Levels of the cancer biomarker CA 19-9 are associated with thrombin generation in plasma from treatment-na?ve pancreatic cancer patients

Mattila, N.

2021-03

Mattila , N , Hisada , Y , Przybyla , B , Posma , J , Jouppila , A , Haglund , C , Seppänen , H , Mackman , N & Lassila , R 2021 , ' Levels of the cancer biomarker CA 19-9 are associated with thrombin generation in plasma from treatment-na?ve pancreatic cancer patients ' , Thrombosis Research , vol. 199 , pp. 21-31 . https://doi.org/10.1016/j.thromres.2020.12.018

http://hdl.handle.net/10138/342267
https://doi.org/10.1016/j.thromres.2020.12.018

Levels of the cancer biomarker CA 19-9 are associated with thrombin generation in plasma from treatment-naïve pancreatic cancer patients

Mattila N[1,2], Hisada Y[3], Przybyla B[2], Posma J[4], Jouppila A[2,5,6], Haglund C[1,7], Seppänen H[1,7], Mackman N[3], Lassila R[2,6,8]

[1]Department of Surgery, Helsinki University Hospital, Helsinki, Finland, [2]Department of Hematology, Coagulation Disorders Unit, Comprehensive Cancer Center, Helsinki University Hospital, Helsinki, Finland; [3]UNC Blood Research Center, Division of Hematology/Oncology, Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA, [4]Laboratory for Clinical Thrombosis and Hemostasis, Department of Internal Medicine and Department of Biochemistry, Cardiovascular Research Institute Maastricht, Maastricht University Medical Center, The Netherlands; [5]Clinical Research Institute HUCH, Helsinki, Finland; Research Programs Unit in [6]Systems Oncology and [7]Translational Cancer Medicine, University of Helsinki, Helsinki, Finland, [8]HUSLAB Laboratory Services, Clinical Chemistry, Helsinki, Finland

Running title: CA 19-9 AND THROMBIN GENERATION IN PANCREATIC CANCER

Word count: 5672

Highlights

- **CA 19-9 and thrombin generation are associated in pancreatic cancer patients**
- **CA 19-9 in patient samples did not associate with coagulation biomarkers**
- **Some commercial CA 19-9 sources are contaminated with tissue factor**

ABSTRACT

Background

Pancreatic ductal adenocarcinoma (PDAC) is associated with a hypercoagulable state and high mortality. Increases in the plasma levels of tumor marker carbohydrate antigen (CA) 19-9 are used in diagnosis and follow-up but have also been reported to precede venous thromboembolism (VTE).

Aims

We examined the association between CA 19-9 and thrombin generation (TG) in plasma from PDAC patients, as well as their association with coagulation biomarkers prior to pancreatic surgery. In addition, we determined the effect of commercial sources of CA 19-9 on TG.

Methods

We collected plasma from 58 treatment-naïve PDAC patients without any signs of VTE. We measured levels of CA 19-9, FVIII, fibrinogen, D-dimer, antithrombin and extracellular vesicle (EV) tissue factor (TF) activity and TG using a Calibrated Automated Thrombogram (CAT). The effect of different commercial sources of CA 19-9 on TG in Standard Human Plasma (SHP) was also studied.

Results

Patient plasma samples were divided into 4 preoperative groups based on the level of CA 19-9: none<2, low=3-200, high=201-1000, and very high>1000 U/mL. CA 19-9 levels were associated with several of the TG parameters, including endogenous thrombin potential, peak, and time to peak. CA

19-9 did not associate with any of the coagulation biomarkers. Spiking of SHP with CA 19-9 increased TG but this was decreased by an anti-TF antibody.

Conclusions

CA 19-9 was associated with TG in patients prior to any pancreatic cancer treatments or signs of VTE. Some commercial sources of CA 19-9 enhanced TG in SHP seemingly due to contaminating TF.

Word count: 249

Key words: CA 19-9 antigen, extracellular vesicles, pancreatic neoplasms, thrombin, tissue factor

List of abbreviations:

CA 19-9 = carbohydrate antigen 19-9
CAT = Calibrated Automated Thrombogram
CV = coefficient of variance
ETP = endogenous thrombin potential
EV = extracellular vesicles
FVIII = Factor VIII
IQR = interquartile range
MP = microparticle
PBS = phosphate buffered saline
PDAC = pancreatic ductal adenocarcinoma
PL = phospholipids
PLA2 = phospholipase A2
PPP = platelet-poor plasma
PRP = platelet-rich plasma
SHP = standard human plasma
Rho = Spearman's correlation coefficient
RT = room temperature
TBS = tris buffered saline
TF = tissue factor
TFPI = tissue factor pathway inhibitor
TTP = time to peak
TG = thrombin generation
VTE = venous thromboembolism

INTRODUCTION

Cancer patients have an increased risk of both arterial and venous thromboembolism (VTE) [1-6]. Cancer patients with thrombosis have increased mortality[7]. Pancreatic cancer, and especially pancreatic ductal adenocarcinoma (PDAC), is one of the most thrombogenic cancer types[8]. PDAC often presents with increased coagulation activity in the form of elevated levels of tissue factor (TF)-bearing extracellular vesicles (EV)[9-14], factor VIII (FVIII)[15, 16], fibrinogen[15, 17], and D-dimer[9], even without visible thrombosis. Therefore, it is important to understand the mechanisms underlying the thrombogenicity, and to identify the patients with the highest thrombotic potential.

The most commonly used tumor-marker for PDAC is carbohydrate antigen (CA) 19-9, the sialylated Lewis a antigen[18]. CA 19-9 is secreted from the epithelial cells of the gastrointestinal tract, such as the pancreatic duct epithelium, and its expression is increased in malignancies. CA 19-9 serves as a diagnostic and follow-up marker for PDAC[19]. In our previous study, we found that combining CA 19-9 with FVIII, fibrinogen, alkaline phosphatase and conjugated bilirubin in a pre-diagnostic panel improved the diagnosis of PDAC in treatment-naïve patients[15].

In plasma and serum, CA 19-9 is present in mucins, especially MUC1, which are high-molecular weight glycoproteins [20, 21]. Mucins form gel-like structures, which capture molecules and can attach to cell surfaces[22]. Mucins have been proposed to contribute to the hypercoagulable state of adenocarcinomas via multiple mechanisms, including via activation of platelets and neutrophils [23-25]. This raises the question if CA 19-9 could also directly promote coagulation.

Thrombin is the key protease in the coagulation cascade and measuring its generation may give insight into the pathways involved in the hypercoagulable state in various diseases[26-28]. Increased TG suggests increased coagulation activity[29, 30]. Malignant cells, especially pancreatic cancer cells, and their TF-bearing EVs enhance TG[31,32]. At present, it is not known if CA 19-9 enhances TG. To this end we chose to study plasma from treatment-naïve PDAC patients to avoid the confounding effects of chemo- and radiation therapy and surgery on coagulation.

The primary goal of the study was to determine if CA 19-9 was a biomarker for increased coagulation activity without any signs of thrombosis in PDAC patients. In addition, we determined the effect of commercial CA 19-9 on TG in standard human plasma (SHP). We also analyzed the correlations between CA 19-9 and TG with biomarkers of coagulation.

PATIENTS AND METHODS

Ethical approval

This study was approved by the Surgical Ethics Committee of the Helsinki University Hospital (Dnro HUS 226/E6/06, extension TMK02 §66 17.4.2013), and was performed according to the Helsinki Declaration. Each patient gave written consent prior to sample collecting.

Patient samples

Citrated (3.2%) plasma samples, prepared from whole blood by a single centrifugation at 2,500 g for 15 minutes, were collected from patients admitted to the Helsinki University Hospital for an upper-gastrointestinal tumor surgery during 2013-2017. Serum was prepared from coagulated blood by centrifugation at 2,000 g for 10 minutes and was used for analysis of CA 19-9. Patient samples were obtained 1-3 days prior to cancer surgery. The samples were stored at -80^{o}Cuntil TG analysis. We included patients having plasma available for reliable TG analysis with a histopathologically confirmed PDAC (n=58), without another active cancer in the previous five years, without established coagulation disorders, without preoperative neoadjuvant or anticoagulant treatments, and without overt thrombotic events to avoid any confounding effects on TG. All patients were considered to have a radiographically resectable disease. If local advancement or distant metastasis was found from frozen sections during a staging laparoscopy, the tumor was deemed non-resectable and the operation was discontinued. Of the 58 patients, 13 had a non-resectable disease, either due to distant metastasis (n=12) or local advancement (n=1). Patients were divided into groups based on their CA 19-9 levels: none<2 (n=4), low=3-200 (n=32), high=201-1000 (n=13), and very high>1000 U/mL (n=9). Patient characteristics are presented in Table 1.

Table 1. Patient characteristics of the different CA 19-9 groups

	None (<2 U/ml)	Low (2-200 U/ml)	High (201-1000 U/ml)	Very high (>1000 U/ml)
CA 19-9 (U/mL), median (IQR)	<2	53 (20-119)	457 (327-807)	7669 (2993-21687)
Number of patients	4	32	13	9
Female, n (%)	1 (25)	17 (53)	8 (62)	5 (56)
Age over 65, n (%)	2 (50)	23 (72)	8 (62)	7 (78)
Operable, n (%)	3 (75)	25 (81)	12 (92)	5 (56)
Stage				
IA	0	3	1	0
IB	0	4	1	0
IIA	0	0	0	0
IIB	0	12	6	2
III	3	7	4	3
IV	1	6	1	4

IQR=interquartile range

Measurement of biomarkers in serum and plasma

Preoperative CA 19-9 levels were measured in serum using the ARCHITECHT CA 19-9$_{XR}$ assay (Abbott Laboratories, Abbot Park, IL, USA) as a part of the routine preoperative laboratory testing. Plasma fibrinogen, antithrombin, D-dimer, and FVIII levels were either analyzed preoperatively as routine testing from citrated (3.2%) plasma, or afterwards from the preoperatively collected

plasma stored at -80°C. Fibrinogen was determined using a Multifibren® U assay (Siemens Healthcare Diagnostics, Marburg, Germany). Antithrombin was measured using a chromogenic assay (Berichrom®Antithrombin III, Siemens), and D-dimer with an immunoturbidimetric assay (Tina-quant D-Dimer®, Roche Diagnostics, Mannheim, Germany). FVIII levels were assessed with a one-stage clotting assay (Pathromtin SL and Coagulation Factor VIII Deficient Plasma, Siemens).

