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Early diagnosis of pancreatic cancer: challenges and new developments

Sukhwinder Kaur[1], **Michael J Baine**[2], **Maneesh Jain**[1], **Aaron R Sasson**[3], and **Surinder K Batra**[1,2,*]

[1]Department of Biochemistry & Molecular Biology, University of Nebraska Medical Center, Omaha, NE, USA

[2]Eppley Institute for Research in Cancer & Allied Diseases, University of Nebraska Medical Center, Omaha, NE, USA

[3]Department of Surgery, University of Nebraska Medical Center, Omaha, NE, USA

Abstract

Pancreatic cancer is a lethal malignancy with its incidence almost equivalent to mortality. The complex pathophysiology, absence of early diagnostic and prognostic markers and unresponsiveness to radiation and chemotherapies are major barriers against successful therapy. Poor performance of therapeutic agents, even in the initial stage of invasive cases, emphasizes the importance of early detection for improved survival. The present review discusses the challenges and advances in biomarkers including serological signatures, circulating tumor cells, autoantibodies, epigenetic markers and miRNAs that are being explored to detect this cancer at early stages. Considering the long time gap between the development of malignant lesions and full-blown primary and metastatic pancreatic cancer, unique opportunities are being contemplated for the development of potential diagnostic and prognostic markers.

Keywords

autoantibody; circulating tumor cell; imaging; pancreatic cancer; serological marker; tumor

Pancreatic cancer (PC) is a lethal malignancy with very high mortality rates. According to the National Cancer Institute (NCI) estimates for 2012, 43,920 Americans (22,090 men and 21,830 women) will be diagnosed and 37,390 deaths will be caused by this deadly disease this year [1]. In the USA, PC is the 11th most common cancer in terms of incidence but the fifth most common cause of cancer-related deaths. Although death rates for cancers of the stomach, lungs, colon and prostate have decreased over the past 40 years, rates for PC has remained unchanged or even increased in elderly individuals (aged >70 years), placing it among the most fatal malignancies. No significant change in the overall mortality rate and similar mortality rates between developing and developed countries clearly suggest poor progress in identifying a cure for PC.

*Author for correspondence: Tel.: +1 402 559 5455, Fax: +1 402 559 6650, sbatra@unmc.edu.

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PC is a group of heterogeneous diseases and includes cancer of the endocrine (islet cell carcinoma, neuroendocrine carcinoma and carcinoma of carcinoid tumors) and exocrine (pancreatic ductal adenocarcinoma and acinar) pancreas. Among these pathologies, pancreatic ductal adenocarcinoma (or PC) accounts for approximately 90% of all cases and is the focus of this review. More than 80% of PC patients present with an unresectable primary tumor along with distant metastasis at the time of diagnosis [1]. Among clinical cases, 74% of patients die within the first year of diagnosis and approximately 94% of patient deaths occur within 5 years of diagnosis, leading to the lowest relative survival rate among all cancers. A median survival of approximately 20 months post-resection and adjuvant chemotherapy has been observed for the remaining 10–15% of patients with tumors confined to the pancreas at the time of presentation. Notably, significantly better outcomes have been reported for smaller tumors detected at an earlier stage. In patients with localized disease with a tumor size less than 20 mm and no lymph node or extrapancreatic metastases, complete surgical resection provides a 5-year survival rate of 30–60%, which further improves to greater than 75% for lesions that are less than 10 mm [2,3]. Furthermore, emerging data from recent deep genomic sequencing of primary and metastatic cases suggest that PC develops slowly over a long time, taking an average of approximately 17 years to progress from tumor initiating cells to the development of metastatic subclones, which is followed by patient death after approximately 2.7 years [4–6]. In a retrospective analysis to better understand the timeline of PC development, Gangi *et al.* reviewed the computed tomography (CT) scans of 114 patients carried out >6 months prior to the clinical detection of PC. Interestingly, PC was either undetectable or fully resectable, signifying that PC is resectable as little as 6 months prior to clinical diagnosis [7]. Thus, the slow development of PC, in conjunction with the better curative response of patients with early disease, implies that an early diagnostic marker can positively impact the outcome of patients. Unfortunately, there are many hurdles to the early diagnosis of PC, and biomarker development remains a challenging endeavor for this malignancy.

Bottlenecks to the early detection of PC

The symptoms of PC, including abdominal or back pain, weight loss, jaundice (yellowing of the skin and eyes), loss of appetite, nausea, light stool color and diabetes, are quite generic and common for multiple abdomen or GI tract pathologies (abdominal aortic aneurysm, ampullary carcinoma, intestinal ischemia, bile duct strictures and bile duct tumors, as well as many other pathologies). While skin cancers can be suspected by external visualization, breast cancers by external palpation, cervical cancers by internal visualization during PAP smears, colorectal cancers by colonoscopies and prostate cancer by digital rectal examination or PSA screening, even after decades of research, no diagnostic examination has proven clinically practical for PC due to the retroperitoneal position of the pancreas. Furthermore, to date, there is no consensus on the use of diagnostic imaging for early detection of PC as the vast majority of imaging modalities fail to accurately detect lesions <3 cm. PC is characterized by dense desmoplasia with dense fibrous tissue made up of extracellular matrix (collagen, fibronectin and hyaluronic acid) and cellular components including cancer-associated fibroblasts, stellate cells and immune cells, with the tumor cells accounting for >40% of the whole lesion, resulting in the dilution of factors secreted by the tumor, which further hinders early detection [8]. Given the rarity of PC, with an incidence rate of 12.1 per 100,000 cases during 2005–2009 from 18 SEER geographic areas, a biological marker with high sensitivity (100%) and specificity (99%) will produce 83 false positives for every positive case of cancer, resulting in the subjugation of a large number of individuals to further unwarranted imaging or more invasive testing [1]. Although the majority of PC cases are sporadic, it is a common consensus that PC screening should be performed within high-risk groups (10% of overall cases) and not within the general population [9–12].

Screening groups

The high-risk groups for PC include individuals with a family history of PC, hereditary pancreatitis, obesity, cases of Peutz–Jeghers disorder, familial atypical multiple mole melanoma, cystic fibrosis and individuals with familial cancer syndrome, including Lynch syndromes, familial adenomatous polyposis (*APC* mutation) and hereditary breast and ovarian cancer syndrome with *BRCA1* and *BRCA2* mutations. Furthermore, current smokers, elderly (over the age of 55 years) and obese individuals carry relatively high risks for developing PC in comparison to the general population. Table 1 details various risk groups and factors and their correlation with relative risk of developing PC. Various clinical trials have been conducted or are ongoing for screening of PC in high-risk groups [10,13–18]. Using endoscopic ultrasonography (EUS), Canto *et al.* observed abnormalities in six patients out of 38 asymptomatic individuals from a familial PC (FPC) group (screening yield: 5.3%) [9]. Furthermore, the same group screened 78 high-risk patients (72 from FPC kindreds and six with Peutz–Jeghers syndrome) and 149 control patients at 12-month gaps with CT scan and EUS. Cases with abnormal EUS were further screened using EUS-fine needle aspirate or endoscopic retrograde cholangiopancreatography (ERCP). During the study, eight patients were confirmed with PC (10% screening yield), six patients with benign intraductal papillary mucinous neoplasm (IPMN), one with malignant IPMN, and one with pancreatic intraepithelial neoplasia [15]. Langer *et al.*, using an EUS/MRI/magnetic resonance cholangiopancreaticogram (MRCP)-based screening program, observed only one case of intraductal papillary mucinous neoplasms after screening 76 individuals (screening yield: 1.3%) from 34 FPC families [10]. Poley *et al.* observed asymptomatic tumorous lesions in three patients (screening yield: 7%) and seven intraductal papillary mucinous neoplasms (screening yield: 16%) using EUS in FPC individuals and carriers of familial atypical multiple mole melanoma syndrome, hereditary pancreatitis, Peutz–Jeghers and *p53*, *BRCA1* and *BRCA2* mutations [17]. Ludwig *et al.* enrolled 309 asymptomatic at-risk relatives of FPC patients into the Familial Pancreatic Tumor Registry and screened them with MRCP, followed by EUS with fine-needle aspiration. MRCP screening revealed radiographic abnormalities in 18 out of 109 patients (16.5%), of which 15 patients were followed by EUS-guided fine-needle aspiration. Six patients among these underwent surgery with overall diagnostic accuracy of 8.3% (nine out of 109), with the higher diagnostic efficacy observed in >65-year-old patients (35%; six out of 17) [11]. During surveillance of p16-Leiden germline mutation carriers (n = 79) via MRI and MRCP, with a median follow-up period of 4 years (range: 0–10 years), precursor lesions (i.e., duct ectasias – cystic lesions of the main duct and side branches – based on MRCP) were observed in nine individuals (screening yield: 11%) and PC in seven patients (screening yield: 9%), showing the ability of MRI/MRCP to detect small lesions as well as duct ectasias [18].

