Full Length Article

Testosterone treatment and association with thrombin generation and coagulation inhibition in Klinefelter syndrome: A cross-sectional study

Simon Chang[a,b,*], Daniel Biltoft[a], Anne Skakkebæk[b,c,d], Jens Fedder[e], Anders Bojesen[d], M. Vakur Bor[a], Claus H. Gravholt[b,c], Anna-Marie B. Münster[a]

[a] *Unit for Thrombosis Research, Department of Regional Health Research, University of Southern Denmark, Department of Clinical Biochemistry, Hospital of South West Jutland, Esbjerg, Denmark*
[b] *Department of Endocrinology and Internal Medicine, Aarhus University Hospital, Aarhus, Denmark*
[c] *Department of Molecular Medicine, Aarhus University Hospital, Aarhus, Denmark*
[d] *Department of Clinical Genetics, Aarhus University Hospital, Aarhus, Denmark*
[e] *Centre of Andrology and Fertility Clinic, Odense University Hospital, Odense, Denmark*

A B S T R A C T

Background: The background for the increased occurrence of thrombosis seen in Klinefelter syndrome (KS) is unknown. The aim was to compare thrombin generation and coagulation inhibition between men with KS and controls, and to investigate whether coagulation in KS was associated with testosterone treatment (TT), and as such, measures of androgen action.
Methods: Untreated men with KS (U-KS) or testosterone treated men with KS (T-KS) were included. KS groups were matched by age and education to groups of control males with no history of TT. Blood samples were collected after overnight fasting. Low tissue factor (1pM) thrombin generation was expressed as lag time (min), time to peak (min), peak (nmol/L), and endogenous thrombin potential (nmol/L × min, ETP). Coagulation inhibitors, sex hormones, and haematocrit were measured. Matched groups were compared by Student's *t*-test or Wilcoxon rank sum test. Among KS, TT status as an outcome predictor was evaluated by linear regression.
Results: 18 U-KS and 27 T-KS with corresponding controls participated. Thrombin generation was not different comparing U-KS and T-KS with respective control groups. Among KS, ETP was lower in T-KS compared with U-KS and inversely associated with testosterone, LH-testosterone ratio and haematocrit.
Conclusion: Neither U-KS nor T-KS expressed a pro-coagulant state compared with controls. Thrombin generation among KS was inversely associated with androgen action and lower in T-KS compared with U-KS. Whether TT is capable of lowering thrombotic risk among men with KS needs to be assessed prospectively.

1. Introduction

Mortality is increased in 47, XXY Klinefelter syndrome (KS) due to overall increased morbidity. This includes an up to 8-fold increased risk of venous thromboembolism (VTE) [1–3] and 2-fold increased risk for arterial thrombosis (ATE) [1,3,4]. Men with KS have hypergonadotropic hypogonadism which in most cases lead to initiation of continuous testosterone treatment [5,6]. The aetiology behind the thrombosis proneness seen in KS has not previously been described in detail. The genetic disposition and the hormonal milieu inducing of a vicious cycle of unfavourable metabolic changes [7,8], as well as later testosterone treatment all might contribute to skewing the haemostatic balance and inducing a pro-coagulant state in men with KS.

Male hypogonadism has been associated with an increased risk of thrombotic events and thrombosis mortality in several studies of non-KS populations [9–13]. Other studies have indicated an increased risk of VTE [14] and ATE [15,16] upon initiation of testosterone treatment

in non-KS males. Thrombin generation, a marker of global coagulation potential reflective of thrombosis risk, has been demonstrated to be augmented in type 2 diabetes [17] and the metabolic syndrome [18]. Both these conditions are well known to increase thrombosis risk [19,20] and are found at higher rates among men with KS [1,7].

On this background the presence of a pro-coagulant state in men with KS can be hypothesized. Testosterone treatment in KS, on one hand, could be protective against coagulation activation by stimulating synthesis of coagulation inhibitors; antithrombin, protein S, protein C, and tissue factor pathway inhibitor (TFPI) [21–23]. On the other hand we recently demonstrated a pro-coagulant state with augmented thrombin generation in current and former anabolic androgenic steroid abusers [21]. This emphasizes the need for more knowledge about the specific effects of testosterone treatment on the haemostatic balance in men with KS.

