Clinical Research Article

Procoagulant Imbalance in Klinefelter Syndrome Assessed by Thrombin Generation Assay and Whole-Blood Thromboelastometry

Rita Indirli,1,2* Emanuele Ferrante,1* Erica Scalabrino,3 Eriselda Profka,2 Marigrazia Clerici,3 Tommaso Lettera,4 Andreea Liliana Serban,1,5 Walter Vena,2,6 Alessandro Pizzocaro,6 Marco Bonomi,7,8 Biagio Cangiano,7,8 Giulia Carosi,1,5 Gherardo Mazzotti,6,9 Luca Persani,7,8 Andrea Lania,6,9 Maura Arosio,1,2 Flora Peyvandi,3 Giovanna Mantovani,1,2 and Armando Tripodi3

1Endocrinology Unit, Fondazione IRCCS Ca’ Granda Ospedale Maggiore Policlinico di Milano, 20122 Milan, Italy; 2Department of Clinical Sciences and Community Health, University of Milan, 20122 Milan, Italy; 3Fondazione IRCCS Ca’ Granda Ospedale Maggiore, Angelo Bianchi Bonomi Hemophilia and Thrombosis Center, 20122 Milan, Italy; 4Laboratorio analisi, Fondazione IRCCS Ca’ Granda Ospedale Maggiore Policlinico, 20122 Milan, Italy; 5Department of Experimental Medicine, Sapienza University of Rome, 00161 Rome, Italy; 6Endocrinology, Diabetology and Andrology Unit, Humanitas Clinical and Research Center IRCCS, Rozzano, 20089 Milan, Italy; 7IRCCS Istituto Auxologico Italiano, Department of Endocrine and Metabolic Diseases and Lab of Endocrine and Metabolic Diseases, 20149 Milan, Italy; 8Department of Medical Biotechnology and Translational Medicine, University of Milan, 20129 Milan, Italy; and 9Humanitas University, Department of Biomedical Sciences, Pieve Emanuele, 20090 Milan, Italy

ORCID numbers: 0000-0001-5642-0563 (R. Indirli); 0000-0002-0556-7650 (E. Ferrante); 0000-0001-5454-6074 (M. Bonomi); 0000-0002-2658-744X (B. Cangiano); 0000-0003-2068-9581 (L. Persani); 0000-0003-3988-3616 (M. Arosio); 0000-0002-9065-3886 (G. Mantovani).

*R.I. and E.F. contributed equally to this work.

Abbreviations: APTT, activated partial thromboplastin time; BMI, body mass index; ETP, endogenous thrombin potential; FVIII, factor VIII; HbA1c, glycated hemoglobin A1c; KS, Klinefelter syndrome; MCF, maximal clot firmness; MPO, myeloperoxidase; NET, neutrophil extracellular trap; PC, protein C; PPP, platelet-poor plasma; PRP, platelet-rich plasma; PT, prothrombin time; rTF, relipidated tissue factor; TGA, thrombin generation assay; TM, thrombomodulin.

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Abstract

Context: Klinefelter syndrome (KS) is a condition at increased risk of thrombosis compared to 46,XY men.

Objective: This work aimed to investigate the coagulation balance of KS patients by thrombin generation assay (TGA) and thromboelastometry.

Methods: An observational, cross-sectional study was conducted at 3 tertiary endocrinological centers in Milan, Italy. Fifty-eight KS patients and 58 age-matched
healthy controls were included. Anticoagulant or antiplatelet therapy and known coagulation disorders were exclusion criteria. TGA was performed in platelet-poor plasma (PPP) and platelet-rich plasma (PRP). Whole-blood thromboelastometry and activities of coagulation factors were assessed. Endogenous thrombin potential (ETP), the area under the thrombin generation curve, assessed with and without thrombomodulin (ETP-TM$^+$ and ETP-TM$^-$), and their ratio (ETP ratio), were considered as indexes of procoagulant imbalance.

**Results:** Patients with KS displayed higher PPP-ETP-TM$^+$ (mean 15.28 vs 0.1315 nM × min; $P < .001$), PPP-ETP ratio (0.78 vs 0.0.70; $P < .001$), factor (F)VIII (135% vs 0.107%; $P = .001$), fibrinogen (283 vs 0.241 mg/dL; $P < .001$), and FVIII/protein C ratio (1.21 vs 0.1.06; $P < .05$) compared to controls. Protein C was comparable in the 2 groups. Similar results were observed in PRP. The ETP ratio was positively associated with FVIII ($\rho = 0.538$, $P < .001$) in KS. Thromboelastometry parameters confirmed evidence of hypercoagulability in KS.

