Combating (pre) malignant pancreatic disease

High time for molecular markers

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COLOFON

The studies described in this thesis were performed at the Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center Rotterdam, The Netherlands.

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Hoog tijd voor moleculaire markers

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General introduction and outline of the thesis

Chapter 1

PANCREATIC CANCER

Pancreatic cancer, frequently referred to as pancreatic ductal adenocarcinoma (PDAC), remains the fourth leading cause of cancer-related deaths in the United States. In 2015 alone, it is estimated that almost 50,000 people will be affected and more than 40,000 deaths will occur [1]. Over time, progress in the field has been slow, with the overall 5-year survival barely improving from 1% between 1950-1954 to 7.8% between 2005 and 2011 [2]. The grim prognosis can be attributed to the late presentation of the disease; most patients present with locally advanced or metastasized disease. Currently, only 15-20% are eligible for curative therapy in the form of surgery, resulting in a 5-year survival of 12-18% in this group [3–5]. The remaining 80-85% have unresectable disease and a median survival between 4.5 and 10.6 months, depending on the stage at diagnosis [6]. Since the latter group is not eligible for curative resection, the only therapeutic option left is systemic chemotherapy, which has been shown to improve survival and alleviate symptoms associated with the disease.

For years, gemcitabine was the only weapon in the arsenal of the physician against PDAC and demonstrated improved 1-year survival of 18% compared to 2% in patients treated with 5-Fluoruracil [7]. Thereafter, many clinical trials followed using gemcitabine as a backbone of multiagent regiments with the majority showing disappointing results. Exception to this rule was the addition of nanoparticle albumin bound (nab)-paclitaxel, which improved median overall survival to 8.5 months compared 6.7 to months when gemcitabine was used as single agent [8]. This however, was accompanied with significant toxicities. Two years after the publication of the phase III results, a modification in the dosing frequency was proposed and preliminary results showed less toxicity, less costs, and improved median survival of 11.1 months [9]. Probably one of the most remarkable breakthroughs in the field has been the trial where FOLFIRINOX (a regimen of 5-FU, leucovorin, irinotecan, and oxaliplatin) was compared to gemcitabine monotherapy. The median overall survival in the FOLFIRINOX group was 11.1 months compared to 6.8 months in patients treated with gemcitabine alone [10]. Other approaches using molecular therapies targeting the MAPK/ERK pathway, such as epidermal growth factor receptor (EGFR) inhibitors [11,12] and MEK-inhibitors [13,14], failed to meet clinically meaningful endpoints. Similarly, mammalian target of rapamycin (mTOR) inhibitors [15,16], vascular endothelial growth factor (VEGF) inhibitors [17,18], and hedgehog inhibitors [19] did not show statistically significant survival benefit.

The risk factors for developing PDAC include various environmental factors, non-hereditary diseases and hereditary genetic syndromes.

Smoking [20] and obesity [21] are well established environmental risk factors associated with PDAC, while data regarding dietary intake [22,23] and non-steroidal anti-inflammatory drug (NSAID) [24,25] intake show conflicting results. Other major risk factors are chronic pancreatitis [26] and diabetes [27,28], although diabetes has been implied to be a consequence of PDAC rather than a cause. The relative risk of developing PDAC is largest when germline mutations are involved in genes such as STK11/LKB1 (Peutz-Jeghers syndrome)[29], p16 (familial atypical multiple mole melanoma [FAMMM]) [30], PRSS1 (hereditary pancreatitis) [31], BRCA and PALB2 (hereditary breast cancer) [32,33], and DNA mismatch repair genes (MLH1, MSH2, and MSH6 all causing Lynch syndrome) [34].

TWO TYPES OF PRECURSOR LESIONS

PDAC can arise from two distinct precursor lesions: Pancreatic Intraepithelial Neoplasia (PanIN) and Pancreatic Cystic Neoplasm (PCN). PanIN lesions can progress from PanIN-1 to PanIN-3, while accumulating genetic mutations along the way, similar to the adenoma to carcinoma sequence described in colorectal cancer **(Figure 1)** [35].

The KRAS oncogene, the human homolog of an oncogene isolated from the Kirsten rat sarcoma virus, is the most early and frequently mutated gene and already found altered in more than 90% of PanIN-1A lesions, suggesting a role in tumor initiation [36]. In transgenic mouse models, KRAS mutations by themselves have been shown to lead to focal lesions resembling PanIN lesions, but not invasive carcinoma [37]. Mutations in KRAS leads to production of a constitutively active Ras protein, resulting in activation of downstream signaling pathways, including RAF-mitogen-activated kinase (MAPK), phosphoinositide-3-kinase (PI3K), and Ral GDS pathways [38]. These effector pathways are involved in many cellular functions and processes, which include proliferation, survival, apoptosis, and cytoskeletal remodeling. Other genes commonly affected during carcinogenesis include inactivation of tumor suppressor genes such as p16/CDKN2A, BRAF, TP53, and SMAD4 [36]. These genes were also found to be altered as a part of the on average 63 genetic mutations found in pancreatic cancers using next generation sequencing. All these genetic alterations can be broadly classified into 12 cellular signaling pathways. These pathways, however, were not uniformly affected in all tumors analyzed, underlining the fact that pancreatic cancer is a heterogeneous disease. Therefore, the authors suggest to abandon strategies aimed to target specific mutated genes, and instead target key nodal points of affected pathways [39]. This principle is the foundation of personalized medicine using molecular profiling in cancer, where treatment is tailored according to genetic status instead of the underlying tumor type.

Similar to PanIN lesions, PCNs are cystic lesions that are considered potential precursors of PDAC [40,41]. Currently, the prevalence of incidental pancreatic cysts (including non-neoplastic cysts), found when imaging of asymptomatic individuals is performed, is estimated to be over 2% [42,43]. Of those, PCNs account for more than 50% and are categorized into 4 types: Serous Cystic Adenoma (SCA), Solid Pseudopapillary Neoplasm (SPN), Intraductal Papillary Mucinous Neoplasm (IPMN), and Mucinous Cystic Neoplasm (MCN) [44–46]. It is well established that SCA scarcely become malignant, and the prognosis is excellent even in the face of metastatic disease [47,48].

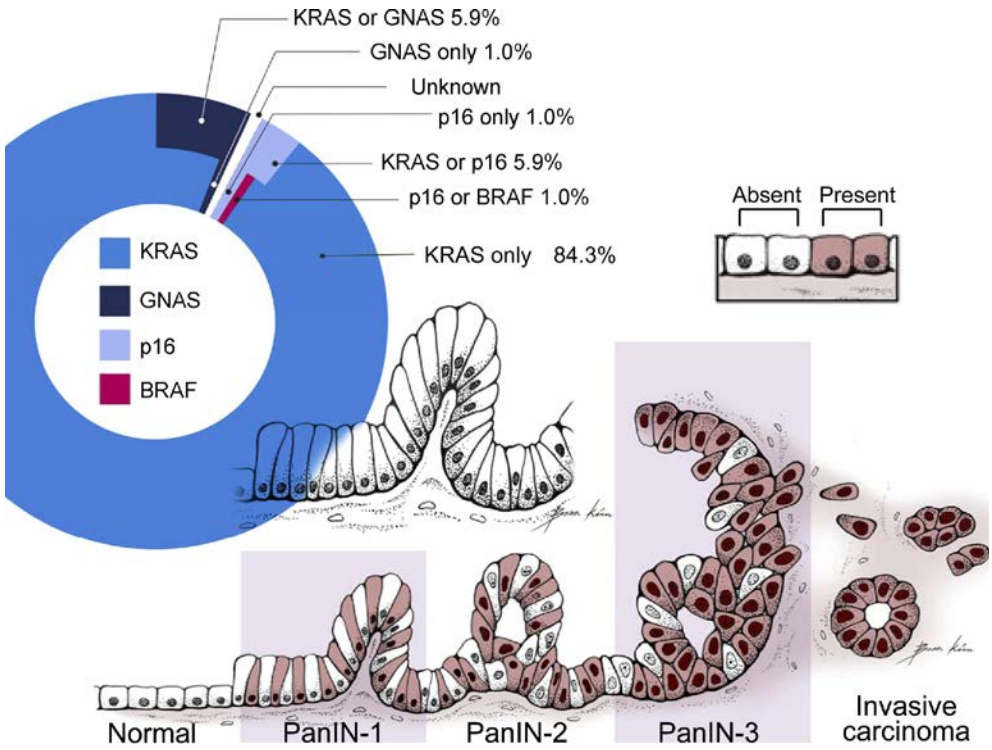

Figure 1. The pie chart indicates the percentage of mutations in each of the genes tested in PanIN-1 lesions. The bottom figure is a schematic model illustrating the increasing percentage of mutant KRAS cells within PanIN lesions as they progress from a low-grade (PanIN-1) to a high-grade PanIN (PanIN-3) and to an invasive ductal adenocarcinoma. Figure adapted from Kanda et al., Gastroenterology. 2012 Apr; 142(4): 730–733.e9.

Therefore, the management of SCAs is guided by the presence of symptoms only. In contrast, SPNs are generally resected due to their malignant potential and because these lesions typically occur in young women. After excision of the SPN, the prognosis is excellent with a disease free survival in 95.6% [49].

As opposed to SCA and SPN, IPMN and MCN confer a substantial risk for developing PDAC and the concomitant grim prognosis. Identical to PanIN lesions, IPMN and MCN has been shown to harbor KRAS mutations. Additionally, mutations in RNF43, TP53, and SMAD4 have been found in both IPMN and MCN [50]. In 2011, Wu et al. discovered recurrent activating mutations at codon 201 of GNAS, an oncogene more common in pituitary tumors, in 66% of IPMN cases studied [51]. These mutations are specific for IPMN, as they were not found in MCN or SCA. Other studies, analyzing cyst fluid or tissue derived from IPMN, found rates of GNAS mutations varying between 41% and 64% [52–55]. Even though the presence of GNAS mutations is highly specific for IPMN, no correlation has been found with the malignant potential of a pancreatic cyst.

CHRONIC PANCREATITIS

In addition to PanIN lesions and PCN, another risk factor for PDAC is chronic pancreatitis (CP), a degeneration of the pancreas due to progressive inflammation-induced fibrosis, resulting in loss of endocrine (acinar cell) and exocrine (islet cell) functions [56]. CP is estimated to occur in 3.5 to 10/100,000 people (the National Pancreas Foundation), and CP patients may be 10 times more likely to develop PDAC as compared to the normal population [57,58]. The most commonly associated risk factor for CP is heavy alcohol use - more than 5 drinks per day appears to be the threshold. However, autoimmune diseases, smoking and genetic predisposition contribute as risk factors for developing CP. Diagnosis is mainly performed by clinical findings and morphology based on radiographic imaging, as there are no useful biochemical tests available [59]. Even though functional tests are useful to diagnose pancreatic insufficiency, they do not specifically diagnose CP as the cause of the insufficiency, making them imperfect diagnostic tools for CP by themselves.The major clinical manifestations are abdominal pain, as well as varying degrees of exocrine pancreatic dysfunction leading to steatorrhea and malnutrition, and endocrine dysfunction leading to diabetes type 3c [60]. While symptoms of impairment of exocrine and endocrine function can be treated by enzyme and hormone replacement therapies [61,62], managing pain complaints in patients remains problematic. Sensory receptors in the visceral tissues (nociceptors) relay sensory information to the central nervous system, which is subsequently perceived as pain

[63]. Importantly, these nociceptors may be sensitized by inflammatory stimuli or tissue damage, thereby decreasing the threshold for signaling, and increasing pain sensation in patients [64]. Importantly, in CP patients receptor sensitization has also been reported, which hampers treatment of these pain complaints. Current treatment consists of opioids, which fails in half of the patients due to the paradoxal nociceptive sensitization induced by these compounds [65]. Anti-depressants and treatments which block nociceptor signal processing by reducing their sensitivity and reducing neurotransmitter release are also used, with pregabalin showing reduction of chronic pain in in PC patients on a randomized, controlled trial [66]. Nevertheless, patients are still undergoing surgery for unresolved pain complaints, and additional research on pain management in CP is needed.

DETECTION OF PANCREATIC DISEASE

Radiographic modalities of preference used in the diagnostic workup of a pancreatic cyst are multidetector Computed Tomography (CT) and Magnetic Resonance Cholangiopancreatography (MRCP), of which MRCP is preferred due to better resolution and the absence of radiation exposure [67–69]. Endoscopic modalities include the use of Endoscopic Retrograde Cholangiopancreatography (ERCP) and Endoscopic Ultrasound (EUS). Endoscopic ultrasound (EUS) has the advantage that aspiration or brush samples may be obtained which can be used for cytology. Pathological evaluation of cytology can be a valuable asset to differentiate between various pancreatic neoplasms including intraductal papillary mucinous neoplasia (IPMN) and mucinous cystic neoplasia (MCN) [70,71]. However, in addition to the differentiation between different cysts, it is even more important to be informed about the presence and/or risk of malignant transformation. Risk stratification in patients with pancreatic cysts is not possible because of the low correlation between morphological features and dysplastic grade [72]. While pancreatic cyst fluid currently has a limited role in clinical decision making towards pancreatic cysts management [73], cyst fluid obtained by EUS-fine needle aspiration (FNA) may contain valuable information to aid in these clinical decisions.

Current criteria for resection of a PCN are based on the Sendai consensus guidelines established in 2006 [74], which were updated in 2012 [73]. The recommendation of surgery as described in these guidelines relies primarily on morphological aspects of PCN acquired by imaging techniques. However, implementation of the 2006 guidelines demonstrates a very low specificity of 23-30%, even though the sensitivity was close to 100% in side-branch intraductal papillary mucinous neoplasm (sb-IPMN),

showing that unnecessary surgery for benign cysts is still being performed on a regular basis [75,76]. IPMN involving main duct are generally resected because of the high risk of malignant transformation, however, it is not possible to predict when and if this will happen. This may lead to surgery and the concomitant morbidity and (possibly) mortality of a pancreaticoduodenectomy or distal pancreatectomy. Vice versa, Fritz et al. pointed out that almost 25% of sb-IPMNs that do not fulfill the guideline criteria already have invasive carcinoma or carcinoma in situ [77], further demonstrating the need for additional markers to improve the diagnostic accuracy of PCN. So far, there are no reliable biomarkers in cyst fluid known to predict which cysts will follow a benign course or which cyst has developed or will develop into a malignancy. Therefore, there is a need for additional predictors to aid in the clinical decision to operate or not.

BIOMARKERS IN PANCREATIC CYSTIC NEOPLASMS

Carcinoembryonic Antigen (CEA)

First discovered in 1965 in human colon adenocarcinoma [78], CEA is a glycoprotein that was found to be expressed about 65-times higher on average in colon carcinoma tissue compared to normal tissue [79]. It is thought to be involved in colon carcinoma cell adhesion by serving as a ligand for E- and L-selectin, and therefore play a role in metastasis [80]. Over the years, the differences in expression of CEA between colorectal carcinoma and normal tissue have led to many studies involving CEA as a tumor marker. Elevated levels of CEA in serum is measurable in numerous benign diseases and gastrointestinal cancers, including pancreatic cancer [81]. The use of serum CEA levels has been studied for the diagnosis and prognosis of pancreatic cancer, but yielded poor sensitivity and specificity [82,83]. False positive results of serum CEA may in part be explained by an increase of circulating CEA in several benign conditions, such as liver disease, pancreatitis, and smoking [84,85]. With the emergence of pancreatic cystic neoplasms as a premalignant condition, the use of CEA was extended towards measurements in pancreatic cyst fluid.

CEA is the most studied tumor marker in pancreatic cyst fluid and can be used to differentiate between mucinous and non-mucinous cysts. Lewandrowski et al. measured CEA levels in 26 pancreatic cyst and found concentrations >367 ng/ml in mucinous cysts, as opposed to concentrations <23 ng/ml in non-mucinous cysts [86]. Larger studies in 111 and 272 cyst fluid samples using a cut-off value of >192 ng/ml diagnosed mucinous cysts with a sensitivity of 75% and 73%, and a specificity of 83.6% and 65%, respectively [87,88]. Other studies employed cut-off values of 30 ng/ml and 109.9 ng/ml

but none of the studies could find a correlation of CEA levels with the presence of malignancy in a pancreatic cyst [89,90]. Thus, even though CEA appear to be useful for the diagnosis of mucinous cysts, it does not correlate with the dysplastic grade of the cystic epithelium, making the applicability in the risk management of pancreatic cysts limited [91]. Nevertheless, combining cyst size with CEA is now recommended to guide clinical decision making [92],

Other protein-based markers

Carbohydrate Antigen 19-9 (CA 19-9) is a glycolipid first found in the serum of colon carcinoma, gastric carcinoma, and pancreatic cancer patients [93]. In the context of pancreatic disease, the use of CA 19-9 is limited for follow-up after curative surgery in pancreatic cancer. The routine use of CA 19-9 in the diagnostics of pancreatic cancer is not recommended due to the limited sensitivity (70-90%) and specificity (68-91%) [94]. Serum CA 19-9 can also be elevated in several other malignancies such as cholangiocarcinoma, hepatocellulair carcinoma, coloncarcinoma, and ovariumcarcinoma. Furthermore, about 10% of the Caucasian population who do not carry Lewis antibodies are unable to produce CA 19-9 [95]. In pancreatic cysts, the use of CA 19-9 was studied by Frossard et al. where a threshold of 50,000 U/ml resulted in an 86% sensitivity and 85% specificity in discriminating malignant MCN compared to other cystic lesions. Another pooled analysis of twelve studies including the previous study, show that CA 19-9 <37 U/ml has a sensitivity of 19% and specificity of 98% in diagnosing SCA and pseudocysts [96]. Other tumor markers studied in pancreatic cyst fluid include CA 72-4, CA 125 and CA 15-3 [86,87,97–99]. Nevertheless, none of the tumor markers discussed with the exception of CEA made it into routine clinical practice for the diagnostic work up of a pancreatic cyst.

Another approach was employed by Streitz et al, who performed 1D gel electrophoresis of cyst fluid samples, and showed that detection of glycoproteins in the sample was able predict mucinous cysts with 85% sensitivity and 100% specificity, although no correlation was made with actual malignant transformation [100]. Jabbar et al. used mass spectrometry to analyze expression of different mucins in pancreatic cyst fluid [101]. They found that mucin profiling discriminated PCN with malignant potential with a 97.5% accuracy, which was better than conventional methods and CEA, although as others point out, this study still did not actually describe the risk of malignant transformation [102]. However, while not distinguishing cysts that will definitely show malignant progression from those that do not, positive identification of mucin in the cyst fluid is a step in the right direction and one that might well be used in clinical practice. Interestingly, the converse might also be possibility – rather than identifying mucinous

cysts, non-mucinous SCA might be identified by the presence of vascular endothelial growth factor (VEGF)-A in the fluid of these cysts [103],

While detection of CEA, Ca 19-9 and mucin detection are now the only markers close to or in use in the clinic, substantial effort is made to identify novel markers with potential to identify malignant cysts. Many of these new techniques use DNA or RNA as basis.

DNA-based biomarkers

It has long been established that patients with cancer often have increased levels of cell free DNA (cfDNA) in their peripheral blood [104], and in pancreatic cancer patients, high levels of cfDNA are associated with poor prognosis [105]. The presence of this circulating cfDNA has been explained by the release of DNA from tumor cells that die through necrosis [106]. However, not all circulating cfDNA is derived from tumor cells, and cfDNA has a half-life of minutes to hours, making it difficult to use the amount of cfDNA as reliable tumor marker [107]. A more promising approach might therefor be to screen the cfDNA for the presence of tumor oncogenes. This approach seems particularly promising in tumors where the mutations are a priori known, or where a large proportion of patients have the same oncogenic transformations, such as the KRAS mutation in colorectal carcinomas [108]. Interestingly, QIAGEN has recently received CE registration for its in vitro diagnostic tool to detect EGF-receptor mutations in non-small cell lung cancers, showing that this is a technique feasible to implement in clinical practice. As KRAS mutations are also common in pancreatic cancer, this would also appear a promising approach to identify malignant pancreatic cysts from non-malignant ones. The largest study to date that has been performed in PCN is the PANDA study, a large multicenter study investigating the use of KRAS mutation detection in pancreatic cyst fluids [109]. They showed that while KRAS mutations were more prevalent in mucinous cysts, the presence of KRAS mutations did not distinguish premalignant from malignant cysts, While additional allelic loss was highly specific for malignant cysts (96%), sensitivity was low (37%). In a small validation study, detection of loss of heterozygosity resulted in a sensitivity of 50% and specificity of 71% [110]. Other studies suggest that while KRAS mutations detected in cyst fluid are not predictive of pancreatic cancer prognosis, KRAS mutations detected in cfDNA from serum are [111], although others even dispute this latter fact [105]. Other mutations have been identified in malignant pancreatic cysts, with GNAS being one of the most frequently observed in IPMN. Together with KRAS, GNAS gives a 98% specificity and 84% sensitivity to detect IPMN malignancy preoperatively, but is not useful for MCN [55]. It appears that combining molecular markers

(i.e. BRAF, CDKN2A, CTNNB1, GNAS, KRAS, NRAS, PIK3CA, RNF43, SMAD4, TP53, and VHL and loss of heterozygosity thereof) together with clinical markers may be needed to improve diagnostic accuracy of PCN [112], In addition to amount of cfDNA, and its mutational status, methylation patterns may distinguish tumor from non-tumor samples [113]. Furthermore, integrity of the DNA may contain information, as tumor DNA is considered to be longer as compared to DNA released by normal cells [114,115]. Thus, DNA may provide a lot of information, though the correct interpretation and implementation for pancreatic disease is as yet far off.

MicroRNA-based biomarkers

microRNAs (miRs) are small non-protein-coding RNA molecules of 20-25 nucleotides long. The first miR was discovered in 1993, while to date, it is estimated that around 4000 miRs may be expressed [116,117]. It is now well established that miRs are produced as a long single stranded primary transcript, which is processed by the RNAse II Drosha/DGCR8 complex to a ~60 nucleotide hairpin structure, and subsequently cleaved by Dicer into ~22 nucleotide long miRNAs [118,119]. The main function of miRs is regulating the expression of genes by binding to the 3'- untranslated regions of specific messenger RNAs (mRNA), thereby effectively regulating all cellular functions [120]. One miR may target multiple mRNAs, thereby interfering with many cellular processes [121]. As such, it is not surprising that more and more evidence points towards a role of deregulated miRs oncogenesis [120], and that miRs are now increasingly being investigated as potential diagnostic tool as well as target for cancer treatment. Combined mRNA profiling and miR profiling in an attempt to identify the deregulated miRs and their target genes in pancreatic cancer showed miR-200c, miR-429 and miT-200b to be differentially regulated between PDAC and healthy individuals [122]. However, many other overexpressed miRs have been identified in PDAC, and more are emerging every year. For instance, Frampton et al showed that overexpressed miR-21, miR-23a, and miR-27a act as inhibitors of PDAC-associated tumor suppressors [123], Vychytilova-Faltejskova and colleagues suggest that miR-21, miR-34a, miR-198 and miR-217 are highly expressed in PDAC and may act as biomarker for diagnosis [124], and Humeau et al show that hsa-miR-21, hsa-miR-23a, hsa-miR-23b and miR-29c are upregulated in saliva of PDAC patients, and have excellent specificity in diagnosing pancreatic cancer patients [125]. So far however, none of these markers have been corroborated thoroughly and studies show conflicting results. A lack of technical reproducibility between laboratories as well as differences in diagnostic scoring may have hampered the corroberation of many of these studies [126]. In addition, miRs have been measured in different bodily samples, including serum, cyst fluid and saliva,

and it is as yet unclear which presents the most promising compartment,

OUTLINE OF THE THESIS

As outlined above, several pressing questions are outstanding in the field of (pre) malignant pancreatic disease. First, we need to identify those pancreatic cysts at risk for malignant transformation, in order to remove them prior to progression to PDAC. However, it is imperative to exclude unnecessary surgery of benign cysts in order to limit surgery-related morbidities. Second, patients with chronic pancreatitis have a highly increased risk of developing PDAC. For these patients, better pain management strategies are required, but limiting inflammatory responses would be beneficial as well. Third, presentation of PDAC is often unexpected, in metastatic state, and novel treatment strategies are urgently needed in order to improve survival rates in these patients. This thesis is divided into two parts. In part one of thesis, we focus on the identification of novel biomarkers for pancreatic malignancy. In part two, we investigate potential targeted therapies for PDAC and CP, with an emphasis on the proliferative and inflammation enhancing signaling capacities of the mTOR-S6 pathway.

Part I: In chapter 2 we make an inventory of the resections performed in our tertiary center for pancreatic cystic neoplasms. We investigate the potential of adherence to Sendai guidelines to preclude unnecessary surgery, and conclude that better diagnostics of malignant and non-malignant cysts is required. The first step in distinguishing these is to identify mucinous cysts, and we demonstrate that assessment of mucinous background in cytopathological smears is a cheap, quick and efficient way to improve mucinous cyst diagnosis. In **Chapter 3** we investigate the potential of microRNA based biomarkers to distinguish malignant from non-malignant cysts, by attempting to validate a 9-miR panel in pancreatic cyst fluid. In **Chapter 4** we focus our attention on DNA-based molecular biomarkers, and investigate whether DNA integrity in pancreatic cysts fluid may serve as a basis to separate malignant cysts from those that do not require surgery.