The aim of this cross-sectional study was to describe the global coagulation profile, expressed as thrombin generation and coagulation

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Table 1

Study participant demographics and outcomes. Normal distributed variables are presented as mean ± SD and compared using Student's *t*-test. Non-normally distributed variables are presented as median (25–75 percentiles) and compared using Wilcoxon rank sum test. U-KS: untreated Klinefelter syndrome, U-C: matched controls for U-KS, T-KS: testosterone treated Klinefelter syndrome, T-C: matched controls for T-KS, BMI: body mass index, BP: blood pressure. P1: U-KS vs. U-C. P2: T-KS vs. T-C. *Fischer exact test.

	U-KS	U-C	P1	T- KS	T-C	P2
Anthropometry and blood pressure						
No. of participants (n)	18	18		27	27	
Age (years)	40.4 ± 10.9	40.6 ± 10.9	1.0	42.7 ± 8.9	42.7 ± 8.9	1.0
Total body fat (%)	34.2 ± 8.5	24.3 ± 7.7	< 0.001	27.4 ± 5.6	24.1 ± 5.5	**0.04**
Systolic BP (mmHg)	122.7 ± 13.0	128.4 ± 13.1	0.2	133.5 ± 12.4	124.2 ± 11.0	**0.01**
Diastolic BP (mmHg)	74.6 ± 7.5	78.6 ± 9.8	0.2	78.3 ± 10.6	74.0 ± 11.2	0.2
Thrombin generation (see also Fig. 1)						
Lag time (min)	5.8 ± 1.0	5.5 ± 0.8	0.4	5.7 ± 0.8	5.5 ± 0.9	0.4
Time to peak (min)	11.3 ± 2.1	11.2 ± 1.5	0.9	11.8 ± 1.7	11.5 ± 1.9	0.6
Coagulation inhibitors						
Antithrombin (IU/mL)	1.08 ± 0.09	1.02 ± 0.06	**0.03**	1.05 ± 0.10	1.02 ± 0.11	0.2
Protein C (IU/mL)	1.13 ± 0.16	1.09 ± 0.15	0.5	1.16 ± 0.20	1.11 ± 0.13	0.3
Free protein S (ratio)	1.11 ± 0.20	1.05 ± 0.22	0.4	1.08 ± 0.16	1.08 ± 0.22	1.0
Free TFPI (ng/mL)	38.7 (34.5–43.1)	32.8 (29.7–38.5)	**0.02**	38.9 (35.4–41.9)	31.5 (29.4–36.7)	**< 0.001**
F-a2M (ng/ml)	388 (320–484)	474 (265–736)	0.4	434 (269–498)	386 (293–1078)	0.4
Metabolism and haematology						
Total cholesterol (mmol/L)	5.2 ± 1.3	5.2 ± 0.8	1.0	5.1 ± 1.0	5.1 ± 0.8	1.0
HDL (mmol/L)	1.3 ± 0.4	1.5 ± 0.3	0.1	1.1 ± 0.2	1.4 ± 0.3	**< 0.001**
Triglycerides (mmol/L)	1.2 (0.8–1.6)	0.9 (0.8–1.2)	0.2	1.4 (1.0–2.0)	1.0 (0.7–1.3)	**0.01**
HbA1C (mmol/mol)	36.6 ± 2.1	36.1 ± 2.9	0.6	36.2 ± 3.1	35.6 ± 2.9	0.4
Haemoglobin (mmol/L)	8.9 (8.2–9.2)	9.6 (9.5–9.8)	< 0.001	10.4 (9.5–10.5)	9.6 (9.1–9.9)	**0.01**
Haematocrit	0.43 (0.41–0.45)	0.46 (0.45–0.46)	< 0.01	0.48 (0.46–0.50)	0.45 (0.45–0.47)	**0.01**
Hormones						
FSH (IU/L)	34.0 (22.0–44.0)	4.4 (3.0–5.4)	< 0.001	4.9 (0.5–28.0)	4.8 (2.9–6.1)	0.3
LH (IU/L)	21.5 (17.4–30.0)	5.8 (5.0–6.6)	< 0.001	2.0 (0.2–22.0)	4.3 (3.4–5.5)	0.5
Total testosterone (nmol/L)	5.6 (4.6–11.7)	19.4 (15.7–22.0)	< 0.001	18.8 (14.5–25.0)	18.9 (16.0–24.0)	0.5
Total estradiol (pmol/L)	64.8 ± 41.0	80.2 ± 23.5	0.2	96.6 ± 40.6	77.3 ± 23.6	**0.04**
SHBG (nmol/L)	48 (33–68)	43 (38–49)	0.6	36 (26–50)	42 (30–57)	0.2
LH/testosterone	3.7 (3.1–5.5)	0.3 (0.2–0.4)	< 0.001	0.2 (0.0–1.1)	0.3 (0.2–0.3)	0.2
Compound risks						
Metabolic syndrome (n(%))	6 (33.3)	6 (33.3)	1.0*	12 (44.4)	6 (22.2)	0.2*
Framingham risk score	1 (−1–9)	5 (−3−10)	0.7	8 (4–12)	4 (0–9)	**0.04**

