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ORIGINAL PAPER

Altered fibrin clot properties in advanced lung cancer: strong impact of cigarette smoking

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Abstract

Background Dense fibrin networks resistant to lysis have been reported in patients at high risk of thromboembolism. Little is known about fibrin clot properties in cancer. We investigated fibrin clot properties and their determinants in patients with inoperable lung cancer.

Methods We enrolled 150 patients with advanced lung cancer prior to therapy and 90 control subjects matched by age, sex, cardiovascular disease, and diabetes. Plasma clot permeability (K_s), turbidimetric analysis of clot formation, clot lysis time (CLT), microparticle-associated tissue factor (MP-TF) activity, thrombin generation, and serum cotinine levels were determined.

Results Lung cancer patients, compared with controls, formed at a faster rate (− 8.1% lag phase) denser plasma fibrin networks (− 27.2% K_s) that displayed impaired lysis (+ 26.5% CLT), along with 19.5% higher MP-TF activity and 100% higher peak thrombin generated, also after adjustment for potential confounders. Cotinine levels were associated with fibrin maximum absorbance ($r = 0.20$, $p = 0.016$) and K_s ($r = -0.50$, $p < 0.0001$) in cancer patients. On multivariate regression analysis, an increase in cotinine levels was a predictor of low K_s (the lower quartile, $< 5.8 \times 10^{-9}$ cm^2; odds ratio = 1.21 per 10 ng/ml, 95% confidence interval 1.02–1.46), but not CLT.

Conclusion Advanced lung cancer is associated with the prothrombotic plasma clot phenotype largely driven by smoking.

Keywords Clot lysis time · Fibrin clot · Lung cancer · Smoking · Thrombin generation

Introduction

Lung cancer is the leading cause of cancer death worldwide [1]. The 5-year survival rate for stage IIIA, IIIB, and IV non-small-cell lung cancer (NSCLC) is about 14%, 5%, and 1%, respectively [2], while 5-year survival rates for stage II, III, and IV small-cell lung cancer (SCLC) are 19%, 8%, and 2%, respectively [3]. Current treatment strategies significantly prolong the life of patients with advanced lung cancer [4].

Venous thromboembolism (VTE) associated with a 50% higher risk of death occurs in approximately 3% of lung cancer patients [5, 6]. The 1-year cumulative incidence of VTE is 10.2% in SCLC [7] and 22% in NSCLC patients [8]. Chemotherapy is associated with threefold higher risk for VTE in lung cancer patients [9]. Moreover, lung cancer increases the stroke risk [10]. Cardiovascular disease observed in 23% of lung cancer patients increases the mortality for stages I–IIIB [11].

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Elevated thrombotic risk in patients with malignancy depends on multiple factors, including the type of cancer, its clinical stage and grade, concomitant diseases, and treatment [12]. Prothrombotic abnormalities in lung cancer involve elevated fibrinogen [12], thrombin generation [13], expression of tissue factor (TF) [14], plasminogen activator inhibitor-1 and -2 [15], cancer procoagulant [13], and impaired activation of the protein C pathway [16]. Increased TF expressing microparticles (MPs) associated with VTE were found in lung cancer and metastatic pancreatic cancer [17, 18].

Formation of compact fibrin networks resistant to lysis predisposes to VTE [19, 20]. Cigarette smoking also impairs fibrin clot properties [21]. Reports on fibrin clot properties in cancer patients are limited [22, 23]. Recently, we have shown that a 3-month chemotherapy improves fibrin clot properties in lung cancer patients [24]. To our knowledge, fibrin clot properties and their determinants in patients with advanced lung cancer before chemotherapy have not been investigated yet.

Materials and methods

Patients

From May to September 2014, we studied 150 white consecutive patients with advanced histologically or cytologically confirmed lung cancer who were recruited at the Department of Oncology, John Paul II Hospital, Cracow, Poland [24]. Patients were categorized into subjects with SCLC and those with NSCLC that included three main subtypes: adenocarcinoma, squamous cell cancer, and not otherwise specified (NOS) carcinoma, involving cases other than adenocarcinoma, squamous cell, large cell carcinoma, or mixed/other histology.

The American Joint Committee on Cancer (AJCC) stages were determined according to the AJCC 7th edition staging system, using available clinical data. The exclusion criteria were any active infections, glomerular filtration rate < 60 ml/min, hypo- or hyperthyroidism, any vascular events within the preceding 3 months, and current anticoagulant therapy. Patients receiving thromboprophylaxis with low-molecular-weight heparins (LMWH) were eligible. The recommended thromboprophylaxis in high-risk patients was administered [25, 26]. Heart failure (HF) was defined as the presence of relevant symptoms and signs and left ventricular ejection fraction ≤ 45%. Arterial hypertension was diagnosed based on a history of hypertension (blood pressure > 140/90 mmHg) or preadmission antihypertensive treatment. Diabetes mellitus was defined as fasting glucose ≥ 7.0 mM on two separate occasions or use of insulin or oral hypoglycemic agents. Coronary artery disease (CAD) was defined as hospitalization for angina, prior documented myocardial infarction, or coronary revascularization. Ischemic stroke was diagnosed based on the World Health Organization criteria. Chronic obstructive pulmonary disease was defined by the presence of irreversible expiratory airflow limitation measured by spirometry. A current smoker was defined as a subject who has smoked 100 cigarettes and currently declares the use of one or more cigarettes per day, while former smoker as a person who has smoked at least 100 cigarettes in a lifetime, but currently (at least within the last month) does not smoke.

