Thrombin generation potential is enhanced in systemic sclerosis: impact of selected endothelial biomarkers

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ABSTRACT

Objective. *Systemic sclerosis (SSc) is a rare immune-mediated heterogenous entity characterised by excessive tissue fibrosis and vascular injury. Recently, increased risk of thromboembolic events has been documented in that disease. Our aim was to investigate prothrombotic plasma properties together with selected laboratory biomarkers of endothelial injury in SSc.*

Methods. *In 56 clinically stable SSc patients and 67 well-matched controls we assessed plasma thrombin generation profile and measured circulating vascular cell adhesion molecule-1 (VCAM-1), cellular fibronectin (cFN), and thrombomodulin, as well as analysed their relationships with disease clinical parameters and autoimmune antibody profile.*

Results. *SSc was characterised by 18.3% increased endogenous thrombin potential (ETP), 14.5% higher thrombin peak (p<0.001 both, also after adjustment for potential confounders), and similar endothelial damage biomarkers, as compared to controls. Surprisingly, raised thrombin generation was related to the lower thrombomodulin and VCAM-1. Inflammatory markers, factor VIII activity, and blood eosinophilia predicted positively ETP, whereas platelet count and thrombomodulin had negative impact on that parameter in a multiple regression model. Intriguingly, patient group had also 6.7% extended lag-time (p=0.01 after adjustment for confounders) which was independently determined by higher thrombomodulin and cFN, as well as lower VCAM-1. Former cyclophosphamide therapy, thus more severe type of the disease was referred to the increased thrombin generation.*

Conclusion. *SSc is characterised by enhanced thrombin generation potential which might contribute to the higher risk of thromboembolic events in that disease. Endothelium may play hereby an additional role, although large observational and experimental studies are needed to verify this hypothesis.*

Introduction

Systemic sclerosis (SSc) is a rare immune-mediated disease characterised by pathological fibrosis of the connective tissue localised in the skin and internal organs as a result of dysregulated repair mechanisms (1, 2). Patients with SSc can be categorised into two major subsets. Cases with proximal involvement are classified as diffuse cutaneous SSc, whereas those with restricted skin changes affecting the distal parts of the limbs, with or without face and neck involvement, are classed as limited cutaneous SSc (1, 3). The first type is related more often to the higher risk of renal injury, pulmonary fibrosis, and generalised skin involvement, while the second might be associated with pulmonary arterial hypertension of vascular origin (3). There is also a small group of patients presenting typical antibodies, but without skin sclerosis, as well as those with systemic sclerosis overlapping other autoimmune-mediated disorders, such as mixed connective tissue disease (4).

Although, pathogenesis of SSc is still not fully understood, the emerging data, particularly those on animal models and using molecular biology, demonstrates overexpression of the genes related to the tissue remodelling and fibrosis, such as transforming growth factor β and platelet-derived growth factor in the sclerotic skin (5). Moreover, it has been documented that SSc is related to the endothelial injury, characterised by impaired arterial flow-mediated dilatation and release of numerous procoagulant molecules,

including tissue factor (TF) and von Willebrand factor into the circulating blood (6, 7). Currently, rising number of reports have indicated that cellular fibronectin (cFN) in peripheral blood might be a novel and specific biomarker of endothelial damage (8, 9). cFN is also produced by activated fibroblasts in tissue repair, thus might be particularly interesting in SSc patients (10).

It has been shown that tissue inflammation in SSc leads to the local activation of coagulation pathway (11). In turn, active coagulation factors, particularly thrombin, might stimulate endothelial cells, blood vessel myocytes, and connective tissue fibroblasts via protease-activated receptors (PARs) (12). Furthermore, it has been documented that in inflammatory tissue thrombin is related to the enhanced collagen accumulation, overexpression of connective tissue growth factor and lower activity of matrix metalloproteinases in extracellular matrix (ECM) (13). On the other hand, initiated coagulation pathway, even in ECM, may contribute to the blood prothrombotic state and increased risk of thromboembolic events reported recently in that disease (14, 15).

