CLINICAL RELATIONSHIP: Can our functional analytes accurately classify grade of dysplasia in pancreatic cysts? The reference samples will be used as an external validation of our microvolumetric enzymatic biomarkers.

BACKGROUND AND SIGNIFICANCE: Pancreatic cystic lesions (PCLs) are incidentally detected in more than a million patients annually in the U.S. and represent an opportunity for early detection of pancreatic adenocarcinoma. The presence of a clinically relevant PCL (>1 cm) is associated with a 10-fold increased likelihood of adenocarcinoma within 5 years.[1] Most of the PCLs with the potential to develop high grade dysplasia/invasive adenocarcinoma ("advanced neoplasia", AN) are lined by epithelial cells that produce an abundance of mucin, and thus are broadly classified as mucinous, and about 75% of mucinous cystic lesions are Intraductal Papillary Mucinous Neoplasms (IPMNs).[1,2] **(Table 1)** The accurate identification of mucinous PCLs with AN that typically warrant surgical intervention represents a critical unmet clinical need.

Diagnostic Category	Cyst Type	Risk of Invasive Carcinoma
Non-Mucinous Cyst	Serous Cystadenoma (SCN)	Minimal
	Pseudocyst (PC)	None
Mucinous	Main Duct (MD) IPMN	40–50%[7]
	Branch Duct (BD) IPMN	10–15%[7]
	Mucinous Cystic Neoplasm (MCN)	15%[8]

Table 1. Classification of cystic lesions of the pancreas and their associated risk of invasive carcinoma

Limitations of Current Clinical Guidelines and the Burden of Overtreatment. Most patients have clinically "indeterminate" cysts in which both the type (mucinous vs. non-mucinous) and histologic grade are radiographically uncertain. There are two major challenges in the management of pancreatic cysts. The first is to identify non-mucinous cysts with no malignant potential (Tier-1), thereby sparing ~25% of patients that undergo surgical resection unnecessarily, and the larger number that are subjected to burdensome prolonged surveillance.[3] The second major challenge is to identify the mucinous cysts with AN for early-stage surgical resection (Tier-2) (**Figure 1**). Patients with indeterminate PCLs are typically managed by surgeons and gastroenterologists according to one of several clinical guidelines that include clinical and imaging features. When we applied the Fukuoka guidelines retrospectively to 251 surgically resected branch-duct IPMNs, the positive predictive value was only 30%.[4] Requiring two or more worrisome features increased guideline specificity, but this came at the cost of reduced sensitivity, which may be deemed unacceptable for such a lethal cancer. Refinements in radiographic imaging and next generation sequencing (NGS) show promise, but have limited results, especially in indeterminate cysts with high grade dysplasia.[5,6,7] We and others have reported that 40-60% of incidentally-detected mucinous cysts removed surgically harbored either no dysplasia or, at most, low grade dysplasia, and most of these patients had no benefit from surgery.[8,9] Pancreatic resections are risky, complex procedures with significant mortality and long-term morbidity, including 15-25% new-onset diabetes and 25-50% exocrine pancreatic insufficiency.[10, 8] Using Markov modeling, we found that surgical decision-making using the current Fukuoka Guidelines has the potential to lead to more deaths than it prevents.[11] *Accurate classification of PCLs is a critical unmet need.*

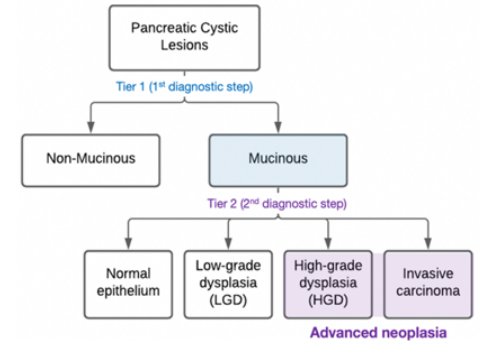

Figure 1. *Two-tier diagnostic classifier for PCLs.*

Current Biomarkers are Inadequate for Pancreatic Cyst Diagnosis and Classification. Clinical management guidelines recommend that patients with PCLs identified on cross-sectional imaging that are >2-3 cm and lack high risk stigmata (the majority) be examined using endoscopic ultrasound with fine-needle aspiration (EUS-FNA) of cyst fluid that is typically sent for carcinoembryonic antigen (CEA) measurement, which is the only routinely used test to identify mucinous cysts.[12] However, this assay requires 500 μL of fluid, which is not always available. *A micro-volumetric assay would improve the diagnostic yield of cyst fluid testing from ~50% to >90% for patients who undergo an invasive EUS-FNA.*[13] CEA at the clinically used cutoff of 192 mg/dL has a specificity at 73-84%, but only modest sensitivity (38-73%).[14,15] *Improvement in the sensitivity of this Tier-1 assay would allow us to identify the ~25% of patients with non-mucinous cysts that may require no further testing, surveillance, or surgery.* Several reports indicate that glucose could improve sensitivity, however