Statistically significant results are highlighted in bold type.

inhibitor levels among men with KS compared with control males. Further, we wanted to assess associations between testosterone treatment, thrombin generation, and coagulation inhibitor concentrations in men with KS.

2. Materials and methods

2.1. Participants

This study is part of a larger study assessing the haemostatic balance and neurocognitive function among men with KS. Men with verified 47, XXY KS were recruited from endocrinology and fertility clinics in Denmark and in corporation with the Danish Klinefelter Association and stratified as either currently being treated with testosterone (T-KS) or untreated (U-KS) with no history of testosterone treatment. Treatment status was evaluated on the basis of patient records, which include continuations giving the indication for treatment, timing of treatment onset, and an overview of dosing and prescriptions. Status as either treated or untreated was further confirmed by the participants upon examination. Controls for U-KS (U-C) and controls for T-KS (T-C), with no history of testosterone treatment, were matched by age and years of education. Controls were recruited among men previously participating as controls at The Department of Endocrinology, Aarhus University Hospital, through posters in public spaces and private businesses or by advertisement online at the official portal for the Danish Healthcare Services; www.sundhed.dk. Men 18–70 years of age were eligible for inclusion. Exclusion criteria applied to all groups were self-reported prior thrombosis current anticoagulation therapy or use of platelet inhibitors, current use of narcotics (e.g. marihuana, amphetamine, cocaine etc.), diabetes mellitus, and prior severe head trauma.

2.2. Ethics

The study was approved by the Central Denmark Regional Committee on Health Research Ethics (1-10-72-131-15) and the Danish Data Protection Agency (1-16-02-472-15). Informed consent was obtained from all participants and the study was registered with ClinicalTrials.gov (NCT02526628).