Thrombin generation assessed in the Calibrated Automated Thrombinography (CAT) is a comprehensive blood coagulation test, which is well recognised and recommended to measure the overall prothrombotic plasma properties (16, 17). Previously, it was analysed in selected inflammatory diseases, including rheumatoid arthritis, asthma and inflammatory bowel disease, indicating unfavourable altered thrombin generation profile with putative prothrombotic plasma properties (18-20).

Given the available data on the increased risk of thromboembolic events and endothelial damage in SSc patients, as well as potential role of thrombin in driving of tissue inflammation and fibrosis we sought to evaluate *in vitro* thrombin generation profile in that disease. We also analysed selected laboratory biomarkers of endothelial damage, such as vascular cell adhesion molecule (VCAM-1), thrombomodulin, and cFN in peripheral blood, as well as studied their relationships with thrombin generation characteristics, clinical parameters of SSc patients and autoantibody profile. Such studies have not been previously performed.

Materials and methods

Participants

We studied 56 consecutive white, 18-70 years old, clinically stable SSc patients recruited at the Department of Allergy and Clinical Immunology, University Hospital, Cracow, Poland from 2016 to 2018. The diagnosis was established according to the 2013 Classification Criteria for Systemic Sclerosis (American College of Rheumatology/European League Against Rheumatism). Stable SSc was defined as no clinical, functional, laboratory or imaging worsening during the preceding 6 months based on physical and laboratory examination, as well as skin and internal organs assessment. All patients were tested for presence of characteristic autoantibodies in peripheral blood.

Control group of 67 subjects, matched for gender, age, body mass index (BMI), smoking habit and comorbidities was recruited from the hospital personnel and their relatives.

The exclusion criteria for both groups included: any acute illness during the last month, previously diagnosed venous thromboembolism, myocardial infarction or stroke, cancer, congestive heart failure, atrial fibrillation, coronary heart disease, current anticoagulant therapy, liver injury or kidney insufficiency. Liver injury was defined as a serum alanine aminotransferase elevated more than twice above the upper limit of the reference range. Kidney insufficiency was defined as an estimated glomerular filtration rate (eGFR) below 60 ml/min/1.73m^2.

Subjects with arterial hypertension, diabetes mellitus, or hypercholesterolemia were eligible. Hypertension was defined as a history of blood pressure >140/90 mmHg or preadmission antihypertensive treatment. Diabetes mellitus was defined as condition treated with insulin or oral hypoglycaemic agents, or fasting serum glucose >7.0 mmol/l. Hypercholesterolemia was defined as previously diagnosed and treated with statins, or serum total cholesterol >5.0 mmol/l. Subjects who stopped smoking 6 months or more before enrolment were included as non-smokers.

SSc-associated interstitial lung disease (ILD) was defined as typical findings on high-resolution computed tomography, such as ground-glass linear or reticular opacifications, traction bronchiectasis, or bronchial cysts (honeycomb). High echocardiographic probability of pulmonary hypertension was defined as a pulmonary artery systolic pressure >45mmHg measured in transthoracic echocardiography (21). Digital ulcers were defined as painful areas of loss of tissue distal to the proximal interphalangeal joints.

The study received approval from the Bioethics Committee of Jagiellonian University Medical College. All subjects were given a thorough description of the methodology and safety protocol before we obtained the informed consent to participate in the study.

Laboratory analysis

Fasting blood samples were drawn between 7:00 and 10:00 AM from the antecubital vein with minimal tourniquet use. Routine laboratory techniques were performed to measure glucose, lipid profile, alanine aminotransferase, urine, creatinine, and complete blood cell counts. Fibrinogen was determined by the Clauss method, while factor VIII activity (FVIII:C) by the one-stage clotting assay (Siemens, Marburg, Germany). Commercially available immunoenzymatic test was used to determine thrombin-antithrombin complex (TAT, Enzygnost TAT micro, Siemens, Marburg, Germany). eGFR was estimated by the Modification of Diet in Renal Disease formula. C-reactive protein (CRP) was assessed by the Cobas Integra System. Blood samples were drawn into tubes containing 0.109 M sodium citrate (vol/vol, 9:1), centrifuged 2000 G for 10 min, at room temperature. The supernatant was frozen in aliquots and stored at -70°C until analysis.