we and others have described limitations in assay performance and reproducibility that may limit clinical usefulness or require high cyst fluid volumes to maintain accuracy.[16,17,18]
For mucinous cysts, clinicians aim to identify and resect cysts with high grade dysplasia (HGD) since 5-year survival is greater than 90% compared to 35-60% for resected cyst-associated invasive cancer.[19] Current diagnostics including radiography, serum biomarkers, and cyst fluid CEA show poor correlation with HGD.[2,20,21] Older NGS-based tests (CompCyst, PancraGen) showed promise but their usefulness has been limited by low specificity of RAS mutations for detection of AN, unproven sensitivity of somatic mutations for detection of HGD, fluid volume/cellularity needs, and cost.[22,23,24] Newer iterations of the NGS-based test (e.g., PancreaSeq) include panels with potentially improved performance for detection of invasive adenocarcinoma, albeit with risks of over-fitting, and with uncertain performance for HGD. Validation and longer follow up for prospective sampling are both needed to determine performance of the latest panel for the target population of indeterminate PCLs at the time of EUS. Importantly, the published NGS panel requires 200-600μL of cyst fluid, which limits clinical usefulness.[7] Cytologic evaluation of aspirated fluid remains the most widely used test for detection of high grade/invasive lesions with a specificity at >95%. However, cytology has poor sensitivity (29-48%).[25,26,27] This low sensitivity for detection of early cancers leaves many clinicians and patients uncomfortable with a "watch and wait" approach and is a major driver of surgical overtreatment and harm.[4,28] *Given the high specificity of cyst fluid cytology, the greatest need is for a low-volume Tier-2 assay that would improve upon the current low sensitivity for detection of radiographically occult AN.*

Global substrate profiling Mass Spectrometry-Based Assay for Identifying Candidate Biomarker Proteases in Cyst Fluid and Principal Component Analysis (PCA) of Proteomics Datasets. Proteases mediate a variety of biological processes critical to the development of various cancers.[29,30] We have shown that *protease activity-based biomarkers have several key advantages*, including representation of current physiological processes from diverse cells in the microenvironment and signal amplification due to *in situ* accumulation of cleavage products.[31] We use a 2-step process for assay development. First, mass spectrometry and shotgun proteomics are used for substrate discovery, then a simple micro-volumetric assay that requires only **5 μl of sample** is prepared for clinical use. To study cyst fluid proteolytic activity in a global and unbiased manner, we used our novel Multiplex Substrate Profiling by Mass Spectrometry (MSP-MS) method.[31] This technology involves a direct cleavage assay with mass spectrometry (MS)-based peptide sequencing for detection of cleaved products in a mixture of synthetic peptides that cover a wide amino acid sequence space (**Figure 2**).

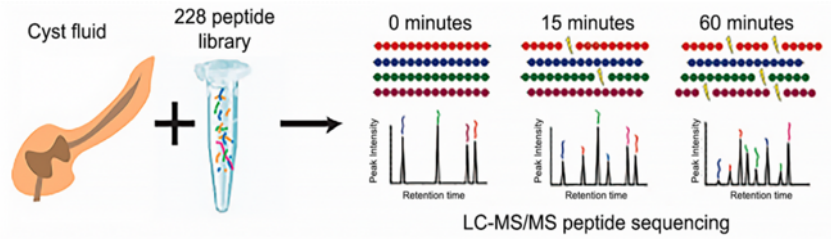

Figure 2 MSP-MS cyst fluid analysis. Cyst fluid is combined with a set of 228 peptides. Aliquots of each reaction are removed at designated time intervals and analyzed using LC-MS/MS peptide sequencing to identify cleavage sites.

PCA is a statistical technique that allows for increased interpretability of large multidimensional datasets. By using PCA tools and plotting protein abundance variation, we found that proteomic profiles differentiated pathologically-confirmed IPMNs from MCNs into independent clusters (**Figure 3**). These PCLs are notoriously difficult to discriminate from one another by current radiographical or cytological approaches despite their clinically distinct management strategies. Our results suggest the presence of biomarkers of specific mucinous PCL subtypes that could be pursued to improve clinical decision-making.

