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Blood biomarkers for differential diagnosis and early detection of pancreatic cancer

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ABSTRACT

Pancreatic cancer is currently the most lethal tumor entity and case numbers are rising. It will soon be the second most frequent cause of cancer-related death in the Western world. Mortality is close to incidence and patient survival after diagnosis stands at about five months. Blood-based diagnostics could be one crucial factor for improving this dismal situation and is at a stage that could make this possible. Here, we are reviewing the current state of affairs with its problems and promises, looking at various molecule types. Reported results are evaluated in the overall context. Also, we are proposing steps toward clinical utility that should advance the development toward clinical application by improving biomarker quality but also by defining distinct clinical objectives and the respective diagnostic accuracies required to achieve them. Many of the discussed points and conclusions are highly relevant to other solid tumors, too.

Introduction

Pancreatic cancer is a major health problem. Globally, 458,918 new cases and 432,242 deaths of pancreatic cancer have been reported in 2018 [1]. Currently, pancreatic cancer is the fourth and seventh most frequent cause of cancer-related death in Western countries and China, respectively, and expected to become second to only lung cancer in Western Europe and North America by 2030 [2]. Despite decades of research and advancement in disease management, the overall 5-years survival remains low at 5–9% of patients. Most patients are older than 50 years. Smoking, diabetes and chronic pancreatitis as well as a family history of cancer are major risk factors for tumor development. Also obesity and heavy alcohol consumption were found to be associated with an increased risk. Pancreatic cancer manifestations are nonspecific, including back pain, weight loss, jaundice, nausea and onset of diabetes. Diagnosis usually relies on imaging such as MRI and multidetector CT as well as pathological examination of a fine-needle aspiration from pancreatic tissue [3].

There are two basic types of pancreatic tumors: endocrine and exocrine. Endocrine tumors are rare and start in hormone-producing cells. Exocrine pancreatic lesions derive from cells that make up the enzyme-producing glands or the ducts of the pancreas. One of them is acinar cell carcinoma, which is a relatively rare tumor. Similarly infrequent are the benign or less malign pancreatic intraepithelial neoplasms (PanINs), intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs). All three may undergo a malignancy transformation process. The most common exocrine neoplasm with 90–95% of all pancreatic cancer cases is pancreatic ductal adenocarcinoma (PDAC) [4]; about 10% of them have a familial background. Because of its frequency and aggressiveness, PDAC is the main reason for the dismal prognosis of pancreatic cancer cases.

Surgery is still the best available therapeutic option and particularly successful for early stages of pancreatic cancer, increasing 5-year survival significantly. However, most PDAC cases are diagnosed at advanced stages so that only 10–20% of tumors are eligible for surgery. Even after decades of clinical research, no reliable diagnostic test has been established that has proven clinical practicality for diagnosing pancreatic cancer early [5]. This is due to the absence of specific signs and symptoms of the disease, a lack of reliable biomarkers and the limited resolution of imaging techniques caused by the retroperitoneal

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position of the pancreas. Modern imaging techniques pick up tumors with a sufficient accuracy at a size of more than one centimeter in diameter [6]. However, early molecular detection should be possible, since neoplastic cells probably take more than a decade to evolve and may require another five years for the acquisition of metastatic ability [7], but it requires the identification of better disease markers. Such biomolecules would also be beneficial for improved diagnostic and prognostic capabilities as well as the definition of possible targets for new treatment options. With appropriate screening processes in place, it may even be possible to pinpoint healthy individuals that may have a molecular predisposition to develop PDAC. The detection of relevant mutations could be an approach to this end. Also changes to cellular functional aspects that may take place prior to transformation, such as epithelial-to-mesenchymal transition [8], could facilitate the identification of people at risk.

Ideal biomarker molecules should meet several characteristics: biological plausibility of the underlying disease-relevant mechanisms; the ability to isolate them by means of a non-invasive and inexpensive process; exhibition of high diagnostic sensitivity, particularly in view of early detection; high specificity to discriminate between different pathological conditions; and robustness so as to allow application in clinical routine. For early detection, identification of pre-neoplastic lesions would be beneficial. Biomarkers can include a broad range of components in a biological specimen, including nucleic acids (e.g., microRNA) and their modifications (e.g., DNA methylation), proteins or peptides and their isoforms and variants, as well as cells (e.g., circulating tumor cells) or exocellular vesicles, such as exosomes.

Human specimens that could serve as a source for biomarker discovery should be readily accessible, sufficiently but not too complex for extracting discriminatory information and exhibit relevant changes. Blood has always been attractive for searching biomarkers due to its stability and accessibility. As a result, much research on biomarker analysis has meanwhile become an investigation of blood-based molecules [9], termed liquid biopsy (Fig. 1). Serological biomarkers have the additional advantage that they can be studied longitudinally during the course of a disease. To date, carbohydrate antigen 19–9 (CA19-9) is the only blood-based biomarker that is being used for the management of pancreatic cancer patients on a routine basis. However, the assay is not really sensitive or specific enough [10]. In addition, CA19-9 is not detectable in 5–10% of patients due to a lack of fucosyltransferase activity in individuals who have homozygous mutations in gene *FUT3*. Other carbohydrate antigens, such as CA242, have been studied, but did not surpass the performance of CA19-9. Therefore, a major objective of recent research activities on PDAC has been the identification of biomarkers in liquid biopsy samples that would complement or even supplement CA19-9. Technological advances actually improved the chances of biomarker identification [11]. Using proteomic approaches, such as mass spectroscopy and antibody microarrays, protein signatures in peripheral blood have been identified [12]. New insights into circulating tumor DNA and cell-free small RNAs as well as epigenetic modifications of nucleic acids were discovered using next generation sequencing or microarray technologies [13]. Flow cytometry and other cell-tracking methods as well as microscopic techniques enable the detection of circulating tumor cells or vesicles, in particular exosomes [14]. In this review, we summarize progress in biomarker discovery in blood samples for differential diagnosis and early detection of pancreatic cancer looking at the different marker types. Also, we discuss processes that may lead to success in this endeavor. Studies conducted in other sample types, such as tissue or pancreatic juice, or by means of animal models or tissue culture, are not covered.

Quality parameters

A frequently used parameter for describing the quality of a biomarker is derived from a receiver operating characteristic (ROC) curve, which illustrates the diagnostic power of an assay [15]. The area under the curve (AUC) value can vary between 0.50 and 1.00, indicating a performance between purely by chance and absolutely accurate. For this review, studies with an AUC of less than 0.75 were not taken into account. Directly related to the AUC are values for diagnostic sensitivity and specificity. The former is defined as the fraction of real positives that are identified by an assay; diagnostic specificity describes the

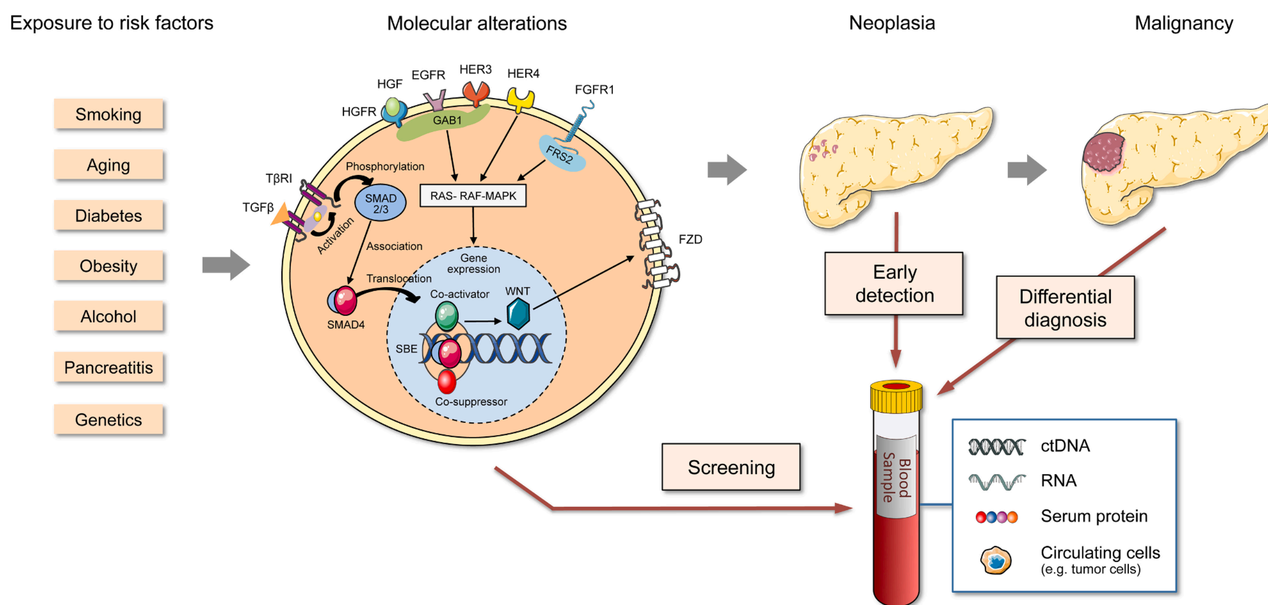

Fig. 1. Aspects of PDAC tumor development and possible application areas of liquid biopsy diagnostics. Exposure to risk factors initiates alterations at the molecular level. During tumorigenesis, relevant pathways are affected and eventually lead to uncontrolled cell proliferation, invasion and migration. Many of the molecular changes that are responsible for this development or result from the ongoing processes may be detectable in peripheral blood and used for screening. After transformation, factors released for niche formation or as part of the molecular communication between cells of the tumor microenvironment could circulate in the blood and permit detection at early stages. Furthermore, such biomarkers could provide valuable information for a differential diagnosis, discriminating malign from benign diseases, for example, or allowing therapy monitoring.

percentage of actual negatives that are called correctly. It has been suggested that a useful diagnostic assay for pancreatic cancer would have to perform with a minimum sensitivity of 88% at a specificity of 85% [16]. We set a more lenient threshold of 80% for both sensitivity and specificity for listing a study in Tables 1 or 2 below; information about studies that do not meet this requirement is available as supplementary material. This does not mean that any marker falling below the 80% thresholds may not be relevant and possibly helpful as part of a diagnostic signature. Inversely, biomarkers for which values above the thresholds have been reported may nevertheless not be applicable as sensitivity, specificity and AUC do not represent the only factors that matter and have to be viewed in perspective of the respective study design.