Interleukin-6 (IL-6), VCAM-1, and soluble thrombomodulin in peripheral blood were measured using standardis ed ELISA (all, R&D Systems, Minne-

apolis, MN, USA), similarly to circulating cFN (Sigma-Aldrich, St. Louis, MO, USA).

Calibrated Automated Thrombogram (CAT) was performed using commercial reagents (Thrombinoscope, BV, Maastricht, Netherlands) as described elsewhere (16, 17). Briefly, 80 µl of platelet poor plasma was mixed with 20 µl of a reagent containing recombinant relipidated TF and phospholipids, with the final concentrations of 5 pmol/l and 4 mmol/l, respectively. The reaction was performed in microtitre well (Thermo Electron, Denmark) after automatic addition of a fresh starting reagent containing calcium chloride (100 mmol/l) and a thrombin specific fluorogenic substrate (Z-Gly-Gly-Arg-AMC) (2.5 mmol/l) in the HEPES buffer. The fluorescence intensity was recorded by the Fluoroskan Ascent® microplate fluorometer (Thermo Fisher Scientific Oy, Vantaa, Finland) using the appropriate software (Thrombinoscope BV, v. 3.0.0.29).

The maximum concentration of thrombin generated during the recording time is defined as the 'thrombin peak' and the range under the curve characterises the 'endogenous thrombin potential' (ETP). 'Lag-time' is the time from the beginning of analysis until thrombin starts to be detected and 'time to thrombin peak' is the time from the start of thrombin generation until the maximum thrombin value is reached. Higher values of ETP and thrombin peak, together with shorter lag time and time to thrombin peak indicate an enhanced and more rapid activation of blood coagulation *in vitro*, thus a prothrombotic state which corresponds to the increased risk of thromboembolic events (16, 17).

Antinuclear antibodies (ANA) were examined in SSc cases using indirect immunofluorescence test (ThermoFisher, Waltham, USA) and antigen-specific ELISA for disease specific autoantibodies (topoisomerase I, centromeres, NOR 90, PM-Scl, RNA polymerase III, Ku, PDGFR, fibrillarin, and Ro-52) (EUROIMMUN, Lübeck, Germany).

Statistical analysis

The results were obtained using STATISTICA Tibco 13.3 software. Data distribution was evaluated by the Shapiro-Wilk test. All continuous variables were non-normally distributed, thus were presented in the manuscript as a median with 95% confidence intervals (CI), and compared by Mann-Whitney U-test, Kruskal-Wallis or multiple repetition test, as appropriate. A one-way covariance analysis (ANCOVA) was performed to adjust for potential confounders, including age, sex and body mass index (BMI). Categorical variables were compared by the Chi^2 test. To evaluate the relationship between continuous variables a Spearman rank correlation test or univariate linear regression model with adjustment for age, sex, and BMI were performed. Independent determinants of ETP and lag time were established in multiple linear regression models. The R^2 was checked for a measure of the variance. To calculate odds ratio (OR) with 95% CI, the cut-off point of ETP and lag-time were calculated based on receiver operating characteristic (ROC) curves. Results that presented *p*-value less than 0.05 were considered statistically significant.

Table I. A summary of demographic, clinical and laboratory characteristics of subjects studied.

