**Procoagulant imbalance in Klinefelter syndrome assessed by thrombin generation assay and whole blood thromboelastometry**

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**Abstract**

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

**Objective.** To investigate the coagulation balance of KS patients by thrombin generation assay (TGA) and thromboelastometry.

**Design.** Observational, cross-sectional study.

**Setting.** Three tertiary endocrinological centers in Milan, Italy.

**Patients or other participants.** 58 KS patients and 58 age-matched healthy controls were included. Anticoagulant or antiplatelet therapy and known coagulation disorders were exclusion criteria.

**Interventions.** TGA was performed in platelet-poor plasma (PPP) and platelet-rich plasma (PRP). Whole-blood thromboelastometry and activities of coagulation factors were assessed.

**Main Outcome Measures.** Endogenous thrombin potential (ETP), i.e. 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 1528*vs.*1315nMxmin; p<0.001), PPP-ETP-ratio (0.78*vs.*0.70, p<0.001), factor (F)VIII (135%*vs.*107%; p=0.001), fibrinogen (283*vs.*241 mg/dL; p<0.001) and FVIII/protein C ratio (1.21*vs.*1.06; p<0.05) compared to controls. Protein C was comparable in the two groups. Similar results were observed in PRP. ETP-ratio was positively associated with FVIII (rho=0.538, p<0.001) in KS. Thromboelastometry parameters confirmed evidence of hypercoagulability in KS.

**Conclusions.** 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

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## Introduction

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 up to 20 folds more frequent in KS subjects of any age (5–9) and are one of the leading causes of death in this condition (8,10). Deep venous thrombosis, pulmonary embolism [collectively known as venous thromboembolism (VTE)], 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 Virchow's triad, VTE can result from hemodynamic changes such as reduction of blood flow or turbulence, endothelial injury or dysfunction, and blood hypercoagulability. A few studies 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 (PAI-1) (16). However, whether and to what extent other hemostasis abnormalities such as the balance between naturally occurring pro- and anticoagulants operating in plasma are influenced by metabolic derangements, hypogonadism, or testosterone treatment is unclear (8).

Coagulation is a complex and integrated mechanism, and the investigation of its individual components (pro- 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 as 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 investigate also 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*.



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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; 2) to evaluate the coagulation potential of KS patients by means of whole blood thromboelastometry.

## Subjects and Methods

*Subjects*

We selected adult male subjects with a cytogenetically confirmed diagnosis of KS among those followed up at three 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 subjects on anticoagulation (parenteral or oral) or antiplatelet treatment.

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

Dyslipidemia was defined as triglycerides >150 mg/dl, and/or HDL-cholesterol <40 mg/dl (20), and/or LDL-cholesterol above the risk category-specific thresholds recommended by 2019 ESC/EAS Guidelines for the management of dyslipidaemias (21), and/or prescription of lipid-lowering treatment. Arterial hypertension was defined as systolic blood pressure >140 mmHg and/or diastolic blood pressure >90 mmHg (22), and/or prescription of antihypertensive treatment. Impaired fasting glucose and diabetes mellitus were defined according to ADA criteria (23). Testosterone therapy was initiated in KS following current recommendations, i.e. when symptoms and signs of testosterone deficiency became evident with endogenous testosterone levels below 12 nmol/L (24–26).

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

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All study procedures were in accordance with the principles set out in the Declaration of Helsinki. The study was approved by 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, Plymouth, UK). For KS patients on testosterone replacement therapy, blood samples were taken two 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 (controlled room temperature) with two different procedures. The first consisted of centrifugation for 20 min at 2,880*g* and separation of PPP. The second consisted of centrifugation for 15 min at 150*g* to obtain PRP. PRP was then diluted into autologous PPP to obtain a standard platelet count of $150x10^9$/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)*

