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THBS2/CA19–9 detecting pancreatic ductal adenocarcinoma at diagnosis underperforms in pre-diagnostic detection: Implications for biomarker advancement

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is often diagnosed too late for effective therapy. The classic strategy for early detection biomarker advancement consists of initial retrospective phases of discovery and validation with tissue samples taken from individuals diagnosed with disease, compared to controls. Using this approach, we previously reported the discovery of a blood biomarker panel consisting of thrombospondin-2 (THBS2) and CA19–9 that together could discriminate resectable stage I and IIa PDAC as well as stages III and IV PDAC, with c-statistic values in the range of 0.96–0.97 in two Phase 2 studies. We now report that in two studies of blood samples prospectively collected from one to fifteen years prior to a PDAC diagnosis (Mayo Clinic and PLCO cohorts), THBS2 and/or CA19–9 failed to discriminate cases from healthy controls at the AUC=0.8 needed. We conclude that PDAC progression may be heterogeneous and for some individuals can be more rapid than generally appreciated. It is important that PDAC early detection

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Author contributions:

G.M.P., A.L.O., W.R.B., and K.S.Z. conceived and designed the study; G.M.P., W.R.B, A.L.O., and D. H. selected the patient and control populations; S.U., N.T., S.Y., E.C., and J.K., designed and performed the THBS2 assays and its automation; W.R.B, A.L.O., G.M.P. analyzed the data and prepared figures and tables; K.S.Z. drafted the paper with G.M.P., A.L.O., and W.R.B.

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studies incorporate high-risk, prospective pre-diagnostic cohorts into discovery and validation studies.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is among the most lethal of cancers because it is usually diagnosed at an advanced stage and the tumors are often challenging to surgically resect. With a 5-year survival rate of less than 10% (1), PDAC may become the second leading cause of cancer death in the United States by the year 2030 (2). The late stage of initial PDAC presentation for the majority of patients has engendered a search for diagnostics to detect PDAC or its precursors early enough to be actionable (3). Since PDAC has an annual incidence of only 13 per 100,000 men and women (4), a diagnostic test must have an exceptionally high ability to detect disease (sensitivity ≥60%) and a comparable ability to truly distinguish individuals without PDAC (specificity ≥99%) to be useful, i.e., ROC AUC ≥0.8 (3). Nonetheless, there is a nearly 2% lifetime risk of PDAC for men and women. This risk is doubled or tripled for individuals who smoke, or who are first degree relatives of PDAC patients, or individuals over age 55 with recent onset diabetes (6–9), and is highest among those with germline mutations in *BRCA1, BRCA2, ATM, CDKN2A, TP53, MLH1, or PALB2* (6,10). Thus, at-risk populations will be the initial beneficiaries of a PDAC diagnostic. In this study, we tested a biomarker panel that detects PDAC at the time of clinical diagnosis (5) for its performance in blood samples collected prior to a PDAC diagnosis. Our results have implications for likely heterogeneity in progression to PDAC and indicate that better strategies may be needed to develop early detection biomarkers.

The most common precursor lesion for PDAC is pancreatic intraepithelial neoplasia (PanIN), which has been designated stages PanIN1, PanIN2, and PanIN3 based upon advancing histopathology (6). While PanIN1 and PanIN2 are considered low-grade lesions with low risk of progression, PanIN3 is considered a high-grade lesion that can lead to PDAC (7). As well, a subset of intraductal papillary mucinous neoplasms (IPMNs) increase risk for PDAC (8). Genomic analysis of PDAC cell populations arising from PanIN and IPMNs reveals common driver mutations in *KRAS, CDKN2A, TP53*, and *SMAD4,* among other genes (9–16). The sporadic accumulation of such mutations in PDAC tumor samples has suggested that precursor lesions could take many years to progress to cancer (17,18).

On the other hand, recent studies indicate that PDAC may progress far more rapidly than previously appreciated. Patients with resectable stage I disease are, on average, only 1.3 years younger than patients with inoperable stage IV disease (19). Venous invasion of PDAC cells and access directly into the liver can explain frequent liver metastases (20–22). The challenge in discerning lesions that are benign from those that will progress to cancer can lead to over-treatment of patients being screened for precursors of disease (3,23). Nonetheless, increased surveillance with endoscopic ultrasound can reveal actionable lesions and increase patient survival (24,25). Only a fraction of high-risk patients undergo invasive screening; thus there is an intense effort to develop blood biomarkers to detect PDAC (3,26).

