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THE ASSOCIATION OF GLOBAL HEMOSTASIS AND ENDOTHELIAL INJURY MARKERS WITH THE RISK OF NEW DIGITAL ULCER ONSET IN SYSTEMIC SCLEROSIS: PROSPECTIVE COHORT STUDY

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ISPITIVANJE POVEZANOSTI POKAZATELJA GLOBALNE HEMOSTAZE I OŠTEĆENJA ENDOTELA SA RIZIKOM ZA NASTANAK NOVIH DIGITALNIH RANICA U SISTEMSKOJ SKLEROZI: PROSPEKTIVNA KOHORTNA STUDIJA

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The difficult is what takes a little time.
The impossible is what takes a little longer!

Fridtjof Nansen

Mojim predivnim roditeljima

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THE ASSOCIATION OF GLOBAL HEMOSTASIS AND ENDOTHELIAL INJURY MARKERS WITH THE RISK OF NEW DIGITAL ULCER ONSET IN SYSTEMIC SCLEROSIS: PROSPECTIVE COHORT STUDY

Abstract

Background: Digital vasculopathy (DV) is common in patients with systemic sclerosis (SSc) and might be connected with the coagulation/fibrinolysis imbalance.

Aims: To evaluate endothelial dysfunction (ED) and hemostasis status in patients with SSc with a focus on the onset of digital ulcers (DUs) over the course of the disease, in relation to the quality of life in these patients.

Methods: Haemostatic parameters were determined in 58 SSc patients and 46 matched healthy controls (HCs) by *Thrombin generation (TG), Overall haemostatic potential* and *Turbidity assays.* ED markers were assessed by *ELISA.* Clot structure was visualized by *Scanning electron microscopy* (SEM). Quality of life was assessed by *SHAQ* and *EQ5D.* A cohort with a history of DUs (hDUs) was followed (1.5 years) in order to assess predictive markers for new DU.

Results: Increased TG, denser fibrin clot (Cmax) and longer clot lysis time (CLT) were found in SSc patients ($p<0.05$, respectively) compared to HC. Active disease was predicted by Cmax (AUC= 0.688, $p<0.05$). ICAM1 was associated with CLT within SSc cohort, hDU cases and patients with novel DUs ($p<0.05$, respectively).CLT itself determined hDUs cases (AUC 0.706, $p<0.05$). Cases with novel DU exhibited longer CLT ($p<0.001$), especially patients with recurrent ulcers ($p<0.05$). The VAS Raynaud phenomenon and CLT were independent predictors of new DUs (OR 1.1, 95% CI 1.0–1.1; OR 1.2, 95% CI 1.1–1.3, respectively). SEM revealed denser clots in cases with new DUs.

Conclusion: Enhanced coagulation and impaired fibrinolysis may have a critical role in SSc pathogenesis. ICAM1 might contribute to impaired fibrinolysis, which could be considered as a key event in DV genesis and progression in patients with SSc.

Keywords: systemic sclerosis, endothelial dysfunction, biomarkers, thrombin generation, clot lysis time, clot density, fibrin clot structure, digital ulcers, quality of life

Scientific Field: Medicine

Scientific subfield: Epidemiology

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Sažetak

Uvod: Oštećenja na nivou krvnih sudova prstiju (digitalna vaskulopatija, DV) javljaju se kod skoro svih pacijenata sa sistemskom sklerozom (SSc) i mogu biti povezana sa poremećajem koagulacije/fibrinolize.

Ciljevi: Ispitati oštećenje endotela (OE) i hemostaze kod pacijenata sa SSc sa posebnim fokusom na pojavu ranica na prstima (DUs) tokom bolesti u odnosu na kvalitet života.

Metod: Parametri hemostaze su odredjivani kod 58 SSc pacijenata i 46 kontrola (K) uparenih po godinama starosti i polu, uz pomoć *Trombin generacije (TG); Sveukupnog hemostatskog potencijal i Turbidimetrijskog eseja.* Markeri OE su analizirani *ELISA*-om. Struktura fibrinskog ugruška je vizualizavana skenirajućim elektronskim mikroskopom (SEM). Kvalitet života je procenjivan *SHAQ i EQ5D* upitnicima. Pacijenata koji su ikada u toku bolesti imali DUs (iDUs) praćeni su (1.5 godinu) kako bi se procenili prediktivni markeri za nastanak nove ranice.

Rezultati: Povišena TG, gušći fibrinski ugrušak (Cmax) i duže vreme lize ugruška (CLT) su imali SSc pacijenti ($p<0.05$, redom) u poredjenju sa K. Cmax je pokazao sposobnost da detektuje aktivnu bolest (AUC= 0.688, $p<0.05$). ICAM1 je bio povezan sa CLT na nivou cele SSc kohorte, grupe sa iDUs i medju pacijentima sa novom DU ($p<0.05$, redom). Pacijenti sa novom DU karakterisali su se produženim CLT-om ($p<0.001$), posebno oni sa rekurentnim ranicama ($p<0.05$). Nezavisnim prediktorima za nove ranice izdvojili su se težina Rejnovog fenomena i CLT (OR 1.1, 95% CI 1.0–1.1 and OR 1.2, 95% CI 1.1–1.3, redom). SEM je potvrdio postojanje gusto pakovanog ugruška kod onih sa novom DU.

Zaključak: Hiperkoagulacija i oštećena fibrinoliza bi mogli imati kritičnu ulogu u patogenezi SSc. ICAM1 može doprineti oštećenoj fibrinolizi, koja bi se mogla smatrati ključnom za nastanak DV i njenu progresiju u SSc.

Ključne reči: sistemska skleroza, oštećenje endotelne funkcije, biomarkeri, stvaranje trombina, struktura fibrinskog ugruška, vreme razgradnje ugruška, gustina ugruška, ranice na prstima, kvalitet života.

Naučna oblast: Medicina

Uža naučna oblast: Epidemiologija

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1 INTRODUCTION

1.1 Definition and history

Systemic sclerosis (SSc) is a life-long autoimmune connective tissue disease, characterized by vascular modifications, activation of immune system and fibroblast dysfunction, with wide-ranging fibrosis of skin and internal organs [1–3].

The etymology of 'scleroderma' comes from the Greek *scleros* + *derma*, meaning 'hardened skin' [4]. The description "thickening of the skin" can be found in some of Hippocrates' notes on his patients as early as 400 BC [5]. The first convincing and detailed report of the disease comes from Carlo Curzio in 1753, who described a 17-year-old women as having "extensive tension and hardness of skin all over her body" [5,6]. As a medical term, 'scleroderma' was introduced in 1836 by Fantonetti and gained acceptance as a clinical entity in the mid-19t^{h} century[7,8]. Maurice Raynaud was the first to notice a relation between exposure to cold and color changes in the fingers and Systemic Sclerosis (SSc), thus the term 'Raynaud's Phenomenon' (RP) was born [7].

1.2 Epidemiology

Epidemiology data are difficult to obtain, mainly due to the rarity of SSc, but also owing to a variety of presentations. SSc may overlap with other rheumatic diseases and be confused with conditions such as "scleroderma mimickers", so reliable estimates of the incidence and prevalence present a great challenge. Over the last few decades, incidence has increased in many parts of the world, and has almost doubled in the United States (US) ,, probably due to improved diagnostics with changes in diagnostic criteria – being the highest in US and Australia and the lowest in Asia and Europe [9-11]. The global incidence of SSc range from 0.6-122 cases per million people (pmp) per year [12], with 1.4 cases pmp according to the latest meta-analysis reported in 2021 [13]. The global prevalence varies from 7 to 489 pmp [14], with 7.2-33.9 pmp in Europe [11]. Many studies have noted the presence of a north-to-south gradient, with a higher rates observed in lower latitudes (France: 158 pmp in 2001;Split, Croatia: 15.6/million people in 2008) and a lower in the north (Iceland: 7.1 pmp in 1994; England: 8.8 pmp in 2004) [14–17]. Recent registry-based studies from Sweden disproved this theory by observing a point prevalence of 305 pmp, estimated on 12.31.2010, using the new classification criteria [18]. Like most autoimmune diseases, SSc affects more females than males, with the incidence gender ratio varying between 1.1/1 to 14/1 with an average of 3/1[14,19]. A peak incidence of SSc occurs in the 25-65 age group [20].

The observations that SSc is clustered in some districts have indicated that environmental exposures, more so than occupational factors such as organic solvents or silica , are important for the pathogenesis of SSc. Individuals exposed to silica had the diffuse cutaneous form of disease (dSSc) more frequently and a lower survival rate [21,22].

Mortality is elevated in SSc and is significantly higher than in the general population with the standardized mortality ratio 2.72 [23,24]. Cohort studies in Europe reported differences in survival regarding SSc subsets and different populations, thus the 10-year survival rate has been reported as the highest in Spain with 86% for dSSc and 95% for limited cutaneous SSc (lSSc), and the lowest in Hungary with 49% for dSSc and 82% for lSSc [25,26]. Survival rates for SSc in Europe seem largely unchanged or even improved, especially in the dcSSc subset as per data from a British retrospective cohort study, where the 5-year survival rate among dSSc had changed from 69-84% [27]. A large study in Pittsburgh, reported a 10-year (1972-2002) survival rate improvement, indicating decreased mortality from 42% to 6% for scleroderma renal crisis (SRC), probably due to improved treatment. However, increased proportions for either pulmonary fibrosis or pulmonary arterial hypertension (PAH) over time have been

also observed [28]. Data from the EUSTAR (*The EULAR Scleroderma Trials and Research Group*) database showed that leading cause of SSc-related death is pulmonary fibrosis (35%) followed by PAH (26%), heart failure, and arrhythmias (26%) [29]. The most common surrogate markers of mortality in SSc are male gender, higher age at Raynaud phenomenon (RP) onset, higher modified Rodnan skin score (mRSS), dSSc subtype, proteinuria, decline in lung function and organ involvement including the presence of PAH, interstitial lung disease (ILD) and digital ulcers (DUs) [12,29–31].

1.3 Pathogenesis

Even though the pathogenesis of SSc still remains elusive, a growing body of evidence has contributed to shifting from the predominately fibrotic concept of disease to a complex syndrome including the interplay between endothelial injury, immune activation, inflammatory response, haemostatic disturbances and fibroblast activation. Today it is well known that all events in SSc pathogenesis result from the cell-cell, cell-cytokine, and cell-matrix interaction [2,32,33].

1.3.1 Vasculopathy

The vasculopathy is considered as a key and first pathological event in SSc mainly affecting microvasculature which adverserely deteriorates over time. Both activated and apoptotic endothelial cells (ECs) could be seen in dermal capillaries before capillary breakdown and skin fibrosis, supporting the hypothesis that vascular injury is the earliest event in SSc pathogenesis [34,35].

Initial triggers for vascular damage are still unknown. The current premise suggests that environmental triggers, such as microbial or occupational, free radicals or chemical agents could directly affect ECs or indirectly by stimulation of the immune system (Figure 1.). Lately it has been observed that a variety of factors and conditions may contribute to EC injury, such as EC cytotoxicity mediated by natural killer cells and antiendothelial cell antibodies, activation and apoptosis, sheer stress and ischemia/reperfusion injury. Independently of the primary trigger, ongoing ECs activation ultimately results in ECs damage and apoptosis [1,2,36].

The vasculopathy is characterized by both structural and functional changes, including EC activation with altered inflammatory features of the cells and EC apoptosis linked with capillary breakdown; smooth cells proliferation and finally vessel occlusion [1,37]. The chain of microvascular alterations could be visualized by nailfold videocapillaroscopy (NVC), from dilated capillaries, refraction of capillaries and microhemorrhages due to capillary damage in the early phase of disease, to severe loss of capillaries with avascular areas characterizing the late stage [38]. Structural abnormalities could be categorized as destructive or proliferative/obliterative. The progressive loss of capillaries followed by hypoxia and fibroblast activation are characteristics of destructive vasculopathy, while proliferation of vascular cells linked with the occlusion of microvasculature and fibroproliferative changes are features of proliferative vasculopathy, seen clinically as DUs, PAH and SRC [35,39]. The imbalance among factors mediating vascular tone is the central characteristic of impaired functional endothelium and is associated with vascular remodeling, onset of tissue hypoxia and repeated ischemia-reperfusion events further affecting progression of structural disorders and fibroblast activation. Namely, nitric oxide generated by ECs, the main vasodilator also involved in the inhibition of adhesion molecules expression, platelet adhesion and aggregation, vascular smooth cell proliferation and defense from oxidative injury, is deficient in SSc along with prostaglandins [37]. On the other hand, endothelin 1 (ET1), a potent endogenous vasoconstrictor, is found to be overexpressed in serum, lung, kidney and skin of SSc, mainly mediating action via upregulated ET1 A receptors leading to increased vascular tone, ECs activation, smooth cellular proliferation, fibroblast differentiation and collagen synthesis [34,40].

Under normal conditions, loss of ECs and ischemia induce both angiogenesis (development of new blood vessels from existing) and vasculogenesis (anew development of blood vessels from endothelial progenitor cells (EPCs)) as compensatory repair processes. In SSc these processes are defective, with rarely seen new capillaries and common presence of avascular areas [41]. Proangiogenic/angiostatic regulators are also increased in SSc, especially VEGF expression in different cell types and their circulating levels. There is no evidence of sufficiently effective capillarogenesis, suggesting that other mechanisms are implicated in defective angiogenesis [42]. In vitro study has demonstrated that fibrinolytic abnormalities like cleavage of urokinase-type plasminogen activator receptor (u-PAR) may also contribute to reduced angiogenesis in SSc [43]. Reduced numbers of bone marrow-derived EPCs have been observed to be associated with active DUs, loss of capillaries and PAH, suggesting their association with impaired vascular repair capacity [44–46].

Taken together, all observed abnormalities lead to intima and media fibroproliferation along with adventitia fibrosis provoking luminal narrowing with the formation of fibrin clots further contributing to tissue hypoxia/anoxia (Figure 1) underlying SSc vascular complications like PAH, CRS, DUs [47,48].

1.3.1.2 Endothelial activation and adhesion molecules

The early SSc stage is characterized by EC activation and injury, opening of the junctions between ECs, transendothelial leukocyte migration and perivascular cell infiltration with inflammatory phenotype, which consists mainly of neutrophils, monocytes, T and B cells, with a predominance of CD4+ T helper lymphocytes [49].

Dynamic interactions between leucocytes and ECs have a pivotal role in the EC permeability onset, allowing leucocytes to leak through the endothelium into the extracellular matrix (ECM), which is mediated by the adhesion molecules [2,33,50]. Three families of adhesion molecules are of specific significance for the leucocytes-ECs interaction, including selectins (P selectin and E selectin), integrins and immunoglobulin superfamily molecules (Intercellular Adhesion Molecule 1-ICAM1 and Vascular Cell Adhesion Molecule 1-VCAM1) [51]. In response to stimuli (tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1) and endotoxin) accompanying inflammation and vascular injury, expression of selectins increases intensely on activated ECs, promoting rolling of leucocytes along the vessel wall and their activation by chemokines. Consequently, β2 integrins are activated on the leucocytes' surface, binding to ICAM1 and VCAM1 and ensuring firm adhesion followed by integrin attachment to platelet-endothelial cell adhesion molecule-1, facilitating extravasation and tissue infiltration of leukocytes, ultimately leading to onset of a proinflammatory environment [52–54] (Figure 1.). Besides for regulating leukocyte migration and vascular permeability, increased levels of ICAM1, VCAM1 and E selectin further provoke ECs activation and ineffective angiogenesis, contributing to chronic and progressive vascular injury [55]. Under normal circumstances, P selectin is stored inside ECs and platelet granules, ICAM1 is expressed in low levels on ECs, while VCAM1 and E selectin do not have constitutive ECs expression at all, thus recent research considers those adhesion molecules as reliable biomarkers of activated ECs [33].

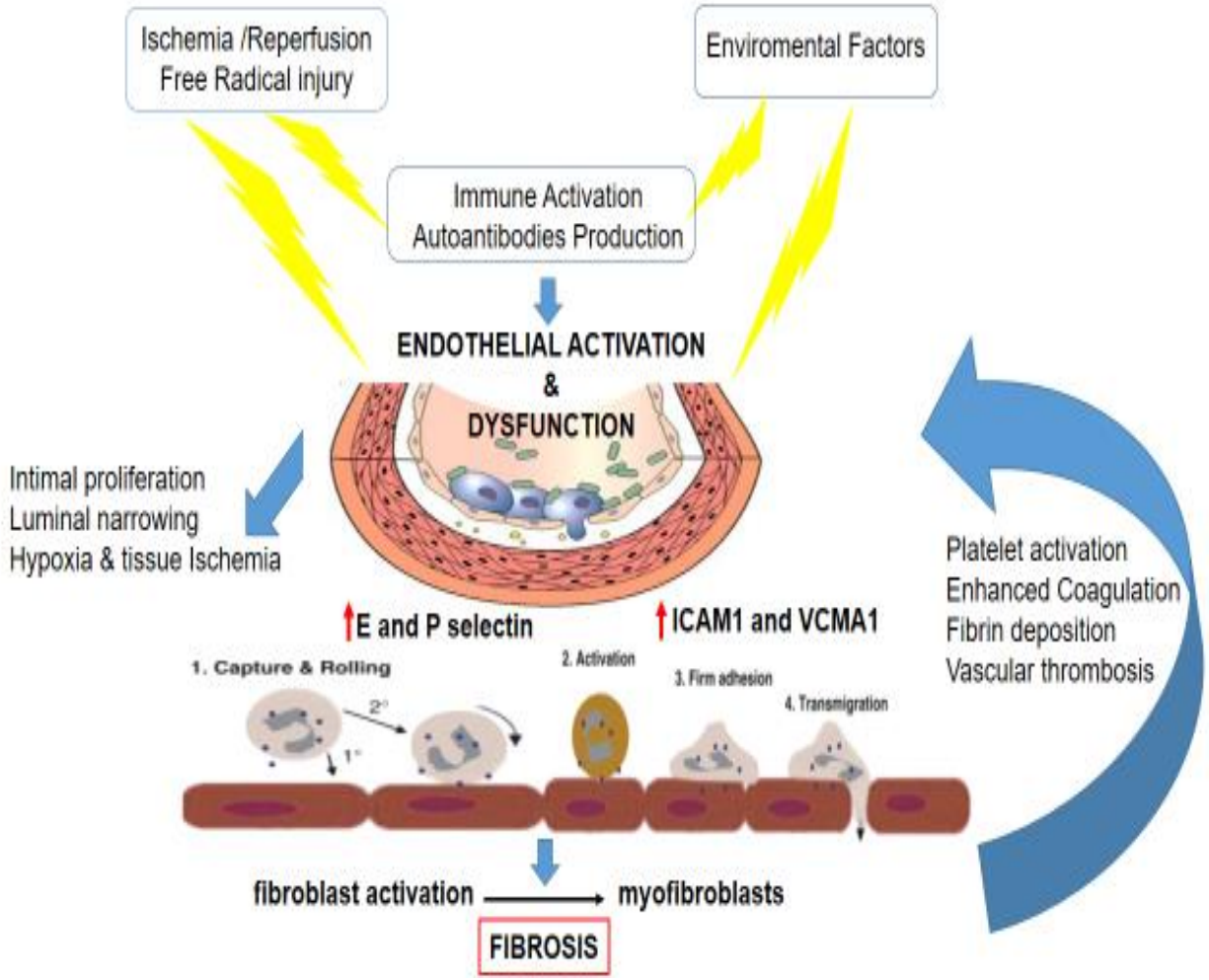


Figure 1. Pathogenesis of systemic sclerosis (Adopted and modified from Matucci-Cerinic et al., Arthritis Rheum. 2013 and Springer et al, 1990). Abbreviations: ICAM1 - Intercellular Adhesion Molecule 1**;** VCAM1 - Vascular Adhesion Molecule 1

Consistently, increased levels of soluble ICAM1, VCAM1, E and P selectins have been found in plasma of SSc patients, especially in those within early inflammatory stage with progressive subset of disease and associated with in situ expression of these molecules on ECs and fibroblasts along with disease activity. Altered ICAM1 and E selectin expression has also been found on the neutrophils, but only in early-stage SSc [56–59]. Further, it is believed that Th1 and Th17 cytokines are implicated in the inflammatory phase of the early disease stages, whereas Th2 cytokines are considered pro-fibrotic [60]. Accumulation of Th2/Th17 cells, macrophages and mast cells is regulated by ICAM1 expressed on ECs, while Th1 cell response is controlled by E and P selectin, giving insight that ECs expressing adhesion molecules not only mediate inflammation but promote profibrotic events as well [61].

1.3.2 Immune activation

Both innate and adaptive immunity play a role in the onset of autoimmunity and are involved in the fibroblast activation and accumulation of ECM. Genetic and functional studies have shown that major elements of the innate immune response, such as monocytes, macrophages and dendritic cells, have been activated in the dermis and lung of patient with early SSc phase, along with the residential cells (EC, fibroblasts), and have the ability to recognize Toll-like receptors and stimulate cytokine/chemokine

production and antigen presentation, ultimately leading to a polarized adaptive immune response. Numerous studies have addressed that polarized Th2/Th17 responses are key triggers of progressive fibrosis in SSc, predominately characterized by IL4, IL5, IL13 and IL17a production linked with alteration of profibrotic mediators, such as transforming growth factor beta (TGFβ).