**Conclusion:** Patients with KS display a procoagulant imbalance expressed by increased thrombin generation both in PPP and PRP, which is at least in part explained by increased FVIII levels. The procoagulant imbalance, which was confirmed by thromboelastometry, may be responsible for the thrombotic events observed in these patients. Further investigation on the benefit/risk ratio of antithrombotic prophylaxis is warranted.

**Key Words:** Klinefelter syndrome, thrombin generation assay, thromboelastometry, thrombosis, hypogonadism, testosterone

Klinefelter syndrome (KS) is the most common chromosomal aneuploidy in men, with an estimated incidence of 1:650 newborn males (1, 2). Increased morbidity and mortality from all causes are described in KS (3), with a reduction in life expectancy of 2.1 years compared with the general population (4). Arterial and venous thromboses are from 3 to 20 times more frequent in KS patients of any age (5-9) and are among the leading causes of death in this condition (8, 10). Deep venous thrombosis, pulmonary embolism (collectively known as venous thromboembolism), mesenteric ischemia, venous insufficiency, leg ulcers (5, 6, 11), as well as cerebrovascular diseases (6, 12) are the main clinical thrombotic events observed in KS. However, the pathophysiological significances underlying this thrombotic predisposition are largely unknown and presumptive mechanisms are surmised mostly from case reports or small studies.

According to the Virchow triad, venous thromboembolism can result from hemodynamic changes such as reduction of blood flow or turbulence, endothelial injury or dysfunction, and blood hypercoagulability. A few studies have investigated endothelial parameters in KS, unveiling increased levels of apoptotic endothelial microparticles, enhanced expression of vitronectin receptor (13), and high concentrations of asymmetric dimethylarginine, a marker of endothelial dysfunction (14). Clues on hypercoagulability come from sparse studies, which suggest increased platelet reactivity (15) and impaired fibrinolysis due to increased levels of plasminogen activator inhibitor-1 (16). However, whether and to what extent other hemostasis abnormalities such as the balance between naturally occurring procoagulants and anticoagulants operating in plasma are influenced by metabolic derangements, hypogonadism, or testosterone treatment are unclear (8).

Coagulation is a complex and integrated mechanism, and the investigation of its individual components (procoagulants and anticoagulants) or the traditional tests, such as the prothrombin time (PT) or activated partial thromboplastin time (APTT), do not reflect the situation occurring in vivo. The thrombin generation assay (TGA) is an in vitro procedure based on the continuous registration of thrombin generation (mediated by procoagulants) and decay (mediated by anticoagulants) and is considered the closest approximation to the process occurring in vivo (17). TGA can be performed with or without the addition of thrombomodulin (TM), the physiological activator of protein C (PC) located on endothelial cells and needed to explore the contribution of this anticoagulant to the overall coagulation balance (17-19). TGA can be performed in platelet-poor plasma (PPP), to assess coagulation factors only, but also in platelet-rich plasma (PRP), to also investigate the contribution of the platelet procoagulant function. Whole-blood thromboelastometry assesses the viscoelastic changes of clotting blood under experimental conditions that mimic the contribution of plasma, blood cells, and platelets in vivo.
The primary aim of this study was to assess the contribution of plasma to thrombin generation performed in PPP in KS patients and age-matched healthy controls. Secondary aims were 1) to evaluate the contribution of the platelet procoagulant activity to thrombin generation performed in PRP, and 2) to evaluate the coagulation potential of KS patients by means of whole-blood thromboelastometry.

Materials and Methods

Participants

We selected adult men with a cytogenetically confirmed diagnosis of KS among those followed up at 3 tertiary endocrinological centers in Milan (Fondazione IRCCS Ca’ Granda Ospedale Maggiore Policlinico; IRCCS Humanitas Clinical and Research Center; IRCCS Istituto Auxologico Italiano). We excluded patients with known hereditary coagulation disorders and individuals on anticoagulation (parenteral or oral) or antiplatelet treatment.

Clinical information regarding smoking habit, arterial hypertension, dyslipidemia, testosterone replacement therapy, and the most recent assessment (ie, within the previous 3 months) of body mass index (BMI), fasting plasma glucose, and glycated hemoglobin A1c (HbA1c) were extracted from hospital records. Total testosterone and gonadotropin concentrations were assessed on blood samples collected during study visits.