We previously established a system to detect candidate protein biomarkers that are secreted or released from human PanIN2/3 lesions harboring common PDAC genetic mutations and

that will progress to an invasive phenotype. Briefly, we reprogrammed late stage, recurrent human PDAC cells harboring mutations in *KRAS, CDKN2A, TP53*, and *SMAD4* to an induced pluripotent stem cell-like state, and re-differentiated the cells to early stage lesions in nonimmune mice that eventually progress to an invasive state (27). Mass spectroscopy analysis of secreted and released proteins from early stage lesion explants revealed Thrombospondin 2 (THBS2) as a candidate biomarker (27). We employed a phased study using prospective-specimen collection, retrospective-blinded-evaluation (PRoBE) design (28,29) that included a Phase 1 plasma discovery phase and two Phase 2 validation studies (5). THBS2 levels were elevated in plasma samples from patients with stage I/II PDAC, with AUCs above 0.8 in the Phase 2 studies. CA19–9, a well-established PDAC biomarker, but lacking sufficient specificity and sensitivity to be clinically useful for early detection (30–34), exhibited AUCs comparable to THBS2 in our Phase 2a/2b studies (5). Yet THBS2 and CA19–9 were partially complementary in detecting PDAC, such that their use as a biomarker panel yielded AUCs for stage I/II PDAC above 0.9 in the Phase studies (5). The 99th percentile value of THBS2 (42 U/mL) had a specificity of 98% and a sensitivity of 87%, which yielded positive and negative predictive values that appeared to be in a clinically useful range for at-risk individuals (5,26,35,36), assuming a second stage of follow-up screening to eliminate false positives. In the present study, we performed a prospective, Phase 3 study, adhering to PRoBE criteria (28,29), to assess the THBS2/CA19–9 panel in blood samples taken prior to a clinical diagnosis of PDAC.

Materials and Methods

For the Phase 3 analysis, we included incident pancreatic cancer cases whose clinical coding were assessed and found to be histologically Pancreatic Ductal Adenocarcinoma (ICD-9 codes 8500–3, 8140), and controls matched for age, sex, race, and follow-up duration. The samples consisted of 179 cases and 475 controls from the PLCO cohort, and 37 cases and 140 controls from the Mayo Clinic cohort. All subjects had THBS2 results available and CA19–9 results were available for all but 2 control samples (1 each from PLCO and Mayo).

PLCO samples:

The PLCO Etiology and Early Markers Studies access committee approved this project (EEMS-2016–0041) in February 2017. The PLCO cohort, which is a resource available to the scientific community for secondary studies, and has been well described https://cdas.cancer.gov/plco/. Briefly, the PLCO cohort was generated from a randomized, controlled trial of screening tests for prostate, lung, colorectal and ovarian cancers. Approximately 155,000 participants were enrolled between November 1993 and July 2001. Data were collected on cancer diagnoses and deaths from all causes that occurred through December 31, 2009. Median follow-up time was 12.4 years. For the Phase 3 study, consented participants with no prior history of pancreatic cancer who provided biospecimens were considered. For each of the 238 participants for whom it was determined through study follow-up to have developed pancreatic cancer within 36 months of blood collection, two subjects (controls), who did not develop pancreatic cancer through a comparable follow-up duration, were matched on gender, age at study entry (<60,60–64,65–69,70+ years), race,

date of blood draw (+/− 365 days). THBS2 (CA19–9) assay results were unavailable for 1 case and 2 controls.

Mayo Clinic samples

The study was reviewed and approved by the Mayo Clinic Institutional Review Board (IRB). Mayo participants in this study provided informed written consent to research under IRB protocols #354–06 and #356–06. The Mayo Clinic IRB is in compliance with the requirements of FDA regulations 21 CFR Parts 50 and 56 and HHS regulations 45 CFR 46, which are guided by the Belmont Report. In addition, the Mayo Clinic IRB complies with ICH guideline on Good Clinical Practices where they are consistent with FDA and HHS regulations. The Mayo Clinic Phase 3 cohort samples consisted of 70 incident PDAC cases and 140 controls. We used two sources of participants who had not been diagnosed with PDAC, recruited from 2001 to 2017: (1) a prospectively collected research registry of at risk individuals (family members of pancreatic cancer families (IRB 355–06), and healthy controls ascertained through primary care clinics (356–06), and (2) the Mayo Clinic Biobank, which consists of 50,000 participants recruited from primary care clinics. From these sources, consented participants who had provided a biospecimen at least one month prior to an incident clinical diagnosis of PDAC were identified. For each of these participants, two controls without cancer were matched on age and gender from their respective cohort sources. One incident cancer case was found not to have sufficient sample available and was excluded. CA19–9 assay results were unavailable for 1 control.

Peri-diagnostic sub-sample of Mayo Clinic participants

Among the Mayo Clinic incident cases, 17 were recruited into the Biospecimen Resource for Pancreas Research (IRB 354–06), previously described) (37). Briefly, this is a clinic-based registry which uses prospective ultra-rapid case ascertainment to recruit patients with PDAC and subsequently, their relatives, with an estimated 80% participation rate. Enrolled participants provided a blood sample at time of PDAC diagnosis, thus enabling analysis of the paired samples for the substudy reported here.