In addition to high-risk groups, various risk factors, including age, chronic pancreatitis (CP), diabetes, obesity and smoking, increase the risk of PC development. The incidence of PC is highest between the ages of 55 and 80 years, with 19.1% of incidences between 55 and 64 years, 25.6% between 65 and 74 years, 30% between 75 and 84 years and 15.5% for those >85 years. PC is rarely seen in people under the age of 40 years [1]. In addition to familial cases, one of the biggest risk factors affecting a very large proportion of the population is new-onset diabetes.

During a meta-analysis of 17 case–control and 19 cohort (or nested case–control) studies, Huxley *et al.* observed a combined age- and sex-adjusted odds ratio (OR) of 1.8 (95% CI: 1.7–1.9) between diabetes and PC [19]. Further analysis revealed that recently diagnosed diabetic cases (<4 years) had a 50% greater risk of PC development compared with individuals who had diabetes for ≥5 years [19–21]. Interestingly, diabetes testing using glucose tolerance tests in PC cases revealed that 45–65% of PC patients were positive for

diabetes in earlier studies [20,22] and that diabetes was diagnosed either simultaneously with PC or approximately 2 years prior to cancer detection, emphasizing the association between the two [20,23]. Furthermore, a meta-analysis from 35 cohort studies from Japan, USA, Europe, Asia and Korea revealed a relative risk ratio of 1.94 (95% CI: 1.66–62.27) between Type 2 diabetes mellitus (T2DM) and PC [24]. To assess the prevalence, onset and delay in diagnosis of T2DM in PC patients in the primary care setting, Aggarwal *et al.* retrospectively reviewed the findings from PC patients for the onset of T2DM. Among 111 PC patients, 52 (47%) had T2DM, of which 30 (58%) had new-onset T2DM and were asymptomatic (no cancer-specific symptoms) at the time of T2DM onset [25]. Moreover, resection of PC abrogated new-onset diabetes in 57% cases, indicating a connection between the two [20,26]. Importantly, new-onset diabetes patients have a substantially increased risk of PC as compared with the general population. It is estimated that 0.8% of patients who are ≥50 years of age and diagnosed with T2DM are likely to develop PC in the next 3 years [20]. Furthermore, evaluation indicates that this might be accurate as approximately 1.9 million people in the USA are diagnosed with T2DM annually and approximately 25% of the 44,000 PC patient are diagnosed with T2DM either prior to or concomitantly with PC [20]. Overall, PC could be detected in 11,000 people if recent-onset T2DM patients were screened, making the overall risk approximately 0.58% for new-onset diabetics if current estimates are accurate. Furthermore, as most cases of diabetes are not diagnosed until they are symptomatic, PC patients could have asymptomatic T2DM at or before diagnosis. Hence, the real risk of the diabetic population is probably between the stated 0.8 and 0.58% from population data, placing the overall risk between 1:125 and 1:172.

Screening strategies

Imaging

Great advances have been observed in various imaging modalities, including EUS, ultrasound (US), CT, MRI, PET and/or ERCP over the last decade. With these advancements, imaging has taken on a central and decisive role in the staging and evaluation of pancreatic disease. Imaging is involved in all aspects of the clinical management of pancreatic diseases, including detection and characterization of the pancreatic mass, identification of any anatomical variants, determination of local and vascular involvement, as well as perineural and lymphatic invasion, margin assessment, detection of distant metastases and assessment of disease resectabilty [27]. Additionally, imaging is centrally involved in the follow-up of surgery and in assessing the response to chemotherapy. In addition to its role in staging and management of disease, in the absence of good screening markers, imaging remains at the forefront of screening for FPC and is routinely being used in the majority of board-approved FPC screening programs in North America and Europe [10,13–18]. Among various imaging modalities, EUS is gaining popularity for: accurately judging deep tumors; EUS-guided fine-needle aspiration, which obtains biopsies of affected tissue by extracting a small number of cells with a very fine needle and has a high diagnostic accuracy (92%) [28]; the ability to detect pancreatic lesions and intraductal papillary mucinous neoplasms <1 cm with a sensitivity greater than that of transabdominal US, CT scan or MRI; and improved sensitivity in detecting lymph node metastasis and vascular infiltration as compared with CT imaging. Further advancement in EUS, including contrast-enhanced EUS (for improved characterization of vessels in the desired lesions, improved differential diagnosis and accurate staging or follow-up of tumor), EUS elastography (for real-time evaluation of tissue stiffness) and hybrid imaging (CT/US, CT/US/MRI) can potentially help improve detection and characterization of focal lesions [29]. In addition to EUS, other imaging modalities, such as CT, MRI and MRCP, are being used either in combination with each other or alongside EUS for screening asymptomatic high-risk cases of PC (discussed in the previous section). Although recent advancements in imaging have improved the overall accuracy, these modalities often fail to detect lesions <1 cm in size,

which differentiates malignant lesions from benign ones, and moreover, have a potential procedure-related risk of acute pancreatitis (ERCP). Additionally, the high cost of these procedures discourages their use for the follow-up of asymptomatic cases. All of these drawbacks press the need to look for highly sensitive and specific surrogate tissue-based screening strategies that can direct patients for further diagnostic confirmation by imaging, while at the same time avoiding invasive diagnostics in asymptomatic cases. To date, there are no methods/molecules available that fulfill all the requirements of a biomarker for PC. Advancing molecular technologies, high-throughput screening strategies and individual molecules that are differentially altered during the development of PC are being studied to identify potential markers for PC. Attempts are being made to identify peripheral blood mononuclear cells (PBMCs), miRNA and epigenetic-based signature sets for the early diagnosis, prognosis and monitoring of PC. Recent studies suggest that circulating tumor cells (CTCs), autoantibodies and epigenetic markers can improve the ability to predict the presence and response to therapy of this deadly disease. The next section details the progress achieved in delineating the role of these marker(s) in predicting the overall presence, stage and therapeutic response to chemotherapy or radiation therapy.