Dyslipidemia was defined as triglycerides greater than 150 mg/dL, and/or HDL cholesterol less than 40 mg/dL (20), and/or LDL cholesterol above the risk category-specific thresholds recommended by the 2019 European Society of Cardiology/European Atherosclerosis Society guidelines for the management of dyslipidemias (21), and/or prescription of lipid-lowering treatment. Arterial hypertension was defined as systolic blood pressure greater than 140 mm Hg and/or diastolic blood pressure greater than 90 mm Hg (22), and/or prescription of antihypertensive treatment. Impaired fasting glucose and diabetes mellitus were defined according to American Diabetes Association criteria (23). Testosterone therapy was initiated in KS following current recommendations, that is, when symptoms and signs of testosterone deficiency became evident with endogenous testosterone levels below 12 nmol/L (24-26).

Healthy adults, recruited among male students and hospital staff in the 3 centers involved in the study, were included as controls. They were matched by age to the patient population and were free from current and past thrombotic events, anticoagulant drugs, or coagulation disorders known to affect TGA.

All study procedures were in accordance with the principles set out in the Declaration of Helsinki. The study was approved by the Milan Area 2 ethics committee (approval ID 1173). Written informed consent was obtained from all individuals included in the study.

Blood sampling and plasma preparation

Blood was collected from an antecubital vein into vacuum tubes containing 1/10 volume of trisodium citrate 109 mM (Becton Dickinson). For KS patients on testosterone replacement therapy, blood samples were taken 2 hours after application of testosterone transdermal gel, or at the end of the dosing interval in case of injectable long-acting testosterone undecanoate (24). One portion of citrated whole blood was used for thromboelastometry and the remaining was centrifuged (at controlled room temperature) with 2 different procedures. The first consisted of centrifugation for 20 minutes at 2880g and separation of PPP. The second consisted of centrifugation for 15 minutes at 150g to obtain PRP. PRP was then diluted into autologous PPP to obtain a standard platelet count of 150 × 10⁹/L. The resultant PRP was eventually tested for thrombin generation immediately after the preparation. PPP was aliquoted in plastic-capped tubes, quickly frozen by immersion in liquid nitrogen, and stored at −70 °C until testing. To limit between-assay variability, an equal number of samples from patients and controls were prepared and tested in the same run. All the experimental procedures were conducted at the Angelo Bianchi Bonomi Hemophilia and Thrombosis Center, Ospedale Maggiore Policlinico, Milan.

Thrombin generation assay

TGA was assessed according to Hemker et al (27) as described (28). Testing was based on the activation of coagulation after addition to plasma of human recombinant relipidated tissue factor (rTF, 1 pM) (Recombiplastin 2G, Werfen) and synthetic phospholipids (PL, 1.0 μM) (Avanti Polar) as coagulation triggers in PPP, or rTF alone in PRP. Testing was performed in 2 plasma aliquots, with and without the addition of rabbit TM (Haematologic Technologies) (2 nM). Registration of thrombin generation was obtained with a fluorogenic substrate (Z-Gly-Gly-Arg-AMC HCl, Bachem) (617 μM) by means of a fluorometer (Fluoroskan Ascent, Thermo Labsystems). The readings were recorded and analyzed with dedicated software (Thrombinoscope, Thrombinoscope BV), which displays the curve of thrombin concentration as a function of time and calculates the following parameters: the time (minutes) between the addition of the triggers and the initiation of thrombin generation (lag time); the thrombin peak (nM); the time (minutes) needed to reach the peak (TT peak); the area under the curve, defined as endogenous
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thrombin potential (ETP) and expressed as nM × min; and the velocity index, defined as [peak/(TT peak – lag time)] and expressed as nM/min. Results were also expressed as ETP ratio, that is, the ratio of ETP measured in the presence of TM (ETP-TM⁺) to the ETP measured in its absence (ETP-TM⁻).

Measurement of individual coagulation parameters

Whole-blood cell counts were performed with Micros-60 (Horiba ABX). PT and APTT were measured with Recombiplastin 2G or ThrombosIL APTT (Werfen). Both tests were performed on an automated coagulometer (ACL Top, Werfen) and results were expressed as the ratio of the patient plasma relative to the pooled normal plasma. PC and antithrombin were measured as chromogenic activity by means of commercial kits (Hemosil antithrombin and Hemosil PC; Werfen). Factor (F)VIII, II, and fibrinogen were measured as described (29) and results reported as percentage activity relative to pooled normal plasma with an (arbitrary) activity of 100%.

Myeloperoxidase

Because a mediator of thrombosis has been identified in extracellular nuclear chromatin, released from dead cells or inflammatory cells, especially in the form of neutrophil extracellular traps (NETs) (30), myeloperoxidase (MPO) was assessed as one of the NET-related variables. Plasma MPO activity was measured by a chromogenic assay employing the enzyme-linked immunosorbent assay (ELISA) kit Myeloperoxidase (human) (Cayman).