B cells are also activated in SSc and are implicating in inflammation and development of fibrosis via production of antibodies, IL6 and TGFβ [40,62–65]. A hallmark of autoimmunity in SSc is present in the evidence that autoantibodies directed against nuclear components (ANA) are found in more than 95% of patients. Some of them, such as antitopoisomerase I (anti Topo I, formerly called Anti Scl 70) antibody, anticentromere (ACA) antibody and anti RNA polymerase III antibody are highly specific for SSc presenting important diagnostic markers that are included in novel classification criteria for SSc [9,66,67]. Additionally, recognition of SSc-specific ANAs is clinically important since they are associated with distinct disease subsets. Thus, ACAs are associated with lSSc, progressive vasculopathy and PAH, while patients with Anti Topo I are more likely to have dSSc, progressive vasculopathy and ILD. A new group of antibodies for functional molecules have also been found in SSc patients showing pathogenic role, such as anti-fibroblast antibodies promoting upregulation of the ICAM1 on cultured human fibroblasts and increasing secretion of different proinflamatory cytokines, inducing fibroblast activation, while anti ICAM1 antibodies might trigger oxidative stress and VCAM1 overexpression on the post capillary ECs, facilitating the infiltration of inflammatory cells and contributing to destructive vasculopathy development [34,39,66,68].

1.3.3 Hemostasis

The endothelium has a crucial role in retaining hemostasis regulating the complex interplay between the coagulation system and the surrounding environment, and is of great importance for wound healing. Upon vascular injury, as a part of the wound healing response and prevention of excessive bleeding, activated and injured ECs release von Willebrand factor (VWF), mediating the adhesion of circulating platelets to subendothelial collagen type I and III and subsequently platelet aggregation (Figure 2.). Furthermore, VWF stabilizes circulating factor VIII, giving insight into its essential role in hemostasis [69,70]. Increased levels of VWF have been found in SSc patients along with its supranormal multimers having prothrombotic properties [33,71–73]. Indeed, plasma levels of VWF and VWF propeptide have been found at higher levels in SSc patients having elevated D dimer and were closely related to thrombotic events [73]. Furthermore, in parallel to platelet aggregation via VWF, activated ECs and vascular smooth cells express tissue factor (TF) triggering thrombin formation through activation of a complex coagulation cascade, consequently inducing an amplification loop of platelet activation and thrombin generation leading to fibrinogen-fibrin conversion and formation of a stable fibrin clot (Figure 2.) [69]. Prevention of unnecessary clot formation underlying thrombotic events is another important side of hemostasis. An intact endothelium express range of anticoagulants is responsible for degradation of coagulation factors, such as TF pathway inhibitor (TFPI), antitrombin, trombomodulin, endothelial protein C receptor (EPCR), and tightly regulates clot formation via protease activated receptor (PAR) along with the release of pro-fibrinolytic factors, such as tissue plasminogen activator (t-PA) and urokinase type plasminogen activator (u-PA), which trigger fibrinolysis through the conversion of plasminogen to plasmin (Figure 2.). Degradation of platelets is mediated by ADAMTS13, which is responsible for cleaving the multimeric VWF, while fibrinolysis is regulated by many other factors including plasminogen activator inhibitors (PAI)[69]. In SSc, enhanced platelet activation with altered tendency to aggregation and activation of a coagulation cascade have been observed along with an increased number of extracellular microvesicles carrying TF [74–76]. Moreover, increased levels of fragments 1+2, thrombin-antithrombin complex, altered thrombin generation, and decreased levels of ADAMTS13 have been found, indicating that SSc is a disease with enhanced coagulation [71,77,78].

However, controversial issues concern the fibrinolytic activity in SSc since depressed fibrinolytic activity, normal and even increased plasma fibrinolytic profiles have been reported [71,77,79–81].

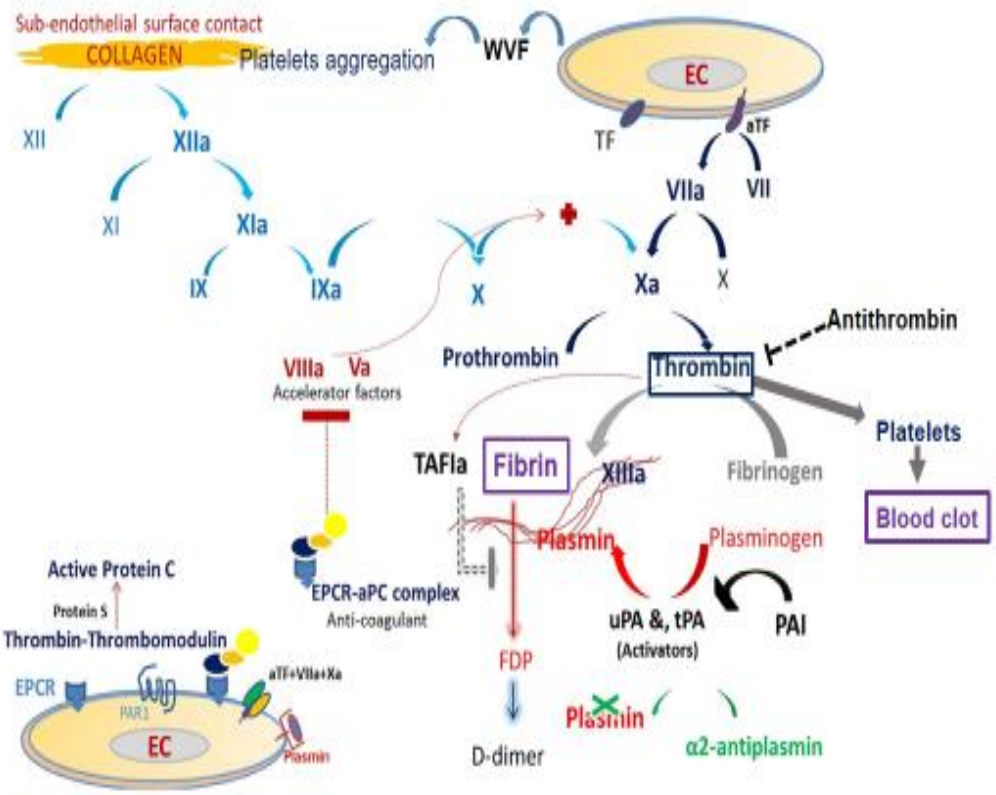


Figure 2. Coagulation cascade and fibrinolysis upon endothelial injury (Adopted and modified by Giris et al., Front. Physiol 2020). Abbreviations: a - activated; EC - endothelial cell; EPCR - endothelial protein C receptor; PAI - plasminogen activator inhibitor; PAR - plasminogen activator receptor; tPA - tissue plasminogen activator; uPA urokinase-type plasminogen activator; TF- tissue factor; TAFI - thrombin activatable fibrinolysis inhibitor; WVF - von Willebrand factor

1.3.4 Fibrosis

Vascular, immune events and haemostatic disturbances further provoke vascular injury and persistent fibroblast activation, leading to progressive loss of ECs and fibrosis. Thus, in later sclerotic disease stages, inflammatory changes fade, while increased accumulation of ECM, obliteration and loss of the microvasculature, and atrophy of skin appendages occur [40].

Fibrosis is the final step of SSc pathogenesis affecting the skin and almost all internal organs including the lungs, the gastrointestinal tract, heart, tendons, and ligaments. It is the result of increased production and impaired degradation of ECM, where the chronic activation of fibroblasts with myofibroblast phenotype is considered as a pivotal event [3,82]. Myofibroblasts may arise from different sources apart from fibroblasts, including perocytes, adipocytes, macrophages, epithelial cells and ECs, via processes named epithelial/endothelial mesenchymal transitions [83–85]. Multiple feed-forward amplification mechanisms may be implicated in myofibroblast activation, such as paracrine signals derived from

leucocytes, ECs and platelets, autocrine factors produced by myofibroblasts, and damage-associated molecular patterns, as a consequence of tissue injury, hypoxia and oxidative stress [40,86]. Profibrotic mediators closely related to SSc pathogenesis include connective tissue growth factor, platelet-derived growth factor, Wnt proteins, IL-4, IL-6, IL-13, monocyte chemoattractant protein-1, ET-1, serotonin and TGFβ, which is considered a master regulator of tissue repair and fibrosis influencing both sides of the fibrotic process, and stimulates the production of ECM and suppresses its degradation [3,40,62]. Many studies have addressed that abnormalities in the fibrinolytic system may be involved in the pathogenesis of fibrosis in SSc, demonstrating that the chronic presence of fibrin depositions may stimulate dermal fibrosis, progressive pulmonary fibrosis and myocardial fibrosis, while, on the other hand, the absence of ICAM1 may suppress development of fibrosis in animal models, emphasizing close association between endothelium injury and fibrosis [61,87,88].

1.4 Classification criteria

In 1980, the American Rheumatism Association (today called *American College of Rheumatology*; ACR) proposed preliminary classification criteria for SSc with sensitivity of 75% and specificity of 72%, in order to obtain a stable and comparable patient cohort with established disease for clinical trials. Based on these criteria, patients with the single major criterion (proximal scleroderma) or ≥ 2 minor criteria (sclerodactyly/pitting scars-fingertips/pulmonary fibrosis) were considered as having SSc [89].The lack of these criteria meant that patients were not recognized in the early stage of disease, which is of paramount importance since detecting disease in the early stages may potentialy prevent irreversible organ damage. Thus, in 2013 a combined ACR/EULAR (*European Alliance of Associations for Rheumatology*) task force developed new classification criteria for SSc with sensitivity 91% and specificity 92%, also taking into account three hallmarks of the disease: presence of autoantibodies, NVC abnormalities, and skin/internal organ fibrosis, which also enables early-phase detection of the disease. These criteria include one sufficient criterion: skin thickening of the fingers extending proximal to the metacarpophalangeal joints, but if that is not fulfilled then the point system is used where subjects with ≥9 points are classified as having SSc [9].

Table 1. The ACR-EULAR criteria for the classification of systemic sclerosis

Items	Sub-items	Weight/score
Skin thickening of the fingers of both hands extending proximal to the metacarpophalangeal joints *(sufficient criterion)*		**9**
Skin thickening of the fingers* (only count the highest score)	Puffy fingers	**2**
	Sclerodactyly of the fingers (distal to the MCP but proximal to the PIPs)	**4**
Fingertip lesions* (only count the highest score)	Digital tip ulcers	**2**
	Fingertip pitting scars	**3**
Telangiectasia		**2**
Abnormal nailfold capillaries		**2**
Pulmonary arterial hypertension and/or interstitial lung disease†	Pulmonary arterial hypertension	**2**
	Interstitial lung disease	
Raynaud's phenomenon		**3**
Scleroderma-related antibodies‡ (any of anti-centromere, anti-DNA-	Anti-centromere	**3**
	Anti-DNA-topoisomerase I	

topoisomerase I (anti-Scl-70), anti-RNA polymerase III	Anti-RNA polymerase III	

*Add up the maximum weight (score) in each category to calculate the total score.
†Maximum score is 2. ‡Maximum score is 3.
ACR/EULAR (American College of Rheumatology/European Alliance of Associations for Rheumatology); MCP - metacarpophalangeal joint; PIP -proximal interphalangeal joint; SSc - systemic sclerosis.

1.5 Clinical presentations of SSc

Today, SSc is considered a syndrome-like disease with a variety of presentations. Skin is almost always an affected organ, and cutaneous sclerosis is considered the key sign of SSc. The degree of skin thickening is the main classification criterion for SSc diagnosis as well as for subset classification. LeRoy and Madsger defined two subsets of the disease: lSSc, where sclerosis is restricted to the skin distal to the elbows and knees, with or without affecting the face, and dSSc, characterized by diffuse skin thickening proximal to the elbows and knees involving other areas in addition to those affected by lSSc. The two subtypes differ in the course, severity and burden of disease with worse prognosis in dSSc [9,90,91]. The reliable and validated tool for assessing the extent of skin sclerosis is the mRSS, which precisely reflects skin biopsy thickness and its higher values are associated with increased mortality, SRC, and other severe organ damage [92-95]. Skin immersion of the hand can range from nearly normal to edematous "puffy" and finally sclerodactyly, as the most severe finding throughout the course of disease, which reflects enhanced fibrosis. The most common musculoskeletal (MS) complications are joint contractures, affecting almost 50% of dSSc patients, which are, along with other complications, such as tendon friction rubs, muscle weakness, and muscle atrophy, more prevalent in dSSc and associated with poor prognosis. The earliest symptoms of disease may be arthralgia and myalgia, indicating that MS affection may be present at diagnosis onset [91,96,97]. Subcutaneous calcinosis is also one of the well-recognized skin manifestations of SSc, and is more often seen in patients with lSSc and associated with longer disease duration and peripheral vascular manifestations like DUs and acroosteolysis [98].

On the other hand, among extracutaneous organ involvement, the gastrointestinal tract (GIT) is the most frequently (90%) affected with predominance of the esophagus, and patients usually complain of reflux, bloating, heartburn, dysphagia, diarrhea, constipation, and fecal incontinency. The severity of GIT disease may also be a marker of unfavorable prognosis [99]. Pulmonary involvement has been seen in more than 70% of SSc patients; presenting as the most common cause of SSc-related death, and its two major manifestations are interstitial lung disease (ILD) and pulmonary vascular disease leading to PAH, respectively related to vasculopathy and interstitial fibrosis [100,101]. Diagnosis of ILD presents a challenge, since the greatest number of patients are asymptomatic in the early stage and by the time fatigue and dyspnea upon exertion with a dry cough are reported, ILD is usually in the more advanced stage.. The most frequent evaluation methods for suspected ILD are pulmonary functional tests (PFTs), high-resolution computed tomography (HRCT), and X-ray. Predicted values >80% for both PFTs parameters, forced vital capacity (FVC %) and diffusing capacity of the lungs for carbon monoxide (DLCO %) are considered "normal" and their decline at the time of initial diagnosis is indicative of ILD. Definitive diagnosis is based on the HRCT results and the most common pathological pattern, fibrotic nonspecific interstitial pneumonia, reflects the finding of ground-glass opacities with peripheral distribution, reticulation and traction bronchiectasis on HRCT [100,102]. The extent of ILD seen on initial HRCT is negatively correlated with both FVC and DLCO, and is a powerful predictor of survival [103-105]. ILD progression has been considered a continual change in PFTs, with FVC decline from a

baseline of ≥10%, or 5-9% along with a DLCO decline of ≥15% and is associated with presence of anti-topo I antibodies [102]. Of note, other processes could be associated with decreased DLCO% including cigarette smoking, chronic obstructive lung disease (COPD), thromboembolic disease and PAH. It has been proposed that the FVC/DLCO ratio > 1.6 is suggestive of the presence of pulmonary vascular disease, addressing more pronounced reduction of gas transfer when comparing to lung volume due to alveolar-capillary remodeling and progressive loss of pulmonary arterial vasculature [106].

PAH is observed in almost 30% of patients with severe ILD; may lead to cor pulmonale and the development of right-sided heart failure. It is more common in lSSc and is associated with higher mortality [107,108]. Based on the *European Society of Cardiology/ European Respiratory Society* guidelines from 2015 for the PAH diagnosis, the Doppler echocardiography with estimation of systolic pulmonary artery pressure (sPAP) should be carried out whenever PAH is suspected, and may be done annually among patients with DLCO ≥80% following *the 6th World Symposium on Pulmonary Hypertension* diagnostic algorithm [109,110]. Moreover, using the two-step DETECT evidence-based algorithm for PAH detection, which is restricted to patients with DLCO <60%, the number of unrecognized PAH cases has decreased to 4% in comparison to 29% if only echocardiography is applied [111]. Still, the gold standard for PAH diagnosis presents right heart catheterization with the finding of a resting mean pulmonary artery ≥ 25 mmHg [112]. Apart from cardiac disease due to pulmonary involvement, pericardial disease, myocardial fibrosis, myocarditis associated with myositis, conduction disturbances, coronary artery disease, and valvular disease could also be seen and are associated with poor prognosis [113].

1.5.1 Peripheral vascular manifestations

1.5.1.2 Raynaud's phenomenon

Vasculopathy of digital blood vessels occurs in almost all SSc patients, ranging in severity from RP to irreversible tissue damage underlying DUs [114,115]. RP represents the earliest clinical presentation of SSc, which is observed in more than 95% of patients, either many years before lSSc or closer to dSSc onset. Along with puffy fingers and positive ANA, RP belongs to "red flag" symptoms indicative of very early diagnosis of systemic sclerosis (VEDOSS) with regard to the proposed diagnostic EUSTAR criteria for SSc [90,116,117]. Typically, the principal symptom of RP is sharply demarcated skin color changes of the digits – from white due to intensive vasoconstriction of the digital microvasculature with reduced blood flow to blue/cyanotic color caused by flow stasis and hemoglobin reduction, and finally red as a result of reactive hyperemia, which may cause severe pain and paresthesia. Apart from the digits, RP may be seen whenever the thermoregulatory skin vessels are distributed including the ears, the tip of nose, tongue, limbs and even the heart when exposed to cold and emotional stress [114,115,118]. Although the RP might be expected to occur more frequently during cold weather conditions, in SSc only 17% of patients experience no RP episodes at all, while the severity of symptoms is reduced by about 50% over the summer, suggesting that its presentation throughout all seasonal variations is underlined by complex vascular injury [119]. Clinically, this is of great importance for distinguishing primary or idiopathic RP, which is characterized by isolated vasospasm followed by reversible ischemia without vasculopathy, more commonly symmetrical without signs of critical ischemia and evidence of any associated disorder, and occurs before age 20 mainly among females, from secondary RP, in which the most important role is the determination of ANA antibodies and NVC alterations [120–122]. Patients with primary RP and capillaroscopic non-specific changes should be monitored, at least once a year, since 10% of them may transition to secondary RP within 4 years [123]. It has been observed that the severity of RP in SSc assessed by patient report outcomes (PROs) is related to the presence of active

DUs, suggesting that advanced ischemia/reperfusion injury is closely related to digital vasculopathy development [124].

1.5.1.3 Digital ulcers

Digital ulcers are one of the main symptom in SSc, and may be experienced by almost half of patients over the course of disease with a point prevalence of 5-10% [125,126]. Data from the prospective EUSTAR study demonstrated that the probability of DU onset was 70% within 10 years, while Hachulla and coworkers, in a single-center retrospective longitudinal study, found that around 75% of patients developed their first DU within 5 years of symptoms other than RP [127,128]. DUs are considered one of the most common visible manifestations of advanced vasculopathy and can often be observed in VEDOSS patients and may also show a relationship with early organ involvement [129]. This is in addition to the fact that their SSc onset is of great importance, since they are included in the new ACR/EULAR classification criteria and the revised EUSTAR disease activity index for SSc [9,130]. So far, three types of digital lesions have been recognized with regard to pathogenesis and localization: fingertips, extensor ulcers, and those developed from calcinosis. Generally, fingertips that are called "pure" DUs are localized at the tips of distal digits (Figure 3.), and are considered ischemic not only due to present digital microvasculopathy but also to the persistent RP and platelet activation with clot formation and tendency to thrombosis development; whereas extensor ulcers located over the extensor surface, particularly over small joints of the hands, are believed to be mechanical as a consequence of intermittent trauma and enhanced skin tension [128]. SSc-related vasculopathy may also affect different macrovascular arteries, such as the ulnar artery, the occlusion of which has been observed in more than 25% of patients, underlying fingertips onset [131,132]. Fingertips are found to be more common than extensor ulcers in some reports, however data from a prospective study showed equal distribution [126,133].

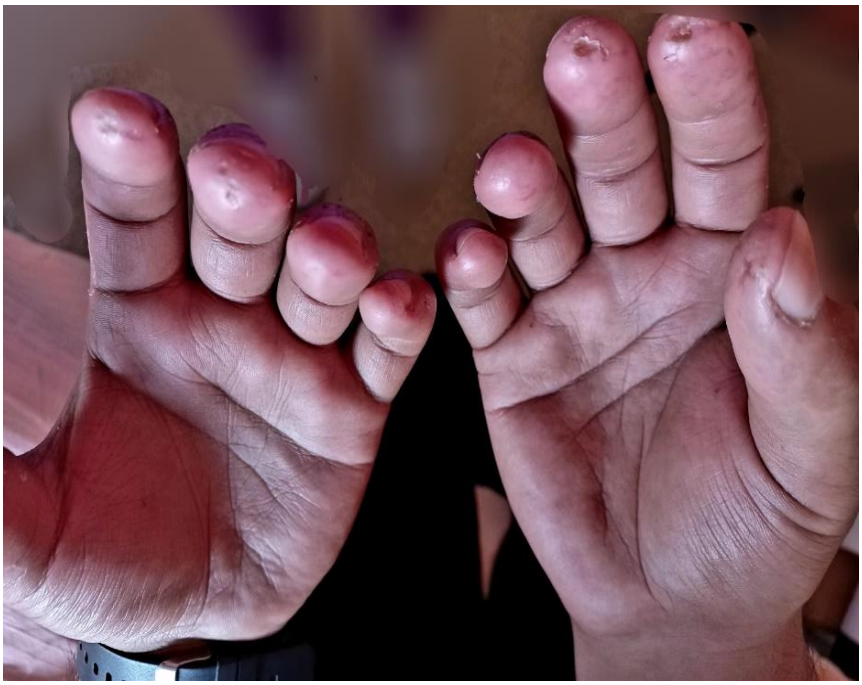


Figure 3. Digital ulcers on the index and middle fingertips of the right hand with multiple digital pitting scars located on the index, middle finger of the left hand and thumb of right hand (*With the permission and courtesy of the patient*)

Although DUs might affect every finger and toe, they are most frequently seen on the thumb, middle and index digits. Furthermore, they are rarely observed at the nail base and on the lateral side of the digits, presenting a challenge in distinguishing them from fissures and paronychia [134]. A widely accepted, consensus-based definition for ulcers proposed by the Scleroderma World Foundation, considers DUs a "loss of epidermal covering with a break in the basement membrane" [135].
DUs are extremely painful and very slow to heal, especially if complications like infection, gangrene or osteomyelitis are present [126,134,136-138]. It has been observed that the mean healing time was 76.2, 25.6 and 281.1 days for fingertips, DUs occurring on pitting scars, and DUs with gangrene, respectively, and almost 5% of patients underwent surgical digital amputation as a consequence of necrotic process [134,139]. Moreover, DUs are associated with severe disease course since they may be predictive of death, a cardiovascular event, or other internal organ complications [31,129]. Nationwide studies revealed that signs of poorer SSc prognosis, such as male gender, younger age at disease onset, dSSc, presence of anti-topo I antibodies, telangiectasia, acroosteolysis, ILD, decreased DLCO and GIT involvement could be considered risk factors for DUs [125,140,141].
Regardless of the amended treatment options for prevention and healing in recent decades, approximately one to two thirds of patients with DUs may still experience progressive vasculopathy with recurrent ulcerations, related to the highest disease burden with increased rate of complications, greater need for hospitalization, and more functional damage [142–144]. Thus, for daily clinical practice, it is still a great challenge to identify the patients at risk of advanced digital vasculopathy and prevent its progression. Cutolo et al. found that the strongest risk factors for future DU onset were capillary density, number of DUs, and history of DUs [145]. Recently, Fridreish et al. proposed the (CIP-DUS) composite score for DUs prediction, combining clinical data (dSSc, PAH, presence of DUs or pitting scars), patient history (history of DUs or pitting scars), and imaging (NVC pattern, fluorescence optical imaging, color Doppler ultrasound) with 100% of sensitivity (Sn) and 74% of specificity (Sp) estimated at ≥ 10 points. As could be seen, biomarkers have not been included in any of the composite scores mentioned above. So far, studies have revealed several haemostatic alterations related to DUs, and some markers linked with thrombotic and inflammation signaling were found predictive for recurrent DUs. Thus, procoagulant activity could be expected to underlay progressive digital vasculopathy, and potential analysis of the global hemostasis profile with a special focus on the fibrin clot structure may represent a more accurate predictive marker for advanced digital vasculopathy that could help define at-risk patients in the future [141,146–150].

