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CLINICAL/NARRATIVE REVIEW

Systematic Review of Pancreatic Cyst Fluid Biomarkers: The Path Forward

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There is significant research interest in developing and validating novel pancreatic cyst-fluid biomarkers given the increasing recognition of the prevalence of pancreatic cysts and their associated malignant potential. Although current international consensus guidelines are helpful, they fail to diagnose with certainty the cyst type and the level of epithelial dysplasia. They also fall short in predicting the future likelihood of malignant transformation. A systematic review was performed with the objective of summarizing cyst-fluid-based biomarkers that have been published in the medical literature over the past 10 years and characterizing the current quality of evidence. Our review demonstrates that there is an increasing interest in this topic with several different and innovative approaches including DNA, RNA, proteomic, and metabolomics profiling. Further techniques to improve upon cytological yield have also been studied. Besides identifying potentially useful clinical biomarkers, these empiric approaches have provided further insight into their pathogenesis. The level of evidence for the vast majority of these studies, however, is limited to retrospective early validation studies. The path forward will be to select out the most promising biomarkers and develop multicenter consortiums capable of capturing adequate sample sizes with appropriate study designs.

INTRODUCTION

Pancreatic cystic lesions are more frequently being identified with routine cross-sectional imaging. Although the exact prevalence in the general population is unknown, the best estimate is ~2% for adults over the age of 40.[1,2] The incidence increases with age with ~10% of patients over 80 having an incidental pancreatic cyst. The most commonly observed cyst types include intraductal papillary mucinous neoplasms (IPMN), mucinous cystic neoplasms (MCN), serous cystic neoplasms (SCN), and pseudocysts (PC).[3] MCNs and IPMNs harbor malignant potential and therefore require an accurate diagnosis with appropriate management. SCNs and PCs have no malignant potential and are subsequently managed differently.

International consensus guidelines were first developed in 2006 and recently updated in 2012 to standardize clinical management.[4,5] These guidelines utilize the available evidence of clinical and imaging characteristics to recommend surgery or surveillance. In the 2006 criteria, surgery was recommended for all clinically suspected MCNs and main duct IPMNs. For cysts suspected of being a branch-duct IPMN, surgery was recommended if they had any of the following: (1) an intracystic mural nodule; (2) an associated dilated main duct; (3) a cyst size >3 cm; (4) associated clinical symptoms; or (5) positive cytology by endoscopic ultrasound (EUS) guided fine needle aspiration (FNA). Although this algorithm demonstrated 100% sensitivity in identifying cysts with

malignancy it had very-low specificity (23–31%) leading to many operations of questionable benefit.[6–8] The revised 2012 guidelines intended to increase the specificity without compromising sensitivity for malignancy. Criteria to proceed directly to surgery were narrowed to those with (1) obstructive jaundice, (2) an enhancing solid component associated with the cyst, and (3) a main duct dilated >10 mm. These differences are highlighted in Table 1. For those with worrisome features, but none of the above high-risk stigmata, EUS is recommended to triage patients for surgery or surveillance.

Although these guidelines are helpful, they exist because there is currently no accurate and reliable method to obtain a definitive diagnosis. The preoperative diagnosis for cyst type is incorrect in 20–30% of cases.[9,10] Imaging modalities including computed tomography and magnetic resonance imaging are also suboptimal with poor interobserver variability.[11,12] Among the imaging modalities, EUS is considered the most sensitive in delineating malignant characteristics such as mural nodules.[13–21] With EUS, FNA of the cyst can be performed for cytology. Because of the low amount of epithelial cells in the cyst fluid however, cytology has had a limited diagnostic yield with a pooled sensitivity of 63% and specificity of 88%.[22] Beyond cytology, the tumor markers such as carcinoembryonic antigen (CEA) have been studied in the cyst fluid and found to differentiate mucinous from non-mucinous cysts with a diagnostic accuracy of 80%.[23]

The accuracy of CEA to define mucinous cysts is not high enough to be widely accepted as a routine diagnostic test.