Part II: In **Chapter 5** we investigate the mTOR-S6 signaling pathway in PDAC patients. This pathway contributes to protein synthesis, and is an important mediator of cell proliferation. We demonstrate that some, but not all PDAC patients show an increased activation of the mTOR-S6 signaling pathway, and more importantly, that inhibition of this signaling pathway is not effective in all pancreatic cancer cells. We set out to develop an in in vitro assay in order to predict which patients respond to inhibitors of mTOR signaling, in order to guide clinical decision making as to the use of

these inhibitors in the treatment of patients. In **Chapter 6**, we describe the secondary outcomes of a clinical trial in which chronic pancreatitis patients are treated with medical cannabinoids in an attempt to alleviate their pain symptoms. In this chapter, we investigate the immune modulatory properties of these cannabinoids by studying pro-and anti-inflammatory mediators in serum from these patients. In **Chapter 7**, we investigate these immune regulatory properties in more detail by analyzing the effect of cannabis on cellular kinome activity, and investigating modulation of the mTOR-S6 pathway in immunocytes from CP patients and healthy controls.

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General introduction and outline of the thesis

Chapter 2

Cytopathological analysis of cyst fluid enhances diagnostic accuracy of mucinous cystic neoplasms of the pancreas

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ABSTRACT

Widespread use of cross-sectional imaging and increasing age of the general population have increased the number of detected pancreatic cystic lesions. However, several pathological entities with a variety in malignant potential have to be discriminated to allow clinical decision making. Discrimination between mucinous pancreatic cystic neoplasms (PCN) and non-mucinous pancreatic lesions is the primary step in the clinical work-up, as malignant transformation is mostly associated with mucinous PCN. We performed a retrospective analysis of all resected PCN in our tertiary center from 2000-2014, to evaluate preoperative diagnostic performance and the results of implementation of the consensus guidelines over time. This was followed by a prospective cohort study of patients with an undefined pancreatic cyst, where the added value of cytopathological mucin evaluation to Carcinoembryonic Antigen (CEA) in cyst fluid for the discrimination of mucinous PCN and non-mucinous cysts was investigated. Retrospective analysis showed 115 patients operated for a PCN with a correct preoperative classification in 96.2% of the patients. High grade dysplasia or invasive carcinoma was observed in only 32.3% of mucinous PCN. In our prospective cohort (n=71), 57.7% of patients were classified as having a mucinous PCN. CEA ⩾192 ng/ml had an accuracy of 63.4%, and cytopathological mucin evaluation an accuracy of 73.0%. Combining these two tests further improved diagnostic accuracy of a mucinous PCN to 76.8%. CEA level and mucin evaluation were not predictive of the degree of dysplasia. These findings show that adding cytopathology to cyst fluid biochemistry improves discrimination between mucinous PCN and non-mucinous cysts.

INTRODUCTION

Pancreatic Cystic Neoplasms (PCN) are potential premalignant lesions which can lead to pancreatic ductal adenocarcinoma or colloid carcinoma [1]. The prevalence of pancreatic cysts, including non-neoplastic cysts, was estimated around 2% in patients undergoing preventive cross-sectional imaging, and increases with age [2]. Four distinct PCN entities with varying malignant potential are recognized according to WHO classification: (i) serous cystic adenoma (SCA), (ii) solid pseudopapillary neoplasm (SPN), (iii) mucinous cystic neoplasm (MCN), and (iv) intraductal papillary mucinous neoplasm (IPMN), with the latter two classified as mucinous PCN [1]. Early differentiation of mucinous PCN and non-mucinous cysts is essential to clinical management, decision to resect, and patient survival. SCA have minimal malignant potential and excellent prognosis even in metastatic disease [3]. SPN is considered as a neoplasm with malignant potential but a rather favorable survival rate exceeding 95% in 5 years [4]. In contrast, MCN has a higher risk of malignant degeneration although the rates of invasive carcinomas are variable in different studies, between 6% and 36% [5,6]. The prognosis of MCN is much improved if the cyst is resected prior to invasion, with a 5-year disease-specific survival of 100% [7]. Because identification of high risk MCN is not possible preoperatively, current guidelines recommend resection of MCN in all surgically fit patients. The frequency of high risk dysplasia and invasive carcinoma in IPMN varies between 24% and 62%, depending on the anatomical involvement of the pancreatic duct, and is categorized in main duct (MD-IPMN), side-branch (SB-IPMN), and mixed type (MT-IPMN) [8]. After adjusting for stage, the prognosis of IPMN-associated adenocarcinoma is similar to that of pancreatic ductal adenocarcinoma [9].

Currently, the management of MCN and IPMN is based upon international consensus guidelines established in 2006 [10], which were updated in 2012 [8]. The algorithm for the management of cystic lesions of the pancreas involves evaluation by imaging such as magnetic resonance cholangiopancreatography (MRCP) or endoscopic ultrasound (EUS). However, the interobserver agreement for both modalities remains moderate at best for characteristics of PCN, and additional markers to discriminate mucinous from non-mucinous PCN may be helpful, particular in hospitals where imaging is less frequently performed [11,12].

We performed a retrospective analysis of all resected PCN in our tertiary referral center, showing an increased number of PCN diagnosed over time, with increased sensitivity of identification of high risk mucinous PCN

upon the introduction of the updated Sendai guidelines. Nevertheless, non-malignant PCN are still frequently resected. In a first step towards identification of PCN with the highest malignant potential, we subsequently show in a prospective cohort, that inclusion of biochemical and cytological analysis of cyst fluid improves accuracy of detection of potentially malignant mucinous lesions.

METHODS

Retrospective Study

From 2000 to 2014, all patients who underwent pancreatic surgery at the Erasmus MC were identified using the 'PALGA', a nationwide network and registry of histo- and cytopathology in the Netherlands. The identified cases were cross-checked with a list of patients who underwent any form of pancreatic surgery during that time period at the Erasmus MC. Only patients who had a histopathologically proven PCN were included. All clinical data were retrospectively collected using the electronic patient files.

Prospective Study

From January 2009 to October 2013 all patients suspected with a pancreatic cyst based on physical complaints or incidental findings, who subsequently underwent endoscopic ultrasound-fine needle aspiration (EUS-FNA), were included. In this cohort, 27 patients underwent surgery, of which 22 patients were part of both the retrospective and prospective cohorts (the remaining 5 were operated in other hospitals). The remainder of the included patients in the prospective cohort did not undergo resection, but only EUS-FNA. Appropriate ethical approval was obtained for all procedures involving patients or patient material, Instutional Review Board (MEC-2008-233).

Definitions

Dysplasia

The WHO classification was used to describe dysplasia ranging from no dysplasia, low grade dysplasia, moderate dysplasia, high grade dysplasia, to invasive carcinoma. The highest grade of dysplasia found throughout the resection specimen was used. High grade dysplasia and invasive carcinoma were then classified as "high risk PCN". SPN were also considered high risk PCN as these lesions are generally recommended to be resected. No dysplasia, low grade dysplasia, and moderate dysplasia were classified as "low risk PCN".

Pancreatic cyst fluid
After EUS-FNA of a pancreatic cyst, CEA levels were measured in pancreatic cyst fluid. Furthermore, cytopathology was evaluated for the presence of neoplastic epithelial cells and mucinous background. Mucinous background refers to the presence of mucin in cytopathological analysis, which is microscopically visible in the May-Grünwald-Giemsa staining.

Outcome
The classification of a pancreatic cysts as a mucinous PCN or non-mucinous cyst was based on the pathology reports after resection, confirmation in EUS-FNA obtained cyst fluid, or the clinical diagnosis when neither were available.

Statistical analysis
Statistical analysis was performed using IBM SPSS Statistics 21. Univariable and multivariable logistic regression were used for the performance of CEA and mucinous background in differentiating between mucinous PCN and non-mucinous cysts. A p-value of <0.05 was considered statistically significant.

Cytopathological analysis of cyst fluid enhances diagnostic accuracy of mucinous cystic neoplasms of the pancreas

RESULTS

Retrospective analysis of PCN resections
Between 2000 and 2014 a total of 115 patients underwent pancreatic resection for a pancreatic cystic lesion. The clinical characteristics of these patients are presented in **table 1**. In total, thirty seven (32.7%; 2 cases could not be classified) were classified as high risk PCN. Of the mucinous PCN, only 30 out of 96 (31.3%) were high risk. Over the years, a significant increase in resections of PCN, in particular of IPMN and MCN (**Figure 1A**), was observed, with the majority occurring in the last 6 years. Of this increasing number of resections, a considerable percentage (34.2%) were asymptomatic lesions found incidentally or during surveillance for familial pancreatic cancer or a genetical predisposition for pancreatic cancer (**Supplementary Figure 1**). Thus, increased frequency of imaging and surveillance tools in recent years have lead to increased incidental finding of pancreatic lesions.

Diagnostic performance of resected PCN
Proper diagnosis of mucinous PCN is essential in order to prevent unnessecary resection of non-mucinous lesions such as SCA and pseudocysts. To determine the accuracy of preoperative diagnosis based on imaging techniques and clinical characteristics, we compared

the preoperative diagnosis with the gold standard of diagnosis; i.e. histopathological assessment of resection specimens. After exclusion of 9 patients with an inconclusive preoperative diagnosis, 106 remained for analysis (**Supplementary Figure 2**). In total, there were 93 mucinous PCN of which 96.8% were diagnosed correctly in the preoperative assesment. In contrast, non-mucinous lesions were diagnosed correctly in only 53.8% of the cases. Overall, there were 9 misdiagnosed cases **(Table 2)**, of which 3 cases (out of 4; 75%) were before 2006 and 6 cases (out of 102; 5.9%) were from 2006 onwards, demonstrating improved diagnosis upon introduction of the initial Sendai guidelines [10]. Potentially serious were case #1, preoperatively diagnosed as SPN, which turned out to be high grade IPMN, and case #2 which was a MCN with invasive carcinoma preoperatively misdiagnosed as pseudocyst. Case #3 was a MCN misdiagnosed as a SCA, and cases #8 and #9 were SPN mistaken for a MCN and cystic panNET, respectively. Four SCA were misdiagnosed before resection, two of which were mistakenly identified as mucinous lesions. In this misdiagnosed cohort, only 2 cases underwent EUS-FNA. Over time, there was a gradual improvement in the diagnostic performance, with up to 96.2% of the mucinous PCN correctly distinguished from non-mucinous cysts between 2012-2014 (**Figure 1B**). However, while the performance in differentiation of mucinous PCN and non-mucinous cysts improved over time, there was a consistent high rate of resections of low risk PCN, with up to 67.7% (31 out of 96 cases) resected without having high grade dysplasia or invasive carcinoma (**Figure 1C and Supplementary Table 1**).

IPMN description

In IPMN specimens involving the main pancreatic duct, main duct–IPMN (MD-IPMN) and mixed type-IPMN (MT-IPMN), the frequency of samples containing high grade dysplasia or invasive carcinoma was 46.8% (22 out of 47). In contrast, the incidence of high risk PCN in SB-IPMN was much lower (26.1%, 6 out of 23). Of note: in 2 cases of MD-IPMN and 7 of SB-IPMN, adenocarcinoma was found adjacent to the IPMN. While in these cases IPMN was scored based on its own malignant state, (e.g. IPMN with low grade dysplasia and adjacent unrelated carcinoma were classified as low risk) resection was of course warranted due to the carcinoma present. Excluding unrelated adjacent carcinoma, the rate of high risk PCN in SB-IPMN was 35.3% (6 out of 17; 1 unknown). In the period between 2009 and 2011, 27.6% of resected IPMN cases were high risk (8 out of 29) whereas between 2012 and 2014 this proportion was 48.6% (18 out of 37) (**Supplementary Figure 3**), suggesting that the updated Sendai guidelines of 2012 result in fewer false positive cases. Of the 71 patients that underwent a pancreatic resection, 1 histology report could not be retrieved, 20 had

low grade dysplasia (28.2%), 21 moderate dysplasia (29.6%), 10 high grade dysplasia (14.1%), 19 invasive carcinoma (26.8%). Forty four out of seventy one (62%) patients presented with symptoms before resection, whereas others were incidental findings or found during surveillance for familial pancreatic cancer.

Histology of IPMN
Of the 71 IPMNs, the distribution of the histologic classification was as follows: 23 were of the intestinal type, 12 of the pancreatobiliary type, 19 of the gastric type, and 2 of the oncocytic type (**Supplementary Figure 4**). Twelve patients were found to have 2 subtypes in their respective pancreatic resection specimen. The pancreatobiliary (58.3%) or oncocytic (100%) types were more associated with high risk PCN. In contrast, the rate of high risk PCN was lower in intestinal (21.1%) and gastric subtypes (26.1%) (**Supplementary Table 1**).

Recurrence of IPMN
The median follow-up after surgery was 12.1 months (IQR 5.7 – 24.9). During follow-up, there was progression of residual IPMN in 9 (12.9%) patients. New onset of IPMN was observed in 3 (4.3%) cases, suggestive of a predisposition for pancreatic lesion development in accordance with the field defect theory in IPMN [8]. In 35 cases (50.0%) no follow-up imaging after surgery was performed, but patients were routinely followed-up at the outpatient department and evaluated based on clinical grounds. In the remaining twenty three cases (32.9%), imaging demonstrated a stable pancreas.

Mucinous Cystic Neoplasm description
A total of 27 MCN were resected from 2000 till 2014. The incidence of high risk PCN was 7.7% (2 out of 26, one case could not be retrieved), while 92.3% was considered a low risk PCN (**Supplementary Table 1**). The majority of the MCN, including the two malignant ones, were located in the tail (92.6%). The average size was 5.8 cm (±2.6). More than half (61.5%) presented with symptoms.

The median follow-up after surgery was 11.9 months (IQR 0.7 – 34.3). After follow-up, 10 out of 27 patients had a demonstrable stable residual pancreas, while in the other 17 patients no imaging was performed.

Prospective analysis of mucinous background addition to diagnosis
While in our specialist tertiary referral center, preoperative diagnosis of resected PCN is almost 100% accurate, global decision making would

	Total (n= 115*)	Low risk PCN (n = 76)+	High risk PCN (n = 37)+
Age, years			
Range	14.5 – 84.4	20.2 – 79.3	14.5 – 84.4
Mean (SD)	60.1 (16.0)	60.4 (14.5)	59.1 (19.2)
Gender, n (%)			
Male	38 (33.0)	23 (30.3)	15 (40.5)
Female*	77 (67.0)	53 (69.7)	22 (59.5)
Histological diagnosis, n (%)			
Main Branch IPMN	24 (20.9)	13 (17.1)	11 (29.7)
Side Branch IPMN*	24 (20.9)	17 (22.4)	6 (16.2)
Mixed Type IPMN	23 (20.0)	12 (15.8)	11 (29.7)
Mucinous Cystic Neoplasm*	27 (23.5)	24 (31.6)	2 (5.4)
Serous Cystadenoma	10 (8.7)	10 (13.2)	0 (0)
Solid Pseudopapillary Neoplasia	7 (6.1)	0 (0)	7 (18.9)
Presentation, n (%)			
Incidental	36 (31.3)	25 (32.9)	11 (29.7)
Abdominal Pain	31 (27.0)	21 (27.6)	10 (27.0)
Jaundice*	13 (11.3)	6 (7.9)	6 (16.2)
Acute pancreatitis	19 (16.5)	14 (18.4)	5 (13.5)
Chronic pancreatitis	1 (0.9)	1 (1.3)	0 (0)
Weight loss	6 (5.2)	4 (5.3)	2 (5.4)
Surveillance	3 (2.6)	1 (1.3)	2 (5.4)
Other	4 (3.5)	3 (3.9)	1 (2.7)
Unknown*	2 (1.7)	1 (1.3)	0 (0)
Other	4 (3.5)	3 (3.9)	1 (2.7)
Unknown*	2 (1.7)	1 (1.3)	0 (0)

*Numbers do not add up because dysplasia of 2 cases could not be retrieved
+ Lesions with adjacent adenocarcinoma were classified based upon dysplasia as found in the cyst.
PCN = Pancreatic Cystic Neoplasm; SD = Standard Deviation; IPMN = Intraductal Papillary Mucinous Neoplasm.

Table 1. Patient characteristics of the retrospective cohort.

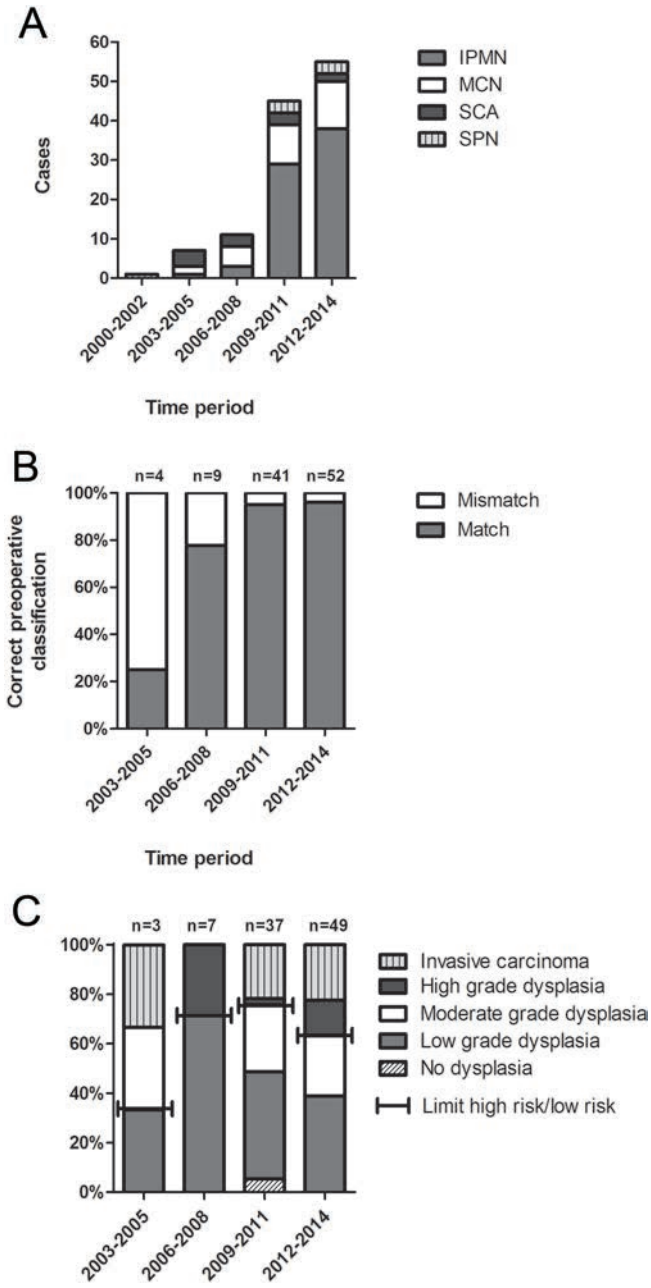

Figure 1. Numbers and presentation of PCN over the years. (A) Graph depicting all PCN resections from 2000 till 2014 (n=115) in the Erasmus MC, Rotterdam, the Netherlands. For all types of PCN, a triennial increase of resections can be noted. (B) The percentage matching preoperative diagnosis compared to the histopathological diagnosis over time in the retrospective cohort. (C) Graph depicting the distribution of dysplasia in resected mucinous PCN over the years.

Case number	Gender	Age	Preoperative diagnosis	Histological diagnosis, dysplasia	Year of resection	EUS-FNA performed
1	Female	45	SPN	SB-IPMN, high grade	2008	No
2	Male	66	Pseudocyst	MCN, invasive carcinoma	2003	No
3	Female	67	SCA	MCN, unknown	2007	Yes
4	Female	37	MD-IPMN	SCA	2004	No
5	Female	77	Cystic PanNET	SCA	2005	No
6	Female	40	Cystic PanNET	SCA	2011	No
7	Male	73	MD-IPMN	SCA	2014	Yes
8	Female	36	MCN	SPN	2009	No
9	Female	24	Cystic PanNET	SPN	2012	No

EUS-FNA = Endoscopic Ultrasound – Fine Needle Aspiration; SPN = Solid Pseudopapillary Neoplasm; SB-IPMN = Side Branch – Intraductal Papillary Mucinous Neoplasm; MCN = Mucinous Cystic Neoplasm; MD-IPMN = Main Duct – Intraductal Papillary Mucinous Neoplasm; SCA = Serous Cystadenoma; PanNET = Pancreatic Neuroendocrine Tumor

Table 2. Characteristics of misdiagnosed patients in retrospective cohort

benefit from additional easily implementable markers distinguishing mucinous PCN and non-mucinous lesions. Pancreatic surgery is associated with important morbidity and mortality, and even incidental unneccesary surgeries should be prevented. We therefore decided to investigate the added value of distinguishing mucinous background in EUS-FNA samples in the discrimination between mucinous PCN and non-mucinous cysts. To this aim, we determined CEA in cyst fluid and performed cytological evaluation of the collected fluid which included rapportage of the presence or absence of mucin and evaluation of the epithelial component. Seventy one (71) subjects were included in this prospective study and underwent EUS-FNA. The patient characteristics are described in **Table 3**.
The median follow-up after EUS-FNA was 13.1 months (IQR 5.3 – 33.4). During that time 38.0% (27 out of 71 patients) were resected of which 4 out of 27 (14.8%) contained high grade dysplasia, meaning the other 85.2% had moderate dysplasia at highest, or were a pseudocyst or SCA.

Carcinoembryonic Antigen (CEA)
The mean CEA level was higher in mucinous PCN (5078 ng/ml; n=41)

compared to non-mucinous cysts (252 ng/ml; n=30). The frequently used cutoff value of 192 ng/ml [13,14] resulted in a sensitivity of 39% and specificity of 96.7%, yielding an overall accuracy of 63.4% to differentiate between mucinous PCN and non-mucinous cysts (**Supplementary Table 2**). Univariable logistic regression yielded an OR of 18.6 (95%CI: 2.3 - 150.0; p=0.006) of having a mucinous PCN (**Table 4**). Evaluating CEA as a predictor for dysplasia did not yield a correlation given the wide range in low and high risk PCN (Low risk PCN: 1.3 – 83,690.0 ng/ml, n=19; high risk PCN: 0.2 – 172.1 ng/ml, n=4; p = 0.33).

Cytopathology and mucin evaluation

Next, we determined the presence of mucin in the EUS-FNA samples, which were available for 67 patients (examples in **Figure 2A-D**). Cytopathology results were available in 52 cases, 15 samples were not diagnostic and another 4 were not available for evaluation. Epithelial cells were present in 8 (11.9%) samples, which actually contributed to diagnosis in only 5 (7.5%) cases. Mucin was present in 24 of the 52 cases, and yielded a sensitivity of 66.6%, specificity of 81.8%, PPV of 83.3%, NPV of 64.3%, and accuracy of 73.0% of predicting a mucinous PCN (Supplementary Table 2). Univariable analysis of the presence of mucinous background had an OR of 9.0 (95%CI: 2.4-33.8; p=0.001) (**Table 4**). Similar to CEA, no association of mucinous background to the degree of dysplasia was observed (p=0.135).

Combining tests

Using both CEA levels and detection of mucin delivered the highest diagnostic properties. The combined test had a sensitivity of 75% and specificity of 79.1% with a diagnostic accuracy of 76.8%. Multivariable logistic regression still indicates both as an independent predictor of mucinous PCN; CEA OR 11.2 (95%CI 1.2 – 105.3; p=0.034) and mucinous background OR 7.7 (1.9 – 31.3; p=0.004) (Table 4). **Figure 2E** shows our proposed diagnostic algorithm using cyst fluid analysis.

DISCUSSION

In this combined retrospective and prospective analysis of pancreatic cystic neoplasms we observed that pancreatic cystic neoplasms, in particular IPMN and MCN, are resected with increasing incidence. Between 2006 and 2008, there were 11 pancreatic resections performed for a PCN, which increased fivefold between 2012 and 2014. The growth in resections of PCN observed in this study is in accordance with the increased detection of pancreatic cysts due to improvement of imaging techniques and the

widespread use of cross-sectional imaging [15].

In our tertiary referral center, the misclassification rate of PCN was lower than 5% in the last 6 years. During the last 3 years 96.2% out of 52 resected PCN were correctly classified preoperatively. Missclassification occured in 9 cases, 5 of which were nevertheless justified resections as these were a high grade SB-IPMN, 2 SPNs, and 2 MCNs. Seven out of nine misclassified cases did not undergo the EUS-FNA which might have avoided false positive surgery. Although diagnostic pancreatic cyst fluid with low levels of CEA and absence of mucin was available in one patient (#7), this was not taken into consideration and the patient was misdiagnosed with an IPMN. Using our currently proposed algorithm, this patient would have more likely been diagnosed with a SCA.

Especially in centers with lower pancreatic cyst volume, the clinical diagnosis may be strengthened by tools that are less hampered by inter-observational differences. Importantly, discrimination between mucinous PCN and non-mucinous pancreatic lesions is the primary step in the clinical work-up, as malignant transformation is mostly associated with mucinous PCN. Routine diagnostics include the cytological evaluation of the epithelial component in cyst fluid. However, in accordance to earlier studies, cytologic evaluation of the epithelial component (without mucinous background evaluation) performed poorly, most likely due to paucicelullarity [13,14]; presence of epithelial cells was detected in only 11.9% of the cases, with diagnostic usefulness limited to a mere half of these.

