJ collection by pancreatic duct cannulation via ERP (endoscopic retrograde pancreatography) or tissue sampling with fine-needle aspiration (FNA) or biopsy (FNB), secretin-stimulated PJ collection from the duodenal lumen is less invasive. Additionally, in contrast to needle biopsy, PJ collection does not rely on a visible mass and PJ potentially contains information on the complete range of tumor clones.[12,13]

Based on available data, several potential biomarkers can be identified. PC progression is associated with a distinct state of inflammation and altered release of cytokines. Interleukin-8 (IL-8), interferon-γ (IFN-γ) and neutrophil gelatinase-associated lipocalin (NGAL) were shown to be overexpressed in dysplastic and/or PC tissue,[14-16] and previous results in either serum of PJ demonstrated that increased concentrations of these proteins are able to distinguish PC cases from controls.[16-20] Mucins are highly glycosylated proteins that are widely expressed in gastrointestinal tissues, yet MUC2 and MUC5AC, specifically, are undetectable in normal pancreas tissue.[21,22] Their diagnostic potential has been suggested in serum as well as FNA specimens, but not yet for PJ.[23-26] As altered expression of mucins is detected in intraductal papillary mucinous neoplasms (IPMNs) and PanIN, increased mucin levels are expected to occur early in the development of PC and may therefore complement cytokines and serum CA19.9 in early detection.[23-26] Previous literature showed that CA19.9 in PJ performed less than the other mentioned proteins in PJ.[20]

To reduce harm to individuals participating in a surveillance program, a new diagnostic tool should be able to rule out malignant progression. For instance, a tool with a (close to) 100% specificity could reliably postpone surgery in individuals with indeterminate features on imaging or reduce surveillance frequency in individuals without relative or absolute indications for surgery.[7] As multiple subtypes of PC have been identified,[27,28] each potentially characterized by distinct biomarkers, it is expected that a combination of biomarkers (rather than one biomarker alone) is needed to reach such high specificity.

In this study, we aimed at developing a biomarker panel that is able to distinguish individuals without PC (high specificity; 'primum non nocere') in order to avoid unnecessary harm to participants in a surveillance population while also detecting PC with considerable sensitivity. To this end, we investigated the diagnostic performance of five biomarkers (IL-8, IFN-γ, NGAL, MUC5AC, MUC2) in both serum and PJ, in addition to CA19.9 in serum.

Materials & methods

Study design and patient inclusion

This is a case-control study including data from three prospective cohort studies performed at the Erasmus MC University Medical Center Rotterdam: 1. KRASPanc-study (MEC-2018-038), concerning patients with (suspected) sporadic PC undergoing diagnostic EUS or fiducial placement for stereotactic radiotherapy; 2. CAPS-study (MEC-2012-448), involving individuals under surveillance for a hereditary predisposition for PC; 3. PACYFIC study (MEC-2014-021), involving individuals undergoing surveillance for suspected neoplastic pancreatic cysts. See **Supplemental Table 1** for in- and exclusion criteria per study. We considered all patients who underwent PJ collection during EUS between August 2018 and May 2020 for inclusion. Samples were excluded if they had undergone a freeze-thaw cycle. As only one patient with high-grade dysplasia was identified in the study cohorts, this patient was excluded from analysis. In case of multiple PJ collections in one patient, the first sample was assessed for this study. In addition, serum samples collected within 3 months of PJ collection were evaluated upon availability.

The institutional Medical Center ethical review board approved the study, and included individuals gave written informed consent before enrolment. The study was carried out according to the ethical principles for medical research involving human subjects from the World Medical Association Declaration of Helsinki.

Clinical data extraction

Clinical variables were collected in the course of prospective studies. EUS-FNB pathology results and CA19.9 values were extracted from patient records if available within 3 months before or after PJ collection.

Biomaterial collection

PJ collection was performed with a linear echoendoscope (Pentax Medical, Tokyo, Japan) by experienced endo-sonographers (L.M.J.W.D, J.W.P, M.J.B). After insertion of the tip of the echoendoscope into the D2 segment of the duodenum, secretion of PJ was stimulated by intravenous injection of human secretin (16 µg/patient, ChiRhoClin, Burtonsville, MD). Suction through the endoscopic channel was applied immediately after injection of secretin for eight minutes by positioning of the tip close to the ampullary orifice.[13] Within ten minutes after collection, juice was aliquoted and snap frozen. Samples were stored at -80°C until use. Serum samples were collected by venipuncture, aliquoted and stored at -80°C until use.

Protein analysis

Concentrations of candidate proteins (IL-8, IFN-γ, NGAL, MUC5AC, MUC2) in serum and PJ were assessed by enzyme linked immune sorbent assay (ELISA) according to the protocol of the manufacturer. Phospholipase A2 Group IB (PLA2G1B; pancreas standard marker) and total protein concentration were assessed to control for collection- or patient-related differences between biomaterials.

For IL-8 and IFN-γ (Thermo Fisher Scientific, Waltham, MA, #88-8086, #88-7316), immunosorb plates were coated with protein-specific capture antibody overnight at 4 °C. Plates were blocked with ELISA diluent for one hour at room temperature, and incubated with PJ (100 µL per well) at 4 °C overnight. Then, biotin-conjugated detection antibody was added at room temperature for 1 hour, followed by avidin-HRP for 30 minutes at room temperature. Antibody binding was visualized by addition of TMB substrate, reactions were terminated by adding sulfuric acid and absorbance was read at 450 nm. Measurements of MUC2, MUC5AC, NGAL (Aviva Systems Biology, San Diego, CA, OKIA00076, OKEH02839, OKEH02840) and PLA2G1B (My Biosource, San Diego, #MBS703283; PJ only) concentration were performed similarly, using pre-coated and pre-blocked plates. Prior to NGAL analysis, PJ was diluted 1:30. To assess the total protein concentration, a Lowry protein assay (Bio-Rad, Hercules, CA) was executed. PJ or serum samples of cases and (different types of) controls were equally distributed among ELISA plates.

Statistical analysis

Depending on distributional properties of measures, descriptive data were expressed as means with standard deviation (SD), medians with range or percentages. Statistical significance was assessed using Mann-Whitney U for continuous data and either Fisher's exact or chi^2-test for categorical data. Individuals were considered to have high-risk features when presenting with one absolute indication for surgery (enhancing mural nodule/solid lesion ≥5mm, caliber change of the main pancreatic duct (MPD) suggesting MPD obstruction, MPD dilation ≥10mm) or two or more relative indications for surgery (MPD 5-9.9 mm, cyst diameter ≥40mm, new-onset diabetes, acute pancreatitis, enhancing mural nodule <5mm)7.

For primary outcome analysis, PC cases and controls were compared with Mann-Whitney U test. Additionally, controls from the PACYFIC cohort with a cystic lesion with absolute or relative indications for surgery[7] were compared with controls without these indications, to evaluate the influence of heterogeneity in the control group (Mann Whitney U). To evaluate if marker concentrations were associated with PJ quality, they were correlated with total protein (ruling out collection-related differences between groups) and PLA2G1B (representing true pancreas-derived material) concentration.

Multiple logistic regression models were created to test if the biomarkers are associated with PC, independent of age, gender and presence of diabetes mellitus (DM). Age and BMI (per group) were correlated with biomarker concentrations using Spearman's correlation. To evaluate the markers association with gender, chemotherapy (treatment naive vs post-treatment), resectabiliy (resectable vs locally advanced pancreatic cancer [LAPC; combination of non- and borderline resectable disease]) and caliber change (as a measure of MPD obstruction), concentrations were compared within groups (cases and controls) by Mann Whitney U test. To rule out influence of common bile duct (CBD) stenting, a sub-analysis was executed, comparing cases and controls without a CBD stent (Mann Whitney U).

Diagnostic performance was evaluated for those biomarkers that significantly differed between cases and controls (Mann Whitney U) and were associated with PC independent of age, gender, DM and BMI (multiple logistic regression described earlier). For these markers, receiver operating characteristic (ROC) curves were created, resulting in an area under the curve (AUC) with 95% confidence interval; a paired-sample analysis was performed to compare AUC-values between panels and markers. Two panels were created: 1. PJ-only panel (IL-8, NGAL, MUC5AC); 2. A combined panel (serum CA19.9, as well as PJ IL-8, NGAL, MUC5AC). Given diagnostic performances of panels are based on the number of biomarkers with a positive test (as indicated in **Table 2**). Since implementation of a biomarker should not result in additional stress or anxiety, unnecessary diagnostic procedures, harm (due to unnecessary surgery/biopsy) and costs, we aimed at high specificity (>85% for individual markers) and positive predictive value (PPV). In this study, selection of cutoff values was based on the specificity. A potential biomarker panel should eventually have a specificity >95% with fair sensitivity (>50%). For CA19.9, a cutoff of ≥37kU/L was used, as is customary in clinical practice7. Confidence intervals of sensitivity, specificity, PPV and accuracy are represented by "exact" Clopper-Pearson confidence intervals. These analyses were performed in SPSS (Statistical Package for the Social Sciences, version 27, SPSS Inc., Chicago, IL); figures were created using GraphPad (GraphPad Prism version 9, GraphPad Software, La Jolla, CA). Survival curves were created and cox regressions were performed using R (Rstudio, PBC, Boston, "ggplot2", "survival", "survminer").

Results

Patient cohort

Patient characteristics are reported in **Table 1**. In total, 185 consecutive individuals met the inclusion criteria. Of these, 59 individuals had histologically confirmed PC. Indications for EUS in this case group were: 1. tissue acquisition for suspected PC (N=38; 63.3%), these cases were treatment-naive; 2. fiducial placement to enable stereotactic radiotherapy (N=21; 35.0%), these patients had undergone chemotherapy prior to PJ collection.

The control group consisted of 126 individuals; 122 undergoing EUS in the course of surveillance for a genetic or familial predisposition (N=71; 56.3%) or for suspected neoplastic pancreatic cysts (N=51; 40.4%) and 4 (3.2%) for an unsubstantiated PC suspicion.

For controls, the median follow-up duration was 31 months (IQR 6 months) during which none of these individuals developed PC. 14 controls had a surveillance duration shorter than 12 months: 10 resigned surveillance (and did not present with PC later in time), 3 patients died and 1 was released from surveillance after an operated mucinous cystic neoplasm (MCN). These controls were not excluded from analysis. In total, four controls underwent pancreatic surgery, of which three had IPMN with low-grade dysplasia (LGD; 2 gastric, 3 intestinal-type) and one had MCN with LGD. Of these, one control died due to abdominal sepsis and multi-organ failure 8 days after resection. During the follow-up period, two controls developed other malignancies, one individual with a hereditary increased risk of PC was diagnosed with stage IV mamma carcinoma 13 months after collection, and another had a melanoma at time of collection and died 7 months after collection.

Relative and absolute indication for surgery

With regard to morphologic changes, 43 controls (34.1%) had no abnormalities on imaging and 80 (63.4%) had a cystic lesion. According to the European guidelines[7,26] controls (20.6%) had a relative or absolute indication for surgery at time of PJ collection (**Table 1**). Of which 10 (7.9% of controls) had one absolute indication or two or more relative indications for surgery and could have undergone surgery according to these guidelines.

TABLE 1 | Clinical characteristics of study participants.

	Cases (N=59)	Controls (N=126)	p-value
Age in years, median (IQR)	68.0 (11.0)	62.0 (16.0)	<0.001
Male gender, n (%)	36 (61.0)	42 (33.3)	<0.001
BMI in kg/m2, median (IQR)	23.2 (4.4)	25.6 (5.1)	<0.001
Familial/genetic predisposition, n (%)	0 (0.0)	71 (56.3)	<0.001
Member of FPC family	.	36 (28.6)	
CDKN2A p16	.	25 (19.8)	
BRCA2 + 2 blood relatives with PC	.	5 (4.0)	
BRCA1 + 2 blood relatives with PC	.	1 (0.8)	
PALB2 + 2 blood relatives with PC	.	1 (0.8)	
BRCA2 + CDKN2A p16	.	1 (0.8)	
STK11/LKB1	.	2 (1.6)	
Diabetes mellitus, n (%)	24 (40.7)	16 (12.7)	<0.001
Indication EUS, n (%)			<0.001
Suspected PC	38 (64.4)	4 (3.2)	
Fiducial placement	21 (35.6)	.	
Surveillance	0 (0.0)	122 (96.8)	
CBD stent in situ, n (%)			<0.001
CBD stent in situ	9 (15.3)	0 (0.0)	
No CBD stent but CBD dilation	14 (23.7)	3 (2.4)	
No CBD stent and no CBD dilation	36 (61.0)	123 (97.6)	
Relative/absolute indications for surgery7, n (%)	59 (100.0)	26 (20.6)	<0.001
Enhancing mural nodule or hypodense lesion	59 (100.0)	4 (3.2)	
Caliber change MPD	44 (74.6)	0 (0.0)	
Diffuse PD dilation > 5mm	0 (0.0)	14 (11.1)	
CA19.9 ≥37 kU/L	37 (62.7)	7 (5.5)	
Cyst size > 40mm	0 (0.0)	2 (1.6)	
New-onset diabetes‡	9 (15.2)	2 (1.6)	
Recent acute pancreatitis†	2 (3.4)	6 (4.8)	
Lymphadenopathy	26 (44.1)	0 (4.0)	
Working diagnosis, n (%)			<0.001
No abnormalities	.	43 (34.1)	
Unspecified cyst	.	12 (9.5)	
SB-IPMN	.	53 (42.1)	
MD/MT-IPMN	.	14 (8.8)	
MCN	.	1 (0.8)	
NET	.	1 (0.8)	
Indeterminate, not suspect for malignancy	.	2 (1.6)	
Resectable PC¶	11 (18.6)	.	
Locally advanced PC	48 (81.4)	.	
Distant metastases (on imaging), n (%)	10 (16.9)	0 (0.0)	<0.001

SD = tandard deviation; CBD = common bile duct; EUS = endoscopic ultrasound; MCN = mucinous cystic neoplasm; MD-IPMN = main-duct intraductal papillary neoplasm; MT-IPMN = mixed-type IPMN; MPD = main pancreatic duct; NET = neuro-endocrine tumor; PC = pancreatic cancer; PD = pancreatic duct; SB-IPMN = side-branch intraductal papillary neoplasm. ‡Development of diabetes mellitus in last 2 years. †Present in the last 2 years; ¶resectable PC (as based on DPCA guideline): Superior mesenteric artery: no contact; celiac axis: no contact; common hepatic artery: no contact; and superior mesenteric vein and portal vein: ≤ 90° contact.

Serum: cases vs controls

Serum samples were available for 116 of 185 patients (48 cases, 68 controls), CA19.9 was determined in 101 of 185 patients (53 cases, 48 controls). Time between PJ and (biobank) serum sampling was ≤3 weeks for all samples; median time between PJ and CA19.9 measurement was 9 days (IQR 14). The total protein concentration did not differ between investigated groups (P=0.21; **Figure 1A**) and none of the investigated serum biomarkers were correlated to the total protein concentration in serum (**Supplemental Figure S1**).

Concentrations of CA19.9 (p<0.0001) and IL-8 (p=0.006) were higher in cases than controls. None of the other serum proteins differed between groups (**Figure 1B-G**). MUC2 was detectable in 15 individuals (7 cases [14%]; 8 controls [12%]) and MUC5AC in five individuals (2 cases [4%]; 3 controls [4%]). Additionally, the presence of serum MUC2 and MUC5AC was not related to the development of other malignancies within the 12 months before or after serum collection.

No differences in serum protein levels were found between controls with and without absolute or relative indications for surgery (p>0.05 for all markers; not shown).[7]

Pancreatic juice: cases vs controls

Total protein (p=0.35) and PLA2G1B (p=0.24) concentration (as a measure of PJ quality) did not differ between cases and controls (**Figure 1H, I**). PLA2G1B levels correlated with the concentration of total protein (r=0.38; p<0.001), IL-8 (r=0.13; p=0.05), IFN-γ (r=0.27; p<0.001), NGAL (r=0.23; p<0.001) and MUC2 (r=0.14; p=0.05), but not with MUC5AC (r=0.01; p=0.85; see **Supplemental Figure S1**).

When comparing cases with controls, IL-8 (p=0.0001) and NGAL (P<0.0001) concentrations were higher for cases, while IFN-γ concentration did not differ from controls (p=0.13; **Figure 1J-L**). Concentrations of the investigated mucins, MUC5AC (P=0.01) and MUC2 (p=0.04), were higher in cases than controls (**Figure 1M, N**). For the control group, none of these markers differed between patients with and without absolute or relative indications for surgery (p>0.05; not shown).[7]

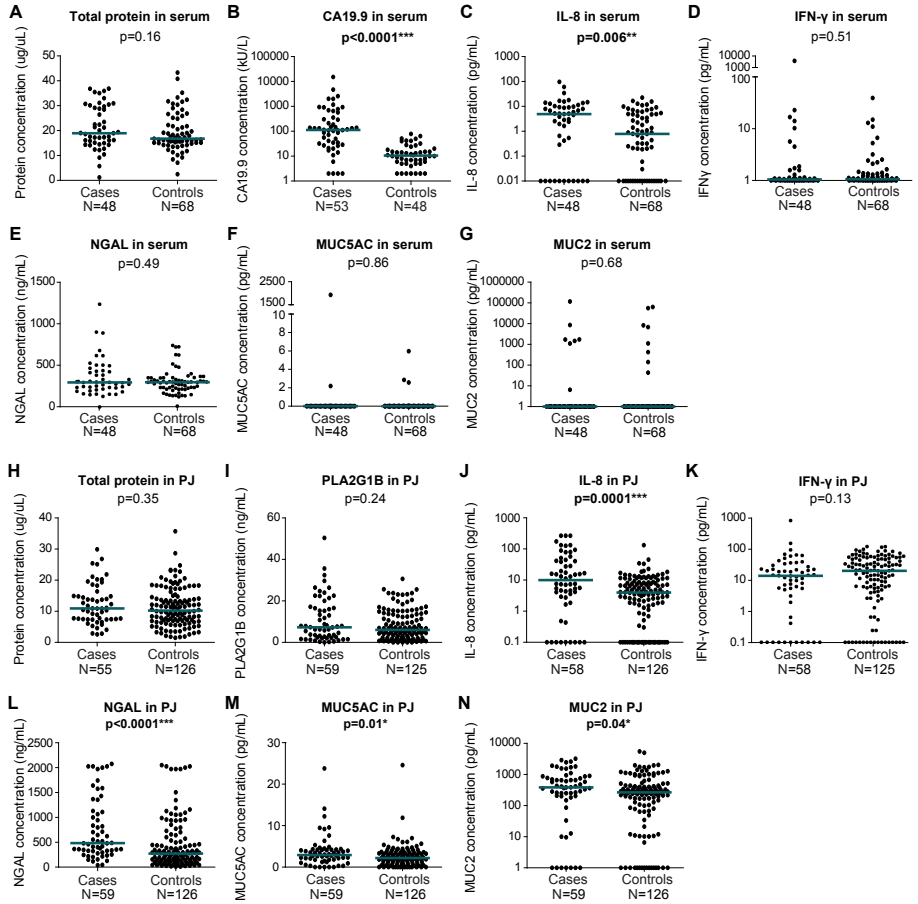


FIGURE 1 | **Serum (A-G) and PJ (H-N) protein levels in cases as compared to controls. A.** Total protein concentration in serum was not different between cases and controls. **B, C.** CA19.9 (B) and IL-8 (C) concentration was higher in serum from cases than from controls. **D-F.** Concentration of IFN-γ (D), NGAL (E), MUC5AC (F), MUC2 (G) in serum did not differ between cases and controls. **H, I.** Based on concentration of total protein (H) and PLA2G1B (B), the quality of pancreatic juice was not different for cases and controls. **J, K.** IL-8 concentration was higher in PJ from cases than from controls, while IFNγ concentration did not differ. **L-N.** Concentrations of NGAL (L), MUC5AC (M), MUC2 (N) were higher in PJ from cases than from controls. Groups were compared with Mann-Whitney U test. The green line indicates the median concentration per group.

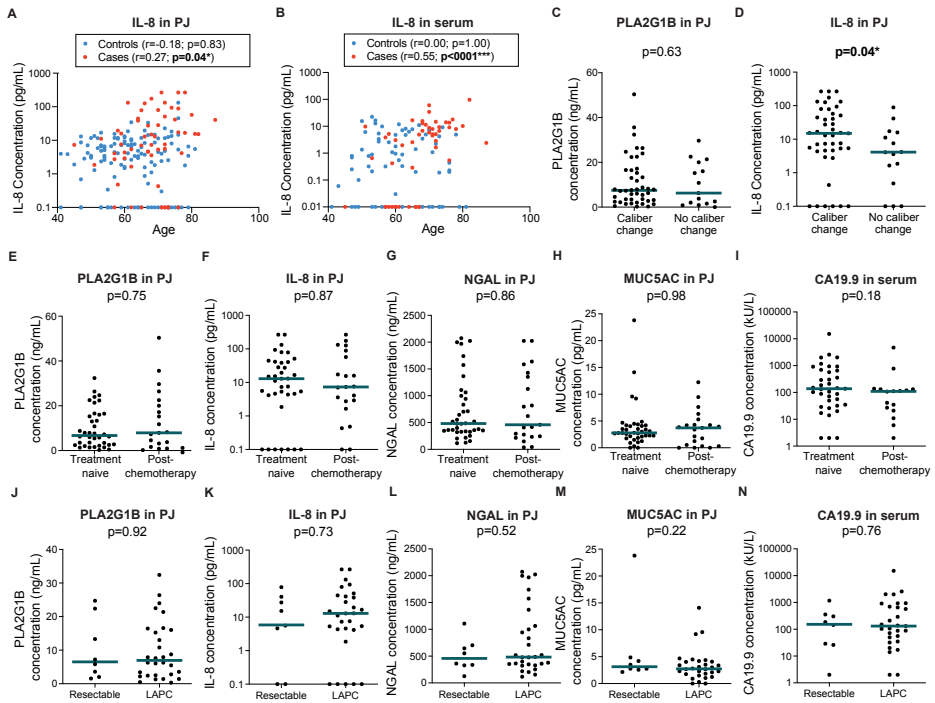


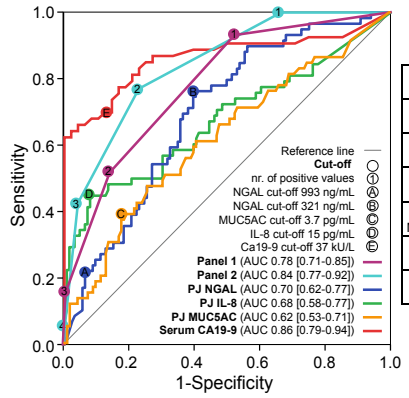
FIGURE 2 | **Association of biomarkers and clinical parameters. A, B.** IL-8 concentration in cases was correlated with age for PJ (A) and serum. (B) This correlation with was not found in controls or for any of the other investigated proteins (not shown). **C, D.** Caliber change (as a measure of pancreatic duct obstruction) did not influence the pancreatic content in PJ (C) while PJ IL-8 concentration was higher in patients with caliber change, than those without (D). This association was not found for the other investigated proteins (not shown). **E-I.** PJ quality and biomarker concentration does not differ between cases with previous chemotherapy and treatment-naive cases. **J-N.** In treatment-naive cases (n=38), marker concentrations were not different in patients with early stage PC ('resectable'), as compared to those with (locally) advanced disease. Significance was tested with Spearman correlation (A, B), Mann-Whitney U (C, D). The green line indicates the median per group (C, D).

Relation of markers to clinical characteristics

As statistical differences in baseline characteristics were present between cases and controls (**Table 1**), we investigated their influence on protein concentrations. IL-8 concentration in PJ (r=0.27; p=0.04; **Figure 2A**) and serum (r=0.55; p<0.0001; **Figure 2B**) was correlated with age for cases, but not for controls. Biomarker levels were similar for males and females and those with or without DM, and did not correlate to BMI (not shown). Multivariable logistic regression showed that IL-8 (p=0.003), NGAL (p=0.003) and MUC5AC (p=0.03) concentrations in PJ, as well as CA19.9 concentration in serum

(p=0.05) were associated with PC, independent of age, gender, and DM (**Supplemental table S2 & 3**).

For cases, a clinical parameter that might affect PJ composition is MPD obstruction (suspected by a caliber change on imaging), as this may decrease pancreatic content in PJ. However, we did not observe lower concentrations of PLA2G1B in such cases (p=0.63; **Figure 2C**). While IL-8 concentration was higher in individuals with a caliber change in the MPD (**Figure 2D**, P=0.05), none of the other investigated PJ markers was influenced by caliber change (not shown). Furthermore, CBD stenting may cause local inflammation and increase biomarker concentrations. Therefore, we performed a sub-analysis after excluding the 9 cases with a CBD stent. Concentrations of IL-8 (p=0.003), NGAL (p<0.0001) and MUC5AC (p=0.04) in juice, and IL-8 (p=0.03) and CA19.9 (p<0.0001) in serum remained higher in cases than controls. Differences in MUC2 in PJ no longer reached significance (p=0.06). As cases who are treatment-naive may show different results (*e.g.*, other inflammatory state, potential tumor reduction) than those that had already undergone chemotherapy, we compared these groups. However, both the PJ quality (as based on the PLA2G1B concentration; **Figure 2E**) and the marker concentrations (**Figure 2E-2I**) were not different between groups. These were also not different for patients with resectable disease (early cancer) or locally advanced disease ('LAPC'; **Figure 2J-N**).



	Serum CA19.9	PJ IL-8	PJ NGAL	PJ MUC5AC	Panel 1	Panel 2
Serum CA19.9						
PJ IL-8	Δ -0.25 **p=0.001****					
PJ NGAL	Δ -0.26 **p=0.001****	Δ 0.22 p=0.62				
PJ MUC5AC	Δ -0.24 **p=0.001****	Δ -0.6 p=0.37	Δ -0.8 p=0.19			
Panel 1	Δ -0.13 **p=0.05***	Δ 0.11 **p=0.02***	Δ 0.08 **p=0.008****	Δ 0.17 **p<0.001****		
Panel 2	Δ -0.02 p=0.7	Δ 0.23 **p<0.001****	Δ 0.24 **p<0.001****	Δ 0.22 **p<0.001****	Δ 0.11 **p<0.001****	

FIGURE 3 | **Diagnostic performance of both individual biomarkers that were associated with PC independent of age, gender and presence of DM, and the created panels (Panel 1: IL-8, NGAL and MUC5AC in PJ; Panel 2: serum CA19.9 and IL-8, NGAL, MUC5AC in PJ). A.** ROC-curves of the different individual markers and panels. For NGAL, two cutoffs are indicated, cutoff A could be used for NGAL as individual marker (high specificity) and cutoff B for the panel (high total diagnostic performance). AUC = area under the curve; given ranges are 95% confidence intervals. The numbers are equal to the number of possible values related to the diagnostic performance on the curve. **B.** Differences (Δ) between AUC-values of the different markers and panels, p-values are generated by a paired-sample analysis.

Diagnostic performance

We generated ROC-curves to assess the diagnostic performance of markers that were independently associated with the presence of cancer (**Figure 3**; serum CA19.9, as well as PJ IL-8, NGAL and MUC5AC). As previously described, we aimed at high specificity. Cutoff values and corresponding sensitivities, specificities, accuracy and PPV of individual markers are presented in **Table 2**. Our results show that CA19.9 (cut off ≥37kU/L) was able to differentiate between cases (n=53) and controls (n=48) with a sensitivity of 69.8% (95% CI 55.7-81.7) and specificity of 85.4 (95% CI 72.2-93.9).

For the biomarkers in PJ, an IL-8 concentration of 15 pg/mL differentiated between cases (n=58) and controls (n=126) with high specificity (92.1%; 95% CI 85.9-96.1%), at a sensitivity of 44.8% (95% CI 31.7-58.5%) and moderate PPV (72.2%; 95% CI 57.3-83.4%). NGAL (993 ng/mL) had a specificity of 87.3% (95% CI 80.2-92.6%), sensitivity of 28.8% (95% CI 17.8-42.1%) and PPV of 51.5% (95% CI 36.6-66.1%) for differentiating between cases (n=59) and controls (n=126; **Table 2**, **Figure 3 & 4**). Serum CA19.9 had a significantly higher AUC than the individual PJ markers (**Figure 3B**; p=0.001). Only one case (and no controls) had a positive test for all four markers (serum CA19.9, as well as PJ IL-8, NGAL, MUC5AC).

Next, two biomarker panels were tested: one combining PJ IL-8, NGAL and MUC5AC (Panel 1) and another that included serum CA19.9 (Panel 2). For PJ markers in these panels, cutoffs with the highest sum (sensitivity + specificity) were selected to increase the overall performance (and not just the specificity). For PJ IL-8 and MUC5AC, these cutoffs had high specificity and were used to calculate diagnostic performance of the individual marker. For NGAL, we selected a different cutoff (321 ng/mL). For Panel 1 (tested in 58 cases and 126 controls), having two or more markers with a positive test was related to a decent diagnostic performance (sensitivity 51.7% [95% CI 38.2-65.1%]; specificity 85.7% [95% CI 78.4-91.3%]; accuracy 75.0% [95% CI 68.1-81.1%]; PPV 62.5% [95% CI 50.4-73.2]). In case of three biomarkers with a positive test, the specificity became close to 100%, yet the sensitivity was 15.5% (95% CI 7.4-27.4%; **Table 2**; **Figure 3A**). With regard to panel 2 (tested in 52 cases and 48 controls), having 3 or more biomarkers with a positive test was able to differentiate between cases and controls with a sensitivity of 42.3% (95% CI 28.7-56.8%); specificity 95.8% (95% CI 85.8-99.5%); accuracy 68.0% [95% CI 57.9-77.0]; PPV 91.7% [95% CI 73.2-97.8%]).

TABLE 2 | **Diagnostic performance of individual markers and generated panels.** For the individual markers that were independently associated with PC (CA19.9, PJ-IL-8, PJ-NGAL), cutoffs were selected aiming specificity (Sensitivity>25%). Two biomarker panels were generated: a PJ-only panel (Panel 1: IL-8 and NGAL) and a combined panel (panel 2: serum CA19.9, PJ IL-8, PJ NGAL). PJ = pancreatic juice, AUC = area under the curve, CI = Confidence interval, NA= not applicable, PPV = positive predictive value.

Protein	Nr. of cases/ controls	AUC (95% CI)	Cutoff Concen-tration	Sensitivity, % (95% CI)	Specificity, % (95% CI)	Accuracy, % (95% CI)	PPV, % (95% CI)
Individual markers							
CA19.9	53/48‡	0.86 (0.79 - 0.94)	37 kU/L	69.8 (55.7 - 81.7)	85.4 (72.2 - 93.9)	77.2 (67.8 - 85.0)	84.1 (72.3 - 91.5)
IL-8 in PJ	58/126‡	0.68 (0.58 - 0.77)	15 pg/mL	44.8 (31.7 - 58.5)	92.1 (85.9 - 96.1)	77.2 (70.4 - 83.0)	72.2 (57.3 - 83.4)
NGAL in PJ	59/126‡	0.70 (0.62 - 0.77)	993 ng/mL	28.8 (17.8 - 42.1)	87.3 (80.2 - 92.6)	68.7 (61.4 - 75.3)	51.5 (36.6 - 66.1)
			321 ng/mL	76.27 (63.4 - 86.4)	59.5 (50.4 - 68.2)	64.9 (57.5 - 71.7)	46.9 (40.6 - 53.2)
MUC5AC in PJ	59/126‡	0.62 (0.53 - 0.71)	3.7 pg/mL	39.0 (26.6 - 52.6)	81.0 (73.0 - 87.4)	67.6 (60.3 - 74.3)	48.9 (37.2 - 60.8)
Panels							
Panel 1: PJ (≥2 positive)†	58/126‡	0.78 (0.71 - 0.85)	*As indicated*†	51.7 (38.2 - 65.1)	85.7 (78.4 - 91.3)	75.0 (68.1 - 81.1)	62.5 (50.4 - 73.2)
Panel 1: PJ (3 positive)†	58/126‡			15.5 (7.4 - 27.4)	99.2 (95.7 - 100)	72.8 (65.8 - 79.1)	90.0 (53.9 - 98.6)
Panel 2: PJ + serum (≥3 positive)†	52/48‡	0.84 (0.77 - 0.92)		42.3 (28.7 - 56.8)	95.8 (85.8 - 99.5)	68.0 (57.9 - 77.0)	91.7 (73.2 - 97.8)

†The number of markers that showed a concentration above the defined cutoffs (CA19.9: 37 kU/L; IL-8: 15 pg/mL; NGAL: 321 ng/mL; MUC5AC: 3.7 pg/mL). ‡Patient numbers were variable, as one concentration of IL-8 in PJ (case) was missing and 85 concentrations of serum CA19.9 were missing (7 cases, 78 controls).

Based on the AUC, both panels performed better than individual PJ markers, but were not superior to CA19.9 alone. Panel 2 performed better than panel 1 (p<0.001; **Figure 3B**). Of note, patient numbers for serum CA19.9 and panel 2 are lower (52 cases, 48 controls) than those for the PJ markers and panel 1 due to missing CA19.9 values.

As a comparison, sensitivity of EUS-FNB (at first try) in our cohort was 73%. The specificity could not be calculated, as none of the controls underwent EUS-FNB at the same procedure. **Figure 4** shows an overview of the investigated biomarkers and panels in relation with (other) patient characteristics.

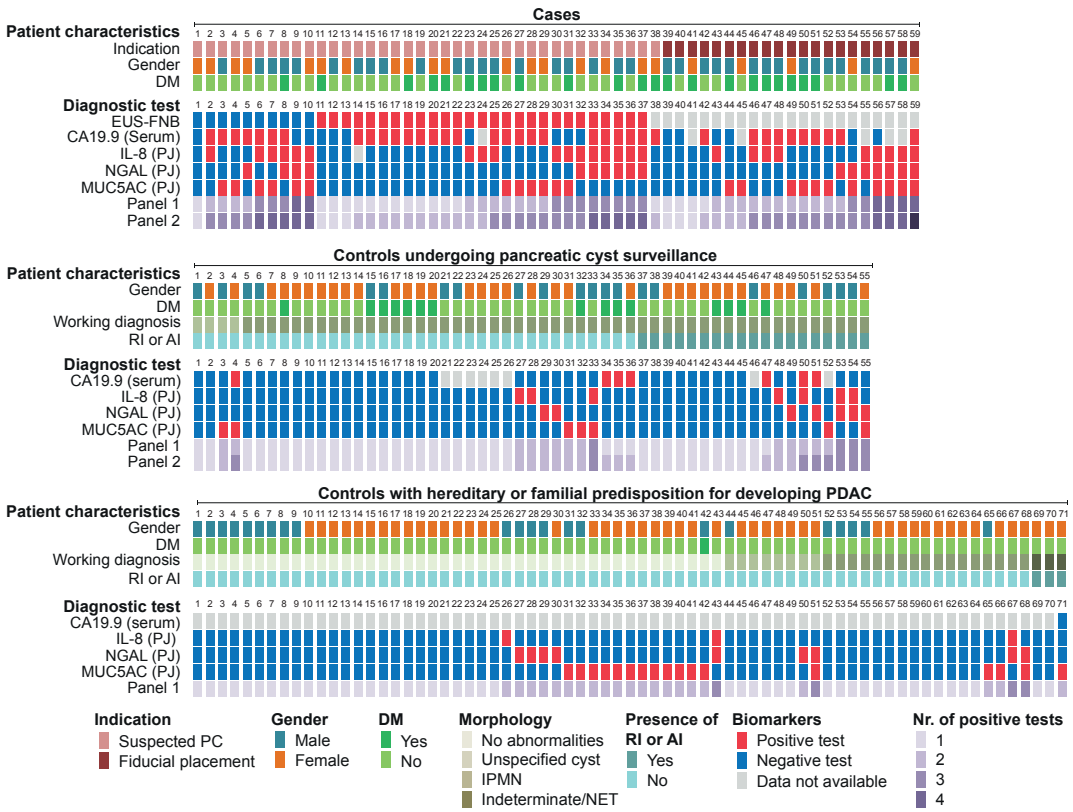


FIGURE 4 | The presence of a positive or negative test of tested individual biomarkers and panels for cases and controls in relation to gender, diabetes mellitus, indication of procedure and working diagnosis based on imaging. For the individual biomarkers and the two panels, the cutoff as shown in Table 2 was used to differentiate between a positive and negative test (for NGAL 993 ng/mL as we aimed for high specificity). Patients with suspected PC are treatment-naive, while patients with indication fiducial placement have undergone chemotherapeutic treatment. The presence of a relative or absolute indication (RI/AI) was based on the European Evidence-based pancreatic cyst guideline.[7] DM = diabetes mellitus, PJ = pancreatic juice.

Predictive value of biomarkers

During a median follow-up period of 30 months (range 19-38 months), 43 (73%) cases died. Survival analysis was only performed for patients who underwent PJ collection (for suspected PC) at time of diagnosis (a.k.a. 'treatment naive', n=38). The other subgroup (who underwent chemotherapy) underwent PJ collection seven months after diagnosis. Cox regression analysis showed that resectability (detected in earlier stage) was not predictive for survival, neither were serum CA19.9, nor PJ IL-8, NGAL and MUC5AC (**Figure 5**) in this patient cohort.

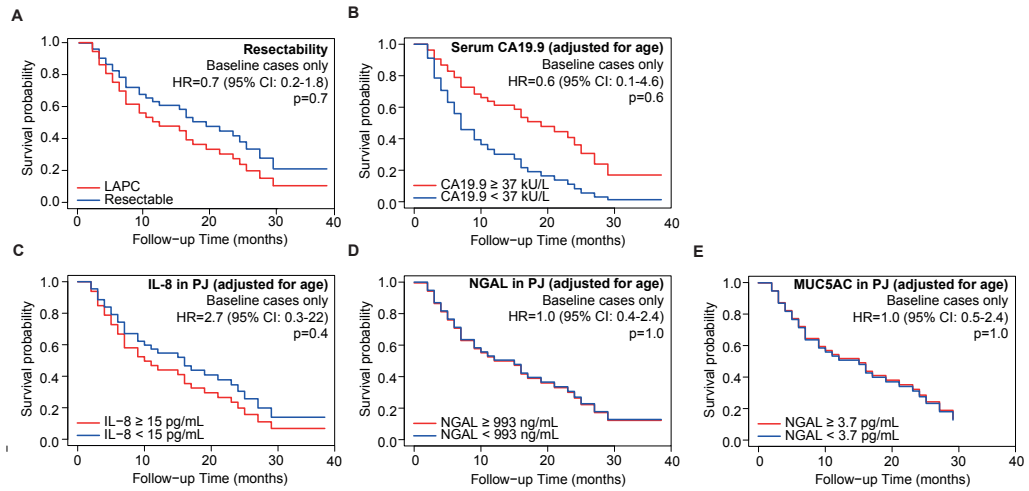


FIGURE 5 | Survival of patients based on resectability (A) and with a positive or negative test for serum CA19.9 (B), PJ IL-8 (C), PJ NGAL (D) and PJ MUC5AC (E). HR = hazard ratio (calculated by cox-regression and adjusted for patient age).

Discussion

Here, we assessed the diagnostic performance of promising protein biomarkers in serum and PJ for PC (and HGD) detection. We showed that concentrations of CA19.9 and IL-8 (not NGAL, MUC5AC, MUC2 and IFN-γ) in serum, and IL-8, NGAL, MUC5AC and MUC2 (not IFN-γ) in PJ are significantly higher in cases than in controls. CA19.9 in serum, and IL-8 and NGAL in PJ were associated with PC, independent of age, gender, BMI and presence of DM. A panel of these three markers was able to differentiate between cases and controls with a specificity and PPV of 100%, which is far higher than achieved with serum CA19.9 alone.

Imaging-based surveillance programs have not yet convincingly shown improved survival in individuals undergoing pancreatic surveillance for hereditary risk. If the decision of surgery would only be based on imaging and clinical features (as is advocated in clinical guidelines), 10 controls (7.9%) with indications of surgery would have undergone surgery, which may have been unnecessary based on that they did not develop PC during the follow-up period of median 16.5 months. Thus, we are in urgent need for tools to improve the early diagnosis of PC, in particular biomarkers which are relatively easy and cheap to determine in laboratories worldwide. Implementation of a biomarker in a surveillance program may serve two goals: 1. Selecting individuals at increased risk that are eligible for increased surveillance, in which case a lower specificity is accepted; 2. To support decision-making regarding additional diagnostic procedures and even treatment including surgery. In the latter case, the marker should have a high specificity to avoid unnecessary harm. The current golden standard (EUS-FNB) is not able to address this due to adverse events that have been related to EUS-FNB (*e.g.*, pancreatitis).

PJ is an attractive biomarker source and a good alternative for serum. Measurement of IL-8, NGAL, MUC5AC and MUC2 in PJ outperformed measurement of these markers in serum. This may be explained by the close contact of PJ with the pancreatic ductal epithelial cell lining, which contains the cells of origin of pancreatic cancer. While serum collection carries a negligible risk, PJ collection – if collected from the duodenal lumen (after secretin stimulation) during EUS (without cannulation or occlusion of the ampullary orifice) – also carries a low and acceptable procedural risk. While contamination of PJ with blood, bile and duodenal fluid may lower detectable concentrations of pancreas-derived biomarkers,[13,29,30] serum may contain biomolecules derived from other tissues as well. In our study, total protein and PLA2G1B in PJ,[10] as measures for PJ dilution, were comparable between our cases and controls.

Serum CA19.9 is currently the only biomarker used in clinical practice for PC, but only for treatment response prediction and detection of disease recurrence. It is less effective for early stage PC detection.[8,31,32] Based on the European evidence-based guideline for cystic lesions,[7] which advocates CA19.9 >37kU/L as a relative indication for surgery, 7 controls of our cohort (none of which developed PC within a 16.5 months follow-up) would have wrongfully undergone surgery. Conversely, in favor of CA19.9 (despite the very low numbers), 4 out of 5 individuals in our surveillance cohort who did undergo surgery had LGD coinciding with serum CA19.9 <37kU/L, while the one individual with HGD had an increased CA19.9 level (60kU/L).

In the current study, PJ NGAL differentiated cases from controls with a sensitivity of 30.5% and a specificity of 87.3, at a cutoff of 993 ng/mL. Kaur *et al.* (2013)[20] discriminated PC (N=58) from controls (N=47) without pancreatic disease (NPNH) with a higher sensitivity (79%; 95% CI 67-89%) and similar specificity (83%; 95% CI 61-95%) at a cutoff of 138 ng/mL. This difference may be caused by the fact that Kaur *et al.* included controls without visible abnormalities and we included a more heterogeneous control group (including individuals at increased risk for PC). Högendorf *et al.* (2016)[33] compared PC (N=21) with chronic pancreatitis (N=15) and found a sensitivity of 71% and specificity of 73% at a cutoff of 27 ng/mL. This low cutoff value may be related to alternative dilution of fluid related to their method of collection (*i.e.*, intraoperatively from the common bile duct). Budzynska *et al.* (2011)[34] compared malignant biliary structures (N=22) due to cholangiocarcinoma or PC with benign biliary strictures (N=18), and found that serum NGAL levels had no discriminative value. However, bile NGAL levels differentiated between the two groups with a sensitivity of 77% and specificity of 72% with cutoff of 459 ng/mL, and similar to our study, a combination of bile NGAL and serum CA19.9 improved the sensitivity to 91% and specificity to 67%. For IL-8 in PJ, our results show a sensitivity of 44% and specificity of 92%, at a cutoff of 15 pg/mL. Our results are comparable to those of Noh *et al.* (2006)[17], where IL-8 in (secretin-stimulated) PJ was able to differentiate between PC (N=38) and controls without abnormalities on EUS (N=41) with a sensitivity of 74% and specificity of 95% at a cutoff of 23 pg/mL.

The variety of biomarker cutoff values in medical literature is a commonly known limitation of biomarker studies and complicates quick study comparisons and meta-analyses. A selected cutoff depends on the goal of the test (high sensitivity or high specificity). For instance, for CA19.9, a cutoff of 37kU/L is generally used. In our study, this cutoff led to a sensitivity of 69.8% and specificity of 85.4%, while, for a surveillance population, a cutoff of 83 kU/L may be preferable (related to a sensitivity of 62.3% and specificity of 100% in our study). It is not feasible to have distinct cutoff values per race or indication, yet it is important to critically appraise cutoffs prior to implementation in guidelines and clinical practice.

This study involved three prospective cohorts (two surveillance cohorts and one cohort with sporadic PC cases). Ideally, to answer if early detection of PC is possible using the selected biomarkers, only PC cases detected in the surveillance cohort would have to be included. However, these cases are rare and it would take years to reach the appropriate sample size. To overcome this problem, we included patients with sporadic PC. A minority of these PC cases was detected early (18% resectable), which is in accordance with the literature on sporadic PC. Extrapolation of the current data to a surveillance population should be done with caution, as high-risk individuals with a hereditary predisposition or pancreatic cystic neoplasms may have a distinct natural disease course (with distinct molecular changes) compared to patients with sporadic PC. Our control group was heterogeneous (comprising of individuals with either a genetic or familial predisposition or suspected neoplastic cystic neoplasms), which may further complicate extrapolation to other groups. Additionally, as we did not have histological confirmation of the control group, we cannot formally exclude the possibility that some of these patients may have had undetectable lesions at time of sampling. However, the fact that none developed PC during 16.5 months of follow-up argues against this. In the current study, survival comparison between patients with a negative and positive test was not the primary objective. Potentially due to an insufficient sample size for this analysis, we did not find a statistical difference. However, the survival curve of serum CA19.9 and PJ IL-8 showed a trend and may become significant in studies with a sufficient sample size for this analysis.

In conclusion, levels of MUC5AC, IL-8 and NGAL in PJ, together with serum CA19.9, provide a biomarker panel for PC detection that is highly specific, fairly sensitive and safe to collect, and could be readily implemented in EUS-based surveillance programs. Future prospective studies are needed to validate this panel in a longitudinal surveillance cohort and investigate if combining these markers with imaging results in increased sensitivity and may allow an earlier identification of PC patients.

References

1 Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. CA: *A Cancer Journal for Clinicians* 2020;70:7-30.

2 Overbeek KA, Levink IJM, Koopmann BDM, et al. Long-term yield of pancreatic cancer surveillance in high-risk individuals. *Gut* 2022;71:1152-1160.

3 Vasen H, Ibrahim I, Ponce CG, *et al*. Benefit of Surveillance for Pancreatic Cancer in High-Risk Individuals: Outcome of Long-Term Prospective Follow-Up Studies From Three European Expert Centers. *J Clin Oncol* 2016;34:2010-9.

4 Canto MI, Almario JA, Schulick RD, *et al*. Risk of Neoplastic Progression in Individuals at High Risk for Pancreatic Cancer Undergoing Long-term Surveillance. *Gastroenterology* 2018;155:740-751.e2.

5 Konings ICAW, Canto MI, Almario JA, *et al*. Surveillance for pancreatic cancer in high-risk individuals. *BJS open* 2019;3:656-665.

6 Yachida S, Jones S, Bozic I, *et al*. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. *Nature* 2010;467:1114-7.

7 European evidence-based guidelines on pancreatic cystic neoplasms. *Gut* 2018;67:789-804.

8 Kim JR, Jang JY, Kang MJ, *et al*. Clinical implication of serum carcinoembryonic antigen and carbohydrate antigen 19-9 for the prediction of malignancy in intraductal papillary mucinous neoplasm of pancreas. *J Hepatobiliary Pancreat Sci* 2015;22:699-707.

9 Le Calvez-Kelm F, Foll M, Wozniak MB, *et al*. KRAS mutations in blood circulating cell-free DNA: a pancreatic cancer case-control. *Oncotarget* 2016;7:78827-78840.

10 Meng Q, Shi S, Liang C, *et al*. Diagnostic and prognostic value of carcinoembryonic antigen in pancreatic cancer: a systematic review and meta-analysis. *OncoTargets and therapy* 2017;10:4591-4598.

11 Zhang Y, Yang J, Li H, *et al*. Tumor markers CA19.9, CA242 and CEA in the diagnosis of pancreatic cancer: a meta-analysis. *Int J Clin Exp Med* 2015;8:11683-91.

12 Choi MH, Mejlænder-Andersen E, Manueldas S, *et al*. Mutation analysis by deep sequencing of pancreatic juice from patients with pancreatic ductal adenocarcinoma. *BMC Cancer* 2019;19:11.

13 Levink IJM, Nesteruk K, Visser DI, *et al*. Optimization of Pancreatic Juice Collection: A First Step Toward Biomarker Discovery and Early Detection of Pancreatic Cancer. *Am J Gastroenterol* 2020;115:2103-2108.

14 Kuwada Y, Sasaki T, Morinaka K, *et al*. Potential involvement of IL-8 and its receptors in the invasiveness of pancreatic cancer cells. *Int J Oncol* 2003;22:765-71.

15 Detjen KM, Farwig K, Welzel M, *et al*. Interferon gamma inhibits growth of human pancreatic carcinoma cells via caspase-1 dependent induction of apoptosis. *Gut* 2001;49:251-262.

16 Moniaux N, Chakraborty S, Yalniz M, *et al*. Early diagnosis of pancreatic cancer: neutrophil gelatinase-associated lipocalin as a marker of pancreatic intraepithelial neoplasia. *Br J Cancer* 2008;98:1540-7.

17 Noh KW, Pungpapong S, Wallace MB, *et al*. Do cytokine concentrations in pancreatic juice predict the presence of pancreatic diseases? *Clin Gastroenterol Hepatol* 2006;4:782-9.

18 Shaw VE, Lane B, Jenkinson C, *et al*. Serum cytokine biomarker panels for discriminating pancreatic cancer from benign pancreatic disease. *Mol Cancer* 2014;13:114.

19 Kaur S, Chakraborty S, Baine MJ, *et al*. Potentials of plasma NGAL and MIC-1 as biomarker(s) in the diagnosis of lethal pancreatic cancer. *PLoS One* 2013;8:e55171.

20 Kaur S, Baine MJ, Guha S, *et al*. Neutrophil gelatinase-associated lipocalin, macrophage inhibitory cytokine 1, and carbohydrate antigen 19-9 in pancreatic juice: pathobiologic implications in diagnosing benign and malignant disease of the pancreas. *Pancreas* 2013;42:494-501.

21 Klöppel G, Kosmahl M. Is the intraductal papillary mucinous neoplasia of the biliary tract a counterpart of pancreatic papillary mucinous neoplasm? *J Hepatol* 2006;44:249-50.

22 Balagué C, Audié JP, Porchet N, *et al*. In situ hybridization shows distinct patterns of mucin gene expression in normal, benign, and malignant pancreas tissues. *Gastroenterology* 1995;109:953-64.

23 Maker AV, Katabi N, Gonen M, *et al*. Pancreatic cyst fluid and serum mucin levels predict dysplasia in intraductal papillary mucinous neoplasms of the pancreas. *Ann Surg Oncol* 2011;18:199-206.

24 Kaur S, Smith LM, Patel A, *et al*. A Combination of MUC5AC and CA19.9 Improves the Diagnosis of Pancreatic Cancer: A Multicenter Study. *Am J Gastroenterol* 2017;112:172-183.

25 Zhang J, Wang Y, Zhao T, *et al*. Evaluation of serum MUC5AC in combination with CA19.9 for the diagnosis of pancreatic cancer. *World J Surg Oncol* 2020;18:31.

26 Wang Y, Gao J, Li Z, *et al*. Diagnostic value of mucins (MUC1, MUC2 and MUC5AC) expression profile in endoscopic ultrasound-guided fine-needle aspiration specimens of the pancreas. *Int J Cancer* 2007;121:2716-22.

27 Son M, Kim H, Han D, *et al*. A Clinically Applicable 24-Protein Model for Classifying Risk Subgroups in Pancreatic Ductal Adenocarcinomas using Multiple Reaction Monitoring-Mass Spectrometry. *Clinical Cancer Research* 2021;27:3370.

28 Birnbaum DJ, Begg SKS, Finetti P, *et al*. Transcriptomic Analysis of Laser Capture Microdissected Tumors Reveals Cancer- and Stromal-Specific Molecular Subtypes of Pancreatic Ductal Adenocarcinoma. *Clinical Cancer Research* 2021;27:2314.

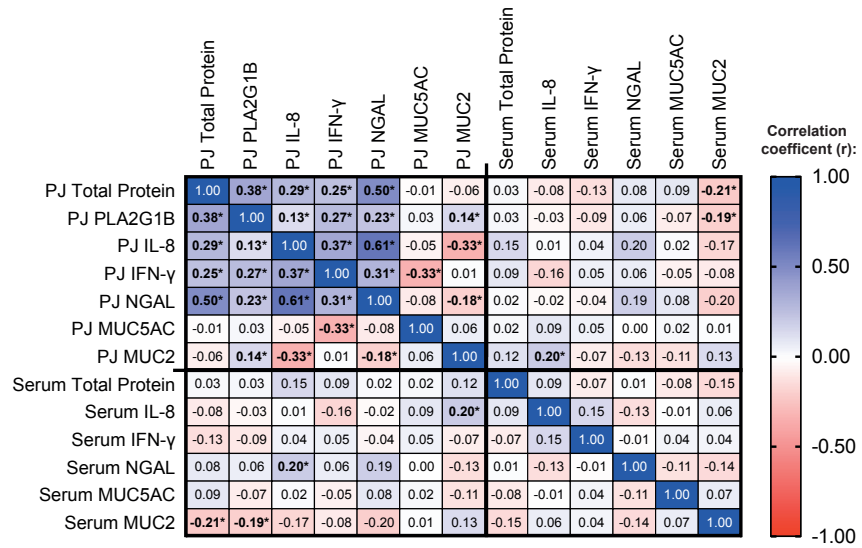
29 Doyle CJ, Yancey K, Pitt HA, *et al*. The proteome of normal pancreatic juice. *Pancreas* 2012;41:186-94.

30 Sadakari Y, Kanda M, Maitani K, *et al*. Mutant KRAS and GNAS DNA Concentrations in Secretin-Stimulated Pancreatic Fluid Collected from the Pancreatic Duct and the Duodenal Lumen. *Clinical and translational gastroenterology* 2014;5:e62-e62.

31 Ciprani D, Morales-Oyarvide V, Qadan M, *et al*. An elevated CA 19.9 is associated with invasive cancer and worse survival in IPMN. *Pancreatology* 2020;20:729-735.

32 Murakami M, Nagai Y, Tenjin A, *et al*. Proposed cutoff value of CA19.9 for detecting pancreatic cancer in patients with diabetes: a case-control study. *Endocr J* 2018;65:639-643.

33 Hogendorf P, Durczyński A, Skulimowski A, *et al*. Neutrophil Gelatinase-Associated Lipocalin (NGAL) concentration in urine is superior to CA19.9 and Ca 125 in differentiation of pancreatic mass: Preliminary report. *Cancer Biomark* 2016;16:537-43.
34 Budzynska A, Nowakowska-Dulawa E, Marek T, *et al*. Differentiation of pancreatobiliary cancer from benign biliary strictures using neutrophil gelatinase-associated lipocalin. *J Physiol Pharmacol* 2013;64:109-14.



SUPPLEMENTAL FIGURE S1 | **Correlations between concentrations of the tested markers.** Numbers indicate the correlation coefficient (Spearman r); bold numbers with asterisk (*) are significant (p<0.05).

SUPPLEMENTAL TABLE S1 | Overview of inclusion criteria of the three prospective cohort studies.

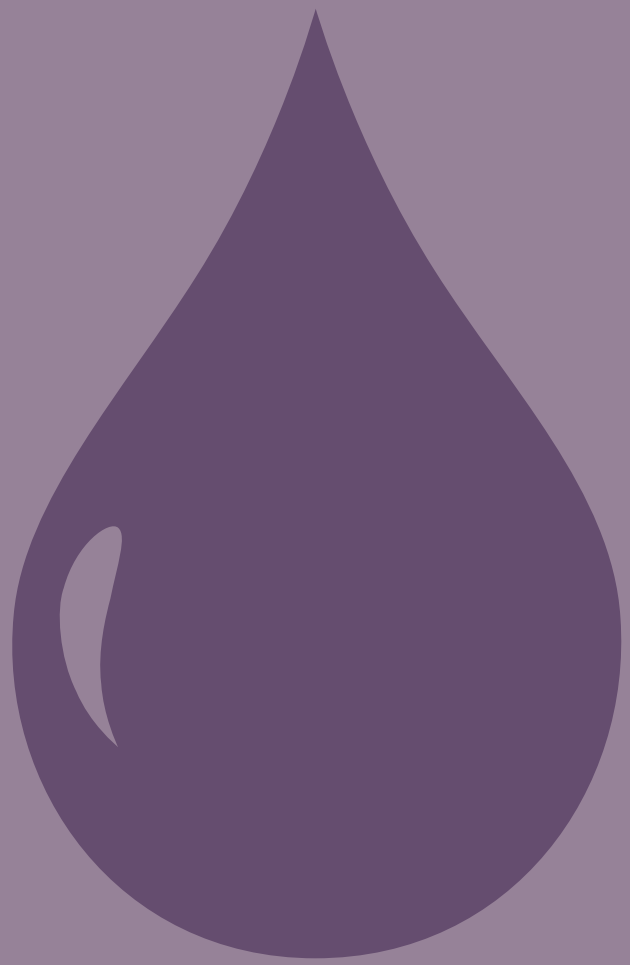
Prospective cohort	Inclusion criteria	Exclusion criteria
KRASPanc	Patients who undergo an EUS for (suspected) PC either as part of a diagnostic process or fiducial placement prior to radiotherapeutic treatment.	Age <18 years
CAPS	Individuals who, after evaluation by a clinical geneticist, have an estimated 10-fold increased risk of developing PC, this includes: (1) Carriers of a gene mutation in *CDKN2A* or *STK11*, regardless of the family history of pancreatic cancer (2) Carriers of a gene mutation in *BRCA1, BRCA2, p53*, or Mismatch Repair Gene with a family history of PC in ≥ 2 family members. (3) Familial PC (FPC) kindreds, defined as individuals with at least (1) 2 first-degree relatives (FDR) with PC, (2) 3 relatives with pancreatic cancer, either FDR or second degree relative (SDR), or (3) 2 SDR relatives with pancreatic cancer of which at ≥ 1 was <50 years at time of diagnosis.	Age <18 years, personal history of pancreatic cancer, individuals unable to provide informed consent, severe medical illness, PRSS1 gene carrier, contra-indication for EUS due to anatomic abnormalities/ surgery
PACYFIC	Individuals with a suspected neoplastic pancreatic cyst (either newly or previously diagnosed, or previously operated upon) for which cyst surveillance is warranted, according to the treating physician.	Age <18 years, history of chronic pancreatitis, suspected pseudocyst (simple, thin-walled cyst that developed in the course of acute pancreatitis, as documented by sequential imaging studies), suspected serous cystadenoma (typical microcystic lesion with lobulated outlines, a calcified central scar and/ or cyst fluid CEA levels < 5ng/ml), Von Hippel-Lindau disease, and limited life expectancy (<2 years).

SUPPLEMENTAL TABLE S2 | Multivariable analysis of markers in pancreatic juice (PJ).

PJ marker	IL-8 (pg/mL)		NGAL (µg/mL)		MUC5AC (pg/mL)		MUC2 (pg/mL)	
	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value
Marker	1.03 (1.01 – 1.05)	**0.003****	2.53 (1.38 – 4.64)	**0.003****	1.16 (1.01 – 1.32)	**0.03***	1.00 (1.00 – 1.00)	0.52
Age (years)	1.04 (1.00 – 1.08)	0.08	1.05 (1.00 – 1.09)	**0.03***	1.06 (1.02 – 1.11)	**0.007***	1.06 (1.01 – 1.10)	**0.01***
Female gender (Y/N)	0.53 (0.25 – 1.09)	0.08	0.41 (0.20 – 0.83)	**0.01***	0.44 (0.22 – 0.89)	**0.02***	0.41 (0.21 – 0.82)	**0.01***
DM (Y/N)	4.14 (1.82 – 9.42)	**<0.001****	4.37 (1.95 – 9.77)	**<0.001***	4.14 (1.86 – 9.21)	**<0.001***	3.66 (1.67 – 8.01)	**0.001****
Constant	0.05	**0.05***	0.04	**0.03***	0.17	**0.01***	0.04	**0.03***

SUPPLEMENTAL TABLE S3 | Multivariable analysis of markers in serum.

Serum marker	IL-8 (pg/mL)		CA19.9 (kU/L)	
	OR (95% CI)	p-value	OR (95% CI)	p-value
Marker	1.08 (1.00 – 1.17)	0.07	1.04 (1.02 – 1.06)	**<001****
Age (years)	1.06 (1.01 – 1.11)	**0.02***	1.03 (0.97 – 1.11)	0.33
Female gender (Y/N)	0.29 (0.12 – 0.70)	**0.006****	0.43 (0.14 – 1.29)	0.13
DM (Y/N)	3.50 (1.26– 9.68)	**0.02***	0.57 (0.17 – 1.96)	0.37
Constant	0.06	0.12	0.08	0.34



Longitudinal Changes of Serum Protein N-Glycan Levels for Earlier Detection of Pancreatic Cancer in High-Risk Individuals

Authors

Iris J.M. Levink*, Derk C.F. Klatte*, Randa G. Hanna-Sawires, Gerda C.M. Vreeker, Isaura S. Ibrahim, Yuri E.M. van der Burgt, Kasper A. Overbeek, Brechtje D.M. Koopmann, Djuna L. Cahen, Manfred Wuhrer, Bert A. Bonsing, Rob A.E.M. Tollenaar, Frank P. Vleggaar, Hans F.A. Vasen, Monique E. van Leerdam, Marco J. Bruno*, and Wilma E. Mesker*

*Authors contributed equally

Pancreatology. 2022 May;22(4):497-506.

Abstract

Background: Surveillance of individuals at risk of developing pancreatic cancer (PC) has the potential to improve survival, yet early detection based on solely imaging modalities is challenging. Alternatively, individuals with lesions progressing to malignancy can be recognized by molecular biomarkers. We aimed to identify changes in serum protein glycosylation levels over time to earlier detect PC in high-risk individuals undergoing surveillance.

Methods: Individuals with a hereditary predisposition to develop PC were followed in two surveillance programs and we included those of which at least two consecutive serum samples were available. Mass spectrometry analysis was performed to determine total *N*-glycome for each consecutive sample. Potentially discriminating N-glycans were selected based on our previous cross-sectional analysis (PC cases versus controls) and relative abundances were calculated for each glycosylation feature.

Results: 165 individuals ("FPC-cohort" N=119; Leiden cohort N=46) were included. In total, 97 (59%) individuals had a genetic predisposition (77 *CDKN2A*, 15 *BRCA1/2*, 5 *STK11*) and 68 (41%) a family history of PC without a known genetic predisposition (estimated >10-fold increased risk of developing PC). From each individual, a median number of 3 serum samples (IQR 3) was collected.
Ten individuals (6%) developed PC during 35 months of follow-up. Upon comparison of these patients with all other individuals, several glycosylation characteristics were increased, namely fucosylation, tri- and tetra-antennary structures, and specific sialic linkage types. Other glycosylation characteristics decreased, such as complex-type diantennary and bisected glycans. The largest change over time was observed for tri-antennary fucosylated glycans, which were able to differentiate cases from controls with a specificity of 92%, sensitivity of 49% and accuracy of 90%.

Conclusion: This study explores the applicability of serum *N*-glycan longitudinal monitoring for early detection in a pancreas surveillance program.

.

Introduction

The majority of patients with pancreatic cancer (PC) are diagnosed at an incurable stage. Due to the lack of early recognizable symptoms, only 20% of patients are eligible for surgery at presentation.[1] Surveillance of patients at risk of developing PC may offer opportunities for early detection and improved survival.[2]

So far, diagnosis of PC has been challenging in surveillance programs for individuals with an hereditary risk of developing PC that combine magnetic resonance imaging (MRI)/ magnetic resonance cholangiopancreatography (MRCP) and endoscopic ultrasound (EUS).[3,4] As a result, resection of abnormalities, which are – upon histological assessment – proven benign, has caused unnecessary harm due to overtreatment.[5] Novel molecular biomarkers are urgently needed to increase our diagnostic capabilities, resulting in more appropriate risk stratification, earlier recognition of malignant progression and personalized clinical management (*i.e.*, intensified/reduced follow-up or surgery). Currently, for diagnosis of PC, fine-needle aspiration (FNA) or biopsy (FNB) is used. Although their diagnostic accuracy is relatively high,[6] they rely on the ability to visualize the lesion and accurate sampling of smaller lesions (< 10 mm) is challenging.[7] A biomarker that shows relative changes over time is specifically beneficial in high-risk individuals undergoing surveillance.

For PC, carbohydrate antigen 19.9 (CA 19.9) is a well-known serum biomarker. Structurally, CA19.9 is sialylated Lewis antigen A, and as such, a well-known example of protein glycosylation. Glycosylation is a post-translational modification of a protein that alters its functional properties. However, its use in clinical practice has been limited thus far to prediction of treatment response and detection of disease recurrence.[8] As a result of its imperfect diagnostic performance,[9] implementation of CA19.9 in a surveillance program should be done with caution, as it may increase harm (due to unnecessary imaging and surgery), patient anxiety and health care costs.

As mass spectrometry (MS)-based protein glycosylation studies have demonstrated that specific glycosylation levels in total serum *N*-glycome profiles have potential in PC detection,[8,10-13] our group set up a pipeline for the development of a glycan panel for surveillance purposes (**Figure 1**). As a first step, we performed a cross-sectional analysis, comparing sporadic PC cases with healthy controls and identified 51 glycosylation traits (combinations of glycan structures according to biosynthetic pathways) that were differentially expressed between these groups. Of these, a preliminary panel of three glycosylation traits (CA2, A3F0L, CFa) was able to differentiate sporadic PC cases with an AUC of 0.81-0.88 (cross-sectionally).[14]

Our next step is validation of glycosylation traits in a longitudinal study to identify markers that are able to earlier detect asymptomatic PC in high-risk individuals undergoing surveillance. For this purpose, we performed *N*-glycome analysis on consecutive serum

samples from high-risk individuals undergoing surveillance, and compared PC cases with controls.

Material and methods

Study design and pancreatic surveillance programs

From our previously identified 51 promising *N*-glycan markers,[14] we evaluated the 13 (25%) best-performing glycosylation markers in serum that were consecutively collected in the course of two Dutch pancreatic surveillance programs (**Figure 1**). Selected participants had at least two blood samples collected at different time points between 2007 and 2018.

The first cohort (FPC-cohort) concerns a collaboration between the University Medical Center Utrecht and the Erasmus MC University Medical Center Rotterdam (EMC), a Dutch branch of the international CAncer of the Pancreas Surveillance (CAPS) consortium.[4] This study was set up in 2007 and includes individuals with an estimated 10% or greater lifetime risk of PC. It encompasses germline mutation carriers of a known PC susceptibility gene (*e.g., CDKN2A, LKB1/STK11, BRCA1/2*), as well as familial pancreatic cancer (FPC) kindreds without a known gene mutation. The latter group was included after genetic testing and detailed evaluation of family history by a clinical geneticist. Individuals in this group have ≥ 2 blood relatives (who are first-degree relatives [FDR] to each other or FDR and/or second-degree relatives [SDR] with ≥ 1 under 50 years of age) or ≥ 3 blood relatives (who are FDR or SDR to each other) with PC. At inclusion, all individuals were 50 to 75 years of age or 10 years younger than the youngest age at which a blood relative developed PC. Individuals included in the FPC-cohort undergo surveillance with both EUS and MRI/MRCP and glucose testing.[4] Additionally, serum samples were stored in the biobank at each follow-up.

The second surveillance cohort is followed by the Leiden University Medical Center (LUMC) from the year 2000 onwards. The vast majority of this population comprises carriers with a proven germline *CDKN2A (p16-Leiden)* mutation, who are at an estimated 15-20% lifetime risk of PC.[15] In addition to *CDKN2A*, 1 high-risk individual was included with 3 family members with PC (1 FDR and 2 SDR). Starting at the age of 45 years, participants were offered annual MRI/MRCP.[2] Since 2012, blood samples were collected at regular screening intervals.

The institutional ethical review boards of participating centers (2007_024, Amsterdam University Medical Center; MEC-2021-448 EMC; MEC P00.107 LUMC) have approved the study, and the included individuals gave written informed consent before enrolment. The study was carried out according to the ethical principles for medical research involving human subjects from the World Medical Association Declaration of Helsinki and Taipei.

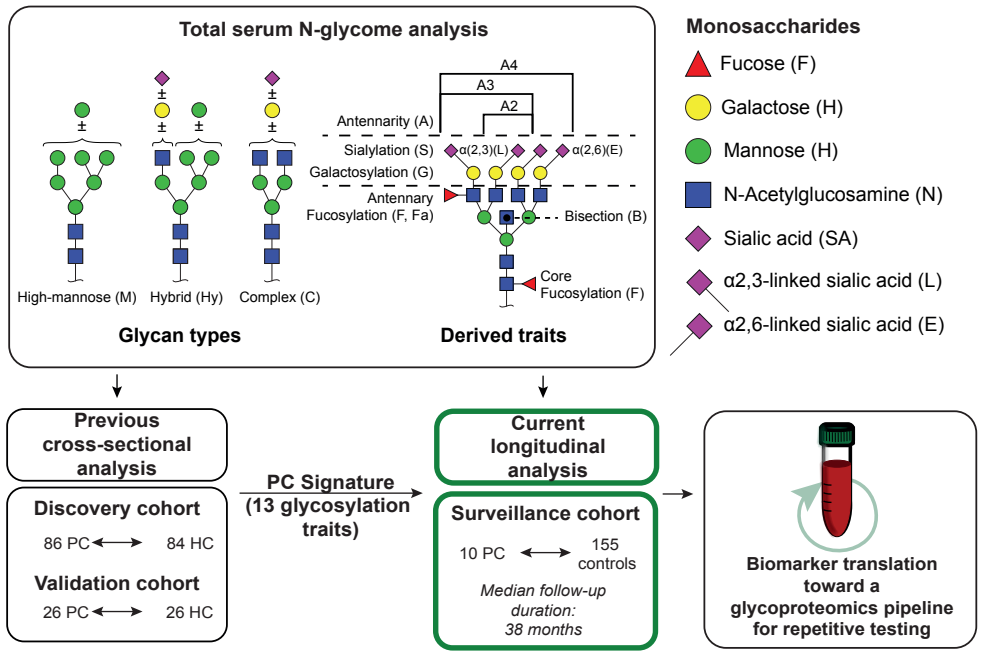


FIGURE 1 | Pipeline showing the development of a glycan-based biomarker panel for surveillance purposes. First, MS-based total *N*-glycome analysis provides relative abundances of single N-glycans from all serum proteins. Three commonly occurring *N*-glycan structures are distinguished, namely high-mannose, hybrid and complex-type. From this data, glycosylation traits are calculated that reflect biosynthetic pathways. The abundance of these glycosylation traits was previously described in a cross-sectional cohort.[1] Blue boxes indicate the current longitudinal analysis. In order to implement glycosylation-based biomarkers in clinical practice (for repetitive monitoring in a surveillance cohort), one or multiple protein-glycosylation trait combinations (glycoproteoform) need to be selected. PC = pancreatic cancer; HC = healthy control.

Serum sample collection and plate design

Serum samples were collected consecutively and processed according to a standardized protocol.[16] Within 4 hours after venipuncture, each blood sample was centrifuged for 10 minutes at 1000xg and collected serum was stored in 1.5 mL aliquots at -80 °C until further analysis. Before measurements were performed (*i.e.*, serum *N*-glycome analysis), each sample was further aliquoted into 60 µL tubes, with one aliquot of each sample relocated into a 96-well plate format. For technical quality control of the spectra, each plate contained a minimum of six in-house standards and two blanks.

