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Impaired Fibrinolysis Is Linked With Digital Vasculopathy and Onset of New Digital Ulcers in Systemic Sclerosis

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ABSTRACT. ***Objective.*** To assess thrombin generation, fibrin formation, and structure together with the fibrinolytic status in patients with systemic sclerosis (SSc) in relation to the occurrence of digital ulcers (DUs) during the course of disease.

Methods. We studied variables of endothelial dysfunction, thrombin generation, overall hemostatic potential, and fibrin clot turbidity in plasma from 58 patients with SSc (39 with DU history and 19 DU-naïve) and 46 matched healthy controls (HCs). Fibrin structure was visualized using scanning electron microscopy (SEM). Finally, 39 patients with a history of DUs were followed for 1.5 years and the predictive value of all investigated markers for new DU onset was explored.

Results. Significantly enhanced endogenous thrombin potential (ETP) and prolonged clot lysis time (CLT) were found in patients with DUs compared to HCs. CLT was prolonged in patients with DUs compared to those without, showing good validity in identifying DUs with an area under the curve of 0.7 (95% CI 0.6–0.8). The levels of ETP and intercellular adhesion molecule 1 were independently associated with CLT. Over the follow-up period, 20 patients developed new DUs. CLT was prolonged ($P < 0.001$) in patients with new DU episodes, especially those with recurrent DUs. Regression analysis showed that the Raynaud phenomenon visual analog scale and CLT were predictors of new DUs (OR 1.1, 95% CI 1.0–1.1 and OR 1.2, 95% CI 1.1–1.3, respectively). SEM confirmed denser fibrin clots in patients with new DUs.

Conclusion. Our results suggest that impaired fibrinolysis might have an emerging role in underlying digital vasculopathy and its progression in SSc.

Key Indexing Terms: digital ulcers, hemostasis, clot lysis time, fibrin structure, systemic sclerosis

Vasculopathy is considered to play a pivotal role in the pathogenesis of systemic sclerosis (SSc), affecting the digital microvascular bed in almost all patients, as clinically manifested by Raynaud phenomenon (RP) to digital ulcers (DUs).[1,2,3]

DUs are associated with a high rate of disability, reduced quality of life, and higher mortality.[4] Despite the improved treatment options for prevention and healing,[5] one-third of patients with DUs may experience progressive vasculopathy with recurrent ulcerations.[6] The most common DUs are on fingertips, mainly as a result of microvasculopathy.[7]

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Endothelial dysfunction is one of the hallmarks of SSc, characterized by upregulation of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), E-selectin, and the von Willebrand factor (vWF). Activation of platelets and leukocytes is also found in association with the loss of endothelial homeostasis, subsequently leading to disturbances in the coagulation/fibrinolysis system, the formation of intravascular fibrin deposits, and a tendency toward microthrombosis.[3,8,9] Several hemostatic alterations have been reported in patients with DUs, suggesting that underlying procoagulant activity might be connected to the progression of digital vasculopathy.[10,11,12,13,14,15] Several biomarkers connected with thrombotic and inflammation signaling are predictive for recurrent DUs.[11]

Since an evaluation of the complex hemostatic process is difficult when assessing single hemostatic factors and/or inhibitors, global hemostatic assays are used to provide an overview of hemostasis. Additionally, analyses of fibrin clot turbidity and structure provide a better understanding of the clot phenotype formed upon a disturbed hemostatic process. Previously, enhanced thrombin generation potential was reported in patients with SSc,[16] whereas prolonged clot lysis time (CLT) was associated with venous thromboembolism (VTE) in patients with primary RP.[17]

However, the unmet clinical need is a better understanding of DU pathogenesis and the determination of predictive markers for advanced digital vasculopathy and recurrent DUs on an

individual level. Thus, our goal with this study was to evaluate underlying coagulation/fibrinolysis disturbances linked to the digital vasculopathy in a cohort of patients with SSc to determine potential predictive biomarkers for new DU onset during 1.5 years of follow-up.

METHODS

Study design. We enrolled 58 patients with SSc from the outpatient clinic of the Institute of Rheumatology, Belgrade, Serbia, between 2017 and 2018. Patients aged > 18 years with a diagnosis of SSc according to the 2013 American College of Rheumatology/European Alliance of Associations for Rheumatology (EULAR) classification criteria,[18] and without previous treatment with endothelin receptor antagonists, phosphodiesterase 5 inhibitors, or prostanoids, were eligible for inclusion. The exclusion criteria included other autoimmune diseases, diabetes mellitus, liver and renal insufficiency, asthma and chronic obstructive pulmonary disease, hemostatic disorders, inflammatory bowel diseases, prior cardiovascular (CV) events, pregnancy, acute infections, and neoplastic diseases. None of the patients had chronic nonhealing DUs, an inflamed active DU, osteomyelitis, or lower-limb ulcers at baseline.

Forty-seven healthy, age- and sex-matched individuals were also included. They were not receiving nonsteroidal antiinflammatory drugs, acetylsalicylic acid (ASA), or anticoagulant drugs prior to the inclusion in the study.

To enrich the occurrence of new DUs, we have prospectively followed up a subgroup of 39 subjects with previous DUs for a 1.5-year period.