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Table 1 Changes in imaging and clinical criteria for recommending surgery between the original consensus guidelines (a.k.a. Sendai criteria, 2006) and revised consensus guidelines (a.k.a. Fukuoka criteria, 2012)

Sendai criteria (2006)	Fukuoka criteria (2012)
Size > 3 cm	Size cutoff abandoned
Presence of mural nodule	Presence of enhanced mural nodule
Dilated main duct > 6 mm	Dilated main duct > 10 mm
Symptomatic	Obstructive jaundice
Positive cytology	Unchanged

Specifically, a low value does not rule out a mucinous cyst and often surveillance is still required.[23–25] The most recent consensus guideline recommends CEA be used in limited settings "in whom additional information will have an impact on the surgical decision making."[14] Given this limit in diagnostic accuracy with current imaging modalities, there remains a growing research interest in discovering and validating novel cyst-fluid biomarkers that may improve upon the performance of CEA. The types of biomarkers available for analysis have been expanding rapidly due to the development of innovative technologies that enable profiling of various bioparticles in the cyst fluid.

In this review, we attempt to comprehensively describe and categorize the published pancreatic cyst-fluid biomarkers. In doing so, we describe the current level of evidence for each biomarker in the context of a proposed framework for further validation to bring it into clinical practice.[26]

METHODS

This systematic review was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. Eligible articles were identified by search of the MEDLINE database for the past 10 years (1 February 2005 to 1 February 2015). The following keywords were used: "pancrea*" AND "cyst*" AND "biomarker", "pancrea*" AND "cyst*" AND "diagnosis", "intraductal papillary mucinous neoplasm" AND ("RNA", OR "DNA", OR "protein" OR "proteomics" OR "cytokine" OR "metabolomics"), "mucinous cystic neoplasm" AND ("RNA", OR "DNA", OR "protein" OR "proteomics" OR "cytokine" OR "metabolomics"), "serous cystadenoma" AND ("RNA", OR "DNA", OR "protein" OR "proteomics" OR "cytokine" OR "metabolomics") and "pseudocyst" AND ("RNA", OR "DNA", OR "protein" OR "proteomics" OR "cytokine" OR "metabolomics").

Reported biomarkers based on immunohistochemistry or those not obtained directly from the pancreatic cyst by FNA were excluded (this includes studies of duodenal fluid). Studies that used cyst fluid obtained at surgery were included as there does not appear to be a difference with cyst fluid obtained during EUS.[27] Studies only in English using human samples were included. Only studies that used histology as the gold standard were included. Review articles were excluded. As CEA is already used in current practice, further validation studies of CEA were excluded. The references of retrieved relevant reviews and articles were reviewed to identify other potential eligible studies. Both authors reviewed eligible studies from the search results. Data abstraction

included the following variables: publication year, study design, sample size, distribution of cyst types, presence of histologic correlation, primary comparison (e.g., mucinous vs. non-mucinous, cancer vs. non-cancer, and serous cystadenoma vs. non-serous cystadenoma), area under the receiver operator curve (ROC), sensitivity, specificity, and diagnostic accuracy.

RESULTS

A total of 2,840 articles were screened. Of these, 203 articles were further assessed for eligibility, and 41 studies were included in this review. Reasons for exclusion include: CEA validation studies ($n = 21$), Immunohistochemistry only studies ($n = 45$), pancreatic juice studies ($n = 13$), review articles ($n = 12$), and not meeting other inclusion/exclusion criteria ($n = 71$).

DNA-based biomarkers. One of the first DNA-based biomarker studies in cyst-fluid was of KRAS mutations, which are present in over 90% of pancreatic adenocarcinomas and represent the most frequent and earliest genetic alterations toward cancer.[28] Khalid *et al.*[29] first described a potential difference in cyst-fluid expression using 36 samples that included 11 malignant cysts and 15 premalignant cysts. Besides studying KRAS mutations, DNA quantity, quality, number and sequence of mutations were also studied. Several subsequent studies followed using these set of DNA parameters (Table 1).[30–37] In sum, these studies showed the presence of a KRAS mutation to be highly specific (80–100%) but limited in sensitivity (33–86%) for diagnosing mucinous cysts (IPMN or MCN). More importantly, the majority of these studies did not find KRAS to be useful for differentiating malignant from premalignant mucinous cysts. However, in combination with other DNA parameters such as allelic loss, a few studies demonstrated decent sensitivity and specificity for diagnosing malignant cysts. Its routine use, consequently, remains controversial and is not widely accepted.