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, Orangeburg, NY) and synthetic phospholipids (PL, 1.0 μM) (Avanti Polar, Alabaster, Alabama) as coagulation triggers in PPP, or rTF alone in PRP. Testing was performed in two plasma aliquots, with and without 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, Bubendorf, Switzerland) (617 μM) by means of a fluorometer (Fluoroskan Ascent®, ThermoLabsystem, Helsinki, Finland). The readings were recorded and analyzed with a dedicated software (ThrombinoscopeTM, Thrombinoscope BV, Maastricht, The Netherlands), which displays the curve of thrombin concentration as a function of time and calculates the following parameters: the time (min) between the addition of the triggers and the initiation of



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thrombin generation (LagTime); the thrombin peak (nM); the time (min) needed to reach the peak (TTPeak); the area under the curve, defined as endogenous thrombin potential (ETP) and expressed as nMxmin; and the velocity index, defined as [Peak/(TTPeak – LagTime)] and expressed as nM/min. Results were also expressed as ETP-ratio, i.e., 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, Montpellier, France). PT and APTT were measured with Recombiplastin 2G or ThrombosIL APTT (Werfen). Both tests were performed on an automated coagulometer (ACL Top, Werfen, Bedford, MA) and results were expressed as 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 protein C (Werfen)). Factor (F)VIII, II, and fibrinogen were measured as described (29) and results reported as percentage activity relatively to pooled normal plasma with an (arbitrary) activity of 100 %.

*Myeloperoxidase*

Since 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 NET-related variables. Plasma MPO activity was measured by a chromogenic assay employing the Elisa kit Myeloperoxidase (human) (Cayman, 1180 E. Ellsworth Rd, Ann Arbor, MI – USA).

*Thromboelastometry*

Whole blood thromboelastometry is an *in vitro* global coagulation procedure, which detects and quantifies the viscoelastic properties of whole blood during clotting and fibrinolysis (31). Thromboelastometry was performed by means of the four-channel ROTEM® Gamma equipment according to instructions and reagents provided by the manufacturer (TEM, Munich, Germany). 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 addition of cytochalasin D (FIBTEM®), to inhibit platelet activity. The main parameters stemming from the procedure are



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(i) the clotting time (CT), which is the time (seconds) needed for starting coagulation; (ii) the clot formation time (CFT), which is the time (seconds) needed for the clot to reach a predefined amplitude; (iii) the alpha angle (degrees), representing the velocity of clot growth and (iv) the maximal clot firmness (MCF), which is the maximal amplitude of the clot (mm) and represents the strength of clot.

*Hormonal assays*

Circulating total testosterone concentrations were assessed by 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 inter- or intra-assay coefficients of variation <5%.

The LH and FSH concentrations were measured by electrochemiluminescence immunoassay 'ECLIA' from Roche Diagnostic (Roche Diagnostics GmbH). LH and FSH assays have a lower limit of detection of 0.1 mIU/mL and a functional sensitivity of 0.2 mIU/mL. The inter- or intra-assay coefficients of variation were <5% in all assays.

*Sample size calculation*

We chose the ETP-ratio as the outcome variable for sample size calculation. Since 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 subjects correspondent to 0.67 (0.11) (32). We then postulated a 10% increase of the ETP-ratio in the KS population relative to controls. The above 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, while variables with a skewed distribution were reported as median and range; qualitative variables were represented as absolute and percentage frequencies. Paired or unpaired Student's t test was performed to compare means of normally distributed variables. Alternatively, the nonparametric Mann-Whitney test was used. Fisher's exact test was employed to compare frequencies of qualitative variables between two groups. Univariate association analysis was performed by linear regression, Pearson correlation test or Sperman's rank correlation test as appropriate. Multivariate analysis was conducted



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by multiple linear regression. Two-sided p-value (p) was considered statistically significant when <0.05. Analysis was performed with IBM SPSS Statistics (version 26; Chicago, IL) 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)] and 58 age-matched controls [median age 37.5 years (22-77), p=0.932] were enrolled in the study. Table 1 summarizes the characteristics of the study population. Controls had a lower frequency of dyslipidemia, a non-significantly lower median BMI, and lower median gonadotropins levels as expected, compared to KS patients, while other explored variables were comparable between the two 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 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 (non-significantly) older than patients not on testosterone replacement therapy, had a higher frequency of impaired fasting glucose (n=7), and higher mean haematocrit levels (Table 1); however, haematocrit was within normal range in all patients and controls. No one presented overt diabetes.