	Patients n=56	Controls n=67	*p*-value
Age, years	57 (50.8-57.2)	50 (49.1-53.1)	0.09
Male gender, number (%)	9 (16.1)	16 (23.9)	0.48
Body mass index, kg/m^2	24.8 (24.4-26.8)	26.9 (25.7-28.1)	0.06
Hypertension, number (%)	18 (32.1)	15 (22.4)	0.62
Diabetes mellitus, number (%)	4 (7.1)	1 (1.5)	0.76
Hypercholesterolemia, number (%)	10 (17.9)	7 (10.4)	0.88
Smoking in the past, number (%)	17 (30.4)	15 (22.4)	0.42
Smoking, packs/years	0 (0-5)	0 (0-10)	0.29
Basic laboratory tests			
Blood platelets, 10^3/µl	214 (213.2-261.7)	211 (191.8-239)	0.78
Red blood cells, 10^6/µl	4.5 (4.3-4.6)	4.56 (4.2-5.0)	0.63
White blood cells, 10^3/µl	6.05 (6.1-7.9)	5.71 (4.7-6.5)	0.33
Total cholesterol, mmol/l	4.45 (3.7-5.2)	5.0 (4.2-5.8)	0.24
High-density lipoprotein cholesterol, mmol/l	1.38 (1.2-1.5)	1.66 (1.4-1.9)	0.07
Low-density lipoprotein cholesterol, mmol/l	2.63 (2.01-3.25)	3.25 (2.55-3.95)	0.01
Triglycerides, mmol/l	1.26 (1.2-1.8)	0.83 (0.3-1.0)	0.01
Glucose, mmol/l	4.9 (4.6-5.1)	4.6 (4.2-5.2)	0.73
Creatinine, µmol/l	65.2 (63.6-79.8)	79.9 (60.8-92.1)	0.27
Urea, mmol/l	4.64 (4.3-5.5)	4.48 (3.5-5.6)	0.76
Alanine aminotransferase, U/l	20.0 (18.1-24)	22.0 (17.2-24.9)	0.64
C-reactive protein, mg/l	5 (5.4-10.7)	1 (0.5-5.8)	0.02
Vascular cell adhesion molecule 1, ng/ml	808.3 (789.3-1063.6)	824.5 (700.4-918.4)	0.79
Interleukin 6, pg/ml	3.44 (2.8-6.3)	1.7 (0.8-2.6)	<0.01
Thrombomodulin, ng/ml	4353.4 (4218.2-5697.0)	4716.1 (4376.2-5360)	0.28
Cellular fibronectin, µg/ml	118.6 (95.6-160)	125.2 (98.5-157.3)	0.81
D-Dimer, ng/ml	429 (0-2055)	232 (103.3-360.7)	0.03
Factor VIII, %	106.2 (69.7-142.7)	115.9 (68-163.8)	0.96
Fibrinogen, g/l	3.5 (2.4-4.6)	3.1 (2.4-3.8)	0.68
Thrombin generation profile in calibrated automated thrombogram			
ETP, nM/min	1758.5 (1717.0.6-1884.7)	1486.1 (1447.4-1575.8)	<0.01
Lag-time, min	3.2 (3.1-4.0)	3 (2.9-3.2)	0.01
Peak TG, nM	330.6 (321.6-360.8)	287.8 (263.7-294.8)	<0.01
Time to thrombin peak, min	5.7 (5.6-6.6)	5.7 (5.7-6.3)	0.98
Thrombin-antithrombin complex formation, µg/l	3.5 (3.7-5.2)	3.6 (3.8-4.7)	0.77

Categorical variables are presented as numbers (percentages), continuous variables as median with 95% confidence interval.

Results

Demographic and laboratory characteristics of SSc patients and control individuals have been provided in Table I. As it has been shown both groups were similar in demographic factors, smoking habit and comorbidities.