In search of other relevant mutations that might provide insight into the pathogenesis of IPMNs, Wu *et al.*[38] performed an exploratory DNA analysis of cyst fluid from 19 patients with IPMNs. They identified recurrent mutations in codon 201 of GNAS (guanine nucleotide-binding protein G subunit alpha isoforms short) that is highly specific for IPMNs. Using a larger cohort of 147 cyst-fluid samples, they observed that 61% of IPMNs had GNAS mutations compared to 0% in SCNs and MCNs. Subsequent studies have since been performed on cyst fluid that validate these earlier findings (Table 2).[39,40] Although these studies were relatively smaller in sample size, they reproduced the observation that GNAS mutations were found only in IPMNs, (but not all IPMNs) and that the presence of either a GNAS or KRAS mutation was highly sensitive for IPMNs. The increasing use of next-generation sequencing technology may further elucidate other clinically useful DNA mutations to aid in the diagnosis and management of premalignant and malignant IPMNs and MCNs.[41] Specifically, DNA mutations that are specific for high-risk cystic lesions (i.e., high-grade dysplasia and/or cancer) are yet to be identified.

Table 2 Summary of DNA-based cyst-fluid biomarkers

Year	First author	Biomarker	Sample size	Sample distribution	Phase	Observations
2005	Khalid et al.[29]	KRAS mutation DNA quantity, quality, no. of mutations, and sequence of mutations	36	15 Premalignant 11 Malignant	2	Difference observed with DNA quantity, quality, no. of mutations, and presence of KRAS mutation plus by LOH
2006	Schoedel et al.[30]	KRAS mutation, LOH	16	IPMN: 4 HGD/Cancer vs. 12 (LGD/MGD)	2	KRAS mutation plus LOH present 50% of HGD/cancers vs. 8% in (LGD/MGD) premalignant IPMNs
2009	Khalid et al.[31]	KRAS mutation, DNA quantity, allelic loss, mutational amplitude, and sequence of mutations	113	40 Malignant 48 Premalignant 25 Benign	2	KRAS mutation had good specificity (96%) but limited sensitivity (45%) for mucinous cysts. KRAS mutations followed by allelic loss had limited sensitivity (37%) but good specificity (96%) for diagnosis of malignancy. Mean allelic loss amplitude (MALA) had the best diagnostic performance for distinguishing malignancy cysts.
2009	Sawhney et al.[32]	KRAS mutation, DNA quantity, allelic loss	19	17 Mucinous 2 Non-mucinous	2	DNA quantity: sensitivity 76% and specificity 100% for diagnosing mucinous cyst.
2009	Shen et al.[33]	DNA Pathfinder TG	35	6 Malignant 15 Pre-malignant, 14 Benign	2	Malignancy: sensitivity 83%, specificity 100% mucinous: sensitivity 86% specificity 93%
2009	Sreenarasim-haiah et al.[34]	DNA Pathfinder TG	20	2 Malignant 7 Pre-malignant 11 Benign	2	Mucinous: sensitivity 33%, specificity 92%
2011	Wu et al.[38]	GNAS and KRAS mutations	147	84 IPMN 21 MCN 42 SCA	2	GNAS mutations: 61% of IPMNs, 0% SCAs, 0% MCNs, KRAS mutations: 82% of IPMNs, 0% in SCAs and 33% MCNs
2012	Talar-Wojnarowski et al.[35]	KRAS mutation	56	17 Mucinous 39 Benign non-mucinous	2	Mucinous: sensitivity 65%, specificity 97%
2013	Siddiqui et al.[39]	GNAS and KRAS mutations	25	9 IPMN 16 non-IPMN	2	KRAS mutation (+) 60–70% Mucinous Cysts GNAS mutation (+) 44% IPMNs, 0% other mucinous cysts. All IPMNs had either KRAS or GNAS.
2013	Al-Haddad et al.[37]	KRAS mutation	48	38 Mucinous 10 Non-mucinous	2	Mucinous: sensitivity 50%, specificity 80%
2013	Nikifovora et al.[36]	KRAS mutation	142	12 Malignant 85 Pre-malignant 45 Benign non-mucinous	2	Mucinous: sensitivity 54%, specificity 100%
2014	Amato et al.[41]	Next-generation sequencing	10	10 IPMNs	1	7 Out of 10 cyst-fluid samples provided adequate DNA for deep sequencing
2014	Singhi et al.[40]	GNAS and KRAS mutations	91	50 IPMNs, 16 MCNs, 25 Non-mucinous	2	Mucinous: GNAS or KRAS mutation sensitivity 65%, specificity 100% IPMNs: GNAS or KRAS mutation sensitivity 84%, specificity 98%.

