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Current Status of Molecular Markers for Early Detection of Sporadic Pancreatic Cancer

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Abstract

Pancreatic cancer (PC) is a highly lethal malignancy with near 100% mortality. This is in part due to the fact that most patients present with metastatic or locally advanced disease at the time of diagnosis. Significantly, in nearly 95% of PC patients there is neither an associated family history of PC nor of diseases known to be associated with an increased risk of PC. These groups of patients who comprise the bulk of PC cases are termed as “sporadic PC” in contrast to the familial PC cases that comprise only about 5% of all PCs. Given the insidious onset of the malignancy and its extreme resistance to chemo and radiotherapy, an abundance of research in recent years has focused on identifying biomarkers for the early detection of PC, specifically aiming at the sporadic PC cohort. However, while several studies have established that asymptomatic individuals with a positive family history of PC and those with certain heritable syndromes are candidates for PC screening, the role of screening in identifying sporadic PC is still an unsettled question. The present review attempts to assess this critical question by investigating the recent advances made in molecular markers with potential use in the early diagnosis of sporadic PC- the largest cohort of PC cases worldwide. It also outlines a novel yet simple risk-factor based stratification system that could be potentially employed by clinicians to identify those individuals who at an elevated-risk for the development of sporadic PC and therefore candidates for screening.

Keywords

diagnosis; PanIN; biomarkers

1. Prologue

It seems that pancreatic cancer (PC) has recently crept further and further into the consciousness of the general public. The news that Luciano Pavarotti, the renowned opera

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singer and one of the legendary “three tenors” had been diagnosed with PC came as a shock to millions of his followers the world over. A year-and-a-half later, the famous actor and heartthrob, Patrick Swayze, also received the diagnosis of PC and succumbed to his disease in just 19 months. Former United States president Jimmy Carter has been intimately affected by PC, having lost his father, mother, brother and two sisters to this malignancy. Perhaps the most well known recent case of PC in the public eye was the case of Dr. Randy Pausch of Carnegie Mellon University who was diagnosed with metastatic pancreatic adenocarcinoma and died from the malignancy in 2008. While his words, “If I don’t seem as depressed or morose as I should be, sorry to disappoint you,” from his “Last lecture” showed the well-grounded courage with which he accepted his disease, his well-publicized plight brought to the attention of the world community the insidious and relentless nature of this malignancy and the need to diagnose PC at an early stage. The objective of this review is to revisit certain aspects of PC screening, highlight the latest advances in the early diagnosis of this cancer, and suggest possible strategies that could help identify high-risk cases among those with no familial or known genetic predisposition to PC.

2. Pancreatic adenocarcinoma: evolution and progression

The pancreas (derived from the Greek words, “*pan*” meaning “all” and “*creas*” meaning “flesh”) is a key organ in the field of medicine due to its association with two diseases, diabetes mellitus and PC [1]. The former accounts for an estimated 24 million cases in the United States alone (around 7.8% of the population) [2], while the latter is the fourth leading cause of cancer related deaths in the United States among both men and women [3]. Although the ductal system comprises a small portion of the exocrine pancreas, 90% of pancreatic neoplasms are ductal in origin, of which more than 80% are invasive adenocarcinomas. It is of interest to note that the term “pancreas cancer” when used in common diction refers to conventional ductal adenocarcinoma (commonly referred to simply as simply “ductal adenocarcinoma”) arising from the gland. However, in the pathological lexicon, this term also encompasses other less common variants of ductal adenocarcinoma (including the foamy gland, large duct and vacuolated patterns) as well as other carcinomas of ductal origin such as colloid, medullary, squamous, adenosquamous, and undifferentiated carcinomas. The origin of the neoplastic cells in ductal adenocarcinoma is still unclear, although several sources have been suggested including the non-neoplastic ductal epithelium, islet cells, and more recently, pancreatic stem cells [4,5]. The progression from non-neoplastic cells to invasive adenocarcinoma has been suggested to occur through a series of pre-malignant lesions characterized by progressively increasing dysplasia. These precursors, termed as pancreatic intraepithelial neoplasia (or PanINs), have well-defined morphological characteristics- PanIN-1A (flat), PanIN-1B (papillary without dysplasia), PanIN-2 (papillary with dysplastic changes) and PanIN-3 (carcinoma *in situ*). Two updates on PanIN lesions were published recently [6],[7].

Adenocarcinoma of the pancreas can sometimes be associated with two other types of pancreatic lesions- intraductal papillary mucinous neoplasm (IPMN) and mucinous cystadenomas of the pancreas. Intraductal papillary mucinous neoplasm or IPMN is a unique category of pancreatic neoplasms which manifests as a massive dilation of the intra-pancreatic ducts either involving the entire duct system or restricted to the main pancreatic duct (main duct IPMN) or its branches (branch duct-type IPMN) [8]. IPMNs are broadly classified into invasive and non-invasive variants. The epithelium lining the non-invasive IPMNs shows a variety of differentiation routes including gastric, intestinal, pancreatobiliary and oncocytic patterns, the latter two being rare subtypes. Intestinal type IPMNs, which are generally found in the main pancreatic duct have a propensity to develop into mucinous non-cystic (colloid) carcinoma, while those in the branch ducts are usually of the gastric type and are commonly benign. Pancreatobiliary IPMNs are generally considered

to be at a greater risk of progressing to PC [9]. From the clinical standpoint, IPMNs are associated with a significant risk of malignancy and hence demand surgical resection [10] (IPMNs have been recently reviewed in [11]). Mucinous cystadenomas represent another important group of pancreatic neoplasms which are distinguished from IPMNs by the absence of a communication between the cyst and the adjacent pancreatic duct. All mucinous cystic neoplasms are generally considered potentially malignant owing to the risk of an associated cystadenocarcinoma. Serous cystic tumors, on the other hand, are completely benign with no reported recurrence following complete surgical resection [12,13].

Pancreatic cancer is one of the few malignancies with nearly 100% mortality once diagnosed. The median survival from the time of diagnosis to death ranges from 3 to 6 months. A major cause for the poor prognosis is the resistance of PC cells to conventional chemotherapy and radiotherapy [14]. Gemcitabine, a pyrimidine analogue that targets cells in the S (synthesis)-phase of the cell cycle, is currently the mainstay of chemotherapy for PC. Recent reports indicate that it is more efficacious when used in combination with Capecitabine (a pro-drug that is converted to 5-fluorouracil inside tumor cells) and platinum-based agents rather than as a monotherapy [15]. Surgery is recommended only for those patients without evidence of metastatic disease and without significant vascular involvement. Generally, malignant tumors of the head and periampullary region (when resectable) are managed by a pancreaticoduodenectomy (with or without preservation of the pylorus), while those involving the body and tail are dealt with by a distal pancreatectomy. Segmental resection of the pancreas is reserved only for benign tumors or pre-malignant lesions in the body of the pancreas. The surgical management of PC has been reviewed by Michalski in a recent article [16].

There have been several recent articles that have discussed various aspects of PC, including the common genetic alterations [17], current management strategies [18] as well as the efforts toward understanding the molecular aspects of this malignancy [19]. Nearly all are in agreement on the point that there is currently no definitive treatment for PC. Hence, there has been an emphasis on identifying one or more markers that can detect cancer of the pancreas at a surgically resectable stage or even earlier (in the stage of dysplasia). Most such studies have been based on the principle that one or more genes (and by extension, proteins) whose expression is upregulated (or appears *de novo*) or diminished (decreased or lost) in localized cancer or the dysplastic lesions (compared to non-neoplastic ductal cells) hold promise as potential biomarkers for early diagnosis. The catch, however, is how to identify patients with PanIN lesions, who could then be subjects in prospective studies, when in nearly all cases they are asymptomatic. An alternative approach has been to prospectively examine samples from patients with clinical conditions known to increase the risk of PC (like CP, Type II Diabetes mellitus, chronic smokers, those with an occupational history of working in the rubber industry, or those with a history of tropical or idiopathic pancreatitis or cystic fibrosis [20], to name a few) or healthy subjects with a family history of PC or PC related syndromes. However, the number of such studies is too few to draw any significant conclusions.

3. Limitations of clinical symptoms in the early diagnosis of pancreatic cancer

It is generally recognized that PC is an insidious disease with no specific early clinical symptoms, except where the primary tumor is located in the head of the pancreas. Then, the patient may present early with signs of biliary obstruction (obstructive jaundice). It is worthwhile to quote two recent studies that examined the utility of clinical symptoms in the early diagnosis of PC. The first study compared the frequency of symptoms reported by 120

patients with PC with that in 180 matched control subjects. An important observation was that most patients with PC reported experiencing symptoms within three years prior to the diagnosis of the malignancy [21]. The most commonly reported symptoms included abdominal pain, unusual bloating, belching or heartburn, altered bowel habits (either constipation or diarrhea), symptoms of biliary obstruction (jaundice, pale stools and pruritus) as well as general constitutional symptoms (fatigue, inability to sleep and weight loss). While the odds ratio for many of these symptoms was quite high (for instance >30 for abdominal pain and pale stools), suggesting that these are reported more commonly by patients with PC than the general population, their specificity remains a major issue as the same symptoms are also reported in many benign conditions. A second study [22] conducted among Brazilian patients found that most patients diagnosed with PC experienced asthenia, weight loss and anorexia which was unrelated to the stage of the cancer. Notably, a longer interval between the onset of symptoms and the initial diagnosis of pancreatic cancer was associated with the disease being first identified at a more advanced stage. Further, none of the patients with a tumor in the body or tail of the pancreas was diagnosed with Stage I disease (nearly 80% were in stage IV at presentation). These studies suggest that while clinical symptoms lack specificity, clinicians should nonetheless maintain a high-index of suspicion for an occult malignancy in patients who meet the criteria of the “high-risk group” (Table 1). Obstructive jaundice appears to be the only condition with some specificity for PC and hence must be carefully investigated by the attending physician to rule out a possible malignancy.

4. Risk factors and their role in pancreatic cancer screening and surveillance

4.1 Modifiable or lifestyle associated risk factors

Several risk factors have been found over the years that are associated with an increased risk of PC. A meta-analysis [23] examining 14 studies (6 case-control and 8 cohort studies) on European or North American individuals found that the risk of PC was not significantly affected by the BMI (relative risk- 1.02 per unit increase in BMI, 95% C.I.: 1.01–1.03). However, the relative risk increased from 1.02 to 1.03 when corrected for smoking ($p=0.04$). Further, a correction for the presence of diabetes did not alter the risk significantly and nor was there a difference in the risk between males (1.03) and females (1.02). However, obese individuals (defined as those with a BMI ≥ 30) did have a slightly higher risk (relative risk: 1.19) of developing PC compared to normal-weight individuals (BMI < 25) (similar findings reported in [24,25]). Men and women with central adiposity were reported to be at a greater risk of PC compared to those who report a peripheral weight gain (relative risk: 1.45; 95% C.I.: 1.02–2.07) [24]. Significantly, there was no relationship between the extent of recreational physical activity and the risk of PC, even when analyzed for the subset of individuals who were aged 40 years and above [24].

Several studies have now shown that smoking is a strong risk factor for PC [27–31]. It has been suggested that smoking contributes to nearly one quarter of all cases of PC, making it the single most prevalent risk factor for this disease [32]. A study of familial PC (FPC) kindreds found that smoking was an independent risk factor for PC (odds ratio: 3.7) and smokers developed cancer of the pancreas nearly one decade earlier than non-smokers (59 yrs vs. 69 yrs) [33]. One population based case-control study [29] among Canadians reported that smokers were at an elevated risk factor of developing PC compared to never smokers. This risk was observed in both of the sexes. Men with a smoking history of ≥ 35 pack years and women who smoked 23 or more pack years were found to be at the highest risk of developing PC (OR: 1.46 and 1.84 respectively). However, neither the amount of total alcohol consumed (even up to one drink every day) nor the type of alcoholic beverage

(wine, beer or liquor) had any effect on their risk of developing ductal adenocarcinoma. Interestingly, this study also found that neither drinking coffee (≥ 4 cups/day) nor the income or the number of years of education had any impact on the risk of PC. A later study by the same group [28] revealed that an increased weekly caloric intake and participation in ≥ 8.2 hrs of strenuous physical activity was associated with a higher and lower risk of PC, respectively (O.R.: 1.68 and 0.59, respectively).