CEA has been described as a diagnostic tool for evaluation of mucinous PCN [13,14]. In our study, diagnostic accuracy of CEA in discriminating between mucinous PCN and non-mucinous cysts was 63.4%, which is comparable to an earlier report [14]. Previous studies using separate mucin staining for diagnosing mucinous PCN found varying results [16,17]. In our study, evaluation of the presence of mucin (without additional staining required) in cytopathological analysis (OR 7.7), additional to CEA, can improve diagnostic accuracy to 76.8%. Thus, we show that a simple evaluation such as mucinous background analysis, which is easy to perform in the routine histopathological practice, can lead to enhanced diagnostic accuracy. We therefore advise pathologists to evaluate and report the background status in cytopathological analysis of pancreatic cyst fluid.

Importantly, our cohorts show that despite improvements in diagnostics and updated guidelines, the rate of resections with no, low, or moderate

	Total (n = 71)	**Non-mucinous (n = 30)**	**Mucinous (n = 41)**
Age, years			
Range	19.9 – 82.0	31.6 – 78.9	19.9 – 82.0
Mean (SD)	60.9 (12.7)	58.7 (13.4)	62.4 (12.0)
Mean CEA (SD), ng/ml	3039 (13390)	252 (237)	5078 (2716)
Gender, n (%)			
Male	28 (39.4)	11 (36.7)	17 (41.5)
Female	43 (60.6)	19 (63.3)	24 (58.5)
Diagnosis, n (%)			
Main Branch IPMN	2 (2.8)	0 (0)	2 (4.9)
Side Branch IPMN	20 (28.2)	0 (0)	20 (48.8)
Mixed Type IPMN	6 (8.5)	0 (0)	6 (14.6)
Mucinous Cystic Neoplasm	13 (18.3)	0 (0)	13 (31.7)
Pseudocyst	13 (18.3)	13 (43.3)	0 (0)
Serous Cystadenoma	16 (22.5)	16 (53.3)	0 (0)
GIST	1 (1.4)	1 (3.3)	0 (0)
Surgery, n (%)			
Yes	27 (38.0)	4 (13.3)	23 (56.1)
No	44 (62.0)	26 (86.7)	18 (43.9)
Dysplasia, of resected (n=27)			
No dysplasia	2 (7.4)		
Low grade dysplasia	13 (48.1)		
Moderate grade dysplasia	4 (14.8)		
High grade dysplasia	4 (14.8)		
Not applicable	4 (14.8)		

SD = Standard Deviation; CEA = Carcinoembryonic Antigen; IPMN = Intraductal Papillary Mucinous Neoplasm; GIST = Gastrointestinal Stromal Tumor

Table 3. Characteristics of patients in prospective cohort

dysplasia remaines high, especially in SB-IPMN and MCN, demonstrating the need for better diagnostic tools for clinical decision making. The occurence of high grade dysplasia and invasive carcinoma in IPMN involving the main duct was 46.8%, and therefore the current management to resect all MD-IPMN and MT-IPMN in surgically fit patients is warranted [18,19]. In contrast, we found that only 26.1% of the resected SB-IPMN were histologically classified as a high risk, while the majority of resected

	Odds Ratio	95% Confidence Interval	P-value
Univariable analysis			
CEA⩾192 ng/ml	18.6	2.3 - 150.0	0.006
Mucin background	9.0	2.4 - 33.8	0.001
Multivariable analysis			
CEA⩾192 ng/ml	11.2	1.2 - 105.3	0.034
Mucin background	7.7	1.9 - 31.3	0.004

CEA = Carcinoembryonic Antigen

Table 4. Logistic regression for the differentiation of mucinous pancreatic cystic neoplasm versus non-mucinous cysts

SB-IPMN showed low grade or moderate dysplasia only. Excluding cases with adjacent carcinoma, the rate of high risk SB-IPMN would only be 35.3%, implying a low correlation of the current guidelines with degree of dysplasia. This low correlation is in accordance with earlier reports and has led to a debate regarding Sendai guidelines and the management of SB-IPMN [20,21]. The incidence of carcinoma in MCN was found to be even lower with 7.7%, similar to an earlier report [6]. In fact, most resected MCN (82.3%) had low or moderate dysplasia only. Currently, there are no reliable predictors for the identification of high risk mucinous PCN, as promising markers in pancreatic cyst fluid such as microRNAs and p53 require clinical validation [22,23]. Furthermore, improved clinical risk stratification is needed to reduce the burden of pancreatic surgery with high morbidity and mortality in selected patients.

We acknowledge several limitations in our study. The retrospective part of the study is unavoidably exposed to selection bias as mainly PCN with high risk stigmata were resected. Additionally, the outcome in our prospective cohort was not always based on the golden standard of histopathology but on clinical diagnosis. However, more than 50% was confirmed histologically and the performance of classifying PCN in this institution based on clinical data was shown to be very accurate in our retrospective data.

CONCLUSIONS

In summary, we show that the number of patients that are diagnosed with PCN is increasing over time. Combination of cytopathology and cyst fluid biochemistry is highly specific and sensitive for discrimination

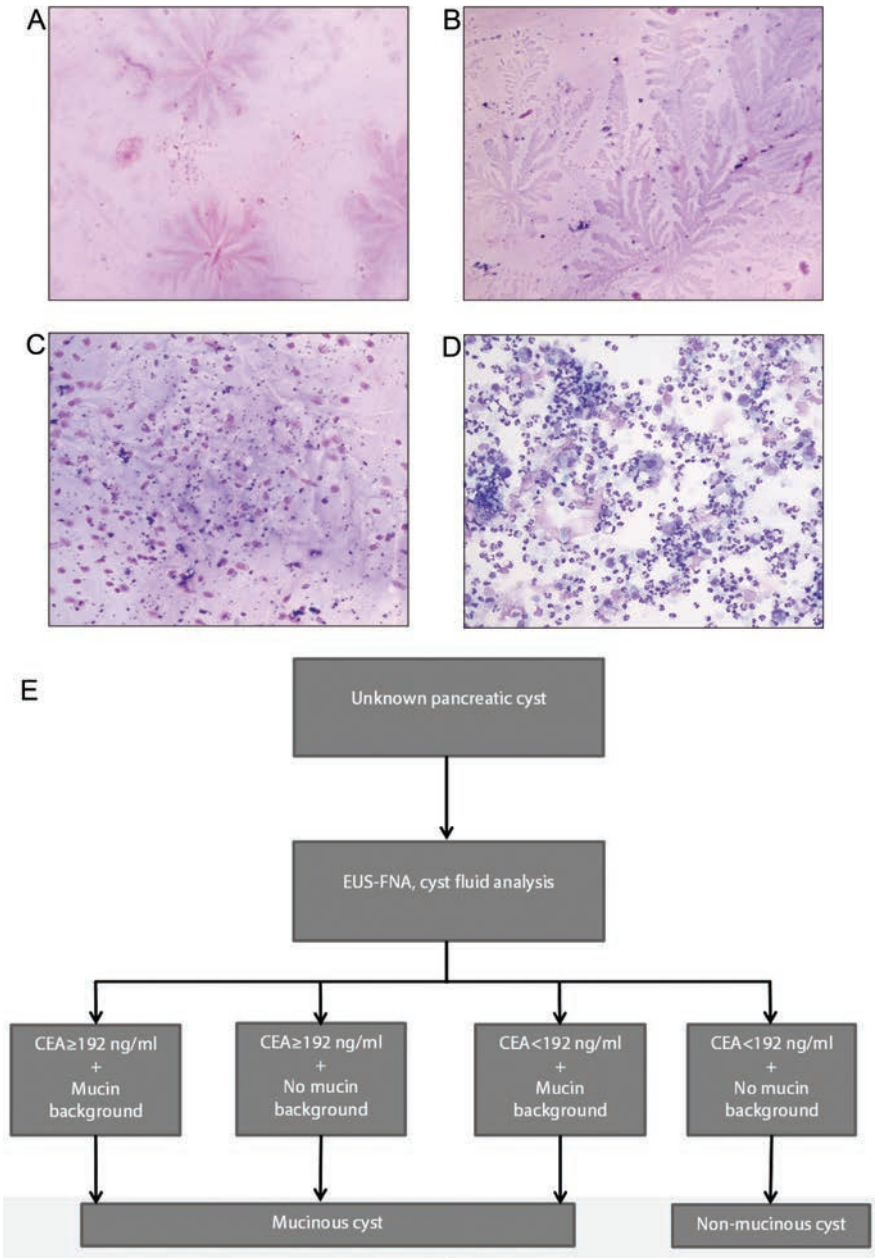

Figure 2. The use of mucinous background in diagnosis of mucinous PCN. Representative microscopical images of mucinous background in cytopathological analysis of pancreatic cyst fluid (A, B, C) and representative image of a reactive background obtained from a pseudocyst without mucin present (D) (200x magnification). (E) proposed diagnostic algorithm incorporating the use of pancreatic cyst fluid obtained by EUS-FNA.

between mucinous PCN and non-mucinous cysts. However, preoperative discrimination of high-grade from low-grade PCN is still problematic and requires novel biomarkers and long term surveillance data to better predict the course of the PCN, especially those with low rate of progression, including MCN and SB-IPMN.

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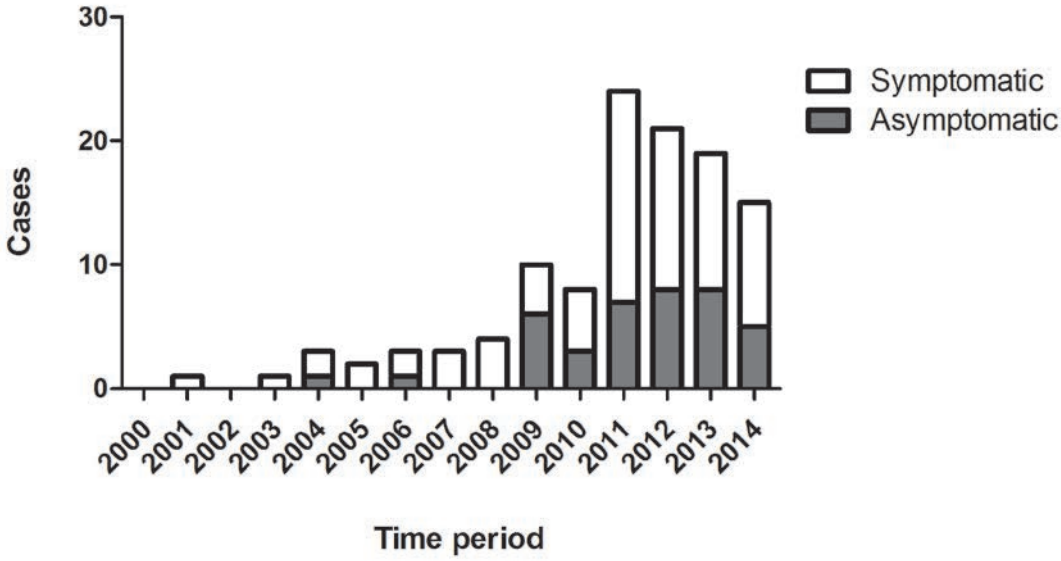
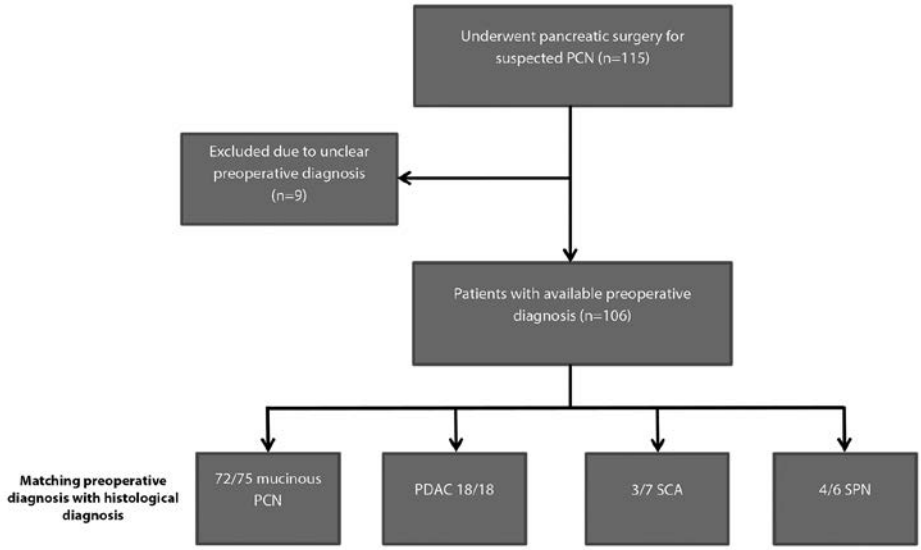
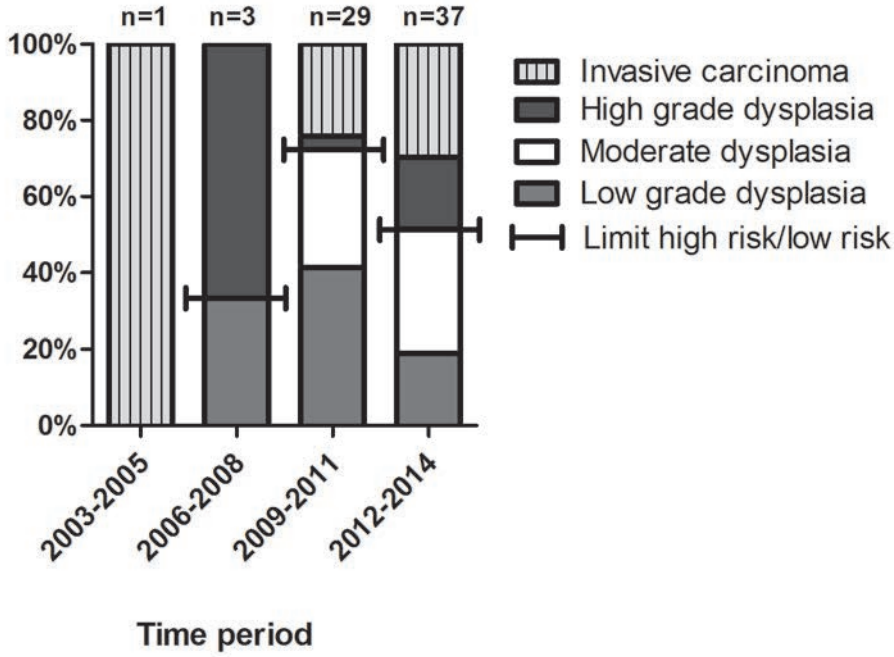
SUPPLEMENTARY MATERIALS



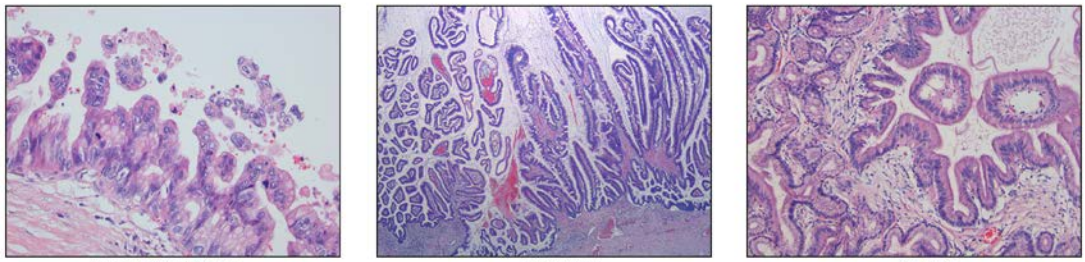
Supplementary Figure 1. Increase in number of PCN over the years. The initial presentation of patients with a PCN was classified in symptomatic or asymptomatic. Patients were considered symptomatic when initial presentation included abdominal pain, jaundice, acute pancreatitis, chronic pancreatitis, weight loss or other complaints. Asymptomatic presentation includes discovery of the lesion incidentally or due to surveillance for familial risk.



Supplementary Figure 2. Flowchart for evaluation of diagnostic performance of all PCN resections. Patients were excluded when preoperative diagnosis was not clearly stated or when multiple imaging modalities contradicted. IPMN and MCN were clustered as mucinous PCN. All preoperatively diagnosed pancreatic ductal adenocarcinoma (PDAC) were histopathologically confirmed as such. Numbers are presented as 'correct diagnosed/total type PCN'.



Supplementary Figure 3. Distribution of dysplasia in resected IPMN over the years. The rate of resections for low risk PCN, while declining in the last few years, remains as high as 51.4% between 2012 and 2014.



Supplementary Figure 4. Different subtypes of IPMN as can be evaluated with regular H&E staining (200x magnification).

	Un-known	No dysplasia	Low grade dysplasia	Moderate dysplasia	High grade dysplasia	Invasive carcinoma	Total	High risk cyst (%)
IPMN								7/12 (58.3)
Pancreatobiliary	0	0	3	2	1	6	12	6/23 (26.1)
Intestinal	0	0	6	11	3	3	23	4/19 (21.1)
Gastric	1	0	10	4	0	4	19	2/2 (100)
Oncocytic	0	0	0	0	1	1	2	2/2 (100)
Tubulo-papillary neoplasia	0	0	0	0	2	0	2	3/5 (60)
Intestinal and Gastric	0	0	0	2	1	2	5	3/5 (60)
Gastric and Pancreatobiliary	0	0	0	2	2	1	5	1/1 (100)
Intestinal and Pancreatobiliary	0	0	0	0	0	1	1	1/1 (100)
Intestinal and Colloid	0	0	0	0	0	1	1	
Unknown	0	0	1	0	0	0	1	
MCN	1	2	20	2	0	2	27	2/26 (7.7%)

Supplementary Table 1. Subtypes and dysplasia of IPMN and MCN patients in retrospective cohort.
In case of adjacent PDAC, the dysplasia of the IPMN was noted.
The dysplasia of 1 case and the subtype of another case could not be retrieved

Variable	Sensitivity, %	Specificity, %	Positive Predictive Value, %	Negative Predictive Value, %	Diagnostic accuracy, %
CEA⩾192 ng/ml	39.0	96.7	94.1	53.7	63.4
Mucin background	66.6	81.8	83.3	64.3	73
CEA⩾192 ng/ml + Mucin background	75.0	79.1	82.7	70.4	76.8

Supplementary Table 2. Diagnostic performance of CEA and the presence of mucin in cytological analysis.

Chapter 3

Validation of a 9-microRNA panel in pancreatic cyst fluid for the risk stratification of pancreatic cysts in a prospective cohort

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Submitted

Chapter 4

DNA integrity as biomarker in pancreatic cyst fluid

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ABSTRACT

Background Identification of pancreatic cysts with malignant potential is important to prevent pancreatic cancer development. Integrity of cell free DNA (cfDNA) has been described as tumor biomarker, but its potential for pancreatic cancer is unclear.

Methods Normal apoptotic cells release uniformly truncated DNA, whereas malignant tissues release long fragments of cell free DNA (cfDNA). We measured 247 base pair (bp) and 115 bp DNA fragments of ALU repeats by qPCR in serum from healthy controls and pancreatic cancer patients, and in cyst fluid from pancreatic cyst patients.

Result No differences in total cfDNA (ALU115) and cfDNA integrity (ALU247/115) were observed between sera from healthy controls (n=19) and pancreatic cancer patients (n=19). Though elevated as compared to serum, no differences in cfDNA were found in cyst fluid between high risk (n=10) and low risk (n=20) cyst patients.

Conclusion cfDNA integrity is not a useful marker to identify (pre) malignant pancreatic lesions.

INTRODUCTION

Pancreatic cystic neoplasms (PCN) can give rise to pancreatic cancer, with intraductal papillary mucinous neoplasms (IPMN) and mucinous cystic neoplasms (MCN) being the most relevant lesions, whereas serous cystic adenomas have no malignant potential [1]. Development of pancreatic cancer may be prevented by resection of the cysts with high risk malignant potential. Unfortunately, current imaging and diagnostic techniques have difficulty distinguishing low risk cysts from high risk cysts, which in some cases leads to unnecessary surgery [2]. Thus, better differential diagnostic tools are urgently needed. Patients with neoplastic diseases often have an increased amount of free circulating, cell-free DNA (cfDNA) in their peripheral blood, which originates from the tumor [3–5]. This cfDNA is not all of an equal length. While apoptotic cells release small, ~180 base pair (bp) DNA fragments, necrotic cells release larger fragments of irregular size [6]. Whereas apoptosis is a normal physiological process occurring in all cells that need to be cleared from the body, necrosis is a potentially harmful form of cell death, which occurs under pathological conditions, including cancer. Thus, the presence of longer DNA fragments in serum is taken as a sign of enhanced necrosis taking place in the body and is thought to be indicative of disease [7]. DNA fragments can be reliably measured by employing the abundant presence in the human genome of DNA ALU repeats - repetitive ~300 bp sequences found in genomic introns [8]. Using different primers, fragments of these ALU repeats can be detected of either >200 bp (indicative of necrotic DNA), or of <200 bp (detecting both necrotic and apoptotic DNA). Detection of these longer cfDNA fragments and their relative abundance compared to short cfDNA fragments in sera appears to be a promising tool for diagnosis and prognostic prediction of malignancies [9–11]. However, the percentage of cfDNA originating from tumor cells has been estimated to range from 10% to 90% of total cfDNA, and applicability of measuring cfDNA length (i.e. DNA integrity) in serum may therefore depend on the type of disease [6]. Thus, while ALU-repeat measurements have been shown to adequately predict colorectal and breast cancer, the presence of pancreatic cancer could not be diagnosed by high length cfDNA fragments in serum [12]. We speculated that pancreatic cyst fluid, coming from a small and enclosed environment comprising of fluid that is solely produced by the pathologic epithelium, would provide a more suitable biological fluid in which to search for tumor markers.

MATERIAL AND METHODS

Pancreatic cyst fluid acquirement
Pancreatic cyst fluid of patients undergoing surgery was collected from two separate biobanks (Erasmus MC Rotterdam, the Netherlands, MEC-2008-233 and MEC-2012-107, and Hôpital Beaujon, Clichy France; DC-2009-938). Fluid was obtained by endoscopic ultrasound-fine needle aspiration (EUS-FNA) or post-resection and stored sterile at -80°C until analysis. Samples were selected so as to represent the different groups of pancreatic cyst based on malignant potential. Cysts with histologically confirmed low grade and intermediate dysplasia were grouped under 'low grade dysplasia', and cysts with high grade dysplasia or invasive carcinoma were considered 'high grade dysplasia'.

Serum acquirement
Patients with pancreatic adenocarcinoma who were eligible for surgery were included at the EMC. Healthy controls (mean age 60±4 years) were collected from the biorepository of the Rotterdam arm of the ERSPC [13,14] (MEC 138.741/1994/152). Serum was obtained by whole blood centrifugation in serum separator tubes (BD-Vacutainer), aliquoted and stored at -80°C until analysis.

Sample preparation and qPCR
After preparation of samples [15], DNA integrity was determined by measuring the presence of ALU repeat fragments of 115 bp size and of 247 bp size, using previously described primers [16]. The ALU115 primers are designed to amplify both the shorter and the longer fragments, and are therefore indicative of total circulating cfDNA (including DNA released from both apoptotic and necrotic cells) whereas the ALU247 primers only amplify the longer DNA fragments, and thus detect of tumor DNA. For full protocol and details see Supplementary Material.

RESULTS

No difference in cfDNA fragment length in sera from pancreatic cancer patients versus controls.
While previous reports were unable to find a relationship between pancreatic cancer and cfDNA length in serum, we wanted to verify this in our own cohort (see **Table 1**). The mean total circulating cfDNA, as represented by ALU115-qPCR values, was 20±3 pg/µl in control sera (n=19) vs 36±14 pg/µl in sera from pancreatic cancer patients (n=19) (p=0.559) (**Figure 1A**), whereas the mean amount of circulating tumor DNA, as

Number of patients	19
Age, years	
Range	24-82
Mean (SD)	65 (13)
Gender, n (%)	
Male	11 (57.9)
Female	8 (42.1)
Disease location (%)	
Pancreas corpus	3 (15.8)
Pancrease head/uncinate	5 (26.3)
Pancreas head	7 (36.9)
Bile duct	3 (15.8)
Papilla	1 (5.2)

Table 1: Patient characteristics of the serum samples

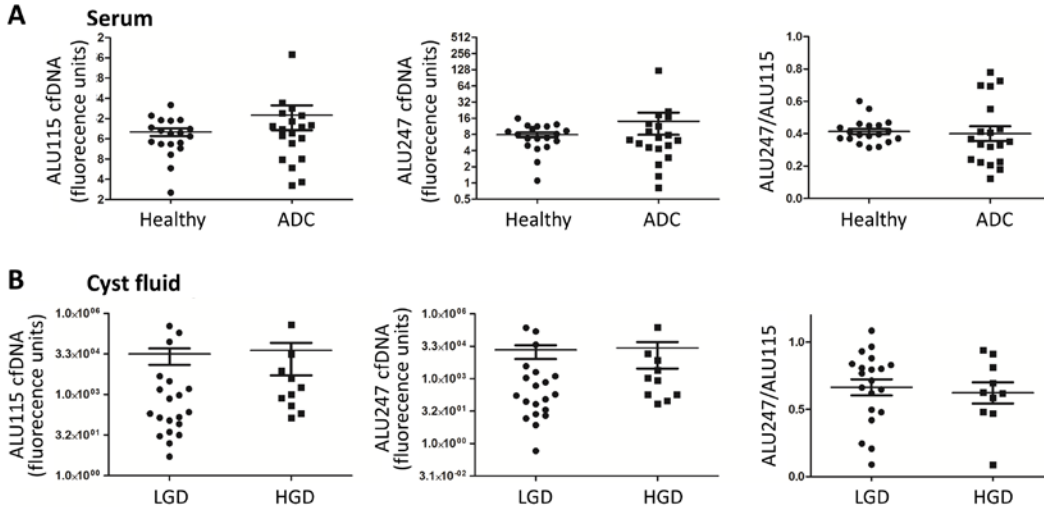

Figure 1. Levels of cfDNA in serum or pancreatic cyst fluid do not identify high risk/ adenocarcinoma patients. PCRs detecting short, apoptotic cell-derived cfDNA (ALU115) and longer cfDNA fragments (ALU247) were performed on (A) serum from healthy donors (n=19) and patients with pancreatic adenocarcinoma (ADC, n=19) and (B) pancreatic cyst fluid from patients with low grade dysplasia (LGD, n=20) and high grade dysplasia (HGD and ADC, n=10). cfDNA was detected by ALU115 and ALU247, and DNA integrity was calculated (ALU247/ALU115). No differences in cfDNA levels and cfDNA integrity were observed.