Extracellular vesicle tissue factor activity

EV TF activity was analyzed as previously described[33]. Four patients were not analyzed for EV TF as we did not have enough plasma. EV TF activity was calculated by reference to a standard curve generated using re-lipidated recombinant human TF in the range of 0–20 pg/mL (Innovin, Siemens, Munich, Germany). The TF-dependent FXa generation (pg/mL) was determined by subtracting the amount of FXa generated in the presence of an anti- TF antibody (HTF-1, BD biosciences, San Jose, CA, USA) from the amount of FXa generated in the presence of the control antibody (Sigma Aldrich, St. Louis, MO, USA), and converted to TF concentration (pg/mL) by reference to the standard curve. Positive controls were generated by treating whole blood from healthy volunteers with *E. coli* lipopolysaccharide (Serotype 0111:B4, Sigma Aldrich) (10 µg/mL) for 5 h at 37 °C with gentle rocking with subsequent isolation of plasma.

Measurement of thrombin generation using a Calibrated Automated Thrombogram

TG was measured using the Calibrated Automated Thrombogram[34] assay (CAT, Fluoroskan Ascent, ThermoFisher, Helsinki, Finland) with the Thromboscan software (Thrombinoscope, Maastricht, The Netherlands). TG was initiated with either 1) Platelet-poor plasma (PPP)-Reagent LOW, containing 1 pM TF and 4 µM phospholipids (PL), 2) PPP-Reagent containing 5pM TF and 4 µM PL, 3) Platelet-rich plasma (PRP)-Reagent containing 1 pM TF and a minimal amount of PL, 4) Microparticle (MP)-Reagent

containing only PL, 5) CA 19-9 at various concentrations or 6) phosphate-buffered saline (PBS) as the activator in patient plasma samples or SHP. First, 20 µL of either activator or Thrombin Calibrator (Thrombinoscope BV, Maastricht, The Netherlands) was pipetted into 96-well microtiter round bottom plates (Mekalasi, Nurmijärvi, Finland). After that, 80 µl of plasma was added to the plates. The plates were then incubated for 10 minutes at 37°C. Finally, FluCa solution was added to detect TG. Various CAT variables, including lag time, endogenous thrombin potential (ETP), peak thrombin and time to peak (TTP) were analyzed.

Effect of CA 19-9 *ex vivo* on thrombin generation in patient plasma measured by Calibrated Automated Thrombogram

Patient plasma was thawed in a 37°C water bath for 15 minutes and then kept at room temperature (RT) until pipetting it into the microtiter plates for TG analysis. PPP-Reagent LOW was used as the activator for TG. The concentration-dependent effect of CA 19-9 on TG was compared in the different patient groups with each other and SHP as control plasma.

Effect of CA 19-9 *in vitro* on thrombin generation in plasma measured by CAT

Six commercial CA 19-9 preparations from different sources were obtained from MyBioSource (San Diego, CA, USA) (Supplement table 1). The concentrations used in the experiments were based on the manufacturer's reported concentration of CA 19-9.

We assessed the effect of CA 19-9 preparations on TG under different conditions. CA 19-9 was diluted in PBS (pH 7.4) to obtain three concentrations of 20, 200 and 900 U/mL. First, the concentration-dependent effect of CA 19-9 on TG was analyzed in the CAT assay with the PPP-Reagent LOW as the activator. Next, CA 19-9 at 900 IU/mL alone was studied as the sole activator of TG, without added TF

or PL, and compared with the TG induced by the commercial activators: 1) PPP-Reagent LOW, 2) PPP-Reagent containing 5pM TF and 4 μM PL, 3) Platelet-rich plasma (PRP)-Reagent containing 1 pM TF and no PL and 4) Microparticle (MP)-Reagent containing only PL. We then assessed the concentration-dependent TG effects of CA 19-9 alone in SHP at 200, 900 and 9000 U/mL without added TF or PL.

To determine if the effect of CA 19-9 on TG in SHP was TF-dependent, we incubated CA 19-9 for one hour at RT with a monoclonal antibody for human TF (anti-TF, REF 4509, Biomedica Diagnostics, Conneticut, USA), or SHP with an antibody against the C-terminus of tissue factor pathway inhibitor (anti-TFPI, Sanquin, Amsterdam, the Netherlands) for one hour prior to CAT analysis. The influence of PLs in the CA 19-9 reagents on TG was analyzed by incubating CA 19-9 with phospholipase A2 (PLA2) from honeybee venom (P9279, Sigma-Aldrich, Germany) for one hour prior to CAT analysis. PBS was used as a control to assess the impact of dilution and incubation time. To dissect the roles of extrinsic and intrinsic pathways on TG, we also tested the effect of CA 19-9 on TG in plasmas deficient in coagulation factor (F) VII, FVIII, FIX, FXI and FXII (Siemens Healthcare, Germany).

Statistical analysis

CAT analysis was performed in 1-4 repeated triplicates depending on the amount of plasma available, and the mean of all repeated measurements was used for statistical analysis. The results of the triplicate measurements were reviewed, and clear outlier curves were discarded. After review of the curves, the intra-triplicate coefficient of variance (CV) was below 20% for all patient plasma measurements. The inter-assay CV of the repeated patient plasma assessments was

maximally 20%. Statistical analysis and graphical work were performed using GraphPad Prism 8 and SPSS Statistics 25. The laboratory values of each group are presented as median values with interquartile ranges (IQR), with the exception of the CA 19-9 none group as only two patients had the variables of coagulation activity available, and thus the results are presented as ranges for this group. Kruskal-Wallis test with post-hoc Dunn's analysis was used to compare the TG and laboratory data between the patient groups and SHP and the dose-dependent effect of CA 19-9 on TG. The effect of coagulation factor deficient plasmas was analyzed both with Kruskal-Wallis test using post-hoc Dunne analysis, and each plasma separately with the reference plasma by using the Mann-Whitney U test. The influence of anti-TF and anti-TFPI antibody and PLA2 were compared with control plasma using the Mann-Whitney U test. Correlations between the biomarkers and thrombin generation values were studied using with Spearman's correlation coefficient (rho). Patients were not followed postoperatively in this study.

RESULTS

CA 19-9 and thrombin generation in treatment-naïve PDAC patients

We determined if there was an association between CA 19-9 and TG variables by analyzing them in all patient plasma samples. We found that CA 19-9 levels positively correlated with both peak TG and ETP (Table 2, rho=0.315, p=0.016 and rho=0.301, p=0.022, respectively).

Table 2. Correlations between CA 19-9 and thrombin generation

		Lag time	ETP	Peak TG	TTP
CA 19-9	Rho	-0.203	.301*	.315*	-0.257
	p-value	0.126	0.022	0.016	0.052
	N	58	58	58	58

*. Correlation is significant at the 0.05 level (2-tailed).

ETP=endogenous thrombin potential
TG=thrombin generation
TTP=time to peak

To analyze the effect of CA 19-9 on TG in more detail, patients were divided into 4 groups based on their different CA 19-9 concentrations: none<2 (n=4), low=3-200 (n=32), high=201-1000 (n=13), and very high>1000 U/mL (n=9) and all groups were compared with each other and with the control plasma, SHP.