The actual number of analyzed samples can have a profound effect on the reliability of the conclusions that can be drawn. For this review, we chose to ignore entirely studies based on less than 50 samples in total or with single-digit sample numbers for either cases or controls; there are a few exceptions for analyses on circulating tumor DNA or tumor cells, and in the case of discriminating PDAC from autoimmune pancreatitis. Another important quality factor is reproducibility, which translates into robustness of an assay. It may vary because of methodological differences. Xie and colleagues, for example, compared ELISA and a time-resolved fluorescence immunological assay (TRFIA) for studying galectin 3 in sera from PDAC patients and healthy donors. Similar specificities of 97% and 90%, respectively, were recorded with the two analytic methods. However, sensitivity was only 32% according to ELISA while TRFIA was 75% sensitive, indicating differences that result from the actual processing rather than the biomedical sample [17]. Testing a biomarker on a second, independent sample set adds a lot to the overall quality of a study. Besides confirming the appropriateness of the methodological approach of detecting the observed variations, it validates the initially merely statistically defined biomarker performance. Another critical factor is the selection of the samples used in the analysis, in particular with respect to controls. Are blood samples from patients with benign tumors better controls than those from patients with chronic pancreatitis or healthy donors? Most studies only looked at a limited spectrum of sample types, frequently even only at tumor and healthy control. Others cover a wider range of tumor forms and different controls [e.g.,18], but may then be more limited with respect to the number of samples for each type. In addition, there might be effects of aspects such as age of the blood donor that could compromise a comparison. Also jaundice has an impact on the performance of biomarkers for pancreatic cancer and thus implications for their clinical translation [19]. With respect to actual utility, it is moreover crucial to know if a biomarker is unique to PDAC or whether it may act as a marker also for other pancreatic or gastrointestinal diseases or for different tumors entities. A lack of disease-specificity may not necessarily lower the quality of a biomarker as such, which may act as a pan-cancer marker of high quality nevertheless. However, without knowledge about this aspect, applicability in a clinical setting is less likely to be successful. While there are investigations that took into account a cross-validation with several other diseases [e.g.,20], most studies did not go that far.

Blood biomarkers for the detection of PDAC

Protein detection

For decades, ELISA has been the gold standard for the detection of protein biomarkers. Furthermore, ELISA platforms provide robust clinical diagnostics. Most ELISA-based studies aimed at PDAC looked at one or only few molecules, since the number of analytes that can be investigated is limited, although technical developments and the availability of antibodies against nearly every human protein [21] have changed this to some extent. A method that circumvents this limitation in numbers is mass spectrometry, which is usually combined with other separation techniques and has been implemented successfully to study the human plasma proteome. There is no bias in selecting the analytes and many of them can be studied simultaneously. A limitation has been technical sensitivity, restricting detection to molecules of relatively high abundance. Also, depletion is required for liquid biopsy samples so as to remove the most abundant proteins, particularly albumin. This processing step may introduce abundance variations for other proteins as well, however. As another method to look at many proteins in parallel, antibody microarrays and related multiplex immunoassays have come into play during the last decade. No depletion is required prior to analyzing serum or plasma samples and a nearly unlimited number of analytes could be studied simultaneously. Detection sensitivity is high, although this aspect partly depends on the affinity of the individual antibodies. Disadvantages are an analyte bias, because of the prior selection of the antibodies used in the assay, and the fact that possibly more than one analyte may bind to a particular antibody. Verifying the specificity of each binding event directly by utilizing a second antibody in an approach similar to ELISA limits the degree of multiplexing.

Individual protein biomarkers

In principle, an individual, sufficiently informative biomarker would be favorable in a clinical setting [22]. Therefore, the search for such molecules has often been at the pinnacle of medical research. However, given the molecular complexity of tumors, and pancreatic cancer in particular, as well as taking into account the variations between the genetic background of individual patients, it is more likely that a panel of markers is required for a highly accurate and robust diagnosis [23]. Nevertheless, numerous ELISA-based analyses on individual proteins have been performed over the years. Many of these markers do not meet our thresholds for sensitivity/specificity (Suppl. Table 1). However, beside their potential utility as part of a marker signature, some could be useful because of particular features. Marten et al., for example, measured the level of siC3b in serum samples of patients with a high risk for developing pancreatic cancer. Although performing diagnostically not as accurate as other proteins, soluble siC3b is an interesting molecule with respect to early diagnosis. The membrane-bound protein activates the complement system during the binding of autoantibodies to tumor cells. In a thorough analysis, it predicted tumor growth four months before radiological evidence became available with an AUC of 0.85 [24].

Protein level alterations that are linked to known disease mechanisms are more likely to act as biomarkers than functionally unrelated molecules. Inflammation, for example, revokes pancreatic cancer cell senescence and contributes to malignancy [25]. Inflammatory factors in peripheral blood could be useful biomarkers, although they might have a lesser accuracy than other molecules in discriminating inflammation and cancer. An example is the high expression of REG proteins, secreted proteins that play a critical role in tissue regeneration and inflammation and were found to be involved in pancreatic cancer metaplasia. REG1A and REG1B were both 92% sensitive and 95% specific in diagnosing pancreatic cancer, and REG4 was 95% sensitive although at only 77% specificity. Extracellular high mobility group box-1 (HMGB1) and cytokine CXCL8 are other examples of functionally characterized molecules from Table 1. HMGB1 is important for tumor growth and metastasis; CXCL8 plays a role in tumor progression and angiogenesis. Also proteins COL6A3 and HSP27 were reported as biomarkers. The former is a major component of the desmoplastic reaction in PDAC; the latter interacts directly with various components of the tightly regulated programmed cell death machinery. Both exhibited an excellent AUC, but the data are less trustworthy because of the low sample numbers, on which the analysis was based. In contrast, GDF-15 was found as a marker in two independent studies with a substantial number of samples each.

Utilizing mass spectrometry, dysbindin (DTNBP1) was studied in a relatively large cohort of patients and healthy donors. Its diagnostic accuracy was maintained in patients, who were CA19-9 negative, a feature that most studies did not look into. The overall most promising

Table 1

Blood-based biomarkers for discriminating PDAC patients from healthy donors. Molecules are listed that were reported with sensitivity and specificity values both being above 80% and an AUC larger than 0.85. The order within each group is according to publication year. Commas (,) link molecules that form a signature; a slash (/) separates molecules that exhibit the indicated values individually. ELISA: enzyme linked immunosorbent assay; FISH: fluorescence *in situ* hybridization; MS: mass spectrometry; NGS: next generation sequencing; PAGE: polyacrylamide gel electrophoresis; qPCR: real-time quantitative polymerase chain reaction; RT-PCR: reverse-transcriptase PCR; ddPCR: digital droplet PCR; RNA: transcriptional profiling; cfDNA: cell-free DNA.

PDAC vs. Healthy										
Biomarker name(s)	No. of PDAC samples	No. of controls	Training & test samples	Cross-validation in other tumors	Sensitivity	Specificity	AUC	Detection Method	Year of publication	Reference
Single Proteins										
HSP-27	35	37	No	No	100%	84%	0.98	ELISA	2007	[95]
HMGB-1	70	70	Yes	No	82%	84%	0.91	ELISA	2012	[96]
COL6A3	44	30	No	No	93%	97%	0.98	ELISA	2014	[97]
GDF-15	152	96	No	No	90%	90%	0.96	ELISA	2014	[98]
GPC1, exosomal	246	120	Yes	Yes	100%	100%	1.00	Flow Cytometry	2015	[99]
PIM-1	90	20	No	No	95%	100%	0.98	ELISA	2016	[100]
REG1A	41	61	No	No	92%	95%	–	ELISA	2016	[101]
REG1B	41	61	No	No	92%	95%	–	ELISA	2016	[101]
DTNBP1	250	150	Yes	No	82%	85%	0.85	MS	2016	[102]
CXCL8	42	34	No	No	98%	95%	0.99	ELISA	2018	[103]
ZIP4, exosomal	24	46	No	No	80%	92%	0.89	ELISA	2018	[104]
Proteins and Carbohydrate Antigen CA19-9										
APOA2, APOC1 & CA19-9	111	105	Yes	No	93%	100%	0.96	MS/ELISA	2010	[105]
TFPI & CA19-9	37	15	No	No	84%	90%	0.94	ELISA	2011	[106]
TFPI, TNC & CA19-9	37	15	No	No	90%	100%	0.99	ELISA	2011	[106]
ICAM-1, OPG & CA19-9	333	227	Yes	Yes	88%	90%	0.93	Immunoassay	2011	[107]
C5, A1BG & CA19-9	22	29	No	No	87%	90%	0.92	MS	2013	[19]
APOA2 isoform & CA19-9	286	217	Yes	Yes	95%	98%	0.96	ELISA	2015	[108]
CEA, CA125, CA242 & CA19-9	52	40	No	No	90%	94%	–	ELISA	2015	[109]
C4BPA & CA19-9	52	40	Yes	Yes	85%	96%	0.93	MS	2016	[110]
IGFBP2, IGFBP3 & CA19-9	101	38	No	Yes	88%	89%	0.89	Immunoassay/ MS	2016	[111]
THBS2 & CA19-9	288	230	Yes	RNA	87%	98%	0.97	MS/ELISA	2017	[112]
TIMP1, LRG1 & CA19-9	187	169	Yes	No	85%	95%	0.95	ELISA	2017	[113]
ALB, CRP, IL-8 & CA19-9	292	383	Yes	Yes	94%	90%	0.98	ELISA	2017	[30]
APOA1, APOE, APOL1, ITIH3 & CA19-9	80	40	Yes	No	95%	94%	0.99	MS	2017	[114]
POSTN, CA242 & CA19-9	213	74	Yes	No	92%	97%	0.98	ELISA	2018	[115]
Peptide Panels or Protein Signatures										
CCL11, HGF, IP-10, MCP-1	54	26	No	No	86%	92%	0.94	ELISA	2005	[116]
18 proteins bound by 18 scFv antibodies	103	30	No	No	88%	85%	0.95	Antibody Microarray	2012	[117]
IIRPC (HPT, C3, C4A, C5, C7, IgG1, IgA1)	122	252	Yes	Yes	92%	91%	0.94	PAGE	2014	[118]
19 proteins bound by 25 scFv antibodies	156	30	No	No	99%	80%	0.98	Antibody Microarray	2015	[119]
Panel of 10 peptides	88	185	Yes	No	92%	95%	0.96	MS	2015	[120]
APOA1, APOE, APOL1, ITIH3	80	40	Yes	No	85%	94%	0.94	MS	2017	[114]
EPHB3, FGF1, ID1, IL2, IL10, IMPDH2, SELL, VCAM1	72	49	Yes	No	89%	91%	0.95	Antibody Microarray	2017	[32]
29 proteins bound by 29 scFv antibodies	586	1107	Yes	No	95%	94%	0.97	Antibody Microarray	2018	[121]
Tumor-Associated Autoantibodies										
anti-CEA-425, anti-EGFR-54, anti-EGFR-479, anti-Pap-112, anti-SART-109	47	42	No	Yes	83%	88%	–	Peptide bead array	2006	[122]
anti-PSCA peptide 2–11, anti-PSCA peptide 86–95, anti-PSCA peptide 109–118	40	28	No	Yes	80%	82%	–	Peptide bead array	2007	[123]
anti-ENOA1,2 & CA19.9	120	40	No	No	97%	92%	0.95	Western blot	2011	[124]
anti-EZR, anti-ENOA1.2 & CA19,9	45	48	No	Yes	100%	92%	0.96	Western blot	2013	[125]
Glycans and Glycosylation										
A1BG response to SNA lectin	22	89	No	Yes	100%	98%	0.99	Antibody-lectin ELISA	2009	[126,127]
Fucosylated haptoglobin	300	315	No	No	85%	82%	0.91	ELISA	2013	[128]
Lectin purified AACT, THBS1, HPT & CA19-9	37	30	No	No	97%	93%	0.99	MS	2014	[129]
sLeA, sLEX variants, sialylated type 1 LacNAc	205	49	Yes	No	85%	90%	0.90	Immunoassay	2016	[130]
Glycosylation of haptoglobin, CE350, VP13A	76	26	No	No	100%	100%	1.00	MS	2017	[131]
25 *N*-glycan structural isomers	32	32	No	No	93%	91%	0.98	MS	2018	[132]