	Total	LGD	HGD
Number of patients	30	20	10
Age, years			
Range	19-85	19-79	52-85
Mean (SD)	59.5 (15.3)	54.7 (15.4)	69.2 (10)
Gender			
Male	10 (33.3%)	3 (15%)	7 (70%)
Female	20 (66.7%)	17 (85%)	3 (30%)
Diagnosis			
IPMN	17 (56.7%)	8 (40%)	9 (90%)
MCN	13 (43.3%)	12 (60%)	1 (10%)
Disease location (%)			
Pancreas head	6 (20%)	2 (10%)	4 (40%)
Pancreas corpus	5 (16.7%)	3 (15%)	2 (20%)
Pancreas tail	18 (60%)	15 (75%)	3 (30%)
Unknown	1 (3.3)	0 (0%)	1 (10%)

Table 2: Patient characteristics of the cyst fluid samples

determined by ALU247-qPCR values, was 8±1 and 14±6 pg/µl (p=0.793). The ratio of ALU247/ALU115, allowing quantification of the integrity of the cfDNA, was 0.41±0.02 and 0.40±0.05 for healthy controls and pancreatic cancer patients, respectively (p=0.267). Thus, no increased total cfDNA or tumor-associated DNA was detected in sera from pancreatic cancer patients.

No difference in cfDNA fragment length in pancreatic cyst fluid between low risk and high risk cyst patients.

Next, we analysed cyst fluid obtained from 40 pancreatic cyst patients, 23 of which had low risk cysts, and 17 had high risk cysts. In 10 of these samples, we were unable to perform a reliable analysis due to the mucinous nature of the fluid. In the remaining samples (**Table 2**), we observed drastically higher levels of cfDNA as compared to sera, presumably due to the enclosed nature of these cysts. However, the mean amount of total and tumor circulating cfDNA did not differ between the high risk (n=10) and low risk (n=20) samples: 44,463±39,228 vs 33,021±20,004 pg/µl for ALU115 (p=0.10) and 27,254±24,2682 vs 22,118±13,774 pg/µl for ALU247 (p=0.18), respectively (**Figure 1B**). Furthermore, no significant differences in ALU247/ALU115 ratio between high risk and low risk cysts (0.63±0.07 vs 0.66±0.06, p=0.34) was seen. Overall, ratios were higher than in serum,

with some samples reaching almost 1. Thus, the nature of cyst fluid makes it a less suitable compartment to determine necrotic/apoptotic cfDNA ratios.

DISCUSSION

Measurement of necrotic cell-derived long cfDNA fragments in serum has been suggested for the early detection of tumors. However, usefulness of this tool in pancreatic diseases has so far not been shown, and we were unable to find increased levels of necrotic cell-derived cfDNA in sera from pancreatic cancer patients. As pancreatic cancer can derive from PCN, we speculated that cyst fluid would present the ideal biological fluid to detect premalignant lesions. Indeed, total levels of cfDNA observed in cyst fluid were almost 1000 fold higher as compared to sera. Nevertheless, we did not observe differences in cfDNA length between high risk and low risk cysts. While the apoptotic process reduces DNA to 180-200 bp fragments, incomplete cleaving of the DNA may result of the presence of multimers of these fragments, which can subsequently also be detected by ALU247 primers. This background 'noise' of 180 bp multimers accounts for the fact that a signal is detected in the ALU247 PCR in samples were no necrotic cfDNA is expected (i.e. healthy serum), and detection of tumor-derived, necrotic DNA depends on a relative increase in the abundance of long cfDNA, and hence a shift in DNA integrity (ALU247/ALU115 ratio). It is conceivable that pancreatic tumor cells produce too little necrotic cfDNA to be detected above background levels. Additionally, in cyst fluid, high levels of total cfDNA levels present may preclude detection of additional long cfDNA fragments.

We acknowledge several limitations to our study. Of the cystic fluid samples selected for this pilot study, 15 of 30 were obtained after resection, with ischemic damage potentially causing necrosis. However, subanalysis of the re-resection and post-resection obtained samples did not show significant differences in total cfDNA levels or ALU247/ALU115 ratios (not shown).

A second limitation is the low number of high risk cyst fluid samples in our analysis. The mucinous nature of the fluid prevented accurate analysis in ~25% of cases. As mucinous cysts show a higher malignant potential, it is not surprising that many of the excluded samples were high risk. sThis means that the intrinsic nature of high risk cyst fluid makes them less suitable for this type of analysis.

In conclusion, despite favorable reports for early tumor detection and the application of pure cyst fluid analysis, our data suggest that cfDNA integrity is of no additional use to discriminate low from high risk pancreatic cysts. This technique therefore does not provide further guidance in the management of patients with asymptomatic pancreatic cysts.

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SUPPLEMENTARY MATERIAL AND METHODS

To digest proteins that might confound results, both cyst fluid and serum were mixed with a buffer containing 25 ml/l Tween 20, 50 mmol/l Tris, 1 mmol/l EDTA, and 0.8 mg/ml proteinase K in a 1:1 ratio. Subsequently, the mix was incubated at 50°C for 20 minutes, followed by heat inactivation at 95°C for 5 minutes. Next, the samples were centrifuged at 10,000g for 5 minutes and 0.2 µl of the supernatant was used in the qPCR reaction. This protocol was also described earlier [13].

To measure the absolute concentration of DNA in the samples, we constructed a calibration curve using genomic DNA derived from Huh7 cell lines at a concentration ranging from 2.97 pg/µl up to 297 ng/µl. The absolute concentration was measured (in the most concentrated sample) using the Nanodrop (Thermo Scientific). This was subsequently used in a serial dilution and used as a template in triplicate on each qPCR-plate measured. The same serial dilutions were used to produce standard curves for all qPCR runs.

For the qPCR reaction of the ALU repeats, previously published primers were used: ALU115 forward, CCTGAGGTCAGGAGTTCGAG; ALU115 reverse, CCCGAGTAGCTGGGATTACA; ALU247 forward, GTGGCTCACGCCTGTAATC; ALU247 reverse CAGGCTGGAGTGCAGTGG[14]. The ALU115 primers are designed to amplify both the shorter and the longer fragments, and are therefore indicative of total circulating cfDNA (including DNA released from both apoptotic and necrotic cells) whereas the ALU247 primers only amplify the longer DNA fragments, and thus detect of tumor DNA. The total volume of the qPCR reaction mix was 25 µl, consisting of 12.5 µl SYBR Green (Life technologies), 2.5 µl 10 µM forward and reverse primer, 9.8 µl Microbial DNA-Free Water (Qiagen), and 0.2 µl template.

The qPCR was run at 95°C for 10 minutes, and subsequently at 95°C for 30 seconds, 64°C for 30 seconds and 72°C for 30 seconds for 40 cycles using the StepOnePlus™ Real-Time PCR System (Life technologies). ALU repeat expression levels were measured in duplicate. The genomic DNA used for the calibration curve and negative controls were measured in triplicate.

Analysis

Using the data obtained from the serial diluted genomic DNA, we constructed a calibration curve on each plate measured using the Graphpad Prism 5. From this, we derived the intercept and slope of the curve using a nonlinear regression model and recalculated the absolute concentration of

the samples from measured Ct values using the following formula:

Absolute concentration = 10^((Ct-intercept)/slope)
Finally, to obtain ALU247/115 ratios, the absolute concentration of ALU247 was divided by the absolute concentration ALU115 measured in the samples. Mean differences were analysed using Mann-Whitney U test. A p-value of <0.05 was considered statistically significant.

DNA integrity as biomarker in pancreatic cyst fluid

Chapter 5

mTOR is a promising therapeutical target in a subpopulation of pancreatic adenocarcinoma

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) remains a highly lethal disease, unusually resistant against therapy. It is generally felt that stratification of patients for personalized medicine is the way forward. Here, we report that a subpopulation of PDACs shows strong activation of the mTOR signaling cassette. Moreover, we show that inhibition of mTOR in pancreatic cancer cell lines showing high levels of mTOR signaling is associated with cancer cell death. Finally, we show using fine needle biopsies the existence of a subpopulation of PDAC patients with high activation of the mTOR signaling cassette and provide evidence that inhibition of mTOR might be clinically useful for this group. Thus, our results define an unrecognized subpopulation of PDACs, characterized by high activation of mTOR and show that identification of this specific patient group in the early phase of diagnosis is feasible.

INTRODUCTION

mTOR is a promising therapeutical target in a subpopulation of pancreatic adenocarcinoma

Pancreatic ductal adenocarcinoma (PDAC) is the 4th leading cause of cancer related death in the world and more than 260,000 people die of this disease every year worldwide [1]. Due to local invasion of vasculature or distant metastasis, only 15-20% of patients are surgical candidates at presentation. Of those operated, the 5-year survival is only 10-15% and adjuvant therapy only improves disease free survival from 5.5% to 16.5% at 5 years but has no impact on survival [2]. The median survival after diagnosis of locally advanced unresectable disease is 4-6 months, and 2-4 months for patients presenting with metastatic disease [3,4]. PDAC is among the most chemoresistant cancers and advancements in traditional chemotherapeutics have been especially disappointing as many targeted therapies have failed to show any benefit. Current palliative therapy is limited to patients with optimal performance score (WHO 0-1) with only 4 months survival benefit in patients with metastatic disease (FOLFIRINOX) [5]. Research efforts are focused on early detection, and multimodality treatment with surgery and chemoradiation. Personalized medicine-based therapy might be another approach to achieve significant long term benefit in patients with PDAC [6].

PDAC is a heterogeneous disease with different pathways affected in different patients. Next generation sequencing and microarray analysis have revealed a set of 12 core cellular signaling pathways and processes that were genetically altered in 67-100% of PDAC tumors. These genetic alterations involve pathways such as apoptosis, KRAS, Hedgehog, WNT/Notch, TGF-beta, and DNA repair pathways [7]. Mutations in the KRAS gene along with activation of EGFR and loss of telomeres are required for the initiation of PDAC [8, 9, 10]. The progression of PDAC requires the constitutive activation of Ras/mammalian target of Rapamycin (mTOR) or Ras/MEK/ERK pathways [11, 12, 13]. In PDAC, levels of phospho S6 (pS6), the activated form of a downstream protein of the mTOR pathway involved in translation initiation, are markedly increased [14,15,16]. Moreover, mTOR pathway activation is shown in pancreatic cancer cell lines, tumor xenografts, human pancreatic tumors, and in a number of other human tumors [17, 18, 19]. Previous studies have shown mTOR pathway activation in PDACs, approximated between 25%-75% [14, 19]. The mTOR pathway consists of two protein complexes, in which mTOR, raptor and mLST8 proteins constitute to form the mTOR Complex 1 (mTORC1) and mTOR, rictor and Sin1 proteins forming mTOR complex 2 (mTORC2). In cellular growth and associated proliferation, mTORC1 plays a vital role by integrating signals from nutrients and energy status. It

regulates several processes like ribosome biogenesis, protein synthesis, metabolism and autophagy [20]. mTORC2 plays a role in cytoskeletal organization through protein kinase C and paxillin [21]. Rapamycin, and its synthetic derivatives (rapalogs), can inhibit the mTOR pathway by binding to FK-binding protein-12, which in turn binds to the mTOR protein, and subsequently preventing the assembly of mTORC1 [22]. Prolonged use of rapalogs has shown to disrupt mTORC2 as well [23]. Several clinical trials involving rapalogs showed clinical benefit in only a minority of pancreatic cancer patients, however none of the studies involved a sensitivity assay of the tumor to rapamycin or rapalogs [17, 24]. In view of the molecular heterogeneity of PDAC, the activity of the Ras/mTOR pathway, and incidental benefit of rapalog treatment in PDAC, we hypothesize that a subpopulation of PDAC patients sensitive for rapalog treatment could be identified using ex vivo biopsies. Hence, the mTOR axis can be a promising target to be included in treatment protocols for PDAC using rapamycin or rapalogs in a subpopulation of patients.

MATERIAL AND METHODS

Cell lines

Pancreatic cancer cell lines BxPC3, Su86.86, HPAF, and HS700T were cultured as confluent monolayers in RPMI-1640 (Gibco) with penicillin and streptomycin (invitrogen) and 7.5% Fetal Calf Serum (FCS) (Sigma-Aldrich) using routine procedures (5% CO2, at 370C). Capan-1 was cultured using IMDM (Gibco) supplemented with 20% FCS. The cell lines were a kind gift of the department of surgery of the Erasmus MC. The cell lines were authenticated by means of a STR-analysis.

Patients and specimens

Appropriate ethical approval was obtained for all procedures involving patients or patient material. We included 64 slides of 39 formalin fixed paraffin embedded (FFPE) specimens from pancreatic surgery with a histologically confirmed PDAC or neuroendocrine tumor. The tissue blocks were collected from a prospectively maintained pathology tissue bank at the Erasmus MC. Specimens were sectioned at 5 µm (Microm HM325 Microtome), incubated at 370C overnight, and stored until used for immunohistochemistry staining.

Endoscopic ultrasound guided fine needle aspiration biopsies (EUS-FNABs) were obtained from patients suspected for pancreatic cancer (or with a pancreatic mass lesion). All patients provided written informed consent. The EUS-FNABs were obtained from the endoscopy department

and transported to the laboratory in RPMI-1640 medium with 10% FCS. The biopsy was washed three times with PBS containing penicillin and streptomycin (Invitrogen). Single cell suspensions were prepared using 0.5 mg/ml collagenase IV (Sigma-Aldrich) and pushed through a 100 µm cell strainer (BD Falcon). The cells were suspended in RPMI-1640 (Gibco) and counted using a slide with counting grids (Kova Glasstic® Slide 10, Hycor Biomedical ltd., Penicuik, UK).

Immunohistochemistry

Tissue sections were deparaffinized in xylene for 5 minutes and rehydrated using ethanol. The endogenous peroxidase activity was blocked with methanol and 3% H2O2 followed by tap water immersion for 5 minutes. Antigen retrieval was performed by boiling in preheated buffer 10 mM citrate buffer pH 7.6 for 10 min at 200W in a microwave. Next, slides were blocked by 10% goat serum in phosphate-buffered saline tween pH 7.4 for 1 hour at room temperature. Primary antibodies rabbit monoclonal anti-pS6 (1:250, Cell Signaling Technology, Beverly, MA, USA) was added and incubated at 4°C overnight. Envision goat anti-rabbit-horseradish peroxidase (DAKO, Denmark) was used as secondary antibody [25]. WKU and VN scored the slides independently in a blinded manner. Five high power fields were counted for each slide. The percentage of cells that stained positive (immunoreactivity above background) in the area was quantified. The pS6 level was scored as follows: a score of 0 for less than 3%, a score of 1 between 3% and 10%, a score of 2 between 10% and 50%, and a score of 3 for more than 50% of positively stained cells (scoring system developed by KB). Pictures were taken using the Zeiss Axioskop20 microscope, Nikon Digital Sight DS-U1 camera and NIS-Elements 3.00 program.

Cell viability assay

To assess the effect of rapamycin on pancreatic cancer cell lines we used a MTT-assay. In short, 10,000 cells from each cell line were incubated with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The purple formazan is measured at 490 nm and 595 nm using a microplate reader (Model 680XR Bio-Rad) at 72 hours. The functional viability of cells was calculated using the mean OD in sample well divided by the mean OD in the control well x 100%. To show the correlation of increasing concentration of rapamycin with cytotoxicity in pancreatic cancer, we used a linear regression to reject the null hypothesis where changes in rapamycin concentration are not associated with increased cell death. The p-value is considered significant when $p<0.01$.

Western blot

Western blotting was performed according to standard fluorescent Odyssey immunoblotting (LI-COR Biosciences, Lincoln, NE, USA). Antibodies specific for p-S6 and light chain 3 (LC3A/B-I and II)(all 1:1000, Cell Signaling Technology) were used. To ascertain equal loading and normalization of the protein for quantification, beta-actin (1:2000, Santa Cruz) was used. The secondary antibodies used for detection were goat anti-rabbit and rabbit anti-mouse (1:5000, LI-COR Biosciences). Transfer membranes were transferred to 50 ml sterile light-protecting centrifuge tubes (Greiner bio-one), incubated with secondary antibodies and washed. Fluorescence Odyssey system (LI-COR Biosciences, Lincoln, NE, USA) was used to visualize and quantify protein expression. Semi quantitative expression data were determined by Odyssey 3.0 software and normalized using beta-actin for reference gene protein expression [25].

Flow cytometry

To assess pS6 expression levels, we analyzed pancreatic cancer cell lines and single cell suspensions prepared from EUS-FNABs as described above, using flow cytometry.

The pancreatic cancer cell lines were washed with 0.1% sodium chloride (Sigma-Aldrich) in PBS and trypsinized with 0.05% trypsin EDTA (Invitrogen). A minimum of 100,000 cells were used for each assay. The cells were divided in the following three conditions during 2 hours: blank unstained cells, RPMI-1640 only (to measure basal pS6 levels), and RPMI-1640 with 0.1 μM rapamycin (to measure inhibition of pS6). The same conditions were applied to single cell suspensions from EUS-FNABs.

Cell permeabilisation was done with a permeabilisation buffer (0.5% saponine, 1% FCS, 0.02% EDTA in PBS). The cells were stained using cytokeratin 8/18 mouse mAb (CK8/18; 1:100; Cell Signaling Technology) and secondary labeled with anti-Mouse IgG eFluor® 660 (1:100; eBioscience, Ltd., UK) to mark epithelial cells. CK8/18 has been shown to be expressed as much as 100% in PDAC [26]. Finally, we stained the samples with V450 Mouse anti-pS6 (1:50; BD Biosciences, Breda, Netherlands). Data was analyzed using FlowJo (v 7.6.5, Treestar, Ashland, OR). Mean Fluorescence Intensity (MFI) was calculated using the geometric mean of the CK8/18+ population. Mean values were compared using a student T-test.

RESULTS

mTOR is a promising therapeutical target in a subpopulation of pancreatic adenocarcinoma

A subpopulation of PDACs is characterized by strong activation of the mTOR signaling cassette

To study activation of the mTOR pathway in pancreatic cancer, we performed an immunohistochemical staining of a set of FFPE specimens that contains 42 normal acinar regions, 42 normal ductal epithelium, 15 PanIN lesions, 7 neuroendocrine tumors, and 39 PDAC regions for the levels of pS6 (**Figure 1**). The proportion of tissue expressing pS6 is 50% in normal ducts, 67% in PanIN lesions, and 82% in adenocarcinoma regions (**Figure 1**). Normal acinar regions show high levels of pS6, while normal duct epithelium exhibits much lower pS6 levels overall (**Figure 2A**). Since it has been shown that the mTOR pathway is deregulated in neuroendocrine tumors (NET), we used NETs as positive controls [27] (**Figure 2B**). Differences in pS6 staining were observed between various patients with PDAC, especially differential staining of dysplastic ducts and stroma can be pronounced (compare **Figure 2C** with **Figure 2D**). The variability in pS6 levels was also seen in various PanIN lesions (**Supplementary Figures 1A and 1B**). Thus, substantial variation in the activation of mTOR pathway exists and our results demonstrate the presence of a subgroup of pancreatic adenocarcinoma that is characterized by very strong activation of the mTOR signaling cassette.

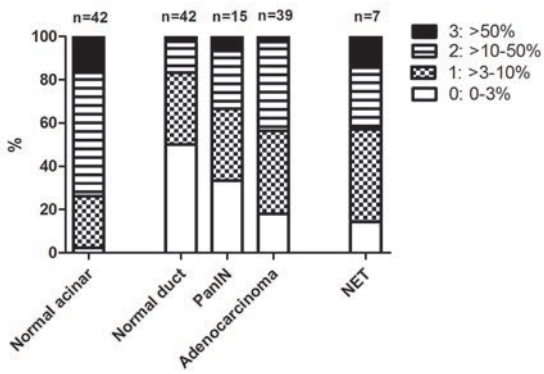

Figure 1. Graph presenting proportion of pS6 levels in various histological conditions. Normal acinar regions were adjacent to PDAC regions. Normal duct represents the fraction of positive ductal epithelium in normal regions. PanIN lesions were also evaluated based on the positive staining of abnormal epithelium. The proportion of samples staining positive for pS6 increases from normal ductal epithelium to PDAC. NETs were used as positive control and had similar distribution to PDACs. The scoring system is described in the materials and methods section.

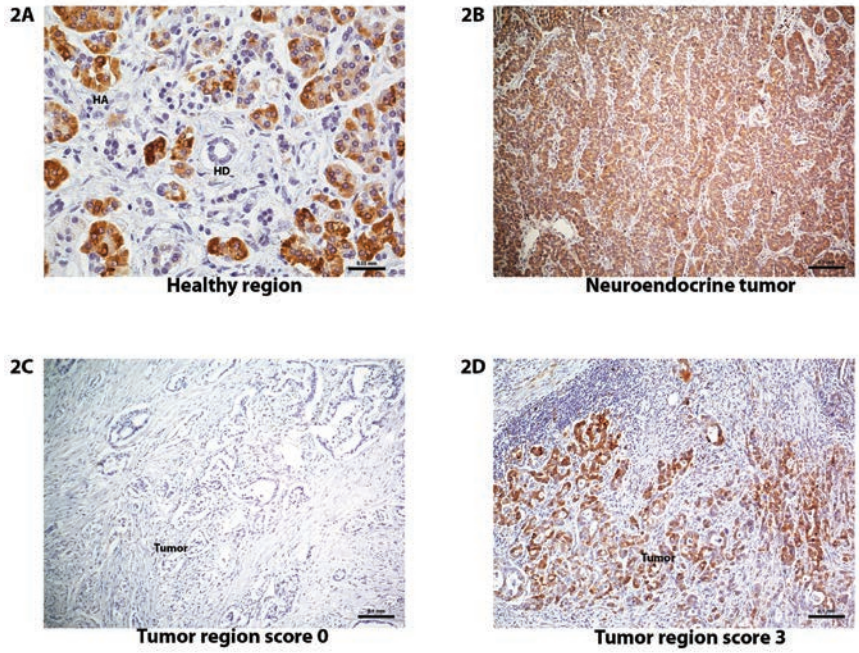

Figure 2A. Representative normal region adjacent of PDAC stained for pS6
Normal duct epithelium (ND) score 0, which was found in 50% of the cases. Normal acinar region (NA) score 3 (20x).

Figure 2B. Representative image of a Pancreatic Neuroendocrine Tumor (NET), score 3 (10x). NETs were stained as positive controls as it has been shown that activation of the mTOR pathway, mediated by IGF-1, is necessary for proliferation in pancreatic NETs [45].

Figure 2C. Representative adenocarcinoma region, score 0 (10x). Staining less than 3% of the adenocarcinoma was observed.

Figure 2D. Representative adenocarcinoma region, score 3 (10x).