1.5.1.4 Digital pitting scars and acroosteolysis

Digital pitting scars (DPS) belong to the spectrum of digital vasculopathy, defined as concave depressions with hyperkeratosis often located on the fingertips (Figure 3) and could be found in 30-50% of SSc patients [134,151]. Multiple DPS reflect long standing severe digital ischemia. Data from a EUSTAR study including 9671 SSc patients have demonstrated that DPS were associated with signs of progressive SSc and may be indicative of active DU ischemia (DUs and gangrene) and possible death [152].

Beyond DUs and DPS, acroostelysis is another manifestation of SSc that may be a consequence of severe vascular ischemia, seen as bone resorption of the distal phalanx, or part thereof, and associated with late NVC pattern, a history of DUs, and gangrene [153–155]

1.6 Management of digital vasculopathy

The management of patients with digital vasculopathy is multifactorial where both conditions – RP and DUs – should be taken into account when deciding on treatment as they represent two sides of the same coin. Generally, besides educating patients in aggravated factors for digital angiopathy, such as cold, stress, and smoking, and recognizing new or worsening ulcers with signs of infection, the treatment approach should combine non-pharmacological, pharmacological, and surgical interventions [114,115]. One of the non-pharmacological interventions that has shown positive effects on healing DUs is hyperbaric chamber treatment, which is reserved for those with chronic DUs and RP refractory to conventional treatment, while occupational therapy could improve functional disability caused by DUs [156–158].

There is a wide range of pharmacological treatments for prevention and treatment of RP and DUs, including vasoactive drugs, prostanoids, endothelin receptor antagonists (ERA), statins, antiplatelet and anticoagulant therapies. Hashulla et al. addressed vasoactive therapy as a central pharmacological option delaying DU onset [128]. Updated EULAR recommendations for the management of SSc proposed vasoactive *dihydropyridine-type* calcium channel blockers (CCB) as a first-line therapy to reduce the frequency and severity of RP events followed by *phosphodiesterase type 5* (PDE5) *inhibitors*, especially in patients with severe RP who do not respond well to CCB. PDE5 inhibitors are also recommended as a therapy for DUs with their main effect on healing, while one clinical trial also reported their preventive effect on new DU onset [142,159]. *Prostanoids*, particularly Iloprost, are considered the most potent vasodilators with anti-proliferative and anti-platelet aggregation effects, and are recommended for the treatment of RP and in healing DUs, while ERA, particularly Bosentan, inhibiting enhanced vasoconstriction and fibroproliferative vasculopathy onset, showed benefit in the treatment of recurrent DUs [142]. A new generation medicines, such as PDE5, prostanoids and ERA, may be considered to potentialy modify vasculopathy progression.

In clinical daily practice, antiplatelet therapy has been widely prescribed to patients with a history of DUs, since it is known that micro clots may be involved in vasculopathy pathogenesis. However there is a lack of strong evidence so far from clinical trials to support this intervention for digital vasculopathy [160].

1.7 Quality of life

Since SSc is a chronic disease with a variety of manifestations that deteriorate over time, and without the currently curative treatment inhibiting the progression of the disease, the patients' quality of life could be affected in many ways. SSc patients may face a range of problems including limitations in the performance of daily activities, mainly due to hand impairments, sleep disorders, depression, anxiety concerning disease progression, decreased self-estimation, sexual dysfunction, fatigue, pain and psychological stress due to aesthetic impairment mostly caused by DUs, RP, facial telangiectasias, hand contractures, and a beak-shaped nose. They perceive these problems as more distressing than internal organ involvement, so a holistic approach including PROs along with physician perspective should be applied in daily clinical practice when making a decision on the treatment [161-163].

Several studies have reported poorer health-related quality of life (HRQoL) in SSc patients compared to the general population, other common chronic diseases, such as hypertension, diabetes mellitus or even other autoimmune diseases including rheumatoid arthritis (RA) and systemic lupus erythematous (SLE) [164-166]. Interestingly, pain is reportedly more severe in SSc patients than in those with RA. Clinical features such as RP and DUs strongly contribute to pain in SSc, where a degree of DU burden is positively associated with analgesic necessity [166-168]. Apart from pain, fatigue and impaired hand function due to skin thickening, RP and DUs greatly impact daily life, work ability and employment status, influencing

not only the economic status but also the social relationships of those patients with greater need for support from others [169,170]. It has been observed that functional impairment was positively related to the number of DUs, while both DUs and RP had a great impact on quality of life over time, addressing their relation to a large burden on global disability. Furthermore, hand disability has been considered more important by patients than the management of other internal organs [168,171]. Although it is well known that progressive digital vasculopathy, including recurrent and chronic DUs, is linked with greater disease burden, so far there is no evidence that any PROs could be predictive of SSc DU [144].

Extensively used and validated PROs for the evaluation of HRQoL in SSc are *Short Form Health Survey* (SF36); *Health Assessment Questionnaire Disability Index* (HAQ DI) and *EuroQol 5-dimensions* (EQ5D), while *Scleroderma Health Assessment Questionnaire Disability Index* (SHAQ-DI) is specific for SSc [161,162].

2 THE OBJECTIVES

The pathogenesis of underlying progressive digital vasculopathy in patients with SS is still unknown. Moreover, there is an unmet clinical need for the predictors of recurrent DUs in individual SSc patients since these patients have high disease activity and impaired quality of life. Thus, the aim of our investigation was:

1. To assess biomarkers of endothelial injury (ICAM-1, VCAM-1, P selectin, E selectin and VWF), parameters of global haemostatic assays (endogenous thrombin generation, overall haemostatic potential, overall coagulation potential, overall fibrinolysis potential) and fibrin clot properties in SSc patients in comparison to healthy controls;

2. To assess if altered fibrin clot properties could be predictive for the onset of new DU episode in SSc patients with a history of DUs during 1.5 years of follow up;

3. To assess predictive value of the quality of life domains in the onset of new DU episode.

3 MATERIAL AND METHODS

3.1 Study design

Prospective cohort study. The research was designed as the combination of cohort, transversal and case-control study.

3.2 Place and period

Study was performed at the Institute of Rheumatology (IR), Belgrade, Serbia in collaboration with Karolinska Institutet (KI), Stockholm, Sweden, in the period 2017.-2020.

3.3 Study population

From 230 consecutive patients, threated and followed through either the outpatient clinic or ward at the IR, Belgrade, Serbia, 170 cases haven't met some of eligibility criteria and in 2 blood samples couldn't be taken, thus fifty-eight patients were enrolled in the study (Figure 4).

Inclusion criteria for initial study were:

- confirmed diagnosis of SSc according to 2013 ACR/EULAR classification criteria [9] ;
- treatment naïve for ERA, PDE5 or prostanoids;
- age ≥18 year old;
- signed written informed consent.

Inclusion criteria for prospective cohort study:

- History of ischemic DU

Exclusion criteria from initial study:

- overlap with other autoimmune diseases including systemic connective tissue disease, Hashimoto thyroiditis, autoimmune hepatitis, primary biliary cholangitis;
- inflammatory bowel diseases;
- liver and renal insufficiency;
- hematological and endocrinology diseases including all types of anaemia, haemostatic disorders, diabetes mellitus;
- previous cerebrovascular and cardiovascular events;
- heart failure (NYHA 2-4);
- asthma or obstructive pulmonary disease;
- pregnancy;
- acute infections and neoplastic disease.

The control group comprised 46 age- and sex matched individuals to SSc cases without self-reported comorbidities and any drug consumption (Figure 4).

Inclusion criteria for the controls:

- age ≥18 year old;
- Signed written informed consent.

Of note, controls who had been ever measured arterial blood pressure more than 140/90mmHg but without any specific therapy were enrolled and considered as having arterial hypertension.

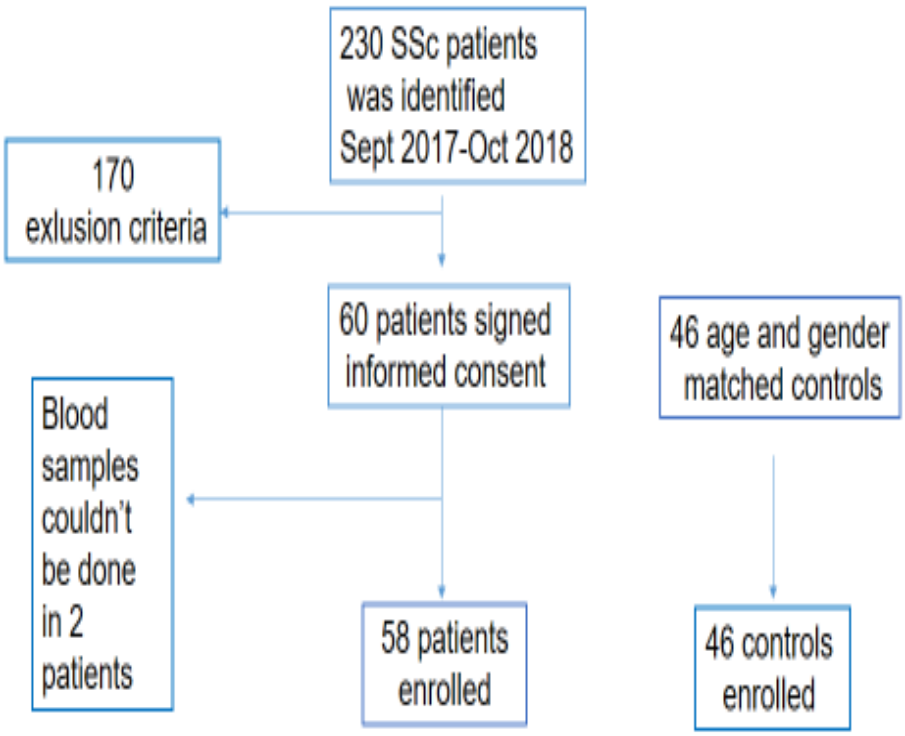


Figure 4. Chart of study participants

3.4 Evaluation instruments

3.4.1 Diagnostic tools

We have used the most recent diagnostic tools (not older than 6 months prior inclusion) for diagnosis of SSc. PFTs were assessed at department for functional diagnostics at the Clinical Center of Serbia, Belgrade. Two dimensional Doppler echocardiography; NVC and lung and hand X-ray were done at IR.

3.4.2 Physical examination

A physical examination was performed to all individuals at inclusion including the measurement of weight and height. Patients were further assessed for skin thickness by the modified Rodnan skin score (mRSS) [172], the presence of sclerodactily, calcinosis, flexion contractures, swollen and tender joints, tendon friction rubs, proximal muscle weakens, telangiectasia's, pitting scars, RP, active DUs and complications of DUs.

An ischemic active DU were considered as a denuded zone located at distal digits with loss of both epidermis and dermis [173].

3.4.3 Score

mRSS is a feasible and sensitive measure of skin thickness [92,172]. During examination patients were in relaxed position avoiding false overestimation the skin score. The total score was obtained by summing the values of 17 examined anatomic region. The thickness range from 0-3, where 0 represent normal skin; 1- mild (easily making skin folds between fingers); 2-moderate (difficulty in making skin folds and without wrinkles) and 3-severe (inability to make skin folds between fingers) thickness.

All data obtained from medical records and physical examination at the initial visit, were entered into the general questioner and questioner related to Systemic sclerosis.

3.4.4 Questioners

General questioner consisted of 4 parts. The first part contained data regarding basic demographic characteristics: age, gender, educational level, employment and reason for retirement (due to SSc or not). The second part was related to the lifestyle (smoking habit) and comorbidities, while third part included data about ongoing therapy; Prednisolone daily doses; previous Cyclophosphamide (CYP) therapy and cumulative dose of CYP.
Subjects were asked are their retirement was connected with disease disability or not.
Smokers were considered those who reported daily smoking habit over a minimum of 60-daysbefore baseline visit. Data regarding the number of cigarettes using per day and duration of smoking were collected. Individuals with BMI above 25kg/m2 were defined as obese.

SSc related questioner consisted of data regarding general clinical characteristics: disease duration-defined as the onset of the first non RP manifestations; age at disease onset; subtype of disease: diffuse or limited cutaneous group [90] presence of SSc specific antibodies and skin involvement assessed by mRSS, followed by data focused on organ involvement: skin manifestations (calcinosis, sclerodactily); musculoskeletal involvement (myalgia, proximal muscle weakness, flexion contractures, arthralgia, arthritis, tendinopathy-tendon friction rubs; cardiopulmonal involvement assessed by: FVC, DLCO, radiological positive chest X-ray findings or ground-glass opacification on HRCT indicative for the ILD; sPAP and EF. Further on, questions according peripheral vascular manifestations present on physical examination were collected: RP, active digital ulcers, pitting scars, teleangiectasias or radiological signs of acroosteolysis along with the information addressing ever presence the renal crisis.
NVC characteristics were collected as early, active and late patter according to Maurizio Cutolo [174] and presence of late pattern signs (loss of capillaries and ramified/bushy capillaries). Further focus was on specific DUs characteristics: onset of first DUs, number of previous episodes; number of active digital ulcers at baseline and their location, presents of complications such as chronic non healing DUs, inflamed

active DU, and osteomyelitis. Finally, data regarding laboratory findings that were sampled at baseline visit were part of these questioner.
SRC was defined as a sudden increase in blood pressure and acute renal failure.

SHAQ-DI comprises twenty items divided into eight domains (dressing, arising, eating, walking, hygiene, reaching, gripping, and activity) and five self-reported VAS scores for pain, RP, DU, lung and gastrointestinal involvement. Each answer has a range of 0 (without disability) to 3 (unable to do).every domain is ranked with the highest scored number. Summation of all ranks divided with 8 represents overall SHAQ DI [10].

EQ5D contains five domains (mobility, self-care, usual activities, pain and discomfort, anxiety and depression) with three levels of severity for each of them: one (without problems), two (some problems) and three (extreme problems), and EQ VAS ranging from 0- 100 millimeters. The VAS highest value mirrors the best state of health[175].Overall utility score was obtained using nation-specific algorithm choosing Europe as variable. Calculator could be found at EQ_5D_index_calculator.xls (live.com). Score of 1 reflects perfect health.

3.4.5 DU diary

DU diary served as a tool for assessing new DU onset, consisting information of date when new DU was occurred over follow up.

3.5 Ethics

The study was permitted by the ethics committee of the IR (No 29/1-110, date 29/11/2017) and was performed in accordance with the principles of the declaration of Helsinki from 2013. Written informed consent was obtained from all recruited participants prior to enrollment.

3.6 Materials

3.6.1 Blood sampling and transportation

Blood sampling was performed at IR, Belgrade, Serbia at enrolment.

Peripheral venous blood was collected into tubes containing clot activator or trisodium citrate after fasting for at least 10 hours and a rest period of at least 20 minutes in calm environment. Vacutainer (BD Blood Collection System) and needle 21G were used. Serum and platelet poor plasma (PPP), were obtained inside one hour of sampling by centrifugation at 2,000 g for 20 minutes (min) at room temperature and then aliquoted into 500ml tubes and frozen at −70°C.

Frozen samples were transported under certified conditions from the IR to the KI for further analysis. Samples were there also kept frozen at −70°C. Of note, all analysis were done within one year of sampling.

3.7 Methods

3.7.1 Routine laboratory analysis and immunology

SSc specific antibodies, antinuclear (ANA); antitopoisomerase I antibody (Anti Topo I); anticentromere (ACA) and, along with complements level (C3, C4), were determined at baseline visit using standard protocols at the IR for all subjects.

Laboratory analyses containing fibrinogen, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), lipid status (total cholesterol and triglycerides) and markers of renal function (urea and creatinine), were determined at baseline visit by standard protocols at the IR for all subjects.

3.7.2 Global haemostatic assays

3.7.2.1 Calibrated automated thrombogram (CAT) assay

The speed and quantity of thrombin generation (TG) in plasma were assessed by CAT assay [176]. Namely, after PPP had been thawed for 4 min at 37°C, a 20µl of TF reagent and 20µl thrombin calibrator were pipetted into each of the 96 wells of round bottom plate, respectively. Coagulation was triggered by calcium (Fluka Solution) and a fluorogenic substrate (2.5 mmol/l, Z-Gly-Gly-Arg-AMC), after 80µl of PPP had been added. The fluorescence was continuously measured every 30 seconds (sec) untill 60 min by a Fluoroskan Ascent fluorometer (Thermo Fisher, Thermolabsystems, Finland). The following parameters were settled using Thrombinoscope software (Thrombinoscope BV, Maastricht, Netherlands):

1. **Lag time**- the time from when the s measurement starts till the detection of TG;
2. **Peak thrombin generation (PT)-**maximal value of TG
3. **ETP-** endogenous thrombin potential, the area under the concentration-time curve;
4. **Time to peak**- the time from when TG starts until the PT is reached.

3.7.2.2 Overall haemostatic potential (OHP) assay

OHP is assessing the overall fibrin formation and fibrinolysis in plasma [177]. Determination of OHP was based on the construction of fibrin aggregation curves using citrated PPP. Coagulation was triggered by adding small amount of thrombin, phospholipids and calcium to the PPP, and (tPA) was added if fibrinolysis was also investigated . When the fibrin aggregation curve occurs, fibrinogen is progressively converted to thrombin-generated by fibrin. At the same time, activation of plasminogen leading to production of plasmin that degrades fibrin. Accordingly, each observed absorbance (Abs) value represents fibrin level at the appropriate time point and the area under the curve (AUC) represents balance between fibrin formation/degradation during the measurement period.

Briefly, assay was performed on microtiter plate (Immulon-IB;Dynex Technologies, Chantilly, Virginia, USA). Brifly, after PPP had been shortly thawed at 37°C, 20µL of phospholipids reagents (0.11 nmol/l; Phospholipid-TGT, Rossix, Sweden) followed by either 140µL PPP or pooled normal plasma were added. Further on, two final puffers were put on: mixture of either thrombin (0.04 U/ml; Sigma-Aldrich, USA) or tPA (300 ng/ml, Boehringer Ingelheim, Germany) with tris HCL and CaCl2 (34mmol/L). The Abs at 405 nm was monitored every 12 sec for 60 min. The AUC was calculated as the sum of the Abs values using spectrophotometry (Multiskan™ FC Microplate Photometer, Thermo Scientific).

The following parameters were calculated (Figure 5):

1. **OHP**- as Abs values summation under the curve.
2. **OCP**- the overall coagulation potential, the summation of Abs values under the fibrin aggregation curve without adding tPA
3. **OFP**- the overall fibrinolysis potential, with the equation OFP = ([OCP–OHP]/ OCP) × 100%.