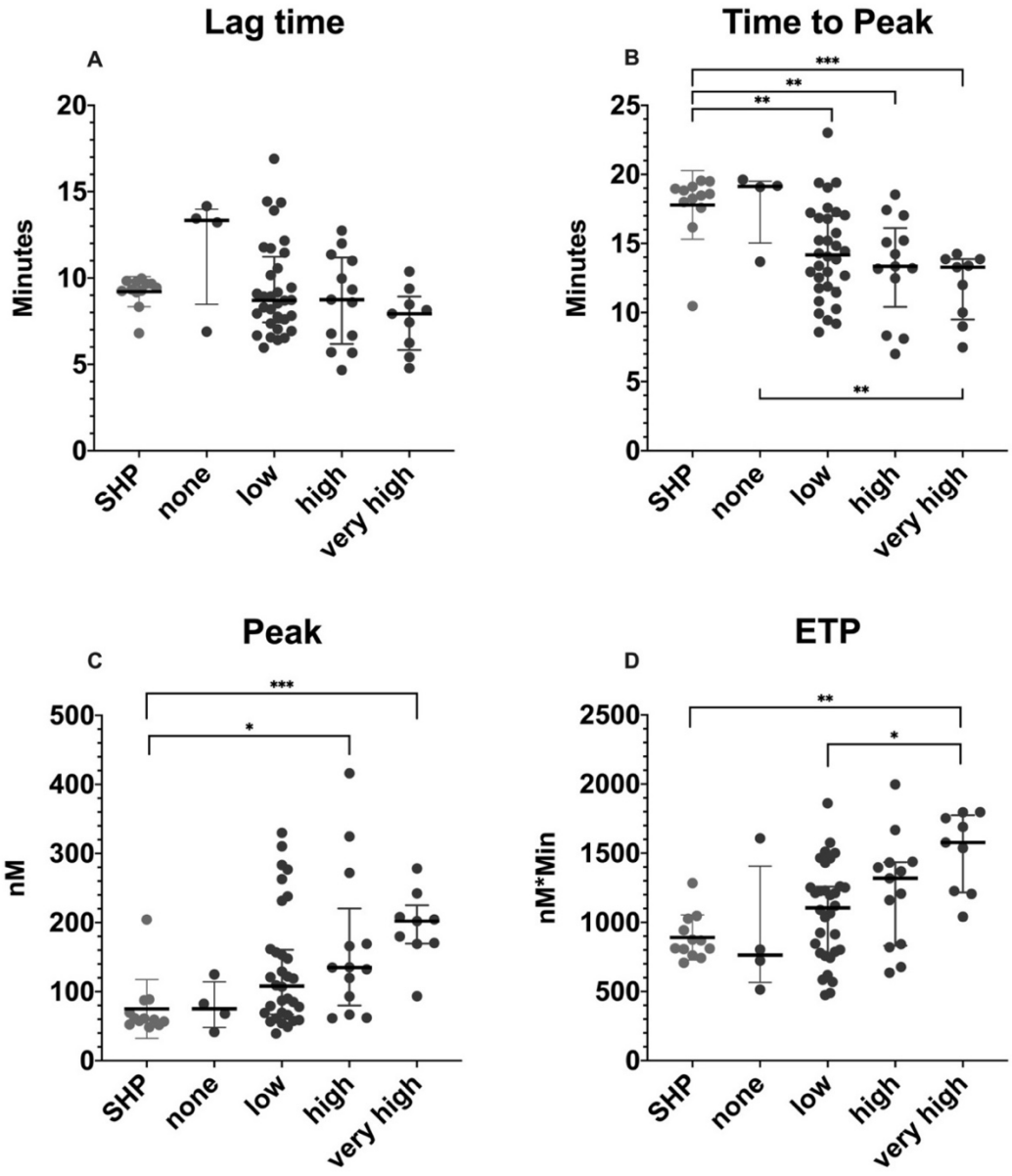

Figure 1. Thrombin generation variables in PDAC patient plasma. *A) Lag time, B) Time to peak, C) Peak, D) ETP. The reagent PPP low (1 pM TF and 4 µM PL) was used as the activator. CA 19-9 groups represent patients with the ranges: none<2 U/mL, low=3-200 U/mL, high=201-1000 U/mL, very high>1000 U/mL. No patients had values 201-299, 974-1738 or above 66357. Each dot represents the mean of each repeated measurement of a single patient. Data are shown as median ± interquartile range. The statistical difference was tested with Kruskall-Wallis test. ETP=endogenous thrombin potential, PDAC=pancreatic ductal adenocarcinoma, PL=phospholipids, PPP=platelet-poor plasma, SHP=standard human plasma, TF=tissue factor. *p<0.05, **p<0.01, *** p<0.001*

All the TG variables, excluding lag time (Fig 1A), were associated with the elevated CA 19-9 levels (Fig 1B-D). Compared with SHP, TTP was shorter in all the CA 19-9 groups, except the group without CA 19-9 (Fig. 1B). Peak TG showed the greatest association and was over 2-fold higher in patients with very

high and high CA 19-9 levels than the controls (3.4-fold and 2.2-fold, respectively, Fig. 1C). Finally, ETP was 1.9-fold higher in the patients with very high CA 19-9 compared with SHP (Fig. 1D). When the different CA 19-9 groups were compared with each other, patients with the very high CA 19-9 had a 1.4-fold shorter TTP than those without measurable CA 19-9 (Fig. 1B) and 1.4-fold higher ETP levels than those with low CA 19-9 (Fig. 1D).

Coagulation biomarkers and CA 19-9

Median FVIII and fibrinogen levels were higher than the normal reference limits in all CA 19-9 groups (Table 3).

Table 3. Levels of coagulation biomarkers in PDAC patients with different levels of CA 19-9

	None		Low		High		Very high		Normal values
	n	Range	n	Median (IQR)	n	Median (IQR)	n	Median (IQR)	
Antithrombin (%)	2	116-122	32	102 (93-112)	13	102 (93-126)	7	101 (84-108)	85-125
FVIII (IU/L)	2	132-227	32	168 (130-231)	13	198 (177-262)	7	167 (152-225)	60-160
Fibrinogen (g/L)	2	4.4-5.0	32	4.3 (3.5-5.2)	13	4.5 (3.3-5.2)	7	4.3 (3.6-4.6)	2.0-4.0
D-dimer (mg/L)	2	<0.2-0.4	32	0.4 (0.3-0.7)	13	0.4 (0.2-0.8)	7	0.4 (0.3-0.6)	<0.5
EV TF activity (pg/mL)	2	0.2-0.3	31	0.4 (0.3-0.6)	13	0.4 (0.2-0.6)	7	0.5 (0.4-0.9)	0.05-0.26

EV=extracellular vesicle, TF=tissue factor, IQR=interquartile range

Likewise, EV TF activity was above the reported reference values of healthy controls[35] in all groups except for the none group (Table 3). D-dimer levels, on the other hand, were within the normal reference values in all CA 19-9 groups. Median antithrombin levels were also within the range of the reference values (Table 3). Levels of CA 19-9, FVIII, fibrinogen, antithrombin, D-dimer and EV TF or the TG variables between patients with resectable or non-resectable disease did not differ (data not shown).

To study the possible contributing or confounding effects of coagulation activity by any other coagulation biomarkers in the different CA 19-9 groups, we compared the levels of the various biomarkers in the four CA 19-9 groups. However, no correlations between CA 19-9 and the coagulation biomarkers were found (Table 4).

Table 4. Correlations of thrombin generation and CA 19-9 with coagulation biomarkers

		EV TF activity	Antithrombin	FVIII	Fibrinogen	D-dimer
CA 19-9	Rho	0.105	-0.101	0.106	-0.125	0.033
	P-value	0.456	0.466	0.444	0.368	0.815
	N	53	54	54	54	54
Lag time	Rho	**-.288***	**.342***	**.288***	**.389****	-0.054
	P-value	**0.037**	**0.011**	**0.035**	**0.004**	0.699
	N	53	54	54	54	54
ETP	Rho	0.095	**-.409****	-0.102	-0.045	0.165
	P-value	0.499	**0.002**	0.464	0.749	0.234
	N	53	54	54	54	54
Peak TG	Rho	0.110	**-.501****	-0.075	-0.185	0.171
	P-value	0.434	**0.000**	0.591	0.180	0.217
	N	53	54	54	54	54
TTP	Rho	-0.197	**.399****	0.252	**.435****	-0.047
	P-value	0.156	**0.003**	0 066	**0.001**	0.736
	N	53	54	54	54	54

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

ETP=endogenous thrombin potential, TG=thrombin generation, TTP=time to peak, EV=extracellular vesicle, TF=tissue factor

Correlation among plasma levels of coagulation biomarkers and thrombin generation variables

To determine if other coagulation biomarkers contributed to TG activity, we analyzed the correlations between the TG variables and antithrombin, FVIII, fibrinogen, D-dimer and EV TF levels. As expected, antithrombin showed a clear correlation with TG variables. Lag time and TTP correlated positively with antithrombin, while ETP and peak TG showed negative correlation (Table 4, $p<0.05$). In contrast, as expected, EV TF showed a negative correlation with the lag time (Table 4).

Effect of exogenous CA 19-9 on thrombin generation

To assess if CA 19-9 enhances TG, we analyzed six commercial CA 19-9 preparations on TG using SHP. After evaluating the effect of adding each preparation in the CAT assay, two of the six preparations, both from colon adenocarcinoma cell lines, showed increased TG when spiked into SHP compared with SHP alone (Supplement Fig. 1).

Dose-dependent effect of the CA 19-9 preparation on thrombin generation

We explored the CA 19-9 preparation MBS537105 from a colon adenocarcinoma cell line in more detail as it expressed the highest TG potential. To determine if the CA 19-9 preparation enhanced TG in a standardized setting, we first added increasing concentrations of the CA 19-9 preparation to SHP with 1 pM TF as the activator (PPP-Reagent LOW). The CA 19-9 preparation dose-dependently increased TG when added to SHP (Fig. 2). A high concentration of CA 19-9 (900 U/mL) decreased lag time, as well as increased peak thrombin, and TTP and ETP compared with SHP ($p<0.05$).