Evaluation instruments. All patients underwent a physical examination at inclusion involving measurement of height, weight, estimation of skin thickness by modified Rodnan skin score (mRSS),[19] and control for the presence of RP, telangiectasias, pitting scars, sclerodactyly, DUs, and contractures. The following data were collected from the medical records: age, sex, smoking habits, comorbidities, disease duration, ever experienced DUs, renal crisis, and ongoing therapy. Patients with SSc were classified into a diffuse cutaneous (dcSSc) or limited cutaneous SSc group.[20] The following analysis was conducted within 6 months prior to inclusion in the study: a pulmonary function test with vital capacity and diffusing capacity of the lung for carbon monoxide, a Doppler echocardiography to estimate systolic pulmonary artery pressure (sPAP), and nailfold videocapillaroscopy (NVC).[21] Presence of interstitial lung disease was defined radiologically by positive chest radiograph findings or a ground glass opacification on high-resolution computed tomography. The functional status of patients with SSc was evaluated with the Health Assessment Questionnaire–Disability Index and visual analog scale (VAS) scores for RP pain, DU pain, and overall pain were recorded.[22]

Blood sampling. Peripheral venous blood was collected in tubes containing clot activator or trisodium citrate. Serum, specifically platelet-poor plasma (PPP), was obtained within 60 minutes of sampling by centrifugation at 2000 g for 20 minutes at room temperature and then aliquoted and frozen at −70 °C. The frozen samples were transported to the Karolinska Institutet, Department of Molecular Medicine and Surgery, Stockholm, Sweden, for further analysis.

Calibrated automated thrombogram assay. The rate and amount of thrombin generation in plasma were measured by calibrated automated thrombogram assay.[23] A 20-μl reagent tissue factor and 20-μl thrombin calibrator were briefly pipetted in a 96-well microplate. After the addition of 80 μl of PPP, coagulation was initiated by calcium and a fluorogenic substrate. The fluorescence was measured every 30 seconds for 60 minutes by a Fluoroscan Ascent fluorometer (Thermo Fisher) and the following variables were determined using Thrombinoscope software (Thrombinoscope BV): lag time (time from start of analysis until detection of thrombin generation), peak thrombin generation, endogenous thrombin potential (ETP; the area under the concentration-time curve), and time to peak (time from start of thrombin generation until the peak thrombin value was achieved).

Overall hemostatic potential assay. To assess the overall fibrin formation and fibrinolysis in plasma, the overall hemostatic potential (OHP) assay was carried out.[24] Calculation of OHP was based on the construction of fibrin aggregation curves using citrated plasma (140 μL) into which thrombin, phospholipids, and calcium were added, with or without a tissue plasminogen activator (tPA; 300 ng/ml). The absorbance (Abs) at 405 nm was monitored every 12 seconds for 60 minutes. The area under the curve (AUC) was calculated as the sum of the Abs values and expressed as OHP, and the overall coagulation potential (OCP) of the summation of Abs values under the fibrin aggregation curve without tPA, as well as the overall fibrinolysis potential (OFP) using the equation OFP = ([OCP–OHP]/OCP) × 100% were also calculated.

Turbidimetric clotting and lysis assays. Clot formation and lysis were monitored by measuring the Abs at 405 nm for 1 hour every 12 seconds. The following variables were calculated: lag time C (the time at which an exponential increase in Abs occurred), the maximum Abs (Cmax; median value of 3 consecutive points where the curve reached plateau less the lag turbidity), lag time L (the time at which an exponential increase in absorbance occurred), and CLT (the time from the initiation of clot formation to the time at which a 50% decrease in Abs from maximum Abs in the lysis assay occurred).

Scanning electron microscopy of fibrin clots. Four clots randomly selected from the OHP assay were fixed with 2% glutaraldehyde in a Hepes-buffered saline for 60 minutes at room temperature and then stored at 4 °C. The specimens were rinsed briefly in distilled water and placed in 70% ethanol for 10 minutes, 95% ethanol for 10 minutes, absolute ethanol for 15 minutes at room temperature, pure acetone for 10 minutes, and then transferred to tetramethylsilane for 10 minutes and air dried. Thereafter, they were attached to an aluminum stub and coated with carbon. The clots were analyzed in an Ultra 55 field emission scanning electron microscope (SEM) at 3 kV.

Serological markers. Antinuclear, anticentromere, and antitopoisomerase I antibodies, complement levels (C3, C4), fibrinogen, and routine laboratory analyses, including C-reactive protein (CRP), erythrocyte sedimentation rate, lipid profile, and urea and creatinine levels, were carried out at inclusion using standard methods at the Institute of Rheumatology for all participants.

Serum levels of ICAM-1, VCAM-1, and E-selectin were analyzed using a commercially available ELISA kit (Quantikine R&D Systems). The concentration of vWF antigen (Ag) was determined according to the Blood Coagulation System (BCS XP 9020687; Siemens Healthcare Diagnostics) protocol.

Follow-up and study outcome. A DU was defined as a denuded area located at distal digits with the loss of both epidermis and dermis, excluding fissures, paronychia, pitting scars, ulcers located over the extensor, and calcium extrusion areas. Occurrence of new DUs was followed for 1.5 years. Each subject was given a "DU diary" for reporting the date of a new fingertip event. All new DUs were recorded by contacting all 39 patients once every 1–3 months during follow-up.

Ethical considerations. The local ethics committee of the Institute of Rheumatology approved this study (No 29/1-110), and written informed consent was signed by all participants prior to enrollment.