EUS, endoscopic ultrasound; FNA, fine needle aspiration; IPMN, intraductal papillary mucinous neoplasms; GNAS, guanine nucleotide-binding protein G subunit alpha isoforms short; LOH, loss of heterozygosity; MALA, mean allelic loss amplitudeic neoplasms; PC, pseudocysts; SCN, serous cystic neoplasms.

Table 3 Summary of RNA-based cyst-fluid biomarkers for cyst-fluid analysis

Year	First author	Biomarker	Sample size	Sample distribution	Phase	Observations
2011	Ryu et al.[44]	Micro-RNA 21	40	24 Mucinous 16 Non-mucinous	1	Micro-RNA 21 had highest ROC for mucinous cysts: sensitivity 76%, specificity 80%
2011	Carrara et al.[45]	Mucin RNA expression	19	IPMNs	1	No unique significant pattern of RNA expression of mucin proteins identified among IPMNs.
2012	Matthaei et al.[42]	Micro-RNA profile	65	30 IPMNs 35 Non-mucinous	1	37 candidate micro-RNA candidates could distinguish high-grade IPMNs. A panel of 9 micro-RNA candidates could differentiate surgically recommended lesions with sensitivity 89%, specificity 100%
2013	Farrell et al.[43]	6 micro-RNA panel	38	4 Malignant 18 Pre-malignant 16 Benign non-mucinous	1/2	Mi-R21 had increasing levels from benign to premalignant to malignant
2014	Wang et al.[46]	Micro-RNA profile	17	3 Malignant 8 High Risk 6 Low Risk	1	Micro-RNA- 216 and 217 were differentially expressed between low grade, high grade, and cancer.

IPMN, intraductal papillary mucinous neoplasms; ROC, region of interest.

RNA-based biomarkers. There were five studies investigating micro-RNA (miRNA) biomarkers from cyst fluid (Table 3).[42–46] Compared with DNA-based studies, this research studies have all been recently published in the last 4 years. While, the majority of these studies were primarily exploratory in nature without an *a priori* hypothesis, the fundamental basis for investigating miRNA stems from earlier observations that they can be involved in tumor initiation and progression.[47] Two of the studies identified miRNA 21 to have clinical utility. Ryu *et al.*[44] showed that miRNA 21 could differentiate mucinous cysts from non-mucinous cysts. Farrell *et al.*[43] also observed that miRNA 21 could differentiate premalignant mucinous cysts from malignant cysts. Other studies identified a panel of miRNAs, and miRNA 216 and 217 as potentially useful for diagnosing surgically recommended cysts.[42,46] These studies suggest that miRNA bear significant potential to become useful clinical tests with further validation.

Exploratory proteomic approaches. Like differential expressions observed in DNA and RNA profiles observed between cancer and normal tissue, differential protein expression may be logical focus for diagnostic test development. Several exploratory studies looking for differential protein expression in pancreatic cyst fluid have been performed (Table 4).[48–55] Like the RNA-based cyst studies, these did not have an *a priori* target and used technical variations of mass spectrometry. The sample sizes were generally small ranging from 8 to 59 samples, and the majority of them reported select protein groups that were differentially expressed in clinically relevant cyst types. For example, Allen *et al.*[49] used a commercially available luminex assay and found a cluster of 14 proteins that differentiated SCNs from IPMNs with 92% accuracy. Although these empirical studies provide indirect insight into the pathogenesis of the various relevant cyst types and may identify candidate targets for further study, translation of these immediate findings into an easily usable diagnostic or predictive test remains a challenge.