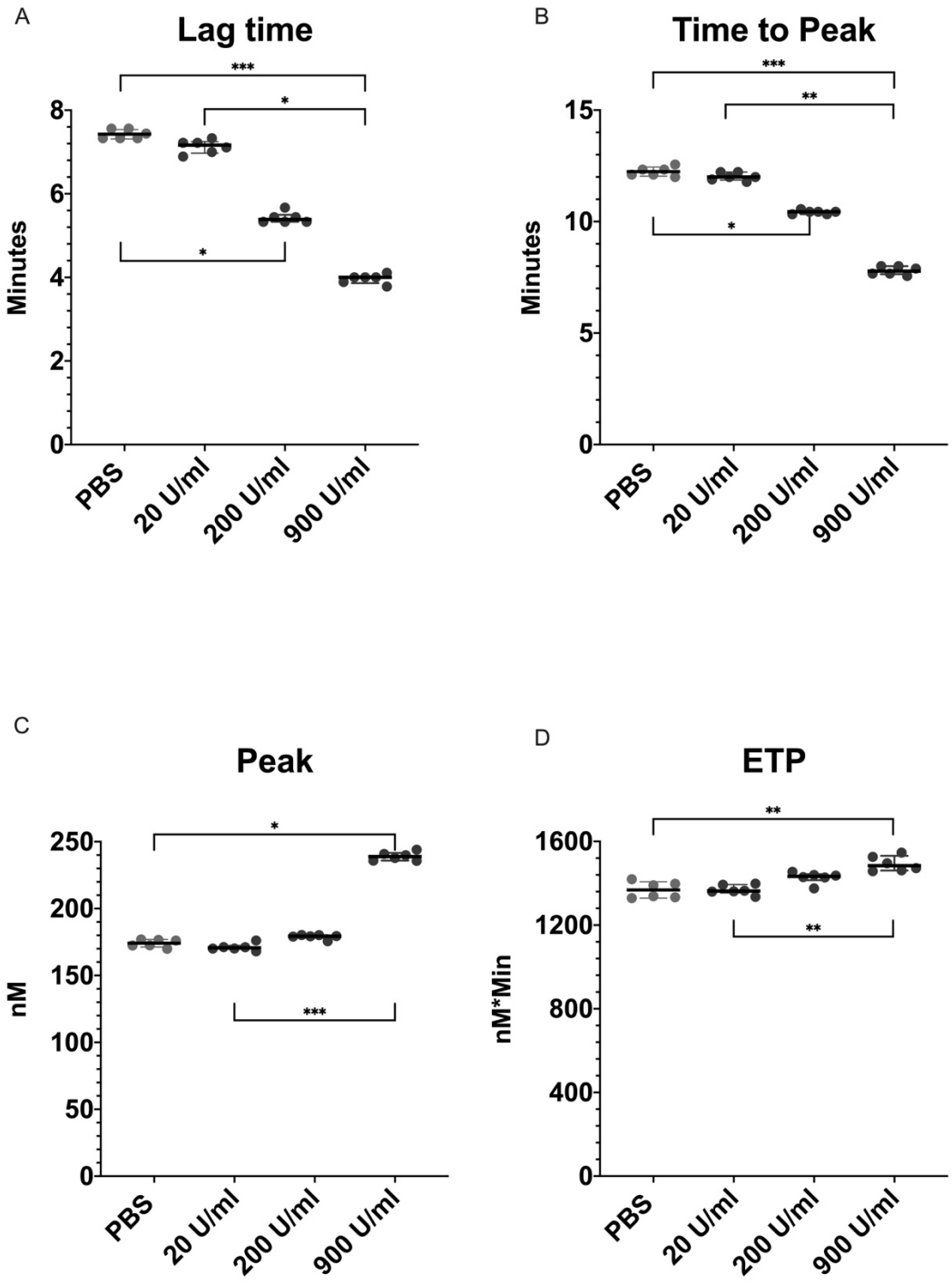

Figure 2. In vitro dose-dependent impact of CA 19-9 on thrombin generation when spiked to SHP in the presence of TF. *A) Lag time, B) Time to peak, C) Peak, D) ETP. CA 19-9 (source, at 20-900 U/mL concentration) was added to SHP and PPP-Reagent LOW with 1 pM TF and 4 μM PL was used as the activator. The dots represent triplicate measurements in the same setting, the line represents median and whiskers the interquartile range. ETP=endogenous thrombin potential, PL=phospholipids, PPP=platelet-poor plasma, SHP=standard human plasma, TF=tissue factor. *p<0.05, **p<0.01, *** p<0.001*

CA 19-9 preparation alone as an activator of thrombin generation *in vitro*

Since the CA 19-9 preparation had a clear effect on TG *in vitro* in the presence of PL and TF, we next determined if the CA 19-9 preparation alone would initiate thrombin generation in plasma. First, we performed CAT analysis using the different commercial activators available (PPP-Reagent, PPP-Reagent LOW, PRP-Reagent and MP-Reagent) and compared the results with CA 19-9 at 900 U/ml without added TF or PL and PBS as a control. PRP-Reagent was chosen to assess the role of PL as we only used plasma (PPP) as the source of TG. MP-Reagent on the other hand, triggers TG without exogenous TF.

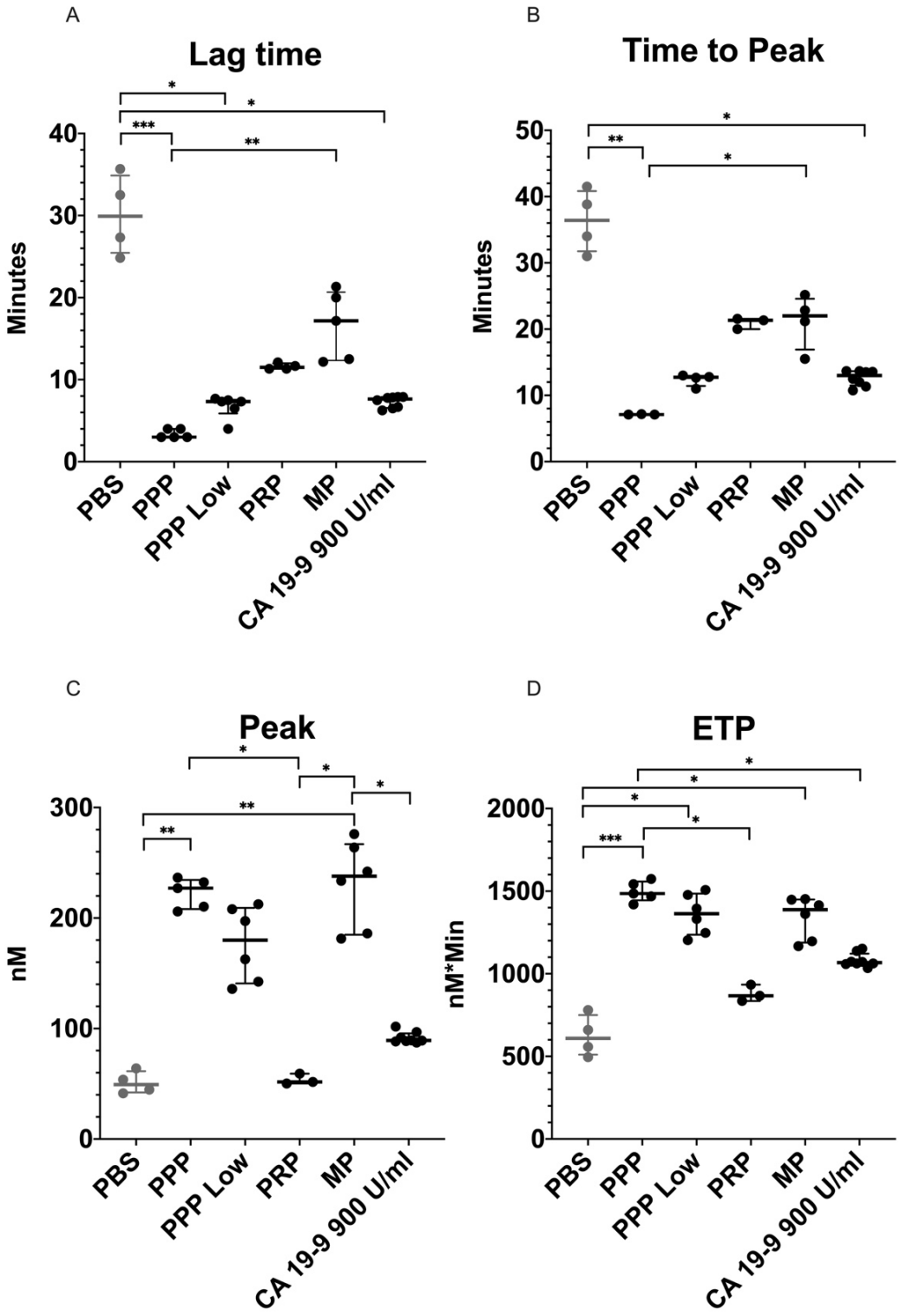

Figure 3. Thrombin generation comparing CA 19-9 as an activator with different commercial activators. *A) Lag time, B) Time to peak, C) Peak, D) ETP. CA 19-9 antigen MBS537105 from MyBioSource was used. The activator reagents introduced in the x-axis were: PBS (n=6), PPP-Reagent (high concentration, 5 pM, of TF and 4 µM PL, n=5), PPP-Reagent LOW (low concentration, 1 pM, of TF and 4 µM PL, n=6), PRP-Reagent (TF without PL, n=3), MP-Reagent (without any TF) and the CA 19-9 at 900 U/ml alone. The dots represent the means of triplicate measurements, the line represents median and whiskers the interquartile range. Statistical differences were analyzed using Kruskal-Wallis test with post-hoc Dunn analysis. ETP=endogenous thrombin potential, MP=microparticle, PL=phospholipids, PPP=platelet-poor plasma, PRP=platelet rich plasma, SHP=standard human plasma, TF=tissue factor. *p<0.05, **p<0.01, *** p<0.001*

CA 19-9 preparation alone shortened lag time and TTP compared with PBS alone ($p<0.05$), but the CA 19-9 preparation and PBS did not differ in their ETP and peak TG values (Fig. 3A-D).

To further analyze the effect of CA 19-9 alone on TG and determine if the effect was concentration-dependent we compared TG in the presence of the CA 19-9 preparation without added TF at three concentrations (200, 900 and 9000 U/mL) or PBS alone (Fig. 4A-D). The CA 19-9 preparation alone acted as an activator of TG in the CAT assay, and the effect was concentration-dependent (Fig. 4A-D). The lag time and TTP were shortest and peak the highest at the highest concentration of the CA 19-9 preparation tested (9000 U/mL) compared with CA 19-9 at 200 U/ml or PBS. There was no concentration-dependent relationship for the ETP.