Study Design and Populations

Procedures followed a biomarker phased design following with PRoBE criteria (28,29). De-identified human plasma samples from the Mayo Clinic pancreas research biospecimen repository or from the PLCO (received at the Mayo Clinic) were shipped to the Zaret laboratory, which performed ELISA analyses blinded to disease status, and then returned coded data to the Mayo Clinic team for statistical analysis and interpretation. An aliquot of serum was assayed for CA19–9 at the Mayo Clinic Immunochemical Core Laboratory as recommended by the ELISA kit manufacturer (Cobas/Roche). Demographic and clinical characteristics in each group are shown in Table 1.

Detecting THBS2 and CA19–9 levels in blood samples

The THBS2 ELISA kit from Bio-Techne (R&D Systems DTSP20) was used as per the recommendations of the manufacturer except that to minimize lot-to-lot variation in the kit's diluent buffer RD1–75, we developed and used our own diluent buffer consisting of 0.2

mg/ml porcine intestinal mucosa heparin (Sigma #H3149) in protein-free phosphate-buffered saline with Tween 20 (Thermo Fisher #37573). Manual THBS2 ELISAs were performed on plasma samples with the new diluent buffer as otherwise described previously (5) and automated ELISAs were performed on a Dynex DS2 device in a pre-clinical biomarker laboratory, working with the Dynex to ensure reproducible and accurate performance (see Figure 1). CA19–9 levels were detected with a clinical ECLIA assay (Roche) at the Mayo Clinic.

Statistical analysis

The primary analysis evaluated the combination of CA19–9 and THBS2 in the combined PLCO and Mayo Clinic cohorts as an early detection marker of pancreatic cancer. Due to use of specimens from time of diagnosis for prior studies and a change of assay, previous studies were not used to establish a fixed algorithm for combining these markers. Thus, a multivariable time to event Cox proportional hazards model assuming incident cases over a range of follow-up times was fit to assess the time varying performance of CA19–9 and THBS2 together (38). CA19–9 was dichotomized as 0=normal (<55 U/mL) or 1=elevated (≥55 U/mL), and THBS2 as a continuous variable. The area under the ROC curve (AUC) was used as a basis for assessing marker performance. Due to the weak performance, a cross validated estimate was not generated. Secondary analyses were performed within PLCO and Mayo Clinic cohorts. Additional exploratory analyses were performed via logistic regression, together with a bootstrap 95% percentile confidence interval (CI) approach. This approach re-sampled the dataset 1000 times, estimating the logistic regression models each time to calculate area under the ROC curve (AUC) on each bootstrapped dataset to approximate the sampling distribution of the AUC. The 2.5^{th} and 97.5^{th} percentiles from this distribution of AUC values were then used as estimates of lower and upper bounds for the 95% CI for the AUC. Subsets of pancreas cancer patients with samples drawn within 3 years (2 years and 1 year) prior to diagnosis are presented. The THBS2 control distribution 99^{th} percentile value from our prior publication (42 U/mL) is indicated on graphs.

RESULTS

Dynex DS2 THBS2 Assay

Given the increased sample size of the Phase 3 study, we migrated our previously developed manual ELISA for THBS2 onto an automated ELISA platform and developed a customized diluent buffer, which ensured assay stability across different lots of the procured THBS2 ELISA kit (see Methods). Using the custom diluent buffer and comparing the original manual ELISA versus the new automated ELISA for THBS2, the automated ELISA exhibited much lower variation among the replicates (Supplemental Figure 1A) with comparable results (Supplemental Figure 1B). We therefore performed testing herein with the automated assay.

Final Sample Selection

PLCO samples included consented participants with no prior history of pancreatic cancer who provided biospecimens. For each of the 238 participants who was determined through study follow-up to have developed pancreatic cancer, two controls (subjects who did not

develop pancreatic cancer through a comparable follow-up duration), were matched by gender, age at study entry (<60, 60–64, 65–69, 70+ years), race, and date of blood draw (+/− 365 days). THBS2 or CA19–9 results were unavailable for 1 or 2 controls, respectively.

Mayo samples included consented participants with incident PDAC who provided biospecimens at least 1 month prior to a clinical diagnosis were identified. For each of these 70 participants, two controls (participants without cancer) were matched on age and gender. One incident PDAC case ended up having insufficient sample available and was excluded. CA19–9 assay results were unavailable for 1 control.

A subset of 615 healthy controls and 216 (PLCO=179, Mayo=37) cases who had PDAC (ICD-O codes 8500–3, 8140), based upon histologic data provided, were considered in the final analysis. All subjects had THBS2 results available and CA19–9 results were available for all but 3 control samples.

