2019

Trefoil Factor(s) and CA19.9: A Promising Panel for Early Detection of Pancreatic Cancer

Rahat Jahan  
*University of Nebraska Medical Center, r.jahan@unmc.edu*

Koelina Ganguly  
*University of Nebraska Medical Center, koelina.ganguly@unmc.edu*

Lynette M. Smith  
*University of Nebraska-Lincoln, lmsmith@unmc.edu*

Pranita Atri  
*University of Nebraska Medical Center, p.atri@unmc.edu*

Joseph Carmicheal  
*University of Nebraska Medical Center, joseph.carmicheal@unmc.edu*

*See next page for additional authors*

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**Recommended Citation**  
Jahan, Rahat; Ganguly, Koelina; Smith, Lynette M.; Atri, Pranita; Carmicheal, Joseph; Sheinin, Yuri; Rachagani, Satyanarayana; Natarajan, Gopalakrishnan; Brand, Randall E.; Macha, Muzafar A.; Grandgenett, Paul M.; Kaur, Sukhwinder; and Batra, Surinder K., "Trefoil Factor(s) and CA19.9: A Promising Panel for Early Detection of Pancreatic Cancer" (2019). *Journal Articles: Biochemistry & Molecular Biology*. 120. https://digitalcommons.unmc.edu/com_bio_articles/120

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Trefoil factor(s) and CA19.9: A promising panel for early detection of pancreatic cancer

Rahat Jahan a, Koelina Ganguly a, Lynette M. Smith b, Pranita Atri a, Joseph Carmicheal a, Yuri Sheinin c, Satyanarayana Rachagani a, Gopalakrishnan Natarajan a, Randall E. Brand d, Muzafar A. Macha e, Paul M. Grandgenett f, Sukhwinder Kaur g, Surinder K. Batra a,f,g,**

a Department of Biochemistry and Molecular Biology, College of Medicine, University of Nebraska Medical Center, Omaha, NE 68198-5870, USA
b Department of Biostatistics, College of Public Health, University of Nebraska Medical Center, Omaha, NE 68198-5870, USA
c Department of Pathology and Microbiology, College of Medicine, University of Nebraska Medical Center, Omaha, NE, USA
d Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA
e Department of Otolaryngology-Head & Neck Surgery, College of Medicine, University of Nebraska Medical Center, Omaha, NE 68198-5870, USA
f Buffett Cancer Center, University of Nebraska Medical Center, Omaha, NE, USA
g Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE, USA

Abstract

Background: Trefoil factors (TFF1, TFF2, and TFF3) are small secretory molecules that recently have gained significant attention in multiple studies as an integral component of pancreatic cancer (PC) subtype-specific gene signature. Here, we comprehensively investigated the diagnostic potential of all the members of trefoil family, i.e., TFF1, TFF2, and TFF3 in combination with CA19.9 for detection of PC.

Methods: Trefoil factors (TFFs) gene expression was analyzed in publicly available cancer genome datasets, followed by assessment of their expression in genetically engineered spontaneous mouse model (GEM) of PC (KrasG12D; Pdx1-Cre (KC)) and in human tissue microarray consisting of normal pancreas adjacent to tumor (NAT), precursor lesions (PanIN), and various pathological grades of PC by immunohistochemistry (IHC). Serum TFFs and CA19.9 levels were evaluated via ELISA in comprehensive sample set (n = 362) comprised of independent training and validation sets each containing benign controls (BC), chronic pancreatitis (CP), and various stages of PC. Univariate and multivariate logistic regression and receiver operating characteristic curves (ROC) were used to examine their diagnostic potential both alone and in combination with CA19.9.

Findings: The publicly available datasets and expression analysis revealed significant increased expression of TFF1, TFF2, and TFF3 in PanINs and PC tissues. Assessment of KC mouse model also suggested upregulated expression of TFFs in PanIN lesions and early stage of PC. In serum analyses studies, TFF1 and TFF2 were significantly elevated in early stages of PC in comparison to benign and CP control group while significant elevation in TFF3 levels were observed in CP group with no further elevation in its level in early stage PC group. In receiver operating curve (ROC) analyses, combination of TFFs with CA19.9 emerged as promising panel for discriminating early stage of PC (EPC) from BC (AUC TFF1+TFF2+TFF3+CA19.9 = 0.93) as well as CP (AUC TFF1+TFF2+TFF3+CA19.9 = 0.93). Notably, at 90% specificity (desired for blood-based biomarker panel), TFFs combination improved CA19.9 sensitivity by 10% and 25% to differentiate EPC from BC and CP respectively. In an independent blinded validation set, the combination of TFFs and CA19.9 (AUC TFF1+TFF2+TFF3+CA19.9 = 0.82) also improved the overall accuracy of CA19.9 to discriminate early stage of PC from benign controls and chronic pancreatitis groups.

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Abbreviations: TFF1, Trefoil Factor 1; TFF2, Trefoil Factor 2; TFF3, Trefoil Factor 3; CA19.9, Cancer Antigen 19.9; AUC, Area under the ROC curve; SN, Sensitivity; SP, Specificity; ROC, Receiver Operating Curve; PC, Pancreatic Cancer; BC, Benign Cancer; CP, Chronic Pancreatitis; EPC, Early Pancreatic Cancer; LPC, Late Pancreatic Cancer.

* Correspondence to: S. Kaur, Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, 985870, Nebraska Medical Center, Omaha, NE, 68198-5870, USA.
** Correspondence to: S. K. Batra, Department of Biochemistry and Molecular Biology, Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, 985870, Nebraska Medical Center, Omaha, NE 68198-5870, USA.

E-mail addresses: skaur@unmc.edu (S. Kaur), sbatra@unmc.edu (S.K. Batra).

https://doi.org/10.1016/j.ebiom.2019.03.056
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Please cite this article as: Rahat Jahan, Koelina Ganguly, L.M. Smith, et al., Trefoil factor(s) and CA19.9: A promising panel for early detection of pancreatic cancer, EBioMedicine, https://doi.org/10.1016/j.ebiom.2019.03.056
Research in context

Evidence before this study

Pancreatic cancer (PC) subtype studies highlight the upregulation of all of the members of Trefoil Factors (TFF1, TFF2, and TFF3) in classical subtypes of pancreatic cancer. Further, global transcriptomic studies indicate differential expression of TFFs in precursor lesion and pancreatic cancer cases. However, to date, there is no comprehensive study exploring the diagnostic potential of all Trefoil Factors in pancreatic cancer. We searched in PubMed and Google Scholar for papers published before Aug 10, 2018, with the terms “biomarker Trefoil Factors in pancreatic cancer” OR “diagnostic significance of trefoil factor in pancreatic cancer” OR “TFF1, TFF2 and TFF3 in pancreatic cancer” with no language restrictions. TFF1 emerged as an urinary biomarker for early detection of PC in combinatorial panel with LYVE-1, REG1A. To our knowledge, there is no published study that explores the diagnostic potential all Trefoil Factors alone or in combination with CA19.9 to diagnose PC utilizing serum/plasma samples.