Serum sample preparation and mass spectrometry analysis of glycans

Six microliter of serum was subjected to analysis according to a previously reported protocol.[16] The procedure consisted of various steps that were carried out in a standardized manner on a Hamilton liquid handling platform, except for the first step of global release of N-glycans using the enzyme PNGase F (Roche Diagnostics, Mannheim, Germany). The procedure consisted of various steps that were carried out in a standardized manner on a Hamilton liquid handling platform. The first step (global release of N-glycans) was an exception, in which we used the enzyme PNGase F (Roche Diagnostics, Mannheim, Germany). All sialic acid residues were chemically derivatized into stable end-products using an in-house developed ethylesterification protocol.[16] Thus, introduced mass differences allow differentiation between α2,3- and α2,6-linked species. Next, the glycans were purified using cotton-based hydrophilic interaction liquid chromatography (HILIC) micro-tips and eluted and premixed with sDHB Matrix-assisted laser desorption/ionization (MALDI) matrix (5 mg/mL in 99% ACN with 1 mM NaOH). The glycans were spotted onto a MALDI target plate (800/384 MTP AnchorChip, Bruker Daltonics, Bremen, Germany) and measured on a Bruker 15T solariX XR Fourier transform ion cyclotron resonance (FTICR) mass spectrometer. The system was controlled by ftmsControl version 2.1.0 and spectra in an m/z-range from 1011.86 to 5000.00 were recorded with 1 M data points (*i.e.*, transient length of 2.307 s). DataAnalysis Software 4.2 (Bruker Daltonics, Bremen, Germany) was used for the visualization and data analysis of all MALDI-FTICR spectra. The relative abundances of different *N*-glycans were determined from MS-data using in-house developed MassyTools (version 0.1.8.1) software,[16] and subsequently combined in glycosylation traits on the basis of common structural features (**Figure 1**; **Supplemental Table S1**).

Data processing and analysis

Clinical characteristics were described as medians with interquartile range (IQR) or percentages. Statistical significance was assessed with Mann-Whitney U test for continuous data and Fisher's exact or χ^2 test for categorical variables.

Candidate glycosylation traits were selected based on published data from our group comparing protein glycosylation in sporadic PC cases and healthy controls.[14] The mean abundance values (relative to total abundance per sample) of selected glycosylation traits were plotted over time per investigated group (cases vs controls) with 95% confidence interval (CI). The median glycosylation levels per investigated group at both baseline (t_0) and last follow-up (before PC development) were assessed and a ratio was calculated by dividing median levels of cases by that of controls at both timepoints. Subsequently, change of glycosylation over time was quantified by calculating the difference between the most recent (before PC development for cases) and first measurement normalized by time between samples (in months). Subsequently, this metric was compared between cases and controls (Mann-Whitney U) for the 13 glycosylation traits (p-values not shown).

P-values < 0.004 were considered significant (Bonferroni correction for multiple testing). To calculate the diagnostic performance of difference over time, receiver operating characteristic (ROC) curves were generated (and area under the curves (AUCs) calculated) for those glycosylation traits that showed a different change over time (Mann-Whitney U; p<0.05). To estimate sensitivity and specificity, two locations on the curve were selected aiming: 1) high sensitivity (minimum specificity ≥ 40%); and 2) high specificity (minimum sensitivity ≥ 40%). CIs for sensitivity, specificity and accuracy are based on the cumulative probabilities of the binomial distribution ("exact" Clopper-Pearson CIs).

Additionally, as changes in glycosylation levels may also reflect the presence of other malignancies, we visualized and compared glycosylation traits over time of cases developing PC, controls with another malignancy, and controls who underwent pancreatic surgery for benign disease.

Data was analyzed with Statistical Package for the Social Sciences (SPSS Inc, Chicago, IL). Data were visualized using R (version 4.0.2; package "Tidyverse") and GraphPad (GraphPad Prism version 9, La Jolla, CA).

Results

Comparison of cohorts

In total, 165 patients met the inclusion criteria, 119 from the FPC-cohort and 46 from the Leiden cohort. Patient characteristics did not differ regarding age (FPC-cohort median 52 years [IQR 14]; Leiden cohort 53 [IQR 14]; p=0.84), gender (FPC-cohort 39% male, Leiden cohort 44%; p=0.60) and BMI (25 kg/m^2 [IQR 5.7]; 26 kg/m2 [IQR 4.8]; p=0.35; **Table 1**). Due to differences in inclusion criteria, the Leiden cohort predominantly consisted of *CDKN2A* mutation carriers (97.8%), while the FPC-cohort was more heterogeneous, containing both mutations carriers (*CDKN2A* 26.9%, *BRCA2* 11.8%, *BRCA1* 0.8%, *STK11* 4.2%) and mutation-negative FPC kindreds (56.3%; **Table 1**). 52.1% of the members of the FPC-cohort had 3 or more family members with PC, this number was lower in the Leiden cohort (26.1%; p=0.001).

At baseline, the majority of patients in both cohorts had no abnormalities on imaging (FPC-cohort n=70 [58.8%]; Leiden cohort n=39 [84.8%]). 7 participants (5.9%) of the FPC-cohort had an indeterminate lesion, for which the follow-up interval was shortened to 3 months, but none of these lesions developed to PC. Participants were followed for a median of 35 months (IQR 61) in the FPC-cohort and 42 months (IQR 37; p=0.23) in the Leiden cohort. During surveillance, more samples were collected in the FPC-cohort (median 4 [IQR 5]) than in the Leiden cohort (median 2 [IQR 0]; **Table 1**)

TABLE 1 | Clinical characteristics.

	CAPS cohort n=119	Leiden cohort n=46	p-value
Baseline information			
Age in years, median (IQR)	52 (14)	53 (14)	0.84
Male sex, n (%)	46 (38.7)	20 (43.5)	0.60
BMI in kg/m2, median (IQR)	25.3 (5.7)	26.2 (4.8)	0.35
Gene mutation, n (%)	52 (34.7)	45 (97.8)	<0.0001
CDKN2A p16	32 (26.9)	45 (97.8)	
BRCA2 + ≥ 2 blood relatives with PC	14 (11.8)	0 (0.0)	
BRCA1 + ≥ 2 blood relatives with PC	1 (0.8)	0 (0.0)	
STK11	5 (4.2)	0 (0.0)	
Total number of any degree blood relatives with PC, n (%)			0.001
0	13 (10.9)	12 (26.1)	
1	9 (7.6)	12 (26.1)	
2	35 (29.4)	10 (21.7)	
3 or more	62 (52.1)	12 (26.1)	
Diabetes mellitus, n (%)	3 (2.5)	0 (0.0)	NA
Working diagnosis at baseline, n (%)			<0.0001
No abnormalities	70 (58.8)	39 (84.8)	
Unspecified cyst	18 (15.1)	2 (4.3)	
SB-IPMN	20 (16.8)	4 (8.7)	
MD-IPMN	1 (0.8)	0 (0.0)	
pNET	1 (0.8)	0 (0.0)	
Duodenum NET	1 (0.8)	0 (0.0)	
Chronic pancreatitis	1 (0.8)	0 (0.0)	
Indeterminate, not suspect for malignancy	7 (5.9)	0 (0.0)	
Suspicion of malignancy	0 (0.0)	1 (2.1)	
Follow-up information			
Number of follow-up visits per individual, median (IQR)	4 (5)	2 (0.3)	<0.0001
Follow-up duration in months, median (IQR)	35 (61)	42 (37)	0.23
Individuals who developed PC, n (%)	3 (2.5)	7 (15.2)	0.005

IQR = interquartile range, IPMN = Intraductal papillary mucinous neoplasm, SB-IPMN = side-branch IPMN, MD-IPMN = main-duct IPMN, (p)NET = (pancreatic) neuroendocrine tumor, PC = pancreatic cancer.

TABLE 2 | Details of individuals who underwent surgery and developed other (non-pancreatic) malignancies.

Patient	Cohort	Pathological outcome	TNM stage AJCC 8th edition	Gender / Age at diagnosis, years	Risk category	Nr. of samples analyzed before resection / total	Time between last blood sample and diagnosis (months)	Time between baseline and diagnosis/surgery (months)
PC cases								
P1	FPC	PC + Cervixca	T1aN0M0	F / 54	*STK11*	3 / 4b	6	25 / 29
P2	Leiden	PC	T1aN0M0	M / 50	*CDKN2A*	2 / 2	16	27 / 31
P3	Leiden	PC	T1aN0M0	F / 62	*CDKN2A*	2 / 2	1	36 / 39
P4	Leiden	PC	T1bN0M0	M / 64	*CDKN2A*	2 / 2	0	3 / 6
P5	FPC	PC	T1cN1M0	M / 50	*CDKN2A*	4 / 4	2	24 / 25
P6	FPC	PC	T2N1M0	M / 55	*CDKN2A*	4 / 4	12	41 / 44
P7	Leiden	PC	T2N2M0	F / 69	*CDKN2A*	2 / 2	1	41 / 42
P8	Leiden	PC	T3N0M0	M / 66	*CDKN2A*	2 / 2	1	8 / 8
P9	Leiden	PC	T3N0M0	M / 70	*CDKN2A*	2 / 2	1	51 / 54
P10	Leiden	PC	T3N1M0	F / 67	*CDKN2A*	2 / 2	0	23 / 24
Benign surgery controls								
B1	FPC	PanIN2 + pNET <2 cm	T1N0M0	F / 49	*CDKN2A*	5 / 10	2	49 / 50
B2	FPC	PanIN2	NA	M / 47	FPC	4 / 11	4	0 / 17
B3	FPC	PanIN1	NA	M / 46	FPC	2 / 11	2	0 / 5
B4	FPC	MD-PMN, IGD	NA	F / 47	*BRCA2*	3 / 6	18	0 / 28
B5	FPC	MT-IPMN, LGD	NA	F / 64	FPC	2 / 4	3	0 / 5
B6	Leiden	No precursor	NA	M / 58	*CDKN2A*	2 / 2	14	23 / 26
B7	Leiden	No precursor	NA	M / 55	*CDKN2A*	2 / 2	0	23 / 26
B8	FPC	No precursor	NA	M / 50	*BRCA2*	2 / 3	2	14 / 16
Other malignancy controls								
M1	FPC	Melanoma	T1aN0M0	F / 50	*CDKN2A*	1 / 11	6	6 / 6
M2	Leiden	Melanoma	T1aN0M0	F / 47	*CDKN2A*	1 / 3	11	11 / 11
M3	Leiden	Melanoma	T1bN0M0	F / 58	*CDKN2A*	2 / 2	3	55 / 55
M4	FPC	Breast cancer	T1N0M0	F / 54	FPC	5 / 9	10	58 / 59
M5	FPC	Breast cancer	TxN0M0	F / 59	FPC	7 / 7	0	83 / 87
M6	FPC	Breast cancer	TxNx	F / 70	FPC	2 / 4	0	12 / NA
M7	FPC	Esophageal cancer	TxN0M0	F / 57	*CDKN2A*	2 / 2	6	18 / 22
M8	FPC	Lymphoma	NA	F / 50	FPC	2 / 3	2	27 / NA
M9	FPC	Duodenal NET <2 cm	T2N0M0	M / 51	CDKN2A	1 / 2	9	0 / 9

PC = pancreatic cancer, MD-IPMN = main-duct intraductal papillary mucinous neoplasm, MT-IPMN = mixed-type intraductal papillary mucinous neoplasm, IGD = intermediate grade dysplasia, LGD = low-grade dysplasia, (p)NET = (pancreatic) neuroendocrine tumor, FPC = mutation-negative familial pancreatic cancer kindred.

Surveillance outcomes

During follow-up, 10 participants (6.1%) developed PC. Nine cases were *CDKN2A* mutation carriers and one was a *STK11* gene mutation carrier (**Table 2**). At baseline, only 1 case (P4 from the Leiden cohort) had a feature suspected for malignancy. All 10 cases underwent surgery within 4 months after diagnosis. Four individuals had stage 1 disease (AJCC 8th edition), while the remainder had stage 2 or higher. One of the cases with stage 1 (P1) had a concomitant primary cancer in the cervix (T2bN1Mx). Two cases were interval cancers, detected because of jaundice. The others were detected at time of surveillance. For the majority of cases (8/10), the last serum collection had been performed less than six months before diagnosis (**Table 2**).

Of 155 controls, eight underwent surgery for falsely suspected PC ('Benign surgery controls'; n=8; 5.2%; **Table 2**). Of these, five individuals had precursor lesions (Pancreatic intraepithelial neoplasia [PanIN] or intraductal papillary mucinous neoplasm [IPMN]) or a pancreatic neuro-endocrine tumor (NET). Benign surgery controls were analyzed as a separate group, as changed glycosylation traits may already be visible in precursor lesions. For 6/8 benign surgery controls, the last collection was performed less than six months before diagnosis. During the study period, 9 controls developed a malignancy other than PC (3 melanoma; 3 breast cancer; 1 esophageal cancer; 1 lymphoma; 1 duodenal NET; **Table 2**).

Comparison of glycosylation traits between cases and controls

Selection of candidate glycosylation traits was based on published data from our group comparing protein glycosylation in sporadic PC cases and healthy controls.[14] In the current study, we evaluated [13] (1st quartile with lowest p-value) of the 51 glycosylation traits that differed between PC cases and controls (≤0.05). Notably, all selected glycosylation traits consisted of 'complex-type' glycans (**Figure 1**).

To evaluate the natural course of these glycosylation traits per investigated group, their mean relative abundance was plotted over time for cases (n=10) and controls (N=155; **Figure 2**). In the case group, values of three glycosylation traits decreased over time (CA2, CB0, A3F0L; **Figure 2A, C, I; Supplemental Figure S1A, C; Supplemental Figure S2C**), whereas ten increased (CA4, CFa, A3F, A3Fa, A2LF, A3LF, A3FE, A4FE, A2F0E, A3F0E; **Figure 2B, D-H, J-M; Supplemental Figure S1B, D-F; Supplemental Figure S2A, B, D-G; Table 3**). These decreases and increases were in agreement with down- and upregulation, respectively, as previously objectified in cross-sectional analysis (sporadic PC vs healthy controls; **Table 3**).[14] The majority of PC cases already showed differences in glycosylation traits 3 to 50 months prior to PC development (t_0; **Table 3**) as compared to controls. The glycosylation levels of two glycosylation traits were higher at the last measurement before PC diagnosis (p≤0.004; A2LF, A3Fa; data not shown). With regard to change over time, the difference between first and last measurement was calculated and normalized

by the time in months. After correction for multiple testing, one glycosylation trait (A3F) showed a different change over time for cases, as compared to controls (p≤0.004; data not shown).

As the majority of the cases harbored a *CDKN2A* germline mutation (9/10), we also visualized the course of glycosylation traits for all cases and controls with a *CDKN2A* germline mutation (**Supplemental Figure S3**). This cohort showed a similar course as that of the total cohort.

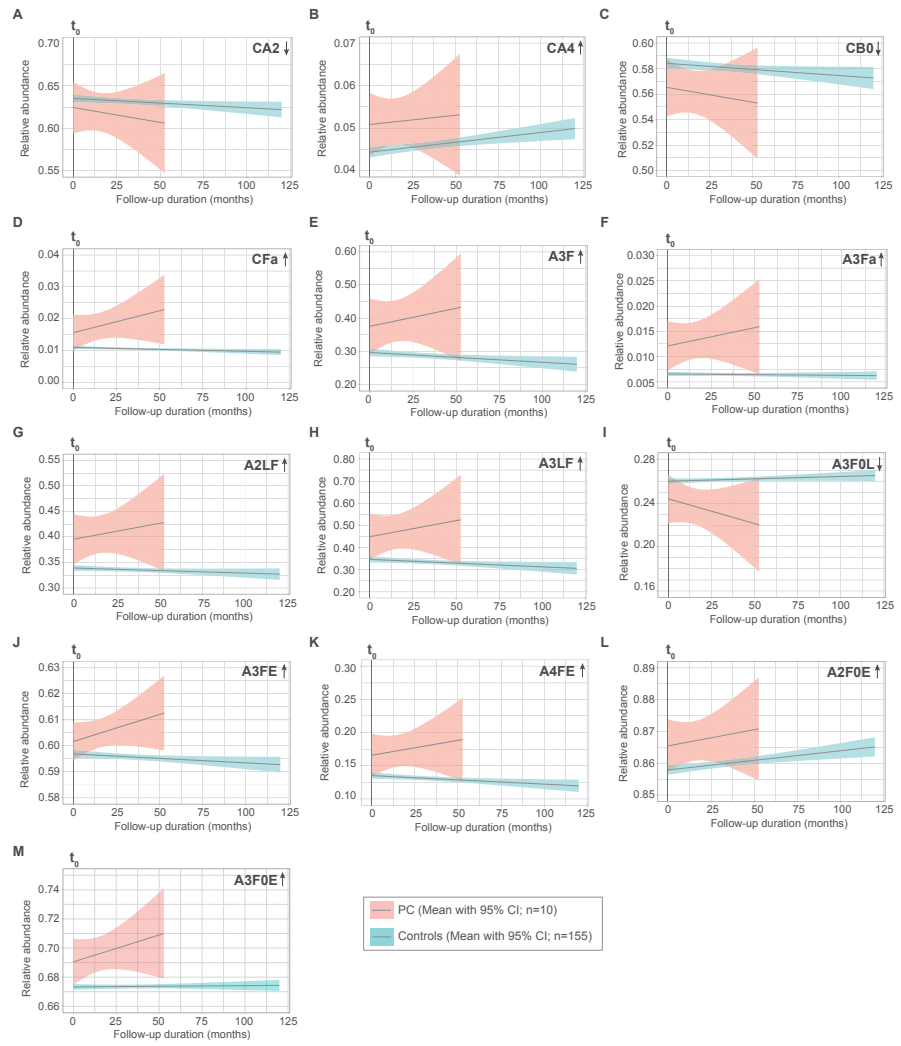


FIGURE 2 | Graphs showing the relative abundance over time for 13 candidate markers. The black lines indicate the mean per group (cases = pink, controls = blue) with 95% confidence interval (CI). Arrows indicate whether the glycosylation trait was up- or downregulated in PC cases in previous cross-sectional analysis[14]).

TABLE 3 | Overview of odds-ratio (OR) at previous cross-sectional analysis[14] as well as the median glycosylation trait level for cases and controls at baseline and at most recent sample. Purple rows indicate a lower value when developing PC in cross sectional analysis.[14] Arrows in the glycosylation in last sample column indicate whether the glycosylation value increased or decreased compared to baseline.

	Cross-sectional analysis[14]		Glycosylation at baseline			Glycosylation in last sample		
	OR[14] (95% CI)	**P-value[14]**	**Cases** Median (IQR)	**Controls** Median (IQR)	**Ratio** Cases/ con-trols	**Cases** Median (IQR)	**Controls** Median (IQR)	**Ratio** Cases/ con-trols
Complex type glycans								
CA2	0.35 (0.25-0.50)	1.052E-08	0.632 (0.094)	0.633 (0.056)	0.998	0.630 (0.050) ↓	0.631 (0.057) ↓	0.998
CA4	6.19 (3.57-10.75)	9.209E-11	0.052 (0.031)	0.043 (0.013)	1.209	0.049 (0.015) ↓	0.045 (0.015) ↑	1.089
CB0	0.39 (0.27-0.54)	5.12E-08	0.565 (0.062)	0.579 (0.057)	0.976	0.571 (0.038) ↑	0.582 (0.055) ↑	0.981
CFa	13.27 (5.68-30.98)	2.306E-09	0.013 (0.008)	0.011 (0.005)	1.182	0.019 (0.019) ↑	0.011 (0.006) =	1.727
Fucosylation (F)								
A3F	2.34 (1.70-3.23)	2.066E-07	0.363 (0.208)	0.282 (0.158)	1.287	0.460 (0.327) ↑	0.281 (0.154) ↓	1.637
A3Fa	5.35 (3.01-9.52)	1.115E-08	0.011 (0.007)	0.006 (0.005)	1.833	0.015 (0.021) ↑	0.006 (0.005) =	2.500
A2LF	2.67 (1.88-3.78)	3.854E-08	0.389 (0.140)	0.337 (0.088)	1.154	0.457 (0.203) ↑	0.332 (0.083) ↓	1.377
A3LF	2.68 (1.91-3.75)	9.323E-09	0.425 (0.265)	0.324 (0.199)	1.312	0.565 (0.421) ↑	0.333 (0.191) ↑	1.697
A2,3-linked sialylation (L)								
A3F0L	0.34 (0.23-0.49)	8.342E-09	0.246 (0.041)	0.260 (0.035)	0.946	0.221 (0.098) ↓	0.260 (0.032) =	0.850
A2,6-linked sialylation (E)								
A3FE	2.44 (1.73-3.43)	3.184E-07	0.601 (0.015)	0.595 (0.019)	1.010	0.610 (0.024) ↑	0.598 (0.018) ↑	1.020
A4FE	3.5 (2.32-5.30)	2.622E-09	0.157 (0.075)	0.133 (0.073)	1.180	0.201 (0.126) ↑	0.132 (0.070) ↓	1.523
A2F0E	3.99 (2.55-6.24)	1.203E-09	0.869 (0.024)	0.858 (0.021)	1.013	0.872 (0.032) ↑	0.859 (0.021) ↑	1.015
A3F0E	2.63 (1.88-3.69)	2.019E-08	0.686 (0.018)	0.672 (0.027)	1.021	0.707 (0.065) ↑	0.674 (0.027) ↑	1.051

OR = odds ratio; 95% CI = 95% confidence interval; IQR = interquartile range.

Resected controls and other malignancies

To evaluate if changes in glycosylation levels (over time) were present in precursor lesions and other malignancies, the mean relative abundance was plotted over time for three subgroups (**Figure 3**): 1. cases (n=10); 2. pathology-proven benign surgery controls (n=8); 3. other malignancy controls (n=9). Due to the limited sample size, we did not perform a statistical analysis, yet graphs indicate a difference between PC cases and benign surgery controls, as well as the other malignancy controls for six glycosylation traits (CFa, A3F, A3Fa, A2LF, A3LF, A4FE; **Figure 3D-H, K**).

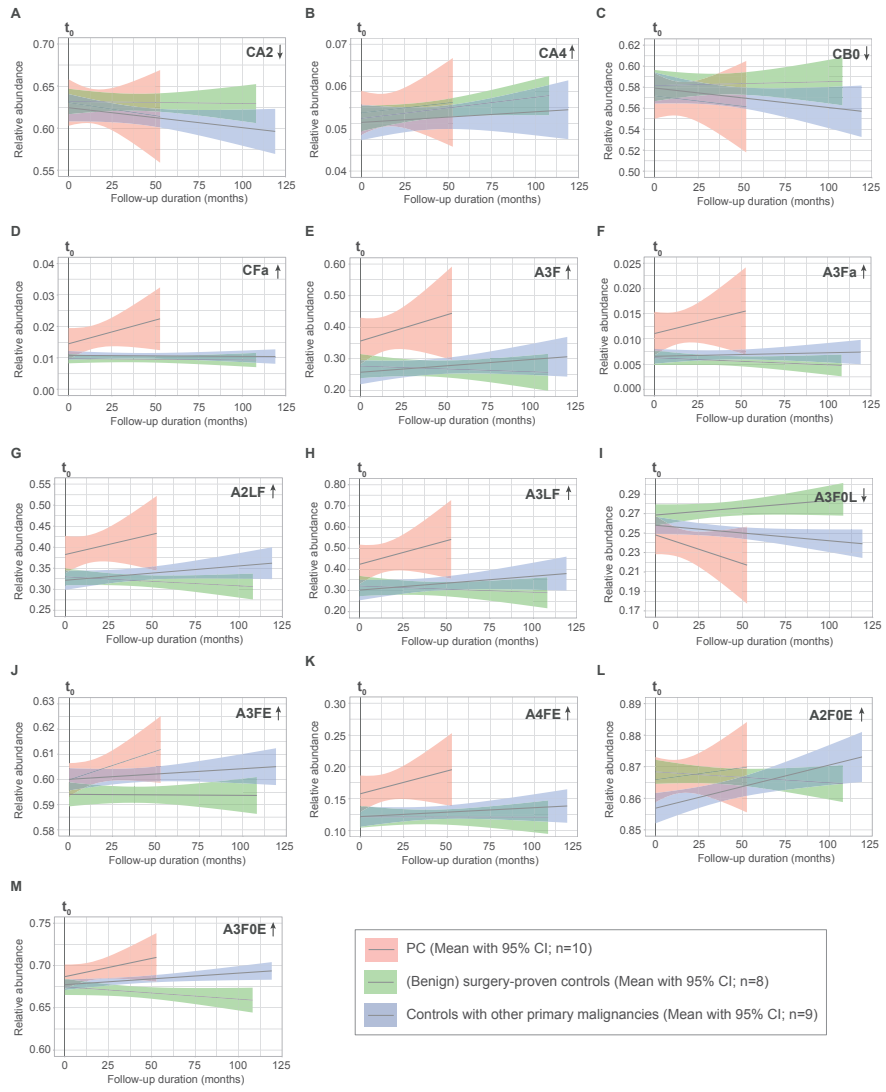
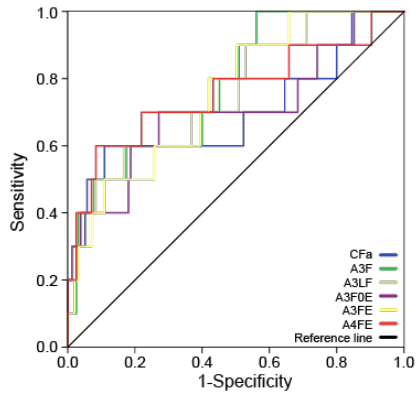


FIGURE 3 | Graphs showing the relative abundance over time for 13 glycosylation traits, comparing three groups: 1. Patients with PC (pink); 2. Patients who underwent pancreatic resection, which appeared to be no/low-grade dysplasia ('benign surgery controls'; green); 3. Patients who develop other types of malignancies during the course of surveillance (blue). The black lines indicate the mean per group, the colored area indicates the 95% confidence interval (CI) per group. Arrows indicate whether the glycosylation trait was up- or downregulated in previous cross-sectional analysis[14]).

Diagnostic performance of change over time

For glycosylation traits that showed a significant difference between cases and controls over time, ROC curves were generated (**Figure 4A**). Overall, A3F performed best (AUC 0.77; 95% CI 0.62-0.92). Per glycosylation trait, two cutoffs were selected, the first aiming at high sensitivity (with specificity ≥ 40%), the second aiming at high specificity (sensitivity ≥ 40%; **Figure 4B**). When aiming high sensitivity, A3F had a sensitivity 90.0% (95% CI 55.5-99.8%) and specificity of 49.0 (95% CI 42.8-59.1%); when aiming high specificity, the sensitivity was 40.0 % (95% CI 12.2-73.8%) and specificity 96.1 (95% CI 91.8-96.6%).

A



B

Derived trait	AUC (95% CI)	Cut-off	Sensitivity, % (95% CI)	Specificity, % (95% CI)	Accuracy, % (95% CI)
CFa	0.70 (0.48-0.91)	0.000003	70.0 (34.8-93.3)	47.7 (39.7-55.9)	49.1 (41.2-57.0)
		0.000141	50.0 (18.7-81.3)	94.2 (89.3-97.3)	91.5 (86.2-95.3)
A3F	0.77 (0.64-0.91)	-0.000108	90.0 (55.5-99.8)	49.0 (44.0-60.8)	54.7 (46.5-62.7)
		0.001986	50.0 (18.7-86.8)	92.3 (86.9-95.9)	89.7 (84.0-93.9)
A3LF	0.76 (0.60-0.92)	-0.000082	90.0 (55.5-99.8)	47.1 (39.0-55.3)	49.7 (41.8-57.6)
		0.002235	50.0 (18.7-81.3)	90.3 (84.5-94.5)	87.9 (81.9-92.4)
A3F0E	0.70 (0.50-0.90)	0.000193	70.0 (34.8-93.3)	72.3 (64.5-79.1)	72.1 (64.6-78.8)
		0.000807	40.0 (12.2-73.8)	96.1 (91.8-98.6)	92.7 (87.6-96.2)
A3FE	0.75 (0.61-0.90)	-0.000006	90.0 (55.5-99.8)	49.7 (41.6-57.8)	52.1 (44.2-50.0)
		0.000341	40.0 (12.2-73.8)	92.9 (87.7-96.4)	89.7 (84.0-93.9)
A4FE	0.76 (0.57-0.95)	0.000039	80.0 (44.4-97.5)	56.8 (48.6-64.7)	58.2 (50.3-65.8)
		0.000594	60.0 (26.2-87.8)	91.6 (86.1-95.5)	89.7 (84.0-93.9)

FIGURE 4 | Diagnostic performance of change over time (between baseline and most recent measurement) for the differentiation between cases and controls. Only those glycosylation traits that were significantly different change (cases vs controls; p<0.05) were shown. A. ROC curves. B. Table showing the sensitivity, specificity and accuracy of individual glycosylation traits. Two cutoffs were selected aiming at: 1. high sensitivity (specificity ≥ 40%); 2. high specificity (sensitivity ≥ 40%). AUC = area under the curve; CI = confidence interval.

Discussion

After our explorative study among symptomatic PC patients, we now evaluated the longitudinal performance of glycosylation traits in a PC surveillance cohort consisting of hereditarily predisposed high-risk individuals. Our results demonstrate that a change of distinct serum glycosylation traits is related to the development of PC. Of the 13 investigated glycosylation traits, A3F showed significant deviations during the course of PC development (after correction for multiple testing). Interestingly, biomarker differences seem to have been present at baseline and increasingly changed during the course of PC development. This may indicate a long window of opportunity to capture malignant progression. This finding is consistent with previous data showing that development of PC takes multiple years[17] and potentially makes these biomarkers useful for surveillance purposes.

Imaging-based surveillance programs have not yet convincingly shown improved survival in individuals undergoing pancreas surveillance for hereditary risk.[18] Diagnosis of PC in surveillance programs for high-risk individuals rely on imaging and FNA/FNB (in case of suspected PC). However, both modalities have a limited discriminative performance for small lesions and the use of FNA/FNB is limited due to potential adverse events in case of repetitive sampling.[19,20] Due to high rates of false positive findings (and therefore potential harm), repetitive CA19.9 monitoring in individuals at risk of developing PC is not recommended by guidelines.[3] Thus, there is an urgent need for more sensitive and specific biomarkers to complement imaging in surveillance programs.[21]

Implementation of a (panel of) biomarkers in a surveillance program could guide risk stratification and select individuals who should undergo intensified surveillance (in case of a test with high sensitivity), yet could also support in decision-making for surgery or additional diagnostic procedures (in case of a test with high specificity). For this reason, we selected two cutoffs for each glycosylation trait aiming at high sensitivity or high specificity. The performance of changes of glycosylation traits over time was promising (accuracy 87.9-92.7%).

For surveillance purposes, a potent biomarker is expected to vary over time, yet, in current literature, data on longitudinal sampling of biomarkers is lacking. This well-defined surveillance cohort offers the unique opportunity to perform this longitudinal analysis as it has a relatively high incidence of PC and surveillance was performed in a standardized fashion.[22] At the same time, due to the time-consuming set-up of a surveillance study (median follow-up 35 months) and the overall prevalence of PC, the absolute number of cases in this cohort was limited and only a first indication of potential differences over time can be extracted. For this reason, we have decided to show our data in a descriptive fashion without extensive statistical analysis. Additionally, as studies have previously shown distinct molecular and transcriptomic subtypes, protein glycosylation may also be heterogeneous between patients. Therefore, it is likely that a panel of biomarkers that complements each other is required.[23] Unfortunately, the low number of cases in this study did not allow analysis of combined biomarkers. Furthermore, we were not able to correct for clinical variables, as these may also influence glycosylation level. One example is the presence of diabetes mellitus, Dotz *et al*. (2018)[24] has previously shown that alpha-2,6-linked sialylation is increased (odds-ratio 1.4; p=5.48E-07) in plasma samples of patients with diabetes mellitus. Another example is the presence of a germline mutation. The majority of the cases (9/10) were *CDKN2A* germline mutation carriers. Therefore, we cannot formally exclude the possibility that the results of this study show the differences between a subgroup of high-risk *CDKN2A* mutation carriers and controls, rather than PC cases and controls, *per se*. However, we do see a similar course of markers for a cohort with only *CDKN2A* mutation carriers (**Supplemental Figure S3**), as for the total cohort. Lastly, another limitation is that the sample collection of one cohort was less standardized, leading to a lower number of consecutive samples per individual. Thus, external validation with a larger cohort (preferably including more longitudinal data

points) is required to evaluate if these biomarkers really enable both risk stratification and early detection of PC (independent of clinical characteristics and how they perform when combined in a panel).[25]

Technical advances in high-throughput protein analysis have enabled identification of several post-translational changes, including glycosylation.[26] These protein alterations are of interest, as such modifications are expected to be more disease-specific than solely a protein concentration. In our previous case-control study,[27] we have identified a PC signature that is able to differentiate PC from controls. This current study shows that a change of A3F is indicative for PC development in a high-risk population. However, at this point, our markers did not outperform the accuracy commonly reported for CA19.9. [28] In order to be successfully implemented in surveillance programs, sensitivity and specificity need improvement. Moreover, future in-depth glycoproteomic analyses are needed to give more detailed information on the protein origin and further specify a PC signature.[29-32] It is foreseen that the determination of these glycosylation changes in a protein-specific manner (*i.e.*, glycoproteomics) will further increase the diagnostic potential (**Figure 1**).[33]

In conclusion, this longitudinal study analyzed the relative abundance of 13 candidate glycosylation markers in consecutively collected serum samples. Our findings demonstrate the potential of specific glycosylation traits that change in the course of PC development. Future serum glycoproteomic analyses, which reveal glycoproteins driving these changes, are necessary and may result in more specific disease markers. Additionally, evaluating these markers in larger prospective studies is warranted to replicate these findings, assess heterogeneity between PC cases (*e.g.*, hereditary vs sporadic) and allow correction for clinical variables to evaluate whether these markers are independently associated with PC development.

References

1 Versteijne E, Suker M, Groothuis K, *et al*. Preoperative Chemoradiotherapy Versus Immediate Surgery for Resectable and Borderline Resectable Pancreatic Cancer: Results of the Dutch Randomized Phase III PREOPANC Trial. *J Clin Oncol* 2020;38:1763-1773.

2 Vasen H, Ibrahim I, Ponce CG, *et al*. Benefit of Surveillance for Pancreatic Cancer in High-Risk Individuals: Outcome of Long-Term Prospective Follow-Up Studies From Three European Expert Centers. *Journal of Clinical Oncology* 2016;34:2010-2019.

3 Goggins M, Overbeek KA, Brand R, *et al*. Management of patients with increased risk for familial pancreatic cancer: updated recommendations from the International Cancer of the Pancreas Screening (CAPS) Consortium. *Gut* 2020;69:7-17.

4 KA Overbeek, IJM Levink, BDM Koopmann, F Harinck, ICAW Konings, MGEM Ausems, A Wagner, P Fockens, CH van Eijck, B Groot Koerkamp, OR Busch, MGBesselink, BAJ Bastiaansen, LMJW van Driel, NS Erler, FP Vleggaar, JW Poley, DL Cahen, JE van Hooft, MJ Bruno, on behalf of the Dutch Familial Pancreatic Cancer Surveillance Study Group. Long-term yield of pancreatic cancer surveillance in high-risk individuals. *Gut* 2021.

5 Corral JE, Mareth KF, Riegert-Johnson DL, *et al*. Diagnostic Yield From Screening Asymptomatic Individuals at High Risk for Pancreatic Cancer: A Meta-analysis of Cohort Studies. *Clin Gastroenterol Hepatol* 2019;17:41-53.

6 Cheng B, Zhang Y, Chen Q, *et al*. Analysis of Fine-Needle Biopsy vs Fine-Needle Aspiration in Diagnosis of Pancreatic and Abdominal Masses: A Prospective, Multicenter, Randomized Controlled Trial. *Clin Gastroenterol Hepatol* 2018;16:1314-1321.

7 Sugiura R, Kuwatani M, Hirata K, *et al*. Effect of Pancreatic Mass Size on Clinical Outcomes of Endoscopic Ultrasound-Guided Fine-Needle Aspiration. *Dig Dis Sci* 2019;64:2006-2013.

8 Kromrey M-L, Bülow R, Hübner J, *et al*. Prospective study on the incidence, prevalence and 5-year pancreatic-related mortality of pancreatic cysts in a population-based study. *Gut* 2018;67:138.

9 Fahrmann JF, Schmidt CM, Mao X, *et al*. Lead-Time Trajectory of CA19.9 as an Anchor Marker for Pancreatic Cancer Early Detection. *Gastroenterology* 2021;160:1373-1383.e6.

10 Balmana M, Gimenez E, Puerta A, *et al*. Increased alpha1-3 fucosylation of alpha-1-acid glycoprotein (AGP) in pancreatic cancer. *J Proteomics* 2016;132:144-54.

11 Krishnan S, Whitwell HJ, Cuenco J, *et al*. Evidence of Altered Glycosylation of Serum Proteins Prior to Pancreatic Cancer Diagnosis. *Int J Mol Sci* 2017;18.

12 Munkley J. The glycosylation landscape of pancreatic cancer. *Oncol Lett* 2019;17:2569-2575.

13 Hanna-Sawires RG, Schiphuis JH, Wuhrer M, *et al*. Clinical Perspective on Proteomic and Glycomic Biomarkers for Diagnosis, Prognosis, and Prediction of Pancreatic Cancer. *Int J Mol Sci* 2021;22.

14 Vreeker GCM, Hanna-Sawires RG, Mohammed Y, *et al*. Serum N-Glycome analysis reveals pancreatic cancer disease signatures. *Cancer Med* 2020;9:8519-8529.

15 Vasen HF, Gruis NA, Frants RR, *et al*. Risk of developing pancreatic cancer in families with familial atypical multiple mole melanoma associated with a specific 19 deletion of p16 (p16-Leiden). *Int J Cancer* 2000;87:809-11.

16 Vreeker GCM, Nicolardi S, Bladergroen MR, *et al*. Automated Plasma Glycomics with Linkage-Specific Sialic Acid Esterification and Ultrahigh Resolution MS. *Analytical Chemistry* 2018;90:11955-11961.

17 Yachida S, Jones S, Bozic I, *et al*. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. *Nature* 2010;467:1114-7.

18 Lu C, Xu CF, Wan XY, *et al*. Screening for pancreatic cancer in familial high-risk individuals: A systematic review. *World J Gastroenterol* 2015;21:8678-86.

19 Sugiura R, Kuwatani M, Hirata K, *et al*. Effect of Pancreatic Mass Size on Clinical Outcomes of Endoscopic Ultrasound-Guided Fine-Needle Aspiration. *Digestive Diseases and Sciences* 2019;64:2006-2013.

20 Walsh RM, Zuccaro G, Dumot JA, *et al*. Predicting success of endoscopic aspiration for suspected pancreatic cystic neoplasms. *Jop* 2008;9:612-7.

21 Pereira SP, Oldfield L, Ney A, *et al*. Early detection of pancreatic cancer. *Lancet Gastroenterol Hepatol* 2020;5:698-710.

22 Saad AM, Turk T, Al-Husseini MJ, *et al*. Trends in pancreatic adenocarcinoma incidence and mortality in the United States in the last four decades; a SEER-based study. *BMC Cancer* 2018;18:688.

23 Llop E, P EG, Duran A, *et al*. Glycoprotein biomarkers for the detection of pancreatic ductal adenocarcinoma. *World J Gastroenterol* 2018;24:2537-2554.

24 Dotz V, Lemmers RFH, Reiding KR, *et al*. Plasma protein N-glycan signatures of type 2 diabetes. *Biochim Biophys Acta Gen Subj* 2018;1862:2613-2622.

25 Capello M, Bantis LE, Scelo G, *et al*. Sequential Validation of Blood-Based Protein Biomarker Candidates for Early-Stage Pancreatic Cancer. *J Natl Cancer Inst* 2017;109.

26 Wang M, Zhu J, Lubman DM, *et al*. Aberrant glycosylation and cancer biomarker discovery: a promising and thorny journey. *Clin Chem Lab Med* 2019;57:407-416.

27 Vreeker GCM, Hanna-Sawires RG, Mohammed Y, *et al*. Serum N-Glycome analysis reveals pancreatic cancer disease signatures. *Cancer medicine* 2020;9:8519-8529.

28 Goonetilleke KS, Siriwardena AK. Systematic review of carbohydrate antigen (CA 19.9) as a biochemical marker in the diagnosis of pancreatic cancer. *Eur J Surg Oncol* 2007;33:266-70.

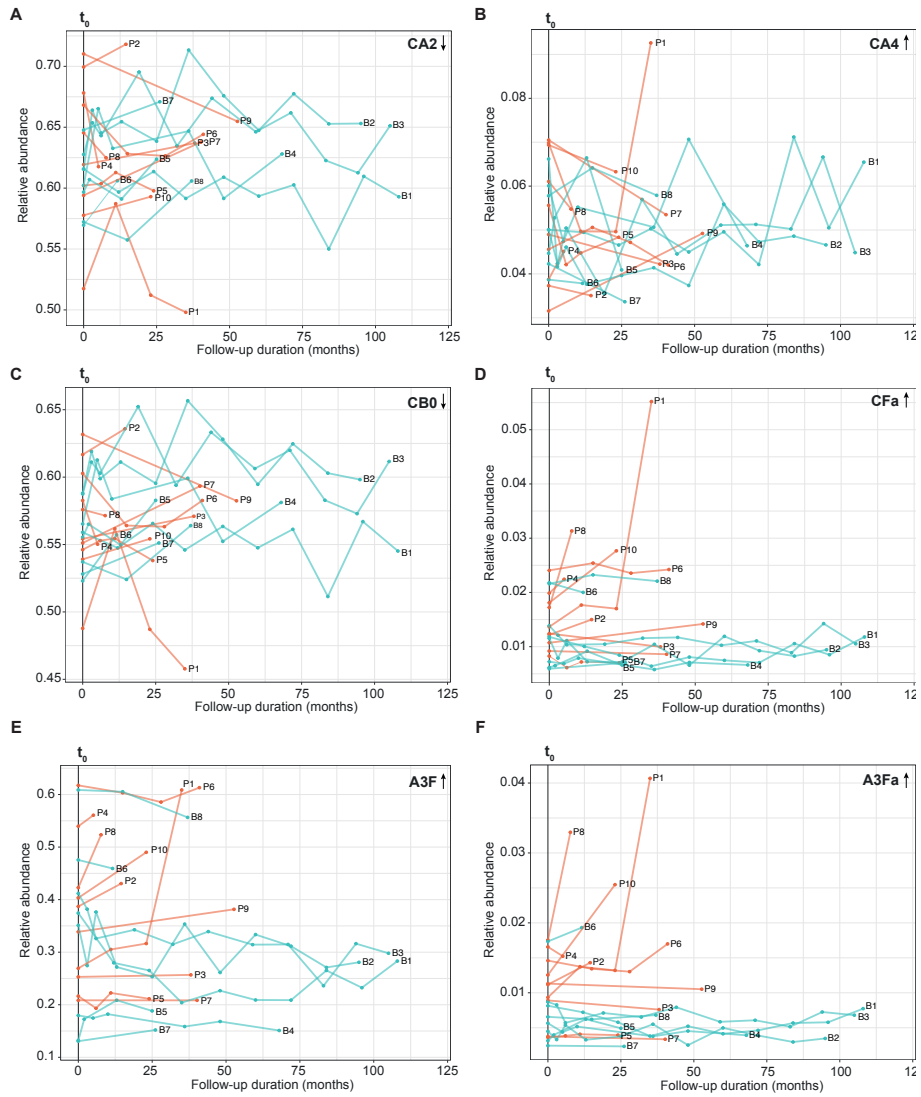
29 Pan S, Chen R, Tamura Y, *et al*. Quantitative glycoproteomics analysis reveals changes in N-glycosylation level associated with pancreatic ductal adenocarcinoma. *J Proteome Res* 2014;13:1293-306.

30 Kontro H, Joenvaara S, Haglund C, *et al*. Comparison of sialylated N-glycopeptide levels in serum of pancreatic cancer patients, acute pancreatitis patients, and healthy controls. *Proteomics* 2014;14:1713-23.

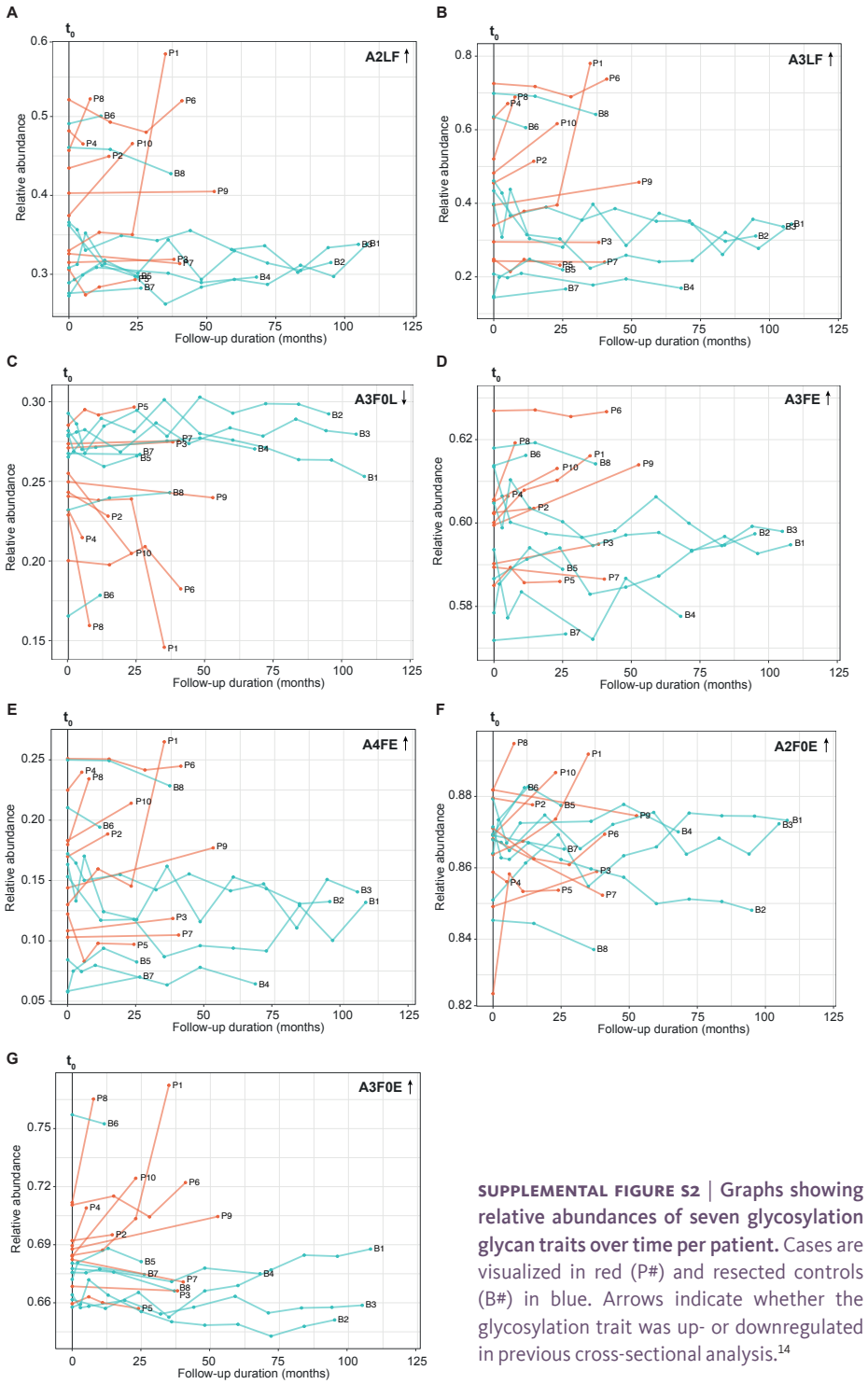
31 Singh S, Pal K, Yadav J, *et al*. Upregulation of glycans containing 3' fucose in a subset of pancreatic cancers uncovered using fusion-tagged lectins. *J Proteome Res* 2015;14:2594-605.

32 Holst S, Belo AI, Giovannetti E, *et al*. Profiling of different pancreatic cancer cells used as models for metastatic behaviour shows large variation in their N-glycosylation. *Sci Rep* 2017;7:16623.

33 Clerc F, Reiding KR, Jansen BC, *et al*. Human plasma protein N-glycosylation. *Glycoconjugate Journal* 2016;33:309-343.



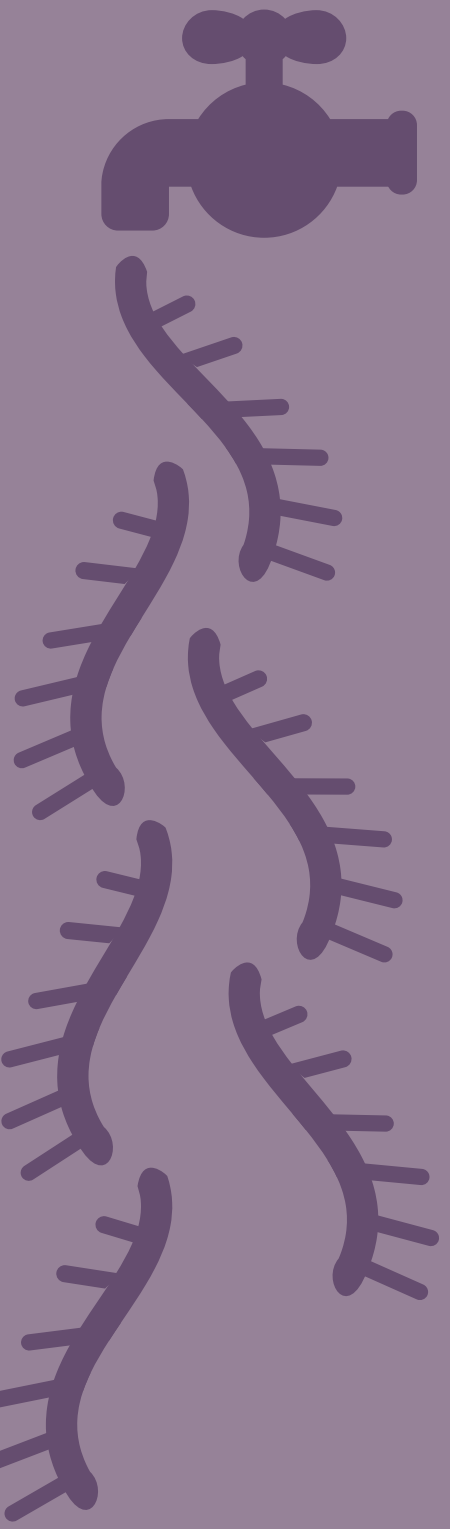
SUPPLEMENTAL FIGURE S1 | Graphs showing relative abundances of specific glycosylation glycan traits over time per patient. Cases are visualized in red (P#) and resected controls (B#) in blue. Arrows indicate whether the glycosylation trait was up- or downregulated in previous cross-sectional analysis.[14]



SUPPLEMENTAL FIGURE S2 | **Graphs showing relative abundances of seven glycosylation glycan traits over time per patient.** Cases are visualized in red (P#) and resected controls (B#) in blue. Arrows indicate whether the glycosylation trait was up- or downregulated in previous cross-sectional analysis.[14]

SUPPLEMENTAL TABLE S1 | Nomenclature of glycans.

	Trait	Description of trait
General	TC	Complex type in total spectrum
	TM	High manose type in total spectrum
	THy	Hybrid type in total spectrum
	CA2	Diantennary species of complex glycans in spectrum
	CA3	Triantennary species of complex glycans in spectrum
	CA4	Tetraantennary species of complex glycans in spectrum
	CF	Fucosylated species of complex glycans in spectrum
	CFa	Antenna-fucosylation of complex glycans
	CB0	Non-bisected species of complex glycans in spectrum
	CB	Bisected species of complex glycans in spectrum
Fucosylation	A2F	Fucosylation of diantennary glycans
	A3F	Fucosylation of triantennary glycans
	A4F	Fucosylation of tetraantennary glycans
	A3Fa	Antenna-fucosylation of triantennary glycans
	A4Fa	Antenna-fucosylation of tetraantennary glycans
	A2SF	Fucosylation of sialylated diantennary glycans
	A2LF	Fucosylation of α2,3-sialylated diantennary glycans
	A3LF	Fucosylation of α2,3-sialylated triantennary glycans
	A4LF	Fucosylation of α2,3-sialylated tetraantennary glycans
	A2EF	Fucosylation of α2,6-sialylated diantennary glycans
	A3EF	Fucosylation of α2,6-sialylated triantennary glycans
	A4EF	Fucosylation of α2,6-sialylated tetraantennary glycans
Bisection	A2B	Bisecting of diantennary glycans
	A2F0B	Bisecting of nonfucosylated diantennary glycans
	A2FB	Bisecting of fucosylated diantennary glycans
Sialylation	A2S	Sialylation of diantennary glycans
	A3S	Sialylation of triantennary glycans
	A4S	Sialylation of tetraantennary glycans
	A2F0S	Sialylation per galactose of nonfucosylated diantennary glycans
	A3F0S	Sialylation of nonfucosylated triantennary glycans
	A4F0S	Sialylation of nonfucosylated tetraantennary glycans
	A2FS	Sialylation of fucosylated diantennary glycans
	A3FS	Sialylation of fucosylated triantennary glycans
	A4FS	Sialylation of fucosylated tetraantennary glycans
α2,3-sialylation	A2L	α2,3-sialylation of diantennary glycans
	A3L	α2,3-sialylation of triantennary glycans
	A4L	α2,3-sialylation of tetraantennary glycans
	A2F0L	α2,3-sialylation of nonfucosylated diantennary glycans
	A3F0L	α2,3-sialylation of nonfucosylated triantennary glycans
	A4F0L	α2,3-sialylation of nonfucosylated tetraantennary glycans
	A2FL	α2,3-sialylation of fucosylated diantennary glycans
	A3FL	α2,3-sialylation of fucosylated triantennary glycans
	A4FL	α2,3-sialylation of fucosylated tetraantennary glycans
	A2GL	α2,3-sialylation per galactose of diantennary glycans



CHAPTER 9

Extracellular Vesicles Derived MicroRNAs in Pancreatic Juice as Biomarkers for Detection of Pancreatic Cancer

Authors
Iris J.M. Levink*, Kateryna Nesteruk*, Esther de Vries, Isis J. Visser, Maikel P. Peppelenbosch, Djuna L. Cahen, Gwenny M. Fuhler, Marco J. Bruno

*these authors contributed equally

Pancreatology, 2022 Jun;22(5):626-635.

Abstract

Introduction: Early detection of pancreatic cancer (PC) at a resectable stage is challenging due to the late presentation of symptoms and limited visibility of sub-centimeter cancers on imaging. MicroRNA (miR) derived from extracellular vesicles (EVs; 'EV-miR') has shown promise as potential biomarker, but has not yet reached clinical implementation. Pancreatic juice (PJ) is a biomarker source, as it is in close contact with ductal cells from which PC arises. This study aims to evaluate the performance of EV-miR derived from PJ (as compared to serum) for the detection of PC.

Methods: In this study, we included all patients with PC, as well as non-malignant controls undergoing surveillance, from whom PJ was available. Serum was also included for a subset of these individuals upon availability. After isolation of EVs from PJ and serum, miR-16, miR-21, miR-25, miR-155 and miR-210 expression were analyzed by qPCR. Serum CA19.9 levels were determined by enzyme immunoassay.

Results: In total, 54 cases and 118 controls were recruited for PJ collection. As compared to controls, cases were older of age (median 68 *vs* 62 years, p=0.001), were more often male (63% vs 35%, p=0.001) and had a lower BMI (24 vs 26 kg/m^2, p=0.001).
Expression of EV-miR-21, EV-miR-25 and EV-miR-16 in PJ was higher in cases than controls, while, in serum, only EV-miR-210 was different between these groups. A panel of PJ-derived EV-miR-21, EV-miR-25, EV-miR-16 and serum-derived miR-210 and CA19.9 has a good performance to detect PC with an area under the curve of 0.91, a specificity of 84% and a sensitivity of 82%.

Conclusion: Detection of miR from EVs in PJ is feasible. A panel of EV-miR-21, EV-miR-25 and EV-miR-16 in PJ, and miR-210 and CA19.9 from serum may be a useful for earlier detection of PC.

Introduction

Pancreatic cancer (PC) has an overall 5-year survival rate of less than 10%.[1] While the 5-year survival rate improves to more than 30% for individuals diagnosed at an operable stage,[2] the majority of patients are diagnosed with inoperable advanced disease due to the late presentation of symptoms.[1,3-5] Additionally, in surveillance programs for individuals with an increased risk of developing PC, present imaging techniques (endoscopic ultrasound [EUS] and magnetic resonance imaging [MRI]) struggle to detect a sub-centimeter cancer mass, even when both modalities are performed concurrently.[6] Conversely, the false-positivity rate of worrisome features is high,[7-9] resulting in resection of lesions that on histological examination appear to be benign.[10,11] Thus, a novel tool that diagnoses PC with a high specificity at an early stage is urgently needed.

One approach is to support clinical diagnosis with molecular markers. Currently, the main biomarker for (recurrence of) PC is serum levels of carbohydrate antigen 19.9 (CA19.9), which has a sensitivity of 70-90%.[12-16] However, this marker is not likely to be positive until PC reaches an advanced stage.[17] Its use in a surveillance setting is disputed as high levels (above the clinically used cutoff of 37kU/L) can be detected in patients with no or low-grade dysplasia.[18] Also, as pancreatic tumors are highly heterogeneous (both within and between individuals), it is conceivable that the diagnostic performance of a single biomarker will not be sufficient, but that a robust panel of biomarkers is required to accurately detect PC at an early stage.[19]

Currently, evidence shows that serum levels of cell-free microRNA (miR)[20-22] and miR isolated from extracellular vesicles EV-miR),[23-25] present in serum (can differentiate between patients with PC and healthy controls. Expression of several of these miRs are altered upon proliferation, angiogenesis, epithelial-to-mesenchymal transition, and metastasis of several human malignancies including PC.[25-35] However, none of these serum miRs have made it to clinical practice yet and it is possible that other biomaterial sources may be more relevant for biomarker detection. Pancreatic juice (PJ) can be safely collected from the duodenal lumen during EUS and, conceivably, contains markers that are more pancreas-specific as compared to blood. Being in direct contact with the potential tumor cells, PJ is also expected to contain information from all tumor clones present. On the other hand, PJ harbors high concentrations of digestive enzymes and represents an abrasive environment which may result in the degradation of promising PC biomarkers.

Interestingly, a recent study showed that, compared to total cell-free miRs in PJ, the diagnostic performance improved when miR was isolated from EVs present in PJ,[36] suggesting that biomarkers in PJ may be protected in EVs. EVs are a group of cellular particles which can be classified based on size and biogenesis. They include: 1. exosomes (<150 nm), which are released from cells through multivesicular bodies in the endosomal pathway; 2. microvesicles (200-500 nm), which arise through budding of the plasma

membrane; and 3. apoptotic bodies (various sizes). On top of these major classes, many specialized EV-subtypes have been described.[37] Cancer cells, specifically, release EVs to create a pre-metastatic niche.[38] Their vesicular contents, which include proteins, DNA, RNA, and microRNAs, are cell-specific and expected to represent a signature of cellular pathology. A previous study showed that the levels of EV-derived miR-21 and miR-155 in PJ discriminated PC cases from controls with an accuracy of 83% and 89% respectively.[36] However, limitations included the low number of subjects involved, and EV-miR expression in PJ and blood has never been directly compared to our best knowledge.

Thus, our study aimed to evaluate the diagnostic performance of PJ- and serum-derived EV-miR for the detection of PC in a larger cohort of patients and controls. Based on the most often described promising microRNAs for PC detection, we selected EV-miR-16, EV-miR-21, EV-miR-25, EV-miR-155, and EV-miR-210 for analysis.[39] We compared the expression of these EV-miRs and their diagnostic performance between PJ and serum and contrasted this against the currently available serum biomarker CA19.9.

Material and Methods

Selection of subjects

This study was executed at the Erasmus University Medical Center. PJ and serum were collected between August 2018 and May 2020 in patients who participate in the following prospective study cohorts: 1) Patients with suspected (sporadic) PC (KRASPanc study, MEC-2018-038); 2) high-risk individuals under surveillance for a hereditary predisposition or familial history of PC (CAPS study, MEC-2012-448, www.caps-registry.com); 3) individuals under surveillance for neoplastic pancreatic cysts (PACYFIC study, MEC-2014-021, www.pacyfic.net). The Erasmus Medical Center ethical review board approved the studies, and the included individuals gave written informed consent before enrolment. The study was carried out according to the ethical principles for medical research involving human subjects from the World Medical Association Declaration of Helsinki.

Inclusion criteria

All inclusion criteria of the prospective cohort studies can be found in **Supplemental Table 1.** Samples were excluded if diagnostic work-up eventually revealed other pancreatic diseases (*e.g.*, pancreatitis). For endpoint analysis, this cohort was divided into a case group (patients with pathologically proven high-grade dysplasia [HGD] or PC) and controls (individuals without HGD or PC). For subgroup analysis, the control group was subdivided into individuals with high-risk morphology who presented with worrisome features or indications for surgery (as described by the European evidence-based guidelines[9]) and those with low-risk morphology and no indications for surgery.[9]

Pancreatic Juice and Serum Collection

PJ was collected from the duodenum as described before.[40] During EUS, PJ collection was performed after visualization of the ampullary orifice. To reduce duodenal contamination, duodenal fluid was aspirated prior to juice collection. Next, a wash-out of PJ was stimulated by intravenous administration of human synthetic secretin (ChriRhoStim, Burtonsville, MD, 16 µg/patient). PJ was collected for up to 8 minutes starting immediately after injection with the endoscope (Pentax Medical, Tokyo, Japan) and assembled in a mucus extractor (Pennine Healthcare, Derby, United Kingdom, 15 mL) attached to the proximal end of the endoscopic channel. PJ was aliquoted to avoid freeze-thaw cycles, snap-frozen within 10 minutes after collection, and stored at -80°C until further use. All serum samples were collected within a 3-week window of PJ collection. CA19.9 data were retrieved from patient records and only measurements performed within 3 weeks preceding or following PJ collection were scored.

Pancreatic juice quality

As a measure of PJ quality, we assessed the concentration of PLA2G1B (pancreas standard marker, MyBiosource, San Diego, USA, MBS703283) by enzyme linked immunosorbent assay (ELISA). Pre-coated, pre-blocked plates were used according to the manufacturer's protocol. Briefly, plates were blocked with ELISA diluent for 1 hour at room temperature. PJ (75 µL per well) was incubated at 4 °C overnight, after which biotin conjugated detection antibody was added at room temperature for 1 hour. Following incubation of avidin-HRP for 30 minutes at room temperature, TMB substrate was added. Reactions were stopped by addition of sulfuric and absorbance was read at 450 nm (Tecan Infinite 200 PRO plate reader). Cases and controls were equally distributed among batches. In addition, we measured the total protein concentration in PJ and EV isolates by Lowry protein assay (Bio-Rad, Hercules, CA, USA) .[41]

EV isolation and analysis

Prior to EV isolation, PJ was centrifuged for 10 min at 4000 RPM 4°C to remove debris. Then, 100 µL of total exosome isolation reagent (Thermo Fisher Scientific, Waltham, MA, #4478359) was added to 200 µL of supernatant and kept on a rollerbank at 4°C overnight. After this, samples were centrifuged for 1h at 14000 RPM and the pellet was resuspended in 400 µL of PBS (filtered with 0.2µM filter). Serum was centrifuged during 30 minutes at 2000g at 4°C to remove debris. 40 µL of Total exosome isolation reagent (for serum; Thermo Fisher Scientific, Waltham, MA, #4478360) was added to 200 µL of supernatant and incubated for 30 minutes at 4°C. Then, samples were centrifuged again at 10 000 g for 10 min at room temperature. Supernatant was discarded and EVs were resuspended in 200 µL of filtered PBS. Concentrated EVs were stored at -80°C until further analysis. The size and concentration of particles were confirmed by NanoSight NS300 (NTA 3.4 Build 3.4.003 software). For nanoparticle tracking analysis samples were diluted 1:1000

in filtered PBS. Quality of EVs was confirmed by electron microscopy and western blot (data not shown).

EVs miR analysis

MicroRNA was isolated from 200 µL of concentrated EVs with QIAzol Lysis Reagent (Qiagen, Hilden, Germany, #79306) and miRNeasy Mini kit (Qiagen, Hilden, Germany, #217004) according to manufacturer's recommendations. MicroRNA-specific cDNA was prepared using the Taqman microRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, #217004; miR-16, miR-21, miR-205, miR-155) as described before.[42,43] Every cDNA reaction consisted of 0.4 µl dNTP mix, 1.35 µl Multiscribe RT enzyme (500U/µL), 2.0 µl 10x RT Buffer, 0.25 µl RNase inhibitor, 1.0 µl of each RT primer, and 5 µl of diluted template RNA. The total reaction volume was adjusted to 20 µl with nuclease-free water. Each qPCR reaction consisted of 6 µl TaqMan Universal PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, #4324018), 0.5 µl microRNA-specific PCR primer and 5.0 µl of the previously 1:5 diluted cDNA. The final volume of every PCR reaction was adjusted to 12 µl with nuclease-free water. All qPCR reactions were performed according to the manufacturer's instructions and carried out in duplicate. MiR expression changes were calculated relative to plate average using the $2^{-\Delta\Delta Ct}$ method[44] and presented as log2 fold change. When expression miR was not detected (only for miR-25 in 6.9% of samples), CT input value of 45 was imputed for quantification.

Statistical analysis

Shapiro–Wilk's test was used to determine data distribution. For normally distributed data, an unpaired Student's t-test was performed to compare two groups. For non-parametric data, a Mann-Whitney U (2 groups) test or a Kruskal-Wallis H test (>2 groups) was performed. Spearman's rank correlation coefficient was used to investigate correlations between biomarkers and continuous variables. A χ^2 test or Fisher's exact probability test was used to evaluate the association between categorical variables. Receiver operating characteristic (ROC) curves and their area under the curve (AUC) were used to assess the diagnostic performance of the biomarkers. The first optimal cutoff values in ROC curves were set to the value that maximizes the Youden index. The Youden index was defined as sensitivity + specificity − 1. For each biomarker, a second cutoff point on the ROC curve was chosen with a specificity of at least 90%, aiming for high specificity to minimize harm due to unnecessary biopsy or surgery in the surveillance population. For CA19.9, the clinically used cutoff of 37kU/L was used. Multiple logistic regression models were generated to test the performance and interaction between a combination of biomarkers. Confidence intervals for sensitivity, specificity, and accuracy are "exact" Clopper-Pearson confidence intervals. All tests were two-sided, and the significance level was set at $p < 0.05$. Excel, Graphpad Prism (GraphPad Prism version 9, GraphPad Software, La Jolla, CA), and IBM SPSS Statistics (Statistical Package for the Social Sciences, version 25, SPSS Inc., Chicago, IL) software were used for the analyses.

TABLE 1 | **Clinical characteristics at time of pancreatic juice collection.**

	Cases (N=54)	Controls (N=118)	P-value
Age in years, median (IQR)	67.5 (10.3)	62.1 (6.0)	0.001
Male gender, n (%)	34 (63.0)	41 (34.7)	0.001
BMI in kg/m², median (IQR)	23.7 (3.7)	25.7 (5.1)	0.001
Familial/genetic predisposition, n (%)	0 (0.0)	66 (55.9)	<0.001
Member of FPC family[a]	.	32 (27.1)	.
CDKN2A p16	.	24 (20.3)	.
BRCA2 + ≥2 blood relatives with PDAC	.	5 (4.2)	.
BRCA1 + ≥2 blood relatives with PDAC	.	1 (0.8)	.
PALB2 + ≥2 blood relatives with PDAC	.	1 (0.8)	.
BRCA2 + CDKN2A p16	.	1 (0.8)	.
STK11/LKB1	.	2 (1.7)	.
Diabetes mellitus, n (%)	21 (38.9)	16 (13.6)	0.001
Indication EUS, n (%)			<0.001
Suspected PDAC	35 (64.8)	4 (3.4)	.
Fiducial placement	18 (33.3)	0 (0.0)	.
Surveillance	1 (1.9)	114 (96.6)	.
CBD stent in situ, n (%)			<0.001
CBD stent in situ	9 (16.7)	0 (0.0)	.
No CBD stent and CBD dilation	14 (25.9)	3 (2.5)	.
No CBD stent and no CBD dilation	31 (57.4)	115 (97.5)	.
Relative or absolute indications for surgery,[9] n (%)	54 (100.0)	26 (22.0)	<0.001
Enhancing mural nodule or hypodense lesion	54 (100.0)	4 (3.4)	.
Caliber change	41 (75.9)	0 (0.0)	.
Diffuse PD dilation > 5mm	0 (0.0)	14 (11.9)	.
CA19.9 ≥37 kU/L	34 (63.0)	7 (5.9)	.
Cyst size > 40mm	0 (0.0)	2 (1.7)	.
New-onset diabetes[b]	9 (16.6)	2 (1.7)	.
Recent acute pancreatitis[c]	2 (3.7)	6 (5.1)	.
Lymphadenopathy	23 (42.6)	0 (0.0)	.
Working diagnosis, n (%)			<0.001
No abnormalities	.	41 (34.7)	.
Unspecified cyst	.	9 (7.6)	.
SB-IPMN	1 (1.9)	50 (42.4)	.
MD-IPMN or MT-IPMN	.	14 (11.9)	.
MCN	.	1 (0.8)	.
NET	.	1 (0.8)	.
Indeterminate lesion, not suspect for malignancy	.	2 (1.7)	.
Resectable PC	10 (18.5)	0 (0.0)	.
Locally advanced PC	43 (79.6)	0 (0.0)	.
Distal mestases (on imaging), n (%)	8 (14.8)	0 (0.0)	<0.001

[a] ≥2 first-degree relatives or 3 relatives (either first or second degree) or ≥2 second-degree relatives of which ≥1 with age <50 years at time of diagnosis. [b] Development of diabetes mellitus in the last 2 years. [c] 3 extra post-ERCP pancreatitis.

Results

Patient characteristics

In total, 54 cases and 118 controls were recruited for PJ collection. A summary of subject characteristics is provided in **Table 1**. Controls tended to be younger (62.1 *vs* 67.5, p=0.001) with a lower proportion of males (63% *vs* 34%, p=0.001) and higher BMI (25.7 kg/m^2 *vs* 23.7 kg/m^2, p=0.001). Cases more often suffered from diabetes mellitus (38.9% *vs* 13.4%, p=0.001). Serum samples were available for 46 cases and 58 controls. Characteristics of this subpopulation are summarized in **Supplemental Table 2**.

Quality of pancreatic juice

As a measure of PJ quality, pancreas-specific PLA2G1B and total protein concentration were determined. Both were similar between controls and cases, indicating a similar pancreatic component in PJ as a result of collection (*P* = 0.24 for total protein and *P* = 0.19 for PLA2G1B; **Figure 1**).