2.3. Blood sampling

Morning blood samples were collected from an antecubital vein after overnight fasting. To minimize contact activation of the coagulation system during blood collection, samples for analysis of thrombin generation and coagulation inhibitors were collected in low-activating 0.106 M citrated tubes (S-Monovette 9NC, Sarstedt, Nümbrecht, Germany). Within 90 min, platelet-poor plasma was prepared at 20 °C by centrifuging at 2000 × *g* for 20 min and frozen at −80 °C. Consecutively, blood samples from the contralateral arm for analysis of hormones, organ markers such as albumin, creatinine etc. were collected in the hospital standard tubes (Vacuette, Greiner Bio-One Int. GmbH, Kremsmünster, Austria).
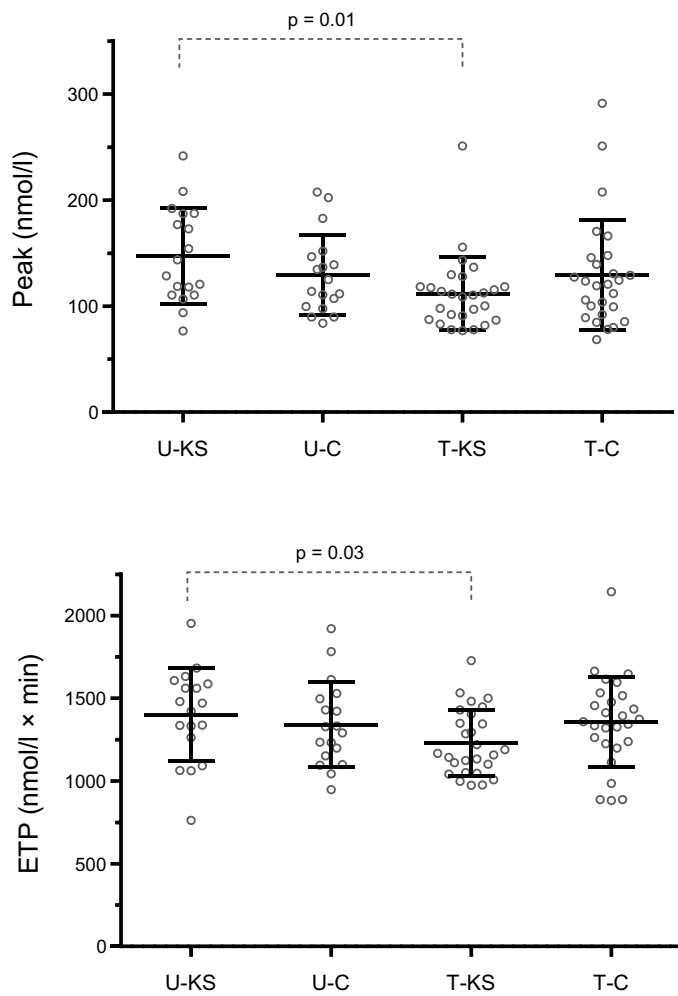
Fig. 1. Peak and endogenous thrombin potential (ETP) in all four groups. Lines indicate mean ± SD. *P*-values reflect the association between either peak or ETP and testosterone treatment (yes/no) among men with Klinefelter syndrome evaluated by univariate linear regression. U-KS: untreated Klinefelter syndrome, U-C: matched controls for U-KS, T-KS: testosterone treated Klinefelter syndrome, T-C: matched controls for T-KS. Mean ± SD peak (nmol/L); U-KS, 147.1 ± 45.0; U-C, 129.5 ± 37.4; T-KS, 111.8 ± 34.8; T-C, 129.3 ± 52.0. Mean ± SD ETP (nmol/L × min); U-KS, 1400.4 ± 280.8; U-C, 1341.6 ± 257.0; T-KS, 1231.3 ± 199.3; T-C, 1356.6 ± 273.6.

Table 2

Univariate linear regression between thrombin generation or coagulation inhibitors and testosterone treatment (yes = 1/no = 0) as a binary covariate among men with Klinefelter syndrome.

Dependent variable	β [95% CI]	p
Thrombin generation		
Lag time (min)	−0.1 [−0.6; 0.5]	0.8
Time to peak (min)	0.5 [−0.7; 1.7]	0.4
Peak (nmol/L)	−35.3 [−60.5; −10.0]	**0.01**
ETP (nmol/L × min)	−169.1 [−322.8; −15.3]	**0.03**
Coagulation inhibitors		
Antithrombin (IU/mL)	−0.02 [−0.08; 0.3]	0.4
Protein C (IU/mL)	0.03 [−0.08; 0.14]	0.5
Free protein S (ratio)	−0.03 [−0.15; 0.08]	0.5
Free TFPI (ng/mL)*	0.03 [−0.10; 0.17]	0.6

Transformation of variables: * log.
Statistically significant results are highlighted in bold type.

2.4. Plasma analysis

Coagulation assays were performed at the Unit for Thrombosis Research, Hospital of South West Jutland, Esbjerg, Denmark, after thawing of plasma samples at 37 °C in a water bath. To the extent possible, batches of samples comprising individuals from all four groups were assayed in parallel to minimize any effect of between-run variation. Sex hormones, lipids, and organ markers were assayed at the Department of Clinical Biochemistry, Aarhus University Hospital, Aarhus, Denmark on the day of participation.

2.5. Thrombin generation

Thrombin generation was analysed by the calibrated automated thrombin generation assay (Thrombinoscope BV, Maastricht, The Netherlands) using a 1 pM tissue factor trigger employing the Fluoroskan Ascent microplate fluorometer (Thermo Fisher Scientific, Hvidovre, Denmark). Measures of thrombin generation: the lag time of the thrombin formation process (lag time, min), the time to peak thrombin concentration was reached (time to peak, min), the peak thrombin concentration (peak, nmol/L), and the endogenous thrombin potential (ETP, nmol/L × min) recording the total amount of thrombin formed was estimated using The Thrombinoscope software package (Thrombinoscope BV).