Among women, four or more pregnancies and an older age at the time of menarche (15 yrs or older) were associated with a reduction in the risk. Additionally, unlike the population as a whole, as seen in the aforementioned studies, caloric intake and physical activity did not appear to alter the risk of PC among women. A history of exposure to environmental tobacco smoke from childhood and onto adulthood, however, translated into a modest elevation in the risk for subsequent development of an adenocarcinoma of the pancreas, even among never-smokers [27]. Further, this risk was amplified in active smokers compared to those with a past history of cigarette use (≥ 25 pack-years).

A hospital based case-control study in northern Italy, while confirming the findings of the Canadian group, also found that the risk of PC among smokers was independent of the number of cigarettes smoked per day (even up to 20 or more per diem). Of interest was the observation that among former smokers, the risk of PC fell to levels comparable to never smokers after only 15 years or more post quitting smoking. Notably, this was independent of the number of cigarettes smoked or the duration of the smoking habit [31].

In one case-control study [34] “heavy smokers” (defined as those with a history of >40 pack years) were at an increased risk of PC if they were carriers of at least one minor allele for the DNA repair gene *XPB/ERCC2* at D312N (O.R.: 2.78, 95% C.I.: 1.3–6) OR D711D (O.R.: 2.2, 95% C.I.: 1–4.7). Thus, the risk of PC associated with smoking appears to be modified by the presence of co-existing genetic alterations in genes that regulate cellular response to DNA damage.

In a large prospective cohort study of over 1 million Americans over a 14-year period [35], black race (>1.5 fold greater risk in both men and women) and history of gallstones (1.3 fold higher risk only among men) were predictive of a risk of PC. The risk was also two fold or higher in current male smokers who smoked 20 or more cigarettes per day (for women, a similar degree of risk was observed with 10 or more cigarettes per day), or smoked for >25 yrs compared to never smokers. Notably, the risk associated with cigarette smoking was not modified by diet or other lifestyle factors examined. The studies also observed no relationship between the levels of education, the consumption of red meat, citrus fruits, juices, coffee or spirits and the risk of PC. From the aforementioned studies, it appears that specific lifestyle traits of an individual have a considerable impact on their risk of developing PC.

A recent nested case-control study among 1,141 PC patients and 7,954 controls British patients found that the use of non-steroidal anti-inflammatory drugs for about 2 years prior to the diagnosis of PC was associated with a significantly decreased risk of PC (O.R. 0.75, 95% C.I.: 0.62–0.97) [36].

4.2 Non-modifiable or genetic risk factors

Although the role of genetic predisposition in sporadic PCs is still unclear, a familial clustering of PC cases has been reported in numerous studies (summarized recently by Landi [37]). Based on these reports, strategies have been recommended to screen asymptomatic individuals belonging to familial PC kindreds [38,39]. Several genetic alterations have been shown to be associated with a hereditary predisposition to PC. Some mutations predispose

the carriers to malignancies in other organs as well. Mutations in the *BRCA1* gene for instance, are associated with an increased risk of breast, ovarian, uterine and fallopian tube malignancy in women and breast and prostate cancer in men. In addition, carriers for a mutation in this gene are also at an increased risk for pancreatic, colon, gastric, lung cancer and melanoma [40]. While genetic changes are more frequent in cases with an associated family history of PC (FPC kindreds), inactivating mutations in *p16^{INK4a}* (also called cyclin dependent kinase A or *CDKN2A*) are frequently observed in sporadic PC [40]. However, mutations in *p16^{INK4a}* are not specific for PC (in which the common mutation is V126D), being also observed in other malignancies such as familial cases of malignant melanoma (also V126D) and breast cancer (associated with an 113insArg mutation). The latter patients are also at an increased risk for PC [41].

In one study involving 18 German families with familial PC [42], each of whom had at least two first degree relatives with PC, and five who had at least one relative with both PC and melanoma, none of the families without an associated melanoma had a mutation in the *p16^{INK4a}* gene, while two of the five families with both PC and melanoma had germline truncating mutations (Q50X and E119X) in the gene. A prospective study among patients enrolled in the National Familial Pancreas Tumor Registry (NFPTR) found that the increase in risk of PC among those with a family history (of PC) was generally confined to patients who were 60 years or older [43].

The *PALB2* (also known as partner and localizer for BRCA2 or FANCN) gene located on chromosome 16 encodes for a protein that stabilizes the BRCA2 oncoprotein in the nucleus [44] and has recently been linked to a possible association with the risk of familial PC. Biallelic inactivating mutations in *PALB2* have been previously linked to Fanconi's anemia, specifically to subtype N which resembles the Fanconi's anemia phenotype caused by a biallelic mutation in the *BRCA2* gene [45]. *PALB2* has also been shown (in several population based studies) to increase the susceptibility of patients to the development of breast cancer (the estimated risk ranging from 2.3-fold to 6-fold for carriers of mutations in the gene) [46]. A recent study comparing tumor DNA from a patient with familial PC with the human reference genome revealed a germline deletion of four base pairs in the *PALB2* gene that translated into a frame shift mutation at codon 58 of the gene [47]. Further sequencing of this gene in 96 patients with a family history of PC revealed truncating mutations in the gene in three patients. Each of the three mutations identified (IVS5-1 G>T, 3116 del A and 3256 C>T) produced a different stop codon. Importantly, no truncating mutations were reported in a cohort of 1084 healthy individuals [48], while only two healthy controls out of 1079 had a mutation (1592delT) in the same gene in another study [46]. A germline missense mutation (P239S) in Palladin, a gene located on the chromosome locus 4q32–34 has also been suggested to be linked to a familial clustering of PC [47]. However, subsequent studies have failed to detect the presence of this mutation in FPC clusters [48–51].

While most PCs with a family history have been described in association with some form of a genetic alteration, an interesting case of a family with six cases of pancreatitis and three of PC in the second generation was recently described wherein the affected members had none of the known genetic alterations [52]. Further, the malignancy in this cohort uniquely spared the head of the pancreas. Instead, a characteristic fatty infiltration involving only the body and the tail was observed. Clearly, the relationship between genetic factors and the environment that modulates the risk of an individual developing PC is highly complex and needs to be elucidated further.

A prospective cohort study of 107,503 U.S. health professionals found that the ABO blood group of an individual could contribute significantly to the risk factor for PC development

[53]. Individuals with non-O blood groups were found to be at a greater risk for the development of PC, with the risk being lower among group A than among group B individuals. Notably, the association between the ABO blood group and the risk of subsequent PC (as measured by the hazard ratio) was not significantly modified when other purported risk factors, like advanced age (≥ 62.5 years), body mass index (≥ 25.7), physical activity (≥ 13.5 metabolic equivalent task hours per week) and smoking (never vs. past/current smokers) were considered. Further, the Rh group did not appear to have an influence on the risk of PC.

Allergies, especially a history of hives, have been linked to the risk of developing cancer, although the direction of the risk varies from study to study. While the presence of an allergy has been shown to increase the risk of hematopoietic malignancies (leukemia, lymphoma and myeloma) [54], it appears to reduce the risk of cancer at other sites [55]. Several epidemiological studies have also examined the association between a history of allergies and PC. One prospective study of over 1 million Americans from 1982 to 2000 found that a history of hay fever was associated with a significantly lower mortality among those diagnosed with PC (compared to those without such a history) [56]. Another hospital based case-control study by Olson et al. found that a history of allergies (regardless of the type of allergen) was associated with a significantly lower risk of developing PC [57], a view supported by a more recent study in Canadians [58]. Interestingly, the presence of a G3017T variation in the IL-4 gene (an inducer of IgE synthesis together with IL-13) was actually associated with a slightly increased risk of PC in allergy-prone individuals (O.R.: 1.47, 95% C.I.: 0.8–2.69), while the risk was reduced in those who did not report any allergies (O.R.: 0.44, 95% C.I.: 0–0.99) [57]. The results of this pilot study seem to suggest that genetic variations in genes encoding for cytokines (including SNPs and mutations) and other mediators of inflammation could also affect the risk of PC, and possibly permit risk stratification for patients.

Polymorphisms in the cytochrome P450 enzyme (CYP2A6), capable of activating several procarcinogens (including the highly carcinogenic nitrosamines) has been linked to an increased risk of sporadic PC (independent of smoking) [59], although further studies are needed to confirm these observations. Single nucleotide polymorphisms (SNPs), which are variations in the DNA sequence (among different individuals) involving a single nucleotide, have been extensively studied for a possible direct association with the risk of developing PC, or alternatively altering the risk of the malignancy among individuals with certain predisposing factors. However, most studies to date have not revealed any significant relationship between a SNP and the risk of PC in the general population. One study even examined the entire mitochondrial DNA for SNPs associated with the risk of PC [60], while another examined for possible correlation with survival [61], but none were observed. A recent study employing 178 PC and 182 healthy controls identified a SNP in the gamma glutamyl transferase (GGT) gene that was significantly ($p < 0.05$) associated with a risk of PC in a separate validation set [62]. A genomewide association analysis employing 3,851 PC and 3,934 healthy controls recently identified eight novel SNPs on chromosome loci 1q32.1 (5 SNPs), 5p15.33 (1 SNP) and 13q22.1 (2 SNPs) that were significantly associated with a risk of developing PC [63]. The SNPs on 1q32.1 map to the *NR5A2* gene that encodes a nuclear receptor normally expressed (among other organs) in the exocrine pancreatic glands, while that on 5p15.3 maps to the *CLPTMIL-TERT* locus encoding two genes that have both been associated with carcinogenesis. The SNP on 1q32.1 however, mapped to a region devoid of any known genes. While these studies provide useful genetic markers to determine the risk of PC, whether (and how) the SNPs results in functional alterations in the protein function and whether they act independently or in association with other risk factors remains to be investigated.

There appear to be an association between SNPs in DNA repair genes and the risk of PC, specifically among smokers. An example is the significantly increased risk of PC, among heavy smokers (defined as those with a history of ≥ 22 pack years) who harbored an Arg¹⁸⁸His polymorphism in the *XRCC2* gene [64], while an Asp³¹²Asn polymorphism in exon 10 of the *XPB* gene significantly reduced the risk (O.R.:0.46, 95% C.I.: 0.2–0.8). The latter did not, however, affect the risk of PC among non-smokers [65]. Further, the presence of both an Asp³¹² polymorphism (on exon 10) and a Lys⁷⁵¹Gln polymorphism (on exon 23) were identified as haplotypes with an elevated risk (OR: 3, 95% C.I.: 1.3–6.9) for PC among smokers (but not among never smokers) in the same study.

As more and more genetic alterations are described, one hope is that, in the future, a chip-based assay (similar to microarrays) can be developed to screen patients with family histories of cancer in general, and PC in particular, to identify mutations that could help identify asymptomatic individuals at high risk for developing a pancreatic malignancy.

4.3 Chronic Pancreatitis

Although the true prevalence of chronic pancreatitis is not known, it is estimated to range between 0.04% and 5% in the normal healthy population [66] (diagnosis, classification and genetics of CP excellently reviewed in [67]). There is a proven association between carcinoma of the pancreas and both the sporadic (chiefly tropical calcifying pancreatitis [68]) and hereditary forms of CP (chiefly due to mutations in the *PRSS1* [69] and cationic trypsinogen gene [70]), the standardized incidence ratio for development of PC in CP cases being 14–18, which is further increased by cigarette smoking [70]. Results from a prospective case-control study [71] seem to suggest that CP may be closely related to PC, although the answer to the question of a cause-and-effect relationship is not clear (the link between CP and PC was discussed in a recent article [72]). A recent meta-analysis has suggested that the interval between CP and PC is nearly 20 years, with pancreatitis occurring within 1–2 years prior to the diagnosis of PC usually resulting from tumor related obstruction of the pancreatic duct [73]. Transgenic mice wherein the human interleukin-1 β gene is expressed under the control of a rat elastase promoter develop features similar to those of severe CP [74]. However, although the older mice developed features of tubular complexes, there were no PanIN lesions or tumors. This model could be useful to examine the relationship between CP and PC in more detail. The possible role of pancreatic stellate cells, fibroblast-like cells that are normally quiescent but get activated during inflammation, in CP-associated carcinogenesis has also been suggested (reviewed in [75]). In support of a possible link between the two pathologies was the observation that abnormalities suggestive of CP were far more common (>70%) in patients who were classified as being at “high-risk” for PC compared to the prevalence of these changes in control subjects (<20%). Further, the study also found that “high-risk” patients, i.e. those with a family history of PC (defined as the presence of PC in at least two first-degree relatives or the presence of an inherited mutation known to predispose to PC) were more likely to have abnormalities suggestive of CP on endoscopic-ultrasound (EUS) than those without such a history (Odds ratio: 17.4). Significantly, the incidence of high-grade CP findings upon EUS examination (graded according to the Cambridge classification [76]) was significantly greater in the “high-risk” patients (53% showed moderate grade and 5% severe grade chronic pancreatitis) compared to the controls (4% moderate grade and none with severe grade chronic pancreatitis). A follow-up of 223 patients with non-hereditary CP (>70% of which were attributed to alcohol) revealed that the incidence of PC among these patients was nearly 6% during a 14-year period [77]. Notably, these patients were also at an increased risk for gastric and esophageal carcinoma, although the risk was less than that for malignancy of the pancreas.