Patients characteristics

In turn, Table II shows clinical parameters and autoantibody profile in SSc group. As it has been demonstrated the median duration of the disease was 4.5 (range: 2-10) years and the majority of patients (n=37, 66%) was diagnosed with a diffuse type of the disease. The Raynaud's phenomenon was the most frequent clinical sign recorded in nearly 90% of the cases. ILD was reported in more than half of them, while other manifestations, including digital ulcers, dysphagia and high echocardiographic probability of pulmonary hypertension were far less frequent. More than a one third of patients were currently treated with steroids, while 28 (50%) subjects received immunosuppressive regimens, such as azathioprine, cyclophosphamide, methotrexate, or mycophenolate mofetil nowadays or in the past. Patients received also other internal medicine medications, of which calcium channel blockers, angiotensin converting enzyme inhibitors or angiotensin receptor antagonists, as well as statins were prescribed most frequently. Moreover, 6 (11%) SSc subjects received aspirin and none therapy for pulmonary arterial hypertension or P2Y12 inhibitors.

Antinuclear antibodies were detected in all patients, whereas anti-topoisomerase I in nearly half of them. Other types of antibodies, such as anti-centromeric, anti-RNA polymerase III, anti-NOR, anti-Ro-52, and anti-Ku had much lower prevalence.

Table II. Clinical characteristics and antinuclear antibodies in systemic sclerosis patients.

	Patients n=56
Duration of the disease (years)	4.5 (2-10)
Limited disease, n (%)	19 (33.9)
Diffuse disease, n (%)	37 (66.1)
The presence of anti-nuclear antibodies, n (%)	56 (100)
Anti-Scl-70 antibodies presence, n (%)	24 (42.9)
Anti-PM/Scl antibodies presence, n (%)	4 (7.1)
Anti-centromeric antibodies presence, n (%)	15 (26.8)
Anti-NOR antibodies presence, n (%)	2 (3.6)
Anti-Ro 52 antibodies presence, n (%)	9 (16.1)
RNA polymerase III antibodies presence, n (%)	4 (7.1)
Anti-Ku antibodies presence, n (%)	1 (1.8)
Organ involvement	
Digital ulcers, n (%)	23 (41.1)
Abnormal nailfold capillaries, n (%)	26 (46.4)
Telangiectasia, n (%)	16 (28.6)
Raynaud's phenomenon, n (%)	49 (87.5)
Dysphagia, n (%)	15 (26.8)
Interstitial lung disease, n (%)	31 (55.4)
High echocardiographic probability of pulmonary hypertension, n (%)	9 (16.1)
Treatment characteristic	
Current steroids therapy, n (%)	20 (35.7)
Current corticosteroid dose, mg per day, recalculated to methylprednisolone	0 (0-6)
Systemic steroids therapy, years	0 (0-2)
Immunosuppressive treatment (currently or in the past)	
Azathioprine, n (%)	6 (10.7)
Cyclophosphamide, n (%)	15 (26.8)
Methotrexate, n (%)	4 (7.1)
Mycophenolate mofetil, n (%)	10 (17.9)
Internal medicine medications	
Angiotensine converting enzyme inhibitors or angiotensin receptor antagonists, n (%)	16 (28.6)
Statins, n (%)	13 (23.2)
Beta-blockers, n (%)	8 (14.3)
Diuretics, n (%)	8 (14.3)
Calcium channel blockers, n (%)	24 (42.9)
Aspirin, n (%)	6 (11)

Categorical variables are presented as numbers (percentages), continuous variables as median with 95% confidence interval. n: number.

Routine laboratory tests and endothelium damage biomarkers

As presented in Table I both studied groups were similar in blood cell counts, liver enzymes, and kidney function. As expected, SSc patients were characterised by higher inflammatory markers, such as CRP and IL-6, as well as raised D-dimer, serum triglycerides and LDL-cholesterol. White blood cell count was associated with plasma fibrinogen and FVIII activity (R=0.4, p=0.003 and R=0.34, p=0.02, respectively).

Patient and control groups did not differ in endothelial damage biomarkers, including circulating thrombomodulin, VCAM-1, and cFN (Table I). In SSc thrombomodulin and VCAM-1 correlated well to each other (R=0.66, p=0.002), as well as to IL-6 (R=0.36, p=0.03 and R=0.39, p=0.02, respectively). Both of them were also related to the kidney function, such as serum concentration of creatinine (R=0.59 p=0.001 and R=0.37, p=0.04, respectively) and urea (R= 0.8 p=0.001 and R=0.45, p=0.01, respectively). cFN remained in positive relationship with fibrinogen (R=0.35, p=0.01). Moreover, interestingly, it was lower in those with interstitial lung disease (125.1 [65.2–185] *vs*.150.8 [99.3–202.3], p=0.03).