Serological markers

Blood-based biomarkers are always preferred over others due to the ease of collection, their relatively noninvasive nature, the ability to easily follow them over the course of the disease and the ability to measure molecules from both tumors and normal body tissue (immune- and nonimmune-based alterations produced as a result of disease development). However, antigens secreted into the blood are diluted many times or overshadowed by highly abundant serum proteins (albumin, immunoglobulin, serotransferrin, haptoglobin, fibrinogen and α-1-antitrypsin constitute 90% of total protein mass), thus losing their potential as markers of disease [30]. Despite these setbacks, CA19.9 (also called sialyl Lewis a), currently the only US FDA-approved marker for following the therapeutic outcome of PC, falls into this category.

CA19.9 was discovered as a tumor-specific antigen reactive with monoclonal antibody N19.9 that was generated against colon cancer cells. N19.9 was subsequently found to react preferentially with the glycoprotein present in the serum of pancreatic and biliary tract cancer patients [31]. While CA19.9 remains the most commonly used tumor biomarker for following the therapeutic outcomes of PC, there are several concerns with its use. These include:

- An elevated expression of CA19.9 in various benign (pancreatitis, cirrhosis and acute cholangitis) and malignant (gastric cancer urothelial carcinoma, uterine squamous cell carcinoma and colorectal cancer) conditions in addition to PC lead to its nonspecificity as a biomarker for PC;
- CA19.9 is only expressed in individuals with Lewis a^+/b^- or Lewis a^+/b^+ genotypes, while 5–10% of the Caucasian population have a Lewis a^-/b^- genotype and cannot express CA19.9;
- Poor-to-moderate sensitivity (69–98%) and specificity (46–98%) in detecting PC;
- Only 65% of patient with resectable PC have elevated levels of CA19.9 [32].

Therefore, extensive efforts are being made to improve the performance of the CA19.9 test. For improving specificity, recent studies have evalauted CA19.9 levels in the context of the specific mucin backbones on which this carbohydrate epitope is present. Using complementary mass spectrometry-based and antibody microarray methods, Yue *et al.* observed the presence of the CA19.9 antigenic epitope on mucins MUC1, MUC5AC and MUC16, as well as on ApoB. While the number of the CA19.9 antigenic epitopes on ApoB

was not influenced by the presence of PC or pancreatitis, the CA19.9 antigen on MUC1, MUC5AC and MUC16 was directly related to the presence of pancreatic pathologies [33]. Similarly, improved sensitivity and specificity was observed when the CA19.9 epitope was measured on specific carrier proteins using antibody arrays. In a subset of patients where no elevation of total CA19.9 using standard tests was observed, there was a definitive elevation in MUC5AC- and MUC16-associated CA19.9. A combined analysis of CA19.9 on specific protein carriers with the total standard CA19.9 test had 67–80% sensitivity with 98% specificity in differentiating PC from controls (with no pancreatic, biliary or liver diseases) [34]. Notably, higher specificity was observed with the antibodies against sialyl Lewis C glycan (glycans with minor alterations to sialyl Lewis a) in diagnosing cancer patients without increasing the detection of pancreatitis patient samples [35]. Furthermore, owing to the suboptimal specificity and sensitivity of CA19.9, the search for other optimal marker(s) is still ongoing. Various molecules have been tested, including α4GnT, α-fetoprotein, pancreatic-associated antigen (SPan-1), CA195, CA242, CA72–74, CA50, CEACAM1, cathepsin D, DUPAN-2, elastase-1, HIP-PAP, integrin B1, haptoglobin, MIC1, MMP-7, ostepontin, PAM4, TIMP1, HSP27, CAM17.1, MCSF, CEA, SAA, TSGF and HSP27. The sensitivity and specificity of important markers and markers identified after the year 2000 are discussed in Table 2. Unfortunately, many putative markers have failed to meet their initial promise and exhibit limited clinical utility. In addition to individual markers, attempts are being made to identify a panel of biomarkers based on genetic, epigenetic, immunological and biochemical changes in PC.

Neoplastic transformation involves complex interplay of the inflammatory microenvironment, immune system and oncogenic mutations. In conjunction with inflammation, immune system mediators are involved in both the initiation and promotion of oncogenic transformation. Moreover, genetic and epigenetic changes in malignant cells create an inflammatory microenvironment supporting tumor development. As immune system-mediated responses are one of the earliest indicators of preneoplastic changes independent of the tumor burden (if the disease burden is low there will be low levels of tumor-originating biomarkers), immunoproteins and molecular changes in the protein profile of circulating immune cells, such as PBMCs, hold immense potential for early diagnosis. Attempts have been made to explore the diagnostic efficacy of PBMCs, proinflammatory and inflammatory cytokines and effectors of immune system autoantibodies for the early detection of PC.

Autoantibodies—Recently, there has been a lot of emphasis on exploring the signature of another important arm of serum proteins, antibodies generated against self-antigens from the tumor, such as autoantibodies. Weak antibody-mediated immune responses against tumor antigens have been observed in multiple cancers including PC [36]. Serum levels of anti-MUC1 IgG antibodies strongly correlated with survival time ($p = 0.0004$). Antibodies against Rad1 were observed in 7% of PC patients. Hong *et al.* observed autoantibodies against calreticulin isoforms in 28% of PC patients, while only 5% of healthy controls (one out of 15) and acute pancreatitis patients (one out of 18) had an antibody response against calreticulin isoforms [37,38]. Furthermore, autoantibodies to two acidic isoforms of enolase (i.e., ENOA1/2) were observed in 62% of PC patients compared with only 4% of controls [39]. Hong *et al.* observed autoantibodies to a vimentin isoform in the sera of 44% PC patients compared with only 5% from pancreatitis patients and none from healthy controls [40]. Although the detailed molecular mechanism for the cause and generation of auto-antibodies against specific tumor antigens is still unclear, it has been suggested that antibodies are formed against mutated, misfolded, overexpressed, aberrantly degraded proteins or differently glycosylated glycoproteins [41]. Since autoantibodies reflect the altered genetic or protein make-up of the affected tissue during disease progression, these could be important means for identifying novel tumor antigens. Furthermore, in addition to a

vehicle for identifying novel tumor-associated antigens, attempts have been made to use autoantibodies as biomarkers for disease diagnosis. Tumor heterogeneity and the low frequency of detecting particular autoantibodies in a specific cancer (10–20%) limits the effective use of autoantibodies against individual molecules as markers for pathology. Recently, in order to identify a panel of molecules responsible for autoantibody production in PC patients, Tomaino *et al.* screened the serum from PC, CP, non-PC tumor patients and healthy controls with proteins from PC cell lines (CF-PAC-1, MiaPaCa-2 and BxPC-3). The autoantibodies against metabolic enzymes (triosephosphateisomerase, retinal dehydrogenase 1, glucose-6-phosphate 1-dehydrogenase, elongation factor Tu, isocitrate dehydrogenase and transgelin) and cytoskeletal proteins (keratin, type I cytoskeletal and Cofilin-11) were observed [42]. Studies are underway for the development of an array of identified proteins for screening the autoantibody response in PC patients and for further validation of identified markers that would help to improve the diagnosis of PC. Furthermore, in order to explore the biomarker potential of autoantibodies, Bracci *et al.* analyzed the autoantibodies against CTDSP1, MAPK9 and NR2E3 (identified as potentially promising biomarkers in exploratory studies) in serum from PC patients (n = 300) and controls (n = 300) in the San Francisco Bay area (CA, USA). A high antibody response was observed in disease cases for CTDSP1 ($p = 0.004$), MAPK9 ($p = 0.0002$) and NR2E3 ($p = 0.0001$) compared with the controls (fourth vs first quartile: CTDSP1 OR: 1.7; MAPK9 OR: 2.5; and NR2E3 OR: 4.0) [43]. Although a good area under the curve (AUC) was not observed for the combined biomarker set, this study is the first milestone in the direction of exploring the role of autoantibodies as diagnostic and prognostic markers for PC. Furthermore, it generates the hope that autoantibodies, with their presence months to years before symptomatic disease develops, could be useful diagnostic and prognostic screening tools.