Statistical analysis. Descriptive statistics were used to summarize the characteristics of the participants. Proportions were shown for categorical variables. ANOVA with the posthoc Bonferroni test was used to examine the difference in continuous normally distributed variables between ≥ 3 groups of participants; as a contrast, the Kruskal-Wallis test was used along with a pairwise posthoc test. *P* values were adjusted for multiple testing. Univariate logistic regression (ULR) was used to compare characteristics between DU groups. Spearman or Pearson tests were used

to test the correlation between CLT and other continuous variables of interest. To summarize the overall discriminatory value of the CLT, a receiver-operating characteristic (ROC) AUC was done. Multiple linear regression analysis was performed to identify predictors of CLT. To evaluate factors associated with the onset of new DUs, we performed ULR and multivariate logistic regression (MLR) analyses. The strength of association between a risk factor and new DU outcome was given by an OR with a 95% CI. All independent continuous variables with $P < 0.15$ in ULR and covariate age at SSc onset were included in the MLR model according to forward-stepwise selection. The Kaplan-Meier method was applied to estimate the DU free survival rate, and the curves were compared by log-rank test, where the cut-off point for CLT was settled according to the quartile analysis (Q2). $P < 0.05$ was considered statistically significant. The data analysis was performed using the IBM SPSS version 26.0 (IBM Corp.).

RESULTS

General patient data at baseline. The characteristics of the initial study population at baseline are described in Supplementary Table 1 (available with the online version of this article). There was no difference between the 2 groups of patients with SSc (those with a history of DUs and those DU-naïve) or the patients with SSc and controls with regard to demographic profile, lipid status, and complement levels.

DUs and laboratory results at baseline. Thirty-nine patients (67.2%) had experienced DUs in the course of their disease. As expected, DUs were associated with younger age at disease onset, higher mRSS, a late NVC pattern, and higher pain. Patients with a history of DUs had significantly higher levels of CRP (Supplementary Table 1, available with the online version of this article) and investigated vascular biomarkers compared to healthy controls (HCs); however, this difference was not observed between the 2 DU groups (Table 1). The results of global hemostatic assays are presented in Table 1.

The most prominent finding was significantly prolonged CLT in the group with a history of DUs compared to both the HC and non-DU groups. In addition, CLT remained independently associated with a history of DUs after adjustment for fibrinogen (OR 1.1, 95% CI 1.0–1.2; data not shown). ROC analysis showed good accuracy for CLT in identifying DUs with an AUC of 0.7 (95% CI 0.6–0.8; Supplementary Figure 1, available with the online version of this article).

In the whole patient group, CLT showed a positive correlation with fibrinogen ($r = 0.4$), CRP ($r = 0.3$), ICAM-1 ($r = 0.5$), vWF-Ag ($r = 0.3$), and ETP ($r = 0.6$). A multiple linear regression model adjusted for fibrinogen revealed that CLT was independently associated with ICAM-1 ($\beta = 0.5$, 95% CI 0.1–0.8) and ETP ($\beta = 0.01$, 95% CI 0.01–0.02; data not shown).

Eighteen patients had active DU (46.1%) at inclusion. The levels of inflammatory markers, investigated vascular markers, and variables of global assays did not differ between patients with active DUs and DUs experienced in the past (data not shown).

Table 1. Vascular markers and hemostatic variables at baseline.

	DU, n = 39	Non-DU, n = 19	HCs, n = 46
Vascular biomarkers			
ICAM-1, ng/mL	30.3 ± 8.7*	27.8 ± 5.8	24.3 ± 4.6[a]
VCAM-1, ng/mL	39.1 (15.4)*	33.5 (14.7)	30.8 (11.9)[a]
E-selectin, ng/mL	5.5 ± 2.1*	4.5 ± 1.5	4.1 ± 1.7[b]
vWF:Ag	1.7 (0.7)*	1.5 (1.5)**	1.2 (0.8)[a]
Fibrinogen, g/L	4.8 (2.6)*,***	3 (2.7)	2.9 (1.3)[a]
Calibrated automated thrombogram assay			
ETP, nMxmin	2020.0 ± 405.3*	1846.0 ± 289.4	1756.3 ± 321.2[b]
Lag time, min	3.2 ± 0.5	3.1 ± 0.7	3.3 ± 0.8[b]
Peak, nM	326.3 ± 87.8	329.0 ± 85.9	293.3 ± 66.8[b]
Time to peak, min	6.2 ± 1.3	6.2 ± 1.7	6.5 ± 1.3[b]
Overall hemostatic potential assay			
OHP, Abs-sum	131.2 ± 78.2	122.1 ± 86.9	106.6 ± 58.9[a]
OCP, Abs-sum	318.4 ± 56.2	290.7 ± 114.2	294.4 ± 63.4[a]
OFP, %	53.1 ± 18.1	58.6 ± 11.8	61.3 ± 12.1[a]
Turbidity assay			
Coagulation			
Lag phase clot, min	5.2 ± 0.9	5.4 ± 1.1	5.7 ± 1.7[b]
Cmax Abs	1.2 ± 0.2*	1.0 ± 0.4	1.0 ± 0.2[a]
Fibrinolysis			
Lag phase lysis, min	5.7 ± 2.0	6.5 ± 3.6	6.2 ± 2.1[b]
CLT, min	37.5 ± 10.5*,***	30.5 ± 6.7	30.7 ± 7.9[a]

Data are presented as mean ± SD or median (IQR) depending on distribution. Differences across the groups were calculated with either [a] Kruskal-Wallis with post hoc analysis or [b] 1-way ANOVA. *P* values were adjusted by the Bonferroni correction for multiple testing. * $P < 0.05$ observed between DU and control group. ** $P < 0.05$ observed between non-DU and HC groups. *** $P < 0.05$ observed between DU and non-DU group. Abs: absorbance; CLT: clot lysis time; Cmax Abs: clot maximum absorbance; DU: digital ulcer; ETP: endogenous thrombin potential; HC: healthy controls; ICAM-1: intercellular adhesion molecule 1; OCP: overall coagulation potential; OFP: overall fibrinolysis potential; OHP: overall hemostasis potential; VCAM-1: vascular cell adhesion molecule 1; vWF:Ag: von Willebrand factor antigen.