Targeted protein-based biomarkers. Twelve studies were identified that reported on a specific protein or group of proteins in an exploratory or validation study for being potentially useful as a cyst-fluid biomarker (Table 5).[56–67] Not surprisingly, a commonly studied protein group in cyst-fluid biomarkers is the secreted mucin proteins. Glycan variants on certain mucin proteins such as MUC5AC, have been described to be particularly sensitive (87–89%) and specific (80–100%) for diagnosing mucinous cysts.[57,66] Maker *et al.*[65] found certain mucin proteins (MUC2 and MUC4) could differentiate dysplasia status in IPMNs. Morris-Stiff *et al.*[64] studied general mucin expression by direct staining of the cyst fluid and observed a sensitivity of 80% and specificity of 40% for diagnosing mucinous cysts. Cao *et al.*[66] also studied glycan alterations on mucin proteins and found that a three-marker panel could differentiate mucinous from non-mucinous cysts with high diagnostic accuracy. Of the different subtypes of mucin proteins, aberrant MUC1 has been strongly associated with pancreatic cancer. Jabbar *et al.*[59] targeted MUC1 and observed it to diagnose malignant

Table 4 Summary of proteomic approaches for cyst-fluid analysis

Year	First author	Biomarker	Sample size	Sample distribution	Phase	Observations
2007	Scarlett et al.[48]	SELDI-TOF MS	10	3 Cancer 7 Non-cancer	1	12 Non-specified protein peaks were differentially expressed between the two groups
2009	Allen et al.[49]	Luminex Assay	59	32 BD-IPMN 12 MCN 15 SCN	1	A cluster of 14 proteins differentiated SCA from IPMN in 92% of patients
2009	Ke et al.[50]	MALDI-TOF MS 2D gel electrophoresis GeLC/MS/MS	20	5 With histology	1	Found potential candidate biomarkers in homologs of amylase, mucin, CEACAM, and S100
2011	Cuoghi et al.[51]	SDS-PAGE LC-MS/MS	8	2 MCN 2 SCN 2 PNET 1 IPMN 1 PC	1	Found unique protein expressions among different cyst types
2012	Corcos et al.[52]	SELDI-TOF MS	43	21 LGD IPMN 22 HGD IPMNs	1	5 Non-specified protein profiles had ROC of 0.88
2012	Lee et al.[53]	89—Cytokine Panel	10	5 BD-IPMNs 5 Pseudocysts	1	GM-CSF and HGF were differentially present in inflammatory cysts
2012	Mann et al.[54]	MALDI-TOF MS LC/MS	20	13 Mucinous 7 Non-mucinous	1	Overexpressed glycoproteins differentially noted in mucinous cysts.
2014	Gbormittah et al.[55]	HP-MLAC SDS-PAGE LC/MS	20	10 Mucinous 10 Non-mucinous	1	Panel of proteins of interest were differentially expressed

BD, branch duct; CEACAM, carcinoembryonic antigen-related cell adhesion molecule; GM-CSF, granulocyte macrophage colony stimulating factor; HGD, high grade dysplasia; HGF, hepatocyte growth factor; IPMN, intraductal papillary mucinous neoplasms; LC-MS/MS, liquid chromatography tandem mass spectrometry; LGD, low grade dysplasia; MALDI-TOF MS, matrix assisted lasor desorption/ionization time of flight mass spectrometry; MCN, mucinous cystic neoplasms; PC, pseudocysts; ROC, region of interest; SCA, serous cystadenoma; SCN, serous cystic neoplasms; SDS-PAGE, sodium dodecyl sulfate - polyacrylamide gel electrophoresis; SELDI-TOF MS, surface enhanced lasor desorption/ionization time of flight mass spectrometry; PNET, pancreatic neuroendocrine tumor.

cysts with a sensitivity of 87.5% and specificity of 92.3% in a sample of 29 cysts.