Cytokines & chemokines—In addition to antibodies, cytokines, chemokines and growth factors released by various components of the immune system play central roles in mediating neoplastic transformation and are considered important diagnostic or prognostic markers. Ingvarsson *et al.* used a recombinant antibody (scFv) microarray directed against 60 serum proteins for identifying differentially expressed proteins in the serum of PC patients compared with healthy controls [44]. This microarray platform was further trained to differentiate short-term (<12 months) from long-term (<24 months) survivors. The training set of 29 analytes reactive against 21 immunoregulatory proteins distinguished these two groups with an AUC of 0.86. During this study, it was observed that IL-1α, -3, -8 and -11 were upregulated in short-term survivors, while upregulation of R ANTES, IL-16, IL-4 and eotaxin were observed in long-term survivors. Wingren *et al.* conducted differential serum expression profiling of PC (n = 34) versus normal (n = 30) individuals using the same antibody array set. The analysis revealed 33 nonredundant protein molecules, predominantly molecules associated with the immune response, including cytokines, chemokines and growth factors. Some of the key molecules, including C1 esterase inhibitor, C3, C5, CD40, CD40 ligand, factor B, GLP-1, IFN-γ, IgM, IL-10, -11, -12, -13, -16, -18, -1-ra, -1α, -3, -5, -6, -7 and -8, integrin-α-11, procathepsin W, sialyl Lewis x, TGF-β1, TNF-α and VEGF, were differentially overexpressed ($p < 0.05$). Furthermore, a signature of 25 markers differentiated PC cases from the combined group of normal, CP and autoimmune pancreatitis cases with sensitivity and specificity of 73 and 75% (AUC: 0.88), respectively, while a sensitivity and specificity of 88 and 85%, respectively, was observed for differentiating PC from normal controls (AUC: 0.95) [45]. Similarly, Zeh *et al.* utilized a panel of markers, including cytokines, chemokines, growth and angiogenic factors, using LabMAP™ technology, and the multicytokine panel in combination with CA19–9 performed better than CA19.9 and differentiated PC from healthy controls with a sensitivity and specificity of 85.7 and 92.3%, respectively, and PC from chronic pancreatitis with a sensitivity and specificity of 98 and 96.4% [46]. In another study, Grote *et al.* evaluated the

levels of C-reactive protein, IL-6 and sTNF-R1/R2 in 455 PC patients and 455 matched controls. Although none of the tested cytokines showed a significant association with PC, an OR of 1.52 was observed between sTNF-R2 (95% CI: −0.97–92.39; highest vs lowest quartile) indicating a weak association between sTNF-R2 level and the development of PC. Furthermore, a strong correlation was observed between sTNF-R2 and diabetes as well as those with a higher BMI [47]. While assessing diagnostic efficacy of angiogenic markers, Pistol-Tanase *et al.* observed significant elevation in expression of VEGF and bFGF in the serum of PC patients (stage I–IV; $p < 0.0001$) in comparison to healthy controls. A strong correlation was observed, between elevated levels of these marker and serum levels of CA19.9. Furthermore, a correlation between elevated expression of these markers and tumor size and stage was observed suggesting that VEGF and bFGF are potential markers for PC diagnosis or prognosis [48]. Considering the critical role of cytokines in the early diagnosis of PC, studies should focus on identifying a panel of critical cytokines using samples from early-stage PC patients and the mouse model of PC (see the 'Conclusion' section) for improved diagnosis, either in combination with CA19.9 or individually.

Peripheral blood mononuclear cells—In addition to autoantibodies and cytokines, circulating mononuclear cells, including monocytes, T cells, B cells and natural killer cells, have emerged as surrogate markers for both benign (e.g., pre-eclampsia and rheumatoid arthritis) and malignant (chronic lymphocytic leukemia and renal cell carcinoma) diseases [49–51]. In order to investigate the diagnostic or prognostic potential of PBMCs, our group recently explored the gene expression from 26 PC patients and 33 matched normal controls by whole-genome cDNA microarray. Interestingly, 383 genes were found to be differentially expressed between PC and healthy controls, with 65 genes, responsible for hematopoietic differentiation, cytokine signaling and natural killer cell activation and $CD8^+$ T-cell cytotoxic response, having at least a 1.5-fold change in expression. An eight-gene predictor set, consisting of *SSBP2*, *Ube2b-rs1*, *CA5B*, *F5*, *TBC1D8*, *ANX A3*, *ARG1* and *ADAMTS20*, was found to distinguish PC patients from healthy controls with an accuracy of 79% from treatment-naive patients and with a sensitivity and specificity of 83 and 75%, respectively [51]. The diagnostic efficacy of these, along with four new genes (*MIC1*, *NGAL*, *MUC1* and *MUC16*), were further validated in 47 healthy controls, 35 CP patients and 95 PC patients using multiplex quantitative real-time PCR. The combination of CA5B, F5, SSBP2 and MIC1 expression levels with CA19.9 significantly improved all aspects of early PC diagnosis for differentiating CP, providing a 2% increase in sensitivity (67 vs 65%) and a 3% increase in specificity (83 vs 80%) over CA19.9 alone [52]. Although considerable improvements were not observed over the sensitivity and specificity of CA19.9, these studies signify the utility of PBMCs in early diagnosis. For improving the diagnostic efficacy of PBMCs, future efforts should be based on analysis of the altered gene profile of the PBMC subpopulation as we have genetically engineered a model of PC that mimics the early stages of PC in a true sense.

Circulating tumor cells

The cells detached from the primary tumor are the most direct indicators of metastatic disease. While working on a mouse model of PC, Rhim *et al.* observed the presence of CTCs, even prior to the presence of evident lesions at the primary site. Moreover, epithelial to mesenchymal transition has been observed in the early PanIN-2 and PanIN-3 lesions in a mouse model of PC [53]. In line with this observation, it is common to spot metastatic lesions years after resection of small primary tumors with no clinically evident metastases at the time of diagnosis. Moreover, metastases of unknown primary tumors account for 4–5% of all clinical metastases, which shows that CTCs originate quite early during the development of the tumor [54,55]. Recently, using antibodies BM7 (anti-MUC1) and VU1D9 (anti-EpCAM), de Albuquerque *et al.* observed the presence of CTCs in 47% of PC

patients (n = 34), whereas none of the healthy controls (n = 40) had CTCs. Furthermore, shorter median progression-free survival was observed in patients positive for CTCs (66 days; 95% CI: 44.8–87.2) compared with patients who were CTC negative (138 days; 95% CI: 124.1–151.9) [56].