New DU episode in 1.5-year follow-up. Over the 1.5 years of follow-up, 20 patients out of 39 (51.3%) experienced at least 1 new ischemic DU (Supplementary Figure 2, available with the online version of this article), with a median time to event of 7.5 (range 4–17) months.

Detailed characteristics are presented in Table 2. Most of the patients with new DUs were female (84.2%), with > 3 previous episodes of DUs (72.2%) and a median age at disease onset 44.4 (SD 10.7; data not shown). Patients with a higher risk of developing new DUs were those with an active DU (OR 5.2, 95% CI 1.3–20.5), dcSSc (OR 5.6, 95% CI 1.5–23.3), RP (OR 4.0, 95% CI 1.1–15.3), sclerodactyly (OR 5.1, 95% CI 1.1–23.4), a higher RP VAS (OR 1.1, 95% CI 1.0–1.2), and DU VAS (OR 1.02, 95% CI 1.00–1.04) at baseline.

Data regarding treatments in each group are shown in Table 2. There was no difference in the mean prednisolone dose or cumulative dose of cyclophosphamide (CYC) at inclusion between the 2 groups. The levels of all investigated variables did not differ between patients treated with ASA (75 mg/d).

Soon after enrollment in the follow-up period, 6 patients started treatment with CYC with similar distribution among groups (new DU, n = 3 [15%] vs those without DU, n = 3 [15.8%]).

The levels of serum vascular markers and the variables of global hemostatic assays are presented in Table 3. Those with new DU onset had higher thrombin generation, increased OHP, and diminished OFP. The turbidimetric assay revealed significantly prolonged CLT in patients with new DUs. In the entire follow-up cohort, CLT was related with ETP ($r = 0.6$), peak of thrombin generation (PT; $r = 0.4$), Cmax ($r = 0.5$), ICAM-1 levels ($r = 0.5$), sclerodactyly (40.4 ± 10.7 vs 32.2 ± 7.4; OR 1.1, 95% CI 1.01–1.14), and dcSSc (41.7 ± 9.8 vs 35.2 ± 10.3; OR 1.04, 95% CI 1.0–1.1), whereas within patients with a new DU episode, CLT remained significantly associated with ETP ($r = 0.6$), PT ($r = 0.5$), and ICAM-1 ($r = 0.5$), and showed a positive correlation with sPAP ($r = 0.4$, $P = 0.06$) that was not significant. A linear regression model including ICAM-1 and ETP showed that CLT remained predicted by ICAM-1 and ETP ($\beta = 0.3$, 95% CI 0.1–0.6 and $\beta = 0.01$, 95% CI 0.01–0.02, respectively) in the whole follow-up cohort (data not shown).

To explore risk factors for new DUs, binary regression was conducted. Final MLR revealed RP VAS and CLT as independent predictors of new DUs (OR 1.1, 95% CI 1.0–1.1 and OR 1.2, 95% CI 1.1–1.3, respectively). Kaplan-Meier analysis of freedom for DUs is shown in Figure 1. Patients with prolonged CLT > 35.4 minutes experienced new DUs within a significantly shorter period of time (log-rank, $P < 0.001$). Comparing patients experiencing episodic (n = 13) or recurrent DUs (n = 7) and the subgroup of patients without DUs during the follow-up period, we observed increased OHP, decreased OFP, and significantly prolonged CLT in the subgroup with recurrent DUs (Figure 2).

There was no correlation between either mean prednisolone dose or cumulative dose of CYC with vascular markers or variables of global assays, except for CYC and Cmax ($r = 0.8$) in the group with new DUs.

Scanning electron microscopy. SEM showed denser fibrin clots with thinner fibers and small pores in patients with a history of DUs compared to either DU-naïve or HC groups and in patients with a new DU onset during the follow-up period with respect to those without (Figure 3).

DISCUSSION

We demonstrate significantly prolonged CLT in patients with a history of DUs and in patients with novel DUs during the follow-up period, implicating impaired fibrinolysis as an underlying mechanism for digital vasculopathy in SSc. Prolonged CLT was associated with enhanced thrombin generation and the endothelial damage measured by ICAM-1. Hypofibrinolysis has emerged as a risk factor especially for recurrent DUs. In addition, prothrombotic fibrin structures were observed in patients with a history of DUs, particularly in patients with novel DU episodes during the follow-up period. Therefore, we postulate that patients with progressive digital vasculopathy in SSc suffer from procoagulant disorder characterized not only by endothelial damage but also by enhanced thrombin generation, formation of altered fibrin clots, and diminished fibrinolysis.

Microvascular disease is a hallmark of SSc, affecting not only the skin but also other organs including heart, lung, kidneys, and the gastrointestinal tract, which underlie life-threatening complications such as pulmonary arterial hypertension (PAH). Novel studies have documented increased risk of CV disease[25] and VTE[26] events in different cohorts of patients with SSc, highlighting that SSc is a systemic vascular disorder.[3]

We have studied hemostatic disturbances in patients with SSc with DUs as the most frequent visible manifestations of peripheral vasculopathy. First DUs develop in 43% of patients with SSc within the first year and in 73% of patients within 5 years after the diagnosis.[27] Moreover, the patients in our cohort had not been previously treated with therapeutic modalities for either DUs or RP in SSc recommended by EULAR,[5] except with calcium channel blockers. Therefore, we postulate that our results reflect the natural course of digital microangiopathy in SSc.

