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Prothrombotic fibrin clot properties are associated with post-discharge venous thromboembolism in acutely ill medical patients

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ABSTRACT

Introduction: Reduced clot permeability and lysability have been reported in patients who experienced venous thromboembolism (VTE) following lower limb injury despite pharmacological thromboprophylaxis. We hypothesized that similarly altered fibrin clot properties characterize patients with post-discharge VTE despite thromboprophylaxis during prior hospitalization due to acute medical illnesses.

Methods: In a case-control study, we assessed 48 patients who developed VTE within 4 weeks post-discharge despite pharmacological thromboprophylaxis during hospitalization (the thromboprophylaxis group) and three age- and sex-matched control groups (n = 48 each): (1) patients who developed VTE following hospitalization without pharmacological thromboprophylaxis (the no-thromboprophylaxis group), (2) patients with unprovoked VTE and (3) individuals without history of VTE (the no-VTE group). Blood samples were obtained following ≥3 months of anticoagulation in VTE patients. Fibrin clot properties, thrombin generation and fibrinolysis activators and inhibitors were assessed.

Results: Compared with the no-VTE group, the thromboprophylaxis group formed denser fibrin networks reflected by lower clot permeability (K_s, −13%) and impaired fibrinolysis, as evidenced by prolonged clot lysis time (CLT, +14%) and lower rate of D-dimer release from clots (D-D_{rate}, −9%) accompanied by elevated high-sensitivity C-reactive protein (hsCRP, +79%), peak thrombin generation (+55%) and α_2-antiplasmin (+10%, all $p < 0.05$). Similar fibrin clot features were observed following unprovoked VTE. The thromboprophylaxis group had also lower K_s (−13%), elevated α_2-antiplasmin (+18%) and higher peak thrombin generation (+25%, all $p < 0.05$) as compared with the no-thromboprophylaxis group.

Conclusions: Unfavorably altered plasma clot properties and increased thrombin generation characterize medical patients with post-discharge VTE despite receiving pharmacological thromboprophylaxis during hospitalization for acute conditions.

1. Introduction

Venous thromboembolism (VTE) encompasses deep vein thrombosis (DVT) and pulmonary embolism (PE), with an overall annual incidence of 104–183 per 100,000 European inhabitants [1,2]. Provoked VTE that accounts for about 40% of all VTE cases is associated with major trauma, surgery, cardiac or respiratory failure, prolonged immobility, presence of central venous lines, acute infection or rheumatic disorder, sometimes with coexistent inherited and acquired thrombophilias [3–5]. Hospitalization represents a common, preventable risk factors

Abbreviations: ΔAb_{max}, maximum absorbance at the plateau phase; APTT, activated partial thromboplastin time; BMI, body mass index; CAT, calibrated automated thrombography; CLT, clot lysis time; COPD, chronic obstructive pulmonary disease; D-D_{max}, maximum D-dimer concentrations; D-D_{rate}, rate of increase in D-dimer levels; DVT, deep vein thrombosis; ETP, endogenous thrombin potential; HDL-C, high-density lipoprotein cholesterol; HRT, hormone replacement therapy; hsCRP, high-sensitivity C-reactive protein; INR, international normalized ratio; IQR, interquartile range; K_s, fibrin clot permeability; LDL-C, low-density lipoprotein cholesterol; LMWH, low molecular weight heparin; NOACs, non-vitamin K antagonist oral anticoagulants; OR, odds ratio; PAI-1, plasminogen activator inhibitor-1 antigen; PCR, polymerase chain reaction; PE, pulmonary embolism; RA, rheumatoid arthritis; SD, standard deviation; SLE, systemic lupus erythematosus; TC, total cholesterol; TF, tissue factor; TG, triglycerides; tPA, tissue plasminogen activator; TTR, the time in therapeutic range; UFH, unfractionated heparin; VTE, venous thromboembolism;

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for VTE [6]. For acutely ill, hospitalized medical patients at increased risk of thrombosis the American College of Chest Physicians recommends thromboprophylaxis (Grade 1B) and advised against extending the duration of thromboprophylaxis beyond the period of patient immobilization or hospital stay (Grade 2B) [7]. The proportion of acutely ill hospitalized patients with VTE risk factors receiving thromboprophylaxis is low [8–10]. Of the biomarkers, only elevated D-dimer on admission has been shown to be related to increased risk of VTE during hospitalization and post-discharge period in acutely ill medical patients [11].

Prophylaxis with low-molecular-weight-heparins (LMWH) and unfractionated heparin (UFH) safely reduces the risk of VTE in patients with acute medical illnesses requiring hospitalization [12]. The prophylaxis failure rates for LMWH and UFH are estimated at 1.10% and 1.17%, respectively [13,14]. In the US Hospital Performance Consortium for VTE 85% of hospitalization-related VTE events occurred in the post-discharge period, with 17–19.5 days to VTE [14–16].

Evidence indicates that there is an association between altered fibrin clot properties and both venous and arterial thrombotic episodes [17]. The so-called prothrombotic clot phenotype is characterized by a dense, compact, poorly lysable fibrin clots with decreased porosity, increased resistance to lysis and it is determined by genetic and environmental factors [17,18]. Unfavorably altered fibrin clot have been reported in individuals with recurrent PE, unprovoked VTE and their first-degree relatives [19–21]. Little is known about fibrin variables in patients with provoked VTE, including those who developed thrombosis following hospitalization.

Goldman et al. [22] have shown recently that patients who experienced DVT despite thromboprophylaxis following lower limb trauma display the prothrombotic fibrin clot phenotype including denser fibrin networks and impaired fibrinolysis. It has been suggested that such patients may benefit from prolonged thromboprophylaxis and might be at risk of recurrent DVT at least following injury [22]. Based on those results, we hypothesized that a prothrombotic state, including increased thrombin generation and unfavorable fibrin clot properties characterize acutely ill medical patients who experienced VTE events within first post-discharge weeks despite receiving pharmacological thromboprophylaxis during hospital stay. The aim of the current study was to assess prothrombotic markers in medical patients with post-discharge VTE in relation to the use of pharmacological prophylaxis (or the lack thereof) during hospital stay and to compare with those with unprovoked VTE and healthy controls.