In order to analyze the dynamic relationship between CTCs and response to therapy, Ren *et al.* assessed the expression of CA19.9 and cytokeratin 8/18 in CTCs from the peripheral blood of patients with advanced PC after the first cycle of chemotherapy [57]. Among 41 patients with advanced-stage PC, 80.5% had more than two CTCs detected in 7.5 ml of peripheral blood before any therapy, with a median number of 16.8 ± 16.0 (range: 0–59). Post-5-flourouracil therapy (after 7 days), only 9.3% (12 out of 41) of these patients had more than two CTCs detected in 7.5 ml of peripheral blood, with a median number of 3.8 ± 7.8 (range: 0–40; p = 0.000). Furthermore, no CTCs were observed in healthy individuals in the same volume of blood (p = 0.000). Henceforth, detection of apoptotic CTCs with positive staining for tumor markers represents a potential avenue for predicting chemotherapy response [57]. Additionally, CTCs have been exploited to identify novel drugs for targeting metastatic cells in PC. Yu *et al.* used $CK^+/CD45^-$ CTCs from blood samples of a genetically engineered mouse model of PC (Pdx1 or P48/LSL-KRASG12D/Tp53lox/1 or Tp53lox/lox) and compared their gene expression profile with primary tumor cells and metastatic ascitic cells of the same mice. Single-molecule RNA sequencing revealed the enrichment of WNT2-positive CTCs compared with primary tumor cells. Furthermore, overexpression of multiple WNT genes (WNT*3A*, *4*, *5A*, *5B*, *6*, *7*, *9*, *10A* and *11B*) was also observed in five out of eight CTCs (CK1EPCAM1/CD452 cells) from human patients' blood. Interestingly, inhibition of MAP3K7 (inhibitor of the WNT-related pathway) by 5Z-7-oxozeaenol abrogated WNT2-induced tumor sphere formation [58]. Considering the significant alteration in the number and distribution of CTCs, their role in the diagnosis, selection of neoadjuvant and adjuvant therapy, detection of recurrent disease, examination of pharmacodynamic biomarkers as well as planning for individualized therapies in the future can be envisioned.

Epigenetic markers

Neoplastic development is an interplay of both genetic and epigenetic changes. Therefore, the development of panels of epigenetically modified genes as markers of disease progression is of immense interest. Epigenetic changes include alterations in the methylation pattern of DNA, physical and chemical changes in chromatin structure and miRNA-mediated regulation of gene expression. Considering the physiological significance of epigenetic changes during tumor progression, extensive studies are underway to explore their role in various malignancies including prostate, renal, breast and bladder cancer. Furthermore, with the emerging methodologies that allow detection of genome-wide DNA methylation in small amounts of sample (biopsy sample, a formalin-fixed paraffin-embedded tissue or a small volume [0.4 ml] of plasma from blood) even with highly fragmented DNA [59], epigenetic-based biomarkers have an immense potential in the diagnosis and prognosis of cancer. In the absence of specific and sensitive serological markers for PC, a number of studies have been performed to evaluate the performance of epigenetic-based biomarkers for PC. Alteration in DNA methylation status of *UCHL1*, *NPTX2*, *SARP2*, *CLDN5*, *LHX1*, *WNT7A*, *FOXE1*, *TJP2*, *CDH3* and *ST14* have been reported [60]. Park *et al.* observed that methylation-specific PCR for plasma *NPTX2* could differentiate PC from benign disease (chronic pancreatitis and biliary stricture) with a sensitivity and specificity of 80 and 76%, respectively [61]. Similarly, methylation profiling of preproenkephalin and p16 promoter showed their hypermethylation in plasma DNA from 30 and 25% of PC patients, respectively [62]. Using microarray coupled with methyl-CpG-targeted transcriptional activation (MeTA-array), Shimizu *et al.* discovered methylation of

TRH, *CYP26A1*, *TMEM204*, *GAD1*, *CSMD2*, *FRG2*, *ARC*, *SLC32A1*, *FOXJ1*, *TBX21*, *HOXA7*, *ANKRD35*, *HBA2*, *SP5*, *TNXB* and *GRASP* in PC cell lines. Among these genes, hypermethylation of *CSMD2*, *SLC32A1* and *TRH* was observed even in primary PC tissues [63]. While analyzing the methylation of circulating plasma DNA of *CCND2*, *SOCS1*, *THBS1* and *PLAU*, Melnikov *et al.* observed a 76% sensitivity and 59% specificity in distinguishing PC from healthy controls [64]. Liggett *et al.* compared the methylation profile of cell-free circulating DNA in 30 samples each from patients with CP, PC and healthy controls, using a microarray-mediated methylation analysis of 56 fragments [65]. The study identified a set of 17 gene promoters (eight for differentiating normal from chronic pancreatitis and 14 for differentiating CP from PC). Using this gene set of 17 promoters, a sensitivity and specificity of 81.7 and 78%, respectively, was observed for differentiating CP from normal individuals, while a sensitivity, specificity and accuracy of 91.2, 90.8 ($p < 0.01$) and >90%, respectively, was observed in differentiating PC from CP. Methylation of certain gene-specific CpG motifs is much higher than genetic defects and is easily detectable. In addition, alterations in DNA methylation occur quite early during the progression of tumor, resulting in the loss/gain of function of critical genes. Therefore, the exploration of epigenetic changes and epigenetic-based screening strategies could lead to the identification of potential diagnostic and prognostic markers in the near future.

miRNAs

miRNAs are small non-protein-coding RNAs of approximately 22 nucleotides in length involved in the regulation of a large number of genes at the post-transcriptional level. Research in the last decade has revealed that miRNAs play a critical role in the initiation, progression and metastasis of cancer. Furthermore, miRNAs are involved in controlling growth, proliferation, tumorigenesis, apoptosis and survival of pancreatic tumor cells (reviewed by Albulescu *et al.* [66]). To date, approximately 900 different miRNA genes have been discovered in the human genome [67] and computational calculations indicate that each miRNA on average recognizes approximately 100 different mRNA targets [68,69]. In light of these facts, it can be speculated that every cellular process is regulated by these small noncoding RNAs. Moreover, in cancer, it has been observed that miRNAs not only control the expression of known protein-coding oncogenes and tumor suppressors, but also act as oncogenes (referred to as oncomirs) and tumor suppressors. Furthermore, the presence of CpG islands in more than half of the miRNA genomic sequences, transcription factor-mediated regulation of miRNA synthesis and hypermethylation of tumor suppressor miRNAs suggest strong epigenetic dysregulation of miRNAs during tumor development [70]. The emerging implication of miRNAs in tumor diagnosis and prognosis can be appreciated from the observation that profiling 217 miRNAs classified various cancer types more precisely than 16,000 mRNA probes [71]. Furthermore, their small size, stability and easy extraction from formalin-fixed, paraffin-embedded tissues allow better applicability for diagnosis and prognosis.