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Table 1 (*continued*)

PDAC vs. Healthy										
Biomarker name(s)	No. of PDAC samples	No. of controls	Training & test samples	Cross-validation in other tumors	Sensitivity	Specificity	AUC	Detection Method	Year of publication	Reference
sTRA & CA19-9	168	176	Yes	No	80%	82%	0.86	Immunoassay	2019	[40]
14 Glycoproteins	45	136	No	No	91%	86%	0.94	MS	2020	[133]
microRNAs										
miR-18a	36	30	No	No	92%	94%	0.93	qPCR	2011	[134]
miR-16, miR-196a & CA19-9	140	179	No	No	92%	96%	0.98	qPCR	2012	[135]
miR-20a, miR-21, miR-24, miR-25, miR-99a; miR-185, miR-191	197	158	Yes	No	94%	95%	0.99	qPCR	2012	[136]
miR-1290	81	39	No	No	92%	94%	0.96	qPCR	2013	[137]
10 microRNAs	409	312	Yes	No	85%	85%	0.93	qPCR	2014	[43]
miR-125a, miR-4294, miR-4476, miR-4530, miR-6075, miR-6799, miR-6836, miR-6880	100	150	Yes	Yes	80%	98%	0.95	Microarray	2015	[138]
12 microRNAs	417	307	Yes	No	85%	90%	0.95	qPCR	2016	[42]
miR-22	35	15	No	No	97%	93%	0.94	qPCR	2017	[139]
miR-642b/miR-885	35	15	No	No	100%	100%	1.00	qPCR	2017	[139]
miR-373	103	50	No	No	81%	84%	0.85	qPCR	2017	[140]
miR-133a	110	64	No	No	91%	87%	0.89	qPCR	2019	[141]
miR-663a, miR-642b, miR-5100, miR-8073	424	2599	Yes	No	99%	88%	0.98	Meta-analysis	2020	[142]
miR-1290, miR-1246 & CA19-9	120	40	No	No	97%	98%	0.99	Meta-analysis	2020	[143]
Long RNAs										
circRNA *LDLRAD3* & CA19-9	31	31	No	No	80%	94%	0.87	qPCR	2017	[144]
lncRNA *ABHD11-AS1* & CA19-9	114	46	No	No	95%	100%	0.98	qPCR	2019	[145]
mRNA content in extracellular vesicles: *CLDN1*, *FGA*, *HIST1H2BK*, *ITIH2*, *KRT19*, *MARCH2*, *MAL2*, *TIMP1*	189	74	Yes	No	96%	100%	0.98	NGS	2020	[146]
ctDNA										
DNA methylation of *ADAMTS1, BNC1*	42	26	No	No	81%	85%	–	Bisulfite qPCR	2013	[147]
Mutations in 92 amplicons from 22 genes	100	29	No	No	82%	100%	–	ddPCR, NGS	2016	[148]
cfDNA quantity	24	38	No	Yes	83%	92%	0.92	Fluorometry	2016	[58]
DNA methylation of *ADAMTS1, BNC1*	39	95	No	No	97%	92%	0.95	Bisulfite qPCR	2019	[149]
DNA methylation of *SST*	30	18	No	Yes, in tissue	93%	89%	0.89	Bisulfite ddPCR	2019	[63]
CTC Detection										
(CTC detection markers)										
CTCs *(CK8, CK18)* & CA19-9	41	20	No	No	80%	100%	–	Immuno-fluorescence	2011	[150]
(expression of C-MET, hTERT, CK20, CEA)	25	15	No	No	100%	100%	–	RT-PCR	2011	[151]
(CD45$^-$, CK8, CK18, CK19)	15	15	No	No	80%	100%	–	Immuno-fluorescence	2015	[152]
(CD45$^-$, CK, DAPI, centromere chromosome 8)	25	20	No	No	88%	90%	0.95	Immuno-fluorescence, FISH	2016	[153]
CTCs *(Vimentin$^+$, CD45$^-$, Hoechst$^+$)* & CA19-9	100	30	No	No	91%	97%	0.97	Immuno-fluorescence	2019	[154]
Metabolites										
1,5-anhydro-D-glucitol, histidine, inositol, xylitol	43	42	No	No	86%	88%	0.93	MS	2013	[155]
Fatty acid PC-594	84	99	No	No	90%	87%	0.93	MS	2015	[156]
PC-594, phosphatidylcholine, sphingomyelin, lysophosphatidylcholine	116	138	No	No	86%	85%	0.93	MS	2016	[157]
Palmitic acid	40	40	No	No	100%	100%	1.00	MS	2016	[158]
PE(18:2), N-succinyl-L-diaminopimelic acid	30	30	No	No	93%	93%	0.95	MS	2017	[159]
5 Amino acids, lactate, LysoPC (18:2), choline, 3-hydrobutyrate, palmitic acid	59	48	No	Yes	93%	96%	0.99	MS	2017	[78]
Exosomal lipid PE (16:0/18:1)	48	40	Yes	No	86%	96%	–	MS	2019	[160]
Panel of 9 metabolites	59	60	No	No	93%	100%	0.99	MS	2020	[74]
Mixed Panels										
DNA methylation, H2Az, H2A1.1, H3K4Me2 in cell-free nucleosomes & CA19-9	25	24	No	No	92%	90%	0.98	ELISA	2015	[161]

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Table 1 (*continued*)

PDAC vs. Healthy										
Biomarker name(s)	No. of PDAC samples	No. of controls	Training & test samples	Cross-validation in other tumors	Sensitivity	Specificity	AUC	Detection Method	Year of publication	Reference
Exosomal molecules: CD44v6, Tspan8, MET, EpCAM, CD104 & miR-1246, miR-4644, miR-3976, miR-4306	131	42	Yes	No	100%	93%	–	Flow Cytometry, qPCR	2015	[162]
miR-21, GDF-15 & CA19-9	164	260	Yes	Yes	88%	99%	0.97	qPCR, ELISA	2016	[163]
miR-25, GDF-15 & CA19-9	164	260	Yes	Yes	84%	100%	0.97	qPCR, ELISA	2016	[163]
Acetylspermidine, diacetylspermine, indole-derivative, LPC(18:0), LPC (20:3) & LRG1, TIMP1 & CA19-9	59	162	Yes	No	90%	88%	0.92	MS	2019	[79]

individual protein biomarker described in literature to date is glypican-1 (GPC1). The membrane-anchored protein is involved in growth factor signaling and overexpressed in PDAC tissue and peritumoral fibroblasts while exhibiting low abundance in chronic pancreatitis tissues and healthy pancreas [26]. It was found at the surface of exosomes released by PDAC tumor cells. Analyzing circulating exosomes in serum by means of antibody binding and ensuing flow cytometry, sensitivity and specificity of diagnosing PDAC were reported to hit an amazing 100% each in a rather large sample cohort. Subsequent analyses could not quite confirm this outstanding performance, however, and have even indicated no discriminatory power at all [e.g.,27,28].