The most prominent finding in our study was diminished fibrinolysis in patients with recurrent DUs, measured by decreased OFP and prolonged CLT. Hypofibrinolysis might be a consequence of the rigid fibrin network, characterized by the thin, highly branched fibers with small intrinsic pores, less susceptible to lysis.[28] SEM confirmed denser clots in patients with previous DUs compared to HC and non-DU patients, and the same characteristics of the clot were noted in patients with new DU episodes. Enhanced thrombin generation and increased fibrinogen concentration are major determinants of denser fibrin clot resistance to lysis. After adjustment for ETP and fibrinogen, CLT remained independently associated with underlying digital vasculopathy and its progression, suggesting alternative mechanisms involved in fibrinolysis impairment in SSc.

Decreased serum levels of the endothelial protein C receptor (EPCR) were previously found in patients with SSc with DUs.[29] EPCR is directly involved in the inactivation of coagulation factors Va and VIIIa, and EPCR levels are inversely related with the levels of the plasmin–α2-plasmin inhibitor complex.[29] The

Table 2. Characteristics of patients with and without new DUs during the 1.5-year follow-up.

	With New DUs, n = 20	Without New DUs, n = 19	OR (95% CI)
Demographic characteristics			
Age, yrs	52.3 ± 11.2	53.5 ± 10.4	1.0 (0.9–1.1)
Sex, F/M, n	17/3	17/2	1.5 (0.2–10.1)
BMI, kg/m^2	23.5 ± 3	23.8 ± 4.2	1.0 (0.8–1.2)
Current smokers	6 (30.0)	7 (36.8)	0.9 (0.4–1.7)
Arterial hypertension	11 (55.0)	10 (52.6)	1.1 (0.3–3.9)
Disease characteristics			
Cutaneous subtype			
Limited	8 (42.1)	15 (83.3)	Ref.
Diffuse	12 (60.0)	4 (16.7)	5.6 (1.5–23.3)
Autoantibody status			
Anticentromere Ab	6 (30.0)	7 (36.8)	0.7 (0.2–2.8)
Anti–Scl-70 Ab	12 (60.0)	7 (36.8)	2.6 (0.7–9.4)
Disease duration, yrs, median (IQR)	5 (14.5)	6 (9.0)	1.0 (0.9–1.1)
mRSS, median (IQR)	13 (8.0)	11 (11.0)	1.0 (0.9–1.0)
FVC% predicted	95.9 ± 20.9	96.5 ± 17.6	1.0 (0.9–1.0)
DLCO% predicted	63.6 ± 13	66.6 ± 20.1	1.00 (1.00–1.02)
ILD	14 (70.0)	8 (42.1)	3.0 (0.9–21.9)
sPAP	30.1 ± 5.4	30.1 ± 7.2	1.0 (0.9–1.1)
Telangiectasia	11 (55.0)	15 (78.9)	0.3 (0.8–1.3)
Sclerodactyly	17 (85.0)	10 (52.6)	5.1 (1.1–23.4)
Contracture	7 (35.0)	6 (31.6)	1.2 (0.3–7.9)
Pitting scars	17 (85.0)	15 (78.9)	1.5 (0.3–20.5)
DU active at baseline	13 (65.0)	5 (26.3)	5.2 (1.3–20.5)
≥ 3 DU episodes	13 (72.2)	8 (42.1)	3.7 (1.0–14.2)
NVC			
Early	2 (10.0)	3 (15.8)	Ref.
Active	10 (50.0)	8 (42.1)	1.9 (0.3–14.1)
Late	8 (40.0)	8 (42.1)	1.5 (0.2–11.5)
SHAQ, median (IQR)	0.4 (0.6)	0.4 (0.4)	1.4 (0.3–6.1)
VAS pain, mm, median (IQR)	43 (45.8)	29 (38.0)	1.00 (1.00–1.03)
RP VAS, mm, median (IQR)	56 (30.3)	22 (24.0)	1.1 (1.0–1.2)
DU VAS, mm, median (IQR)	46.6 (65.0)	0 (31.0)	1.02 (1.00–1.04)
Treatment			
Ongoing treatment			
GCs	6 (30.0)	10 (52.6)	0.4 (1.0–1.4)
MTX	4 (20.0)	5 (26.3)	0.7 (0.2–3.1)
Chloroquine	2 (10.0)	1 (5.3)	2.0 (0.2–24.1)
AZA	2 (10.0)	4 (22.2)	0.4 (0.1–2.4)
ACE inhibitor	5 (25.0)	9 (47.4)	0.4 (0.9–1.4)
Calcium channel blocker	9 (45.0)	10 (52.6)	0.7 (0.2–2.6)
Beta blockers	4 (20.0)	4 (21.1)	0.9 (0.2–4.4)
ASA	9 (45.0)	4 (21.1)	3.1 (0.7–12.6)
Previous treatment			
CYC	10 (51.3)	8 (42.1)	1.4 (0.4–4.9)
Hyperbaric oxygen	10 (50.0)	6 (31.6)	2.2 (0.6–7.9)
Laboratory variables			
CRP, mg/L, median (IQR)	6 (5.6)	4.8 (10.0)	1.0 (0.9–1.1)
ESR, mm/h, median (IQR)	19 (29.3)	18 (26.0)	1.00 (1.00–1.04)
Cholesterol, mmol/L	6.7 ± 1.7	6.5 ± 1.4	1.1 (0.7–1.7)
Triglycerides, mmol/L	1.5 ± 0.6	1.8 ± 0.7	0.4 (0.1–1.2)
Urea, mmol/L	5.4 ± 1.9	4.7 ± 1.3	1.3 (0.9–2.0)
Creatinine, mmol/L	76.6 ± 9.5	73.5 ± 13.1	1.0 (1.0–1.1)
C3, g/L	1.3 ± 0.3	1.3 ± 0.3	1.1 (0.1–10.4)
C4, g/L	0.2 ± 0.1	0.2 ± 0.1	49.1 (10^{-3} to 19×10^5)