Differential expression profiles of various miRNAs have been observed in PC. Studies on individual miRNAs have reveled that miR-21, -34, -146a, -155, -196a-2, -200a/b, -221, -181b, -100 and -196a show a significantly higher expression in PC compared with the non-neoplastic pancreas; by contrast, miR-217 is normally found in the pancreas, but has a consistently decreased or undetectable expression in adenocarcinomas [72]. Ren *et al.* evaluated the miRNA signature (miR-16, -21, -155, -181a, -181b, -196a and -210) in stool samples collected from PC ($n = 29$) and CP ($n = 22$) patients, and healthy controls ($n = 13$). Among these, miR-181b and -210 discriminated PC from normal individuals with receiver operator characteristic curves and AUCs of 0.745 and 0.772, respectively, and a significant correlation was observed between tumor diameter and miR-196a expression (Spearman's rank: 0.516; $p = 0.041$) [73]. While looking for an miRNA profile for the early detection of

PC, Yu *et al.*, in a comprehensive miRNA analysis, evaluated 700 miRNAs in PanIN lesions via quantitative real-time PCR. An altered expression of 107 miRNAs was observed in different PanIN grades and 35 miRNAs exhibited differential expression in PanIN-3, including overexpression of let-7f/g, miR-101, -103, -106b, -146a, -15b, -155, -18a, -182, -190, -193b, -194, -196b, -200a/b, -203, -21, -222, -29a/b/c, -31, -338-3p, -429, -486-3p, -93 and -95, and loss or weakened expression of miR-107, -139-3p/5p, -216a/b, -217, -218 and -483-5p. An exclusive expression of miR-196b was also observed in PanIN-3 lesions and PC cases compared with PanIN-1 and PanIN-2 lesions [74]. Liu *et al.* assessed the differentiation efficacy of 95 miRNAs, selected on the basis of their potential roles in tumor development and apoptosis, in tissues from pancreatitis and PC cases as well as PC cell lines (n = 3). Among all the tested miRNAs, a panel of eight miRNAs (miR-196a, -190, -186, -221, -222, miR-200b, -15b and -95) was found to be differently overexpressed (three- to 2018-fold change in expression) in PC tissues and cell lines [75]. A combination of CA19.9, miR-16 and -196a differentiated PC patients from healthy controls with 92.0% sensitivity and 95.6% specificity and an AUC of 0.979, while a combination demarcated CP patients from PC with a sensitivity, specificity and AUC of 88.4, 96.3 and 0.956%, respectively. Furthermore, miR-216 has been repeatedly identified to be pancreas-specific and normally observed to be lost during the progression of PC. Looking at the plethora of miRNAs differently expressed in PC, it is important to profile them according to stage, grade and cellular origin. Studies should be focused on miRNA profiling in fine-needle aspirates to improve the diagnostic performance. A greater understanding of their tissue specificity, redundancy between closely related miRNAs and the influence of polymorphisms on their levels and their targets can significantly improve the chances of realistic use of miRNAs as biomarkers [76]. Furthermore, technical issues including data normalization and the effect of contaminating cells have to be resolved. Studies should be focused on understanding the mechanism(s) by which miRNAs enter the circulation, developing improved quantification and analytic approaches for delineating the prognosis and diagnostic efficacy of miRNAs identified by high-throughput sequencing, and elucidating the biologic significance of circulating miRNAs. Overall, considering the robust involvement of miRNAs in various oncological developments, miRNA profiling in serum, pancreatic juice, cyst fluid and brushings from ERCP are regarded as the best potential avenue for miRNA-based biomarker development.

Somatic gene mutations

The development and progression of PC from PanIN-1 to full-blown pancreatic ductal adenocarcinoma involves accumulation of multiple genetic alterations. More than 100 mutations have been associated with PC. Global genomic analysis of 23,219 transcripts, representing 20,661 protein-coding genes from advanced pancreatic adenocarcinoma cases (n = 24) revealed accumulation of 48 nonsilent mutations, six amplifications and eight homozygous deletions [77]. A set of 69 genes was found to be genetically altered in the vast majority of cases and among these, 31 genes belonging to 12 core signaling pathways were altered in 67–100% of 24 cases analyzed [77]. These included genetic alterations in major players including KRAS2, CDKN2A/p16, SMAD4/DPC4 and TP53. Mutations in SMARC4A, CDH1, EPHA3, FBXW7, EGFR, IDH1 and NF1.7 were also observed. Unlike immunoregulatory proteins that are indirectly associated with tumor development, somatic mutations appear directly in tumor cells and are thus most specific diagnostic target(s). Among various genetic alterations, activating point mutations in the *KRAS* oncogene is one of the earliest genetic events and is prevalent in 80–100% of cases [78]. Given its prevalence and occurrence in early stages of PC, *KRAS* mutational status is considered to be a promising marker for gene-based early diagnosis. Caldas *et al.*, while analyzing stool specimens for mutated *KRAS* sequences, observed mutated *KRAS* in six out of 11 patients with pancreatic adenocarcinoma, from two out of three patients with cholangiocarcinoma

and from one out of three patients with chronic pancreatitis [79]. In addition, *KRAS* mutations are detected in pancreatic tissue, pancreatic juice, bile and plasma [77]. Parker *et al.* performed a meta-analysis of 34 studies for analyzing the diagnostic utility of *KRAS* mutations [80]. However, considerable variability in the sensitivity for detecting *KRAS* mutation (0–100%) was observed in these studies, whereas the specificity ranged from 58 to 100%. Interestingly, the highest diagnostic accuracy was observed for cytohistochemcial samples (76.5% sensitivity and 91.8% specificity) while lower accuracy was observed in clinical samples (patients series similar to those who would receive the test in practice; 68.4%) compared with case–control designs (patient set with definitive diagnosis and control group of healthy individuals; 82.7%). Furthermore, highly sensitive technologies are being applied to precisely quantify somatic mutations at the DNA and protein levels from patient samples. While analyzing the potential of mutated alleles as cancer-specific markers, Wang *et al.* detected and quantified peptides from normal and mutated *KRAS* alleles by selected reaction monitoring using a triple–quadruple mass spectrometer in clinical specimens from colorectal and pancreatic tumor tissues as well as in premalignant pancreatic cyst fluid and cancer cell lines [81]. Although in its infancy, this approach, in which missense mutations can be quantified and compared with the wild-type, provides a new avenue for diagnosing PC at early stages.

Conclusion

PC is a lethal malignancy with limited treatment options. Early diagnostic markers with the ability to detect the disease in its preinvasive stage can change the fate of PC patients. A plethora of biomarkers have been tested for the detection of PC. However, the skewed nature of the discovery dataset (>50% positive cases used for potential testing of biomarker), along with poor AUC, results in the failure of biomarker(s) in subsequent validation studies. Furthermore, the sensitivity of biomarkers is reduced due to the asymptomatic nature of precursor lesions and the low level of secretory molecules in the surrogate tissues/biological fluids. One major issue that complicates the search for early diagnostic marker(s) is the unavailability of early-stage tissue and serum samples from asymptomatic PC patients harboring noninvasive precursor lesions. Thus, specific and sensitive marker(s) for early diagnosis of PC remain a distant dream. In the present scenario, mouse models of PC that spontaneously develop ductal adenocarcinoma and reproducibly recapitulate both preneoplastic and neoplastic changes observed during human PC have emerged as important tools for circumventing the lack of early-stage PC patients, understanding tumor initiation, development and progression as well as the role of inflammation and the tumor microenvironment. Furthermore, they are becoming important models to study new therapeutics. Among these, the LSL-KRASG12D/Pdx1-cre mouse model, which expresses oncogenic KRASG12D in the pancreas and develops various PanINs (PanINs 1, 2 and 3), as well as the LSL-KRASG12D/TP53/Pdx1-Cre model, which spontaneously produces ductal adenocarcinoma of the pancreas and mimics histology, pathology, preneoplastic and neoplastic developments of human PC, are models with the greatest potential for studying early stages of PC. Additionally, models with key mutations, including the *p16/CDKN2A* TGF-β pathway components, *DPC4/SMAD4*, *TGFβRI* and *TGFβRII*, in combination with *KRAS* and *p53*, are quite important for predicating the diagnostic and prognostic efficacy of identified markers during later stages of disease. Pdx1-Cre *Ink4a/Arflox/lox* and KRASG12D *Ink4a/Arflox/lox* mouse models allowed identification of the markers for the early- and late-stage proteome of PC using mass spectrometry [82]. The identified marker sets were validated on human samples. The study revealed the differential expression of *ALCAM*, *ICAM1* and *TIMP1*. Gruner *et al.* observed significantly increased expression of thymosin-β4 in sera of mice with PanIN lesions [83]. These models will aid in both identifying new potential biomarkers as well as understanding the kinetics of their release. Furthermore, the immunocompetent nature of these models will help to delineate both tumor

and non-tumor-based biomarkers. These studies highlight the potential of mouse models to serve as a source of early-stage samples for the identification of biomarkers for early diagnosis.