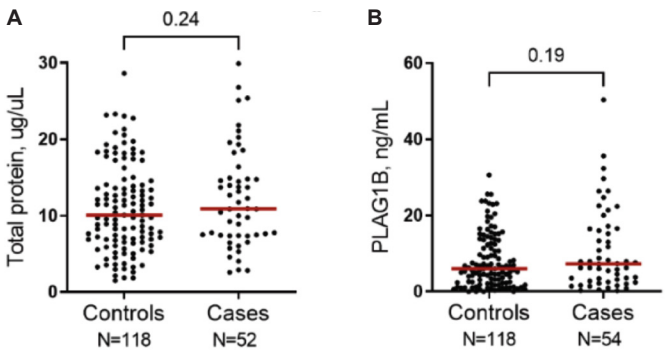


FIGURE 1 | No difference was found between controls and cases in concentration of total protein (A) and phospholipase A2 group IB (PLA2G1B; as a measure of pancreatic origin) (B), indicating similar composition of PJ.

EV-miR expression

Nanoparticle tracking analysis revealed abundant small vesicles in PJ with a mode size of 116 nm and an overall concentration of 8.42*10^{11} particles/mL (range 1.85*10^{11} to 4.28*10^{12}, data not shown).

EV-miR-16, EV-miR-21, EV-miR-155, and EV-miR-210 were detectable in all subjects for both PJ and serum, while EV-miR-25 was detectable in 161/172 (93.6%) PJ samples and all serum samples. When comparing cases with controls, EV-miR-21 (*P* = 0.002), EV-miR-25 (*P* = 0.005), EV-miR-210 (*P* = 0.02), and EV-miR-16 (*P* = 0.004) were significantly overexpressed in PJ, while no difference was found for EV-miR-155 (*P* = 0.11; **Figure 2 A-E**). In serum, the concentration of CA19.9 (P<0.001) and expression of EV-miR-210 (P=0.03) levels were higher in cases than controls (**Figure 2 F-K**). Expression of EV-miRs was highly correlated with each other in both PJ and serum but no correlation was found between PJ and serum (**Supplemental Tables 3 and 4**). After correction for age, gender, BMI and diabetes mellitus, EV-miR-21, EV-miR-25, and EV-miR-16 remained significantly overexpressed in cases vs controls in PJ (**Table 2**) and EV-miR-210 in serum, thus showing an independent association with PC.

In subgroup analysis, expression of EV-miR-21 (*P* = 0.07) and EV-miR-210 (*P* = 0.04) in PJ tend to be higher in high-risk controls, who harbor worrisome features, as compared to low-risk controls, while no difference was found between high-risk controls and PC (**Supplemental Figure 1**). CA19.9 was increased in PC compared to both control groups (**Supplemental Figure 1**).

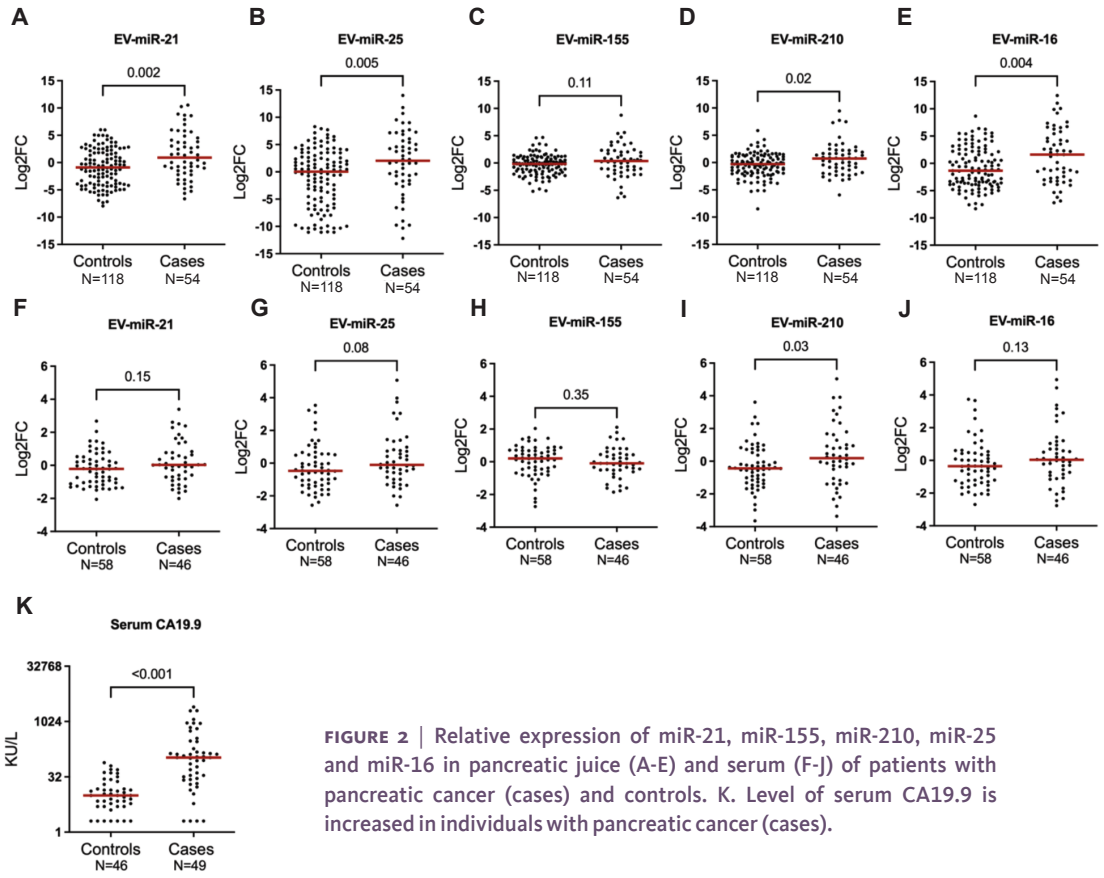


FIGURE 2 | Relative expression of miR-21, miR-155, miR-210, miR-25 and miR-16 in pancreatic juice (A-E) and serum (F-J) of patients with pancreatic cancer (cases) and controls. K. Level of serum CA19.9 is increased in individuals with pancreatic cancer (cases).

TABLE 2 | Crude odds ratio (OR) and adjusted OR for biomarkers and clinical variables within the pancreatic juice and serum cohorts

Parameter	Crude OR (95% CI)	*P-value*	Adjusted OR (95% CI) * (age, gender, BMI, diabetes)	*P-value*
Pancreatic juice				
Age	1.070	0.001	1.064	0.028
Gender	3.193	0.001	3.523	0.009
BMI	0.827	0.002	0.778	<0001
Diabetes mellitus	0.246	<0001	0.332	0.52
PJ EV-miR-21	1.173	0.001	1.152	0.03
PJ EV-miR-25	1.095	0.005	1.117	0.016
PJ EV-miR-210	1.224	0.006	1.183	0.094
PJ EV-miR-16	1.141	0.001	1.146	0.017
Serum				
Age	1.080	0.01	1.070	0.042
Gender	4.890	<0001	7.071	0.003
BMI	0.814	0.0083	0.757	0.006
Diabetes mellitus	0.261	0.0043	0.464	0.323
Serum EV-miR-210	1.317	0.0359	1.571	0.033

*ORs with 95% confidence intervals (CIs) and p-value determined by multivariate logistic regression analysis, adjusted for age, gender, BMI and diabetes.

Diagnostic performance of analyzed miRs

ROC curves were constructed for independently associated EV-miRs in PJ and serum to compare their diagnostic value for PC detection. AUC values for individual overexpressed EV-miRs in PJ ranged from 0.61 to 0.64. For serum, EV-miR-210 achieved an AUC of 0.62 for PC prediction, while for CA19.9 an AUC of 0.85 was reached (**Table 3**). While performance of individual EV-miRs was poor, when combining PJ EV-miR-21, EV-miR-25, and EV-miR-16 with serum EV-miR-210 and CA19.9, the sensitivity and specificity were 84.2% and 81.5% (AUC = 0.91) respectively, compared to 85.7% and 73.3% for CA19.9 alone (**Figure 3**).

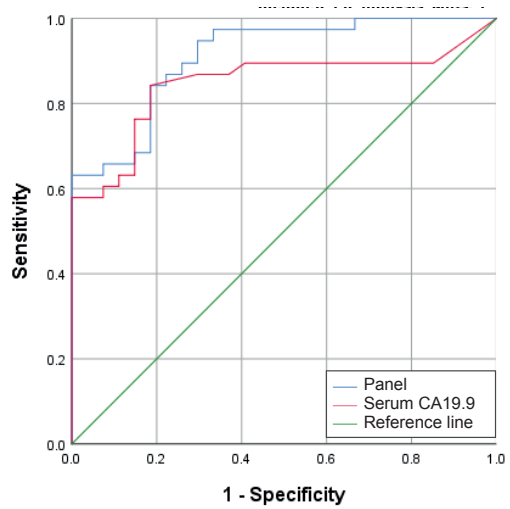


FIGURE 3 | Receiver operation curve showing the diagnostic performance of CA19.9 level and the tested panel (PJ: EV-miR-21, EV-miR-25, EV-miR-16 and serum: EV-miR-210 and CA19.9).

TABLE 3 | Diagnostic performance of analyzed miRs and panels.

			Cutoff 1 (Specificity=100%)		Cutoff 2 (Youden Index)	
	Controls / cases	ROC-AUC (95% CI)	Sensitivity, % (95% CI)	Specificity, % (95% CI)	Sensitivity, % (95% CI)	Specificity,% (95% CI)
Individual markers						
EV-miR-21 in PJ	118/54	0.64 (0.55-0.73)				
EV-miR-25 in PJ	118/54	0.63 (0.54-0.73)				
EV-miR-16 in PJ	118/54	0.64 (0.55-0.73)				
EV-miR-210 in serum	58/49	0.62 (0.51-0.74)				
CA19.9 in serum	45/49	0.85 (0.77-0.93)	59.2 (44.2-73.0)	100.0 (92.1-100)	85.7 (72.8-94.1)	73.3 (58.1-85.4)
Panels						
PJ EV-miR-21, PJ EV-miR-25, PJ EV-miR-16, Serum CA19.9	45/49	0.89 (0.82-0.95)	63.3 (48.3-76.6)	100.0 (92.1-100)	75.5 (61.1-86.7)	86.7 (73.2-95.0)
PJ EV-miR-21, EV-miR-25, EV-miR-16, Serum CA19.9, Serum EV-miR-210	27/38	0.91 (0.84-0.98)	63.2 (46.0-78.2)	100 (87.2-100)	84.2 (68.6-94.0)	81.5 (61.9-93.7)

ROC = receiver operating curve, AUC = area under the curve CI = confidence interval, EV = extracellular vesicle, PJ = pancreatic juice.

Discussion

In the present study, we extracted EVs from serum and PJ and investigated EV-miR expression of 54 malignant cases and 118 non-malignant controls. Five EV-miRs were selected based on their promise inferred from literature (see **Supplemental Table 5**): EV-miR-21, EV-miR-25, EV-miR-210, EV-miR-155, EV-miR-16. Of these, EV-miR-21, EV-miR-25, EV-miR-16 were overexpressed in PJ from cases compared to controls, independently from other clinical characteristics. EV-miR-210 was the only miR to be overexpressed in serum from cases compared to controls. A combined panel of PJ EV-miR-21, EV-miR-25, EV-miR-16, and serum EV-miR-210 and CA19.9 is able to distinguish cases from controls undergoing surveillance with a specificity of 81.5% and sensitivity of 84.2%.

MiRs are short non-coding RNAs composed of 18–25 nucleotides which are functional regulators of gene expression. In PC cells, miR-21 regulates gene expression of *MMP2, MMP9,* and *VEGF* to enhance cellular proliferation and invasion.[45] MiR-21 expression in PC tumor-associated fibroblasts is also linked with decreased overall survival.[46] MiR-25 expression promotes cell proliferation by targeting the regulator of actin cytoskeleton dynamics *ABI2* in PC.[47] Zhang *et al*. have found that cigarette smoke-induced miR-25-3p excessive maturation promotes the development and progression of pancreatic cancer.[48] MiR-210 is a hypoxia marker in PC[49] and has been shown to mediate epithelial-mesenchymal transition induced by HIF-1α under hypoxic conditions by inhibition of HOXA9 in PC cell line.[50] Luciferase reporter assays suggested that miR-16 post-transcriptionally regulates *Bcl-2* expression in PC cells by targeting sites of the 3′ untranslated region of this gene.[51] Thus, the overexpressed miRs are clearly involved in pathogenesis and progression of PC, explaining why these molecules have been targeted for investigation as potential biomarkers.

Cell-free miR can either be isolated from whole biofluid, or from EVs obtained from these biobluids. To our best knowledge, only one study investigated EV-miR in PJ before.[36] Nakamure *et al*. demonstrated overexpression of EV-miR-21 and EV-miR-155 in PC cases (N=27) compared to chronic pancreatitis controls (N=8).[36] While we obtained similar results for EV-miR-21, no difference in expression was found for EV-miR-155, likely due to different expressions in control groups (cystic lesions and pancreas without abnormalities in our study). Technical differences may also account for different study results, including: 1) the method of PJ collection (endoscopic retrograde pancreatography vs less invasive EUS in our study), 2) the method of EV extraction (ultracentrifugation vs more clinically applicable method with Invitrogen kit in our study), and 3) normalization method for RNA expression (miR-16 vs PJ volume in our study). Other studies mostly concentrated on total miR[20,52-54] or EV-miR[23,55-57] in blood, but some also investigated total,[58] EV-miR[36] or cellular miR[59] in PJ (**Supplemental table 5**). The expression profile of miRs in EVs is not the same as the corresponding cell-free total miRs, indicating that miRs have a strict sorting mechanism.[60] When side by side comparison of EV-miR with miR from the whole biofluid

was performed, EV-miR was superior as a biomarker of PC in both serum[55] and PJ,[36] and as such EV-miRs may be more useful and stable as biomarkers for PC detection compared to total miR in body fluids, including PJ. However, EV-miR in PJ is clearly under-investigated in this respect.

Here we found that EV-miR expression patterns do not correlate between serum and PJ. Thus, PJ may contain biomarkers that are not present in serum and *vice versa*. For instance, EV-miR-210 was overexpressed in serum of cases, but not in PJ after adjusting for clinical parameters. Aberrant high expression of miR-210 has been detected in many tumors[61] making it in theory a less specific PC biomarker when detected in blood. EVs in PJ are larger in size when compared to serum-derived EVs (unpublished data) and as size is linked to biogenesis this might also explain the different molecular cargo of these EVs. Thus, differences may be related to different subtypes of EVs present in these bodily fluids or the fact that most EVs in serum are not pancreas derived. Selection of candidate miRs for our study were based on available data which predominantly exists for blood. It has been estimated that each exosome can accommodate 70–25,000 small RNA or protein molecules.[62] Taking into account our findings on different expression of EV-miRs in blood and PJ, a more systematic approach is needed to determine miR composition of EVs in PJ and identify good candidate miRs for PC diagnosis in PJ.

The ideal biomarker (or panel) should be able to discriminate PC patients not only from heathy controls but more importantly also from other non-malignant pancreatic and other organs conditions. We previously found that a minority (38%) of individuals with a solid lesion on imaging and a minority (35%) of individuals undergoing surgery for a suspicious lesion had malignancy or high-grade dysplasia in the resected specimen.[6] To prevent unnecessary surgeries, it is important to test a potential biomarker on a relevant control group with non-malignant pancreatic masses. For this reason, we included individuals under surveillance for familiar or genetic predisposition to pancreatic cancer and for neoplastic pancreatic cysts. 59% of controls had non-malignant pancreatic abnormalities. This is also agreement with the current consensus[63,64] that screening for pancreatic cancer should not be aimed at the general population but at individuals at increased risk of developing pancreatic cancer. In subgroup analysis, splitting low-risk controls and high-risk controls (based on the presence of worrisome features and indications for surgery[9]), a gradual increase in the expression of EV-miR-21, EV-miR-25 and EV-miR-16 was seen from low-risk controls to high-risk controls and PC in PJ, but not serum. Although high-risk controls were not statistically different from either low-risk controls or PC, low-risk controls were significantly different from PC. Our data in line with studies on total miR in PJ, demonstrating a decrease in expression levels from PC to chronic pancreatitis to non-pancreatic disease controls, with differences between PC and chronic pancreatitis patients not being significant.[58] Including only healthy individuals in a control group may lead to the overestimation of diagnostic performance of candidate biomarker. Indeed, it has been reported that the diagnostic performance of serum Ca19.9 for discriminating PC is higher when including healthy controls compared to

benign pancreatic disease cases.[65] A drawback of our approach is that high-risk controls may theoretically harbor as yet undetected cancer, thus resulting in an underestimated performance of candidate biomarkers. However, the probability of this occurring is low, with a follow-up of a minimum of one year for all controls in our cohort. Most published studies (**Supplemental table 4**) include heathy controls and chronic pancreatitis patients, with some adding other cancers or non-healthy controls without pancreatic disease. High diagnostic values have been reported for heathy subjects vs PC, but at the same time increased miR levels were seen in cases with IPMN[20] or chronic pancreatitis.[52] Chronic pancreatitis is a risk factor for PC as they share many clinical symptoms, making a clear distinction between the two diseases difficult, particularly during the early stages of pancreatic cancer development. Thus, there is clinical need in distinguishing malignant and non-malignant abnormalities of the pancreas, which should be reflected in design of studies aiming to estimate diagnostic values of candidate biomarkers for detection of pancreatic cancer.

In conclusion, a panel of PJ-derived EV-miR-21, EV-miR-25, EV-miR-16 and serum miR-210 and CA19.9 may be a useful diagnostic biomarker set for detection of PC.

References

1 Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. *CA Cancer J Clin* 2019;69:7-34.
2 Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. *CA: A Cancer Journal for Clinicians* 2020;70:7-30.
3 Konings I, Canto MI, Almario JA, *et al*. Surveillance for pancreatic cancer in high-risk individuals. *BJS Open* 2019;3:656-665.
4 Konings I, Cahen DL, Harinck F, *et al*. Evolution of features of chronic pancreatitis during endoscopic ultrasound-based surveillance of individuals at high risk for pancreatic cancer. *Endosc Int Open* 2018;6:E541-E548.
5 Konings IC, Harinck F, Poley JW, *et al*. Prevalence and Progression of Pancreatic Cystic Precursor Lesions Differ Between Groups at High Risk of Developing Pancreatic Cancer. *Pancreas* 2017;46:28-34.
6 Overbeek KA, Levink IJM, Koopmann BDM, *et al.* Long-term yield of pancreatic cancer surveillance in high-risk individuals. *Gut* 2022;71:1152-1160.
7 Yu S, Takasu N, Watanabe T, *et al*. Validation of the 2012 Fukuoka Consensus Guideline for Intraductal Papillary Mucinous Neoplasm of the Pancreas From a Single Institution Experience. *Pancreas* 2017;46:936-942.
8 Canto MI, Harinck F, Hruban RH, *et al*. International Cancer of the Pancreas Screening (CAPS) Consortium summit on the management of patients with increased risk for familial pancreatic cancer. *Gut* 2013;62:339-47.
9 European Study Group on Cystic Tumours of the P. European evidence-based guidelines on pancreatic cystic neoplasms. *Gut* 2018;67:789-804.
10 Pereira SP, Oldfield L, Ney A, *et al*. Early detection of pancreatic cancer. *Lancet Gastroenterol Hepatol* 2020;5:698-710.
11 Valsangkar NP, Morales-Oyarvide V, Thayer SP, *et al*. 851 resected cystic tumors of the pancreas: a 33-year experience at the Massachusetts General Hospital. *Surgery* 2012;152:S4-12.
12 Skulimowski A, Durczyński A, Strzelczyk J, *et al*. Comparison of clinical usefulness of serum Ca125 and CA19-9 in pancreatic adenocarcinoma diagnosis: meta-analysis and systematic review of literature. *Biomarkers* 2021:1-9.
13 Goonetilleke KS, Siriwardena AK. Systematic review of carbohydrate antigen (CA 19.9) as a biochemical marker in the diagnosis of pancreatic cancer. *Eur J Surg Oncol* 2007;33:266-70.
14 Xing H, Wang J, Wang Y, *et al*. Diagnostic Value of CA 19.9 and Carcinoembryonic Antigen for Pancreatic Cancer: A Meta-Analysis. *Gastroenterol Res Pract* 2018;2018:8704751.
15 Satake K, Chung YS, Yokomatsu H, *et al*. A clinical evaluation of various tumor markers for the diagnosis of pancreatic cancer. *Int J Pancreatol* 1990;7:25-36.
16 Nazli O, Bozdag AD, Tansug T, *et al*. The diagnostic importance of CEA and CA 19.9 for the early diagnosis of pancreatic carcinoma. *Hepatogastroenterology* 2000;47:1750-2.
17 Sawabu N, Watanabe H, Yamaguchi Y, *et al*. Serum Tumor Markers and Molecular Biological Diagnosis in Pancreatic Cancer. *Pancreas* 2004;28.

18 Kim JR, Jang JY, Kang MJ, *et al*. Clinical implication of serum carcinoembryonic antigen and carbohydrate antigen 19-9 for the prediction of malignancy in intraductal papillary mucinous neoplasm of pancreas. *J Hepatobiliary Pancreat Sci* 2015;22:699-707.

19 Jenkinson C, Earl J, Ghaneh P, *et al*. Biomarkers for early diagnosis of pancreatic cancer. *Expert Rev Gastroenterol Hepatol* 2015;9:305-15.

20 Yu Y, Tong Y, Zhong A, *et al*. Identification of Serum microRNA-25 as a novel biomarker for pancreatic cancer. *Medicine (Baltimore)* 2020;99:e23863.

21 Guz M, Jeleniewicz W, Cybulski M, *et al*. Serum miR-210-3p can be used to differentiate between patients with pancreatic ductal adenocarcinoma and chronic pancreatitis. *Biomed Rep* 2021;14:10.

22 Zhu Y, Wang J, Wang F, *et al*. Differential MicroRNA Expression Profiles as Potential Biomarkers for Pancreatic Ductal Adenocarcinoma. *Biochemistry (Mosc)* 2019;84:575-582.

23 Que R, Ding G, Chen J, *et al*. Analysis of serum exosomal microRNAs and clinicopathologic features of patients with pancreatic adenocarcinoma. *World J Surg Oncol* 2013;11:219.

24 Madhavan B, Yue S, Galli U, *et al*. Combined evaluation of a panel of protein and miRNA serum-exosome biomarkers for pancreatic cancer diagnosis increases sensitivity and specificity. *Int J Cancer* 2015;136:2616-27.

25 Ariston Gabriel AN, Wang F, Jiao Q, *et al*. The involvement of exosomes in the diagnosis and treatment of pancreatic cancer. *Mol Cancer* 2020;19:132.

26 Peng Y, Croce CM. The role of MicroRNAs in human cancer. *Signal Transduction and Targeted Therapy* 2016;1:15004.

27 Rawat M, Kadian K, Gupta Y, *et al*. MicroRNA in Pancreatic Cancer: From Biology to Therapeutic Potential. *Genes* 2019;10:752.

28 Daoud AZ, Mulholland EJ, Cole G, *et al*. MicroRNAs in Pancreatic Cancer: biomarkers, prognostic, and therapeutic modulators. *BMC Cancer* 2019;19:1130.

29 Elewaily MI, Elsergany AR. Emerging role of exosomes and exosomal microRNA in cancer: pathophysiology and clinical potential. *J Cancer Res Clin Oncol* 2021;147:637-648.

30 Wang X, Luo G, Zhang K, *et al*. Hypoxic Tumor-Derived Exosomal miR-301a Mediates M2 Macrophage Polarization via PTEN/PI3Kγ to Promote Pancreatic Cancer Metastasis. *Cancer Res* 2018;78:4586-4598.

31 Chang J, Li H, Zhu Z, *et al*. microRNA-21-5p from M2 macrophage-derived extracellular vesicles promotes the differentiation and activity of pancreatic cancer stem cells by mediating KLF3. *Cell Biol Toxicol* 2021.

32 Uysal-Onganer P, D'Alessio S, Mortoglou M, *et al*. Peptidylarginine Deiminase Inhibitor Application, Using Cl-Amidine, PAD2, PAD3 and PAD4 Isozyme-Specific Inhibitors in Pancreatic Cancer Cells, Reveals Roles for PAD2 and PAD3 in Cancer Invasion and Modulation of Extracellular Vesicle Signatures. *Int J Mol Sci* 2021;22.

33 Li M, Guo H, Wang Q, *et al*. Pancreatic stellate cells derived exosomal miR-5703 promotes pancreatic cancer by downregulating CMTM4 and activating PI3K/Akt pathway. *Cancer Lett* 2020;490:20-30.

34 Jiang MJ, Chen YY, Dai JJ, *et al*. Dying tumor cell-derived exosomal miR-194-5p potentiates survival and repopulation of tumor repopulating cells upon radiotherapy in pancreatic cancer. *Mol Cancer* 2020;19:68.

35 Sonohara F, Yamada S, Takeda S, *et al*. Exploration of Exosomal Micro RNA Biomarkers Related to Epithelial-to-Mesenchymal Transition in Pancreatic Cancer. *Anticancer Res* 2020;40:1843-1853.

36 Nakamura S, Sadakari Y, Ohtsuka T, *et al*. Pancreatic Juice Exosomal MicroRNAs as Biomarkers for Detection of Pancreatic Ductal Adenocarcinoma. *Annals of Surgical Oncology* 2019.

37 Maas SLN, Breakefield XO, Weaver AM. Extracellular Vesicles: Unique Intercellular Delivery Vehicles. *Trends in Cell Biology* 2017;27:172-188.

38 Costa-Silva B, Aiello NM, Ocean AJ, *et al*. Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. *Nat Cell Biol* 2015;17:816-26.

39 Xue J, Jia E, Ren N, *et al*. Circulating microRNAs as promising diagnostic biomarkers for pancreatic cancer: a systematic review. *Onco Targets Ther* 2019;12:6665-6684.

40 Levink IJM, Nesteruk K, Visser DI, *et al*. Optimization of Pancreatic Juice Collection: A First Step Toward Biomarker Discovery and Early Detection of Pancreatic Cancer. *Am J Gastroenterol* 2020;115:2103-2108.

41 Lowry OH, Rosebrough NJ, Farr AL, *et al*. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265-75.

42 Verhoeven CJ, Farid WRR, de Ruiter PE, *et al*. MicroRNA profiles in graft preservation solution are predictive of ischemic-type biliary lesions after liver transplantation. J*ournal of Hepatology* 2013;59:1231-1238.

43 Farid WRR, Pan Q, van der Meer AJP, *et al*. Hepatocyte-derived microRNAs as serum biomarkers of hepatic injury and rejection after liver transplantation. *Liver Transplantation* 2012;18:290-297.

44 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402-8.

45 Moriyama T, Ohuchida K, Mizumoto K, *et al*. MicroRNA-21 modulates biological functions of pancreatic cancer cells including their proliferation, invasion, and chemoresistance. *Mol Cancer Ther* 2009;8:1067-74.

46 Kadera BE, Li L, Toste PA, *et al*. MicroRNA-21 in pancreatic ductal adenocarcinoma tumor-associated fibroblasts promotes metastasis. *PLoS One* 2013;8:e71978.

47 Lu H, Zhang L, Lu S, *et al*. miR-25 expression is upregulated in pancreatic ductal adenocarcinoma and promotes cell proliferation by targeting ABI2. *Exp Ther Med* 2020;19:3384-3390.

48 Zhang J, Bai R, Li M, *et al*. Excessive miR-25-3p maturation via N. *Nat Commun* 2019;10:1858.

49 Ho AS, Huang X, Cao H, *et al*. Circulating miR-210 as a Novel Hypoxia Marker in Pancreatic Cancer. *Transl Oncol* 2010;3:109-13.

50 Ni J, Zhou S, Yuan W, *et al*. Mechanism of miR-210 involved in epithelial-mesenchymal transition of pancreatic cancer cells under hypoxia. *J Recept Signal Transduct Res* 2019;39:399-406.

51 Basu A, Jiang X, Negrini M, *et al*. MicroRNA-mediated regulation of pancreatic cancer cell proliferation. *Oncol Lett* 2010;1:565-568.

52 Liu J, Gao J, Du Y, *et al*. Combination of plasma microRNAs with serum CA19-9 for early detection of pancreatic cancer. *Int J Cancer* 2012;131:683-91.

53 Liu G, Shao C, Li A, *et al*. Diagnostic Value of Plasma miR-181b, miR-196a, and miR-210 Combination in Pancreatic Cancer. *Gastroenterol Res Pract* 2020;2020:6073150.

54 Deng T, Yuan Y, Zhang C, *et al*. Identification of Circulating MiR-25 as a Potential Biomarker for Pancreatic Cancer Diagnosis. *Cell Physiol Biochem* 2016;39:1716-1722.

55 Goto T, Fujiya M, Konishi H, *et al*. An elevated expression of serum exosomal microRNA-191, -21, -451a of pancreatic neoplasm is considered to be efficient diagnostic marker. *BMC Cancer* 2018;18:116.

56 Kawamura S, Iinuma H, Wada K, *et al*. Exosome-encapsulated microRNA-4525, microRNA-451a and microRNA-21 in portal vein blood is a high-sensitive liquid biomarker for the selection of high-risk pancreatic ductal adenocarcinoma patients. *Journal of Hepato-Biliary-Pancreatic Sciences* 2019;26:63-72.

57 Wu L, Zhou WB, Zhou J, *et al*. Circulating exosomal microRNAs as novel potential detection biomarkers in pancreatic cancer. *Oncol Lett* 2020;20:1432-1440.

58 Wang J, Raimondo M, Guha S, *et al*. Circulating microRNAs in Pancreatic Juice as Candidate Biomarkers of Pancreatic Cancer. *Journal of Cancer* 2014;5:696-705.

59 Sadakari Y, Ohtsuka T, Ohuchida K, *et al*. MicroRNA expression analyses in preoperative pancreatic juice samples of pancreatic ductal adenocarcinoma. *Jop* 2010;11:587-92.

60 Squadrito ML, Baer C, Burdet F, *et al*. Endogenous RNAs modulate microRNA sorting to exosomes and transfer to acceptor cells. *Cell Rep* 2014;8:1432-46.

61 Feng S, He A, Wang D, *et al*. Diagnostic significance of miR-210 as a potential tumor biomarker of human cancer detection: an updated pooled analysis of 30 articles. *Onco Targets Ther* 2019;12:479-493.

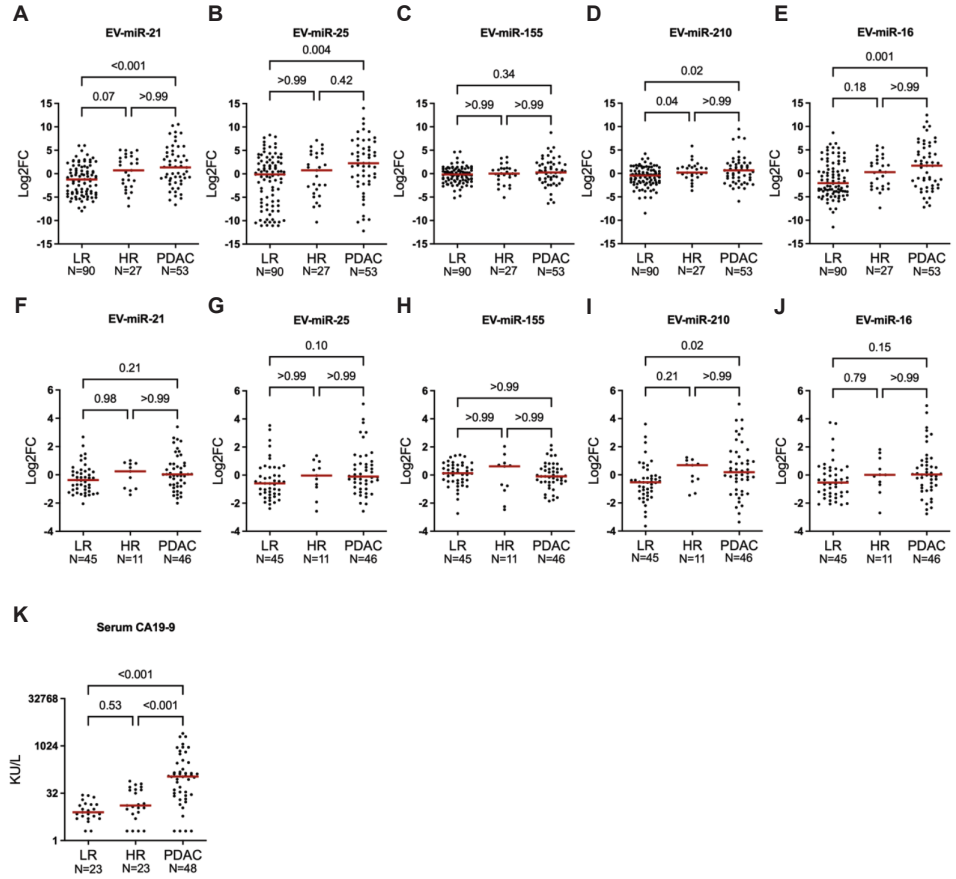
62 Li M, Zeringer E, Barta T, *et al*. Analysis of the RNA content of the exosomes derived from blood serum and urine and its potential as biomarkers. *Philos Trans R Soc Lond B Biol Sci* 2014;369.

63 Goggins M, Overbeek KA, Brand R, *et al*. Management of patients with increased risk for familial pancreatic cancer: updated recommendations from the International Cancer of the Pancreas Screening (CAPS) Consortium. *Gut* 2020;69:7.

64 Poruk KE, Firpo MA, Adler DG, *et al*. Screening for pancreatic cancer: why, how, and who? *Annals of surgery* 2013;257:17-26.

65 Poruk KE, Gay DZ, Brown K, *et al*. The clinical utility of CA 19.9 in pancreatic adenocarcinoma: diagnostic and prognostic updates. *Curr Mol Med* 2013;13:340-51.

66 Yu Q, Xu C, Yuan W, *et al*. Evaluation of Plasma MicroRNAs as Diagnostic and Prognostic Biomarkers in Pancreatic Adenocarcinoma: miR-196a and miR-210 Could Be Negative and Positive Prognostic Markers, Respectively. *Biomed Res Int* 2017;2017:6495867.



SUPPLEMENTAL FIGURE 1 | Relative expression of EV-miR-21, EV-miR-155, EV-miR-210, EV-miR-25, and EV-miR-16 in pancreatic juice (A-E) and serum (F-J) of individuals with pancreatic cancer (PC), low-risk controls (LR) and high-risk controls (HR). K. Level of serum CA19.9 is increased in individuals with pancreatic cancer (PDAC).

SUPPLEMENTAL TABLE S1 | Inclusion criteria prospective cohort studies.

KRASPanc study	
Inclusion	Patients (≥ 18 years of age) who undergo an EUS for (suspected) PC either as part of a diagnostic process or fiducial placement prior to radiotherapeutic treatment.
CAPS study	
Inclusion	Individuals (≥ 18 years of age) who, after evaluation by a clinical geneticist, have an estimated 10-fold increased risk of developing PC, this includes: (1) Carriers of a gene mutation in *CDKN2A* or *STK11*, regardless of the family history of pancreatic cancer (2) Carriers of a gene mutation in *BRCA1, BRCA2, p53,* or Mismatch Repair Gene with a family history of PDAC in ≥ 2 family members. (3) Familial PC (FPC) kindreds, defined as individuals with at least (1) 2 first-degree relatives (FDR) with PC, (2) 3 relatives with PC, either FDR or second degree relative (SDR), or (3) 2 SDR relatives with PC of which at ≥ 1 was <50 years at the time of diagnosis.
PACYFIC study	
Inclusion	Individuals (≥ 18 years of age) with a neoplastic pancreatic cyst (either newly or previously diagnosed, or previously operated upon) for which cyst surveillance is warranted, according to the treating physician.

[a] Received previous chemotherapy, [b] One can have developed multiple worrisome features, ⟶
[c] Development of diabetes mellitus in two years before biomaterial collection [d] Acute pancreatitis in 2 years before biomaterial collection (not related to performed ERCP).

SUPPLEMENTAL TABLE S2 | Clinical characteristics (serum).

	Cases (N=46)	Controls (N=58)	*P*-value
Age in years, median (IQR)	68 (10.5)	60 (7.3)	0.001
Male gender, n (%)	29 (63.0)	15 (25.9)	<0.001
BMI in kg/m², median (IQR)	23.2 (3.2)	25.7 (5.2)	0.003
Familial/genetic predisposition, n (%)	0 (0.0)	29 (50.0)	<0.001
Member of FPC family	.	13 (22.4)	
CDKN2A p16	.	10 (17.2)	
BRCA2 + ≥ 2 blood relatives with PDAC	.	3 (5.2)	
BRCA1 + ≥ 2 blood relatives with PDAC	.	0 (0.0)	
PALB2 + ≥ 2 blood relatives with PDAC	.	1 (1.7)	
BRCA2 + CDKN2A p16	.	1 (1.7)	
STK11	.	1 (1.7)	
Diabetes mellitus, n (%)	19 (41.3)	9 (15.5)	0.004
Indication EUS, n (%)			<0.001
Suspected PDAC	28 (60.9)	3 (5.2)	
Fiducial placement[a]	18 (39.1)	0 (0.0)	
Surveillance	0 (0.0)	55 (94.8)	
CBD stent in situ, n (%)			<0.001
CBD stent in situ	8 (17.4)	0 (0.0)	
No CBD stent and CBD dilation	10 (21.7)	0 (0.0)	
No CBD stent and no CBD dilation	28 (60.9)	58 (100.0)	
Relative or absolute indications for surgery,[b] n (%)	46 (100.0)	11 (19.0)	<0.001
Enhancing mural nodule or hypodense lesion	46 (100.0)	0 (0.0)	
Caliber change	35 (76.1)	0 (0.0)	
Diffuse PD dilation > 5mm	0 (0.0)	7 (12.1)	
CA19.9 ≥37 kU/L	27 (58.7)	4 (6.9)	
Cyst size > 40mm	0 (0.0)	2 (3.4)	
New-onset diabetes[c]	7 (15.2)	1 (1.7)	
Recent acute pancreatitis[d]	2 (4.3)	4 (6.9)	
Lymphadenopathy	21 (45.7)	0 (0.0)	
Working diagnosis, n (%)			<0.001
No abnormalities	.	18 (31.0)	
Unspecified cyst	.	10 (17.2)	
SB-IPMN	.	23 (39.7)	
MD-IPMN	.	2 (3.4)	
MT-IPMN		5 (8.6)	
MCN	.	0 (0.0)	
NET	.	0 (0.0)	
Indeterminate, not suspect for malignancy	.	0 (0.0	
Resectable PDAC	11 (23.9)	.	
Locally advanced PDAC	35 (76.1)	.	
Distal metastases (on imaging), n (%)	6 (13.0)	0 (0.0)	<0.001
Time in days between serum and PJ sample collection, median (IQR)	0 (0.0)	0 (9.3)	0.003

SUPPLEMENTAL TABLE S3 | EV-miR expression levels in cases (N=42) correlate with each other in serum and in PJ, but not between these two biomarker sources. (*P<0.05)

Spearman R					
	Serum EV-miR-21	Serum EV-miR-25	Serum EV-miR-155	Serum EV-miR-210	Serum EV-miR-16
Serum EV-miR-21		0.81 (*)	0.40 (*)	0.85 (*)	0.75 (*)
Serum EV-miR-25			0.16	0.83 (*)	0.96 (*)
Serum EV-miR-155				0.23	0.18
Serum EV-miR-210					0.80 (*)
Serum EV-miR-16					
	PJ EV-miR-21	PJ EV-miR-25	PJ EV-miR-155	PJ EV-miR-210	PJ EV-miR-16
PJ EV-miR-21		0.90 (*)	0.83 (*)	0.86 (*)	0.93 (*)
PJ EV-miR-25			0.78 (*)	0.76 (*)	0.93 (*)
PJ EV-miR-155				0.85 (*)	0.86 (*)
PJ EV-miR-210					0.84 (*)
PJ EV-miR-16					
	PJ EV-miR-21	PJ EV-miR-25	PJ EV-miR-155	PJ EV-miR-210	PJ EV-miR-16
Serum EV-miR-21	-0.11	-0.03	0.00	0.02	-0.16
Serum EV-miR-25	0.06	0.20	0.05	0.13	0.02
Serum EV-miR-155	-0.03	-0.03	0.11	0.03	-0.01
Serum EV-miR-210	0.00	0.09	0.06	0.08	-0.03
Serum EV-miR-16	0.02	0.19	0.03	0.08	0.00
p-value					
	Serum EV-miR-21	Serum EV-miR-25	Serum EV-miR-155	Serum EV-miR-210	Serum EV-miR-16
Serum EV-miR-21		1.09E-10	0.01	1.67E-12	8.09E-09
Serum EV-miR-25			0.31	6.33E-12	1.76E-23
Serum EV-miR-155				1.48E-01	2.54E-01
Serum EV-miR-210					2.57E-10
Serum EV-miR-16					
	PJ EV-miR-21	PJ EV-miR-25	PJ EV-miR-155	PJ EV-miR-210	PJ EV-miR-16
PJ EV-miR-21		3.01E-16	1.15E-11	1.73E-13	5.72E-19
PJ EV-miR-25			1.47E-09	4.84E-09	8.90E-19
PJ EV-miR-155				1.29E-12	5.07E-13
PJ EV-miR-210					3.26E-12
PJ EV-miR-16					
	PJ EV-miR-21	PJ EV-miR-25	PJ EV-miR-155	PJ EV-miR-210	PJ EV-miR-16
Serum_miR-21	0.48	0.83	0.98	0.88	0.30
Serum_miR-25	0.73	0.20	0.76	0.40	0.91
Serum_miR-155	0.83	0.84	0.48	0.85	0.97
Serum_miR-210	1.00	0.57	0.72	0.61	0.85
Serum_miR-16	0.91	0.22	0.86	0.63	0.99

SUPPLEMENTAL TABLE S4 | EV-miR expression levels in cases (N=55) correlate with each other in serum and in PJ, but not between these two biomarker sources. (*P<0.05)

Spearman R					
	Serum EV-miR-21	Serum EV-miR-25	Serum EV-miR-155	Serum EV-miR-210	Serum EV-miR-16
Serum EV-miR-21		0.81 (*)	0.47 (*)	0.83 (*)	0.84 (*)
Serum EV-miR-25			0.37 (*)	0.88 (*)	0.97 (*)
Serum EV-miR-155				0.43 (*)	0.44 (*)
Serum EV-miR-210					0.89 (*)
Serum EV-miR-16					
	PJ EV-miR-21	PJ EV-miR-25	PJ EV-miR-155	PJ EV-miR-210	PJ EV-miR-16
PJ EV-miR-21		0.83 (*)	0.62 (*)	0.58 (*)	0.94 (*)
PJ EV-miR-25			0.67 (*)	0.53 (*)	0.88 (*)
PJ EV-miR-155				0.49 (*)	0.61 (*)
PJ EV-miR-210					0.63 (*)
PJ EV-miR-16					
	PJ EV-miR-21	PJ EV-miR-25	PJ EV-miR-155	PJ EV-miR-210	PJ EV-miR-16
Serum EV-miR-21	-0.06	-0.09	-0.25	-0.03	-0.05
Serum EV-miR-25	0.03	-0.06	-0.22	0.01	0.04
Serum EV-miR-155	0.19	0.12	0.26 (*)	0.29	0.16
Serum EV-miR-210	0.08	0.00	-0.18	0.12	0.09
Serum EV-miR-16	0.02	-0.07	-0.20	0.04	0.03
p-value					
	Serum EV-miR-21	Serum EV-miR-25	Serum EV-miR-155	Serum EV-miR-210	Serum EV-miR-16
Serum EV-miR-21		4.92E-14	2.61E-04	6.51E-15	1.14E-15
Serum EV-miR-25			0.01	3.27E-19	4.15E-33
Serum EV-miR-155				1.18E-03	9.00E-04
Serum EV-miR-210					2.10E-19
Serum EV-miR-16					
	PJ EV-miR-21	PJ EV-miR-25	PJ EV-miR-155	PJ EV-miR-210	PJ EV-miR-16
PJ EV-miR-21		5.30E-15	3.69E-07	3.32E-06	1.56E-25
PJ EV-miR-25			2.52E-08	3.77E-05	1.31E-18
PJ EV-miR-155				1.30E-04	7.11E-07
PJ EV-miR-210					2.03E-07
PJ EV-miR-16					
	PJ EV-miR-21	PJ EV-miR-25	PJ EV-miR-155	PJ EV-miR-210	PJ EV-miR-16
Serum_miR-21	0.66	0.52	0.06	0.82	0.71
Serum_miR-25	0.85	0.69	0.11	0.91	0.78
Serum_miR-155	0.17	0.40	0.05	0.03	0.24
Serum_miR-210	0.55	0.97	0.19	0.37	0.49
Serum_miR-16	0.87	0.63	0.14	0.78	0.81

SUPPLEMENTAL TABLE S5 | Summary of studies with selected miR.

Ref	miR	Biofluid	Isolated EVs or total biofluid	N Cases / Controls	Control group	Sensitivity (%)	Specificity (%)	Normalization method
20	miR-25	Serum	Total	80 / 91	HC	83	94	Absolute quantification
52	miR-16	Serum	Total	140 / 111+68	CP + HC	NG AUC=0.75	NG	cel-miR-39
52	miR-21	Serum	Total	140 / 111+68	CP + HC	NG AUC=0.78	NG	cel-miR-39
52	miR-155	Serum	Total	140 / 111+68	CP + HC	NG AUC=0.70	NG	cel-miR-39
52	miR-210	Serum	Total	140 / 111+68	CP + HC	NG AUC=0.76	NG	cel-miR-39
54	miR-25	Serum	Total	303 / 600 + 160	NM + 40 CP, 20 GC, 20 LuC, 20 EC, 20 CRC, 20 LiC, 20 BC	76	93	Serum volume
55	miR-21	Serum	EV and total	32 / 22	NM + NPNH	81 (for total: 54)	81 (for total: 76)	NG
23	miR-21	Serum	EVs	22 / 27	6 benign pancreatic, 7 AC, 6 CP, 8 HC	82	96	RNU6B
23	miR-155	Serum	EVs	22 / 27	6 benign pancreatic, 7 AC, 6 CP, 8 HC	Low expression	NG	RNU6B
57	miR-21	Serum	EVs and Total	30 / 10	CP	80% (for total 73%)	90% (for total 70%)	cel-miR-39
57	miR-210	Serum	EVs and Total	30/10	CP	83% (for total 76%)	90% (for total 70%)	cel-miR-39
66	miR-21, miR-210, miR-155, miR-25	Plasma	Total	31 / 28	HC	NG, AUC miR-21: 0.85 miR-210: 0.69 miR-155: 0.82 miR-25: 0.76	NG	absolute quantification and miR-39
53	miR-210	Plasma	Total	40 / 40	HC	83	80	U6
56	miR-21	Plasma	EVs	55 / 20	HC	73 (peripheral blood 55)	73 (peripheral blood 64)	RNU6B
58	miR-210	PJ	Total	50 / 19+19	NPNH + CP	76	95	RNU6B
36	miR-21	PJ	EVs and Total	27 / 8	CP	81 (for total AUC=0.71)	88	miR-16
36	miR-155	PJ	EVs and Total	27 / 8	CP	89 (for total AUC=0.56)	88	miR-16
59	miR-21	PJ	Cell pellet	16 / 5	CP	NG	NG	RNU6B, miR-191
59	miR-155	PJ	Cell pellet	16 / 5	CP	NG	NG	RNU6B, miR-191

Ref = reference, AC = ampullary cancer, BC = breast cancer, CP = chronic pancreatitis, CRC = colorectal cancer GC = gastric cancer, HC = healthy control, LiC = liver cancerLuC = lung cancer, NG = not given, NM = non-malignant, NPNH = non-pancreatic non healthy.

CHAPTER 10

Size and Concentration of Extracellular vesicles in Pancreatic Juice from Pancreatic Ductal Adenocarcinoma Patients

Authors
Kateryna Nesteruk, Iris J.M. Levink, Natasja F.J. Dits, Djuna L. Cahen, Maikel P. Peppelenbosch, Marco J. Bruno, Gwenny M. Fuhler

Clinical and Translational Gastroenterology, 13(3): p e00465, March 2022.

Introduction

Detection of pancreatic cancer (PC) at a curable stage is challenging due to the late presentation of symptoms and limited visibility of sub-centimeter lesions on imaging. Therefore, accurate biomarkers for early detection are urgently needed. In recent years, extracellular vesicles (EVs) have gained interest as potential disease biomarkers. EVs carry a unique molecular cargo to communicate between cells and are expected to represent a cell-specific signature.[1] Cancer cells release EVs to form a pre-metastatic niche.[1] Thus, detection of cancer-derived EVs based on their content may predict the presence of disease.

While blood-born EVs are most frequently studied in this context, for PC, pancreatic juice (PJ) may be a promising biomarker source, as it is in close contact with ductal cells from which PC arises. Indeed, detection of microRNA molecules from EVs derived from PJ was able to distinguish PC from controls.[2] Interestingly, the concentration of EVs determined by nanoparticles tracking analysis in bile discriminated patients with malignant (including PC) from non-malignant common bile duct stenosis with 100% accuracy.[3] These data suggest that cancer cells emit elevated numbers of EVs in the extracellular space,[3] which may be exploited to detect the presence of cancer without studying specific EV content and would greatly simplify testing. Here, we characterize the size and concentration of EVs in PJ and serum of PC patients and controls, to establish whether this may present a promising biomarker for early detection of PC.

Methods

Selection of subjects

This study was executed at the Erasmus University Medical Center and analyzed PJ and serum was collected between August 2018 and May 2020 in patients who participate in the following prospective study cohorts: 1) Patients with suspected (sporadic) PC (KRASPanc study, MEC-2018-038); 2) high-risk individuals under surveillance for a hereditary predisposition or familiarly history of PC (CAPS study, MEC-2012-448, www.caps-registry.com);[4] 3) individuals under surveillance for neoplastic pancreatic cysts (PACYFIC study, MEC-2014-021, www.pacyfic.net). The Erasmus Medical Center ethical review board approved the studies, and the included individuals gave written consent before enrolment. The study was carried out according to the ethical principles for medical research involving human subjects from the World Medical Association Declaration of Helsinki.

PJ collection

PJ was collected as described before5. In short, during EUS, PJ collection was performed after visualization of the ampullary orifice. To reduce duodenal contamination, duodenal fluid was aspirated prior to juice collection. Next, a wash-out of PJ was stimulated by intravenous administration of human synthetic secretin (ChriRhoStim, Burtonsville, MD, 16µg/patient). PJ was collected for up to 8 minutes starting immediately after injection with the endoscope (Pentax Medical, Tokyo, Japan) and assembled in a mucus extractor (Pennine Healthcare, Derby, United Kingdom, 15 mL) attached to the proximal end of the endoscopic channel. PJ was aliquoted, snap frozen within 10 minutes after collection and stored at -80°C until further use.

EV analysis

PJ was centrifuged for 10 min at 4000 RPM 4°C to remove debris. Then, 100 µL of Total Exosome Isolation Reagent (Thermo Fisher Scientific, Waltham, MA, #4478359) was added to 200 µL of PJ supernatant and kept on a rollerbank overnight at 4°C. After this, samples were centrifuged for 1 h at 14000 RPM and the pellet was resuspended in 400 µL of PBS (pre-filtered with 0.2 µM filter).

Serum was centrifuged during 30 min at 2000 g at 4°C. 40 µL of Total Exosome Isolation (Thermo Fisher Scientific, Waltham, MA, #4478360) were added to 200 µL of serum supernatant and incubated 30 min at 4°C. Then, samples were centrifuged 10 min at 10000 g and pellet was resuspended in 200 µL of filtered PBS. EVs were stored at -80°C until further analysis.

The total protein concentration was determined by Lowry assay (Bio-Rad, Hercules, CA, USA).[6] For Nanoparticle Tracking Analysis (NTA), samples were diluted 1:1000 in filtered PBS. The size and concentration were detected by NanoSight NS300 (NTA 3.4 Build 3.4.003 software). Two measurements of each sample were performed. EVs were visualized with Transmission electron microscopy (TEM). For this, 10 µL droplets were deposited on formvar/carbon coated 400 Mesh Cu grids and incubated for 10 min. Thereafter, remaining liquid was drained with filter paper, samples were stained with a drop of Uranyless stain for 1 minute. Remaining liquid was drained, and grids allowed to air dry. Grids were observed under the electron microscope Talos L120C TEM from Thermofisher Scientific at 120 kV. For Western blot, total proteins were extracted in 300 µl Laemmli Buffer [SDS 4%, glycerol 20%, Tris-Cl (pH 6.8) 120 mM, bromophenol blue 0.02% (w/v) and DTT 0.1 M]. Proteins were resolved by SDS-PAGE and blotted onto Immobilon FL PVDF membranes (Millipore, Bedford, MA, USA). Membranes were blocked in Odyssey Blocking Buffer (Thermo Fisher Scientific) and incubated overnight at 4 °C with primary antibody (Caveolin-1 [Cell Signaling Technology #3238], CD81, GAPDH [Santa Cruz Biotechnology #sc-51906]), followed by the appropriate Alexa-linked secondary antibodies, at 1:5000 dilution, in Odyssey Blocking Buffer for 1 h. The fluorescent bands were detected using fluorescent Odyssey Imaging System and densitometric analysis was performed with Image Studio Lite Ver.5.2.

TABLE 1 | Clinical characteristics at time of pancreatic juice collection.

	Cases (N=54)	Controls (N=117)	*P*-value
Age in years, median (IQR)	67.5 (10.3)	62.0 (6.0)	0.001
Male gender, n (%)	34 (63.0)	40 (34.2)	<0.001
BMI in kg/m², median (IQR)	23.7 (3.7)	25.7 (5.0)	0.001
Familial/genetic predisposition, n (%)	0 (0.0)	66 (56.4)	<0.001
Member of FPC family[a]	.	31 (26.5)	.
CDKN2A p16	.	24 (20.5)	.
BRCA2 + ≥ 2 blood relatives with PC	.	5 (4.3)	.
BRCA1 + ≥ 2 blood relatives with PC	.	1 (0.9)	.
PALB2 + ≥ 2 blood relatives with PC	.	1 (0.9)	
BRCA2 + CDKN2A p16	.	1 (0.9)	.
STK11/LKB1	.	2 (1.7)	.
Diabetes mellitus, n (%)	21 (38.9)	16 (13.7)	0.001
Indication EUS, n (%)			<0.001
Suspected PC	35 (64.8)	4 (3.4)	.
Fiducial placement[b]	18 (33.3)	0 (0.0)	.
Surveillance	1 (1.9)	114 (96.6)	.
CBD stent in situ, n (%)			<0.001
CBD stent in situ	9 (16.7)	0 (0.0)	.
No CBD stent and CBD dilation	14 (25.9)	3 (2.5)	.
No CBD stent and no CBD dilation	31 (57.4)	115 (97.5)	.
Relative or absolute indications for surgery,[c,7] n (%)	54 (100.0)	26 (22.2)	<0.001
Enhancing mural nodule or hypodense lesion	54 (100.0)	4 (3.4)	
Caliber change	41 (75.9)	0 (0.0)	
Diffuse PD dilation > 5mm	0 (0.0)	14 (12.0)	
CA19.9 ≥37 kU/L	34 (63.0)	7 (6.0)	
Cyst size > 40mm	0 (0.0)	2 (1.7)	
New-onset diabetes[d]	9 (16.6)	2 (1.7)	
Recent acute pancreatitis[e]	2 (3.7)	6 (5.1)	
Lymphadenopathy	23 (42.6)	0 (0.0)	
Working diagnosis, n (%)			<0.001
No abnormalities	.	41 (35.0)	.
Unspecified cyst	.	9 (7.7)	.
SB-IPMN	1 (1.9)	50 (41.9)	.
MD-IPMN or MT-IPMN	.	14 (12.0)	.
MCN	.	1 (0.9)	
NET	.	1 (0.9)	.
Indeterminate lesion, not suspect for malignancy	.	2 (1.7)	.
Resectable PC	10 (18.5)	0 (0.0)	.
Locally advanced PC	43 (79.6)	0 (0.0)	.
Distal metastases (on imaging), n (%)	8 (14.8)	0 (0.0)	<0.001

[a] ≥2 first-degree relatives or 3 relatives (either first or second degree) or ≥2 second-degree relatives of which ≥1 with age <50 years at time of diagnosis; [b] Received previous chemotherapy; [c] One can have developed multiple worrisome features; [d] Development of diabetes mellitus in two years before biomaterial collection; [e] Acute pancreatitis in 2 years before biomaterial collection (not related to performed ERCP); 13 extra post-ERCP pancreatitis.

Statistical analysis

Graphpad Prism 9 (GraphPad Software Inc.) and SPSS (version 25, SPSS Inc., IBM, Chicago, IL) software were used for the generation of graphs and statistical analyses. The Shapiro–Wilk test was used to determine data distribution; the Mann-Whitney U test was performed to compare 2 groups.

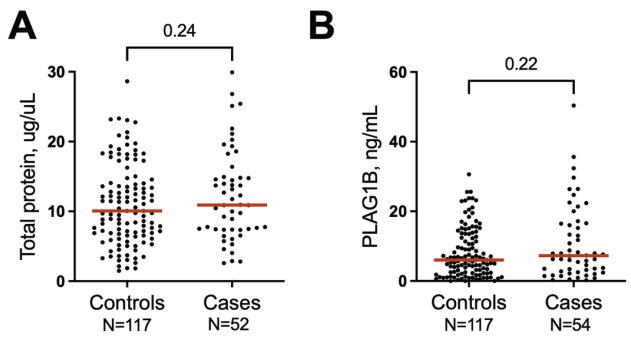


FIGURE 1 | **Comparison of protein concentrations between cases and controls.** No difference was seen between controls and cases in pancreatic juice concentration of total protein (A) and phospholipase A2 group IB (PLA2G1B) (B), as a measure of pancreatic origin, indicating similar composition of pancreatic juice.

Results

A summary of clinical characteristics is provided in **Table 1**. Pancreas-specific PLA2G1B was detected in PJ of all PC patients and 115/117 controls, and concentrations of PLA2G1B (p=0.22) and total protein content (p=0.24) did not differ between cases and controls, indicating similar PJ quality (**Figure 1)**.

For both PJ and serum, isolated EV fractions showed round double-membrane vesicle-like structures, typical of EVs (**Figure 2A**), which express membrane and cytoplasmic EV markers such as CD81, caveolin-1 and GAPDH (**Figure 2B**). NTA analysis allowed visualization of heterogeneous populations of spherical nanoparticles moving under Brownian motion (**Figure 2C**). The concentration of EVs was significantly higher in serum (median $3.28*10^{12}$ particles/ml; 95% CI $2.85*10^{12}$-$3.68*10^{12}$) than in PJ (median $8.42*10^{11}$ particles/ml; 95% CI $7.53*10^{11}$-$9.49*10^{11}$, p<0.001).

When comparing cases and controls, the total protein content in EVs isolated from PJ or serum are not different for serum (p=0.35) and PJ (p=0.38; **Figure 2D**). Similarly, NTA showed no difference in particle concentrations between cases and controls in PJ

(median $7.73*10^{11}$ [95% CI $5.92*10^{11}$-$1.145*10^{12}$] for cases and $8.71*10^{11}$ particles/ml [95% CI $7.67*10^{11}$-$9.86*10^{11}$] for controls, *P* = 0.41), and serum (median $3.28*10^{12}$ [95% CI $2.82*10^{12}$-3.96*1012] vs $3.34*10^{12}$ [95% CI $2.71*10^{12}$-$3.94*10^{12}$; p=0.84], respectively; **Figure 2E**).

PJ-derived EVs appeared larger than serum EVs in TEM analysis, which was confirmed by NTA analysis (mode diameter of 116 nm [95% CI 114.2-120.2] for PJ and 82 nm [95% CI 80.2-84.1] for serum, *P* < 0.0001). No difference in mode diameter was observed between cases and controls for either PJ (**Figure 2F**) or serum (**Figure 2G**). However, when comparing the concentration of EVs according to their size distribution, a significant difference between cases and controls was seen in PJ, but not in serum. In PJ, particles with the sizes 102.5 nm, 355.5-385.5 nm, 534.5-629.5 nm, 631.5-642.5 nm and 645.5-647.5 nm reached significantly higher concentrations (p<0.05) in cases, as compared to controls (**Figure 2H**). When selecting a threshold of 350 nm, cases had a higher proportion of large EVs (size >350 nm) in PJ as compared to controls (*P* < 0.001, **Figure 2I**).

Discussion

This study shows that, as compared to serum, EVs from PJ are larger while their absolute concentration is lower, indicating a distinct proportional composition of EV-subtypes in PJ. We, and Severino *et al.* (2017),[3] did not find differences in EV-concentrations between PC cases and controls in serum, where the vast majority of EVs may be of non-tumor origin. In contrast to previous reports for bile,[3] showing higher EV-concentrations in bile in cases with cholangiocarcinoma and PC with biliary stenosis as compared to controls, we did not find differences in PJ-derived EV-concentrations between cases and controls.

However, the proportion of large EVs (>350 nm) in PJ of PC cases was significantly increased, suggesting a different prevalence of distinct subtypes of EVs in these groups. The number of large EVs correlated with pancreas-specific PLA2G1B levels (not shown), implying that these larger-sized EVs are of pancreatic origin, and that EV-size may be a promising tool to discriminate PC patients from controls. This also confirms the findings by Severino *et al.* (2017),[3] who showed that bile-derived EVs are larger in cancer patients compared to patients with chronic pancreatitis. However, this study did not reveal the exact size distributions.[3]

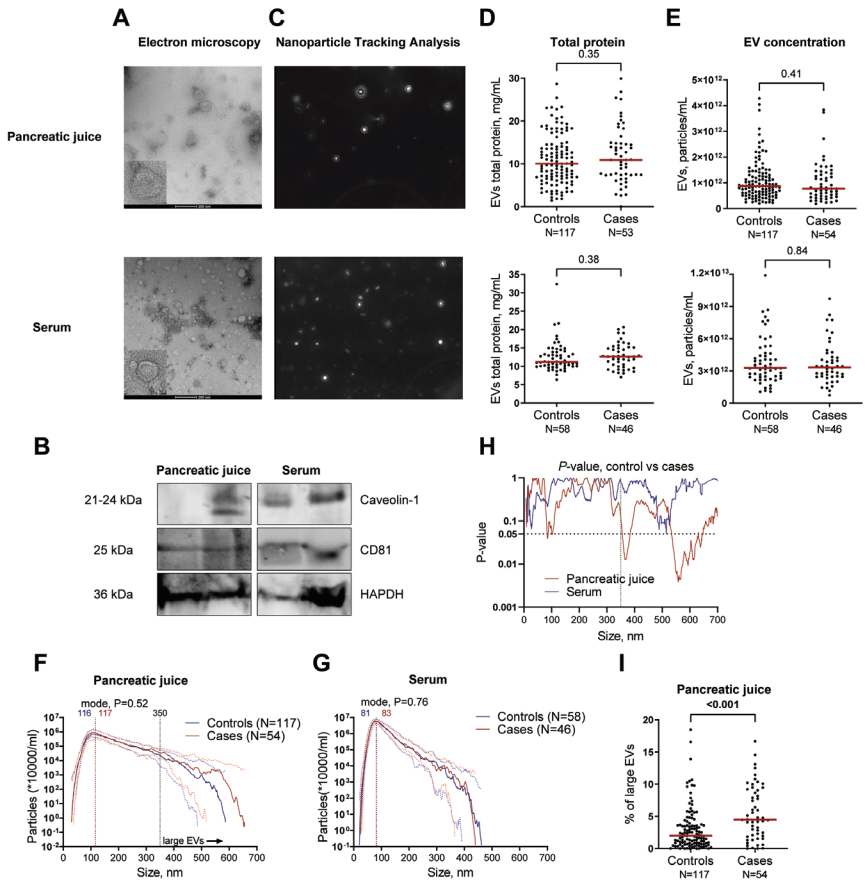


FIGURE 2 | Analysis of EVs in PJ of controls and individuals with PC (cases) shows a different size distribution between these groups. A. Representative images of EVs extracted from pancreatic juice (PJ) and serum by transmission electron microscopy (TEM) showing the presence of double membrane vesicles. Notice the larger size of EVs in PJ compared to serum. **B.** Western blot analysis of typical EV markers commonly found in exosome subpopulations. **C.** Representative NTA images for pancreatic juice (PJ) and serum. **D, E.** Total protein content in EVs isolated from PJ (upper panel) or serum (lower panel) are equal between controls and cases and NTA showed no difference in particle concentrations between controls and cases in PJ (E, median concentration of $8.71*10^{11}$ particles/ml [95% CI $7.67*10^{11}$-$9.86*10^{11}$] for controls vs $7.73*10^{11}$ [95% CI $5.92*10^{11}$-$1.145*10^{12}$] for cases, $P = 0.41$) or serum (E, median $3.28*10^{12}$ [95% CI $2.82*10^{12}$-$3.96*10^{12}$] and $3.34*10^{12}$ [95% CI $2.71*10^{12}$-$3.94*10^{12}$; p=0.84], respectively. **F, G.** Median concentration of EVs of different sizes (from 0 to 750 with stepwise increments of 0.5 nm) in PJ (F) and serum (G). For PJ, vertical lines indicate mode size of 116 and 117 nm for controls and cases respectively ($P = 0.52$). A threshold line of 350 nm indicating large size particles is indicated, with cases having more EVs with diameter > 350 nm then controls. For serum, vertical lines indicate mode diameter of 81 and 83 nm for controls and cases respectively ($P = 0.76$). Scattered lines indicate IQR. **H.** Comparison of the concentration of EVs of different sizes (from 0 to 750 with stepwise increments of 0.5 nm) between controls and cases. *P*-values for PJ are indicated in red, serum in blue. While the number of EVs in serum is similar between cases and controls across the size ranges, cases present significantly more EVs in the larger range as compared to controls in PJ. Dotted line indicates significance threshold level of p=0.05. **I.** Percentage of large EVs (>350 nm) in PJ is higher in cases *vs* controls.

EVs are classified based on size and their biogenesis: exosomes (<150 nm) are released through multivesicular bodies (MVBs) in the endosomal pathway, microvesicles (200-500 nm) are formed by budding from the plasma membrane, and apoptotic bodies of various sizes derive from programmed cell death. In addition, many other specialized EVs subtypes have been described.[8] Due to a significant overlap in size, similarities in composition and lack of specific markers, it is difficult to assign individual EVs to one of the biogenesis pathways, but the nature of the large EVs found in PJ of PC patients represents an interesting research question.

Furthermore, we show that EV extraction from PJ with isolation kits requiring microcentrifuges yields similar concentrations as reported for extraction with ultracentrifugation.[2,9] As microcentrifuges are commonly available in laboratories, this finding facilitates the application of EVs as a clinical biomarker.

In summary, we characterized vesicular composition of PJ in cases with PC and controls undergoing surveillance and found that PJ from individuals with PC harbor increased amounts of large EVs, which may be useful for future biomarker development.

References

1 Costa-Silva B, Aiello NM, Ocean AJ, *et al*. Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. *Nat Cell Biol* 2015; 17: 816-826.

2 Nakamura S, Sadakari Y, Ohtsuka T, *et al*. Pancreatic Juice Exosomal MicroRNAs as Biomarkers for Detection of Pancreatic Ductal Adenocarcinoma. *Ann Surg Oncol* 2019; 26: 2104-2111.

3 Severino V, Dumonceau JM, Delhaye M, *et al*. Extracellular Vesicles in Bile as Markers of Malignant Biliary Stenoses. *Gastroenterology* 2017; 153: 495-504.e498.

4 Overbeek KA, Levink IJM, Koopmann BDM, *et al*. Long-term yield of pancreatic cancer surveillance in high-risk individuals. *Gut* 2022; 71: 1152-1160.

5 Levink IJM, Nesteruk K, Visser DI, *et al*. Optimization of Pancreatic Juice Collection: A First Step Toward Biomarker Discovery and Early Detection of Pancreatic Cancer. *Am J Gastroenterol* 2020; 115: 2103-2108.

6 Lowry OH, Rosebrough NJ, Farr AL, *et al*. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193: 265-275.

7 European evidence-based guidelines on pancreatic cystic neoplasms. *Gut* 2018; 67: 789-804.

8 Maas SLN, Breakefield XO and Weaver AM. Extracellular Vesicles: Unique Intercellular Delivery Vehicles. *Trends Cell Biol* 2017; 27: 172-188. 2016/12/17.

9 Zheng J, Hernandez JM, Doussot A, *et al*. Extracellular matrix proteins and carcinoembryonic antigen-related cell adhesion molecules characterize pancreatic duct fluid exosomes in patients with pancreatic cancer. *HPB (Oxford)* 2018; 20: 597-604.

CHAPTER 11

Systematic Review and Meta-analysis: Diagnostic Performance of DNA Alterations in Pancreatic Juice for the Detection of Pancreatic Cancer

Authors
Iris J.M. Levink*, Isis J. Visser*, Maikel P. Peppelenbosch, Gwenny M. Fuhler, Marco J. Bruno, Djuna L. Cahen

*These authors contributed equally

Pancreatology, 2022 Nov;22(7):973-986.

Abstract

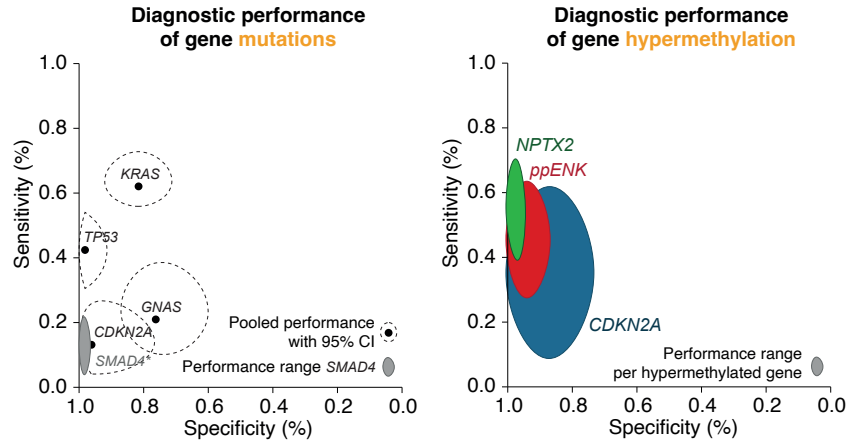
Background and Aims: Pancreatic cancer has a dismal prognosis. So far, establishing an early enough diagnosis by imaging has been proven difficult. Thus, biomarkers are urgently needed for early detection and improved survival. Our aim was to evaluate the pooled diagnostic performance of DNA alterations in pancreatic juice.

Methods: A systematic literature search was performed in EMBASE, MEDLINE Ovid, Cochrane CENTRAL and Web of Science for studies concerning the diagnostic performance of DNA alterations in pancreatic juice to differentiate patients with high-grade dysplasia or pancreatic cancer from controls. Study quality was assessed using QUADAS-2. The pooled prevalence, sensitivity, specificity and diagnostic odds ratio were calculated.

Results: Studies mostly concerned cell-free DNA mutations (32 studies: 939 cases, 1678 controls) and methylation patterns (14 studies: 579 cases, 467 controls). *KRAS, TP53, CDKN2A, GNAS* and *SMAD4* mutations were evaluated most. Of these, *TP53* had the highest diagnostic performance with a pooled sensitivity of 42% (95% CI: 31-54%), specificity of 98% (95%-CI: 92%-100%) and diagnostic odds ratio of 36 (95% CI: 9-133). For DNA methylation, hypermethylation of *CDKN2A, NPTX2* and *ppENK* were studied most. Hypermethylation of *NPTX2* performed best with a sensitivity of 39-70% and specificity of 94-100% for distinguishing pancreatic cancer from controls.