Added value of this study

Comprehensive analyses of gene datasets, human pancreatic precursor lesions, and tumor tissues revealed differential expression of all of the members of TFF family, i.e. TFF1, TFF2, and TFF3, in precursor lesions and during the progression of PC. Differential expression of TFFs is observed in pancreatic precursor lesions from genetically engineered mouse models of PC progression. In addition to pancreatic precursor lesions and tumor tissues, elevated levels of TFFs were observed in serum samples from early and late stage PC cases. Combination of TFFs and CA19.9 has improved overall diagnostic accuracy with increased sensitivity and specificity for detecting early stage of PC in comparison to CA19.9 alone both in training and independent validation sets.

Implications of all the available evidence

The multimarker panel combining of TFF1, TFF2, TFF3, and CA19.9 holds the potential to predict the lethal PC at an early stage. Overall, these findings are clinically relevant, as early diagnostic marker for PC is urgently needed not only for disease detection but also to lead for more targeted approaches for treating patients with this disease.

1. Introduction

Pancreatic cancer (PC) is an aggressive disease with a five-year overall survival rate of ~8%. It is the third-leading cause of cancer-related deaths worldwide, and by 2030, it is projected to escalate to second rank of cancer-related death [1,2]. While the five-year survival rate of patients with localized PC is 34.3%, unfortunately, only 10% of total PC patients are diagnosed at an early stage. Approximately 52% of cases are diagnosed at late/metastasized stage, with a worsened five-survival rate of only 2.7% [2]. Considering these dire statistics, early detection is key to improved PC patient survival. Therefore, identification of early diagnostic biomarkers may result in a timely therapeutic intervention and lead to improve patient prognosis.

To characterize a prospective diagnostic signature for PC, a compendium of several secretory and membranous proteins was enlisted as potential biomarker candidates that demand methodological validation for clinical effectiveness [3]. Among the identified 160 secretory molecules, trefoil factors (TFF1, 2 and 3 (TFFs)) were recognized as potential markers for PC [3]. TFFs are small, secretory mucin-associated proteins known to protect epithelial cells from various environmental insults [4]. Although under physiological conditions they protect the gastric mucosa from inflammation, the oncogenic role of TFFs has been observed in multiple malignancies, including breast, prostate, ovarian, and colon cancers [5]. The secretory nature of TFFs, and their high resistance to proteolytic digestion, acid, and heat degradation qualify them as advantageous from a biomarker perspective.

We aimed to explore the individual and combined diagnostic potential of TFFs alone and in combination with CA19.9 in PC. Although they were previously recognized as promising biomarkers, there has been no comprehensive study assessing the diagnostic capability of TFFs for early detection of PC. To evaluate this potential, we explored publicly available datasets of PC, followed by validation of expression via immunohistochemistry (IHC) in the genetically engineered spontaneous mouse model of PC progression and human tissues comprising of normal pancreas adjacent to tumor (NAT), PC precursor lesions (PanIN), and PC tissues. We further evaluated circulating TFF levels in the sera obtained from training and validation clinical cohorts of PC patients and control samples and analyzed the biomarker potential of individual TFFs in combination with CA19.9. This study reports a potential diagnostic biomarker panel to identify early-stage PC with improved sensitivity (SN) and specificity (SP).

2. Materials and methods

2.1. Ethics statement

Training and validation sets were obtained from University of Pittsburgh Medical Center (UPMC, IRB number PRO070300072), with written consent from all patients before enrollment in the study. Tissue micro-arrays were obtained from Rapid Autopsy Program (RAP), University of Nebraska Medical Center (IRB-#091-01) with written consent from the patients before tissue collection.

2.2. Trefoil factor expression in publicly available PC datasets

GEO datasets containing PC-specific gene expression and patient clinical information were used to assess expression of TFFs. Further, to make the comparisons statistically significant and reduce any chance of method-induced statistical bias, two independent datasets (GSE16515 and GSE43288) were selected containing normal pancreas, PanIN, and PC samples. For dataset GSE43288, samples were profiled using Affymetrix Human Genome U133A Array (Affymetrix, Inc., Santa Clara, CA), which contains normal pancreas (n = 3), PanIN (n = 13) and PC (n = 4) tumor tissues, while dataset GSE16515 samples were profiled using Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Inc., Santa Clara, CA) and contain normal pancreas (n = 16) and PC (n = 36) tumor tissues [6]. Briefly, the raw CEL files were first downloaded and background-corrected. Expression was calculated using the quantile normalization method robust multi-array average (RMA) within the same package for each dataset. The normalized gene expression values (RMA) for TFFs in normal, PanIN, and PC were plotted using MedCalc software. We also analyze all TFFs expression from cbioPortal database, a freely available dataset which is comprised of published and provisional TCGA datasets consisting of 169 studies from 30 different tumor types.

2.3. Tissue immunohistochemistry (IHC) and immunofluorescence for TFF1, TFF2 and TFF3

Immunohistochemistry analyses were performed on commercial tissue microarray (TMA) (Biomax, USA) as well as TMA from the rapid autopsy program (RAP) at UNMC. Tissue spots on the Biomax TMA (BC14011, OD-CT-DgPan03-001, OD-CT-DgPan01-006) were examined by their own pathologists to determine the pathological grading