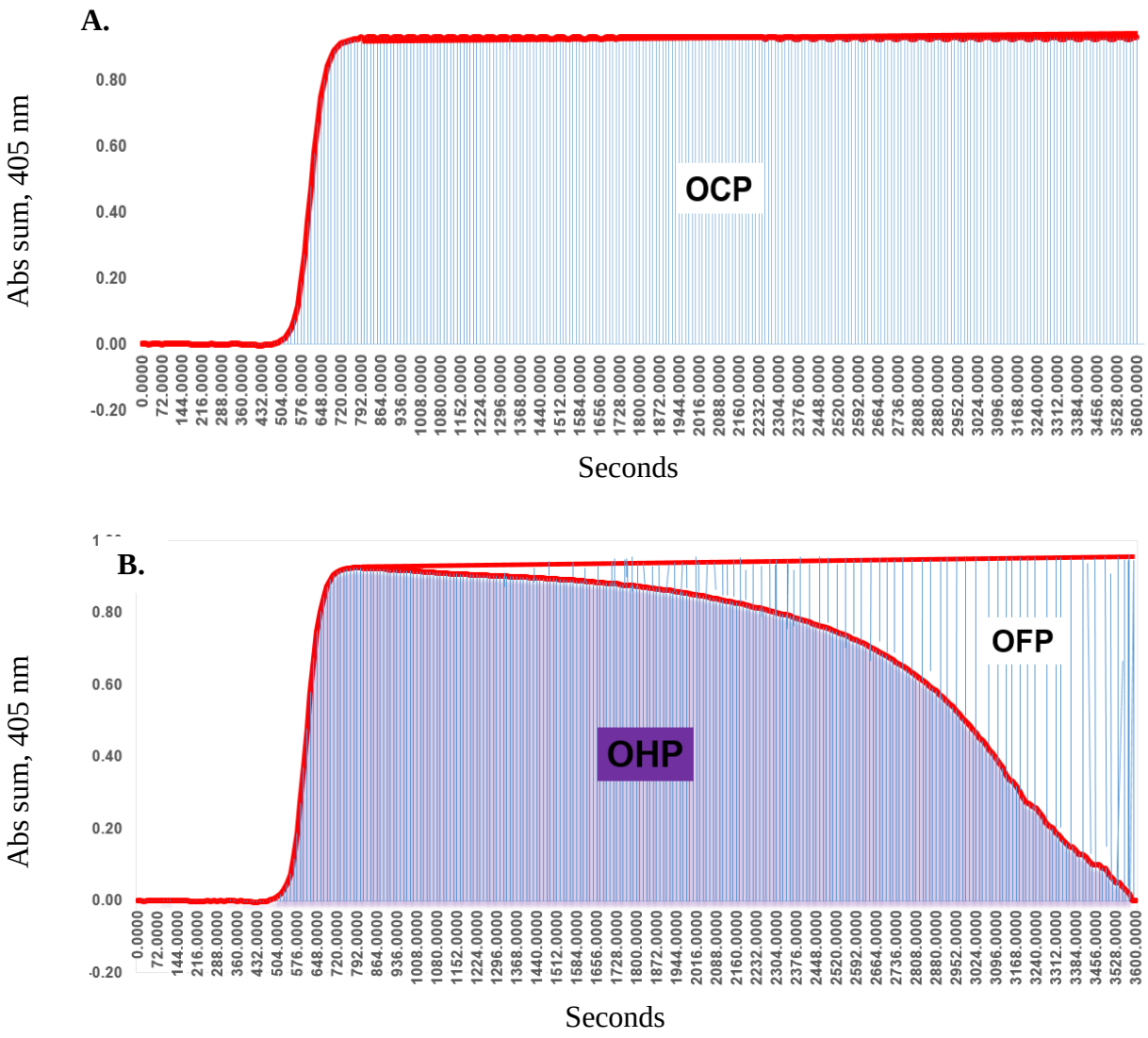


Figure 5. Graphic presentation of A. OCP- The overall coagulation potential and B. OHP –overall haemostatic potential along with OFP- the overall fibrinolysis potential

3.7.2.3 Turbidimetric assay

Turbidimetric assay is modified OHP assay, evaluting the density of the fibrin clot and the rate of fibrin decomposition [178]. Clot formation and lysis were examined by measuring the Abs at 405nm every 12 sec for 60 min. The following parameters were calculated (Figure 6):

1. Clotting assay

a) **Lag C** - Lag time coagulation, the time at which an exponential increase in Abs occurred;
b) **Cmax** - the Max Abs -median value of three consecutive points where the curve reached plateau less the lag turbidity;

2. *Fibrinolysis assay*

a) **Lag L -** Lag time lysis, the time at which an exponential increase in absorbance occurred;

b) **CLT**- clot lysis time, the time from the initiation of clot formation to the time at which a 50% fall in Abs from Max Abs in the lysis assay occurred.

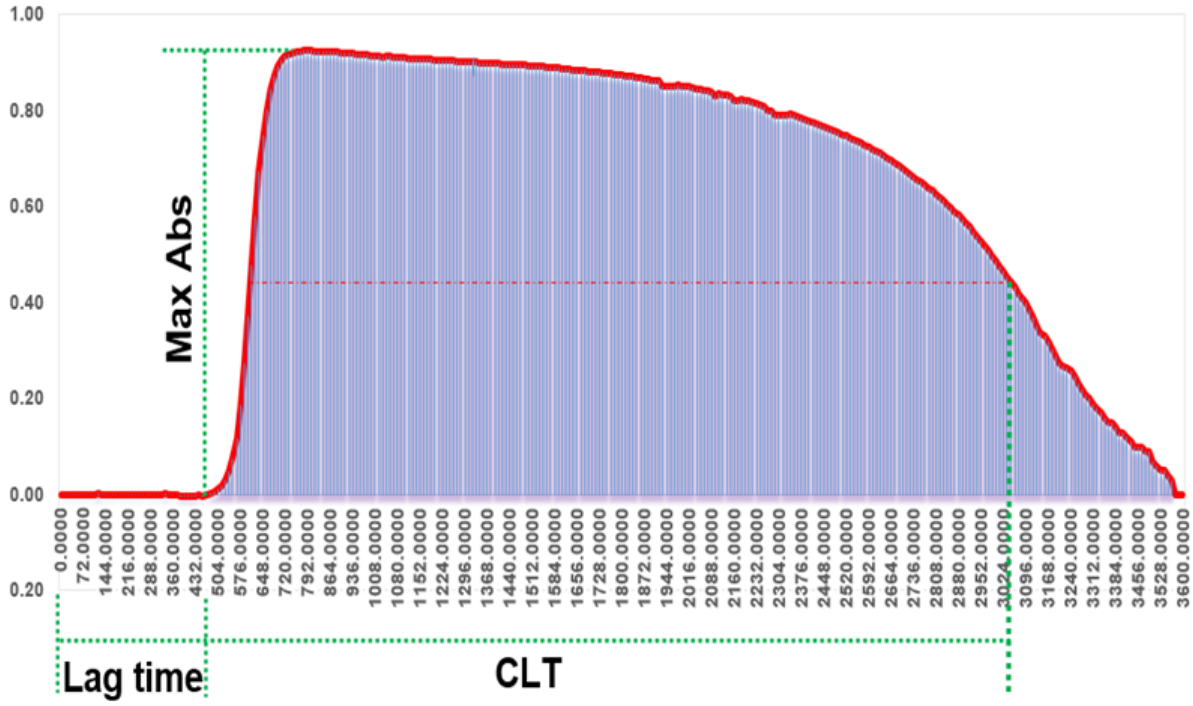


Figure 6. Graphic presentation of turbidity parameters. CLT-clot lysis time

3.7.3 Scanning electron microscopy (SEM) of fibrin clots

Clots for SEM were prepared after OHP analysis was done. Namely, fibrin clots randomly selected from the OHP assay were rinsed with PBS (phosphate-buffered saline), followed by phosphate puffer and were fixed with 2% glutaraldehyde in a Hepes buffered saline (Medicago, Uppsala, Sweden) for 60 min at room temperature and then stored at 4ºC. Before SEM analyses, the specimens were rinsed in distilled water and placed in 70% ethanol for 10min, 95% ethanol for 10min, absolute ethanol for 15min at room temperature, pure acetone for 10 min and then transferred to tetramethylsilane for 10 min and air-dried. Afterward, clots were attached to an aluminum stub and coated with carbon (Bal-Tec MED 010, Lichtenstein). The clots were analysed by electron microscope (Ultra 55 field emission SEM, Carl Zeiss, Oberkochen, Germany) at 3 kV.

3.7.4 Determination of ICAM1 levels by enzyme linked immunosorbent assays (ELISA) method

The quantitative measurement of Intercellular Adhesion Molecule-1 (ICAM1) levels was determined using the standard method *Human ICAM-1/CD54 Non Allele-specific Quantikine ELISA Kit (catalog number DCIMOO, R&D system, Inc., Minneapolis, USA),* according to the manufacture's protocol. Namely, after preparation of all reagents, working standards and samples, 100 µL serum samples or ICAM1 standards were added to an *Human ICAM-1 Non Allele-specific* conjugate preabsorbed microplate. After a 1 hour incubation, *Substrate Solution* was added, followed by a brief incubation. Sample/standard absorbance was read at 450/620nm wavelength. The ICAM1 concentration in serum

samples was determined using the standard ICAM1 curve and the results were expressed as ng/ml of ICAM1.

3.7.5 Determination of VCAM1 levels by ELISA method

For the quantitative determination of VCAM1 concentration in serum, standard method *Human VCAM-1/CD106 Quantikine ELISA Kit (catalog number DVC00, R&D system, Inc., Minneapolis, USA)* was performed according to the manufacture's protocol. Namely, after preparation of all reagents, working standards and samples, 100 μL serum samples or *VCAM1 standards* were added to an *Human VCAM-1 Conjugate* preabsorbed microplate. After a 1.5 hour incubation, *Substrate Solution* was added. Sample/standard absorbance was read at 450/620nm wavelength. The VCAM1 concentration in serum samples was determined using the standard VCAM1 curve and the results were expressed as ng/ml of VCAM1.

3.7.6 Determination of E selectin levels by ELISA method

The quantitative measurement of E selectin concentration in serum was performed using the standard method *Human E-Selectin/CD62E Immunoassa (catalog number DSLE00, R&D system, Inc., Minneapolis, USA),* according to the manufactures protocol. Namely, after preparation of all reagents, working standards and samples, *Assay Diluent RD1W* and 100 μL serum samples or E selectin standards were added to a *Human E-Selectin conjugate* preabsorbed microplate. After a 2 hour incubation, *Substrate Solution* was added. Sample/standard absorbance was read at 450/620nm wavelength. The E selectin concentration in serum samples was determined using the standard E selectin curve and the results were expressed as ng/ml of E selectin.

3.7.7 Determination of P selectin levels by ELISA method

The quantitative measurement of P selectin concentration in serum was performed using the standard *Human P-Selectin/CD62P Quantikine ELISA Kit (catalog number DPSE00, R&D system, Inc., Minneapolis, USA),* according to the manufactures protocol. Namely, after preparation of all reagents, working standards and samples, 100 μL serum samples or P selectin standards were added to an *Human P-Selectin Conjugate* preabsorbed microplate. After a 1 hour incubation, *Substrate Solution* was added. Sample/standard absorbance was read at 450/620nm wavelength. The P selectin concentration in serum samples was determined using the standard P selectin curve and the results were expressed as ng/ml of P selectin.

3.7.8 Determination of VWF antigen

The concentration of VWF antigen (Ag) was assesed in accordance to the Blood Coagulation System (*BCS XP 9020687, Siemens Healthcare Diagnostics*) KI protocol. Automated method with latex particles was used. Reagent for analysis were VWF Ag (*catalog number OPAB03*) containing Latex reagent solution, diluent and buffer, and Owner buffer (OVB; *catalog number B4234-25*). After the plasma samples had been thawed for 5-10 minutes in a water bath at 37 C, centrifugation for 10 min at 14000 RPM was done. First, 15 mL of plasma and 15 mL OVB were mixed followed by addition of 60 mL WVF buffer. After incubation of the mixture for 230 sec, 90 mL Latex WVF reagents was added. Measurement took place at 660 nm. Full method program is available in Sysmex CS 2500 under Assay Group setting. Biologic WVF Ag range is 0.60-1.60 KIE/L.

3.8 Follow up

All cases with history of DU were followed up eighteen months from inclusion and were instructed to report only new ischemic DU. To ensure that the new DU onset happened, regardless of whether it was filled in the diaries or not, all 39 subjects were contacted once in every 1 to 3 months over follow-up period and data were recorded as yes or no along with the date of event. Further, medical records of all follow up cohort were checked for the new DU status.

If more than one DUs were recorded during follow up, it was considered as recurrent, otherwise episodic [144].

3.9 Statistical analysis

After completing database with all variables of interest for both patients and controls statistical analysis was performed.
In this study, descriptive statistics was used to summarize the characteristics of the participants, as mean ± standard deviation (SD) or median (minimum-maximum) for continuous variables depending on the normality distribution. Distribution was tested by using Kolmogorov–Smirnov test. Categorical variables were reported as frequency (%).
Differences in examined continuous variables between patients and controls; different clinical features; were checked using the independent sample *Student t-test* or the nonparametric *Mann–Whitney U* test as appropriate, while categorical data were assessed by either *Pearson's $\chi 2$* test or *Fisher exact test*.
The difference in markers of endothelial dysfunction and parameters of global hemostasis assays between cases and controls was evaluated by odds ratio (OR) and 95% confidence intervals (CI), computed from multivariate logistic regression (MLR) analysis. OR was adjusted by continuous potential confounders (age, BMI, pack-years).
The difference between patients with history of DU and those DU naïve was assessed by OR and 95%CI as well.
The differences in continuous normally distributed variables with homogeneity of variance between three groups of participants was tested by analysis of variance - *ANOVA* with the post-hoc test or by the *Kruskal–Wallis* test with a pairwise post hoc Bonferroni test.
Pearson's or *Spearman's* coefficients of linear correlation were used for analysing the association of investigated endothelial markers to each other or the association between haemostatic parameters with either: demographic/lifestyle/clinical/therapy/laboratory/quality of life continuous data; endothelial markers or to each other.
To summarize the overall discriminatory value of global haemostatic assays parameters among different patients groups, receiver-operating characteristic (ROC) analysis was used.
Multiple linear regression analysis was applied to identify predictors of ETP, Cmax and CLT.
To evaluate predictive factors related with history of DU and onset of new ischemic DU univariate and MLR analyses were performed. The strength of association between a predictive factor and outcome was given by the OR with its 95% CI. Predictors of DU history were explored via 2 step multivariable models, first including laboratory significant variables (ICAM1 and fibrinogen) and second with added clinical variables (age at disease onset and disease duration). All independent continuous factors from univariate analysis with $P < 0.15$ along with covariate age at disease onset were enter in multivariate logistic analysis using forward-stepwise model for assessing independent predictive factors for new DU onset. ROC analysis was done for assessing value of final predictive model.
Survival analysis was performed using the Kaplan-Meier method to estimate the digital ulcers free survival rate. The long-rank test was applied for the assessment of differences in the curves presenting binary CLT values, with cut-off point settled according to the quartile analysis (Q2).

The data analysis was done using the the IBM SPSS version 26.0 (IBM Corp. Released 2020. IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp.), and figures were designed in GraphPad Prism 9 (Version for Windows, GraphPad Software, La Jolla California, USA).

4 RESULTS

4.1 General characteristics of study cohort

The selected baseline demographic characteristics of 58 SSc patients and 46 controls are presented in Table2. As indicated, there was no significant difference with regard to age and gender distribution between SSc patients and controls. Mean age in SSc cohort was 54.3±11.1, where the youngest patient was 30 years old and the oldest 69 years at the baseline. From 58 patients, 51 (88%) were female and 7 (12%) male, with the gender ratio of 7:1. Significant difference was observed between SSc cohort and controls in terms of education level and employment status ($p<0.001$, respectively). High school educational level was predominant within patients (79%), while controls had mostly higher education degree (65%). The unemployment was higher in SSc (n=21; 36%) compared to healthy group (n=2; 4%). Further, the frequency of those who are retired was higher in SSc group, while employed status had 24% patients and 85% controls. The most common reason for retirement within SSc cohort was due to burden of disease (n=23; 65%-data has not shown).

Table 2**.** Baseline general demographic characteristic of study cohort

	SSc patients (58)	**Controls (46)**	**p-value**
Age (years) [a]	54.29±11.05	51.28±9.34	0.143[d]
Gender, n (%)			
Male	7 (12)	5 (11)	
Female	51 (88)	41 (89)	0.849[e]
Education level, n (%)			
Elementary school	2 (3)	0(0)	
High school	46 (79)	16(35)	
Higher education	10(17)	30 (65)	<0.001[f]
Employment status, n (%)			
Unemployed	21 (36)	2 (4)	
Employed	14(24)	39(85)	
Retirement	23(40)	5 (11)	<0.001[f]
Lifestyle and comorbidities			
Smoking, n (%)			
Never	42 (72)	36 (78)	
Ever[b]	16 (28)	10 (22)	0.494[e]
Pack-years[c]	0 (0-60)	0(0-40)	0.480[g]
BMI (kg/m2)[a]	23.6±3.5	23.1±2.8	0.516[d]
Obesity, n (%)			
BMI<25	35 (60)	33(72)	
BMI≥25	23 (40)	13(28)	0.300[e]
Arterial hypertension, n (%)			
No	27 (47)	24 (52)	
Yes	31 (53)	22 (48)	0.569[e]

[a]Mean ±SD; [b] minimum of 60-day period any time prior to the study onset; [c] Median (Min-Max); [d]Student T test; [e] X^2 test; [f] Fisher exact test; [g]Mann Whitney U test

The distribution of arterial hypertension, ever smokers and obesity was similar between SSc and control group. In our SSc cohort 40% patients were obese; 28% ever smokers and 53% suffered from arterial hypertension. Although none of the healthy controls reported suffering from any disease or taking medicines, 22 (4%) cases mentioned that arterial pressure > 140/90mmHg had been measured at least once in a past 1 years, so lifestyle recommendations have been followed.

4.2. Clinical characteristics

The general clinical characteristics of the cases with SSc are summarized in Table 3. The mean age at disease onset was 48.25±12.07, where the youngest patient was 20 years old and the oldest 69 years old. Median disease duration was 4.5 years at inclusion ranging from 0-29 years. Sixteen patients (28%) had disease onset within one year prior to baseline. Regarding the extent of skin involvement, lSSc was present in 36 (62%), while 22 (38%) patients had dSSc. Regarding SSc specific autoantibody status the majority of cases had Anti-topo I antibody, while ACA were positive in 38% patients. Within dSSc subgroup 18 (82%) had anti Topo I antibodies and all lSSc patients in our cohort were ACA positive. Modified Rodnan skin score varied from 6-34 with a mean value 12.58±7.19. In addition, during physical examination the presence of specific SSc skin manifestation were recorded, so sclerodactily was present in more than one third of patients.

Table 3. General clinical characteristics and skin manifestations of 58 SSc patients

General clinical characteristics	**SSc patients**
Age at disease onset, years[a]	48.25 ± 12.07
Disease duration, years[b]	4.5 (0-29)
Cutaneous subtype, n (%)	
Limited	36 (62)
Diffuse	22 (38)
Autoantibody status, n (%)	
Anti-centromere	22 (38)
Anti-topoisomerase I	25 (43)
mRSS	10 (3-31)
mRSS >14,n(%)	22 (38)
Skin manifestations,n (%)	
Sclerodactily	36 (62)
Calcinosis	13(22)

[a]Mean ±SD; [b] Median (Min-Max); mRSS-modifed Rodnan skin score

The most common affected extra cutaneous organ system in our cohort was the musculoskeletal. The range of system involvement is presented in Table 4.

Table 4. Clinical features of extra cutaneous organ involvement in 58 SSc patients

Extra cutaneous organ involvement	**SSc patients**
Musculosceletal, n (%)	
Contracture	16(28)
Atralgia	24 (41)
Arthritis	5 (9)
Tendinopathy	2 (3)
Cardiopulmonal	

FVC %[a]	95.6±18.8
FVC <80 %, n (%)	11(19)
DLCO % [a]	65.2±16.3
DLCO <70%, n (%)	35 (60)
FVC/DLCO %> 1.6, n(%)	20 (35)
ILD, n (%)	28(48)
sPAP, (mmHg)[a]	30.4±7.5
Left ventricular ejection fraction %[a]	62.2±5.3

[a]Mean ±SD; FVC-forced vital capacity; DLCO- diffusing capacity for carbon monoxide; ILD-interstitial lung disease; sPAP- systolic pulmonary artery pressure

The high majority of cases had arthralgia and contracture as skeletal manifestations. Physical assessment hasn't revealed any patient with muscle weakness and no one has reported myalgia.

PFTs revealed that more than half of the patients had decreased DLCO below 70%, but only 19% of patients had FVC value below 80%. Twenty-eight patients suffered from interstitial lung disease. Evaluating the risk for PAH occurrence, FVC%/DLCO% ration was calculated and 35% of our SSc cohort had result more than 1.6. Doppler echocardiography data have demonstrated that minority of patients had elevated sPAP more than 30 mmHg and no one had reduces left ventricular ejection fraction below 50%. Only 3/58 subjects had sPAP more than 45mmHg and they performed heart catheterization but PAH wasn't confirmed. None of the patients in our cohort suffered from SSc renal crises.

Peripheral vascular manifestations may be triggered by cold, especially RP and DUs. In order to avoid seasonal impact on occurrence of new DUs, our cohort was collected over four seasons and distribution of their enrollments is presented in Figure 7.

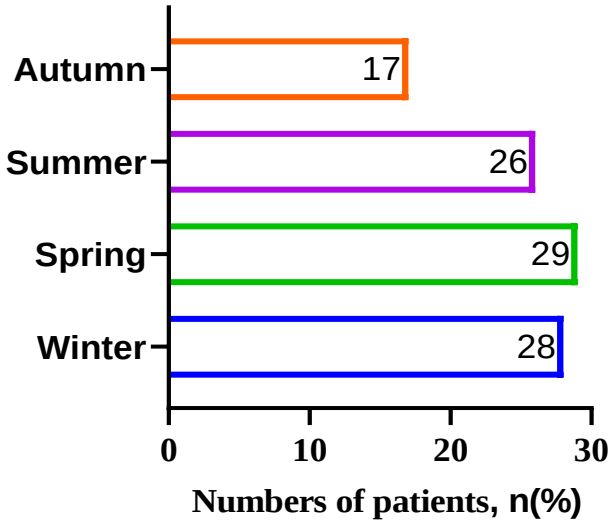


Figure 7. Distribution of patients based on four seasons

Clinical signs of peripheral vasculopathy are shown in Table 5. Almost half of our SSc cohort had any sign of vasculopathy at physical examination. The most frequent were telangiectasia, followed by pitting scars, RP, active DU and acro-osteolysis. The median value of telangiectasia score was 3 (range 0-16).

Table 5. Peripheral vascular manifestations of 58 SSc patients

Peripheral vascular manifestations	SSc patients, n(%)
RP present	28(48)
Telangiectasia present	39(67)
Pitting scars	33(57)
Active digital ulcers	18 (31)
Acroosteolysis	13(22)

RP-Raynaud phenomena

Of note, majority of patients with active DU had RP at baseline physical examination (Figure 8).