Among the other studies, there are a few that appear to be promising and worth further validation primarily because of their potential to directly impact surgical decision-making. Maker et al.[61] looked at cytokine expression differences and observed that IL-1B, an important pro-inflammatory mediator, was significantly higher in IPMNs with high-grade dysplasia and malignancy than low grade IPMNs (ROC 0.92). Amphiregulin, a secreted epidermal growth factor receptor ligand that is overexpressed in pancreatic cancer, has also been shown to differentiate high-grade dysplastic and cancerous cysts with an ROC of 0.76.[58,68] Raty et al.[67] observed that cyst-fluid SPINK1 levels in pancreatic cysts could differentiate surgically recommended lesions (MCN, and main/mixed IPMNs) with an ROC of 0.94. More recently, Das et al.[62] reported on the clinical utility of a monoclonal antibody reactive against a colonic phenotype of epithelium in pancreatic cysts. Monoclonal antibody Das-1 could differentiate high-risk IPMNs that might require surgery from low-risk IPMNs. This particular analysis was unique in that definition of high-risk incorporated IPMN histological subtype. The majority of these biomarkers focuses on identifying which lesions are high risk and necessitate immediate surgery. In contrast, Yip-Schneider et al.[63] showed that cyst-fluid VEGF could diagnose SCNs with an ROC > 0.99. Such a biomarker would remain valuable as these cysts do not need require surgery or need to be followed.

Metabolomics. If DNA profiling explains "what can happen", RNA profiling explains "what appears to be happening", and protein profiling explains "what makes it happen", then metabolite profiling explains "what has happened and is happening".[69] To date, there is one study that profiles metabolites in the cyst fluid (Table 6). Park et al. used a semi-targeted method and identified four metabolites that were differentially expressed in mucinous cysts compared to non-mucinous cysts. With mass spectrometry, two were identified as glucose and kynurenine. The latter is a tryptophan metabolite that has been associated with cancer development.[70] With a sample size of 45 cysts, both glucose (ROC 0.92) and kynurenine (0.94) demonstrated high diagnostic accuracies for mucinous cysts.[71]

Cytology revisited. One of the major impetuses for identifying novel cyst-fluid biomarkers stems from the poor diagnostic accuracy of cyst-fluid cytology. For the diagnosis of high-grade dysplasia or cancer, the specificity is high, while the sensitivity is low making it of limited value for surgical decision-making.[72] This has been attributed to the scant quantity and quality of aspirated cells from the cyst cavity. To make cytological-based analysis more clinically useful, Pitman et al. devised a modified cytological system that included a category of atypical epithelial cells (AEC). AECs were defined by the presence of a single or cluster of cells with a relatively increased nuclear to cytoplasmic ratio and enlarged irregular nuclei. With this new classification system, they showed that in a sample of 18 patients, 83% (5 out of 6) of those with high-grade dysplasia or higher had the presence of AECS compared to 33% (4 out of 12) that did not.[73] Using a larger sample cohort of 112 patients with mucinous cysts (39 of which were malignant), AEC sensitivity was 72% and specificity was 85% for diagnosing malignant cysts (Table 6).[74] While this is significantly improved from the standard cytological classification system, its generalizability