Control subjects ($n = 90$) matched for age, sex, cardiovascular disease, and diabetes included members of the hospital staff and their family. The Bioethics Committee of the District Medical Chamber in Cracow approved the study (31/KBL/OIL/2013), and participants provided informed consent in accordance with the Declaration of Helsinki. A written informed consent was obtained from all the participants.

Laboratory investigations

Fasting blood samples were drawn from antecubital vein using minimal stasis. Blood samples were drawn at the time of enrolment before starting therapy. Patients who received prophylactic LMWH were drawn at least within 12 h post injection. Complete blood count, glucose, and creatinine were assayed by routine laboratory techniques. Fibrinogen was determined using the Clauss method. Plasma D-dimer was measured with the Innovance D-dimer assay (Siemens, Marburg, Germany). Immunoenzymatic assays were used to determine the TF-bearing MP (MP-TF) activity (Hyphen BioMed, Neuville sur Oise, France) and cotinine levels (Calbiotech, El Cajon, CA, USA).

Fibrin clot properties

In citrated plasma (vol/vol, 9:1 of 3.2% sodium citrate), the following variables describing a plasma clot formation, structure, and lysability were determined in duplicate by technicians blinded to the origin of the samples (intraassay and interassay coefficients of variation, 5–7%).

Clot permeability and scanning electron microscopy

Permeation of plasma fibrin clots was determined as described [27]. In brief, 20 mM calcium chloride and 1 U/mL human thrombin (Sigma-Aldrich, St. Louis, MO, USA) were added to citrated plasma. Tubes containing the clots were connected to a reservoir of a Tris-buffered saline, and its volume flowing through the gels was measured. A permeation coefficient (K_s), which indicates the pore size, was calculated from the equation: $K_s = Q \times L \times \eta / t \times A \times \Delta p$, where Q is the flow rate in time t, L is the length of a fibrin

gel, η is the viscosity of liquid, t is percolating time, A is the cross-sectional area, and Δp is a differential pressure.

After K_s measurement, clots ($n=6$) were fixed using 2.5% glutaraldehyde, then removed from tubes, washed with distilled water, dehydrated in graded water–ethanol solutions, dried by the critical point procedure, and sputter coated with gold. Samples were scanned in six different areas (microscope JEOL JCM-6000; JEOL Ltd., Tokyo, Japan) at magnification of 5,000x to determine a fibrin diameter of at least 50 fibers per clot using the ImageJ software (US National Institutes of Health, Bethesda, MD, USA).

Turbidity measurements

Plasma citrated samples were mixed 2:1 with a TBS containing 0.6 U/mL human thrombin (Sigma-Aldrich) and 50 mM $CaCl_2$ to initiate polymerization. Absorbance was read at 405 nm with a Perkin-Elmer Lambda 4B spectrophotometer (Molecular Devices). The lag phase of the turbidity curve, which reflects the time required for initial protofibril formation and maximum absorbance at the plateau phase (ΔAbs), indicating an average fibrin fiber size, fibrin branching, and clot density, was recorded [27].

Clot lysis assay

Clot lysis time (CLT) was measured as described previously [27]. Briefly, to 75 µl of citrated plasma we added TF (dilution 105 times; Innovin, Dade Behring, Deerfield, IL, USA), $CaCl_2$ (final concentration, 17 mM), tissue plasminogen activator (tPA, final concentration, 30 U/ml; Boehringer Ingelheim, Ingelheim, Germany), and phospholipid vesicles [28] (final concentration, 10 mM). HEPES buffer was added to make a total volume of 150 µl.

Thrombin generation assay

To assess the thrombin generation profiles, we used the assay previously described [29, 30]. In brief, corn trypsin inhibitor was added to citrated plasma (0.1 mg/mL, final concentration) and 80 µL of the sample was mixed with relipidated TF (5 pM, final concentration), 2.5 mM Z-GGR-AMC/90 mM $CaCl_2$ solution, and a 120 µM phospholipid vesicles solution. Hydrolysis of the AMC substrate (at 370/460 nm) was followed over a 3600s period. Changes in fluorescence were converted to thrombin concentration using a calibration curve built by sequential dilutions of human thrombin.

Statistical analysis

The study was powered to have a 90% chance of detecting a 10% difference in CLT using a P value of 0.01, based on the previous study [31]. In order to demonstrate such a difference, or a greater one, 32 patients or more were required in each group. In turn, to demonstrate such a difference, or a greater one, in K_s using a P value of 0.05 at least 31 patients were required in each group [28].

Variables were presented as mean and standard deviation, median, and interquartile range or otherwise stated. The normality of distribution was checked using Shapiro–Wilk test. The Spearman's rho correlation coefficient was computed to measure the relationship between continuous variables. For testing association for categorical variables, the Fischer's Exact test was used. The t test or ANOVA were used for means comparison, whereas the nonparametric U Mann–Whitney or Kruskal–Wallis tests were used for comparison of nonnormally distributed variables. For paired data, the paired Student's t test or the Wilcoxon signed-rank tests were used as appropriate.

Odds ratios of having the lower quartile for K_s ($\leq$Q1 vs. $>$Q1) and the upper quartile for CLT ($\geq$Q3 vs. $<$Q3) were estimated using multivariate logistic regression. The models were adjusted for age, sex, body-mass index, and fibrinogen. In the final models, only variables with p values <0.05 were retained. All statistical analyses were performed with software JMP®, Version 12.2.0 (SAS Institute INC., Cary, NC, USA).