Rapamycin-dependent cytotoxicity in pancreatic cancer cell lines correlates with the level of mTOR-dependent signaling

A logical question arising from our discovery of a mTOR hyperactivated subset of PDAC is whether cancer survival is dependent on activation of this signaling cassette in such cancers. To this end, we compared pancreatic cancer cell lines exhibiting different levels of mTOR activation for their sensitivity to the mTOR inhibitor. We determined pS6 levels in BxPC3, Su86.86, HS700T, HPAF, and Capan-1 and its inhibition after 2,

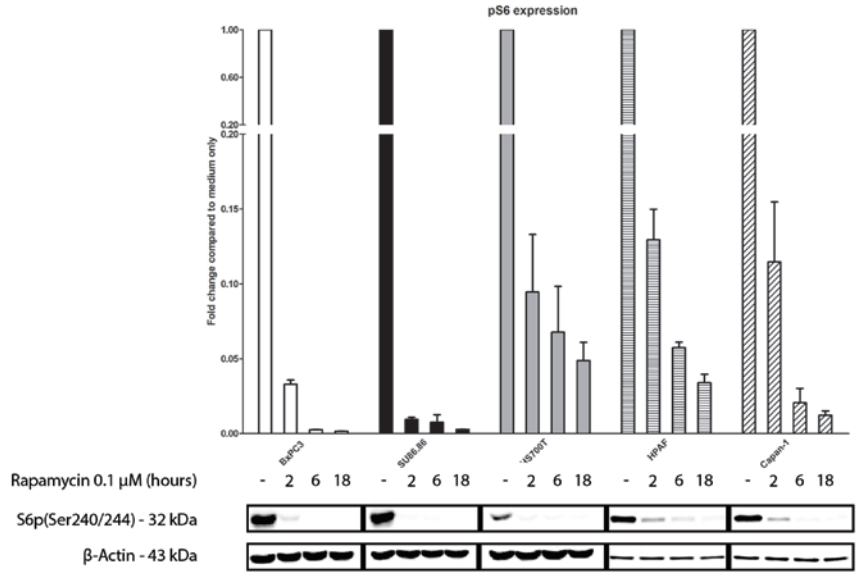

Figure 3. Western blot analysis and quantification of phosphorylated S6 in BxPC3, SU86.86, HS700T, HPAF, and Capan-1. The cell lines were exposed to increasing duration of 0.1 µM rapamycin. Lysates were subjected to pS6 antibodies and subsequently quantified and normalized against beta-actin. Higher basal expression of pS6 is seen in BxPC3 and SU86.86 compared to HS700T. In BxPC3, rapamycin inhibits phosphorylation of S6 by 30, 392, and 655-fold after 2, 6, and 18 hours respectively with rapamycin. Similarly, in SU86.86 this effect is 106, 132, and 355-fold in the same conditions. HS700T is less rapamycin-sensitive as seen by a less pronounced decrease of pS6 expression (11, 15, and 20-fold decrease). The inhibition of pS6 levels in HPAF and Capan-1 were also lower, exhibiting a 8, 17, and 29-fold decrease in HPAF, and a 8, 48, 82-fold decrease in Capan-1. Experiment was performed in duplicate, one representative blot is shown.

6, and 18 hours incubation with 0.1 µM rapamycin. BxPC3 and SU86.86 are most sensitive to rapamycin with a decrease of pS6 expression up to 655 and 355-fold ($p<0.01$) in Western blot respectively (**Figure 3**), and thus, display most mTOR signaling activity. Rapamycin induced decrease of pS6 expression was less pronounced in Capan-1, HPAF, and HS700T cells, with up to a 10-fold decrease after 2 hours (**Figure 3**), and therefore, these cell lines are characterized by a substantial lower degree of rapamycin sensitivity. The level of rapamycin-sensitive mTOR activity correlated well with the cytotoxic effects of rapamycin. In BxPC3 (**Figure 4A**) and SU86.86 (**Figure 4B**) 12.5 nM rapamycin decreases cell viability to 69%(±10.0%) and 75%(±4.0%), respectively. In contrast, HS700T, HPAF, and Capan-1 pancreatic cancer cells show much less pronounced mTOR activation. Accordingly, Capan-1 (**Figure 4C**) and HPAF (**Figure 4D**) show

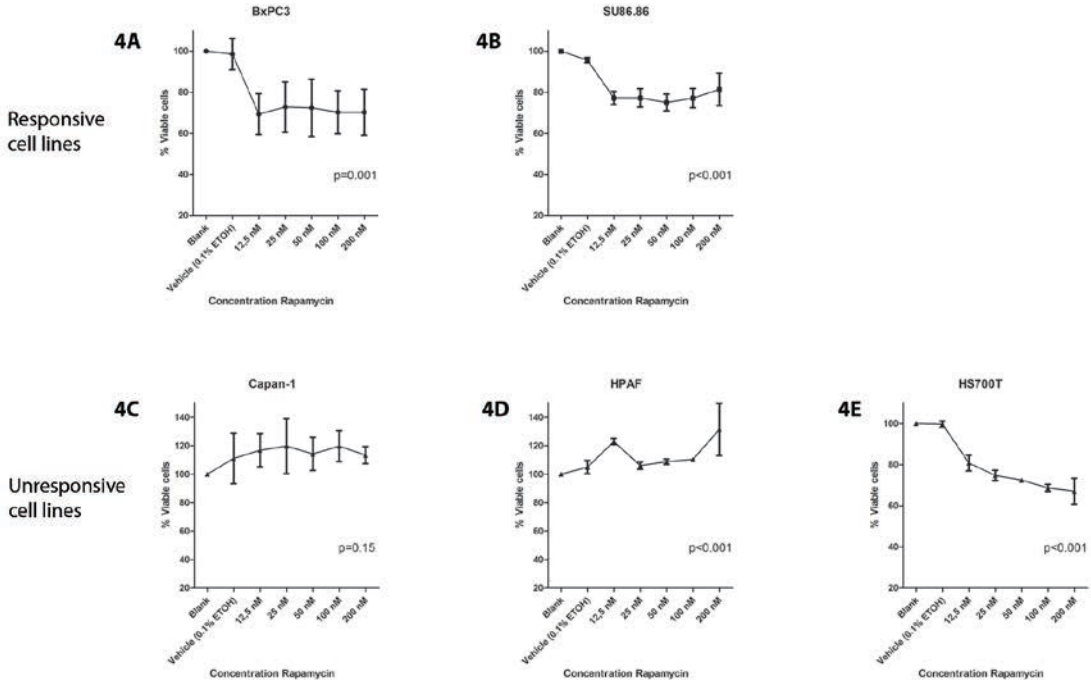

Figure 4. Graphs depicting the effect of rapamycin on cell viability in pancreatic cancer cell lines. BxPC3 (A), SU86.86 (B), Capan-1 (C), HPAF (D), and HS700T (E) were treated with increasing doses of rapamycin for 72 hours. Mean values are averages from 3 independent experiments performed in triplicate.

no response to rapamycin, exhibiting cell viabilities of 113.4%(±6.0%) and 131.4%(±18.4%) at 200 nM concentration. HS700T cells shows 80.8%(±1.9%) cell viability in the presence of 12.5 nM rapamycin and more than 50 nM rapamycin is needed to obtain a comparable decrease in cell viability as in BxPC3 cells (**Figure 4E**). Thus, high activation of mTOR shows a correlation with increased sensitivity to mTOR inhibition in pancreatic cancer. To illustrate the correlation between pS6 inhibition and cell viability we plotted both parameters for all cell lines. The mean pS6 inhibition after 2 hours incubation with 0.1 μM rapamycin of both responsive and non-responsive cell lines (derived from western blot) was plotted against the percentage of cell viability as measured by MTT-assay with the same concentration of rapamycin (**Figure 5**). Sensitivity to mTOR inhibition is correlated with decreased cell viability for the rapamycin responsive cell lines but not for the rapamycin unresponsive cell lines.

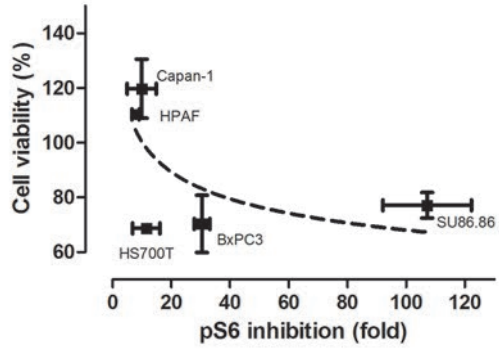

Figure 5. Scatter plot showing the correlation of pS6 levels and predicted cytotoxicity. On the x-axis is the fold pS6 inhibition (retrieved from western blot with 2 hours 0.1 μM rapamycin) and cell viability on the y-axis (retrieved from MTT-assay with 0.1 μM rapamycin). A non-linear regression curve was fitted through the available datapoints indicating that higher inhibition of pS6 levels correlate with higher rapamycin-dependent cytotoxicity. Responsive cell lines (BxPC3 and SU86.86) are in the higher range of pS6 inhibition, corresponding with lower cell viabilities. In contrast, lower pS6 inhibition in cell lines correspond to better survival of pancreatic cancer cells(Capan-1 and HPAF). HS700T however, exhibit declining cell viability without proper mTOR inhibition, most likely due to off-target effects.

Rapamycin induces autophagy in rapamycin sensitive pancreatic cancer cell lines

Subsequently, we were interested in the molecular mechanism of cytotoxicity in mTOR-proficient pancreatic cancer cell lines. In the canonical response to rapamycin, induction of autophagy and apoptosis are considered the major effectors here. To assess the role of autophagy and apoptosis in rapamycin induced decrease of cell viability of pancreatic cancer cell lines, we performed Western blot analysis on LC3A/B-I and II. The expression of LC3A/B-II increased 2.4 fold in both BxPC3 and SU86.86, after 18 hours incubation with rapamycin (**Figure 6**). In contrast to BxPC3 and SU86.86, we did not observe a significant increase of LC3A/B-II in HS700T, HPAF, or Capan-1 (**Figure 6**). These results support a role for hyperactivated mTOR for maintaining survival in a subpopulation of PDAC.

Sensitivity to rapamycin in pancreatic cancer cell lines can be measured using flow cytometry

We have shown in Western blot that rapamycin reduces phosphorylation of S6 in pancreatic cancer cell lines. To translate this into an ex vivo sensitivity assay, to a protocol useful for personalized medicine in clinical practice, we measured phosphorylation of S6 in pancreatic cancer cell lines

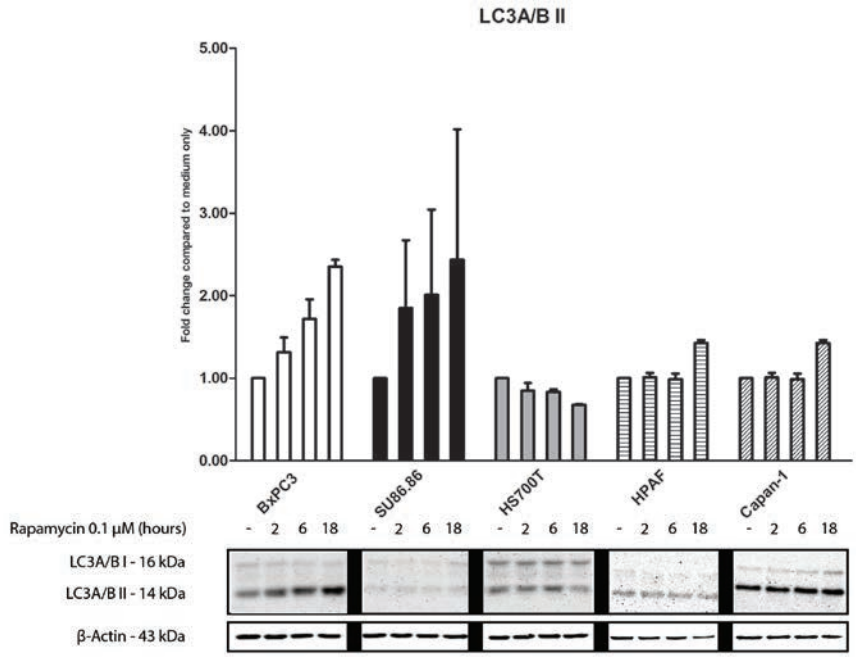

Figure 6. Western blot analysis and quantification of autophagy marker LC3A/B in BxPC3, SU86.86, HS700T, HPAF, and Capan-1. The cell lines were treated with increasing duration of 0.1 µM rapamycin. Next, we prepared lysates and these were subjected to LC3A/B antibodies. The expression of LC3A/B-II increases during prolonged incubation with rapamycin in rapamycin-sensitive cell lines. We quantified the expression of LC3A/B-II, normalized against beta-actin, as indicator of autophagy.

using flow cytometry. Rapamycin resulted in an absolute reduction of CK 8/18+ pS6+ cells by more than 40% in BxPC3 (**Figure 7A**) and SU86.86 (**Supplementary Figure 2**). In contrast, there was a minimal reduction of less than 1% within the CK8/18+pS6+ population in the rapamycin-unresponsive cell lines; HS700T (**Figure 7B**), Capan-1 (**Supplementary Figure 3A**), and HPAF (**Supplementary Figure 3B**). We observed the same effect of rapamycin on the amount of pS6 per cell as shown in **Figure 7C**. Thus, flow cytometry is in principle useful for determining the sensitivity of pancreatic cancer cells to mTOR inhibition.

The mTOR pathway is activated in a subfraction of EUS-FNABs from pancreatic cancer patients and is potently inhibited by rapamycin
Finally, we determined ex vivo mTOR pathway activation in biopsies obtained through endoscopic ultrasonography in patients suspected for pancreatic cancer. Two hours of incubation with 0.1 µM rapamycin inhibited

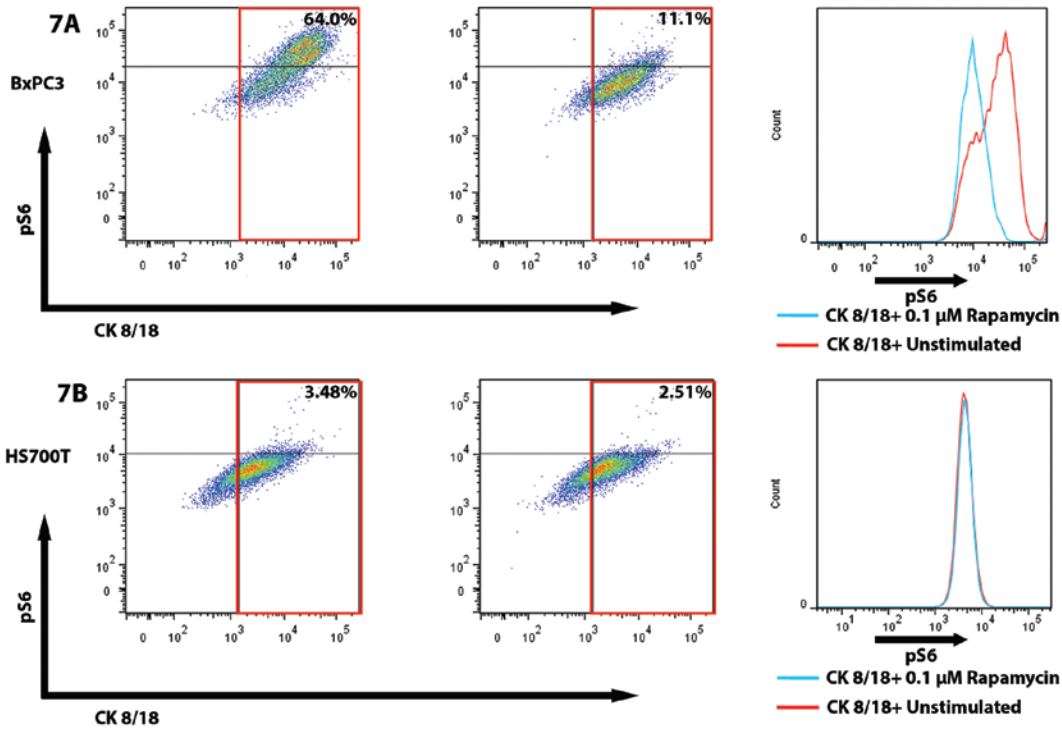

Figure 7. Flow cytometry scatter plots and corresponding overlay histograms of a responsive cell line – BxPC3 (A) and a non-responsive cell line – HS700T (B). In accordance with our western blot data, BxPC3 display higher basal mTOR activation in the CK8/18+ population (64.0%). This CK8/18+pS6+ population was effectively reduced after 2 hours incubation with 0.1 µM rapamycin to 11.1%. In contrast, basal mTOR activation in HS700T was markedly lower (3.48%) and the effect of rapamycin on the CK8/18+pS6+ population was therefore diminished. The overlay graphs are a representation of the count (y-axis), in the CK8/18+ population (marked by the red box), within the pS6-channel (x-axis).

pS6 levels in CK8/18+ cells in two out of nine samples (22%). In those two samples we identified as 'potential responders', rapamycin led to a relative reduction of more than 90% (94.2% and 98.4%) in the amount of pS6 expressing cells. Figure 8 depicts two representative scatter plots and histograms of a patient with high levels of pS6 and response to rapamycin treatment (**Figure 8A**) and a patient with no response to rapamycin (**Figure 8B**). To measure the phosphorylation of S6 per cell, the pS6 MFI of CK8/18+ cells was calculated. Potential responders (n=2) showed a mean reduction in MFI of 81.3% (±2.7%), while the average MFI change in non-responders (n=7) was 8.8% (±6.1%) ($p<0.01$) (**Figure 8C**). Thus, EUS-FNAB and flow cytometry are useful for identifying potential responders to rapamycin therapy.

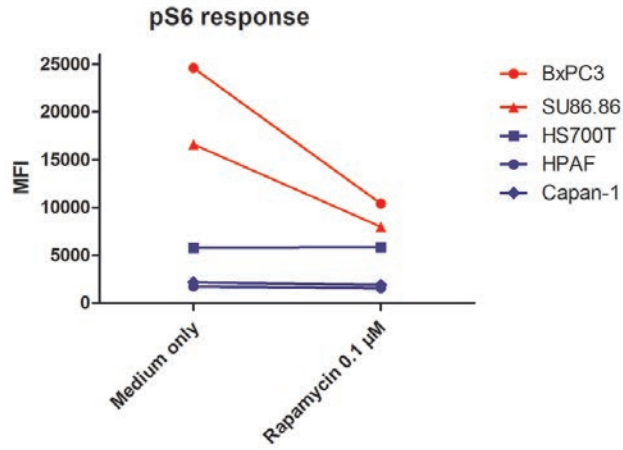

Figure 7C. Graphical overview of the mean fluorescence intensity (MFI) of all cell lines incubated with and without 0.1 µM rapamycin during 2 hours. The MFI change in rapamycin-sensitive cell lines was 57.7% and 51.9% for BxPC3 and SU86.86, respectively. In HS700T the change in MFI was 0.8%. Capan-1 and HPAF exhibited reductions of 9.8% and 13.0%, respectively. The MFI was calculated within the CK8/18+ population only.

DISCUSSION

Our ex vivo analysis on EUS guided FNABs of patients shows that 22% of PDAC patients potentially benefits from treatment with rapalogs. This number is in accordance to what was observed in a preclinical setting in mice, where rapamycin induced regression in 4 out of 17 xenografts (23.5%) [14]. However, the presence of feedback loops after mTOR inhibition can induce therapy resistance in these patients [28]. Consequently, the question remains whether these patients can be treated with rapalog monotherapy or whether rapalog will be part of a treatment protocol. In the coming years it will become clear whether strategies involving combination therapy with second generation mTOR inhibitors which inhibit mTORC1 and mTORC2 [29], dual mTOR/PI3K inhibitors which block the PI3K/Akt feedback activation [30], or the addition of JAK2/STAT5 inhibitors [31] will prove beneficial in PDAC.

Pancreatic cancer is a heterogeneous disease, with various different mutations in individual genes among a diversity of pathways [7]. The idea of tumor heterogeneity in pancreatic cancer is a concept that has been established decades ago [32]. In our immunohistochemistry data we confirmed heterogeneity in terms of mTOR activation. During tumor genesis we observe a gradual increase in mTOR activation from normal

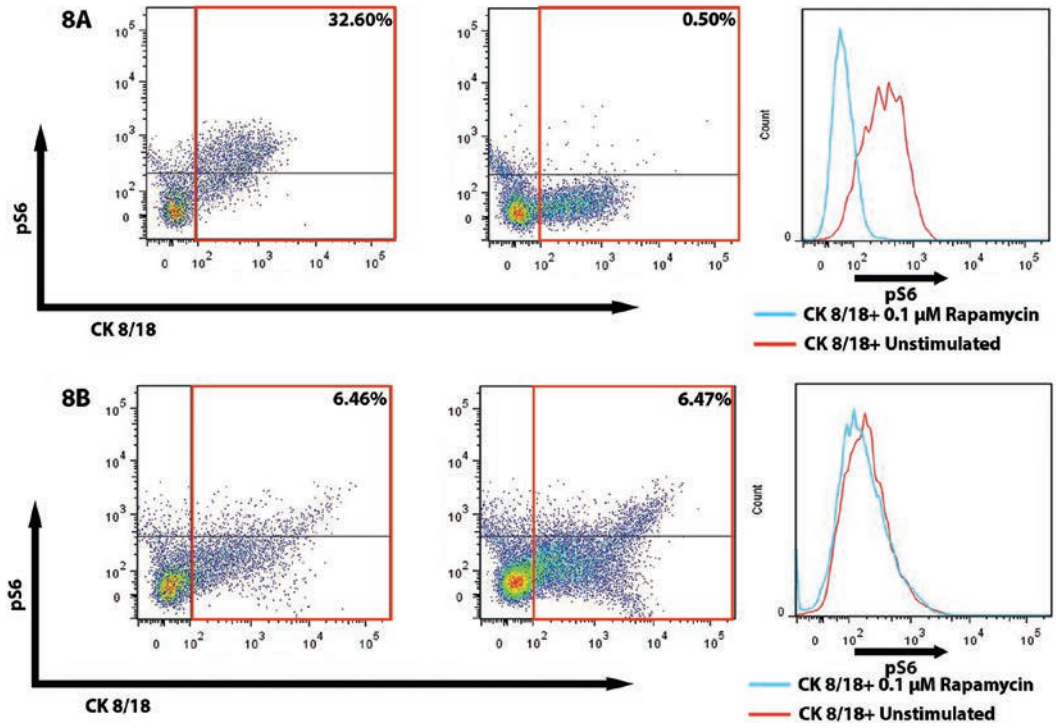

Figure 8. Representative flow cytometry scatter plots and corresponding overlay histograms of a potential responder (A) and non-responder (B). The potential responder exhibited activation in the mTOR pathway which responded to rapamycin. The non-responder showed no change in the CK8/18+pS6+ population after treatment with rapamycin. The overlay graphs are a representation of the count (y-axis), in the CK8/18+ population (marked by the red box), within the pS6-channel (x-axis).

ducts, to PanIN lesion, and finally in PDAC. This heterogeneity is also observed between PDACs, which makes selection of patients necessary to avoid futile treatment. Moreover, we also observed high mTOR activation in normal acinar region. However, the normal acinar regions were taken adjacent to adenocarcinoma so it is debatable whether morphological normal areas are actually normal, considering the yet unknown origin of PDAC [33].

The data from the western blot and MTT-assay show increased cytotoxicity in pancreatic cancer cell lines with hyperactivated mTOR pathway after the addition of rapamycin. Both responsive cell lines (BxPC3 and SU86.86) show significant correlation of increasing dose of rapamycin with cytotoxicity ($p \leqslant 0.001$). In comparison, unresponsive cell lines do not show a clear threshold effect but rather a dose response effect as can be

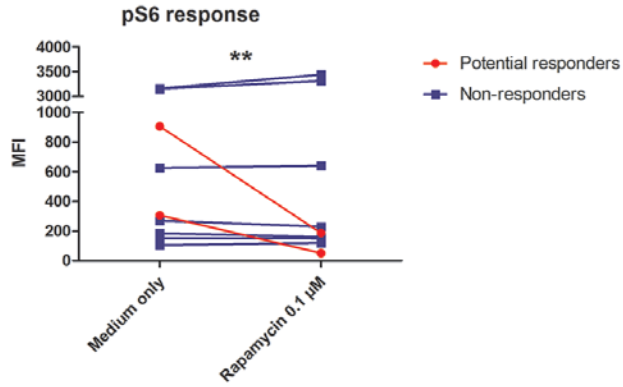

Figure 8C. Graph depicting changes in MFI in potential responders and non-responders. Single cell suspensions from EUS-FNABs were measured using flow cytometry. Each sample was incubated without and with 0.1 µM rapamycin for 2 hours. We selected the CK8/18+ population and calculated the MFI in each condition. Two samples displayed a substantial reduction in MFI by an average of 81.3%. Seven samples were classified as non-responders due to the minimal change in MFI after treatment with rapamycin.

observed in HS700T(most likely due to off-target effects).To further study the mechanism behind increased cytotoxicity, we analyzed autophagy (type 2 cell death) in our pancreatic cancer cell lines. Interestingly, we observed that rapamycin only induced autophagy in rapamycin sensitive pancreatic cancer cell lines. In contrast, some pancreatic cancer cell lines (HPAF, Capan-1, and HS700T) seem unsensitive to rapamycin, which is also reflected in a lower degree of pS6 inhibition and failure to induce autophagy. Rapamycin has been shown to induce autophagy pancreatic cancer cell lines before [34]. In this context however, autophagy was most likely activated due to the antitumor effect of rapamycin rather than a protective response [34, 35]. Whether autophagy in cancer is a prosurvival process or part of antitumor effects is still point of discussion. Yang et al. found that in the case of pancreatic cancer, autophagy is needed for tumorigenic growth [36]. Therefore, they recommend trials in PDAC using drugs targeting autophagy, such as chloroquine.

Using flow cytometry, we show that the responsiveness of a tumor for rapamycin can be quantitatively assessed. Our pancreatic cancer cell line data indicate that hyperactivation of the mTOR pathway in PDAC can be potently inhibited by tolerable concentrations of rapamycin. Hyperactivation of the mTOR pathway is also seen in patients with the Peutz-Jeghers syndrome where mutations in LKB1 can lead to inactivation of the LKB1/AMPK/TSC axis and thereby activating mTORC1, and

eventually leading to the development of tumors [37]. Interestingly, 3-35% of PDACs have been shown to have loss of LKB1 expression in multiple studies [38, 39] and most likely subsequent mTOR activation. To further support the rationale of the use of mTOR inhibitors, Klümpen et al. reported successful use of a rapalog (everolimus) in the treatment of a patient with advanced pancreatic cancer suffering from Peutz-Jeghers syndrome [40], supporting the need for individualized treatment. The question remains how to obtain tumor samples for stratification, and we believe EUS guided FNAB could be helpful in this situation. Phenotyping of these EUS-FNABs using flow cytometry shows that the determination of rapamycin sensitivity in clinical setting is possible, paving the way for personalized medicine.

mTOR is a promising therapeutical target in a subpopulation of pancreatic adenocarcinoma

Our study has some limitations pertaining the translation into an in vivo situation. We could not directly correlate our flow cytometry data of EUS-FNABs with immunohistochemical staining of the same samples due to insufficient material. Furthermore, in our sensitivity assays of EUS-FNABs, we did not include measurements of cell viability. Therefore, solely blocking the mTOR pathway in a patient might not be sufficient to combat the tumor, regardless of sensitivity towards rapamycin or rapalogs.