2.6. Coagulation inhibitors

Plasma antithrombin activity was determined using an automated chromogenic assay kit (Liquid Antithrombin, HemosIL, Instrumentation Laboratory, Bedford, MA, USA) employing the ACL9000 Coagulation Analyzer (Instrumentation Laboratory). Plasma free protein S antigen was quantitatively determined using an immuno-turbidometric assay kit (STA-Liatest Free Protein S, Stago, Asnieres-sur-Seine, Paris, France) employing the STA-R coagulation analyzer (Stago). Plasma protein C activity was determined using an automated chromogenic assay kit (Protein C, HemosIL, Instrumentation Laboratory) employing the ACL9000 Coagulation Analyzer (Instrumentation Laboratory). Plasma free tissue factor pathway inhibitor (TFPI) antigen was quantitatively determined using a sandwich enzyme immunoassay (ELISA) (Human
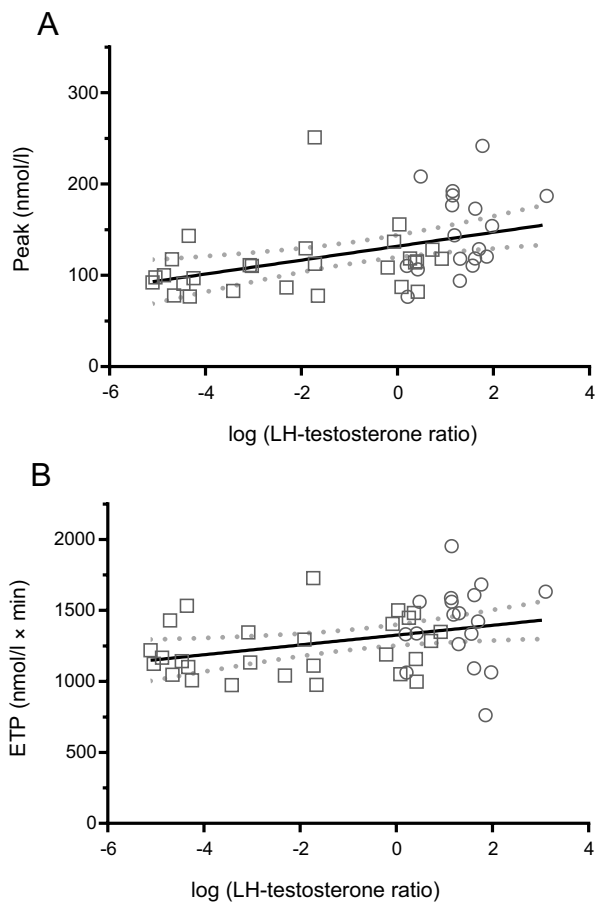
Fig. 2. Associations between LH-testosterone ratio and (A) peak or (B) endogenous thrombin potential (ETP) among (o) untreated Klinefelter syndrome and (□) testosterone treated Klinefelter syndrome patients. Associations obtained by linear regression are represented by solid lines with 95% confidence interval (dotted lines). Peak: β [95% CI], 7.62 [2.78;12.46], p = 0.003. ETP: β [95% CI], 34.64 [5.22;64.06], p = 0.02.

TFPI, Quantikine ELISA, R&D systems, Inc., Minneapolis, MN, USA) employing a microplate reader (Sunrise, Tecan Trading AG, Basle, Switzerland).

To assess activation of the internal coagulation pathway, a potential confounder when evaluating thrombin generation data, fast form α-2-macroglobulin (F-α2M) was measured using a specific monoclonal antibody applying an ELISA setup as described previously [24].

2.7. Sex hormones

Follicle stimulating hormone (FSH), luteinizing hormone (LH), and sex hormone binding globulin (SHBG) were quantitatively determined by immunoassay employing the Cobas e601 electrochemiluminescence measuring unit (Cobas, Roche Diagnostics Limited, Rotkreuz, Switzerland).

Testosterone and oestradiol were measured by liquid chromatography tandem mass spectrometry. The limit of detection was 0.1 nmol/L, and the working range was 0.2–100 nmol/L with a coefficient of variation of < 10%. Remaining biochemical variables were assayed using standard methods available at Department of Clinical Biochemistry, Aarhus University Hospital, Aarhus, Denmark.