*Thrombin generation in platelet poor plasma (PPP)*

Mean ETP-TM$^{+}$ (SD) as measured in PPP was significantly higher in KS than controls [1527 (358) *vs.* 1315 (351) nMxmin; p<0.001]. No significant difference was observed in TGA performed in absence of TM (Fig.1). The median (min-max) ETP-ratio (with/without TM) was significantly increased in KS compared to controls [0.78 (0.43-0.99) *vs.* 0.70 (0.19-0.88); p<0.001] (Fig.1 and Table 2). KS displayed also higher thrombin peak (p=0.001) and velocity index than controls (p=0.014) (Fig.2), with no significant difference in LagTime and TTPeak (Fig.2).

Higher ETP-TM$^{+}$ (median 1567, min-max 939-2176 nMxmin), ETP-ratio (median 0.81, min-max 0.43-0.99), and non-significantly increased ETP-TM$^{-}$ (mean 2028, SD 282 nMxmin) as compared to controls (p=0.0002, p<0.0001 and p=0.0575, respectively) were still observed in the KS group after exclusion of patients with



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mosaic 46,XY/47,XXY and 48,XXXY karyotypes. In the latter two categories, ETP-TM$^{+}$, ETP-TM$^{-}$ and ETP-ratio were within the non-mosaic 47,XXY range.

*Thrombin generation in platelet rich plasma (PRP)*

PRP was available in 52 KS and 43 controls. In PRP assays, KS displayed again higher median ETP-TM$^{+}$ compared to controls [1543 (1046-2379) *vs* 1399 (781-1742) nMxmin, p=0.004]. ETP-TM$^{-}$ was comparable in the two groups. Median ETP-ratio was higher in KS than controls (Table 2). The relative difference of the median ETP-ratio values between KS and controls did not increase in PRP assay compared to PPP (Table 2).

*Other coagulation parameters*

PT, APTT and platelet count did not differ in the two groups. Higher FVIII [135% (78-430) *vs* 107% (47-380); p=0.001], fibrinogen [283 mg/dL (179-424) *vs* 241 mg/dL (170-359) p<0.001] and FVIII/PC ratio [1.21 (0.65-3.85) *vs* 1.06 (0.48-3.28); p<0.05] were observed in KS compared to controls. The levels of the remaining pro- and anti-coagulant factors were similar in the two groups.

In KS patients with mosaic 46,XY/47,XXY or 48,XXXY karyotypes, FVIII and fibrinogen levels and FVIII/PC ratio were within the min-max range of non-mosaic 47,XXY patients.

*Myeloperoxidase (MPO)*

MPO activity was not significantly different between KS [27 (9-66) ng/ml] and controls [23 (9-82) ng/ml, p=0.394]. The mean percent (SD) of neutrophils was similar in the two groups, as well [KS 61.1 (7.4); controls 59.4 (7.2); p=0.326].

*Thromboelastometry*

Thromboelastometry performed in 52 KS 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=0.013) were observed when testing for EXTEM in KS patients compared to controls (Fig.3). When Cytochalasin D was added to inhibit platelet activity, a significant trend towards hypercoagulability was again evident in the KS group compared to controls in terms of greater MCF (p<0.001) and alpha angle (p<0.001) (Fig.3).