Conclusions: This meta-analysis shows that in pancreatic juice, the presence of distinct DNA mutations (*TP53, SMAD4* or *CDKN2A*) and *NPTX2* hypermethylation have a high specificity (close to 100%) for the presence of high-grade dysplasia or pancreatic cancer. However, the sensitivity of these DNA alterations is poor to moderate, yet may increase if they are combined in a panel.

Graphical abstract



Pooled and summary diagnostic performance of genes that were investigated in three or more studies. A. Pooled performance with 95% interval for genes described in five or more studies. **B.** Range of sensitivity and specificity for genes investigated in 3-4 studies.

Introduction

Pancreatic cancer (PC) is a leading cause of cancer-related death, with a dismal five-year survival rate of 9%.[1] As early detection provides the best chance for cure, surveillance is advocated in high-risk groups. According to guidelines, hereditary predisposed Individuals and those with a neoplastic pancreatic cyst are being followed by imaging with endoscopic ultrasound (EUS) and/or magnetic resonance imaging (MRI).[2,3] Unfortunately, even when both modalities are applied concurrently, high-grade dysplasia (HGD) and early cancer are being missed.[3] Thus, novel diagnostic methods are needed.

In the last decades, DNA analysis techniques have greatly improved. This resulted in numerous studies investigating its potential for diagnostic testing, characterization of the tumor genome, personalized therapy and monitoring of therapeutic efficacy[4-6] (mostly in blood). The majority of these studies evaluated the value of gene mutations and (hyper) methylation. The latter, (epi) genetic changes, cause malignant development through silencing of tumor suppressor genes.

Pancreatic juice (PJ) seems a promising alternative DNA source, as it is produced by (and in close contact with) the ductal cells from which PC originates. Its excretion can be stimulated by intravenous secretin infusion during EUS allowing non-invasive collection. Our group has already shown that PJ harbors 50-250x higher concentrations of cell-free DNA than blood.[7] As tumor progression takes months to years, circulating tumor DNA

(ctDNA) is likely to be detectable from an early stage,[8] creating a window of opportunity for detection. In clinical practice, PJ DNA analysis may differentiate PC or HGD from individuals with a neoplastic cyst with low-grade dysplasia (LGD) or inflammation (*e.g.*, groove, chronic or auto-immune pancreatitis).[9]

Multiple promising genetic and epigenetic DNA alterations have been identified in PJ. The aim of this systematic review and meta-analysis was to calculate the pooled diagnostic performance (diagnostic odds ratio [DOR], sensitivity, specificity) of frequently investigated DNA alterations in order to establish DNA alterations that promise for future research and implementation in clinical practice. Detection of a marker with a specificity close to 100% for detection of HGD or PC would prompt for surgery; lower is not acceptable as it would cause unnecessary harm to those who appear to be benign after surgery. Also, during surveillance, changes in PJ DNA markers over time may warrant intensified surveillance or (in case of high specificity) prompt for surgery.

Materials and Methods

Literature search and screening strategy

In November 2020, a systematic literature search was performed for studies concerning the diagnostic performance of DNA alterations in PJ for detection of PC or HGD. The search was executed in EMBASE, MEDLINE Ovid, Cochrane CENTRAL and Web of Science (core collection). The keywords were: DNA, biomarkers, pancreatic juice, pancreatic ductal adenocarcinoma/cancer, intraductal pancreatic mucinous neoplasm (IPMN). The full search string can be found in **Supplemental Materials and Methods Table 1**. Reference lists of recent reviews were assessed for any missed studies. The review was written according to the Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) statement. The protocol for this systematic review was registered on PROSPERO (ID: CRD42020176792).

Study selection

Based on title and abstract, articles were considered eligible in case of original research with PC or HGD as investigated group, evaluation of DNA alterations in PJ, and involvement of both a case and control group of at least ten individuals. DNA alterations were restricted to the three most frequently evaluated, namely mutations, methylation and chromosomal instability. Studies were included if they reported sensitivity and specificity, or metrics from which these could be derived. Articles published in another language than English, reviews, case reports, letters-to-editor and conference abstracts were excluded. If a research group had published multiple articles and duplication of data could not be ruled out, the most recent article was included. Screening was executed by two authors (I.V. and I.L.) independently. In case of inconsistency, studies were discussed until consensus was reached.

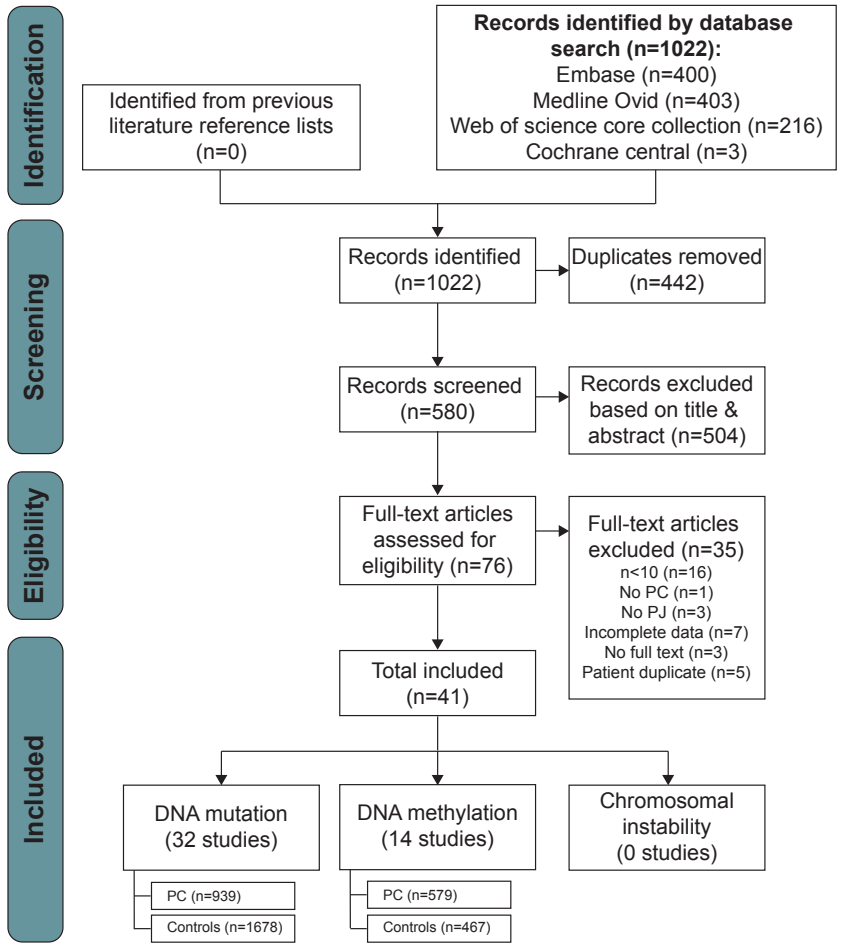


FIGURE 1 | PRISMA flow-chart showing the selection of literature. Five articles investigated both DNA mutation and methylation. PC = pancreatic cancer; PJ = pancreatic juice.

Data extraction and quality assessment

Patient characteristics, DNA alteration type, methods of PJ collection, methods of DNA analysis and diagnostic performance per DNA alteration were extracted. Patients with either HGD, invasive IPMN, carcinoma in situ or PC were classified as cases. Controls were stratified in: all controls (AC), non-pancreatic controls (NP; *e.g.*, patients with biliary stones or healthy controls), chronic pancreatitis (CP), hereditarily predisposed high-risk individuals (HRI) and precursor lesions (PL; Mucinous Cystic neoplasm [MCN], IPMN and pancreatic intraepithelial neoplasia [PanIN]). The latter group was evaluated as one heterogeneous group, as the type of 'precursor lesion' was not always defined in studies, metrics of the IPMN-subgroup were extracted if possible. To avoid inclusion of PC or HGD

in the control group, control groups that may harbor HGD (due to lack of information), were excluded. Additionally, patients with other cancer types (*e.g.*, esophageal cancer) and individuals that did not fit in abovementioned groups were excluded.

Study quality was assessed using the Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2).[10] Risk of bias was scored in four domains: patient selection, index tests, reference standard, flow and timing. A total score was generated as described in **Supplemental Figure 1**. The importance of the different domains was quantified with an in-house scoring system.

Statistical analysis

The pooled performance for differentiation of HGD and PC from the different control groups (AC, NP, CP, HRI, PL) of each DNA alteration (investigated ≥5 studies) was assessed by calculating the prevalence, sensitivity, specificity and diagnostic odds ratio (DOR) from published metrics extracted from the included studies. First, forest plots were constructed and meta-analyses were performed using the Midas tool in STATA (version 16.1, Stata corporation, College station, TX, USA) resulting in a pooled sensitivity, specificity and DOR. Second, statistical heterogeneity was measured using I^2 statistics. Third, a hierarchical summary receiver operating curve (HSROC) was generated. This model jointly analyzes the diagnostic performance of biomarkers while accounting for heterogeneity within and in between studies.[11] For DNA alterations that were reported in less than five studies, outcomes were described systematically. For gene methylation, a cross table was used to calculate the sensitivity and specificity per study (SPSS; Statistical Package for the Social Sciences, version 27, SPSS Inc., Chicago, IL); confidence intervals were presented as "exact" Clopper-Pearson confidence intervals. The prevalence of both *TP53* and *KRAS* mutations were compared between subgroups (secretin-induces vs not secretin-induced; Mann Whitney U) and visualized by Graphad (GraphPad Prism version 9, GraphPad Software, La Jolla, CA). A p-value of <0,05 was considered statistically significant.

Results

Overview of included studies

The initial search identified 580 studies, of which 504 were excluded based on title and abstract. After full-text review, 41 studies were included: 32 concerning DNA mutations, 14 DNA methylations and five describing both[12-52] (**Figure 1**). For each study, distinct collection methods and DNA analysis methods were reported. For DNA mutations, all included studies used a targeted approach, while for DNA methylation, four studies used an unbiased approach (*e.g.*, whole genome sequencing).[4,18,22,37] For further details see **Table 1 and 2**.

Quality assessment

Based on the QUADAS-2 quality assessment, the quality of the included studies was overall acceptable, but varied between studies (**Figure 2**). Notably, earlier studies did not describe their DNA analysis techniques as explicitly as the more recent studies. As a consequence, the definition of a positive test was not always evident. For instance, in earlier studies (before 2001) polymerase chain reaction (PCR)-techniques were used that score the presence of a mutation 'upon visualization of bands' (**Table1**); a metric that highly depends on interpretation. Whereas in the recent studies using next generation sequencing (NGS), thresholds of allelic frequency (or 'NGS-scores') were given in the majority of studies (**Table 1**). Important other quality remarks are that none of the studies reported on the interval between the index test (PJ analysis) and reference test (histopathological diagnosis) and the majority had not used the same reference test for cases (pathology-proven in 36 of 41 studies) and controls (based solely on clinical and imaging characteristics in 37 of 41 studies).

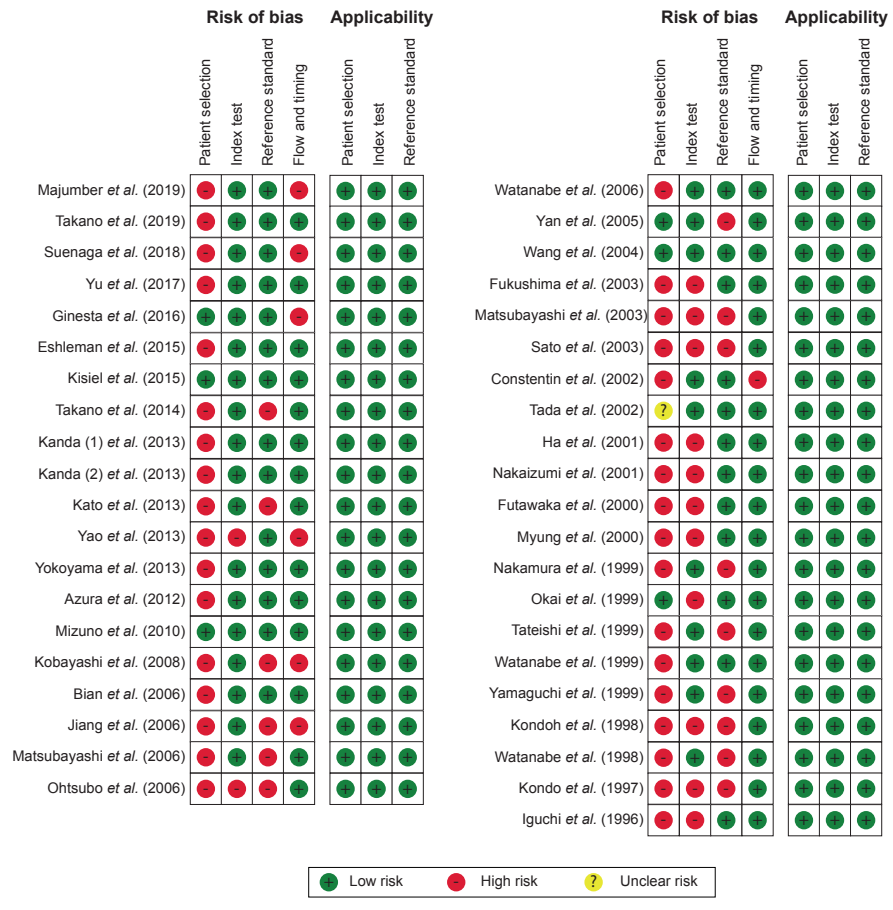


FIGURE 2 | Results of Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2).

TABLE 1 | Overview of included patients of studies investigating DNA Mutations (n=32).

Study (year of publication)	Cases			Controls		Procedure of PJ collection	Ampulla Cannulation (Y/N)	Secretin induced (Y/N)	Laboratory analysis	Definition of mutation	Investigated gene
	Definition of cases	Cases, n	PA-Proven (Y/N)	Definition of controls	Controls, n						
Majumber *et al*. (2019)‡	HGD / PC	38	Y	NP / benign ¶	32 / 41	END	N	Y	NR	NR	*KRAS*
Takano *et al*. (2019)	Inv. IPMN	15	Y	PL (IPMN)	13	ERCP	Y	N	NGS	AF > 0.5%	*KRAS; TP53; GNAS*
Suenaga *et al*. (2018)	HGD / PC	18	Y	NP / PL (IPMN + PanIn) / HRI	9 / 9 (7+2) / 31	EUS	N	Y	NGS	NGS-score ≥ 12▲	*KRAS; GNAS; TP53; SMAD4; RNF43; CDKN2A; TGFBR2; BRAF; PIK3CA*
Yu *et al*. (2017)	PC	34	Y	NP / PL (IPMN)	24 / 57	EUS	N	Y	NGS	NGS-score ≥ 12▲	*KRAS; GNAS; TP53; SMAD4; RNF43; CDKN2A; TGFBR2; BRAF; PIK3CA*
Ginesta *et al*. (2016)‡	PC / inv. IPMN	85 / 3	Y	PL (IPMN) / CP	9 / 14	Surgery	NR	N	qPCR	NR	*KRAS*
Eshleman *et al*. (2015)	PC	30	NR	NP / CP / PL (IPMN) / HRI	22 / 9 / 17 / 194	EUS	N	Y	DHRM + pyroseq.	Mutation score ≥ 3	*KRAS; GNAS*❖
Kisiel *et al*. (2015)‡	PC	61	Y	NP / CP	19 / 22	EUS / EGD	N	Y	QUART	NR	*KRAS*
Takano *et al*. (2014)	PC / inv. IPMN	39 / 17	NR	NP / CP / PL (IPMN)	9 / 22 / 65	ENPD	Y	N	NGS	AF>1%	*KRAS; GNAS; TP53; SMAD4*
Kanda *et al*. (1; 2013)	PC	14	Y	NP / CP / PL (PMN) / HRI	20 / 20 / 76 / 123	EUS	N	Y	DHRM + pyroseq.	A fluorescence difference of 3%	*GNAS*
Kanda *et al*. (2; 2013)	PC / HGD	43 / 8	Y	NP / CP / PL (IPMN + PanIn)	34 / 24 / 14	EUS	N	Y	DHRM + pyroseq.	Detection by both DHRM and pryoseq	*TP53*
Azura *et al*. (2012)	PC	42	Y	CP	6	Surgery	NR	N	Nanofluidic dPCR	AF>0.05%	*KRAS*
Mizuno *et al*.(2010)	Inv. IPMN	12	Y	PL (IPMN)	41	ERCP	Y	N	PCR-PHFA	NR	*KRAS*
Kobayashi *et al*. (2008)	PC	21	NR	Benign ¶ / PL (IPMN)	20 / 19	ERCP	Y	N	PCR-PHFA/-SSCP + seq.	Upon visualization of peaks	*KRAS; CDKN2A; TP53*
Bian *et al*. (2006)	PC	20	Y	NP / CP	8 / 8	Surgery / ERCP♦	Y	N	PCR-TGCE	Upon visualization of peaks	*TP53; CDKN2A*
Othsubo *et al*. (2006)‡	PC	28	Subgroup^	CP	20	EGD	Y	Y	SSCP + sequencing	Upon visualization of bands	*TP53*
Yan *et al*. (2005)	PC	48	Subgroup^	NP / CP	49 / 49	ERCP	Y	N	RT-PCR	< 98% confidence / >0;25% red colonies	*KRAS; TP53*
Wang *et al*. (2004)	PC	21	Subgroup^	CP	25	EGD	Y	Y	MASA (KRAS)/ PCR-SSCP + seq. (TP53)	NR	KRAS❖; TP53
Constentin *et al*. (2002)	PC	18	Y	CP / benign ¶	20 / 19	ERCP	Y	N	PCR-RFLP / PCR-CMA	Upon visualization of bands	*KRAS; CDKN2A (p16); DPC4*
Tada *et al*. (2001)	PC	23	Subgroup^	NP / CP / PL (IPMN)	23 / 13 / 15	ERCP	NR	Y	PCR-ELMA	Mutation score 2+ (2 – 20% of the mutant)	*KRAS*
Ha *et al*. (2001)	PC	19	Subgroup^	CP	25	EGD	Y	Y	PCR-MASA	Detection rate >0.01-0.001% of total DNA	*KRAS*
Nakaizumi *et al*. (2001)	PC	19	Y	NP / PL (cyst)	8 / 34	EGD	Y	Y	PCR-PHFA	NR	*KRAS*
Futawaka *et al*. (2000)	PC / inv. IPMN	12 / 4	Y	NP / CP	10/10	ERCP	Y	N	PCR-RFLP	Mutant:WT ratio >1:300	*KRAS*
Myung *et al*. (2000)	PC	12	Y	NP / CP	8 / 11	EGD	Y	Y	PCR-RFLP	Upon visualization of bands	*KRAS*
Okai *et al*. (1999)	PC	19	Subgroup^	CP	11	END	Y	Y	PCR-RFLP	Upon visualization of bands	*KRAS*
Tateishi *et al*. (1999)	PC	20	NR	NP / CP / PL (cyst)	21 / 6 / 14	ERCP	Y	Y	PCR-ELMA	Mutation score 2+ (sensitivity 80-98%)	*KRAS*
Watanabe *et al*. (1999)	PC	38	Subgroup^	NP / CP	62 / 38	Aspirator device	N	N	PCR-MASA	Detection rate >0.01-0.001% of total DNA	*KRAS*
Yamaguchi *et al*. (1999)	PC	26	Subgroup^	CP	16	EGD	Y	Y	PCR-SSCP	Upon visualization of bands	*KRAS❖; TP53*
Nakamura *et al*. (1999)	PC	23	Subgroup^	CP / PL (cyst + MCN)	4 / 8 (3+5)	ERCP	Y	Y	PCR-PAGE	Upon visualization of bands	*KRAS*
Watanabe *et al*. (1998)	PC	29	Subgroup^	NP / CP	11 / 26	END	NR	NR	PCR-PHFA	Upon visualization of peaks (chemi-luminescence >11.020 RLUs)	*KRAS*
Kondoh *et al*. (1998)	PC / inv. IPMN	10 / 5	N	CP / PL (cyst)	9 / 3	ERCP	Y	N	PCR-SSCP	Upon visualization of bands	*KRAS*
Kondo *et al*. (1997)	PC / inv. IPMN	43 / 4	NR	NP / CP	28 / 22	EGD	Y	Y	NRI-SSCP-PCR	NR	*KRAS*
Iguchi *et al*. (1996)	PC	19	Y	CP	29	Dreiling tube	N	Y	PCR-SSCP	Mutant:WT ratio between 1:1024 and 1:2048	*KRAS*

‡ Study describes both DNA mutations and methylation; ^ Only a subgroup was histologically proven; ¶ Benign pancreatic diseases, but impossible to subdivide into clear subgroups ▲ NGS-score is related to the count of NGS aliquotes with a mutation; ❖ mutation was not included in meta-analysis; as presence in PC cases was not described; ♦ PJ of PC-group collected during surgery; of control group during ERCP. Y = yes; N = No; PC = pancreatic cancer, HGD = high-grade dysplasia; AC = all controls; IPMN = intraductal papillary mucinous neoplasm; NP = non-pancreatic (including healthy controls and indivividuals with an extrapancreatic disease, extrapancreatic malignancies are excluded); PL = precursor lesions (including benign IPMN, Mucinous cystic neoplasm, PanIn, unspecified cyst); CP = chronic pancreatitis; HRI = (hereditarily predisposed) high-risk individuals; END = Endoscopic; EUS = endoscopic ultrasound; ERCP = endoscopic retrograde cholangiopancreatography; EGD = esophagogastroduodenoscopy; ENPD = endoscopic nasopancreatic drainage; AF = Allele frequency; NGS = next generation sequencing; qPCR = quantitative polymerase chain reactive; DHRM = Digital high-resolution melt-curve; QUART = Quantitative real-time allele-specific target and signal amplification; PHFA = Preferential homoduplex formation assay; SSCP = Single-strand conformation polymorphism; LD-PCR = limiting dilution PCR; TGCE = temperature gradient capillary electrophoresis; RFLP = restriction fragment length polymorphism; CMA = comparative multiplex assay; ELMA = enzyme-linked mini-sequence assay; MASA = mutant allele specific amplification; PAGE = polyacrylamide gel electrophoresis; WT = wild type; NR = not recorded.

DNA mutations

For DNA mutations, 2617 individuals (939 cases and 1678 controls) were included from 32 studies (**Table 1**). Different groups served as controls; 25 studies used CP, 20 NP, 15 PLs and three HRIs with a hereditary predisposition for PC. *KRAS* mutations were most frequently investigated (25 studies). Other frequently investigated DNA mutations were in *TP53* (11 studies), *CDKN2A* (5 studies), *GNAS* (5 studies) and *SMAD4* (3 studies). Mutations in *RNF43, TGFBR2, FBXW7, ARID1A, BRAF, PIK3CA* and *COL12A1* were only investigated in one or two studies.

The studies evaluating *KRAS* involved 729 cases and 1178 controls. Mutated *KRAS* was detected in 60% of cases and 27% of controls (8% of NP, 19% of CP, 52% of HRI and 48% of PL). The presence of mutated KRAS discriminated cases from controls with a sensitivity of 62% (95% CI 56-68%; I^2 63% [95% CI 47-79%]; **Figure 3A**), specificity of 81% (95% CI 72-89%; I^2 90% [95% CI 86-93%]; **Figure 3B**), DOR of 7 (95% CI 4.5-11; **Figure 3C**) and AUC of 0.74 (95% CI 0.70-0.77; **Figure 3D**). When comparing cases with individual control groups, *KRAS* mutations were able to differentiate cases from NP (n=323) with a sensitivity of 57% (95% CI; 48-65%; I^2 32% [95% CI 0-65%]) and specificity of 96% (95% CI 88-99%; I^2 49% [95% CI 11-69%]; **Figure 3E**), cases from CP (n=337) with a sensitivity of 60% (95% CI 51-68%; I^2 65% [95% CI 50-80%]) and specificity of 87% (95% CI; 77-93%; I^2 55% [95% CI 35-75%]; **Figure 3F**), and cases from PL (n=220) with a sensitivity of 61% (95% CI 51-70%; I^2 0% [95% CI 0-58%]) and specificity of 47% (95% CI 36-59%; I^2 0% [95% CI 0-58%]; **Figure 3G**).The most prevalent *KRAS* mutations were G12D (42% of cases, 20% of controls, sensitivity 40% [95% CI 31-50%], specificity 82% [95% CI 71-89%]), G12V (29% of cases, 16% of controls, sensitivity 26% [95% CI 16-39%], specificity 93% [95% CI 79-98%]) and G12R (16% of cases, 8% of controls, sensitivity 13% [95% CI 8-20%], specificity 94% [95% CI 88-97%]). 24% of cases and 14% of controls had multiple types of *KRAS* mutations (**Supplemental Table S2** and **Supplemental Figure S2**).

TP53 was investigated in 320 cases and 445 controls. A mutation was detected in 43% of cases and 7% of controls (0% of NP, 2% of CP, 23% of HRI and 22% of PL). *TP53* mutational status performed best in discriminating cases from controls, based on the DOR (36 [95% CI 9-133; **Figure 4C**] and the AUC (0.77 [95% CI 0.73-0.80; **Figure 4D**]). The sensitivity was 42% (95% CI 31-54; I^2 73% [95% CI 58-89%]; **Figure 4A**) and the specificity 98% (95% CI 92-100; I^2 77% [95% CI 65-90%]; **Figure 4B**). *TP53* was able to differentiate cases from NP (n=133) with a sensitivity of 43% (95% CI 26-62%; I^2 68% [95% CI 48-67%]) and specificity of 99% (95% CI 94-100%; I^2 0% [95% CI 0-88%]; **Figure 4E**), cases from CP (n=168) with a sensitivity of 40% (95% CI 27-55%; I^2 64% [95% CI 41-86%]) and specificity of 98% (95% CI 95-99%; I^2 0% [95% CI 0-88%]; **Figure 4F**), and cases from individuals with PL (n=93) with a specificity of 79% (95%CI 0.61-0.90; I^2 0% [95% CI 0-88%]) and sensitivity of 62% (95%CI 53-70%; I^2 0% [95% CI 0-88%]; **Figure 4G**).The most prevalent *TP53* mutations were R248W (5.1% of cases, 1.1% of controls), R248Q (3.8% of cases, 1.1% of controls)

and R175H (3.8% of cases and 1.1% of controls). 5.6% of cases and 0.7% of controls had multiple *TP53* mutations (**Supplemental Table S2**).

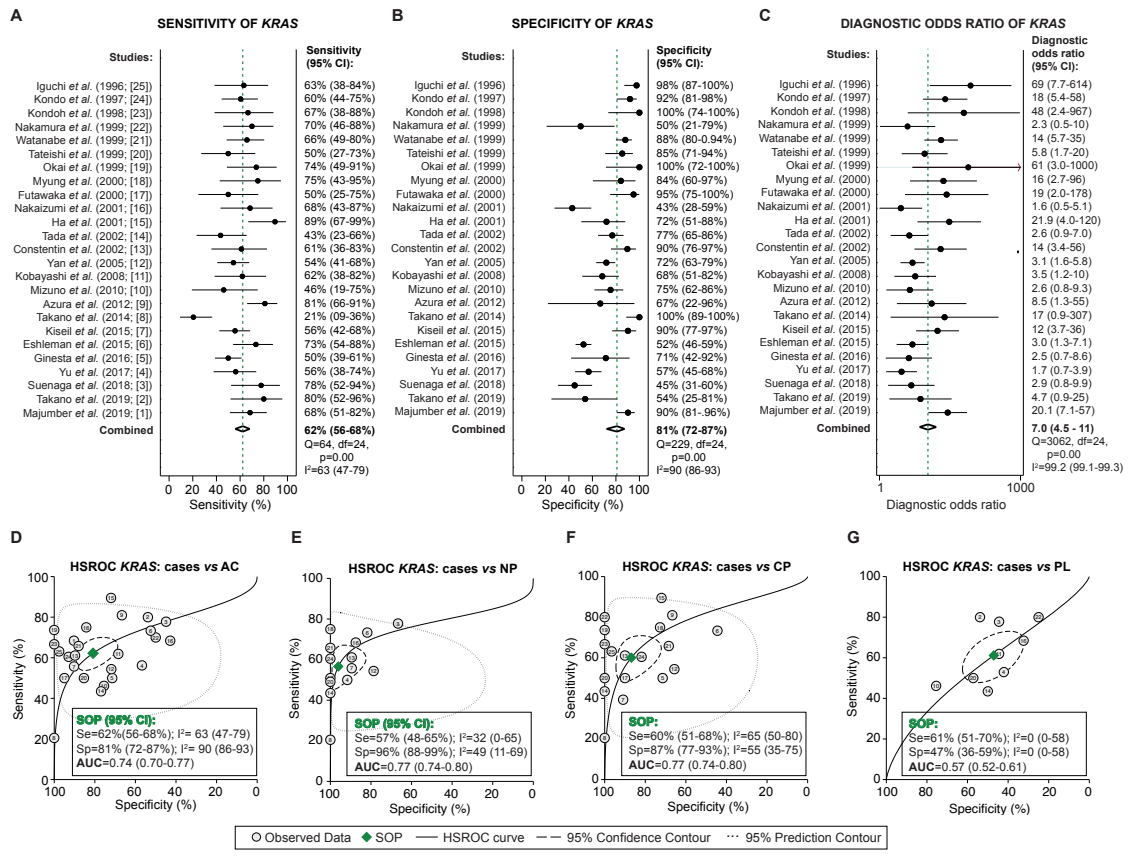


FIGURE 3 | The diagnostic performance of the presence of a KRAS mutation in PJ for the differentiation between PC (n=950) and controls (n=1732). A-C. Forrest plots of the sensitivity (A); specificity (B) diagnostic odds ratio (C) for differentiation between PC and all controls. The dashed line indicates the combined sensitivity (A), specificity (B) and odds ratio (C). **D-G** Hierarchical Summary receiver operating curves (HSROCs) representing the performance of the presence of a *KRAS* mutation for the differentiation between PC and all controls (AC; D); non-pancreatic (NP; n=323; E); chronic pancreatitis (CP; n=337; F); precursor lesions (PL; n=220; G). Numbers in figures D-G are linked to numbered references in figure A. SOP = summary operating point, Se = sensitivity, Sp = specificity; AUC = area under the curve; CI = confidence interval.

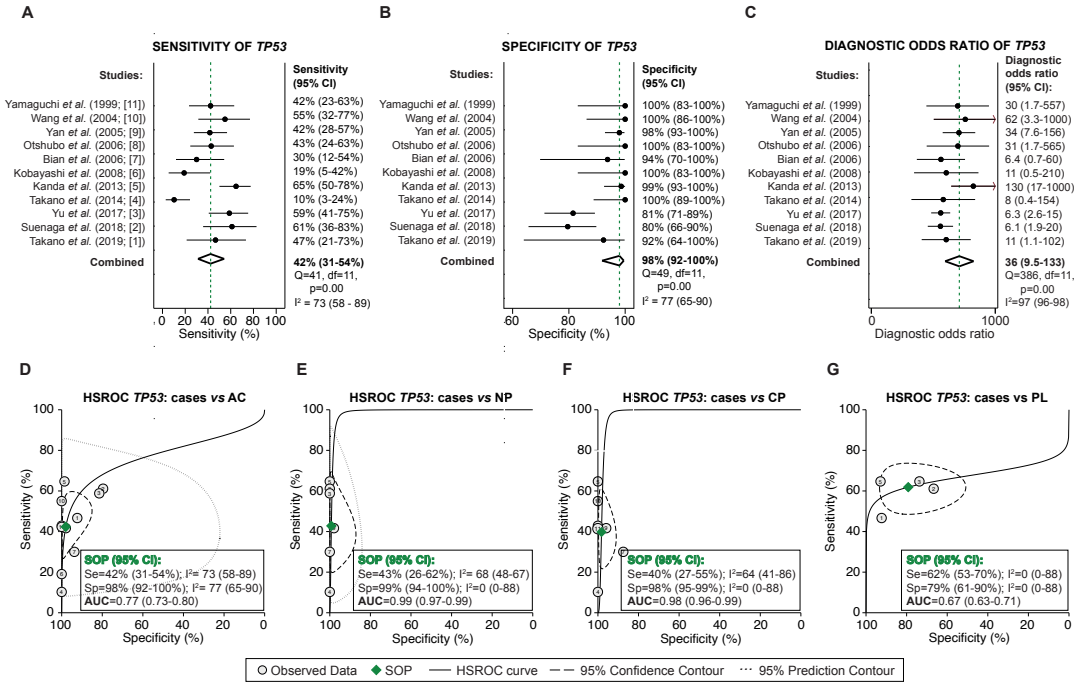


FIGURE 4 | The diagnostic performance of the presence of a *TP53* mutation for the discrimination between cases (n=320) and controls (n=445). Forrest plots of the sensitivity (A); specificity (B) diagnostic odds ratio (C) for differentiation between pancreatic cancer (PC) and all controls (AC). **D-G** Hierarchical summary receiver operating curves (HSROCs) representing the performance of the presence of a TP53 mutation for the differentiation between PC and all controls (AC; D); non-pancreatic (NP n=133; E); chronic pancreatitis (CP n=168; F); precursor lesions (PL n=93; G). Numbers in figures D-G are linked to numbered references in figure A. SOP = summary operating point, Se = sensitivity, Sp = specificity; AUC = area under the curve; CI = confidence interval.

CDKN2A was investigated in 111 cases and 146 controls. The prevalence of a *CDKN2A* mutation was 17% in the case and 13% in the control group (9% of NP, 36% of CP, 3% of HRI and 0% of PL). The sensitivity for differentiating cases from controls was 13% (95% CI 4-36; I^2 87% [95% CI 77-97%]; **Figure 5A**) and the specificity 96% (95% CI 77-99; I^2 92% [95% CI 87-97%]; **Figure 5B**). Consequently, the overall performance was poor, with a DOR of 3.4 [95% CI 0.4-29.4]; **Figure 5C**) and AUC 0.61 (95% CI 0.00-1.00; data not shown). For a specification of detected gene mutations see **Supplemental Table S2**.

Studies investigating *GNAS* involved 116 cases and 384 controls, of which 218 had IPMN. A *GNAS* mutation was detected in 22% of cases and 26% of controls (10% of NP, 2% of CP, 8% of HRI and 55% of PL). The sensitivity for differentiating cases from controls was 21%

(95% CI 11-38; I^2 67% [95% CI 55-97%]; **Figure 5D**), the specificity 76% (95% CI 60-87; I^2 80% [95% CI 62-97%]; **Figure 5E**), DOR 0.86 (95% CI 0.45-1,6; **Figure 5F**), and AUC 0.48 (95% CI 0.17-0.80; not shown). The most prevalent *GNAS* mutations were R201C (25% of cases, 27% of controls) and R201H (8.5% of cases, 20% of controls), 1.4% of cases and 5.3% of controls had multiple *GNAS* mutations (**Supplemental Table S2**).

Mutated *SMAD4* was present in 17% of the 111 cases and 10% of the 168 controls (0% of NP, 0% of CP, 3% of HRI and 0% of PL). The sensitivity for differentiating cases from controls ranged from 3 to 22% and specificity from 97 to 100%. For a specification of detected gene mutations see **Supplemental Table S2**.

As oncogenic mutations may already be detected at non-tumorigenic stages (*e.g., KRAS* mutations in pancreatic cysts), combining gene mutations in a panel may improve diagnostic performance of mutational screening. Two studies investigated such panels, including *KRAS, GNAS, TP53, SMAD4, RNF43, CDKN2A* and *TGFBR2*.[15,16] The panels showed similar high specificities (>90%), yet higher sensitivities of 72% and 85%, as compared to each of the mutations alone. In addition, the combined panel also showed a high specificity in differentiating cases from controls with IPMN (86%).

Mutated *KRAS* was not detected more often in secretin-stimulated samples (from cases), as compared to samples not collected after secretin stimulation (p=0.22; Supplemental **Figure S3**). For *TP53*, the prevalence of a mutation was higher in PJ collected after secretin stimulation. No difference in prevalence was found when comparing cannulation with no cannulation, or NGS with no NGS (data not shown).

DNA methylation

For DNA methylation, 1046 patients (579 cases and 467 controls) were included from 14 studies (**Table 2**). As controls, 11 studies included CP patients, 5 included NP patients, PLs were included in 10 studies, and one study included HRI. All studies evaluated (promoter) CpG island methylation, a common mechanism for silencing of genes during tumorigenesis. No single genes were investigated in ≥ 5 studies, so no meta-analysis was performed. For a more detailed overview of prevalence and diagnostic performance of investigated methylated gene (promotors) for each study, see Table 3 (cases vs AC) and **Supplemental Table S3** (controls divided in the subgroups IPMN, CP and HRI).

CDKN2A (encoding for p16) hypermethylation was investigated most frequently (4 studies).[22,32,35,38] In total, these studies included 175 cases and 168 controls; *CDKN2A* was hypermethylated in 23% of cases and 11% of controls (7% of NP, 11% of CP, 5% of HRI and 32% of IPMN). *CDKN2A* is able to differentiate cases from controls with a sensitivity of 9-62% (3/4 studies had a very low sensitivity: 9%, 11% and 27%) and specificity of 73-100%.

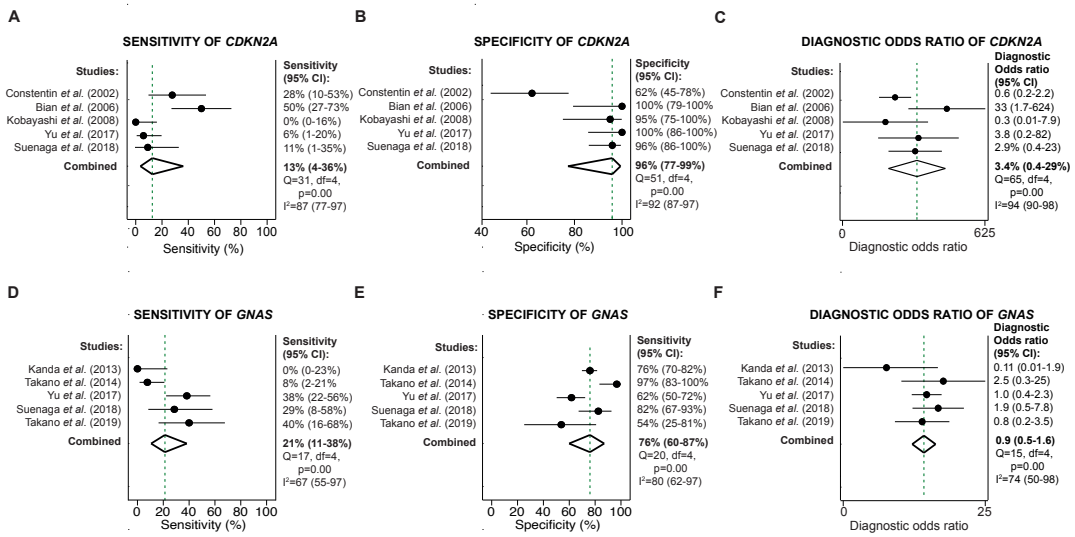


FIGURE 5 | **The diagnostic performance of the presence of a *CDKN2A* (A-C; G) and *GNAS* (D-F; H) mutation fthe differentiation between PC and all controls. A-F.** For *CDKN2A* (111 cases, 168 controls) and *GNAS* (116 cases, 384 controls); forest plots of the sensitivity (A; D); specificity (B; E) diagnostic odds ratio (C; F). CI = confidence interval.

NPTX2 promotor hypermethylation was investigated in three studies, which included 128 cases and 126 controls.[21,32,37] *NPTX2* was hypermethylated in 51% of cases and 5% of controls (0% of NP, 5% of CP, 5% of HRI and 7% of IPMN). The sensitivity for distinguishing cases from all controls was 39-70% and specificity from 94-100%. Although not significant (based on confidence intervals; **Table 3**), *NPTX2* has the highest diagnostic performance for detecting HGD or PC.

ppENK methylation was investigated in three studies,[31,32,38] and present in 45% of the 144 cases and 8% of the 138 controls. For controls, hypermethylation of *ppENK* was found in 9% of NP, 3% of CP, 9% of HRI and 15% of IPMN. *ppENK* methylation status was able to differentiate cases from controls with a sensitivity ranging from 27 to 63% and a specificity ranging from 86 to 100%.

APC[17,22] (in total, 98 cases and 39 controls), *cyclin D* (86 cases, 89 controls),[32,52] *SARP2* (67 cases, 46 controls)[28,37] and *TFPI2* (110 cases, 117 controls)[32,33] were each investigated in two studies. *APC* hypermethylation was present in 67% of cases, and 31% of controls (14% of NP, 7% of CP, and 56% of IPMN). The sensitivities (cases *vs* AC) were 47% and 71%, the specificities were 80% and 63%. Hypermethylation of *Cyclin D* was present in 21% of cases and 1% of controls (0% of NP, 0% of CP, 0% of HRI and 11% of IPMN). The sensitivities were 14% and 41%, while the specificities were 99% and 100%. With regard to *SARP2*, hypermethylation was present in 69% of cases, 20% of controls (0% of NP, 4% of CP, and 89% of IPMN). Sensitivities for distinguishing cases from controls were 48% and 80%, specificities were 100% and 76%. Heterogeneity in specificity was predominantly related to inclusion of IPMN in the control group, as almost all IPMN controls harbored *SARP2* hypermethylation. *TFPI2* hypermethylation was present in 35% of cases and 11% of controls (9% of NP, 9% of CP, 12% of HRI and 25% of IPMN).

In five studies, methylation of more than one gene was investigated.[14,17,20,32,37] These panels showed a higher diagnostic performance than the DNA methylations individually (sensitivities ranging from 72-87% and specificities from 80-100%).

TABLE 2 | Overview of the studies investigating DNA methylation (n=14).

Author	Cases			Controls		Procedure PJ collection	Ampullary orifice cannulation (Yes/No)	Secretin-induced (yes/no)	DNA-analysis	Definition of positive test	Tested genes	Target and region
	Definition of cases	Nr. of cases	PA-Proven (Y/N)	Type of controls	Nr. of controls							
Majumber *et al.* (2019)‡	PC / HGD	35 / 3	Yes	NP / benign°	32 / 41	END	No	Yes	QUART	Percentage methylation >10%	*C13orf18, FER1L4, BMP3*	CpG island hypermethylation
Ginesta *et al.* (2016)‡	PC / inv. IPMN	85 / 3	Yes	IPMN / CP	7 / 14	Surgery	NR	No	MS-MCA	NR	*APC, HRH2, CDH13, SPARC, EN-1*	Promoter hypermethylation
Kisiel *et al.* (2015)‡	PC	61	Yes	NP / CP	19 / 22	EUS / EGD	No	Yes	qMSP	NR	*CD1D, KCNK12, CLEC11A, NDRG4, IKZF1, PRKCB*	CpG island hypermethylation
Kato *et al.* (2013)	PC	15	NR	NP / IPMN	7 / 8	ERCP / PTCD	Yes	No	MethyLight assay	PMR > 4	*UCHL1, RUNX3, CDKN2A, IGF2, CACNA1G, AHRR, SFRP1, MGMT, APC, NEUROG1*	CpG island hypermethylation
Yao *et al.* (2013)	PC / inv. IPMN	31 / 10	Subgroup^	CP / IPMN	23 / 6	EGD	Yes	Yes	MSP	PTX2:MYOD1 ratio > 1.39/2.35	*NPTX2*	Promoter hypermethylation
Yokoyama *et al.* (2013)	PC	15	NR	IPMN	28	ENPD / ERCP	NR	No	MSE	NR	*MUC1, MUC2, MUC4*	NR
Matsubayashi *et al.* (2006)	PC / in situ	56 / 8	NR	NP / CP / IPMN / HRI	11 / 26 / 9 / 43	Surgery / ERCP	Yes	No (surgery) / Yes (ERCP)	Q-MSP	>1% methylated DNA	*CDKN2A (p16), Cyclin D2, TFPI2, NPTX2, ppENK, FOXE1*	CpG island hypermethylation
Jiang *et al.* (2006)	PC / inv. IPMN	36 / 10	Subgroup^	CP / IPMN	21 / 7	EGD	Yes	Yes	Q-MSP	TFPI-2:MYOD1 ratio > 2.5	*TFPI2*	Hypermethylation of CpG island promoter region
Ohtsubo *et al.* (2006)‡	PC	26	Subgroup^	CP / IPMN	20 / 15	EGD	Yes	Yes	MSP (supernatant)	>1 methylated band	*ppENK*	Hypermethylation of CpG island promoter region
Watanabe *et al.* (2006)	PC / inv. IPMN	33 / 11	Subgroup^	CP / IPMN	19 / 9	EGD	Yes	Yes	Q-MSP	Similar melting curve (cutoff 8*10-11) and Tm as MiaPaCa-2	*SARP2*	Hypermethylation of CpG island promoter region
Yan et al. (2005)‡	PC	42	Subgroup^	NP / CP	24 / 26	ERCP	Yes	No	MSP	Methylation Index > 12%	*CDKN2A (P16INK4a)*	Hypermethylation of CpG island promoter region
Fukushima *et al.* (2003)	PC / inv IPMN / in situ	45 / 5 / 4	Subgroup^	CP	12	Surgery / END	No	Yes	MSP	MSP > 10–20 copies of DNA	*ppENK, CDKN2A (P16)*	Hypermethylation of CpG island promoter region
Matsubayashi *et al.* (2003)	PC	22	Yes	CP	10	Surgery	Yes	Yes	MSP	MSP > 0.2 ng of DNA	*Cyclin D2*	Hypermethylation of CpG island promoter region
Sato *et al.* (2003)	PC	23	NR	CP	8	Surgery	NR	NR	MSP	NR	*NPTX2, SARP2, CLDN5*	Hypermethylation of CpG island promoter region

‡ Study describes both DNA mutations and methylation; ^ Only a subgroup was histologically proven. °Benign group consists of CP (17), IPMN with low-grade dysplasia (2), unspecified cysts (3), fatty pancreas (4), imaging finding indeterminate for chronic pancreatitis (15). PC = pancreatic cancer, HGD = high-grade dysplasia; AC = all controls; IPMN = intraductal papillary mucinous neoplasm; NP = non-pancreatic (including healthy controls and indivividuals with an extrapancreatic disease, extrapancreatic malignancies are excluded); PL = precursor lesions (including benign IPMN, Mucinous cystic neoplasm, PanIn); CP = chronic pancreatitis; HRI = (hereditarily predisposed) high-risk individuals; END = Endoscopic; EUS = endoscopic ultrasound; ERCP = endoscopic retrograde cholangiopancreatography; EGD = esophagogastroduodenoscopy; ENPD = endoscopic nasopancreatic drainage; PTCD = percutaneous transhepatic cholangiography drainage; QUART = quantitative allele specific real time target and signal amplification; MS-MCA = methylation specific-melting curve analysis; (q)MSP = (quantitative) methylation-specific polymerase chain reaction; MSE = Methylation-specific electrophoresis; PMR = percentage methylated reference; NR = not recorded.

TABLE 3 | Diagnostic performance of investigated hypermethylated genes in included studies. Specificities ≥ 80% and sensitivities ≥ 70% were marked bold. If metrics were available, both cases and controls were included as one group (for diagnostic performances of subgroups, see **Supplemental Table S3**). For Mayumber et al. (2019) was not included in this table, as the metrics of the individual genes were not available.

Methylated gene	Reference	Cases (n)	Sensitivity, % (95% CI)	Controls(n)	Specificity, % (95% CI)
AHRR	Kato *et al.* (2013)	PC (15)	13 (1.7-40)	NP (7) + PL (8)	**88** (64-99)
APC	Ginesta *et al.* (2016)	PC (83)	**71** (61-80)	CP (14) + PL (10)	63 (41-81)
	Kato *et al.* (2013)	PC (15)	47 (21-73)	NP (7) + PL (8)	**80** (52-96)
CACNA1G	Kato *et al.* (2013)	PC (15)	13 (1.7-40)	NP (7) + PL (8)	**88.2** (64-99)
CD1D	Kisiel *et al.* (2015) ‡	PC (61)	**84** (72-91)/**79** (67-87)	CP (22) / NP (19)	**90** (NR)
CDH13	Ginesta *et al.* (2016)	PC (77)	57 (46-67)	CP (14) + PL (9)	**78** (56-93)
CDKN2A	Kato *et al.* (2013)	PC (15)	27 (8-55)	NP (7) + PL (8)	**73** (45-92)
	Matsubayashi *et al.* (2006)	PC (56)/in situ (8)	9 (3.5-19)	CP (26) + HRI (43) + NP (11) + PL (9)	**89** (80-94)
	Yan *et al.* (2005)	PC (42)	62 (46-76)	CP (26) + NP (24)	**90** (78-97)
	Fukushima *et al.* (2003)	PC (45)/inv. IPMN (5) /in situ (4)	11 (4-23)	CP (12) + PL (2)	**100** (77-100)
CLDN5	Sato *et al.* (2003)	PC (22)	45 (24-68)	CP (8)	**100** (63-100)
CLEC11A	Kisiel *et al.* (2015) ‡	PC (61)	53 (40-65)/67 (55-78)	CP (22) / NP (19)	**90** (NR)
Cyclin D	Matsubayashi *et al.* (2006)	PC (56)/in situ (8)	14 (6.6-25)	CP (26) + HRI (43) + NP (11) + PL (9)	**99** (94-100)
	Matsubayashi *et al.* (2003)	PC (22)	41 (21-64)	CP (12)	**100** (83-100)
EN-1	Ginesta *et al.*2016	PC (78)	37 (27-48)	CP (14) + PL (10)	**75** (53-90)
FOXE1	Matsubayashi *et al.* (2006)	PC (56) / in situ (8)	44 (31-57)	CP (26) + HRI (43) + NP (11) + PL (9)	**99** (94-100)
HRH2	Ginesta *et al.* (2016)	PC (82)	65 (54-74)	CP (14) + PL (10)	54 (33-74)
IGF2	Kato *et al.* (2013)	PC (15)	6.7 (0.2-32)	NP (7) + PL (8)	80 (52-96)
IKZF1	Kisiel*et al.* (2015) ‡	PC (61)	54 (42-66)/62 (50-73)	CP (22) / NP (19)	**90** (NR)
KCNK12	Kisiel *et al.* (2015) ‡	PC (61)	46 (34-58)/**79** (67-87)	CP (22) / NP (19)	**90** (NR)
NDRG4	Kisiel *et al.* (2015) ‡	PC (61)	67 (55-78)/**72** (60-82)	CP (22) / NP (19)	**90** (NR)
MGMT	Kato *et al.* (2013)	PC (15)	33 (12-62)	NP (7) + PL (8)	53 (27-79)
NEUROG1	Kato *et al*. (2013)	PC (15)	40 (16-68)	NP (7) + PL (8)	**87** (60-98)
NPTX2	Yao *et al.* (2013)	PC (31)/inv. IPMN (10)	59 (42-74)	CP (23) + PL (6)	**94** (79-99)
	Matsubayashi *et al.* (2006)	PC (56)/in situ (8)	39 (27-52)	CP (26) + HRI (43) + NP (11) + PL (9)	**96** (89-99)
	Sato *et al.* (2003)	PC (23)	**70** (47-87)	CP (8)	**100** (63-100)
ppENK	Matsubayashi *et al.* (2006)	PC (56) / in situ (8)	27 (16-39)	CP (26) + HRI (43) + NP (11) + PL (9)	**93** (86-97)
	Othsubo *et al.* (2006)	PC (26)	46 (27-67)	CP (20) + PL (15)	**86** (70-95)
	Fukushima *et al.* (2003)	PC (45)/inv. IPMN (5) / in situ (4)	63 (49-76)	CP (12) + PL (2)	**100** (77-100)
PRKCB	Kisiel *et al.* (2015) ‡	PC (61)	38 (27-50)/67 (55-78)	CP (22) / NP (19)	**90** (NR)
RUNX3	Kato *et al.* (2013)	PC (15)	53 (27-79)	NP (7) + PL (8)	**93** (68-100)
SARP2	Watanabe *et al.* (2006)	PC (33)/inv. IPMN (11)	**80** (65-90)	CP (19) + NP (10) + PL (9)	**76** (60-89)
	Sato *et al.* (2003)	PC (23)	48 (27-69)	CP (8)	**100** (63-100)
SFRP1	Kato *et al.* (2013)	PC (15)	53 (27-79)	NP (7) + PL (8)	**73** (27-79)
SPARC	Ginesta *et al.* (2016)	PC (72)	49 (37-60)	CP (13) + PL (9)	68 (45-86)
TFPI2	Matsubayashi *et al.* (2006)	PC (56) / in situ (8)	25 (15-37)	CP (26) + HRI (43) + NP (11) + PL (9)	**89** (80-94)
	Jiang *et al.* (2006)	PC (36) inv. IPMN (10)	50 (35-65)	CP (21) + PL (7)	**93** (77-99)
UCHL1	Kato *et al.* (2013)	PC (15)	67 (38-88)	NP (7) + PL (8)	**100** (78-100)

‡Specificity was set on 90% by Kisiel (2015) et al. and metrics to calculate sensitivity of the total cohort were not available. PC = pancreatic cancer; IPMN = intraductal papillary mucinous neoplasm; NP = non-pancreatic; PL = precursor lesions (including benign IPMN; Mucinous cystic neoplasm, PanIn); CP = chronic pancreatitis; HRI = (hereditarily predisposed) high-risk individuals; NR = not reported.

Discussion

This systematic review provides the first comprehensive overview of all current literature on DNA alterations in PJ. It may serve as a guide to those who aim to set up a pipeline for PC molecular diagnostic analysis. We show that mutations in *TP53*, *SMAD4* and *CDKN2A*, and aberrant methylation of *NPTX2* in PJ are highly specific (close to 100%) for the presence of PC. However, individually, the sensitivity of these DNA alterations is poor. Based on two studies, combining DNA alterations in a panel increases sensitivity.

As imaging-based surveillance programs have not shown genuine survival benefits, biomarkers may play a pivotal role in early detection (and decrease mortality rates). Implementation of a biomarker in a surveillance program may serve two goals: 1. Selection of individuals at increased risk that are eligible for increased surveillance (EUS and MRI in combination with biomarker analysis), in which case a lower specificity is accepted; 2. Supporting decision-making regarding additional diagnostic procedures and treatment (including surgery). In the latter case, the marker should have a specificity close to 100% to avoid unnecessary harm.

Biomarkers determined in PJ are expected to be more (pancreas-)specific, as this fluid constitutes a wash-out of the pancreatic ductal system and has been in close contact with the ductal cells from which PC originates. As compared to either PJ collection by pancreatic duct cannulation via ERP (endoscopic retrograde pancreatography) or tissue sampling with fine-needle aspiration (FNA) or biopsy (FNB), secretin-stimulated PJ collection from the duodenal lumen is less invasive. Additionally, in contrast to needle biopsy, PJ collection does not rely on a visible mass and PJ potentially contains information on the complete range of tumor clones.[7,53] In individuals who undergo regular EUS-procedures as part of a surveillance program, the additional patient burden of PJ collection is relatively low. Conversely, challenges are that currently PJ can only be reliably collected during endoscopy and quick snap freezing of PJ samples is required (expectedly in 10 minutes) to prevent PJ enzymes to digest different components that may serve as biomarkers.[7]

TP53 is a tumor suppressor gene that is activated by cellular stressors, leading to inhibition of cell cycle progression, promotion of senescence or apoptotic cell death. Both loss-of-function and gain-of-function mutations have been described in a wide variety of cancers. Of the DNA alterations investigated in this study, *TP53* mutation reached the highest overall performance (DOR 36). Large NGS studies have shown the prevalence of mutated *TP53* in PC tissue to range from 70% to 79% in PC cases,[54-57] and in blood from 17% to 55%.[57,58] Our review shows a lower prevalence in PJ (43%) than in tissue. Caution should be taken while interpreting the results, as the prevalence of a *TP53* mutation was high in HRI (23%) and PL (22%) and low in NP (0%) and CP (2%). This may implicate that *TP53* mutations are already present in (early) precursor lesions (as previously shown by Noe *et al.* [2021]), but would contrast the findings of Hosoda *et al.* (2017), who reported

a low prevalence of *TP53* mutations in HGD.[59,60] Another explanation could be that HRI/PL groups in these studies already harbored HGD/PC (as histology was not obtained in 90% of studies).[59] Ideally, while challenging, this (and other) PJ marker(s) should be investigated in individuals without a visible lesion on imaging to investigate this, to push boundaries of early detection and evaluate the role of even earlier intervention on both surgery- and cancer-related morbidity and mortality. At last, as shown in **Supplemental table S2**, there is no locus on the *TP53* gene that is specific for PC and generation of a test targeting specific *TP53* loci would not be feasible.

KRAS encodes the GTPase K-RAS, which regulates proliferation, differentiation, cell survival and migration via the RAS/MAPK signaling pathway.[61] It is one of the most prevalent cancer mutations and was also the most frequently mutated gene in PJ of PC patients (60% of cases). This percentage is lower than that found in tissue (88-93% of samples[54-56,62]), which suggests that not all mutations are captured when analyzing PJ with the current techniques or just one clone is being drained into PJ. Nevertheless, *KRAS* mutation analysis in PJ seems more valuable than that in serum or cyst fluid, in which they were observed in 47% and 41% of samples, respectively.[63,64] Direct comparisons of biomaterials (including PC tissue) are needed to evaluate if *KRAS* mutation analysis in PJ is indeed superior. The specificity of *KRAS* in PJ (PC *vs* controls) was considerably lower than that of *TP53, SMAD4* and *CDKN2A*. This may be explained by the assumption that *KRAS* is a driver of malignant progression, and thus present from the beginning of this process.[65] For this reason, *KRAS* differentiates better between PC and NP (sensitivity 57%; specificity 96%), than between PC and PL (sensitivity 61%; specificity 47%; **Figure 3E, G**). This poor performance inhibits *KRAS* role as an independent diagnostic marker. However, adding *KRAS* G12D to a panel may improve the diagnostic accuracy (**Supplemental Figure S2**). Our findings are consistent with earlier meta-analyses on *KRAS* performance in PJ; Parker *et al*. (2011)66 showed a similar pooled sensitivity and specificity, while Yang *et al*. (2014)67 showed a higher specificity (87%), as compared to our findings.

SMAD4 is a tumor suppressor gene that inhibits oncogenesis via the TGF-β signaling pathway.[68] Based on previous literature,[54-56] a *SMAD4* mutation is present in 22-29% of PC tissue samples. In the current systematic review, a mutation was found in 17% of PJ samples from cases and 10% controls (0% of controls when excluding the HRIs), resulting in high specificity. This was expected, based on previous data showing that *SMAD4* predominantly occurs in invasive disease.[59, 60] Based on Suenaga *et al*. (2018), Combining *SMAD4* with *TP53* may further enhance accuracy, with a sensitivity of 61% (substantially higher than *TP53* alone in this systematic review), specificity of 96% and AUC of 0.82.[15]

GNAS encodes the stimulatory Gα-subunit of heterotrimeric G-proteins and stimulates tumor growth via cAMP-PKA signaling and cooperates with *KRAS* to develop IPMN.[69-71] The prevalence of a *GNAS* mutations in cases was lower than that in (IPMN) controls. However, as only 8.5% of cases and 20% of controls harbored a *GNAS* R201H mutation (as compared to 25% and 27% for R201C, respectively), the absence of a R201H mutation

may be more specific for PC, than the presence/absence of an unspecified *GNAS* mutation. Kawabata *et al* (2022)[72] performed CRIPSPR/Cas9-mediated *GNAS* R201H silencing in IPMN-associated (PC) cells and showed that oncogenic *GNAS* induced mucin production via MUC2 and MUC5AC, which may be the reason of increase of the size of cystic lesions. Interestingly, in line with our results, they showed that a *GNAS* mutation limits tumor aggressiveness and, thus, a *GNAS* mutation may be protective against PC development.

CDKN2A is a tumor suppressor gene that encodes two proteins; p16 which regulates cell cycle arrest at G1, and p14arf which stabilizes p53 which in turn regulates cell cycle progression. The current systematic review shows *CDKN2A* mutations in PJ of 17% of cases. This is comparable to PC tissue samples, where the prevalence of *CDKN2A* mutations was reported to be 18-27%.[54-56] The specificity for distinguishing cases from controls was high, yet the DOR was low (as expectedly due to the low prevalence). Remarkably, a *CDKN2A* mutation was present in a high percentage (36%) of included patients with CP, which makes this marker unreliable for the differentiation between CP and PC, yet low patient numbers preclude drawing definitive conclusions. No relation was found between mutations on distinct loci of the gene and the presence of PC (**Supplemental Table S2**). Hypermethylation of the promotor of the *CDKN2A* gene was present in 23% of investigated cases in PJ and 29-31% of cases in blood.[73,74] In the current systematic review, the sensitivity for detecting PC was low. However, as the specificity (73-100%) is high, it may be valuable as part of a biomarker panel.

NPTX2 is a tumor suppressor gene, the expression of which is downregulated through hypermethylation. In the three studies included in this systematic review, *NPTX2* hypermethylation was present in 51% of PJ samples from cases. This prevalence is lower than described for plasma (79-84%), tissue (100%) and cytology (73-81%),[75-79] but head-to-head comparisons are needed. As for *KRAS* and *TP53*, the use of *NPTX2* hypermethylation as diagnostic test for PC may potentially be hampered by its occurrence in early malignant development (PanIn1).[80] However, this is not seen in the current systematic review; 1 of 15 patients with IPMN harbored *NPTX2* hypermethylation (there is no data on *NPTX2* hypermethylation in PJ from patients with PanIN).

A limitation of this systematic review is the heterogeneity (I^2) of the diagnostic performances. Comparison the presence of DNA alterations in cases with HGD and PC with those in control subgroups (NP, CP, HRI, PL) showed that this may be the result of heterogeneous controls groups (as less heterogeneity was found in subgroup analysis), each harboring a different risk of having HGD or PC. Additionally, as expected, DNA alterations in PJ were less potent in differentiating PC from PL than from NP or CP. The control group 'PL' should be interpreted with caution as it consists of different lesions (PanIn, IPMN, MCN, unspecified pancreatic cyst), which were generally not proven by histology. Although we aimed for exclusion of individuals for whom it was unclear if they harbored HGD/PC, PA-unproven precursor lesions may harbor (microscopic) HGD/PC. Furthermore, studies that included HRI as controls showed a lower diagnostic

performance (especially specificity). This might be caused by the presence of germline mutations (*e.g., CDKN2A, BRCA1, BRCA2*) in this group, rendering these individuals more prone to accumulation of additional mutations.

Furthermore, PJ collection methods, DNA analyses and statistical analyses varied between studies and may have caused heterogeneity. In the past, PJ was collected more often by direct cannulation of the ampullary orifice. Nowadays, secretin infusion enables non-invasive collection from the duodenal lumen during EUS.[7] For instance, studies that performed PJ collection after secretin stimulation show a higher prevalence of mutated *TP53* than studies that did not. This is similar to results published by Doyle *et al.* (2012)[81] on proteins, which show a one- to two-fold higher concentration of proteins in PJ collected by pancreatic duct cannulation as compared to collection from the duodenal lumen (after secretin stimulation). In addition, the PJ collection methods varied, which may have influenced results.[7] With regard to the DNA analysis, the choice of material, the concentration of input DNA or the selected thresholds can differ. We recommend to interpret the described data as relative and not absolute values.

In conclusion, *TP53, SMAD4,* and *CDKN2A* mutations and *NPTX2* methylation provide the highest specificity, yet are associated with a low sensitivity. Likely, combining these markers in panels will improve their diagnostic accuracy. Further prospective studies are needed to directly compare these (panels of) DNA alterations in PJ of PC patients and well-defined control groups. Additionally, longitudinal studies are required prior to implementation in a surveillance program to evaluate if changes over time (detection of new mutations or higher concentrations) may inform about cancer development in individuals undergoing surveillance and their diagnostic value in patients with sub-centimeter cancer.

References

1 Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. *CA Cancer J Clin* 2020;70:7-30.

2 Canto MI, Harinck F, Hruban RH, *et al*. International Cancer of the Pancreas Screening (CAPS) Consortium summit on the management of patients with increased risk for familial pancreatic cancer. *Gut* 2013;62:339-47.

3 Overbeek KA, Levink IJM, Koopmann BDM, *et al.* Long-term yield of pancreatic cancer surveillance in high-risk individuals. *Gut* 2022;71:1152-1160.

4 Lee JH, Long GV, Menzies AM, *et al*. Association Between Circulating Tumor DNA and Pseudoprogression in Patients With Metastatic Melanoma Treated With Anti-Programmed Cell Death 1 Antibodies. *JAMA Oncol* 2018;4:717-721.

5 Lin KK, Harrell MI, Oza AM, *et al*. BRCA Reversion Mutations in Circulating Tumor DNA Predict Primary and Acquired Resistance to the PARP Inhibitor Rucaparib in High-Grade Ovarian Carcinoma. *Cancer Discov* 2019;9:210-219.

6 Zulato E, Attili I, Pavan A, *et al*. Early assessment of KRAS mutation in cfDNA correlates with risk of progression and death in advanced non-small-cell lung cancer. *Br J Cancer* 2020;123:81-91.

7 Levink IJM, Nesteruk K, Visser DI, *et al*. Optimization of Pancreatic Juice Collection: A First Step Toward Biomarker Discovery and Early Detection of Pancreatic Cancer. *Am J Gastroenterol* 2020;115:2103-2108.

8 Yachida S, Jones S, Bozic I, *et al*. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. *Nature* 2010;467:1114-7.

9 European Study Group on Cystic Tumours of the P. European evidence-based guidelines on pancreatic cystic neoplasms. *Gut* 2018;67:789-804.

10 Whiting PF, Rutjes AW, Westwood ME, *et al*. QUADAS-2: a revised tool for the quality assessment of diagnostic accuracy studies. *Ann Intern Med* 2011;155:529-36.

11 Lee J, Kim KW, Choi SH, *et al*. Systematic Review and Meta-Analysis of Studies Evaluating Diagnostic Test Accuracy: A Practical Review for Clinical Researchers-Part II. Statistical Methods of Meta-Analysis. *Korean J Radiol* 2015;16:1188-96.

12 Takano S, Fukasawa M, Kadokura M, *et al*. Mutational patterns in pancreatic juice of intraductal papillary mucinous neoplasms and concomitant pancreatic cancer. *Pancreas* 2019;48:1032-1040.

13 Takano S, Fukasawa M, Kadokura M, *et al*. Next-Generation Sequencing Revealed TP53 Mutations to Be Malignant Marker for Intraductal Papillary Mucinous Neoplasms That Could Be Detected Using Pancreatic Juice. *Pancreas* 2017;46:1281-1287.

14 Majumder S, Raimondo M, Taylor WR, *et al*. Methylated DNA in Pancreatic Juice Distinguishes Patients With Pancreatic Cancer From Controls. *Clin Gastroenterol Hepatol* 2019.

15 Suenaga M, Yu J, Shindo K, *et al*. Pancreatic juice mutation concentrations can help predict the grade of dysplasia in patients undergoing pancreatic surveillance. *Clin Cancer Res* 2018;24:2963-2974.

16 Yu J, Sadakari Y, Shindo K, *et al*. Digital next-generation sequencing identifies low-abundance mutations in pancreatic juice samples collected from the duodenum of patients with pancreatic cancer and intraductal papillary mucinous neoplasms. *Gut* 2017;66:1677-1687.
17 Ginesta MM, Diaz-Riascos ZV, Busquets J, *et al*. APC promoter is frequently methylated in pancreatic juice of patients with pancreatic carcinomas or periampullary tumors. *Oncol Lett* 2016;12:2210-2216.
18 Kisiel JB, Raimondo M, Taylor WR, *et al*. New DNA methylation markers for pancreatic cancer: Discovery, tissue validation, and pilot testing in pancreatic juice. *Clin Cancer Res* 2015;21:4473-4481.
1 Eshleman JR, Norris AL, Sadakari Y, *et al*. KRAS and guanine nucleotide-binding protein mutations in pancreatic juice collected from the duodenum of patients at high risk for neoplasia undergoing endoscopic ultrasound. *Clin Gastroenterol Hepatol* 2015;13:963-969.
20 Yokoyama S, Kitamoto S, Higashi M, *et al*. Diagnosis of pancreatic neoplasms using a novel method of DNA methylation analysis of mucin expression in pancreatic juice. *PLoS ONE* 2014;9.
21 Yao F, Sun M, Dong M, *et al*. NPTX2 hypermethylation in pure pancreatic juice predicts pancreatic neoplasms. *Am J Med Sci* 2013;346:175-180.
22 Kato N, Yamamoto H, Adachi Y, *et al*. Cancer detection by ubiquitin carboxyl-terminal esterase L1 methylation in pancreatobiliary fluids. *World J Gastroenterol* 2013;19:1718-1727.
23 Kanda M, Knight S, Topazian M, *et al*. Mutant GNAS detected in duodenal collections of secretin-stimulated pancreatic juice indicates the presence or emergence of pancreatic cysts. *Gut* 2013;62:1024-1033.
24 Kanda M, Sadakari Y, Borges M, *et al*. Mutant TP53 in duodenal samples of pancreatic juice from patients with pancreatic cancer or high-grade dysplasia. Clin Gastroenterol Hepatol 2013;11:719-730.e5.
25 Azuara D, Ginesta MM, Gausachs M, *et al*. Nanofluidic digital PCR for KRAS mutation detection and quantification in gastrointestinal cancer. *Clin Chem* 2012;58:1332-1341.
26 Mizuno O, Kawamoto H, Yamamoto N, *et al*. Single-pattern convergence of k-ras mutation correlates with surgical indication of intraductal papillary mucinous neoplasms. *Pancreas* 2010;39:617-621.
27 Kobayashi N, Inamori M, Fujita K, *et al*. Characterization of K-ras gene mutations in association with mucinous hypersecretion in intraductal papillary-mucinous neoplasms. *J Hepato-Biliary-Pancreatic Surg* 2008;15:169-177.
28 Watanabe H, Okada G, Ohtsubo K, *et al*. Aberrant methylation of Secreted Apoptosis-Related Protein 2 (SARP2) in pure pancreatic juice in the diagnosis of pancreatic neoplasms. *Pancreas* 2006;32:382-389.
29 Watanabe H, Ha A, Hu YX, *et al*. K-ras mutations in duodenal aspirate without secretin stimulation for screening of pancreatic and biliary tract carcinoma. *Cancer* 1999;86:1441-1448.