Kras mutations have been demonstrated to occur in only a small percentage (4.4 percent of 429 micro dissected lesions) of non-hereditary/sporadic CP (in the absence of any mutated

p53) in a study of 30 resected specimens. Acute inflammation of the pancreas (acute pancreatitis) in the setting of a pre-existing activating *Kras*(G12D) mutation, however, resulted in rapid progression of PanIN lesions and accelerated development of pancreatic adenocarcinoma in a mouse model of PC [78]. This suggests that there is interplay between *Kras* activation and the inflammatory cascade (possibly through cytokines) that enhances the risk of malignant transformation in the pancreas. Activating mutations in *Kras* have been identified in the pancreatic juice and tissues from CP cases [79]. It would be of interest to examine whether patients with CP who have mutations in *Kras* and develop recurrent attacks of pancreatitis are at an accelerated risk of developing invasive adenocarcinoma. This information would be crucial to make a decision on surgical removal of the diseased pancreas in these individuals.

Several studies have found that CP and PC are very similar in the profile of genes expressed in them [80,81]. One study [82] found that a set of seven genes could discriminate between the two pathologies with an accuracy of 92% in a randomly assigned training set. Alpha integrin ($\alpha 6\beta 4$ integrin) was shown to be highly expressed in PC and PanIN lesions but weakly in the normal pancreatic ducts and CP tissues, suggesting its potential use as tissue based marker to distinguish the two closely related pathologies. Proteomics based approaches have revealed that Maspin [83], MUC4-p53 combination [84] Annexin-2 and Insulin-like growth factor binding protein 2 (IGFBP-2) [85] are differentially overexpressed in PC compared to CP. A Western blot array (Powerblot, BD Biosciences) analysis of pooled protein samples from normal, CP and PC derived pancreatic tissues identified more than 50 proteins that were upregulated in PC compared to CP, while an almost equal number were downregulated [86]. Figure 2 lists genes that were differentially expressed between PC and CP by a magnitude of at least five-fold.

However, several of these studies suffer from limitations, mostly related to the study design (small sample size, no distinction between early and late or resectable vs. unresectable PC) and validation of potential markers. In most cases, only a small percentage of the markers identified were validated in a test set (which sometimes was too small in size). Thus, it is important that the results of these studies be first validated in blinded samples at multiple centers to identify the most promising molecules.

4.4 Type-II Diabetes mellitus

The relationship between adult onset diabetes mellitus, especially within three years post the initial diagnosis, and the development of PC has been quite murky. The central question remains: which came first, the diabetes or the cancer? While there is no denying the fact that the two do seem related, there does not seem to be a simple relationship between them. A study by Egawa et al. comparing patients with a family history of diabetes with those without showed that pre-existing diabetes (≤ 3 years duration), was more likely to be associated with or lead to PC. Further, PC patients with a family history of diabetes in a first degree relative were on average at least 5 years younger at diagnosis (61 ± 9 vs. 65 ± 11 years), had masses predominantly involving the body and tail of the pancreas (consistent with the higher concentration of beta cells in these regions), and had cancer of a non-tubular type (including intraductal papillary mucinous cancer, adenosquamous carcinoma and mixed ductal-endocrine cancer) [87]. In a nested case-control study in a population of residents of Rochester (Minnesota) aged 50 years and above [88], Chari and co-workers identified that among newly diagnosed type-II diabetics, the risk of developing PC within the first three years was almost eight-fold higher than that in the general population. Perhaps, the most significant finding from the viewpoint of surveillance was that 10 of the 18 patients diagnosed with PC in the study were diagnosed within six months of meeting the criteria for diabetes. Further, only seven of the 18 diabetics who developed PC had a family history of diabetes. Notably, nine of the 18 patients had symptoms suggestive of malignancy at the

time when they first met the criteria for diabetes, though only three of the 18 cases were resectable at the time of presentation. The proportion of ever smokers was also considerably higher among those diabetics who developed PC (95% vs. 69% in those without PC) in this study. A relative lack of a link, however, between adult onset diabetes and the risk of PC among FPC kindreds was also reported elsewhere [33]. The case control study in Italy alluded to earlier [31] also examined the risk of PC among diabetics, specifically those who were on treatment. Diabetic patients who were on treatment but did not develop PC were taken as controls in this study. The presence of diabetes emerged as a significant risk factor for subsequent diagnosis of PC (relative risk, R.R.: 2.89 (95% CI= 1.71–4.86). A key observation was that while the mean age of onset of diabetes mellitus (55.7 yrs vs. 55.4 yrs) and the duration of the disease (6.6 yrs vs. 9.2 yrs) were comparable between cases and controls, PC cases were more likely to have received treatment for diabetes within 2 yrs before the diagnosis of PC (RR: 4.61, (95% CI 1.99–11.53)). Further, the risk of PC decreased progressively with the time elapsed between the diagnosis (and treatment) of DM and the diagnosis of PC (R.R: 2.41 for cases diagnosed 3–5 yrs before diagnosis of PC and 2.06 for cases diagnosed more than 5 yrs before the diagnosis of PC). Notably, the type of treatment also appeared to influence the risk of PC with patients on insulin at a significantly higher risk than those treated with oral hypoglycemics (RR: 7.68, 95% C.I.: 1.27–17.22). Most significantly, diabetic patients treated with insulin for more than five years were at a significant risk for developing PC (RR: 6.21, 95% C.I.: 1.61–23.96). However, patients treated with oral hypoglycemics did not show any increase in risk, even after five years of treatment.

4.5 Cystic neoplasms of the pancreas

Cystic tumors of the pancreas can be divided into two principal types- serous and mucinous cystic neoplasms. Serous neoplasms are generally benign. Mucinous cystic tumors, on the other hand, are potentially malignant. Invasive cancer arising in a mucinous cyst (mucinous cystadenocarcinoma) is a lethal disease with a prognosis similar to that of pancreatic adenocarcinoma [89]. In one study, all patients identified with unresectable adenocarcinoma in cystic mucin producing tumors of the pancreas were dead within 2–20 months of detection. Conversely, those with benign tumors (hyperplasia or adenoma) were alive without recurrence after surgery. In another retrospective analysis of 84 cases of cystic neoplasms at a single center, none of the 77 patients without invasive carcinoma developed recurrence following surgical resection of the lesion [89]. In sharp contrast, five of the six who survived surgical resection for cystadenocarcinoma died of recurrence of the carcinoma within five years of their surgery. All of these five patients had 5–10 cm sized tumors. The sole survivor had a singly microscopic focus of invasive carcinoma. In another single center study, 20% of patients with symptomatic cysts were found to have an associated adenocarcinoma on histopathologic examination of the resected specimen in comparison to only 5% for incidental cysts [90]. Further, in this study, EUS was able to identify cysts in eight cases that were missed by both CT and MRI. Thus, the incidence of neoplastic changes is nearly four times greater in symptomatic cysts than silent ones, suggesting that patients in this group are good candidates for close long-term follow-up.

Consequently, early detection of cystic tumors of the pancreas is essential for continuous follow-up to identify nascent changes associated with malignancy. However, hyperplastic and adenomatous tumors are unremarkable in their features, being almost always less than 3 cm in size, associated with a main pancreatic duct (MPD) diameter less than 3mm, have a characteristic absence of mural nodules and lack of mucin extrusion from the ampulla of Vater. Cytology is also non-informative and serum CA 19–9 as well as CEA are usually within normal limits. However, in intra-ductal tumors (IDT), pancreatoscopy during

Endoscopic Retrograde Cholangiopancreatography (ERCP) can visualize the MPD and also measure its diameter.

5 Role of imaging in the early diagnosis of pancreatic cancer

5.1 Endoscopic-Ultrasound

CP is considered a risk factor for PC. However, most cases of PC probably arise sporadically in a pancreas afflicted by changes of CP [91,92]. PC in the setting of CP presents a diagnostic challenge owing to several reasons. First, CP often mimics changes seen in ductal cancer and the converse is also true. Further, the chronic inflammation accompanying CP can often obscure a small malignant focus. These reduce the sensitivity and specificity of even EUS guided FNA, necessitating additional criteria or markers to distinguish foci of malignant change in the background of CP. One clue comes from the observation that CP seldom presents with obstructive jaundice [93]. So, the presence of concomitant jaundice in a patient with risk factors for CP (e.g. male, age <50 years, African-American descent with/without a history of alcohol intake) should alert the radiologist to search carefully for minute foci of malignant change. Measures that increase the chances of detecting isolated dysplastic/neoplastic foci is to increase the number of passes of the needle during EUS-guided FNA and surgical exploration in FNA-negative cases with a high index of suspicion. However, EUS-FNA should not be performed in those patients with CP without an apparent mass, due to the risk of procedure related pancreatitis.

A prospective study by Le Blanc et al. [94] showed that the minimum number of FNA passes to get an optimal diagnosis of pancreatic tumor is seven, while it is five in case of lymph nodes. By using this approach, they achieved a sensitivity of 83.3%, with specificity, positive (PPV), and negative predictive value (NPV) of 100%, 100% and 50%. In the case of lymph nodes, the sensitivity, specificity, PPV, and NPV of five or more passes of the needle were 77%, 100%, 100% and 81%, respectively. This study suggests that the lack of a cytopathologist at the bedside is one of the key reasons for an inadequate smear. For instance, usually FNA is taken from the edge of a pancreatic mass as the central regions could be necrotic. However, sometimes even an FNA taken from such a location may contain necrotic debris which cannot be interpreted by the cytopathologist, necessitating a change in the site of aspiration. Surgical histopathology and a one year clinical follow-up were used as reference standards for this study [94].

Despite advances in other imaging techniques, EUS-FNA has increasingly become the gold standard for confirming diagnosis without subjecting the patient to surgery. A recent study [95] examined the performance of contrast-enhanced power Doppler (CEPD) and real-time sonoelastography (RTSE) in distinguishing between CP and PC. While a combination of the two techniques diagnosed PC with a sensitivity, specificity, accuracy, PPV and NPV of 76%, 95%, 96%, 71% and 83% respectively, EUS-FNA still performed better (comparative performance 88%, 100%, 100%, 84% and 93% respectively).

Early detection of pancreatic neoplasms would logically require the detection of small lesions. In a retrospective study of over 1000 FNAs conducted at one institution, it was reported that EUS guided FNA was more sensitive (86.5% vs. 80%), had comparable specificity (97.3% vs. 100%), PPV (97% vs. 100%), higher NPV (87.8% vs. 50%), fewer false negatives (6.8% vs. 16.7%) and higher accuracy (91.9% vs. 83.3%) than US-guided FNA in detecting intra-pancreatic lesions less than 3cm in diameter. Moreover, the rate of inadequate specimens was significantly reduced by EUS guided FNA (2%) as compared to percutaneous US or CT guided biopsy (18.5%). Several factors, including the presence of an on-site cytopathologist as well as the experience of the endoscopist and radiologist, can influence the accuracy of this technique. Given the considerations of time and cost, it is

recommended that for patients at risk of PC (from history, and presence of risk factors), EUS-FNA is the screening test of choice to detect lesions smaller than 3cm, even if US or the CT scan is negative. For larger lesions, there was no obvious difference in the three techniques, so a US or CT guided percutaneous FNA to obtain tissue from suspicious masses is advisable in the interests of cost, time, and availability of trained personnel. It is further recommended that all cases of atypical/inconclusive and suspicious FNA should be closely followed by repeat biopsy or surgery if necessary given that a significant number of them progress to ductal cancer (82.2% and 58.6%, respectively, in one study) [96].