Results

Patient characteristics

Baseline characteristics of 150 lung cancer patients and 90 control subjects are summarized in Table 1. At enrolment 61 (40.7%) patients were diagnosed with the SCLC and 89 (59.3%) with the NSCLC. Metastatic lung cancer was diagnosed in 92 (61.3%) patients, while in 58 (38.7%) locally advanced inoperable lung cancer was recognized. The SCLC group included 34 (55.7%) patients with limited disease (LD, stage IIIAB) and 27 (44.3%) with extended disease (ED, stage IV).

Among NSCLC patients, 27 (30%) individuals had metastases to lungs, 10 (11.2%) to the pleura, 9 (10.1%) to the liver, 14 (15.7%) to adrenals, 10 (11.2%) to bones, 10 (11.2%) to the brain, and 10 (11.2%) to other locations. In patients with SCLC, lung metastases were present in 4 (6.6%) patients, metastases to the pleura in 2 (3.3%), to the liver in 13 (21.3%), to adrenals in 4 (6.6%), to bones in 8 (13.1%), to the brain in 2 (3.3%), and to other locations in 3 (4.9%) patients. Metastasis in more than one location was detected in 29 (19.3%) patients. Patients with lung cancer, compared with controls, had lower body mass index (BMI) and were more often smokers (Table 1). We observed 27.9% higher fibrinogen and 126% higher D-dimer, together with 19.5% higher MP-TF activity in patients compared with

Table 1 Baseline characteristics of the control subjects and lung cancer patients

Variable	Control subjects ($n=90$)	Lung cancer patients ($n=150$)	*P* value
Age, years	64.1 ± 7.2	64.2 ± 7.0	0.92
Male, *n* (%)	69 (76.7)	102 (68.0)	0.15
Body mass index, kg/m^2	27.3 ± 3.5	25.2 ± 4.8	< 0.001
Clinical characteristics, *n* (%)			
Current smoking, *n* (%)	9 (10.0)	82 (54.7)	< 0.001
Previous stroke, *n* (%)	8 (8.9)	6 (4.0)	0.12
CAD, *n* (%)	19 (21.1)	23 (15.3)	0.25
Arterial hypertension, *n* (%)	50 (55.6)	71 (47.3)	0.22
Diabetes, *n* (%)	7 (7.8)	18 (12.0)	0.30
COPD, *n* (%)	12 (13.3)	19 (12.7)	0.88

Data are given as number (percentage), mean ± SD, or median (interquartile range)

COPD chronic obstructive lung disease, *CAD* coronary artery disease, *HF* heart failure, *ACEI*, angiotensin-converting enzyme inhibitors

controls (Table 2). Lung cancer patients with ED compared to those with LD were characterized by 91.7% higher D-dimer levels ($p<0.0001$) and 42.8% higher MP-TF activity ($p=0.014$; Table 3).

Cotinine serum levels in lung cancer patients

Smoking intensity in lung cancer patients was high (median, 40, range 25–45 pack-years), especially in those with SCLC compared with the remainder [40 (30–50) vs. 30 (20–4) pack-years, $p=0.0073$]. Cotinine was detected in 95% of lung cancer patients, and its levels were higher in patients who declared current smoking compared to those who declared former smoking ($n=55$) [66.5 (59–74) vs. 33 (12–58) ng/ml, $p<0.0001$], and never smoking ($n=13$) [66.5 (59–74) vs. 5 (1–8) ng/ml, $p<0.0001$), respectively. Cotinine serum levels [median, 58.5 (23–70) ng/ml] correlated with the smoking pack-years ($r=0.28$, $p=0.0014$) and fibrinogen ($r=0.27$, $p<0.001$), but showed no associations with demographic variables or clinical factors.

Thrombin generation

Lung cancer patients had 87% longer lag time and 100% higher peak thrombin generated than controls, while time to thrombin peak (TTPeak) and endogenous thrombin potential (ETP) were similar in both groups (Table 2). There were no associations of thrombin generation with demographic or clinical factors, including CAD or diabetes.

Thrombin generation variables were similar among patients with different histological types of lung cancer and in those with LD or ED (Table 3). Lag time and TTPeak

Table 2 Laboratory tests and fibrin clot variables in control subjects and lung cancer patients

Variable	Control subjects ($n=90$)	Lung cancer patients ($n=150$)	*P* value*
RBC, 10^6/μl	4.35 ± 0.4	4.47 ± 0.5	0.87
WBC, 10^3/μl	6.0 (5.2–7.2)	9.2 (7.4–12.0)	< 0.001
Platelets, 10^3/μl	237 (198–303)	309 (244–366)	< 0.001
Hemoglobin, g/dl	13.9 ± 1.4	12.8 ± 1.6	< 0.001
Glucose, mM	4.7 (4.4–5.0)	5.3 (4.9–5.9)	< 0.001
Creatinine, μM	68 (62–77)	74 (62–84)	0.064
Fibrinogen, g/l	2.47 (2.24–3.08)	3.16 (2.70–3.95)	< 0.001
D-dimer, ng/ml	218 (193–261)	493 (308–893)	< 0.001
MP-TF activity, pg/ml	1.33 (0.98–1.9)	1.59 (1.00–2.40)	0.020
K_s, 10^{-9}cm^2	9.2 (7.5–9.9)	6.7 (5.8–7.5)	< 0.001
Lag phase, s	43.16 ± 4.56	39.65 ± 4.06	0.006
ΔAbs (405 nm)	0.80 (0.75–0.86)	0.85 (0.80–0.89)	0.69
Clot lysis time, min	76.9 ± 14.3	97.3 ± 16.8	< 0.001
Lag time, s	631 (566–729)	1180 (1031–1453)	< 0.001
Peak thrombin generated, nM	73 (60–159)	146 (105–221)	0.001
TTPeak, s	2006 (1654–2253)	2134 (1854–2519)	0.098
ETP, nM*s	82,836 ± 27,965	95,423 ± 38,411	0.23