Thromboelastometry

Whole-blood thromboelastometry is an in vitro global coagulation procedure that detects and quantifies the viscoelastic properties of whole blood during clotting and fibrinolysis (31). Thromboelastometry was performed by means of the 4-channel ROTEM Gamma equipment according to instructions and reagents provided by the manufacturer (TEM). Partial thromboplastin from rabbit origin, ellagic acid, and calcium chloride (INTEM) were employed to activate the intrinsic pathway of coagulation; in another aliquot, rTF was added (EXTEM) to assess the extrinsic coagulation pathway. Another aliquot was tested with the addition of cytochalasin D (FIBTEM) to inhibit platelet activity. The main parameters stemming from the procedure are (i) the clotting time, which is the time (seconds) needed for starting coagulation; (ii) the clot formation time, which is the time (seconds) needed for the clot to reach a predefined amplitude; (iii) the α angle (degrees [°]), representing the velocity of clot growth; and (iv) the maximal clot firmness (MCF), which is the maximal amplitude of the clot (in millimeters [mm]) and represents the strength of clot.

Hormonal assays

Circulating total testosterone concentrations were assessed by an Elecsys Testosterone II (Calibrator reference: 05200067190) test marketed by Roche Diagnostics. This method is standardized via isotope dilution–gas chromatography/mass spectrometry. The assay has a lower limit of detection of 0.087 nmol/L, a functional sensitivity of 0.4 nmol/L, and interassay or intra-assay coefficients of variation of less than 5%.

Luteinizing hormone and follicle-stimulating hormone concentrations were measured by the electrochemiluminescence immunoassay “ECLIA” from Roche Diagnostics (Roche Diagnostics GmbH). Luteinizing hormone and follicle-stimulating hormone assays have a lower limit of detection of 0.1 mIU/mL and a functional sensitivity of 0.2 mIU/mL. The interassay or intra-assay coefficients of variation were less than 5% in all assays.

Sample size calculation

We chose the ETP ratio as the outcome variable for sample size calculation. Because no previous data were available for ETP ratio in KS, we elected to use the mean ETP ratio (SD) value obtained in a group of healthy participants corresponding to 0.67 (0.11) (32). We then postulated a 10% increase of the ETP ratio in the KS population relative to controls. These assumptions led to a sample size of 58 patients and 58 controls when setting a power of 90% and a 5% probability of type I error.

Statistical analysis

Distribution of quantitative variables was assessed by Shapiro-Wilk test. Normally distributed quantitative variables were expressed as mean and SD, whereas variables with a skewed distribution were reported as median and range; qualitative variables were represented as absolute and percentage frequencies. Paired or unpaired t test was performed to compare means of normally distributed variables. Alternatively, the nonparametric Mann-Whitney test was used. The Fisher exact test was employed to compare frequencies of qualitative variables between 2 groups. Univariate association analysis was performed by linear regression, Pearson correlation...
test or Spearman rank correlation test as appropriate. Multivariable analysis was conducted by multiple linear regression. A 2-sided \( P \) value was considered statistically significant when less than .05. Analysis was performed with IBM SPSS Statistics (version 26) or GraphPad Prism (version 9).

**Results**

From April 2018 to December 2019, 58 patients affected by KS (median age, 39.7 years [range, 18-78 years]) and 58 age-matched controls (median age, 37.5 years [range, 22-77 years], \( P = .93 \)) were enrolled in the study. Table 1 summarizes the characteristics of the study population. Controls had a lower frequency of dyslipidemia, a nonsignificantly lower median BMI, and lower median gonadotropin levels as expected, compared to KS patients, while other explored variables were comparable between the 2 groups.

One KS patient reported one past episode of pulmonary embolism, another had experienced a transient ischemic attack several years earlier when he was on testosterone treatment, and 3 patients suffered from chronic venous insufficiency in the lower limbs.

Thirty-five patients were receiving testosterone replacement therapy at study entry; 12 hypogonadal patients were tested before starting testosterone treatment, while the remaining were testosterone-naïve patients with still normal endogenous testosterone levels. Patients treated with testosterone were (nonsignificantly) older than patients not on testosterone replacement therapy, had a higher frequency of impaired fasting glucose \( (n = 7) \), and higher mean hematocrit levels (see Table 1); however, hematocrit was within normal range in all patients and controls. No one presented with overt diabetes.