2.8. Other quantities

Metabolic syndrome was assessed according to NCEP ATPIII criteria as the presence of three or more risk factors; large waistline, high triglyceride levels, low high-density lipoprotein (HDL), high blood pressure or high fasting blood sugar [25]. Cardiovascular risk was assessed by ATPIII/Framingham Risk Score [26].

Dual-energy X-ray absorptiometry (DXA) was applied using a Hologic QDR2000/w osteodensitometer (Hologic, Inc., Waltham, MA, USA) assessing total fat percent.

Blood pressure was measured at the right arm using a fully automated blood pressure monitor (UA-852, A&D Medical, Tokyo, Japan) and given as the lowest values obtained from three repeated measurements.

2.9. Sample size calculation

We chose ETP as the outcome variable for sample size calculation. No previous data were available for ETP in KS. Based on data from healthy individuals, we estimated a predicted mean ETP of 1500 nmol/L × min with a standard deviation of 200 nmol/L × min. There is no consensus on a universally clinical relevant change in ETP. We have previously demonstrated a > 12% increase in ETP among populations with increased VTE risk [27,28]. With a 15% change in ETP as the effect, determining power at 80% with an error rate of 5% estimating 2-sided equality of means from two groups (e.g. U-KS vs. U-C), sample size needed was estimated at 13.

2.10. Statistical analysis

Distributions of variables were evaluated by histograms and quantile-quantile plots. Data are presented as mean ± standard deviation (SD) or median (25th–75th percentile). Between groups comparison of normal distributed variables were performed by Student's *t*-test and non-normal distributed variables were compared between groups using Wilcoxon rank sum test. Age was compared across all four groups using

Table 3

Univariate linear regression in men with Klinefelter syndrome with thrombin generation peak or endogenous thrombin potential (ETP) as outcomes and markers of metabolism and androgen action as covariates. Transformation of variables:* inverse, † log, ‡ cubic, # square root. BP: blood pressure, HDL: high-density lipoprotein.

Covariate	Peak		ETP	
	β [95% CI]	p	β [95% CI]	p
Total body fat (%)	0.86 [−0.84;2.55]	0.3	8.32 [−1.34;17.98]	0.1
Systolic BP (mm Hg)	−0.48[−1.45;0.49]	0.3	−4.78 [−10.31;0.75]	0.1
HDL (mmol/L)	31.92 [−11.61;75.44]	0.2	−18.95[−278.36;240.46]	0.9
Triglycerides (mmol/L)*	28.12 [−5.29;61.52]	0.1	69.66 [−129.88;269.20]	0.5
HbA1C (mmol/mol)	−2.16 [−6.92;2.60]	0.4	−4.96 [−32.89;22.96]	0.7
Haemoglobin (mmol/L) †	−123.94 [−228.67;-19.20]	**0.02**	−433.99 [−1068.32;200.33]	0.2
Haematocrit ‡	−623.00 [−1064.59;-181.41]	**0.01**	−2685.46 [−5360.36;-10.56]	**0.049**
Total testosterone (nmol/L) #	−12.57 [−21.43;-3.72]	**0.01**	−58.14 [−111.40;-4.88]	**0.03**
Total oestradiol (pmol/L)	−0.34 [−0.64;-0.05]	**0.02**	−0.88 [−2.66;0.90]	0.3

one-way ANOVA. Categorical variables were evaluated by Fischer's exact test. The association between testosterone treatment status and various outcomes among men with KS was assessed by univariate linear regression with testosterone treatment status as a binary covariate. Associations between thrombin generation, coagulation inhibitors, and other outcomes were evaluated by univariate linear regression. For regression analyses, transformation of all non-normally distributed variables was performed as indicated in relevant tables. A *p*-value < 0.05 was considered significant. Analysis was performed using StataIC 15 (StataCorp LLC, College Station, TX, USA). Figures were made using Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Participants demographics and characteristic

From November 2015 to June 2017, 18 U-KS, 18 U-C, 27 T-KS, and 27 T-C were included. Among T-KS, 20 were treated with testosterone undecanoate injections (Nebido, Bayer), 6 with testosterone gel (Tostran, Kyowa Kirin; Testim, Ferring) and 1 with injections of combined testosterone propionate and enanthate (Testoviron, Bayer). The median duration of testosterone treatment among T-KS was 10 years (full range, 4–39 years). Use of prescription medications was not different between U-KS and T-KS (Supplemental Table 1). The total age span for the study sample was from 19.7 to 61.9 years. Age was not different comparing all four groups (*p* = 0.6, one-way ANOVA).