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and staging. This array contained spots from healthy normal or normal adjacent to the pancreatic tumor, chronic pancreatitis (CP), and PC of different stages and grades. For immunohistochemical staining of mouse TFF1, TFF2 and TFF3 in the paraffin-embedded mouse tissues, 10, 20, and 30 week old floxed KrasG12D Pdx1-Cre mouse tissues (positive for both Kras and Pdx1-Cre), and their age-matched littermate controls (n = 3 animals per age group), were processed using methods describing previously [7]. Briefly, after deparaffinization with xylene and subsequent rehydration with ethanol, epitope retrieval was achieved by boiling the slides in citrate buffer (pH 6.0) for 15 min. Endogenous peroxidase activity was quenched by immersing the TMAs in hydrogen peroxidase solution [0.3% hydrogen peroxidase in 1:1 solution of methanol: water] for 1 h at room temperature, in the dark. Tissues were next blocked with horse serum (ImmPRESS Universal antibody kit; Vector Laboratories, Burlingame, CA) for 2 h at room temperature. Subsequently, the TMAs were incubated with individual primary antibodies for TFF1 (1:200, ab92377, Abcam, Cambridge, MA), TFF2 (Protein tech, 1:500) and TFF3 (ITF Antibody (FL-80): sc-28927, Santa Cruz for human tissue, for mouse tissue TFF3 antibody was kindly provided by Daniel K. Podolsky, UT Southwestern Medical Center, Dallas, TX). After overnight incubation with primary antibodies, the slides were washed four times with PBS, followed by incubation with horse-radish peroxidase (HRP)—conjugated secondary antibody (ImmPRESS Universal antibody kit; Vector Laboratories, Burlingame, CA) for 1 h. Following secondary antibody incubation, the TMAs were washed with PBS and color was developed using DAB solution (3,3'-diaminobenzidine solution) (DAB substrate kit; Vector Laboratories, Burlingame, CA). Once the reddishbrown precipitate was developed, the peroxidase reaction was quenched using distilled water, and sections were counterstained with hematoxylin for 1–2 min. After that, dehydration in an increasing percentage of ethanol followed by three consecutive washings with xylene were performed. Finally, the sections were mounted using Vecta-mount mounting medium (Vector Laboratories). Each tissue spot was evaluated by pathologist for the H-score, which is the product of the percentage of cells positive in the area for each TFF and intensity of staining on a 0–3 scale (0 = no staining, 1 = weak staining, 2 = moderate staining and 3 = strong staining). Colorectal cancer tissues were used as positive controls and isotype control was used as negative control (image not shown). The slides were scanned with Ventana iScan HT from (Ventana Medical Systems, Inc.; Roche Group, Tucson, AZ).

For tissue immunofluorescence studies on TFFs, the initial steps up to blocking were similar to those of IHC. Following blocking, the slides were incubated with an appropriate dilution of primary antibodies (1:25, 1:50 and 1:25 for TFF1, TFF2, and TFF3 respectively, the same antibody used for immunohistochemistry) overnight at 4 °C. Primary antibody detection and visualization of TFF1–3 was achieved by Alexa Fluor 488 labeled goat anti-rabbit IgG2b (Invitrogen Molecular Probes, Eugene, OR, diluted 1:200), incubated for one hour and then washed with PBS three times. Finally, the sections were mounted using an anti-fading mounting medium containing DAPI (Vectashield H–1500; Vector Laboratories). The stained sections were analyzed by confocal scanning laser microscopy, using an LSM 510 Meta laser scanning microscope (Carl Zeiss, Jena, Germany).

2.4. Sandwich Enzyme-Linked Immunosorbent Assay (ELISA)

Individual TFF levels in serum were measured quantitatively by sandwich ELISA in accordance with manufacturer instructions, using the DuoSet ELISA kit with few modifications (R&D Systems, Catalog for TFF1: DY5237, for TFF2: DY4077, and for TFF3: DY4407). Before performing the analysis, assay optimization was performed to select appropriate positive controls and the sample dilution factor to be used for ELISA. Standard curves were produced from standard provided with the kit. TFF1 and TFF2 standards were serially (log2) diluted from 250 pg/ml to 0.4 pg/ml, and TFF3 standard from 750 pg/ml to 1.46 pg/ml. A detailed ELISA protocol is provided in the supplementary section. For the detection step, instead of using the manufacturer provided streptavidin-HRP, we used Pierce Streptavidin Poly-HRP (Thermo Scientific, USA), (diluted to 0.4 μl/ml in 1% BSA) and incubated for 20 min in the dark at room temperature. ELISA plates were read at 450 nm with an absorbance correction at 540 nm. Appropriately diluted sample lysates from TFF expressing cell lines were used as a positive control (MCF7 breast cancer cells for TFF1, LS174T colon cancer cell line for TFF2 and AsPC-1 PC cell line for TFF3). According to manufacturer’s datasheet, there is no cross-reactivity across TFFs in ELISA assays. CA19.9 serum levels were measured using DRG® CA 19–9 ELISA (ELISA-3940) kit (DRG International, Inc., NJ, USA). Serum samples were diluted (50 fold) appropriately to obtain absorbance in the linear range of the assay. The Area Under Curve (AUC) for CA19.9 analysis was performed with the standard clinical cutoff value (37 U/ml) [8]. All samples were tested in duplicate. Data were analyzed using SOFTMAX PRO software (Molecular Devices Corp., Sunnyvale, CA).

2.5. Study cohorts

Two independent sets of samples (training and validation sets) were used to assess the diagnostic performance of TFFs in PC patient serum. The training set (n = 304) included benign control (BC, n = 104), chronic pancreatitis (CP, n = 47), and PC (n = 153). Within the PC group, samples from early stage (EPC, Stage 1 and 2, n = 80) and late stage PC (LP, Stage 3 and 4, n = 73) were segregated for further analysis. Serum samples were shipped from UPMC by overnight mail to the University of Nebraska Medical Center (UNMC) for all experimental analyses. PC staging was determined surgically, based on operative pathology, biopsy of metastatic disease, or radiographic imaging studies. The grade, location of the tumor and stage were based on reviewed hospital records. Patients with benign pathologies such as duodenal ulcers, cholecystochele, common bile duct stones, benign stricture, biliary dilation, or abnormal imaging on computed tomography or magnetic resonance imaging scans of the pancreas were categorized as BC. The independent blinded validation set was comprised of BC, CP, and PC for which serum samples were collected and stored at UPMC. In both training and validation cohort, all samples from UPMC were collected pretreatment (pre-surgery, pre-chemotherapy). Diagnostic significance of TFFs were further assessed in an independent blinded validation set (n = 58) containing BC (n = 8), CP (n = 27) and PC (n = 23). In our validation cohort, the PC samples majority consisted of EPC (n = 18). Patient demographic information for both training and validation datasets is included in the Supplementary Table ST1a-b.