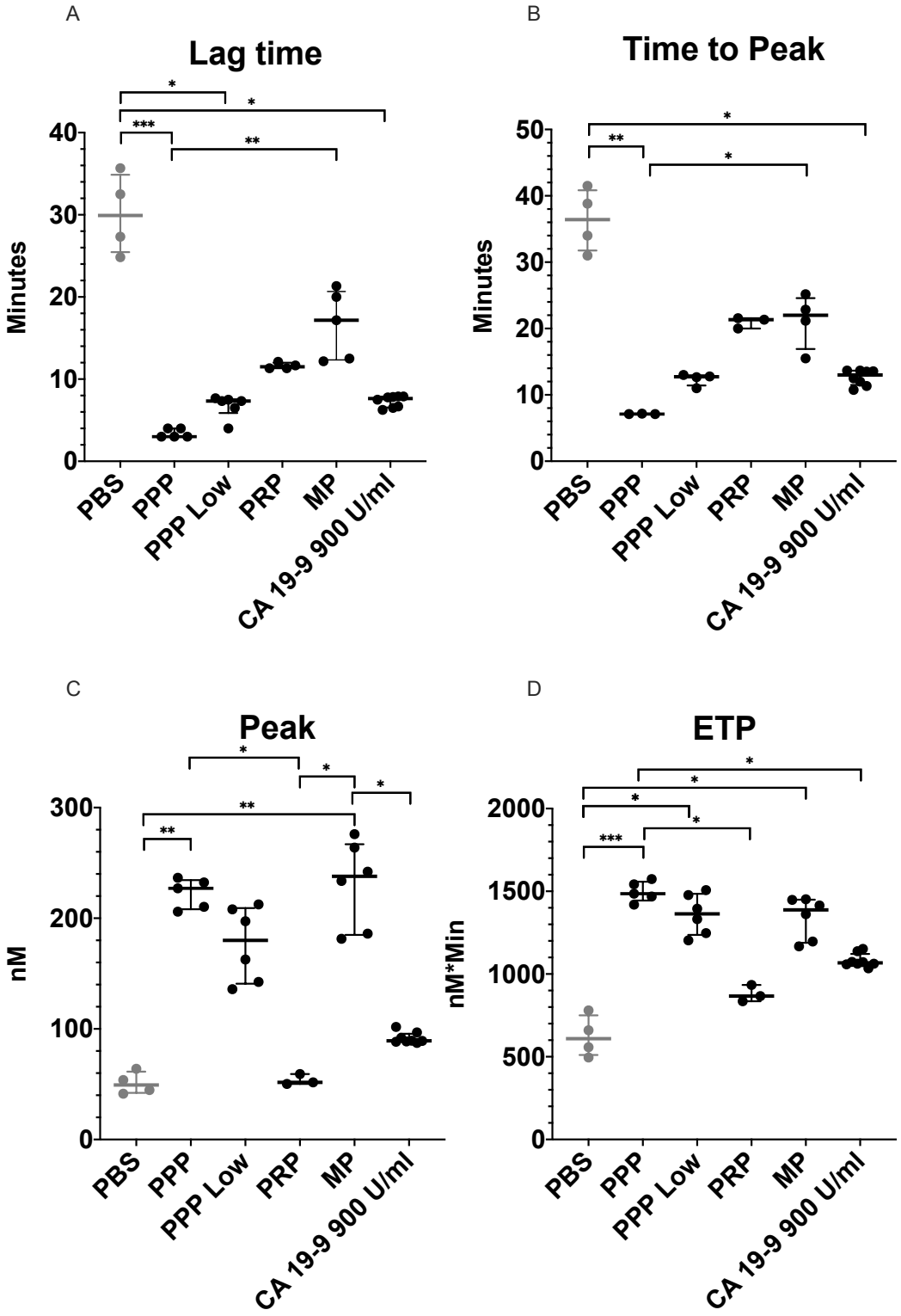

Figure 4. In vitro dose-dependent effect of CA 19-9 as an activator of thrombin generation without added TF in SHP*. A) Lag time, B) Time to peak, C) Peak, D) ETP. CA 19-9 antigen MBS537105 from MyBioSource was used. SHP was used as the plasma. CA 19-9 at different concentrations was used alone as the activator. The dose-dependent relationship of CA 19-9 on TG was analyzed. PBS without added TF was used as a control. The dots represent the means of triplicate measurements, the line represents median and whiskers the interquartile range. Statistical differences were analyzed using Kruskal-Wallis test with post-hoc Dunn analysis. ETP=endogenous thrombin potential, PBS=phosphate buffered saline, SHP=standard human plasma, TF=tissue factor. *p<0.05, **p<0.01.*

Effect of the CA 19-9 preparation as an activator of thrombin generation in coagulation factor-deficient plasmas

To better characterize the TG triggered by the CA 19-9 preparation, we studied five different coagulation factor (FVII, FVIII, FIX, FXI and FXII) deficient plasmas. To study the intrinsic activation route, we activated FIX, FXI and FXII deficient plasmas (Fig. 5 A-D) with the CA 19-9 preparation.

The peak TG and ETP values were lower in all three plasmas compared with SHP, without differences in the lag time or TTP. In FVIII-deficient plasma peak TG and ETP decreased, and both the lag time and TTP were prolonged compared to SHP. The most prominent difference, however, was observed in FVII-deficient plasma in which TG clearly diminished, depicted in all CAT parameters (Fig. 5 A-D).

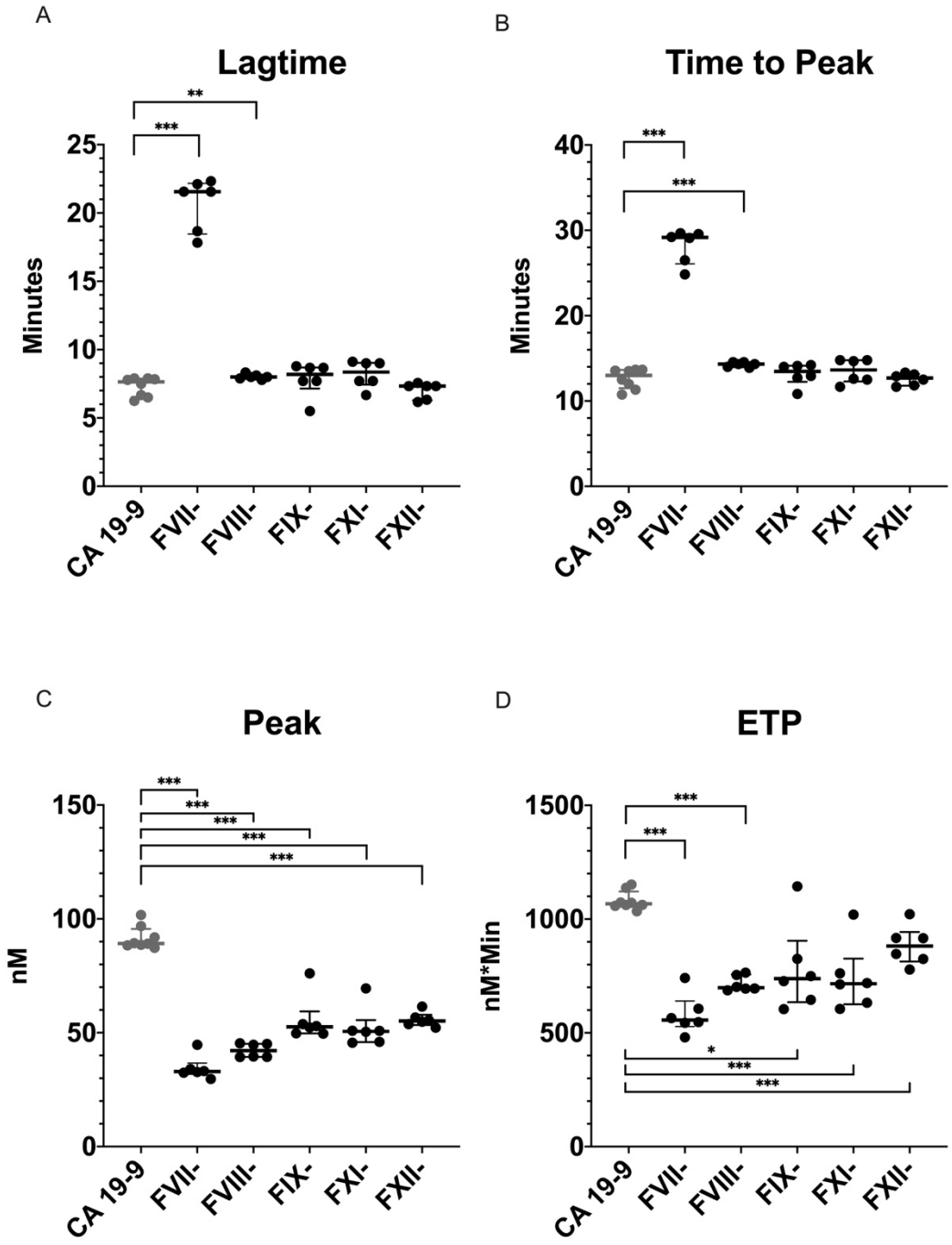

Figure 5. In vitro triggered thrombin generation with the CA 19-9 preparation alone (900 U/mL) in different coagulation factor deficient plasmas*. A) Lag time, B) Time to peak, C) Peak, D) ETP. CA 19-9 antigen MBS537105 from MyBioSource was used. Coagulation factor (F) VII, VIII, IX, XI and XII plasmas were used. Dots are the means of triplicate measurements, the lines are median and error bars are interquartile ranges. Each plasma was compared with SHP using Mann-Whitney U analysis. ETP=endogenous thrombin potential, SHP=standard human plasma *p<0.05, **p<0.01, *** p<0.001*

Effect of an anti-TF antibody, an anti-TFPI antibody and PLA2 on thrombin generation initiated by the CA 19-9 preparation

In contrast to the data with CA 19-9 in plasma from PDAC patients, the exogenous CA 19-9 preparation shortened the lag time of the TG. Since TF is known to shorten the lag time of the TG[36], we determined if the CA 19-9 preparation contained functional TF by treating the CA 19-9 preparation with an anti-TF antibody prior to CAT analysis. The presence of the anti-TF antibody prolonged the lag time of TG and TTP, as well as decreased the peak TG and ETP triggered by the CA19-9 preparation (Fig. 6A-D, $p<0.001$). However, an anti-TFPI antibody did not influence the thrombin potential of CA 19-9 alone (data not shown).