2. Materials and methods

2.1. Patients

In a case-control study we recruited 4 age- and sex-matched groups. The cases comprised 48 consecutive adult patients who developed VTE within 4 weeks since hospitalization in internal medicine wards due to acute medical illnesses and exacerbations of chronic diseases and were referred to our center for additional laboratory work-up (further referred to as thromboprophylaxis group). Patients hospitalized for an acute heart failure (NYHA class III or IV), exacerbation of asthma or chronic obstructive pulmonary disease (COPD), pneumonia or exacerbation of rheumatic diseases (rheumatoid arthritis, systemic lupus erythematosus or others) or combinations thereof were eligible. All patients received pharmacological thromboprophylaxis (enoxaparin 40 mg or dalteparin 5000 units daily) during the entire hospital stay starting from the first day. Injections were performed under supervision of a nurse.

All patients were recruited at the Center for Coagulation Disorders in Cracow, Poland from October 2010 until June 2017, after at least 3 months of anticoagulation treatment, mainly with LMWH at therapeutic doses followed by the vitamin K antagonists (VKA) or non-vitamin K antagonist oral anticoagulants (NOAC). Patients who received LMWH for 3 months based on their preferences were also eligible.

We enrolled three control groups matched for age and sex (48 individuals in each group):

1. The no-thromboprophylaxis group included patients hospitalized in internal medicine wards without pharmacological thromboprophylaxis ordered by the managing physicians and developed VTE within 4 weeks since the end of hospitalization.
2. The unprovoked-VTE group included patients following the VTE event of an unknown cause. Exclusion criteria were hospitalization > 3 days within the last month, known cancer, major trauma, surgery, oral contraceptive use or hormone replacement therapy and pregnancy or delivery within the last 3 months.
3. The no-VTE group included individuals without any history of VTE or other vascular events.

The exclusion criteria for all four groups were as follows: age above 65 years, known malignancy, acute coronary syndrome, ischemic stroke, major trauma or surgery within the previous 3 months, chronic kidney disease stage 4 or 5, high-risk thrombophilia (i.e. antiphospholipid syndrome, deficiency of antithrombin, protein C or protein S and homozygous prothrombotic mutations or the combined abnormalities), international normalized ratio (INR) > 1.2 on the day of blood samples drawn and pregnancy or postpartum period.

The diagnosis of PE was based on the presence of typical symptoms and positive result of high resolution spiral computed tomography. The diagnosis of DVT was established by a positive finding of color duplex sonography (visualization of an intraluminal thrombus in calf, popliteal, femoral or iliac veins).

Smoking was defined as the inhalation of the smoke of burning tobacco encased in at least one cigarette daily. Diabetes mellitus was defined in accordance with the American Diabetes Association criteria. The diagnosis of arterial hypertension was established by a history of hypertension (consistent blood pressure ≥ 140/90 mmHg). Family history of VTE was defined as confirmed VTE episode in a first-degree relative. Obesity was defined as body mass index (BMI) ≥ 30 kg/m^2. Heart failure was defined as the presence of relevant symptoms and signs and left ventricular ejection fraction ≤40%. Chronic obstructive pulmonary disease was diagnosed based on the signs and symptoms and results of spirometry in medical records. The diagnosis of asthma was established based on a history of recurrent respiratory symptoms (shortness of breath, chest tightness, wheeze and cough) and documented post bronchodilator increase in FEV_1 of at least 200 mL and 12% from the baseline. All patients with rheumatic diseases fulfilled the American College of Rheumatology criteria for diagnosing them [23,24].

The Jagiellonian University Medical College Ethical Committee approved the study and all participants provided informed consent in accordance with the Declaration of Helsinki.

2.2. Laboratory investigations

Fasting blood samples were drawn using a minimal stasis and atraumatic venipuncture from an antecubital vein between 8 and 10 AM after a minimum 3 months of anticoagulant treatment. Patients treated with VKA were switched to an LMWH for 10 to 14 preceding days and blood samples were collected 16 to 24 h after the last subcutaneous injection. To confirm negligible if any residual anti-Xa activity, this parameter was determined in patients receiving LMWH prior to blood collection (Siemens, Marburg, Germany). The remaining patients treated with NOAC were examined after at least 24 h since the last dose of the drug. Blood cell count, glucose, creatinine, lipid profile, INR and activated partial thromboplastin time (APTT) were measured using routine laboratory techniques. The time in therapeutic range (TTR) was used to monitor anticoagulation quality. Fibrinogen was determined using the von Clauss method. High-sensitivity C-reactive

protein (hsCRP) was measured by immunoturbidimetry (Roche Diagnostics GmbH, Mannheim, Germany). An Innovance assay was used to determine plasma D-dimer levels (Siemens, Marburg, Germany). Plasminogen and α_2-antiplasmin activities were analyzed by chromogenic assays (STA Stachrom antiplasmin and STA Stachrom plasminogen, Diagnostica Stago, Asnieres, France). Plasminogen activator inhibitor-1 antigen (PAI-1) was measured by enzyme-linked immunosorbent assays (Hyphen Biomed, Neuville Sur Oise, France). Blood samples were mixed with 3.2% trisodium citrate solution (vol/vol, 9:1) and centrifuged at 2000 ×*g* for 10 min within 30 min of the draw. After centrifuging, the supernatant was aliquoted and stored at −80 °C until the analysis of the clot properties and thrombin generation. All measurements were determined by technicians blinded to the origin of the samples (intra-assay and inter-assay coefficients of variation, 5% to 7%).

2.3. Thrombin generation

To assess peak thrombin generation (maximum concentration of thrombin formed during the recording time), time to peak and endogenous thrombin potential (ETP; area under the curve) we used the calibrated automated thrombography (CAT) with computational model of thrombin dynamics (Thrombinoscope BV, Maastricht, the Netherlands). According to the manufacturer's instructions in the 96-well plate fluorometer (Ascent Reader, Thermo Lab Systems OY, Helsinki, Finland) equipped with the 390/460 filter set at a temperature of 37 °C duplicate plasma samples were analyzed. Briefly, 80 µl of platelet-poor plasma was diluted with 20 µL of a tissue factor (TF)-based activator (Diagnostica Stago, Asnieres, France) containing 5 pmol/L recombinant TF, 4 micromolar phosphatidylserine/phosphatidylcholine/phosphatidylethanolamine vesicles and 20 µL of FluCa solution (Hepes, pH 7.35, 100 nmol/L CaCl2, 60 mg/mL bovine albumin and 2.5 mmol/L *Z*-Gly-Gly-Arg-amidomethylocoumarin).