Comorbidities had no impact on vascular biomarkers, albeit SSc subjects with diabetes mellitus were characterised by higher fibrinogen levels (5.23 [4.14–6.32] *vs*. 3.42 [2.47–4.37], p=0.01), while those with hypercholesterolemia had raised FVIII activity (135.5 [95.7–175.3] *vs*. 107.6 [74.1–141.1] p=0.04).

Thrombin generation assay

Patients with SSc had a significantly altered thrombin generation profile as compared to controls (Table I). SSc was related to the 18.3% increased ETP and 14.5% higher thrombin peak (p<0.001 both, also after adjustment for potential confounders), in comparison to control individuals. Interestingly, patient group was also characterised by 6.7% extended lag time (p=0.01) but similar time to peak. SSc cases had markedly higher OR (2.45 [95%CI: 1.65–3.64]) of increased ETPs, defined as values above the cut-off point of 1614.3 nmol/l thrombin x min, as well as 1.67

(95%CI: 1.16–2.41) higher risk of prolonged lag-time (defined as values above the cut-off point of 3.07 min), compared with control individuals.

In SSc ETP remained in strong positive association with fibrinogen (β=0.36 [95%CI: 0.19–0.52]) and triglycerides (β=0.35 [95%CI: 0.14–0.56]), as well as in weaker with CRP (β=0.2 [95%CI: 0.04–0.38]) and eGFR (β=0.22 [95%CI: 0.05–0.4]). In turn, peak thrombin generated was related to FVIII activity (β=0.42 [95%CI: 0.26–0.58]), fibrinogen level (β=0.23 [95%CI: 0.06–0.4]), white and red blood cell counts (β=0.4 [95%CI: 0.23–0.56] and β=0.26 [95%CI: 0.1–0.43], respectively) and eGFR (β=0.46 [95%CI: 0.29–0.61]). Interestingly, both parameters describing thrombin generation potential, such as ETP and peak thrombin, correlated inversely with thrombomodulin (β=-0.26 [95%CI: -0.47 to -0.05] and β=-0.45 [95%CI: -0.64 to -0.25], respectively) and VCAM-1 (β=-0.48 [95%CI: -0.67 to -0.29] and β=-0.49 [95%CI: -0.67 to -0.3], respectively), both endothelial damage parameters.

In a multiple regression model, as expected, ETP was positively determined by FVIII and inflammatory markers, including CRP and IL-6, but also by the blood eosinophilia. In turn, circulating thrombomodulin, blood platelet count and TAT predicted ETP negatively in that model. All aforementioned parameters explained 61% of the ETP variability (Table III).

In turn, lag time remained in potent positive relationship with fibrinogen (β=0.52 [95%CI: 0.37–0.67]), cFN (β=0.47 [95%CI: 0.31–0.62]), triglycerides (β=0.39 [95%CI: 0.19–0.6]), and glucose level (β=0.35 [95%CI: 0.19–0.52), whereas the only negative and weak association was demonstrated with VCAM-1 (β=-0.24 [95%CI: -0.45 to -0.03]). In a multiple regression model longer lag time was predicted by increased circulating thrombomodulin and cFN, raised blood glucose, and intriguingly, longer disease duration. Moreover, VCAM-1 and D-dimer had a negative impact on that parameter (Table III).

Localised and diffused SSc did not differ in thrombin generation profile. However, those with digit ulcers were characterised by extended lag time (3.59 [2.75–4.43] n=20 *vs*. 3.09 [3.0–3.99] n=36, *p*=0.04). Moreover, interestingly, patients who were previously treated with cyclophosphamide had markedly higher risk of having a greater ETP, defined as values above the cut-off point of 1697.3 nmol/l thrombin x min (OR 2.78 [95%CI: 1.23–6.28], *p*=0.01), compared with the remaining cases.