2.6. Statistical analysis

Serum concentration of each protein was calculated using GraphPad software (GraphPad Software, Inc., San Diego, CA). Logarithm transformation was applied to all serum analyses. If the biomarker had a zero level, before log transformation, the zero was changed to half the next lowest value for that marker. Biomarker levels were compared among assay groups with ANOVA. If the overall test was significant, then pairwise comparisons were conducted, adjusting for multiple comparisons with Tukey’s method. Patient characteristics were compared by diagnosis using chi-square or Fisher’s exact test for categorical variables and ANOVA for continuous variables. TFF1, TFF2, and TFF3 levels were compared with patient clinicopathological characteristics using t-tests or ANOVA. We examined the correlation between markers using Pearson correlation. Univariate and multivariate logistic regression and ROC curves were used to test individual markers and combinations as predictors of disease status. ROC curves were used to determine the optimal marker cut points for discriminating the potential of an individual protein. SAS software version 9.3 was used for data analysis (SAS Institute Inc., Cary, NC).
3. Results

3.1. Expression of TFFs in PanIN lesions and PC from publicly available cancer genome dataset

We began our exploration with the analysis of TFF1, 2, and 3 expressions in PC using publicly available data sets (GSE43288, GSE16515). Analyzing both data sets, we observed differential expression of all TFFs in PanINs and PC compared to normal controls (Fig. 1 a&b). Significant upregulation of TFFs was observed in PanIN (TFF1, P < .005; TFF2, P < .005 and TFF3 P < .05, Mann-Whitney U Test) as compared to normal control (Fig. 1a). Similar upregulation of TFFs was observed in PC samples compared to normal control (Fig. 1b). In line with these results, our analysis of the TCGA genome database from cbioPortal (http://www.cbioportal.org) also showed that TFFs are widely expressed in a variety of cancers, predominantly pancreas, colorectal, breast, and prostate (Supplementary Fig. S1a-c) [9,10]. Interestingly, TFF1 was found to be most differentially expressed in PC followed by breast and other malignancies (Supplementary Fig. S1a). Similarly, the highest expression of TFF2 was observed in PC followed by colorectal cancer (Supplementary Fig. S1b). Slightly deviating from TFF1 & TFF2, the highest expression of TFF3 was observed in colorectal cancer, followed by PC (Supplementary Fig. S1c). All members of the TFF family were highly expressed in pancreatic tumors, in comparison to other malignancies. Based on the differential upregulation of TFFs from the genomic data, we next sought to comprehensively analyze the expression of TFFs in a panel of PanIN and PC tissues as well as in serum samples.

3.2. Expression of TFFs in human clinical samples and spontaneous mouse model of PC

After observing upregulation of TFFs in PC using available data sets, we analyzed their expression in TMAs representing human NAT, PanIN, and PC tissues (Fig. 2a). In corroboration with our genomic data, we observed significant upregulation of all TFFs in PanIN lesions and different grades of PC. No expression of any TFF was detected in NAT. Strong expression of TFFs was observed in PanINs, well-differentiated (WD), and moderately differentiated (MD) PC tissues, with moderate expression in poorly differentiated (PD) tissues (Fig. 2a). The H-score of TFFs was found to be significantly higher for PanIN-I-III (P < .0005 for TFF1, P < .005 for TFF2 and P < .05 for TFF3), WD (P < .0005 for TFF1, P < .005 for TFF2 and TFF3) and MD (P < .0005 for TFF1, P < .005 for TFF2 and TFF3), as compared to normal pancreatic tissue adjacent to tumor (NAT) (Welch’s t-Test) (Fig. 2a). Similar results of differential TFFs expression in well-differentiated tumor were also observed using immunofluorescence (Supplementary Fig. S2a). We also observed strong positive staining of all TFFs in metastatic liver tissues (5/5) (Supplementary Fig. S2b). Strong expression of TFF3 was observed in the islet of Langerhans (Supplementary Fig. S2c) while no expression of other two TFFs were observed. Overall, our results demonstrated elevated expression of all the TFFs in PanINs, well-differentiated and moderately differentiated tumors in comparison to normal ducts. Given the significant overexpression in early stages of PC development, we reasoned that TFFs can have the potential for early diagnosis of PC, even before the onset of symptoms. Considering the lack of early-stage tumor tissue, we next analyzed the expression of TFF1, 2, and 3 in a well-characterized spontaneous PC mouse model (KrasG12D, Pdx1-Cre) (KC) that recapitulates genetic and histopathological features of early stages of PC [11]. Using IHC analysis, we observed differential expression of all mouse TFF1, 2, and 3 in KC animal at various weeks representing precursor lesion and early stage of PC respectively (Fig. 2b).

3.3. Circulating levels of TFFs in clinical samples

To investigate the diagnostic potential of TFFs in PC, we analyzed their levels in PC patient serum samples, using ELISA. The demographic and clinical characteristics of patients were detailed in methods and materials section. The training set is comprised of BC, CP, EPC, and LPC patient serum samples. We observed that the median serum levels of TFF1 in patients with BC, CP, EPC, and LPC were 257 pg/ml (Inter Quartile Range, IQR: 156–616 pg/ml), 270.26 pg/ml (IQR: 185–574 pg/ml), 370.41 pg/ml (IQR: 214–1002 pg/ml), and 303 pg/ml (IQR: 186–589 pg/ml) respectively. The median serum levels of TFF2 in patients with BC, CP, EPC, and LPC were 3768 pg/ml (IQR: 2510–5322 pg/ml), 3683 pg/ml (IQR: 2679–7450 pg/ml), 5792 pg/ml (IQR: 3518–8932 pg/ml), and 4807 pg/ml (IQR: 2757–7556 pg/ml) respectively. The median serum levels of TFFs in patients with BC, CP, EPC, and LPC were 9348 pg/ml (IQR: 6728–13,223 pg/ml), 11,945 pg/ml (IQR: 7452–19,149 pg/ml), 11,168 pg/ml (IQR: 7756–19,865 pg/ml), and 9183 pg/ml (IQR: 6329–14,851 pg/ml) respectively. TFFs levels are plotted on a logarithm scale (Fig. 3 a-c). The median serum level of TFF1 was significantly higher in EPC as compared to BC (P < .005) and CP (P < .005, ANOVA) (Fig. 3a). Serum levels of TFF2 were also significantly higher in the EPC group compared to BC and CP (Fig. 3b). In contrast, a significant elevation in TFF3 was observed in the CP group compared to the BC group (P < .01, ANOVA), with no further change observed during PC development (Fig. 3c). Of interest, differential circulating levels of TFFs were observed in late stages of PC as well in comparison to BC and CP, however, their levels were comparatively lower than the early stage cases (Fig. 3a-c).

We also investigated the possible correlations between TFFs and clinicopathological variables such as gender, age, race, bilirubin level, and alcohol history. No apparent difference was observed in the mean value of TFF levels across gender, alcohol history, race (African American, Asian & Caucasian) and bilirubin level. We noted that levels of TFFs have a strong correlation with age (P < .0001, P = .0004 and P = .0014 for TFF1, TFF2, and TFF3, respectively, ANOVA) (Supplementary Table ST2). Circulatory levels of all TFFs were significantly high in patients aged >64 (Supplementary Table ST2).