2.4. Fibrin permeation

A pressure-driven system was used to assess fibrin clot permeation. $CaCl_2$ (20 mmol/L) and human thrombin (1 U/mL) (Sigma, St Louis, MO, USA) were added to 120 µL citrated plasma. Samples were incubated for 120 min in a wet chamber. Tubes containing the clots were connected via plastic tubing to a reservoir of a buffer (0.01 M Tris, 0.1 M NaCl, pH 7.4) and its flowing through the gel was measured within 60 min. We calculated a permeation coefficient (K_s), which indicates the pore size, from the equation: $K_s = (Q \times L \times \mu)/(t \times A \times \Delta p)$ (where Q is the volume of the buffer flowing through the gel in time t; L is the length of a fibrin gel (13 mm); μ is the viscosity of liquid (in poise); A is the cross-sectional area ($0.049 cm^2$); Δp is the differential pressure (in dyne/cm^2); t is the percolating time). K_s is a measure of density of fibrin network which represents a mean surface of the average pore size.

2.5. Turbidity measurements

To initiate the polymerization we mixed plasma citrated samples 2:1 with a Tris buffer containing 0.6 U/mL human thrombin (Sigma) and 50 mM $CaCl_2$. The absorbance was read at 405 nm. We recorded a lag phase of the turbidity curve, which reflects the time required for initial protofibril formation, and maximum absorbance at the plateau phase (ΔAb_{max}) indicating an average fibrin fiber thickness.

2.6. Lysis Assays

Two methods were used to assess the susceptibility to in-vitro fibrinolysis.

Assay 1. Citrated plasma was mixed with 15 mmol/L $CaCl_2$, 10,000 diluted human TF (Innovin, Siemens) with a final concentration of 0.6 pM, 12 µM phospholipid vesicles and 60 ng/mL recombinant tissue plasminogen activator (tPA) (Boehringer Ingelheim, Ingelheim, Germany). The turbidity was measured at 405 nm at 37 °C. Clot lysis time (CLT) was defined as the time from the midpoint of the clear-to-maximum-turbid transition (representing the clot formation) to the midpoint of the maximum-turbid-to-clear transition (representing the lysis of the clot).

Assay 2. Fibrinolysis of the plasma clots, formed as for the permeability evaluation, was assessed during their perfusion with a Tris buffer containing 0.2 µmol/L rtPA (Boehringer Ingelheim). D- dimer levels were measured every 20 min in the effluent using an ELISA (American Diagnostica). When the fibrin gel collapsed under the pressure, usually after 80–120 min, the experiment was ended. Maximum rate of increase in D-dimer levels ($D\text{-}D_{rate}$) and maximum D-dimer concentrations ($D\text{-}D_{max}$) were measured.

2.7. Genotyping

The factor XIII Val34Leu (FXIII Val34Leu), α-fibrinogen Thr312Ala, factor V Leiden (FV Leiden) and prothrombin 20210A polymorphism were determined by the polymerase chain reaction (PCR) followed by restriction fragment length polymorphism analysis.

2.8. Statistical analysis

The study was powered to have a 90% chance of detecting a 10% difference in clot lysis time using a *p*-value of 0.05, based on the values of clot lysis time in the published article [25]. In order to demonstrate such a difference or greater, 23 patients were required in each group. To demonstrate such a difference or greater in K_s, using a *p*-value of 0.05, at least 31 patients were required in each group [26].

Continuous variables are presented as a mean and standard deviation (SD) or as a median and interquartile range (IQR). Categorical variables are presented as numbers and percentages. To identify the normal distribution, the Shapiro-Wilk test was applied. The χ^2 test, Fisher exact test, Student *t*-test and Mann-Whitney *U* test were used, as appropriate, for groups comparison. To investigate intergroup differences between multiple groups Kruskal-Wallis test was used. The Pearson correlation test (Pearson's r) were calculated for the correlation of parametric variables and the Spearman's rank correlation test was used for nonparametric variables. The logistic regression was applied to assess odds ratio (OR) with 95% confidence intervals (CI). To adjust for confounders: age, BMI, sex or fibrinogen all non-normal distributed data were log-transformed and a one-way analysis of covariance (ANCOVA) was performed.

The level of significance for the two sided *p*-value was set below 0.05. Statistical analyses were performed with STATISTICA 13.0 Software (Statsoft Inc., Tulsa, Oklahoma, USA).

3. Results

3.1. Patient characteristics

Characteristics of the four groups are presented in Table 1.

There were no intergroup differences in demographic or clinical variables except for a prevalence of type 2 diabetes (Table 1). In the thromboprophylaxis group there were 8 (16%) diabetics, in the no-VTE group 3 (6%), 2 (4%) in the unprovoked-VTE group and none in the no-thromboprophylaxis group (p = 0.005). There were no differences with regard to the comorbidities and causes of hospitalization (Table 1). In the thromboprophylaxis group patients were hospitalized due to decompensated heart failure: 15 (31%), exacerbation of asthma: 4 (8%), COPD: 10 (21%), pneumonia: 4 (8%), exacerbation of rheumatoid arthritis (RA): 8 (17%), systemic lupus erythematosus (SLE): 3 (6%), other rheumatic diseases: 3 (6%) or combinations thereof: 1 (2%). In the no-thromboprophylaxis group respectively: decompensated HF: 19

Table 1
Characteristics of the studied groups.