To study if the CA 19-9 antigen carried cancer-cell derived phospholipids, which could enhance the coagulation potential, we treated the CA 19-9 antigen with PLA2 to degrade the phospholipids prior to the CAT analysis (Fig. 6A-D). Indeed, PLA2 at 0.1 U/mL decreased the peak TG ($p<0.001$) but did not impact the other CAT variables, when CA 19-9 was used as the sole activator.

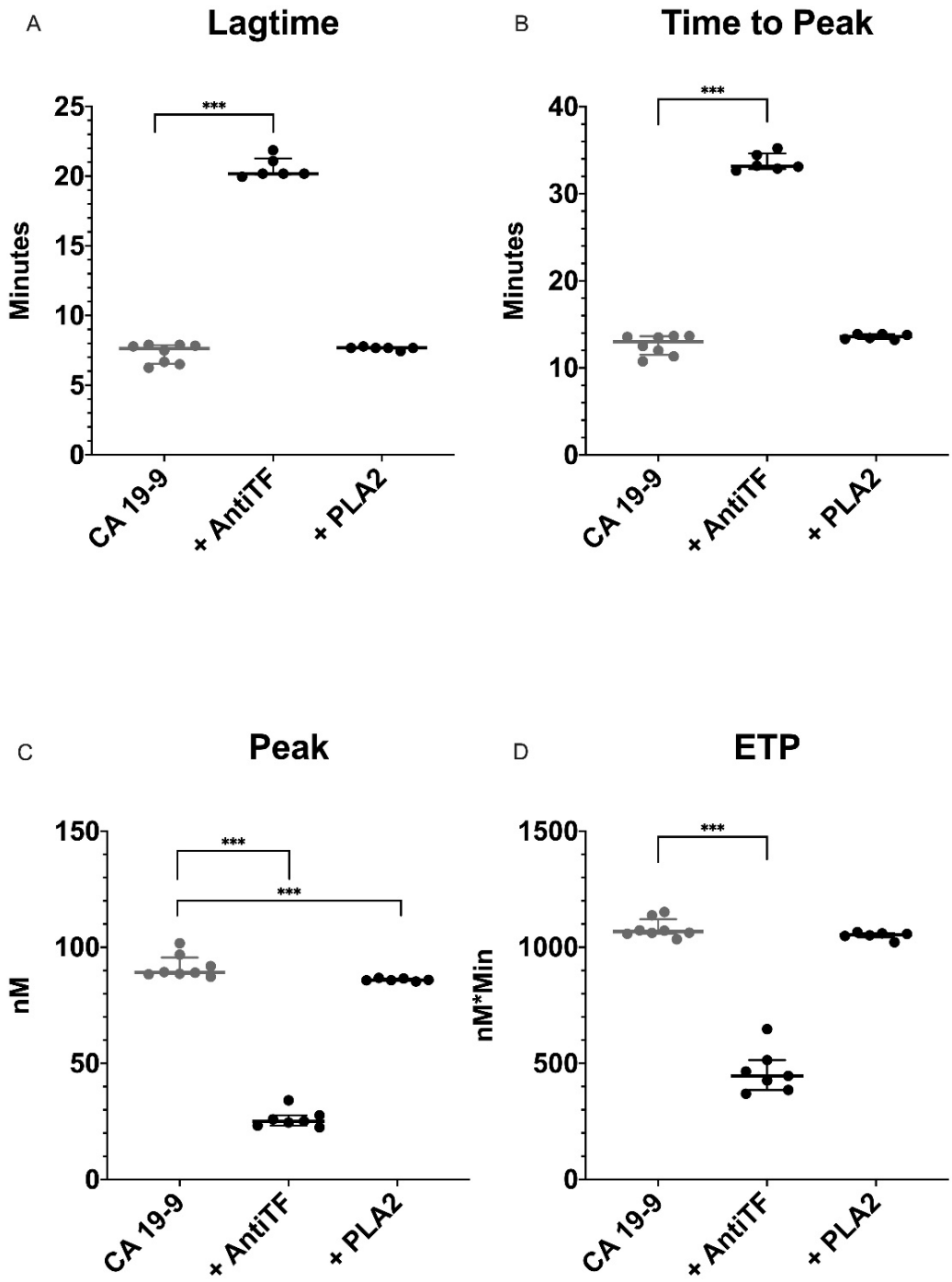

Figure 6 Effect of anti-TF antibody (10 ug/mL) and phospholipase A2 (PLA2) (0.1 U/mL) on thrombin generation stimulated by CA19-9 without added TF. *A) Lag time, B) Time to peak, C) Peak, D) ETP.CA 19-9 antigen MBS537105 from MyBioSource was used. CA 19-9 was incubated with either anti-TF or PLA2 at room temperature for one hour and the effect on CA 19-9 induced TG was analyzed by comparison with CA 19-9 alone. Each dot represents the mean of a triplicate measurement, the line represents median and error bars interquartile ranges. Mann Whitney U test was used for statistical comparison. ETP=endogenous thrombin potential, PLA2=phospholipase A2, TF=tissue factor, TG=thrombin generation. *p<0.05, **p<0.01, *** p<0.001*

DISCUSSION

In this study, we analyzed the association between the tumor marker CA 19-9 and TG in treatment-naïve PDAC 1-3 days prior to surgery. A concentration-dependent effect of CA 19-9 on TG in plasma was observed in patient samples. Some commercial CA 19-9 preparations also generated thrombin, but this was due to functional TF contamination of the preparation rather that CA 19-9 itself.

CA 19-9 has been shown to rise during disease progression[37-39], and thus it is used routinely for the follow-up of PDAC. We chose the cut-off values for the CA 19-9 groups of the patients based on the previously reported predictive cut-off limits of CA 19-9 of approximately 200 U/mL pre- and postoperatively on advanced stage and 1000 U/mL on poor prognosis[38], with the addition of the group who are proposed to be sialyl Lewis a negative as they do not secrete measurable amounts of CA 19-9. A clear dose-dependent effect of CA 19-9 on TG was observed *ex vivo* from patient samples, as patients with high CA 19-9 expressed more TG than those with low CA 19-9. This effect did not relate to disease burden, as the CA 19-9 levels did not differ between patients with resectable or non-resectable disease.

A dose-dependent response on TG was also seen using a CA 19-9 preparation *in vitro.* However, the TG enhancing effect of the CA 19-9 preparation *in vitro* was inhibited in the presence of anti-TF antibody and in FVII-deficient plasma, suggesting that the CA 19-9 antigen contains functional TF. Novakovic and Gilbert recently found that the procoagulant effect of purified skeletal and cardiac muscle myosin appeared to be due to contaminating TF [40]. The fact that the CA 19-9 preparation contains TF confounds the interpretation of the results with these CA 19-9 preparations.

Tumor cells of solid tumors, especially adenocarcinomas such as PDAC, are known to release TF-positive EVs into the circulation[41, 42]. EV TF activity is associated with VTE in PDAC, but not in other forms of cancer, such as brain, colorectal, gastric and ovarian cancer[9, 12, 13, 43, 44]. Here we show that, in the absence of VTE, EV TF activity negatively correlated with lag time, which is consistent with the role of TF in the initiation phase of TG. EV TF has been linked with CA 19-9 as Thaler *et al* showed a strong correlation between EV TF and CA 19-9[45] and another study found a weak but significant correlation between CA 19-9 and EV TF activity (r=0.44, P=0.00011) (Dr. Woei-A-Jin personal communication). We did not observe a correlation between the CA 19-9 levels and EV TF activity in the patient plasmas. However, the above studies are not comparable with our study since we included only treatment-naïve patients, whereas the Thaler study included patients who had undergone chemo- and radiation therapies prior to inclusion.

Mucins, which may contain CA 19-9, can increase coagulation activity by activating platelets through selectins, especially P-selectin[23, 46]. Platelets possess heparinase activity, and it has been shown that increased heparinase activity promotes TF expression[47]. As mucins increase coagulation activity through platelets, this may provide a link between CA 19-9 and TF. When the CA 19-9 preparation alone was analyzed in TG, PLs within the CA 19-9 preparation were involved in enhancing the thrombin peak because PLA2 reduced the peak triggered with the CA 19-9 preparation alone. In the *ex vivo* patient analysis, however, we used PPP, largely eliminating the role of platelets, and thus the TG potential of CA 19-9 suggests additional plasma-derived thrombin inducing properties. However, our plasma was centrifuged only once, which may allow residual platelets or their remnants to be left in the plasma and confound the PL-dependent TG.

The only variables correlating with the peak TG and ETP were CA 19-9 and antithrombin. The fact that antithrombin levels had a negative correlation with both peak TG and ETP, as well as a positive correlation with lag time and TTP may be expected but emphasizes the importance of the natural anticoagulant antithrombin to TG. FVIII is observed to increase in PDAC and especially in metastasized disease[15, 17, 48]. This was also evident in the patients included in this study, as the median FVIII levels were above the reference value in all CA 19-9 groups. The levels of FVIII and fibrinogen correlated with the initiation phase of TG, however, they did not correlate with the CA 19-9 levels. Elevated FVIII levels have been linked to increased coagulation activity in PDAC in various states of chemotherapy and other cancer treatments, similar to D-dimer, and also to VTE[16, 49-53].