Data are given as mean ± SD or median (interquartile range)

RBC red blood cells, *WBC* white blood cells, *MP-TF* Tissue Factor-bearing microparticles procoagulant activity, K_s fibrin clot permeability, *ΔAbs (405 nm)* maximum absorbance of fibrin gel at 405 nm, *TTPeak* time to thrombin peak, *ETP* endogenous thrombin potential

*Adjusted for body mass index, creatinine, glucose, and fibrinogen

Table 3 Characteristics of lung cancer patients with regard to the histological type of cancer and the cancer stage

Variable	Histological type of cancer				Cancer stage	
	SCLC ($n=61$)	NSCLC				
		Squamous cell cancer ($n=37$)	Adenocarcinoma ($n=39$)	NOS carcinoma ($n=13$)	LD ($n=58$)	ED ($n=92$)
Age, years	65 (60–68)	65 (61–68)	63 (58–67)	63 (59–66)	64 ± 7	65 ± 7
Male, *n* (%)	39 (64)	26 (70)	27 (69)	10 (77)	39 (67)	63 (69)
Body mass index, kg/m^2	24.9 (22.4–28.7)	24.5 (21.1–27.6)	25.0 (21.7–26.8)	24.1 (22.0–25.6)	25.9 ± 4.8	24.8 ± 4.8
Current smoking, *n* (%)	35 (57)	19 (51)	19 (49)	9 (69)	32 (55)	50 (54)
RBC, 10^6/µl	4.53 (4.08–4.80)	4.39 (4.11–4.60)	4.38 (4.15–5.0)	4.80 (4.47–5.10)	4.5 ± 0.5	4.5 ± 0.5
WBC, 10^3/µl	8.96 (7.28–11.3)	9.40 (7.88–12.30)	8.81 (6.73–11.40)	12.90 (8.99–16.50)	9.30 (7.02–11.70)	9.11 (7.59–12.40)
Platelets, 10^3/µl	300 (215–342)	334 (267–413)	299 (237–374)	315 (254–434)	321 (270–380)	292 (226–364)
Hemoglobin, g/dl	13.2 (11.6–14.0)	12.4 (11.4–13.6)	12.8 (12.2–14.3)	12.9 (12.4–13.9)	13.0 (12.2–14.0)	12.7 (11.6–14.0)
Glucose, mM	5.2 (4.9–5.6)	5.2 (5.0–7.0)	5.6 (5.1–6.0)	6.4 (5.2–7.6)	5.2 (4.9–5.8)	5.4 (5.0–6.1)
Creatinine, µM	74 (62–86)	69 (54–88)	74 (63–82)	70 (58–79)	72 (62–84)	74 (62–85)
Fibrinogen, g/l	3.13 (2.70–3.90)	3.32 (2.61–4.0)	3.38 (3.04–4.10)	2.90 (2.55–3.00)	3.11 (2.65–3.80)	3.22 (2.71–4.10)
D-dimer, ng/ml	446 (254–691)	445 (357–1167)	663 (351–1174)	491 (303–572)	324 (238–494)	621 (399–1231)
MP-TF activity, pg/ml	1.43 (0.93–2.18)	1.63 (1.00–2.52)	1.91 (1.24–2.47)	1.31 (1.08–1.43)	1.31 (0.89–2.17)	1.87 (1.21–2.48)
Cotinine, ng/ml	63 (33–72)	45 (22–70)	46 (18–70)	57 (49–59)	57 (27–67)	61 (23–73)
K_s, 10^{-9}cm^2	6.8 (5.8–7.4)	6.2 (5.8–7.5)	6.5 (5.8–7.0)	7.8 (6.5–7.9)	6.5 ± 1.0	6.8 ± 1.1
Turbidimetric lag phase, s	39 (37–42)	40 (38–43)	39 (36–40)	39 (38–42)	39 (37–43)	40 (37–42)
ΔAbs (405 nm)	0.85 (0.81–0.90)	0.86 (0.80–0.90)	0.85 (0.80–0.90)	0.90 (0.80–0.90)	0.85 ± 0.06	0.85 ± 0.06
Clot lysis time, min	94 (84–104)	99 (89–107)	101 (91–111)	96 (81–104)	99 (85–107)	98 (89–107)
Lag time, s	1210 (1047–1418)	1204 (1067–1574)	1151 (924–1372)	1011 (927–1219)	1229 (1050–1485)	1135 (979–1379)
Peak thrombin generated, nM	138 (102–188)	161 (130–233)	139 (97–208)	209 (154–273)	154 (116–213)	144 (100–224)
TTPeak, s	2139 (1854–2614)	2243 (1996–2566)	2115 (1749–2490)	1949 (1749–2134)	2215 (1915–2552)	2105 (1825–2504)
ETP, nM*s	90,767 (62,099–111,559)	90,699 (65,796–128,937)	94,234 (70,243–128,012)	123,087 (83,329–144,520)	94,687 (67,917–119,202)	91,221 (63,066–129,663)

Data are given as mean ± SD or median (interquartile range)

SCLC small cell lung cancer, *NSCLC* non-small cell lung cancer, *NOS carcinoma* not otherwise specified carcinoma, *LD* limited disease, *ED* extended disease, for other abbreviations see Table 2

correlated with platelet count ($r=0.25$, $p=0.0022$ and $r=0.26$, $p=0.0019$, respectively), while peak thrombin generated and ETP showed associations with WBC count ($r=0.20$, $p=0.014$ and $r=0.18$, $p=0.028$). We observed no associations of thrombin parameters with MP-TF or cotinine.