30 Watanabe H, Yamaguchi Y, Ha A, *et al*. Quantitative determination of K-ras mutations in pancreatic juice for diagnosis of pancreatic cancer using hybridization protection assay. *Pancreas* 1998;17:341-347.
31 Ohtsubo K, Watanabe H, Yao F, *et al*. Preproenkephalin hypermethylation in the pure pancreatic juice compared with p53 mutation in the diagnosis of pancreatic carcinoma. *J Gastroenterol* 2006;41:791-797.
32 Matsubayashi H, Canto M, Sato N, *et al*. DNA methylation alterations in the pancreatic juice of patients with suspected pancreatic disease. *Cancer Res* 2006;66:1208-1217.
33 Jiang P, Watanabe H, Okada G, *et al*. Diagnostic utility of aberrant methylation of tissue factor pathway inhibitor 2 in pure pancreatic juice for pancreatic carcinoma. *Cancer Sci* 2006;97:1267-1273.
34 Bian Y, Matsubayashi H, Pin-Li C, *et al*. Detecting low-abundance p16 and p53 mutations in pancreatic juice using a novel assay: Heteroduplex analysis of limiting dilution PCRs. *Cancer Biol Ther* 2006;5:1392-1399.
35 Yan L, McFaul C, Howes N, *et al*. Molecular analysis to detect pancreatic ductal adenocarcinoma in high-risk groups. *Gastroenterology* 2005;128:2124-2130.
36 Wang Y, Yamaguchi Y, Watanabe H, *et al*. Detection of p53 Gene Mutations in the Supernatant of Pancreatic Juice and Plasma from Patients with Pancreatic Carcinomas. *Pancreas* 2004;28:13-19.
37 Sato N, Fukushima N, Maitra A, *et al*. Discovery of novel targets for aberrant methylation in pancreatic carcinoma using high-throughput microarrays. *Cancer Res* 2003;63:3735-3742.
38 Fukushima N, Walter KM, Uek T, *et al*. Diagnosing pancreatic cancer using methylation specific PCR analysis of pancreatic juice. *Cancer Biol Ther* 2003;2:78-83.
39 Tada M, Tateishi K, Kawabe T, *et al*. Quantity of mutant K-ras gene in pancreatic secretions for diagnosis of pancreatic carcinoma with different assays: Analysis of 100 patients. *Clin Chim Acta* 2002;324:105-111.
40 Costentin L, Pagès P, Bouisson M, *et al*. Frequent deletions of tumor suppressor genes in pure pancreatic juice from patients with tumoral or nontumoral pancreatic diseases. *Pancreatology* 2002;2:17-25.
41 Nakaizumi A, Oka T, Takenaka A, *et al*. Frequent multiple c-Ki-ras oncogene activation in pancreatic juice from patients with benign pancreatic cysts. *Dig Endosc* 2001;13:77-81.
42. Ha A, Watanabe H, Yamaguchi Y, *et al*. Usefulness of supernatant of pancreatic juice for genetic analysis of K-ras in diagnosis of pancreatic carcinoma. *Pancreas* 2001;23:356-363.
43 Myung SJ, Kim MH, Kim YS, *et al*. Telomerase activity in pure pancreatic juice for the diagnosis of pancreatic cancer may be complementary to K-ras mutation. *Gastrointest Endosc* 2000;51:708-713.
44 Futakawa N, Kimura W, Yamagata S, *et al*. Significance of K-ras mutation and CEA level in pancreatic juice in the diagnosis of pancreatic cancer. *J Hepatobiliary Pancreat Surg* 2000;7:63-71.
45 Yamaguchi Y, Watanabe H, Yrdiran S, *et al*. Detection of mutations of p53 tumor suppressor gene in pancreatic juice and its application to diagnosis of patients with pancreatic cancer: Comparison with K-ras mutation. *Clin. Cancer Res*. 1999;5:1147-1153.

46 Tateishi K, Tada M, Yamagata M, *et al*. High proportion of mutant K-ras gene in pancreatic juice of patients with pancreatic cystic lesions. *Gut* 1999;45:737-740.

47 Okai T, Watanabe H, Yamaguchi Y, *et al*. EUS and K-ras analysis of pure pancreatic juice collected via a duodenoscope after secretin stimulation for diagnosis of pancreatic mass lesion: A prospective study. *Gastrointest Endosc* 1999;50:797-803.

48. Nakamura Y, Onda M, Uchida E. Analysis of K-ras codon 12 point mutations using duodenal lavage fluid for diagnosis of pancreatic carcinoma. *Pancreas* 1999;18:133-140.

49 Kondoh S, Kaino M, Okita S, *et al*. Detection of Ki-ras and p53 gene mutations in tissue and pancreatic juice from pancreatic adenocarcinomas. *J Gastroenterol* 1998;33:390-396.

50 Kondo H, Sugano K, Fukayama N, *et al*. Detection of K-ras gene mutations at codon 12 in the pancreatic juice of patients with intraductal papillary mucinous tumors of the pancreas. *Cancer* 1997;79:900-905.

51 Iguchi H, Sugano K, Fukayama N, *et al*. Analysis of Ki-ras codon 12 mutations in the duodenal juice of patients with pancreatic cancer. *Gastroenterology* 1996;110:221-226.

52 Matsubayashi H, Sato N, Fukushima N, *et al*. Methylation of cyclin D2 is observed frequently in pancreatic cancer but is also an age-related phenomenon in gastrointestinal tissues. *Clin Cancer Res* 2003;9:1446-1452.

53 Choi MH, Mejlænder-Andersen E, Manueldas S, *et al*. Mutation analysis by deep sequencing of pancreatic juice from patients with pancreatic ductal adenocarcinoma. *BMC Cancer* 2019;19:11.

54 Sausen M, Phallen J, Adleff V, *et al*. Clinical implications of genomic alterations in the tumour and circulation of pancreatic cancer patients. *Nat Commun* 2015;6:7686.

55 Waddell N, Pajic M, Patch AM, *et al*. Whole genomes redefine the mutational landscape of pancreatic cancer. *Nature* 2015;518:495-501.

56 Bailey P, Chang DK, Nones K, *et al*. Genomic analyses identify molecular subtypes of pancreatic cancer. *Nature* 2016;531:47-52.

57 Patel H, Okamura R, Fanta P, *et al*. Clinical correlates of blood-derived circulating tumor DNA in pancreatic cancer. *Journal of hematology & oncology* 2019;12:130-130.

58 van der Sijde F, Azmani Z, Besselink MG, *et al*. Circulating TP53 mutations are associated with early tumor progression and poor survival in pancreatic cancer patients treated with FOLFIRINOX. *Ther Adv Med Oncol* 2021;13:17588359211033704.

59 Noe M, Niknafs N, Fischer CG, *et al*. Genomic characterization of malignant progression in neoplastic pancreatic cysts. *Nat Commun* 2020;11:4085.

60 Hosoda W, Chianchiano P, Griffin JF, *et al*. Genetic analyses of isolated high-grade pancreatic intraepithelial neoplasia (HG-PanIN) reveal paucity of alterations in TP53 and SMAD4. *J Pathol* 2017;242:16-23.

61 di Magliano MP, Logsdon CD. Roles for KRAS in pancreatic tumor development and progression. *Gastroenterology* 2013;144:1220-1229.

62 Witkiewicz AK, McMillan EA, Balaji U, *et al*. Whole-exome sequencing of pancreatic cancer defines genetic diversity and therapeutic targets. *Nat Commun* 2015;6:6744.

63 Faias S, Pereira L, Luís Â, *et al*. KRAS in Cyst Fluid Obtained by Endoscopic Ultrasound-Fine-Needle Aspiration in Pancreatic Cystic Lesions: A Systematic Review and Meta-analysis. *Pancreas* 2019;48:749-758.

64 Zhuang R, Li S, Li Q, *et al*. The prognostic value of KRAS mutation by cell-free DNA in cancer patients: A systematic review and meta-analysis. *PLoS One* 2017;12:e0182562.

65 Mas L, Lupinacci RM, Cros J, *et al*. Intraductal Papillary Mucinous Carcinoma Versus Conventional Pancreatic Ductal Adenocarcinoma: A Comprehensive Review of Clinical-Pathological Features, Outcomes, and Molecular Insights. *Int J Mol Sci* 2021;22.

66 Parker LA, Lumbreras B, Lopez T, *et al*. How useful is it clinically to analyse the K-ras mutational status for the diagnosis of exocrine pancreatic cancer? A systematic review and meta-analysis. *Eur J Clin Invest* 2011;41:793-805.

67 Yang J, Li J, Zhu R, *et al*. K-ras mutational status in cytohistological tissue as a molecular marker for the diagnosis of pancreatic cancer: a systematic review and meta-analysis. *Dis Markers* 2014;2014:573783.

68 Ahmed S, Bradshaw AD, Gera S, *et al*. The TGF-β/Smad4 Signaling Pathway in Pancreatic Carcinogenesis and Its Clinical Significance. *J Clin Med* 2017;6.

69 Patra KC, Kato Y, Mizukami Y, *et al*. Mutant GNAS drives pancreatic tumourigenesis by inducing PKA-mediated SIK suppression and reprogramming lipid metabolism. *Nature Cell Biology* 2018;20:811-822.

70 O'Hayre M, Vázquez-Prado J, Kufareva I, *et al*. The emerging mutational landscape of G proteins and G-protein-coupled receptors in cancer. *Nat Rev Cancer* 2013;13:412-24.

71 Wu J, Matthaei H, Maitra A, *et al*. Recurrent GNAS mutations define an unexpected pathway for pancreatic cyst development. *Sci Transl Med* 2011;3:92ra66.

72 Kawabata H, Ono Y, Tamamura N, *et al*. Mutant GNAS limits tumor aggressiveness in established pancreatic cancer via antagonizing the KRAS-pathway. *J Gastroenterol* 2022;57:208-220.

73 Jiao L, Zhu J, Hassan MM, *et al*. K-ras mutation and p16 and preproenkephalin promoter hypermethylation in plasma DNA of pancreatic cancer patients: in relation to cigarette smoking. *Pancreas* 2007;34:55-62.

74 Park JW, Baek IH, Kim YT. Preliminary study analyzing the methylated genes in the plasma of patients with pancreatic cancer. *Scand J Surg* 2012;101:38-44.

75 Park JK, Ryu JK, Yoon WJ, *et al*. The role of quantitative NPTX2 hypermethylation as a novel serum diagnostic marker in pancreatic cancer. *Pancreas* 2012;41:95-101.

76 Singh N, Rashid S, Rashid S, *et al*. Clinical significance of promoter methylation status of tumor suppressor genes in circulating DNA of pancreatic cancer patients. *J Cancer Res Clin Oncol* 2020;146:897-907.

77. Zhang L, Gao J, Li Z, *et al*. Neuronal pentraxin II (NPTX2) is frequently down-regulated by promoter hypermethylation in pancreatic cancers. *Dig Dis Sci* 2012;57:2608-14.

78 Parsi MA, Li A, Li CP, *et al*. DNA methylation alterations in endoscopic retrograde cholangiopancreatography brush samples of patients with suspected pancreaticobiliary disease. *Clin Gastroenterol Hepatol* 2008;6:1270-8.

79 Park JK, Ryu JK, Lee KH, *et al*. Quantitative analysis of NPTX2 hypermethylation is a promising molecular diagnostic marker for pancreatic cancer. *Pancreas* 2007;35:e9-15.

80 Sato N, Fukushima N, Hruban RH, *et al*. CpG island methylation profile of pancreatic intraepithelial neoplasia. *Mod Pathol* 2008;21:238-44.

81 Doyle CJ, Yancey K, Pitt HA, *et al*. The proteome of normal pancreatic juice. *Pancreas* 2012;41:186-94.

A. Overview of the questions

Question/ applicability	Domains			
	Patient selection	Index test	Reference standard	Flow and timing
Signaling question 1	Was a consecutive or random sample of patients enrolled?	Were the index test results interpreted without knowledge of the results of the reference standard?	Is the reference standard likely to correctly classify the target condition	Was there an appropriate interval between index test and reference standard?
Signaling question 2	Was a case-control design avoided?	If a threshold was used; was it pre-specified?	Were the reference standard results interpreted without knowledge of the results of the index test?	Did all patients receive the same reference standard?
Signaling question 3	Did the study avoid inappropriate exclusions?	-	-	Were all patients included in the analysis?
Applicability	Are there concerns that the included patients and setting do not match the review question?	Are there concerns that the index test; its conduct; or interpretation differ from the review question?	Are there concerns that the target condition as defined by the reference standard does not match the question?	

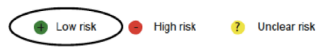
B. In-house scoring system

Risk of bias	Domains			
	Patient selection	Index test	Reference standard	Flow and timing
low risk (* -1)	consecutive random order patient samples +1	blinding dna test and reference standard +1	reference test correct classify target condition +2	timing interval between tests +1
unclear risk (* 0)	case control design avoided +2	threshold pre-specified +2	blinding reference standard and dna test +1	cases and control same test +1
high risk (*+1)	inappropriate exclusion avoided +1	-	-	all patient included in analysis +2

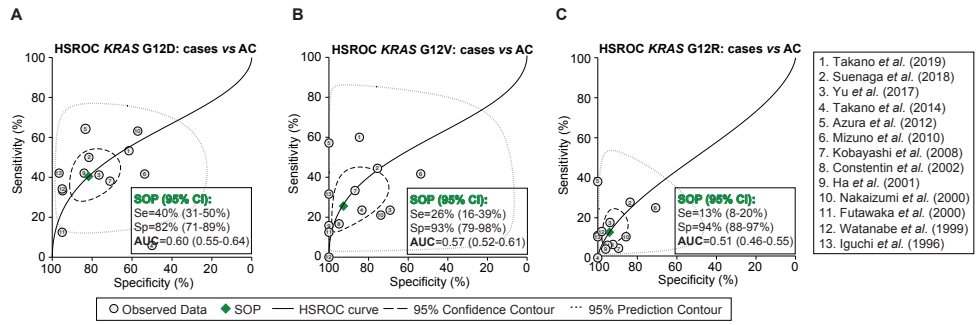
C. Example of scoring patient selection

Article x; patient selection:
- Unclear risk of consecutive order patient samples (=0*1)
- Low risk of case-control design (avoided = -1*2)
- High risk of inappropriate exclusion (not avoided = 1*1)

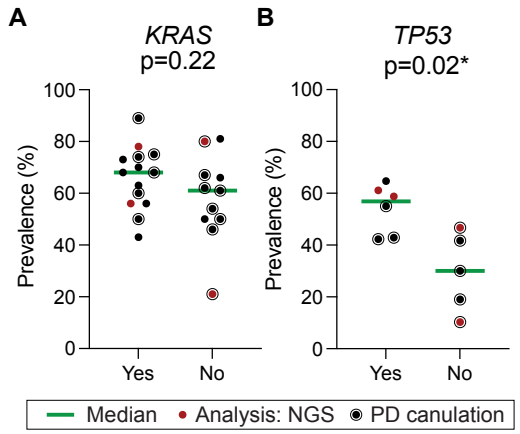
SUM = 0*1 + -1*2 + 1*1 = -1 (low risk)



SUPPLEMENTAL FIGURE S1 | Overview of questions of Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2; A) and in-house scoring system (B, C).



SUPPLEMENTAL FIGURE S2 | **Hierarchical summary receiving operating curves (HSROCs) showing the diagnostic performance for detection PC with *KRAS* G12D (A), G12V (B), and G12R (C) mutation.** PC = pancreatic cancer, AC = all controls, SOP = summary operating point, Se=sensitivity, Sp=specificity; AUC = area under the curve.



SUPPLEMENTAL FIGURE S3 | **The prevalence of detected gene mutations for KRAS (A) and TP53 (B) for secretin-induced samples (Yes), as compared to those which were not secretin-induced (No). Green lines indicate the median.** The figure also indicates the influence of next-generation sequencing (NGS) and PD canulation on detecting a gene mutation.

SUPPLEMENTAL TABLE S1 | Search string and results in different databases.

Search string and results in different databases	Initial search	After duplicates removed
Embase.com		
('pancreas juice'/exp OR (((pancrea* NEAR/3 juice)) OR (pancrea* NEAR/3 fluid AND secretin*)):ab;ti) AND ('biological marker'/exp OR 'nucleic acid'/exp OR 'nucleic acid metabolism'/exp OR 'biological factor'/exp OR 'mutation'/exp OR (((biological*) NEAR/3 (marker* OR factor*)) OR biomarker* OR nucleic-acid OR dna OR cfdna OR cf-dna OR mutation*):ab;ti) AND ('pancreas tumor'/exp OR neoplasm/de OR 'pancreas dysplasia'/de OR (tumo* OR cancer* OR carcinoma* OR adenoma* OR adenocarcinoma* OR neoplas* OR dysplasia* OR ipma OR ipmn OR precancer* OR premalign* OR precursor*):ab;ti) NOT ('case report'/de OR 'case report':ti) NOT ([Conference Abstract]/lim AND [1800-2017]/py)	400	392
Medline Ovid		
(Pancreatic Juice / OR (((pancrea* ADJ3 juice)) OR (pancrea* ADJ3 fluid AND secretin*)).ab;ti.) AND (Biomarkers/ OR exp Nucleic Acids/ OR exp Biological Factors/ OR Mutation/ OR (((biological*) ADJ3 (marker* OR factor*)) OR biomarker* OR nucleic-acid OR dna OR cfdna OR cf-dna OR mutation*). ab;ti.) AND (Pancreatic Neoplasms / OR Neoplasms / OR (tumo* OR cancer* OR carcinoma* OR adenoma* OR adenocarcinoma* OR neoplas* OR dysplasia* OR ipma OR ipmn OR precancer* OR premalign* OR precursor*).ab;ti.) NOT (case reports/ OR case report.ti.)	403	140
Web of science core collection		
TS=(((((pancrea* NEAR/2 juice)) OR (pancrea* NEAR/2 fluid AND secretin*))) AND ((((biological*) NEAR/2 (marker* OR factor*)) OR biomarker* OR nucleic-acid OR dna OR cfdna OR cf-dna OR mutation*)) AND ((tumo* OR cancer* OR carcinoma* OR adenoma* OR adenocarcinoma* OR neoplas* OR dysplasia* OR ipma OR ipmn OR precancer* OR premalign* OR precursor*)))) NOT TI=("case report") AND DT=(article	216	47
Cochrane CENTRAL		
((((pancrea* NEAR/3 juice)) OR (pancrea* NEAR/3 fluid AND secretin*)):ab;ti) AND ((((biological*) NEAR/3 (marker* OR factor*)) OR biomarker* OR nucleic-acid OR dna OR cfdna OR cf-dna OR mutation*):ab;ti) AND ((tumo* OR cancer* OR carcinoma* OR adenoma* OR adenocarcinoma* OR neoplas* OR dysplasia* OR ipma OR ipmn OR precancer* OR premalign* OR precursor*):ab;ti)	3	1
Total:	**1022**	**580**

SUPPLEMENTALTABLE S2 | Locations of somatic mutations identified per study: cases *vs* controls. Specific somatic mutations were not specified in Majumber *et al.* (2019); Ginesta *et al.* (2016); Eshleman *et al.* (2015); Kisiel *et al.* (2015); Bian *et al.* (2006); Othsubo *et al.* (2006); Wang *et al.* (2004); Tada *et al.* (2001); Myung *et al.* (2000); Okai *et al.* (1999); Yamaguchi *et al.* (1999); Nakamura *et al.* (1999); Watanabe *et al.* (1998); Kondoh *et al.* (1998); Kondo *et al.* (1997) and therefore not shown in this table.

Study (year of publication)	DNA mutation investigated	Patient cohort	*KRAS*	*TP53*	*CDKN2A*	*SMAD4*	*GNAS*
Takano *et al.* (2019)	*KRAS; TP53; GNAS*	Cases (n=15)	9 G12V; 8 G12D; 2 G61H; 1 G12R; *7 multiple*†	1 Y234del; 1 E346G; 1 S183T; 1 I162S; 1 C242fs; 1 L130I; 1 N288D; 1 V272G; 1 E287G; *2 multiple*†	NI	NI	NR
		Controls (n=13)	5 G12D; 2 G12V; 1 G12R; 1 G13V; *3 multiple*†	1 T170R	NI	NI	NR
Suenaga *et al.* (2018)	*KRAS; GNAS; TP53; SMAD4; RNF43; CDKN2A; TGFBR2; BRAF; PIK3CA*	Cases (n=18)	9 G12D; 8 G12V; 5 G12R; 1 G12C; 1 G13R; 1 Q61R; 1 Q61K; *8 multiple*†	2 S315Y; 2 R273C; 1 R248Q; 1 R248W; 1 H365fs; 1 R283H; 1 W91X; 1 V274A; 1 H193Y; 1 N288D; 1 N310D; 1 G266fs; 1 Q331fs; 1 R175H; *4 multiple*†	1 R128W; 1 D108V	1 G299fs; 1 Y434fs; 1 G89fs; 1 D537G; 1 L533R; *1 multiple*†	6 R201C
		Controls (n=49)	12 G12V; 9G12D; 8 G12R; 5 Q61H; 5 Q61R; 2 G12S; 2 G13D; 1 G13R; 1 G12A; 1 Q61L; *11 multiple*†	2 R273H; 1 H214R; 1 H179R; 1 R248W; 1 R306X; 1 R282Q; 1 M237fs; 1 G245S; 1 R248Q; 1 c.375+2A>AG; 1 559.2A>AG; *2 multiple*†	1 G67S; 1 F90L	1 C499R	8 R201H; 7 R201C; *3 multiple*†
Yu *et al.* (2017)	*KRAS; GNAS; TP53; SMAD4; RNF43; CDKN2A; TGFBR2; BRAF; PIK3CA*	Cases (n=34)	14 G12D; 8 G12V; 6 G12R; 6 G12S; 4 Q61H; 6 G13D; 2 Q61L; 2 Q61R; 2 G12C; 1 G13C; 1 Q61P; *13 multiple*†	2 R196X; 4 R175H; 3 R248Q; 4 R248W; 1 L194R; 2 Y220C; 1 C141G; 1 R181C; 1 L130Sfs; 1 R273C; 1 C277R; 1 Y163C; 1 Y220C; *2 multiple*†	NR	1 Q311X; 1 Q256X; 1 T273fs; 1 A457V; 1 M543T; 1 K50Kfs; *1 multiple*	8 R201C; 5 R201H; 1 Q227R; *1 multiple*†
		Controls (n=81)	25 G12V; 19 G12D; 11 Q61R; 10 Q61H; 9 G12S; 9G13D; 6 Q61L; 6 G12C; 5 G12R; 1 G12A; *27 multiple*†	3 R175H; 2 R248W; 1 R196L; 2 R248Q; 1 Q165X; 1 Q136X 1 K291R; 1 M246V; 2 R282W; 1 G245S	NR	1 W524R	19 R201C; 14 R201H; 6 Q227R; 1 Q227P; *6 multiple*†
Takano *et al.* (2014)	*KRAS; GNAS; TP53; SMAD4*	Cases (n=17)	4 G12V; 1 G12D	NR	NI	NR	3 R201C
		Controls (n=6)	3 G12D; 1 G12V; 1 Q61H	NR	NI	NR	3 R201H
Kanda *et al.* (2013)	*GNAS*	Cases (n=2)	NI	NI	NI	NI	1 R201H; 1 R201C
		Controls (n=109)	NI	NI	NI	NI	39 R201C; 25 R201H;1 R201S; 1 R201G; *4 multiple*†
Kanda *et al.* (2013)	*TP53*	Cases (n=43)	NI	2 C242Y; 2 R248W; 2 R282W; 1 T155P; 1 R156C; 1 R158C; 1 A159T; 1 S166L; 1 T170M; 1 R174S; 1 R175H; 1 L194R; 1 D207E; 1 R213L; 1 H214D; 1 P219S; 1 Y220C; 1 Y234C; 1 C242S;1 M246T; 1 R248Q; 1 V272M; 1 R273C; 1 K292E; 1 E298D; 1 R290C; 1 12609Tdel; 1 12417Adel; *2 multiple*†	NI	NI	NI
		Controls (n=28)	NI	1 R175C; 1 L188M; 1 P222T; 1 S261N; 1 C275R	NI	NI	NI

Study (year of publication)	DNA mutation investigated	Patient cohort	*KRAS*	*TP53*	*CDKN2A*	*SMAD4*	*GNAS*
Azura *et al.* (2012)	*KRAS*	Cases (n=42)	27 G12D; 24 G12V; 20 G12S; 19 G12C; 16G12R; 1 G12A; *24 multiple*†	NI	NI	NI	NI
		Controls (n=6)	2 G12C; 1G12D; 1 G12S; *1 multiple*†	NI	NI	NI	NI
Mizuno *et al.* (2010)	*KRAS*	Cases (n=12)	5 G12D; 5 G12V; 3 G12R; 8 *multiple*†	NI	NI	NI	NI
		Controls (n=41)	19 G12D; 19 G12V; 12 G12R; 8 *multiple*†	NI	NI	NI	NI
Kobayashi *et al.* (2008)	*KRAS; CDKN2A; TP53*	Cases (n=21)	8 G12D; 7 G12V; 1 G12R; *2 multiple*†	NR	NR	NI	NI
		Controls (n=38)	11 G12D; 5 G12V; 4 G12R; *4 multiple*†	NR	NR	NI	NI
Yan *et al.* (2005)	*KRAS; TP53*	Cases (n=48)	NR	2 C242R; 1 L194F; 1 D218G; 1 G245D; 1 R248Q; 1 R248W; 1 R273C; 1 R283C; 1 R181P; 1 R209T; 1 R213L; 1 M237K; 1 T256K; 1 L264R; 1 E271stop; 1 E271A	NI	NI	NI
		Controls (n=98)	NR	1 Y236G; 1 E271stop	NI	NI	NI
Constentin *et al.* (2002)	*KRAS; CDKN2A (p16); DPC4*	Cases (n=18)	6 G12D; 3 G12V; 2 G12R	NI	NR	NI	NI
		Controls (n=39)	2 G12D; 2 G12V	NI	NR	NI	NI
Ha *et al.* (2001)	*KRAS*	Cases (n=19)	8 G12D; 5 G12C; 3 G12V; 1 G12R	NI	NI	NI	NI
		Controls (n=25)	4 G12D; 2 G12C; 1 G12R	NI	NI	NI	NI
Nakaizumi *et al.* (2001)	*KRAS*	Cases (n=19)	12 G12D; 4 G12V; 3 G12A; 2 G12R; G12S; 7 *multiple*†	NI	NI	NI	NI
		Controls (n=42)	18 G12D; 11 G12V; 5 G12A; 6 G12R; 1 G12C; 1 G12S; *13 multiple*†	NI	NI	NI	NI
Futawaka *et al.* (2000)	*KRAS*	Cases (n=16)	2 G12D; 2 G12V; 1 G12R	NI	NI	NI	NI
		Controls (n=20)	1 G12R; 1 G12A; 1 G12D; *1 multiple*†	NI	NI	NI	NI
Watanabe *et al.* (1999)	*KRAS*	Cases (n=38)	13 G12D; 5 G12R; 4 G12S; 3 G12C	NI	NI	NI	NI
		Controls (n=98)	5 G12D; 4 G12S; 2 G12R; 1 G12C	NI	NI	NI	NI
Iguchi *et al.* (1996)	*KRAS*	Cases (n=19)	8 G12D; 6 G12V; 2 G12R	NI	NI	NI	NI
		Controls (n=29)	1 G12D	NI	NI	NI	NI

‡Number of patients of which the specific somatic mutation was shown. †Indicated for those patients who harbored multiple mutations in the specific gene (e.g., KRAS); the given numbers of mutations per location on the gene are including these mutations (e.g., 12 times G12D may be the sum of 11 patients having G12D (alone) * 10 + G12D * 1 (from patient that also had G12V). NI = not investigated; NR = Not reported (in reference of origin).

SUPPLEMENTAL TABLE S2 | Diagnostic performance of investigated methylated gene panels in included studies. Specificities ≥ 80% and sensitivities ≥ 70% were marked bold. Kisiel *et al.* (2015), Kato *et al.* (2013), Yao *et al.* (2013), Jiang *et al.* (2006), Othsubo *et al.* (2006), Watanabe *et al.* (2006), Yan *et al.* (2005), Fukushima *et al.* (2003) and Matsubayashi *et al.* (2003) did not present any results on gene panels and were therefore not presented in this table.

Methylated genes	Reference	Cases (n)	Sensitivity, % (95% CI)	Controls	Specificity, % (95% CI)
C13orf18, FER1L4, BMP3	Majumber *et al.* (2019)	PC (35) + HGD (3)	**74** (57-87)	NP (32) + benign (41)	**99** (NR)
APC, HRH2, CDH13, SPARC, EN-1	Ginesta *et al.* (2016)	PC (83)	**72** (61-80)	CP (14)	**93** (68-99)
MUC1, MUC2, MUC4	Yokoyama *et al.* (2013)	PC (15)	**87** (NR)	PL (28)	**80** (NR)
CDKN2A (p16), Cyclin D2, TFPI2, NPTX2, ppENK, FOXE1	Matsubuyashi *et al.* (2006)	PC (56) + in situ (8)	**82** (NR)	CP (26) + HRI (43) + NP (11) + PL (9)	**100** (NR)
NPTX2, SARP2, CLDN5	Sato *et al.* (2003)	PC (24)	69 (48-86)	CP (8)	**100** (63-100)

[a]Benign group consists of CP n=17, intraductal papillary mucinous neoplasm (IPMN) with low-grade dysplasia n=2 , unspecified cysts n=3, fatty pancreas n=4, imaging finding indeterminate for chronic pancreatitis n=15. PC = pancreatic cancer; NP = non-pancreatic; PL = precursor lesions (including benign IPMN, Mucinous cystic neoplasm, PanIn); CP = chronic pancreatitis; NR = not reported (in reference of origin).

CHAPTER 12

Mutation Analysis of Pancreatic Juice and Plasma for the Detection of Pancreatic Cancer

Authors
Iris J.M. Levink, Maurice P.H.M. Jansen, Zakia Azmani, Wilfred van IJcken, Ronald van Marion, Maikel P. Peppelenbosch, Djuna L. Cahen, Gwenny M. Fuhler, Marco J. Bruno

International Journal of Molecular Sciences. 2023; 24(17):13116.

Abstract

Background and aims: Molecular profiling may enable earlier detection of pancreatic cancer (PC) –including in high-risk individuals undergoing surveillance – and allow for personalization of treatment. We hypothesized that the detection rate of DNA mutations is higher in pancreatic juice (PJ) than that in plasma due to its closer contact with the pancreatic ductal system from which pancreatic cancer cells originates and higher overall cell-free DNA (cfDNA) concentrations.

Methods: In this study, we included patients with pathology-proven PC or intraductal papillary mucinous neoplasm (IPMN) with high-grade dysplasia (HGD) from two prospective clinical trials (KRASPanc and PACYFIC), for whom both PJ and plasma were available. We performed next generation sequencing on PJ, plasma and tissue samples and described the presence (and concordance) of mutations in these biomaterials.

Results: We included 26 patients (25 PC, 1 IPMN with HGD), of which seven were female (27%), with a median age of 71 years (IQR 12), and median BMI of 23 kg/m^2 (IQR 4). Ten patients with PC (40%) were (borderline) resectable at baseline. Tissue was available from six patients (resection n=5, biopsy n=1). A median volume of 2.9 mL plasma (IQR 1.0 mL) and 0.7 mL PJ (IQR 0.1 mL, p<0.001) was used for DNA isolation. PJ had a higher median cfDNA concentration (2.6 ng/µL [IQR 4.2]) than plasma (0.29 ng/µL [IQR 0.40]). A total of 41 unique somatic mutations were detected; 24 mutations in plasma (2 *KRAS*, 15 *TP53*, 2 *SMAD4*, 3 *CDKN2A* 1 *CTNNB1*, 1 *PIK3CA*), 19 in PJ (*3 KRAS, 15 TP53, 1 SMAD4*) and 8 in tissue (2 *KRAS*, 2 *CDKN2A*, 4 *TP53*). The mutation detection rate (and the concordance with tissue) did not differ between plasma and PJ.

Conclusions: Whilst the concentration of cfDNA was indeed higher in PJ than in plasma, the mutation detection rate was not different. A few cancer-associated genetic variants were detected in both biomaterials. Further research is needed to increase the detection rate and assess the performance and suitability of plasma and PJ for PC (early) detection.

Introduction

Pancreatic cancer (PC) is a leading cause of cancer-related death due to asymptomatic progression leading to diagnosis at an unresectable stage in approximately 80% of patients,[1,2] whilst radical surgery of early-stage cancer, or preferably high-grade precursor lesions, is the only chance for long-term survival. So far, extensive imaging-based surveillance programs – following individuals at a high risk of developing PC – have not lived up their expectations.[3] Use of biomarkers may lead to earlier detection in these programs and, concurrently, support personalization of treatment.

Cancers, including PC, arise as a consequence of accumulating gene mutations. Molecular profiling of tissue collected by fine-needle biopsy (FNB) – to assess these mutations – is less applicable for surveillance purposes, as it is invasive, relies on a visible mass and is expected to obtain information from a single clone. Additionally, collection during endoscopic ultrasound (EUS) has been challenging due to low tumor cellularity and limited yield of tissue.[4,5] To date, for PC, carbohydrate antigen 19.9 (CA19.9) is the only serum marker implemented in clinical practice. However, for surveillance purposes, CA19.9 is controversial, as elevated values are regularly observed in patients with no or low-grade dysplasia, and low values do not rule out progression. A panel of biomarkers (from different sources) may increase the diagnostic performance.

Gene mutations may be investigated in circulating tumor DNA (ctDNA), which consists of short cell-free DNA-fragments released into body fluids by cancer cells due to apoptosis, necrosis and secretion. Multiple trials have aimed to diagnose PC (and its precursor lesions) based on plasma ctDNA, as it is non-invasively collected and expected to contain information from all present clones.[6,7] However, in patients with PC, ctDNA concentrations in plasma are often below the limits of detection (especially in precursor lesions and early stages).[8-10] Also, detected alterations in plasma are not specific for the pancreas, as they may originate from another organ.

Pancreatic juice (PJ) may serve as an alternative biomarker source. A wash-out from the pancreas can be stimulated by secretin and collected from the duodenal lumen during endoscopy (without pancreatic duct cannulation or tissue sampling). As compared to FNB, PJ does not rely on a visible mass and is expected to contain information on the complete range of tumor clones.[11,12] As compared to blood, biomarkers determined in PJ are likely more pancreas-specific, as this fluid was in continuous close contact with the pancreatic ductal system from which PC originates. Also, PJ may harbor more ctDNA due to a higher overall cell-free DNA (cfDNA) concentration. On the other hand, it contains enzymes, rendering it important to snap-freeze the sample immediately after collection.[12]

We hypothesized that the cfDNA concentration and mutation detection rate PJ by next generation sequencing (NGS) are higher in PJ than in blood plasma. This study aims to compare the detection rate of gene mutations in (cf) DNA from tissue, plasma and PJ.

Methodology

Study design and patient inclusion

Plasma, PJ and tissue samples were prospectively collected at the Erasmus University Medical Center in Rotterdam, as part of two clinical studies: 1. KRASPanc-study (MEC-2018-038), concerning patients with (suspected) sporadic PC undergoing diagnostic EUS or fiducial placement for stereotactic radiotherapy; 2. PACYFIC study (MEC-2014-021), involving individuals undergoing surveillance for suspected neoplastic pancreatic cysts. See **Supplemental Table 1** for a description of the KRASPanc and PACYFIC study cohorts.

Participants of these clinical studies were included for the present study based on presence of (pathology-proven) high-grade dysplasia (HGD) or PC, and availability of both a plasma and PJ sample. Patients who had undergone radiotherapy or pancreatic resection prior to PJ collection were excluded. Availability of tissue samples was not an in- or exclusion criterion, and selection of tissue for next-generation sequencing (NGS) was based upon availability of fresh-frozen tissue (from FNB or tissue) that had been stored after clinical work up. No formal sample size analysis was performed due to the explorative nature of the study.

The institutional Medical Center ethical review board approved the studies, and included individuals gave written informed consent before enrolment. The studies were carried out according to the ethical principles for medical research involving human subjects from the World Medical Association Declaration of Helsinki.

Biomaterial collection

PJ collection was performed with a linear echoendoscope (Pentax Medical, Tokyo, Japan) by experienced endo-sonographers. After insertion of the tip of the echoendoscope into the D2 segment of the duodenum, secretion of PJ was stimulated by intravenous injection of human secretin (16 µg/patient, ChiRhoClin, Burtonsville, MD). Suction through the endoscopic channel was applied immediately after secretin injection for eight minutes, by positioning of the tip close to the ampullary orifice.[12] Within 10 minutes after collection, juice was aliquoted and snap frozen. Samples were stored at -80°C until use.

Plasma samples were collected by venipuncture in CellSave tubes (CellSearch, Bryn Athyn, PA, USA, #7900005) on the same day as the PJ collection. After collection, centrifugation at 1600 rpm for 10 min was performed, and samples were aliquoted and stored at -80°C until use. Before cfDNA isolation, samples were centrifuged at 16000 rpm for 10 min and DNA was isolated from the supernatant. Tissue samples were freshly frozen after resection (patient #1-4, #6) or FNB (#5) at the same day. Routine hematoxylin and eosin (H&E)-stained sections from tissue samples were assessed for tumor cellularity by a pathologist and areas enriched for tumor cells were identified and manually micro-dissected.

DNA isolation

For cfDNA isolation, the automatic bead-based Maxwell RSC cfDNA Plasma kit (Promega, Fitchburg, WI, AX1115) was used according to manufacturer's instructions. The choice of kit was based on previous results by our group.[12] To quantify the concentration of total (double stranded) DNA, the Quant-iT dsDNA High-Sensitivity Assay Kit was employed, according to manufacturer's instructions. For mutational cfDNA analysis of plasma and PJ samples, DNA libraries were prepared using 25 ng cfDNA input from PJ. From plasma samples, all available cfDNA (up to 25 ng) was used.

To prepare the tumor tissue samples for sequencing, the tissue was washed twice with PBS, and subsequently treated with 550 µl lysisbuffer and 20 µl protease overnight at 37-55 ºC. 3 µl RNase A was added and incubated for 15-60 minutes at 37 ºC. Subsequently, Chemagic MSM1 isolation robot (PerkinElmer Chemagen Technology, Baesweiler, Germany) was used to isolate the DNA according to manufacturer's recommendations.

Deep sequencing and data analysis

First, we performed a pilot and evaluated solely the plasma and PJ samples of four patients (#1-4) using the Oncomine Colon cfDNA Assay (Thermo Fisher Scientific, Waltham, MA, **Supplemental Table S2**), with library preparation according to manufacturer's recommendations. Results were contrasted against sequencing data derived for clinical purposes from tissue (patients #1-6) using an in-house generated pan-cancer AmpliSeq (ThermoFisher Scientific) panel covering 100% of *CDKN2A, KEAP1, PTEN, STK11, TP53,* and hotspots in other cancer genes (*BRAF, CTNNB1, EGFR, ERBB3, FBWX7, GNAS, KRAS, PIK3CA, SF3B1, SMAD4*, among others; **Supplemental Table S2**). Again, libraries were prepared according to manufacturer's recommendations. Subsequently, for all three biomaterials, template preparation was performed using an Ion Chef system, and sequencing was performed using the Ion GeneStudio S5 Prime System on 540 chips with the Ion 540 Chef Kit. Data were analyzed with the Variant Caller v5.10.0.18 and annotated using ANNOVAR.[13]

Secondly, we performed sequencing on an extended panel of genes to evaluate the presence of additional mutations in a larger group. For plasma and PJ samples of patients #5-26, cfDNA concentrations were first measured by real-time-qPCR using Alu115 primer pairs (forward: 5'- CCTGAGGTCAGGAGTTCGAG-3' and reverse: 5'-CCCGAGTAGCTGGGATTACA-3') using initial denaturation (at 95°C for three minutes – at 95°C for five seconds – at 62°C for 30 sec; Swift Biosciences, Ann Arbor, MI, USA). Additionally, real-time qPCR was performed using Alu247 primers (forward: 5'-GTGGCTCACGCCTGTAATC-3' and reverse: 5'-CAGGCTGGAGTGCAGTGG-3') primers using the same PCR program as for Alu115. As cfDNA exhibits a narrow size distribution of ±167 bp, Alu115-qPCR results quantifies the total amount of cfDNA, while the Alu247/Alu115 ratio illustrates the cfDNA integrity. DNA libraries were prepared by

multiplex PCR using the Accel-Amplicon 57G Plus Pan-Cancer Profiling Panel (Swift Biosciences, Ann Arbor, MI, USA) which covers 286 amplicons of 57 genes, amplified for 25 cycles in total (as per protocol), followed by the ligation of Illumina adaptors with sample specific indices. See **Supplemental Table S2** for the similarities and differences between used panels. Indexed sequencing libraries of PJ, plasma and tissue cfDNA were pooled and 250 paired-end or 300 base-pair sequenced on two flow cells of an Illumina MiSeq sequencer (Illumina, San Diego, CA, USA). From the reads, adapter sequences were trimmed and aligned to the human GRCh38 reference genome using BWA mem. Variant calling was performed using BCFtools combined with in-house scripts.

Non-synonymous variants were considered as true mutations if these had a variant allele frequency (AF) >0.1%, ≥5 mutated reads and if they are ≥5 times described as somatic mutation in PC patients in the databases of COSMIC, OncoKB and http://www.cancerhotspots.org. Mutations with allele frequencies higher than 49% were considered as homozygous or heterozygous germline mutations, not ctDNA mutations, and were excluded. The prevalence of *TP53* P72R homozygosity and heterozygosity was noted, as it may modify the effect of *TP53* hotspots mutants (whether it is associated with an increased risk of cancer remains highly controversial).[14-17] The potential pathogenicity of somatic mutations was evaluated using ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) and COSMIC (http://grch38-cancer.sanger.ac.uk/cosmic). Variants judged as benign were not tabulated. Recurrent mutations occurring in ≥10 samples were compared with prevalence in PC in COSMIC database, in case of an unusual higher (100x) prevalence, PCR errors (*e.g.*, polymerase slippage) and sequencing errors (*e.g.*, substitution errors and pseudogenes) were considered. These variants were only considered in case of AF >0.5%. For instance, this applied to *TP53* p.G245D, which was present in 25 of 34 samples (74%) analyzed with Accel-Amplicon 57G Plus Pan-Cancer Profiling Panel, yet present in only 9/2829 (0.3%) investigated patients in COSMIC database. This also applied to *SMAD4* p.R135* present in 17/34 (50%) samples (COSMIC: 7/2829) and *PIK3CA* p.E545A in 10/34 (29%) samples (COSMIC: 4/2273).

Statistical analysis

All statistical analyses were performed with SPSS (Statistical Package for the Social Sciences, version 27, SPSS Inc., Chicago, IL); figures were generated using GraphPad (GraphPad Prism version 9, GraphPad Software, La Jolla, CA). p-values <0.05 were considered significant.

Descriptive data were expressed as medians with IQR or percentages. Further statistical analyses were performed using a Wilcoxon paired-samples test or Spearman's rank order correlation for continuous variables.

TABLE 1 | **Patient characteristics (all inclusions).**

	Total cohort (n=26)
Age, median (IQR)	71 (12)
Sex, n female (%)	7 (27)
BMI¶, median in kg/m² (IQR)	23 (4)
Smoking, n (%)	
No	6 (23)
Currently	7 (31)
Former (>2 years ago)	11 (42)
Unknown	1 (4)
Diabetes mellitus, n present (%)	10 (39)
Any symptom, n (%)	21 (81)
Jaundice	8 (31)
Epigastric pain	13 (50)
Weight loss	10 (39)
CA19.9 >37 kU/L, n (%)	20 (77)
Treatment naive, n (%)	21 (81)
Resectability of PC, n (%)	
Resectable	8 (31)
Borderline resectable	2 (8)
Locally advanced PC	16 (62)

¶BMI is missing for 5 patients. IQR = interquartile range, PC = pancreatic cancer, CBD = common bile duct.

Results

Patient cohort

In this study, 26 patients were included with histologically-proven HGD (n=1) or PC (n=25) were included. These individuals had a median age of 71 years (interquartile range [IQR] 12), a median BMI of 23 kg/m² (IQR 4) and seven were female (27%; **Table 1**). At time of plasma and PJ collection, diabetes mellitus was present in 10 individuals (39%) and 21 (81%) had symptoms of either biliary obstruction (n=8; 31%; all had a common bile duct [CBD] stent in situ), epigastric pain (n=13; 50%) and weight loss (n=10; 39%) at time of PJ collection. Eight patients had resectable, two borderline-resectable and 16 (locally) advanced disease. The majority had a CA19.9 level >37 kU/L (n=20; 77%) and did not undergo chemotherapy before plasma and PJ collection (n=22; 85%). The four individuals who had previously undergone chemotherapy received treatment with either FOLFIRINOX (#18 and #25 for 3 and 8 cycles) or gemcitabine combined with nab-paclitaxel (#16 and #26 for 4 cycles).

Tissue was available from six patients; H&E-stained slides can be found in **Supplemental Figure S1**. Fresh-frozen tissue was available and analyzed from five patients (1 HGD: #1;

4 PC: #2-4, #6) and the time between plasma/PJ collection and surgery was 2 months for HGD #1 and 4-6 months for PC #2-4 and #6. FNB biopsy specimen was available and analyzed from one patient (#5); this sample was collected on the same day as the PJ/plasma sample.

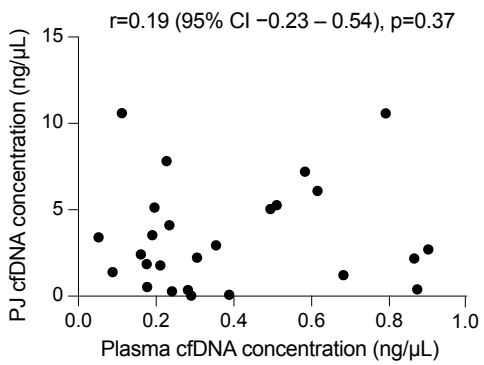


FIGURE 1 | **The DNA concentration in pancreatic juice (PJ) is not correlated to that in plasma.**
Displayed correlation coefficients are Spearman's rank order correlations.

Cell-free DNA concentration and fragment length

A median volume of 2.9 mL (IQR 1.0 mL) plasma and 0.7 mL (IQR 0.1 mL, p<0.001) PJ was used for DNA isolation, which resulted in a median yield of 16ng cfDNA from plasma (IQR 13 ng; median concentration 0.29 ng/µL [IQR 0.40 ng/µL]) and 1630ng from PJ (IQR 3048ng, p=<0.001; 2.6 ng/µL [IQR 4.2 ng/µL], p=<0.001). As compared to plasma, PJ contained more long DNA fragments, as indicated by the long (247 bp) to short-fragment (115 bp) ratio (median 0.26 [IQR 0.07] vs 0.42 [0.55], respectively, p=0.03; **Supplemental Table S3**). The concentration of cfDNA in plasma was not correlated to that in PJ (p=0.37; **Figure 1**), yet to the plasma 247/115 ratio (r=-0.61, 95% CI =0.82- -0.23; p=0.003),

The cfDNA concentration (of plasma and PJ) was not associated to age, sex, BMI, previous chemotherapy, location of the solid mass, presence of a CBD stent and resectability.

Mutation detection rate in PJ and plasma

For NGS, all isolated cfDNA (up to 25ng) from plasma samples (range 4.7-25ng) and 25ng from PJ was used. For the Oncomine panel (#1-4), the number of reads ranged from 4.8 million to 7.0 million. For the Swift panel, the median number of used sequence reads was 2.8 million (IQR 1.4) for plasma and 2.6 million (IQR 0.9 million) for PJ (p=0.23). For the number of reads per panel and coverage, see **Supplemental Table S3**.

A total of 41 unique somatic mutations were detected (**Figure 2**); 24 in plasma (2 *KRAS,* 15 *TP53*, 2 *SMAD4*, 3 *CDKN2A* 1 *CTNNB1*, 1 *PIK3CA*), 19 in PJ (3 *KRAS*, 15 *TP53*, 1 *SMAD4*) and 8 in tissue (2 *KRAS*, 2 *CDKN2A*, 4 *TP53*). At least one mutation was present in 17 (65%) plasma samples, 16 (61%) PJ samples and 5 (83%) tissue samples (p=0.22). The median number of mutations was 2 (IQR 1) for plasma, 1 for PJ (IQR 1) and 2 for tissue (IQR 2; p=0.74). The number of mutations per sample was not associated with the input cfDNA concentration, number of reads, Alu247/Alu115 ratio, age, gender, BMI, concentration of CA19.9, presence of DM, previous chemotherapy, resectability, CBD-stent in situ or the location of the solid mass (p>0.05; **Figure 2**).

KRAS mutations were present in 13 of 26 patients (50%): 5 plasma samples (19%), 9 PJ samples (35%) and 4 tissue samples (67%; **Figure 3**). The detection rate in tissue was higher than that in plasma (p=0.02), no difference in detection rate was found between PJ and plasma. As compared to tissue, plasma was concordant in one patient (#6, *KRAS* p.G12D), PJ was concordant in two patients (#4 and # 6, *KRAS* p.G12D). This concordance and detection rate were not related to resectability or the previous administration of chemotherapy. Concordance of *KRAS* mutations between PJ and plasma was seen in three patients (#6, #17: G12D, #16 G12V; **Figure 2**).

TP53 mutations were most prevalent and detected in 19 of 26 patients (73%): in 12 plasma samples (46%), 13 PJ samples (50%) and 4 tissue samples (67%; p>0.05). For *TP53* no concordance between tissue and plasma was seen, yet in two patients, concordance between PJ and plasma was seen (#13: R273H and #20: R248Q). The *TP53* P72R homozygous germline variant (R72/R72) was present in 11 patients (5 [45%] had ≥1 TP53 mutation), and the *TP53* P72R heterozygous germline variant (P72/R72) in 12 patients (12 [100%] had ≥1 *TP53* mutation). Three patients had no P72R variant (P72/P72; 2 [67%] had ≥1 *TP53* mutation). As expected for a germline variant, the concordance between plasma and PJ was 100%.

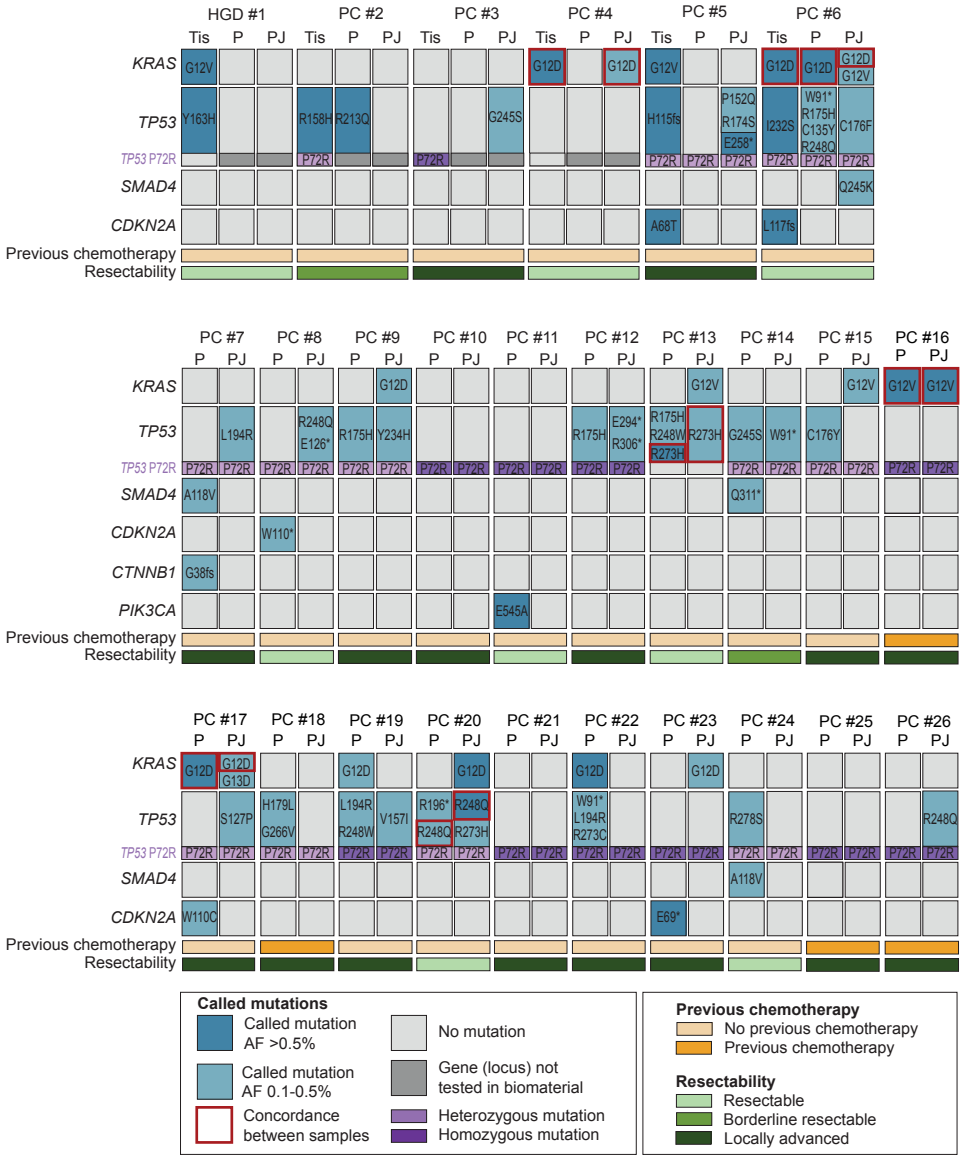


FIGURE 2 | Schematic overview of gene mutations in plasma (P) and pancreatic juice (PJ) for 1 patient with high-grade dysplasia (HGD; #1) and 25 patients with pancreatic cancer (PC; #2-#26). Only genes showing an alteration are presented. Gene mutations in plasma and PJ #1-4 were evaluated using the Oncomine Colon cfDNA Assay and for # 5-26 the Accel-Amplicon 57G Plus Pan-Cancer Profiling Panel was used. For the tissue (#1-4, #6) and biopsy samples (#5) an in-house generated pan-cancer panel was used. Concordance of *KRAS* between PJ and plasma was seen in three patients (#6, #16, #17), whilst concordance of *TP53* between PJ and plasma was seen in two patients (#13, #20). Furthermore, patients with a P72R heterozygous variant had a higher TP53 detection rate than those with a homozygous variant. HGD = high-grade dysplasia, PC = pancreatic cancer, Tis = tissue, P = plasma, PJ = pancreatic juice, AF = allelic frequency.

Discussion

This study compares the detection rate of DNA mutations in PJ and plasma, which were (unexpectedly) found to be similar. Whilst the concordance of the *TP53* P72 variants was 100%, the concordance between biomaterials for variants with a lower allelic frequency was small. Additionally, both the total cfDNA concentration and the Alu247/Alu115 were higher in PJ than in plasma.

cfDNA analyses in plasma and secretin-stimulated PJ could serve as non-invasive method for repetitive (longitudinal) sampling in a surveillance program. As (cancer) cells shed information to surrounding tissues and body fluids, it is expected that biomarkers are able to detect PC at an earlier stage. Additionally, in case of a suspicion of PC, obtaining a pancreatic biopsy is often challenging and requires multiple sampling efforts. Currently available biomarkers (such as CA19.9) have a high false-positive rate and are therefore not able to fulfill this need.[18,19] Additionally, as an increasing number of targeted therapies are being developed, cfDNA analysis may be able to identify cancers eligible for personalized approaches (such as HER2 or BRAF targeted therapy or platinum-based chemotherapy for *BRCA2* mutated PC).

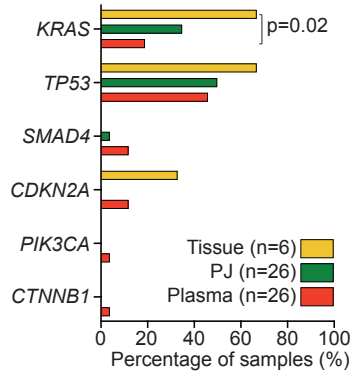


FIGURE 3 | Detection rate of mutations in tissue (yellow), pancreatic juice (PJ; green) and plasma (orange). *KRAS* mutations were detected significantly more often in tissue as compared to plasma; *KRAS* detection rate between PJ and plasma was not different. Percentages were compared with a Kruskal Wallis test.

While several studies have described ctDNA analyses in plasma or PJ for PC detection, we are the first to compare these two sources head-to-head. We hypothesized the superiority of PJ for the detection of PC (based on a higher concentration of DNA). Indeed, the current study showed a significantly higher cfDNA concentration as well as Alu247/Alu115 ratio in PJ than in plasma. This means that the cfDNA in PJ may potentially be contaminated with high concentrations of genomic DNA (as a result of cell decay during collection). Conversely, the higher Alu247/Alu115 ratio in PJ may be due to its close relation with

PC, and may be a measure of cell necrosis (rather than apoptosis), as necrosis produces longer cfDNA fragments.[20-23] If so, Alu247/Alu115 ratio in PJ may be a measure of tumor volume, and response to therapy. Also, smaller lesions may be earlier detectable in PJ than in plasma. However, further research is needed to verify this.

For *KRAS*, our analysis showed a low *KRAS* detection rate in PJ (35%, as compared to 60% reported in literature),[24] as well as serum. In the current cohort, none of the patients had metastatic disease, and therefore may have had insufficient advanced disease to generate an allelic frequency above the limit of detection (while potentially present as driver mutation in the tumor). Unfortunately, this would limit the use of this analysis for detection of lesions prior to them becoming visible on imaging. We do not believe that this has been caused by the use of secretin-stimulated PJ (as compared to the 'purer' PJ collected by ampullary cannulation), because other studies showed similar *KRAS* detection rate when using secretin-stimulated PJ or pure PJ (collected by ampullary canulation). However, no head-to-head comparison was performed in any of these studies.

For *TP53*, this study shows a detection rate of 50% in PJ, which is higher than the detection rate in PJ described previously (43%)14. This may have been caused by our strict selection criteria to call a mutation. For plasma samples, the TP53 detection rate was 46%. In patients >65 years (23 of 26 patients in this cohort), there is a potential for confounding plasma *TP53* variants due to clonal hematopoiesis, but the low allelic frequency makes this unlikely.[25] Our data implies that the prevalence of somatic *TP53* mutations is higher in patients with a germline heterozygous *TP53* P72R (P72/R72) variant than in those with a homozygous variant (R72/R72). This is consistent with De Souza *et al.* (2021),[14,15] who showed enrichment of *TP53* genes in ovarian cancer cells harboring the P72 variant. Furthermore, van der Sijde *et al.* (2021)[26] showed that a P72R homozygous variant was associated with early tumor progression and poor overall survival. More research is required to evaluate the role of these P72 variants in the development and prognosis of PC.

This study has several limitations. Tissue (either resection specimen or fine needle biopsy) was only available in six patients (for logistic reasons). Thus, we were unable to evaluate which of the observed mutations (in plasma or PJ) are actually reflective of tumor tissue. A few patients underwent previous chemotherapy, which may have caused a lower detection rate for these samples. To evaluate performance of a test, a case-control design would be more valuable than the current design comparing detection rates. Another limitation of this study is the use of different kits for different samples. Based on our data, it is unclear which of the used kits performs better. At last, sample handling was different between plasma and PJ samples. Plasma was stabilized in EDTA anticoagulant and a cell preservative (CellSave) – proven to preserve the cfDNA quality and somatic variant detection ability in serum for at least 96 hours[24] – and PJ was snap frozen. This may have caused difference in results between plasma and PJ.

In conclusion, this head-to-head comparison of plasma and PJ confirms a higher DNA concentration and Alu247/Alu115 ratio in PJ, yet does not show superiority of PJ over plasma in the detection rate of PC-related mutations. The exploratory nature of these results demands further research on sample handling (*i.e.*, the used volumes, the use of preservation tubes to reduce genomic DNA for PJ, the possibilities of scavenging background noise or the role of different DNA sequencing kits) to increase the mutation rate in these samples and evaluate the potential applicability of PJ for molecular analysis. Furthermore, research with a case-control design is needed to compare accuracy of mutations in PJ and plasma.

References

1 Rawla P, Sunkara T and Gaduputi V. Epidemiology of Pancreatic Cancer: Global Trends, Etiology and Risk Factors. *World J Oncol* 2019; 10: 10-27. 2019/03/06.

2 Siegel RL, Miller KD and Jemal A. Cancer statistics, 2020. *CA Cancer J Clin* 2020; 70: 7-30. 2020/01/09.

3 Overbeek KA, Levink IJM, Koopmann BDM, *et al*. Long-term yield of pancreatic cancer surveillance in high-risk individuals. *Gut* 2022;71:1152-1160.

4 Razzano D, Bouza SJ, Hernandez PV, *et al*. Comprehensive molecular profiling of pancreatic ductal adenocarcinoma in FNA, biopsy, and resection specimens. *Cancer Cytopathol* 2022; 130: 726-734. 2022/05/06.

5 Bang JY, Hebert-Magee S, Navaneethan U, *et al*. EUS-guided fine needle biopsy of pancreatic masses can yield true histology. *Gut* 2018; 67: 2081.

6 Yang X, Xu W, Tian X, *et al*. Diagnostic and prognostic value of KRAS mutations in circulating pancreatic ductal adenocarcinoma tumor DNA. *Translational Cancer Research* 2018; 7: 622-633.

7 Herreros-Villanueva M, Bujanda L, Ruiz-Rebollo L, *et al*. Circulating tumor DNA tracking in patients with pancreatic cancer using next-generation sequencing. *Gastroenterol Hepatol* 2022; 45: 637-644. 2022/01/30.

8 Singh N, Gupta S, Pandey RM, *et al*. High levels of cell-free circulating nucleic acids in pancreatic cancer are associated with vascular encasement, metastasis and poor survival. *Cancer Invest* 2015; 33: 78-85. 2015/02/04.

9 Earl J, Garcia-Nieto S, Martinez-Avila JC, *et al*. Circulating tumor cells (Ctc) and kras mutant circulating free Dna (cfdna) detection in peripheral blood as biomarkers in patients diagnosed with exocrine pancreatic cancer. *BMC Cancer* 2015; 15: 797. 2015/10/27.

10 Takai E, Totoki Y, Nakamura H, *et al*. Clinical utility of circulating tumor DNA for molecular assessment in pancreatic cancer. *Sci Rep* 2015; 5: 18425. 2015/12/17.

11. Choi MH, Mejlænder-Andersen E, Manueldas S, *et al*. Mutation analysis by deep sequencing of pancreatic juice from patients with pancreatic ductal adenocarcinoma. *BMC Cancer* 2019; 19: 11. .

12 Levink IJM, Nesteruk K, Visser DI, *et al*. Optimization of Pancreatic Juice Collection: A First Step Toward Biomarker Discovery and Early Detection of Pancreatic Cancer. *Am J Gastroenterol* 2020; 115: 2103-2108. 2020/10/27.

13 Wang K, Li M and Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. *Nucleic Acids Res* 2010; 38: e164. 2010/07/06.

14 De Souza C, Madden JA, Minn D, *et al*. The P72R Polymorphism in R248Q/W p53 Mutants Modifies the Mutant Effect on Epithelial to Mesenchymal Transition Phenotype and Cell Invasion via CXCL1 Expression. *Int J Mol Sci* 2020; 21 2020/11/01.

15 De Souza C, Madden J, Koestler DC, *et al*. Effect of the p53 P72R Polymorphism on Mutant TP53 Allele Selection in Human Cancer. *J Natl Cancer Inst* 2021; 113: 1246-1257. 2021/02/09.

16 Dahabreh IJ, Schmid CH, Lau J, *et al*. Genotype misclassification in genetic association studies of the rs1042522 TP53 (Arg72Pro) polymorphism: a systematic review of studies of breast, lung, colorectal, ovarian, and endometrial cancer. *Am J Epidemiol* 2013; 177: 1317-1325. 2013/06/05.

17 Doffe F, Carbonnier V, Tissier M, *et al*. Identification and functional characterization of new missense SNPs in the coding region of the TP53 gene. *Cell Death & Differentiation* 2021; 28: 1477-1492.

18 Levink IJM, Visser IJ, Koopmann BDM, *et al*. Protein biomarkers in pancreatic juice and serum for identification of pancreatic cancer. *Gastrointest Endosc* 2022; 96: 801-813.e802. 2022/05/11.

19 Levink IJM, Jaarsma SC, Koopmann BDM, *et al*. The additive value of CA19.9 monitoring in a pancreatic cyst surveillance program. *United European Gastroenterology Journal* 2023; 11: 601-611.

20 Salem R, Ahmed R, Shaheen K, *et al*. DNA integrity index as a potential molecular biomarker in colorectal cancer. *Egyptian Journal of Medical Human Genetics* 2020; 21: 38.

21 Shen Q, Cen H, Jiang J, *et al*. The level and integrity of plasma circulating cell-free DNA in patients with primary multiple myeloma. *Translational Cancer Research* 2022; 11: 4137-4147.

22 Zhu F, Ma J, Ru D, *et al*. Plasma DNA Integrity as a Prognostic Biomarker for Colorectal Cancer Chemotherapy. *Journal of Oncology* 2021; 2021: 5569783.

23 Yu D, An G and Xu L. Investigation of Efficacy Evaluation Comparison of cfDNA and CEA in Colorectal Cancer. *Clin Lab* 2016; 62: 1947-1953. 2017/02/07.

24 Visser IJ, Levink IJM, Peppelenbosch MP, *et al*. Systematic review and meta-analysis: Diagnostic performance of DNA alterations in pancreatic juice for the detection of pancreatic cancer. *Pancreatology* 2022; 22: 973-986. 2022/07/22.

25 Chen S and Liu Y. p53 involvement in clonal hematopoiesis of indeterminate potential. *Curr Opin Hematol* 2019; 26: 235-240. 2019/05/03.

26 van der Sijde F, Azmani Z, Besselink MG, *et al*. Circulating TP53 mutations are associated with early tumor progression and poor survival in pancreatic cancer patients treated with FOLFIRINOX. *Ther Adv Med Oncol* 2021; 13: 17588359211033704. 2021/08/24.

SUPPLEMENTAL TABLE S1 | In- and exclusion criteria per prospective cohort study.

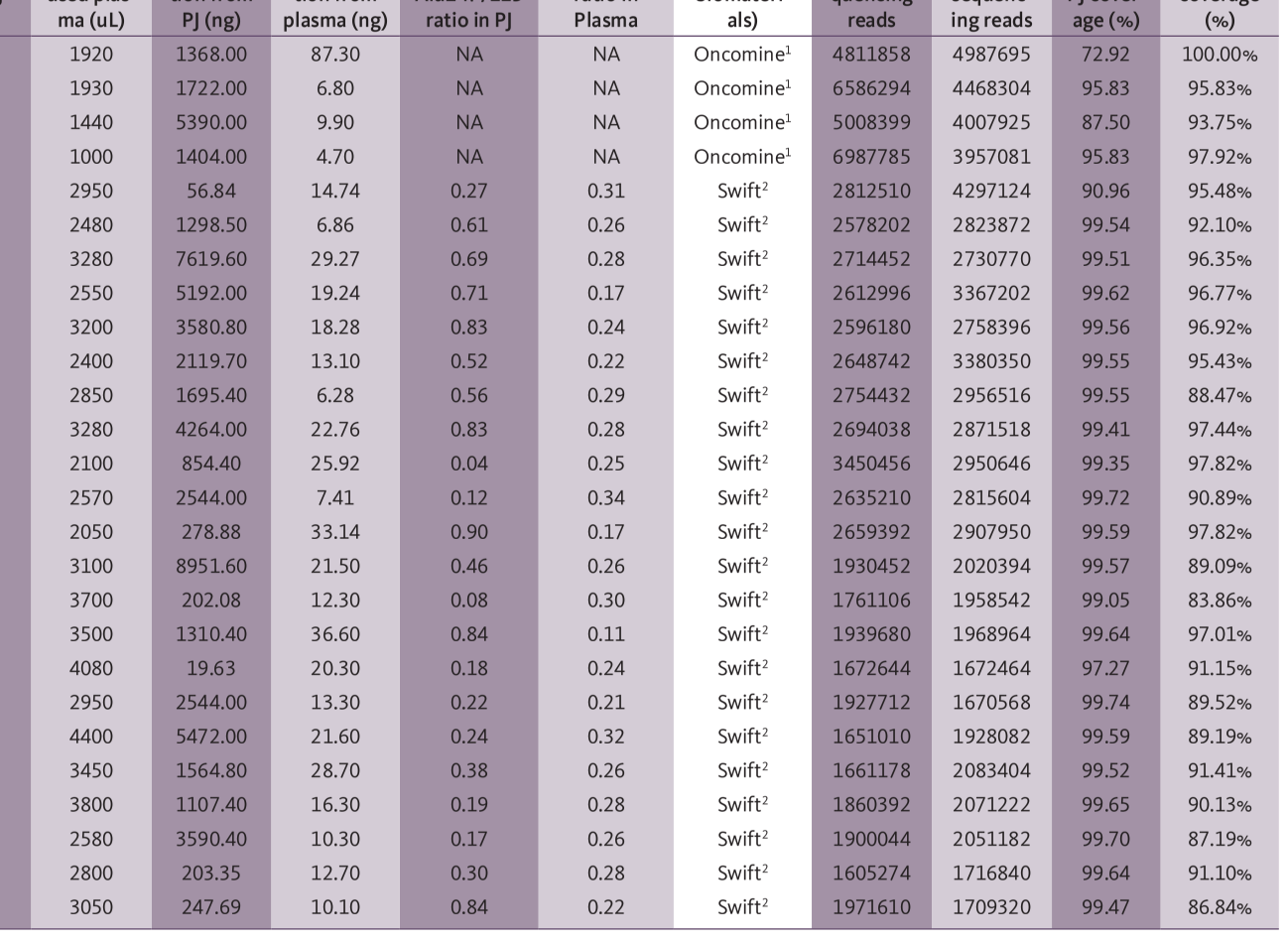
Prospective cohort	Inclusion criteria	Exclusion criteria
KRASPanc	Patients who undergo an EUS for (suspected) PC either as part of a diagnostic process or fiducial placement prior to radiotherapeutic treatment.	Age <18 years
PACYFIC	Individuals with a suspected neoplastic pancreatic cyst (either newly or previously diagnosed, or previously operated upon) for which cyst surveillance is warranted, according to the treating physician.	Age <18 years, history of chronic pancreatitis, suspected pseudocyst (simple, thin-walled cyst that developed in the course of acute pancreatitis, as documented by sequential imaging studies), suspected serous cystadenoma (typical microcystic lesion with lobulated outlines, a calcified central scar and/or cyst fluid CEA levels < 5ng/ml), Von Hippel-Lindau disease, and limited life expectancy (<2 years).

SUPPLEMENTAL TABLE S1 | Panels used for next-generation sequencing.

Panel	Samples tested	Genes (with hotspot coverage)
Oncomine Colon cfDNA Assay	Plasma and PJ samples of patients #1-4	*AKT1, BRAF, CTNNB1, EGFR, ERBB2, FBXW7, GNAS, KRAS, MAP2K1, NRAS, PIK3CA, SMAD4, TP53, APC*
in-house created pan-cancer AmpliSeq panel	Tissue of patients #1-6	*CDKN2A* (100%), *KEAP1* (100%), *PTEN* (100%), *STK11* (100%), *TP53* (100%). Mutatie hotspots: *AKT1* (exon 3), *AKT2* (3), *AKT3* (2), *ALK* (20, 22-25), *APC* (16), *ARAF* (7), *BRAF* (11, 12, 14, 15), *CDK4* (2, 4, 7, 8), *CHEK2* (3, 4, 11, 12), *CTNNB1* (3, 7, 8), *DDR2* (14-19), *EGFR* (12, 18-21), *EIF1AX* (1, 2), *HER2* (8, 17-21), *ERBB3* (3, 6-10, 21, 23), *ESR1* (4, 5, 7, 8), *EZH2* (16), *FBWX7* (9, 10), *FGFR1* (4, 7, 12-14), *FGFR2* (7, 9, 12), *FGFR3* (7, 9, 14, 15), *FOXL2* (1), *GNA11* (4, 5), *GNAQ* (4, 5), *GNAS* (8, 9), *HRAS* (2-4), *IDH1* (4), *IDH2* (4), *JAK2* (14), *JAK3* (4, 16), *KIT* (8, 9, 11, 13-18), *KNSTRN* (1), *KRAS* (2-4), *MAP2K1* (1-6), *MET* (2, 14, 19, 20), *MTOR* (30, 39, 40, 43, 47, 53, 56, 57), *MYD88* (5), *NFE2L2* (2), *NOTCH1* (26, 27), *NRAS* (2-4), *OXA1L* (1), *PDGFRA* (12, 14, 18), *PIK3CA* (2, 5, 8, 10, 14, 21), *POLD1* (6, 8, 12, 15-17, 24), *POLE* (9-14, 21, 25), *RAC1* (2), *RAF1* (7), *RET* (11, 16), *RHOA* (2), *RIT1* (4, 5), *RNF43* (2-10), *ROS1* (36-41), *SF3B1* (14, 15), *SMAD4* (3, 9, 12).
Accel-Amplicon 57G Plus Pan-Cancer Profiling Panel	Plasma and PJ samples of patients #5-26	*ABL1, AKT1, ALK, APC, ATM, BRAF, CDH1, CDKN2A, CSF1R, CTNNB1, DDR2, DNMT3A, EGFR, ERBB2, ERBB4, EZH2, FBXW7, FGFR1, FGFR2, FGFR3, FLT3, FOXL2, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, KRAS, MAP2K1, MET, MLH1, MPL, MSH6, NOTCH1, NPM1, NRAS, PDGFRA, PIK3CA, PTEN, PTPN11, RB1, RET, SMAD4, SMARCB1, SMO, SRC, STK11, TP53* (full exon coverage), *TSC1, TSC2, VHL*

SUPPLEMENTAL TABLE S3 | The input volume (and yield of DNA) and responding Alu247/11p ratio, number of reads and coverage per biomaterial and patient.