**Thrombin generation in platelet-poor plasma**

PRP was available in 52 KS patients and 43 controls. In PRP assays, KS again displayed higher median ETP-TM\(^+\) compared to controls \( (1543 \pm 1046-2379 \text{ vs } 1399 \pm 781-1742 \text{ nM} \times \text{min}, P = .004) \). ETP-TM\(^-\) was comparable in the 2 groups. Median ETP ratio was higher in KS patients than controls (see Table 2). The relative difference of the median ETP ratio values between KS patients and controls did not increase in the PRP assay compared to PPP (see Table 2).

**Other coagulation parameters**

PT, APTT, and platelet count did not differ in the 2 groups. Higher FVIII \( (135\% \text{ [range, } 78\%-430\%] \text{ vs } 107\% \text{ [range, } 47\%-380\%]; P = .001) \), fibrinogen \( (283 \text{ mg/dL} \text{ [range, } 179-424 \text{ mg/dL}] \text{ vs } 241 \text{ mg/dL} \text{ [range, } 170-359 \text{ mg/dL}] \text{ P < .001}) \), and FVIII/PC ratio \( (1.21 \text{ [range, } 0.65-3.85] \text{ vs } 1.06 \text{ [range, } 0.48-3.28]; P < .05) \) were observed in KS patients compared to controls. The levels of the remaining procoagulant and anticoagulant factors were similar in the 2 groups.

In KS patients with mosaic 46,XY/47,XXY or 48,XXXXY karyotypes, FVIII and fibrinogen levels and FVIII/PC ratio were within the minimum to maximum range of nonmosaic 47,XXY patients.

**Myeloperoxidase**

MPO activity was not significantly different between KS patients \( (27 \text{ ng/mL} \text{ [range, } 9-66 \text{ ng/mL}] \)) and controls \( (23 \text{ ng/mL} \text{ [range, } 9-82 \text{ ng/mL}] \); \( P = .39 \)). The mean percentage \( (SD) \) of neutrophils was similar in the 2 groups, as well \( (KS 61.1 \pm 7.4\%; \text{ controls } 59.4 \pm 7.2\%; P = .33) \).

**Thromboelastometry**

Thromboelastometry performed in 52 KS patients and 43 controls showed a procoagulant imbalance in the former. A greater \( \alpha \) angle and MCF with only the former reaching a statistical significance \( (P = .01) \) were observed when testing for EXTEM in KS patients compared to controls (Fig. 3).
| Table 1. Clinical and biochemical characteristics of Klinefelter syndrome patients and controls |
|---------------------------------------------------------------|
| Controls | KS, all | P controls vs KS, all | T-treated KS | Non–T-treated KS | P T-treated vs non–T-treated KS |
| No. | 58 | 58 | – | 35 | 23 | – |
| Age, y, median (range) | 37.5 (22-77) | 39.7 (18-78) | .93 | 41 (20-58) | 27 (18-78) | .08 |
| Nonmosaic 47,XXY | – | – | – | 21 | – | – |
| n (%) | – | 53 (91.4) | – | 32 | – | – |
| Mosaic 46,XY/47,XXY | – | 21 | – | 2 | – | – |
| n (%) | 4 (6.9) | 2 | 2 | 0 | .66 |
| 48, XXXY | – | 1 (1.7) | – | 1 | – | – |
| BMI, kg/m², median (range) | 24.6 (18.5-34.1) | 26.1 (16.1-36.4) | .06 | 26.5 (18.3-36.4) | 25.3 (16.1-32.0) | .47 |
| Fasting plasma glucose, mg/dL, mean (SD) | NA | 93.5 (6.5) | – | 94.3 (7.3) | 92.3 (4.9) | .43 |
| Glycated hemoglobin mmol/mol, mean (SD) | NA | 34 (3.96) | – | 36 (3.7) | 35 (4.4) | .59 |
| Impaired fasting glucose, n (%) | 3 (5%) | 7 (12) | .73 | 7 (20) | 0 (0) | .03 |
| Dyslipidemia, n (%) | 5 (9%) | 26 (45) | .001 | 18 (51) | 9 (39) | .43 |
| Arterial hypertension, n (%) | 5 (9%) | 3 (5.2) | .26 | 3 (1) | 0 (0) | .27 |
| Smoking habit, n (%) | 9 (16%) | 15 (26) | .81 | 10 (29%) | 5 (22) | .76 |
| T levels nmol/L, median (range) | 16.7 (12.1-28.4) | 15.8 (0-9.35) | .59 | 20.2 (3.7-35.7) | 11.8 (0.9-33.6) | .001 |
| LH mIU/mL, median (range) | 5.8 (2.3-9.2) | 16.0 (0-52.2) | .01 | 3.8 (0-43.1) | 25.4 (13.1-52.2) | < .001 |
| FSH mIU/mL, median (range) | 3.8 (0.8-10.6) | 18.9 (0-75.0) | < .001 | 6.6 (0-22.9) | 38.9 (15.1-75.0) | < .001 |
| T replacement therapy, n (%) | 0 | 35 (60) | < .001 | 35 | 0 | – |
| T formulation | – | – | – | – | – | – |
| Long-acting injectable T undecanoate, n (%) | – | 24 (69) | – | 24 | – | – |
| Daily 2% transdermal T gel, n (%) | – | 11 (31) | – | 11 | – | – |
| Hematocrit, %, mean (SD) | 39.6 (3.0) | 40.9 (4.1) | .10 | 41.8 (3.6) | 39.2 (4.6) | .04 |
| Arterial thrombotic events, n (%) | 0 | 1 (1.7) | > .99 | 1 | 0 | > .99 |
| Venous thrombotic events, n (%) | 0 | 1 (1.7) | > .99 | 0 | 1 | .40 |
| Chronic venous insufficiency, n (%) | 0 | 3 (5.2) | .24 | 2 | 1 | > .99 |