Values are n (%) or mean ± SD unless otherwise indicated. Ab: autoantibody; ACE: angiotensin-converting enzyme; ASA: acetylsalicylic acid; AZA: azathioprine; CRP: C-reactive protein; CYC: cyclophosphamide; DLCO: diffusing capacity for carbon monoxide; DU: digital ulcer; ESR: erythrocyte sedimentation rate; FVC: forced vital capacity; GC: glucocorticoid; ILD: interstitial lung disease; mRSS: modified Rodnan skin score; MTX: methotrexate; NVC: nailfold videocapillaroscopy; OR: odds ratio; RP: Raynaud phenomenon; SHAQ: Scleroderma Health Assessment Questionnaire; sPAP: systolic pulmonary artery pressure; VAS: visual analog scale.

Table 3. Vascular markers and hemostatic parameters in patients with and without new DUs during the 1.5-year follow-up.

	With New DUs, n = 20	Without New DUs, n = 19	Univariate Logistic Regression OR (95% CI)
Vascular biomarkers			
ICAM-1, ng/mL	32.5 ± 8.6	27.9 ± 8.4	1.1 (1.0–1.2)
VCAM-1, ng/mL	41.1 ± 9.8	41.3 ± 16.8	0.9 (0.9–1.1)
E-selectin, ng/mL	5.6 ± 2.2	5.4 ± 2.1	1.0 (0.8–1.4)
vWF:Ag, median (IQR)	1.7 (0.7)	1.75 (0.8)	1.8 (0.7–4.8)
Fibrinogen, g/L	5.3 ± 2.0	4.4 ± 1.8	1.3 (0.9–1.8)
Calibrated automated thrombogram assay			
ETP, nMxmin	2134.7 ± 426.1	1899.2 ± 353.7	1.002 (1.000–1.003)
Lag time, min	3.1 ± 0.5	3.3 ± 0.5	0.4 (0.1–1.5)
Peak, nM	347.5 ± 79.5	303.9 ± 92.6	1.00 (1.00–1.01)
Time to peak, min	5.9 ± 1.4	6.5 ± 1.2	0.7 (0.4–1.2)
OHP	184.8 ± 70.4	115.5 ± 48.8	1.02 (1.01–1.04)
OCP	330.0 ± 65.9	306.1 ± 42.3	1.0 (0.9–1.0)
OFP	43.9 ± 18.2	62.8 ± 12.1	0.9 (0.8–0.9)
Turbidity assay			
Coagulation			
Lag phase clot, min	4.9 ± 1	5.2 ± 0.8	0.7 (0.3–1.5)
Cmax Abs	1.2 ± 0.3	1.1 ± 0.2	22.7 (0.9–566.9)
Fibrinolysis			
Lag phase lysis, min	5.4 ± 2	6.0 ± 1.9	0.8 (0.6–1.2)
CLT, min	44.1 ± 9.6	31.4 ± 6.8	1.2 (1.1–1.3)

Values are mean ± SD unless otherwise indicated. CLT: clot lysis time; Cmax Abs: clot maximum absorbance; DU: digital ulcer; ETP: endogenous thrombin potential; ICAM-1: intercellular adhesion molecule 1; OCP: overall coagulation potential; OFP: overall fibrinolysis potential; OHP: overall hemostasis potential; VCAM-1: vascular cell adhesion molecule 1; vWF:Ag: von Willebrand factor antigen.

plasmin–α2-plasmin inhibitor complex reflects an in vivo generation of plasmin, the key enzyme in the fibrinolytic process.[30] Thus, the loss of anticoagulant EPCR function during the course of SSc may be related to enhanced coagulation and diminished fibrinolysis. Further, increased expression of the plasminogen activator inhibitor 1 (PAI-1), the main inhibitor of the fibrinolytic system, was previously detected in patients with SSc.[31] Żuk et al have shown association between augmented PAI-1 levels and both prolonged CLT and decreased clot permeability in patients with RP and VTE.[17]

We demonstrate a clear association between CLT and ICAM-1 levels in patients with a history of DUs, as well as in patients experiencing new DU episodes during the follow-up. This finding, to the best of our knowledge, has not been elucidated before. An activated/dysfunctional endothelium upregulates ICAM-1 to facilitate leucocyte adhesion and migration across endothelium, a process that may be fostered by PAI-1.[32,33] ICAM-1 could have an effect on fibrin structure, since it can be upregulated by thrombin and can bind fibrinogen.[34,35] Our results further point toward the role of endothelial injury in the formation of abnormal fibrin clots in patients with digital vasculopathy.

When interpreting our results, clinical aspects of the study cohort should be considered. A higher number of previous DUs at enrollment had an effect on the risk of future DUs.[36] Further, 65% of patients with new DUs had RP at physical examination, and the severity of RP assessed by the Scleroderma Health Assessment Questionnaire RP VAS was strongly associated with new DU episodes. Thus, the coexistence of the vasospasm and underlying vasculopathy might contribute to a more severe breakdown of microcirculation, causing recurrent ulcers. Because of a positive association between CLT and sPAP in patients with new DU episodes, we speculate that these patients are at higher risk of developing PAH, as previously shown.[4] Concerning ongoing treatment, the prophylactic dose of ASA (75 mg/daily) showed a lack of effect on CLT, suggesting that higher doses may be needed to influence clot permeability.[37] The cumulative dose of CYC was associated with denser clots in patients with novel DU episodes, which is probably linked to increased severity of disease including extensive skin and lung involvement.