EUS has emerged as the frontrunner among various imaging modalities tested for screening patients to detect indolent pancreatic neoplasms. In a prospective study on high-risk patients aimed at detecting asymptomatic PCs [71], EUS was more sensitive than CT-scan and endoscopic retrograde cholangiopancreatography (ERCP) in identifying silent pancreatic neoplasms. This study also confirmed the safe nature of EUS, with mild post-procedural pain as the only major complication. It is interesting to note that branch-type intraductal papillary mucinous neoplasm (IPMN) was the most common pancreatic neoplasm identified by EUS. Further, the IPMN adenomas were most frequently associated with PanIN lesions of varying grades and CP (10 out of 12 patients with IPMN in another study had symptoms of CP [97]). In one high-risk patient, the IPMN lesion (obtained after surgical resection) was associated with carcinoma-in situ. It has previously been reported [98] that several genes that are highly upregulated in PC are also similarly deregulated in IPMNs (including lipocalin-2, galectin-3, claudin-4 and cathepsin E). These studies strengthen the premise that IPMN is a precursor of invasive adenocarcinoma. A second high-risk patient in this study had a branch-type IPMN lesion with multiple foci of PanIN-3 (high grade dysplasia) and what appeared like adenocarcinoma. In a case report, Nüssler and co-workers reported that a 54-year old woman with margin-negative, non-invasive IPMN treated by segmental resection, subsequently developed an adenocarcinoma in the tail of the pancreas three years later [99]. A prospective study of 12 patients with IPMN (referred to earlier [97]) observed extensive multifocal intraductal changes involving most or all of the pancreas in six of the ten patients who underwent surgery for removal of the pancreas. The study concluded that while IPMNs in general have a favorable prognosis, a diffuse dilation of the main pancreatic duct usually indicates widespread involvement of the duct by the tumor and warrants total pancreatectomy.

A study focusing on cystic neoplasms of the pancreas reported that IPMNs were the most common diagnoses among patients undergoing surgery for a cystic lesion (either due to symptoms or other factors including increasing size, patient anxiety, or doubtful findings upon imaging), being present in nearly 50% of the resected specimens [100]. Notably, 38% of the main duct-IPMNs had an associated invasive component.

5.2 Recent advances in pancreatic imaging

Many modalities are available to image the pancreas including non-invasive techniques like ultrasound, contrast-enhanced multi-detector computed tomography (CECT), magnetic resonance imaging (MRI), integrated positron emission tomography/computed tomography, and invasive techniques, like endoscopic retrograde cholangiopancreatography (ERCP) and endoscopic ultrasound (EUS) (reviewed in [101] and [102]). Advances in cytology including digital image processing and fluorescence in situ hybridization (FISH) are emerging as useful adjuncts to conventional cytology in the early detection of PC. In a study of 446 patients, 80% of those with polysomic signal on FISH (on ERCP brushings) were found to have PC by cytology, and nearly all were diagnosed with PC within 2 years of identification of a polysomy on FISH [103]. Semiconductor nanocrystals or quantum dots (QDs) have emerged as novel agents for bio-imaging owing to several advantages over traditional organic dye based imaging including a higher quantum yield, greater stability and an wide

range of excitation wavelengths (ranging from the visible to near-infrared) that can be easily modulated by changing physical characteristics (size, shape and composition of the nanoparticle). A major development has been the recent development of non-cadmium based QDs which were non-toxic to PC and immortalized cells over a wide concentration range [104]. These indium phosphide-zinc sulphide QDs can be conjugated to biomolecules like antibodies and have been shown *in vitro* to target specifically to the cells expressing the specific antigen. Plectin-1, recently identified as a marker for PC, was recently shown to be a specific marker to distinguish PC from chronic pancreatitis immunohistochemically, while plectin-1-targeted peptides conjugated to magnetofluorescent nanoparticles (PTP-NP) allowed the detection of small PC by *ex vivo* MRI in genetically engineered mouse models [105]. Manganese-(Mn) doped quantum dots (MnQDs), which emit light in the near-infrared region (700nm-1µm) and can be solubilized stably in aqueous media (by surface functionalization with lysine) and also conjugated to biomolecules, have also been recently developed [106]. These MnQDs conjugated with anti-claudin 4, anti-mesothelin or anti-PSCA (prostate stem cell antigen) monoclonal antibodies were internalized into the PC cells Panc-1 and MiaPaca without significant toxicity to the cells. When injected into mice, the MnQDs produced a peak at 828 nm, which was distinct from the autofluorescence produced by the mouse tissues. Importantly, a dose nearly five times the routine dose for QDs used in imaging breast cancer cells in small animals did not produce any toxicity or behavioral change in mice even 12 weeks after a single injection. These studies, although preliminary, point toward exciting new developments that are poised to provide better resolution imaging (employing QDs conjugated to proteins that are expressed by high-grade dysplasia, the stage of precursor lesion most definitely linked to risk of infiltrating adenocarcinoma). Low-coherence enhanced backscattering (LEBS) and elastic light scattering fingerprinting (ELF) based optical measurements of the apparently healthy periampullary duodenal tissue has shown that high-risk cases (including those with a family history) had signals that lay in between that obtained from healthy individuals and those with PC [107], suggesting the utility of field-effects as useful tools to identify early malignancy.

6. PanIN lesions as a source of potential biomarkers

Analysis of differentially expressed genes in various PanIN lesions isolated from patient tissue samples by micro dissection revealed that PanIN-2, and not PanIN-1 is the first stage in the development of cancer that is associated with significant genetic changes (PanIN- 1B, 2, 3 and ductal cancer showed 47, 438, 578 and 610 differentially expressed genes respectively) [108]. Three main groups of differentially expressed genes could be recognized in this study: those whose expression was lost from normal ducts very early during pathogenesis [including the genes for putative cytokine high in normal 1(*SCGB3A1*), amiloride sensitive cation channel 2 (*ACCN2*), and the transmembrane mucin *MUC13*], genes whose expression was elevated beginning from the stage of PanIN-1B and maintained until PC [including S100 calcium-binding protein (*S100P*), the trefoil factors 1 and 2, and matrix metalloproteinase 1 (*MMP 1*)], and those for which an increased expression was first detected only in the late stages of dysplasia (i.e. PanIN-2,3 or PC), which included the serine threonine kinase 11, fibronectin 1 and plasmin 3 genes. Further, two additional patterns of gene expression were identified by the authors. A transient change in the expression of some genes was detected in the early PanIN stages that returned to levels comparable to normal tissue in the later stage of pathogenesis. A more persistent pattern of differential gene expression was also noted, particularly for genes important in maintaining the cellular structure and those important in development/differentiation. The transient change in gene expression can be explained by an activation of counter-regulatory genes that are turned on in response to early mutations that impair normal cellular functions. However, in the later stages of cancer progression, many of the normal control mechanisms in the cells are inactivated, presumably accounting for the reversal of the genetic changes seen in the earlier

PanIN stages. The more persistent changes in expression usually effect genes that are important in the remodeling of the extracellular matrix, an important change during tumorigenesis (these include upregulation of MMP 7, fibronectin 1, and type 3 collagen as well as downregulation of MMP17). Based on the premise that PanIN-2 is the earliest precursor of invasive ductal adenocarcinoma, the authors selected 22 genes that were significantly upregulated in PanIN-2 compared to the normal ducts as potential biomarkers for early diagnosis. However, a major drawback of this study was the lack of any chronic pancreatitis tissues in the analysis. As we see later, CP shares several markers in common with PC (and PanINs) and thus one of the goals of biomarker discovery for the early diagnosis of PC is to exclude proteins that are common to CP. One of the earliest altered oncoproteins is the mucin MUC1. Immunostaining with the PAM-4 monoclonal antibody against MUC-1 specifically labeled the dysplastic ducts but not the normal ones, with a progressive increase from low to high grade PanINs in one study [109].

An ideal biomarker, from the standpoint of both the patient and the physician, is one that can also predict the prognosis following an early diagnosis. One such possible marker is the epithelium specific marker cytokeratin-20 (CK-20). Belonging to the category of acidic intermediate filaments, CK-20 is not expressed in normal fetal and adult pancreas, but is aberrantly expressed in 30–60% of pancreatic ductal adenocarcinomas [110]. Further, it is also expressed in PanIN lesions, but only when the associated cancer is also positive for CK-20. It is more commonly expressed in higher grade PanIN's (88% in PanIN-II and III combined vs. 60% in PanIN-I). This suggests that in a reasonable fraction of ductal cancers, the aberrant expression of CK-20 precedes the development of invasive cancer. Screening high risk patients for CK-20 expression in the pancreatic juice or in FNA specimens could identify a subgroup of individuals with asymptomatic changes of PanIN in their pancreas. CK-20 also has a prognostic role, as CK-20 expressing cancers appear to have a significantly poor prognosis. In one study, none of the patients who expressed CK-20 in their cancers were alive beyond 26 months [111]. CEACAM-6 is another protein which is strongly expressed in the PanIN-3 lesions, and like CK-20, an inverse relationship is observed between CEACAM-6 expression and survival of PC patients, with non-expression (in the primary tumor) translating into absence of nodal metastasis and a longer postoperative survival [111]. Class-III β tubulin (TUBB3) has also been shown to be absent in non-neoplastic ducts, but increasingly expressed with advancing grades of PanIN, reaching the highest expression in adenocarcinoma [112].

A recent proteomic analysis [113] using micro dissected cells from PanIN lesions of nine PC patients identified 31 proteins that were differentially expressed at one or more stages of intraepithelial neoplasia (compared to the non-neoplastic ducts). Immunohistochemical validation of five of these proteins (MVP, AGR2, 14-3-3-sigma, annexin-4 and S100A10) was also done suggesting that these markers in combination could be used to correctly identify PanIN lesions, most pertinently in FNAs and cytopathological specimens. A similar approach (but using gross rather than micro dissected specimens of normal pancreas, chronic pancreatitis, PanIN-3 and PC followed by mass spectrometry) was adopted by Pan et al. [114]. Using a cut-off of a ≥ 1.75 fold change in PanIN-3 compared to the normal pancreas, 70 proteins were found to be upregulated and 133 downregulated in PanIN-3 (compared to the normal ducts). Interestingly, most of the enriched proteins dysregulated in PanIN-3 lesions were related to cellular motility and remodeling of the cytoskeleton. As the actin cytoskeleton plays a central role in cellular invasion, motility and metastases, the findings of this study suggest that dysregulation of proteins associated with cellular invasion (a change typical of cancer) begins early on, i.e. in the high-grade dysplastic lesions. Particularly interesting was the dysregulation of 18 proteins that are known to directly interact with *c*-MYC, a well-known oncogene which is involved in nearly one-fifth of all human cancers. One of the proteins, β -tubulin, which was shown to be upregulated both in PanIN and

chronic pancreatitis tissues, has been shown to be differentially expressed during the progression of PC in an earlier study [115]. Some of the proteins were also found to be dysregulated in a direction similar to that reported by another study by Sitek and others [113] (e.g. *TPM2*, *EEF1A1*). However, while Sitek and co-workers had observed a downregulation of Annexin A-IV (*ANXA4*), the other study [114] found an upregulation (≈ 2.5 fold) in the gene in PanIN lesions. The study [114] also validated the overexpression of Laminin- $\beta 1$, actinin-4 and galectin-1 by immunohistochemistry which revealed that while all three were expressed in the stroma (actinin-4 only in PC and galectin-1 in both PanIN and PC stroma), only actinin-4 was also overexpressed by the ductal epithelium. Figure 1 depicts the proteins that were upregulated specifically in PanIN-3 (but not in CP) by ≥ 3 -fold. Claudin-18, which belongs to the family of tight junctional proteins, is not expressed by the non-neoplastic ductal cells, but is expressed in low grade PanINs (PanIN-1) with a progressive increase in expression until infiltrating adenocarcinoma [116]. Further, a strong and diffuse expression of claudin-18 by the PCs is associated with a better survival in the patients.

While detection of biomarkers for PanIN lesions is the correct approach toward realizing the dream of one or more sensitive and specific biomarkers for pancreatic dysplasia, it is important to realize two things. First, PanIN lesions are quite frequent among patients with chronic pancreatitis (and in one report in serous cystadenoma [117], a benign cystic tumor of the pancreas).. Second, the time from the onset of PanIN lesions to the development of invasive adenocarcinoma and the absolute risk with a given grade of PanIN is still unknown. In one study [118], only one out of 9 patients with PanIN-3 lesions in the setting of sporadic chronic pancreatitis developed invasive adenocarcinoma after nearly 10 years. In the same study, none of the patients with PanIN-2 (n=11) or PanIN-1 (n=31) developed ductal adenocarcinoma during follow-up. Further, the mean duration of chronic pancreatitis in this cohort of patients was 8 years. This suggests that it takes nearly two decades from the time of onset of chronic pancreatitis to develop invasive adenocarcinoma and that only a few patients (about 10%) with high-grade dysplasia go on to develop invasive malignancy. More studies are needed to clarify the association between chronic pancreatitis, incidental pancreatic dysplasia and the risk of developing invasive adenocarcinoma.