Patient	Volume used PJ (uL)	Volume used plasma (uL)	DNA yield after isolation from PJ (ng)	DNA yield after isolation from plasma (ng)	Alu247/115 ratio in PJ	Alu247/115 ratio in Plasma	Used sequencing panel (both biomaterials)	PJ number of sequencing reads	Plasma number of sequencing reads	PJ coverage (%)	Plasma coverage (%)
HGD #1	500	1920	1368.00	87.30	NA	NA	Oncomine[1]	4811858	4987695	72.92	100.00%
PC #2	500	1930	1722.00	6.80	NA	NA	Oncomine[1]	6586294	4468304	95.83	95.83%
PC #3	500	1440	5390.00	9.90	NA	NA	Oncomine[1]	5008399	4007925	87.50	93.75%
PC #4	1000	1000	1404.00	4.70	NA	NA	Oncomine[1]	6987785	3957081	95.83	97.92%
PC #5	720	2950	56.84	14.74	0.27	0.31	Swift[2]	2812510	4297124	90.96	95.48%
PC #6	700	2480	1298.50	6.86	0.61	0.26	Swift[2]	2578202	2823872	99.54	92.10%
PC #7	720	3280	7619.60	29.27	0.69	0.28	Swift[2]	2714452	2730770	99.51	96.35%
PC #8	720	2550	5192.00	19.24	0.71	0.17	Swift[2]	2612996	3367202	99.62	96.77%
PC #9	700	3200	3580.80	18.28	0.83	0.24	Swift[2]	2596180	2758396	99.56	96.92%
PC #10	720	2400	2119.70	13.10	0.52	0.22	Swift[2]	2648742	3380350	99.55	95.43%
PC #11	700	2850	1695.40	6.28	0.56	0.29	Swift[2]	2754432	2956516	99.55	88.47%
PC #12	700	3280	4264.00	22.76	0.83	0.28	Swift[2]	2694038	2871518	99.41	97.44%
PC #13	700	2100	854.40	25.92	0.04	0.25	Swift[2]	3450456	2950646	99.35	97.82%
PC #14	720	2570	2544.00	7.41	0.12	0.34	Swift[2]	2635210	2815604	99.72	90.89%
PC #15	700	2050	278.88	33.14	0.90	0.17	Swift[2]	2659392	2907950	99.59	97.82%
PC #16	1700	3100	8951.60	21.50	0.46	0.26	Swift[2]	1930452	2020394	99.57	89.09%
PC #17	380	3700	202.08	12.30	0.08	0.30	Swift[2]	1761106	1958542	99.05	83.86%
PC #18	600	3500	1310.40	36.60	0.84	0.11	Swift[2]	1939680	1968964	99.64	97.01%
PC #19	700	4080	19.63	20.30	0.18	0.24	Swift[2]	1672644	1672464	97.27	91.15%
PC #20	620	2950	2544.00	13.30	0.22	0.21	Swift[2]	1927712	1670568	99.74	89.52%
PC #21	700	4400	5472.00	21.60	0.24	0.32	Swift[2]	1651010	1928082	99.59	89.19%
PC #22	700	3450	1564.80	28.70	0.38	0.26	Swift[2]	1661178	2083404	99.52	91.41%
PC #23	620	3800	1107.40	16.30	0.19	0.28	Swift[2]	1860392	2071222	99.65	90.13%
PC #24	700	2580	3590.40	10.30	0.17	0.26	Swift[2]	1900044	2051182	99.70	87.19%
PC #25	730	2800	203.35	12.70	0.30	0.28	Swift[2]	1605274	1716840	99.64	91.10%
PC #26	700	3050	247.69	10.10	0.84	0.22	Swift[2]	1971610	1709320	99.47	86.84%



SUPPLEMENTAL FIGURE S1 | H&E staining of tissue and biopsy material showing the cancer cellularity at time of resection for patient #1 with HGD (A-B) and patients #2 (C), #4 (D), #6 (E) with PC, and at time of biopsy for patient #5 (F) with PC. No slide was available of patient #3 at time of writing. HGD = high-grade dysplasia, PC = pancreatic cancer. The used magnification is 10x.

CHAPTER 13

An 8q24 Gain in Pancreatic Juice is a Candidate Biomarker for the Detection of Pancreatic Cancer

Authors
Iris J.M. Levink, Malgorzata I. Srebniak, Walter G. De Valk, Monique M. van Veghel-Plandsoen, Anja Wagner, Djuna L. Cahen, Gwenny M. Fuhler, Marco J. Bruno

International Journal of Molecular Sciences. 2023; 24(6):5097.

Abstract

Background and aims: Secretin-stimulated pancreatic juice (PJ), collected from the duodenum, presents a valuable biomarker source for the (earlier) detection of pancreatic cancer (PC). Here, we evaluate the feasibility and performance of shallow sequencing to detect copy number variations (CNVs) in cell-free DNA (cfDNA) from PJ for PC detection.

Methods: First, we confirmed the feasibility of shallow sequencing in PJ (n=4), matched plasma (n=3) and tissue samples (n=4, microarray). Subsequently, shallow sequencing was performed on cfDNA from PJ of 26 cases (25 sporadic PC, 1 high-grade dysplasia) and 19 controls with a hereditary or familial increased risk of PC.

Results: 40 of the 45 PJ samples met the quality criteria for cfDNA analysis. Nine individuals had an 8q24 gain (oncogene MYC; 23%; 8 cases [33%] and 1 control [6%], p=0.04), and six both a 2q gain (STAT1) and 5p loss (CDH10; 15%; 4 cases [17%] and 2 controls [13%], p=0.72). The presence of an 8q24 gain differentiated cases and controls with a sensitivity of 33% (95%CI 16-55%) and specificity of 94% (95%CI 70-100%). The presence of either an 8q24 or 2q gain with 5p loss was related to a sensitivity of 50% (95%CI 29-71%) and specificity of 81% (95%CI 54-96%).

Conclusions: Shallow sequencing of PJ is feasible. The presence of an 8q24 gain in PJ shows promise as a biomarker for detection of PC. Further research is required with a larger sample size and consecutively collected samples in high-risk individuals prior to implementation in a surveillance cohort.

Introduction

Pancreatic cancer (PC) has a poor prognosis, with incidence rates closely mirroring mortality rates. Surveillance of individuals at risk of developing PC aims at detecting disease at an earlier (resectable) stage, but this has been proven challenging based on imaging alone.[1] As development of PC from the first genetic alteration to cancer is expected to take years, biomarkers may enable detection of resectable cancer stages, or preferably premalignant lesions, not yet visible on imaging.

The presence of chromosomal aberrations is a common molecular feature in human cancer including loss of tumor suppressor genes or gain of oncogenes, driving oncogenic signaling and cancer development. Specifically, in PC, amplification of genes involved in DNA repair and tyrosine kinase signaling are associated with poor survival.[2] Detection of such alterations in cell-free DNA (cfDNA) released from tumor cells (circulating tumor DNA [ctDNA]) has shown promise for several cancers in (pre) clinical studies.[3,4]

Clinical testing for chromosomal aberrations in cfDNA is currently routinely performed during pregnancy by noninvasive prenatal testing (NIPT). One of the approaches is based on shallow whole-genome sequencing of cfDNA present in maternal plasma, which consists of both maternal and fetal DNA. Whilst a NIPT-test aims to diagnose chromosomal aberrations in fetal DNA, different chromosomal patterns in maternal DNA have been incidentally detected, which raised the suspicion of maternal malignancy.[5,6] The majority of these cancers were of hematologic origin, yet solid tumors such as colorectal, gastric, breast and ovarian cancer were also detected.[5-7] The recently published TRIDENT-2 study showed a prevalence of 70% maternal malignancy in individuals with two or more CNVs.[8] Remarkably, these aberrations originated from undiagnosed maternal cancer, suggesting that this test may serve as an early screening tool for cancer.

For detection of pancreatic cancer, pancreatic juice (PJ) may provide a promising biomarker source, as it is in close contact with pancreatic ductal cells, which are the cells of origin for more than 90% of PCs.[9,10] PJ harbors higher concentrations of cfDNA than plasma[11] and, in contrast to fine-needle biopsy (FNB), it is expected to contain information from all tumor clones present in the pancreatic ductal system. A wash-out of PJ from the pancreatic ductal system can be provoked by secretin and collected by suction through an endoscope from within duodenum, which is relatively non-invasive and carries a very low procedural risk as compared to tissue sampling by endoscopic ultrasound (EUS)-guided FNB or PJ collection through direct pancreatic duct cannulation.

The aim of this study was to evaluate the feasibility of shallow sequencing of cfDNA obtained from PJ and plasma from patients with PC by using the clinically available and robust NIPT pipeline. Subsequently, we compared the presence of chromosomal aberrations in PJ between PC cases to controls undergoing surveillance for a hereditary predisposition of PC.

Methodology

Study design and patient inclusion

This study included data and biomaterial that were prospectively collected at the Erasmus Medical Center in Rotterdam as part of three clinical studies: 1. KRASPanc-study (MEC-2018-038), concerning patients with (suspected) sporadic PC undergoing diagnostic EUS or fiducial placement for stereotactic radiotherapy; 2. PACYFIC study (MEC-2014-021), involving individuals undergoing surveillance for suspected neoplastic pancreatic cysts; 3. FPC-study, including individuals with an hereditary increased risk of pancreatic cancer undergoing surveillance. See **Supplemental Table 1** for in- and exclusion criteria per study. The institutional ethical review board approved these studies and they were carried out according to the ethical principles for medical research involving human subjects from the World Medical Association Declaration of Helsinki. Participants gave written informed consent before enrolment. All authors had access to the study data and had reviewed and approved the final manuscript.

The current study consisted of two phases. The first ('feasibility') phase was executed to evaluate the feasibility of chromosomal aberration detection in cfDNA isolated from PJ by comparing results with matched plasma and/or tissue samples (analyzed by chromosomal microarray) from three patients with pathology-proven PC and one individual with pathology-proven intraductal papillary mucinous neoplasm (IPMN) harboring high-grade dysplasia (HGD).

The second ('experimental') phase included PJ samples from individuals from the feasibility phase, additional cases with sporadic PC who underwent PJ collection as part of the KRASPanc and controls undergoing pancreas surveillance without morphologic changes on imaging as part of the FPC-study (also known as CAPS-study). Sample inclusion was based on the availability of PJ and, for controls, the absence of morphologic aberrations (no major Rosemont criteria and at maximum 1 minor Rosemont criterion, side or main duct <5mm, no absolute or relative indications for surgery) or clinical symptoms. No formal sample size analysis was performed due to the explorative nature of the study.

Biomaterial collection

PJ collection was performed with a linear echoendoscope (Pentax Medical, Tokyo, Japan) by experienced endo-sonographers. Secretion of PJ was stimulated by intravenous injection of human secretin (16 µg/patient, ChiRhoClin, Burtonsville, MD) after positioning the tip of the scope close to the ampullary orifice. Suction through the endoscopic channel was applied (without occluding the ampullary orifice) for eight minutes directly after secretin infusion.[11] After collection, PJ was aliquoted and snapfrozen. Samples were stored at -80°C until use. On the same day as PJ samples, plasma samples were collected by venipuncture in Cellsave tubes, centrifuged, aliquoted and stored at -80°C until use. Tissue was snapfrozen within hours after pancreatic resection.

cfDNA analysis

To assess presence of chromosomal aberrations in plasma and PJ, a diagnostic routine NIPT pipeline was used. As PJ samples typically harbor higher cfDNA concentrations than blood plasma, PJ samples were diluted with ice cold PBS ensuring that an input of ca. 1ng cfDNA was used for the automated pipeline. When necessary, the input was diluted, so that the pipeline could accept the sample for the run. After samples were cold centrifuged at 1600g and 4 ºC for 10 minutes, an automated NGS workflow was performed using the VeriSeq NIPT microlab Star robot (Hamilton, Gräfelfing, Germany). In short, the plate was sealed and re-centrifuged at 5600g and 20 ºC for 10 minutes. DNA from 900 µl supernatant was extracted and the sequence library was created using the VeriSeq NIPT solution (Illumina, Cambridge, United Kingdom). Subsequently, a unique synthetic DNA 'barcode' (index) was attached to each sample and the library product was quantified using a fluorescent dye and comparison of the results with a DNA standard curve. At last, shallow whole genome sequencing was performed on a NextSeq500 sequencer yielding 2x36 paired-end reads in a 48-plex reaction. The SeqFF model was used as a surrogate marker to assess the percentage of short fragmented cfDNA that are likely of tumor origin in the DNA pool (herein called the percentage tumor cfDNA).[63]

Microarray analysis

To prepare the tumor tissue samples for CNV analysis, the tissue was washed twice with PBS, and subsequently treated with 550 µl lysisbuffer and 20 µl protease overnight at 37-55 ºC. 3 µl RNase A was added and incubated for 15-60 minutes at 37 ºC. Subsequently, Chemagic MSM1 isolation robot (PerkinElmer Chemagen Technology, Baesweiler, Germany) was used to isolate the DNA according to manufacturer's recommendations. Genotyping was performed on an SNP array (Illumina GSAMD-24v3 chip) according to manufacturer's recommendations (Illumina, Cambridge, United Kingdom).

Five PJ samples (1 case, 4 controls) were excluded from further analysis due to technical failure of the Veriseq NIPT microlab Star robot even upon repeated measurement. These samples were not included in described analyses. In addition, samples with <5 million reads were included for fragment length analysis, yet excluded from further CNV (and sensitivity) analysis, as this was considered indicative of insufficient number of reads for calling chromosomal aberrations. Fragment size (insert size) distribution within the samples was evaluated (and compared) to that in blood plasma. All sequencing results were visualized on Wisecondor graphs and present gains and losses were assessed by an experienced reader (MIS).[64] Findings were divided into four categories according to the type of chromosomal aberrations: 1. No significant CNVs; 2. Samples with an 8q gain

and/or 10p loss; 3. Samples with a 2q and/or 5p loss; 4. Samples with chaotic aberrations spread over multiple chromosomes without a clear relation to each other. The genome reference database used in this study was GRCh37.

Statistical analysis

Descriptive data were expressed as medians with interquartile range (IQR; continuous data) or percentages (categorical data). Statistical significance was assessed using Mann-Whitney U and χ^2-test, respectively. Sensitivity, specificity and accuracy were calculated by a receiver operating curve (ROC) analysis and the confidence interval (CI) was represented as an "exact" Clopper-Pearson confidence interval. A two-sided p-value <0.05 was considered statistically significant. Analyses were performed in SPSS (Statistical Package for the Social Sciences, version 27, SPSS Inc., Chicago, IL); figures were created using GraphPad (GraphPad Prism version 9, GraphPad Software, La Jolla, CA).

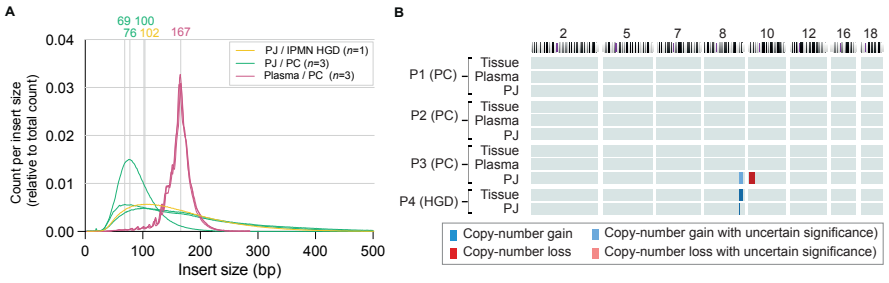


FIGURE 1 | **Results of pilot samples. A.** The fragment lengths distributions (insert size) in pilot PJ and plasma samples. No clear difference was seen between intraductal papillary mucinous neoplasm (IPMN; yellow) with high-grade dysplasia (HGD) and pancreatic cancer (PC; green). PJ samples showed a mode of 69-102 base pairs (bp), whereas the fragment peak for plasma samples (all PC) was 167 bp. **B.** Chromosomal aberrations in pilot samples. No significant aberrations were seen in plasma samples, whereas – in pancreatic juice (PJ) – an 8q gain with (P3) or without (P4) 10p loss was seen. For P4, this aberration was also found in tissue.

Results

Feasibility phase

To investigate the feasibility of detecting chromosomal instability in PJ, a pilot study was performed including four patients with HGD (n=1) or PC (n=3). The concentration of the three PC plasma samples ranged from 0.05 to 0.11 ng/µl and of the four PJ samples from 11 to 33 ng/µl. After multiple concentration (for plasma) and dilution (for PJ) steps, the input cfDNA concentration of the three plasma samples ranged from 52 to 74 ng/µl and that of the four PJ samples from 19 to 398 pg/µl (3/4 had a concentration of 185 pg/µl

or more). cfDNA from plasma samples harbored mostly fragments of 167bp (mode size) with the expected sawtooth pattern of 11 bp related to helical periodicity of DNA,[12,13] whereas fragments in PJ did not show this typical pattern and were generally shorter of length (peak prevalence at 68-102; **Figure 1A**).

The number of reads in PJ ranged from 10,000,000 to 116,000,000 (1.8-3.6% duplicates), whereas those in plasma ranged from 14,630,000 to 17,500,000 (2.5-3.8% duplicates). The fraction percentage of tumor cfDNA was 0.7-1.9% for plasma, which was too low to call chromosomal aberrations in cfDNA. The %cfDNA in PJ samples ranged from 6-8%.

Two (PC #3, HGD #4) out of four samples from PJ showed an amplification on the q-arm of chromosome 8 (both chr8:128,000,000-140,000,000; 8q24), with PC #3 also showing a deletion on 10p (chr10:10,000,000-21,000,000; 10p14-10p21.31; **Figure 1B**). To exclude technical errors generated by usage of different biomaterial (*e.g.*, DNA fragmentation), these findings were compared to microarray results in tumor tissue (**Supplemental Figure S1** for H&E stainings). The 8q24 gain (chr8: 128,491,792-130,491,752) of HGD (#P4) was also the only chromosomal aberration detected in tissue of this patient. Tissue of PC #3 showed a very noisy profile without significant chromosomal aberrations. However, for this patient, time between PJ collection and pancreatic resection had been four months, during which the patient had undergone neoadjuvant chemoradiotherapy. A partial response to treatment was seen as a severely fibrotic area of 3 cm (ypT2N1), which may have hampered tumor-DNA extraction from this tissue.

Based on these results, we decided to evaluate the feasibility of shallow sequencing of PJ in a larger group.

Experimental phase

PJ samples from 45 individuals: 26 cases (25 PC, 1 HGD) and 19 controls were included in the experimental phase of the study (**Table 1**). Cases were of older age (69 [IQR 9] *vs* 60 [IQR 9] years, p<0.001) and more often males (n=18 [69%] *vs* n=7 [37%], p=0.04). The majority of cases was treatment-naive and underwent PJ collection at time of diagnosis (n=23, 88%), whilst three had undergone their last chemotherapy 2-4 weeks before collection (#PC08, #PC10, #PC15) and underwent PJ collection during an EUS procedure that was indicated for fiducial placement prior to radiotherapy (and surgery). Sixteen cases (62%) had locally advanced disease, fifteen (58%) had a solid mass located in the pancreatic head and seven (27%) had a common bile duct stent *in situ* at time of collection. After 35 months (IQR 11) of follow-up, 11 cases (42%) had undergone surgery and 20 (77%) had died of PC.

TABLE 1 | Patient characteristics.

	Cases (n=26)	Controls (n=19)	p-value
Age, median (IQR)	69 (9)	60 (9)	<0.001
Sex, n male (%)	18 (69)	7 (37)	0.04
BMI, median in kg/m2 (IQR)	23 (4)	24 (5)	0.23
Diabetes mellitus, n present (%)	11 (42)	0 (0)	<0.001
Hereditary predisposition, n (%)	0 (0)	19 (100)	<0.001
Member of FPC family		7 (16)	
CDKN2A germline mutation		8 (18)	
BRCA1/2 germline mutation		3 (7)	
PALB2 germline mutation		1 (2)	
History of malignancy, n (%)	3 (12)	8 (42)	0.02
Breast cancer	0 (0)	1 (5)	
Melanoma	0 (0)	7 (37)	
Other	3 (12)	0 (0)	
Any symptom, n (%)	21 (81)	0 (0)	<0.001
Jaundice	8 (31)		
Epigastric pain	15 (58)		
Weight loss	11 (42)		
CA19.9 >37 kU/L, n (%)	19 (73)	NA	NA
Treatment naive, n (%)	23 (88)	NA	NA
Resectability of PC, n (%)			
Resectable	8 (31)	NA	NA
Borderline resectable	2 (8)		
Locally advanced PC	16 (62)		

IQR = interquartile range, FPC = familial pancreatic cancer, PC=pancreatic cancer, CBD=common bile duct. NA = not applicable.

Of nineteen controls, twelve had a proven germline mutation: eight (18%) *CDKN2A* (p16), three (7%) *BRCA1/2* and one (2%) *PALB2*. The remaining seven controls were deemed at risk because they had multiple family members with PC without proven gene mutations (as previously investigated by germline genetic testing; see **Supplemental Table S1** for the in- and exclusion criteria). None of the controls had symptoms or a history of diabetes mellitus. Seven out of eight individuals with a *CDKN2A* germline mutation previously underwent curative treatment for a melanoma (<T1). One individual (#Co07) developed breast cancer two years before PJ collection, seemed in remission during collection but died of metastases (pathology-proven breast cancer) one year after study inclusion. During 25 months (IQR 12) of follow-up, none of the controls developed cancer, morphologic (pancreatic) abnormalities on imaging, or worrisome symptoms like new-onset diabetes, jaundice or acute pancreatitis.

Copy number variants in pancreatic juice

For CNV analysis of PJ samples, the median cfDNA input concentration was 161 pg/µl (IQR 276), and 7.6 million (IQR 9.1) read pairs were generated. These values were not different between cases and controls (p>0.05). Controls less often had a fragment length (also known as insert size) of 133-135bp, as compared to cases (p=0.05; **Figure 2A**).

After exclusion of five individuals, who did not meet the >5 million read pairs criterion, CNV results of the remaining 40 individuals (24 cases, 16 controls) were further evaluated. Twenty samples (50%; 9/24 cases [38%], 11/16 controls [69%]; p=0.05) had no significant CNVs. Of these, 50% showed a clear sawtooth pattern on the insert size graph (**Figure 2B**) with a peak in fragment size prevalence between 68-81 bp. The absence of a sawtooth pattern did not differentiate between cases and controls (p<0.05). Samples of five individuals (13%; 3/24 cases [13%], 2/16 controls [13%]) showed complex chaotic aberrations spread over multiple chromosomes without a clear relation to each other. Besides this complex chaotic profile, DNA of these samples was severely fragmented and did not show a sawtooth pattern (**Figure 2C**). Fifteen individuals (12/24 cases, 3/16 controls) showed clues for chromosomal aberrations that could be subdivided in either an 8q24 subgroup of samples showing at least an 8q24 gain with or without a 10p loss and/or 18q loss, and a subgroup showing at least a 2q gain combined with a 5p loss. Additional aberrations present in this latter subgroup were a 7q gain, 12q gain and/or 16q loss. Chromosomal aberrations in 8q24 and 2q-5p were mutually exclusive.

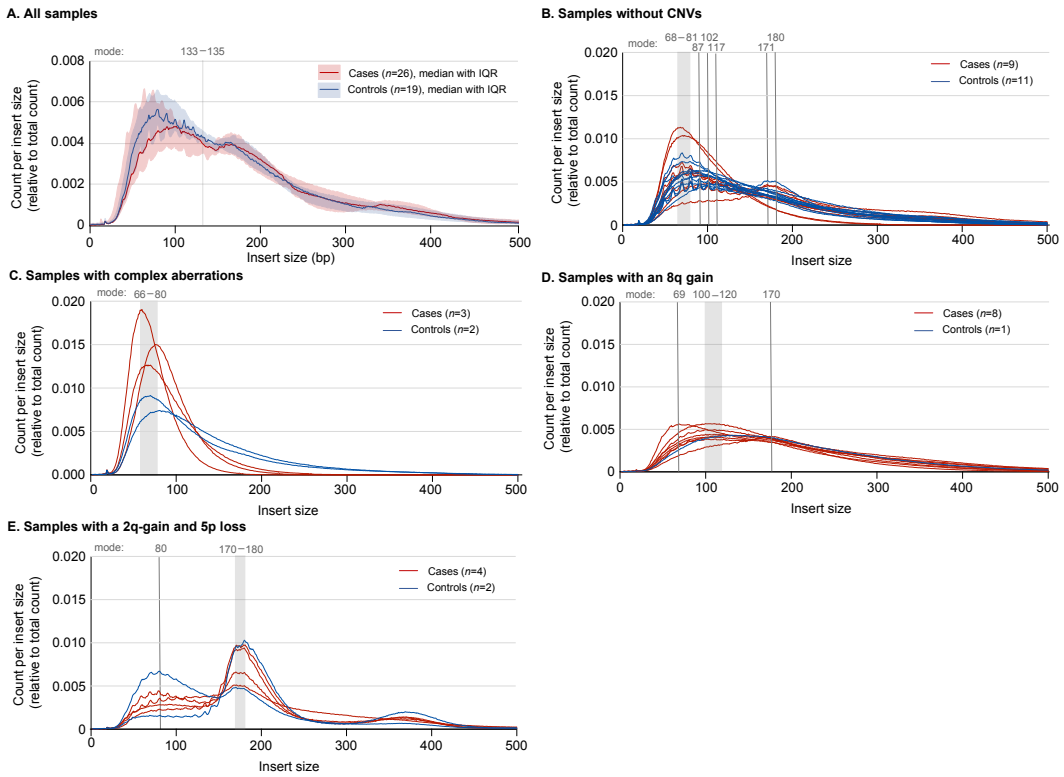


FIGURE 2 | The fragment lengths (insert size) distributions in pancreatic juice (PJ) samples. A. Cases (red) in comparison with controls (blue); fragments with a length of 133-135 base pairs (bp) were more prevalent in controls than cases (p=0.05; Mann-Whitney U). IQR = interquartile range. **B.** Samples without a copy number variation (CNV) on the Wisecondor image. Peak prevalence varied between 65-117, 171 and 180 bp. 10 out of 20 samples had a sawtooth pattern (50%), which al showed a showed a second peak at 170-180 bp. **C.** Samples with complex (chaotic) aberrations on the Wisecondor image. Each sample harbors mostly short fragments (mode 65-80 bp). The presence of short fragments and the complex (chaotic) aberrations may be related. **D.** Samples from individuals with an 8q-gain showed similar graph with a moderate (or no) sawtooth pattern. Six out of eight individuals had a mode between 100 and 120 bp. **E.** Samples with a 2p gain and 5q loss: each line shows two peaks (at ±80 bp and 170-180 bp); the first peak has a clear saw-tooth pattern.

8q24 subgroup

Nine individuals (23%; 8 cases [33%], 1 control [6%], p=0.04) had at least an 8q24 gain (chr8:128,000,000-146,000,000; **Figure 3D**, I). DNA insert size mode was 100-120bp in 7/9 samples (69bp and 170 bp in the other two) and all graphs showed a smooth shape (**Figure 2D**). Other CNVs present in this subgroup were 10p loss (n=5; chr10: 8,000,000-25,000,000; 10p11-1p15; **Figure 3E, I**), 18q loss (n=2; chr18: 45,000,000-67,000,000; 18q21-18q22; **Figure 3H**) and a single 5q gain (chr5: 143,000,000-155,000,000; 5q31-5q33). The presence of an 8q24 gain differentiated cases from controls with a sensitivity of 33% (95% CI 16-55%), specificity of 94% (95% CI 70-100%) and accuracy of 57% (95% CI 41-73%; **Figure 3I** & **Table 2**).

Whilst genetic variation of the 8q24.21 band has been associated with various cancer types, protein-coding genes within this band are sparse (**Supplemental Figure S2A, B**). *MYC*, a well-described oncogene got our specific interest, as it is overexpressed in 44% of PC tissues.[14] *MYC* is downstream of the RAS/RAF pathway, but also other oncogenic pathways (WNT-β-Catenin, JAK/STAT, TGF-β, Notch). *MYC* expression, in turn, drives cell growth and proliferation by binding to the enhancer box of transcription factors and acting as transcription factor to oncogenes such as *BCL2* (loss of this gene was seen in two individuals from the 8q24 subgroup), *TP53* and *p19ARF*. Tumorigenic effects of the 8q24 gain in PC may also be caused by neighboring oncogenic sequences coding for long noncoding RNAs (lncRNAs; PCAT1, CASC19, PRNCR1, CCAT1, CASC8, CCAT2, CASC11, PVT1, TMEM75, CCDC26, all associated with various cancers) or microRNAs (miRNAs/miR; miR-1204, miR-1205, miR-1206, miR-1207-5p, miR-1207-3p, and miR-1208). For instance, miR1208 was shown to be overexpressed in PJ from PC patients as compared to controls.[15] Another candidate gene (located next to *MYC*) is *PVT1*, a transcriptional activator of *MYC*. Another amplified region on chromosome 8 in PJsamples (yet not in #P4 tissue sample) is band 8q24.3, the location of multiple protein-coding genes. Examples are *PARP10, PSCA, HSF1* and *PLEC1*, the protein levels of which are shown to be overexpressed in PC tissue (as compared to healthy tissue) and promote tumorigenesis (by different pathways). Currently, efficacy of therapy against proteins, these genes code for, is being investigated for PC and multiple other cancer types.[16-21]

Our data also showed a 10p loss (10p15.1-10p11.22; **Supplementary Figure S2A, C**) in these PJ samples. This region contains multiple genes that have been related to carcinogenesis, whilst one gene located on 10p12.2 may be of particular interest: the transcription factor *PTF1A*. Silencing of *PTF1A* protects acinar cells during injury, which allows them to recover.[22] However, in case of oncogenic insults, loss of *PTF1A* potentiates acinar-to-ductal metaplasia and development of PanIN.[23-25] *BMI1*, a candidate gene located close to *PTF1A*, is a key player in regulation of pancreatic β- and acinar cell proliferation. The gene is required for regeneration (*e.g.*, after pancreatitis)[26-28] and inhibition of *BMI1* has been shown to upregulate production of reactive oxygen species,[29] which is a an essential step for the onset of pancreatic carcinogenesis.[30]

Two samples showed a 18q21 loss. This band, which is frequently altered in gastrointestinal cancers,[31] harbors *SMAD4* (**Supplemental Figure S2A, D**). *SMAD4* expression is decreased in 58% of PC cases and loss of *SMAD4* via homozygous deletion or mutation often occurs in late-stage PC.[32, 33] Loss of *SMAD4* promotes carcinogenesis by the stimulation of TGF-β signaling. *BCL2*, also located on 18q21, is an anti-apoptotic protein under modulation of

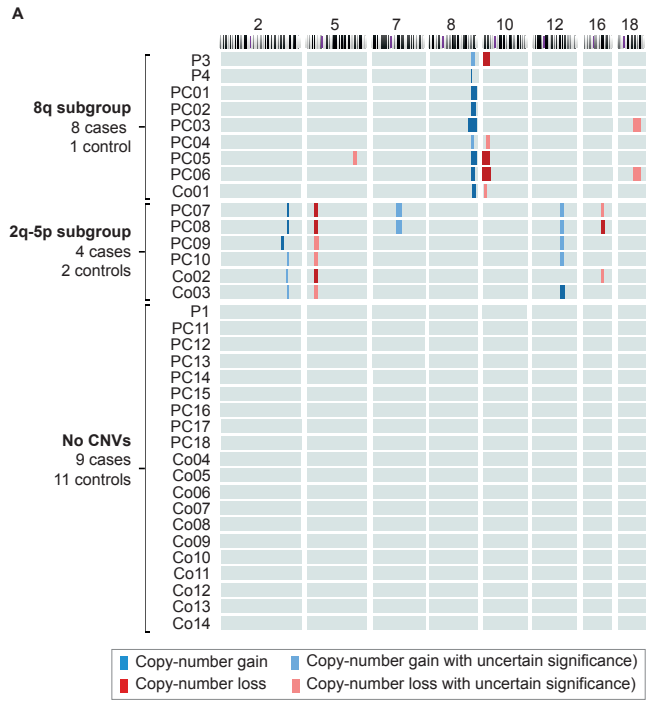
TP53, a gene that is mutated in 71% of patients with PC.[34, 35] Its expression is found to be downregulated in PC tissue, and loss of *BCL2* has been associated to a poor survival.[36,37] A third gene of interest in this region is *DCC*, a gene that codes for the netrin-1 receptor, which has been elaboratively investigated as tumor suppressor gene for colorectal cancer and has been associated with tumor stage in PC.[38]

2q-5p subgroup

Six individuals (15%; 4 cases [17%], 2 controls [13%]; p=0.72) had a 2q gain (with two different areas of amplification; chr2:150,000,000-168,000,000 and 183,000,000-195,000,000; 2q23-2q24, 2q32; **Figure 3A, I**) and 5p loss (chr5:19,000,000-33,000,000; 5p13-5p14; **Figure 3B, I**). DNA fragments had a mode size of 170-180bp, this peak prevalence is preceded by a plateau that shows a notable sawtooth pattern (**Figure 2E**). Of these, two samples showed an additional 7q gain (chr7: 75,000,000-90,000,000; 7q11-7q21; **Figure 3C, I**), three a 12q gain (ca. chr12: 83,000,000-95,000,000; 12q21-12q23; **Figure 3F, I**) and three a 16q loss (chr16:55,000,000-68,000,000; 16q12-16q22; **Figure 3G, I**). The ability to differentiate cases from controls was lower than for the 8q24 group (sensitivity: 17% [95% CI 5-38%], specificity: 87% [95% CI 62-98%]; accuracy: 45% [95% CI 29-62%]; **Table 2**). The presence of either an 8q24 or 2q-5p profile had a sensitivity of 50% (95% CI 29-71%), specificity of 81% (54-96%) and accuracy of 63% (95% CI 46-77%).

Six individuals had a 2q gain, located on 2q23-2q24 or 2q32 (**Figure 3A; Supplemental Figure S3A,B**). The 2q24 band houses two genes, *FAP* and *GALNT3*, involved in the desmoplastic and immunosuppressive microenvironment respectively; two major hurdles to be crossed in PC research. *FAP* has been shown to be expressed in PC cells and fibroblasts and plays a pivotal role in PC desmoplasia.[39-41] *GALNT3* encodes for an enzyme involved with O-linked glycosylation. It is shown to be overexpressed in well-differentiated PC tissue, yet downregulated in poorly differentiated PC, and may be a marker for prognosis. *STAT1* and *STAT4* are genes located on the 2q32 band, which encode for important components of the JAK-STAT signaling pathway that is (among other processes) involved in apoptosis and oncogenesis, having both tumor suppressive and tumor promoting functions.[42] For PC, increased expression of *STAT1* has been related to a favorable prognosis[43,44.]

Lastly, examples of potentially important genes that have been related to carcinogenesis and are located on the other aberrant bands (5p24, 7q11-7q21,12q21-12q23 and 16q12-16q22; **Supplemental Figure S3A,C-F**) are: *CDH6, CDH9, CDH10, CDH12* and *CDH18* (located on 5p24, which are involved in cell differentiation, loss of heterozygosity of *CDH10* is present in 24% of cases with PC[45]), *HGF* (7q21; mediates the interaction between cancer cells and pancreatic stellate cells),[46,47] *DUSP6* (12q21; a tumor suppressor and key player in the RAS/ERK signaling pathway)[48] and *HSF4* (16q22; a transcriptional factor critical for the activation of NF-κB signaling).[49]



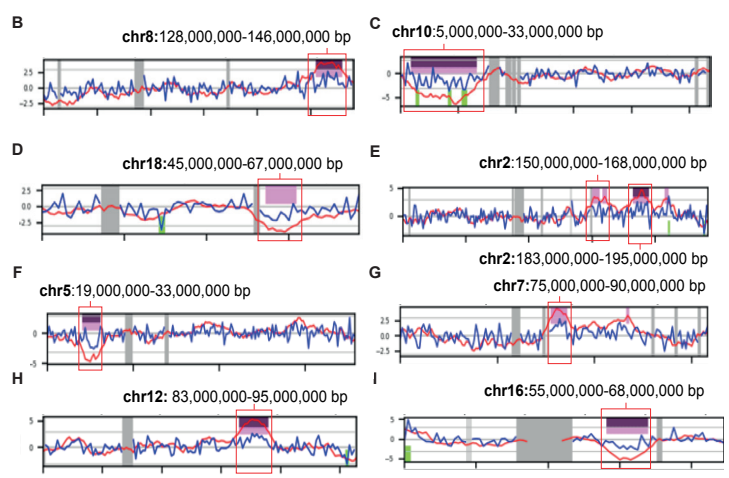


FIGURE 3 | Copy-number gains (blue) and losses (red) per chromosome (A) and patient (B) of all pancreatic juice (PJ) samples included in the study. A-H. Snapshots of Wisecondor images. Purple = aberration called by software, pink = aberration of uncertain significance. Locations on the chromosomes are given in base-pairs (bp). **I.** Nine individuals had an 8q gain (with or without a 10p loss and/or 18 loss) and six individuals had both a 2q gain and 5p loss (with or without a 7q gain, 12q gain and/or 16q loss). Aberrations in subgroups did not overlap. P = pilot, PC = PC case, Co = control. Chromosomes without a significant aberration and individuals with a 'chaotic profile' (P2, PC19, PC20, Co15, Co16) were not shown.

TABLE 2 | Diagnostic performance of chromosomal profiles.

	Present/total	AUC (95% CI)	Sensitivity in %	Specificity in %	Accuracy in %
8q-10p profile	9/40	0.64 (0.46-0.81)	33 (16-55)	94 (70-100)	57 (41-73)
2q-5p profile	6/40	0.52 (0.34-0.70)	17 (5-38)	87 (62-98)	45 (29-62)
8q-10p or 2q-5p profile	15/40	0.66 (0.48-0.83)	50 (29-71)	81 (54-96)	63 (46-77)

AUC = area under the curve, CI = confidence interval.

Discussion

This study shows that shallow sequencing using the robust NIPT-pipeline is feasible for cfDNA isolated from PJ collected from the duodenal lumen after secretin stimulation, but not for plasma. The most notable finding was the high prevalence of an 8q24 gain in the PC group. This was even seen in a patient with HGD, suggesting that this is a relatively early aberration that may hold promise for early detection of PC. Interestingly, individuals with an 8q24 also tended to have a distinct 'smooth' fragment length profile with rather short DNA fragments. This CNV was seen in combination with loss of 10p11-10p15 and/or 18q21-18q22. The detection of an 8q24 gain was highly specific for PC (94%), yet had low sensitivity (33%). Another prevalent finding was the combination of a gain of 2q and loss of 5p in patients with a distinct DNA fragmentation pattern (longer fragments with a sawtooth pattern). The presence of either an 8q24 or 2q-5p profile had a sensitivity of 50%, specificity of 81% and accuracy of 63%.

The aim of this study was to find biomarkers that enable earlier detection of PC in individuals who undergo surveillance. Moreover, the overall presence of CNVs and that on distinct locations may guide risk stratification and frequency of surveillance. Secretin-stimulated PJ collection is, as compared to fine-needle biopsy or PJcollection by cannulation of the pancreatic duct during endoscopic retrograde pancreatography (ERP), less invasive. In our opinion, the safety profile potentiates repetitive collections (*e.g.*, yearly) and allows monitoring of (early chromosomal) changes over time that are indicative for malignant transformation. Additionally, these changes in PJ may predict response to therapeutic agens.[50,51] For instance, PARP-inhibitors and platinum agents have shown to be effective in solid tumors bearing an unstable genome (including PC).[52,53] Another example is BCL2 downregulation which is associated with restoration of sensitivity to gemcitabine.[54]

The two identified patient subgroups with CNVs showed distinct features on the fragment size graphs. The 8q24 graphs were smooth with a mode size of 100-120 bp, whereas the 2q-5p subgroup had a saw tooth patter with mode size of 170-180 bp. The graph of the

later also showed a distinct plateau prior to the peak. cfDNA is generally produced by apoptosis and has a modal size of ±167 bp, which corresponds to 147 bp of DNA wrapped around a nucleosome plus the stretch of DNA on Histone H1 that links two nucleosome cores. The shorter fragments in these samples may be due to cleavage by enzymes in the PJ. However, that does not explain the difference between subgroups. This pattern is not expected to be a result of necroptosis or cellular secretion, as DNA fragments generated in these processes are generally larger (up to >10,000 bp for necroptosis and 1000-3000 bp for secretion).[53] Different experiments have demonstrated smaller fragment sizes (90-150bp) for tumor-derived cfDNA than wild-type cfDNA,[55,56] which may clarify the fragment size distributions in our samples. Additionally, mitochondrial DNA (mtDNA) has shown to be more fragmented (30-100 bp).[57,58]

Previous studies investigating chromosomal aberrations in tissue showed an 8q24 gain in 24-45% of patients with PC and 27% of those with HGD.[59-61] Therefore, the presence of this CNV may serve as a marker to detect HGD or early PC. The combination of an 8q gain and loss of 10p and 18q has been shown before in tissue, however loss of 18q and 10p has also been seen without 8q gain[59,61] We are the first to describe chromosomal aberrations in PJ that was collected non-invasively from the duodenum after stimulation by secretin. Mateos *et al*. (2019)[62] performed whole-exome sequencing on PJ samples collected during ERP or endoscopic nasopancreatic drainage of 39 patients with IPMN. They found 11 significantly amplified regions and four deleted regions. Of these, gain of 7q21 (1/8 low-grade dysplasia [LGD], 1/20 HGD, 7/11 PC), 8q24 (2/8 LGD, 1/20 HGD, 6/11 PC) and 12q21(2/8 LGD, 3/20 HGD, 1/11 PC) match our results.

We acknowledge several limitations to our study. Tissue samples were available only for patients included in the feasibility phase. For the three PC cases in this phase, there was an ample time between PJ collection and surgery (4-10 months), during which two underwent chemotherapy (eight cycles of FOLFIRINOX). For the patient with HGD (and 8q24 gain), only two months passed between PJ collection and surgery. Therefore, we do not have a confirmation of the breakpoints on the chromosomes. However, we were able to link our results to gene expression results currently present in literature. The low sensitivity (33%) may implicate that 8q24 gain represents a subtype rather than a general biomarker of PC. For instance, patients with germline mutations (*e.g., CDKN2A, BRCA2*) or IPMN may have distinct molecular mechanisms for carcinogenesis. A combination of different cfDNA aberrations (*e.g.*, mutations and chromosomal aberrations) may result in a panel of markers with higher sensitivity. Thus, while this case-control study is not able to conclusively prove a role for CNV testing in early PC detection, it highlights its potential and should be regarded as the starting point for further research in these specific surveillance cohorts.

In conclusion, shallow sequencing using the robust NIPT-pipeline is feasible for PJ cfDNA analysis. We identified 8q24 and the 2q-5p combination as hotspots, which seem specific for PC. Future studies in larger sample sizes are required, which include parallel testing

of tissue and cfDNA analysis of PJ-samples. Additionally, to evaluate the role of PJ cfDNA analysis in pancreas surveillance, analysis of consecutive samples, to assess the evolution of cfDNA changes over time, is essential.

References

1 Overbeek KA, Levink IJM, Koopmann BDM, *et al*. Long-term yield of pancreatic cancer surveillance in high-risk individuals. *Gut* 2022;71:1152-1160.

2 Zhan Q, Wen C, Zhao Y, *et al*. Identification of copy number variation-driven molecular subtypes informative for prognosis and treatment in pancreatic adenocarcinoma of a Chinese cohort. *eBioMedicine* 2021;74.

3 Keller L, Belloum Y, Wikman H, *et al*. Clinical relevance of blood-based ctDNA analysis: mutation detection and beyond. *British Journal of Cancer* 2021;124:345-358.

4 Bonner ER, Harrington R, Eze A, *et al*. Circulating tumor DNA sequencing provides comprehensive mutation profiling for pediatric central nervous system tumors. *npj Precision Oncology* 2022;6:63.

5 Amant F, Verheecke M, Wlodarska I, *et al*. Presymptomatic Identification of Cancers in Pregnant Women During Noninvasive Prenatal Testing. *JAMA Oncol* 2015;1:814-9.

6 Bianchi DW, Chudova D, Sehnert AJ, *et al*. Noninvasive Prenatal Testing and Incidental Detection of Occult Maternal Malignancies. *JAMA* 2015;314:162-169.

7 Moellgaard MH, Lund ICB, Becher N, *et al*. Incidental finding of maternal malignancy in an unusual non-invasive prenatal test and a review of similar cases. *Clin Case Rep* 2022;10:e6280.

8 Heesterbeek CJ, Aukema SM, Galjaard RH, *et al*. Noninvasive Prenatal Test Results Indicative of Maternal Malignancies: A Nationwide Genetic and Clinical Follow-Up Study. *J Clin Oncol* 2022:Jco2102260.

9 Kleeff J, Korc M, Apte M, *et al*. Pancreatic cancer. *Nat Rev Dis Primers* 2016;2:16022.

10 Ducreux M, Cuhna AS, Caramella C, *et al*. Cancer of the pancreas: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2015;26 Suppl 5:v56-68.

11 Levink IJM, Nesteruk K, Visser DI, *et al*. Optimization of Pancreatic Juice Collection: A First Step Toward Biomarker Discovery and Early Detection of Pancreatic Cancer. *Am J Gastroenterol* 2020;115:2103-2108.

12 Klug A, Lutter LC. The helical periodicity of DNA on the nucleosome. *Nucleic Acids Res* 1981;9:4267-83.

13 Han DSC, Ni M, Chan RWY, *et al*. The Biology of Cell-free DNA Fragmentation and the Roles of DNASE1, DNASE1L3, and DFFB. *Am J Hum Genet* 2020;106:202-214.

14. Schleger C, Verbeke C, Hildenbrand R, *et al*. c-MYC Activation in Primary and Metastatic Ductal Adenocarcinoma of the Pancreas: Incidence, Mechanisms, and Clinical Significance. *Modern Pathology* 2002;15:462-469.

15 Wang J, Raimondo M, Guha S, *et al*. Circulating microRNAs in Pancreatic Juice as Candidate Biomarkers of Pancreatic Cancer. *J Cancer* 2014;5:696-705.

16 Schleicher EM, Galvan AM, Imamura-Kawasawa Y, *et al*. PARP10 promotes cellular proliferation and tumorigenesis by alleviating replication stress. *Nucleic Acids Res* 2018;46:8908-8916.

17 Teng K-Y, Mansour AG, Zhu Z, *et al*. Off-the-Shelf Prostate Stem Cell Antigen–Directed Chimeric Antigen Receptor Natural Killer Cell Therapy to Treat Pancreatic Cancer. *Gastroenterology* 2022;162:1319-1333.

18 Bausch D, Thomas S, Mino-Kenudson M, *et al*. Plectin-1 as a novel biomarker for pancreatic cancer. *Clin Cancer Res* 2011;17:302-9.

19 Wernitznig D, Meier-Menches SM, Cseh K, *et al*. Plecstatin-1 induces an immunogenic cell death signature in colorectal tumour spheroids. *Metallomics* 2020;12:2121-2133.

20 Liang W, Liao Y, Zhang J, *et al*. Heat shock factor 1 inhibits the mitochondrial apoptosis pathway by regulating second mitochondria-derived activator of caspase to promote pancreatic tumorigenesis. *J Exp Clin Cancer Res* 2017;36:64.

21 Carpenter RL, Gökmen-Polar Y. HSF1 as a Cancer Biomarker and Therapeutic Target. *Curr Cancer Drug Targets* 2019;19:515-524.

22 Jakubison BL, Schweickert PG, Moser SE, *et al*. Induced PTF1a expression in pancreatic ductal adenocarcinoma cells activates acinar gene networks, reduces tumorigenic properties, and sensitizes cells to gemcitabine treatment. *Mol Oncol* 2018;12:1104-1124.

23 Kondratyeva LG, Chernov IP, Zinovyeva MV, *et al*. Expression of master regulatory genes of embryonic development in pancreatic tumors. *Dokl Biochem Biophys* 2017;475:250-252.

24 Krah NM, De La OJ, Swift GH, *et al*. The acinar differentiation determinant PTF1A inhibits initiation of pancreatic ductal adenocarcinoma. *Elife* 2015;4.

25 Campos ML, Sánchez-Arévalo Lobo VJ, Rodolosse A, *et al*. ICAT is a novel Ptf1a interactor that regulates pancreatic acinar differentiation and displays altered expression in tumours. *Biochem J* 2013;451:395-405.

26 Sangiorgi E, Capecchi MR. Bmi1 lineage tracing identifies a self-renewing pancreatic acinar cell subpopulation capable of maintaining pancreatic organ homeostasis. *Proc Natl Acad Sci U S A* 2009;106:7101-6.

27. Fukuda A, Morris JPt, Hebrok M. Bmi1 is required for regeneration of the exocrine pancreas in mice. *Gastroenterology* 2012;143:821-831.e2.

28 Dhawan S, Tschen SI, Bhushan A. Bmi-1 regulates the Ink4a/Arf locus to control pancreatic beta-cell proliferation. *Genes Dev* 2009;23:906-11.

29 Bednar F, Schofield HK, Collins MA, *et al*. Bmi1 is required for the initiation of pancreatic cancer through an Ink4a-independent mechanism. *Carcinogenesis* 2015;36:730-8.

30 DeNicola GM, Karreth FA, Humpton TJ, *et al*. Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis. *Nature* 2011;475:106-9.

31 Barberá VM, Martín M, Mariñoso L, *et al*. The 18q21 region in colorectal and pancreatic cancer: independent loss of DCC and DPC4 expression. *Biochim Biophys Acta* 2000;1502:283-96.

32 Ritterhouse LL, Wu EY, Kim WG, *et al*. Loss of SMAD4 protein expression in gastrointestinal and extra-gastrointestinal carcinomas. *Histopathology* 2019;75:546-551.

33 Wang JD, Jin K, Chen XY, *et al*. Clinicopathological significance of SMAD4 loss in pancreatic ductal adenocarcinomas: a systematic review and meta-analysis. *Oncotarget* 2017;8:16704-16711.

34 Hemann MT, Lowe SW. The p53-Bcl-2 connection. Cell Death Differ 2006;13:1256-9.

35 Waddell N, Pajic M, Patch AM, *et al*. Whole genomes redefine the mutational landscape of pancreatic cancer. *Nature* 2015;518:495-501.

36 Song S, Wang B, Gu S, *et al*. Expression of Beclin 1 and Bcl-2 in pancreatic neoplasms and its effect on pancreatic ductal adenocarcinoma prognosis. *Oncol Lett* 2017;14:7849-7861.

37 Evans JD, Cornford PA, Dodson A, *et al*. Detailed tissue expression of bcl-2, bax, bak and bcl-x in the normal human pancreas and in chronic pancreatitis, ampullary and pancreatic ductal adenocarcinomas. *Pancreatology* 2001;1:254-62.

38 An XZ, Zhao ZG, Luo YX, *et al*. Netrin-1 suppresses the MEK/ERK pathway and ITGB4 in pancreatic cancer. *Oncotarget* 2016;7:24719-33.

39 Shi M, Yu DH, Chen Y, *et al*. Expression of fibroblast activation protein in human pancreatic adenocarcinoma and its clinicopathological significance. *World J Gastroenterol* 2012;18:840-6.

40 Lo A, Li CP, Buza EL, *et al*. Fibroblast activation protein augments progression and metastasis of pancreatic ductal adenocarcinoma. *JCI Insight* 2017;2.

41 Lee H-O, Mullins SR, Franco-Barraza J, *et al*. FAP-overexpressing fibroblasts produce an extracellular matrix that enhances invasive velocity and directionality of pancreatic cancer cells. *BMC Cancer* 2011;11:245.

42 Meissl K, Macho-Maschler S, Müller M, *et al*. The good and the bad faces of STAT1 in solid tumours. *Cytokine* 2017;89:12-20.

43 Zhang J, Wang F, Liu F, *et al*. Predicting STAT1 as a prognostic marker in patients with solid cancer. *Therapeutic Advances in Medical Oncology* 2020;12:1758835920917558.

44 Sun Y, Yang S, Sun N, *et al*. Differential expression of STAT1 and p21 proteins predicts pancreatic cancer progression and prognosis. *Pancreas* 2014;43:619-23.

45 Jinawath N, Shiao MS, Norris A, *et al*. Alterations of type II classical cadherin, cadherin-10 (CDH10), is associated with pancreatic ductal adenocarcinomas. *Genes Chromosomes Cancer* 2017;56:427-435.

46 Xu Z, Pang TCY, Liu AC, *et al*. Targeting the HGF/c-MET pathway in advanced pancreatic cancer: a key element of treatment that limits primary tumour growth and eliminates metastasis. *British Journal of Cancer* 2020;122:1486-1495.

47 Pothula SP, Xu Z, Goldstein D, *et al*. Hepatocyte growth factor inhibition: a novel therapeutic approach in pancreatic cancer. *British Journal of Cancer* 2016;114:269-280.

48 Kidger AM, Saville MK, Rushworth LK, *et al*. Suppression of mutant Kirsten-RAS (KRASG12D)-driven pancreatic carcinogenesis by dual-specificity MAP kinase phosphatases 5 and 6. *Oncogene* 2022;41:2811-2823.

49 Guo F, Cheng X, Jing B, *et al*. FGD3 binds with HSF4 to suppress p65 expression and inhibit pancreatic cancer progression. *Oncogene* 2022;41:838-851.

50 Franko J, Krasinskas AM, Nikiforova MN, *et al*. Loss of heterozygosity predicts poor survival after resection of pancreatic adenocarcinoma. *J Gastrointest Surg* 2008;12:1664-72; discussion 1672-3.

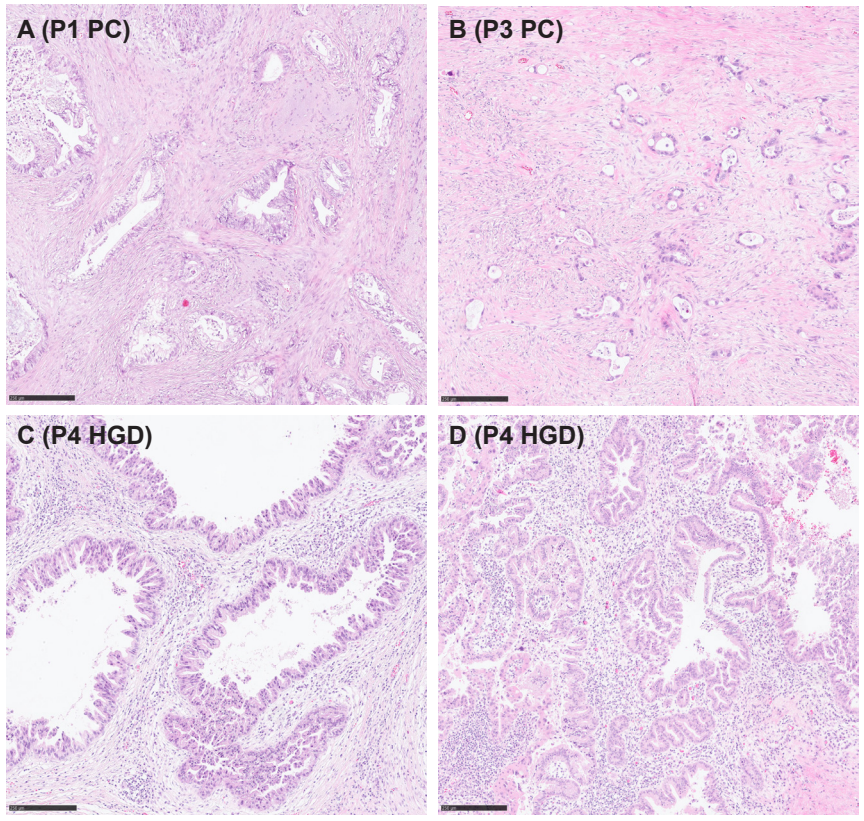
51 Kluzek K, Srebniak MI, Majer W, *et al*. Genetic characterization of Polish ccRCC patients: somatic mutation analysis of PBRM1, BAP1 and KDMC5, genomic SNP array analysis in tumor biopsy and preliminary results of chromosome aberrations analysis in plasma cell free DNA. *Oncotarget* 2017;8:28558-28574.

52 Chi J, Chung SY, Parakrama R, *et al*. The role of PARP inhibitors in BRCA mutated pancreatic cancer. *Therap Adv Gastroenterol* 2021;14:17562848211014818.

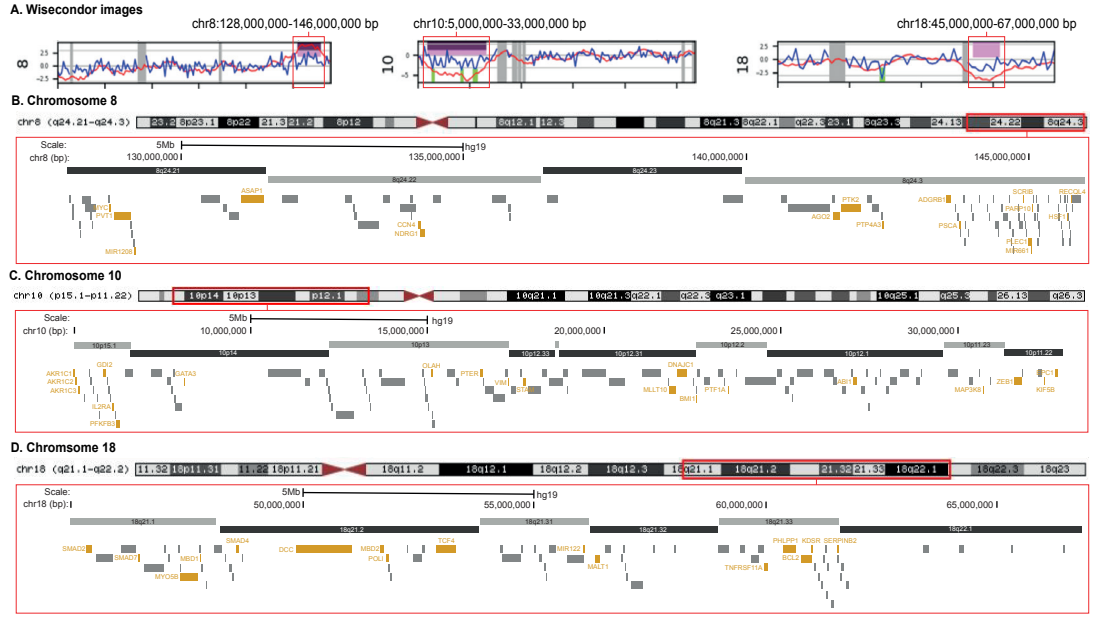
53 Emelyanova M, Pudova E, Khomich D, *et al*. Platinum-based chemotherapy for pancreatic cancer: impact of mutations in the homologous recombination repair and Fanconi anemia genes. *Ther Adv Med Oncol* 2022;14:17588359221083050.
54 Wang M, Lu X, Dong X, *et al*. pERK1/2 silencing sensitizes pancreatic cancer BXPC-3 cell to gemcitabine-induced apoptosis via regulating Bax and Bcl-2 expression. *World Journal of Surgical Oncology* 2015;13:66.
55 Mouliere F, Chandrananda D, Piskorz AM, *et al*. Enhanced detection of circulating tumor DNA by fragment size analysis. *Sci Transl Med* 2018;10.
56 Underhill HR, Kitzman JO, Hellwig S, *et al*. Fragment Length of Circulating Tumor DNA. *PLoS Genet* 2016;12:e1006162.
57 Zhang R, Nakahira K, Guo X, *et al*. Very Short Mitochondrial DNA Fragments and Heteroplasmy in Human Plasma. *Scientific Reports* 2016;6:36097.
58 Mair R, Mouliere F, Smith CG, *et al*. Measurement of Plasma Cell-Free Mitochondrial Tumor DNA Improves Detection of Glioblastoma in Patient-Derived Orthotopic Xenograft Models. *Cancer Research* 2019;79:220-230.
59 Witkiewicz AK, McMillan EA, Balaji U, *et al*. Whole-exome sequencing of pancreatic cancer defines genetic diversity and therapeutic targets. Nature Communications 2015;6:6744.
60 Hata T, Suenaga M, Marchionni L, *et al*. Genome-Wide Somatic Copy Number Alterations and Mutations in High-Grade Pancreatic Intraepithelial Neoplasia. *Am J Pathol* 2018;188:1723-1733.
61 Schleger C, Arens N, Zentgraf H, *et al*. Identification of frequent chromosomal aberrations in ductal adenocarcinoma of the pancreas by comparative genomic hybridization (CGH). *J Pathol* 2000;191:27-32.
62 Mateos RN, Nakagawa H, Hirono S, *et al*. Genomic analysis of pancreatic juice DNA assesses malignant risk of intraductal papillary mucinous neoplasm of pancreas. *Cancer Med* 2019;8:4565-4573.
63 Kim SK, Hannum G, Geis J, *et al*. Determination of fetal DNA fraction from the plasma of pregnant women using sequence read counts. *Prenat Diagn* 2015;35:810-5.
64 Straver R, Sistermans EA, Holstege H, *et al*. WISECONDOR: detection of fetal aberrations from shallow sequencing maternal plasma based on a within-sample comparison scheme. *Nucleic Acids Res* 2014;42:e31.

SUPPLEMENTAL TABLE S1 | In- and exclusion criteria per prospective cohort study. PC = pancreatic cancer, EUS = endoscopic ultrasound, FPC = familial pancreatic cancer, FDR = first-degree relative, SDR = second-degree relative, CEA = carcinoembryonic antigen.

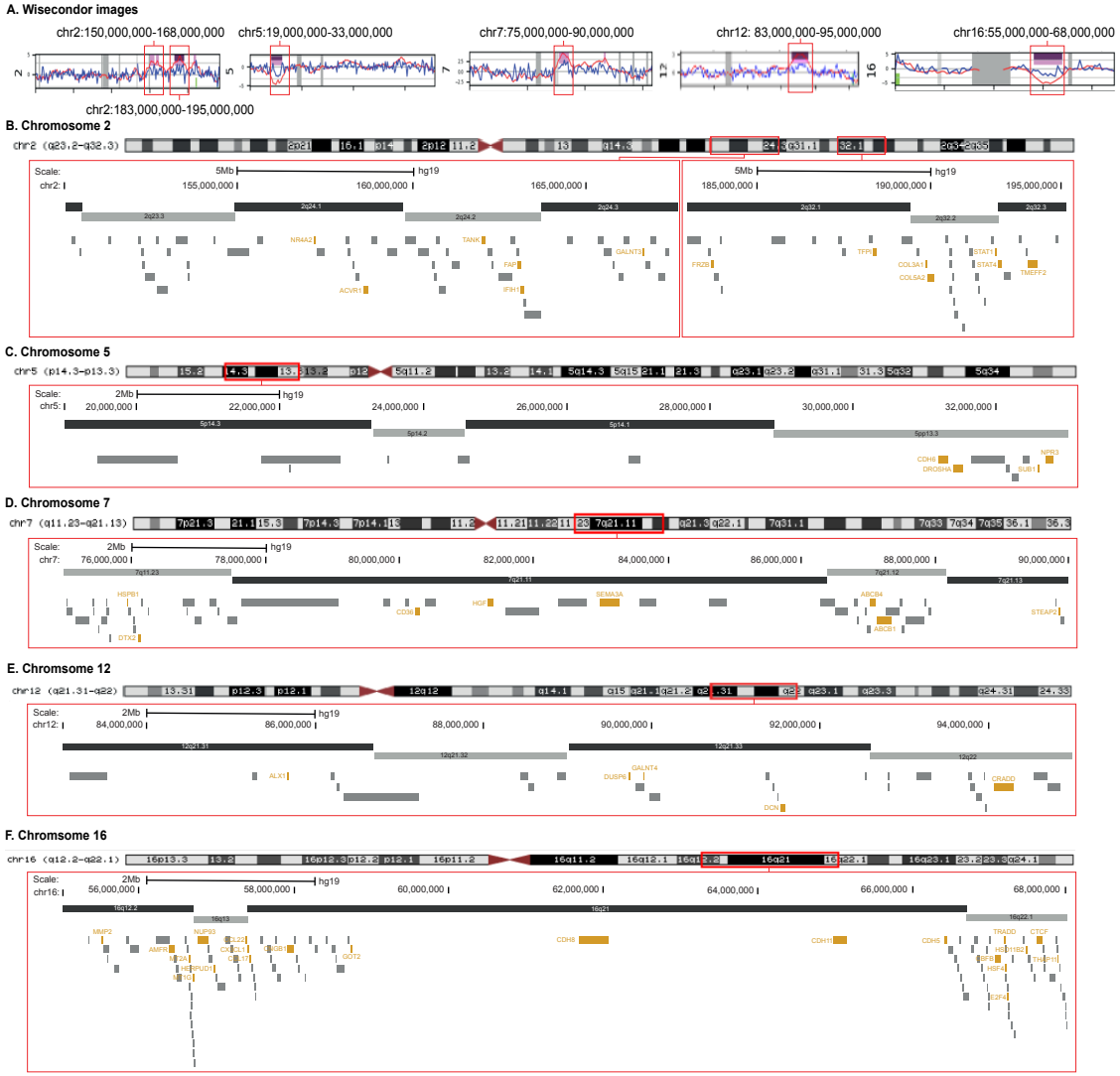
Prospective cohort	Inclusion criteria	Exclusion criteria
KRASPanc	Patients who undergo an EUS for (suspected) PC either as part of a diagnostic process or fiducial placement prior to radiotherapeutic treatment.	Age <18 years
CAPS	Individuals who, after evaluation by a clinical geneticist, have an estimated 10-fold increased risk of developing PC, this includes: (1) Carriers of a gene mutation in *CDKN2A* or *STK11*, regardless of the family history of PC (2) Carriers of a gene mutation in *BRCA1, BRCA2, p53*, or Mismatch Repair Gene with a family history of PC in ≥2 family members. (3) FPC-kindreds, defined as individuals with at least (1) two FDRs with PC, (2) 3 relatives with pancreatic cancer, either FDR or SDR, or (3) two SDR relatives with PC of which at ≥1 was <50 years at time of diagnosis.	Age <18 years, personal history of pancreatic cancer, individuals unable to provide informed consent, severe medical illness, *PRSS1* gene carrier, contra-indication for EUS due to anatomic abnormalities/surgery.



SUPPLEMENTAL FIGURE S1 | H&E staining of tumor slides showing the cancer cellularity at time of resection for P1 (A; PC), P3 (B; PC) and P4 (C-D; HGD). No slide was available of P2 at time of writing. PC = pancreatic cancer; HGD = high-grade dysplasia.



SUPPLEMENTAL FIGURE S2 | An overview of copy number variations seen in the 8q subgroup and genes located on the aberration segments. A. Snapshot of the Wisecondor images showing an 8q gain and 10p loss and 18q loss. Aberrant segments are marked with a red box. Purple = significant copy number variations called by the software. Pink = copy number variations of uncertain significance. **B-C** Genes located on the aberrant chromosome segments. Yellow = genes associated with cancer development according to literature.



SUPPLEMENTAL FIGURE S3 | An overview of copy number variations seen in the 2q-5p subgroup and genes located on the aberration segments. A. Snapshot of the Wisecondor images showing a 2q gain and 5p loss (with or without 7q gain, 12q gain or 16q loss). Aberrant segments are marked with a red box. Purple = significant copy number variations called by the software. Pink = copy number variations of uncertain significance. **B-F** Genes located on the aberrant chromosome segments. Yellow = genes associated with cancer development according to literature.

PART IV

General discussion & appendices

CHAPTER 14

Summary, general discussion and future perspectives

Rationale behind this thesis

Pancreatic cancer (PC) is a lethal disease, for which timely detection and early surgical resection provide the only chance of cure. In individuals with an increased risk of developing PC (high-risk individuals), including those with a hereditary predisposition or neoplastic pancreatic cyst, surveillance may lead to earlier detection and improved survival. However, it is debatable whether these benefits weigh up to its drawbacks, such as patient anxiety, harm by overtreatment and increased healthcare expenses.

While malignant transformation is difficult to identify with current imaging modalities, (cancer) cells constantly shed various materials to surrounding tissues. We postulate that measuring these substances in blood and pancreatic juice (PJ) may serve as indicators for high-grade dysplasia (HGD) or early-stage cancer. The detection of a biomarker signature in blood and PJ may stratify high-risk individuals by cancer risk and bring forth a surveillance program with tailored intervals and modalities. The ultimate goal of such a marker panel is to detect PC at a curable stage, while minimizing surveillance-related harms.

This thesis critically appraises current recommendations for pancreas surveillance (**PART I**) and evaluates the potential of biomarker analysis in blood and PJ (**PART II**).

PART I: Pancreas surveillance: the current practice

Current guidelines

Pancreatic cysts are increasingly being incidentally discovered on diagnostic imaging performed for unrelated conditions. Around half of these are neoplastic intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs), which have a malignant potential.[1,2] Therefore, individuals with these cystic lesions are candidates for surveillance.

Four major guidelines have been published on pancreatic cyst management; a European guideline (2018; **Table 1**),[3] an 'international' (Fukuoka) guideline (2017; **Table 1**),[4] and two American guidelines (ACG, 2018; AGA, 2015).[5,6] These guidelines overlap in their use of distinct, mostly morphologic features to stratify the risk of malignancy, and differ on the role and threshold of cyst size and growth speed, the use of serum CA19.9 monitoring, surveillance intervals, duration of surveillance, and when to perform surgery. These differences stem from a lack of solid evidence, and recommendations are mostly based on expert opinion and retrospective studies. The latter generally involves surgical cases and/or surrogate endpoints.

TABLE 1 | Overview of relative and absolute indications for surgery according to the European evidence-based guideline on pancreatic cystic neoplasms (2018)[3] and high-risk stigmata and worrisome features according to the Fukuoka guidelines (2017).[4] HGD = high-grade dysplasia, MPD = main pancreatic duct.