Variable	The thromboprophylaxis group (n = 48)	The no-thromboprophylaxis group (n = 48)	The unprovoked VTE group (n = 48)	The no-VTE group (n = 48)	p-Values
Age, y	52.0 (10.5)	56.5 (10.0)	56.0 (10.0)	55.0 (10.5)	0.43
Male sex, n (%)	30 (63)	25 (52)	28 (58)	25 (52)	0.67
Body mass index, kg/m^2	27.4 (5.6)	26.8 (3.4)	26.2 (5.4)	25.8 (3.9)	0.07
Risk factors and comorbidities, n (%)					
Smoking	15 (31)	18 (38)	21 (44)	17 (35)	0.64
Oral contraceptives or HRT[a]	9 (50)	7 (30)	0 (0)[b]	5 (22)	0.51[b]
Diabetes mellitus	8 (16)	0 (0)	2 (4)	3 (6)	0.005
Arterial hypertension	17 (35)	13 (27)	13 (27)	14 (29)	0.78
Family history of VTE	13 (27)	7 (15)	3 (6)	6 (13)	0.04
Obesity	13 (27)	9 (19)	11 (23)	5 (10)	0.20
Causes of hospitalization, n (%)					
Respiratory disease	18 (38)	15 (31)	–	–	0.52
Decompensated HF	15 (31)	19 (40)	–	–	0.39
Exacerbation of rheumatic disorder	14 (29)	14 (29)	–	–	1.00
COPD + HF	1 (2)	–	–	–	–
Clinical presentation of venous thromboembolism, n (%)					
Isolated DVT	29 (60)	19 (40)	27 (56)	0 [c]	0.09[c]
Isolated PE	16 (33)	20 (42)	14 (29)	0 [c]	0.42[c]
DVT with PE	3 (6)	9 (19)	7 (15)	0 [c]	0.18[c]
Time since discharge from hospital (days)	9 ± 5	9 ± 3	–	–	0.48
Laboratory parameters					
Haemoglobin, g/L	13.85 ± 1.21	13.93 ± 1.18	13.77 ± 1.11	13.89 ± 0.98	0.91
Platelets, 10^9/L	215.5 (95.0)	228.0 (72.0)	210.0 (78.0)	229.5 (64.0)	0.96
White blood cells, 10^9/L	6.66 ± 1.24	6.62 ± 1.54	7.07 ± 1.31	6.98 ± 1.49	0.28
Creatinine, μmol/L	69.5 (20.5)	70.7 (19.6)	69.0 (17.4)	65.0 (11.0)	0.43
Glucose, mmol/L	4.95 (0.90)	5.00 (0.69)	4.76 (0.87)	5.2 (1.05)	0.01
TG, mmol/L	1.17 (0.99)	1.13 (0.90)	1.58 (1.18)	1.18 (0.75)	0.02
TC, mmol/L	5.03 ± 1.17	4.94 ± 1.00	5.57 ± 1.01	4.78 ± 0.84	0.001
HDL-C, mmol/L	1.51 (0.58)	1.47 (0.48)	1.29 (0.46)	1.36 (0.46)	0.25
LDL-C, mmol/L	2.95 ± 0.86	2.92 ± 0.82	3.49 ± 0.86	2.84 ± 0.66	0.0003
hsCRP, mg/L	2.47 (4.58)	1.73 (1.15)	2.1 (1.39)	1.38 (1.31)	< 0.0001
INR	1.01 (0.13)	0.98 (0.14)	0.98 (0.20)	0.97 (0.15)	0.86
D-dimer, ng/mL	290.5 (160.5)	283.0 (98.5)	232.5 (86.0)	278.0 (81.0)	0.002
Fibrinogen, g/L	3.06 (1.36)	2.62 (1.07)	3.01 (1.76)	2.70 (0.91)	0.06
APTT, s	29.25 ± 3.06	29.55 ± 2.00	28.54 ± 2.91	28.07 ± 2.96	0.04
Genetic polymorphisms, n (%)					
Factor XIII Val34Leu	24 (50)	24 (50)	21 (44)	12 (25)	0.04
α-Fibrinogen Thr312Ala	28 (59)	18 (38)	24 (50)	15 (31)	0.03
Factor V Leiden	7 (15)	4 (8)	5 (10)	10 (21)	0.3
Prothrombin 20210A mutation	1 (2)	0 (0)	2 (4)	4 (8)	0.1
Therapy at blood collection, n (%)					
Compression	36 (75)	24 (50)	28 (59)	0	0.037
Aspirin	10 (21)	3 (6)	8 (17)	0	0.09
Anticoagulant therapy, n (%)					
VKA switched to LMWH	17 (35)	15 (31)	19 (40)	–	0.095
NOACs[d]	18 (38)	20 (42)	26 (54)	–	
LMWH	13 (27)	13 (27)	3 (6)	–	

Data are given as mean ± standard deviation, median (interquartile range) or number (percentage).
Abbreviations: COPD, chronic obstructive pulmonary disease; DVT, deep vein thrombosis; HDL-C, high-density lipoprotein cholesterol; HF, heart failure; HRT, hormone replacement therapy; hsCRP, high-sensitivity C-reactive protein; INR, international normalized ratio; LDL-C, low-density lipoprotein cholesterol; LMWH, low molecular weight heparin; NOACs, non-vitamin K antagonist oral anticoagulants; PE, pulmonary embolism; TC, total cholesterol; TG, triglycerides; VTE, venous thromboembolism;
[a] Females only.
[b] Contraceptives was an exclusion criterion in the unprovoked-VTE group (p-Value refers to the comparison of three other groups).
[c] Comparison for 3 groups except the unprovoked-VTE group with no such cases.
[d] Rivaroxaban or dabigatran.