Fibrin clot properties

Lung cancer patients had 27.2% lower K_s, 8.1% shorter turbidimetric lag phase, and 26.5% longer CLT than control subjects (Fig. 1), while ΔAbs did not differ between both groups (Table 2). Intergroup differences in fibrin variables remained significant after adjustment for body mass index, creatinine, glucose, and fibrinogen. Thinner plasma fibrin fibers were observed in lung cancer patients compared with controls (87.1 ± 9.9 vs. 116.7 ± 13.2 nm, $p<0.0001$; Fig. 2).

Patients with lung cancer and concomitant CAD had 6.3% longer CLT than those without CAD [102 (95–118) vs. 96 (85–106) min, $p=0.026$]. There were no differences in fibrin clot properties regarding other clinical or demographic variables (data not shown).

Interestingly, lung cancer patients who declared smoking intensity > 20 pack-years had 6.8% lower K_s compared with the remainder (6.5 ± 1.0 vs. 7.0 ± 1.0 × 10^{-9}cm^2, $p=0.026$). Fibrin features were similar among patients with different histological types of lung cancer and in patients with ED compared with those with the LD (Table 3). We observed no associations of fibrin variables with MP-TF or plasma

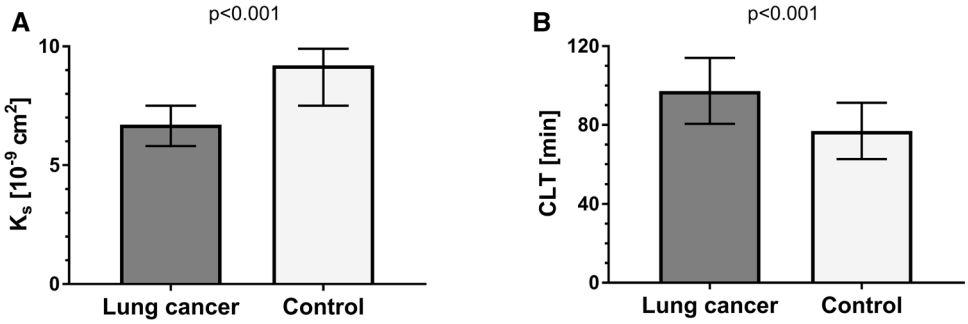

Fig. 1 Fibrin clot permeability (**a**) and clot lysis time (**b**) in lung cancer patients and controls

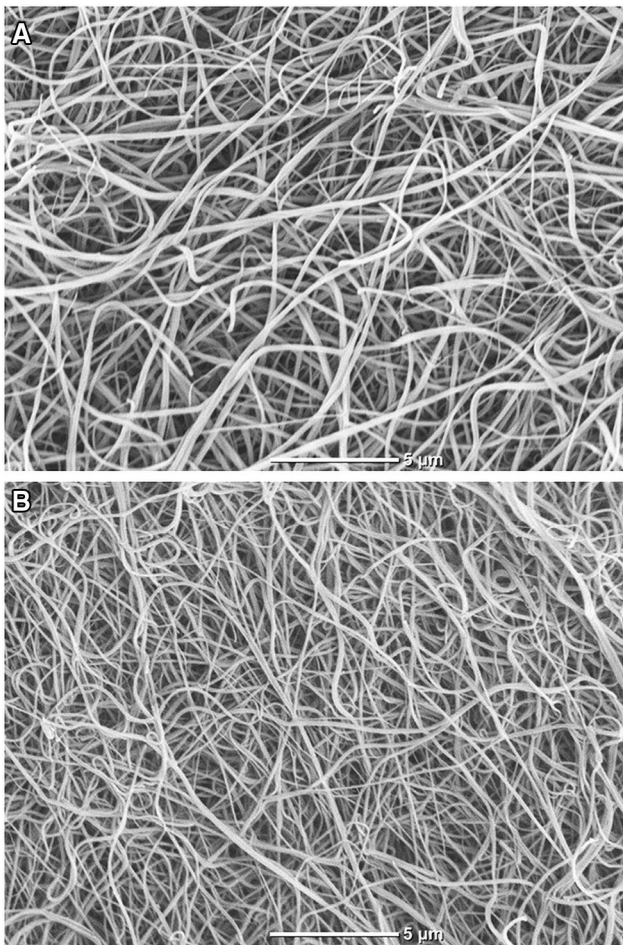

Fig. 2 Representative scanning electron microscopy (SEM) images of fibrin networks in control (**a**) and lung cancer patient (**b**). Magnification, 5000x; fibrinogen concentration, 3 g/L

D-dimer. There was a weak positive correlation between CLT and ETP in cancer patients ($r = 0.18$, $p = 0.029$), but no other associations between thrombin generation parameters and fibrin clot variables were noted. Cotinine levels were associated with ΔAbs ($r = 0.20$, $p = 0.016$) and K_s ($r = -0.50$, $p < 0.0001$).