Hisa et al. [119] in a study on small (≤ 2 cm diameter) ductal carcinoma of the pancreas found that PanIN-3 lesions were commonly spread out within 2.5 cm from the edge of the main mass (less than 25% were beyond 1cm from the edge of the tumor). On the other hand, PanIN-2 lesions, although found adjacent to the mass (only 50% were more than 1cm beyond the mass edge), were discontinuous with the mass and/or the PanIN-3 lesions. PanIN-1 lesions were found to be distributed in a sporadic manner throughout the pancreas (nearly 90% of PanIN-1a and 95% of PanIN-1b were more than 1cm from the edge of the cancer). This study suggested that given their close proximity to invasive adenocarcinoma, PanIN-3 lesions represent an intraductal extension of the cancer and hence, for small (≤ 2 cm) malignant masses, a margin of at least 11mm (from the mass edge) is required.

A second model proposed for the development of PC is the transformation of acinar cells into malignant ductal cells [120]. According to this model, pancreatic acini transform gradually into ductules, a process accompanied by a loss of their enzyme-producing cell characteristics (including loss of prominent endoplasmic reticulum and zymogen granules, and loss of chymotrypsin reactivity), a reduction in cell height (manifests as an enlargement of the lumen) and accompanied by a fibro inflammatory reaction in the surrounding stroma. However, tubular complexes (as the precursor lesions are called) are also observed in chronic pancreatitis and the benign serous cystadenoma. Interestingly, while PanIN-1 and 2 lesions were also observed in the same sections, PanIN-3 lesions were found only in association with PC and not in any of the 42 chronic pancreatitis and 18 serous cystadenoma

sections examined in the same study [120]. This model received further credence when a recent study [121] showed that knockout of an acinus restricted transcription factor *Mist1* in *Kras^{G12D}* expressing mice led to an accelerated development of mPanIN (mouse PanIN) lesions in the pancreas and transformation of acinar cells into a ductal cell phenotype *in vitro* associated with an activation of epidermal growth factor receptor (EGFR) and Notch signaling pathways in the transforming cells.

7. Biomarkers in body fluids

7.1 Serum and plasma

Serum and plasma (serum without fibrinogen and other clotting factors) remain the most easily accessible tissues for diagnostic testing and, hence are an attractive medium for biomarker testing to screen for early stage disease. An advantage of using serum is that it represents the proteins released from both the tumor cells and stroma, thereby increasing the number of potential markers. However, studies to identify potential markers of disease have been hampered by the difficulty in isolating and identifying the low-abundance proteins. Immunodepletion of the 12 most abundant proteins, followed by fluorometric 2-DIGE (2-dimensional gel electrophoresis) and mass spectrometry on plasma samples collected from patients with an established diagnosis of PC (two with stage I, seven with stage II and one stage III disease) at three time points- just before surgery, 10 weeks post-operative and just before commencement of chemotherapy, identified two sets of proteins with immense prognostic significance [122]. The first set was comprised of 14 proteins that correlated positively with the tumor burden (these decreased after removal of the tumor), while the second set included eight proteins that were selectively elevated in patients who had progression of the disease (defined as either recurrence of the tumor or death) compared to those without any detectable tumor one year after surgery (summarized in figure 3). Notably, while both the stage I patients were alive at one-year, four of the stage II patients were dead from recurrence, reinforcing the urgency of an early diagnosis of PC. Another study identified mannose-binding lectin-2 (MBL2) and myosin light chain kinase (MLCK) as being significantly upregulated proteins in the serum of PC patients by fluorometric 2-DIGE followed by tandem MS and validation by western blotting [123]. The major drawback of this study was that it only included one stage 1 patient who showed significant elevation of MLCK but not MBL2 in his serum. An analysis of serum samples from patients with PC (stages I-IV), benign pancreatic diseases (including pancreatitis, serous cystadenoma, pancreatic pseudocyst, ampullary adenoma and diverticulitis), and healthy controls (high-risk individuals from FPC kindreds undergoing surveillance) by antibody microarrays (using a two-color rolling circle amplification) revealed that a set of proteins present in the serum could distinguish samples of PC cases from those with benign diseases (anti-protein induced Vitamin K antagonist-II (PIVKA-II) and CA15-3) and from healthy individuals (C-reactive protein, PIVKA-II, α 1 antitrypsin, IgA, cathepsin D and alkaline phosphatase) with more than 90% sensitivity and specificity [124].

Another recent study identified elevated phospho-ERK1/2 levels in the serum of PC patients as a potential adjunct to CA19-9 in the diagnosis of PC. However, there was considerable overlap in the serum levels of ERK-1/2 and other phosphoproteins between patients with PC and those with pancreatitis, which together with the lack of a specific cut-off for levels of phosphoproteins and the small sample size of patients with resectable PC suggest the need for validation of the role of phosphoproteins as early markers of PC [125]. These studies, though preliminary have established that antibody microarrays, like gene microarrays, are a valuable tool to identify differentially expressed proteins with diagnostic potential in serum.

An observation often reported in literature is the discrepancy between the level of expression of a protein and that of its transcript for a given type of cell. A good example is a study to

identify the secretome of PC cells (Panc-1 chosen as prototype) by comparing it with that of normal pancreatic ductal cells (HPDE cells) [126], wherein nearly 50% of the proteins did not show a correlation between their mRNA and protein levels (overall correlation between RNA and protein expression in this study was 0.28). Certain proteins may also show an inverse variation in mRNA and protein levels. For instance, the CD9 antigen which was nearly eight-fold upregulated in the Panc-1 secretome was downregulated two-fold at the mRNA level. One explanation for this discrepancy between transcript and protein level could be the relative preponderance of a post-transcriptional regulation in the case of certain proteins (e.g. cytokines), which might explain the relative difference in the abundance of the two forms. From the diagnostic standpoint, this observation underscores the importance of performing an analysis of both the transcriptome and the secretome, each of which has a unique set of differential expression.

Using multivariate analysis procedures including classification and regression tree and logistic regression, it was demonstrated [127] that surface-enhanced laser detection/ionization time-of flight mass spectrometry (SELDI TOF-MS) could be used to generate fingerprints of cancer cells from serum samples. In the aforementioned study, this model could correctly classify all of the PC serum samples in a randomly assigned “test” set, while the specificity was about 94%. Notably, the individual discriminatory proteins were not identified. Fingerprinting readily accessible body fluids from cancer patients and comparing it with a database of similar data from a large base of normal samples could ultimately provide the solution to early identification of small neoplasms that would otherwise be missed on imaging.

Like the pancreatic juice, the presence of a biliary obstruction can alter the proteomic expression in patients with benign pancreatic diseases, most exemplified by the false elevation of serum CA19-9 in patients with obstructive jaundice. However, this distinction is not made by most studies. One study by Bloomston et al. [128] identified fibrinogen γ as being highly expressed in sera of PC patients (mean concentration: 51 mg/dl in 32 PC cases vs. no detectable levels in healthy controls). However, no correction was made for serum bilirubin levels nor did the authors discuss the stage of malignancy in their study population. A study among Chinese patients [129] came to the conclusion that the traditional proteomic technique of 2-DE followed by matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry is rather inefficient in identifying low abundance proteins in the plasma. Most of the differentially expressed proteins in their study were acute phase reactants and high abundance proteins (haptoglobin, Immunoglobulin-J, hemoglobin and α 1-antitrypsin). SELDI-MS is designed to overcome these problems. In this technique, the proteins (as little as 20 μ l of serum/plasma) are captured, concentrated and purified on the small chemical surface of a SELDI chip, followed by the measurement of the molecular weight (obtained from the mass/charge ratio) and the relative intensity of each protein captured on the chip by sensitive time of flight (TOF)-MS. Using this technique and a high resolution QqTOF instrument, four proteins, producing mass peaks of 8,766 m/z, 17,272 m/z, 28,080 m/z and 14,779 m/z were found to discriminate PC patients from healthy controls with high sensitivity (97%) and specificity (95%) [130]. Upon validation in a training cohort of PC (including 1 stage I and 4 stage II patients of whom four, including the stage I were correctly identified) and healthy controls at two institutions, this set of proteins had a sensitivity and specificity of 91%. When combined with measurement of CA19-9, all the PC samples in the validation set were correctly identified, while six of the 39 controls were also positive (false positive rate of 15%). The identity of the four peaks remains unresolved, chiefly owing to the difficulty in purifying the proteins from these low intensity peaks without contamination from the surrounding proteins. The limitations of this study included a lack of samples from patients with either pancreatic inflammation (acute or chronic) or benign pancreatic diseases. Analyzing serum samples from 126 PC, 61 CP, 24 type 2

diabetics and 12 healthy controls by SELDI-TOF/MS Navaglia and co-workers identified 219 peaks corresponding to low molecular weight proteins (m/z range 1007–9255) [131]. A decision tree employing the binary recursive partitioning method identified three peaks with a m/z ratio of 1526, 1211 and 3519 that could correctly classify 100% of healthy controls (from CP, PC and type 2 DMs), 73% of type 2 diabetics (from CP and PC) and 70% of CPs (from PC patients). However, a receiver operating characteristic (ROC) curve analysis revealed that this combination of SELDI-TOF/MS peaks was not better than CA19-9 either alone or in combination in discriminating PC patients from the other groups (AUC for CA19-9 being 0.89, for SELDI-TOF/MS peaks alone 0.81 and for the combination 0.86). When patients with type 2 DM alone were considered, a combination of CA19-9 and the three SELDI-TOF/MS peaks could correctly identify 100% of patients ($n=24$) who had type 2 DM in absence of CP or PC. However, while 97% of PC cases were correctly classified, only 77% of CP cases were accurately discriminated by the combination. It remains to be seen whether the proteins identified by these studies can be detected by other less technically demanding techniques or serum/plasma based assays for possible application as screening biomarkers.

A serum based assay to identify the circulating mucin MUC1 reactive to the monoclonal antibody PAM4 revealed that the assay was able to discriminate between patients with PC and pancreatitis/healthy controls with high specificity [132]. However, the study had several limitations. It was not clear as to whether the PC patients tested had early or advanced disease. There was also no mention of whether the specificity of the assay for patients with PC vs. those with benign pancreatic disease with obstructive jaundice was examined. Moreover, while the assay was highly specific, its sensitivity was still low, making it a better confirmatory than a screening serological test.

Serum levels of tumor specific growth factor (TSGF) above a cut-off of >71 U/ml was found to be 92% sensitive (83% specific) for PC, which was higher than that of CA242 and CA19-9 [133]. Significantly, when the performance of the three markers was compared across different stages of PC, a general trend emerged wherein the markers were least sensitive in detecting stage I and most sensitive in detecting stage IV cases. Several factors could explain this observation. Most studies on biomarker discovery employ stage II-IV patients, with a pooling of the samples, hence selecting for markers that are elevated in advanced cancer. Further, the larger tumor burden with increasing stage could translate into greater production of the tumor marker and hence its appearance in the serum. TSGF was still the most sensitive in detecting stage I patients (sensitivity of 60% compared to 30% for CA242 and 40% for CA19-9), suggesting that it could be potentially useful in identifying early stage disease. Interestingly, the level of two serum markers (TSGF and CA242) was higher in patients with cancer of the head of the pancreas compared to those involving the body, tail or whole gland. PCs in the head are known to present earlier owing to obstruction of biliary outflow, while those in the body and tail are usually silent until the advanced stages. When the three markers were combined together (TSGF >71 U/ml, CA242 >20 U/ml and CA19-9 >37 U/ml) they were 100% specific for the diagnosis of PC, while no marker alone could reach perfect specificity. The only major limitation of this study was not investigating the specificity of these markers in the presence of benign conditions causing obstructive jaundice.

Macrophage inhibitory cytokine (MIC-1), a member of the TGF- β family is elevated in the serum of patients with PC. However, it is not very useful to distinguish PC (specifically resectable cases) from CP (specificity of 44% compared to 86% for CA19-9 in one study [134]). The presence of diabetes or jaundice could not explain its low specificity in the cases examined. However, it was highly sensitive in identifying PC cases (90% each in distinguishing PC from normal and chronic pancreatitis, respectively) which was better than

that of CA19-9 (62% sensitivity). Hence, MIC-1 could hold potential as an initial screening test in high-risk patients. The authors of this study also pointed out a key issue: the need for a consistent assay for a given marker to avoid the issues of variability from one study to another.