Abbreviations: BMI, body mass index; FSH, follicle-stimulating hormone; KS, Klinefelter syndrome; LH, luteinizing hormone; NA, not available; T, testosterone.
When cytochalasin D was added to inhibit platelet activity, a significant trend toward hypercoagulability was again evident in the KS group compared to controls in terms of greater MCF ($P < .001$) and $\alpha$ angle ($P < .001$) (see Fig. 3).

**Association between procoagulant imbalance and clinical and biochemical characteristics**

In the univariate analysis conducted in the KS patient group, a positive linear relationship was observed between ETP-TM+ and FVIII ($\rho = 0.356$, $P = .006$), ETP ratio and FVIII ($\rho = 0.538$, $P < .001$), and ETP ratio and FVIII/PC ratio ($\rho = 0.448$, $P < .001$). Table 3 summarizes the correlation coefficients and statistical significance for the examined variables. Overall, no relationship was found for testosterone replacement therapy or serum testosterone levels with TGA parameters and coagulation factor concentrations. Conversely, age showed a positive association with ETP-TM+, ETP ratio, and fibrinogen (Fig. 4). When patients were divided according to testosterone treatment (yes or not) and age range ($\leq 29$ years, $30-49$ years, $\geq 50$ years), younger patients on testosterone treatment showed lower thrombin generation and fibrinogen concentrations than untreated ones, but these differences did not reach statistical significance (see Fig. 4). Moreover, ETP (assessed with or without TM), ETP ratio, and fibrinogen were variably associated with dyslipidemia, BMI, and HbA1c (see Table 3), whereas no such association was observed for FVIII or FVIII/PC ratio. Smoking habit, hematocrit, and arterial hypertension were not associated with the coagulation profile.

In a multivariable analysis in KS patients that included as independent variables age, testosterone concentrations, testosterone replacement therapy (yes/no), BMI, HbA1c, dyslipidemia, and hematocrit, only age was confirmed as a significant predictor of ETP-TM+ ($P = .009$, $\beta = 21.72$), whereas no parameter could predict ETP ratio or fibrinogen concentrations.
Finally, in a multivariable analysis involving both KS patients and controls that included KS diagnosis (yes/no), total testosterone concentrations, testosterone replacement therapy (yes/no), BMI, and dyslipidemia as independent variables, the KS diagnosis was the only variable significantly associated with ETP-TM+ (P = .02, β = 317.4) and ETP ratio (P = .005, β = .1421).

**Discussion**

KS patients are at increased risk of arterial/venous thrombosis (5-8) and death from thrombosis (8, 10) compared to the general population. A number of risk factors have been described encompassing autoimmune disorders like antiphospholipid syndrome (33), genetic predisposition (34-39), or increased levels of procoagulant and antifibrinolytic factors (36, 40). However, evidence relies on small series or anecdotal reports evaluating single components of the coagulation cascade (17). The only study available on TGA in KS patients has been reported by Chang et al (41), who used PPP. The authors did not find a significant difference in TGA parameters between controls and KS patients, irrespective of testosterone replacement therapy, and concluded that platelet activation, rather than plasmatic factors, are affected in KS individuals. However, in that study TGA was performed in the absence of TM. We performed TGA in the
The presence of exogenous TM, which was aimed to mimic the relevant contribution of endothelial cells in the activation of PC as it occurs in vivo. Notably, these experimental conditions are sensitive to the FVIII-PC balance (17), hence it is not surprising that increased FVIII (the procoagulant) or decreased PC (the anticoagulant), make the ETP ratio with/TM much more reliable than the TGA performed/TM to truly represent the conditions operating in vivo (17). This is further substantiated if one considers that activated PC is the physiological inhibitor to FVIII (17, 42).