(40%), asthma: 4 (8%), COPD: 9 (19%), pneumonia: 2 (4%), RA: 9 (19%), SLE: 4 (8%) and other rheumatic diseases: 1 (2%). Twenty-eight (58,3%) patients from the thromboprophylaxis group and 24 (50%) patients from the no-thromboprophylaxis group (p = 0.41) were hospitalized for the first time. Mean duration of hospitalization in the thromboprophylaxis group was 10 ± 2 days. In medical records non-compliance was not reported in any patients from the thromboprophylaxis group. On the day of discharge from the hospital none of the patients were advised to use thromboprophylaxis and none of them were immobilized or transferred to another hospital. There were no intergroup differences with regard to hsCRP on discharge between the thromboprophylaxis group and no-thromboprophylaxis group (median

[IQR]; 2.91 [2.38] vs 2.70 [2.39] mg/L, respectively, p = 0.46). Symptoms of VTE appeared after 9 ± 5 days (range from 2 to 20) after discharge in the thromboprophylaxis group and after 9 ± 3 days (range from 5 to 17) in the no-thromboprophylaxis group (p = 0.48). The duration of anticoagulation therapy after VTE diagnosis to enrolment was shorter in the thromboprophylaxis group (median [IQR]; 4 [2] months) and the no-thromboprophylaxis group (4 [2] months) than in the unprovoked-VTE group (9.5 [5] months, both p < 0.0001). Family history of VTE occurred more frequently in the thromboprophylaxis group versus the unprovoked-VTE group (p = 0.01).

In the thromboprophylaxis group glucose level was slightly lower than in the no-VTE group and higher than in the unprovoked-VTE group (Table 1). The thromboprophylaxis group showed lower triglycerides, total cholesterol and low-density lipoprotein cholesterol when compared with the unprovoked-VTE group (all p < 0.05). Patients with thromboprophylaxis had higher hsCRP compared with the no-VTE group (+79%; p = 0.0001) and the no-thromboprophylaxis group (+43%, p = 0.007) with a borderline significance when compared to the unprovoked-VTE group (p = 0.054). The thromboprophylaxis group and the no-thromboprophylaxis group showed higher D-dimer levels (by +25%, p = 0.014 and by +22%, p = 0.002) compared with the unprovoked-VTE group, but there were no differences when compared with the no-VTE group (both $p > 0.05$). Other laboratory tests, including fibrinogen, were similar in all groups (Table 1).

There was a higher prevalence of FXIII Val34Leu allele carriers in the thromboprophylaxis group and in the no-thromboprophylaxis group than in the no-VTE group. α-Fibrinogen Thr312Ala allele carriers occurred more frequent in the thromboprophylaxis group when compared with the no-thromboprophylaxis group and the no-VTE group. A similar prevalence of factor V Leiden and prothrombin 20210A mutations was observed in all 4 groups (Table 1).

We found no differences with regard to the anticoagulant used (Table 1 and Supplementary Table 1). In 20 of 80 subjects on LMWH prior to blood collection (25%), detectable anti-Xa activity was found (maximum, 0.11 IU/ml; median [IQR], 0.06 [0.048–0.085] IU/ml). Detectable anti-Xa was found in 6 patients of the 30 subjects on LMWH in the thromboprophylaxis group (0.07 [0.053–0.095] IU/ml), while 7 patients of the 28 subjects in the no-thromboprophylaxis group (0.06 [0.050–0.085] IU/ml) and 7 out of 22 patients in the unprovoked VTE group (0.05 [0.035–0.090] IU/ml). No differences in fibrin clot properties and thrombin generation were found between the 20 patients with detectable anti-Xa and the remainder (Supplementary Table 2).

3.2. Thrombin generation

ETP was larger by 7% in the thromboprophylaxis group, by 7% in the no-thromboprophylaxis group and by 10% in the unprovoked-VTE group compared with the no-VTE group (all p < 0.001). There were no differences in ETP among the three VTE patient groups (Table 2; Fig. 1A).

In the thromboprophylaxis group the time to peak was 39% longer than in the no-thromboprophylaxis group and 25% longer than in the unprovoked-VTE group (both p < 0.001) but it was similar to the value observed in the no-VTE group ($p = 0.91$; Table 2).

Peak thrombin generation was higher by 55% in the thromboprophylaxis group, by 25% in the no-thromboprophylaxis group and by 31% in the unprovoked-VTE group compared with the no-VTE group (all p < 0.0001; Table 2). Unexpectedly, the thromboprophylaxis group had higher peak thrombin generation when compared with three other groups (all p < 0.005). Intergroup differences in thrombin generation parameters remained significant after adjustment for age, sex, BMI, fibrinogen, the anticoagulant used and clinical presentation of VTE.

Analysis of thrombotic and fibrinolysis markers in subgroups formed according to the clinical presentation of venous thromboembolism shown no differences (Supplementary Table 3).

In the thromboprophylaxis group 60% of patients had increased peak thrombin generation defined as a value above 90th percentile observed in the no-VTE group (> 244 nmol/L; OR, 4.1; 95% CI; 2.28–7.38; p < 0.0001). Corresponding ORs for the unprovoked VTE patients and those who developed post-discharge VTE without thromboprophylaxis during hospital stay were lower (OR, 2.57; 95% CI; 1.43–4.63; p = 0.002 and OR, 2.13; 95% CI; 1.17–3.87; p = 0.014; respectively).

3.3. Fibrinolysis activators and inhibitors

No differences between groups with regard to plasminogen were found.

α_2-antiplasmin was higher in the thromboprophylaxis group versus the no-VTE group (+10%, p = 0.0002), versus the no-thromboprophylaxis group (+18%, p < 0.001) and versus the unprovoked-VTE group (+9%, $p = 0.004$) (Fig. 1B). When elevated α_2-antiplasmin level was defined as an α_2-antiplasmin level above 90th percentile of the no-VTE group (> 115%), 40% of patients from the thromboprophylaxis group had elevated α_2-antiplasmin.

PAI-1 was lower in the thromboprophylaxis group versus the no-VTE group (−32%, p < 0.001), versus the no-thromboprophylaxis group (−27%, p < 0.001) and versus the unprovoked-VTE group (−44%, p < 0.001) (Table 2).

As expected, in the thromboprophylaxis group α_2-antiplasmin correlated positively with BMI ($r = 0.35$, p < 0.05) and plasminogen ($r = 0.4$, p = 0.005).

All the differences in fibrinolysis activators and inhibitors remained significant after adjustment for age, sex, BMI, fibrinogen, the anticoagulant used and clinical presentation of VTE.

3.4. Fibrin clot features

K_s was decreased in the thromboprophylaxis group compared with the no-VTE group (−13%, p < 0.0001) and the no-thromboprophylaxis group (−13%, p = 0.049), but it was similar to the values measured in the unprovoked-VTE group (Fig. 1C). Similarly, patients with unprovoked-VTE had lower K_s (−11%, p < 0.0001) when compared with the no-VTE group.