Comorbidities, smoking status, and internal medicine drugs had no impact on thrombin generation profile.

Table III. Multiple linear regression models for a relative increase of endogenous thrombin potential (ETP) and lag time in SSc patients. Presented variables have been documented as independent determinants of both studied parameters, explaining 61% of their variability.

	β (95% CI)	R^2
Endogenous thrombin potential, nmol/l thrombin x min		
C-reactive protein, mg/l	0.76 (0.55–0.96)	
Interleukin 6, pg/ml	0.39 (0.24–0.54)	
Factor VIII, %	0.28 (0.13–0.43)	
Eosinophils, 10^3/μl	0.22 (0.06–0.37)	
Blood platelets, 10^3/μl	-0.7 (-0.93– -0.48)	0.61
Thrombomodulin, ng/ml	-0.79 (-0.95– -0.59)	
Thrombin-antithrombin complexes, μg/l	-0.18 (-0.34– -0.02)	
Adjustment statistics	F=4.63 *p*=0.003	
Lag-time, min		
Thrombomodulin, ng/ml	0.69 (0.46–0.92)	
Glucose, mmol/l	0.47 (0.32–0.62)	
Cellular fibronectin, μg/ml	0.41 (0.27–0.56)	0.61
Interleukin 6, pg/ml	0.23 (0.07–0.38)	
Vascular cell adhesion molecule 1, ng/ml	-0.45 (-0.65– -0.25)	
D-dimer, ng/ml	-0.37 (-0.54– -0.19)	
Systemic scleroderma duration, years	0.27 (0.12–0.42)	
Adjustment statistics	F=4.43, *p*=0.004	

The resulting standardised regression coefficient (β) with 95% confidence interval (95%CI) for a factor (independent variable) indicates the increase/decrease in standard deviations (SDs) of dependent variable (ETP or lag time), when that particular factor increases with 1 SD and all other variables in the model remain unchanged.

Discussion

In the present study we have documented for the first time that SSc is related to the enhanced *in vitro* plasma thrombin generation, characterised by higher ETP and peak thrombin concentration, as compared to matched controls. Our observation might indicate prothrombotic state in that disease, which corresponds to the lately reported epidemiological data demonstrating increased risk of pulmonary embolism and deep vein thrombosis among SSc individuals. The evaluation of thrombin generation in performed by us CAT assay is widely recommended and provides a comprehensive insight into the overall prothrombic plasma properties (20, 22). Moreover, research to date has shown that raised thrombin generation profile in that assay refers to the reliable higher risk of thromboembolic complications in many diseases, including diabetes, coronary heart disease, and deep vein thrombosis (23-28). Furthermore, activated coagulation factors may contribute to the SSc pathology, particularly in the skin microvasculature.

Our outcomes mirror results obtained for other rheumatic diseases. Enhanced thrombin generation has been previously demonstrated in rheumatoid arthritis and systemic lupus erythematous (19, 29), emphasising once again pathogenetic relationships between inflammatory and coagulation pathways. We expanded that knowledge to SSc, another autoimmune-mediated inflammatory disease, characterised by enhanced tissue fibrotic response. To date, only Solfietti *et al*. (30) analysed thrombin production in SSc patients using similar to us, but not automated fluorometric method. However, they compared results with rheumatoid arthritis patients and did not include controls into the analysis. Furthermore, the number of SSc subjects in that study was relatively small (25 subjects). Our SSc group

was twice as large, while comparison to control individuals makes the report novel and interesting. Moreover, we for the first time performed the well validated and widely recommended automated CAT assay, as well as analysed thrombin generation profile in both limited and disseminated SSc subtypes demonstrating no difference between them.