Overview of the Phase 3 Analysis of the PLCO+Mayo Clinic Cohorts

We evaluated THBS2 in plasma and CA19–9 in serum in 238 pre-diagnostic case samples and 475 controls from the PLCO, and 69 pre-diagnostic case samples and 140 controls from the Mayo Clinic. Similar to our approach in the Phase 1 and 2a/2b studies (5), we used all 615 healthy controls and 216 PDAC samples (Table 1) to directly assess performance of these validated assays in the Phase 3 samples in Table 2, in contrast to other studies that split samples into training and test sets (39). Participants who were incident cases, developing PDAC, were matched to controls who did not develop PDAC across the 15-year time periods in each study (Table 1). CA19–9 data had been obtained from clinical assays of matched serum or plasma samples.

Phase 3 pre-diagnostic biomarker analysis: PLCO samples

The PLCO Phase 3 cohort samples consisted of 179 incident PDAC cases and 475 healthy controls, where two controls were each matched to a case on age, sex and time on study in the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening cohort. Scatterplot analysis showed that CA19–9 results on average appear elevated up to a few years prior to a PDAC diagnosis (Figure 1A), with THBS2 less clearly so (Figure 1B). For comparison, we plotted the biomarker levels in control samples taken at various time points prior to study exit, illustrating the occasional elevated signals among the controls (Figure 1C, D). While CA19–9 and THBS2 exhibited AUCs for incident pancreatic cancer of 0.557 and 0.547, respectively, across the entire PLCO dataset and of 0.587 when combined (n = 179) (Table 3A), CA19–9 exhibited AUCs of 0.649 and 0.697 three (n = 64) and two years (n = 44) prior to a PDAC diagnosis, respectively, with THBS2 AUCs of 0.536 and 0.568, respectively, and only elevating CA19–9 in combination to 0.713 two years prior to a PDAC diagnosis (Table 3A).

Phase 3 pre-diagnostic biomarker analysis: Mayo Clinic samples

The Mayo Clinic Phase 3 cohort samples consisted of 37 incident PDAC cases and 140 controls, where 2 controls were matched to a case on age, sex and time on study. These individuals had been recruited into the Mayo Clinic pancreatic cancer family studies or from

primary care clinics from 2001 to 2017. An analysis of the Mayo Clinic Phase 3 pre-diagnostic samples is shown in Figure 1E,F (cases). Scatterplot analysis of CA19–9 and THBS2 levels showed sporadic positive values, i.e., above the 99th percentile value from Phase 2a/2b studies, and no marked trend towards elevated signals within one or two years prior to a PDAC diagnosis (Figure 1E, 1F). These impressions were confirmed by a statistical analysis, which showed that CA19–9 and THBS2 exhibited AUCs of 0.537 and 0.552, respectively, across the entire dataset (Table 3B, n = 37). The combination of the two markers only elevated the AUC to 0.585 (Table 3B). More specifically, for the samples taken three years prior to a PDAC diagnosis (n = 16), the markers yielded AUCs below 0.6 alone or in combination, while samples taken two years prior to a diagnosis (n = 10) yielded AUCs of 0.596 and 0.548 for CA19–9 and THBS2, respectively, and of 0.665 for the combination (Table 3B).

Weak overall biomarker panel performance in the combined PLCO+Mayo Clinic Phase 3 study

The combination of the dichotomized CA19–9 and THBS2 in the overall PLCO+Mayo Clinic cohort generated an AUC of 0.572 (0.53, 0.61) (Table 3C). While our work to date has used CA19–9 with a 55 U/ml case vs. control cutoff, employing CA19–9 levels as a continuous variable yielded an AUC of 0.591 alone and 0.595 with THBS2 (Table 3C). When assessing AUCs for the combined biomarker panel as they occur within 10 years prior to a diagnosis, a modest upward trend was observed starting about 4 years prior to PDAC diagnosis, but it did not reach a clinically useful level (Figure 2A). More specifically, CA19–9 alone exhibited AUCs of 0.679 and 0.752 at two years (n = 54) and one year (n = 29), respectively, prior to a PDAC diagnosis; combining the data with that for THBS2 one year prior did not increase the AUC (0.746) (Table 3D).

Rise in THBS2 and CA19–9 levels comparing paired pre-diagnostic and diagnostic samples from the Mayo Clinic

For a pre-diagnostic study, it is helpful to demonstrate that a biomarker from a Phase 2 study performs at the time of diagnosis in a Phase 3 study, given the differences in sample collection, storage, and freeze/thaws over time. Unfortunately, matched diagnostic samples of incident PDAC patients in cohorts are rarely obtained or made accessible; such was the case for the PLCO cohort. In the Mayo Clinic biorepository, specimens from before and at the time of diagnosis were available for only 17 patients (Supplemental Table 1). While THBS2 levels did increase from pre-diagnosis to time of diagnosis for all but 3 patients, there is no trend of the pre-diagnostic values increasing before diagnosis (Figure 2B, C). We conclude that THBS2 exhibited an upward trend at PDAC diagnosis, but did not exhibit a trend of increased levels prior to the diagnosis in the samples studied.