Table 5 Summary of protein-based biomarkers for cyst-fluid analysis

Year	First author	Biomarker	Sample size	Sample distribution	Phase	Observations
2008	Schmidt et al.[56]	Prostaglandin E2	58	29 IPMN 11 MCN 12 PDAC 6 Non-Mucinous	2	Prostaglandin E2 was elevated in IPMNs compared with MCNs
2010	Haab et al.[57]	Glycosylation Variants	53	32 Mucinous 21 Non-mucinous	2	Glycan variant MUC5AC sensitivity 78% specificity 80% for mucinous cysts
2010	Morris-Stiff et al.[64]	Mucin	128	86 Mucinous 42 Non-mucinous	2	Mucinous cysts: sensitivity 80% specificity 40%, when combined with CEA, sensitivity 83% specificity 65%
2011	Maker et al.[65]	MUC1, MUC2, MUC4, MUC5AC	40	21 LGD IPMN 19 HGD/Cancer IPMN	2	MUC 2 & 4 elevated in HGD IPMN compared with LGD IPMN. Intestinal type had increased expression of MUC2
2011	Doyle et al.[60]	TGF-alpha	46	26 IPMN 9 MCN 6 SCN 5 PC	1	Only IPMN cysts had any levels above 95 pg/ml
2011	Maker et al.[61]	Cytokine Panel Assay	40	21 LGD IPMN 19 HGD/Cancer IPMN	1	IL—1beta had high diagnostic accuracy (ROC 0.92) for HGD/Cancer IPMNs compared with LGD IPMNs
2012	Tun et al.[58]	Amphiregulin	33	12 Malignant 15 Mucinous 6 Benign non-mucinous	1	Level > 300 pg/ml sensitivity 83%, specificity 73% for malignancy (ROC 0.76)
2013	Cao et al.[66]	MUC5AC-WGA, MUC5AC-BGH, Endorepellin-WGA	44	27 Mucinous 17 Non-mucinous	1	Mucinous: sensitivity 89% specificity 100%
2013	Raty et al.[67]	SPINK1	61	33 Mucinous 28 Non-mucinous	2	Surgically recommended lesions (MCN and main duct IPMN) vs. SCA or branch-duct IPMN = ROC 0.94
2014	Jabbar et al.[59]	MUC1	29	16 Malignant 13 Non-malignant	2	Malignancy: sensitivity 87.5% and specificity 92.3%
2014	Das et al.[62]	mAb Das-1	38	27 IPMN 11 Non-mucinous	1	High-Risk IPMNs: Sensitivity 89% specificity 100% (High risk = IPMN-gastric with HGD, any IPMN-intestinal, and IPMN-pancreatico-biliary, any IPMN-oncocytic, and invasive IPMN.)
2014	Yip-Schneider et al.[63]	VEGF	87	17 SCN 70 Non-SCN	2	VEGF-A had an ROC of > 0.99 for SCNs. With a cutoff set at 8,500 pg/ml, VEGF-A provides 100% sensitivity and 97% specificity as a biomarker for benign SCN lesions. VEGF-C had a similar diagnostic performance

CEA, carcinoembryonic antigen; IPMN, intraductal papillary mucinous neoplasms; MCN, mucinous cystic neoplasms; PDAC, pancreatic ductal adenocarcinoma; ROC, region of interest; SCN, serous cystic neoplasms; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

Table 6 Summary of metabolomic and modified cytology techniques for cyst-fluid analysis

Year	First author	Biomarker	Sample size	Sample distribution	Phase	Observations
2008	Pitman et al.[73]	AECs	18	6 Malignant 12 Non-malignant	2	5/6 (83%) malignant cysts+AEC vs. 4/12 (33%) non-malignant cysts+AEC
2010	Pitman et al.[74]	AECs	112	39 Malignant 73 Pre-malignant	2	Malignancy: AEC sensitivity 72%, specificity 85%
2013	Park et al.[71]	Metabolomics: glucose and kynurenine	45	31 Mucinous 14 Non-mucinous	1	Mucinous cysts: glucose ROC 0.92 and kynurenine ROC 0.94)

AEC, atypical epithilial cell; ROC, region of interest.

remains a significant barrier as substantial experience is required to achieve fair agreement among pathologists.[75]

DISCUSSION

Our systemic review identified a substantial number of promising and novel cyst-fluid based biomarkers. From 2005 to 2015, the number of publications by time period demonstrates a growth in interest. From 2005 to 2009, there were 11 studies within our inclusion and exclusion criteria. In the latter half, there were 30 studies. This does not include numerous studies that were based on immunohistochemistry expression. The vast majority of the studies included in this review were single center retrospective studies and required histology as the gold standard for comparison. This latter part includes a selection bias that may limit the generalizability of
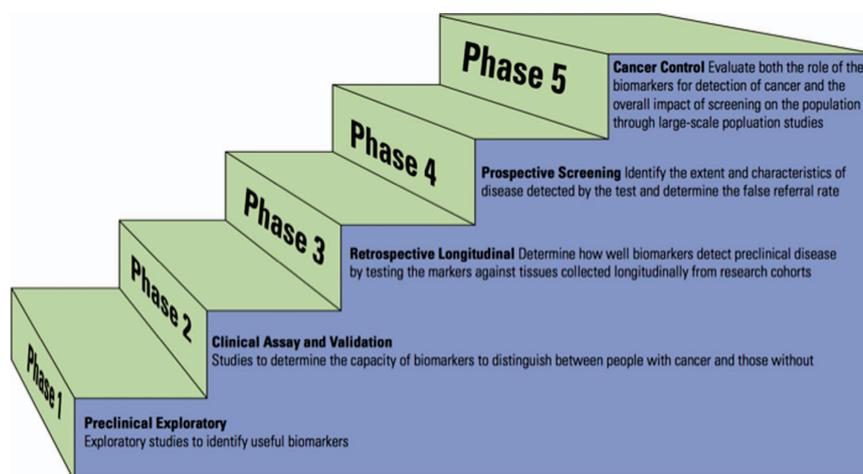
Figure 1 Graphic of the five proposed phases for diagnostic test discovery and validation as defined by Pepe *et al.*[26]