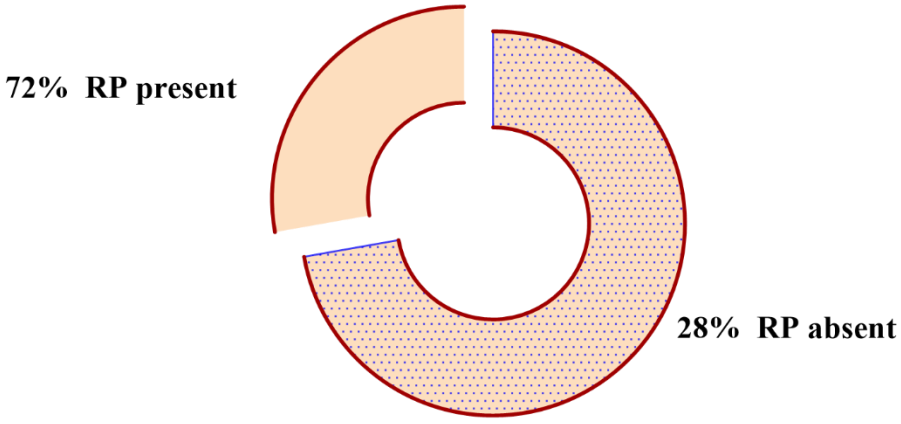


Figure 8. Distribution of Raynaud phenomena within active digital ulcer group.

Further, microvascular disease was assessed with NVC and as indicated in Figure 9 the most frequent NVC pattern was active.

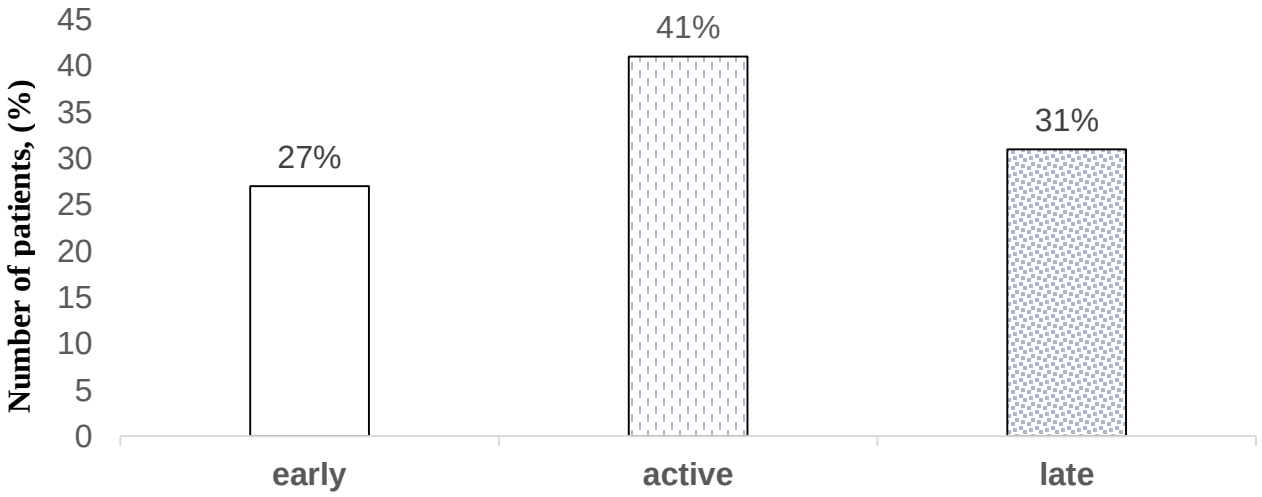


Figure 9. Distribution of capillaroscopy patterns in 58 SSc patients

In addition, the most specific characteristics of SSc microangiopathy have been evaluated by cappilaroscopy showing that the majority of cases had moderate loss of capillaries, while severe loss and ramified/ bushy capillaries were found in almost one third of patients, as indicated in Table 6.

Table 6. Specific NVC features of 58 SSc patients

Specific NVC characteristics	SSc patients, n(%)
Loss of capillaries	
No	10(17)
Few	6(10)
Moderate	23 (41)
Severe	18(31)
Ramified bushy capillaries	17(29)

NVC: nailfold videocapillaroscopy

4.2.1. Detailed characteristics of digital ulcers

Thirty nine patients had ever DU in their history of disease and distribution of DUs in our SSc cohort is shown in Figure 10. The median time from first DU onset was 4.5 years with a range from 0-29 year prior inclusion.

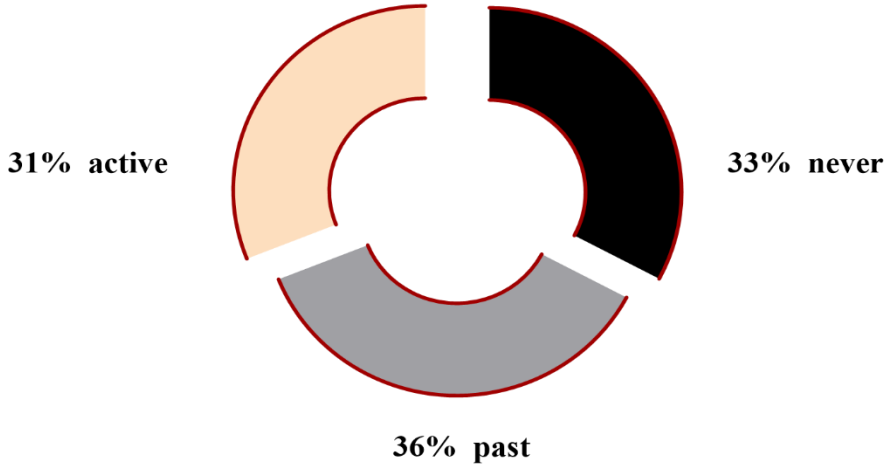


Figure 10. Distribution of patients according to DU status. DU-digital ulcer

More than half of patients with DUs history have had more than 3 episodes of DU. The majority of patients with active DU had one DU at baseline, as shown in Table 7.

Table 7. Distribution of patients according to the number of previous DU episodes and active DUs

Variable	SSc patients, n (%)
Episodes of DUs	
1	5 (13)
2	13(33)
≥3	21(54)
Number of active DUs at baseline	
1	12 (67)
2	1 (6)
3	2 (11)
4	2 (11)
10	1 (6)

DUs- Digital ulcers

Middle finger at right hand was predominantly affected as presented in Figure 11.

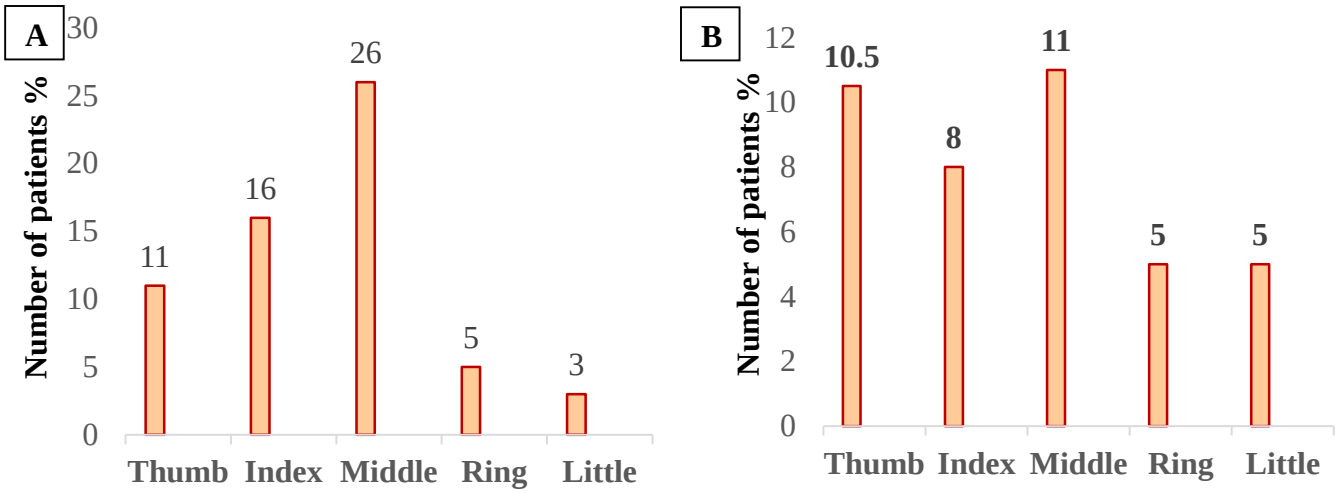


Figure 11. Distribution of active DUs **A.** right hand**; B**. left hand. DUs-digital ulcers

None of the patients had inflamed active DUs, osteomyelitis, chronic not-healing DUs, or lower-limb ulcers at inclusion.

4.2.2. Therapy

Data concerning therapy modalities among SSc patients are presented in Figure 12. Regarding ongoing immunosuppressive therapy, majority of patients were taking Methotrexate (MTX) with median dose 10 mg per week (range 10-15mg). Glucocorticoids were administered in 38% of cases with median dose of 5mg (range 2.5-10mg) daily. Half of patients were treated with CCB and 40% had acetylsalicylic acid (ASA) of 75 mg daily. Twenty four subjects had been receiving CYP in the past with cumulative median dose of 12.25g (range 4.8-25g).

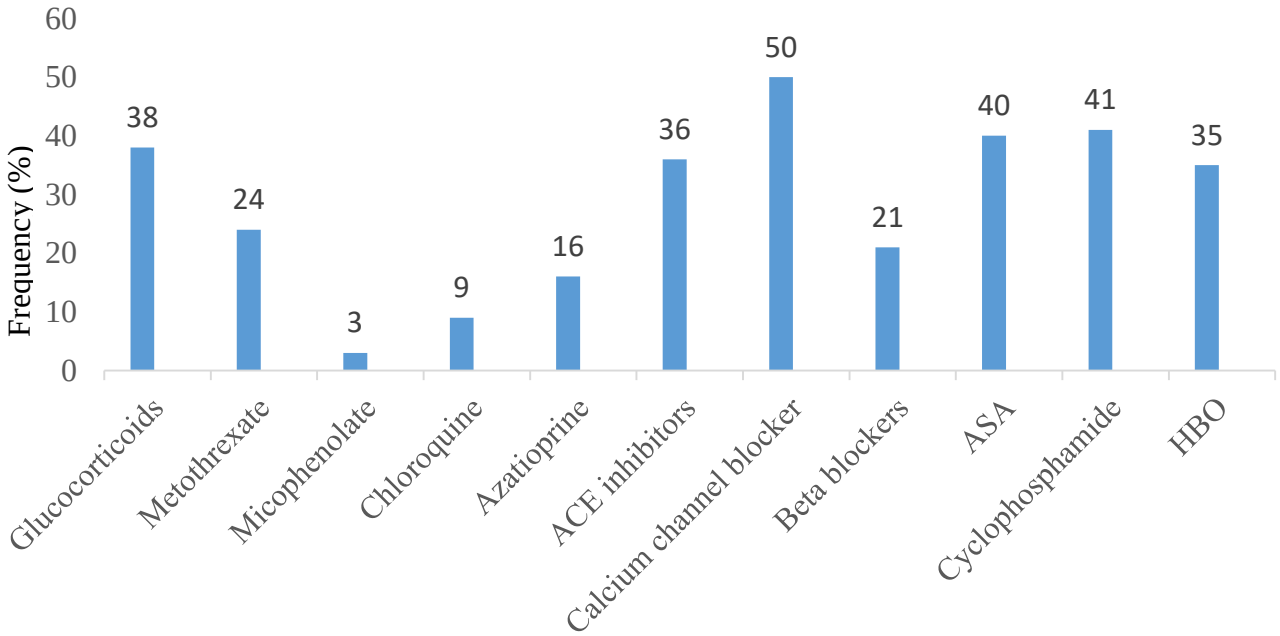


Figure 12. Distribution of therapeutic modalities among 58 patients. ASA: acetylsalicylic acid, HBO-hyperbaric oxygen therapy

4.2.3. Quality of life

Range of overall disability assessed by SHAQ DI was 0-2.6, with median 0.37. The most impaired SHAQ DI domains were eating and gripping, while the less affected were arising and hygiene. VAS data indicated that presence of RP and pain were the most limited for daily activities by the opinion of our patients (Table 8).

Table 8. Baseline values of 58 SSc patients for the SHAQ DI domains

SHAQ DI domains	**Median**	**Range (min-max)**
Dressing	0	0-3
Arising	0	0-2
Eating	1	0-3
Walking	0	0-3
Hygiene	0	0-2
Reaching	0	0-2
Gripping	1	0-3
Activity	0	0-3
Overall	0.37	0-2.6
VAS scale, (mm)		
Pain	24.5	0-91
RP	33	0-81
DU	0	0-89
Lung	0	0-90
GIT	0	0-52

SHAQ DI- Scleroderma Health Assessment Questionnaire disability index; VAS- visual analog scale; RP- Raynaud phenomenon; DU- digital ulcer; GIT- gastrointestinal tract

Anxiety and depression impacted the most quality of life analyzed by EQ5D, while mobility and self-care domains were the most preserved. The worst value of VAS overall health was estimated as 16mm and the best 100mm (Table 9).

Table 9. Baseline values of 58 SSc patients for the EQ5D domains

EQ5D domains	**Mean ±SD**
Mobility	1.1±0.4
Self-care	1.1±0.4
Usual activities	1.4±0.5
Pain and discomfort	1.6±0.6
Anxiety and depression	1.7±0.6
Overall	6.5±1.7
VAS scale, mm	
EQ	0.73±0.18

EQ5D- EuroQol 5 Dimension; VAS- visual analog scale

4.3. Laboratory markers in study cohort

Regarding general laboratory analysis, markers of renal function, lipids and complement levels (C3 and C4 components) were not statisticaly different between SSc cohort and HC (Table 10). Of note, the levels of complement components were within the reference range for healthy population. On the other hand, serum levels of all inflammatory markers, including CRP, ESR and fibrinogen, were significantly elevated in patients compared to HC (p <0.001, respectively).

Table 10. General laboratory analysis in study cohort

Laboratory parameters	**SSc patients (58)**	**Controls (46)**	p-value
Markers of renal function			
Urea, (mmol/L)[a]	5.3±1.7	4.8±1.7	0.165 [c]
Creatinine, (ηmol/L) [a]	77.5±19.0	74.2±9.1	0.276 [c]
Lipid panel			
Cholesterol, (mmol/L) [a]	6.7±1.6	6.1±2.1	0.107 [c]
Triglycerides, (mmol/L) [a]	1.5±0.7	1.3±0.6	0.183 [c]
Inflammatory markers			
CRP (mg/l) [b]	4.9(0-30)	2.1(0.0-12.4)	**<0.001 [d]**
ESR,(mm/hr) [b]	18(4-66)	10(2-42)	**<0.001 [d]**
Fibrinogen, (g/L) [b]	4.5(2.0-10.3)	2.9(1.5-4.3)	**<0.001 [d]**
Complement level			
C3, (g/L) [a]	1.3±0.3	1.3±0.2	0.783 [c]
C4, (g/L) [a]	0.21±0.06	0.23±0.16	0.207 [c]
Values of haemostatic parameters			
aPTT,(s) [a]	27.9±6.9	28.0±4.3	0.886 [c]
PT (INR) [a]	1.2±0.1	1.2±0.1	0.473 [c]

[a] Mean ±SD; [b] Median (Min-Max); [c] Student T test; [d] Mann Whitney U test; CRP- C reactive protein; ESR –erythrocyte sedimentation rate; aPTT- activated partial thromboplastin time

Of note, the highest values of inflammatory markers were found in patients with recent disease onset (≤1 year from inclusion) compared to other cases (fibrinogen 4.9 (2.4-10.3) vs. 4.2(2.0-9.8), p=0.141; ESR 19 (4-60) vs. 18 (4-50), p=0.644; CRP 5.7(0.8-30.6) vs. 3.9 (0-16.4), p=0.154), but without statisticaly significant difference.

4.3.1. Endothelial dysfunction

As indicated in Table 11 serum levels of ICAM1, VCAM1, E selectin and VWF:Ag were significantly increased in SSc patients respect to HC (p<0.05, respectively). After adjustment for variables that could have impact on endothelial function (age, BMI, pack/years), the difference of investigated markers between patients and controls remain significant for ICAM1, VCAM1, E selectin, VWF:Ag, while trend of higher P selectin levels in patients was observed.

Table 11. Baseline values of serum vascular biomarkers in study cohort

Vascular biomarkers	**SSc patients(58)**	**Controls(46)**	**p-value**	**OR[e] (95%CI)**	**p-value**
ICAM1, (ng/ml)[a]	29.5±7.9	24.3±4.6	<0.001[c]	1.1(1.01-1.21)	**0.030**
VCAM1, (ng/ml)[b]	37.9(16.7-138.5)	30.8(20.8-47.4)	0.001[d]	1.1(1.03-1.21)	**0.006**

E selectin, (ng/ml)[a]	5.1±2.0	4.1±1.7	0.030[c]	1.5(1.03-2.03)	**0.032**
P selectin, (ng/ml)[b]	5.5(0-24.8)	5.3(0-9.9)	0.131[d]	1.1(1-1.3)	*0.073*
VWF:Ag[b]	1.7(0.7-7.6)	1.2 (0.6-3.3)	0.001[d]	4.6(1.89-10.91)	**0.001**

[a] Mean ± SD; [b] Median (Min-Max); [c] Student T test; [d] Mann Whitney U test; [e] OR-odds ratio adjusted to age, BMI, pack years; CI- confidence interval; ICAM-1- intercellular adhesion molecule 1; VCAM1- vascular adhesion molecule 1; VWF:Ag- von Willebrand factor antigen

Within our SSc cohort, there was significant moderate correlation between ICAM1 - VCAM1 (ρ=0.428, p=0.001) and ICAM1 - E selectin (r=0.502, p<0.001), while weak positive relation was found between: ICAM1-vWF antigen (ρ=0.333, p=0.011), E selectin - VCAM1 (ρ=0.376, p=0.004) and VCAM1–vWF Ag (ρ=0.373, p=0.004), suggesting that interplay of endothelial markers is important for SSc pathogenesis (Figure13).

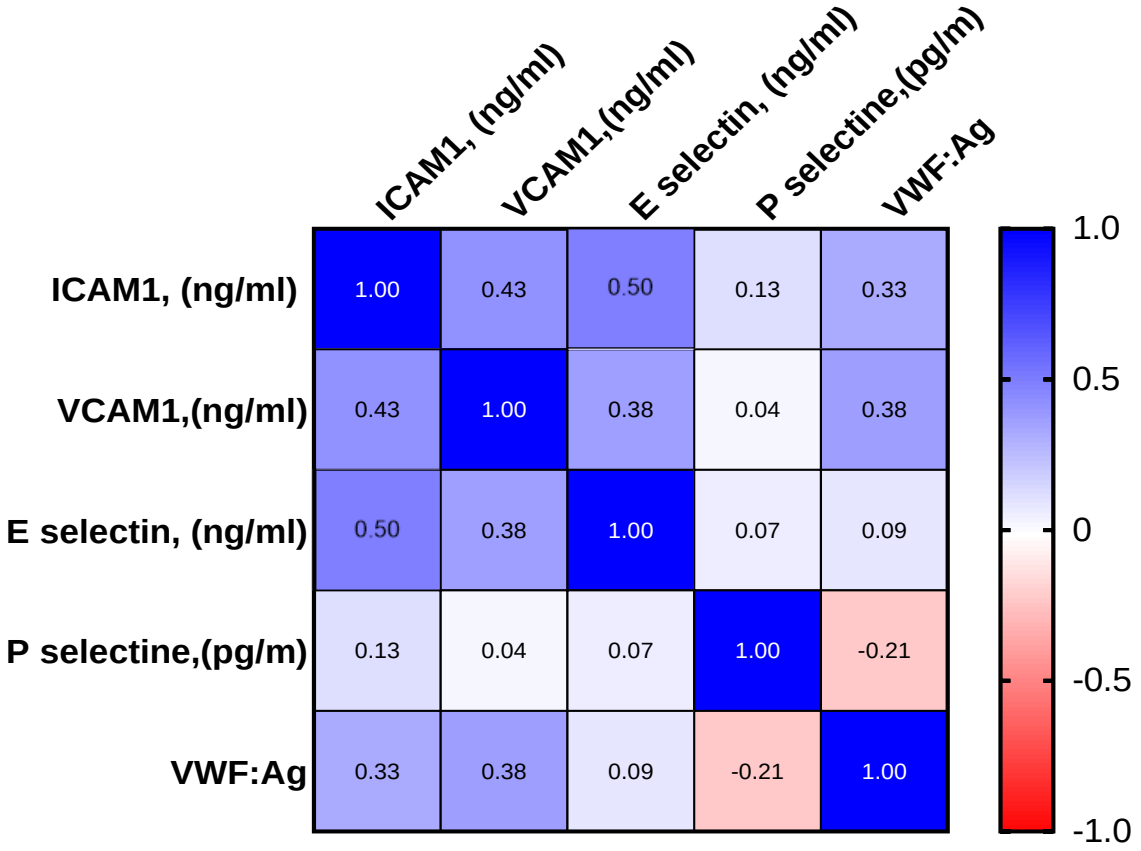


Figure 13. Correlations between markers of endothelial dysfunction.
ICAM-1- intercellular adhesion molecule 1; VCAM1- vascular adhesion molecule 1; VWF:Ag- von Willebrand factor antigen

4.4. Hemostasis

Adjusted OR of various CAT assay parameters between SSc patients and controls are presented in Table 12. SSc cohort had significantly higher ETP and PT along with faster time to peak. A trend of shorter lag time was found in patients, indicating faster thrombin generation.