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

In univariate analysis conducted in the KS patients group, a positive linear relationship was observed between ETP-TM$^+$ and FVIII (rho=0.356, p=0.006), ETP-ratio and FVIII (rho=0.538, p<0.001), and ETP-ratio and FVIII/PC ratio (rho=0.448, p<0.001). Table 3 summarizes 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 factors 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 (≤29 years old, 30-49 years old, ≥50 years old), younger patients on testosterone treatment showed lower thrombin generation and fibrinogen concentrations then untreated ones, but these differences did not reach statistical significance (Fig.4). Moreover, ETP (assessed with or without TM), ETP-ratio and fibrinogen were variably associated with dyslipidemia, BMI and HbA1c (Table 3), while no such association was observed for FVIII or FVIII/PC ratio. Smoking habit, haematocrit and arterial hypertension were not associated with the coagulation profile.

In a multivariate analysis in KS patients that included as independent variables age, testosterone concentrations, testosterone replacement therapy (yes/no), BMI, HbA1c, dyslipidemia, haematocrit, only age was confirmed as a significant predictor of ETP-TM$^+$ (p=0.0087, β=21.72), while no parameter could predict ETP-ratio or fibrinogen concentrations.

Finally, in a multivariate 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=0.0177, β=317.4) and ETP-ratio (p=0.0052, β=0.1421).

## 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 the anti-phospholipid syndrome (33), genetic predisposition (34–39), or increased levels of pro-coagulant and anti-fibrinolytic 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 has been reported by Chang et al. (41) who used platelet poor plasma. The authors did not find significant difference in



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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. However, in that study TGA was performed in the absence of TM. We performed TGA in 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/without TM much more reliable than the TGA performed without 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 higher ETP-ratio compared to controls, while ETP did not differ significantly between the two groups when assessed in the absence of TM. The latter findings are in line with data by Chang et al. (41). Overall, our results indicate that KS patients possess a plasma procoagulant imbalance, likely due to increased levels of FVIII (i.e., 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 showed by TGA in KS was not mirrored by shortened PT or APTT, which were comparable to controls. This is not surprising as these tests are instrumental for the diagnosis of haemorrhagic 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 a more rapid clot formation and greater clot strength as shown by the increased alpha angle and MCF that were observed in KS compared to controls.

The 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 recognised risk factors for cardiovascular outcomes. Interestingly, while advancing age and some metabolic features displayed association with fibrinogen and thrombin generation in univariate analysis in the KS group, only advancing age was confirmed as a significant predictor of ETP (but not of ETP-ratio) in multivariate analysis. Overall, these findings suggest that coagulation abnormalities, including raised FVIII and fibrinogen concentrations should be regarded as intrinsic features of KS, with only a minor contribution, if any, of the



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metabolic background. We can speculate that these features may hypothetically mirror an altered gene expression. Several genes, located on both autosomal and sexual chromosomes, are differentially expressed in KS 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 (e.g., fat mass, inflammatory status, etc.), which 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 factors 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 (non-significantly) associated with lower incidence rates of venous thrombosis and thrombotic deaths (8), thus indirectly confirming that the replacement therapy does not negatively impact on the procoagulant imbalance of KS subjects. Whether and in which way restoration of normal testosterone concentrations may exert a protective role in KS has not been thoroughly investigated so far. Male hypogonadism has been associated with unfavourable pro-coagulant and anti-fibrinolytic 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 (non-statistically 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 ($\beta = -0.412$, $p = 0.001$) in KS patients (14). Therefore, it is possible that testosterone acts on targets (e.g., 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 subjects.

Besides coagulation factors, platelets may play a direct role in the thrombosis risk of KS. Platelets in fact possess a dual function in haemostasis. First, they adhere to the subendothelial matrix at the site of vessel wall injury (or perturbation) and then aggregate one to the other to form the platelet plug; this is collectively known as the haemostatic function of platelets. Second, platelet membranes upon activation express negatively charged phospholipids that help assembling the coagulation factors, which are instrumental to generate thrombin; this is collectively known as the platelet procoagulant function. The platelet haemostatic function is explored by tests of adhesion/aggregation and/or by the measurement of biochemical markers secreted by activated platelets. The



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procoagulant function is investigated by TGA performed in PRP, where the phospholipids needed for coagulation factors 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 haemostatic 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 F2a and 11-dehydro-thromboxane-$B_2$, and require exposure to lower concentrations of arachidonic acid to reach adequate levels of aggregation (15). Norris et al. 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). The above 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 naïve patients (53). The results on the haemostatic 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 haemostasis (i.e., thrombin generation) is unlikely as 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 in 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 (i.e., NETs). It is however unlikely that NETs are increased in our cohort, as 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).