The results of our study suggest that it is possible to identify a subpopulation of pancreatic cancer patients with mTOR activation that are eligible for treatment with rapalogs. The lack of this test in previous studies with rapalogs might be an explanation for the disappointing results; only 20% would have been sensitive to rapalog treatment. Similarly, some pancreatic cancer cell lines seem totally resistant to mTOR inhibition, which may explain the failure of rapamycin therapy in unselected pancreatic cancer patients. Consistent with previous literature, we found an activation of the mTOR pathway in cell lines, resection specimens, and EUS-FNABs of PDAC [19, 41]. This activation in pancreatic cancer cell lines and EUS-FNABs was effectively blocked by rapamycin in western blot and flow cytometry analysis. Furthermore, inhibition of pS6 levels by rapamycin are a good predictor of later cytotoxicity of the drug or its analogues. Targeting mTOR using rapalogs has recently shown to be efficacious in several neoplastic diseases such as pancreatic neuroendocrine tumors [42], hormone receptor positive breast cancer [43], and renal cell carcinoma after the failure of treatment with sunitinib or sorafenib [44].

We conclude that tumor tissue obtained in a minimal invasive way by means of endoscopic ultrasound guided fine-needle biopsy in chemotherapy naive PDAC patients can be used ex vivo to identify a

subpopulation of approximately 22% of patients that are potentially responsive to rapalog treatment. Such selection of patients for targeted treatment avoids futile treatment and potentially improves the outcome of existing chemotherapeutic regimens. Given our findings, future research should aim for combining ex vivo drug sensitivity analysis using pS6 flow cytometry analysis as biomarker for therapeutic effect with in vivo patient responses to therapy.

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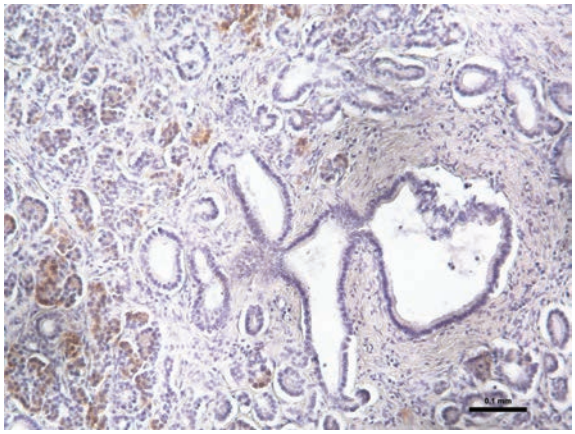
SUPPLEMENTARY MATERIAL

A



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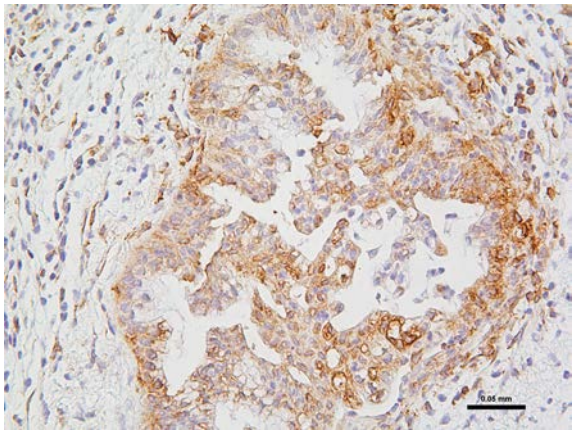
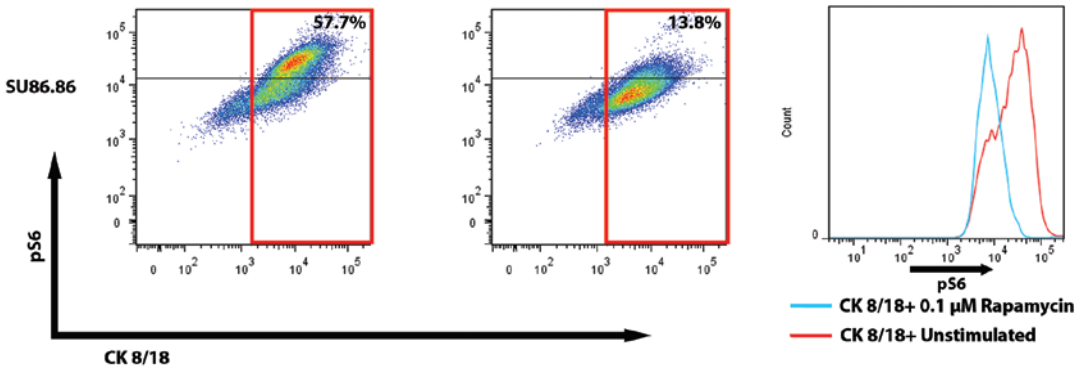
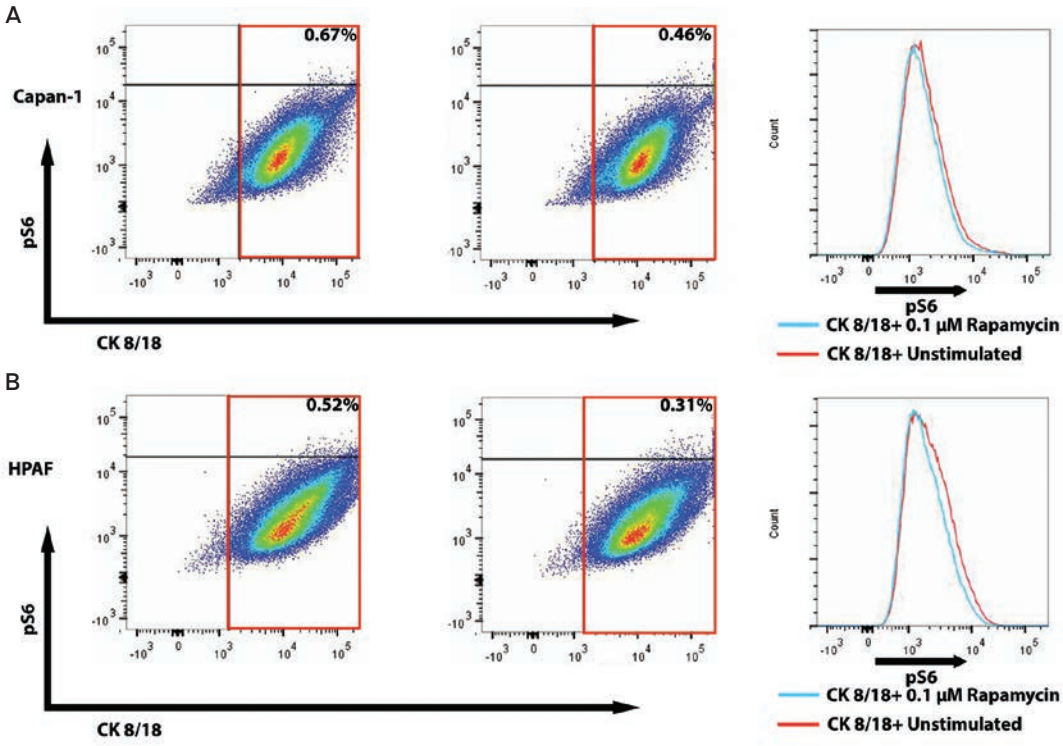


Supplementary Figure 1. Immunohistochemical staining of PanIN lesions. The variability of pS6 expression is also seen in premalignant lesions. PanIN lesions with a score 0 (S1A)(10x) and PanIN lesions with a score of 3 (S1B)(20x).



Supplementary Figure 2. Scatter plot of flow cytometry analysis and corresponding overlay histogram of SU86.86 with and without rapamycin. As seen in BxPC3 (figure 6A), basal mTOR activation is high. Consistently, rapamycin led to a marked reduction of the CK8/18+pS6+ population from 57.7% to 13.8% in SU86.86. This effect of rapamycin can also be observed in the corresponding histogram. The overlay graphs are a representation of the count (y-axis), in the CK8/18+ population (marked by the red box), within the pS6-channel (x-axis).



Supplementary Figure 3. Flow cytometry scatter plot of other rapamycin insensitive cell lines Capan-1 (A) and HPAF (B). In Capan-1 and HPAF we observed similar absolute reductions of 0.21% in the CK8/18+pS6+ population after the addition of 0.1 µM rapamycin. The overlay graphs are a representation of the count (y-axis), in the CK8/18+ population (marked by the red box), within the pS6-channel (x-axis).

Chapter 6

Cannabinoid receptor agonist Namisol does not affect cytokine levels in chronic pancreatitis patients

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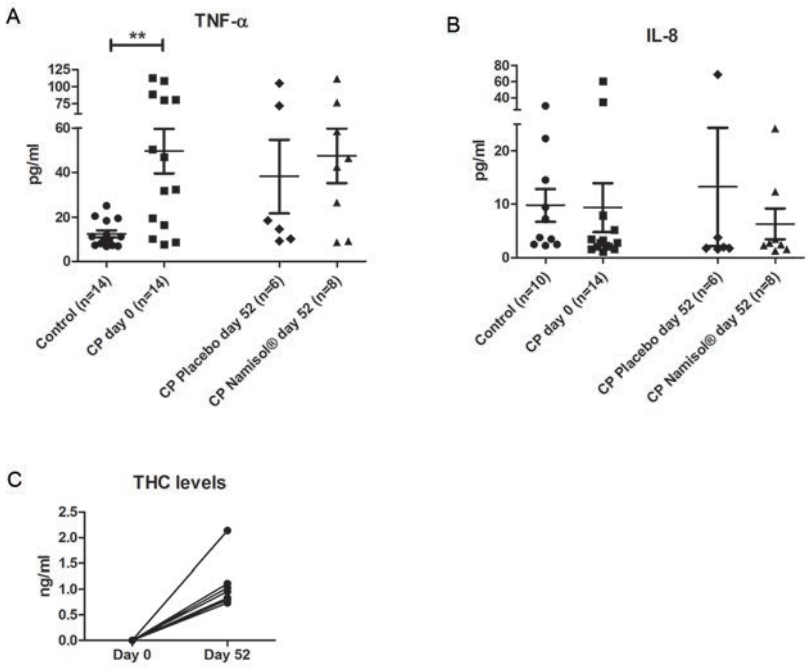
To the Editor: We read with interest the review from Gerich et al. in which they presented an excellent overview supporting the use of medical marijuana for gastrointestinal disorders (1). They report on 5 currently published randomized controlled trials performed for marijuana in gastrointestinal disease, using either dronabinol, a tablet containing >95% Δ9-tetrahydrocannabinol (THC), or smoked marijuana, and conclude that marijuana could be a promising modifier of gastrointestinal symptoms. The molecular mechanisms through which marijuana modulates gastrointestinal disease remain obscure from this paper. Gerich et al refer to in vitro studies showing inhibition of tumor necrosis factor-α (TNF-α)-induced interleukin-8 (IL-8) release in epithelial cells after activation of the cannabinoid receptor (CB) 2, suggesting that intestinal inflammatory responses may be dampened by CB2 mediated alterations in pro-and anti-inflammatory cytokine profiles. However, we now have strong indication that this mechanism, i.e. modulation of cytokine profiles, is not likely to contribute to the effect of THC in vivo.

Recently, a phase II randomized double blinded placebo controlled study was conducted using Namisol® (a tablet containing pure, natural THC [dronabinol])(2), in chronic pancreatitis (CP) patients to relief pain (clinicaltrials.gov: NCT01551511), with CP patients randomized to receive either Namisol® or placebo for 50-52 days. While the primary study outcomes on pain remain to be reported, here we report one of the secondary outcomes, which was to investigate cytokine profiles after ingestion of Namisol®. Plasma samples were collected before start of study treatment, and after Namisol® or placebo treatment. Cytokine levels were measured by enzyme-linked immunosorbent assay (ELISA). We demonstrate that while baseline levels of the pro-inflammatory cytokine TNF-α were significantly increased in CP patients as compared to healthy controls (p=0.0012), use of the CB1 and CB2 agonist Namisol® did not modify these levels, i.e., no differences were observed in TNF-α levels between patients receiving Namisol® or placebo (Figure 1A). Similarly, levels of IL-8, which were not significantly increased in CP patients compared to healthy controls, were not changed after Namisol® administration (**Figure 1B**). Similar results were observed for levels of the anti-inflammatory cytokine IL-10 (data not shown). Positive plasma THC levels in patients receiving Namisol® were confirmed by liquid chromatography-tandem mass spectometry (ABL BV, Assen) (**Figure 1C**), and did not correlate with cytokine levels. Thus, activation of the CB1 and CB2 receptors by Namisol® (THC) does not seem to affect levels of the pro- and anti-inflammatory cytokines TNF-α, IL-8, and IL-10 in vivo, in this subset of patients with chronic pancreatitis. Possible anti-inflammatory effects



Cannabinoid receptor agonist Namisol does not affect cytokine levels in chronic pancreatitis patients

Figure 1. Levels of TNF-α and IL-8 are unchanged upon Namisol® intake, while THC plasma levels are increased.

TNF-α (A) and IL-8 (B) in healthy controls and chronic pancreatitis (CP) patients at baseline and three hours after Namisol® intake at day 52 of the placebo randomised control trial. THC plasma levels (C) at baseline and 3 hours after Namisol® intake.

of medical marijuana may stem from other cannabinoids or mechanisms of immunoregulation, such as apoptosis or inhibition of immune cell proliferation, are currently under investigation. Further research using cannabinoids or selective CB receptor agonists or antagonists are needed to elucidate the mechanisms by which the compounds exert their various effects in different gastrointestinal disorders.

Chapter 6

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Cannabinoid receptor agonist Namisol does not affect cytokine levels in chronic pancreatitis patients

Chapter 7

Modulation of human inflammatory signaling through cannabinoid receptor activation

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Chapter 8

Summary and general discussion

Chapter 8

PART 1: IDENTIFICATION OF HIGH RISK PANCREATIC CYSTS

Diseases of the pancreas, such as chronic pancreatitis (CP) and pancreatic cysts pose a common health problem. In addition to harboring their own pathogenic features and clinical challenges, these diseases have the associated disadvantage of conferring an increased risk for development of pancreatic cancer (**Figure 1**). The incidence of CP is estimated to be around 12/100,000, and may lead to pooled relative risk for pancreatic cancer of 13.3, and in the cases of hereditary pancreatitis even 60 [1,2]. In addition, the prevalence of asymptomatic pancreatic cysts may be as high as 2%, and is increasing with age. Pancreatic cystic neoplasms (PCN), which encompass more than 50% of all pancreatic cysts, are considered pre-malignant lesions, which can progress to pancreatic cancer and therefore are often resected as a preventative measure. However, in truth, only solid pseudopapillary neoplasm (SPN), mucinous cystic neoplasms (MCN) and intraductal papillary mucinous neoplasms (IPMN) show a high tendency to transform, whereas serous cystic adenomas (SCAs) are benign [3]. Thus, there is an urgent need to find accurate, reliable, non-invasive markers, which can distinguish these high risk and low risk cysts, and aid clinical decision making. While several markers have been suggested, and some are implemented in the clinic (see General Introduction, Chapter 1), the rate of unnecessary surgery is still too high, leading to unnecessary risks to patients. In the first part of this thesis, we aimed validate some of the biomarkers suggested in the literature, in order to find a clinically implementable tool contributing to an improved risk assessment.

In **Chapter 2** of this thesis, we first set out to make an inventory of the pancreatic resections performed in our institute, and to determine whether these resections were justified. We found that there was an increasing incidence of pancreatic cyst resections, most likely due to improved imaging techniques available. The Sendai guidelines (which were updated in 2012 from their previous version of 2006 [4]) suggest surgical resection of all MCN in surgically fit patients. In addition, resection is recommended for main duct-IPMN in all surgically fit patients, with resection of side branch-IPMN recommended when 'worrisome features' and 'high-risk stigmata' are present. Thus, a correct diagnosis between mucinous and non-mucinous cysts is required in order to follow these guidelines. While in our tertiary referral center, pre-operative diagnosis of mucinous cysts was relatively good, and improved over time, non-mucinous cysts were only correctly diagnosed in 54% of cases. This suggests that patients with benign cysts are still undergoing unnecessary surgery. One can

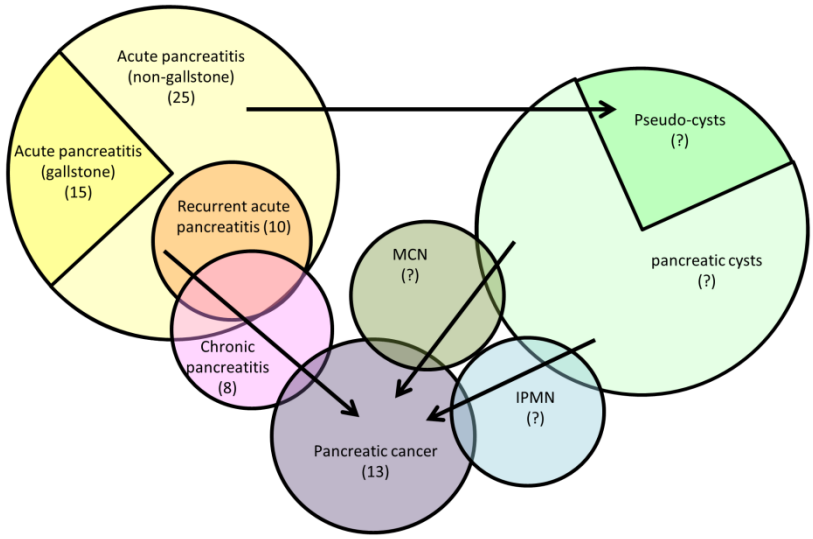

Figure 1. inter-relationships between pancreatic diseases. Where known, approximate yearly incidence rates per 100,000 persons are indicated. Arrow indicates relationship between diseases. Figure adapted from Yadav and Lowenfels, Gastroenterology 2013 Jun:144(6):1252-1261.

imagine that in centers with a lower patient load and less experience in the required diagnostic imaging procedures, these numbers might be even higher. We therefore investigated whether diagnostic accuracy between mucinous and non-mucinous PCN could be improved by assessment of mucinous background in cytological preparations from endoscopic ultrasound-fine needle aspirates (EUS-FNA). Indeed, while measurement of the commonly used marker carcinoembryonic antigen (CEA) resulted in a sensitivity of 39%, specificity of 96.7% and overall accuracy of 63.4%, adding mucinous background information to this analysis improved these to a 75% sensitivity, a 79.1% specificity and overall accuracy of 76.8%. In our cohort, implementation of mucinous background could have prevented misdiagnosis in several cases, thus showing the benefit of implementing this analysis. Other markers to distinguish mucinous from non-mucinous cysts have also been suggested. For example, the measurement of glucose in cyst fluid showed a sensitivity of 100%, but a specificity of only 33% and requires an additional test [5]. Thus, the evaluation of mucus in the cyst fluid during cytology, which is non-labor intensive and does not infer extra costs, may be one of the most easily implementable assets.

While a better identification of non-mucinous cysts is definitely a step in the right direction, a better characterization of the mucinous cysts themselves is also warranted. In **Chapter 2**, we go on to show that only

35% of the resected mucinous PCN demonstrate malignant transformation by histological analysis, suggesting that surgery could have safely been postponed or even deferred indefinitely in some of these patients. While several studies have shown that the 2006 Sendai guidelines had a low positive predictive value, resulting in the resection of many benign cysts, the 2012 revision of these guidelines aimed to reduce this false positivity rate by stratifying side branch-IPMN in 3 instead of 2 categories – high risk, worrisome risk and low risk [6]. Nevertheless, our data show that the current Sendai guidelines are still not specific enough, and result in a large number of false positives. These results are consistent with others, showing that implementation of the new guidelines have not eradicated resections of low risk cysts [7,8]. It has even been suggested that the guidelines are not safe, in that malignant cysts are sometimes missed [9]. We and others [10] show that neither CEA nor the presence of mucin in FNA is able to accurately predict malignant transformation of cysts. Thus it is clear that additional tumor markers to further identify high risk and low risk cysts are urgently needed.

Several biomarkers for high risk cysts have been suggested, based on DNA, RNA or protein alterations, although few have been validated or made it into clinical practice [11,12]. One of the most promising novel strategies for identification of pathological conditions is the measurement of microRNAs (miRs) in bodily fluids [13]. MiRs are very stable and alternatively expressed in many tumor types. Serum or tissue miRs have also been suggested as potential marker for pancreatic ductal adenocarcinoma [14]. A recent publication suggests that the individual measurement of miR-223 in serum may distinguish between malignant and non-malignant IPMN with an area under the curve (AUC) of 0.834, a sensitivity of 62.0%, a specificity of 94.1% and an accuracy of 77.7% [15]. Combining several miRNAs may be an even more promising approach, as Matthaei et al. showed that a 9 miR panel was able to discriminate between high risk and low risk cysts with a sensitivity of 89%, a specificity of 100% and an impressive AUC of 1 [16]. This being the most promising potential marker panel published to date, we aimed to validate the use of these miR measurements in cyst fluid for the identification of high risk cysts in **Chapter 3**.

We therefore measured 9 individual miRs in 52 low risk cysts and 10 high risk EUS-FNA obtained cyst fluids, and combined these into 7 distinctive diffpairs by extracting the Ct value of one miR from the other. While expression of none of the individual miRs differed between low risk and high risk cysts, expression of one diffpair ([miR106b;miR92a]) was significantly decreased in high risk cysts. We next combined these

7 diffpairs into a risk profile based on a logistic regression model, representing (as described by Matthaei et al.) the need for resection, i.e. differentiating between high risk and low risk cysts. In our cohort, this risk profile indeed showed significant differences between cysts with high or low malignant potential. However, using a cut-off level of 50%, specificity of this marker panel was 100%, but sensitivity was a meagre 10.0%. While dropping the cut-off level improved diagnostic accuracy, we concluded that, as it stands, this miR model is not yet suitable for clinical implementation. One potential problem with the clinical implementation of this miR panel, is that in the logistic regression model, a different weight is attributed to each of the individual diffpairs. This relative weight is based on the relative differences of the diffpairs between high risk and low risk cysts (i.e., the more distinguishing power a diffpair has, the higher its weight-factor in the model) – however, these weight-factors are based on the differences observed by Matthaei et al, in their cohort, and may not be the same in other cohorts. While they were able to validate their model in another cohort, we obviously were not.

We therefore continued our search for suitable biomarkers for malignant transformation of pancreatic cysts in **Chapter 4**. Whereas in Chapter 3 we hoped to use RNA as a marker, in Chapter 4 we focused our attention on DNA profiles. We wanted to take advantage of one of the tumor hallmarks – their tendency to release long fragments of DNA into their surroundings. During normal cellular and tissue homeostasis, aged cells will die through a carefully coordinated mechanism. During this programmed cell death, cells shrink, DNA condenses, the cell membrane starts to show irregular shapes (blebbing) and the nucleus collapses (**Figure 2**) [17]. Cellular components are subsequently wrapped up in pieces of the plasma membrane, forming apoptotic bodies. These can be taken up by phagocytes, which degrade the contents of these apoptotic bodies through lysosomal degradation [18,19]. During apoptosis, an internal cascade of enzymatic reactions is activated, which help degrade the intracellular components. One of the proteins activated by this caspase cascade is DNA fragmentation factor (DFF) [20], which cleaves DNA at internucleosomal sites. As DNA is wound twice around a nucleosome, with a total length of 146bp, the nucleosomal fragments produced by DFF are generally of a uniform size of 180-200 bp in length. This physiological process of programmed cell death prevents cell content from dying cells from spilling into the surrounding environment, where they would otherwise elicit an inflammatory response [21]. Under pathological conditions, however, cells can also die by necrosis, during which cells swell, and intracellular components are released upon rupture of the plasma membrane. At this time, long fragments of cellular

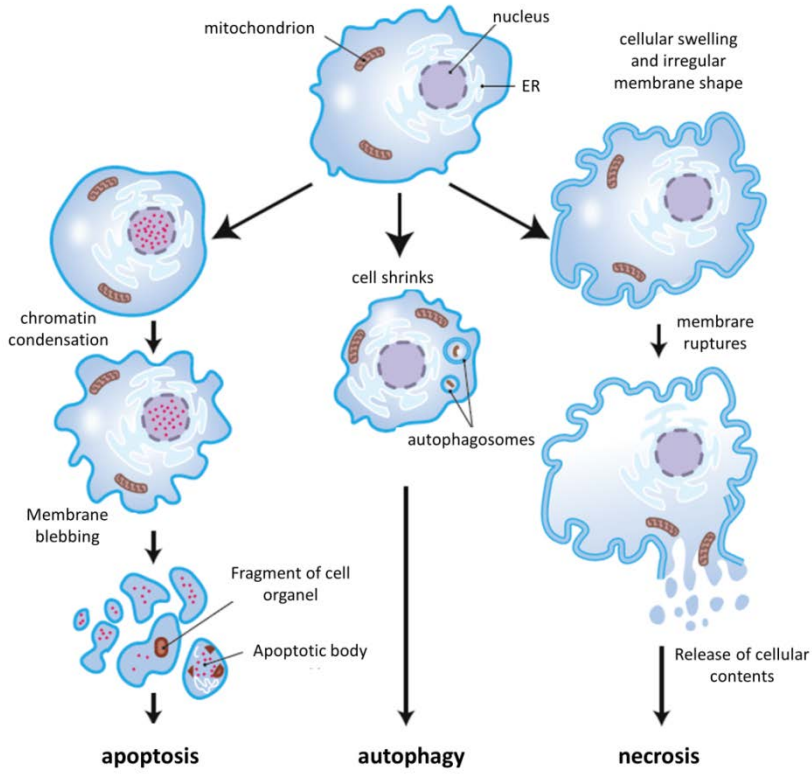

Figure 2. Characteristics of the three major forms of cell death. The macroscopic differences between apoptosis, necrosis and autophagy are presented. ER; endoplasmic reticulum. Image adapted from Basisboek Medische Celbiologie, with permission from the authors.