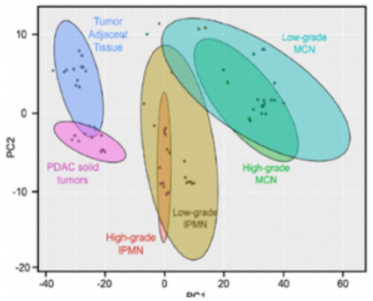

Figure 3. Principal component analysis of proteomic datasets from different types of pancreatic lesions. PCA tools were used to categorize pancreatic lesions in individual clusters based on their proteomic profiles. Each oval represents a 95% confidence interval that characterizes a cluster, which each colored dot represents one proteomic dataset used in the PCA analysis. Patient n = 23, Proteomic datasets n = 69 (three technical replicates for each patient).

To develop our Tier-1 assay, we used the **Prospective specimen collection, Retrospective Blinded Evaluation (PRoBE) study design** and applied an

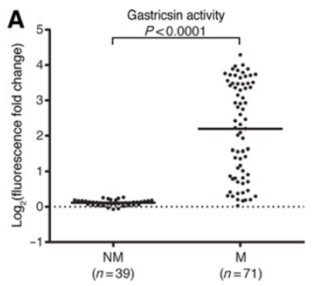

Figure 4. Performance of gastricsin for identification of mucinous cysts

unbiased screening of pancreatic cyst fluid to identify specific proteolytic signatures for cyst classification.[31] We analyzed 35 pancreatic cyst fluid samples during the discovery profiling screen: 7 non-mucinous (SCAs and pseudocysts) and 28 IPMN (14 HGD, 14 LGD) mucinous samples (**Figure 4**).[32] Mucinous cysts had a marked increase in specific cleavage of MSP-MS library peptides using pH 3.5 assay conditions. We identified more than 20 proteolytic enzymes in IPMN cyst fluid with discriminating activity profiles and the top performer was the aspartyl protease, gastricsin, which exhibited increased activity in mucinous but not in non-mucinous cysts.[32] Gastricsin was confirmed to be more abundant by label-free quantitative MS and western blot in mucinous cysts compared to non-mucinous cysts.[33]

While MSP-MS is a powerful discovery tool, it is not a practical clinical diagnostic test, so we developed a high-throughput, cost-effective, micro-volumetric and easily exportable diagnostic tool for pancreatic cyst stratification. Using the MSP-MS cleavage pattern, we developed internally quenched Fluorescence Resonance Energy Transfer (FRET) substrates that yield, following cleavage by proteases, a fluorescence signal that can be easily detected with a simple benchtop microplate reader, and the assay requires 5 μL of cyst fluid. *Importantly, the use of a benchtop microplate reader increases exportability of this assay to routine clinical laboratories.* The small fluid requirement mitigates current diagnostic tests' limitations imposed by small biospecimen volumes or low cellularity in cyst fluid. In a validation cohort of 110 convenient pancreatic cyst samples from resected tumors, we found that at the optimal cutoff of a 1.23-fold change in fluorescence gastricsin demonstrated specificity for mucinous at 100% and sensitivity of 93% (**AUC 0.979**) and with a limit of detection for gastricsin was 50 ng/mL[33] (**Figure 5).**

Figure 5. Determination of the limit of detection of enzymatic activity (RFU/sec) using internally quenched florescent substrate for gastricsin.

To discriminate grade of dysplasia, Tier-2, we again used our MSP-MS and targeted proteomic analyses and identified a pepstatin-insensitive lysosomal serine tri-aminopeptidase, TPP1, that had a demonstrated an AUC of 0.65, sensitivity of 89% and specificity of 40% for AN, with a limit of detection 95 pg/mL.[34] With further MSP-MS we have also identified more candidate markers, ELANE, a head-to-head validation of this marker with TPP1 will be explored in this proposal.