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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 finding 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.

**Data Availability**

Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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**Figure legends**

**Fig.1. Comparison of endogenous thrombin potential (ETP) between controls (HC, white boxes) and patients with Klinefelter syndrome (KS, grey boxes).** Panel A: ETP assessed without addition of thrombomodulin ($TM^-$). Panel B: ETP assessed with addition of TM ($TM^+$). Panel C: ETP-ratio calculated as [$ETP\text{-}TM^+$ / $ETP\text{-}TM^-$].

* p<0.05; ** p<0.01; *** p<0.001

**Fig.2. Comparison of other thrombin generation parameters between healthy controls (HC, white boxes) and patients with Klinefelter syndrome (KS, grey boxes).** Panel A: Peak-thrombin. Panel B: LagTime, the time (minutes) from the addition of the trigger to the initiation of thrombin generation. Panel C: Time-to-Peak (minutes), the time needed to reach the peak-thrombin. Panel D: Velocity Index (VelInd), which depends on thrombin peak, the LagTime and the Time-to-Peak.

* p<0.05; ** p<0.01; *** p<0.001

**Fig.3**. **Thromboelastometry results in healthy controls (HC, white boxes) and patients with Klinefelter syndrome (KS, grey boxes).** Panels A-C, clotting time, clot formation time, alpha angle and maximal clot firmness, respectively when measuring by the extrinsic pathway (EXTEM). Panels E-G: clotting time, alpha angle and maximal clot firmness, respectively when assessed upon platelets activity inhibition (FIBTEM).

* p<0.05; ** p<0.01; *** p<0.001

**Fig.4. Association between age (years) and coagulation parameters in KS patients.**

Dark grey dots represent KS patients with total testosterone concentrations < 12 nmol/L, light grey dots indicate KS patients with total testosterone concentrations > 12 nmol/L (25). Bars differentiate KS patients not taking any testosterone therapy at study entry (white bars, median) and patients on testosterone treatment (black bars, median) for each age-range, i.e. ≤ 29 years old, 30-49 years old, and ≥ 50 years old. Correlation coefficient and p-value are reported.

$ETP\text{-}TM^+$, endogenous thrombin potential as assessed in platelet poor plasma after addition of thrombomodulin. ETP-ratio, the ratio of ETP assessed with thrombomodulin to ETP assessed without thrombomodulin. TRT, testosterone replacement treatment.

**Fig.5**. **Hypothetical interactions of platelets function and components of the coagulation cascade in Klinefelter syndrome.** FVIII, factor VIII. PC, protein C. TM, thrombomodulin.



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**Table 1 Clinical and biochemical characteristics of Klinefelter syndrome patients and controls.** T, testosterone. LH, luteinizing hormone. FSH, follicle stimulating hormone.