In order to make diagnostic and prognostic marker(s) for PC a reality, a lot of efforts are being made to identify a set of sensitive and specific markers(s) from unconventional sources, such as pancreatic juice, stool, tumoral or peritumoral tissue as well as other less directly implicated body fluids such as urine and bile (reviewed by Chakraborty *et al.* [84]). Studies should focus on identifying altered genetic and molecular expression profiles of immune proteins, including autoantibodies, cytokines and PBMCs for diagnosing disease early in its asymptomatic phase. Similarly, screening for epigenetic modifications, altered miRNAs and circulating tumor cells can lead to good diagnostic and prognostic tools in the near future. Furthermore, the advent of genetically engineered mouse models of PC, recapitulating early stages of PC, has helped to decipher the intricacies of highly complex and lethal PC at the earliest stages of development. Importantly, such models can serve as a valuable source of early-stage tissue and serum samples to identify novel biomarkers that can be subsequently validated in clinical specimens. Overall, with recent advancements in the understanding of PC biology, improved methods to analyze alterations in its molecular profile, and the advent of the genetically engineered mouse models of PC, hopes are high to diagnose this malignancy early in a preinvasive state for improved treatment and prognosis for PC patients.

Future perspective

Biomarkers are needed to improve the diagnostic ability, provide prognostic information and for following disease progression and treatment response in PC. A set of sensitive and specific markers(s) should be identified and validated for diagnostic efficacy from unconventional sources, such as pancreatic juice, stool, tumoral or peritumoral tissue, as well as other, less directly implicated body fluids such as urine and bile. Looking at the prognostic significance of CA19.9, investigations should be performed to validate the diagnostic accuracy of the CA19.9 epitope in the context of the specific mucin backbones as well as different antibodies against various modifications of the silayl Lewis epitope (the carbohydrate epitope of CA19.9 antibody). Validation should be performed on the previously identified biomarker panel from proteomic and genomic studies. There is a need to exploit genetically engineered mouse models of PC for the discovery and validation of preinvasive-stage diagnostic marker(s). Validation of identified markers should be performed on asymptomatic cases from high-risk groups. Studies should focus on identifying the altered genetic and molecular expression profiles of immune proteins, including autoantibodies and cytokines as well as a genetic and expression profile of circulating PBMCs for the diagnosing disease early in its asymptomatic phase.

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Executive summary

Need for a biomarker of pancreatic cancer

- Pancreatic cancer (PC) is a lethal malignancy with the lowest overall survival among all cancers and a 5-year survival rate of <6%. Biomarkers are required for diagnosis, prognosis and prediction of therapeutic outcomes.

Early diagnosis equals better survival

- Significantly better outcomes have been observed for smaller tumors detected at an earlier stage. The 5-year survival rates of 10–20, 30–60 and >75% have been observed in patients with resected pancreatic tumors of 3, ≤2 and ≤1 cm in diameter, respectively. This clearly indicates that detecting the disease early can change the fate of PC patients.

Screening of high-risk individuals

- The rarity of PC in the general population (12.1 incidences per 100,000 cases) makes diagnostic screening an unmanageable venture. Even a highly sensitive (100%) and specific (99%) marker (ideal biomarker) will detect 83 false positives for every true PC diagnosis. In this scenario, screening should be restricted to a high-risk group, including individuals with a family history of PC, hereditary pancreatitis, obesity, cases of Peutz–Jeghers disorder, familial atypical multiple mole melanoma, cystic fibrosis and individuals with familial cancer syndrome, including Lynch syndromes, familial adenomatous polyposis (*APC* mutation) and hereditary breast and ovarian cancer syndrome (*BRCA1* and *BRCA2* mutations) and new-onset diabetes cases.

Diagnostic imaging

- In the absence of a surrogate marker, the diagnosis of high-risk groups is restricted to imaging modalities, including endoscopic ultrasonography, ultrasound, computed tomography, MRI, PET and/or endoscopic retrograde cholangiopancreatography. Endoscopic ultrasonography-guided fine-needle aspiration, with high sensitivity to detect a lesion <1 cm, is gaining popularity in familial PC programs.

Surrogate markers

- Due to the invasive, costly and nonspecific nature of invasive modalities, a surrogate marker capable of confirming the findings of imaging and directing asymptomatic patients for screening is highly necessary. Serological markers, including autoantibodies, cytokines and gene expression profiling of peripheral blood mononuclear cells, can be of immense significance in the diagnosis and prognosis of PC.

Epigenetic modifications & miRNAs

- Epigenetic modifications and the miRNA profile provide a new avenue for identifying novel markers for PC.

Circulating tumor cells

- Recent studies suggest that circulating tumor cells appear quite early during the development of the pancreatic tumors and are useful prognostic tools for predicting the therapeutic outcome.

Table 1

Association of various risk factors/groups with pancreatic cancer.