PRELIMINARY DATA AND METHODS: ELANE was tested in pancreatic cysts from our Biorepository. Among all mucinous PCLs (22 IPMNs and 7 MCNs) ELANE had an AUC of 0.71, sensitivity of 82% (CI 58-93%), specificity of 72% (CI 43-90%). When we stratified the results by subtype of cyst, ELANE performed better in IPMNs than MCNs. **Among 22 main duct, branch duct, and mixed duct IPMNs, ELANE had an AUC of 0.94, sensitivity of 93% (CI 70-99%), and specificity of 85% (CI 48-99%)** (**Figure 6, manuscript in preparation**). Among the 8 MCNs, ELANE had an AUC of 0.62, sensitivity of 100% (CI 17-100%), and specificity of 50% (CI 8-91%) (**Figure 7**). To better identify AN in MCNs, we plan to test performance of additional enzymes we have identified using MSP-MS (including our published analyte TPP1) to determine if specificity is improved using a panel of analytes for MCNs. We will then use the reference set to validate the MCN panel. We also plan to test the hypothesis that the Tier-1 assay improves the performance of the Tier-2 assay by identifying the clinically relevant mucinous cysts.

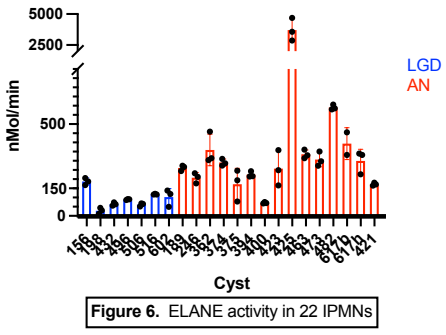

Figure 6. ELANE activity in 22 IPMNs

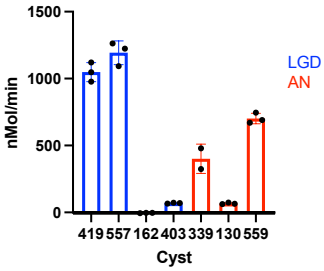

Figure 7. ELANE activity in 7 MCNs

The proteolytic activity of our analytes in cyst fluid samples is analyzed using FRET peptide substrates are incubated with 5μl cyst fluid, PBS, DNase diluted into assay buffer (50 mM Tris, 1 M NaCl, 0.05% (w/v) Brij-35, pH 7.5) in black, round-bottom 384-well plates. The increase in fluorescent signal after proteolytic cleavage by the analytes is monitored with a Biotek Synergy HT plate reader (excitation 328 nm/emission 393nm). The slopes from this assay are converted to uM product/time with a standard curve of known concentrations of the fluorescent molecule MCA. We will run the assays normalized to volume, since our previously published data shown that volume normalization is equivalent to signal normalized to protein concentration.

Pilot testing of ELANE was done using samples from the Kirkwood Pancreatic Cyst Fluid Biorepository. The biorepository began 7 years ago and has been collecting cyst fluid samples, serum, plasma, urine, and whole blood from patients who undergo both EUS and surgical resection of pancreatic cysts at UCSF. Cyst fluid, serum, and plasma samples are collected according to EDRN protocol. Each cyst fluid sample underwent a maximum of two freeze-thaw cycles.

DATA ANALYSIS PLAN:

We request all samples (current n=275; 54 nonmucinous, 130 mucinous with low grade dysplasia, 91 mucinous with high grade dysplasia). To compute the power to test for non-inferiority margin of our gastricsin classifier compared to CEA, we used the R package EQUIVNONINF[35], assuming alpha=0.05, power=0.8, and the discordance p01=p10. If we assume a non-inferiority margin of .05, then we have >80% power for discordances of <.04 for tier 1 (n=221 mucinous, n=54 nonmucinous) and <.03 for tier 2 (n=130 low grade mucinous, and n=91 high grade mucinous).

This proposal is supported by a statistician Thomas Hoffman, PhD and epidemiologist Paige Bracci, PhD for study design planning and data analysis.

FUTURE DIRECTIONS:

Do you plan to approach EDRN for funding and collaboration in proceeding to a Phase Ⅱ validation study? If not, do you have other resources where validation can be accomplished? Describe clearly other resources at your disposal and how they are sufficient to complete a larger Phase Ⅱ validation study if you will not seek help from the EDRN.

Yes. We have proposed to work with the EDRN in our PCDC application (impact score 23).

Are you amenable to working within the collaborative framework of EDRN in proceeding to Phase Ⅱ studies?

Yes, we are amenable to working collaboratively with the EDRN.

If deemed beneficial, will you be amenable to including your biomarker into a larger panel of biomarkers for Phase Ⅱ validation?

Yes, we anticipate this biomarker will be incorporated into a larger panel for the detection of advanced neoplasia.

If refinements will improve the performance of the biomarker test, will you concur with further development of the test? Will it be advantageous to include resources of EDRN for this purpose?

Yes, we anticipate refinements to this protease and others relevant to MCNs and EDRN resources will be beneficial.

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