In the present study, we found that KS patients display increased ETP measured in the presence of TM, as well as

### Table 3. Association of thrombin generation test parameters and clinical and biochemical characteristics of Klinefelter syndrome patients in univariate analysis

| Age, y | BMI | HbA1c | Dyslipidemia | Hematocrit | TT levels | Treatment yes/no |
|--------|-----|-------|--------------|------------|-----------|------------------|
| 0.232  | 0.464e | 0.474e | 0.331e | 0.226 | 0.309 | 0.385e |
| 0.263  | 0.457 | 0.327e | 0.307 | 0.309 | 0.385e | 0.342 |
| 0.439e | 0.474e | 0.144 | 0.194 | 0.103 | 0.094 |

**Correlation coefficients are reported.**

**Abbreviations:** BMI, body mass index; ETP, endogenous thrombin potential; ETP ratio, [ETP-TM+]/[ETP-TM–]; ETP-TM–, endogenous thrombin potential without thrombomodulin; ETP-TM+, endogenous thrombin potential with thrombomodulin; FVIII, factor VIII; FVIII/PC, factor VIII:protein C ratio; HbA1c, glycated hemoglobin A1c; T, testosterone; TT, serum total testosterone.

**Significance levels:** *P* less than .05; **P** less than .01.
higher ETP ratio compared to controls, whereas ETP did not differ significantly between the 2 groups when assessed in the absence of TM. The latter findings are in line with data by Chang and colleagues (41). Overall, our results indicate that KS patients possess a plasma procoagulant imbalance, likely due to increased levels of FVIII (ie, one of the most potent triggers of thrombin generation) and to the increased FVIII/PC ratio. Interestingly, FVIII and FVIII/PC ratio are also directly correlated with ETP-TM and ETP ratio. Increased FVIII in KS had already been described in previous reports (34, 36, 43). However, unlike other series investigating these changes in patients experiencing thrombotic events, we documented that they could occur in the whole KS population as a hallmark of a generalized prothrombotic state.

The procoagulant imbalance TGA showed in KS was not mirrored by shortened PT or APTT, which were comparable to controls. This is not surprising because these tests are instrumental for the diagnosis of hemorrhagic diseases but much less suitable to investigate prothrombotic conditions subsequent to congenital deficiency of naturally occurring anticoagulants (17). The procoagulant imbalance in KS was further supported by the results of thromboelastometry, which points at more rapid clot formation and greater clot strength as shown by the increased α angle and MCF that were observed in KS compared to controls.

Metabolic syndrome is another circumstantial determinant of thrombosis. Indeed, the prevalence of metabolic syndrome is greatly increased in KS (44). Furthermore, diabetes, obesity, and dyslipidemia are recognized risk factors for cardiovascular outcomes. Interestingly, whereas advancing age and some metabolic features displayed an association with fibrinogen and thrombin generation in the univariate analysis in the KS group, only advancing age was confirmed as a significant predictor of ETP (but not of ETP ratio) in the multivariable analysis. Overall, these findings suggest that coagulation abnormalities, including increased FVIII and fibrinogen concentrations, should be regarded as intrinsic features of KS, with only a minor contribution, if any, of the metabolic background. We can speculate that these features may hypothetically mirror altered gene expression. Several genes, located both on autosomal and sexual chromosomes, are differentially expressed in KS patients compared to 46,XX women and 46,XY men (16), probably as a result of epigenetic changes (45, 46), and in association with syndromic features. Admittedly, coagulative changes may still be related to other metabolic features (eg, fat mass, inflammatory status) that were not specifically addressed in the present study.