No differences among the four groups were observed with regard to ΔAb_{max} (Fig. 1D).

In the thromboprophylaxis group lag phase was shorter than in the no-VTE group (−7%, p = 0.001), but it was longer than observed in the unprovoked-VTE group (+16%, p = 0.003) (Fig. 1E). Lag phase was shorter comparing the unprovoked-VTE group compared with controls (−20%, p < 0.0001).

Longer CLT was observed in the thromboprophylaxis group when compared with the no-VTE group (+14%, p = 0.012), but there were no differences between the former group and the 2 remaining ones (Fig. 1F). Patients with unprovoked-VTE had longer CLT versus the no-VTE group (+11%, p = 0.03).

Compared with the no-VTE group patients with post-discharge VTE despite thromboprophylaxis during prior hospitalization were associated with lower rate of D-dimer release from clots ($D\text{-}D_{rate}$, −9%, p < 0.0001) (Fig. 1G). Slightly lower $D\text{-}D_{rate}$ was also observed in the no-thromboprophylaxis group (−6%, p = 0.02) and the unprovoked-VTE group (−7%, p = 0.008). There were no differences between other groups.

$D\text{-}D_{max}$ was lower in the thromboprophylaxis group versus the unprovoked-VTE group (−12%, p < 0.001) (Fig. 1H). $D\text{-}D_{max}$ was higher in the unprovoked-VTE group versus the no-VTE group (+12%, p = 0.012) and versus the no-thromboprophylaxis group (+17%, p < 0.001).

No differences in prothrombotic markers were observed in relation to the reason for hospitalization and time of VTE event since discharge (data not shown). All the differences in fibrin clot features remained

Table 2
Thrombotic and fibrinolysis markers.

Variable	The thromboprophylaxis group (n = 48)	The no-thromboprophylaxis group (n = 48)	The unprovoked VTE group (n = 48)	The no-VTE group, (n = 48)	p-Values
Thrombin generation					
Endogenous thrombin potential, nmol/L thrombin x min	1600.5 (199.0)	1591.5 (139.5)	1636.0 (124.5)	1492.5 (113.5)	< 0.0001
Time to peak thrombin concentration, s	351.0 (121.0)	252.5 (88.0)	281.5 (114.0)	348.0 (59.0)	< 0.0001
Thrombin peak, nmol/L	265.5 (134.0)	213.0 (69.5)	224.5 (58.0)	171.0 (50.5)	< 0.0001
Fibrinolysis activators and inhibitors					
Plasminogen, %	103.0 (20.0)	100.0 (20.5)	101.5 (18.0)	100.0 (21.0)	0.84
α_2-antiplasmin, %	111.5 (17.5)	94.5 (17.5)	102.0 (22.5)	101.7 (12.7)	< 0.0001
PAI-1 antigen, ng/mL	8.11 (2.80)	11.05 (9.30)	14.4 (8.75)	11.9 (6.05)	< 0.0001
Fibrin clot features					
K_s, 10^{-9} cm^2	6.45 (1.35)	7.40 (2.45)	6.60 (1.60)	7.40 (1.05)	< 0.0001
ΔAb_{max}, 405 nm	0.82 (0.14)	0.80 (0.13)	0.80 (0.11)	0.80 (0.10)	0.89
Lag phase, s	44.0 (11.0)	43.0 (5.5)	38.0 (5.5)	47.5 (6.0)	< 0.0001
CLT, min	91.7 ± 22.5	83.0 ± 17.1	88.9 ± 15.9	80.1 ± 14.5	0.006
D-D_{rate}, mg/L/min	0.069 (0.008)	0.072 (0.011)	0.071 (0.009)	0.076 (0.010)	0.003
D-D_{max}, mg/L	3.78 (0.47)	3.69 (0.83)	4.30 (0.72)	3.85 (0.59)	0.0001

Values are given as median (interquartile range), mean ± standard deviation (only CLT), p-Values refer to Kruskal-Wallis test.
Abbreviations: ΔAb_{max}, maximum absorbance at the plateau phase; CLT, clot lysis time; D-D_{max}, maximum D-dimer concentrations; D-D_{rate}, rate of increase in D-dimer levels; K_s, fibrin clot permeability; PAI-1, plasminogen activator inhibitor-1 antigen; VTE, venous thromboembolism;

significant after adjustment for age, sex, BMI, fibrinogen, the anticoagulant used and clinical presentation of VTE.

The FXIII Val34Leu allele carriers with a history of VTE showed longer CLT ($p < 0.001$) and higher ΔAb_{max} ($p < 0.001$), whereas Val34Val homozygous subjects had higher K_s ($p = 0.014$) and D-D_{rate} ($p = 0.02$). In α-fibrinogen Thr312Ala allele carriers with a history of VTE, higher D-D_{max} was observed ($p = 0.036$), whereas higher D-D_{rate} was measured in Thr312Thr homozygous subjects ($p < 0.001$). The presence of factor V Leiden and prothrombin 20210A mutation showed no associations with clot properties and thrombin generation in VTE patients.

4. Discussion

In the present study we show for the first time that acutely ill medical patients, who experienced VTE in the post-discharge period despite receiving pharmacological thromboprophylaxis during prior hospitalization, display a prothrombotic state as reflected by increased thrombin generation and prothrombotic fibrin clot phenotype with measured in vitro hypofibrinolysis. Faster formation of denser fibrin networks resistant to lysis characterized patients who developed VTE despite prior thromboprophylaxis as well as those after unprovoked VTE, who are considered at the highest risk of VTE recurrence after anticoagulation withdrawal [27]. Our findings suggest that medical patients who experienced VTE within 4 weeks since hospital discharge despite the use of LMWH as in-hospital thromboprohylaxis represent a special at-risk group displaying several prothrombotic alternations detectable a few months of anticoagulant treatment since the event. It might be speculated that this subset could require longer, post-discharge thromboprophylaxis. This issue deserves further investigation.