Variable	Details	Association with pancreatic cancer	Ref.
High-risk group			
Family history	RR of developing PC increases with increasing numbers of first-degree relatives diagnosed with PC	1 first-degree relative: 4.6-fold increased risk (95% CI: 0.5–16.4); ≥2 first-degree relatives: 6.4-fold increased risk (95% CI: 1.8 –16.4); ≥3 first-degree relatives: 32-fold increased risk (95% CI: 10.2–74.7)	[85]
CP	An incidence ratio of 14–18 observed for the development of PC in CP cases, which is further increased by cigarette smoking	Recent meta-analysis suggested a gap of 20 years between CP and PC, with pancreatitis occurring within 1–2 years prior to the diagnosis of PC	[86,87]
Hereditary pancreatitis	Repeated attacks of acute pancreatitis beginning in childhood, pancreatic insufficiency caused by mutation in cationic trypsinogen (*PRSS1*) gene	A 53-fold (95% CI: 23–105) increased risk for developing PC and a lifetime risk (age 70 years) of PC of 30–40% in comparison to normal. RR increases further in smokers	[88,89]
Cystic fibrosis	Autosomal recessive disorder caused by a defect in protein CFTR	Analysis of PC patients (n = 949) and controls (n = 13,340) for *CFTR* mutation revealed that 5.3% of patients and 3.8% of controls carried a mutation (W1282X nonsense mutation) in the *CFTR* gene (OR: 1.40; 95% CI: 1.04–1.89). No correlation was observed by Matsubayashi *et al.*	[12,16]
Familial atypical multiple mole melanoma	Autosomal-dominant disorder characterized by the familial occurrence of malignant melanoma of the skin and multiple atypical precursor lesions, germline mutation in *p16* (*CDKN2A*) in a quartile of patients	Increased risk of developing PC. *p16* is somatically inactivated in 95% of sporadic cases of PC and 40% of familial melanoma	[90]
Peutz–Jeghers	Autosomal-dominant inherited disorder (>80 cases due to a mutation in the *STK11* tumor suppressor gene)	132-fold (95% CI: 44–261) increased risk of PC compared with the general population	[91,92]
Lynch syndromes	Autosomal-dominant hereditary disease with mutation in DNA mismatch repair genes including *hMSH2*, *hMLH1*, *hPMS1*, *hPMS2* and *hMSH6*/*GTBP*	8.6-fold (95% CI: 4.7–15.7) increased risk for developing PC compared with the general population. An estimated 3.68% (95% CI: 1.45–45.88%) lifetime (age 70 years) risk of PC	[93,94]
HBOC	HBOC syndrome has a germline mutation in the *BRCA2* and *BRCA1* genes	*BRCA2* germline mutation carriers have a 5% lifetime risk of PC in comparison to 1.78% for controls. *BRCA1* mutation is 2.26-times that of the normal population	[95,96]
Risk factors			
Age	Incidence of PC increases with age	Ages 55–64 years: 20.7% of cases; ages 65–74 years: 25.8% of cases; ages 75–84 years: 27.8% of cases; age 85+ years: 13.3% of cases	[1]
Gender	More in men than women	The incidence rate is 13.8 per 100,000 men and 10.8 per 100,000 women	[1]
Race	African–Americans more prone to developing PC than Asians/Pacific Islanders	15.5 males and 12.6 females per 100,000 in African–Americans, while 8.4 males and 6.9 females per 100,000 for Asians/Pacific Islanders	[1]
Smoking	Most established risk factor for PC. Risk increases significantly with greater intensity: ≥30 cigarettes/day (OR: 1.75; 95% CI: 1.27–22.42); duration ≥50 years (OR: 2.13; 95% CI: 1.25–3.62); and cumulative smoking dose ≥40 pack-years (OR: 1.78, 95% CI: 1.35–2.34)	Pooled data from the international Pancreatic Cancer Cohort Consortium nested case–control study (1481 cases, 1539 controls) showed that current smokers had a significantly elevated risk (OR: 1.77; 95% CI: 1.38–2.26)	[97]
Obesity	A strong association has been observed between obesity and increased risk of PC	Obese individuals (BMI ≥30) have a slightly higher risk (RR: 1.19) of developing PC compared with normal-weight individuals (BMI <25)	[98]

Variable	Details	Association with pancreatic cancer	Ref.
Diabetes mellitus	Newly onset diabetes mellitus is considered as a risk for PC, but whether it is the cause or effect of PC is still unclear	Meta-analysis from 35 cohort studies from Japan, USA, Europe, Asia and Korea revealed a RR ratio of 1.94 (95% CI: 1.66–62.27) between T2DM and PC. 40–100% increases in the risk of PC are observed with established diabetes, while new-onset diabetes is associated with a four- to seven-fold increase in risk, such that 1–2% of patients with recent-onset diabetes will develop PC within 3 years	[24,98]

CP: Chronic pancreatitis; HBOC: Hereditary breast ovarian cancer syndrome; OR: Odds ratio; PC: Pancreatic cancer; RR: Relative risk; T2DM: Type 2 diabetes mellitus.

Table 2

Diagnostic efficacy of various biomarkers and biomarker panels tested for pancreatic cancer.

Marker	Screening efficacy	Ref.
CA19.9	Discussed in 'Serological markers' section	
α4GnT	Glycosyltransferase is involved in the biosynthesis of α1,4-GlcNAc-capped *O*-glycans. The mRNA from PBMCs of 76.4% of the PC patients was positive, while 40% of CP patients and 17.1% of normal individuals expressed it	[99]
SPan-1	Sialylated epitope present on mucins. A total of 81.3% of PC cases were positive for SPan-1, while nonspecific elevation was observed in liver cirrhosis and chronic hepatitis. A SN of 81.3% and SP of 75.6% were observed in comparison to other groups. It was found to have an 82% SN and 85% SP, and performed equally as well as CA19.9; however, higher levels were observed in biliary insufficiency than CA19.9	[100, 101]
CEACAM1	Also known as biliary glycoprotein 1. Had an 85% SN and 98% SP in differentiating normal from PC cases. Failed to differentiate CP from PC	[102]
MIC-1	Member of the TGF-β superfamily. MIC-1 performed better than CA19.9 in differentiating PC from HC (AUCs: 0.99 and 0.78, respectively; p = 0.003), but failed to differentiate cancer from CP (AUCs: 0.81 and 0.74, respectively; p = 0.63)	[103]
HIP-PAP	Pancreatic juice HIP-PAP-I concentrations were associated with an increased risk of PC and found to have a 75% SN and 87% SP in differentiating PC from nonpancreatic diseases	[104]
Osteopontin	Transformation-associated protein. Exhibited 80% SN and 97% SP for differentiating PC from normal cases. Performed poorly in differentiating CP from PC in later studies	[105]
PAM4	Monoclonal antibody PAM4 is reactive against MUC1. It was found to have an improved SN and SP for differentiating PC from pancreatitis than CA19.9, with an AUC of 0.89 (95% CI: 0.82–0.93) for differentiating PC from CP with a SP of 95.4%	[106]
TSGF	TSGF had a 91.6% SN (n = 88) and 93% SP, and improved diagnostic efficacy was observed in combination with CA242 and CA19.9	[107]
Biomarker panel		
ALCAM, ICAM-1, LCN2, TIMP-1, REG1A, REG3 and IGFBP4	ALCAM, ICAM-1, LCN2, TIMP-1, REG1A, REG3 and IGFBP4, with or without the addition of CA19–9, differentiated PC cases from controls with an AUC of 0.96 in comparison to CA19–9 (AUC: 0.79)	[82]
83 circulating proteins tested in training set and validation set	CA19.9, ICAM-1 and OPG had an 88% SN and 90% SP for differentiating PC from HC; CA19.9, CEA and TIMP-1 had a 76% SN and a 90% SP for differentiating PC from benign cancer; CA19.9, ICAM-1 and OPG had a 78% SN and a 94% SP for differentiating PC from benign cancer; CA19–9, CEA and TIMP-1 had a 71% SN and an 89% SP. The CA19–9, ICAM-1 and OPG panel is selective for PC and did not recognize breast (SP = 100%), lung (SP = 97%) or colon (SP = 97%) cancer	[108]
VNN1 and MMP-9	PBMC-based Affymetrix microarray analysis had a 95.8% SN and 76% SP for differentiating PC-associated Type 2 diabetes mellitus patients from long-lasting Type 2 diabetes mellitus patients (>5 years)	[109]
CA19-9, SAA and haptoglobin	Haptoglobin had an 82.7% SN and 71.1% SP, and SAA had a 34.7% SN and 90.2% SP for differentiating PC from non-PC cases. A combination of all three provided an overall SP of 91.3% and SN of 81.3%	[110]
CA19-9, CA50, CA242 and CA72–4 antigen	The combination with CA19–9, CA242 and CA72–4 showed a sensitivity of 89.2% and a specificity of 62.7% in differentiating the PC from non-PC group	[111]

AUC: Area under the curve; CP: Chronic pancreatitis; HC: Healthy control; PBMC: Peripheral blood mononuclear cell; PC: Pancreatic cancer; SAA: Serum amyloid A protein; SN: Sensitivity; SP: Specificity; SPan-1: Pancreatic-associated antigen; VNN1: Vanin-1.