Discussion

We adhered to the recommended PRoBE criteria for three phases of biomarker validation (Figure 3A, B) (28,29), following a biomarker discovery platform to detect proteins that are secreted or released from early stage PanIN lesions that harbor common PDAC mutations and that will progress to invasive PDAC (27). We found that neither CA19–9 nor THBS2,

nor the combination of the two, performed sufficiently in a Phase 3, prospective study of two different cohorts of samples. The results have implications for future study designs to discover biomarkers for relatively rare diseases such as pancreatic cancer, and for screening for PDAC.

Strengths of our experimental design include access to plasma samples from the Mayo Clinic and the PLCO Cancer Screening Trial (40); samples were collected 1 to 15 years prior to a PDAC diagnosis; the availability of time-of-diagnosis samples of a subset of the incident Mayo Clinic cases; the development of an automated ELISA assay for THBS2 that reduces variability among replicates, and operator blinding during sample identification and analysis. Factors that could have affected our work were the advanced age of numerous frozen plasma specimens, multiple freeze-thaw cycles (41), and that only a small number of patients had paired pre-diagnostic and time of diagnosis samples, limiting the ability to validate biomarker stability over the time frame. Still, the fraction of THBS2-high patients at the time of PDAC diagnosis in the Mayo cohort was about the same as seen in our Phase 2a/2b studies (Figure 2B, C). We suggest that pre- and time-of-diagnosis paired samples be employed for future Phase 3 studies to validate the utility of the biomarker with the particular sample set. We did not have access to data on the incident PDAC patients to explore biomarker levels with tumor characteristics.

Interestingly, CA19–9 considered across the entire Phase 3 study, i.e., Mayo and PLCO together, performed at the level of modest discrimination (AUC-0.75) in the subset of 29 cases one year prior to PDAC diagnosis (Table 3D). In the Phase 3 study with the Mayo Clinic samples, THBS2, CA19–9, and the combination was not able to discriminate PDAC cases prior to a diagnosis. That THBS2 increased in the Mayo Clinic diagnostic samples (Figure 2B, C), albeit with a small sample size, suggests that the negative results with THBS2 in the Mayo pre-diagnostic samples are valid. Similarly, THBS2 performed poorly with the PLCO pre-diagnostic samples and only raised the AUC with CA19–9 (0.697 alone) to 0.713, for two years prior to a diagnosis. The CA19–9 discrimination is similar to the AUC of 0.656 found by Nolan et al. with a different subset of a PLCO samples (42).

We suggest that the high dynamic range of CA19–9 signals in the PDAC population, compared to the modestly changing THBS2 (Figure 3B, see Phase 2a/2b), makes CA19–9 a more sensitive indicator of emerging PDAC, though with fewer samples exhibiting elevated signals. Thus, it may be relevant to focus on new biomarkers that, like THBS2, are partially complementary in detecting PDAC, but have a wider dynamic range in affected individuals compared to controls.

The 17 matched pre- and peri-diagnostic samples provided a qualitative sense of THBS2 biomarker dynamics longitudinally. Our observations suggest that PDAC progression can be heterogeneous. Indeed, the difference in biomarker concentration over time could be more informative for detecting disease than the absolute level over a general threshold, suggesting that future Phase 3 studies would benefit from serial samples. In addition, our findings underscore the importance of discovering biomarkers in patient samples as close as possible to targeted clinical utility, for screening goals.

The most recent recommendations for high-risk patient screening, based on the International Cancer of the Pancreas Screening (CAPS) consortium, is for annual surveillance in the absence of concerning lesions (24,25). Based on the ability of the THBS2/CA19–9 panel to detect PDAC in early stage I/II patients (5) but underperformance in the Phase 3 pre-diagnostic studies described here, including plasma samples at all stages from one source (Mayo Clinic), it is possible that some tumors develop more rapidly than appreciated. If the progression of PDAC is more rapid than generally appreciated, more research is needed on frequency of screening in high-risk individuals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Synopsis:

The contrast of a successful biomarker panel on blood sampled at the time of diagnosis of pancreatic cancer, but not on blood sampled pre-diagnostically, indicates that renewed attention to design and construction of biospecimen sets is critical to advance early detection.