We also documented a lack of association between testosterone treatment or circulating testosterone levels and thrombin generation or coagulation factor concentrations. Although the association between testosterone treatment and increased thrombotic risk in the general male hypogonadal population is controversial (47), recent studies focusing on KS patients do not support such an association. In the study by Chang et al (41), thrombin generation was lower in treated than untreated KS patients. The same authors in a recently published KS cohort reported that testosterone replacement was (nonsignificantly) associated with lower incidence rates of venous thrombosis and thrombotic deaths (8), thus indirectly confirming that replacement therapy does not negatively affect the procoagulant imbalance of KS individuals. Whether and in which way restoration of normal testosterone concentrations may exert a protective role in KS have not been thoroughly investigated so far. Male hypogonadism has been associated with unfavorable procoagulant and antifibrinolytic changes (48, 49), while testosterone may enhance anticoagulant activity (50) and modulate platelet activation (51). Although similar data in KS patients are lacking, in the present study coagulation parameters showed no correlation with the androgenic state except for a (nonstatistically significant) reduction of thrombin generation and fibrinogen concentrations among young testosterone-treated patients. However, in a study by Haymana et al, concentrations of asymmetric dimethylarginine (a marker of endothelial dysfunction) were negatively associated with total testosterone levels (β = -.412, P = .001) in KS patients (14). Therefore, it is possible that testosterone acts on targets (eg, endothelial function) other than coagulation factors in KS. Further investigations are needed to assess the effects of testosterone treatment on the thrombotic profile of KS individuals.

Besides coagulation factors, platelets may play a direct role in the thrombosis risk of KS. Platelets in fact possess a dual function in hemostasis. First, they adhere to the subendothelial matrix at the site of vessel wall injury (or perturbation) and then aggregate to each other to form a platelet plug; this is collectively known as the hemostatic function of platelets. Second, platelet membranes on activation express negatively charged phospholipids that help assemble the coagulation factors that are instrumental to generating thrombin; this is collectively known as the platelet procoagulant function. The platelet hemostatic function is explored by tests of adhesion/aggregation and/or by the measurement of biochemical markers secreted by activated platelets. The procoagulant function is investigated by TGA performed in PRP, in which the phospholipids needed for coagulation factor alignment are provided by the platelets themselves. In thromboelastometry, platelet contribution can be assessed by comparison of parameters obtained in EXTEM and in FIBTEM, but the hemostatic and the procoagulant functions cannot be differentiated.
Di Minno et al reported that platelets from KS patients express higher levels of the activation markers 8-iso-prostaglandin F2\(\alpha\) and 11-dehydro-thromboxane-B\(_2\), and require exposure to lower concentrations of arachidonic acid to reach adequate levels of aggregation (15). Norris and colleagues described the case of a 47,XXY patient with leg ulcers and evidence of enhanced platelet aggregation in response to a low concentration of adenosine diphosphate (52). These findings are consistent with a greater platelet hemostatic reactivity. However, in the KS cohort reported by Chang et al, platelet aggregation was not increased in testosterone-naive patients (53). The results on the hemostatic function of platelets when combined with the results of the present TGA study in PRP and PPP are consistent with the hypothesis depicted in Fig. 5, whereby the (enhanced) platelet hemostatic function (although still controversial) could play a role in the thrombotic risk observed in KS, but their contribution to secondary hemostasis (ie, thrombin generation) is unlikely because it is related much more to plasma than to platelets.

Our study has some limitations. First, owing to the relatively small sample size and consequently small number of thrombotic events, we could not assess whether there are TGA difference in KS patients with or without thrombosis. Second, we did not systematically investigate our cohort for the presence of prothrombotic mutations and polymorphisms, which may contribute to thrombosis. Metabolic parameters were not recorded at the time of study blood sampling but up to 3 months earlier, and this may have introduced potential bias to the association analysis. Third, we did not investigate extensively for the possible contribution to the heightened thrombin generation brought about by the molecular species released in plasma following neutrophil activation (ie, NETs). It is unlikely however that NETs are increased in our cohort because the levels of MPO (a surrogate marker of NETs) were similar in patients and controls. Similarly, we did not investigate for the possible contribution of microparticles derived from platelets, monocytes, and endothelial cells, which disseminate in the circulation the procoagulant asset derived from the parent cells (54, 55). Elevated levels of circulating microparticles are observed in a variety of diseases (54), and it has been shown that increased levels of microparticles are likely to contribute to thrombosis (56).

In conclusion, this study shows a plasma procoagulant imbalance in patients with KS as indicated by increased thrombin generation in the presence of TM, which is driven (at least in part) by increased levels of FVIII. The procoagulant imbalance is also substantiated by alterations of the parameters of thromboelastometry. These biochemical findings are in line with the relatively high thrombotic risk observed in these patients. Whether antithrombotic prophylaxis should be considered in these patients should be investigated by appropriate clinical trials.

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Additional Information

Correspondence: Giovanna Mantovani, MD, PhD, Fondazione IRCCS Ca’ Granda, Ospedale Maggiore Policlinico, Department: Endocrinology Unit, Via Francesco Sforza, 35, Milan, 20122, Italy. Email: giovanna.mantovani@unimi.it.

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