Combining protein biomarkers with carbohydrate antigens

With CA19-9 being established as a marker in clinical routine, it could be expected that its combination with other markers could enhance diagnostic robustness and accuracy, providing better sensitivity by detecting cases missed by CA19-9 alone while maintaining specificity. One example for this is a group of four proteins – APOA1, APOE, APOL1 and ITIH3 – selected from more than 1000 proteins that were looked at in a proteome quantification based on mass spectrometry. They already served as a useful biomarker panel on their own. Combination with CA19-9, however, improved sensitivity from 85% to 95% while specificity remained unchanged at 94%. The utility of apolipoproteins for diagnostics was corroborated in other investigations [29]. In addition, a cancer-specific isoform of APOA2 resulted in high diagnostic accuracy together with CA19-9 in a thorough analysis of over 500 samples, which included an evaluation of samples from patients with other tumor entities. The relevance of this cross-checking of a biomarker in other tumor forms is highlighted by a study, in which again many samples were looked at. One panel – ALB, CRP, IL-8 and CA19-9 – showed high accuracy for PDAC. However, it actually exhibited a diagnostic value also in samples from breast, cervical, colorectal, gastric, prostate and lung cancer patients, although at a substantially lower level [30].

PanINs are early lesions during the development of PDAC. Focusing on initially 107 proteins that were secreted selectively by human PanIN organoids, THBS2 was found to be highly informative for PDAC diagnosis in conjunction with CA19-9. The combination also exhibited good discrimination of PDAC vs. chronic pancreatitis or IPMN. The same is true for C4BPA, which binds to key molecules involved in inflammatory and coagulation pathways; its direct interaction with CD40 induces B cell proliferation. C4BPA levels also differentiated PDAC from other gastrointestinal tumors. A combination of CA19-9 with TFPI or OPG and ICAM-1 achieved good results as well; OPG exhibited very high specificity (>97%) for PDAC as compared to breast, lung and colon cancer. Also C5, A1BG and CA19-9 did result in good discrimination, as did TIMP1, LRG1 and CA19-9 – based on considerably more samples – and IGFBP2, IGFBP3 and CA19-9. The last combination also discriminated PDAC reasonably against esophagus, gastric and colon cancer. Periostin (POSTN) was jointly acting as a biomarker with the two carbohydrate antigens CA242 and CA19-9. Last, also four carbohydrate antigens alone – CA19-9, CEA, CA125 and CA242 – yielded promising results. Successfully supplementing CA19-9 diagnostics has been reported for other proteins, too, but these markers did not pass our performance thresholds (Suppl. Table 1).

Protein biomarker signatures

Combining markers could improve diagnostic accuracy and increase reliability and robustness, since each individual marker contributes only part of the overall information. Any deficiency of a particular molecule because of technical or biological reasons could thus be compensated. Also, biomarkers of high specificity frequently exhibit relatively low sensitivity. A combination of such molecules, however, could increase sensitivity substantially without compromising specificity. The more biomarkers are put together, the more likely it is that a signature is gaining in power. This is offset, however, by the fact that ever more molecules have to be analyzed for diagnosis. Also, just adding biomarkers to a signature does not necessarily lead to overall better diagnosis (e.g., Fig. 2).

In a very large study, sera from 1366 patients with lung, colorectal, pancreatic, gastric, and thyroid cancer, 376 patients with related benign diseases and initially 3707 healthy controls were analyzed. Additionally, samples from 103 patients with seven other epithelial cancers (esophagus, throat, liver, prostate, kidney, uterus and breast) and 38 from patients with multiple myeloma were included. Overall, 8 circulating immunoinflammation-related protein complexes (IIRPCs) were identified that comprised complement components (C), immunoglobulins (Ig), haptoglobin (HPT), apolipoprotein A-1 (APOA1), and transferrin besides other proteins. One IIRPC made of HPT, C3, C4A, C5, C7, IgG1 and IgA1 showed high discrimination power. However, it was also good in

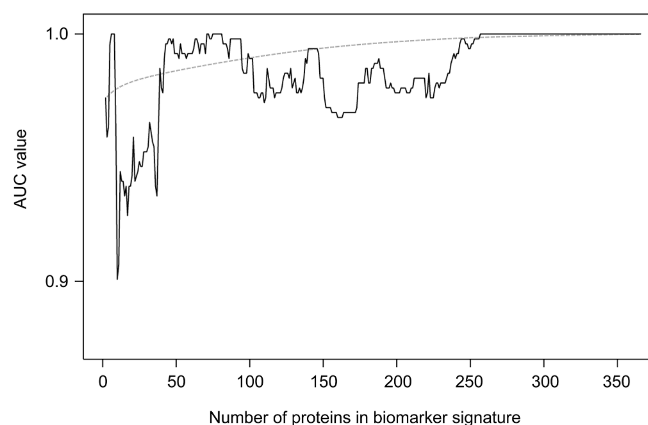

Fig. 2. Cumulative effect of biomarkers on diagnostic accuracy indicated as AUC value. A panel of 364 proteins exhibited significant differential abundance in sera from PDAC patients and healthy individuals [32]. Starting with a protein that exhibited in the training set of samples an AUC value of 0.97 on its own, proteins were added one by one, creating biomarker signatures made of two to eventually 364 proteins. Each added biomarker was selected at random from the remaining proteins. Rather than a continuous increase in accuracy (dotted line), profound variations in the overall AUC occurred.

discriminating other cancers from relevant controls and functioned more as a marker for several diseases. An analysis on 31 cytokines, chemokines, growth and angiogenic factors yielded a panel of four markers – HGF, MCP-1, IP-10, CCL11 – of high accuracy, although without validation in a second sample cohort. In another study, 507 binders targeting cytokines were applied on antibody microarrays, but the achieved accuracy was not as high (Suppl. Table 1). Velstra et al. studied serum samples by high-resolution mass spectrometry and identified an initial set of seven peptides that differentiated PDAC patients from healthy individuals. Based on these promising results, they evaluated further the use of peptides for diagnosis and came up with a signature of 10 molecules. Another group used a microarray made of 121 human recombinant single-chain variable fragment (scFv) antibodies, targeting 57 mainly immunoregulatory biomolecules; they found an informative 18-analyte signature. Adding CA19-9 levels to the overall signature actually decreased performance. Subsequently, the same group analyzed samples from a multicenter study on 293 scFv antibodies directed against 98 antigens. This time, they defined a diagnostic panel of 25 antibodies. Next, they applied 350 scFv binders to staging PDAC plasma samples and achieved with 25 antibodies AUC-values of 0.71, 0.86, 0.90 and 0.93, respectively for discriminating healthy individuals from patients with PDAC stages I to IV [31]. Utilizing the same 350 antibodies to analyze a very large cohort of 1693 samples, a panel of 29 antibodies that bind to 29 proteins was selected that had a combined AUC value of about 0.97 (Tab. 1). For the selection of a much smaller but nevertheless informative panel of antibodies, Mustafa and colleagues took the approach of studying both the secretome of 16 cancer cell lines and patient sera for specifically secreted proteins. The assumption was that proteins, which are released by tumor cells but not by non-tumor cells, should be detectable specifically in cancer patient sera. On an array of 1439 antibodies, 8 of the 112 secretome proteins that were specific for cancer cells exhibited similar abundance variations in the serum profile specific for PDAC patients, which was composed of altogether 189 proteins. These 8 markers – EPHB3, FGF1, ID1, IL2, IL10, IMPDH2, SELL and VCAM1 – shared by secretome and serum did not show a superior performance as markers individually. Combined, however, they yielded an AUC of 0.95, while any combination of serum-only markers produced substantially less accurate results [32].

Tumor-associated autoantibodies

Another option of detecting PDAC-related proteins takes advantage of the immune system, which reacts even at low antigen concentrations. It should therefore produce antibodies against tumor-associated proteins already early during tumorigenesis, although it is not fully understood how the immune system is triggered by tumors [33]. PDAC-derived exosomes have been shown to display a large repertoire of tumor antigens that induce autoantibodies, suggesting that they exert a decoy function by inducing inhibition of complement-dependent cytotoxicity towards cancer cells [34]. Irrespective of the actual reason for their formation, tumor-associated autoantibodies could have diagnostic utility. However, a thorough data review found that reported autoantibodies exhibited on average a rather low sensitivity of 14% at a simultaneously high specificity of 95% [35]. As one could expect, therefore, no autoantibody against an individual PDAC antigen was reported that met our sensitivity threshold, apart from two molecules in inadequate analyses with single-digit sample numbers. As we entirely disregarded reports with sensitivities of less than 50%, since the respective AUC would not meet our 0.75 threshold, there are only five studies listed in Suppl. Table 1. In combination with CA19-9 and each other, however, autoantibodies against three antigens – the ENOA1,2 isoforms and EZR – actually performed well (Tab. 1). Additionally, they exhibited specificity to PDAC compared to breast, colon and lung cancer. An earlier study had revealed that autoantibodies against five out of 63 peptides with HLA-A2 or HLA-A24 binding motifs were able to discriminate PDAC from healthy. The same laboratory found a very similar result for three peptides that represent the amino acids at positions 2–11, 85–95 and 109–118 of the prostate stem cell antigen. As opposed to the ENOA1,2 isoforms and EZR, however, both peptide panels were not tumor-specific but acted as markers also for colon and gastric cancer. Lack of tumor specificity was also reported for autoantibody levels against p53 in combination with the CA19-9 level (Suppl. Table 1).

Glycan profiling

Glycosylation plays a crucial role in many biological processes including cellular adhesion and the activation of cellular receptors [36]. As glycans strongly influence all kinds of cellular interactions, the development and progression of cancer is bound to result in substantial changes in glycosylation patterns at cell surfaces and of secreted glycoproteins. Changes observed in pancreatic cancer involve sialyl Lewis antigens, truncated *O*-glycans, branched and fucosylated *N*-glycans, specific proteoglycans as well as *O*-linked *N*-acetylglucosamine [37]. Many early findings about glycans as biomarkers in serum or plasma were focused on examining the changes at particular proteins [38]. Frequently, they were based on very small patient numbers or lacking appropriate controls [39]. But also not all studies with an adequate number of samples produced performance parameters that met our thresholds (Suppl. Table 1). An interesting example in this group are glycans containing 3′ fucose, which might be able to complement the use of CA19-9 by providing information on the patient subgroup that does not express CA19-9. Other investigations report results that meet our standards (Tab. 1). Rather than analyzing merely glycan abundance, Liu et al. widened the detection window and identified 25 *N*-glycan structural isomers that act as potential biomarkers and achieve a high diagnostic accuracy if applied in combination. Slightly less precise was another reported panel made of three glycans: sLeA, sLeX variants and sialylated type 1 LacNAc. As discussed in the section on proteins, functional relevance could point toward good biomarkers and help in their identification. The hypothesis had been put forward that tumors, which do not overproduce CA19-9, are different from those that do and may produce alternate but structurally similar glycans instead [40]. The glycan sTRA (sialylated tumor-related antigen) was found to be produced and secreted by pancreatic tumors and PDAC models that did not

produce CA19-9. Its abundance in blood distinguished samples from PDAC and healthy donors, although only narrowly passing our sensitivity and specificity thresholds.