European guidelines	
Absolute indications	**Relative indications**
Positive cytology for malignancy/HGD	Growth-rate ≥5 mm/year
Solid mass	Increased levels of serum CA 19.9 (>37 U/mL) *in the absence of jaundice*
Jaundice (tumor related)	MPD dilatation between 5 and 9.9 mm
Enhancing mural nodule ≥5 mm	Cyst diameter ≥40 mm
MPD dilatation ≥10mm	New onset of diabetes mellitus
	Acute pancreatitis (caused by IPMN)
	Enhancing mural nodule (<5 mm)
Fukuoka guidelines	
High-risk stigmata	**Worrisome features**
Obstructive jaundice	Pancreatitis
Enhancing mural nodule ≥5 mm	Cyst size ≥30 mm
MPD size of ≥10 mm	Thickened/enhancing cyst walls
	MPD size 5-9 mm
	Abrupt change in calibre of pancreatic duct with distal pancreatic atrophy
	Lymphadenopathy
	Increased serum level of CA19.9
	Cyst growth rate ≥5mm per 2 years

The PACYFIC-registry

Proof of efficacy of pancreatic cyst surveillance is urgently needed. To fill this gap, the PACYFIC-registry was instigated in 2015. This international collaboration prospectively investigates the yield and management of surveillance in a mixed population harboring low- and high-risk cysts from academic and community hospitals in Europe and North America. The study provides a unique insight in the way pancreatic cyst surveillance is performed and enables critical appraisal of current cyst management. Physicians record data pertaining their patients prospectively in an eCRF, equipped with restrictions, obligatory values, and notifications, to prevent missing values as much as possible.

However, the PACYFIC-registry also faces challenges. Surveillance studies are labor-intensive and take years before robust conclusions can be drawn. For example, whilst being set up in 2015, the median follow-up time of PACYFIC-participants was 25 and 26 months at the time **Chapters 4** and **5** were written. Thus, we were unable to evaluate

the HGD and PC risk on the longer term. Also, over time, required variables may change due to new insights. For instance, an elevated serum CA19.9 had not yet been defined as a relative indication for surgery at the start of the PACYFIC-registry. Therefore, CA19.9 values were not determined at first. Additionally, cyst management (such as surveillance intervals and imaging modality) within the PACYFIC-registry is being performed upon the discretion of the treating physician. Hence, the registry is prone to intra- and inter-individual differences. This may influence results, but also offers opportunities, as it allows comparison of different modalities. Furthermore, whilst the PACYFIC registry aims to represent the surveillance population at large, half of the participating hospitals are academic, which may result in overrepresentation of high-risk cysts. At last, a general limitation of cyst surveillance studies is their inclusion of other (non-neoplastic) lesions (*e.g.*, SCAs, lymphoepithelial cysts, pseudocysts), as morphological features may be misleading. Therefore, the results from the PACYFIC-registry cannot be extrapolated explicitly to IPMNs.

Identification of reassuring features

Current surveillance strategies pose a high burden on patients and health care resources. A less intensive surveillance strategy – with reduced surveillance frequency and duration – may limit financial expenditure of health care resources, increase patient adherence, and lower surgical mortality without increasing surveillance related mortality.[7] Identification of 'reassuring features' may enable identification of those individuals amenable for a low-intensive surveillance strategy. Therefore, a paradigm shift is required from the (unilateral) selection of high-risk cysts, to a bilateral approach based on both 'reassuring' and 'worrisome' features that will divide the cyst population into three groups: low-, moderate- and high-risk.

Potential 'reassuring features' are a small size and absence of growth. In **Chapter 4**, we used data from the PACYFIC-registry to investigate the risk of malignancy according to size. Indeed, small cysts (<20mm) and slow growth rate (<5mm/year) were associated with a lower risk of developing HGD/PC. For such 'trivial cysts', a less intensive follow-up regime may suffice.

The Fukuoka guidelines (2017)[4] propagate an incremental size-based approach. After cyst size being stable for six-months, they recommend two-yearly follow-up for cysts <10mm, yearly for cysts 10-20mm, and every 3-6 months for larger cysts, with possible lengthening of intervals when cysts remain stable. For future guidelines,[3] we would opt for a similar size-based approach, with gradual prolongation of intervals in case of stability in size and absence of relative and absolute indications for surgery. For instance, an individual with a newly detected cyst could undergo follow-up every six-months in the first year, yearly in the subsequent three years, and if stable (as compared to baseline), every two-years thereafter (**Figure 1**).

The cyst size threshold for reassurance at 20mm is not set in stone. **Chapter 4** shows that individuals with a cyst size of 20-29mm do not have a significantly higher risk of developing HGD or PC, as compared to those <10mm (p=0.70). Further modelling studies are required to calculate the (cost-) effectiveness of low-intensity surveillance programs at different size thresholds. Additionally, one should take in account cyst stability over time, as longitudinal size stability and absence of indications for surgery may be a stronger predictor than size alone.

'Reassuring features' may also aid identifying those eligible for termination of surveillance. In 2015, the AGA published the first guideline that opted for discontinuation of surveillance after five years for cysts without significant change.[4] This recommendation was controversial due to lack of evidence, and instigated several studies on the prevalence of PC after five years of follow-up.[8] A recent meta-analysis by Chhoda *et al*. (2023)[8] showed that individuals with a stable cyst being followed for 5 years or more had a pooled incidence of worrisome features or high-risk stigmata of 1.9%, and advanced neoplasia of 0.2% per patient year. More recently, proposals for discontinuation of cyst surveillance have been published. For instance, Marchegiani *et al*. (2023)[9] showed that the incidence of PC decreased significantly with age and cyst size. Therefore, they suggested an age-dependent strategy with stopping surveillance in individuals with cysts <30mm that have been stable over five years at the age of 75 years and at the age of 65 with stable cysts <15mm. A stimulation model by Lobo *et al*. (2020)[7] evaluating cost-effectiveness of surveillance in low risk cysts implicates that an annual risk of <0.12% of malignant progression is most likely outweighed by the costs of surveillance, risks of surgery and death from unrelated causes.[7] More interestingly, they showed that a less-intensive surveillance protocol would result in a similar number of deaths, but at lower costs. However, a formal analysis using prospective data with longer follow-up duration is required to confirm this.

Furthermore, fitness for surgery needs to be strictly assessed. In clinical practice, physicians are often hesitant to discontinue surveillance even in unfit patients. Either because they fail to acknowledge a patient's frailty or because of a strong patient wish to continue.[10] An evidence-based algorithm that assists physicians in assessing fitness and that supports risk/benefit discussions with patients may be of great value.

This thesis did not discuss the modality of choice (EUS or MRCP) to measure cyst size. However, a consistent choice of modality at each follow-up is important to reduce modality-related size differences and increase the reliability of cyst stability assessments. In short, MRCP may be the modality of choice for large (multilocular) cysts (± >50mm), as capturing them in one field, with subsequent measurements, may be challenging with EUS.[11] Conversely, solid components and smaller cysts (>10 and < ±50mm) are better detected by EUS.[11]

Potential other 'reassuring features' may be the presence of a solitary cyst or absence of connection of the cyst with the main pancreatic duct, pancreatic steatosis and EUS features related to chronic pancreatitis ('Rosemont criteria').[12] However, more research is needed to verify their relation to malignant transformation. Similarly, biomarkers could serve as 'reassuring features'. For instance, the presence of a *GNAS* mutation in PJ, absence of a *KRAS, TP53, CDKN2A* or *SMAD4* mutation or a low concentration of CEA and amylase may indicate benign disease.[13] Importantly, fine-needle aspiration should not be performed routinely in low-risk cysts due to its associated pancreatitis risk.

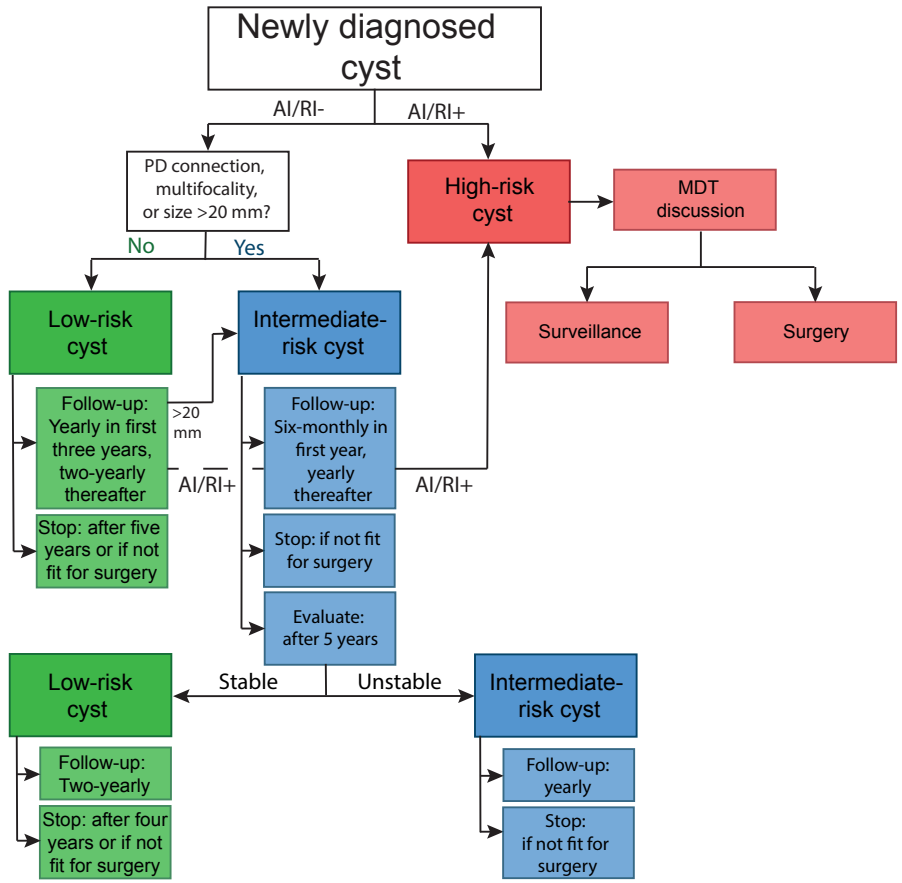


FIGURE 1 | Alternative surveillance algorithm for low-, intermediate- and high-risk pancreatic cysts. MDT = multidisciplinary team.

CA19.9 and the risk of overtreatment

Serum CA19.9 is the single biomarker widely used in clinical practice for PC, but only for monitoring of treatment response and disease recurrence and not for early detection.[14-17] However, in the latest update of the European guidelines (2018),[18] serum CA19.9 elevation was introduced as a relative indication for surgery, meaning that surgery should be considered when another relative indication for surgery is present. However, high rates of false-positive values in participants with benign disease have raised concerns. In **Chapter 5**, we aimed to evaluate the performance of CA19.9 monitoring in a cyst surveillance population. We showed that serum CA19.9 at a cutoff level of 37 kU/L or as continuous variable was not independently associated with the development of HGD or PC. Moreover, when used as recommended by the European guidelines,[3] it led to unnecessary intensified surveillance and surgery.

Based on these data, we concluded that CA19.9 monitoring in its present form has limited additive value in a cyst surveillance program. However, its performance may be improved by using a different cutoff. In **Chapter 5** and **7**, we show that a higher cutoff (87 kU/L, 97 kU/L,[19] 100 ku/L[14] or 133 kU/L[20]) increases the specificity, and thus may decrease the risk of overtreatment. Chapter 7 evaluates a panel of biomarkers, of which serum CA19.9 performed best. However, this cohort contained only 11 patients with resectable disease. It is conceivable that for early onset disease, other biomarkers (*e.g.*, MUC5A, NGAL, IL-8) will outperform CA19.9. In conclusion, the role of CA19.9 should be critically appraised, as our data suggests it has low sensitivity in early-stage cancer, and limited specificity to prevent unnecessary harm.

Other future perspectives

Identification of low-risk cysts for which limited or no surveillance will suffice should be a focal point of attention for future studies. Trade-offs between more- and less-intensive strategies should be made based on comprehensive cost-effectiveness analyses, rather than consensus. Research on larger cohorts with longer follow-up duration will be required to draw definite conclusions. Additionally, ethicists can help to establish moral values pertaining acceptable benefits and harms of pancreas surveillance. Last but not least, patients should be involved in this process to help them understand the rationale behind de-intensification of surveillance.

Within the coming years, ongoing prospective studies such as the PACYFIC-study will provide a better insight into the long-term risk of PC in those having low-, intermediate, and high-risk cysts based on prospective cohort studies. Additionally, information is needed on the natural course of IPMN, which is difficult to gain due to lack of tissue from cysts that do not require surgery. Future technical developments of cross-sectional imaging and EUS (*e.g.*, based on artificial intelligence) may result in more detailed images to allow appreciation of in-vivo transformation on a cellular (or even molecular) level.

PART II: Potential biomarkers and pancreatic juice

Cancer development results from a cascade of molecular changes in cells and affects a complex web of biochemical pathways. Tumor-derived information, in the form of molecules and particles, is constantly released in the surrounding tissue and blood stream as the resultant of cellular secretion, apoptosis and necroptosis. This information has the potential to serve as biomarker for early cancer detection. In **PART II**, we evaluate the role of PJ as a biomarker source, aiming to identify biomarkers in blood and PJ for earlier detection of PC in a surveillance cohort.

Selection of potential biomarkers

The holy grail would be to identify biomarkers that can detect early PC (or, preferably, HGD) before a mass becomes visible on imaging. Such biomarkers would have to meet the following criteria:

1. Sample collection should be easy and safe, as it is performed repetitively.
2. Analysis should be robust, cheap and easy to access, and, ideally, available around the globe.
3. Biomarkers should be:
   a associated with a low number of false-positive values (high specificity/positive predictive value) to prevent unnecessary harm by overtreatment.
   b. associated with a low number of false-negative values (high sensitivity) to avoid missing cases.

Many potential biomarkers for PC have been described in literature. Rather than repeating unbiased approaches, we set out to validate previously identified potent biomarkers. Whilst unbiased approaches (such as whole genome sequencing) reveal the full cancer signature and are especially amenable if the molecular signature of a disease that is unknown, they are expensive, elaborate, require a large sample size and generate noise due to cancer-unrelated aberrations. Therefore, we used existing genomics,[21-30] transcriptomics[31,32] and (glyco) proteomics[33-35] data to identify potent biomarkers. Additionally, we selected the most potent markers for validation based on the whole range of DNA,[36-38] RNA,[39,40] protein [41,42]and cellular components.[43,44] By casting our net wide, we appraised a variety of markers and laboratory analyses to select those eligible for further longitudinal evaluation in a surveillance cohort. Additionally, as PC can be divided into subtypes, based on molecular, transcriptomic, and epigenetic characteristics,[45,46] likely, a panel of biomarkers that complement each other will be required to catch all different subtypes, rather than a single biomarker.

Pancreatic juice as biomarker source

PJ is a promising biomarker source, as it constitutes a wash-out of the pancreatic ductal system and has been in close contact with the ductal cells from which PC originates. Thus far, PJ evaluation has not been widely investigated due to lack of standardized PJ collection methods and, until recently, a shortage of human secretin. Historically, PJ was collected after direct cannulation of the ampullary orifice during ERCP. Although this technique ensures PJ purity,[33] it poses a risk of pancreatitis, reported to be as high as 25%.[47,48] In healthy participants undergoing surveillance, such risk of pancreatitis is unacceptable. Therefore, PJ collection from the duodenal lumen, without cannulation, seems more viable.

As a panel of biomarkers will likely be most predictive, we aimed to establish the most effective way to collect secretin-stimulated PJ suitable for the detection of a variety of biomarkers. Contamination of PJ with blood, bile and duodenal fluid may lower concentrations of pancreas-derived biomarkers.[33,49,50] In **Chapter 6**, we compared an abundance of biomarkers in PJ between two collection techniques: through-the-scope aspiration with a catheter vs suction through the endoscopic channel. In contrast to Suenaga *et al.* (2017),[51] we did not use a distal cap on the tip of a forward viewing endoscope, as this may cause pancreatitis by occluding the ampullary orifice and requires reintroduction of the scope. We hypothesized that a through-the-scope catheter decreases duodenal contamination by precise positioning of the tip close to the ampullary orifice, while with suction through the endoscopic channel, we expected to collect higher volumes. Indeed, we obtained more PJ when performing suction through the endoscopy channel. Unexpectedly however, this fluid also tended towards a purer consistency, based on the concentration of the pancreas-specific protein PLA2G1B. Culturing PJ-derived organoids was more successful with juice collected through the catheter. Concentrations of other biomarkers did not differ between collection methods.

We also investigated the best timeframe for PJ collection after secretin injection. The first flush may consist of stagnant remnants and be unsuitable for biomarker detection. Alternatively, this first wash-out may be more concentrated whilst prolonged collection may dilute the biomarkers of interest. Additionally, the optimal time of collection may depend on the biomarker of interest. For instance, for soluble proteins, a larger volume may dilute beyond the optimal time point. In contrast, for collection of cellular fractions (*e.g.*, for organoid growth), it is likely that larger PJ volumes provide a higher yield, as these fractions may be concentrated by centrifugation. Our results indicate that most measured constituents (*i.e.*, DNA, mRNA, diverse proteins and cells) have optimal detection ranges within the 0-4 or 4-8-minute timeframes, while collection beyond 8 minutes resulted in more blood contamination and lower biomarker levels. In addition, PJ collected from 8 to 15 min after secretin injection contained more longer segment DNA (as a potential measure of genomic DNA or cell necrosis). Another disadvantage of longer collection times is that they impose more strain on endoscopy programs. Similarly,

Suenaga *et al.* (2018)[52] detected most cell-free DNA (cfDNA) mutations in PJ collected 5-10 min after secretin infusion.

Thus, in **Chapter 6** we set a first step towards standardization of PJ collection for biomarker detection. Based on these results, we now biobank and analyze PJ samples collected between 4 to 8 minutes after secretin infusion by suction through the endoscopic channel. To prevent enzyme-induced digestion and subsequent loss of valuable information, samples are snap-frozen within 10 minutes of collection.

Evaluated biomarkers in this thesis

As a next step, we dived into the literature to select and, subsequently, analyze levels of promising biomarkers (proteins, extracellular-vesicle-derived-microRNA [EV-miR], and DNA) in PJ and blood from cancer cases and controls.

Proteins

PJ is rich in proteins such as mucins and cytokines, and proteomic analysis has shown differences between PC and healthy controls.[33] Mucins form the outer layer of the epithelial surface of the pancreatic ductal system and play an important role in its protection. Mucins are highly glycosylated and of particular interest in PC due to their aberrant expression and glycosylation in this condition.[53,54] It is thought that mucins serve as cell-surface sensors that interact with components of the microenvironment and cell surface receptors, conducting intracellular signals that enhance tumorigenicity, invasiveness and metastasis. Mucins have shown to be differentially expressed in tissue, cyst fluid and PJ of patients with PC.[53,55-57] MUC2 and MUC5AC for instance is present at low levels in healthy pancreas, and higher levels in different subtypes of IPMN.[58,59] Their diagnostic potential for the detection of early PC has been suggested in serum as well as fine-needle aspiration (FNA) specimens, but not yet in PJ.[55-57,60]

Like mucins, inflammatory mediators such as cytokines and immune cell components can be easily detected by ELISA. PC progression is associated with a distinct inflammatory state and altered cytokine release. Interleukin-8 (IL-8), interferon-γ (IFN-γ) and neutrophil gelatinase-associated lipocalin (NGAL) have been shown to be overexpressed in dysplastic and/or PC tissue,[61-63] and previous results in either serum or PJ demonstrated that increased concentrations of these proteins are able to distinguish PC cases from controls.[41,42,63-65]

Chapter 7 evaluates the diagnostic performance of three inflammatory mediators (IL-8, IFN-γ, and NGAL) and two mucins (MUC5AC, MUC2) in PJ and serum in differentiating sporadic PC (cases) from controls undergoing surveillance for familial/genetic predisposition or neoplastic pancreatic cysts. Alongside this panel, we evaluated serum CA19.9, as a known PC biomarker. We showed that concentrations of CA19.9 and IL-8

(but not NGAL, MUC5AC, MUC2 and IFN-γ) in serum, and IL-8, NGAL, MUC5AC and MUC2 (and not IFN-γ) in PJ were significantly higher in cases than in controls. CA19.9 in serum, and IL-8, NGAL and MUC5AC in PJ were associated with PC, independent of age, gender, BMI and presence of diabetes mellitus. MUC5AC, IL-8 and NGAL in PJ, together with serum CA19.9 constitute a biomarker panel for PC detection that safe to collect, highly specific (96%, as compared to 85% for serum CA19.9 alone) and fairly sensitive (42%, as compared to 70% for CA19.9 alone).

Protein glycosylation

Glycosylation is a post-translational protein modification that is characterized by attachment of a sugar side-chain ('glycan'). Together they form an important cellular mechanism via which protein stability, folding and function is regulated. Glycosylation can be subclassified in *O*-glycosylation (attachment of glycan to the oxygen atom of serine or threonine residues) and *N*-glycosylation (glycan linkage to the nitrogen atom of an asparagine side chain).[66] Such post-translational protein modifications are increasingly being investigated, as in the last decade technical advances in high-throughput protein analysis have enabled robust identification.[67] In PC, a wide range of glycan alternations have been observed in serum.[68-71] Vreeker *et al*.[72] showed that the protein *N*-glycosylation profile (assessed by mass spectrometry) can differentiate sporadic PC cases from controls.

To evaluate the role of *N*-glycosylation analysis in early detection, we evaluated consecutive serum samples from our hereditary predisposed high-risk cohort, and compared samples from those who developed PC with those who did not (**Chapter 8**). Potentially discriminating *N*-glycans were selected based on the cross-sectional analysis by Vreeker *et al*.[72] using a 'biased approach'. Interestingly, the study showed that biomarker differences were already present at baseline and increasingly changed prior to PC development, which indicates a reassuring long window to capture malignant progression. In PC patients, an increase of distinct sialic linkage types (fucosylation, tri- and tetra-antennary structures) and a decrease of complex-type di-antennary and bisected glycans were observed. The largest change over time was seen for tri-antennary fucosylated glycans (A3F); a combination of these glycans was able to differentiate cases from controls with a specificity of 92%, sensitivity of 49% and accuracy of 90%.

Similar studies are required for PJ. Also, future research is needed to assess why A3F is more abundant than other glycans. Investigations may focus on the protein that contributes most to this altered profile (*e.g.*, mucins), or on the enzyme that is involved in this post-translational modification. For instance, MGAT4 and MGAT5 have been reported to enhance branching of *N*-glycans.[73] Additionally, evaluation of these markers in larger prospective studies is warranted to replicate findings, assess heterogeneity between PC cases (*e.g.*, hereditary vs sporadic) and allow correction for confounders.

MicroRNAs

MicroRNAs (miRs) are short non-coding RNAs composed of 18-25 nucleotides that regulate gene expression via mRNA degradation and translatory inhibition. Approximately 2300 miRs have been identified on the human genome,[44] and over 50 have been identified to be differentially expressed in PC tissue.[74-76] miR can be secreted from a cell in its 'free' form or within exosomes. Extracellular vesicles are classified based on size and their biogenesis: exosomes (<150 nm) are released through multivesicular bodies in the endosomal pathway, microvesicles (200-500 nm) are formed by budding from the plasma membrane, and apoptotic bodies (various sizes) derive from programmed cell death. However, many other specialized subtypes have been described.[77] Exosomes – and the miRs they contain – modulate processes that interfere with tumor growth, cancer invasion, metastasis, angiogenesis, immunity and microenvironment.

Chapter 9 evaluates the diagnostic accuracy of a selection of extracellular-vesicle-derived-miRs (EV-miR) extracted from serum and PJ for the differentiation between sporadic PC and controls. We chose to evaluate EV-miR (rather than free miR) as this is expected to reduce the risk of rapid degradation of miRs by enzymes during collection, freezing and storage. Different normalization approaches were considered, namely the use of exosome count, plate average and EV-miR-16, as these may lead to different outcomes.[78-80] However, normalization by plate average was the method of choice, as this reduces the batch effect, and not EV-miR-16, as it has been shown to be differently expressed in PC81. We show that the expression of EV-miR-21, EV-miR-25, EV-miR-210 and EV-miR-16 in PJ, and EV-miR-210 (not EV-miR-155) in serum were significantly higher in cases than controls. A combined panel of PJ EV-miR-21, EV-miR-25, EV-miR-16, and serum EV-miR-210 and CA19.9 distinguished PC cases from controls with a sensitivity of 84% and specificity of 82%.

In **Chapter 10**, we hypothesized that malignant cells shed more extracellular vesicles (EVs) than non-malignant cells. Therefore, we characterized the size and concentration of EVs in PJ and serum of sporadic PC cases and controls. In serum, we did not find differences in EV concentrations between cancer patients and controls, which may implicate that the vast majority of EVs in the bloodstream do originate from other organs.[78] Conversely, whilst overall being larger than in serum, PJ-derived EVs from cases were more often over 350 nm. Perhaps in PC, particular larger EV subtypes, such as apoptotic bodies, are more prevalent, and these may be easier detectable within PJ.

Cell-free DNA mutations

DNA analysis techniques have greatly improved over the last decades. This resulted in numerous studies investigating its potential for diagnostic testing, tumor characterization, personalized treatment and response monitoring.[82,83] Molecular analyses of plasma and cytology is already part of clinical practice. Analysis of PJ may be

even more informative, as it harbors a 50-250x higher cfDNA concentration than blood, as shown in **Chapter 6**. In this thesis, we performed meta-analyses to evaluate the pooled diagnostic performance of DNA alterations in PJ (**Chapter 11**). We compared PJ and plasma as biomaterial for molecular analysis (**Chapter 12**) and evaluated the feasibility of shallow sequencing for the assessment of chromosomal instability in PJ and plasma (**Chapter 13**).

In **Chapter 11**, we identified 32 studies (939 cases, 1678 controls) that evaluated cfDNA mutations in PJ and 14 that investigated methylation patterns (579 cases, 467 controls). This meta-analysis showed that *TP53, SMAD4*, and *CDKN2A* mutations and *NPTX2* hypermethylation provide the highest specificity for differentiation between PC cases and controls, yet with (independently) low sensitivity. Unfortunately, incorporated studies were heterogeneous regarding control groups, PJ collection methods, DNA analysis techniques, choice of kits, sequencing depth, concentration of input DNA or selected thresholds, and applied statistical analyses. Nevertheless, these results may guide the selection of genetic alterations for future longitudinal analysis.

In **Chapter 12**, we hypothesized that the detection rate of DNA mutations is higher in PJ than in plasma, presumably due to a closer contact with the pancreatic ductal system and higher overall cfDNA concentration. Whilst the total cfDNA concentration and the Alu247/Alu115 were higher in PJ, unexpectedly, the detection rate of DNA mutations was found to be similar in PJ and plasma, and the concordance between these biomaterials was low. Changes in sample handling may increase detection, for instance use of cell preservation tubes to reduce genomic DNA in PJ, or use of a different DNA sequencing kit.

Chromosomal alterations (loss of tumor suppressor genes or gain of oncogenes) are common molecular features in human oncogenic signaling and cancer development. Clinical testing for chromosomal alterations in cfDNA is routinely performed during pregnancy by shallow sequencing, known as non-invasive prenatal testing (NIPT). Whilst a NIPT-test aims to diagnose foetal DNA alterations, incidental maternal chromosomal abnormalities have led to the detection of maternal malignancy.[84-87]

Chapter 13 shows that shallow sequencing by the NIPT pipeline of cfDNA is feasible in PJ but not in plasma, as the concentration of plasma circulating tumor DNA (ctDNA) is too low. Subsequently, we compared the presence of chromosomal alterations in PJ between cases (25 PC and 1 HGD) and 19 controls undergoing surveillance for a hereditary predisposition of PC. The most notable finding was the high prevalence of an 8q24 gain (33%) in the case group, as compared to 5% in controls. This alteration was also seen in the one patient with HGD (both PJ and tissue), suggesting that this is a potential early alteration that holds promise for early detection. These results are supported by those from previous analyses in tissue showing a relatively high prevalence of 8q24 gain in both HGD (27%) and PC (24-45%).[29,88,89]

Notably in our experiments, fragment length patterns seemed to be associated with the presence of an amplification or deletion. It is unlikely that shortening of cfDNA fragments was a result of lysis by enzymes, as the fragment length in PJ was longer than in plasma. In **Chapter 12**, we also detected longer cfDNA fragments (higher Alu247/Alu115 ratio) in PJ than in plasma. This may implicate that the cfDNA in PJ may be contaminated with high concentrations of genomic DNA (due to cell decay during collection). Conversely, the higher Alu247/Alu115 ratio in PJ may be due to its close relation with PC, being a measure of cell necrosis rather than apoptosis. Apoptosis namely produces a modal size of ±167 bp, and necrosis produces longer cfDNA fragments.[90-93] If Alu247/Alu115 ratio in PJ is a measure of tumor volume (and therefore response to therapy) should be further investigated. Knowledge of fragment length may also differentiate circulating tumor DNA and non-tumor cfDNA in PJ, and therefore increase sensitivity of tests.[94] Further research is needed to evaluate the implication of these fragment length patterns in PJ and their role in the diagnostic pathway.

Challenges to overcome

In this thesis, we performed multiple studies to identify the most promising biomarkers for PC detection. Together, they reveal new relationships that can be studied in more detail in the future. However, the cross-sectional design of our studies makes it impossible to draw conclusions on causality.

Histology was often unavailable for both patients with PC and controls. Therefore, in the control group, we cannot rule out that an undetected lesion was present at time of sampling. However, the fact that none developed PC during >12 months of follow-up makes this unlikely. Currently, tissue sampling is restricted to selective cases with high PC suspicion. Therefore, the dysplasia grade in pancreatic tissue is generally unknown until resection. This complicates making associations between biomarker values and real-time cancer progression. In **Chapter 8**, we aimed to overcome this problem by consecutive measurements, to detect gradual changes over time in biomarker signature in those individuals who developed PC. Some studies have tried to overcome this by micro-dissection of tissue and comparison of molecular characteristics of different grades of dysplasia in a single tissue sample.[95] However, this does not allow for comparison with the biomarker status. In the future, *in vivo* microscopy (such as fluorescence-guided endoscopy) or the generation of high-definition 3D images of the pancreas by artificial intelligence may allow sequential tissue assessments.

Ideally, to answer the question whether early detection of PC is feasible in a surveillance population using selected biomarkers, only PC cases detected in the surveillance cohorts of interest should have been included (like in **Chapter 6**). However, these cases are rare, and it would take years to reach the appropriate sample size to draw robust conclusions. To overcome this, we set up a prospective cohort study (KRASPanc-trial) to collect biomaterial (blood and PJ) in patients with sporadic PC. In this cohort, only a minority of

PC cases is detected at an early (resectable) stage (±18%, unpublished data), and high-risk individuals with a hereditary predisposition or pancreatic cystic neoplasms may have a distinct natural disease course (with distinct molecular changes) compared to patients with sporadic PC. Thus, extrapolation of the data to a surveillance population should be done with caution. Additionally, the control groups for these studies were heterogeneous (*i.e.*, individuals with either a genetic or familial predisposition or suspected neoplastic cystic neoplasms), which may also complicate extrapolation to respective other groups.

Is PJ ready for prime time?

Our data show that PJ collection from the duodenum is feasible, safe and extracts biomarkers that can distinguish PC from controls with considerable diagnostic performance. However, in the current state, PJ monitoring is not ready for clinical implementation and further studies are needed.

Chapter 7 shows no additional benefit of enzyme inhibitors (*i.e.*, superase and protease inhibitors) on the detection or concentration of relevant markers. However, similar to EDTA and cell preservative available in CellSave tubes, PJ may require similar buffers to stabilize biomarkers for better detection. This requires further investigation.

As mentioned, rather than a single marker, a panel will likely increase the diagnostic performance. However, clear thresholds and handling choices should be made, as an increase of sensitivity may reduce specificity in case of lenient criteria for a 'positive test'. Possible ways to solve this are: giving each mutation a distinct 'weight', combining biomarkers in a single test (*e.g.*, MethylSaferSeqS[96]), or by drawing conclusions based on expert consensus (*e.g.*, in a 'tumor mutational board') taking in account a combination of clinical, imaging, molecular characteristics. Eventually, in case of PC suspicion, molecular analysis of PJ may replace a pancreatic biopsy as it does not require multiple sampling efforts.[97,98] However, the false-positive rate of currently available biomarkers (such as CA19.9) is too high to replace pancreatic biopsy and fulfill these needs.

Longitudinal studies are needed to evaluate if changes over time inform about cancer development in individuals undergoing surveillance, and to determine the diagnostic value in patients with sub-centimeter cancer. Likely, longitudinal assessments are more valuable than snapshot determinations. The current NIPT pipeline in the Erasmus MC is robust and this analysis in PJ has shown its feasibility and performance. For instance, **Chapter 13** can be used as the starting point for a longitudinal analysis in surveillance cohorts.

Furthermore, it is unknown if PJ sampling is cost-effective. Once we have assessed the performance of biomarkers in PJ, methodologists can assist in building a prediction model to establish the cost-effectiveness of surveillance protocols with and without PJ sampling.

What else is out there?

Several alternative approaches for the detection of HGD or early PC have not been discussed in this thesis. One is cytology, which has been extensively investigated by others. In 2019, Tanaka *et al.*[43] published a meta-analysis evaluating 193 papers that described and compared the diagnostic performance of biomarkers from cyst fluid, serum and PJ of patients for the differentiation between non-malignant and malignant IPMN. They showed that cytology in PJ has the highest pooled performance (area under the curve [AUC] 0.84, sensitivity 54% and specificity 91%) to diagnose malignant IPMN, similar to serum CA19.9 (AUC 0.81, sensitivity 45%, and specificity 90%) and cyst fluid cytology (AUC 0.82, sensitivity 57% and specificity 84%). However, these trials collected PJ by direct cannulation and studies are needed to evaluate if PJ obtained with secretin-simulation provides similar outcomes.

Some methods can increase the diagnostic value of circulating tumor cells (and molecular analysis) in PJ; one is single-cell sequencing. Sequencing analysis is generally performed on a mixed sample of millions of cells from different subtypes, resulting in the dilution of genetic material from tumor cells with other cells such as fibroblasts and immune cells. Conversely, single-cell sequencing enables assessment of distinct (single) cells, which may increase the specificity of the test. Additionally, it enables identification of different clones with distinct growth rates, drug sensitivities or interactions with the tumor microenvironment. Thus, this may not only facilitate (early) detection of PC, but also personalized treatment.[99]

Also, enhancing the cellular yield may improve diagnostic performance of PJ. In theory, organoids are an unlimited source of cells for diagnosis (DNA analysis, staining, immunohistochemistry) and treatment response prediction (DNA, *in vitro* sensitivity testing). In **Chapter 7**, we showed that establishment of organoid cultures from PJ is feasible. Gene expression analysis revealed that they were of pancreatic origin and had disease-specific characteristics. Whilst further studies are necessary, these results open up the tantalising possibilities of capturing premalignant lesions and personalising treatment.

Another promising field is the human microbiome. Recent studies have associated dysbiosis of (gut) microbiota with several gastrointestinal cancers. The microbiome is implicated to play a role in carcinogenesis by the production and attraction of various inflammatory cytokines and immune cells that promote tumor development. For PC, Geller *et al.* (2017)[100] showed that 76% of all PC tissue samples are colonized with bacteria, as compared to 15% of normal pancreas controls. Recently, Kartal *et al.* (2022)[101] evaluated the microbiome in saliva, feces, and tumor tissue in 57 treatment naive patients with PC and 50 matched controls. They identified a fecal microbial signature containing 27 species that differentiated PC from controls with an AUC of 0.84. Timely adjustment of the microbiome (*e.g.*, with fecal microbiota transplantation [FMT]) may modulate the

microbiome, change tumor microenvironment, and reduce tumor growth.[102] However, global differences in the microbiome challenge extrapolation of results to other continents.

Cancer is characterized by the ability of aerobic glycolysis, which is thought to be a consequence of reprogrammed mitochondrial metabolism induced by activation of distinct pathways (such as, PI3K/Akt).[103] These metabolites can be measured in different bodily fluids (such as, blood, urine and the exhaled breath), and have shown to enable differentiation between PC cases and controls.[104,105] Analysis of these metabolites in PJ may be valuable and seems feasible.[106]

Conclusion

This thesis describes the current state of pancreassurveillance and critically appraises currently used patient management tools and strategies. The use of biomarkers may enable earlier detection of PC, and, concurrently, select those individuals that are unlikely to benefit from surveillance. PJ is a promising biomarker source, as collection is feasible and safe, and likely contains potent biomarkers. With this thesis, we have made a comprehensive start in this exciting novel field, yet more research is needed in consecutive samples to identify if biomarkers are truly able to detect HGD or early PC. It is our hope that in the coming years biomarker analysis in PJ will proof to be a reliable source for risk stratification and management of patients with a pancreatic cyst, while posing an acceptable burden on both patients and the health care system.

References

1 Oyama H, Tada M, Takagi K, *et al*. Long-term Risk of Malignancy in Branch-Duct Intraductal Papillary Mucinous Neoplasms. *Gastroenterology* 2020; 158: 226-237.e225. 2019/09/02.

2 Reddy RP, Smyrk TC, Zapiach M, *et al*. Pancreatic mucinous cystic neoplasm defined by ovarian stroma: demographics, clinical features, and prevalence of cancer. *Clin Gastroenterol Hepatol* 2004; 2: 1026-1031. 2004/11/20.

3 The European Study Group on Cystic Tumours of the P. European evidence-based guidelines on pancreatic cystic neoplasms. *Gut* 2018; 67: 789.

4 Tanaka M, Fernández-Del Castillo C, Kamisawa T, *et al*. Revisions of international consensus Fukuoka guidelines for the management of IPMN of the pancreas. *Pancreatology* 2017; 17: 738-753. 2017/07/25.

5 Elta GH, Enestvedt BK, Sauer BG, *et al*. ACG Clinical Guideline: Diagnosis and Management of Pancreatic Cysts. *Official journal of the American College of Gastroenterology* | ACG 2018; 113.

6 Vege SS, Ziring B, Jain R, *et al*. American Gastroenterological Association Institute Guideline on the Diagnosis and Management of Asymptomatic Neoplastic Pancreatic Cysts. *Gastroenterology* 2015; 148: 819-822.

7 Lobo JM, Scheiman JM, Zaydfudim VM, *et al*. Clinical and Economic Outcomes of Patients Undergoing Guideline-Directed Management of Pancreatic Cysts. *Am J Gastroenterol* 2020; 115: 1689-1697. 2020/06/20.

8 Chhoda A, Singh S, Sheth AH, *et al*. Benefit of Extended Surveillance of Low-Risk Pancreatic Cysts After 5-Year Stability: A Systematic Review and Meta-Analysis. *Clin Gastroenterol Hepatol* 2023; 21: 1430-1446. 2022/05/15.

9 Marchegiani G, Pollini T, Burelli A, *et al*. Surveillance for Presumed BD-IPMN of the Pancreas: Stability, Size, and Age Identify Targets for Discontinuation. *Gastroenterology*.

10 Overbeek KA, Kamps A, van Riet PA, *et al*. Pancreatic cyst surveillance imposes low psychological burden. *Pancreatology* 2019; 19: 1061-1066. 2019/10/05.

11 Overbeek KA, Levink IJM, Koopmann BDM, *et al*. Long-term yield of pancreatic cancer surveillance in high-risk individuals. *Gut* 2022; 71: 1152-1160. 2021/04/07.

12 Catalano MF, Sahai A, Levy M, *et al*. EUS-based criteria for the diagnosis of chronic pancreatitis: the Rosemont classification. *Gastrointest Endosc* 2009; 69: 1251-1261. 2009/02/27.

13 Cancer risks in BRCA2 mutation carriers. *J Natl Cancer Inst* 1999; 91: 1310-1316. 1999/08/05.

14 Ciprani D, Morales-Oyarvide V, Qadan M, *et al*. An elevated CA 19.9 is associated with invasive cancer and worse survival in IPMN. *Pancreatology* 2020; 20: 729-735.

15 van Manen L, Groen JV, Putter H, *et al*. Elevated CEA and CA19-9 serum levels independently predict advanced pancreatic cancer at diagnosis. *Biomarkers* 2020; 25: 186-193. Article.

16 Jang J-Y, Park T, Lee S, *et al*. Proposed Nomogram Predicting the Individual Risk of Malignancy in the Patients With Branch Duct Type Intraductal Papillary Mucinous Neoplasms of the Pancreas. *Annals of Surgery* 2017; 266.

17 Kim JR, Jang J-Y, Kang MJ, *et al*. Clinical implication of serum carcinoembryonic antigen and carbohydrate antigen 19-9 for the prediction of malignancy in intraductal papillary mucinous neoplasm of pancreas. *Journal of Hepato-Biliary-Pancreatic Sciences* 2015; 22: 699-707.

18 European evidence-based guidelines on pancreatic cystic neoplasms. *Gut* 2018; 67: 789-804. 2018/03/27.

19 Fahrmann JF, Schmidt CM, Mao X, *et al*. Lead-Time Trajectory of CA19-9 as an Anchor Marker for Pancreatic Cancer Early Detection. *Gastroenterology* 2021; 160: 1373-1383. e1376. 2020/12/14.

20 Levink IJM, Jaarsma SC, Koopmann BDM, *et al*. The additive value of CA19.9 monitoring in a pancreatic cyst surveillance program. *United European Gastroenterology Journal*. 2023 Sep;11(7):601-611.

21 Klein AP, Wolpin BM, Risch HA, *et al*. Genome-wide meta-analysis identifies five new susceptibility loci for pancreatic cancer. *Nat Commun* 2018; 9: 556. 2018/02/10.

22 Amundadottir L, Kraft P, Stolzenberg-Solomon RZ, *et al*. Genome-wide association study identifies variants in the ABO locus associated with susceptibility to pancreatic cancer. *Nat Genet* 2009; 41: 986-990. 2009/08/04.

23 Childs EJ, Mocci E, Campa D, *et al*. Common variation at 2p13.3, 3q29, 7p13 and 17q25.1 associated with susceptibility to pancreatic cancer. *Nat Genet* 2015; 47: 911-916. 2015/06/23.

24 Petersen GM, Amundadottir L, Fuchs CS, *et al*. A genome-wide association study identifies pancreatic cancer susceptibility loci on chromosomes 13q22.1, 1q32.1 and 5p15.33. *Nat Genet* 2010; 42: 224-228. 2010/01/27.

25 Wolpin BM, Rizzato C, Kraft P, *et al*. Genome-wide association study identifies multiple susceptibility loci for pancreatic cancer. *Nat Genet* 2014; 46: 994-1000. 2014/08/05.

26 Campa D, Gentiluomo M, Obazee O, *et al*. Genome-wide association study identifies an early onset pancreatic cancer risk locus. *International Journal of Cancer* 2020; 147: 2065-2074.

27 Nodari Y, Gentiluomo M, Mohelnikova-Duchonova B, *et al*. Genetic and non-genetic risk factors for early-onset pancreatic cancer. *Dig Liver Dis* 2023 2023/03/28.

28 Waddell N, Pajic M, Patch AM, *et al*. Whole genomes redefine the mutational landscape of pancreatic cancer. *Nature* 2015; 518: 495-501. 2015/02/27.

29 Witkiewicz AK, McMillan EA, Balaji U, *et al*. Whole-exome sequencing of pancreatic cancer defines genetic diversity and therapeutic targets. *Nature Communications* 2015; 6: 6744.

30 Bailey P, Chang DK, Nones K, *et al*. Genomic analyses identify molecular subtypes of pancreatic cancer. *Nature* 2016; 531: 47-52. 2016/02/26.

31 Collisson EA, Sadanandam A, Olson P, *et al*. Subtypes of pancreatic ductal adenocarcinoma and their differing responses to therapy. *Nat Med* 2011; 17: 500-503. 2011/04/05.

32 Yonemori K, Kurahara H, Maemura K, *et al*. MicroRNA in pancreatic cancer. *Journal of Human Genetics* 2017; 62: 33-40.

33 Doyle CJ, Yancey K, Pitt HA, *et al*. The proteome of normal pancreatic juice. *Pancreas* 2012; 41: 186-194. 2011/12/02.

34 Law HCH, Lagundžin D, Clement EJ, *et al*. The Proteomic Landscape of Pancreatic Ductal Adenocarcinoma Liver Metastases Identifies Molecular Subtypes and Associations with Clinical Response. *Clinical Cancer Research* 2020; 26: 1065-1076.

35 Cao L, Huang C, Cui Zhou D, *et al*. Proteogenomic characterization of pancreatic ductal adenocarcinoma. *Cell* 2021; 184: 5031-5052.e5026.

36 Takano S, Fukasawa M, Kadokura M, *et al*. Mutational Patterns in Pancreatic Juice of Intraductal Papillary Mucinous Neoplasms and Concomitant Pancreatic Cancer. *Pancreas* 2019; 48: 1032-1040. 2019/08/14.

37 Choi MH, Mejlænder-Andersen E, Manueldas S, *et al*. Mutation analysis by deep sequencing of pancreatic juice from patients with pancreatic ductal adenocarcinoma. *BMC Cancer* 2019; 19: 11. 2019/01/07.

38 Mateos RN, Nakagawa H, Hirono S, *et al*. Genomic analysis of pancreatic juice DNA assesses malignant risk of intraductal papillary mucinous neoplasm of pancreas. *Cancer Med* 2019; 8: 4565-4573. 2019/06/22.

39 Wang J, Raimondo M, Guha S, *et al*. Circulating microRNAs in Pancreatic Juice as Candidate Biomarkers of Pancreatic Cancer. *J Cancer* 2014; 5: 696-705. 2014/09/27.

40 Nakamura S, Sadakari Y, Ohtsuka T, *et al*. Pancreatic Juice Exosomal MicroRNAs as Biomarkers for Detection of Pancreatic Ductal Adenocarcinoma. *Ann Surg Oncol* 2019; 26: 2104-2111. 2019/03/02.

41 Kaur S, Baine MJ, Guha S, *et al*. Neutrophil gelatinase-associated lipocalin, macrophage inhibitory cytokine 1, and carbohydrate antigen 19-9 in pancreatic juice: pathobiologic implications in diagnosing benign and malignant disease of the pancreas. *Pancreas* 2013; 42: 494-501.

42 Noh KW, Pungpapong S, Wallace MB, *et al*. Do cytokine concentrations in pancreatic juice predict the presence of pancreatic diseases? *Clin Gastroenterol Hepatol* 2006; 4: 782-789. 2006/05/23.

43 Tanaka M, Heckler M, Liu B, *et al*. Cytologic Analysis of Pancreatic Juice Increases Specificity of Detection of Malignant IPMN - A Systematic Review. *Clin Gastroenterol Hepatol* 2019.

44 Alles J, Fehlmann T, Fischer U, *et al*. An estimate of the total number of true human miRNAs. *Nucleic Acids Res* 2019; 47: 3353-3364. 2019/03/02.

45 Bailey P, Chang DK, Nones K, *et al*. Genomic analyses identify molecular subtypes of pancreatic cancer. *Nature* 2016; 531: 47-52.

46 Lomberk G, Blum Y, Nicolle R, *et al*. Distinct epigenetic landscapes underlie the pathobiology of pancreatic cancer subtypes. *Nature Communications* 2018; 9: 1978.

47 Yamakawa K, Masuda A, Nakagawa T, *et al*. Evaluation of efficacy of pancreatic juice cytology for risk classification according to international consensus guidelines in patients with intraductal papillary mucinous neoplasm; a retrospective study. *Pancreatology* 2019; 19: 424-428.

48 Nicholson JA, Greenhalf W, Jackson R, *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: 260-265. 2014/12/02.

49 Levink IJM, Nesteruk K, Visser DI, *et al*. Optimization of Pancreatic Juice Collection: A First Step Toward Biomarker Discovery and Early Detection of Pancreatic Cancer. *Am J Gastroenterol* 2020; 115: 2103-2108. 2020/10/27.

50 Sadakari Y, Kanda M, Maitani K, *et al*. Mutant KRAS and GNAS DNA Concentrations in Secretin-Stimulated Pancreatic Fluid Collected from the Pancreatic Duct and the Duodenal Lumen. *Clin Transl Gastroenterol* 2014; 5: e62-e62.

51 Suenaga M, Sadakari Y, Almario JA, *et al*. Using an endoscopic distal cap to collect pancreatic fluid from the ampulla (with video). *Gastrointest Endosc* 2017; 86: 1152-1156. e1152. 2017/03/06.

52 Suenaga M, Dudley B, Karloski E, *et al*. The Effect of Pancreatic Juice Collection Time on the Detection of KRAS Mutations. *Pancreas* 2018; 47.

53 Kaur S, Kumar S, Momi N, *et al*. Mucins in pancreatic cancer and its microenvironment. *Nat Rev Gastroenterol Hepatol* 2013; 10: 607-620. 2013/07/17.

54 Hollingsworth MA and Swanson BJ. Mucins in cancer: protection and control of the cell surface. *Nat Rev Cancer* 2004; 4: 45-60. 2003/12/19.

55 Maker AV, Katabi N, Gonen M, *et al*. Pancreatic cyst fluid and serum mucin levels predict dysplasia in intraductal papillary mucinous neoplasms of the pancreas. *Ann Surg Oncol* 2011; 18: 199-206. 2010/08/19.

56 Kaur S, Smith LM, Patel A, *et al*. A Combination of MUC5AC and CA19-9 Improves the Diagnosis of Pancreatic Cancer: A Multicenter Study. *Am J Gastroenterol* 2017; 112: 172-183. 2016/11/16.

57 Zhang J, Wang Y, Zhao T, *et al*. Evaluation of serum MUC5AC in combination with CA19-9 for the diagnosis of pancreatic cancer. *World J Surg Oncol* 2020; 18: 31. 2020/02/08.

58 Klöppel G and Kosmahl M. Is the intraductal papillary mucinous neoplasia of the biliary tract a counterpart of pancreatic papillary mucinous neoplasm? *J Hepatol* 2006; 44: 249-250. 2005/12/20.

59 Balagué C, Audié JP, Porchet N, *et al*. In situ hybridization shows distinct patterns of mucin gene expression in normal, benign, and malignant pancreas tissues. *Gastroenterology* 1995; 109: 953-964. 1995/09/01.

60 Wang Y, Gao J, Li Z, *et al*. Diagnostic value of mucins (MUC1, MUC2 and MUC5AC) expression profile in endoscopic ultrasound-guided fine-needle aspiration specimens of the pancreas. *Int J Cancer* 2007; 121: 2716-2722. 2007/08/22.

61 Kuwada Y, Sasaki T, Morinaka K, *et al*. Potential involvement of IL-8 and its receptors in the invasiveness of pancreatic cancer cells. *Int J Oncol* 2003; 22: 765-771. 2003/03/13.

62 Detjen KM, Farwig K, Welzel M, *et al*. Interferon gamma inhibits growth of human pancreatic carcinoma cells via caspase-1 dependent induction of apoptosis. *Gut* 2001; 49: 251-262.

63 Moniaux N, Chakraborty S, Yalniz M, *et al*. Early diagnosis of pancreatic cancer: neutrophil gelatinase-associated lipocalin as a marker of pancreatic intraepithelial neoplasia. *Br J Cancer* 2008; 98: 1540-1547. 2008/04/09.

64 Shaw VE, Lane B, Jenkinson C, *et al*. Serum cytokine biomarker panels for discriminating pancreatic cancer from benign pancreatic disease. *Mol Cancer* 2014; 13: 114. 2014/06/03.

65 Kaur S, Chakraborty S, Baine MJ, *et al*. Potentials of plasma NGAL and MIC-1 as biomarker(s) in the diagnosis of lethal pancreatic cancer. *PLoS One* 2013; 8: e55171. 2013/02/06.

66 Schjoldager KT, Narimatsu Y, Joshi HJ, *et al*. Global view of human protein glycosylation pathways and functions. *Nature Reviews Molecular Cell Biology* 2020; 21: 729-749.

67 Wang M, Zhu J, Lubman DM, *et al*. Aberrant glycosylation and cancer biomarker discovery: a promising and thorny journey. *Clin Chem Lab Med* 2019; 57: 407-416. 2018/08/24.

68 Yue T, Goldstein IJ, Hollingsworth MA, *et al*. The prevalence and nature of glycan alterations on specific proteins in pancreatic cancer patients revealed using antibody-lectin sandwich arrays. *Mol Cell Proteomics* 2009; 8: 1697-1707. 2009/04/21.

69 Park HM, Hwang MP, Kim YW, *et al*. Mass spectrometry-based N-linked glycomic profiling as a means for tracking pancreatic cancer metastasis. *Carbohydr Res* 2015; 413: 5-11. 2015/06/10.

70 Zhao J, Qiu W, Simeone DM, *et al*. N-linked glycosylation profiling of pancreatic cancer serum using capillary liquid phase separation coupled with mass spectrometric analysis. *J Proteome Res* 2007; 6: 1126-1138. 2007/01/26.

71 Munkley J. The glycosylation landscape of pancreatic cancer. *Oncol Lett* 2019; 17: 2569-2575. 2019/03/12.

72 Vreeker GCM, Hanna-Sawires RG, Mohammed Y, *et al*. Serum N-Glycome analysis reveals pancreatic cancer disease signatures. *Cancer Medicine* 2020; 9: 8519-8529.

73 Yin B, Gao Y, Chung CY, *et al*. Glycoengineering of Chinese hamster ovary cells for enhanced erythropoietin N-glycan branching and sialylation. *Biotechnol Bioeng* 2015; 112: 2343-2351. 2015/07/15.

74 Shams R, Saberi S, Zali M, *et al*. Identification of potential microRNA panels for pancreatic cancer diagnosis using microarray datasets and bioinformatics methods. *Scientific Reports* 2020; 10: 7559.

75 Ma J, Sun S, Song C, *et al*. Screening potential microRNAs associated with pancreatic cancer: Data mining based on RNA sequencing and microarrays. *Exp Ther Med* 2020; 20: 2705-2715. 2020/08/09.

76 Vicentini C, Calore F, Nigita G, *et al*. Exosomal miRNA signatures of pancreatic lesions. *BMC Gastroenterology* 2020; 20: 137.

77 Maas SLN, Breakefield XO and Weaver AM. Extracellular Vesicles: Unique Intercellular Delivery Vehicles. *Trends Cell Biol* 2017; 27: 172-188. 2016/12/17.

78 Severino V, Dumonceau JM, Delhaye M, *et al*. Extracellular Vesicles in Bile as Markers of Malignant Biliary Stenoses. *Gastroenterology* 2017; 153: 495-504.e498. 2017/05/10.

79 Faraldi M, Gomarasca M, Sansoni V, *et al*. Normalization strategies differently affect circulating miRNA profile associated with the training status. *Scientific Reports* 2019; 9: 1584.

80 Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; 25: 402-408.

81 Gao L, He SB and Li DC. Effects of miR-16 plus CA19-9 detections on pancreatic cancer diagnostic performance. *Clin Lab* 2014; 60: 73-77. 2014/03/08.

82 Lee JH, Long GV, Menzies AM, *et al*. Association Between Circulating Tumor DNA and Pseudoprogression in Patients With Metastatic Melanoma Treated With Anti-Programmed Cell Death 1 Antibodies. *JAMA Oncol* 2018; 4: 717-721. 2018/02/10.
83 Anandappa G, Starling N, Peckitt C, *et al*. TRACC: Tracking mutations in cell-free DNA to predict relapse in early colorectal cancer—A randomized study of circulating tumour DNA (ctDNA) guided adjuvant chemotherapy versus standard of care chemotherapy after curative surgery in patients with high risk stage II or stage III colorectal cancer (CRC). *Journal of Clinical Oncology* 2020; 38: TPS4120-TPS4120.
84 Amant F, Verheecke M, Wlodarska I, *et al*. Presymptomatic Identification of Cancers in Pregnant Women During Noninvasive Prenatal Testing. *JAMA Oncol* 2015; 1: 814-819. 2015/09/12.
85 Bianchi DW, Chudova D, Sehnert AJ, *et al*. Noninvasive Prenatal Testing and Incidental Detection of Occult Maternal Malignancies. *Jama* 2015; 314: 162-169. 2015/07/15.
86 Moellgaard MH, Lund ICB, Becher N, *et al*. Incidental finding of maternal malignancy in an unusual non-invasive prenatal test and a review of similar cases. *Clin Case Rep* 2022; 10: e6280. 2022/10/18.
87 Heesterbeek CJ, Aukema SM, Galjaard RH, *et al*. Noninvasive Prenatal Test Results Indicative of Maternal Malignancies: A Nationwide Genetic and Clinical Follow-Up Study. *J Clin Oncol* 2022; 40: 2426-2435. 2022/04/09.
88 Hata T, Suenaga M, Marchionni L, *et al*. Genome-Wide Somatic Copy Number Alterations and Mutations in High-Grade Pancreatic Intraepithelial Neoplasia. *Am J Pathol* 2018; 188: 1723-1733. 2018/04/24.
89 Schleger C, Arens N, Zentgraf H, *et al*. Identification of frequent chromosomal aberrations in ductal adenocarcinoma of the pancreas by comparative genomic hybridization (CGH). *J Pathol* 2000; 191: 27-32. 2000/04/18.
90 Salem R, Ahmed R, Shaheen K, *et al*. DNA integrity index as a potential molecular biomarker in colorectal cancer. *Egyptian Journal of Medical Human Genetics* 2020; 21: 38.
91 Shen Q, Cen H, Jiang J, *et al*. The level and integrity of plasma circulating cell-free DNA in patients with primary multiple myeloma. *Translational Cancer Research* 2022; 11: 4137-4147.
92 Zhu F, Ma J, Ru D, *et al*. Plasma DNA Integrity as a Prognostic Biomarker for Colorectal Cancer Chemotherapy. *Journal of Oncology* 2021; 2021: 5569783.
93 Yu D, An G and Xu L. Investigation of Efficacy Evaluation Comparison of cfDNA and CEA in Colorectal Cancer. *Clin Lab* 2016; 62: 1947-1953. 2017/02/07.
94 Underhill HR, Kitzman JO, Hellwig S, *et al*. Fragment Length of Circulating Tumor DNA. *PLOS Genetics* 2016; 12: e1006162.
95 Noë M, Niknafs N, Fischer CG, *et al*. Genomic characterization of malignant progression in neoplastic pancreatic cysts. *Nature Communications* 2020; 11: 4085.
96 Wang Y, Douville C, Cohen JD, *et al*. Detection of rare mutations, copy number alterations, and methylation in the same template DNA molecules. *Proceedings of the National Academy of Sciences* 2023; 120: e2220704120.

97 Mencel J, Feber A, Begum R, *et al*. Liquid biopsy for diagnosis in patients with suspected pancreatic and biliary tract cancers: PREVAIL ctDNA pilot trial. *Journal of Clinical Oncology* 2022; 40: 522-522.
98 Bunduc S, Gede N, Váncsa S, *et al*. Prognostic role of cell-free DNA biomarkers in pancreatic adenocarcinoma: A systematic review and meta-analysis. *Crit Rev Oncol Hematol* 2022; 169: 103548. 2021/11/30.
99 Xu J, Liao K, Yang X, *et al*. Using single-cell sequencing technology to detect circulating tumor cells in solid tumors. *Mol Cancer* 2021; 20: 104. 2021/08/21. 100 Geller LT, Barzily-Rokni M, Danino T, *et al*. Potential role of intratumor bacteria in mediating tumor resistance to the chemotherapeutic drug gemcitabine. *Science* 2017; 357: 1156-1160. 2017/09/16.
101 Kartal E, Schmidt TSB, Molina-Montes E, *et al*. A faecal microbiota signature with high specificity for pancreatic cancer. *Gut* 2022; 71: 1359-1372. 2022/03/10.
102 Riquelme E, Zhang Y, Zhang L, *et al*. Tumor Microbiome Diversity and Composition Influence Pancreatic Cancer Outcomes. *Cell* 2019; 178: 795-806.e712. 2019/08/10.
103 Ward PS and Thompson CB. Metabolic reprogramming: a cancer hallmark even warburg did not anticipate. *Cancer Cell* 2012; 21: 297-308. 2012/03/24.
104 Mayerle J, Kalthoff H, Reszka R, *et al*. Metabolic biomarker signature to differentiate pancreatic ductal adenocarcinoma from chronic pancreatitis. *Gut* 2018; 67: 128-137. 2017/01/22.
105 Markar SR, Brodie B, Chin ST, *et al*. Profile of exhaled-breath volatile organic compounds to diagnose pancreatic cancer. *Br J Surg* 2018; 105: 1493-1500. 2018/07/19.
106 Cortese N, Capretti G, Barbagallo M, *et al*. Metabolome of Pancreatic Juice Delineates Distinct Clinical Profiles of Pancreatic Cancer and Reveals a Link between Glucose Metabolism and PD-1(+) Cells. *Cancer Immunol Res* 2020; 8: 493-505. 2020/02/06.

CHAPTER 15

Nederlandse samenvatting en discussie

Rationale voor dit proefschrift

Per jaar worden in Nederland ongeveer 3000 patiënten gediagnosticeerd met alvleesklierkanker (pancreas carcinoom). Ondanks medische ontwikkelingen is de kans op overleving bij deze ziekte nog steeds klein. Dit komt door het lang uitblijven van symptomen en een tekort aan effectieve therapieën. Op dit moment is alvleesklierkanker alleen te genezen wanneer de diagnose in een vroeg stadium wordt gesteld. Volledige chirurgische verwijdering is namelijk alleen mogelijk als de tumor klein is, beperkt is doorgegroeid in omliggende weefsels of bloedvaten, en niet verspreid is naar andere organen.

Alvleeskliersurveillance heeft als doel alvleesklierkanker in een vroeg en operabel stadium te ontdekken, om zo de kwaliteit van leven en overleving van patiënten met deze ziekte te verbeteren. Op dit moment wordt dit soort surveillance uitgevoerd bij twee verschillende risicogroepen; personen met een erfelijke belasting, bijvoorbeeld door een aangeboren genetische afwijking, en personen met een alvleeskliercyste.

Dit proefschrift focust zich hoofdzakelijk op het verbeteren van de vroegdiagnostiek voor de tweede groep. Van de volwassenen tussen de 50 en 80 jaar heeft bijna 50% een (vaak kleine) alvleeskliercyste, en het aantal gevonden cystes stijgt door toenemende precisie van beeldvormende technieken. Deze cysten worden hoofzakelijk bij toeval ontdekt op beeldvormend onderzoek uitgevoerd om andere redenen. Een subgroep van deze cysten, intraductale papillaire mucineuze neoplasieën (IPMNs), heeft ongeveer 3% kans op het ontwikkelen tot alvleesklierkanker.[1] Om deze reden adviseren richtlijnen jaarlijkse surveillance middels inwendige echoscopie of MRI-scan ('MRCP'), zolang een persoon fit genoeg is voor een operatie. Hierbij wordt gekeken of er zorgelijke kenmerken (ook wel 'worrisome features' of 'indications for surgery' genoemd) aanwezig zijn. Afhankelijk van het bestaan van deze kenmerken en/of symptomen kan surveillance vaker plaatsvinden of gekozen worden voor (gedeeltelijke) chirurgische verwijdering van de alvleesklier.

Tot op heden voldoen deze surveillanceprogramma's niet aan de verwachtingen, omdat: 1. Na chirurgie regelmatig geen sprake blijkt te zijn van kwaadaardigheid. Dit is extra kwalijk omdat het grote operaties betreft, die gepaard kunnen gaan met ernstige complicaties, langdurige ziekenhuisopnames en zelfs overlijden; 2. Personen desondanks soms toch te laat worden gediagnosticeerd, terwijl bij voorgaande onderzoeken geen zorgelijke kenmerken gezien werden; 3. De vele onderzoeken belastend zijn voor de patiënt en het zorgstelsel.

Er zijn dus betere diagnostische testen nodig die alvleesklierkanker vroeger kunnen diagnosticeren. We weten dat kankercellen constant stoffen uitscheiden naar omliggende weefsels. Deze moleculen bevatten informatie over de ziekte en noemen we 'biomarkers'. Wij denken dat het meten van dit soort markers in bloed of alvleeskliersap kan leiden tot een vroegere diagnose van alvleesklierkanker, of, liever nog, van een voorstadium hiervan

dat 'hooggradige dysplasie' wordt genoemd. Op basis van deze biomarkers zou er meer maatwerk geleverd kunnen worden ten aanzien van zowel vorm, frequentie en duur van surveillance, als ook de timing van eventuele operatie.

In dit proefschrift toetsen we de huidige Europese richtlijn[2] voor alvleeskliersurveillance bij personen met een alvleeskliercyste (**DEEL I**) en evalueren we of biomarkers in bloed en alvleeskliersap zouden kunnen bijdragen aan het vroeger ontdekken van alvleesklierkanker bij personen die surveillance ondergaan (**DEEL II**).

Surveillance van alvleeskliercysten, de huidige klinische praktijk

Alvleeskliercyste richtlijnen

De vier belangrijkste richtlijnen over alvleeskliercysten zijn een Europese, een 'internationale' en twee Amerikaanse richtlijnen. In **hoofdstuk 3** beschrijven we hoe deze richtlijnen overeenkomen wat betreft hun adviezen ten aanzien van diagnostiek en beleid. Terwijl al deze richtlijnen hun beleid laten afhangen van de aanwezigheid van zorgelijke kenmerken, zijn er duidelijke verschillen in naamgeving van deze kenmerken, hun consequenties, de rol van biomarkers, en de frequentie van surveillance.[2-5]

Deze richtlijnen zijn gebaseerd op studies die geopereerde patiënten betroffen. Deze subgroep met een hoog-risico cyste (anders was niet voor operatie gekozen) is niet representatief voor de groep cyste-dragers als geheel. Vanwege dit hiaat in wetenschappelijke kennis, geven de richtlijnen geen harde adviezen, maar slechts aanbevelingen die gebaseerd zijn op overeenstemming tussen experts. Prospectief onderzoek naar het nut en de juiste vorm van cystesurveillance is dan ook hoognodig.

Het PACYFIC-registratiesysteem

In 2015 is daarom een internationaal registratiesysteem opgezet: de PACYFIC-registry (www.pacyfic.net). Dit betreft een samenwerking tussen meer dan 40 Europese en Noord-Amerikaanse ziekenhuizen. In dit registratiesysteem worden personen geïncludeerd met een cyste die volgens hun behandelend arts in aanmerking komt voor controle. Op basis van prospectief ingevoerde gegevens kan niet alleen het nut van cystesurveillance worden getoetst, maar ook een unieke inkijk worden verkregen in hoe de surveillance wordt uitgevoerd in de dagelijkse praktijk.

Cyste grootte en stabiliteit als geruststellende kenmerken

Slechts een klein percentage (±3%) van de alvleeskliercystes ontwikkelt zich tot kanker.[1] Om de belasting van surveillance op de patient en het zorgsysteem te beperken, is het van belang om niet alleen verontrustende kenmerken te identificeren, maar juist ook

geruststellende. Op basis hiervan kan mogelijk de intensiteit, frequentie en duur van surveillance worden verlaagd. Potentieel geruststellende kenmerken zijn een klein formaat en stabiliteit over tijd. In **hoofdstuk 4** laten we zien dat mensen met kleinere cysten (<20mm), die niet of langzaam groeien (<5mm/jaar) en geen kwaadaardige kenmerken hebben, inderdaad een lager risico hebben op het ontwikkelen van alvleesklierkanker in de twee daaropvolgende jaren. Dit impliceert dat stabiele, kleine cysten mogelijk minder vaak gecontroleerd hoeven worden. Toekomstig onderzoek is nodig om te bepalen of deze gepersonaliseerde aanpak ook op de lange termijn veilig en kosteneffectief is, en of surveillance voor deze groep misschien zelfs helemaal gestaakt kan worden.

De rol van CA19.9 concentratie in bloed bij alvleeskliercystesurveillance

CA19.9 is een tumor marker in het bloed die gebruikt wordt om het ziektebeloop van personen met alvleesklierkanker te voorspellen. De huidige cysterichtlijnen adviseren een operatie bij een verhoogde CA19.9 waarde (>37 kU/L) in combinatie met aanwezigheid van een ander zorgelijk kenmerk. Echter, CA19.9 kan ook verhoogd zijn bij andere aandoeningen.

In **hoofdstuk 5** beschrijven we 685 personen die cystesurveillance ondergaan, en laten zien dat een verhoogd CA19.9 (>37 kU/L) het risico op het ontwikkelen van hooggradige dysplasie en kanker binnen twee jaar overschat. In deze groep personen resulteerde het gebruik van deze marker in onnodige extra controles en zelfs chirurgie. Bij het verhogen van de drempelwaarde naar 133 kU/L zou het aantal foutieve diagnoses afnemen. Dit betekent dat CA19.9 monitoring met de huidige drempelwaarde geen toegevoegde waarde heeft bij cystesurveillance. Kritische beschouwing van de indicatie en drempelwaarden voor CA19.9 is nodig voorafgaand aan implementatie in toekomstige richtlijnen.

Uitdagingen alvleeskliercystesurveillancestudies

Het duurt tientallen jaren voordat er harde conclusies kunnen worden getrokken op basis van surveillance studies. Een andere uitdaging van de PACYFIC-studie is dat deelnemende artsen zelf bepalen hoe ze de surveillance uitvoeren. Dit kan leiden tot verschillen in aanpak, waardoor het samenvoegen van data voorzichtigheid vraagt. Ook kunnen verschillende beeldvormende technieken de grootte van een cyste net anders inschatten. Daarnaast is het niet altijd mogelijk de cyste te karakteriseren, waardoor een aantal deelnemers misschien geen IPMN hebben, maar een cyste die eigenlijk geen surveillance behoeft, zoals een pseudocyste of sereus cysteadenoom. Tot slot zijn niet alle variabelen altijd verzameld. CA19.9 wordt bijvoorbeeld pas structureel bepaald sinds het bestempeld is als relatieve indicator voor chirurgie.[1]

Biomarkers en de rol van alvleeskliersap als bron

Bronnen voor biomarkers in de huidige klinische praktijk

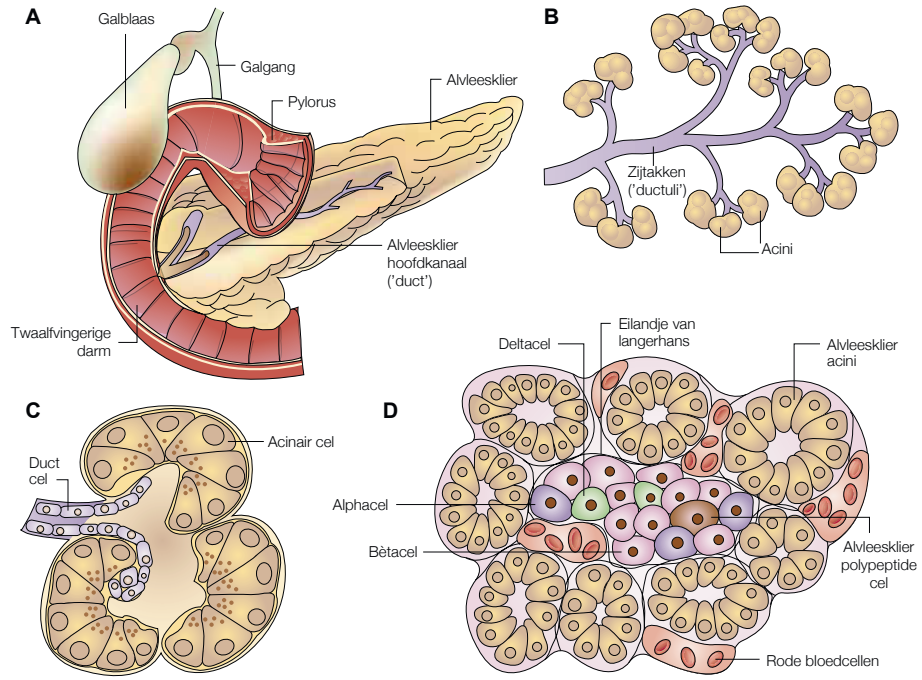
Een biomarker is een meetbare stof in bloed of een andere lichaamsvloeistof die iets zegt over de aanwezigheid van een ziekte of conditie (elders) in het lichaam. De gedachte is dat cellen een reeks (genetische) veranderingen ondergaan tijdens hun ontwikkeling tot kanker en informatie hierover (in de vorm van bijvoorbeeld eiwitten, RNA en DNA) afgeven aan het omgevend weefsel en vloeistoffen. Door deze informatie te meten kan de aanwezigheid van kanker of voorstadia daarvan worden voorspeld.