7.2 Pancreatic juice

The pancreatic juice has recently received a lot of interest as a potential source of biomarkers of early stage neoplasia owing to its direct relationship to the ductal system of the pancreas [135,136]. One study [137] found that the levels of certain heavy metals (present most commonly in cigarette and environmental smoke) were significantly elevated in pancreatic juice from patients with PC. Of significance was the observation that an increase in levels of chromium (mean ($\pm$ SE) level in healthy subjects: 9 ± 14 μ g/ml) by one standard deviation above that seen in healthy controls was associated with a nearly three-fold increased risk of PC (95% C.I.: 1.2–7.8). Further, if the mean levels of chromium and selenium (mean ($\pm$ SE) level in healthy subjects: 43 ± 13 μ g/ml) were added, an increase of 20 μ g/ml in this sum was associated with the greatest risk of PC (O.R.: 5.8, 95% C.I.: 1.5–22).

The pancreatic juice contains a variety of cells. These include erythrocytes, neutrophils, lymphocytes and ductal cells. It has been suggested that analysis of whole tissue for gene expression studies like microarray might yield erroneous results owing to the over representation of a more particular cell type(s) in the specimen [138]. To overcome this, one study [138] employed immunomagnetic separation of ductal cells (both normal and malignant) by selecting cells that express the mucin MUC1 (a known epithelial marker) on their surface. Microarray analysis performed using these fractionated cells yielded a set of genes that distinguished healthy subjects from those with pancreatic adenocarcinoma with the greatest accuracy. What was apparent from this study was the fact that a single marker cannot distinguish between two conditions owing to heterogeneity of expression between normal and diseased cells. Hence, a combination of markers, possibly arrayed onto a chip can be used to screen fractionated epithelial cells (instead of the whole specimen) and a criterion established (based on analysis of large number of samples) that distinguishes between the various disease states with the greatest accuracy.

A comparison of the proteome of pancreatic juice from patients with PC with those diagnosed with other diseases (including IPMN, islet cell tumor, chronic pancreatitis and serous cystadenoma of the pancreas) yielded a 16.57 kDa protein, HIP/PAP-1 (hepatocarcinoma-intestine-pancreas/pancreatitis associated protein-1) [139]. ELISA performed on pancreatic juice validated that this protein, which is normally released from the acini during pancreatitis, is elevated in the pancreatic juice of patients with PC but not in those with non-malignant pancreatic diseases. HIP/PAP-1 expression in the acini (but not the ducts) was also confirmed by immunohistochemistry. However, the main drawback of the study was that of the 28 patients with PC examined, only one was of stage I, and two were of stage 2. While the stage 1 patient had high levels of HIP/PAP-1 in the pancreatic juice, both stage 2 patients had HIP/PAP-2 levels that overlapped with that of IPMN cases. Another recent study identified 20 proteins that were significantly elevated ($p<0.05$) at least 2-fold or greater in pancreatic juice collected from three patients with PanIN-3 lesions compared to a control sample comprising pooled pancreatic juice from five patients with benign pancreatic diseases (sphincter of Oddi dysfunction and CP) [140]. Some of these have also been previously shown to be elevated in PanIN tissues including anterior gradient-2, MUC5AC, cytoplasmic actin and Annexin A4 (Figure 1). An important observation in this study was the lack of correlation between elevation of secreted proteins (specifically AGR-2) levels between the pancreatic juice and serum from the same patient, possibly due to circulating levels that are too low to be detected by ELISA.

A global mRNA expression analysis on the pancreatic juice from patients (on two different types of chips-U133A and X3P) revealed that intact RNA could be isolated from pancreatic juice and that between 40–130 genes were increased three-fold or more in PC samples compared to non-cancer controls (healthy individuals and CP), depending on the platform used [141].

Human telomerase reverse transcriptase (hTERT) is a major catalytic subunit of human telomerase and its mRNA levels correlate closely with the activation of telomerase and has been suggested to have a possible diagnostic role. An examination of hTERT expression in pancreatic juice samples (obtained after secretin stimulation) from patients with PC and IPMN revealed that while cytology was 47% sensitive and 57% accurate in distinguishing malignant cells from benign cells, a positive staining for hTERT was nearly 85% sensitive and over 80% accurate in achieving this distinction [142]. Significantly, among the PC cases, hTERT staining correctly identified 17 out of 21 cases that were negative for malignant cells by cytology. Among patients with invasive IPMN, hTERT positivity correctly identified eight out of eight cases negative by cytology, while among IPMN cases with carcinoma-*in-situ*, 14 of the 17 cases negative by cytology were positive for hTERT staining in the cells. There appeared to be no preference for hTERT expression among different grades of PC or between main vs. branch duct IPMN. Importantly, the pathologists reviewing the slides did so without knowledge of the diagnosis and none of the cells obtained from patients with benign pancreatic disease (IPMN with adenoma and CP) were positive for hTERT. The diagnostic performance of applying hTERT immunostaining to cytology negative cases as a method to identify early malignancy and pre-malignant lesions (especially in high-risk individuals) appears to show great promise and needs to be examined in larger studies.

Using a combination of 2-DIGE and tandem MS, a comparison of the proteome of pooled pancreatic juice from nine PC and nine control samples (included CP, gallstone induced pancreatitis, benign cystic neoplasm and cystic fibrosis), three proteins, namely matrix metalloproteinase-9, oncogene DJI and α -1B-glycoprotein precursor were identified as being differentially upregulated in PC patients [143]. While the study validated the expression of these three proteins in pancreatic juice by Western blotting, validation of their expression in tissues did not include any of the control conditions that were used for the initial discovery. Instead, normal pancreas was used as the control. Analysis of MMP-9 levels in serum revealed that the levels were significantly higher in PC patients than controls (CP and healthy individuals). However, four of the five PC patients in this study had metastatic disease, while the TNM stage of the remaining five was not discussed. It is possible that the high levels of MMP-9 are representative of the metastatic stage and hence need to be confirmed in early stage patients.

The composition of pancreatic juice can be influenced considerably by the presence of co-existing biliary obstruction. One study [144] found that by 2-DE, there were only seven spots that were more prominent consistently in pancreatic juice from patients with cancer of the head of the pancreas compared to those with non-malignant disease of the pancreas but with coexistent obstruction of the biliary tract. In another study [145], apolipoprotein A1, transthyretin and apolipoprotein E were significantly altered in PC patients (compared to controls-CP and benign biliary disease) without considering the effect of biliary obstruction (measured as the level of total serum bilirubin). However, after accounting for elevation of bilirubin ($>17\mu\text{mol/l}$), only transthyretin levels (which is decreased in PC patients) remained associated with the risk of PC.

7.3 Other body fluids

Urine has been examined as another non-invasive biological source of potential biomarkers. One study that employed 2-DIGE separation followed by mass spectrometry revealed that nearly 60 proteins were differentially expressed in PC compared to controls (CP and healthy controls) [146]. However, the results could not be validated by Western blotting. One of the reasons suggested has been that proteins in urine might undergo extensive post-translational modification (chiefly glycosylation) and fragmentation which could account for the failure of antibodies to identify them. However, this study did not distinguish PC by stage, necessitating further studies to examine the utility of urinary biomarkers in the diagnosis of PC.

Bile collected by ERCP from patients who presented with biliary stenosis of various etiologies when examined by mass spectrometry was found to contain 127 protein fragments [147]. Of these, several including MUC-1, MMP7 and NGAL [148] have been reported to have a role in the pathogenesis of PC. Two proteins, CEACAM-6 and MUC-1 (using monoclonal antibody that detects the CA19-9 antigen) were subsequently validated by Western blot. CEACAM-6 is a membrane receptor linked to glycosylphosphatidylinositol (GPI), which has been shown to be elevated in several cancers. MUC-1, on the other hand, is a membrane bound mucin expressed by normal and tumor cells of the digestive tract which carries the cancer associated CA19-9 epitope (2,6 sialosyl-fucosyl lactotetraose). Both these proteins could be detected by Western blot in the supernatant (obtained after centrifugation of the bile) rather than in the cell pellet, suggesting that these are secreted into the bile. An observation worthy of note is that most of the cholangiocarcinoma specimens included in this study were also positive for CECAM6 and CA19-9 (3/3 for CECAM6 and 2/3 for CA19-9), suggesting that the two malignancies share common dysregulated proteins. Given the dismal prognosis for patients with cholangiocarcinoma [149], it would be useful to include samples from patients with this malignancy in subsequent studies together with those from PC when examining for early diagnostic markers (role of bile as a source of biomarkers for hepatobiliary malignancies has been recently reviewed [150]).

8. Advances in molecular diagnosis of pancreatic cancer: role of microRNAs

In the recent years, an ever strengthening link has been demonstrated between the altered expression, mutations, and mature microRNA processing and the susceptibility to, or progression of, cancers. *MirNA-21* has been demonstrated to be significantly overexpressed in both PC cell lines and tissues relative to normal pancreatic tissue [151]. Further, an A to G germline mutation was identified in the genomic DNA 59bp upstream of the region encoding for the pre-miRNA in one PC patient. However, luciferase based assays revealed no change in the transcription of the mature miRNA-21 as a result of this mutation. Given that a germline mutation in the primary precursor of *miR-16-1- miR-15a* has been linked to a familial variant of CLL [152], it is possible that mutations in certain human miRNAs could be linked to an increased risk of developing PC. Further, the same could also be linked to familial clustering of PC cases and to the survival and response of pancreatic tumors to therapy.

Mir-155 and *miR-21* transcripts were shown to be significantly upregulated (mean:12-fold) in areas of IPMN without invasion (by locked nucleic acid *in-situ* hybridization), while *miR-155* transcripts were detected in the pancreatic juice of six out of 10 IPMN cases but in none of the controls (patients with non-neoplastic pancreato-biliary disorders) [153]. Small non-coding RNAs have recently been investigated for the diagnostic role in PC. One small study [154] comprised of 15 adenocarcinoma, eight chronic pancreatitis and nine normal

adjacent tissue samples identified a signature of 24 non-coding RNAs that discriminated PC from the remaining two groups. The implication of these findings will need to be assessed in larger studies.

Using primary malignant tumor specimens from six different sites (breast, colon, pancreas, prostate, stomach and lungs) and a microarray platform, a set of microRNAs have been identified that are shared by more than one type of solid tumor as well as those that are specifically upregulated in PC tissues [155]. Lee et al. [156] used microarray to profile the microRNA precursors overexpressed in PC tissues compared to pancreatitis, benign adjacent pancreas, and normal pancreatic tissue. They also identified a set of microRNAs that could classify correctly 28 out of 28 PDACs, six out of six normal pancreatic tissue and 11 out of 15 benign pancreatic disease tissues of a “test set.” Interestingly, exocrine pancreatic neoplasms appear to have a miRNA expression profile distinct from pancreatic endocrine tumors (PETs) [157]. The mature form of *miR-155*, which is upregulated in PC, was found to be downregulated in PETs (comparing with normal pancreas). Further, upregulation of *miR-204* was found to be specific for insulinomas (no significant change in PC). Additionally, *miR-21* was found to correlate with metastases of PETs to the liver and also distinguished acinar cell carcinomas from non-tumor pancreatic tissue. Given *miR-21*'s overexpression in several cancers, including pancreatic [158] and breast [159], and its correlation with advanced stage, metastases [159] and chemoresistance [158], it appears that it could emerge as a general indicator of poor prognosis in most patients with malignant tumors.