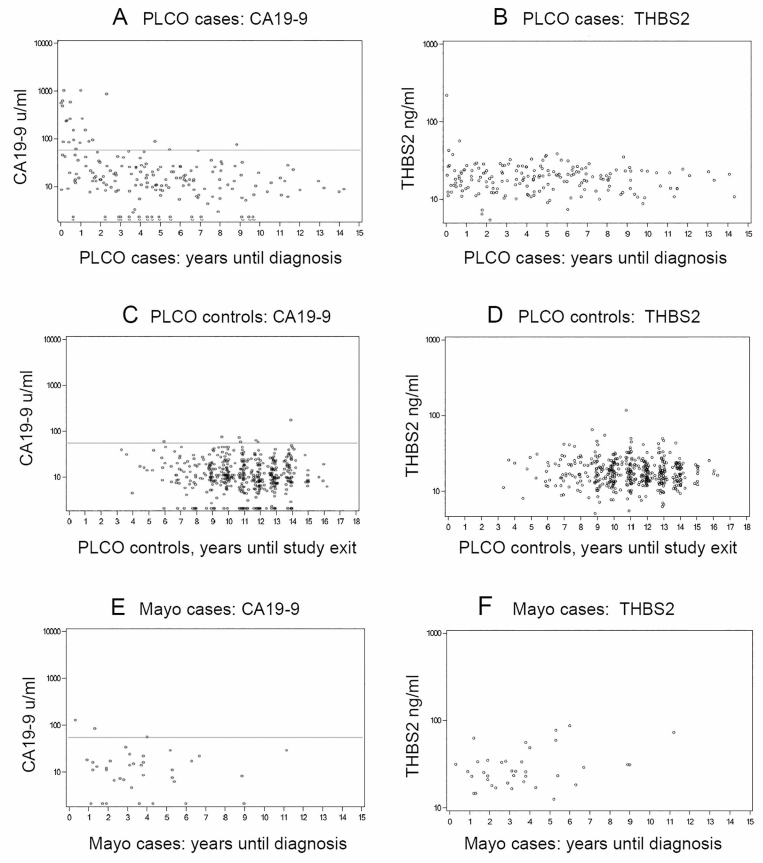

Figure 1. THBS2 and CA19–9 levels across pre-diagnostic PDAC cohorts.

(A,B) Marker values of a pre-diagnostic PDAC population from the PLCO at various times of the 15 year study. Scatterplots depict cases for CA19–9 in serum (A; line depicts 55 u/ml threshold) and THBS2 in plasma (B) by proximity to a diagnosis of PDAC (n.b.,our original Phase 2 study threshold for THBS2 was provisional (5)). (C, D) Marker values of PLCO controls, years prior to study exit for CA19–9 (serum, panel C; line depicts 55 u/ml threshold) and THBS2 (plasma, panel D). (E,F) Marker values of a pre-diagnostic PDAC population from the Mayo Clinic at various times of the 15 year study. Scatterplots depict cases for CA19–9 in serum (E; line depicts 55 u/ml threshold) and THBS2 in plasma by proximity to a diagnosis of PDAC (n.b., our original Phase 2 study threshold for THBS2 was provisional (5)).

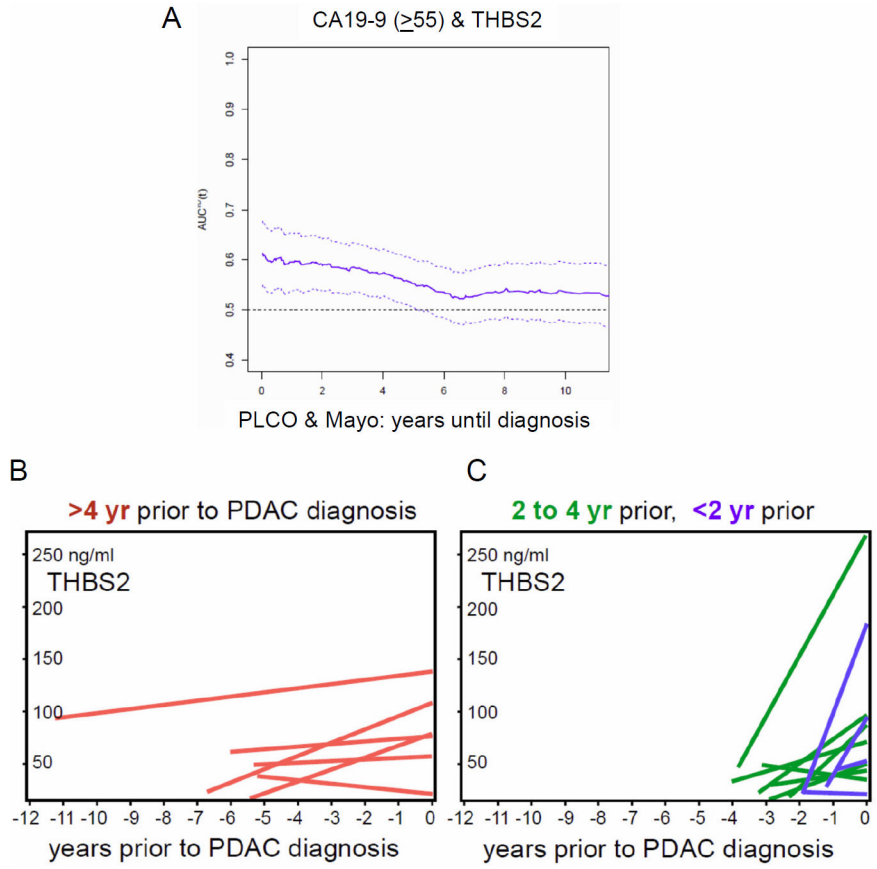

Figure 2. THBS2 and CA19–9 levels across the combined PLCO and Mayo Clinic datasets and THBS2 levels in matched pre- and peri-diagnostic Mayo Clinic samples.