Our findings indicate that post-discharge VTE in acutely ill medical patients could be related to similar abnormalities previously demonstrated in patients who experienced DVT despite thromboprophylaxis following lower limb trauma [22]. In that study performed in patients at similar age with similar sex distribution, patients following the prophylaxis, when compared with controls, exhibited 12.8% lower K_s, 46.2% prolonged CLT and 8% lower D-D_{rate} which is in line with our observations except less impaired hypofibrinolysis reflected by CLT in the current report (+14%). The two studies suggest that individuals in whom the standard prophylaxis with LMWH failed, regardless of the clinical setting requiring its use, have blood prothrombotic characteristics, which may have practical implications.

The occurrence of VTE in patients without thromboprophylaxis and without so-called prothrombotic clot phenotype deserves a comment. At the time when the data were collected a proportion of acutely ill hospitalized patients receiving thromboprophylaxis was low. Secondly, this prothrombotic state may be observed only in a special at-risk group developing unprovoked VTE or despite thromboprophylaxis. In addition, there may be other still unknown factors that contribute to such observations. This issue deserves further investigation.

Given various anticoagulant drugs used in our study, residual anticoagulant effects as a potential confounder should be ruled out. Detectable levels of anti-Xa activity at below 0.11 IU/mL (far below therapeutic and prophylactic ranges) [28] were not associated with differences in prothrombotic markers including clot properties in our study. As previously shown [19], such low anti-Xa activity does not affect K_s or CLT using the same methodology. Moreover, we found no differences in the studied parameters between patients treated with VKA and switched to LMWH before blood collection versus those on NOAC. This suggests that > 24 h since the last dose of NOACs is sufficient to abolish anticoagulant effects detectable in the assays used. For rivaroxaban, we documented that low levels of this drug do not alter clot properties [29]. Even given controversial data on the rebound hypercoagulability after cessation of oral anticoagulant therapy, it appears unlikely that the intergroup differences reported here can be explained by this effect [30].

PAI-1 is a key regulator of the fibrinolytic system. Elevated levels of PAI-1 are observed in a large variety of pathologic conditions such as infections, stroke, myocardial infarctions and diabetes [31]. We observed decreased PAI-1 antigen level in the thromboprophylaxis group. The assay that we used for PAI-1 antigen measurement does not reflect the PAI-1 activity and does not discriminate between complexed PAI-1 and free PAI-1. Other fibrinolysis modulators that we did not study also might have been of some importance. Finally, α_2-antiplasmin level was higher in patients, who experienced VTE in the post-discharge period despite receiving pharmacological thromboprophylaxis during prior hospitalization when compared with other groups. It is the final component inhibiting the speed and abundance of fibrinolysis through direct plasmin inactivation and it was shown to play a major role in controlling fibrinolysis [32]. Interestingly, we found a positive correlation between α_2-antiplasmin and CLT and negative correlation between α_2-antiplasmin and D-D_{rate} suggesting that the main

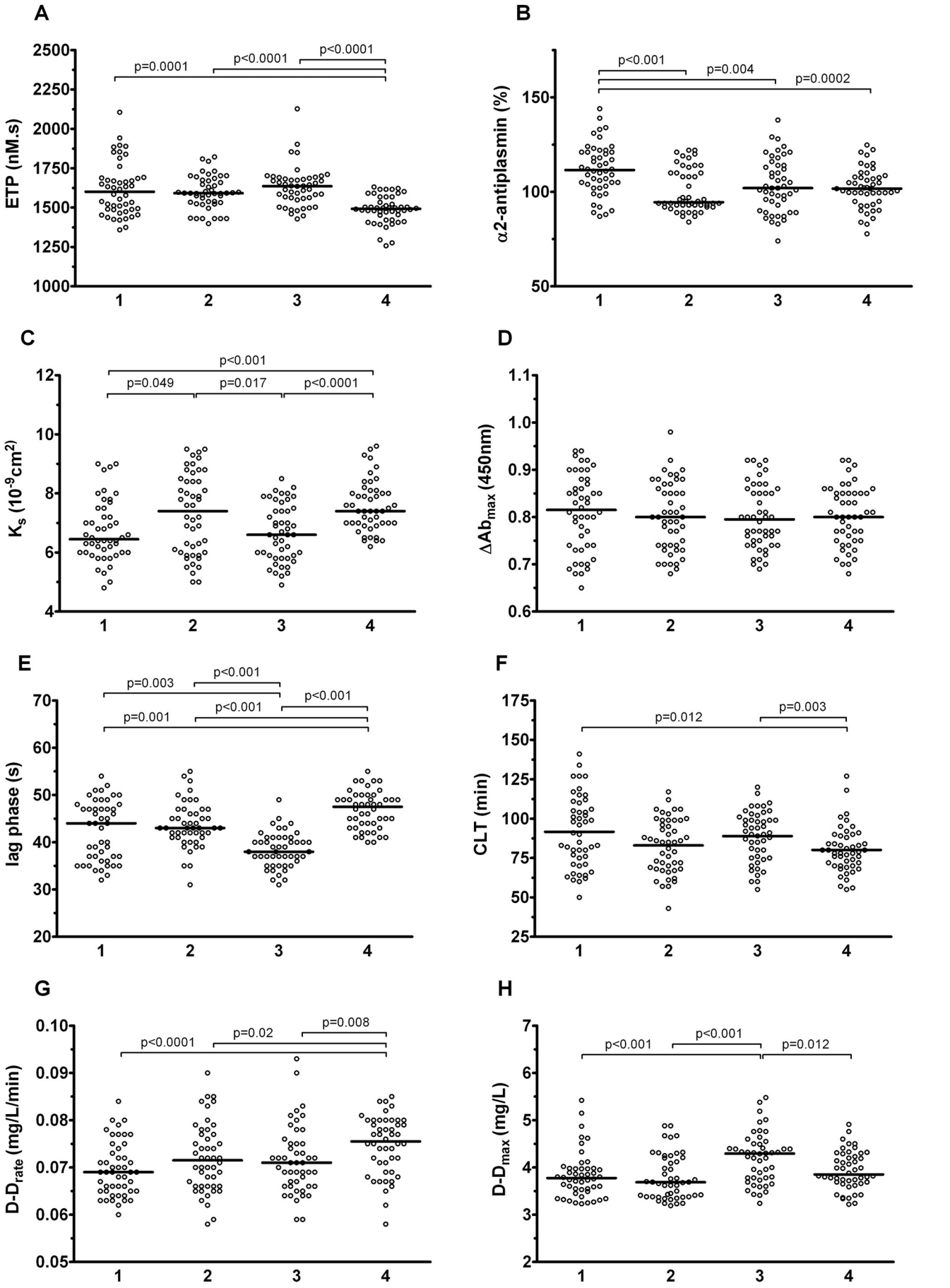

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Fig. 1. Comparison of selected parameters in the four studied groups. Horizontal lines represent medians or means (only CLT) of each groups.
1- The thromboprophylaxis group.
2- The no-thromboprophylaxis group.
3- The unprovoked VTE group.
4- The no-VTE group.