On univariate analysis, the presence of HF ($n = 8$, 5.3%), hyperlipidemia ($n = 23$, 15.3%), and cotinine levels were associated with a large density of plasma fibrin clots defined as the lower quartile of K_s [$< 5.80 \times 10^{-9}$ cm^2; odds ratio (OR) = 12.78, 95% confidence interval (CI) 2.44–66.82, $p = 0.0025$, OR 2.76, 95% CI 1.07–7.12, $p = 0.036$, and OR 1.027 per 1 ng/ml of cotinine level increase, 95% CI 1.011–1.045, $p = 0.0013$, respectively] in lung cancer patients. In multivariate analysis, the increase in cotinine level independently predicted the lower quartile of K_s (OR 1.21 per 10 ng/ml, 95% CI 1.02–1.46, $p = 0.037$). HF was associated with hypofibrinolysis defined as the upper quartile of CLT (≥ 107 min) on univariate and multivariate regression analyses (OR 6.22, 95% CI 1.41–27.53, $p = 0.016$, and OR 5.83, 95% CI 1.22–27.90, $p = 0.027$, respectively).

Discussion

We demonstrated that the prothrombotic plasma clot phenotype, including faster formation of fibrin clots, reduced fibrin network porosity, and impaired clot lysability, characterizes patients with advanced lung cancer. All the differences between the groups were observed also after adjustment for fibrinogen, a major determinant of clot properties, which was increased in cancer patients as expected. These prothrombotic alterations showed no relationship with increased plasma D-dimer or thrombin generation, except the weak association between CLT and ETP, suggesting the impact of other factors on fibrin clot structure and function in cancer. Of note, there were no differences in the thrombin generation or fibrin properties between patients with disseminated vs. locally advanced inoperable lung cancer. Our study provides evidence that a hypercoagulable state observed in advanced lung cancer patients involves unfavorably altered structural and functional characteristics of fibrin clots, which might implicate difficulties in effective thromboprophylaxis and have practical implications in thrombotic risk assessment among cancer patients given data on a value of K_s and CLT in predicting thromboembolic events [32, 33].

We found that current cigarette smoking has the most potent effect on clot density tested in vitro. Our study demonstrated that active smoking is associated with unfavorably altered plasma clot properties, including K_s and ΔAbs, which exceed beyond potential effects of higher thrombin generation or MP-TF activity. Barua et al. have demonstrated that acute cigarette smoke exposure in healthy subjects impaired clot lysis ability measured in whole blood using thromboelastography by 23.9% and 21.6% compared to nonsmokers or with smokers after an overnight abstinence from smoke exposure [21]. Previously, we have reported that plasma fibrin clots prepared from plasma of apparently healthy smokers were characterized by 21.2% reduced K_s and 35.4% prolonged fibrinolysis when compared to never-smokers [34]. Interestingly, smokers with diagnosed CAD had 24% lower fibrin porosity than healthy smokers [34]. In the current study, active smoking was independently associated with the compact clot structure reflected by low K_s in lung cancer patients. This finding suggests that smoking has an incredibly strong prothrombotic effect in lung cancer. Cotinine level, as a marker of smoking exposure, showed that most of the so-called former smokers are still smoking and provided false information. A recent meta-analysis indicated that the proportion of patients with lung cancer who continue to smoke after diagnosis ranged from 6 to 83% [35]. Mechanisms behind the association between smoking and fibrin clot properties in cancer patients remain to be established; however, most likely increased oxidative stress contributes to our observation [36]. Oxidative stress reflected by 8-epi-prostaglandin $F_{2\alpha}$ was enhanced in heavy smokers compared to never-smokers and strongly correlated with impaired fibrin clot properties in the former group [34]. The current study provides new insights into a hypercoagulable state in lung cancer, suggesting altered fibrin clot structure largely determined by the smoking status which is potent enough to preserve its impact on fibrin properties even in the presence of strong cancer-associated activation of blood coagulation.

Since fibrin clot variables in advanced lung cancer patients were similar to those reported in subjects with digestive tract cancer [22], the present study suggests that the prothrombotic clot phenotype could be a common feature of patients with various solid tumors. Indeed, Shoji et al. have shown that deposits of crosslinked fibrin occur within the stroma of 80% lung tumors [37] and can enhance cell proliferation, neovascularization, and protect tumor tissue by making an impermeable barrier [38]. Clinical implications of these observations remain to be established not only in terms of thromboembolic risk but also the disease itself. Previous studies demonstrated that cancer is associated with elevated platelet count, MP-TF activity, thrombin generation, and D-dimer [39]. We confirmed these observations. There is evidence that disturbed hemostasis plays a role in the metastatic capacity of solid tumors [40]. Increased activation of the blood coagulation along with the subsequent fibrinolysis evidenced in our cohort of lung cancer patients by higher MP-TF activity and thrombin leads to elevated D-dimer levels in patients with ED, related to poor prognosis in several studies on patients with solid tumors, including lung cancer [41]. These observations are in line with the study by Zhang et al. [42]. Thrombin generation markers and D-dimer have been shown to be implicated in the metastatic potential of various cancers [43]. In addition, thrombin and TF have been postulated to represent key mediators of cancer-related thrombosis in general [44]. However, we expanded the published data by demonstrating that fibrin is another important contributor to thrombotic tendency in cancer, and this feature is largely unrelated to the previously reported prothrombotic alterations. The current study provides additional evidence that a thrombotic potential of TF-bearing MPs might contribute to the multifactorial hypercoagulability in lung cancer patients, however, without any clear association with fibrin characteristics in the advanced inoperable disease. Our study indicates that the cancer-related prothrombotic clot phenotype cannot be explained by elevated fibrinogen or increased thrombin formation. Although these two factors are well known as those rendering the plasma fibrin clot more prothrombotic [19, 20], we found only a week association between CLT and ETP, but differences between cancer and control groups remained significant after adjustments. It might be speculated that other factors disturb associations between thrombin generated and fibrin properties. Identification of its determinants deserves further investigation.