Table 12. Thrombin generation assay parameters in study cohort

CAT assay	SSc patients(58)	Controls(46)	OR[b] (95%CI)	p-value
ETP, (nM/min)[a]	1962.9±377.8	1756.3±321.2	1.0(1.001-1.003)	**0.003**
Lag time, (min)[a]	3.2±0.6	3.3±0.8	0.54 (0.28-1.02)	*0.057*
Peak, (nM)[a]	327.2±86.5	293.3±66.8	1.01(1.00-1.01)	**0.020**
Time to peak,(min)[a]	6.2±1.4	6.5±1.3	0.68 (0.49-0.94)	**0.018**

[a]Mean±SD; OR[b] odds ratio adjusted to age, BMI, pack-years; CI- confidence interval; ETP-endogenous thrombin potential

In Figure 14, ROC curve of ETP in SSc patients is shown. The best estimated ETP cut off point for discriminating SSc cases from controls was 1804.68 nM/min, with sensitivity 71.7% and specificity 65.5% (AUC 0.696, 95%CI 0.594 - 0.798). When applying dummy variable in regression analysis adjusted for age, BMI, pack years, SSc cases had 5 fold higher chance having enhanced ETP (OR 4.75, 95%CI 2.02-11.18) in respect to controls.

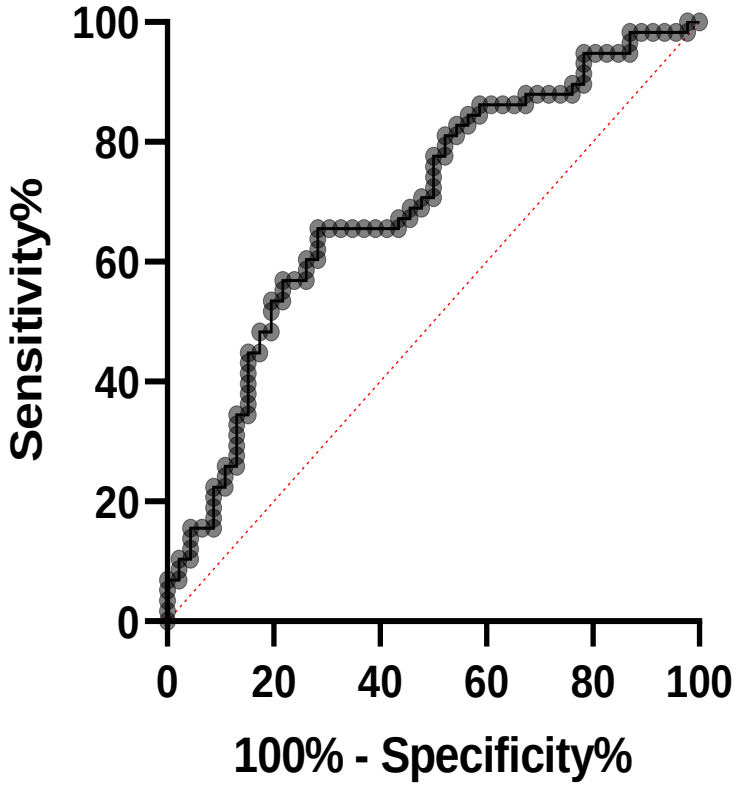


Figure 14.The ROC curve of endogenous thrombin potential

When analyzing data from OHP assay, summarized in Table 13, it has been observed that OHP was increased, while OFP was significantly decreased in cases versus controls.

Table 13. Overall haemostatic assay parameters in study cohort

OHP assay	SSc patients(58)	Controls(46)	OR[b] (95%CI)	p
OHP,(Abs-sum)[a]	123.5(24.1-318.2)	106.6(41.8-214.5)	1.01(1.00-1.02)	*0.087*
OCP,(Abs-sum)[a]	314.8(70.2-446.5)	308.0(184.6-419.3)	1.00(0.99-1.01)	0.482
OFP, (%)[a]	60.7(14.4-77.0)	64.5(19.9-81.1)	0.97(0.94-0.99)	**0.041**

[a]Median (Min-Max); OR[b] - odds ratio adjusted to age, BMI, pack-years; CI- confidence interval; OHP- overall haemostasis potential; OCP - overall coagulation potential; OFP-overall fibrinolysis potential

Finally, turbidity assay revealed altered clot properties in patients with higher Cmax and significantly prolonged CLT compared to HC, along with shorter time to clotting in coagulation assay (Table 14)

Table 14. Turbidity assay parameters in study cohort

Turbidity assay	SSc patients(58)	Controls(46)	OR[b] (95%CI)	p-value
Coagulation				
Lag phase clot, (min)[a]	5.26±0.9	5.7±1.7	0.72(0.52-0.99)	**0.040**
Cmax, (Abs)[a]	1.1±0.3	1.0±0.2	5.09 (1.00-25.89)	**0.050**
Fibrinolysis				
Lag phase lysis, (min)[a]	5.9±2.7	6.2±2.1	0.96(0.82-1.13)	0.639
CLT, (min)[a]	35.5±9.9	30.7±7.9	1.06 (1.01-1.11)	**0.023**

[a]Mean ± SD; OR[b] - odds ratio adjusted to age, BMI, pack-years; CI- confidence interval; Cmax Abs-clot maximum absorbance; CLT-clot lysis time.

The most appropriate cut off points with Sn, Sp, AUC for both Cmax (1.081Abs, 63%, 63.8%, 0.647 95% CI 0.541 - 0.754) and CLT (28.20 min, 53.5%, 72.4%, 0.637 95% CI 0.531 - 0.744) were calculated from ROC analysis presented in Figure 15. SSc patients had around 3 fold higher risk of either altered Cmax (OR (adjusted for age, BMI, pack/years) 2.71, CI 95% 1.19-6.17, p=0.018) or prolonged CLT (OR (adjusted for age, BMI, pack/years) 3.06, CI 95% 1.27-7.35, p=0.012), taking cut off points as discriminative values.

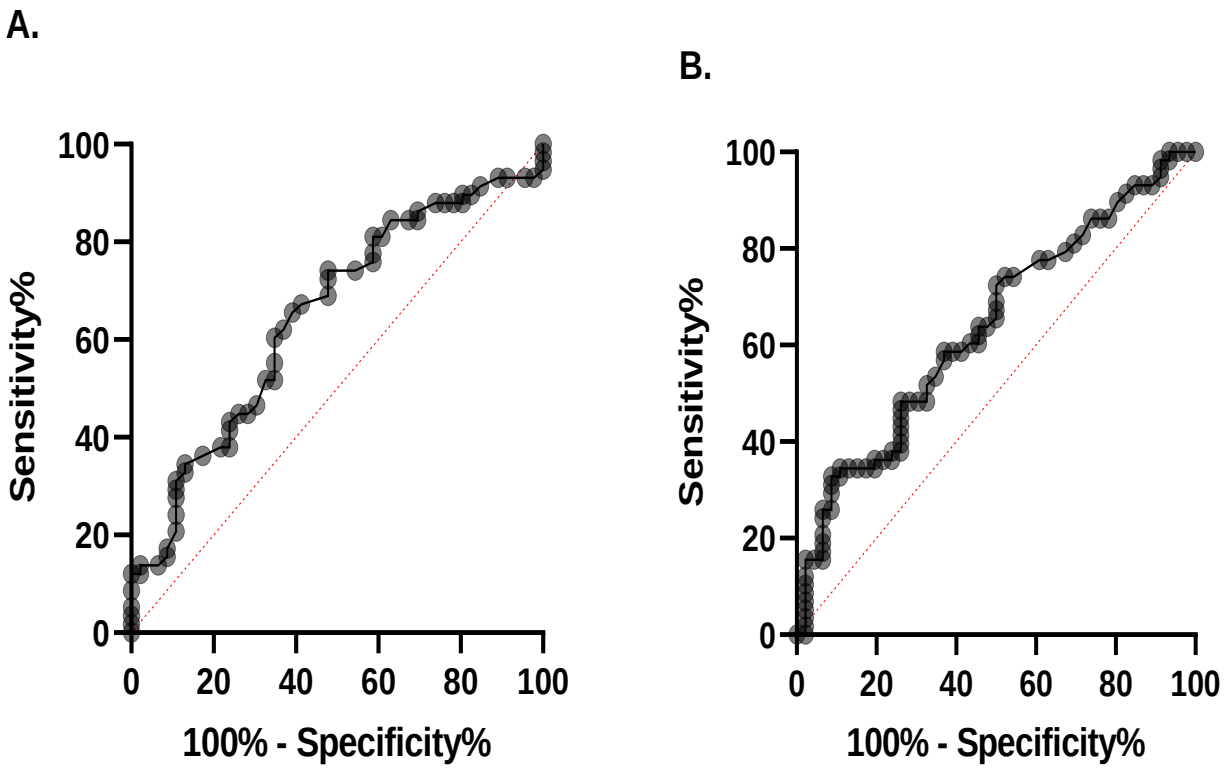


Figure 15.The ROC curve of A. Cmax and B. CLT. Cmax Abs- clot maximum absorbance; CLT- clot lysis time

Correlation of different haemostatic parameters with general demographic, clinical characteristics and continues lifestyle variable are shown in Table 15. It can be observed that only positive week association was found between CLT and pack-years along with a trend of week relation between Cmax and pack-years.

Table 15. Correlation between ETP, Cmax, CLT and general demographic, lifestyle and clinical characteristics

		General demographic characteristics		**Lifestyle**	**General clinical characteristics**	
Haemostatic parameters		**Age (years)**	**BMI (kg/m2)**	**Pack-years**	**Age at disease onset (years)**	**Disease duration (years)**
ETP, (nM/min)	r	0.127	0.161	0.150	0.095	0.372
	p	0.341	0.226	0.262	0.477	0.156
Cmax, (Abs)	r	0.031	0.028	0.238	-0.023	0.010
	p	0.818	0.832	*0.072*	0.864	0.942
CLT, (min)	r	-0.002	0.123	0.290	-0.010	-0.054
	p	0.986	0.357	**0.027**	0.942	0.687

r- correlation coefficient; ETP-endogenous thrombin potential; CLT-clot lysis time; Cmax Abs- clot maximum absorbance

When assessing the difference between patients with recently onset of SSc respect to others, even enhanced thrombin generation was observed in cases with early disease statistical significance wasn't found (Table 16).

Table 16. Difference between SSc patients with disease duration less than 1 year and others in regard to CAT assay parameters

CAT assay	SSc patients ≤ 1(16)	SSc patients >1 (42)	p-value
ETP, (nM/min)	2026.1±367.6	1940.6±380.8	0.465
Lag time, (min)	3.2±0.6	3.3±0.4	0.427
Peak, (nM)	339.8±68.7	322.4±92.6	0.626
Time to peak,(min)	6.2±1.6	6.3±0.9	0.486

Results are presented as Mean± SD; SSc-systemic sclerosis; CAT- Calibrated automated thrombogram; ETP- endogenous thrombin potential

Comorbidities and smoking status had no impact on haemostasis (Table 17).

Table 17. Levels of ETP, Cmax, and CLT according to smoking status of comorbidities.

	Lifestyle		Comorbidities			
Haemostatic parameters	**Smoking**		**Arterial hypertension**		**Obesity**	
	No	**Yes**	**No**	**Yes**	**No**	**Yes**
ETP, (nM/min)[a]	2042.6± 459.9	1932.6± 343.0	2061.2± 413.4	1877.4± 326.9	1960.8± 383.6	1969.3± 364.6
p-value[b]	value		0.064		0.934	
Cmax, (Abs)[a]	1.2±0.3	1.1±0.3	1.1±0.3	1.1±0.3	1.1±0.2	1.1±0.3
p-value[b]	0.317		0.957		0.693	
CLT, (min)[a]	38.8±8.8	34.2±10.2	33.8±9.9	37±9.9	35.5±10.1	35.5±9.9
p-value[b]	0.121		0.227		0.999	

[a]Mean ± SD;[b] T test; ETP-endogenous thrombin potential; CLT-clot lysis time; Cmax Abs- clot maximum absorbance

4.4.1. Haemostatic parameters in regard to clinical features

As shown in Table 18, all haemostatic parameters were significantly higher in patients with active/very active disease respect to HC (ETP p=0.001, Cmax p=0.001, CLT p= 0.008). Presence of active disease was characterized with significantly higher mean value of Cmax and trend of longer CLT (p=0.004, p=0.074, respectively).

Table 18. Differences in haemostatic parameters according to presence of active disease

Haemostatic parameters	EUSTAR			p value
	inactive/ moderately active [29]	**active/ very active [29]**	**Controls [n = 46]**	
ETP, (nM/min)[a]	1893.3±360.9	2035.1±383.6*****	1756.3±321.2	**0.001 [b]**
Cmax, (Abs)[a]	1.0±0.3	1.2±0.2***,¶**	1.0±0.2 [b]	**<0.001 [b]**
CLT, (min)[a]	33.1±8.9	37.9±10.5*****	30.7±7.9 [b]	***0.011*** **[c]**

[a]Mean ±SD; [b] one way ANOVA with post hoc analysis; [c] Kruskal Wallis with post hoc analysis; p values were adjusted by the Bonferroni correction for multiple testing.***** p<0.05 observed between active/very active and control group; ¶p <0.05 observed between two EUSTAR groups ; ETP-endogenous thrombin potential; CLT-clot lysis time; Cmax Abs- clot maximum absorbance

ROC analysis revealed that Cmax showed good discriminatory abilities in recognizing those with active/very active disease (AUC 0.688 95% CI 0.552 - 0.824, p=0.014) (Figure 16).

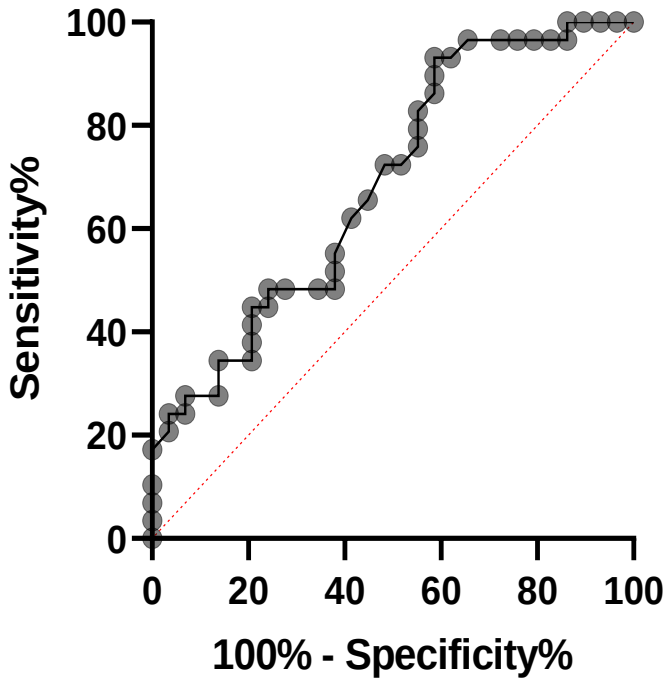


Figure 16. ROC analysis for Cmax using active/very active disease=1. Cmax Abs- clot maximum absorbance

Differences in haemostatic parameters within disease subtypes are presented in Table 19. Although all investigated haemostatic parameters were increased in dSSc patients, only Cmax showed a trend of statistical significance.

Table 19. Differences in haemostatic parameters regarding cutaneous subtype

	Cutaneous subtype		
Haemostatic parameters	**Limited(36)**	**Diffuse(22)**	**p value[b]**
ETP, (nM/min)[a]	1920.8 ±373.8	2031.9±382.8	0.281
Cmax, (Abs)[a]	1.1±0.3	1.2±0.2	*0.053*
CLT, (min)[a]	33.9±9.4	38.0±10.6	0.133

[a]Mean ±SD; [b] T test; ETP-endogenous thrombin potential; CLT-clot lysis time; Cmax Abs- clot maximum absorbance

On the other hand, presence of specific SSc antibodies haven't shown significant association with either thrombin generation or fibrin clot properties (Table 20).

Table 20. Differences in haemostatic parameters according to presence of SSc specific antibodies

Haemostatic parameters	SSc specific antibodies			
	Anti Topo I		ACA	
	absent (33)	present (25)	absent (22)	present (36)
ETP, (nM/min)[a]	1962.2±364.9	1963.9±401.8	1999.9±365.0	1902.6±399.0
p value[b]	0.986		0.346	
Cmax, (Abs) [a]	1.1±0.3	1.2±0.3	1.2±0.2	1.1±0.3
p value[b]	0.285		0.069	
CLT, (min) [a]	33.9±9.4	37.6±10.5	36.0±9.8	34.6±10.4
p value[b]	0.161		0.604	

[a]Mean ±SD; [b] T test; Anti Topo I- anti topoisomerase I antibody; ACA- anticentromere antibody; ETP-endogenous thrombin potential; CLT-clot lysis time; Cmax Abs- clot maximum absorbance

Even though, all investigated haemostatic parameters were higher in patients with severe skin involvement, defined as mRSS ≥ 14, significant difference wasn't observed between groups (Table 21).

Table 21. Differences in haemostatic parameters regarding extension of skin involvement

Haemostatic parameters	Extension of skin involvement		
	mRSS		
	<14 (36)	≥14 (22)	p value[b]
ETP, (nM/min)[a]	1959.4±386.4	1972.1±367.1	0.902
Cmax, (Abs)[a]	1.1±0.3	1.2±0.2	0.145
CLT, (min)[a]	34.2±9.9	37.6±9.8	0.213

[a]Mean ±SD; [b] T test; mRSS- modified Rodnan skin score; ETP-endogenous thrombin potential; CLT-clot lysis time; Cmax Abs- clot maximum absorbance

However, concerning skin manifestations, presence of sclerodactily was significantly associated with prolonged fibrinolysis, while calcinosis haven't shown relation with any of investigated hemostatis parameters as it is shown in Table 22.

Table 22. Differences in haemostatic parameters according to presence of skin manifestations

Haemostatic parameters	Skin manifestations			
	Sclerodactily		Calcinosis	
	absent (22)	present (36)	absent (45)	present (13)
ETP, (nM/min)[a]	1994.2±406.9	1912.0±327.2	1971.9±351.9	1937.9±465.1
p value	0.427[b]		0.461[c]	
Cmax, (Abs)[a]	1.1±0.3	1.1±0.3	1.2±0.3	1.1±0.2
p value	0.793[b]		0.417[c]	
CLT, (min)[a]	31.8±7.3	37.7±10.8	35.2±9.9	36.5±10.3
p value	**0.016**[b]		0.602[c]	

[a]Mean ±SD; [b] T test ; [c] Mann Whitney U test. ETP-endogenous thrombin potential; CLT-clot lysis time; Cmax Abs- clot maximum absorbance

Table 23.presents mean values of haemostatic parameters observed in cases with contractures and those without. Although enhanced thrombin generation and prolonged fibrinolysis were observed in patients

with the most frequent skeletal manifestation in our cohort, significant difference was only found for Cmax.

Table 23. Differences in haemostatic parameters according to presence of contractures

	Skeletal involvement		
Haemostatic parameters	**Contracture**		
	absent (36)	**present (22)**	**p value[b]**
ETP, (nM/min)[a]	1939.0±389.2	2025.8±350.1	0.439
Cmax, (Abs)[a]	1.1±0.3	1.3±0.1	**0.036**
CLT, (min)[a]	34.8±9.9	37.3±10.1	0.386

[a] Mean ±SD; [b] Mann Whitney U test . ETP-endogenous thrombin potential; CLT-clot lysis time; Cmax Abs- clot maximum absorbance

Correlation between haemostatic parameters and either PFTs or echocardiography findings are tabulated in Table 24. Interestingly, Cmax was significantly inversely correlated with both markers of ILD severity while longer values of CLT were negatively related with DLCO%. Indeed, testing both FVC% and DLCO% as dichotomous variable as indicating in Table 25., it has been noted that significantly increased clot density and diminished fibrinolysis were present in cases with DLCO <70%**,** while patients with FVC<80% had significantly higher Cmax.

Table 24. Correlation of ETP, Cmax, CLT with DLCO%, FVC% and sPAP

		Pulmonary functional tests		**Echocardiography**
Haemostatic parameters		**DLCO%**	**FVC%**	**sPAP**
ETP, (nM/min)	r	-0.153	-0.094	0.015
	p	0.252	0.484	0.909
Cmax, (Abs)	r	-0.422	-0.371	0.129
	p	**0.001**	**0.004**	0.336
CLT, (min)	r	-0.270	0.030	0.136
	p	**0.040**	0.821	0.308

r- correlation coefficient; ETP-endogenous thrombin potential; CLT-clot lysis time; Cmax Abs- clot maximum absorbance; DLCO- diffusing capacity for carbon monoxide; FVC-forced vital capacity; sPAP: systolic pulmonary artery pressure

Table 25. Differences in haemostatic parameters according to ILD severity parameters

Haemostatic parameters	**DLCO% (n)**		**FVC% (n)**	
	<70 (35)	**≥70 (22)**	**<80 (11)**	**≥80 (47)**
ETP, (nM/min)[a]	2022.9±396.4	1871.8±335.6	2084.5±291.3	1934.5±392.5
p value	0.138[b]		0.159[c]	
Cmax, (Abs)[a]	1.2±0.2	0.9±0.3	1.3±1.9	1.1±0.3
p value	**0.001[b]**		**0.005[c]**	
CLT, (min)[a]	38.1±9.8	31.6±9.1	36.6±10.1	35.2±10.0
p value	**0.014[b]**		0.677[c]	

[a]Mean ±SD; [b] T test; [c] Mann Whitney U test. ETP-endogenous thrombin potential; CLT-clot lysis time; Cmax Abs- clot maximum absorbance; DLCO- diffusing capacity for carbon monoxide; FVC-forced vital capacity

Further on, significant correlation was not observed between haemostatic parameters and echocardiography sign of PAH, sPAP (Table 26). However, patients with FVC/DLCO ratio >1.6 had a trend of longer CLT compared to those without, as shown in Table 26.