Analyzing glycoproteins represents a kind of mixed signature, consisting of changes in both glycan and protein abundance, although they are not necessarily unrelated factors. In a very recent investigation, a panel of 504 glycan motifs was scrutinized, representing 73 proteomic markers. Fourteen of them could be combined to a classification model of high relevance. In a study that took also into account several controls beyond samples from healthy individuals, *Aleuria aurantia* lectin was used to extract fucosylated glycoproteins. Analyzed by mass spectrometry, the fucosylated proteins AACT, THBS1 and HPT were found to show high diagnostic potential. Combined with CA19-9, they distinguished PDAC from all other conditions analyzed (normal, diabetes, cystic tumors, chronic pancreatitis, obstructive jaundice) with an AUC of 0.92. But not all fucosylated proteins function as biomarkers of the same quality. Fucosylated MUC5AC, for instance, did not meet our standards (Suppl. Table 1). Also other results with fucosylated HPT vary, most likely due to the quality of the antibodies used in the different measurements; only one more report surpassed our quality threshold. HPT features also in another panel of three glycoproteins: HPT, CE350 and VP13A. In cases in which all three were glycosylated, all samples came from PDAC patients and none from healthy people, thereby providing highly accurate information, although only to a subgroup of patients.

RNA level variations

The use of microRNAs as biomarkers attracted attention because of the defined and relatively small number of molecules and thus the ability to analyze comprehensively an entire level of regulative activity. In addition, microRNAs exhibit good biochemical stability as opposed to mRNAs, making their detection less error-prone [41]. Similar to studying proteins in liquid biopsy samples, different processes are available for microRNA analysis. Quantitative PCR (qPCR) frequently targets individual markers, but high-throughput layouts with many microRNAs are also available. DNA-microarrays as well as next-generation sequencing look at basically all microRNA molecules. There are two basic analysis formats: one deals with serum or plasma samples, the other looks at the microRNA content of whole blood. It is well established that vesicles in serum or plasma, such as exosomes, contain large quantities of microRNA. Exosomal profiles vary according to the cell types from which the exosomes are released. However, their microRNA content does not necessarily resemble the intracellular profile. In whole-blood analyses, additional microRNA originates from blood cells, in particular erythrocytes, which contain substantial amounts of microRNA despite their high degree of differentiation and lack of nuclei. While no direct comparison between serum/plasma and whole-blood samples has been performed, there are two studies with serum and whole-blood samples, respectively, which used similar sample numbers (724 vs. 721), the same detection mode (qPCR) and an analogous study design. Johansen et al. [42] found 34 discriminating microRNAs in serum, whereas Schultz et al. [43] identified 38 such molecules. Also, they defined panels of 12 or 10 microRNAs, respectively, which could distinguish PDAC from healthy samples with very similar performance parameters (Tab. 1). However, only two of the detected microRNAs were in common – miRNA-26b and miRNA-345 – and the derived diagnostic panels do not share any molecule at all.

In a plasma study, the combined use of only two microRNAs – miR-16 and miR-196a – in plasma as well as CA19-9 in related serum samples yielded good discrimination. Maybe more interesting, however, is the fact that miR-21 was found as a potential biomarker in this as well as many other, entirely unrelated investigations on plasma, serum or exosomal microRNA. However, miR-21 was frequently not the best marker in the respective study, or the studies do not pass our performance thresholds (Suppl. Table 1). In an investigation with few samples, a diagnostic accuracy of up to 100% was reported for miR-22, miR-642b and miR-885. Another study identified miR-18a as a good marker molecule. Interestingly, the authors had performed longitudinal testing and found high miR-18a levels in plasma before surgical resection of the tumor, which dropped significantly thereafter. Additionally, there were indications that the level increased again upon tumor recurrence. Regrettably, the last result was anecdotal and not compared to other processes of detecting recurrence. Other microRNAs that yielded good diagnostic power were miR-133a, miR-373 and miR-1290 as well as a panel of 8 molecules – miR-125a, miR-4294, miR-4476, miR-4530, miR-6075, miR-6799, miR-6836 and miR-6880. Several other microRNAs or microRNA signatures did not perform according to our standards (Suppl. Table 1).

Recently, a meta-analysis was performed that took advantage on several data sets, thus achieving good coverage especially for the number of healthy individuals. With a panel of just four biomarkers – miR-663a, miR-642b, miR-5100, and miR-8073 – high accuracy was achieved. In another meta-analysis, a combination of miR-1246, miR-1290 and CA19-9 was found to be highly informative, too. A rather global experimental set-up studied microRNAs for their ability to discriminate not just PDAC but between different cancers and non-cancer diseases [44]. In total, the level of 848 miRNAs was measured in 1,049 whole-blood samples. The investigated cohort included healthy controls as well as patients diagnosed with one of 10 cancer entities or 9 non-cancer diseases. For each disease, all other samples acted as controls. Both common cancer markers as well as molecule signatures for each individual illness were identified. Not surprising, however, the sensitivity and specificity values were lower than in many other studies, since the approach resembled much more a complex real-life clinical situation. Nevertheless, accuracies of 94% for discriminating cancer from non-cancer patients and 90% for the specific detection of PDAC were achieved. Finally, in terms of using plasma microRNA for determining the risk of developing PDAC, microRNA levels in 225 pre-diagnostic samples and 225 matched controls were investigated. Level variations of a panel of six microRNAs – miR-10a, miR-10b, miR-21, miR-30c, miR-155 and miR-212 – were associated with risk, but fell clearly short of an accuracy degree that would be required for early diagnosis (Suppl. Table 1).

More recently, not just variations in the concentration of microRNAs were reported but also changes seen for longer RNA molecules, namely long non-coding RNA (lncRNA), messenger RNA (mRNA) and circular RNA (circRNA). An lncRNA that yielded good results was *ABHD11-AS1*, exhibiting very high accuracy in combination with the CA-19-9 level. In another study, the content of mRNA packaged in extracellular vesicles was analyzed. RNA in extracellular vesicles is probably better protected from degradation than freely circulating molecules. Performed on a relatively large set of samples, high diagnostic accuracy was achieved with a panel of 8 mRNA molecules – *CLDN1*, *FGA*, *HIST1H2BK*, *ITIH2*, *KRT19*, *MARCH2*, *MAL2* and *TIMP1*. This was also true for samples from patients, who were CA19-9-negative, indicating that the panel has the potential to supplement CA19-9 diagnosis. This is supported by the fact that adding CA19-9 to the signature did not influence performance substantially. With regard to circular RNA [45], circRNA *LDLRAD3* was a molecule found to be informative, again in combination with CA19-9.

Circulating tumor DNA

Tumors leave evidence of their presence in a human body by releasing circulating tumor DNA (ctDNA). In blood, ctDNA has a short half-life. Therefore, it very much represents the actual situation and is likely to reflect dynamic molecular changes during tumor development [46,47]. Ever since an inaugural publication [48], ctDNA has been looked at for understanding the mutational landscape of tumors including PDAC, for the identification of disease-relevant signaling pathways and for its utilization for diagnosis [49]. It was considered as a novel process of PDAC tumor classification by looking at mutations in genes that affect several molecular pathways [50]. Many studies were

performed that document the high frequency of mutations in known pancreatic cancer driver genes, such as *KRAS, TP53*, *SMAD4* and *CDKN2A*, of which particularly *KRAS* mutations were used for diagnostic approaches [51 and citations therein]. For example, in one study, mutations in 54 genes were assessed in ctDNA samples from 26 pancreatic cancer patients in comparison to the tumor tissue sequences. The conclusion was that ctDNA mutations in five genes – *KRAS*, *TP53*, *APC*, *FBXW7* and *SMAD4* – should be able to detect PDAC accurately [52]. However, the reported frequencies of detecting ctDNA mutations in patients vary widely; for *KRAS*, they range from about 30% to 92% of PDAC patients [53]. Therefore, it appears as if a highly accurate diagnostics could not be based on this information alone [54 and references therein], in particular when taking into account the fact that other tumor entities also show mutations in the very genes altered in PDAC [55]. Still, mutational analysis of ctDNA could contribute diagnostic value together with other marker molecules (see mixed panel section below), but may be better suited to provide prognostic information and allow disease monitoring.

In a rather different approach, it was reported that simply the amount of ctDNA isolated from plasma samples was able to discriminate PDAC from control samples, irrespective of the ctDNA's mutational status [56–58], although it is unclear how specific this may be. Analyses of ctDNA work at the detection limit [54] and ctDNA amounts are higher in patients with a larger tumor volume and metastases [59]. Given that many of the studied samples were from patients with late PDAC stages, the increased ctDNA levels in patients may only reflect tumor size while differences between healthy individuals and patients with early PDAC stages may not exist since both may fall below the detection limit. Measuring variations of ctDNA concentrations after tumor resection, however, indicated the potential usefulness of this approach for checking for recurrence. It predicted clinical relapse 6.5 months earlier than CT imaging did [60].