3.4. Diagnostic performance of TFF1-3 individually and in combination in a training cohort

Considering upregulated expression of TFFs across disease groups, we next sought to explore their diagnostic potential either alone or in combination to differentiate various stages of PC from benign controls. The diagnostic performance of TFFs alone was assessed by using ROC curve analysis (Table 1). In discriminating BCs from PC, individual TFFs showed moderate discriminatory potential with SN/SP values for TFF1, TFF2, and TFF3 being 0.72/0.46, 0.52/0.77 and 0.53/0.64, respectively, and AUCs 0.61, 0.64, and 0.58, respectively (Table 1, Fig. S3a). For differentiating CP from PC, SN/SP values for TFF1, TFF2, and TFF3 were 0.84/0.36, 0.68/0.53, and 0.46/0.65, respectively, and AUCs of 0.62, 0.58, and 0.55, respectively (Table 1, Supplementary Fig. S3a). We also analyzed whether the ratio of circulating individual TFF levels that could differentiate between disease groups. Among all possible ratios, TFF1/TFF3 showed the most promising potential to differentiate between PC vs. CP (AUC = .71) and EPC vs. CP (AUC = .73) (Supplementary Fig. S3b).

We next analyzed the diagnostic performance of TFFs in combination of two in each group (Supplementary Table ST3a). For this, we first assessed correlation across TFFs and disease group. Our results suggested that the Pearson correlation coefficient was significantly higher between TFF1 and TFF3 (r = 0.514, P = .0003) in the CP group (Supplementary Fig. S4). Interestingly, this dual combination of TFF1 and TFF3 could distinguish PC and EPC from CP, AUCs of 0.71 and 0.72, respectively (Supplementary Table ST3a). As discussed earlier, TFFs has shown age-dependent variation, we performed the overall analysis with age-adjustment taking into account those samples who are >40 years and surprisingly, we have seen combination of TFF1 and TFF3 made an overall improvement of differentiating PC and EPC from
Fig. 2. Differential expression of TFF1–3 in tissues from precursor lesions, PC tissues from spontaneous PC mouse models and human PC. (a) Immunohistochemical analysis of all mouse TFF (mTFF1, mTFF2, & mTFF3) protein expression levels throughout the progression of PC in the spontaneous Kras<sup>G12D</sup>Pdx1-Cre (KC) mouse model, from 10 weeks to 30 weeks. Expression of TFF1, TFF2, and TFF3 was progressively increased from 10 to 30 weeks. Scale bars are 100 μm (n = 3 animals/age groups). The interquartile range (IQR) for TFFs expression is presented by box and whisker plot (Middle horizontal line represents the median value). *<i>P</i> < .05 is determined by Student t-test.

Fig. 1. Differential expression of TFF1, TFF2, and TFF3 in PC genomic datasets. (a) Representative box and whisker plots depict the comparison of normalized expression of TFF1–3 mRNA in GSE43288 dataset across normal pancreas (n = 31), PC precursor lesions i.e. PanIN (n = 13) and pancreatic tumor (PC) tissues (n = 4). (b) Representative box and whisker plots comparing normalized expression of TFF1–3 mRNA in GSE16515 dataset across normal pancreas (n = 16), and pancreatic tumor tissues (n = 36). The interquartile range (IQR) for TFF1–3 expression is presented by box and whisker plot (horizontal line represents the 25th percentile, median and 75th percentile and whisker represents 5th and 95th percentile). Publicly available datasets were obtained from Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/). Significantly elevated expression of all the TFFs were observed in pancreatic tumors. Further, the elevation in TFFs was found to be significantly higher across precursor lesions of PC. P values were determined using the Mann-Whitney U test (two-tailed). Red triangle represents outlier value.
A combination of all TFFs demonstrated an AUC of 0.66 (95% CI, 0.59–0.74) with SN/SP of 0.73/0.54 to segregate PC from BC. A similar prediction trend was observed for this panel to distinguish EPC from BC. Compared to any single TFF, the combination panel of TFFs showed significant improvement in differentiating PC from CP, with an AUC of 0.76 and SN/SP of 0.47/0.92 (Table 2). Similarly, to distinguish early-stage PC from CP, the panel achieved an AUC value of 0.76 with SN/SP of 0.51/0.90. Values of AUC and sensitivity/specificity at optimal cutoffs are presented in Table 2. Our analysis suggested that, with age adjustment combination of all TFFs has shown better performance in differentiating PC or EPC from CP (AUC of 0.76 without age adjustment and 0.85 with age adjustment) (Table 2 and Table ST4b). Values of AUC and SN/SP at optimal cutoffs for dual combinations of TFFs without and with age adjustment are presented in Supplementary Table ST3a and ST3b respectively.

3.5. The combination of TFFs with CA19.9 improves diagnostic performance

The Pearson correlation coefficient (r) was used to delineate the correlation between TFF and CA19.9 serum levels (Supplementary Fig. S4). A positive correlation was observed for TFF1 and CA19.9 in BC (r = 0.217, P = .033) and a non-significant positive correlation observed in EPC (r = 0.226, P = .6). Significant positive correlation was also observed for TFF2 and CA19.9 (r = 0.389, P = .013) in the CP group (Supplementary Fig. S4). Of note, a significant negative correlation was observed between TFF2 and CA19.9 in EPC (r = -0.186, P = .017), whereas a significant positive correlation was observed in LPC (r = 0.276, P = .055). Additionally, a positive but not significant correlation was observed for TFF3 and CA19.9 in all the groups (Supplementary Fig. S4). Our correlation analysis suggested that these markers can be complementary to each other in various groups, which can improve the overall efficacy of diagnosis.

As individual TFF showed moderate discriminatory potential and positive correlation with CA19.9, we next investigated the diagnostic performance of all TFF in combination with CA19.9. Our purpose was to analyze whether various combinations of TFFs can improve the diagnostic ability of CA19.9. CA19.9 differentiated PC from BC with SN/SP of 0.86/0.81 (Fig. 4a, Table 2). Combining TFFs with CA19.9 showed improved efficiency to distinguish PC from BC with AUC of 0.94 (95% CI, 0.87–0.99) compared to CA19.9 alone, AUC of 0.91 (95% CI, 0.87–0.95) (Fig. 4a, Table 2). Sensitivity also increased from 0.76 to 0.85 (at 90% specificity), to discriminate between PC and BC (Table 2). To discriminate between EPC and BC, the panel showed an interesting 10% increase in sensitivity (at 90% specificity) compared to CA19.9 alone (Table 2). In the case of discerning PC from CP, the AUC value escalated from 0.91, (95% CI, 0.86–0.95) to 0.94, (95% CI, 0.90–0.98) (Table 2). The combination of all four markers dramatically improved SN/SP of CA19.9 from 0.87/0.81 to 0.92/0.92 to differentiate EPC from CP (Table 2). Moreover, a 16% rise in sensitivity (at 90% specificity) was observed to distinguish PC from CP. Analysis of EPC and CP also demonstrated a sharp increase of sensitivity from 0.67 to 0.92 (at 90% specificity) after the addition of all TFFs to CA19.9 (Table 2). In the case of differentiating LPC from BC or CP, the panel reasonably improved sensitivity and specificity as well as the overall AUC value (Table 2). AUC values and SN/SP of this panel for the different groups (at optimal cutoffs as well as sensitivity at 90% specificity) for the panel are presented in Table 2. Optimal cutpoints and models to determine SN/SP for CA19.9 and combination of TFFs and CA19.9 are presented in Supplementary Table ST4a. Again, when we analyzed our sample with age-adjustment, we observed similar results to differentiate EPC from CP as compared to without age adjustment (Supplementary Table ST4b). The overall performance of all possible combination of TFF with CA19.9 without age adjustment are depicted in Supplementary Fig. S5 and Supplementary Table ST4c.