Table 26. Differences in haemostatic parameters according to FVC/DLCO ration

Haemostatic parameters	**FVC/DLCO (n)**		
	≤1.6 (36)	**>1.6 (20)**	**p value[b]**
ETP, (nM/min)[a]	1941.9±324.8	2003.0±469.3	0.607
Cmax, (Abs)[a]	1.1±0.3	1.2±0.3	0.250
CLT, (min)[a]	33.7±9.7	38.9±9.8	0.056

[a]Mean ±SD; [b] T test. ETP-endogenous thrombin potential; CLT-clot lysis time; Cmax Abs- clot maximum absorbance; DLCO- diffusing capacity for carbon monoxide; FVC- forced vital capacity

In a line with above observed results regarding PFTs, ILD was characterized with notably increased clot density and its prolonged lysis. Moreover, when adjusting for pack years, levels of Cmax and CLT remained significantly associated with ILD, emphasizing that with each increases of Cmax for one value, risk of having ILD is around 16 fold higher (Table 27).

Table 27. Differences in haemostatic parameters according to ILD presence

Haemostatic parameters	**ILD (n)**		**p value[b]**	**OR[c] (CI 95%)**	**p value**
	Without (30)	**With (28)**			
ETP, (nM/min)[a]	1981.8±370.2	1942.8±391.5	0.698	1.00(0.99-1.00)	0.752
Cmax, (Abs)[a]	1.1±0.3	1.2±0.2	0.024	16.25 (1.43-185.25)	**0.025**
CLT, (min)[a]	32.6±9.1	38.6±10.1	0.020	1.08 (1.01-1.12)	**0.019**

[a]Mean ±SD; [b] T test,[c] OR- odds ration adjusted for pack years; CI- confidence interval ETP-endogenous thrombin potential; CLT-clot lysis time; Cmax Abs- clot maximum absorbance; ILD-interstitial lung disease

As presented in Table 28.among patients without ILD, Cmax showed significant negative correlation with DLCO% and trend of relation with FVC%, while prolongation of lysis time was associated with decreasing of FVC%. On the other side, only within ILD patients enhanced thrombin generation was negatively related FVC%.

Table 28. Correlation of haemostatic parameters with DLCO%, and FVC% in regard to presence of ILD

Without ILD			
		Pulmonary functional tests	
Haemostatic parameters		**DLCO%**	**FVC %**
ETP, (nM/min)	r	-0.147	0.097
	p	0.439	0.612
Cmax, (Abs)	r	-0.432	-0.357
	p	**0.017**	*0.053*
CLT, (min)	r	-0.132	-0.364
	p	0.487	**0.048**

With ILD			
		Pulmonary functional tests	
Haemostatic parameters		**DLCO%**	**FVC%**
ETP, (nM/min)	r	-0.288	-0.391
	p	0.137	**0.040**
Cmax, (Abs)	r	-0.180	-0.191
	p	0.360	0.330
CLT, (min)	r	-0.115	-0.053
	p	0.560	0.790

r- correlation coefficient; ILD-interstitial lung disease; ETP-endogenous thrombin potential;

CLT-clot lysis time; Cmax Abs- clot maximum absorbance; DLCO- diffusing capacity for carbon monoxide; FVC- forced vital capacity

Assessment of the association between thrombin generation and fibrin clot features with the presence of peripheral vascular manifestation demonstrated that only pitting scars were related with significantly altered thrombin generation and prolonged lysis time as demonstrated in Table 29.

Table 29. Haemostatic parameters within different peripheral vascular manifestations

	Peripheral vascular manifestations			
Haemostatic parameters	**Pitting scars (n)**		**Acroosteolisis (n)**	
	No (25)	**Yes (33)**	**No (45)**	**Yes (13)**
ETP, (nM/min)[a]	1841.3±273.2	2055.1±421.9	1958.7±333.7	1977.6±519.1
p value	**0.023[b]**		0.787 [c]	
Cmax, (Abs) [a]	1.1±0.3	1.19±0.2	1.14±0.3	1.14±0.2
p value	0.120 [b]		0.356 [c]	
CLT, (min)[a]	31.3±7.3	38.67±10.6	34.65±9.1	38.4±12.4
p value	**0.003 [b]**		0.391 [c]	

[a]Mean ±SD; [b] T test; [c] Mann Whitney U test. ETP-endogenous thrombin potential; CLT-clot lysis time; Cmax Abs- clot maximum absorbance

In order to assess more specifically hemostasis in microvascular disease, differences in haemostatic parameters across NVC patterns have been explored (Table 30). Although an increase in mean values of Cmax and CLT were noticed with progression of capillaroscopy findings, significant difference was not found between the groups.

Table 30. Levels of haemostatic parameters according to NVC patterns

	NVC patterns (n)			
Haemostatic parameters	**Early (16)**	**Active (24)**	**Late (18)**	**p value[b]**
ETP, (nM/min) [a]	1880.3±276.7	1997.6±435.7	1990.3±381.2	0.521
Cmax, (Abs) [a]	1.1±0.3	1.1±0.3	1.2±0.3	0.411
CLT, (min)[a]	32.5±8.3	35.4±10.5	38.3±10.3	0.242

[a]Mean ±SD; [b] one way ANOVA. ETP-endogenous thrombin potential; CLT-clot lysis time; Cmax Abs-clot maximum absorbance; NVC- nailfold videocapillaroscopy

However, when more specific sign of late NVC pattern including loss of capillaries were analysed (Table 31.), it has been found that Cmax levels significantly differed between subgroups, with the highest value observed in patients with severe loss followed by those with moderate loss in respect to patients without/few loss.

Table 31. Levels of haemostatic parameters according to loss of capillaries

Haemostatic parameters	Loss of capillaries (n)			
	no/few (16)	Moderate (24)	Severe (18)	p value
ETP, (nM/min)[a]	1881.7±285.3	1999.1±435.9	1987.0±375.5	0.595[b]
Cmax, (Abs)[a]	0.9±0.3	1.2±0.2[*]	1.2±0.3[*]	**0.006[b]**
CLT, (min)[a]	31.2±6.9	35.9±10.8	38.8±10.3	0.116[c]

[a]Mean ±SD; [b] one way ANOVA adjusted by the Bonferroni correction for multiple testing; [c]Kruskal Wallis.[*] p<0.05 observed between no/few with either sever and moderate loss. ETP-endogenous thrombin potential; CLT-clot lysis time; Cmax Abs- clot maximum absorbance

Moreover, cases with ramified/bushy capillaries had significantly higher mean values of Cmax (Table 32.) versus those without, confirming that denser fibrin clots are present within late NVC pattern.

Table 32. Differences in haemostatic parameters according to presence of ramified/bushy capillaries

Haemostatic parameters	ramified/bushy capillaries (n)		p value[b]
	Absent (41)	Present (17)	
ETP, (nM/min)[a]	1920.8±355.9	2064.7±419.8	0.297
Cmax, (Abs)[a]	1.1±0.3	1.3±0.3	**0.040**
CLT, (min)[a]	34.1±9.4	38.9±10.8	0.137

[a]Mean ±SD; [b]Man Whitney U test; ETP-endogenous thrombin potential; CLT-clot lysis time; Cmax Abs-clot maximum absorbance

Concerning therapeutic modalities, significantly higher ETP levels were found in individuals with GC therapy, while those taking MTX had trend of increased values. In contrary, CYP therapy showed potential beneficial effect on thrombin generation, since trend of lower values was observed in patients treated previously with this therapy option even though higher values of Cmax and CLT were also noticed within same group but without reaching statistical significance (Table 33).

Table 33**.** Levels of haemostatic parameters in patients with different therapeutic modalities

Haemostatic parameters	Therapeutic modalities					
	Glucocorticoids (n)		Methotrexate(n)		Cyclophosphamide(n)	
	No(36)	Yes(22)	No(44)	Yes(14)	Never (34)	Ever(24)
ETP, (nM/min)[a]	1875.8±262.8	2108.9±483.0	1897.4±330.4	2174.2±442.9	2036.3±351.9	1861.9±392.5
p value	**0.046[b]**		*0.054 [c]*		*0.089 [b]*	
Cmax, (Abs) [a]	1.1±0.3	1.2±0.2	1.1±0.3	1.2±0.2	1.1±0.3	1.2±0.3
p value	0.277 [b]		0.284 [c]		0.517 [b]	
CLT, (min)[a]	34.8±8.8	36.6±11.7	35.2±11.5	35.6±9.6	35.0±9.7	26.2±10.5
p value	0.557 [b]		0.637[c]		0.663 [b]	

[a]Mean ±SD; [b] T test, [c] Mann Whitney U test; ETP-endogenous thrombin potential; CLT-clot lysis time; Cmax Abs-clot maximum absorbance

Further on, when daily prednisolone and cumulative CYP doses were taken into account (Table 34.), it has been observed that the cumulative dose of CYP had moderate positive relation with levels of Cmax and a trend of week correlation with CLT.

Table 34**.** Correlation of haemostatic parameters with either Prednisolone or Cyclophosphamide doses

Haemostatic parameters		**Prednisolone, (mg/day)**	**Cyclophosphamide cumulative dose,(g)**
ETP, (nM/min)	r	0.050	0.121
	p	0.826	0.575
Cmax, (Abs)	r	0.062	**0.412**
	p	0.783	**0.045**
CLT, (min)	r	-0.031	0.318
	p	0.891	*0.066*

r- correlation coefficient; ETP-endogenous thrombin potential; CLT-clot lysis time; Cmax Abs- clot maximum absorbance

Moreover, it hasn't been found statisticaly significant beneficial impact of taking antiplatelet therapy (ASA 75mg/per day) on both thrombin generation and fibrin clot properties (Table 35).

Table 35. Differences in haemostatic parameters regarding ASA therapy

Haemostatic parameters	**Acetylsalicylic Acid (n)**		**p -value[b]**
	No (35)	**YES (23)**	
ETP, (nM/min)[a]	1967.1±399.3	1956.6±351.3	0.918
Cmax, (Abs)[a]	1.1±0.3	1.2±0.3	0.071
CLT, (min)[a]	34.3±9.7	37.4±10.3	0.253

[a]Mean ±SD; [b] T test; ETP-endogenous thrombin potential; CLT-clot lysis time; Cmax Abs- clot maximum absorbance

4.4.2. Association of Inflammation and endothelial dysfunction with hemostasis

As it shown in Table 36, nearly all investigated haemostatic parameters were significantly positively correlated with inflammatory markers including CRP, ESR and fibrinogen, however only between ETP and ESR correlation haven't shown statisticaly significance.

Table 36. Correlation between haemostatic parameters and inflammatory markers

		Inflammatory markers		
Haemostatic parameters		**ESR,(mm/h)**	**Fibrinogen,(g/L)**	**CRP,(mg/l)**
ETP, (nM/min)	r	0.206	0.324	0.351
	p	0.122	**0.013**	**0.007**
Cmax, (Abs)	r	0.428	0.393	0.330
	p	**0.001**	**0.002**	**0.012**
CLT, (min)	r	0.295	0.340	0.264
	p	**0.025**	**0.009**	**0.045**

r- correlation coefficient; ETP-endogenous thrombin potential; CLT-clot lysis time; Cmax Abs- clot maximum absorbance; ESR- sedimentation rate; CRP- C reactive protein

When correlation between haemostatic parameters and vascular markers was explored (Table 37), significant positive correlation between ICAM1 and all investigated haemostatic parameters was observed. Lysis time had also week significant positive association with VWF:Ag and trend of relation with either VCAM1 or E selectin.

Table 37. Correlation between haemostatic parameters and vascular markers

		Vascular markers				
Haemostatic parameters		**ICAM1 (ng/ml)**	**VCAM1 (ng/ml)**	**E selectin (ng/ml)**	**P selectin (ng/ml)**	**VWF:Ag**
ETP, (nM/min)	r	0.282	-0.169	0.010	0.172	0.197
	p	**0.034**	0.204	0.940	0.198	0.138
Cmax, (Abs)	r	0.375	0.073	0.085	0.072	0.174
	p	**0.004**	0.589	0.527	0.592	0.192
CLT, (min)	r	0.470	0.238	0.228	0.144	0.279
	p	**<0.001**	0.072	0.085	0.280	**0.034**

r- correlation coefficient; ETP-endogenous thrombin potential; CLT-clot lysis time; Cmax Abs- clot maximum absorbance; ICAM-1- intercellular adhesion molecule 1; VCAM1- vascular adhesion molecule 1; VWF:Ag- von Willebrand factor antigen

When analyzing interrelation between assessed haemostatic parameters, it was found that ETP had positive correlation with both CLT and Cmax (r=0.476, p<0.001; r=0.551, p<0.001, respectively) and as expected with increasing of clot density, the clot lysis time was longer (r=0.500, p<0.001).

Further on, focusing on results previously observed within ILD patients, correlation analysis between ETP and both markers of inflammation and endothelial injury was done in order to explore if any of them could have impact on ETP. Significant relation ETP with CRP, fibrinogen and WVF Ag was observed as presented in Table 38.

Table 38. Correlation analysis between ETP and both markers of inflammation and endothelial injury within ILD patients

Haemostatic parameters		**CRP (mg/l)**	**Fibrinogen (g/l)**	**ICAM1 (ng/ml)**	**VCAM1 (ng/ml)**	**E selectin (ng/ml)**	**P selectin (ng/ml)**	**WVF Ag**
ETP, (nM/min)	r	0.439	0.451	0.307	0.080	-0.047	0.063	0.376
	p	**0.019**	**0.016**	0.112	0.687	0.811	0.751	**0.049**

r- correlation coefficient; ETP-endogenous thrombin potential; CRP- C reactive protein; ICAM-1- intercellular adhesion molecule 1; VCAM1- vascular adhesion molecule 1; VWF:Ag- von Willebrand factor antigen

4.4.3. Regression analysis for haemostatic parameters

To identify variables with independent impact on chosen haemostatic parameters multivariable linear regression was done. Even CRP showed positive relation with both ETP and Cmax (β=15.4, 95%CI -1.56-32.300, p=0.074; β=0.01, 95%CI 0.002-0.03, p=0.026, respectively) after adjustment for fibrinogen, the association wasn't stay significant (β=7.467, 95%CI -10.81-25.74, p=0.416; β=0.007, 95%CI -0.01-0.02, p=0.317, respectively). As presented in Table 39, fibrinogen was independently positively

associated with levels of ETP, while Cmax was positively predicted by both ICAM1 and fibrinogen (Table 40).

Table 39. Linear regression analysis for ETP as dependent variable

	ETP			
Variable	**Univariable linear regression**		**Multivariable linear regression**	
	β (95% CI)	**p**	**β (95% CI)**	**p**
ICAM1, ng/ml	13.39(1.19-25.59)	0.032	9.41(-3.07-21.89)	0.137
Fibrinogen, g/l	60.01(14.26-105.75)	0.011	48.85 (1.25-96.46)	**0.044**

β –unstandardized coefficient; CI –confidence interval; ETP- endogenous thrombin potential; ICAM-1- intercellular adhesion molecule 1

Table 40. Linear regression analysis for Cmax as dependent variable

	Cmax			
Variable	**Univariable linear regression**		**Multivariable linear regression**	
	β (95% CI)	**p**	**β (95% CI)**	**p**
ICAM1, ng/ml	0.01(0.01-0.02)	0.004	0.01(0.001-0.02)	**0.033**
Fibrinogen, g/l	0.06(0.02-0.09)	0.001	0.05 (0.01-0.08)	**0.008**

β –unstandardized coefficient; CI –confidence interval; ICAM-1- intercellular adhesion molecule 1; Cmax Abs- clot maximum absorbance

All variables previously significantly correlated with CLT were adjusted for fibrinogen, and only ICAM1 and ETP remained predictive (Table 41.). Further when model with ICAM1, ETP and fibrinogen was run, explaining 42% of CLT variability, ICAM1 and ETP still were independently positively associated with CLT (β=0.400, 95%CI 0.119-0.682, p=0.002;β=0.011, 95%CI 0.005-0.017,p<0.001; respectively).

Table 41. Linear regression analysis for CLT as dependent variable

	CLT	
	Multivariate linear regression	
Variable	**β [a] (95% CI)**	**p-value**
CRP, mg/l	-0.02(-0.50-0.47)	0.945
ICAM1, ng/ml	0.51(0.20-0.82)	**0.002***
VWF:Ag	-0.04(-2.64-2.56)	0.975
ETP, (nM/min)	0.01(0.01-0.02)	**<0.001***

β [a] - unstandardized coefficient adjusted for fibrinogen;* Multivariable analysis (model: ICAM1, ETP, fibrinogen); ETP-endogenous thrombin potential; CLT-clot lysis time; CRP-C reactive protein; ICAM-1- intercellular adhesion molecule 1; VWF:Ag- von Willebrand factor antigen

4.5. Endothelial dysfunction and hemostasis imbalance in patients with a history of DUs

In our cohort, 67% patients had history of DUs. Baseline demographic characteristics of patients with history of DUs compared to either DU naïve or controls in the case control study are displayed in Table 42. As it can be observed study groups did not differ in terms of demographic, lifestyle and comorbidities characteristics including age, gender, prevalence of obesity, smoking and arterial hypertension.

Table 42. Baseline characteristics of SSc patients with history of DU, DU naïve and controls.

Baseline characteristics	DU(39)	DU naïve(19)	Controls(46)	p-value
Age (years) [a]	52.9±10.7	57.2±11.5	51.3±9.3	0.116 [d]
Gender, n(%)				
Male	5(13)	2(10)	5 (11)	
Female	34(87)	17(90)	41 (89)	0.950[e]
BMI (kg/m2)[a]	23.8±3.8	23.4±3.4	23.1±2.8	0.782[b]
Obesity, n(%)				
BMI<25	25(64)	10(53)	33(72)	
BMI≥25	14(36)	9(48)	13(28)	0.568 [e]
Smoking, n(%)				
Never	26(67)	16(84)	36 (78)	
Ever[b]	13(33)	3(16)	9 (20)	0.277 [e]
Pack-years[c]	0(0-60)	0(0-40)	0(0-40)	0.292[f]
Arterial hypertension, n (%)				
No	18(46)	9(47)	24 (52)	
Yes	21(54)	10(53)	22 (48)	0.847 [e]

[a]Mean±SD; [b] at lest of 60-day period any time prior to the study onset; [c]Median (Min-Max); [d] one way ANOVA; [e]X^2 test; [f]Kruskal-Wallis test; DU-digital ulcer

Regarding disease characteristics, DUs were associated with younger age at SSc onset and longer duration of disease. (Table 43). The frequency of active and late NVC pattern were higher in DUs patients than in DU naïve. Cases with active pattern were at 6.6-fold higher risk while those with late NVC pattern were at 17.60 fold higher risk of DUs development over course of disease compared to those with early pattern. Further, individuals with ILD were 2.8 times more susceptible to DUs, but this association wasn't statistically significant.

Table 43. Disease characteristics between DU groups

Disease characteristics	DU(39)	DU naïve (19)	OR 95%(CI)	p
Age at disease onset, (years)[a]	45.2±10.8	53.3±12.4	0.93 (0.88-0.98)	**0.018**
Disease duration, (years)[b]	6(0-29)	3(0-11)	1.12(1.00-1.25)	**0.047**
Cutaneous subtype, n (%)				
Limited	23(59)	13(68)	1.00[c]	
Diffuse	16(41)	6 (32)	1.50(0.47-4.80)	0.488
mRSS[b]	11(4-31)	7(3-29)	1.1 (1.00-1.20)	*0.086*
Autoantibody status, n (%)				
Anti-centromere Ab	13 (33)	9 (47)	0.55(0.18-1.70)	0.304
Anti-topoisomerase I Ab	19 (49)	6 (32)	2.05(0.64-6.52)	0.220
Sclerodactily, n (%)	27(69)	9(47)	2.5(0.81-7.73)	0.111
Calcinosis, n (%)	11(29)	2(11)	3.34(0.66-16.92)	0.145
Contracture, n (%)	13 (33)	3 (16)	2.66(0.66-10.83)	0.170
FVC, (%)[a]	96.2±19.1	94.3±18.3	1.01(0.98-1.04)	0.711
DLCO, (%)[a]	64.2±16.9	67.2±14.9	0.99(0.95-1.02)	0.502
ILD, n(%)	22(79)	6(32)	2.8(0.88-8.91)	*0.080*
sPAP, (mmHg)[a]	30.1±6.3	30.9±9.7	0.99(0.92-1.06)	0.714

RP present, n (%)	19(49)	9(47)	1.06(0.34-3.16)	0.923
Telangiectasia present, n(%)	26(67)	13(68)	0.92(0.28-2.99)	0.894
NVC pattern, n(%)				
Early	5(13)	11(58)	1.00[c]	
Active	18(46)	6 (32)	6.60(1.62-26.87)	**0.008**
Late	16(41)	2(11)	17.60(2.88-107.61)	**0.002**

[a] Mean±SD; [b] Median (Min-Max); [c] reference group; OR-odds ration; CI - confidence interval; DU-digital ulcer; mRSS –modified Rodnan skin score; FVC-forced vital capacity; DLCO- diffusing capacity for carbon monoxide; ILD- interstitial lung disease; sPAP- systolic pulmonary artery pressure; NVC-nailfold videocapillaroscopy;

Assessing HRQL by SHAQ DI, it was observed that patients with history of DUs had much higher level of pain, slightly impaired gripping and trend of more severe RP in respect to those without (Table 44).