physiological inhibitor of plasmin contributes to the risk of post-discharge VTE in medical patients. It is known that elevated α_2-antiplasmin levels have been associated with increased thrombotic risk [33,34]. This current finding is consistent with the study by Goldman et al. [22]. Of note, α_2-antiplasmin level was 18% higher in acutely ill medical patients receiving the thromboprophylaxis with post-discharge VTE versus the no-thromboprophylaxis group. A surprising role of α_2-antiplasmin in hypofibrinolysis among medical patients who developed VTE needs to be clarified in further studies.

We found no differences with regard to ΔAb_{max} between the groups. Higher maximum clot absorbance is considered to be a marker of thicker fibers or increasing branching. However, plasma clot from patients with thrombotic manifestations are typically composed of thinner fibers, although several studies showed similar or larger thickness of fibrin fibers as compared to the controls even if the clots were less permeable and poorly lysable which suggests that plasma clots possess a far more complex structure in terms of its functional consequences [35]. To assess thickness of fibers other techniques are needed, and the ΔAb_{max} appears to be not sensitive enough to detect differences given comparable fibrinogen concentrations among groups, a key factor affecting this variable.

Goldman et al. have shown that individuals who experienced DVT despite thromboprophylaxis following lower limb trauma, when compared with controls, exhibited 5.9% higher ETP and 53.2% increased peak thrombin, which is in the line with results that we obtained in acutely ill medical patients (7% higher ETP and 55% increased peak thrombin) [22]. Interestingly, patients who developed VTE despite thromboprophylaxis had increased thrombin generation when compared with individuals after unprovoked VTE, which was not observed by Goldman et al. [22]

Comparison of prothrombotic variables in well-matched acutely ill medical patients with post-discharge VTE who received or not LMWH during hospital stay deserves a comment. We found that a specific prothrombotic clot phenotype associated with impaired clot lysability, in part driven by elevated α_2-antiplasmin, characterized patients who were prone to develop VTE following acute medical conditions activating blood coagulation after prophylactic benefits of heparins disappeared. Patients with VTE following hospitalization without thromboprophylaxis appeared to have “better” coagulation parameters and the use of heparins is likely to provide sufficient protection against VTE also in the post-discharge period.

Unexpectedly, we also found that post-discharge VTE in acutely ill medical patients despite receiving prophylaxis might be attributed to genetic factors. FXIII Val34Leu and α-fibrinogen Thr312Ala allele carriers tended to be overrepresented in the thromboprophylaxis group and displayed more prothrombotic clot features. It has been showed that at low fibrinogen concentration, similar to that in our study, FXIII Val34Leu is associated with less permeable clots which are more resistant to lysis, suggesting that protection against thrombosis by FXIII Val34Leu occurs only at elevated fibrinogen levels [17]. Moreover, higher prevalence of FXIII Val34Leu allele carriers in the thromboprophylaxis group and in the no-thromboprophylaxis group than in the no-VTE group was of borderline significance ($p = 0.04$). Possible protection against thrombosis could have been balanced by numerous risk factors related to hospitalization and acute illness.

The Thr312Ala allele has been associated with thicker fibrin fibers and increased α-chain cross-linking by FXIIIa [36]. Taken together, our study suggests a novel role of the two common polymorphisms as risk factors for VTE in medical patients with hospitalization-related thrombosis. This concept is worth further investigation.

Our study has several limitations. The size of the groups was relatively small. Given a 1% rate of VTE while using LMWH as recently demonstrated in the MARINER trial and according to our knowledge, analysis of such a cohort appears to be unique [13]. Moreover, the study was sufficiently powered to demonstrate intergroup differences among well-matched groups. Our case-control study has drawbacks inherent to this design. The current results cannot be likely extrapolated to hospitalized, elderly patients with a high probability of prothrombotic diseases, e.g. cancer and/or medications interfering with the tested parameters [18]. All the parameters were analyzed after VTE, which might per se alter clot phenotype, therefore it remains to be established whether prior to hospitalization they displayed a more prothrombotic alterations. We failed to observe any association between the time since the event and the magnitude of prothrombotic alterations, hence in our opinion the impact of the VTE event itself in the current study is negligible and if any, it cannot explain the intergroup differences in coagulation variables reported here. Non-compliance to LMWH during hospitalization in the thromboprophylaxis group is unlikely to largely contribute to the post discharge occurrence of the VTE and affect the results. Finally, clinical relevance of our findings needs to be further explored, however it might be hypothesized that patients following post-discharge VTE episode despite prophylaxis who have such prothrombotic clot characteristics are at risk of recurrent VTE as demonstrated by us recently [19,20]. Optimal thromboprophylaxis in this subset of patients remains to be established.

5. Conclusion

Our study demonstrates that patients receiving pharmacological thromboprophylaxis during hospitalization due to acute medical illnesses, who develop VTE in post-discharge period, exhibit denser fibrin clot formation and impaired clot lysis together with elevated α_2-antiplasmin. It might be speculated that altered thromboprophylaxis protocol could be beneficial for patients with abnormal clot characteristics in the presence of transient VTE risk factors such as hospitalization.

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Declaration of competing interest

A.U. received lecture honoraria from Bayer, Boehringer Ingelheim and Pfizer; other authors none declared.

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