A study by Szafranska et al. [160] identified a different set of mature miRNAs that were differentially expressed in chronic pancreatitis and PC (seven stage 2 and three stage 3) and compared to normal pancreatic tissue (summarized in Table 2). Further, the difference between raw Ct (threshold cycle in real-time PCR) values of only two miRNAs (*miR-196* and *miR-217*) provided a simple index that could distinguish diseased pancreatic tissues (CP or PC) from normal pancreas independent of the total quantity of RNA sample. A subsequent analysis [161] confirmed that miRNA signatures can be used to discriminate between normal, CP, and PC in frozen fine needle aspirate specimens (*miR-217*, *148130b*, and *375* were downregulated, while *miR-196a* and *miR-210* were upregulated in PC relative to CP). Further, an index similar to the one used for tissues earlier ($C_t \text{ miR-196} - C_t \text{ miR-217}$) was able to correctly classify pancreatic tissues into normal, CP and PC. *miR-196a* expression was also demonstrated to be specific for malignant ductal cells, being expressed in none of the non-pathologic ductal and acinar cells, but with increasing expression seen in PanIN lesions, culminating in 100% positivity in the malignant ductal cells. This study suggested for the first time that *miRNA-196a* expression is turned on during the progression of pancreatic adenocarcinoma and thereby could be useful in the early diagnosis of PC. Expression by the primary tumor of certain miRNAs also has a bearing on patient survival, with some (*miRNA-452*, *105*, *127*, *518-2*, *187* and *miR-30a-3p*) being associated with longer survival, while others (*miR-196-a* and *miR-219*) are associated with reduced survival in a cohort of PC patients with metastasis to lymph nodes [162]. A strong expression of *miR-21* is also associated with shorter survival (15 vs. 27 months in one study [163]), although no association with tumor stage or grade was observed. Significantly, the expression of the miRNAs discussed in a further study (in [162]) did not correlate with expression of any of the genetic abnormalities (inactivation of *TP53*, *CDKN2*, *SMAD4* or activating mutations of *KRAS*), suggesting a unique role for miRNAs in the epidemiology of PC.

9. Screening as a tool for early detection of pancreatic cancer

Screening for cancer requires markers with high sensitivity [164]. Implementation of screening programs appears to be currently the only way to reduce the mortality associated with PC, given the paucity of options for treatment beyond surgical resection. However, it is not currently in place for the general population owing to the relatively low incidence of this malignancy and the lack of accurate, inexpensive and non-invasive diagnostic tests for early disease [71]. For the purpose of screening, two groups (kindreds) of at-risk individuals are considered: those with at least one first degree relative diagnosed with PC, termed as familial PC kindreds (FPC) and those without any relatives affected with PC, termed sporadic PC kindreds (SPC). The latter group comprises the majority of PC diagnosed annually in the United States. Several studies have shown that the risk of PC among the FPC group increases with the number of affected first degree relatives with PC (fivefold, six-fold and 32-fold increase in risk with 1, 2 and 3 affected first degree relatives, respectively, in one study [165]).

A consensus guideline on the management of patients at high risk for the development of PC recommended secondary screening on a research basis for patients with hereditary pancreatitis, families with multiple cases of PC, individuals with even one documented case of a mutation known to predispose to PC in the family, and individuals from kindreds of Peutz-Jeghers syndrome [166]. Knowledge of risk factors that predispose to the development of PC, especially for aggressive neoplasms, would help us stratify risk criteria more effectively and, in turn, tailor screening and surveillance strategies, better targeting those at high risk and away from those at low risk who could be examined with less invasive methods or not at all. Genetic factors influence the risk of developing PC considerably, as evidenced by the racial difference in susceptibility to PC. Further, about 10% of PC cases have a family history of the disease, suggesting that an altered expression or activity of one or more genes determines the risk of developing PC in a subset of PC cases [167,168]. However, most syndromes associated with PC are rare and usually associated with an increased prevalence of other cancers. Some of these conditions include FAP (familial adenomatous polyposis, mutation of adenomatous polyposis coli tumor suppressor gene (TSG); 4.5 fold greater risk) [170], FAMMM (familial atypical multiple mole melanoma; mutations in the TSG *CDKN2A/p16* or in minority of cases in *CDK4*), Li-Fraumeni syndrome (recessive mutations in *p53* TSG or *CHK2*), Peutz-Jeghers syndrome (autosomal dominant mutations in the *STK11* gene) and the Lynch syndrome-II (mutations in mismatch repair genes) [170]. Some individuals with mutations in the *BRCA2* TSG also have an increased risk of PC [171]. It is interesting to quote the results of a prospective study which included two high-risk patients [71], one with and the other without a *6174 delT BRCA2* mutation in the germline. The first patient, with an Ashkenazi Jewish ancestry and a history of ovarian and breast cancer, was normal during baseline evaluation but found to have a 6-mm cyst in the uncinate process communicating with the pancreatic duct one year after the initial evaluation. Within the next six months, multiple lesions appeared in the liver, and a diagnosis of metastasis from a primary pancreatic adenocarcinoma was made. In the second patient, there was a family history of *BRCA2* mutation (although she herself was negative), an IPMN adenoma together with multiple high-grade PanIN lesions was found. This study also highlighted the complementary role of EUS and CT scan in screening patients suspicious of harboring an occult neoplasm of the pancreas. In another interesting report [172], PC surveillance by EUS and MRI resulted in the detection of asymptomatic PC in two members (a mother and daughter) of a Dutch family with p16-Leiden mutation (19 bp deletion in exon 2 of the *CDKN2A* gene) and with an atypical presentation of the FAMMM syndrome (presence of PC and melanoma in two members of the same family). Two other family members were discovered to be carriers for the p16-Leiden, of which one was treated for melanoma and carcinoma of the cheek while the other was healthy. Three other family

members who were screened turned out negative for the mutation. In this study, the index case, a 76-year old woman, was found to have atrophy of the pancreatic tail and dilation of the pancreatic duct tapering to an ill-defined mass on EUS. A CT-scan revealed a hypodense lesion in the tail of the pancreas. Histologic examination of the resected pancreas revealed a poorly differentiated adenocarcinoma of the tail with perineural and nodal invasion (4/8 adjacent nodes) but with no distant metastases. Upon follow-up, the patient was doing well following chemotherapy. Her 51-year-old daughter had a small (1cm) hypoechoic mass in the body of the pancreas at the level of the splenic artery which was suspicious for malignancy. During surgery, the regional lymph nodes were found to be free of cancer and histology revealed a moderately differentiated adenocarcinoma. It is notable that MRI and CT scans performed in the same patient did not pick up any abnormality in the pancreas. One of the carriers of the mutation was also screened by EUS and MRI and found to have side branch IPMN.

It has been posed that EUS is a cost-effective method of screening asymptomatic patients, at least those from familial PC kindreds. A model-based analysis employing a hypothetical cohort of 100 patients from a FPC kindred found that if every person who was found to have pancreatic dysplasia on screening agreed to undergo total pancreatectomy, a pretest probability of at least 16% (for occurrence of pancreatic dysplasia) was required for a significant survival benefit resulting from endoscopic screening (by EUS and ERCP) [173]. Survival benefit was measured as years of survival following pancreatectomy and was found to be dependent on the sensitivity of the test being employed. Thus, a sensitivity of less than 84% for EUS and 68% for ERCP was predicted to actually result in fewer life-years for the patients if screening was undertaken. This study also estimated that a screening procedure was not likely to produce an improvement in survival if the life-expectancy if the patient was less than 27 years at the time of screening. A temporal relationship was also evident between the time of onset of pancreatic dysplasia and death from PC. It was estimated that for a given malignancy, screening high-risk cases would not be appropriate if this period was more than 12 years (for PC, it has been estimated that the time from the onset of dysplasia to onset of invasive carcinoma is 10 years [174], while the median survival after diagnosis has been estimated to be 0.8 years [175]). Inflation and costs of pancreatic surgery did not appear to affect the overall cost-effectiveness of screening patients by endoscopy (EUS followed by ERCP). Rather, the two key factors that determined the cost-effectiveness of a screening test were the pretest probability of pancreatic dysplasia and the sensitivity of the test. Importantly, as the pre-test probability of dysplasia increased, the screening test could be less sensitive for the screening strategy to still remain effective (the study assumed a 20% prevalence of pancreatic dysplasia in their hypothetical cohort and 90% sensitivity of EUS and ERCP in identifying dysplastic lesions in the pancreas).

Germline mutations occur in only a small percentage of sporadic PC cases (7% of apparently sporadic cases had a germline mutation in *BRCA2* in one hospital-based study) [176]. This argues against implementing screening for genetic mutations in patients without a suggestive family history or known inherited syndrome who are considered at a high-risk for PC (e.g. chronic pancreatitis and those diagnosed with type-II diabetes within the last three years). Further, germline deletions can be missed, even with standard sequencing techniques, potentially leading to false-negative results [177]. However, among asymptomatic patients with a family history of PC or an inherited mutation, the story is different. Up to 12% of patients with at least one first degree relative with PC [178], and nearly 18% cases with three or more relatives with PC, have a germline mutation in *BRCA2* [177]. Thus, employing a screening test for genetic mutations would be suitable as a first-line screening test in these patients.

10. Management of the high risk patient

Cystic neoplasms of the pancreas, when diagnosed (usually by imaging), should be completely resected (except when a clear diagnosis of serous cystic neoplasm is made, when a wait and watch policy is recommended [179]) and a thorough histopathologic examination should be performed. If no evidence of tissue invasion is detected, the chances of recurrence are negligible and prolonged follow-up is not recommended. However, if invasion is identified (invasive cystadenocarcinoma), the prognosis is generally poor [12]. One of the largest prospective studies undertaken to examine the effects that influenced mortality from PC [35] reported an inverse association between consumption of vegetables (carrots, tomatoes, squash/corn, green leafy vegetables, raw vegetables, and cabbage, broccoli, and Brussels sprouts) and the risk of PC. However, this reduction in risk was observable only among men. Another case-control study reported a reduction in risk with intake of fruits [180]. This finding could be useful as lifestyle modification advice to patients who are determined to be at an increased risk of PC owing to their lifestyle and dietary habits.

One study [181] aimed at examining the role of EUS and associated guided FNA in the detection of pancreatic neoplasms represents a potentially useful strategy that can be applied for screening. In this study, 110 patients who were suspicious for possible PC based on an enlargement of the head of the pancreas (HOP) or a dilation of the pancreatic duct (by CT or MRI), but who did not have any detectable mass (by any of these two modalities) were screened by EUS. Cases where a focal lesion was identified by EUS were subjected to FNA and a final diagnosis was made based on the cytopathological examination. Out of 43 patients with a dilated pancreatic duct (irrespective of presence or absence of a dilated CBD), a focal pancreatic lesion was identified in 18, all of whom underwent FNA. Four were subsequently diagnosed with adenocarcinoma. A dilation of the pancreatic duct with an abrupt proximal cut-off was more commonly associated with an adenocarcinoma of the pancreas (four cases of PC out of 15 cases) compared to a diffuse dilation of the pancreatic duct (no PCs among 28 patients). Among patients with an enlarged HOP (n=67), a focal pancreatic lesion was identified in 14 of which two were found to be primary pancreatic adenocarcinoma, one a metastatic tumor to the pancreas, while 10 were benign cases (by FNA). 17 patients also had features of CP, of which 12 had no focal lesion while five had a concomitant focal lesion. In this study, nearly 17% of all patients who underwent CT or MRI for suspected PC had either an enlarged HOP (6%) or a dilated pancreatic duct (11%). The diagnostic accuracy of a EUS with or without FNA was 99% (95% C.I.: 97%–100%). Due to the fact that obstructive jaundice can also cause dilation of the pancreatic duct in the absence of PC [182], the authors of this study excluded all patients with serum total bilirubin $\geq 1\text{mg/dl}$ at the time of initial presentation. In this study, based at the Saint Louis University Hospital, all patients who underwent EUS were rigorously followed through telephone calls and correspondence with referring and primary care physicians. Those without any evidence of cancer underwent repeat imaging by EUS/CT/MRI three, six or 12 months after the initial procedure. Interestingly, there were no specially trained radiologists employed to interpret the CT/MRI findings in this study. The NPV of EUS in this study was 99% (95% C.I.: 0.97–1.0) strengthening its role as the most useful imaging modality for screening suspicious patients.

11. Conclusions and Perspectives

There is little doubt that a general screening for PC in the populace is not practical, chiefly owing to the extremely low incidence of PC (9 per 100,000 per year) [183]. About 5–10% of PCs report a family history in a close family member and this constitutes an attractive cohort for screening purposes (reviewed in [184]). Thus, the majority of cases of PC are sporadic. There is a need to identify certain “indicators” which can alert the attending physician to the

possibility of an occult pancreatic neoplasm. The suspicious cases can then be screened by a CT-scan or EUS (the latter being preferable given its high sensitivity and specificity). Those patients in whom a suggestive lesion is discovered can then be investigated further by EUS-guided FNA to identify the nature of the lesion. Applying EUS to screen asymptomatic individuals of a FPC kindred found that most individuals of this kindred had findings that were strongly suggestive of chronic pancreatitis (most commonly the presence of multiple hypoechoic foci, generally in the head or the tail region). As these findings are also observed in patients who have chronic pancreatitis secondary to prolonged alcohol consumption, it has been recommended that the presence of EUS features suggestive of chronic pancreatitis in the setting of a positive history of chronic alcoholism entails a repeat of the same procedure after at least six months of complete abstinence. If the findings are still suspicious, an ERCP (pancreatogram) is recommended.