One of the drawbacks of standard CA 19.9 is that 5–10% people do not express CA19.9 and therefore possess a risk of false negative results [12]. Our finding demonstrates differential correlation between CA19.9 and TFF1–3, which suggests that their addition should complement CA19.9 to identify PC. In light of this, we next sought to identify the diagnostic role of TFFs in low expressing CA19.9 (<37 U/ml) and high expressing CA19.9 (>37 U/ml) PC patient samples, assuming a likely possibility that low CA19.9 PC patients are Lewis negative. We grouped the patients based on the well-established and recommended cut-off value for CA19.9, 37 U/ml [13]. We found that a combination of TFF1–3 can better discriminate EPC from BC in low CA19.9 expressing group, AUC- 0.74, than in high CA19.9 expressing group, AUC 0.69 (Fig. 4b). The ability to discriminate between EPC and CP was also improved in low vs. high expressing CA19.9 groups, AUC 0.87 vs. 0.71, and SN/SP 1.62 vs. 0.92/0.46 (Fig. 4b, Supplementary Table ST5a and b). As demonstrated with these correlation and ROC curve results, a combination of TFF1–3 can complement CA19.9 to determine PC status.

Additionally, as we have observed age-dependent elevation of TFFs, we performed age-matched analysis of diagnostic potential of TFFs in
low and high CA19.9 group. Surprisingly, after age adjustment in both high and low CA19.9 groups, combination of TFFs has demonstrated an AUC of 0.92 with SN/SP of 0.80/0.91 to differentiate PC (n = 10) from CP (n = 23) in low CA19.9 (<37 U/ml) group, whereas, in high CA19.9 group it showed an AUC of 0.81 with SN/SP 0.83/0.82 to distinguish PC (n = 88) from CP (n = 11) (Supplementary Table ST6a & b). Improved diagnostic potential to differentiate EPC from BC or CP in low CA19.9 as compared to high CA19.9 groups by combination of TFFs is also evident from the analysis (Supplementary Table ST6a & b).

### 3.6. Diagnostic performance of TFF1-3 in the validation cohort

Next, we performed an independent blinded study for TFFs and CA19.9 to validate their discriminatory potential in an independent serum sample cohort consisting of BC (n = 27) and CP (n = 23). We also want to note that, our PC group in validation cohort majorly consisted of EPC (n = 18). In accordance with our earlier results, we observed the diagnostic performance of combination of all TFFs with an AUC value of 0.76, 0.84, 0.76, 0.82 in PC vs BC, PC vs CP, EPC vs BC and EPC vs CP (Supplementary Fig. S6). While in validation set CA19.9 demonstrated AUC value of 0.85, 0.71, 0.82, 0.66, interestingly combination of TFFs and CA19.9 showed an AUC value of 0.85, 0.85, 0.82 to differentiate PC vs BC, PC vs CP, EPC vs BC and EPC vs CP, respectively, suggesting that in our validation set also, combination panel of TFFs has improved the diagnosing ability of CA19.9 in detecting EPC from BC or CP which corroborates with our earlier result from training set (Supplementary Fig. S6). Sensitivity and specificity value of individual and combination of TFFs along with CA19.9 using same cutpoint as top differentially expressed genes in the classical subtype of PC [14,15]. From the biomarker point of view, TFF1, along with Lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1) and Regenerating Family Member 1 Alpha (REG1A), has shown promising results as urinary markers for PC [16]. Moreover, findings have suggested that TFF1 originates from PC, since its level sharply decreases after surgical removal of the tumor [16]. The potential of TFFs to determine disease status is also well evident in other cancers. For instance, TFF3 has been demonstrated as a promising biomarker in colorectal cancer and gastric cancers compared to conventional markers [17-19]. Though TFFs have been proposed as potential diagnostic markers for PC in many studies, to our knowledge, there has been no comprehensive study of all trefoil family members for diagnosing this disease. Here, we analyzed the expression of TFFs along PC progression and evaluated their potential to improve diagnosis of PC at early stage with better accuracy.

### 4. Discussion

Trefoil Factors have recently emerged as a prominent player in PC pathogenesis. They have been identified by multiple individual studies as top differentially expressed genes in the classical subtype of PC [14,15]. From the biomarker point of view, TFF1, along with Lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1) and Regenerating Family Member 1 Alpha (REG1A), has shown promising results as urinary markers for PC [16]. Moreover, findings have suggested that TFF1 originates from PC, since its level sharply decreases after surgical removal of the tumor [16]. The potential of TFFs to determine disease status is also well evident in other cancers. For instance, TFF3 has been demonstrated as a promising biomarker in colorectal cancer and gastric cancers compared to conventional markers [17-19]. Though TFFs have been proposed as potential diagnostic markers for PC in many studies, to our knowledge, there has been no comprehensive study of all trefoil family members for diagnosing this disease. Here, we analyzed the expression of TFFs along PC progression and evaluated their potential to improve diagnosis of PC at early stage with better accuracy.