The present study has several potential limitations. Even though the prospective cohort study design is primarily used, our study could have benefited from a larger sample size and a longer follow-up period. A rather low number of patients in the investigated groups did not allow us to include clinical categorical data in multivariable analysis for assessing predictive factors of new DU episodes. We decided to exclude DU-naïve cases from prospective study for explanatory purposes, since the incidence of the new DU was expected to be low, causing a slight selection bias in our design. Further, broad exclusion criteria have restricted assessing the effect of confounders on clot properties

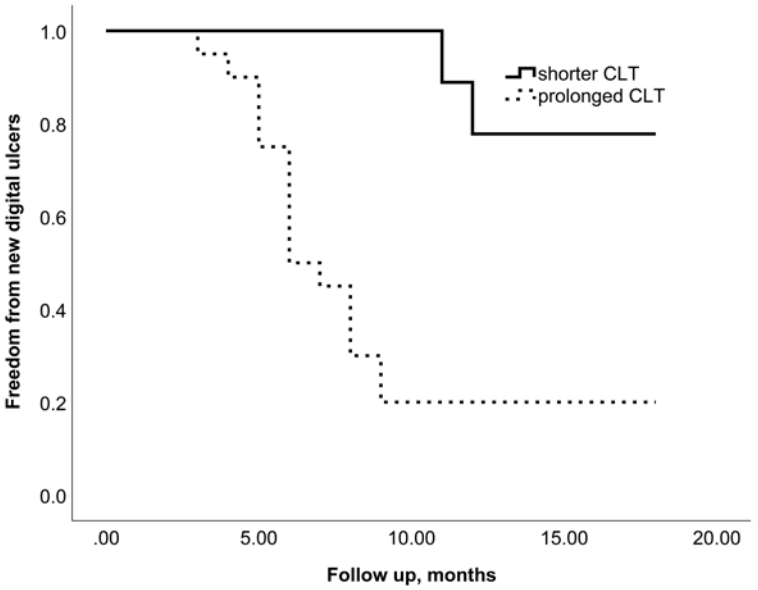

Figure 1. Kaplan-Meier analyses of freedom from new DUs in an 18-month follow-up of 39 patients with SSc with previous DUs. The curve is shown for short CLT (≤ 35.4 min) or prolonged (> 35.4 min) value. CLT: clot lysis time; DU: digital ulcer; SSc: systemic sclerosis.

and digital vasculopathy progression. Last, the measurement of investigated variables has not been repeated at the time of new DU onset; thus, we were unable to further explore the relation of the variables to new DUs.

Our study provides evidence that impaired fibrinolysis is not only critical in the pathogenesis of digital vasculopathy but it also represents a significant prognostic factor for worse disease outcome in patients with SSc. Our findings need to be replicated in larger cohorts, allowing for a timely therapeutic approach for patients with progressive vasculopathy.

ONLINE SUPPLEMENT

Supplementary material accompanies the online version of this article.

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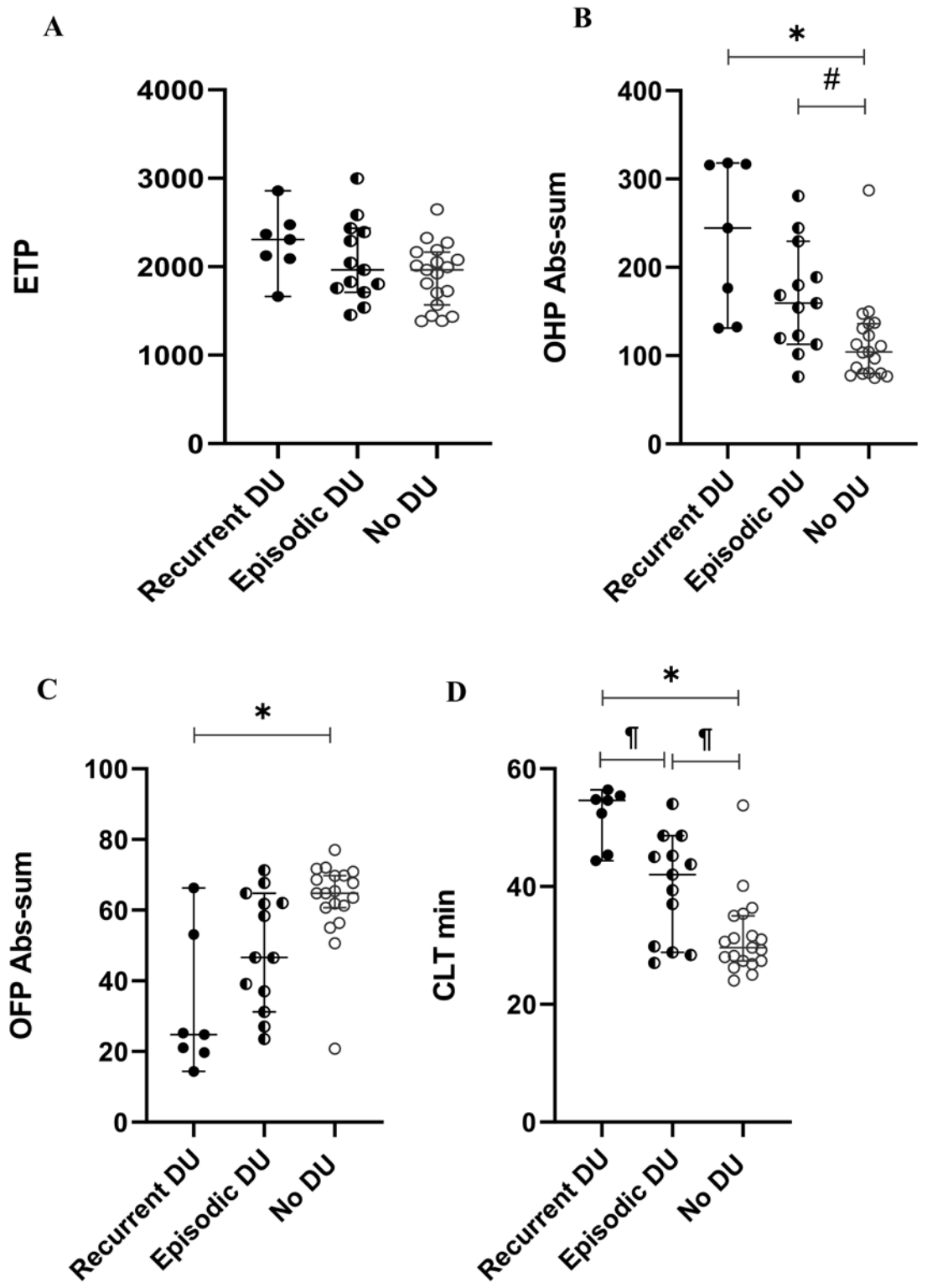