As expected, in SSc cases increased ETP was determined by higher fibrinogen and FVIII but also by inflammatory markers and blood eosinophilia, indicating complex regulation of prothrombotic plasma alterations in that disease. Although, positive association with blood eosinophilia seems surprising, it can be easily explained by the immunomodulatory effect of thrombin, which promotes the Th2 profile of inflammation (31). In turn, demonstrated negative relationship between thrombomodulin and ETP is consistent with the expected. Thrombomodulin acts as a trigger of protein C system and plays the anticoagulant role. Thus, although circulating thrombomodulin was similar in both studied groups, negative association with thrombin potential in SSc patients suggests simultaneous activation of both coagulation and anticoagulant mechanisms also *in vivo*. Furthermore, since thrombomodulin is produced by endothelial cells, one might speculate that vascular endothelium plays a crucial role in the prothrombotic/anticoagulant balance. That is also a novel and worth attention finding of our study. On the other hand, negative relationship of ETP with platelet count is an intriguing finding and merits a comment. One possible explanation of that association is an increased platelet consumption in the fibrotic skin, followed by activation of the coagulation pathway (32). Notably, in our study particularly low platelet count has been demonstrated in those with high echocardiographic probability of pulmonary hypertension together with increased CRP (data not shown).

The next issue, which deserves a comment is an extended lag time in SSc patients. The lag time refers to the clotting time and if shorter indicates prothrombotic plasma properties. In our data thrombomodulin and cFN, both likely of endothelial origin, had the most important impact on prolonged lag time with favourable anticoagulant mode of action. Moreover, subjects with digital ulcers, thus more severe microvascular skin changes were characterised by its higher values. These observations point once again the likely protective role of endothelium in thrombotic plasma balance in SSc. On the other hand, lag time duration does not automatically contain an information on the amount of thrombin formed in the following peak and ETP (17). Hemker *et al*. (17)have previously demonstrated that delay of lag time is pleiotropic and associated with, among others, presence of antiphospholipid antibodies (17, 33). Unfortunately, we did not analyse the presence of antiphospholipid antibodies in our group of patients due to the lack of clinical indications. However, it has been previously reported that they might be present even in 10-45% of SSc patients (34, 35). Thus, we are not able to exclude that some of our cases were positive for them, particularly after long time of disease duration, which again correlated with the lag time. Noteworthy, the profile of autoantibodies in SSc often varied over time, and their effect on the pathogenesis of disease phenomena is still not fully understood (3). Further studies considering the presence of antiphospholipid antibodies in SSc patients without a history of thromboembolism could provide more detail on unfavourable changed thrombin generation curve in these patients.

There are ambiguous reports regarding effects of immunosuppressive treatment, including cyclophosphamide on endothelial status and thrombotic balance. These medications are related to the cell damage (36), but at the same time may also inhibit inflammatory process. In our study, former cyclophosphamide therapy was associated with increased thrombin formation, however, we are not able to exclude that those subjects had also more severe and aggressive form of the disease (37).

Study limitation

The limited number of SSc patients declines the strength of our results. However, our study group is one of the largest evaluated in the literature so far regarding thrombin generation in SSc. A substantial number of patients presented comorbidities such as diabetes mellitus, hypertension and hypercholesterolemia. However, we matched our patients with appropriate controls and analysed impact of comorbidities in adequate statistical tests. We did not analyse fibrinolytic mechanisms, as well as did not identify other possible coagulation modulators, such as factor V Leiden mutation which might contribute to the extended lag time. Some presented associations might be accidental, and do not reflect the causal relationships. Particularly subgroup analysis must be analysed with the caution. Nonetheless, we believe that our outcomes reflect intergroup differences in thrombin generation profile.

In a summary, we have demonstrated that patients with SSc are characterised by *in vitro* enhanced thrombin generation, which might explain increased risk of thromboembolic events in that disease. Moreover, activated coagulation factors, particularly thrombin, may contribute to the progression of tissue fibrosis. However, large observational and experimental studies are needed to verify in which extent unfavourable plasma properties might be related to the thromboembolic complications or disease progression.

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