| | Controls | KS (all) | P Controls vs KS (all) | T-treated KS | Non T-treated KS | P T-treated vs. non T-treated KS |
|---|---|---|---|---|---|---|
| **Number** | 58 | 58 | - | 35 | 23 | - |
| **Age** years, median (range) | 37.5 (22-77) | 39.7 (18-78) | 0.932 | 41 (20-58) | 27 (18-78) | 0.0825 |
| **Non mosaic 47,XXY** n (%) | - | 53 (91.4%) | - | 32 | 21 | 0.6583 |
| **Mosaic 46,XY/47,XXY** n (%) | - | 4 (6.9%) | - | 2 | 2 | |
| **48,XXXY** n (%) | - | 1 (1.7%) | - | 1 | 0 | |
| **BMI** Kg/m$^2$, median (range) | 24.6 (18.5-34.1) | 26.1 (16.1-36.4) | 0.0610 | 26.5 (18.3-36.4) | 25.3 (16.1-32.0) | 0.4680 |
| **Fasting plasma glucose** mg/dl, mean (SD) | N/A | 93.5 (6.5) | - | 94.3 (7.3) | 92.3 (4.9) | 0.4271 |
| **Glycated haemoglobin** mmol/mol, mean (SD) | N/A | 34 (3.96) | - | 36 (3.7) | 35 (4.4) | 0.5880 |
| **Impaired fasting glucose** n (%) | 3 (5%) | 7 (12%) | 0.7331 | 7 (20%) | 0 (0%) | **0.0349** |
| **Dyslipidemia** n (%) | 5 (9%) | 26 (45%) | **0.0010** | 18 (51%) | 9 (39%) | 0.4259 |
| **Arterial hypertension** n (%) | 5 (9%) | 3 (5.2%) | 0.2597 | 3 (9%) | 0 (0%) | 0.2695 |
| **Smoking habit** n (%) | 9 (16%) | 15 (26%) | 0.8084 | 10 (29%) | 5 (22%) | 0.7604 |
| **Testosterone levels** nmol/L, median (range) | 16.7 (12.1-28.4) | 15.8 (0.9-35.7) | 0.5927 | 20.2 (3.7-35.7) | 11.8 (0.9-33.6) | **0.0011** |
| **LH** mIU/mL, median (range) | 5.8 (2.3-9.2) | 16.0 (0-52.2) | **0.0101** | 3.8 (0-43.1) | 25.4 (13.1-52.2) | **<0.0001** |
| **FSH** mIU/mL, median (range) | 3.8 (0.8-10.6) | 18.9 (0-75.0) | **<0.0001** | 6.6 (0-22.9) | 38.9 (15.1-75.0) | **<0.0001** |
| **T replacement therapy** n (%) | 0 | 35 (60%) | **<0.0001** | 35 | 0 | - |
| **T formulation** Long-acting injectable T undecanoate, n (%) | - | 24 (69%) | - | 24 | - | - |
| Daily 2% transdermal T gel, n (%) | - | 11 (31%) | - | 11 | - | - |
| **Haematocrit** %, mean (SD) | 39.6 (3.0) | 40.9 (4.1) | 0.0961 | 41.8 (3.6) | 39.2 (4.6) | **0.0357** |
| **Arterial thrombotic events** n (%) | 0 | 1 (1.7%) | >0.9999 | 1 | 0 | >0.9999 |
| **Venous thrombotic events** n (%) | 0 | 1 (1.7%) | >0.9999 | 0 | 1 | 0.3966 |
| **Chronic venous insufficiency** n (%) | 0 | 3 (5.2%) | 0.2435 | 2 | 1 | >0.9999 |

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**Table 2. Comparison of median (min-max) ETP-ratio between Klinefelter syndrome patients and healthy controls in assays performed on platelet poor plasma (PPP) and platelet rich plasma (PRP).** Δ%, the percent relative difference of median ETP-ratio between KS and controls, calculated as {[(median ETP ratio in KS) – (median ETP ratio in controls)] / (median ETP ratio in controls)}.

ETP (endogenous thrombin potential) ratio: the ratio of ETP measured in the presence of thrombomodulin to the ETP measured in its absence.

| | **ETP-ratio** | | | |
|---|---|---|---|---|
| | **Controls** | **Klinefelter** | **Δ%** | **p-value** |
| **PPP** | 0.70 (0.19-0.88) | 0.78 (0.43-0.99) | 11% | **<0.001** |
| **PRP** | 0.72 (0.45-0.93) | 0.79 (0.51-0.99) | 10% | **0.009** |

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**Table 3. Association of thrombin generation test parameters and clinical and biochemical characteristics of Klinefelter syndrome patients in univariate analysis.** Correlation coefficients are reported.