Figure 2. (A) ETP; (B) OHP; (C) OFP, and (D) CLT in patients with recurrent DUs, episodic DUs, and those who did not experience DUs during follow-up. *P* values were adjusted by the Bonferroni correction for multiple testing. * $P < 0.05$, # $P = 0.05$, and ¶ $P = 0.07$. CLT: clot lysis time; DU: digital ulcer; ETP: endogenous thrombin generation; OFP: overall fibrinolytic potential; OHP: overall hemostatic potential.

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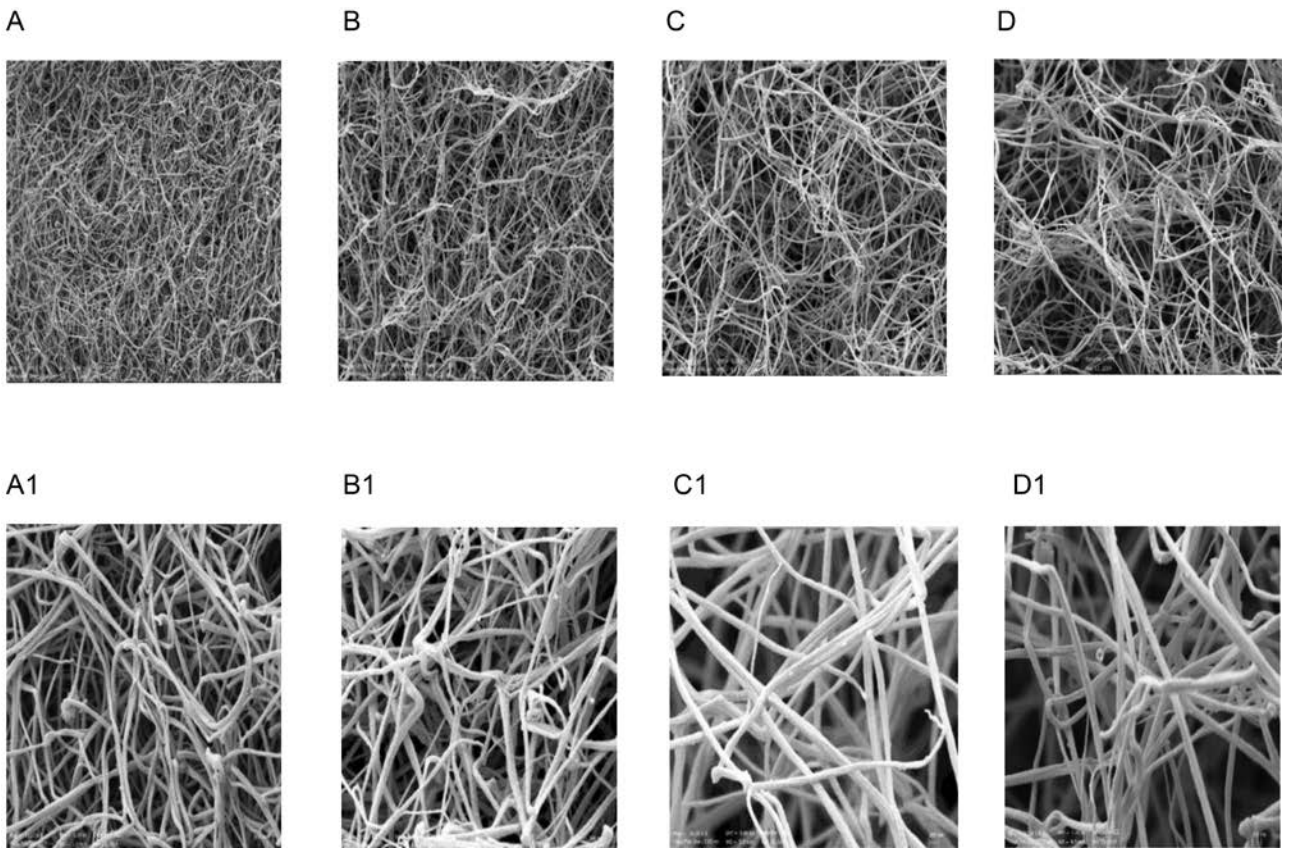

Figure 3. Representative scanning electron micrographs of fibrin clots. (A) Patient with new DU. (B) Patient without new DU during the follow-up. (C) DU-naïve patient. (D) Healthy control. The magnification 1 μm was used for Figures A–D and 300 nm for Figures A1–D1. DU: digital ulcer.

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