these studies. Specifically, these sample cohorts select for a higher proportion of high-risk cystic lesions, which will likely impact the diagnostic performance characteristics in routine clinical settings when the majority of lesions tested will be low-risk cysts.

A framework for advancing biomarkers from the laboratory to the bedside has been proposed.[26] This proposed pathway for the discovery and validation of cancer biomarkers has five phases: preclinical exploratory (phase 1), clinical assay and validation (phase 2), retrospective longitudinal (phase 3), prospective screening (phase 4), and cancer control (phase 5). (Figure 1) Preclinical exploratory studies typically compare tumor tissue from nontumor tissue to look for difference in tumor markers or gene expression levels. A clinical assay uses non-invasive methods to obtain specimens from cases as well as control subjects or subjects with benign growths to estimate the sensitivity and specificity to evaluate if biomarkers can be used for screening purposes. The third phase is a retrospective longitudinal study with aims to understand as a function of time before clinical diagnosis, the capacity of the biomarker to detect preclinical disease and identify the criteria for a positive screening test. The fourth phase is a prospective study where the diagnostic test is applied to individuals, with a standardized protocol for definitive treatment (i.e., surgery) to those testing positive. The final phase is a population-based cancer control study, which investigates whether testing reduces the burden of cancer on the population. Not all phases are required to bring biomarkers to clinical use but at least successful performance in a phase 3 study is required for validation.

In the context of this framework, the studies described in this review are in phases 1 or 2 (listed in Tables 2–6). The next steps for new cyst-fluid biomarkers to enter clinical practice will be to complete phase 2 validation studies for those that have only undergone phase 1 and begin planning a phase 3 studies of the most promising biomarkers. Phase 3 studies will be based on repositories from centers that have already been banking pancreatic cyst fluid. These samples will be collected only by EUS that in the future (but not immediately) will have undergone surgery. The goal will be to assess the biomarker's

capacity to predict disease and will be assessed retrospectively. Sample sizes with adequate power will need to be collated and will require a multicenter collaboration. With proper design and application, retrospective longitudinal studies can allow for rapid clinical uptake of novel biomarkers. Successful phase 3 studies should be enough to enable implementation into clinical practice. These studies should include minimally acceptable true- and false- positive rates for testing defined by community consensus. Based on what is known of the indolent nature for most of these premalignant cysts, phase 4 prospective studies, while important, will require a very large sample with longitudinal follow-up that may not be practical or feasible to define as the threshold prior to introduction into clinical practice.

In conclusion, there are several promising cyst-fluid biomarkers in the literature that warrant a concerted coordinated multicenter effort. Among those discussed, the most promising include KRAS (V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog), GNAS, miRNA 21, and glucose to differentiate non-mucinous from mucinous cyst types. MUC1, amphiregulin, IL-1B, SPINK1, mAb Das-1, and miRNA 21 should be further studied to differentiate low-risk from high-risk cyst types (those harboring high-grade dysplasia, or invasive cancer). VEGF should be further validated for its ability to diagnose serous cystic neoplasms. Phase 3 retrospective longitudinal study designs represent the next rational step forward. As this will require significant pooling of resources, careful consideration in the planning stages should be made to allow the most number of potential biomarkers to be studied, both alone and in combination with each other. This will also ensure that we reach our shared goal of bringing new diagnostic tests and improving the management of patients with pancreatic cysts.

CONFLICT OF INTEREST

Potential competing interests: None.

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