An alternative to DNA mutations could be variations in DNA methylation. It has already demonstrated its high potential for routine classification of tissue samples from central nervous system tumors [61], for instance. One could expect that it may be a means for identifying and discriminating other tumor types and that the variations may also be reflected in the methylation status of ctDNA. A methylation panel in the promoters of the genes *ADAMTS1* and *BNC1* produced good sensitivity and specificity in plasma ctDNA in two reports, separated by six years (Tab. 1). Other investigations came up with DNA methylation markers of lesser accuracy (Suppl. Table 1). A study that looked into the frequency of 5-hydroxymethylcytosine (rather than the usual 5-methylcytosine) in ctDNA of several tumor entities showed good values for both colorectal and gastric cancer, but the results for PDAC indicated a lack of any diagnostic potential [62]. As with mutations, disease specificity of DNA methylation is also an issue. Recently, *SST* hypermethylation has been reported for PDAC, for example, and relevant changes were also reflected in ctDNA. However, rather than being specific for tumors of the pancreas, *SST* hypermethylation could be observed across a wide range of tumor entities, thus qualifying as a strong pan-cancer marker but lacking PDAC specificity [63]

Circulating tumor cells

Apart from circulating DNA, also intact cells are shed by tumors and can be detected in peripheral blood [64] even before metastasis occurs [8]. Usually a mixture of processes is used for their identification. CD45-depletion is applied to remove white blood cells. Positive enrichment of circulating tumor cells (CTCs) is achieved by selecting for epithelial cell adhesion molecule (EPCAM) or size-based filtration. The actual CTC recognition is then mostly done by looking at cell morphology, by measuring the transcript levels of particular marker genes or their expression as proteins, and by immunofluorescence staining, utilizing molecules that are assumed to be specific for CTCs. A marker that was repeatedly applied as a CTC reporter was the creatine kinase family [54] either on its own or in combination with other detection modes.

A large body of publications exists about the frequency of CTC occurrence in PDAC blood samples [54,65,66]. Most studies, however, were based on very small samples numbers. Also, analyses on blood from cancer-free control samples were frequently missing. Partly, reasonable sensitivities were recorded but without any information about specificity. In some studies, it was assumed that no CTC-positive signal could be generated with blood from cancer-free individuals. However, CTC-like cells have been observed in samples from healthy donors, partly even surpassing commonly applied threshold levels of 1–3 cells per milliliter [e.g.,67]. For this review, we ignored analyses without controls from healthy individuals, which went through the very CTC enrichment and detection processes used on the patient samples. Only relatively few studies met our requirements (Table 1; Suppl. Table 1). Overall, the frequency, with which CTCs can be detected in blood samples, varies enormously. This may also dependent on the different sample processing and CTC detection modes. The variation underlines the relatively low value of using the mere presence or absence of CTCs for diagnostics. Furthermore, while there are studies that report clear differences between malign and benign pancreatic diseases [e.g.,68], others point to the opposite, namely that rates of CTC identification were similar in patients with benign, premalignant, and malignant lesions. Cauley and colleagues, for example, identified cells in the samples of 49% of PDAC, 64% of neuroendocrine tumor, 62% of IPMN and 46% of chronic pancreatitis patients [69]. The real value of CTC analysis may lie elsewhere. Even more than ctDNA, CTCs provide a valuable representation of the individual tumor and reflect the variations occurring during its development. Thus, they make available information for risk stratification [70], predict liver metastasis several months prior to imaging [71] and are related to cancer recurrence [72] and shorter survival [73], although there are also reports with the contradictory finding that CTCs in PDAC patients were not associated with poor prognosis [69].

Metabolites

The conversion of a normal to a cancerous cell is accompanied by a reprogramming of metabolic pathways. In addition, cancer grows in a usually hypoxic environment and is deprived of nutrition because of poor angiogenesis. In PDAC, there is also a particularly dense fibrotic stroma, much of which consisting of extracellular matrix. Therefore, it is likely that the presence of a tumor does influence the abundance of metabolites circulating in the blood [74]. Techniques such as the measurement of nuclear magnetic resonance (NMR) and mass spectrometry allow a relatively broad and in principle unbiased search for metabolic biomarkers and have been used to such ends. Some of the reports – in particular but not only early ones – were about analyses on very few samples and were not considered.

Several studies yielded high accuracy in discriminating PDAC and healthy (Tab. 1). One of them probed 45 water-soluble metabolites and found 18 of them to be informative, although none exhibited sensitivity and specificity values that were both above 80%. However, combining four metabolites – xylitol, 1,5-anhydro-d-glucitol, histidine, and inositol – resulted in a diagnostic model that improved performance considerably. In another investigation, phosphatidylethanolamine PE(18:2) and N-succinyl-L-diaminopimelic acid also exhibited good discrimination power. A very recent, untargeted investigation identified a panel of 9 metabolites that distinguished PDAC from healthy. Concerning single marker detection, Ritchie et al. showed that the lipid acid PC-594 allowed good discrimination. Adding three choline metabolites as markers in a follow-on study and analyzing more samples did not impact performance. However, it validated PC-594 as a marker. Exceptionally high scores for individual molecules were reported by Di Gangi and colleagues, who looked at altogether 206 metabolites. Sixteen of them were informative. Best performing was palmitic acid with actually perfect discrimination power. Interestingly, a palmitic acid derivative was found in another investigation. Some 270 lipids that belong to 20

lipid species showed some potential as biomarkers. The best one was exosomal lipid PE (16:0/18:1), which consists of one chain of palmitic acid at the C-1 and one chain of oleic acid at the C-2 position.

Other studies failed to meet our thresholds (Suppl. Table 1). Taking into account the concentration of all 20 amino acids as determined by mass spectrometry is one of them. This work is of interest nevertheless because of the actual procedure used for analyzing the abundance variation data. Rather than following the usual path of pairwise comparisons (e.g. PDAC vs. healthy), a multi-class evaluation strategy was applied instead, by which basically any number of disease categories could be compared simultaneously and accurately [75]. In another study on 360 PDAC samples and 8372 samples from healthy donors, diagnosis was based on six amino acids: Ala, Asn, His, Ile, Ser and Trp. At 80% specificity, a sensitivity of 77% was determined. Betaine, choline, glutamate, methylguanidine and 1,5-anhydro-d-glucitol are a further group of possible marker molecules. They enabled an accurate calling of disease in a training group of 200 US-samples, but validation in a Chinese cohort of the same size yielded a much lower accuracy. Concerning early diagnosis, a nicely arranged multi-national study was conducted with 1243 samples in total, representing either plasma from patients, who were diagnosed with PDAC between 1 month and 17 years after blood sampling, or age- and sex-matched controls. While metabolomic biomarkers with predictive values were obtained, they did not allow a prediction of practical value [76]. The work could also not confirm a finding that branched-chain amino acids may indicate an increased risk and are associated with early stage PDAC [77].

As seen in other molecules classes, the reproducibility of marker identification between studies is limited also for metabolites, although 12 amino acids as well as choline, glutamate, lactate, LysoPC(18:2), 1,5-anhydro-d-glucitol, 3-hydroxybutyrate, palmitic acid and the fatty acid PC-594 were found more than once [78]. Many analyses focused on particular molecule classes and may therefore have missed other biomarker types. If identical molecules were identified, comparability is usually hampered, since the actual purification, separation and detection processes differed from study to study. A re-analysis, evaluating 10 of the above metabolites, showed very high accuracy in discriminating PDAC from healthy (AUC = 0.99; Table 1). Plasma samples from patients with colorectal cancer yielded an identical AUC value. Also the discrimination of type-2 diabetes and healthy samples resulted in an AUC of 0.96. The markers allowed a clear cut between type-2 diabetes und PDAC (AUC = 0.99), but they failed to separate colorectal from pancreatic cancer (AUC = 0.65) [78], suggesting that many metabolite variations may be common to several carcinomas or tumor entities.

Mixed panels

In an informative biomarker panel, molecules are supporting each other best, if they are functionally unrelated. Level variations of enzymes that belong to the same metabolic pathway and are co-regulated, for example, may convey the same information but are less likely to have a much better performance as a group than just one of them might have alone. The same could be true across molecule classes. Methylation variations in a promoter region are bound to lead to a change in transcription, for instance. Therefore, only the respective methylation or transcription of a gene should be used as part of a marker signature. Preferably, biomarker groups should consist of molecules that do not have any functional connection.

Several mixed panels have been reported that yielded highly promising results (Table 1). One of them required the isolation of exosomes from serum samples. The frequency of five cell markers – CD44v6, Tspan8, EpCAM, MET and CD104 – was then determined by flow cytometry and combined with the levels of four miRNAs – miR-1246, miR-4644, miR-3976 and miR-4306 – in the exosomes. Put together, absolute sensitivity and high specificity was achieved. In a study, in which the biomarkers may actually not be entirely functionally independent, variations of four epigenetic features that are associated with cell-free nucleosomes circulating in serum – the level of DNA methylation as well as the abundances of histone modification H3K4Me2 and histone sequence variants H2AZ and mH2A1.1 – were combined with CA19-9 levels, achieving a highly relevant degree of diagnostic power. Very interesting were variations in protein GDF-15 abundance and CA19-9 level combined with changes in the quantities of miRNAs miR-21 or miR-25, respectively. Besides yielding highly accurate discrimination of PDAC and healthy in both a training and validation cohort, there was also good distinction between PDAC and other gastrointestinal tumors (AUC of about 0.87), documenting the signatures' PDAC-specificity. In a fourth investigation, another mixed signature was created by joining three sets of biomarkers that on their own did not meet our thresholds – namely a panel of five metabolites [79], the proteins LRG1 and TIMP1 [80] as well as CA19-9.

The performance of an assay analyzing *KRAS* mutations in ctDNA, CA19-9 levels and abundance variations of CEA as well as proteins HGF and OPN yielded a sensitivity of 64% at a specificity of 99% for discriminating PDAC from healthy (Suppl. Table 1). Following up this result, a slightly improved accuracy level was obtained in a subsequent, much larger study. The blood samples of 812 healthy individuals were compared to 1005 samples from patients with 8 different tumors, of which 93 samples were from PDAC patients. Sixty-one amplicons on 16 genes (*NRAS, CTNNB1, PIK3CA, FBXW7, APC, EGFR, BRAF, CDKN2A, PTEN, FGFR2, HRAS, KRAS, AKT1, TP53, PPP2R1A, GNAS*) were produced from ctDNA and studied with respect to their mutational status. This information was combined with the levels of the carbohydrate antigens CA-125, CEA, CA19-9 as well as the proteins PRL, HGF, OPN, MPO and TIMP-1. The combined power of these optimized 69 biomarkers improved sensitivity to 72% at 99% specificity [55]. While lower in mere numbers than other results, this study is probably more significant than many, since taking into account the existence of other tumor entities. This is likely to reduce the relevance of quite a few marker molecules. The analysis is therefore much closer to the real clinical situation since not dealing with carefully selected samples of one tumor entity only. However, even in this set-up, the reported specificity value represents an overestimation as it only takes into account the number of false positives (cancer) in the healthy cohort, ignoring falsely assigned cancer samples.