HbA1c, glycated haemoglobin. T, testosterone. TT, serum total testosterone. ETP-$TM^-$, endogenous thrombin potential without thrombomodulin. ETP-$TM^+$, endogenous thrombin potential with thrombomodulin. ETP ratio, [(ETP-$TM^+$) : (ETP-$TM^-$)] ratio. FVIII, factor VIII. FVIII/PC, factor VIII : protein C ratio.

* p<0.05; ** p<0.01; *** p<0.001

| | Age | BMI | HbA1c | Dyslipidemia | Haematocrit | T treatment yes/no | TT levels |
|---|---|---|---|---|---|---|---|
| **ETP-$TM^-$** | 0.232 | **0.464**** | **0.474*** | 0.214 | 0.176 | -0.039 | 0.077 |
| **ETP-$TM^+$** | **0.331*** | 0.226 | **0.457*** | 0.263 | -0.045 | -0.049 | 0.217 |
| **ETP ratio** | **0.285*** | -0.047 | 0.309 | **0.322*** | -0.086 | -0.040 | 0.161 |
| **Fibrinogen** | **0.438**** | **0.474**** | **0.385*** | **0.375*** | -0.003 | -0.103 | -0.029 |
| **FVIII** | 0.144 | 0.194 | 0.201 | 0.242 | -0.135 | 0.110 | -0.055 |
| **FVIII/PC** | 0.094 | 0.103 | -0.228 | 0.078 | -0.210 | 0.015 | 0.004 |