Het huidige diagnostisch traject van alvleesklierkanker en cysten maakt al gebruik van biomarkers, onder andere glucose, HbA1c, en CA19.9 in bloed, en CEA, amylase en DNA-mutaties in cystevloeistof. Bloed is relatief makkelijk te verkrijgen via venapunctie, maar bepalingen hierin blijken weinig specifiek. Cystevloeistof kan met een naald worden opgezogen middels een punctie gedurende een inwendige endoscopie. Dit materiaal is specifieker voor de alvleesklier, maar de te puncteren afwijking dient dan wel goed zichtbaar te zijn en bijvoorbeeld minstens 5mm groot. Ook riskeert men hierbij complicaties zoals een bloeding en acute pancreatitis. Dit zijn complicaties die in ongeveer 0.3% van de procedures voorkomen.[6]

Een alternatieve diagnostische test voor een surveillance populatie moet veilig, goedkoop, en makkelijk te bepalen zijn. Ook moet de specificiteit hoog zijn, zodat gebruik niet leidt tot fout-positieve resultaten en onnodige schade door meer onderzoeken of interventies (zoals chirurgie). Aan de andere kant kunnen fout-negatieve waarden leiden tot gemiste diagnoses.

Alvleeskliersap en methode van collectie

Alvleeskliersap is een veelbelovende bron van biomarkers, aangezien het in nauw contact staat met de ductale cellen waaruit alvleesklierkanker ontstaat (**Figuur 1**). De afgifte van sap vanuit de alvleesklier aan de dunne darm wordt gestimuleerd door de aanwezigheid van voedsel in de maag en dunne darm. Alvleeskliersap bevat spijsverteringsappen (‘enzymen’) die nodig zijn om voedsel te verteren. Tijdens een inwendige echoscopie kan de afgifte van dit sap worden gestimuleerd door infusie van het hormoon secretine. Enkele seconden hierna komt het sap vrij in de twaalfvingerige darm. Hier kan het opgezogen worden (**Figuur 2**). Een uitdaging hierbij is dat het sap zo snel mogelijk moet worden ingevroren, aangezien het enzymen bevat die de inhoud kunnen afbreken. Daarnaast mag de papil nooit worden afgesloten, want dat kan in sommige gevallen resulteren in een alvleesklierontsteking. Alvleeskliersap zelf is transparant en kleurloos, tijdens het collecteren (en analyseren) moet rekening worden gehouden door de mogelijkheid van bijmenging van gal, sappen vanuit de maag of twaalfvingerige darm, en bloed vanuit de wand van de twaalfvingerige darm dat vrijkomt uit minibloedinkjes door zuigdefecten.

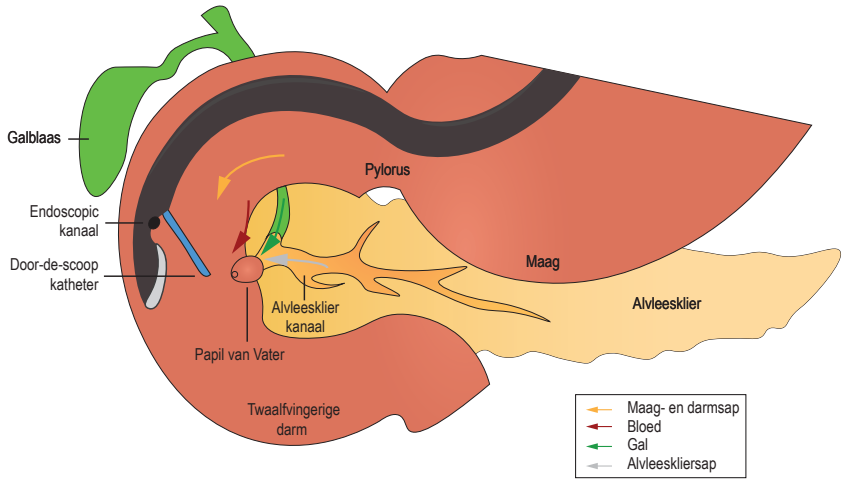


FIGUUR 1 | Grafische weergave van de anatomie van de alvleesklier A. De alvleesklier is gelegen achter de maag en bestaat uit parenchym (‘orgaanweefsel’) met daar tussendoor kleine kanaaltjes (‘ductuli’). Zijtakjes monden uit in het alvleesklier hoofdkanaal, dat op zijn beurt weer uitmondt in de twaalfvingerige darm, samen met de galgang. **B, C.** Het overgrote deel van het klierweefsel bestaat uit exocriene cellen, die clusters vormen (‘acini’). Acini produceren spijsverteringsenzymen en scheiden deze af in het kanaalsysteem. **D.** Tussen de acini bevinden zich bloedvaten en de eilandjes van Langerhans, bestaande uit alfa-, beta- en deltacellen. Deze cellen produceren hormonen, zoals insuline en glucagon, die onder andere de bloedsuikerspiegel reguleren. Deze figuur is met goedkeuring verkregen en vertaald uit een artikel gepubliceerd in Nature in 2002 geschreven door Bardeesy en collega’s.[7]

Secretine was tot op heden beperkt beschikbaar, waardoor deze niet invasieve vorm van sapcollectie wereldwijd weinig werd uitgevoerd. De optimale collectiemethode was dan ook nog onduidelijk. In **hoofdstuk 6** hebben wij twee verschillende manieren van alvleeskliersapcollectie getoetst; opzuigen via de tip van de endoscoop zelf of middels een door het endoscopiekanaal opgevoerde katheter. Wij speculeerden dat collectie met behulp van een katheter zou zorgen voor minder bijmenging van vocht uit de twaalfvingerige darm, en daardoor mogelijk hogere concentraties van biomarkers zou kunnen bevatten. Onze resultaten lieten echter zien dat zonder de katheter een groter volume kon worden verzameld, maar ook dat de concentratie van het alvleesklier specifieke eiwit PLA2G1B niet lager was. Hieruit concludeerden wij dat dit sap niet méér vervuild is met vocht uit de twaalfvingerige darm. Meer onderzoek is nodig om

te evalueren of het sap gecollecteerd met de katheter mogelijk meer 'steriel' is (en we daarom iets succesvoller waren bij het groeien van celkweken ['organoids'] uit dit materiaal).

Ook keken wij naar de optimale timing en duur van sap collectie. Hierbij bleek dat het sap gecollecteerd gedurende 4-8 minuten na secretine infusie meer pancreas-specifieke moleculen bevatte (op basis van PLA2G1B concentratie) dan sap verzameld gedurende 0-4 minuten of 8-15 minuten. De bijmenging van bloed (op basis van IgG concentratie) nam toe naarmate er langer verzameld werd. Bovendien was de opbrengst van biomarkers (geteste microRNAs en eiwitten) lager in sap verzameld tussen 8-15 minuten. Kortom, er bleek geen voordeel van het gebruik van een katheter, noch van een collectieduur langer dan 8 minuten.

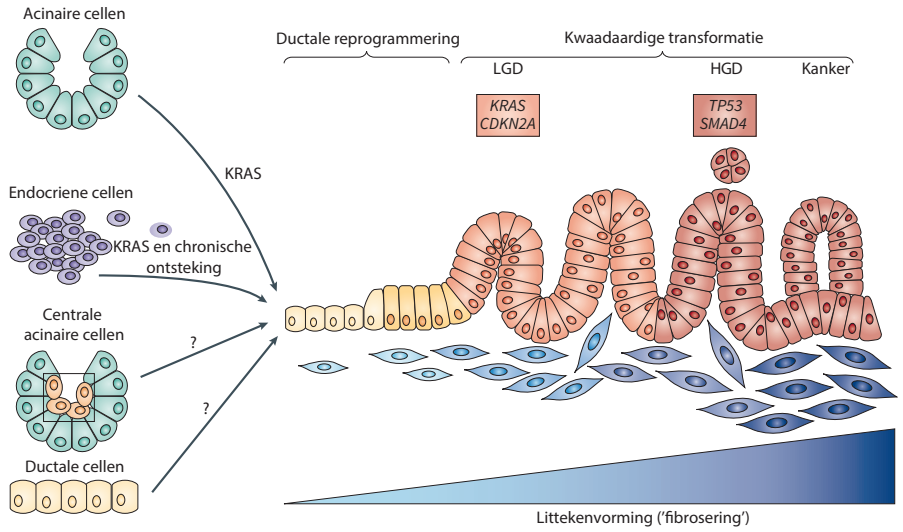


FIGUUR 2 | **Grafische weergave van een alvleeskliersapcollectie.** Bij een collectie met behulp van het endoscopiekanaal wordt de scoop (zwart) direct voor de papil geplaatst en het sap opgezogen. Bij collectie met een katheter wordt deze opgevoerd door het endoscopiekanaal, de kathetertip (blauw) voor de papil geplaatst en sap opgezogen via deze katheter. Dit figuur is overgenomen en vertaald uit **hoofdstuk 6** van dit proefschrift.[8]

De identificatie van biomarkers in alvleeskliersap

Het percentage personen dat alvleesklierkanker ontwikkelt binnen de surveillance-populaties is zeer laag. Het duurt dan ook jaren voordat genoeg kankergevallen zijn ontstaan om het beloop te analyseren. Om deze reden hebben we eerst een studie opgezet bij patiënten met alvleesklierkanker die geen surveillance ondergingen (KRASPanc-studie). Voor deze studie werden personen benaderd die verdacht werden van het hebben van alvleesklierkanker en een inwendige echoscopie zouden ondergaan. Van deze personen werd alvleeskliersap en bloed verzameld, waarin we vervolgens op

zoek gingen naar kanker biomarkers. De resultaten van de subgroep die daadwerkelijk kanker bleek te hebben werden in **hoofdstukken 7, 9, 10 en 13** vergeleken met die van de personen die surveillance ondergingen vanwege een cyste (PACYFIC-studie) of een genetisch (dan wel familiair) verhoogd risico op alvleesklierkanker (FPC-studie). Bij deze personen wordt sinds respectievelijk 2015 en 2012 al bloed verzameld en in 2018 werd gestart met alvleeskliersapcollecties. In dit proefschrift werden de meeste analyses in bloed en alvleeskliersap uitgevoerd, zodat we naast de waarde van de biomarkers ook die van het biomateriaal konden testen.



FIGUUR 3 | **Grafische weergave van de ontwikkeling tot alvleesklierkanker.** Alvleeskliercellen kunnen door een aanpassing ('mutatie') in een gen gereprogrammeerd worden ('metaplasie'). Dit kan leiden tot veranderingen in het gedrag van de cel en ontremde celdeling. Normaal weefsel verandert hierbij gradueel in laaggradige dysplasie (LGD), hooggradige dysplasie (HGD) en kanker. Ook neemt hierdoor het aantal litteken-veroorzakende cellen ('fibroblasten') in de alvleesklier toe ('fibrosering'). Dit figuur representeert een voorbeeld van kwaadaardige transformatie, maar dit verloopt niet bij iedere persoon hetzelfde. Ook zijn er verschillen tussen patiënten die alvleesklierkanker ontwikkelen uit intraductale papillaire mucineuze neoplasie (IPMN), mucineus cysteuze neoplasie (MCN) en intra-epitheliale neoplasie (PanIn). *KRAS*, *CDKN2A*, *TP53* en *SMAD4* zijn voorbeelden van genen die gemuteerd kunnen zijn tijdens de kwaadaardige transformatie. Dit figuur is met goedkeuring verkregen en vertaald uit een artikel gepubliceerd in Nature in 2010 geschreven door Moris en collega's.[9]

Eiwitten in alvleeskliersap en bloedserum

Alvleeskliersap is rijk aan eiwitten. De aanwezigheid of concentratie van specifieke eiwitten zouden informatie kunnen geven over processen in de alvleesklier. Twee groepen eiwitten trokken onze aandacht: mucines en cytokines. Mucines spelen een belangrijke

rol in het beschermen van de oppervlakte cellen van het ductale systeem. Het is bekend dat er al vroeg in het transformatieproces naar dysplasie en kanker andere concentraties van mucines worden uitgescheiden.[10] Cytokines zijn signaaleiwitten die een belangrijke rol spelen in de regulatie van het immuunsysteem, het primaire verdedigingsmechanisme van het lichaam tegen infecties, maar ook tegen de vorming van kanker (**Figuur 3**). Daarnaast kunnen verschillende cytokines dienen als graadmeter voor de mate van littekenvorming in de alvleesklier.

In **hoofdstuk 7** hebben wij het onderscheidend vermogen van vijf eiwitten (drie eiwitten betrokken bij het immuunsysteem: IL-8, IFN-γ en NGAL; en twee mucines: MUC5AC en MUC2) getoetst in alvleeskliersap en bloed van 59 patiënten met alvleesklierkanker en 126 controles die surveillance ondergaan. CA19.9 waarden werden daarnaast geëxtraheerd uit het patiëntendossier. Deze studie liet zien dat de concentraties van CA19.9 en IL-8 in bloed, alsmede IL-8, NGAL, MUC5AC en MUC2 in alvleeskliersap, hoger waren bij alvleesklierkankerpatiënten dan bij controles. Echter bleken alleen CA19.9 in bloed en IL-8, NGAL en MUC5AC in alvleeskliersap onafhankelijk geassocieerd te zijn met de aanwezigheid van kanker na correctie voor leeftijd, geslacht en het bestaan van suikerziekte. Een combinatie van deze vier biomarkers kon het bestaan van alvleesklierkanker onderscheiden met een specificiteit van 96%, hetgeen hoger is dan de specificiteit van CA19.9 alleen (85%). Echter ging dit wel ten koste van de sensitiviteit (van 70% naar 42%). Vergelijkbaar met **hoofdstuk 5**, liet **hoofdstuk 7** ook zien dat het verhogen van de CA19.9 drempelwaarde de specificiteit van de test zou verbeteren. Dit is met name voordelig, omdat een hogere specificiteit mogelijk het risico op schade door (onnodige) intensivering van surveillance en chirurgie vermindert.

Serum eiwit N-glycosylering als potentiële biomarker.

Hoofdstuk 8 verschilt in opzet met de andere studies in **DEEL II**. Terwijl we in de meeste hoofdstukken personen met alvleesklierkanker op één tijdspunt vergelijken met controles, beschrijft dit hoofdstuk de expressie van een biomarker (*N*-glycome) in de tijd. Glycanen zijn suikerketens die gebonden kunnen worden aan verschillende eiwitten. Ze kunnen hiermee de structuur en functie van een eiwit wijzigen. Een team uit Leiden (Vreeker en collega's[11]) had al laten zien dat aanwezigheid van glycanen in de bloedbaan ons mogelijk in staat zou stellen om patiënten met alvleesklierkanker van gezonde controles te onderscheiden.

Wij onderzochten derhalve of glycanen de ontwikkeling van alvleesklierkanker ook vroegtijdig kan voorspellen. Hierbij werkten wij samen met teams uit Leiden en Utrecht, en gebruikten een eerder beschreven platform[5] om de expressie van glycanen te meten in 165 personen uit ons erfelijk belast surveillance cohort. Wij toonden aan dat de expressie van bepaalde glycanen gradueel toeneemt in de maanden tot jaren voordat alvleesklierkanker zich openbaart. Het grootste verschil werd gezien bij een bepaalde groep glycanen: A3F. Deze kon het bestaan van alvleesklierkanker voorspellen met

een accuratesse van 90%. Een limitatie van deze studie is dat slechts tien personen alvleesklierkanker ontwikkelden. Hierdoor hebben we de invloed van andere factoren (zoals leeftijd, geslacht, het bestaan van een bepaalde genetische kiembaan mutatie of suikerziekte) niet kunnen testen. Het trekken van robuuste conclusies was dan ook niet mogelijk en vervolgonderzoek is noodzakelijk.

MicroRNAs en extracellulaire vesikel s in alvleeskliersap en bloedserum

Een andere potentiële groep biomarkers zijn microRNAs. Dit zijn korte RNA-moleculen die bestaan uit 18-25 nucleotiden. Meer dan 2300 verschillende micro-RNAs zijn geïdentificeerd, waarvan ongeveer 50 verschillend tot expressie komen in alvleesklierkanker in vergelijking met gezonde controles.[12-14] MicroRNAs komen vaak verpakt in blaasjes ('extracellulaire vesikels') voor in verschillende lichaamsvloeistoffen. Extracellulaire vesikels zijn onder te verdelen in verschillende soorten met specifieke formaten, voorbeelden zijn: exosomen (die een formaat hebben van <150nm), microvesikels (200-500nm) en apoptose lichamen ontstaan door celdood (deze kunnen verschillende formaten aannemen). MicroRNAs en extracellulaire vesikels ('EV-miRs') zijn samen in staat om tumorgroei en de vorming van uitzaaiingen te moduleren.

In **hoofdstuk 9** hebben wij vijf, op basis van literatuur veelbelovende, microRNAs geselecteerd en onderzocht in alvleeskliersap en bloedserum. De studie liet zien dat een combinatie van EV-miR-21, EV-miR-25, en EV-miR-16 in alvleeskliersap en EV-miR-210 in serum in staat was om alvleesklierkanker van controles te onderscheiden met een sensitiviteit van 84% en een specificiteit van 82%. Deze betrouwbaarheid was iets beter dan dat van CA19.9 alleen (sensitiviteit 86% en specificiteit 73%). Ook was het opvallend dat de biomarkers, gemeten in alvleeskliersap, beter presteerden dan wanneer deze gemeten werden in serum.

Ook verwachtten wij dat de concentratie en het soort extracellulaire vesikels in serum en bloed mogelijk verschillend zouden zijn. **Hoofdstuk 10** laat zien dat bloedserum een hogere concentratie extracellulaire vesikels heeft dan alvleeskliersap, maar er waren geen concentratieverschillen tussen patiënten met alvleesklierkanker en controles. Wel bleken bij patiënten met kanker de vesikels in sap vaker groter (>350nm) te zijn dan bij controles. Het zou kunnen dat bepaalde extracellulaire vesikels meer voorkomen in sap van patiënten met alvleesklierkanker (zoals apoptose lichamen), maar deze hypothese hebben we nog niet kunnen toetsen.

Celvrij DNA in alvleeskliersap

Bij celdood komt DNA vrij in het omgevende weefsel. Deze informatie kan gemeten worden in verschillende lichaamssappen. De concentratie en aanwezigheid van DNA in alvleeskliersap is in het verleden verschillende keren gemeten in losse (kleinere) patiëntenpopulaties. Wij hebben in **hoofdstuk 11** een literatuurstudie uitgevoerd,

waarbij we aanwezige studies over DNA-bepaling in alvleeskliersap gezamenlijk hebben geanalyseerd. Hierbij keken we naar DNA-wijzigingen ('mutaties'), moleculen ('methylgroepen') die aan het DNA kunnen binden en de functie wijzigen ('methylatie'), en de stabiliteit van het DNA ('chromosomale instabiliteit').

Wij includeerden 32 studies uit bestaande literatuur met samen 939 patiënten en 1678 controles, waarbij mutaties in celvrij DNA waren getest in alvleeskliersap. Wij zagen dat mutaties in de genen *TP53, SMAD4* en *CDKN2A* patiënten met alvleesklierkanker het beste onderscheidde van controles met een (gezamenlijke) sensitiviteit van 13-42% en specificiteit van 97-100%. Daarnaast vonden we 14 studies die de betrouwbaarheid van DNA-methylatie onderzochten in 579 patiënten en 467 controles. Hieruit bleek dat een verhoogd methylatiepatroon van *NPTX2* ('hypermethylatie') het meest voorspellend was voor het bestaan van alvleesklierkanker, met een sensitiviteit van 39-70% en specificiteit van 94-100%.

Er voldeden geen studies naar chromosomale instabiliteit aan onze inclusiecriteria. Opvallend was dat de specificiteit van deze DNA-afwijkingen voor het herkennen van alvleesklierkanker erg hoog was. De kans is dan ook klein dat een positieve test schade veroorzaakt door overdiagnostiek of overbehandeling.

Celvrij DNA in alvleeskliersap in vergelijking met bloedplasma

Superioriteit van DNA-analyse in alvleeskliersap over bloedplasma is noodzakelijk alvorens er, in de klinische praktijk, gekozen zal worden voor alvleeskliersap als bron voor biomarkers. In **hoofdstuk 6** zagen wij dat de concentratie van celvrij DNA in alvleeskliersap ongeveer 50-250x hoger is dan in bloedplasma. Aangezien sap meer in contact staat met de alvleesklier dan bloed, verwachtten wij dat DNA hierin de aanwezigheid van kanker beter voorspelt.

In **hoofdstuk 12** bevestigden we niet alleen de hogere concentratie van DNA in sap dan in bloedplasma, maar we zagen ook langere DNA-moleculen ('Alu247/Alu115 ratio') in sap. Dit kan verklaard worden door een van de volgende hypotheses: 1. Deze langere moleculen komen niet uit kankercellen maar uit gezonde cellen (zoals witte bloedcellen) en deze gezonde cellen verliezen in alvleeskliersap meer DNA dan in bloedplasma; 2. De DNA-moleculen komen vrij door necrose van cellen (celdood gestimuleerd door externe factoren zoals ontsteking of zuurstoftekort, vaak gezien bij tumoren maar minder gebruikelijk bij gezonde cellen) in plaats van apoptose (celdood geïnitieerd door de cel zelf als verdedigingsmechanisme). Necrose is mogelijk beter te detecteren dichtbij de bron.

Het is dan ook onduidelijk welk materiaal meer representatief is voor het bestaan van kanker. Uiteindelijk bleek de detectiegraad van afwijkingen in het DNA laag (en vergelijkbaar) in zowel bloedplasma als alvleeskliersap. We concludeerden dat het

mogelijk belangrijk is om het alvleeskliersap onder bepaalde condities te bewaren, waarbij gezonde cellen minder makkelijk hun DNA loslaten. Verlagen van deze 'achtergrondruis' maakt het mogelijk makkelijker circulerend tumor DNA aan te tonen.

Chromosomale instabiliteit in alvleeskliersap en bloedplasma

Het meten van chromosomale instabiliteit in alvleeskliersap heeft mogelijk ook de potentie om alvleesklierkanker te diagnosticeren. Door verhoogde celdeling (en de verminderde controle hierop) in kankercellen kunnen deze namelijk fouten maken in de verdubbeling van het DNA. Terwijl in een gezonde cel elk chromosoom in principe twee keer voorkomt, kunnen delen van het chromosoom (of het chromosoom in zijn geheel) vaker ('amplificatie') of minder vaak ('deletie') voorkomen bij kwaadaardige transformatie. Waarbij gezonde cellen bij dit soort fouten doodgaan, hebben kankercellen mechanismes ontwikkeld om dergelijke fouten te overleven. Het meten van dit soort instabiliteit wordt gebruikt bij het opsporen van foetale chromosoomafwijkingen tijdens de zwangerschap ('NIPT-test'), Als 'bijvangst' werd hierbij soms ook een chromosomale afwijking gevonden in het DNA afkomstig van de moeder, wat leidde tot de ontdekking van kanker.[15]

In **hoofdstuk 13** gebruikten wij de pijplijn van de NIPT-test van de afdeling klinische genetica om chromosomale instabiliteit te testen in weefsel, sap en bloedplasma van patiënten met alvleesklierkanker dan wel een hooggradig voorstadium en controles uit onze surveillancecohorten. We zagen dat een bepaald deel van chromosoom 8 ('8q24') vaker geamplificeerd was in sap van patiënten met kanker dan in controles. Opvallend was dat deze afwijking ook aanwezig was in sap en weefsel van de persoon met het hooggradig voorstadium. Hierdoor zou de test mogelijk gebruikt kunnen worden voor vroege ontdekking van afwijkingen. De onderzochte groep personen was echter klein in deze studie en vervolgstudies zijn dan ook noodzakelijk om dit verder te onderzoeken. De concentratie circulerend tumor DNA in plasma was te laag en bleek niet betrouwbaar te analyseren te zijn. Voor het gebruik van deze pijplijn was alvleeskliersap dan ook superieur.

Conclusie

Dit proefschrift evalueert de huidige staat van alvleeskliercystesurveillance, introduceert 'geruststellende kenmerken' die de behandelend arts kan ondersteunen bij het herkennen van laag-risico cysten, en benadrukt de mogelijkheid dat klinisch handelen op basis van herhaaldelijke CA19.9 bepalingen mogelijk leiden tot meer kwaad dan goed. Prospectieve studies met langere follow-up tijd zijn noodzakelijk om het nut en van surveillance verder te beoordelen. Modellen die de kosteneffectiviteit simuleren kunnen hierbij helpen.

Vroege herkenning van alvleesklierkanker middels beeldvorming is uitdagend gebleken. Het identificeren van nieuwe biomarkers is noodzakelijk om de kans op vroege herkenning te verhogen, maar ook om die personen te selecteren die mogelijk geen voordeel hebben

van surveillance. Alvleeskliersap is een waardevolle bron van biomarkers en collectie is veilig. Biomarkers uit dit sap kunnen kanker voorspellen met redelijke individuele betrouwbaarheid. In dit proefschrift onderzochten wij de biomarkers alleen nog in sporadische kankers, bij gebrek aan kankergevallen in ons hoog-risico cohort. Ook waren de patiëntengroepen relatief klein. De testen van biomarkers in alvleeskliersap zijn dan ook nog niet klaar voor gebruik in de praktijk en vervolgstudies zijn nodig waarbij biomarkers worden bepaald over tijd in hoog-risico personen met en zonder kanker ter evaluatie of bepalingen ook leiden tot vroegere detectie, betere kwaliteit van leven en overleving. Bovendien verwachten we dat verschillende biomarkers elkaar kunnen aanvullen. Daarom is het noodzakelijk om biomarkers ook in combinatie te testen. Uiteindelijk zal hiermee een algoritme ontwikkeld moeten worden dat het hebben (en ontwikkelen) van alvleesklierkanker voorspelt.

Referenties

1 Oyama H, Tada M, Takagi K, *et al*. Long-term Risk of Malignancy in Branch-Duct Intraductal Papillary Mucinous Neoplasms. *Gastroenterology* 2020; 158: 226-237.e225. 2019/09/02.

2 The European Study Group on Cystic Tumours of the P. European evidence-based guidelines on pancreatic cystic neoplasms. *Gut* 2018; 67: 789.

3 Tanaka M, Fernández-Del Castillo C, Kamisawa T, *et al*. Revisions of international consensus Fukuoka guidelines for the management of IPMN of the pancreas. *Pancreatology* 2017; 17: 738-753. 2017/07/25.

4 Elta GH, Enestvedt BK, Sauer BG, *et al*. ACG Clinical Guideline: Diagnosis and Management of Pancreatic Cysts. *Official journal of the American College of Gastroenterology* | ACG 2018; 113.

5 Vege SS, Ziring B, Jain R, *et al*. American Gastroenterological Association Institute Guideline on the Diagnosis and Management of Asymptomatic Neoplastic Pancreatic Cysts. *Gastroenterology* 2015; 148: 819-822.

6 van Riet PA, Larghi A, Attili F, *et al*. A multicenter randomized trial comparing a 25-gauge EUS fine-needle aspiration device with a 20-gauge EUS fine-needle biopsy device. *Gastrointest Endosc* 2019; 89: 329-339. 2018/10/28.

7 Bardeesy N and DePinho RA. Pancreatic cancer biology and genetics. *Nature Reviews Cancer* 2002; 2: 897-909.

8 Levink IJM, Nesteruk K, Visser DI, *et al*. Optimization of Pancreatic Juice Collection: A First Step Toward Biomarker Discovery and Early Detection of Pancreatic Cancer. *Official journal of the American College of Gastroenterology* | ACG 2020; 115.

9 Morris JPt, Wang SC and Hebrok M. KRAS, Hedgehog, Wnt and the twisted developmental biology of pancreatic ductal adenocarcinoma. *Nat Rev Cancer* 2010; 10: 683-695. 2010/09/04.

10 Wang S, Zheng Y, Yang F, *et al*. The molecular biology of pancreatic adenocarcinoma: translational challenges and clinical perspectives. *Signal Transduction and Targeted Therapy* 2021; 6: 249.

11 Vreeker GCM, Hanna-Sawires RG, Mohammed Y, *et al*. Serum N-Glycome analysis reveals pancreatic cancer disease signatures. *Cancer Medicine* 2020; 9: 8519-8529.

12 Shams R, Saberi S, Zali M, *et al*. Identification of potential microRNA panels for pancreatic cancer diagnosis using microarray datasets and bioinformatics methods. *Scientific Reports* 2020; 10: 7559.

13 Ma J, Sun S, Song C, *et al*. Screening potential microRNAs associated with pancreatic cancer: Data mining based on RNA sequencing and microarrays. *Exp Ther Med* 2020; 20: 2705-2715. 2020/08/09.

14 Vicentini C, Calore F, Nigita G, *et al*. Exosomal miRNA signatures of pancreatic lesions. *BMC Gastroenterology* 2020; 20: 137.

15 Heesterbeek CJ, Aukema SM, Galjaard RH, *et al*. Noninvasive Prenatal Test Results Indicative of Maternal Malignancies: A Nationwide Genetic and Clinical Follow-Up Study. *J Clin Oncol* 2022; 40: 2426-2435. 2022/04/09.

Appendices

Abbreviations

ACG	American College of Gastroenterology
AGA	American gastroenterological association
AI	Absolute indication for surgery
AC	All controls
AUC	Area under the curve
BMI	Body mass index
BD-IPM	Branch-duct intraductal papillary mucinous neoplasm
Bp	Base-pair
CA19.9	Carbohydrate antigen 19.9
CATH	Catheter (collection method)
CBD	Common bile duct
cDNA	Complementary DNA
CEA	Carcinoembryonic antigen
cfDNA	Cell-free DNA
CI	Confidence interval
CNV	Copy number variant
CP	Chronic pancreatitis
CT	Conventional tomography
ctDNA	Circulating tumor DNA
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DNase	Desoxyribonuclease
DOR	Diagnostic odds ratio
dPCR	digital PCR
eCRF	Electronic case record form
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immuno Sorbent Assay
EMC	Erasmus Medical Center
END	Endoscope suction channel
ERCP	Endoscopic retrograde cholangiopancreatography
ERP	Endoscopic retrograde pancreatography
EUS	Endoscopic ultrasound
EV	Extracellular vesicle
FAM	Carboxyfluorescein
FAMM	Familial cutaneous malignant melanoma
FDR	First-degree relative
FMT	Fecal microbiota transplantation
FNA	Fine-needle aspiration
FNB	Fine-needle biopsy
FPC	Familial pancreatic cancer

FTICR	Fourier transform ion cyclotron resonance
H&E	Hematoxylin and eosin
HEX	5'-Hexachloro-Fluorescein-CE Phosphoramidite
HGD	High-grade dysplasia
HR	Hazard ratio
HRI	High-risk individual
HRS	High-risk stigmata
HSROC	Hierarchical summary receiver operating curve
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
IPMN	Intraductal papillary mucinous neoplasm
IPNB	Intrapapillary neoplasm of the bile duct
IQR	Interquartile range
LGD	Low-grade dysplasia
LUMC	Leiden University Medical Center
MALDI	Matrix-assisted laser desorption/ionization
MD-IPMN	Main-duct IPMN
miR/miRNA	MicroRNA
MCN	Mucinous cystic neoplasm
MPD	Main pancreatic duct
MRI	Magnetic resonance imaging
MRCP	Magnetic resonance cholangiopancreatography
MS	Mass spectrometry
MT-IPMN	Mixed-type IPMN
mtDNA	Mitochondrial DNA
MVB	Multivesicular body
muKRAS	Mutated KRAS
NA	Not applicable
nCLE	Needle-based confocal laser endomicroscopy
NET	Neuro-endocrine tumor
NGAL	Neutrophil gelatinase-associated lipocalin
NGS	Next generation sequencing
NIPT	Non-invasive prenatal testing
NP	Non-pancreatic controls
NPV	Negative predictive value
NTA	Nanoparticle Tracking Analyzer
OR	Odds ratio
PanIN	Pancreatic intraepithelial neoplasia
PBS	Phosphate buffered saline
PC	Pancreatic cancer
PCR	Polymerase chain reaction
PJ	Pancreatic juice
PL	Precursor lesion
PLA2G1B	Phospholipase A2 Group IB
PPV	Positive predictive value
qPCR	Quantitative PCR
QUADAS-2	Quality Assessment of Diagnostic Accuracy Studies-2
RI	Relative indication for surgery
RNA	Ribon ucleic acid
RNase	Ribonuclease
ROC	Receiver operating curve
RR	Relative risk
SCA	Serous cyst adenoma
SD	Standard deviation
SDR	Second-degree relative
SeqFF	Fetal DNA fraction
SPN	Solid pseudopapillary neoplasm
TEM	Transmission electron microscopy
WF	Worrisome features
WT	Wildtype

Contributing authors

Zakia Azmani
Center for Biomics,
Erasmus University Medical Center
Rotterdam, The Netherlands

Neville Azopardi
Department of Gastroenterology & Hepatology
Mater dei Hospital
Msida, Malta

Jilling F. Bergmann
Department of Gastroenterology & Hepatology
HagaZiekenhuis
The Hague, The Netherlands

Anne-Marie van Berkel
Department of Gastroenterology & Hepatology
Noordwest ziekenhuisgroep
Alkmaar, The Netherlands

Georg Beyer
Department of Gastroenterology & Hepatology
Ludwig-Maximilians-University of Munich
Munich, Germany

Bert A. Bonsing
Department of Surgery
Leiden University Medical Center
Leiden, The Netherlands

Marco J. Bruno
Department of Gastroenterology and Hepatology
Erasmus University Medical Center
Rotterdam, The Netherlands

Yuri E.M. van der Burgt
Center for Proteomics and Metabolomics
Leiden University Medical Center
Leiden, The Netherlands

Djuna L. Cahen
Department of Gastroenterology and Hepatology
Erasmus University Medical Center
Rotterdam, The Netherlands

Silvia Carrara
Department of Gastroenterology
IRCCS Humanitas Research Hospital
Milan, Italy

Riccardo Casadei
Department of Surgery
Bologna University
Bologna, Italy

Laszlo Czacko
Department of Gastroenterology & Hepatology
University of Szeged
Szeged, Hungary

Natasja F.J. Dits
Department of Urology
Erasmus University Medical Center
Rotterdam, The Netherlands

Lydi M.J.W. van Driel
Department of Gastroenterology and Hepatology
Erasmus University Medical Center
Rotterdam, The Netherlands

Megan M.L. Engels
Department of Gastroenterology & Hepatology
Mayo Clinic
Jacksonville, Florida, USA

Department of Gastroenterology & Hepatology
Leiden University Medical Center
Leiden, The Netherlands

Celio J.C. Fernandes
Department of Gastroenterology and Hepatology
Erasmus University Medical Center
Rotterdam, The Netherlands

Gwenny M. Fuhler
Department of Gastroenterology and Hepatology
Erasmus University Medical Center
Rotterdam, The Netherlands

Myrte Gorris
Department of Gastroenterology & Hepatology
Amsterdam UMC
Amsterdam, The Netherlands

Randa G. Hanna-Sawires
Department of Surgery
Leiden University Medical Center
Leiden, The Netherlands

Chantal Hoge
Department of Gastroenterology & Hepatology
Maastricht University Medical Center
Maastricht, The Netherlands

Pieter Honkoop
Department of Gastroenterology & Hepatology
Albert Schweitzer Hospital
Dordrecht, The Netherlands

Jeanin E. van Hooft
Department of Gastroenterology & Hepatology
Leiden University Medical Center
Leiden, The Netherlands

Department of Gastroenterology & Hepatology
Amsterdam UMC
Amsterdam, The Netherlands

Sanne A. Hoogenboom
Department of Gastroenterology & Hepatology
Amsterdam UMC
Amsterdam, The Netherlands

Department of Gastroenterology & Hepatology
Mayo Clinic
Jacksonville, Florida, USA

Isaura S. Ibrahim
Department of Gastroenterology & Hepatology
Leiden University Medical Center
Leiden, The Netherlands

Wilfred van IJcken
Center for Biomics
Erasmus University Medical Center
Rotterdam, The Netherlands

Carlo Ingaldi
Department of Surgery
Bologna University
Bologna, Italy

Sanne Jaarsma
Department of Gastroenterology and Hepatology
Erasmus University Medical Center
Rotterdam, The Netherlands

Maurice P.M.H. Jansen
Department of Medical Oncology
Erasmus University Medical Center
Rotterdam, The Netherlands

Derk C.F. Klatte
Department of Gastroenterology and Hepatology
Leiden University Medical Center
Leiden, The Netherlands

Brechtje D.M. Koopmann
Department of Gastroenterology and Hepatology
Erasmus University Medical Center
Rotterdam, The Netherlands

Department of Public Health
Erasmus University Medical Center
Rotterdam, The Netherlands

Johanna Laukkarinen
Department of Surgery
Tampere University Hospital
Tampere, Finland

Monique E. van Leerdam
Department of Gastroenterology and Hepatology
Leiden University Medical Center
Leiden, The Netherlands

Department of Gastrointestinal Oncology
Netherlands Cancer Institute
Amsterdam, the Netherlands

Ivonne Leeuwenburgh
Department of Gastroenterology & Hepatology
Franciscus Gasthuis
Rotterdam, The Netherlands

Ronald van Marion
Department of Pathology
Erasmus University Medical Center
Rotterdam, The Netherlands

Wilma E. Mesker
Department of Surgery
Leiden University Medical Center
Leiden, The Netherlands

Jihane Meziani
Department of Gastroenterology and Hepatology
Erasmus University Medical Center
Rotterdam, The Netherlands

Kateryna Nesteruk
Department of Gastroenterology and Hepatology
Erasmus University Medical Center
Rotterdam, The Netherlands

Kasper A. Overbeek
Department of Gastroenterology and Hepatology
Erasmus University Medical Center
Rotterdam, The Netherlands

Elizabeth Pando
Department of Surgery
Vall d'Hebron Institute of Research
Barcelona, Spain

Maikel P. Peppelenbosch
Department of Gastroenterology and Hepatology
Erasmus University Medical Center
Rotterdam, The Netherlands

Jan-Werner Poley
Department of Gastroenterology and Hepatology
Erasmus University Medical Center
Rotterdam, The Netherlands

Marcin Polkowski
Department of Gastroenterology, Hepatology, and Clinical Oncology
The Maria Sklodowska-Curie National Research Institute of Oncology
Warsaw, Poland

Rutger Quispel
Department of Gastroenterology & Hepatolog
Reinier de Graaf hospital
Delft, The Netherlands

Priscilla A. van Riet
Department of Gastroenterology and Hepatology
Erasmus University Medical Center
Rotterdam, The Netherlands

Gemma Rossi
Pancreato-Biliary Endoscopy and Endosonography Division
Pancreas Translational and Clinical Research Center
San Raffaele Scientific Institute IRCCS
Vita Salute San Raffaele University
Milan, Italy

Erik J. Schoon
Department of Gastroenterology & Hepatology
Catharina Hospital, Eindhoven
The Netherlands

Matthijs P. Schwartz
Department of Gastroenterology & Hepatology
Meander Medical Center
Amersfoort, The Netherlands

Anieta M. Sieuwerts†
Department of Medical Oncology
Erasmus University Medical Center
Rotterdam, The Netherlands

Marianne E. Smits
Department of Gastroenterology & Hepatology
Tergooi, Hilversum
The Netherlands

Ellert van Soest
Department of Gastroenterology & Hepatology
Spaarne Gasthuis
Haarlem, The Netherlands

Marloes L.J.A. Sprij
Department of Gastroenterology and Hepatology
Erasmus University Medical Center
Rotterdam, The Netherlands

Malgorzata I. Srebniak
Department of Clinical Genetics
Erasmus University Medical Center
Rotterdam, The Netherlands

Trevor Tabone
Department of Gastroenterology & Hepatology
Mater dei Hospital
Msida, Malta

Adriaan C.I.T.L. Tan
Department of Gastroenterology & Hepatology
Canisius Wilhelmina hospital
Nijmegen, The Netherlands

Rob A.E.M. Tollenaar
Department of Surgery
Leiden University Medical Center
Leiden, The Netherlands

Walter G. De Valk
Department of Clinical Genetics
Erasmus University Medical Center
Rotterdam, The Netherlands

Hans F.A. Vasen
Department of Gastroenterology and Hepatology
Leiden University Medical Center
Leiden, The Netherlands

Monique M. van Veghel-Plandsoen
Department of Clinical Genetics
Erasmus University Medical Center
Rotterdam, The Netherlands

Niels G. Venneman
Department of Gastroenterology & Hepatology
Medisch Spectrum Twente
Enschede, The Netherlands

Frederike G.I. van Vilsteren
Department of Gastroenterology & Hepatology
University Medical Center Groningen
Groningen, The Netherlands

Dido I. Visser
Department of Gastroenterology and Hepatology
Erasmus University Medical Center
Rotterdam, The Netherlands

Isis J. Visser
Department of Gastroenterology and Hepatology
Erasmus University Medical Center
Rotterdam, The Netherlands

Frank P. Vleggaar
Department of Gastroenterology and Hepatology
University Medical Center Utrecht
Utrecht, The Netherlands

Marlies Vornhülz
Department of Gastroenterology & Hepatology
Ludwig-Maximilians-University of Munich
Munich, Germany

Patrick M. Vos
Department of Radiology
St. Pauls hospital
Vancouver, Canada

Gerda C.M. Vreeker
Center for Proteomics and Metabolomics
Leiden University Medical Center
Leiden, The Netherlands

Laurens A. van der Waaij
Department of Gastroenterology & Hepatology
Martini Hospital
Groningen, The Netherlands

Anja Wagner
Department of Clinical Genetics
Erasmus University Medical Center
Rotterdam, The Netherlands

Michael B. Wallace
Department of Gastroenterology & Hepatology
Mayo Clinic
Jacksonville, Florida, USA

Manfred Wuhrer
Center for Proteomics and Metabolomics
Leiden University Medical Center
Leiden, The Netherlands

Bibliography

Iris J.M. Levink. "Barrett's Esophagus: A Short Overview." *Radboud Annals of Medical Students* (2018).

Juan E. Corral, Omar Y. Mousa, Murli Krishna, **Iris J.M. Levink,** Khela R. Pursell, Mohammad Afsh, Paul T. Kröner, Denise M. Harnois, Herbert C. Wolfsen, Michael B. Wallace, and Frank J. Lukens. "Volumetric Laser Endomicroscopy in the Biliary and Pancreatic Ducts: A Feasibility Study with Histological Correlation." *Endoscopy* 50.11 (2018): 1089-1094.

Iris J.M. Levink, Marco J. Bruno, and Djuna L. Cahen. "Management of Intraductal Papillary Mucinous Neoplasms: Controversies in Guidelines and Future Perspectives." *Current Treatment Options in Gastroenterology* 16.3 (2018): 316-332.

Iris J.M. Levink, Gursimran Kochhar, and Michael B. Wallace. "Endoscopic Evaluation of Lesions with White-Light Endoscopy and Chromoendoscopy." *Advanced Techniques for Endoscopic Resection in the GI Tract,* SLACK Incorporated (2019).

Iris J.M. Levink, Herbert C. Wolfsen, Peter D. Siersema, Michael B. Wallace, and Guillermo J. Tearney. "Measuring Barrett's Epithelial Thickness with Volumetric Laser Endomicroscopy as a Biomarker to Guide Treatment." *Digestive Diseases and Sciences* 64.6 (2019): 1579-1587.

Iris J.M. Levink, and Michael B. Wallace. "Barrett's Esophagus." *Encyclopedia of Gastroenterology 2nd edition*, Academic Press (2020), p. 248-258.

Patrick P.C. Boor, Kostandinos Sideras, Katharina Biermann, Hosein Aziz, **Iris J.M. Levink,** Shanta Mancham, Nicole S. Erler, Xudong Tang, Casper H. van Eijck, Marco J. Bruno, Dave Sprengers, Xingxing Zang, and Jaap Kwekkeboom. "HHLA2 is Expressed in Pancreatic and Ampullary Cancers and Increased Expression is Associated with Better Post-Surgical Prognosis." *British Journal of Cancer* (2020), Apr; 122(8):1211-1218.

Iris J.M. Levink*, Kateryna Nesteruk*, Dido I. Visser, Anieta M. Sieuwerts, Celio J.C. Fernandes, Maurice P.H.M. Jansen, Lydi M.J.W. van Driel, Jan-Werner Poley, Maikel P. Peppelenbosch, Djuna L. Cahen, Gwenny M. Fuhler, and Marco J. Bruno. "Optimization of pancreatic juice collection: A first step toward biomarker discovery and early detection of pancreatic cancer." *American Journal of Gastroenterology* (2020), 115.12: 2103-2108.

Iris J.M. Levink, Guillermo J. Tearney, Nicole S. Erler, Herbert C. Wolfsen, Peter D. Siersema, and Michael B. Wallace. "Barrett's Epithelial Thickness, Assessed by Volumetric Laser Endomicroscopy, Is Associated with Response to Radiofrequency Ablation." *Clinical Gastroenterology and Hepatology* (2021), 19.6: 1160-1169.

Iris J.M. Levink, Lodewijk A.A. Brosens, Sander S. Rensen, Merel R. Aberle, Steven S.W. Olde Damink, Djuna L. Cahen, Sonja I. Buschow, Gwenny M. Fuhler, Maikel P. Peppelenbosch, and Marco J. Bruno. "Neoantigen Quantity and Quality in Relation to Pancreatic Cancer Survival.", *Frontiers in Medicine* (2022), Feb 9;8:751110.

Kateryna Nesteruk, **Iris J.M. Levink,** Natasja F.J. Dits, Djuna L. Cahen, Maikel P. Peppelenbosch, Marco J. Bruno, and Gwenny M. Fuhler. "Size and Concentration of Extracellular Vesicles in Pancreatic Juice from Patients with Pancreatic Ductal Adenocarcinoma.", *Clinical and Translational Gastroenterology* (2022), Mar; 13(3): e00465.

Kasper A. Overbeek, Michael G. Goggins, Mohamad Dbouk, **Iris J.M. Levink,** Brechtje D.M. Koopmann, Miguel Chuidian, Ingrid C.A.W. Konings, Salvatore Paiella, Julie Earl, Paul Fockens, Thomas M. Gress, Margreet G.E.M. Ausems, Jan-Werner Poley, Nirav C. Thosani, Elizabeth Half, Jesse Lachter, Elena M. Stoffel, Richard S. Kwon, Alina Stoita, Fay Kastrinos, Aimee L. Lucas, Sapna Syngal, Randall E. Brand, Amitabh Chak, Alfredo Carrato, Frank P. Vleggaar, Detleft K. bartsch, Jeanin E. van Hooft, Djuna L. Cahen, Marcia Irene Canto, and Marco J. Bruno; International Cancer of the Pancreas Screening Consortium. "Timeline of Development of Pancreatic Cancer and Implications for Successful Early Detection in High-Risk Individuals.", *Gastroenterology* (2022), Mar; 162(3):772-785.e4.

Rachid Bouzid*, Amy L. Kessler*, **Iris J.M. Levink*,** Thierry P.P. van den Bosch, Sonja I. Buschow, Gwenny M. Fuhler, Maikel P. Peppelenbosch, Jerome Cros, Philippe Levy, Marco J. Bruno*, and Michail Doukas*. "Three Distinct Stroma Types in Human Pancreatic Cancer Identified by Image Analysis of Fibroblast Subpopulations and Collagen – Letter." *Clinical Cancer Research* (2022), 28.2: 425-426.

Iris J.M. Levink*, Derk C.F. Klatte*, Randa G. Hanna-Sawires, Gerda C.M. Vreeker, Isaura S. Ibrahim, Yuri E.M. van der Burgt, Kasper A. Overbeek, Brechtje D.M. Koopmann, Djuna L. Cahen, Manfred Wuhrer, Bert A. Bonsing, Rob A.E.M. Tollenaar, Frank P. Vleggaar, Hans F.A. Vasen, Monique E. van Leerdam, Marco J. Bruno, and Wilma E. Mesker. "Longitudinal Changes of Serum Protein N-Glycan Levels for Earlier Detection of Pancreatic Cancer in High-Risk Individuals.", *Pancreatology* (2022), May;22(4):497-506.

Kasper A. Overbeek, **Iris J.M. Levink,** Brechtje D.M. Koopmann, Femme Harinck, Ingrid C.A.W. Konings, Margreet G.E.M. Ausems, Anja Wagner, Paul Fockens, Casper H van Eijck, Bas Groot Koerkamp, Olivier R.C. Busch, Marc G. Besselink, Barbara A.J. Bastiaansen, Lydi M.J.W. van Driel, Nicole S. Erler, Frank P. Vleggaar, Jan-Werner Poley, Djuna L. Cahen, Jeanin E. van Hooft and Marco J. Bruno; Dutch Familial Pancreatic Cancer Surveillance Study Group. "Long-term yield of pancreatic cancer surveillance in high-risk individuals.", *Gut* (2022), Jun; 71(6):1152-1160.

Iris J.M. Levink*, Kateryna Nesteruk*, Esther de Vries, Isis J. Visser, Maikel P. Peppelenbosch, Djuna J. Cahen, Gwenny M. Fuhler, and Marco J. Bruno. "Extracellular Vesicles Derived Micrornas in Pancreatic Juice as Biomarkers for detection of Pancreatic Ductal Adenocarcinoma.", *Pancreatology* (2022), Jun;22(5):626-635.

Iris J.M. Levink, Isis J. Visser, Brechtje D.M. Koopmann, Lydi M.J.W. van Driel, Jan-Werner Poley, Djuna L. Cahen, Marco J. Bruno, and Gwenny M. Fuhler. "Protein Biomarkers in Pancreatic Juice and Serum for Identification of Pancreatic Cancer.", *Gastrointestinal endoscopy* (2022), Nov; 96(5):801-813.e2.

Isis J. Visser*, **Iris J.M. Levink*,** Maikel P. Peppelenbosch, Gwenny M. Fuhler, Marco J. Bruno, and Djuna L. Cahen. "Diagnostic Performance of DNA Alterations in Pancreatic Juice for the Detection of Pancreatic Cancer: A Systematic Review and Meta-analysis.", *Pancreatology* (2022), Nov; 22(7):973-986.

Iris J.M. Levink, Ian Chau. "The Link Between Microenvironment and Immunotherapy Response in Cholangiocarcinoma and Pancreatic Ductal Adenocarcinoma.", *ASCO Daily News* (2023), January 26.

Alberto Balduzzi, Claudia Campani, Ana Dugic, Omar Elshaarawy, **Iris J.M. Levink,** Anthea Pisani, Joana Roseira. "The upward trajectory of the UEG journal: Next Generation Trainee Editors." *United European Gastroenterology Journal* (2023), Feb;11(1):131-133.

Giovanni Marchegiani, **Iris J.M. Levink,** Alberto Balduzzi – "Young GI Angle: Clinical Trials 101: Unpacking the Fundamentals - Part 3.", *United European Gastroenterology* (2023).

Iris J.M. Levink, Malgorzata I. Srebniak, Walter G. De Valk, Monique M. van Veghel-Plandsoen, Djuna L. Cahen, Gwenny M. Fuhler, and Marco J. Bruno. "An 8q24 Gain in Pancreatic Juice Is a Candidate Biomarker for the Detection of Pancreatic Cancer." *International Journal of Molecular Sciences* (2023), Mar 7;24(6):5097.

Iris J.M. Levink, Maurice P.H.M. Jansen, Zakia Azmani, Wilfred van IJcken, Ronald van Marion, Maikel P. Peppelenbosch, Djuna L. Cahen, Gwenny M. Fuhler, and Marco J. Bruno. "Mutation Analysis of Pancreatic Juice and Plasma for the Detection of Pancreatic Cancer." *International Journal of Molecular Sciences* (2023), Aug 23;24(17):13116.

Iris J.M. Levink, Sanne C. Jaarsma, Brechtje D.M. Koopmann, Priscilla A. van Riet, Kasper A. Overbeek, Jihane Meziani, Marloes L.J.A. Sprij, Riccardo Casadei, Carlo Ingaldi, Marcin Polkowski, Megan M.L. Engels, Laurens A. van der Waaij, Silvia Carrara, Elizabeth Pando9, Marlies Vornhülz, Pieter Honkoop, Erik J. Schoon, Johanna Laukkarinen, Jilling F. Bergmann, Rossi Gemma, Frederike G.I. van Vilsteren, Anne-Marie van Berkel, Trevor Tabone, Matthijs P. Schwartz, Adriaan C.I.T.L. Tan, Jeanin E. van Hooft, Rutger Quispel, Ellert van Soest, Laszlo Czacko, Marco J. Bruno, and Djuna L. Cahen. On behalf of the PACYFIC-registry work group. "The additive value of CA19.9 monitoring in a pancreatic cyst surveillance program.", *United European Gastroenterology Journal* (2023), Sep; 11(7):601-611.

Iris J.M. Levink, Alberto Balduzzi, and Joost P.H. Drenth. "Beyond the numbers: United European Gastroenterology journal's mission to build a community and connect.", *United European Gastroenterology Journal* (2023), Sep; 11(7):596-598.

*Shared authorship

PhD portfolio

Name PhD student:	Iris J.M. Levink
PhD period:	December 2017 – October 2023
Erasmus MC Department:	Gastroenterology & Hepatology
Promotors:	Marco J. Bruno
Co-promotors:	Djuna L. Cahen and Gwenny M. Fuhler

Attended scientific courses and workshops

Good Clinical Practice, The Royal Marsden NHS Foundation Trust	2023
Integrity in Scientific Research, Erasmus MC	2020
Biomedical English Writing Course, Erasmus MC	2020
Advanced Immunology, MolMed, Erasmus MC	2019
Survival analysis course, MolMed, Erasmus MC	2019
Biostatistical Methods I: Basic Principles Part A (CC02a), NIHES	2018
Basic course on 'R', MolMed, Erasmus MC	2018
Gene expression data analysis using 'R': How to make sense out of your RNA-seq and micro-array data, MolMed, Erasmus MC	2018
Basic introduction in SPSS, MolMed, Erasmus MC	2018
Photoshop and Illustrator CC Workshop, Erasmus MC	2018
OpenClinica, Erasmus MC	2018
BROK Course, Consultatiecentrum Patientgebonden Onderzoek (CPO)	2018
Endnote Workshop, Erasmus MC Library	2018
Systematic Literature Retrieval in Pubmed Workshop, Erasmus MC Library	2018
Systematic Literature Retrieval in Other Databases, Erasmus MC Library	2018
Certificate in Advanced English, Radboudumc	2017
Interpretation of Volume Laser Endomicroscopy (VLE), Bedford, Massachusetts	2017

Attended (inter)national conferences

United European Gastroenterology Week (UEGW)	2017-2020, 2022, 2023
European Pancreatic Club (EPC)	2018-2020, 2023
Digestive Disease Week (DDW), USA	2017-2019
Dutch Tumor Immunology Meeting (DTIM), Breukelen	2018, 2019
Digestive Disease Days, NVGE, Veldhoven	2018, 2019
OESO, Geneva	2017
2nd Annual GI Advances in Endoscopy and Minimally Invasive Surgery, Florida	2017
'Coassistenten Congres', Egmond aan Zee	2015

Chaired scientific sessions at (inter)national conferences

Best of Hepatology in UEG Journal *UEGW, Copenhagen*	2023
Lower GI Cancer: Screening, Follow-up and Beyond *UEGW, Copenhagen*	2023
Oncology, Transplantation and Encephalopathy *UEGW, Copenhagen*	2023
New Molecules and Tools in GI Cancer *UEGW, Copenhagen*	2023
Pancreatitis *EPC, Alpbach*	2023

Oral presentations at (inter)national conferences

Small Cyst Size and Slow Growth Rate Are Reassuring Features in Individuals with Pancreatic Cysts Undergoing Surveillance: Results from the PACYFIC Trial *EPC, Alpbach*	2023
YOUPPIE Basic Science: Biomarkers for Pancreatic Cancer Screening (invited Lecture) EPC, Alpbach	2023
Methodology of Pancreatic Juice Collection for Biomarker Analysis *UEGW, Barcelona*	2019
Methodology of Pancreatic Juice Collection for Biomarker Analysis *Digestive Disease Days, NVGE, Veldhoven*	2019
Measuring Barrett's Epithelial Thickness with Volumetric Laser Endomicroscopy (VLE) as a Biomarker to Guide Treatment Choice *OESO, Geneva*	2017

Poster presentations at (inter)national conferences

A Combination of Biomarkers in Serum and Pancreatic Juice Allows Identification of Pancreatic Cancer with Higher Specificity *Digestive Disease Week, virtual*	2021
Optimization of Pancreatic Juice Collection: a First Step Towards Biomarker Discovery and Early Detection of Pancreatic Cancer *Digestive Disease Week, virtual*	2020
Measuring Barrett's Epithelial Thickness with Volumetric Laser Endomicroscopy (VLE) as a Biomarker to Guide Treatment *DDW, Washington*	2018
Correlation Between Barrett's Epithelial Thickness, Assessed by Volumetric Laser Endomicroscopy, and Response to Radiofrequency Ablation DDW, Washington	2018
Measuring Barrett's Epithelial Thickness with Volumetric Laser Endomicroscopy (VLE) as a Biomarker to Guide Treatment *UEGW, Barcelona*	2017
The Association Between the Thickness of Barrett's Epithelium, assessed by Volumetric Laser Endomicroscopy, and Response to Radiofrequency Ablation: The Methods *2nd Annual GI Advances in Endoscopy and Minimally Invasive Surgery, Mayo Clinic, Florida*	2017
The Association Between the Thickness of Barrett's Epithelium, assessed by Volumetric Laser Endomicroscopy, and Response to Radiofrequency Ablation: The Methods *University of Central Florida - Mayo Clinic symposium, Orlando, Florida*	2017

Masterclasses, meetings and symposia

Erasmus Liver Day	2023
Regionaal Onderwijs Interne Geneeskunde (ROIG)	2023
NETs for Newcomers: An Introduction to NET Medicine, UKI NETs, Birmingham	2023
Post-graduate Course, UEG	2019, 2023
Advanced Life Support, Reinier de Graaf Gasthuis, Delft	2023
Masterclass on Chronic pancreatitis - United European Gastroenterology (UEG), Vienna	2022
Fundamental Critical Care Support, FCCS, NVIC, Houten	2021
Pancreas Research Meetings, Department of Gastroenterology and Hepatology, Erasmus MC	2018-2020
Journal Clubs, Department of Gastroenterology and Hepatology, Erasmus MC	2017-2020
EUS live in Amsterdam 22nd Annual Course, Amsterdam	2019
'Casuïstische conferentie', NVGE, Utrecht	2019

Masterclasses, meetings and symposia (continuation)

'Casuïstische conferentie', NVGE, Utrecht	2019
Medical business Masterclasses	2018, 2019
DPCG-meeting, Utrecht/Gouda	2017-2019
Diner Pensant Hepatology/Pancreatology, Rotterdam	2017-2019
University of Central Florida - Mayo Clinic symposium, Orlando, Florida	2017

Prices and honored funds

Best Manuscript Award, Gastrointestinal Endoscopy	2022
IELTS-certificate of advanced English	2022
NVGE Gastrostart	2020
Best Presented Abstract, UEGW	2019

Memberships

European Pancreatic Club (EPC)
United European Gastroenterology (UEG)
American Society for Gastrointestinal (ASGE)
Dutch Society of Gastroenterology (NVGE)
De Jonge Dokter
De Jonge Specialist
Promeras

Peer review activities

United European Gastroenterology Journal (UEGJ)
American Journal of Gastoenterology (AJG)
BMJ Open Gastroenterology
Diseases of the Esophagus
Scientific Reports
RAMS, Radboudumc

Educational activities

Supervisor Master Student: Sanne Jaarsma	2020
Lecture for minor 'HPB'	2019
Supervisor Master Student: Isis Visser	2019
Teach the Teacher I, Erasmus MC	2019
Workshop 'hoorcollege geven'	2019
Tutor of First year Medical Students	2018-2019
Training 'omgaan met groepen', Erasmus MC	2018
Supervisor Master Student: Dido Visser	2018

Extracurricular activities

Trainee Editor for UEG Journal	2023
Editor for 4 GH	2020
Member of Platform Innovation 'MDL' (PLATINUM)	2018-2020

About the author

Iris J.M. Levink was born in Vlijmen, The Netherlands, where she grew up as the youngest of four children. She completed her secondary school in 's-Hertogenbosch (Stedelijk Gymnasium) and studied Medicine at the Radboud University in Nijmegen.

Her enthusiasm in Gastroenterology was triggered during a research internship at the Department of Gastroenterology and Hepatology of Mayo Clinic Florida under supervision of Prof. Dr. Michael B. Wallace. Together with Prof. Dr. Guillermo J. Tearney, she developed an algorithm to measure thickness of Barrett's esophagus and evaluated if this was associated with efficacy of radiofrequency ablation.

She received her medical degree in November 2017. In December 2017, she started her full-time PhD at the Department of Gastroenterology and Hepatology of Erasmus Medical Center under supervision of Prof. Dr. Marco J. Bruno, Dr. Djuna L. Cahen and Dr. Gwenny M. Fuhler. Her thesis focused on early detection of pancreatic cancer by biomarkers in blood and pancreatic juice. She was also given the opportunity to coordinate (and design) clinical trials (PACYFIC-study, FPC-study and KRASPanc-study) and to get more insight in tumor immunology and tyrosine kinases. Under her supervision, three medical students successfully defended their master thesis during this period.

In 2021 and 2022, she has worked as a resident (not in training) at the National Cancer Institute Antoni van Leeuwenhoek and Amsterdam UMC. From August 2022 on, she has worked for one year as a clinical research fellow at the Gastrointestinal Cancer Department of Royal Marsden NHS trust in London. More recently, in September 2023, she started her residency in Internal Medicine training at Reinier de Graaf Hospital, under supervision of Dr. Henk Boom. She will continue her training in Gastroenterology in 2025.

Parallel to her clinical work, she currently works as trainee editor at United European Gastroenterology Journal, at which she is member of the medical guideline taskforce. She also reviews articles submitted to the journal in the Gastrointestinal Oncology section. Next to that, Iris likes to perform sports (running, cycling, hiking, field hockey and skiing), and is passionate about travelling.

Dankwoord

Dit proefschrift is tot stand gekomen dankzij de bijdrage van veel mensen aan wie ik veel dank verschuldigd ben.

Beste **Marco**, ruim zes jaar geleden ontmoette ik jou voor het eerst. Ik kwam net terug van mijn stage in het buitenland, het onderzoek smaakte mij naar meer en jij had een interessante onderzoekslijn te bieden. Dit was het startschot van een leerzame tijd, waarin jij mij ondergedompeld hebt in het leven van een onderzoeker. Bedankt voor je fijne begeleiding, vertrouwen en kritische blik. Van jou heb ik geleerd hoe ik effectief kwalitatief onderzoek kan leveren met impact. Mijn ideeën werden altijd serieus genomen en dat waardeer ik enorm.

Lieve **Djuna**, we leerden elkaar kort voor de start van mijn promotietraject kennen tijdens UEGW in Barcelona. Zoals altijd straalde je warmte en positiviteit uit en ik voelde gelijk een klik. Als team gingen we van start om mijn promotietraject en de verschillende studies tot een succes te maken. Hierbij was kwaliteit het hoogste doel. Voor mij was jij niet alleen een mentor in de wetenschap, maar ook daarbuiten. Discussies over methodiek of schrijfwijzen hebben mij verder doen ontwikkelen als onderzoeker. Daarnaast kon ik twijfels over onder andere levens- of carrièrekeuzes altijd bij je kwijt. We noemden je niet voor niets vaak gekscherend 'Mama Pancreas'. Met veel plezier kijk ik terug naar de gezellige diners en borrels tijdens congressen, waar we veel hebben gelachen. Lieve Djuna, bedankt voor je begeleiding, luisterend oor, gezelligheid en nuchterheid.

Lieve **Gwenny**, door het translationele karakter van mijn onderzoekslijn, werd het al gauw duidelijk dat jij onmisbaar was in het promotieteam. Jij leerde mij de fijne kneepjes van het lab en ik voel me bevoorrecht dat ik een kijkje heb mogen nemen in de keuken. Ik ben je nog steeds dankbaar dat je me hebt geadopteerd toen het nodig was. Je gaf me ruimte om zaken uit te zoeken, maar je hielp me altijd waar nodig. Dit heeft me doen ontwikkelen tot waardig translationeel onderzoeker. Met plezier kijk ik terug naar de gezellige videomeetings tijdens corona met op de achtergrond de fluitende vogels of jouw konijn. Lieve Gwenny, bedankt voor je tijd, rust en handige oplossingen.

*I would like to thank the **Assessment Committee** for the assessment of my thesis: dr. Groot Koerkamp, prof. dr. Julia Mayerle and prof. dr. Drenth. I am especially grateful to Prof. Dr. Julia Mayerle for taking the time and effort to be present at my PhD defence.*

Lieve **Sonja**, jij hebt me ondergedompeld in de wereld van de immunotherapie. Ik heb het altijd jammer gevonden dat ik niet de tijd en de ruimte had om deze onderzoekslijn verder uit te bouwen. Bedankt voor je begeleiding, het delen van je kennis en de gezelligheid. Beste **Michael**, bedankt voor je vele ideeën en slimme oplossingen. Ik waardeer het enorm dat jouw deur altijd openstond. Beste **Lydi** en **Jan-Werner**, bedankt voor alle tijd die jullie hebben gestoken in de pancreassapcollecties. Ik kan me voorstellen dat het niet

altijd makkelijk is geweest, als ik (of een van de studenten) weer eens binnenkwam tijdens een druk endoscopieprogramma. Ik waardeer jullie rust en bereidheid enorm.

Prof. dr. Michael Wallace *and* ***dr. Herbert Wolfsen****, I feel honored and grateful for the opportunity to work on my master thesis in Jacksonville. You paved the path for my scientific career and taught me the essentials of gastroenterology and science.* ***Prof. dr. Peter Siersema****, thank you for making this possible.*

Lieve **Carla** en **Andrea**, bedankt voor de gezelligheid en alles wat jullie geregeld hebben tijdens mijn promotietijd. Deze ondersteuning was onmisbaar.

Graag wil ik alle **co-auteurs** bedanken. Ieder hoofdstuk in dit proefschrift komt voort uit een samenwerking met ieder zijn eigen verhaal, waar ik veel van heb genoten en geleerd. De volgende personen wil ik speciaal bedanken. *Dear PACYFIC-group members, your hard work was of great importance to bring pancreatic cyst surveillance to another level. I would like to thank you for your devotion, time and input. Dear* ***Kateryna****, thank you for the great collaboration and the time you spent in the lab on micro-RNA and organoids in pancreatic juice.* Beste **Derk**, hoofdstuk 8 is een echte teamprestatie. Bedankt voor je enthousiasme, R-codes en online gezelligheid tijdens de COVID-periode. Beste **Yuri**, bedankt voor de spoedcursus *glycomics* en de tijd die je hebt besteed om het project tot een goed einde te brengen. Beste **Gosia**, jij hebt mij hoogstpersoonlijk geïntroduceerd in de wereld van de chromosomale instabiliteit en de NIPT-testen. Bedankt voor de tijd en moeite die je in dit project hebt gestopt.

Lieve **Kasper** en **Brechtje**, 'Team Pancreas', samen deelden wij de coördinatie van onze klinische pancreas studies. Bedankt voor de fijne samenwerking en de onwijze gezelligheid tijdens congressen, waar ik met veel plezier aan terugdenk. Ook wil ik mijn voorgangers (**Priscilla van Riet, Ingrid Konings** en **Femme Harinck**) en opvolgers bedanken voor al het werk dat jullie hebben verricht aan de FPC-, PACYFIC- en KRASPanc studie. **Jihane** en **Marloes**, door jullie harde werk en hernieuwde energie zijn deze studies in goede handen.

Ook ben ik veel dank verschuldigd aan de geneeskundestudenten die veel tijd hebben gestopt in het verzamelen van biomateriaal en data voor dit proefschrift. **Dido**, samen met jou heb ik de eerste laboratoriumanalyses op het pancreassap uitgevoerd. Bedankt voor je ondersteuning, nuchterheid en gezelligheid. **Isis**, jij staat aan de basis van twee manuscripten in dit proefschrift. Tijdens de COVID-periode hebben we vele uurtjes online doorgebracht om toch zoveel mogelijk voortgang te maken. Bedankt voor je doorzettingsvermogen en ongelimiteerde enthousiasme. **Sanne (Jaarsma)**, jij hebt hard gewerkt aan de PACYFIC-database. Ook jij hebt je stage tijdens de COVID-periode gelopen. Bedankt voor je tomeloze inzet, precisie en het bewaken van het fort, toen ik weg was. Ook ben ik veel dank verschuldigd aan **Lieke, David, Amber, Yasmine, Anniek** en **Sanne (van de Vondervoort)** voor de ondersteuning bij de pancreassapcollecties. Jullie waren onmisbaar.

Bovendien wil ik mijn **MDL-collega's** bedanken, omdat jullie de promotietijd zo leuk hebben gemaakt. Ik kijk met veel plezier terug op alle koffiepauzes, skireizen, borrels en congressen met jullie!

Collega's van het Reinier de Graaf, bedankt voor het warme bad waar ik me sinds september 2023 in bevind.

En dan mijn lieve vriendinnen, bedankt voor alle afleiding en heerlijke momenten samen. Lieve **Flufs, Charlotte, Fran, Funda, Lotte, Marije, Marlous** en **Stella**, wat een vermakelijke avonturen beleef ik telkens weer met jullie. We kennen elkaar door en door, en zullen elkaar daardoor altijd begrijpen. Lieve Proletariërs, **Celeste, Floyd, Kim, Lisa, Lianne, Lisanne, Maud, Mijntje, Schel** en **Stèfanie**, wat ben ik blij dat ik jullie 12.5 jaar geleden heb leren kennen. De borrels, etentjes, festivals en weekendjes maken me elke keer weer gelukkig.

Lieve **Fran** en **Steffi,** mijn paranimfen, wat een eer dat jullie tijdens de dag van mijn verdediging naast mij zullen staan. Lieve **Steffi**, als PhD-collega van het eerste uur begonnen we zes jaar geleden samen op 'het DAK'. Samen zochten we uit hoe we protocollen moesten indienen en vierden we hoogtepunten. Het is bijzonder hoe jij altijd het beste voor iedereen voor hebt. Lieve **Fran**, op jou kan ik al bijna 20 jaar bouwen. Je brengt plezier in mijn leven met je tomeloze energie, je goede dansmoves en je bizarre geheugen. Op naar nog veel meer avonturen samen!

Lieve **(schoon) familie**, ik dank jullie voor jullie vertrouwen in en betrokkenheid bij mijn promotie in de afgelopen jaren. **Mama, papa**, bedankt voor jullie grenzeloze steun. **Jasper, Merel** en **Pepijn**, bedankt voor de ongelimiteerde dosis gezelligheid en (soms ongevraagde) reflectie.

Lieve **Stino**, ik kan jou niet genoeg bedanken voor jouw onvoorwaardelijke geloof en liefde. Jij brengt muziek in mijn leven. Ik kijk uit naar een hele lange toekomst samen.