A pertinent question that often comes to mind is how much has the early diagnosis by imaging got to do with the observer-to-observer variation in interpretation of the results? One study [185] tried to examine this same question by assessing the inter-observer agreement in interpretation of EUS findings among 17 experienced endosonographers before and after a workshop to draft consensus EUS findings for the early diagnosis of PC in high-risk persons. The results revealed that except for cysts, inter-observer agreement for all other conditions (including masses, chronic pancreatitis lesions and even normal pancreas) was rather poor. Several factors, including poor video quality and experience of the endosonographer in identifying the subtle changes often observed in high risk patients, were suggested as possible causes for the lack of agreement. Thus, better resolution instruments and training of sonographers in identifying the changes associated with high-risk cases appears to be a pressing requirement needed to improve detection rates for early PC lesions.

A key point that comes to light from a review of the literature is the importance of a careful history, specifically relating to the enumeration of risk factors for PC. Table 6 lists some of the points from the history which can be helpful pointers for identifying patients at a high risk for PC.

Another question often asked is whether it is economically viable to perform an invasive procedure like EUS or an expensive one like CT on an asymptomatic individual. A suggestion has been that to keep the screening procedure viable, asymptomatic at-risk cases should be screened by a highly sensitive and relatively inexpensive test (preferably serological). Positive cases should then be screened by more specific tests (again preferably serological) before proceeding to more expensive imaging techniques like EUS [186]. Although the exact frequency of EUS based screening has not yet been established, it has been suggested (in [187]) that patients from FPC kindreds undergo EUS based screening annually, beginning 10 years earlier than the age of onset of PC in the youngest case. Those patients (from FPC kindreds) with normal EUS findings are recommended to be followed-up at 2–3 year intervals. This interval is likely to be longer for patients without a family history of PC or other contributing genetic conditions. Patients who are assessed to be at high risk following screening have been offered the option of pancreatectomy (either total or distal) [187]. In the Washington experience [187], total pancreatectomy was offered to family members of the high risk FPC kindred, while distal pancreatectomy was offered to all other asymptomatic patients with pancreatic dysplasia (non-FPC). Total pancreatectomy in the latter cohort was limited only to those patients with documented high-grade dysplasia in the pancreas.

Counseling is an important aspect of management of patients (both FPC and SPC types) in order to emphasize that currently there is no definitive test to detect early stage PC or pre-cancer. It is also important to understand the factors that may influence the desire of

asymptomatic individuals to seek advice for cancer screening. The experience from a study in Korea to understand the factors associated with screening seeking behavior in gastric cancer [188] found three main factors that determined this behavior: 1. anxiety about developing cancer, 2. having a family member who was having regular check-ups and 3. Being educated about the importance of cancer screening tests (this last factor was the strongest factor linked to the desire to seek screening).

Alexander Graham Bell, the great inventor, once remarked, “Great discoveries and improvements invariably involve the cooperation of many minds. I may be given credit for having blazed the trail, but when I look at the subsequent developments I feel the credit is due to others rather than me.” In this age of ever growing information, the emphasis has been on sharing information and resources. Inter-disciplinary and inter-institutional collaboration holds the key to success in our endeavor to conquer this seemingly incurable malignancy. An example in this direction was the recent creation of an open access database (<http://www.pancreasexpression.org/>) [189] which contains gene expression measurements from various PC types, PanIN lesions and chronic pancreatitis. This form of public databases could serve as repositories of information to enable a systematic unraveling of the pathways underlying PC initiation and progression.

As pointed out earlier, a large percentage of the studies aimed at discovery of biomarkers for PC either did not separate the patients according to stage, history of chemotherapy, family history, and presence or absence of important risk factors (e.g. smoking and diabetes) or employed too small a sample size [135]. In some cases, there was a discrepancy in the results of two similar studies [162,160]. Thus, another important need is to pay careful attention to the design of studies. Ideal groups of PC patients would be those in stage I or II (which are considered to be resectable stages) [190] who have not yet received any form of therapy (as this might modify expression of genes/proteins) and who have no family history of PC (SPC). Validation of the potential markers identified from a “training” set of carefully chosen subjects should then be validated in a “test” set who should be as similar to the “training” set as possible. This is not surprising as it often happens that markers identified by global analysis fail validation (e.g. although cyclin-I was one of the most upregulated proteins in sera of PC patients by MS, several benign samples expressed the protein on subsequent validation by Western blot on a separate set of samples [191]). Where reports are conflicting (as in the C677T polymorphism in the methylene tetrahydrofolate reductase gene which has been linked to an increased risk of PC in the setting of folate deficiency [192]), careful studies are needed to clear the controversy.

Thus, in conclusion, it would be fair to say that we have a large and diverse armamentarium of biomarkers ranging from the short miRNAs to the huge mucin glycoproteins which, combined with existing and emerging imaging techniques, has the potential to identify this lethal malignancy at an early, potentially resectable stage. Exciting new developments continue to be made including the discovery of new tumor suppressor genes like *WWOX* (WW-domain-containing oxidoreductase) [193] and the development of mouse models that are bringing us ever closer to understanding the cellular origin of PC itself and to the dream of identifying occult PC in every pre-symptomatic individual.

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Figure 1. Recent update on genes with a differential expression in the pre-malignant pancreatic intraepithelial neoplasia (PanIN) lesions

Pancreatic cancer develops from a series of premalignant lesions termed as PanINs. There are four grades of PanIN-PanIN 1a, 1b, 2 and 3. Genes that are differentially expressed in PanIN lesions hold significant potential in the early detection of adenocarcinoma of the pancreas. The figure shows genes that show a significant up- or downregulation in these precursor lesions compared to the non-neoplastic ducts of the pancreas. The gene name in the figure refers to its Entrez Gene ID (<http://www.ncbi.nlm.nih.gov/gene>). Those genes with a “*” sign beside them were identified as being upregulated three-fold or more in PanIN-3 lesions compared to the normal pancreas and were not expressed in chronic pancreatitis tissues. €MUC5AC is strongly expressed in PanIN-1 lesions and this is maintained until invasive adenocarcinoma. ¥ Based on reactivity to the PAM-4 monoclonal antibody to MUC1. £ Examination of S100P mRNA in micro dissected PanIN and PDAC tissues did not show any difference between PanIN and PDAC (Adapted from [6,185,194–202])

Figure 2. Recent update on proteins differentially expressed between chronic pancreatitis and pancreatic cancer

A major problem in the early diagnosis of pancreatic cancer is the difficulty in distinguishing foci of adenocarcinoma in the setting of an underlying chronic pancreatitis. Further, the two processes share many genes that show a similar pattern of expression. The figure shows the genes that are differentially upregulated specifically in chronic pancreatitis (left) and pancreatic cancer (right). The symbols represents the HUGO names of the respective proteins (<http://www.genenames.org/>). Only proteins that were found to be up to fivefold or more are included. * Proteins whose expression was validated by quantitative real time RT-PCR. ¥ Proteins that were validated by immunohistochemistry (Adapted from [203]).

Figure 3. Recent update on proteins whose expression correlates with tumor burden and clinical outcome in patients with pancreatic cancer

The prediction of tumor burden and early identification of recurrence remain two key challenges in patients with established pancreatic cancer. Proteins that can predict these events are immensely useful in devising prognostic algorithms, tailoring existing treatment strategies and devising new strategies for the treatment of pancreatic adenocarcinoma. The figure depicts proteins that have been identified as indicators of tumor burden (left) and recurrence or progression of disease (right) in pancreatic cancer. The unique NCBI identifiers for the proteins are as follows: CC3 (*Protein accession number: NP_000055.2*), C4A (*GenBank: AAA51855.1*), CFH (*Swiss-Prot: P08603.4*), A1BG (*PIR: 69990*), GC (*NP_000574*), APOA4 (*GenBank: AAA51748.1*), SERPINF1 (*GenBank: AAA60058.1*), HPX (*GenBank: AAH05395.1*), β -2 microglobulin (*GenBank: CAA23830.1*), α -2 microglobulin (*PRF: 224053*), α -2 microglobulin (*GenBank: CAI15899.1*), Plasminogen (*GenBank: AAH60513.1*), α -2 HS glycoprotein (*GenBank: BAA22651.1*), Serum albumin precursor (*GenBank: AAF01333.1*), and C1q B-chain precursor (*GenBank: CAA26880.1*) (Adapted from [204])

Table 1

Risk factor based stratification to identify patients at risk of developing sporadic pancreatic cancer

Risk group	Risk factor
High-risk (OR[¥]≥2)	<ul style="list-style-type: none"> i. Obesity (BMI ≥30) ii. Current smokers iii. Former or current smokers with an Arg¹⁸⁸His (on <i>XRCC2</i>) or a combination of Asp³¹² (on exon 10) and a Lys⁷⁵¹Gln polymorphism in the <i>XPB</i> gene iv. Type-II Diabetes mellitus of recent onset (<3 years) v. History of pancreatitis^a ≥1 year back vi. Chronic pancreatitis vii. History of symptoms suggestive of pancreatic cancer^b for ≥3 years viii. Endoscopic ultrasound findings of high grade (moderate to severe) chronic pancreatitis^c
Intermediate risk (OR ≥1.5 but <2)	<ul style="list-style-type: none"> i. Blood group AB and B ii. Overweight (BMI ≥25 but <30) iii. Calories adjusted intake of saturated fat >25 g/day^d iv. Race: African Americans
Low risk (OR<1.5)	<ul style="list-style-type: none"> i. Blood group A ii. History of gallstones iii. Childhood exposure to environmental smoke
No significant risk	<ul style="list-style-type: none"> i. Former smokers who quit ≥ 15 years ago, irrespective of the number of pack years smoked ii. Alcohol (independent of type and duration)^e iii. Non-alcoholic beverages: tea, coffee, juices iv. Red meat v. Education status or income
Decreased risk of pancreatic cancer	<ul style="list-style-type: none"> i. Age at menarche ≥15 years ii. ≥4 pregnancies iii. Allergies (hay fever, seasonal allergies and allergy to animals^f)
Possible risk factors^g	<ul style="list-style-type: none"> i. Patients with an attack of acute pancreatitis in the presence of a pre-existing Kras^{G12D} mutation ii. Marital status (widowed or never married versus currently married)^h

[¥] Odds ratio;

^a No distinction made between acute or chronic pancreatitis;

^b Includes abdominal pain, unusual bloating, belching or heartburn, altered bowel habits, symptoms of biliary obstruction, general constitutional symptoms (fatigue, inability to sleep, anorexia and weight loss);

^c according to the Cambridge criteria;

^d only in males;

^e could contribute indirectly through alcohol-induced chronic pancreatitis;

^firrespective of the type of animal;

^gThese are the factors reported in single studies to be associated with an increased risk of PC and need further confirmation;

^hThe former group were shown to be at an elevated risk of pancreatic cancer in one study

Table 2

Mature microRNA signatures expressed differentially in the normal pancreas, chronic pancreatitis and pancreatic cancer tissues (modified from [13] and [137]) ^a

Expression in the normal pancreas (compared to PDAC)			
miRNA ID[2]	Fold change	miRNA ID [1]	Fold change
miR_217	269.8	miR_148a	5.5
miR_216	188.8	miR_148b	3.2
miR_375	14.7	miR_375	2.2
miR_494	8.1	miR_221	-3.4
miR_29c	7.1	miR_181a	-3.0
miR_96	5.8	miR_21	-3.0
miR_30a_3p	5.2	miR_155	-2.1
miR_223	-10	miR_210	-3.0
miR_31	-10	miR_181b	-2.9
Expression in chronic pancreatitis (compared to PDAC)			
miRNA ID	Fold change	miRNA ID	Fold change
miR_217	77.4	miR_148a	4.5
miR_216	52.9	miR_148b	3.0
miR_375	6.2	miR_375	2.2
miR_196b	-5	miR_203	-4.1
miR_196a	-5	miR_221	-2.5
miR_210	-8	miR_181d	-2.2
Expression in normal pancreas (compared to chronic pancreatitis)			
miRNA ID	Fold change	miRNA ID	Fold change
miR_150	5.6	miR_497	2.0
miR_96	-6.5	miR_494	-4.7
miR_148b	-8.5	miR_100_1/2	-3.3

^a Highly-selected microRNAs with a 5 -fold or greater differential expression between two types of pancreatic tissues compared (with a p-value of <0.0001).