(A) Plot of AUC levels for the combined CA19–9 (continuous) and THBS2 panel across the combined PLCO and Mayo Clinic datasets as a function of time, from 10 years prior to a diagnosis. The dark and dashed lines represent the local point estimate and 95% confidence intervals, respectively. (B, C) Lines connect THBS2 levels (ng/ml) determined in matched pre- and peri-diagnostic samples from the Mayo Clinic Phase 3 cohort. B. Red lines, pre-diagnosis draw was over 4 years prior to a PDAC diagnosis. C. Green lines, pre-diagnosis draw was above 2 years to 4 years prior to a PDAC diagnosis, and blue lines, pre-diagnosis draw was earlier than 2 years prior to a PDAC diagnosis. The upward trajectory of many of the samples, comparing pre- and peri-diagnostic samples, indicates an increase in THBS2 in this limited serial sample, longitudinal analysis.

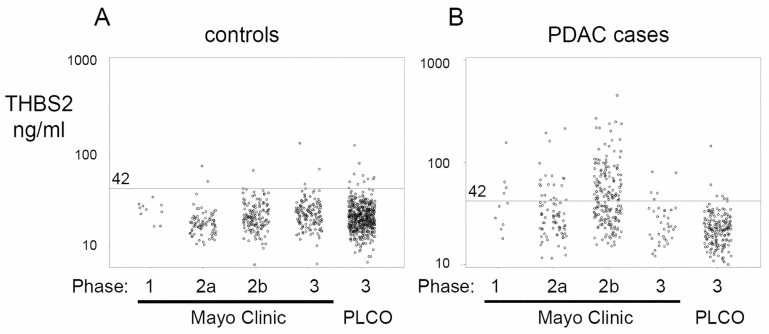

Figure 3. Summary of Phase 1, 2a, 2b, 3 studies

(A,B) Dots indicate THBS2 levels (ng/ml) in control (A) or PDAC (B) samples across three Phases of a PRoBE design study, comparing data from our prior work on Phase 1, 2a, and 2b (Kim et al. 2017) with the current Phase 3 study.

Table 1:

Characteristics of samples used in this Study, drawn from the National Cancer Institute's Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Cohort, and from the Mayo Clinic[a]

	Total Incident PDAC Cases (N=216)		Total Controls (N=615)	
	PLCO (N=179)	Mayo (N=37)	PLCO (N=475)	Mayo (N=140)
Age (SD)[b]	63.4 (5.1)	68.9 (9.2)	63.9 (5.2)	67.0 (10.2)
Male (%)	103 (58)	23 (62)	289 (61)	86 (61)
White, non-Hispanic (%)	158 (88)	11 (100)	419 (88)	61 (100)
Body Mass Index[c] (SD)	27.3 (4.8)	29.9 (84)	27.2 (4.8)	28.4 (5.3)
Personal history of diabetes mellitus (%)	22 (12)	5 (45)	36 (8)	9 (15)
Ever smoker (%)	105 (58)	5 (50)	245 (52)	24 (44)
Pack years smoked[d] (SD)	22.8 (30.9)	24.7 (11.6)	17.7 (26.7)	21.1 (21.6)

[a] In the Mayo Clinic sample, data were not available on race, BMI, personal history of diabetes mellitus, and smoking status on 26 cases and 78 controls

[b] SD = Standard Deviation

[c] Body Mass Index or BMI = weight(kg)/ height(m)^2

[d] Pack years = number of cigarette packs smoked per day x number of years smoked

Table 2.