Blood biomarkers that discriminate between PDAC and chronic pancreatitis

In clinical reality, the discrimination between different diseases of the pancreas is a major issue as it is prerequisite to a quick application of the appropriate treatment option. This is particularly relevant for PDAC, given the small time window during which treatment might have a significant effect. Chronic pancreatitis (CP) is a progressive inflammatory disease characterized by recurrent abdominal pain, pancreatic stones and calcification, ductal atrophy and pancreatic insufficiency [81]. About 5% of CP cases progress to become pancreatic cancer. Besides being a major risk factor, CP obscures the diagnosis of PDAC by presenting as a cancer-like mass that standard imaging techniques may not be able to differentiate [6]. Furthermore, many molecular changes compared to healthy pancreas are shared between PDAC and CP. In consequence, both sensitivity and specificity of distinguishing pancreatic cancer and CP are commonly around 65% only [82]. Apart from possible failure to diagnose PDAC, also falsely diagnosing actual CP as PDAC can have serious implications as it leads to unnecessary surgery. It has been reported that CP was found in 9.9% of cases that were presumed to be pancreatic cancer and underwent surgical resection [83]. More conclusive diagnostic results require invasive tissue sampling, currently. For simpler and less risky diagnosis, reliable non-invasive processes would be advantageous. While quite a few studies of this kind were performed, many were done as part of investigations that had their focus on comparing PDAC and healthy; frequently, the differences between CP and healthy were analyzed for avoiding inflammation

markers in PDAC signatures. In other investigations, CP samples were mixed with other “benign” samples rendering it impossible to extract a specific result. Only relatively few studies looked at the discrimination of PDAC and CP specifically and used a sufficiently large number of samples.

Protein biomarkers

Efforts to define informative blood biomarkers yielded relatively few proteins. YKL-40 and IL-6, for instance, were found in a large study on 639 samples but did not pass our quality thresholds (Suppl. Table 2). Also the diagnostic indices of ALCAM, CFB, CSPG4, DKK1, GDF-15, HSP70, LRG1, NGAL and PIM-1 as single markers did not meet our criteria and were inferior to CA19-9 [84]. Likewise, two marker panels – albumin, CRP and IGF-1 as well as a set of 29 proteins – did not perform well enough. One of the better markers (Table 2) is ULBP2, which plays a significant role in tumor cell recognition by binding to NKG2D receptors on immune cells, initiating an immune response against the tumor. Even on its own, variation of the ULBP2 concentration was able to discriminate PDAC and healthy samples. It performed diagnostically even better in combination with GDF-15 and CA19-9. Also, exosomal ZIP4 yielded good accuracy. In a targeted study, Saraswat et al. found 13 proteins that met our sensitivity/specificity thresholds [85]; the three best performing molecules were AMBP, ITIH2 and ATP6V1B1. However, the study was performed on very few sera only, casting doubt on its robustness. A protein signature of IFNγ, IL-8, IP-10, TNFα and TNFRII was found to be highly accurate in separating PDAC and CP samples. Similarly, a marker set made of 25 proteins yielded good performance.

CA19-9 is elevated in both pancreatic cancer and CP compared to samples from healthy individuals. However, it nevertheless functions as a biomarker that is able to discriminate the two diseases to some extent [84]. Therefore, it may help to enhance differential diagnosis. Molecules

Table 2

Blood-based biomarkers for discriminating PDAC patients from patients with chronic pancreatitis (CP) or autoimmune pancreatitis (AIT). Molecules are listed that were reported with sensitivity and specificity values both being above 80% and an AUC value larger than 0.85. The order within each group is according to publication year. Commas (,) link molecules that form a signature; a slash (/) separates molecules that exhibit the indicated values individually. ELISA: enzyme linked immunosorbent assay; MS: mass spectrometry; NGS: next generation sequencing; qPCR: real-time quantitative polymerase chain reaction; cfDNA: cell-free DNA.

PDAC vs. CP

Biomarker name(s)	No. of PDAC samples	No. of CP samples	Training & test samples	Sensitivity	Specificity	AUC	Method	Year of publication	Reference
Single Proteins									
ULBP2	152	91	No	88%	82%	0.92	ELISA	2014	[98]
ZIP4, exosomal	24	32	No	84%	91%	0.89	ELISA	2018	[104]
Proteins and Carbohydrate Antigens									
PIGR, REG3A & CA19-9	38	39	No	84%	80%	0.89	MS	2013	[19]
ULBP2, GDF-15 & CA19-9	152	91	No	93%	94%	0.98	ELISA	2014	[98]
IL-8, IP-10, IL-6 & CA19-9	127	49	Yes	87%	88%	0.91	Immunoassay	2014	[164]
Albumin, IGF-1 & CA19-9	47	20	Yes	94%	95%	0.96	ELISA	2016	[165]
THBS2 & CA19-9	288	83	Yes	82%	83%	0.87	MS/ELISA	2017	[112]
Protein Signatures									
IFNγ, IL-8, IP-10, TNFα, TNFRII	54	22	No	98%	96%	0.98	ELISA	2005	[116]
25 Proteins	72	43	Yes	100%	88%	0.97	Antibody Microarray	2017	[32]
Glycosylation									
AACT, THBS1, HPT & CA19-9	37	30	No	87%	91%	0.96	MS	2014	[129]
Other biomarkers									
miR-20a, miR-21, miR-24, miR-25, miR-99a; miR-185, miR-191	120	82	No	99%	98%	0.99	qPCR	2012	[136]
miR-16, miR-196a & CA19-9	140	179	No	88%	96%	0.96	qPCR	2012	[135]
DNA methylation in promoter of *CCND2, CDKN1C, CDKN2B, DAPK1, ESR1, HMLH1, MGMT, MUC2, MYOD1, PGK1, PGR, RARb, RB1, SYK*	30	30	No	91%	91%	–	Microarray	2010	[166]
9 Metabolites (ceramide, histidine, isocitrate, phosphatidylcholine, proline, pyruvate, sphinganine-1-phosphate, sphingomyelin, sphingomyelin) & CA19-9	271	282	Yes	90%	91%	0.94	MS	2018	[167]
mRNA content in extracellular vesicles: *CLDN1*, *FGA*, *HIST1H2BK*, *ITIH2*, *KRT19*, *MARCH2*, *MAL2*, *TIMP1*	186	55	Yes	94%	81%	0.92	NGS	2020	[146]
Mixed Panels									
GDF-15, miR-25 & CA19-9	76	22	No	90%	82%	0.94	qPCR & ELISA	2016	[163]
THBS2, CA19-9, cfDNA quantity	52	32	Yes	93%	91%	0.94	ELISA and Qubit assay	2019	[168]

PDAC vs. AIP

Biomarker name(s)	No. of PDAC samples	No. of AIT samples	Training & test samples	Sensitivity	Specificity	AUC	Method	Year of publication	Reference
IgG4	45	24	No	84%	89%	–	ELISA	2014	[169]
IgG4 & CA19-9	53	33	No	84%	100%	0.92	Immunoassay	2014	[170]
miR-181d	69	15	No	81%	80%	0.88	qPCR	2016	[171]

that performed well in combination of CA19-9 were PIGR and REG3A (Table 2), although this is only true for PDAC cases in presence of jaundice. With jaundice being absent, the power of discriminating PDAC and CP fell substantially. Another study revealed that THBS2 and CA19-9 led to a reasonable differentiation. Adding to this marker pair a quantification of cell-free DNA created a composite biomarker of superior performance. Also mixing CA19-9 levels with glycosylation variations on the proteins AACT, THBS1 and HPT yielded good results. Finally, cytokines IL-6, IL-8, IP-10 were found to be informative with CA19-9. Several other markers have been reported but did not pass our sensitivity and specificity thresholds (Suppl. Table 2).

Other biomarker types

In an interesting setup, a direct comparison of different diagnostic processes on one sample set put their potential into perspective. Next to endoscopic ultrasound-guided fine needle aspiration as a kind of current gold standard, the presence of CTCs in plasma, *KRAS* mutations in ctDNA as well as the CA19-9 level were studied in 52 tumor and 9 pancreatitis samples [86]. The reported respective sensitivities and specificities for PDAC diagnosis were 73% and 88% for fine needle aspiration, 67% and 80% for CTCs, 65% and 75% for the *KRAS* mutations as well as 79% and 93% for CA19.9. Besides the fact that the number of CP samples was too low for inclusion, the accuracy values were not above our thresholds either. Still, the study provides another glimpse at the fact that not one method alone but their combination may make the difference in discriminating samples with a higher accuracy than what is possible today. To such an end, also biomarkers other than proteins were investigated for their diagnostic capacities. For microRNAs, a combination of miR-16 and miR-196 with CA19-9 yielded good results, for example, as did a panel of altogether 7 microRNAs – namely miR-20a, miR-21, miR-24, miR-25, miR-99a, miR-185 and miR-191 – which had already shown to discriminate PDAC and healthy sera. A three-microRNA combination – miR-106b, miR-126 and miR-486 – resulted in a slightly less accurate diagnosis (Suppl. Table 2). Yuan et al. showed that level changes in the levels of miR-25, GDF-15 and CA19-9 made accurate discrimination possible. Also the mRNA content of extracellular vesicles was informative. Concerning methylation variations in ctDNA, 14 gene promoters were found in an analysis of just 56 genes. Put together, they achieved discrimination of sufficient specificity and sensitivity. A group of 8 other gene promoters were looked at in a much larger sample number; they actually failed to reach the quality values of the aforementioned panel (Suppl. Table 2). Also methylation changes in the *NPTX2* promoter were found to be interesting, in particular since the result was confirmed independently; both studies did not meet our requirements, however. An analysis of 477 metabolites that belong to 10 ontology classes was performed in one study; about two-thirds of the molecules were complex lipids, fatty acids and related metabolites. A prediction panel of 9 metabolites together with CA19-9 was able to differentiate between PDAC and CP.