DNA are released into the system. Pathological conditions during which necrosis takes place include cancer, when cells become resistant against apoptosis, and employ necrosis when they do die [22,23]. In cancer patients, increased levels of long fragments of free circulating cell free DNA (cfDNA) were shown to be present in the peripheral blood, and mutational analysis showed this DNA to originate from the original tumor. Therefore, analysis of the integrity of cfDNA by comparing the ratios of long length to short length DNA fragments in serum from cancer patients versus controls is thought to be a promising tool in the diagnosis and prediction of cancer [24,25]. In **Chapter 4**, we therefore analysed the integrity of cfDNA in serum from patients with pancreatic carcinoma and healthy controls, but did not observe any significant differences between sera from patients and controls. These results were perhaps not surprising, as previous studies were also unable to show necrosis-derived DNA fragments in serum from

patients with pancreatic cancer [26]. We speculated that the enclosed nature of the pancreatic cysts prevents dissemination of released necrotic DNA, thereby precluding their detection in the peripheral blood above background levels. We therefore decided to analyse 115 bp (apoptotic-cell derived) and 247 bp (necrotic cell derived) DNA fragments in pancreatic cyst fluids, in the hope to distinguish between low risk and high risk cysts. Our results show that DNA levels are almost a thousand fold higher in cyst fluids as compared to sera, which appears to confirm the isolated compartment of the cyst interior. However, we did not observe an increased presence of long DNA fragments in cyst fluid from patients with high risk cysts, showing that unfortunately, measurement of DNA integrity is not a good diagnostic tool for the detection of high risk pancreatic cysts. As the amount of necrosis within a tumor is depend on the tumor size [27], it is conceivable that the tumor load of the premalignant lesions is simply not large enough to trigger enough necrosis to make this assay a valuable tool for pancreatic cancer screening. While these results are disappointing, they do indicate that concentrations of DNA, and possibly therefore RNA and proteins, are high in pancreatic cysts, suggesting that other markers may be found in this compartment to help clinical decision making.

One challenge we encountered in the course of these studies is that the number of low risk cysts outweighs that of the high risk cysts, making statistical comparisons challenging. In addition, the mucinous nature of the cyst fluid makes their handing difficult and resulted in further loss of samples in our analyses. Another impediment in these studies is that cyst fluids were collected from patients undergoing EUS-FNA in the course of clinical patient care. Thus, there were considerable differences in the length of time that passed between the collection of the cyst fluid and histological confirmation of malignancy. It is therefore theoretically conceivable that malignant features that were not present at time of EUS-FNA had developed by the time of resection. In such hypothetical cases the cysts fluid, now classified as high risk, should have been classified as low risk. However, the studies as presented accurately describe the clinical practice – it is impossible to predict when a lesion may transform and the only possible way to allow for these temporal issues would be to subject patients to an EUS-FNAs on a regular basis. Such (bi-)annual screening would present a high burden on both patients and the health care system, and cost effectiveness studies would need to show whether such screenings would be worthwhile.

Conclusion Part 1

Thus, in part one of this thesis we show that despite the implementation

of the revised Sendai guidelines in 2012, unnecessary surgery is still performed for benign cysts. While identification of mucinous cysts can be improved by including mucinous background reads of cytology smears in diagnostic work-up, markers to distinguish high risk mucinous cysts from those with low malignant potential are still needed. Cyst fluid may be an ideal compartment to detect such biomarkers. However, our analyses show that neither a previously described 9-miR panel, nor measurement of DNA integrity, is a suitable instrument for high risk cyst diagnosis.

PART 2: TARGETED TREATMENT OF PANCREATIC DISEASE

Not all pancreatic ductal adenocarcinomas arise as a secondary consequence of cysts or pancreatitis – pancreatic intraepithelial neoplasia (PanIN) lesions are also a common precursor of PDAC, which develops in a stepwise progression from low to high risk. The total incidence of pancreatic cancer in the SEER database of the National Cancer Institute suggests that around 1.5% of all people will be diagnosed with pancreatic cancer at some point in their life. Thus, pancreatic cancer is one of the most frequent cancers worldwide. The prognosis for pancreatic cancer is poor, with a relative 5-year survival rate of around 25% for PDAC isolated in the pancreas. However, upon lymph node involvement and widespread metastasis, the 5-year survival drops to a dramatic 9.9% and 2.3%, respectively [28]. Unfortunately, >90% cases are detected when the cancer has already spread to regional or distant sites, making the overall 5-year survival rate around 5%. Until better markers and early diagnostic tools are found, the question remains: how best to treat these patients?

Treatment of pancreatic cancer is currently based on 5 standard options: surgery, radiation therapy, chemotherapy, chemoradiation therapy and targeted therapy. In addition, pain treatment is often performed. Chemotherapy consists of an arsenal of antimetabolites (e.g. 5-Fluorouracil, Gemitabine), DNA crosslinking agents (e.g. Mitomycin C) and anti-microtubule agents (e.g. Paclitaxel). These chemotherapeutics target all proliferating cells, including normal cells. Recent decades have seen the development of targeted therapies for many cancers, including PDAC. Targeted therapy options, where specific molecules that are important for cancer cell growth and survival are inhibited, include Sunitinib, a multi-receptor tyrosine kinase (RTK) inhibitor and Erlotinib, an EGFR inhibitor, which also inhibits other RTKs in PDAC [29]. Another targeted therapeutic approved for PDAC treatment is Everolimus, a

specific inhibitor of the mTOR pathway. The mTOR pathway controls many cellular processes, and enhanced activity of this pathway has been shown in many tumors, including PDAC [30–32]. Activation of mTOR through receptor activation by growth factors is a multi-step process. First, receptor activation leading to activity of phosphatylidinositol-3(OH) kinase results in activity of Akt. Akt subsequently phosphorylates the tuberous sclerosis complex (TSC1/2), thereby inactivating its ability to switch on the GTPase activity of Rheb. Rheb is therefore unable to hydrolyze its bound GTP to GDP, becomes activated and activates mTOR. This kinase then activates p70S6 kinase, which phosphorylates the ribosomal protein S6. Activity of the mTOR-S6 pathway is important for protein synthesis and cell growth and mediates development of pancreatic cancer [33]. Inactivation of mTOR can occur upon nutrient deficiency and is mediated through AMP-activated protein kinase (AMPK). This process causes autophagy, whereby the cell tries to outlive temporary nutrient shortage by recycling cellular components but which, on prolonged duration, leads to cell death (**Figure 2**). Chemical activation of AMPK by Metformin has therefor been suggested as potential treatment for cancer (**Figure 3**) [34]. Interestingly, in patients with diabetes mellitus, the use of insulin has been associated with an increased risk, while using metformin is associated with a lower risk of developing pancreatic cancer [35]. This is consistent with the finding that the Insulin-like Growth Factor I (IGF-1) receptor is overexpressed in pancreatic cancer [36].Thus, targeting mTOR with small molecule inhibitors appears a promising approach in the treatment of PDAC. Nevertheless, clinical trials with Everolimus in PDAC have so far been disappointing, with only a small groups of patients benefitting from the use of this mTOR inhibitor [37,38]. However, pancreatic cancer is a heterogenic disease, with different patients showing activation of different intracellular growth pathways [30]. Studies with targeted therapy in other cancers have shown that effectiveness of treatment may depend on the signaling pathways activated in tumors [39]. We therefore hypothesized that effectiveness of mTOR-inhibitor treatment would depend on mTOR activity status in the tumor.

In **Chapter 5**, we investigated whether molecular response to mTOR inhibition could be used as an in vitro tool to predict cell death in response to treatment. We first showed that some, but not all PDAC patients show a strong activation of the mTOR pathway, as determined by S6 phosphorylation. While over-activation of mTOR in cancer can be caused by mutations in the TSC genes, a more common cause of mTOR upregulation is inactivating mutations in the cell cycle control gene TP53 [40]. However, over-activation of mTOR in pancreatic cancer can also be a

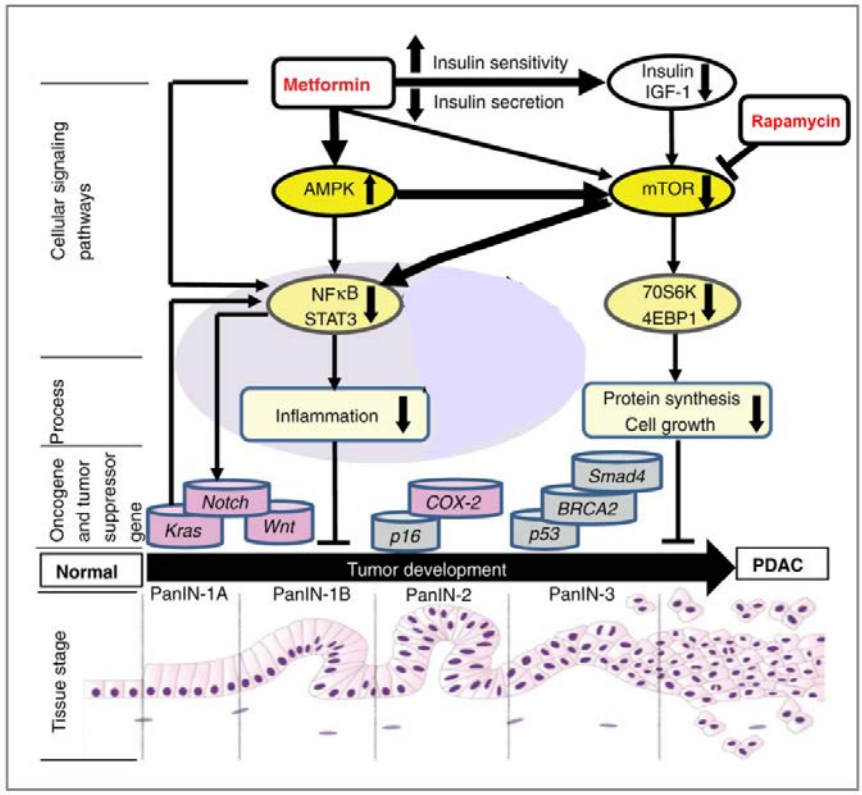

Figure 3. mTOR signaling in pancreatic cancer. mTOR signaling is involved in inflammation – a positive trigger for PDAC development – as well as protein synthesis and cell growth. Inhibition of mTOR, either directly (through Rapamycin analogues) or indirectly (through Metformin-induced AMPK activation) may inhibit inflammation and tumor development in PDAC. Figure adapted from Yue et al, Cancer Prev Res 2014;7:388-397

direct result of oncogenic Ras-ERK signaling, in which case dual inhibition of ERK and mTOR is more effective than only inhibiting the downstream partner mTOR [41]. Indeed, KRAS PTEN tumors were shown to be much more sensitive to mTOR inhibitor treatment than KRAS TP53 driven tumors [42]. Thus, different tumors may depend on different oncogenic signaling pathways, which in turn may determine the cells' sensitivity to inhibitors of these pathways. Importantly, most tumors, including PDAC, have multiple mutations interacting with similar pathways, and downstream mutations may hamper the effectiveness up upstream inhibitors [43]. As not all mutations are a priori known in PDAC patients, it would be useful to have an in vitro test to determine the potential response of patients to mTOR inhibitor treatments. We analyzed different pancreatic cancer cell lines for the presence of S6 phosphorylation by Western blot

analysis, and their sensitivity to Rapamycin treatment by cell killing assays. We showed that while all 5 cell lines tested showed high constitutive S6 phosphorylation patterns, S6 phosphorylation was drastically decreased upon Rapamycin treatment in only 2 of these cell lines, while the remaining three were more resistant. Interestingly, resistance as determined by S6 phosphorylation corresponded to resistance of these cell lines to Rapamycin-induced cell death, thereby opening up the possibility that in vitro analysis of phospho-S6 reduction by mTOR inhibitors might be a proxy for their cytotoxic effect. We went on to show that this reduction in S6 phosphorylation could be quantifiably measured by flow cytometric analysis – a huge benefit, as this subsequently allowed the analysis of S6 phosphorylation in individual cell populations rather than all cells combined. We were subsequently able to determine S6 phosphorylation specifically in epithelial cells present in EUS-FNA from pancreatic cancer patients. While constitutive S6 phosphorylation varied between patients, we demonstrated that in vitro treatment of cells with Rapamycin reduced S6 phosphorylation in 2 out of 9 patients. We concluded that measurement of sensitivity of the mTOR-S6 pathway to Rapamycin may be a promising new in vitro tool to predict response of pancreatic cancer patients to mTOR inhibitor treatment.

Our results suggest that the use of mTOR modulators is a promising strategy for pancreatic cancer, at least in a subset of patients. While direct growth inhibition is most likely the main mechanism for tumor eradication, inhibition of mTOR mediated pro-inflammatory processes may be another (**Figure 3**). One of the risk factors for pancreatic cancer development is chronic pancreatitis – a finding attributed to the fact that ongoing inflammation causes DNA damage through the release of reactive oxygen species and pro-inflammatory mediators [44,45]. It is therefore conceivable that inhibition of the pro-inflammatory signaling in CP patients may reduce their risk to develop cancer. In the last two chapters of this thesis, we investigated the potential anti-inflammatory properties of medicinal marijuana, which was investigated in a clinical trial for pain relief in CP patients.

While cannabis has been used in the clinic for many centuries, its main purpose has been to reduce nausea and combat anorexia and pain. However, immune cells express high levels of the cannabinoid receptor CB2, which suggests that one of the effects of cannabis might be the modulation of the immune system. Indeed, a link between cannabis use and susceptibility to bacterial infections in mice and men suggest that THC may have an immune-depressing effect [46]. In addition, THC treatment

was shown to alleviate acute pancreatitis in a mouse model [47]. While the exact mechanisms of CB2-mediated anti-inflammatory actions remain obscure, it is becoming clear that THC may have a direct effect on T-cells [48], by inhibiting mixed lymphocyte reactions and CD3-stimulated T-cell proliferation as well as suppressing CD8 T-cell cytolytic activity [49,50]. In **Chapter 6**, we investigated the cytokine profiles in sera from CP patients taking part in a clinical trial investigating the pain-alleviating capabilities of medicinal cannabis in CP. Patients were asked to ingest Namisol®, a 98% pure THC preparation, for 52 consecutive days, and blood was obtained at day 0, 15 and 52. We showed that levels of the pro-inflammatory cytokine tumor necrosis factor α (TNFα) were significantly elevated in CP patients prior to the start of the trial as compared to healthy controls. However, treatment with Namisol® did not reduce these TNFα levels when compared to placebo-treated patients. Furthermore, no differences were observed in either levels of pro-inflammatory interleukin 8 (IL-8) or the anti-inflammatory IL-10 between patients receiving Namisol® or placebo. Thus, these data showed that THC does not have a direct effect on cytokine levels in patients with CP.

To gain further insight into the potential anti-inflammatory properties of THC, we went on to investigate the intracellular, molecular consequences of THC on immune cells in **Chapter 7**. We started off by making a profile of the kinomic changes occurring in vivo in healthy subjects upon THC intake. This approach allows the unbiased analysis of kinomic activity towards 1024 described kinome substrates in one sample, thereby allowing identification of novel signaling links without a priori assumption of the pathways involved in the biological system tested. As we were interested in THC effects in the context of an inflammatory setting, we stimulated peripheral blood mononuclear cells (PBMCs) with the immune trigger lipopolysaccharide (LPS) prior to kinome analysis. Our data showed that THC lowered several signaling pathways in LPS-stimulated PBMCs, including ERK activity, Akt-S6 activity, Calcium signaling and Wnt signaling. Most noticeably however, was a downregulation of innate stress/inflammatory signaling, including JNK and p38 activity, without an effect on NFκB. We confirmed that these changes were indeed induced by a direct effect of THC on PBMCs, by demonstrating that in vivo treatment of PBMCs with THC also reduced activity of these pathways in LPS-stimulated PBMCs. LPS is a membrane component of Gramm-negative bacteria, and as such stimulates mainly the innate immune system (which is the first line of defense against bacterial infections) [51]. Some reports have suggested that T-cells may express the LPS sensor TLR4 as well, making it difficult to attribute the kinomic changes directly to individual

innate or adaptive immune cell compartments [52]. However, comparison of LPS and T-cell specific (αCD3/CD28 stimulated) signaling in PBMCs showed large differences between these two: whereas LPS induced a strong p38 phosphorylation and limited S6 activity, αCD3/CD28 triggered a massive S6 phosphorylation, and almost no p38 signaling. Analysis of individual cell subsets confirmed the innate immune function of LPS by showing that it did not activate S6 phosphorylation in T-cells. In subsequent experiments, we therefore used CD3/CD28 in order to show that THC also reduced S6 phosphorylation in activated T-cells. In light of the anti-inflammatory signaling observed in healthy individuals, we subsequently investigated mTOR-S6 signaling in patients taking medicinal cannabis for symptoms of pain. Again, we showed that phosphorylation of S6 was reduced within 1 to 5 hours after THC intake, in both T-cell and monocytes. However, we also demonstrated that prolonged treatment with THC increased basal S6 levels in innate and adaptive immune cells in two patients.

A differential role of mTOR-S6 signaling in monocytes and T-cells has been suggested. While inhibition of mTOR clearly mediates anti-inflammatory aspects in T-cells, mTOR is suggested to limit inflammatory responses in monocytes. Thus, a decrease in mTOR signaling in monocytes might theoretically promote inflammation in patients taking THC. However, in light of the limited S6 signaling in monocytes as compared to T-cells, and the relative importance of p38 in these cells, it seems more likely that the reduction in p38 and S6 phosphorylation immediately following THC treatment together elicit an anti-inflammatory effect. Nevertheless, our data also suggest that while incidental use of marijuana may reduce inflammatory responses, prolonged treatment with THC may increase inflammatory signaling in T-cells at least. It would be interesting to investigate the long term effect of THC on p38 phosphorylation in innate-immune cells. Interestingly, there have now been several case studies demonstrating that regular cannabis use may lead to acute pancreatitis [53–55]. We postulate that an increase in mTOR-S6 pro-inflammatory signaling may have contributed to these cases, and argue that caution should be taken in the use of medical cannabis.

Conclusion Part 2

In part 2 of this thesis, we demonstrate that mTOR inhibition as a targeted therapy for pancreatic cancer may be of use, but would benefit from in vitro screening of those patients who are eligible for this treatment, based on a reduction in cellular S6 phosphorylation upon Rapamycin treatment. Targeted treatment for chronic pancreatitis may include the use of medical

marijuana. We demonstrate that medical cannabis has strong anti-inflammatory properties directly after intake, as determined by a reduction in p38 and mTOR-S6 pro-inflammatory signaling, but that prolonged use of cannabis may enhance inflammation, which could potentially contribute to tumorigenesis. Thus, caution should be taken when using cannabis for medicinal purposes.

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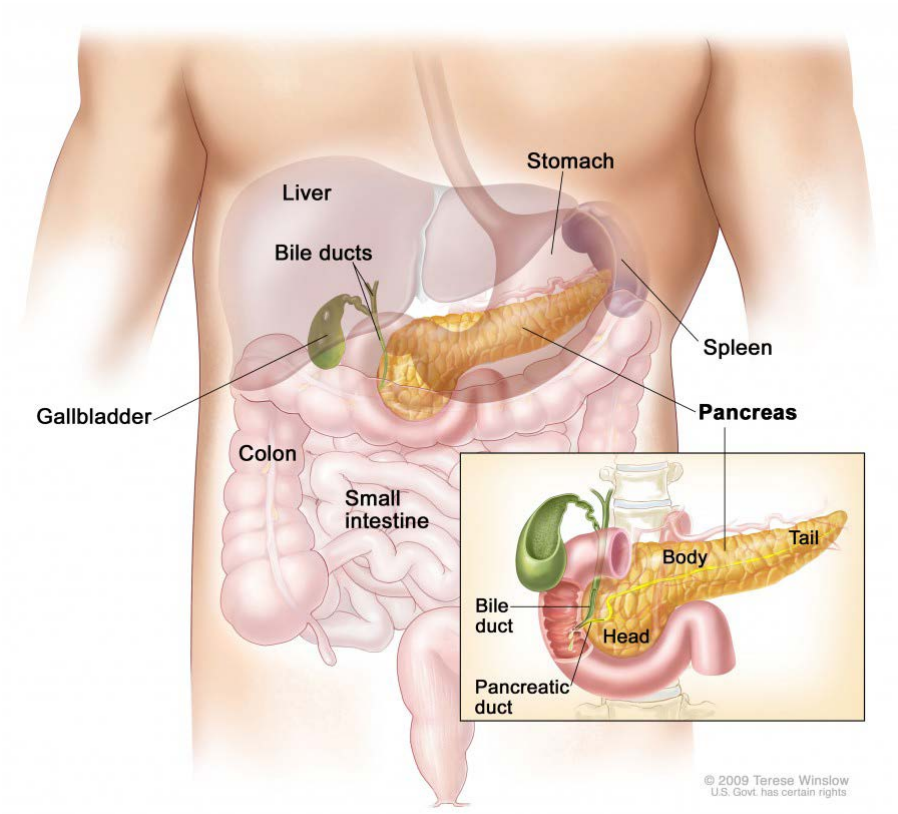
Chapter 9

Nederlandse samenvatting

Chapter 9

DE ALVLEESKLIER

De alvleesklier is een orgaan van ongeveer 14 cm lang en 2 cm dik, dat zich achter de maag in de buikholte bevindt. De medische naam van de alvleesklier is pancreas. De belangrijkste functies van de alvleesklier zijn het produceren van spijsverteringssappen die helpen bij de afbraak van voedsel en de aanmaak van hormonen die de suikerspiegels in het bloed reguleren. De spijsverteringssappen die worden aangemaakt bevatten enzymen die in staat zijn de suikers, vetten en koolhydraten in ons eten af te breken. Deze sappen worden aangemaakt in zogenaamde 'exocriene' cellen, die deze sappen afgeven aan een netwerk van afvoergangen in de alvleesklier. Deze eindigen uiteindelijk via de alvleesklierbuis (pancreatic duct) in de twaalfvingerige darm, alwaar de spijsverteringssappen zich



Figuur 1. De alvleesklier (pancreas) bevindt zich achter de maag (stomach) en ligt tegen de milt (spleen) aan. Het bestaat uit een kop(head), lichaam (body) en staart (tail). Verteringssappen die in de pancreas worden geproduceerd worden afgevoerd naar de twaalfvingerige darm via de ductus pancreaticus (pancreatic duct).

kunnen mengen met het voedsel in ons maag-darm kanaal en hier kunnen bijdragen aan de vertering. Dagelijks produceert de alvleesklier ongeveer 1,2 liter spijsverteringssap. De tweede, endocriene functie van de pancreas is het produceren van insuline en glucagon. Deze hormonen worden aangemaakt in eilandjes van cellen die in de alvleesklier gelegen zijn, de eilandjes van Langerhans. Ten tijde van een overschot aan suiker (glucose) in het bloed worden β-cellen in de eilandjes van Langerhans aangezet tot de productie van insuline dat via de bloedbaan naar de rest van het lichaam wordt getransporteerd, waar het onder andere vet- en spiercellen aanzet tot het opnemen van glucose uit het bloed. Wanneer glucose bloedspiegels te laag zijn produceren α-cellen in de eilandjes van Langerhans het hormoon glucagon, dat lever-, vet- en spiercellen vertelt dat ze glucose moeten afgeven aan het bloed. Deze hormoonafgifte zorgt ervoor dat de bloedsuikerspiegels gedurende de hele dag constant blijven.