Increased levels of CA 19-9 have also been reported to be associated with VTE. Peippo *et al* reported that the exponential increase of CA 19-9 was associated with developing VTE[54], and Woei *et al* reported that patients with VTE have higher CA 19-9 levels than those without[14]. Faille *et al* also reported an association between higher levels of CA 19-9 and VTE occurrence[55].. We collected the blood samples prior to any interventions and the absence of symptomatic VTE. The time of blood collection in relation to the timing of diagnosis in the Faille study was not defined. Frere *et al*, on the other hand, investigated the role of biomarkers on VTE and did not find an association between CA 19-9 and VTE in PDAC[6]. However, in their study the CA 19-9 levels were measured when patients were included in the study and not before the VTE diagnosis, possibly allowing the association to diminish. Although we did not investigate VTE, our data showing an association between CA 19-9 and TG support the notion that higher levels of CA 19-9 may increase the risk of VTE in PDAC patients.

Ay *et al.* showed that increased TG is a risk factor for VTE in cancer patients[56]. Hence, our findings, along with the previous studies on CA 19-9 and VTE[14, 54, 55] propose that CA 19-9 values gathered from PDAC patients during their follow-up could be used as a marker for increased thrombotic activity and thus perhaps thrombotic risk integrated to the established risk scores, such as the Khorana score[57]. However, as our patients were treatment naïve, we would like to point out that PDAC itself, without clinical confounders of chemotherapy or recent surgery, is associated with TF-related coagulation activity. The patients were devoid of signs of VTE prior to the cancer surgery or 3 months afterwards, of which time the first month was covered with thromboprophylactic low-molecular weight heparin.

A limitation of our study is the inherent variability of the CAT assay. We observed a CVof around 20% for all measurements. We minimized this variability by performing measurements in duplicate and triplicate. This variability may be due, in part, to the contribution of unknown amounts of EVs in the samples, as the centrifugation steps of the patient plasma handling followed the routine clinical chemistry practice and were not performed according to the guidance of EV studies[58]. Another limitation was the small patient numbers in each group after dividing the patients into groups according to their CA 19-9 levels. Also, in patient samples, multiple other confounding factors, such as medications and comorbidities, may influence TG. To minimize the confounding effects, these PDAC patients were all treatment-naïve. Indeed, the samples were collected in a standardized fashion a couple of days before the operation without overt thrombosis or influence of anticoagulants, previous radiation or chemotherapy, providing strength to our study.

In conclusion, CA 19-9 levels were concentration-dependently associated with increased TG in blood samples obtained from treatment-naïve PDAC patients a couple of days prior to surgery.

Further studies are warranted to assess if CA 19-9 could be used as a biomarker to identify PDAC patients at greater risk of cancer progression via TG, and perhaps benefit from targeted anticoagulant treatments. Interestingly, we found that some commercial CA 19-9 preparations contained functional TF.

ACKNOWLEDGEMENTS

This work was supported by grants from the Finnish Governmental Funding (VTR), The Heart Research Foundation, the Sigrid Jusélius Foundation, the Cancer Foundation Finland, Inkeri and Mauri Vänskä foundation, Japanese Society on Thrombosis and Hemostasis (Y.H.), National Institutes of Health (Y.H. T32 HL007149 and N.M. R01 HL147149), and the John C. Parker Professorship (N.M.). None of the foundations were involved in the study design.

REFERENCES

1. Epstein AS, Soff GA, Capanu M, et al. Analysis of incidence and clinical outcomes in patients with thromboembolic events and invasive exocrine pancreatic cancer. *Cancer* 2012; 118: 3053-3061. 2011/10/13. DOI: 10.1002/cncr.26600.
2. Ishigaki K, Nakai Y, Isayama H, et al. Thromboembolisms in Advanced Pancreatic Cancer: A Retrospective Analysis of 475 Patients. *Pancreas* 2017; 46: 1069-1075. 2017/08/09. DOI: 10.1097/MPA.0000000000000889.
3. Blom JW, Osanto S and Rosendaal FR. High risk of venous thrombosis in patients with pancreatic cancer: a cohort study of 202 patients. *Eur J Cancer* 2006; 42: 410-414. 2005/12/03. DOI: 10.1016/j.ejca.2005.09.013.
4. Ouaissi M, Frasconi C, Mege D, et al. Impact of venous thromboembolism on the natural history of pancreatic adenocarcinoma. *Hepatobiliary Pancreat Dis Int* 2015; 14: 436-442. 2015/08/11. DOI: 10.1016/s1499-3872(15)60397-6.
5. Menapace LA, Peterson DR, Berry A, et al. Symptomatic and incidental thromboembolism are both associated with mortality in pancreatic cancer. *Thromb Haemost* 2011; 106: 371-378. 2011/06/30. DOI: 10.1160/TH10-12-0789.
6. Frere C, Bournet B, Gourgou S, et al. Incidence of Venous Thromboembolism in Patients with Newly Diagnosed Pancreatic Cancer and Factors Associated With Outcomes. *Gastroenterology* 2019 2019/12/18. DOI: 10.1053/j.gastro.2019.12.009.
7. Posch F, Riedl J, Reitter EM, et al. Hypercoagulabilty, venous thromboembolism, and death in patients with cancer. A Multi-State Model. *Thromb Haemost* 2016; 115: 817-826. 2016/01/08. DOI: 10.1160/TH15-09-0758.
8. Petterson TM, Marks RS, Ashrani AA, et al. Risk of site-specific cancer in incident venous thromboembolism: a population-based study. *Thromb Res* 2015; 135: 472-478. 2014/12/31. DOI: 10.1016/j.thromres.2014.12.013.
9. Thaler J, Ay C, Mackman N, et al. Microparticle-associated tissue factor activity, venous thromboembolism and mortality in pancreatic, gastric, colorectal and brain cancer patients. *J Thromb Haemost* 2012; 10: 1363-1370. 2012/04/24. DOI: 10.1111/j.1538-7836.2012.04754.x.
10. Zwicker JI, Liebman HA, Neuberg D, et al. Tumor-derived tissue factor-bearing microparticles are associated with venous thromboembolic events in malignancy. *Clin Cancer Res* 2009; 15: 6830-6840. 2009/10/29. DOI: 10.1158/1078-0432.CCR-09-0371.
11. Tesselaar ME, Romijn FP, Van Der Linden IK, et al. Microparticle-associated tissue factor activity: a link between cancer and thrombosis? *J Thromb Haemost* 2007; 5: 520-527. 2006/12/15. DOI: 10.1111/j.1538-7836.2007.02369.x.
12. Khorana AA, Francis CW, Menzies KE, et al. Plasma tissue factor may be predictive of venous thromboembolism in pancreatic cancer. *J Thromb Haemost* 2008; 6: 1983-1985. 2008/09/18. DOI: 10.1111/j.1538-7836.2008.03156.x.
13. Bharthuar A, Khorana AA, Hutson A, et al. Circulating microparticle tissue factor, thromboembolism and survival in pancreaticobiliary cancers. *Thromb Res* 2013; 132: 180-184. 2013/07/17. DOI: 10.1016/j.thromres.2013.06.026.
14. Woei AJFJ, Tesselaar ME, Garcia Rodriguez P, et al. Tissue factor-bearing microparticles and CA19.9: two players in pancreatic cancer-associated thrombosis? *Br J Cancer* 2016; 115: 332-338. 2016/07/13. DOI: 10.1038/bjc.2016.170.
15. Mattila N, Seppanen H, Mustonen H, et al. Preoperative Biomarker Panel, Including Fibrinogen and FVIII, Improves Diagnostic Accuracy for Pancreatic Ductal Adenocarcinoma. *Clin Appl Thromb Hemost* 2018; 24: 1267-1275. 2018/06/06. DOI: 10.1177/1076029618779133.

16. Vormittag R, Simanek R, Ay C, et al. High factor VIII levels independently predict venous thromboembolism in cancer patients: the cancer and thrombosis study. *Arterioscler Thromb Vasc Biol* 2009; 29: 2176-2181. 2009/09/26. DOI: 10.1161/ATVBAHA.109.190827.
17. Guo Q, Zhang B, Dong X, et al. Elevated levels of plasma fibrinogen in patients with pancreatic cancer: possible role of a distant metastasis predictor. *Pancreas* 2009; 38: e75-79. 2009/03/12. DOI: 10.1097/MPA.0b013e3181987d86.
18. Scara S, Bottoni P and Scatena R. CA 19-9: Biochemical and Clinical Aspects. *Adv Exp Med Biol* 2015; 867: 247-260. 2015/11/05. DOI: 10.1007/978-94-017-7215-0_15.
19. Dong Q, Yang XH, Zhang Y, et al. Elevated serum CA19-9 level is a promising predictor for poor prognosis in patients with resectable pancreatic ductal adenocarcinoma: a pilot study. *World J Surg Oncol* 2014; 12: 171. 2014/06/04. DOI: 10.1186/1477-7819-12-171.
20. Magnani JL, Steplewski Z, Koprowski H, et al. Identification of the gastrointestinal and pancreatic cancer-associated antigen detected by monoclonal antibody 19-9 in the sera of patients as a mucin. *Cancer Res* 1983; 43: 5489-5492. 1983/11/01.
21. Yue T, Goldstein IJ, Hollingsworth MA, et al. The prevalence and nature of glycan alterations on specific proteins in pancreatic cancer patients revealed using antibody-lectin sandwich arrays. *Mol Cell Proteomics* 2009; 8: 1697-1707. 2009/04/21. DOI: 10.1074/mcp.M900135-MCP200.
22. Hollingsworth MA and Swanson BJ. Mucins in cancer: protection and control of the cell surface. *Nat Rev Cancer* 2004; 4: 45-60. 2003/12/19. DOI: 10.1038/nrc1251.
23. Shao B, Wahrenbrock MG, Yao L, et al. Carcinoma mucins trigger reciprocal activation of platelets and neutrophils in a murine model of Trousseau syndrome. *Blood* 2011; 118: 4015-4023. 2011/08/24. DOI: 10.1182/blood-2011-07-368514.
24. Varki A. Trousseau's syndrome: multiple definitions and multiple mechanisms. *Blood* 2007; 110: 1723-1729. 2007/05/15. DOI: 10.1182/blood-2006-10-053736.
25. Pineo GF, Regoeczi E, Hatton MW, et al. The activation of coagulation by extracts of mucus: a possible pathway of intravascular coagulation accompanying adenocarcinomas. *J Lab Clin Med* 1973; 82: 255-266. 1973/08/01.
26. Tripodi A. Thrombin Generation Assay and Its Application in the Clinical Laboratory. *Clin Chem* 2016; 62: 699-707. 2016/03/10. DOI: 10.1373/clinchem.2015.248625.
27. Hemker HC, Al Dieri R, De Smedt E, et al. Thrombin generation, a function test of the haemostatic-thrombotic system. *Thromb Haemost* 2006; 96: 553-561. 2006/11/03.
28. Lance MD. A general review of major global coagulation assays: thrombelastography, thrombin generation test and clot waveform analysis. *Thromb J* 2015; 13: 1. 2015/05/06. DOI: 10.1186/1477-9560-13-1.
29. Eichinger S, Hron G, Kollars M, et al. Prediction of recurrent venous thromboembolism by endogenous thrombin potential and D-dimer. *Clin Chem* 2008; 54: 2042-2048. 2008/10/25. DOI: 10.1373/clinchem.2008.112243.
30. Hron G, Kollars M, Binder BR, et al. Identification of patients at low risk for recurrent venous thromboembolism by measuring thrombin generation. *JAMA* 2006; 296: 397-402. 2006/07/27. DOI: 10.1001/jama.296.4.397.
31. Hellum M, Franco-Lie I, Ovstebo R, et al. The effect of corn trypsin inhibitor, anti-tissue factor pathway inhibitor antibodies and phospholipids on microvesicle-associated thrombin generation in patients with pancreatic cancer and healthy controls. *PLoS One* 2017; 12: e0184579. 2017/09/15. DOI: 10.1371/journal.pone.0184579.