Summary of all study phases performed to discover and validate THBS2

Study Phase	group	n	Mean THBS2 (ng/ml)	std	P_25	median	P_75
PHASE 1		20	37.3	30.7	23.1	27.6	38.6
PHASE 2a		189	28.3	25.8	16.8	20.3	29.7
PHASE 2b		537	40.3	43.1	19.9	27.6	42.6
PHASE 3: Mayo		177	24.9	12.9	18.0	22.5	27.6
PHASE 3: PLCO		654	22.2	10.0	16.6	20.7	25.3
PHASE 1	PDAC	10	49.7	40.2	24.7	38.6	56.6
PHASE 1	PDAC (I/II)	6	64.3	47.0	37.2	53.3	64.3
PHASE 1	PDAC (III/IV)	4	27.8	9.2	21.4	26.6	34.2
PHASE 1	Controls	10	24.8	5.4	22.5	25.6	27.9
PHASE 2a	Pancreatitis	28	24.7	17.3	15.9	19.5	26.5
PHASE 2a	PDAC	81	38.8	34.5	22.1	29.7	41.1
PHASE 2a	PDAC (I/II)	58	33.5	18.6	20.3	26.3	40.4
PHASE 2a	PDAC (III/IV)	23	52.4	56.3	23.1	31.5	47.7
PHASE 2a	Controls	80	18.9	8.4	15.0	17.5	20.2
PHASE 2b	IPMN	115	27.6	12.2	19.4	24.7	32.5
PHASE 2b	Islet Cell	30	60.3	84.3	22.2	30.0	45.7
PHASE 2b	Pancreatitis	55	34.9	32.3	18.9	26.9	37.4
PHASE 2b	PDAC	197	59.0	53.5	28.8	43.3	70.3
PHASE 2b	PDAC (I/II)	88	57.5	57.8	29.1	40.0	64.1
PHASE 2b	PDAC (III/IV)	109	60.3	50.0	27.8	45.8	72.8
PHASE 2b	Controls	140	22.0	8.0	17.0	20.4	25.9
PHASE 3: Mayo	Pre-dx PDAC	37	28.5	15.9	18.0	23.5	33.9
PHASE 3: Mayo	Controls	140	23.9	11.9	17.9	22.2	26.8
PHASE 3: PLCO	Pre-dx PDAC	179	23.5	12.0	17.0	21.9	26.6
PHASE 3: PLCO	Controls	475	21.7	9.1	16.4	20.2	24.8

Table 3.

Data for PLCO and Mayo Clinic Phase 3 studies

A AUC (95% CI)	PLCO (Cases=179)	PLCO: within 3 years of DX (Cases=64)	PLCO: within 2 years of DX (Cases=44)	
Ca19–9 ≥ 55	0.557 (0.54,0.57)	0.649 (0.60,0.69)	0.697 (0.64,0.75)	
THBS2 - Auto	0.547 (0.48,0.59)	0.536 (0.48,0.59)	0.568 (0.50,0.64)	
Ca19–9 ≥ 55 + THBS2 - Auto	0.587 (0.51,0.63)	0.640 (0.59,0.72)	0.713 (0.62,0.78)	
B **AUC (95% CI)**	**Mayo (Cases=37)**	**Mayo: within 3 years of DX (Cases=16)**	**Mayo: within 2 years of DX (Cases=10)**	
Ca19–9 ≥ 55	0.537 (0.51,0.57)	0.559 (0.51,0.62)	0.596 (0.51,0.70)	
THBS2 - Auto	0.552 (0.48,0.64)	0.505 (0.46,0.65)	0.548 (0.45,0.72)	
Ca19–9 ≥ 55 + THBS2 - Auto	0.585 (0.51,0.67)	0.569 (0.50,0.72)	0.665 (0.51,0.85)	
C **AUCs, combined PLCO & Mayo data**	**Overall (Cases = 216, Controls = 615)**			
Ca19–9 ≥ 55	0.554 (0.54,0.57)			
THBS2	0.529 (0.49,0.56)			
Ca19–9 ≥ 55 + THBS2	0.572 (0.53,0.61)			
Ca19–9 - Continuous	0.591 (0.55, 0.63)			
Ca19–9 + THBS2	0.595 (0.56, 0.63)			
D **AUC (95% CI), combined PLCO & Mayo data**	**Overall (Cases=216)**	**≤3 years of DX (Cases=80)**	**≤ 2 years of DX (Cases=54)**	**≤ 1 year of DX (Cases=29)**
Ca19–9 ≥ 55	0.554 (0.54,0.57)	0.631 (0.59,0.67)	0.679 (0.63,0.73)	0.752 (0.68,0.82)
THBS2 - Auto	0.529 (0.49,0.56)	0.522 (0.47,0.58)	0.549 (0.49,0.62)	0.539 (0.47,0.62)
Ca19–9 ≥ 55 + THBS2 - Auto	0.572 (0.53,0.61)	0.620 (0.58,0.70)	0.699 (0.61,0.76)	0.746 (0.67,0.84)

A. Area under the curve (AUC) analysis by proximity to diagnosis of CA19–9, THBS2, and the combination of the two over the entire PLCO dataset and for samples either three or two years prior to a PDAC diagnosis. DX, diagnosis. Case numbers as shown, compared to 475 controls.

B. Area under the curve (AUC) analysis by proximity to diagnosis of CA19–9, THBS2, and the combination of the two over the entire Mayo Clinic dataset and for samples either three or two years prior to a PDAC diagnosis. Case numbers as shown, compared to 140 controls.

C. Area under the curve (AUC) analyses of combined PLCO and Mayo Clinic datasets using either our standard 55 U/ml cutoff for CA19–9 levels or CA19–9 levels as a continuous variable.

D. AUC analysis by proximity to diagnosis of CA19–9, THBS2, and the combination of the two over the entire combined PLCO and Mayo Clinic datasets and for samples either three, two, or one year prior to a PDAC diagnosis. Case numbers as shown, compared to a total of 615 controls.