ZIEKTE VAN DE PANCREAS

Pancreas kanker is een van de meest dodelijke vorm van kanker, en staat op de vijfde plek wat betreft kanker-gerelateerde doodsoorzaken in Europa. Slechts 5% van de mensen met pancreaskanker overleeft langer dan 5 jaar na diagnose. Een belangrijke reden voor deze slechte kans is dat pancreaskanker zelf weinig symptomen veroorzaakt, waardoor de ziekte vaak pas ontdekt wordt in vergevorderd stadium, als deze al is uitgezaaid in het lichaam. Een van de belangrijkste risicofactoren voor het ontwikkelen van pancreaskanker is roken of gerookt hebben. Daarnaast kan ook het gebruik van alcohol (> 3 glazen per dag) de kans op kanker van de alvleesklier aanzienlijk verhogen. Een belangrijke reden hiervoor is dat alcohol chronische ontsteking van de pancreas kan veroorzaken, wat op zichzelf weer een risicofactor is voor het ontstaan van pancreaskanker. Chronische ontstekingsreacties veroorzaken productie van toxische stoffen die DNA schade kunnen veroorzaken, waardoor cellen oncogene mutaties kunnen oplopen die weer kunnen resulteren in een groeivoordeel. Naast chronische pancreatitis zijn er ook andere ziekten van de pancreas die kunnen bijdragen aan een verhoogde kans op pancreaskanker. Pancreascysten bijvoorbeeld, zijn holtes gevuld met vocht in de pancreas die soms kunnen ontaarden in kanker. Hoe vaak cysten in de pancreas voorkomen is niet precies bekend, aangezien ze niet altijd symptomen veroorzaken, en vaak per toeval ontdekt worden. Een groot deel van de cysten in de pancreas zijn goedaardig, en vormen geen risico voor de patiënt. Neoplastische pancreascysten daarentegen dragen wel een risico met zich mee en deze worden, afhankelijk van het risico, operatief verwijderd. Binnen deze groep van cysten kan

onderscheid gemaakt worden tussen hoog risico en laag risico cysten. Waar intraductale papillaire mucineuze neoplasma (IPMN) en mucineuze cystische neoplasma (MCN) premaligne cysten zijn, is de prognose bij solide pseudopapillaire tumoren en sereuze cystische adenomas relatief goed. Het onderscheid tussen deze typen cysten is echter op basis van de huidige beeldvormende technieken niet goed te maken. De klinische besluitvorming is erbij gebaat indien hoog risico cysten vroegtijdig zouden kunnen worden onderscheiden van de laag risico cysten, om hiermee onnodige chirurgische ingrepen met alle potentiële complicaties van dien, te voorkomen. In deel 1 van dit proefschrift hebben wij geprobeerd moleculaire biomarkers te identificeren die zouden kunnen helpen bij de vroegtijdige opsporing van hoog risico cystische laesies. In deel twee van dit proefschrift hebben we gekeken naar mogelijke behandeling van pancreaskanker met remmers van oncogene signalering, en de rol van deze signalering bij behandeling van ontstekingen van de pancreas.

DEEL 1: IDENTIFICATIE VAN HOOG RISICO PANCREASCYSTEN

In **Hoofdstuk 2** van dit proefschrift hebben we geïnventariseerd hoeveel patiënten in het Erasmus MC zijn geopereerd tussen 2000 en 2014 voor de aanwezigheid van een premaligne pancreas cyste, en of deze operaties gerechtvaardigd waren. We laten zien dat er een stijgende lijn is in het aantal operaties dat werd uitgevoerd in dit tijdsbestek, met slechts 2 resecties tussen 2000 en 2002 en meer dan 50 operaties tussen 2012 en 2014. Dit is waarschijnlijk het gevolg van betere beeldvormende technieken, waardoor cysten vaker per toeval worden gevonden. Aangezien mucineuze cysten vaker uitmonden in pancreas tumoren, zouden volgens de richtlijnen alle IPMN en MCN moeten worden verwijderd. Het is dus van belang dat mucineuze en nonmucineuze cysten goed van elkaar worden onderscheiden. In ons centrum werd de diagnose voor mucineuze laesies in bijna alle gevallen goed gesteld vóór de operatie. Van de nonmucineuze laesies werd echter bijna de helft gediagnostiseerd als mucineus, en verwijderd terwijl dat wellicht (nog) niet nodig was geweest. In sommige gevallen wordt voor de diagnose van de laesies een biopt genomen uit de cyste, met een holle naald. Het materiaal dat op deze manier verzameld wordt, wordt op een glaasje uitgesmeerd en gekleurd, waarbij de eventueel aanwezige cellen door een patholoog worden beoordeeld op hun maligniteit. In **Hoofdstuk 2** laten wij zien dat de aanwezigheid van mucine in dit cystevloeistof ook te detecteren is op deze preparaten, en dat het scoren van de aanwezigheid van mucine de diagnose voor mucineuze

versus non-mucineuze cystes had kunnen verbeteren. Wij raden dan ook aan om in de toekomst dergelijke informatie mee te nemen in de diagnose van pancreas laesies.

Hoewel het onderscheid tussen mucineuze en niet-mucineuze cysten een stap in de goede richting is, zijn we er hier mee nog zeker niet. Mucineuze cysten hebben dan wellicht een grotere kans om te ontaarden in een maligniteit, dit gebeurt niet in alle gevallen, en niet altijd even snel. Uit histologische analyse van chirurgisch verwijderde laesies bleek in **Hoofdstuk 2**, dat slechts 35% van de gereseceerde cysten ook daadwerkelijk kenmerken van maligniteit vertoonden. Deze operaties hadden dus naar waarschijnlijkheid uitgesteld kunnen worden of waren wellicht niet nodig. Een betere identificatie van hoog risico cysten is dus nog steeds nodig.

Er zijn al verschillende moleculaire biomarkers voorgesteld om het onderscheid tussen hoog risico en laag risico pancreas laesies te kunnen maken, maar tot nu toe is nog geen van deze markers in de kliniek in gebruik genomen, aangezien validatie van deze markers in andere studies nog niet is uitgevoerd. In **Hoofdstuk 3** hebben wij gekeken of een eerder gepubliceerde biomarker ook in ons eigen patiënten cohort in staat zou zijn om hoog en laag risico pancreascysten van elkaar te onderscheiden. Hierbij maakten we gebruik van het feit dat cellen in staat zijn om kleine stukjes RNA los te laten in hun omgeving. Deze RNA moleculen, de zogenaamde microRNAs (miRs) reguleren in een cel welke eiwitten vanuit het genomisch DNA worden aangemaakt. Ieder celtype maakt een specifieke set miRs aan, en ook tumor cellen hebben een voor hun kenmerkend miR patroon. Deze miRs kunnen door cellen worden losgelaten, en zijn dan te meten in lichaamsvloeistoffen zoals serum, maar ook pancreas cystevloeistof. Wij hebben een panel van 9 van deze miRs gemeten in cystevloeistof afkomstig uit hoog risico en laag risico cysten, en vonden dat de relatieve hoeveelheden van deze miRs inderdaad verschilden tussen de verschillende risico klassen. Echter, in tegenstelling tot wat eerder beschreven was in andere pancreas cyste cohorten, vonden wij dat het onderscheidend vermogen van deze test niet gevoelig genoeg was om met voldoende zekerheid te kunnen zeggen of een pancreascyste kwaadaardig was of niet. Derhalve raden wij deze assay dan ook niet aan als klinische bepaling waarop het behandelplan van pancreas laesies kan worden aangepast.

In **Hoofdstuk 4** hebben wij een nog niet eerder voor pancreas cystevloeistof beschreven biomarker getest, ditmaal gebaseerd op de integriteit van DNA

dat door tumor cellen wordt losgelaten. Wanneer cellen in het lichaam doodgaan, gebeurt dit normaliter volgens een nauwkeurig gereguleerd proces, waarbij het genomisch DNA in kleine stukjes wordt geknipt, die vervolgens vrij kunnen komen in het lichaam. Bij niet-fysiologische celdood, die onder andere optreedt bij tumor cellen, wordt het DNA niet op deze nauwgezette methode afgebroken, en komen dus langere DNA fragmenten vrij uit de stervende cel. De ratio tussen lange en korte DNA fragmenten (de DNA integriteit) in het serum, kan bij sommige vormen van kanker dan ook worden aangewend om vast te stellen dat er zich een tumor in het lichaam bevind. In hoofdstuk 4 hebben wij de lange en korte DNA fragmenten in het bloedserum van gezonde mensen en patiënten met pancreaskanker gemeten, maar wij konden geen verschil aantonen in de DNA integriteit tussen deze twee groepen. Mogelijkerwijs is de pancreas tumor te klein om het DNA wat er uit vrij komt op te pikken in het perifere bloed, of kunnen de lange DNA fragmenten niet uit de cyste treden naar de bloedbaan. We hebben daarom ook de DNA integriteit gemeten in het cystevloeistof van patiënten met pancreascysten. We vonden dat de totale concentratie van DNA in dit vloeistof tot wel 1000 maal hoger was dan de concentratie DNA in het perifere bloed, wat erop duidt dat het binnenste van een cyste inderdaad een afgesloten compartiment is. Helaas konden we echter geen verschil aantonen in de mate van DNA integriteit tussen hoog risico en laag risico cysten, en hebben wij moeten concluderen dat DNA integriteit geen goede marker is om kwaadaardige pancreascysten vroegtijdig op te sporen.

DEEL 2: GERICHTE THERAPIE VAN PANCREASAANDOENINGEN

Aangezien het opsporen en diagnosticeren van pancreas maligniteiten nog steeds problematisch is, hebben we in **Hoofdstuk 5** onze aandacht gericht op de mogelijke behandeling van pancreas kanker. Er zijn meerdere therapeutica beschikbaar in het arsenaal van de behandelende arts, maar tot nu toe is de overlevingsduur van pancreaskanker patiënten nog steeds beperkt, en nieuwe therapieën zouden welkom zijn. Het wordt steeds duidelijker dat niet alle pancreas tumoren op elkaar lijken, en dat intracellulaire processen die betrokken zijn bij de celdeling en metastasering, bij deze tumoren kunnen verschillen. Er wordt dan ook steeds meer ingezet op 'personalized medicine' – een individuele benadering van de behandeling, gebaseerd op de kenmerken van de tumor in een patiënt. Dergelijke kenmerken komen voort uit het feit dat niet alle tumoren ontstaan uit dezelfde oncogene mutaties, en dat de intracellulaire signaleringsmoleculen die geactiveerd worden binnen in de cel, per tumor

kunnen verschillen. Remmers van dergelijke signaleringsroutes worden in rap tempo ontwikkeld, en hun mogelijke toepassing in de kliniek wordt nu in klinische trials getest. Een van de signaal transductie route remmers die getest is in pancreastumoren is rapamycine. Dit medicijn remt de activiteit van het enzym mTOR, wat betrokken is bij celgroei en eiwitsynthese. Klinische trials met rapamycine in pancreaskanker zijn tot nu toe echter nog niet succesvol gebleken. In **Hoofdstuk 5** tonen wij aan dat er een groot verschil bestaat in de mate van mTOR activatie in verschillende pancreastumoren, en dat er bovendien een verschil is in de mate waarin dit enzym door rapamycine geremd wordt. We tonen aan dat een in vitro test waarin we kijken naar de hoeveelheid remming van mTOR voorspelt hoeveel remming van de cel overleving er plaatsvindt. Deze resultaten geven een mogelijke verklaring voor het falen van rapamycine in klinische trials – immers niet alle tumoren hebben evenveel actief mTOR en niet in alle tumorcellen wordt mTOR evenveel geremd door rapamycine. Ons onderzoek suggereert echter dat we met behulp van een eenvoudige test zouden kunnen onderzoeken of een patiënt baat heeft bij het gebruik van rapamycine, en dat dit medicijn dus mogelijk een goede aanvulling zou kunnen zijn in een subpopulatie van pancreaskanker patiënten.

Naast een rol in celdeling bij kanker, speelt mTOR ook een belangrijke rol in inflammatie, doordat het immuun cellen (met name T-cellen) kan aanzetten tot deling. Zoals eerder vermeld, geeft chronische ontsteking van de pancreas een verhoogd risico op het ontwikkelen van pancreaskanker. Chronische pancreatitis (CP) wordt gekenmerkt door klachten van pijn in de bovenbuik, misselijkheid, gewichtsverlies en vettige ontlasting. Afgezien van chirurgische verwijdering van de pancreas is er geen genezende behandeling mogelijk van deze ziekte, en is men gelimiteerd tot pijnbestrijding. Een mogelijke methode om eetlust op te wekken alsmede pijn te bestrijden is het gebruik van medicinale cannabis. Naast de psychotropische effecten van THC, het werkzame bestanddeel van cannabis, wordt ook gespeculeerd dat THC een direct effect heeft op het immuun systeem. In **Hoofdstuk 6** onderzochten wij de cytokine profielen in het serum van CP patiënten die deel namen aan een klinische trial waarin pijn verlichtende effecten van medicinale cannabis werden bestudeerd. Patiënten werden gevraagd Namisol®, een preparaat van 98% THC, in te nemen gedurende 52 opeenvolgende dagen, en bloed werd afgenomen op dag 0, 15 en 52. Wij toonden aan dat de niveaus van de pro-inflammatoire cytokine tumornecrosefactor α (TNFα) significant verhoogd zijn in CP patiënten ten opzichte van gezonde controles op dag 0. Echter, behandeling met Namisol® veranderde deze TNFα spiegels niet meer dan in patiënten behandeld met placebo. Bovendien werden er geen verschillen

waargenomen in spiegels van het pro-inflammatoire interleukine 8 (IL-8) of het anti-inflammatoire IL-10 tussen patiënten die Namisol® of placebo kregen. Dus, uit deze gegevens lijkt dat THC geen direct effect heeft op cytokine spiegels in patiënten met CP.

Om meer inzicht te krijgen in de mogelijke anti-inflammatoire eigenschappen van THC, hebben wij naar de intracellulaire effecten van THC op immuuncellen gekeken in **Hoofdstuk 7**. Allereerst zijn wij begonnen met het in kaart brengen van veranderingen in het zogenaamde kinoom profiel, waarin signaleringsroutes middels 1024 kinase substraten werden geanalyseerd. Hiermee onderzochten wij de in vivo veranderingen na inname van THC in gezonde proefpersonen. Omdat wij met name geïnteresseerd waren in de effecten van THC in het kader van een inflammatoire omgeving, stimuleerden wij perifere bloed mononucleaire cellen (PBMC's) met een immuun activator, lipopolysaccharide (LPS) voorafgaand aan de kinoom analyse. Onze data liet zien dat THC de activiteit van verschillende signaaltransductieroutes in LPS gestimuleerde PBMC's verlaagde, waaronder ERK, Akt-S6, calcium signalering en Wnt-signalering. Opvallend was de remming van stress/inflammatoire signaleringroutes, zoals JNK en p38, zonder dat er een effect was op NFκB. Wij bevestigden dat deze veranderingen werden veroorzaakt door een direct effect van THC op PBMC's, door aan te tonen dat in vitro stimulatie van PBMC's met THC ook verminderde activiteit van deze signaleringsroutes in LPS-gestimuleerde PBMC's liet zien. In het kader van de anti-inflammatoire signalering die wij hebben waargenomen in gezonde proefpersonen, onderzochten we vervolgens mTOR-S6 signalering in patiënten die medicinale cannabis kregen vanwege pijnklachten. Hierin tonen wij nogmaals aan dat fosforylering van S6 werd verlaagd binnen 1 tot 5 uur na inname van THC in zowel T-cellen als monocyten. Maar wij toonden tevens aan dat langdurige behandeling met THC kan leiden tot verhoogde basale niveaus van S6 in het aangeboren en adaptieve immuuncompartiment van twee patiënten.

Deze gegevens suggereren een duale rol van mTOR-S6 signalering in monocyten en T-cellen. Hoewel het duidelijk is dat remming van mTOR een anti-inflammatoire effect heeft in T-cellen, is er gesuggereerd dat mTOR in monocyten inflammatie tegengaat. Zo zou een afname van mTOR signalering in monocyten theoretisch juist een ontsteking kunnen bevorderen in patiënten die THC innemen. Echter, gezien de beperkte S6 signalering in monocyten in vergelijking met T-cellen en het relatieve belang van p38 in deze cellen, lijkt het waarschijnlijk dat de afname van p38 en S6 fosforylering na THC behandeling samen het anti-inflammatoir

effect bewerkstelligen. Desalniettemin suggereren onze gegevens ook dat, ondanks dat het incidenteel gebruik van marihuana ontstekingsreacties kan verminderen, langdurige behandeling met THC inflammatoire signaleringsroutes in T-cellen kan verhogen.

Al met al laat het werk beschreven in dit proefschrift zien dat zowel behandeling alsook diagnose van (pre-)maligne afwijkingen in de pancreas verbetering behoefd en dat het mogelijk is dit middels moderne experimentele technieken te onderzoeken. Ook is echter duidelijk dat dergelijke nieuwe benaderingen niet onmiddellijk een pasklaar resultaat opleveren, maar dat er nog veel werk zal moeten gebeuren.

Chapter 10

Appendix

I Dankwoord
II Publication list
III PhD portfolio
IV About the author

Chapter 10

DANKWOORD

Poehpoeh! Opeens ben ik dan toch aanbeland bij het meest (en soms enig) gelezen stuk van mijn boekje. In de aanloop van het tot stand komen van dit proefschrift ben ik vele mensen mijn dank verschuldigd. De afgelopen vier jaar waren zeker niet hetzelfde geweest zonder de aanwezigheid van eenieder hieronder genoemd, met ieder zijn eigen inbreng.

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Angela
Elvira
Sergey
Dowty
Pratika
Team dakduiven
Priscilla, Lisanne, WP, Raoel, Esmee, Jihan, Mitchell, Wim, Margo, Heng,

Team Whisky
Han, Arthur, Vincent, Jerome, Maykel, Luc, Sander, Chris, Wu, Ralf, Johan, jaarlijkse uitje

Team Oosterlicht

Lindsey

Chapter 10

LIST OF PUBLICATIONS

1. Utomo WK, Gabbe BJ, Simpson PM, Cameron PA. Predictors of in-hospital mortality and 6-month functional outcomes in older adults after moderate to severe traumatic brain injury. Injury. 2009 Sep;40(9):973-7.

2. de Mare-Bredemeijer EL, Mancham S, Utomo WK, de Canck I, van Thielen M, de Meester E, Rossau R, van der Laan LJ, Hansen BE, Tilanus HW, Kazemier G, Janssen HL, Metselaar HJ, Kwekkeboom J. Genetic polymorphisms in innate immunity receptors do not predict the risk of bacterial and fungal infections and acute rejection after liver transplantation. Transpl Infect Dis. 2013 Apr;15(2):120-33.

3. Verhaar AP, Hoekstra E, Tjon AS, Utomo WK, Deuring JJ, Bakker ER, Muncan V, Peppelenbosch MP. Dichotomal effect of space flight-associated microgravity on stress-activated protein kinases in innate immunity. Sci Rep. 2014 Jun 27;4:5468.

4. Utomo WK, Narayanan V, Biermann K, van Eijck CH, Bruno MJ, Peppelenbosch MP, Braat H. mTOR is a promising therapeutical target in a subpopulation of pancreatic adenocarcinoma. Cancer Lett. 2014 May 1;346(2):309-17.

5. Utomo WK, Braat H, Bruno MJ, van Eijck CH, Groot Koerkamp B, Krak NC, van de Vreede A, Fuhler GM, Peppelenbosch MP, Biermann K. Cytopathology in addition to cyst fluid CEA improves diagnostic accuracy of mucinous neoplasms of the pancreas. Medicine (Baltimore). 2015 Jun;94(24):e988.

6. Utomo WK, de Vries M, van Rijckevorsel DC, Peppelenbosch MP, van Goor H, Fuhler GM. Cannabinoid recepor agonist Namisol® does not affect cytokine levels in chronic pancreatitis patients. Am J Gastroenterol. 2015 Aug;110(8):1244-5.

7. Utomo WK, Looijenga LH, Hansen BE, Bruno MJ, Peppelenbosch MP, Fuhler GM, Braat H. Validation of a 9-microRNA panel in pancreatic cyst fluid for the risk stratification of pancreatic cysts in a prospective cohort. Submitted.

8. Utomo WK, de Vries M, Braat H, Bruno MJ, Parikh K, Comalada M, Peppelenbosch MP, van Goor H, Fuhler GM. Modulation of human

inflammatory signaling through cannabinoid receptor activation. Submitted.

9. Utomo WK, Janmaat VT, Verhaar AP, Lévy P, Cros J, Ruszniewski P, Vredenbregt-van den Berg MS, Jenster G, Bruno MJ, Braat H, Fuhler GM, Peppelenbosch MP. DNA integrity as biomarker in pancreatic cyst fluid. Submitted.

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PhD PORTFOLIO

Name PhD student:	Wesley Kristian Utomo
Erasmus MC department:	Gastroenterology and Hepatology
PhD period:	September 2011-Augustus 2015
Promotors:	Prof. Dr. Maikel P. Peppelenbosch
	Prof. Dr. Marco J. Bruno
Co-promotors:	Dr. Gwenny M. Fuhler
	Dr. Henri Braat

General courses

2011 Erasmus Summer Programme, Netherlands Institute for Health Sciences, Erasmus MC
Study Design (CC01), Netherlands Institute for Health Sciences, Erasmus MC
Clinical Epidemiology (CE02), Netherlands Institute for Health Sciences, Erasmus MC
Biostatistical Methods I: Basic Principles (CC02), Netherlands Institute for Health Sciences, Erasmus MC

2012 Dutch liver week 2012, Dutch Liver Association
Laser Capture Microdissection, Erasmus MC
Basic real time PCR Training, Life technologies, Leiden
Erasmus Winter Programme, Netherlands Institute for Health Sciences, Erasmus MC
Scientific Writing in English for Publication, Netherlands Institute for Health Sciences, Erasmus MC

2013 Erasmus Winter Programme, Netherlands Institute for Health Sciences, Erasmus MC

Conferences - Oral presentations

2014 United European Gastroenterology Week 2014 (UEGW), Vienna, Austria
Potential mechanisms of therapeutic cannabis use in chronic pancreatitis

2015 Spring Meeting of Dutch society for Gastroenterology (NVGE), Veldhoven, the Netherlands
Validation of a 9-microRNA panel in pancreatic cyst fluid for the risk stratification of pancreatic cysts in a prospective cohort

Conferences - Poster presentations

2013 17th Annual Day of the Molecular Medicine Postgraduate School, Rotterdam, the Netherlands Feasibility of pathway analysis in

EUS-FNA obtained tissue of pancreatic cancer patients: a step towards personalized based medicine

2013 Spring Meeting of Dutch society for Gastroenterology (NVGE), Veldhoven, the Netherlands Feasibility of pathway analysis in EUS-FNA obtained tissue of pancreatic cancer patients: a step towards personalized based medicine

2013 United European Gastroenterology Week 2013, Berlin, Germany Feasibility of pathway analysis in EUS-FNA obtained tissue of pancreatic cancer patients: a step towards personalized based medicine

2013 Digestive Disease Week 2013, Orlando, United States of America Feasibility of pathway analysis in EUS-FNA obtained tissue of pancreatic cancer patients: a step towards personalized based medicine

2014 Digestive Disease Week 2014, Chicago, United States of America Potential mechanisms of therapeutic cannabis use in chronic pancreatitis

2014 Spring Meeting of Dutch society for Gastroenterology (NVGE), Veldhoven, the Netherlands Potential mechanisms of therapeutic cannabis use in chronic pancreatitis

2015 Digestive Disease Week 2015, Washington, United States of America
Validation of a 9-microRNA panel in pancreatic cyst fluid for the risk stratification of pancreatic cysts in a prospective cohort

Scientific awards and grants

2013 Erasmus Trustfonds Travel Grant

2013 Dutch Society for Gastroenterology Travel Grant

2014 Erasmus Trustfonds Travel Grant

2014 Oral free paper prize UEGW 2014 for 'Potential mechanisms of therapeutic cannabis use in chronic pancreatitis'

2014 UEGW Travel Grant

Teaching activities

2012 Lecture 'Diagnostic research', minor Medicine, Medical Delta

2013 Lecture 'Diagnostic research', minor Medicine, Medical Delta
Lecture 'Personalized based medicine in cancer', minor Gastroenterology and Hepatology, Medicine

2014 Lecture 'Personalized based medicine in cancer', minor Gastroenterology and Hepatology, Medicine

2014 Supervision research thesis, Adriaan van de Vreede, Bachelor student Medicine

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Other activity

2012 Maser 12 Rocket Launch, Esrange Space Center, Kiruna, Sweden

List of Publication

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ABOUT THE AUTHOR

Wesley Kristian Utomo was born on March 2nd 1985 in Delft, the Netherlands. He was raised by his beloved parents Suryohonggo and Melanie Utomo, and grew up together with his little sister, Lindsey Utomo. After having lived for 4 years in Indonesia, he started the 2nd year of secondary school at the Oosterlicht college in 1998. In 2003, he completed his gymnasium secondary school and went on to study biomedical sciences at the University of Utrecht. After one year, he started medical school at the Erasmus University Rotterdam. In 2008, he performed his graduation research at the Emergency and Trauma Center, the Alfred Hospital, Melbourne, Australia under the supervision of Prof. Peter Cameron and dr. Belinda Gabbe. His research was entitled "Predictors of in-hospital mortality and 6-month functional outcomes in older adults after moderate to severe traumatic brain injury". After two years of clinical internships, he received his medical degree in 2011 and started his PhD project under the supervision of Dr. Henri Braat, Dr. Gwenny Fuhler, prof. dr. Marco Bruno, and prof. dr. Maikel Peppelenbosch. During the first years of his PhD project, he also completed a Master of Science degree Clinical Research at the Netherlands Institute for Health Sciences. Currently, he works at the department of Internal medicine of the Ikazia hospital, Rotterdam.