The special case of autoimmune pancreatitis

Autoimmune pancreatitis (AIT) is a relatively rare form of chronic pancreatitis and consists of two subtypes [87]. AIT symptoms are particularly similar to those of PDAC. An important diagnostic criterion is actually the success of a treatment with steroids. As opposed to PDAC patients, AIP patients show a dramatic response to this therapy. Still, the distinction between AIP and especially AIP type 2 (AIT-2) and PDAC is challenging. A multicenter study showed that 60% or 78% of AIT-1 or AIT-2 cases, respectively, were diagnosed only retrospectively after a pancreas resection had been performed under the suspicion that the disease was PDAC [88]. As with CP, AIP misdiagnosis has negative implications each way, either by missing the opportunity for curative surgery in case of PDAC or by performing surgery unnecessarily, which accounts for approximately 8% of all pancreatectomies performed under the suspicion of malignancy. This overtreatment is generally accepted in the light of the risk of missing pancreatic cancer and the lack of a sufficient method for discriminating between both entities [87].

A commonly accepted biomarker in sera of patients with AIT-1 is the elevated blood level of IgG4, which has been found in several independent studies [89]. A diagnostic quality that meets our standards has been reported for IgG4 alone or in combination with CA19-9 (Table 2). In addition, there have been studies that confirmed its biomarker status but reported sensitivity and specificity values below our thresholds (Suppl. Table 2); third, there were also analyses with good results that were performed on too few samples to be included here. A review and meta-analysis of the performance of 15 IgG4 and 8 IgG datasets [90] reported an altogether 73% sensitivity and 93% specificity for the former and 51% and 94% for the latter. Concerning markers other than IgG4, Felix et al. found the ratio of the enzymes MMP9 over MMP2 to be informative. The same group analyzed also cytokines and concluded that a combination of IL-7 and G-CSF had a diagnostic value (Suppl. Table 2). In a study that used full blood instead of serum or plasma, a combination of the percentage of eosinophils, which were substantially reduced in PDAC blood samples, with the levels of globulin, Hb and CA19-9 only narrowly missed our threshold for specificity at high sensitivity (Suppl. Table 2). Finally, the microRNA miR-181d, which is associated with mitogen-activated protein (MAP) kinase pathways, was found to distinguish AIT and PDAC (Table 2). Three other microRNAs showed promise as well (Suppl. Table 2).

Conclusions and prospects

All kinds of marker molecule classes have been studied and a large number of analyses have been performed over the years. However, comparison between them or drawing overall conclusions is not easily achieved. For a start, only few biomolecules were looked at in many investigations. Even more global biomarker discovery procedures, such as for proteins mass spectrometry or antibody-microarrays, analyzed merely up to a few thousand molecules, which is only a small portion of the total number of expressed proteins even under the incorrect assumption that one gene encodes only one protein. Therefore, many studies have no or only little overlap with others. Furthermore, it is likely that discrepancies between studies were introduced by using different sample preparation protocols and detection methodologies, although the influence of this is difficult to quantify. The already mentioned comparison of the microRNA content in plasma or whole-blood samples [42,43] highlights the effect this can have. A biological rather than technical complication has its origin in the inherent fact that blood circulates through the entire body. Changes in biomarker levels observed in tumor secretome may be less easily detectable in blood, since many other cells apart from the cells in the tumor could release the respective molecules as well, thereby obscuring the result. This influence could also differ from person to person. It has been suggested that collecting blood from the portal vein rather than from blood vessels at the periphery may remedy the problem, but few data exist so far [91] and any such approach would make blood collection more difficult and invasive.

Another restriction toward utility is the actual definition of many biomarkers. Diagnostics could be more accurate if not variation in the concentration of a particular molecule would be used as a marker but changes that are more binary in nature, present in cancer but absent in the case of healthy pancreas or vice versa, such as specific mutations, methylation variations in DNA or RNA or the occurrence of circulating tumor cells or protein isoforms. DNA methylation has demonstrated its power in the diagnosis of other tumor entities and may thus be a strong candidate also for PDAC analysis. To date, only relatively little data is available, however. On the other hand, much of today's literature indicates that accurate diagnosis based on ctDNA and CTC analysis may be difficult to achieve for the lack of sufficient sensitivity and specificity but may have great potential for stratification, prognosis and disease

monitoring [92]. They may also be fit for accurate diagnosis, once a profiling process by other means has reduced the degree of freedom for the particular patient that is being looked at [93]. Concerning cell-based analysis, mainly CTCs have been used for tumor diagnosis, prognosis and treatment monitoring. However, many cell types within the microenvironment and even well beyond the histological tumor boundary change substantially at the molecular level [94]. They also could be utilized for diagnostic ends, once processes for their enrichment next to that of tumor cells would be in place, providing a much more complete and personalized picture of the individual tumor.

The exploration of the molecular basis of PDAC development and the characterization of precursor lesions are crucial steps toward biomarker discovery and evaluation. For blood-based analysis, emphasis should be on intercellular communication and how molecules may affect a recipient cell after uptake rather than focusing on intracellular processes and pathways. The relevant molecules are more likely to end up in the blood at a sufficient quantity for discriminatory detection. Given the high aggressiveness of PDAC, the identification of changes early during tumorigenesis – possibly even prior to cell transformation [8] – may be required to catch developing pancreas tumors in time for a successful intervention. Also, the influence of co-existing clinical conditions such as diabetes and obstructive jaundice on biomarkers performance should be taken into consideration more.

We split studies in two performance classes, apart from ignoring altogether studies that do not meet minimal requirements in accuracy or sample number. One needs to keep in mind, however, that some of the studies that surpassed our thresholds exhibited relatively high sensitivity and specificity values but were lacking validation in a second, independent sample set (as indicated in the tables). The performance reported for some other investigations actually fell short of the threshold during exactly this validation. Also, it is a common feature across the studies and analyzed molecule classes that the reported diagnostic accuracy of a test has a tendency to decrease the more samples are being analyzed. This indicates one discrepancy in the design of many studies and the real-life situation of clinical diagnosis. Another one is the fact that in a clinic setting there is the need to identify patients who actually have PDAC within a group of people who are under suspicion of having pancreatic cancer. Only few studies tried to mirror this in some form. Unfortunately, they mostly looked at a limited number of samples only. In addition, they differed in their definition of the non-cancer group. Therefore, no real conclusion could be drawn.

Discrimination from other diseases and tumor entities adds substantially to the complexity, since particularly other tumors may partly exhibit similar if not identical molecular changes. It is likely that for many major cancers a comparable amount of data on potential biomarkers of overall similar quality has been accumulated. Taking all this data in account instead of concentrating on one tumor entity may relatively quickly identify biomarkers that are either common to several cancers or unique to PDAC. This implies also changes in the basic data analysis process. In an interesting approach that goes beyond the classic pairwise comparison, a three-class study design was used for comparing samples from patients with PDAC or CP and from healthy donors. Thereby, a volume under the surface (the tree-dimensional equivalent of AUC) of 0.89 for discriminating the samples was achieved [75], suggesting that multi-class evaluation strategies could produce highly selective marker panels. The process is based on established mathematical algorithms that permit to study any number of patient (sub)groups this way.

To move on from the currently still relatively sectioned research toward an analysis that is oriented on the actual requirements in a clinical setting, the above points suggest a pipeline that consists of a kind of triple-meta analysis followed by large-scale experimental validation. First, the most powerful markers in each molecular category get selected. In combination, all marker types of a particular tumor entity, e.g. PDAC, get combined so as to define the overall most informative markers; they may eventually consist of just few marker types down to one molecular class, such as DNA methylation. Third, all this will be compared to similar data obtained from other tumor entities. The result of this overall process should then be subjected to validation in an international multi-center study on a really large number of different samples that are collected under routine clinical conditions. The involved processes should be standardized; best would be procedures that are already established in routine diagnostics, but technical improvements should nevertheless be taken into consideration, if they are likely to improve performance. Unfortunately, the scientific landscape is not shaped to make this approach easily possible as it would be located somewhere in between research and clinical application. In addition, this type of study is frequently considered to be rather trivial and not fancy enough to gather the kind of support needed for the scale that is prerequisite to success. Without such a process, however, a successful selection from the very many marker molecules found in research projects and their translation into a realistic diagnostic process that could be applied to patients is rather unlikely to be accomplished.

Because of the apparent molecular diversity of PDAC, it is probable that a marker panel will be required for sufficient accuracy and robustness, raising another obstacle. Any molecular signature will only be accepted in clinical routine diagnosis, if it clearly outperforms existing procedures, particularly CA19-9-based diagnosis, and would be available in a format that fits to the existing clinical processes and structures. For achieving clinical utility, it may be useful to proceed in a stepwise manner with respect to the accuracy that is asked for. For this, a clear definition of the diagnostic objectives is warranted. Starting with the easiest before advancing to the more difficult ones could facilitate acceptance. For PDAC, picking up tumor recurrence after surgical resection may be a less stringent application. It deals with a very defined patient group, so tumor specificity is not much of an issue. Also, most patients with recurrence will experience this within two years postoperatively. Therefore, even diagnosing recurrence with relatively low accuracy may be enough to warrant clinical intervention. A difficulty could be that not all markers may react immediately to the removal of the primary tumor or reappear upon recurrence. The highest diagnostic precision would be required in screening approaches. However, again there would be varying stringency requirements. Analyzing cancer-free family members of patients with suspected familial PDAC is probably less demanding than a screening of patients with CP. A basic, age-dependent random screening of individuals with no risk or strong suspicion of disease would be the most difficult objective. However, even this may turn out to be clinically relevant in view of the enormous expected burden by pancreatic cancer becoming the second most cancer-related cause of death in the near future.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ctrv.2021.102193.

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