Precinical exploration of differentially expressed genes from microarray and GEO datasets, in conjunction with previously published reports, suggest that TFFs are differentially upregulated in PC. Our findings from cBioportal also showed very high expression of TFF1, 2, and 3 in PC compared to 30 other malignancies listed in the databases. Genes and proteins overexpressed in PanIN lesions hold the potential to detect PC at early stages. While investigating early genetic aberrations during PC pathogenesis, Guo et al. identified TFF1 overexpression in the PanIN lesions [20]. Earlier, the transcriptomic analysis also revealed that TFF1 was one of the top upregulated molecules in sporadic family members for diagnosing this disease. Here, we analyzed the expression of TFFs along PC progression and evaluated their potential to improve diagnosis of PC at early stage with better accuracy.
Fig. 4. Evaluation of the diagnostic significance of TFFs in combination with CA19.9 (a) To evaluate diagnostic significance of the TFFs in combination with CA19.9 for the training set, ROC curves and AUC analyses were carried out for TFF1, TFF2, TFF3 and is presented to distinguish different control groups from PC. The value of the area under curve (AUC) is represented in each box. BC, benign control group; CP, chronic pancreatitis; EPC, early-stage pancreatic cancer (stage 1 and 2); PC, pancreatic cancer. The ROC curve shows comparable performance among all individual TFFs to distinguish different control groups from PC. ROC curves and AUC values for the combination of TFF3 and CA19.9 to distinguish different control groups from PC. (b). Evaluation of the diagnostic performance of TFF1-3 in CA19.9 low (37 U/ml) and high (>=37 U/ml) groups, ROC curves and AUC values for the combination of TFF1-3 in low CA19.9 (<37 U/ml) and high CA19.9 (>37 U/ml) groups, to distinguish different control groups from PC. The value of the area under curve (AUC) is represented in each box. The ROC curve showed that combination of TFF1–3 retains their diagnostic potential in the setting of low CA19.9, to differentiate between PC and control groups. The AUC is represented in each box. BC, benign control group; CP, chronic pancreatitis; EPC, early-stage pancreatic cancer (stage 1 and 2); PC, pancreatic cancer. The ROC curve showed that combination of TFFs and CA19.9 has better diagnostic accuracy over CA19.9 to distinguish different control groups from PC as well as EPC.

**Table 4.** Evaluation of the diagnostic significance of TFFs in combination with CA19.9 for the training set, ROC curves and AUC analyses were carried out for TFF1, TFF2, TFF3 and CA19.9 to distinguish different control groups from PC. The value of the area under curve (AUC) is represented in each box. BC, benign control group; CP, chronic pancreatitis; EPC, early-stage pancreatic cancer (stage 1 and 2); PC, pancreatic cancer. The ROC curve shows comparable performance among all individual TFFs to distinguish different control groups from PC. ROC curves and AUC values for the combination of TFF3 and CA19.9 to distinguish different control groups from PC. (b). Evaluation of the diagnostic performance of TFF1-3 in CA19.9 low (37 U/ml) and high (>=37 U/ml) groups, ROC curves and AUC values for the combination of TFF1-3 in low CA19.9 (<37 U/ml) and high CA19.9 (>37 U/ml) groups, to distinguish different control groups from PC. The value of the area under curve (AUC) is represented in each box. The ROC curve showed that combination of TFF1–3 retains their diagnostic potential in the setting of low CA19.9, to differentiate between PC and control groups. The AUC is represented in each box. BC, benign control group; CP, chronic pancreatitis; EPC, early-stage pancreatic cancer (stage 1 and 2); PC, pancreatic cancer. The ROC curve showed that combination of TFFs and CA19.9 has better diagnostic accuracy over CA19.9 to distinguish different control groups from PC as well as EPC.

Please cite this article as: R. Jahan, K. Ganguly, L.M. Smith, et al., Trefoil factor(s) and CA19.9: A promising panel for early detection of pancreatic cancer, EBioMedicine, https://doi.org/10.1016/j.ebiom.2019.03.056
The strength of our study is the comprehensive evaluation of TFFs alone or in combination with CA19.9 as potential PC biomarkers by employing human tissues, large cohort of serum samples, and the use of a mouse progression model of PC. While our study strongly suggests that a combination of TFF-1 and CA19.9 disciminate early stage PCIs from BCS with improved sensitivity and specificity, we also observed decreased TFFs expression in late stages of PC. Though the underlying mechanisms for this downregulation are still unknown, alteration in methylation patterns between the well and poorly differentiated PCs might be one of the reasons [40]. Earlier studies have shown hypomethylated TFF2 promoter in 84% of PC tissues and treatment with DNA methyltransferase inhibitor and histone deacetylase inhibitor in PC cell line, where TFF2 is silenced by methylation, led to the activation of this gene [41]. Recent studies using spontaneous mouse model demonstrated that loss of TFF1 and TFF2 enhanced PanIN progression along with PC, which reveals their tumor-suppressing role [42,43].

Nevertheless, TFF1 and TFF2 have been shown to increase PC cell proliferation and migration [24,44]. Moreover, higher TFF1 expression was found in central tumors while decreased expression was observed in the invasion front of human PDAC however, retained TFF1 expression in the invasion front was associated with positive lymphatic invasion, lymph node metastasis and poor survival of PC patients [45]. However, based on our findings and earlier reports, we believe that TFFs are very critical factors in PC pathogenesis and that they warrant precise study with mouse models and in vivo lineage tracing studies.

The limitations of our study include the small sample size of BC in the validation cohort. Although, analysis in our validation cohort has demonstrated that combination of TFFs and CA19.9 (AUC = 0.82) improves the overall efficacy of CA19.9 (AUC = 0.66) to differentiate EPC from CP [Supplementary Fig. S6, Supplementary Table S7]), however, addition of TFFs could neither add nor reduce the diagnostic power of CA19.9 to differentiate PC from BC which differed from our analysis in training set. This finding further necessitates an extensive blinded validation study with a large number of BC samples in the future. In addition, CA19.9 and TFFs are shown to be elevated in other cancers, but our study has investigated the diagnostic potential of this biomarker panel in PC only, and therefore further research in other cancers as also warranted [5,31]. Furthermore, multi-institutional validation and cross-validation for this panel are needed to make it a reliable multimarker panel. Moreover, TFF1 has shown to be a promising urine biomarker for PC [16]. Based on previous studies and our analysis from the training and validation sets also suggest that TFF1 should be given more emphasis for future biomarker and functional studies, as it performed better in both datasets to differentiate between EPC and CP. Moreover, it will be interesting to investigate their diagnostic performance in pancreatic juice and urine as well. Alongside the prognostic significance, ability of TFFs to predict recurrence and drug-response in PC patients, are interesting aspects to investigate in the future as CA19.9 has been observed to predict post-operative recurrence as well as response to chemotherapy in PC patients [13]. Another limitation in our study was a very small sample number in age-adjusted low CA19.9 (<37 U/ml) group, though our interesting finding has suggested that combination of TFFs has the ability to differentiate EPC from CP in age-adjusted low CA19.9 group with an AUC value of 0.94 (Supplementary Table S7a). However, considering the limitation of small sample number and their diagnostic potential in those groups warrants investigating the potential of combination of TFFs in low and high CA19.9 group with age adjustment in a larger cohort of patient sample in future. Moreover, we have analyzed individual TFFs expression in patient sample BC - > 1.2 mg/dL and BC < 1.2 mg/dL samples to identify whether TFFs can differentiate between non carcinoma and carcinoma related jaundice. We didn't observe any significant difference of TFF1 and TFF3 expression with high and low bilirubin levels. At the same time, we observed differences in CA19.9 and TFF2 (P < .05, Mann–Whitney U Test) across these groups suggesting TFF1 or TFF3 could be potential markers that are not be affected by acute biliary obstructions or non-carcinoma related jaundice. Thus, evaluating TFF 1 and 3 in conjunction with CA19.9 can improve the performance of the differentiation of non-cancer related jaundice or acute biliary obstruction from early stage PC (Data not shown). Thus, evaluating TFFs clinical utility in differentiating cancer-related jaundice and non-cancer related jaundiced with a large cohort of clinical samples would be next logical step as CA19.9 gets elevated even in non-carcinoma conditions like obstructive jaundice and produce false positive result [46].