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Figure 1

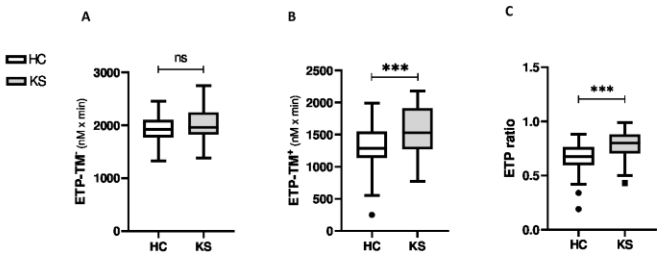


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Figure 2

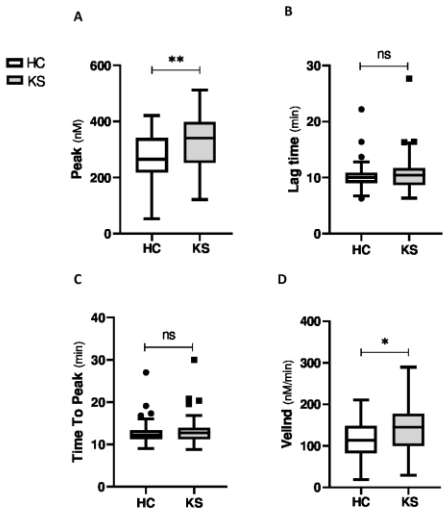


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Figure 3

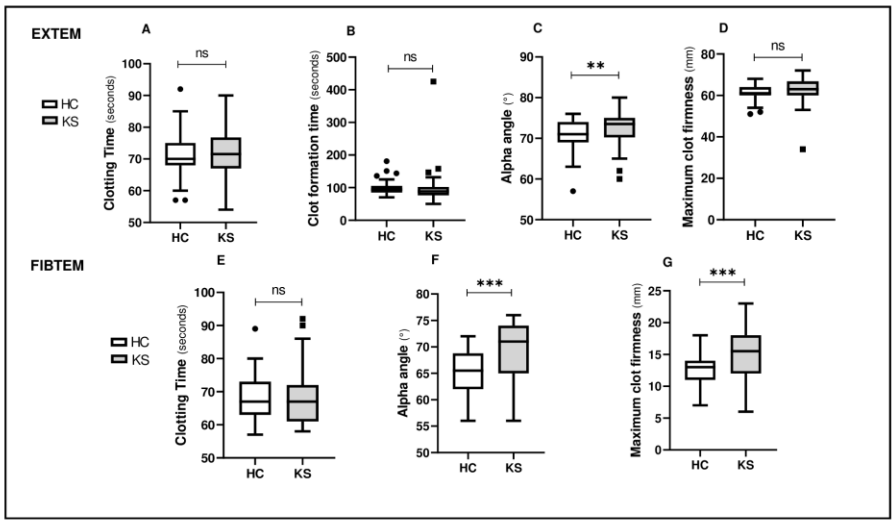


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Figure 4

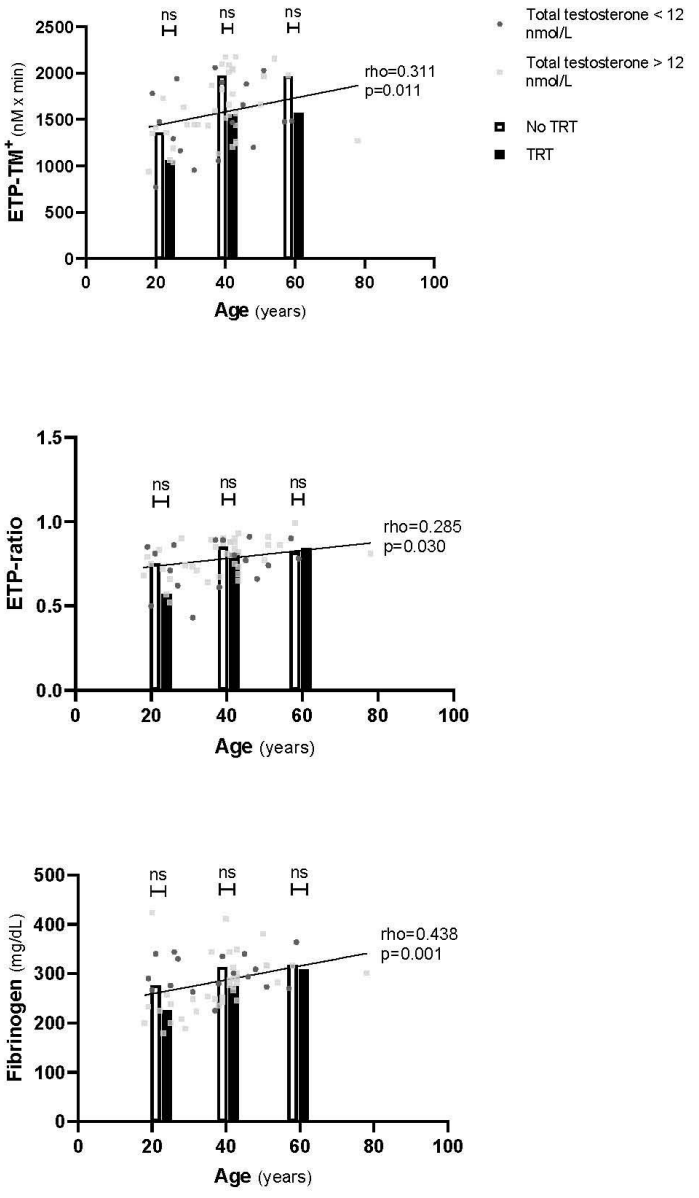


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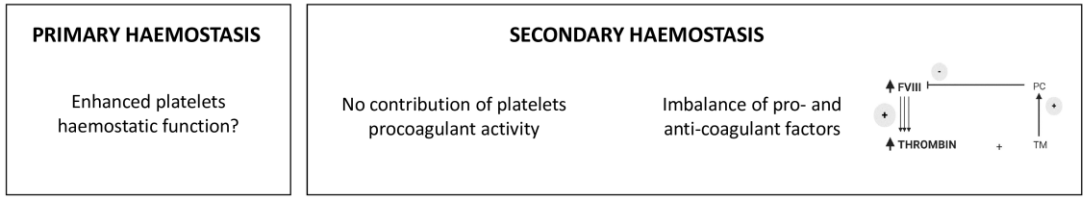
Figure 5

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