U-KS compared with U-C expressed a state characterized by lower androgen action while hormone levels were overall comparable between T-KS and T-C (Table 1).

Among KS, testosterone treatment was associated with lower gonadotropins and higher testosterone and oestradiol levels (p ≤ 0.02 for all, Supplemental Table 2). Further, testosterone treatment among KS was associated with lower body fat percentage, lower HDL, higher systolic blood pressure, higher haemoglobin, higher haematocrit, and higher Framingham risk score (*p* ≤ 0.02 for all, Supplemental table 2).

3.2. Thrombin generation

Peak or ETP were not different comparing U-KS and U-C or comparing T-KS with T-C (Fig. 1). Among KS, testosterone treatment was associated with lower peak (*p* = 0.01) and ETP (*p* = 0.03) (Fig. 1). Lag time, time to peak or F-α2M were not different between groups (Table 1) and were not associated with testosterone treatment status among KS (Table 2).

Thrombin generation was associated with measures of androgen action in men with KS. As such thrombin generation peak was inversely associated with haemoglobin, haematocrit, total testosterone and total oestradiol and positively associated with LH-testosterone-ratio applying univariate linear regression (p ≤ 0.01 for all, Fig. 2, Table 3). Likewise, ETP among KS was inversely associated with haematocrit, and total testosterone and positively associated with LH-testosterone ratio (*p* < 0.05 for all, Fig. 2, Table 3).

3.3. Coagulation inhibitors

U-KS had higher antithrombin activity and free TFPI antigen compared with U-C (*p* ≤ 0.03 for all, Table 1). T-KS had higher free TFPI antigen compared with T-C (*p* < 0.001). In univariate linear regression antithrombin activity was associated with lag time ((β (95% CI), 0.02 (0.002–0.05), *p* = 0.002). Likewise, free TFPI antigen was associated with lag time (β (95% CI), 0.15 (0.10–0.20), *p* < 0.0005) and time to peak (β (95% CI), 0.06 (0.03–0.08), *p* < 0.0005). Among KS, testosterone treatment was not associated with coagulation inhibitor concentrations (*p* ≥ 0.4 for all, Table 2). Neither antithrombin activity nor free TFPI antigen was associated with total testosterone or LH-testosterone ratio in univariate linear regression including either the whole

study sample or restricted to patient- or control groups separately (data not shown).

4. Discussion

This was the first study to investigate global thrombin generation and coagulation inhibitor levels in men with KS. Thrombin generation is associated with thrombosis risk [29,30], but there is no established direct relation between a certain change in ETP and a corresponding change in thrombosis risk. We found that thrombin generation in both T-KS and U-KS was comparable to groups of age-matched controls, indicating that a pro-coagulant state might not be universally present among men with KS. We demonstrate that thrombin generation among men with KS was inversely associated with testosterone treatment status and measures of androgen action such as sex hormone levels and haematological parameters indicating a less pro-coagulant global coagulation profile with increasing androgen levels in these patients. The inverse association between global coagulation and testosterone treatment in KS provides the first biochemical indication that testosterone treatment in KS is not associated with the introduction of a severe pro-coagulant shift in the haemostatic balance potentially increasing the clinical risk of thrombosis.

A previous study found no change in ETP after 52 weeks treatment with testosterone undecanoate in 13 men with low baseline total testosterone [32], supporting the notion that testosterone treatment in men with hypogonadism is not associated with a pro-coagulant state. Contrary to this, we have previously demonstrated a marked increase in ETP among men with extremely high testosterone levels due to abuse of anabolic androgenic steroids [21]. Taken together these findings of apparently opposite associations between global coagulation and increasing testosterone levels could indicate that there is a form of physiological optimum for this association. This notion is supported by the findings among elderly men of a J-shaped association between testosterone levels and arterial thrombosis [33] and an U-shaped association between dihydrotestosterone and cardiovascular disease [34] and further by the increased risk of ischemic heart disease found among women with either extremely high or extremely low sex hormone levels [35].