This study has several limitations. First, the sample size was limited. The study was, however, adequately powered to detect intergroup differences in clot variables, although the subgroup analyses should be interpreted with caution. Second, we did not measure several potential fibrin clot modifiers including homocysteine, lipoprotein(a), and fibrinolysis inhibitors [45], but their effects are likely to be of minor importance in cancer patients.

In conclusion, advanced lung cancer is associated with impaired plasma clot characteristics. Our study provides evidence for a strong effect of cigarette smoking on fibrin-related variables despite the advanced lung cancer. Thus, smoking might be a key modulator of prothrombotic clot alterations in this disease. Clinical relevance of the current findings remains to be explored.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

References

1. Ridge CA, McErlean AM, Ginsberg MS. Epidemiology of lung cancer. Semin Intervent Radiol. 2013;30:93–8.
2. American Cancer Society. Non-small cell lung cancer survival rates, by stage. 2018. http://www.cancer.org/cancer/lungcancer-non-smallcell/detailedguide/non-small-cell-lung-cancer-survival-rates. Accessed 1 May 2018.
3. American Cancer Society. Small cell lung cancer survival rates, by stage. 2018. http://www.cancer.org/cancer/lungcancer-smallcell/detailedguide/small-cell-lung-cancer-survival-rates. Accessed 1 May 2018.
4. Bansal P, Osman D, Gan GN, Simon GR, Boumber Y. Recent advances in targetable therapeutics in metastatic non-squamous NSCLC. Front Oncol. 2016;6:112.
5. Chew HK, Davies AM, Wun T, Harvey D, Zhou H, White RH. The incidence of venous thromboembolism among patients with primary lung cancer. J Thromb Haemost. 2008;6:601–8.
6. Walker AJ, Baldwin DR, Card TR, Powell HA, Hubbard RB, Grainge MJ. Risk of venous thromboembolism in people with lung cancer: a cohort study using linked UK healthcare data. Br J Cancer. 2016;115:115–21.
7. Lee YG, Lee E, Kim I, Lee KW, Kim TM, Lee SH, et al. Cisplatin-based chemotherapy is a strong risk factor for thromboembolic events in small-cell lung cancer. Cancer Res Treat. 2015;47:670–5.
8. Numico G, Garrone O, Dongiovanni V, Silvestris N, Colantonio I, Di Costanzo G, et al. Prospective evaluation of major vascular events in patients with nonsmall cell lung carcinoma treated with cisplatin and gemcitabine. Cancer. 2005;103:994–9.
9. Huang H, Korn JR, Mallick R, Friedman M, Nichols C, Menzin J. Incidence of venous thromboembolism among chemotherapy-treated patients with lung cancer and its association with mortality: a retrospective database study. J Thromb Thrombolysis. 2012;34:446–56.
10. Chen PC, Muo CH, Lee YT, Yu YH, Sung FC. Lung cancer and incidence of stroke: a population-based cohort study. Stroke. 2011;42:3034–9.
11. Kravchenko J, Berry M, Arbeev K, Lyerly HK, Yashin A, Akushevich I. Cardiovascular comorbidities and survival of lung cancer patients: Medicare data based analysis. Lung Cancer. 2015;88:85–93.
12. Van Wersch JW, Tjwa MK. Coagulation/fibrinolysis balance and lung cancer. Haemostasis. 1991;21:117–23.
13. Ruf W, Mueller BM. Thrombin generation and the pathogenesis of cancer. Semin Thromb Hemost. 2006;32(Suppl 1):61–8.
14. Goldin-Lang P, Tran QV, Fichtner I, Eisenreich A, Antoniak S, Schulze K, et al. Tissue factor expression pattern in human non-small cell lung cancer tissues indicate increased blood thrombogenicity and tumor metastasis. Oncol Rep. 2008;20:123–8.
15. Ruf W, Yokota N, Schaffner F. Tissue factor in cancer progression and angiogenesis. Thromb Res. 2010;125(Suppl 2):36–8.
16. Sohn RH, Deming CB, Johns DC, Champion HC, Bian C, Gardner K, et al. Regulation of endothelial thrombomodulin expression by inflammatory cytokines is mediated by activation of nuclear factor-kappa B. Blood. 2005;105:3910–7.
17. Tseng CC, Wang CC, Chang HC, Tsai TH, Chang LT, Huang KT, et al. Levels of circulating microparticles in lung cancer patients and possible prognostic value. Dis Markers. 2013;35:301–10.
18. Thaler J, Koder S, Kornek G, Pabinger I, Ay C. Microparticle-associated tissue factor activity in patients with metastatic pancreatic cancer and its effect on fibrin clot formation. Transl Res. 2014;163:145–50.
19. Undas A, Ariens RAS. Fibrin clot structure and function: a role in the pathophysiology of arterial and venous thromboembolic diseases. Arterioscler Thromb Vasc Biol. 2011;31:e88–99.
20. Ząbczyk M, Undas A. Plasma fibrin clot structure and thromboembolism: clinical implications. Pol Arch Intern Med. 2017;127:873–81.
21. Barua RS, Sy F, Srikanth S, Huang G, Javed U, Buhari C, et al. Acute cigarette smoke exposure reduces clot lysis – association between altered fibrin architecture and the response to t-PA. Thromb Res. 2010;126:426–30.
22. Gronostaj K, Richter P, Nowak W, Undas A. Altered plasma fibrin clot properties in patients with digestive tract cancers: links with the increased thrombin generation. Thromb Res. 2013;131:262–7.
23. Undas A, Zubkiewicz-Usnarska L, Helbig G, Woszczyk D, Kozińska J, Dmoszyńska A, et al. Altered plasma fibrin clot properties and fibrinolysis in patients with multiple myeloma. Eur J Clin Invest. 2014;44:557–66.
24. Królczyk G, Ząbczyk M, Czyżewicz G, Plens K, Prior S, Butenas S, et al. Altered fibrin clot properties in advanced lung cancer: impact of chemotherapy. J Thorac Dis. 2018;10:6863–72.
25. Zamorano JL, Lancellotti P, Muñoz DR, Aboyans V, Asteggiano R, Galderisi M, et al. 2016 ESC Position Paper on cancer treatments and cardiovascular toxicity developed under the auspices of the ESC Committee for Practice Guidelines. [in Polish]. Kardiol Pol. 2016;74:1193–233.
26. Krasiński Z, Krasińska B, Dzieciuchowicz Ł, Urbanek T, Gabriel M. Heparins in cancer-associated venous thrombosis. Pol Arch Med Wewn. 2016;126:419–29.
27. Undas A, Zawilska K, Ciesla-Dul M, Lehmann-Kopydłowska A, Skubiszak A, Ciepłuch K, et al. Altered fibrin clot structure/function in patients with idiopathic venous thromboembolism and in their relatives. Blood. 2009;114:4272–8.
28. Lisman T, de Groot PG, Meijers JC, Rosendaal FR. Reduced plasma fibrinolytic potential is a risk factor for venous thrombosis. Blood. 2005;105:1102–5.
29. Mann KG, Whelihan MF, Butenas S, Orfeo T. Citrate anticoagulation and the dynamics of thrombin generation. J Thromb Haemost. 2007;5:2055–61.
30. Hemker HC, Giesen P, Al Dieri R, Regnault V, de Smedt E, Wagenvoord R, et al. Calibrated automated thrombin generation measurement in clotting plasma. Pathophysiol Haemost Thromb. 2003;33:4–15.
31. Meltzer ME, Lisman T, de Groot PG, Meijers JC, le Cessie S, Doggen CJ, et al. Venous thrombosis risk associated with plasma