To translate this diagnostic panel from bench to bedside, more effort should be made to uncover the molecular landscape of TFFs in PC. Specifically, it would be interesting to explore whether increased levels of TFFs, both in serum and tissue, are the drivers or the consequence of disease progression. TFF1 was previously correlated with increased PC cell proliferation and metastasis [44], and recombinitant treatment with TFF2 has induced PC cell migration [24]. By contrast, loss of TFF2 from a newly defined progenitor compartment in PC, coined the pancreatic duct gland, has shown to accelerate IPMN formation [42].

Recently, Collisson et al. identified three PC subtypes: classical, quasi-mesenchymal, and exocrine-like, based on gene signatures from human and mouse PC samples. They observed that classical subtype is more gemicitabine-resistant compared to other subtypes [14]. In another study, the Moffitt group identified two subgroups, ‘classical’ and ‘basal-like’, where basal-like tumors showed a strong trend toward a better response to adjuvant therapy. Surprisingly, both groups have discovered family members of TFFs to be critical contributors in the classical subtype of PC. Identification of PC subtyping has created a new avenue for PC precision medicine. This will pave the way to improved clinical outcomes and therapeutic response based on intrinsic molecular variabilities among patient groups that clinically progress at different rates and may respond differently to administered therapies [14,15]. We believe that untangling the complex mechanism of PC progression, as well as understanding the genomic landscape of PC subtypes, is thus urgently required for the development of novel screening strategies and chemopreventive approaches for PC. Uncovering the role of subtype-specific molecules like TFFs is much needed.

The identification of an early diagnostic marker is gaining unprecedented attention not only because it provides insight into disease occurrence but also provides the impetus for developing novel strategies for therapeutic intervention. Importantly, both our training and validation sets analysis suggested that combination of TFFs and CA19.9 are able to differentiate EPC from chronic pancreatitis, a perplexing state which lead to difficulties in identifying PC at early stages. Our combination of TFFs has demonstrated an AUC value of 0.76 (without age adjustment) and 0.85 (with age adjustment) to differentiate EPC, a surgically resectable stage from CP (Fig. 2 and Supplementary Fig. S4a). Our results indicated that the combination of CA19.9 and TFFs can differentiate early stages of pancreatic cancer, which is the stage for surgically resectable pancreatic cancer, from benign controls and chronic pancreatitis.

Thus, we anticipate that combination of TFF1, 2, 3 may not be a useful marker for identifying tumor resectability with high sensitivity and specificity but combination of TFFs and CA19.9 will have the clinical utility for early detection of surgically resectable PC. While still in its infancy, validation of TFFs in combination with CA19.9 in serum will not only predict the presence of PC at early stage but may also have utility in stratifying patients for appropriate therapeutic regimen selection, given that TFFs have been shown to be highly upregulated in classical subtype of PC. Apart from protein biomarkers, very recently, circulating tumor DNA (ctDNA), circular RNA, miRNAs, IncRNA, exosomes have gained immense attention as minimally invasive tool for early detection and diagnosis of cancer, however efforts are still going on to improve their isolation techniques, stability, reliability, sensitivity, cost of
analysis, storage measures for bringing them into clinics [47–50]. Furthermore, recent evidence of conventional biomarker coupled with KRAS mutations in ctDNA has provided optimism as a diagnostic and prognostic marker for PC. However, protein-based biomarkers still stand out as better diagnostics than other emerging biomarkers, primarily because of their superior stability, enhanced availability in the serum and well-established protocols. More studies are highly desirable to combine the benefit of both protein-based biomarkers along with genetic marker for diagnosis of lethal PC [51].

Based on our knowledge from the published literature, this study is the first of its kind to demonstrate the potential role of TFF1, TFF2, and TFF3 as serum-based markers for diagnosing early-stage PC. However, the establishment of a clinically valuable biomarker panel requires exhaustive validation and cross-sectional multicenter studies, and this is our ongoing research focus for this panel. Our results suggest compelling evidence from publicly available datasets, tissue, and serum analysis that TFF1, TFF2, and TFF3, along with CA19.9, can be a useful biomarker for identifying PC.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.03.056.

Conflicts of interest
SKB is one of co-founders of Sanguine Diagnostics and Therapeutics, Inc. No potential conflicts of interest were disclosed by the other authors.

Writing assistance
We thank Dr. Adrian E. Koesters, Research Editor at UNMC, for her editorial contribution to the manuscript supported by UNMC and our startup package.

Author contribution
R.J.: Study concept and design, generation and collection of data, analysis, and interpretation of data and drafting of the manuscript, study supervision.
S.K.: Study concept and design, analysis and interpretation of data, critical revision of the manuscript, study supervision.
L.M.S.: Statistical analysis, critical revision of the manuscript.
Y.S.: Analysis and interpretation of data.
P.A.: Bioinformatics analysis.
K.G.N.: Data acquisition and critical revision of the manuscript.
J.C. and M.A.M.: Critical revision of the manuscript.
S.K: Study concept and design, analysis and interpretation of data, critical revision of the manuscript, study supervision.

Funding sources
This study/authors were supported, in part, by grants from the NIH P30CA36727, 5R01CA211462, 1U01CA210240 and U01 CA200466.

Acknowledgments
We greatly appreciate the kind technical help of Ms. Kavita Mallya, UNMC. We also thank Ms. Melissa Holzapfel from Tissue Science facility, UNMC for assisting in tissue imaging. We cordially thank Dr. Daniel Podolsky, (UT Southwestern Medical Center, Dallas, TX) for sharing TFF3 antibody with us.

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