Thrombin generation was associated with an array of androgen action related variables. However, a potential testosterone driven mechanism affecting thrombin generation in men with KS cannot be derived from the present study. Increased androgen availability from testosterone treatment in KS is likely giving rise to a multitude of physiological changes, that could have a direct or indirect effect of global coagulation. Thus, our findings could be affected by pathways, that are not investigated in the current manuscript. However, correction of the unfavourable metabolic profile seen in men with KS [6,8] induced by testosterone treatment could be contributing to the inverse association between coagulation and androgen levels. The metabolic syndrome or a long duration of type 2 diabetes mellitus has been demonstrated to increase thrombin generation [17,18]. However, we found no difference in HbA1c between U-KS and T-KS, and although the two KS groups demonstrated different total body fat percent, systolic blood pressure, and HDL levels, these variables or the presence of the metabolic syndrome were not associated with thrombin generation. However, the metabolic profile among T-KS resulted in a higher Framingham risk score compared with both their controls and U-KS. The Framingham risk score has not previously been applied in KS research and the findings, and clinical implications hereof, need to be validated. However, dyslipidaemia is frequently described in KS[6] and testosterone treatment in men with KS has previously been demonstrated to decrease HDL [38,39].

The present study confirms the positive association between thrombin generation lag time and TFPI as previously demonstrated by ourselves, and others [21,31,36]. TFPI was higher in both KS groups compared with controls, but contrary to other studies, not associated

with testosterone levels or other markers reflecting androgen action including the measured metabolic traits [31,37]. In a recent study we demonstrated TFPI levels increased by > 50% and associated with significantly longer thrombin generation lag time and time to peak among current abusers of anabolic androgenic steroids, who express extremely high testosterone levels, compared with former users and controls [21]. Compared to this, the increase in TFPI among men with KS in the present study is quite modest and perhaps for this reason does not extend to cause differences in lag time or time to peak comparing men with KS and controls. TFPI is synthesised mainly from the vascular endothelium, which could support that endothelial function is affected in KS. Also the overall increased inhibitor levels with non-differential thrombin generation in men with KS compared with controls indicate that although the haemostatic balance is preserved in KS, systems working on both in sides of the equation are upregulated seemingly unaffected by sex hormone levels. This could indicate that some other mechanism associated with the KS phenotype directly affects the haemostatic balance.

The cross-sectional design does not allow for any conclusion regarding causality and the findings could be affected by unmeasured confounding. So far, epidemiological studies [1,2] on KS morbidity and mortality have not been able to distinguish U-KS and T-KS, and although prospective clinical studies are notoriously difficult to carry out in rare disorders, they are important to perform to elucidate whether testosterone treatment indeed is modifying thrombosis risk among men with KS. In this context, it is noted that haematocrit was higher among T-KS compared with T-C or U-KS, and it has been shown that erythrocytosis, elevated haematocrit, and hyperviscosity may contribute to the multifactorial aetiology of thrombosis [40–42]. Injecting testosterone undecanoate is associated with higher testosterone levels in the period following immediately after the injection relative to the period leading up to the injection. We did not record data regarding the timing of testosterone injection and further included also patients using testosterone gel. Therefore, we are not able to tell if timing of testosterone injections relative to blood sampling could have had an impact on our findings.

Low tissue factor thrombin generation is more susceptible to bias from unwanted activation of the contact pathway due to pre-analytical conditions. The present study applied a standardized blood sampling and plasma preparation procedure to minimize contribution from the contact activation pathway. Further, as an expression of contact pathway activation we evaluated levels of F-a2M finding no difference between the groups and overall low levels. This increases the validity of our findings. In addition, the use of two matched control groups increases the robustness of our findings.

Due to an only 25% detection rate for KS [44], identification and inclusion of newly diagnosed and untreated cases is challenging. However, pre-inclusion sample size calculations and post hoc power analysis point to the study being sufficiently powered regarding the observed difference in ETP. As with all studies in KS, the results presented might not be reflective of what could be found among the overwhelming 75% of expected KS cases that are never diagnosed. In addition, in men with KS, platelet activation could be affected [43]. The current methodology, applying platelet free plasma samples, is insensitive to contributions from activated platelet during in vivo blood clotting.

5. Conclusion

In this cross-sectional study thrombin generation in men with KS was inversely associated with testosterone and androgen action. T-KS expressed a less pro-coagulant thrombin generation profile compared with U-KS. Whether testosterone treatment in KS affects thrombosis risk in KS needs to be evaluated from longitudinal studies.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2019.08.011.

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