32. Ollivier V, Wang J, Manly D, et al. Detection of endogenous tissue factor levels in plasma using the calibrated automated thrombogram assay. *Thromb Res* 2010; 125: 90-96. 2009/04/07. DOI: 10.1016/j.thromres.2009.03.003.
33. Hisada Y and Mackman N. Measurement of tissue factor activity in extracellular vesicles from human plasma samples. *Res Pract Thromb Haemost* 2019; 3: 44-48. 2019/01/19. DOI: 10.1002/rth2.12165.
34. Hemker HC, Giesen P, AlDieri R, et al. The calibrated automated thrombogram (CAT): a universal routine test for hyper- and hypocoagulability. *Pathophysiol Haemost Thromb* 2002; 32: 249-253. 2003/09/19. DOI: 10.1159/000073575.
35. Hisada Y, Alexander W, Kasthuri R, et al. Measurement of microparticle tissue factor activity in clinical samples: A summary of two tissue factor-dependent FXa generation assays. *Thromb Res* 2016; 139: 90-97. 2016/02/27. DOI: 10.1016/j.thromres.2016.01.011.
36. Duchemin J, Pan-Petesch B, Arnaud B, et al. Influence of coagulation factors and tissue factor concentration on the thrombin generation test in plasma. *Thromb Haemost* 2008; 99: 767-773. 2008/04/09. DOI: 10.1160/TH07-09-0581.
37. Karachristos A, Scarmeas N and Hoffman JP. CA 19-9 levels predict results of staging laparoscopy in pancreatic cancer. *J Gastrointest Surg* 2005; 9: 1286-1292. 2005/12/08. DOI: 10.1016/j.gassur.2005.06.008.
38. Ferrone CR, Finkelstein DM, Thayer SP, et al. Perioperative CA19-9 levels can predict stage and survival in patients with resectable pancreatic adenocarcinoma. *J Clin Oncol* 2006; 24: 2897-2902. 2006/06/20. DOI: 10.1200/JCO.2005.05.3934.
39. Maithel SK, Maloney S, Winston C, et al. Preoperative CA 19-9 and the yield of staging laparoscopy in patients with radiographically resectable pancreatic adenocarcinoma. *Ann Surg Oncol* 2008; 15: 3512-3520. 2008/09/11. DOI: 10.1245/s10434-008-0134-5.
40. Novakovic V and Gilbert GE. Procoagulant Activities of Skeletal and Cardiac Muscle Myosin depend on contaminating phospholipid. *Blood* 2020 2020/07/01. DOI: 10.1182/blood.2020005930.
41. Callander NS, Varki N and Rao LV. Immunohistochemical identification of tissue factor in solid tumors. *Cancer* 1992; 70: 1194-1201. 1992/09/01. DOI: 10.1002/1097-0142(19920901)70:5<1194::aid-cncr2820700528>3.0.co;2-e.
42. Zacharski LR, Schned AR and Sorenson GD. Occurrence of fibrin and tissue factor antigen in human small cell carcinoma of the lung. *Cancer Res* 1983; 43: 3963-3968. 1983/08/01.
43. Mirdell R, Farnebo S, Sjoberg F, et al. Accuracy of laser speckle contrast imaging in the assessment of pediatric scald wounds. *Burns* 2018; 44: 90-98. 2017/08/12. DOI: 10.1016/j.burns.2017.06.010.
44. Gezelius E, Flou Kristensen A, Bendahl PO, et al. Coagulation biomarkers and prediction of venous thromboembolism and survival in small cell lung cancer: A sub-study of RASTEN - A randomized trial with low molecular weight heparin. *PLoS One* 2018; 13: e0207387. 2018/11/10. DOI: 10.1371/journal.pone.0207387.
45. Thaler J, Ay C, Mackman N, et al. Microparticle-associated tissue factor activity in patients with pancreatic cancer: correlation with clinicopathological features. *Eur J Clin Invest* 2013; 43: 277-285. 2013/02/13. DOI: 10.1111/eci.12042.
46. Wahrenbrock M, Borsig L, Le D, et al. Selectin-mucin interactions as a probable molecular explanation for the association of Trousseau syndrome with mucinous adenocarcinomas. *J Clin Invest* 2003; 112: 853-862. 2003/09/17. DOI: 10.1172/JCI18882.

47. Nadir Y, Brenner B, Zetser A, et al. Heparanase induces tissue factor expression in vascular endothelial and cancer cells. J Thromb Haemost 2006; 4: 2443-2451. 20060913. DOI: 10.1111/j.1538-7836.2006.02212.x.
48. Sun W, Ren H, Gao CT, et al. Clinical and Prognostic Significance of Coagulation Assays in Pancreatic Cancer Patients With Absence of Venous Thromboembolism. *Am J Clin Oncol* 2015; 38: 550-556. 2014/01/10. DOI: 10.1097/01.coc.0000436088.69084.22.
49. Ay C, Vormittag R, Dunkler D, et al. D-dimer and prothrombin fragment 1 + 2 predict venous thromboembolism in patients with cancer: results from the Vienna Cancer and Thrombosis Study. *J Clin Oncol* 2009; 27: 4124-4129. 2009/07/29. DOI: 10.1200/JCO.2008.21.7752.
50. Kondo S, Sasaki M, Hosoi H, et al. Incidence and risk factors for venous thromboembolism in patients with pretreated advanced pancreatic carcinoma. *Oncotarget* 2018; 9: 16883-16890. 2018/04/24. DOI: 10.18632/oncotarget.24721.
51. Reitter EM, Kaider A, Ay C, et al. Longitudinal analysis of hemostasis biomarkers in cancer patients during antitumor treatment. *J Thromb Haemost* 2016; 14: 294-305. 2015/12/15. DOI: 10.1111/jth.13218.
52. Kyrle PA, Minar E, Hirschl M, et al. High plasma levels of factor VIII and the risk of recurrent venous thromboembolism. *N Engl J Med* 2000; 343: 457-462. 2000/08/19. DOI: 10.1056/NEJM200008173430702.
53. Koster T, Blann AD, Briet E, et al. Role of clotting factor VIII in effect of von Willebrand factor on occurrence of deep-vein thrombosis. *Lancet* 1995; 345: 152-155. 1995/01/21. DOI: 10.1016/s0140-6736(95)90166-3.
54. Peippo MH, Kurki S, Seppanen H, et al. CA 19-9 doubling time in pancreatic cancer as a predictor of venous thromboembolism: a hospital database study. *Acta Oncol* 2019: 1-5. 2019/10/28. DOI: 10.1080/0284186X.2019.1679881.
55. Faille D, Bourrienne MC, de Raucourt E, et al. Biomarkers for the risk of thrombosis in pancreatic adenocarcinoma are related to cancer process. *Oncotarget* 2018; 9: 26453-26465. 2018/06/15. DOI: 10.18632/oncotarget.25458.
56. Ay C, Dunkler D, Simanek R, et al. Prediction of venous thromboembolism in patients with cancer by measuring thrombin generation: results from the Vienna Cancer and Thrombosis Study. *J Clin Oncol* 2011; 29: 2099-2103. 2011/04/06. DOI: 10.1200/JCO.2010.32.8294.
57. Khorana AA, Kuderer NM, Culakova E, et al. Development and validation of a predictive model for chemotherapy-associated thrombosis. *Blood* 2008; 111: 4902-4907. 2008/01/25. DOI: 10.1182/blood-2007-10-116327.
58. Lacroix R, Judicone C, Mooberry M, et al. Standardization of pre-analytical variables in plasma microparticle determination: results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop. *J Thromb Haemost* 2013 2013/04/05. DOI: 10.1111/jth.12207.