hypofibrinolysis is explained by elevated plasma levels of TAFI and PAI-1. Blood. 2010;116:113–21.
32. Zabczyk M, Plens K, Wojtowicz W, Undas A. Prothrombotic fibrin clot phenotype is associated with recurrent pulmonary embolism after discontinuation of anticoagulant therapy. Arterioscler Thromb Vasc Biol. 2017;37:365–73.
33. Cieslik J, Mrozinska S, Broniatowska E, Undas A. Altered plasma clot properties increase the risk of recurrent deep vein thrombosis: a cohort study. Blood. 2018;131:797–807.
34. Undas A, Topór-Madry R, Tracz W, Pasowicz M. Effect of cigarette smoking on plasma fibrin clot permeability and susceptibility to lysis. Thromb Haemost. 2009;102:1289–91.
35. Parsons A, Daley A, Begh R, Aveyard P. Influence of smoking cessation after diagnosis of early stage lung cancer on prognosis: systematic review of observational studies with meta-analysis. BMJ. 2010;340:b5569.
36. Karademirci M, Kutlu R, Kilinc I. Relationship between smoking and total antioxidant status, total oxidant status, oxidative stress index, vit C, vit E. Clin Respir J. 2017. https://doi.org/10.1111/crj.12757.
37. Shoji M, Hancock WW, Abe K, Micko C, Casper KA, Baine RM, et al. Activation of coagulation and angiogenesis in cancer: immunohistochemical localization in situ of clotting proteins and vascular endothelial growth factor in human cancer. Am J Pathol. 1998;152:399–411.
38. Komurcuoglu B, Ulusoy S, Gayaf M, Guler A, Ozden E. Prognostic value of plasma D-dimer levels in lung carcinoma. Tumori. 2011;97:743–8.
39. Ay C, Pabinger I. Predictive potential of haemostatic biomarkers for venous thromboembolism in cancer patients. Thromb Res. 2012;129(Suppl 1):6–9.
40. Gale AJ, Gordon SG. Update on tumor cell procoagulant factors. Acta Haematol. 2001;106:25–32.
41. Ay C, Ünal UK. Epidemiology and risk factors for venous thromboembolism in lung cancer. Curr Opin Oncol. 2016;28:145–9.
42. Zhang C, Jia Y, Jia Y, Zhang X, Li K. Prognostic and predictive value of plasma D-dimer levels in patients with small-cell lung cancer. Int J Clin Oncol. 2018;23:1070–5.
43. Kwon HC, Oh SY, Lee S, Kim SH, Han JY, Koh RY, et al. Plasma levels of prothrombin fragment F1 + 2, D-dimer and prothrombin time correlate with clinical stage and lymph node metastasis in operable gastric cancer patients. Jpn J Clin Oncol. 2008;38:2–7.
44. Rickles FR, Patierno S, Fernandez PM. Tissue factor, thrombin, and cancer. Chest. 2003;124:58S–68S.
45. Gronostaj K, Richter P, Nowak W, Undas A. Determinants of hypofibrinolysis in patients with digestive tract cancer. Prz Gastroenterol. 2016;11:104–10.