Table 44. Difference between groups according to SHAQ DI domains

SHAQ DI variable	**DU(39)**	**DU naïve (19)**	**OR 95%(CI)**	**p**
Dressing	0(0-2)	0(0-3)	1.40 (0.62-3.16)	0.416
Arising	0(0-2)	0(0-2)	0.67(0.24-1.88)	0.448
Eating	1(0-3)	1(0-3)	1.45(0.73-2.87)	0.289
Walking	0(0-2)	0(0-3)	0.53(0.25-1.12)	*0.096*
Hygiene	0(0-2)	0(0-2)	1.15(0.29-4.49)	0.845
Reaching	0(0-2)	0(0-2)	1.34(0.55-3.28)	0.514
Gripping	1(0-3)	0(0-3)	1.98(0.92-4.29)	*0.082*
Activity	0(0-2)	0(0-3)	1.07(0.53-2.15)	0.860
Overall	0.37(0-2)	0.25(0-26)	1.35(0.42-4.26)	0.628
VAS scale, mm				
Pain	36(0-91)	10(0-88)	1.03(1.01-1.06)	**0.021**
RP	35(0-81)	19(0-72)	1.02(0.99-1.05)	*0.082*
Lung	0(0-90)	0(0-74)	1.00(0.98-1.03)	0.748
GIT	0(0-52)	0(0-36)	1.04(0.98-1.10)	0.219

Results are presented as Median (Min-Max); OR-odds ratio, CI-confidence interval; SHAQ-Scleroderma Health Assessment Questionnaire; DU-digital ulcers; VAS- visual analog scale; RP-Raynaud phenomenon; GIT-gastrointestinal tract

When EQ5D was analyzed, in a line with previous observation, pain impacted significantly patients with DUs. Further, presence of DUs was associated with slightly higher level of anxiety and depression and with significantly reduced overall quality of life (Table 45).

Table 45. Difference between groups according EQ5D domains

EQ5D domains	**DU(39)**	**DU naïve (19)**	**OR (95%CI)**	**p-value**
Mobility[a]	1(1-2)	1(1-2)	0.78(0.17-3.69)	0.759
Self-care[a]	1(1-2)	1(1-2)	1.55(0.28-8.49)	0.617
Usual activities[a]	1(1-3)	1(1-2)	1.31(0.44-3.90)	0.634
Pain and discomfort[a]	2(1-3)	1(1-2)	4.25(1.36-13.27)	**0.013**
Anxiety and depression[a]	2(1-3)	2(1-2)	2.76(0.95-8.01)	0.061
EQ5D utility index[b]	0.68±0.17	0.82±0.18	0.01(0.00-0.34)	**0.012**

VAS scale, mm				
Overall health[b]	62.3±21.2	71.4±25.8	0.98(0.96-1.01)	0.159

[a]Median (Min-Max); [b] Mean±SD; OR-odds ratio, CI-confidence interval; DU-digital ulcers; VAS-visual analog scale

Distribution of therapy modalities among DU groups is presented in Table 46. As indicated, there were no differences in the therapy regimes between the groups.

Table 46. Treatment modalities between DU groups

Treatment, n (%)	DU(39)	DU naïve(19)	OR 95% (CI)	p
I. Ongoing treatment				
Glucocorticoids	16(41)	6(32)	1.51 (0.47-4.80)	0.488
Metothrexate	9(23)	5(26)	0.84 (0.23-2.93)	0.787
Chloroquine	3(8)	2(11)	0.71 (0.11-4.64)	0.719
Azathioprine	6(16)	3(17)	0.94 (0.21-4.26)	0.933
ACE inhibitor	14(36)	7(37)	0.96 (0.31-2.91)	0.944
Calcium channel blocker	19(49)	10(53)	0.84 (0.28-2.53)	0.780
Beta blockers	8(21)	4(21)	0.96 (0.25-3.73)	0.962
ASA	13(33)	10(53)	0.44 (0.14-1.33)	0.162
II. Previous treatment				
Cyclophosphamide	18(46)	6(32)	1.85 (0.58-5.80)	0.293

OR-odds ration; CI - confidence interval; ACE-angiotensin converting enzyme; ASA – Acetylsalicylic acid; DU-digital ulcers

As presented in Table 47. , there was no difference between observed groups according markers of either renal function, lipid profile or complement levels. All investigated inflammatory markers were significantly elevated in patients with DUs compared to controls, while ESR was increased in DU naïve group compared to controls. On the other hand, only fibrinogen was significantly elevated in DUs versus naïve cases.

Table 47. Differences between groups according laboratory parameters

Laboratory parameters	DU(39)	DU naïve(19)	Controls(46)	p
Markers of renal function				
Urea, (mmol/L)[a]	5.0±1.6	5.7±1.9	4.8±1.7	0.129 [c]
Creatinine, (ηmol/L) [a]	75.1±11.4	82.6±28.8	74.2±9.1	0.121 [c]
Lipid panel				
Cholesterol, (mmol/L) [a]	6.6±1.5	6.7±1.7	6.1±2.1 [a]	0.274 [c]
Triglycerides, (mmol/L) [a]	1.6±0.6	1.4±0.7	1.3±0.6 [a]	0.216 [c]
Inflammatory markers				
CRP, (mg/l) [b]	5.4 (0.2-16.4)*****	3.4 (0-30.6)	2.5 (0-12.4) [b]	0.001[d]
ESR,(mm/hr) [b]	18 (4-66)*****	22 (4-60)#	10 (2-42)	<0.001[d]
Fibrinogen, (g/L) [a]	4.8±2.0 ***,¶**	3.6±2.1	2.9±0.7	<0.001[d]
Complement level				
C3, (g/L) [a]	1.3±0.3	1.3±0.3	1.3±0.2 [a]	0.934 [c]
C4, (g/L) [a]	0.2±0.1	0.2±0.1	0.2±0.1 [a]	0.170 [d]

[a] Mean±SD; [b] Median (Min-Max); [c] one way ANOVA with post hoc analysis; [d] Kruskal Wallis with post hoc analysis; 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 control group; ¶p <0.05 observed between two DUs groups; DUs-digital ulcers; CRP- C reactive protein; ESR –erythrocyte sedimentation rate

4.5.1. Differences in endothelial vascular markers and haemostatic parameters across the groups

Differences in levels of various vascular biomarkers across different groups are summarized in Table 48.Nearly all investigated biomarkers including ICAM1, VCAM1, E selectin and VWF:Ag, differed across compared groups with the highest levels in the patients with DU compared to controls subjects (p=0.006 ,p=0.001, p=0.016, p<0.001, respectively). However, despite elevated levels of all markers of endothelial dysfunction in DU group compared to DU naïve patients, the difference has not reached statistical significance

Table 48. Vascular markers between DUs groups and controls

Vascular biomarkers	DU (39)	DU naïve (19)	Controls (46)	p value
ICAM1, (ng/ml)[a]	30.3±8.7*****	27.8±5.8	23.3±4.6	**0.008[c]**
VCAM1, (ng/ml)[b]	39.1(16.7-1.3)*****	33.5(20.2-138.5)	30.8 (20.8-47.4)	**0.002[c]**
E selectin, (ng/ml)[a]	5.5±2.1*****	4.5±1.5	4.1±1.7	**0.013[d]**
P selectin, (ng/ml)[b]	6 (0-24.8)	4.9 (0-23.8)	5.3 (0-9.9)	0.194[c]
VWF:Ag[b]	1.7 (0.9-7.6)*****	1.5 (0.7-2.9)[#]	1.2 (0.6-3.3)	**<0.001[d]**

[a] Mean±SD; [b] Median (Min-Max); [c] Kruskal Wallis with post hoc analysis; [d] one 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 control group; DU-digital ulcers; ICAM1- intercellular adhesion molecule 1; VCAM1- vascular adhesion molecule 1; vWF:Ag- von Willebrand factor antigen

The levels of haemostatic parameters assessed by overall haemostatic assays are presented in Table 49. CAT assay showed that ETP significantly varied across groups with notably enhanced levels in individuals with DUs versus controls. Although, parameters from OHP assay did not significantly differ between groups, altered fibrin clot properties with higher Cmax and shorter CLT were observed in DUs subjects compared to controls. In addition, a trend of lag time extension in coagulation assay across groups was found, with the shortest average time in DUs groups. The most prominent result was prolonged CLT in DU compared to naïve patients.

Table 49. Difference between groups according haemostatic parameters

Haemostatic parameters	DU (39)	DU naïve (19)	Controls (46)	p value
Calibrated automated thrombogram assay				
ETP, (nM/min)[a]	2020.0±405.3*****	1846.0±289.4	1756.3±321.2	**0.001[b]**
Lag time, (min) [a]	3.2±0.5	3.2±0.7	3.3±0.8	0.499[b]
Peak, (nM) [a]	326.31±87.8	329.0±85.9	293.3±66.8	*0.075[b]*
Time to peak, (min) [a]	6.2±1.3	6.2±1.7	6.5±1.3	0.248[b]
Overall haemostatic potential assay				
OHP, (Abs-sum) [a]	131.2 ±78.2	122.1±86.9	106.6±58.9	*0.072[c]*

OCP, (Abs-sum) [a]	318.4±56.2	290.7±114.2	294.4±63.4	0.312[c]
OFP, (%)[a]	53.1±18.1	58.6±11.8	61.3±12.1	0.178[c]
Turbidity assay				
Coagulation				
Lag phase clot, (min) [a]	5.2±0.9	5.4±1.1	5.7±1.7	0.096[b]
Cmax, (Abs) [a]	1.2±0.2*****	1.0±0.4	1.0±0.2	**0.030[c]**
Fibrinolysis				
Lag phase lysis, (min) [a]	5.7±2.0	6.5±3.6	6.2±2.1	0.387[b]
CLT,(min) [a]	37.5±10.5***,¶**	30.5±6.7	30.7±7.9	**0.002[c]**

[a]Mean ±SD; [b] one way ANOVA; [c] Kruskal Wallis with post hoc analysis; 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 two DU groups; DU-digital ulcers; ETP-endogenous thrombin potential; OHP- overall haemostasis potential; OCP - overall coagulation potential; OFP-overall fibrinolysis potential; Cmax Abs- clot maximum absorbance; CLT-clot lysis time

In order to assess whether inflammatory status, endothelial dysfunction and hemostasis might underly healing process the difference between patients with active and case without DUs was analyzed. As indicated, there was no significant differences between groups (Table 50).

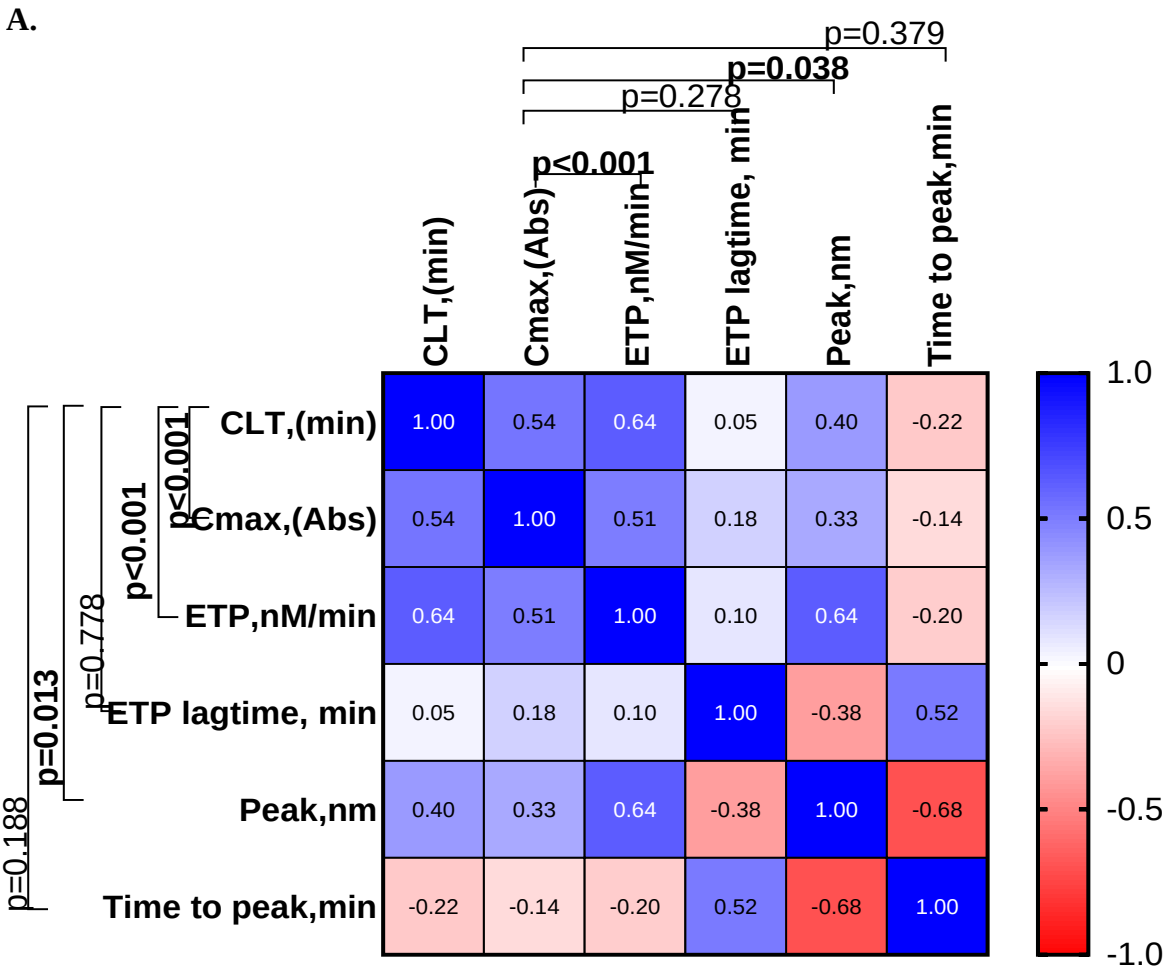
Table 50. Inflammatory, vascular markers and haemostatic parameters in patients with- and without active DUs at baseline

Variable	**Active DU (18)**	**Non active DU (21)**	**OR 95%(CI)**	**p value**
Inflammatory biomarkers				
ESR, (mm/hr) [a]	35(4-66)	16 (4-58)	1.04(0.98-1.08)	0.192
CRP, (mg/l) [a]	6.9(0.3-12.2)	4 (0.2-16.4)	1.01(0.87-1.16)	0.932
Fibrinogen, (g/l) [b]	5.1±2	4.7±2	1.09(0.79-1.52)	0.573
Vascular biomarkers				
ICAM1,(ng/ml) [b]	.29.3±9.9	30.6±7.8	0.99(0.92-1.07)	0.806
VCAM, (ng/ml) [b]	41.4±9.4	41.1±16.4	1.00(0.95-1.05)	0.944
E selectin, (ng/ml) [b]	5.5±2.1	5.5±2.2	0.99(0.74-1.33)	0.954
P selectin, (pg/ml) [a]	6.5(0-24.8)	5.9(0.9-12.6)	1.09(0.95-1.26)	0.204
VWF: Ag [a]	1.7 (0.9-7.6)	1.8 (1-3.3)	1.20(0.65-2.23)	0.560
CAT assay				
ETP, (nM/min) [b]	1902.9±352	2120.3±428.7	0.99(0.99-1.00)	0.101
Lag time, (min) [b]	3.2±0.5	3.2±0.4	0.98(0.26-3.71)	0.973
Peak, (nM) [b]	307.6±86.6	342.3±87.7	0.99(0.99-1.00)	0.218
Time to peak, (min) [b]	6.2±1.6	6.2±1.1	1.04(0.65-1.67)	0.879
OHP assay				
OHP, (Abs-sum) [b]	148.1±71.8	153.5±69.2	0.99(0.99-1.01)	0.808
OCP, (Abs-sum) [b]	309.8±60	320.3±49.9	0.99(0.99-1.01)	0.819
OFP, (%) [b]	53.7±17.9	52.5±18.5	1.00(0.97-1.04)	0.830
Turbidity assay				
Lag phase clot, (min) [b]	4.9±1	5.1±1	0.85(0.41-1.75)	0.661
Cmax, (Abs) [b]	1.2±0.3	1.2±0.2	1.97(0.13-30.24)	0.627
CLT, (min) [b]	38.9±10.5	37.1±10.6.	1.02(0.96-1.08)	0.590

OR-odds ration; CI - confidence interval; [a] Median (min-max);[b] Mean ±SD ;DU-digital ulcer; CRP -C reactive protein; ESR - erythrocyte sedimentation rate, ICAM1- Intercellular Adhesion Molecule 1; VCAM1-Vascular cell adhesion protein1; CAT- Calibrated automated thrombogram; ETP- endogenous thrombin potential; OHP- overall haemostasis potential, OCP - overall coagulation potential; OFP-overall fibrinolysis potential; Cmax Abs- maximum clot absorbance; CLT-clot lysis time

4.5.2. Association between endothelial dysfunction and hemostasis in patients with history of DUs

As presented in Figure 17, both Cmax and CLT showed positive correlation with either ETP or peak of thrombin generation, while as expected Cmax and CLT remained significantly positively associated within those with DUs as they were in whole patients cohort. Regarding vascular markers, also in a line with previous observation, CLT and Cmax were positively correlated with levels of ICAM1.



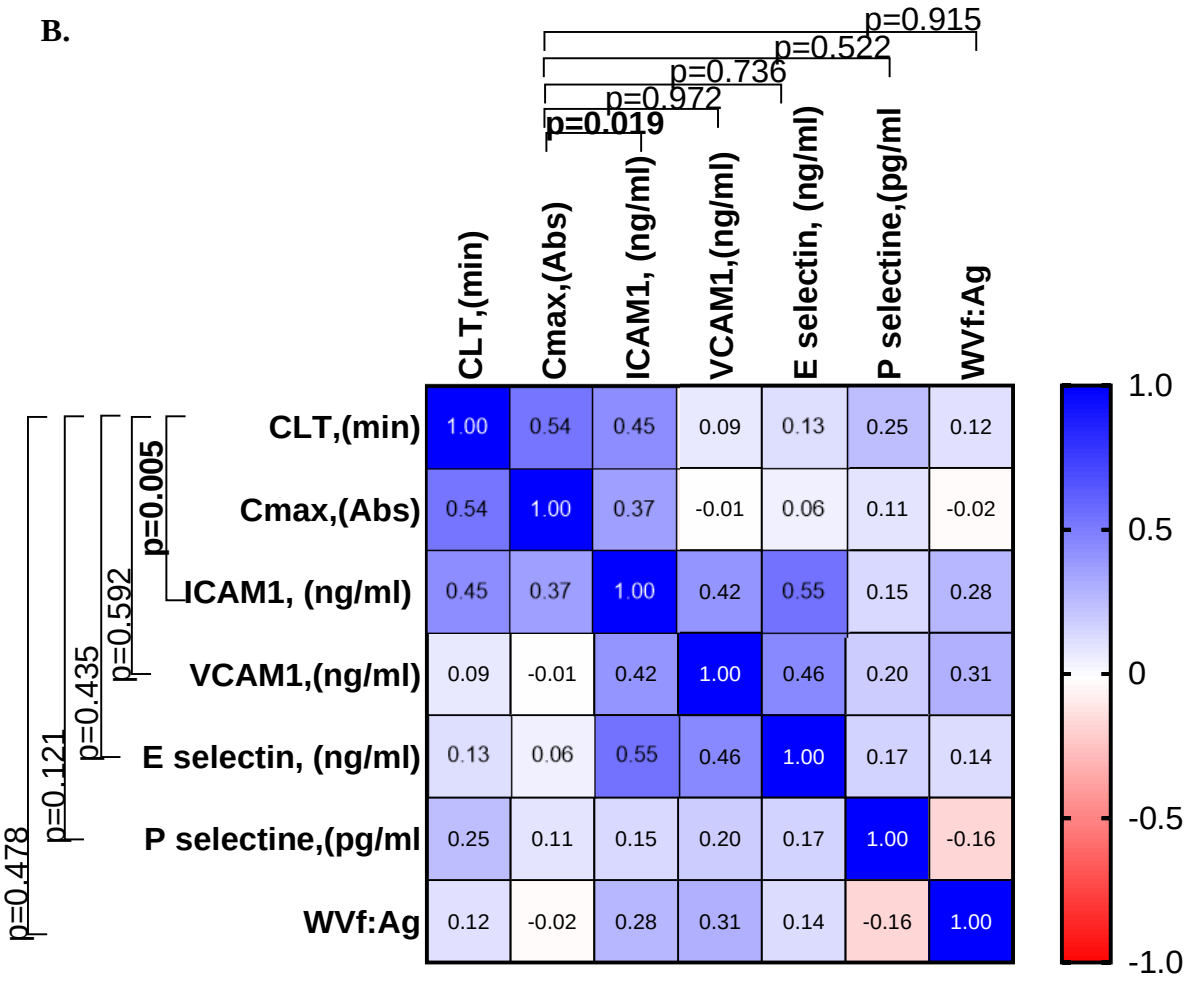


Figure 17. Correlation matrix between either Cmax or CLT with **A.** haemostatic parameters**; B.** vascular biomarkers within patients with history of DUs. DUs-digital ulcers; ETP-endogenous thrombin potential; Cmax Abs-clot maximum absorbance; CLT-clot lysis time; ICAM1- intercellular adhesion molecule 1; VCAM1- vascular adhesion molecule 1; VWF:Ag- von Willebrand factor antigen

4.5.3. Risk factors for digital ulcers

Table 51.summarizes the results of multivariable logistic regression analysis regarding risk to DUs. In the first model laboratory variables that previously showed significant association with DUs were included, thus CLT controlled for fibrinogen remained independently associated with DUs. Further, when clinical characteristics such as age at disease onset and VAS pain, were added into Model 2, patients with higher CLT values were still significantly susceptible to DUs, while age at disease onset showed significant negative trend of association.

Table 51. Multivariable logistic regression regarding risk to DUs

Variable	**Model 1**		**Model 2**	
	OR (95% CI)	p	OR (95% CI)	p
Laboratory				
Fibrinogen, (g/l)	1.27 (0.90-1.80)	0.170	1.17 (0.79-1.872)	0.420

CLT, (min)	1.08 (1.002-1.17)	**0.045**	1.11 (1.02-1.22)	**0.022**
Clinical				
Age disease onset, (years)			0.94 (0.88-0.99)	**0.038**
VAS Pain, (mm)			1.02(0.99-1.05)	**0.221**

OR-odds ratio; CI - confidence interval; CLT-clot lysis time

Furthermore, ROC analysis confirmed that CLT may discriminate patients with history of DU with high probability (AUC= 0.706, 95%CI 0.567-0.845, p=0.012) (Figure 18).

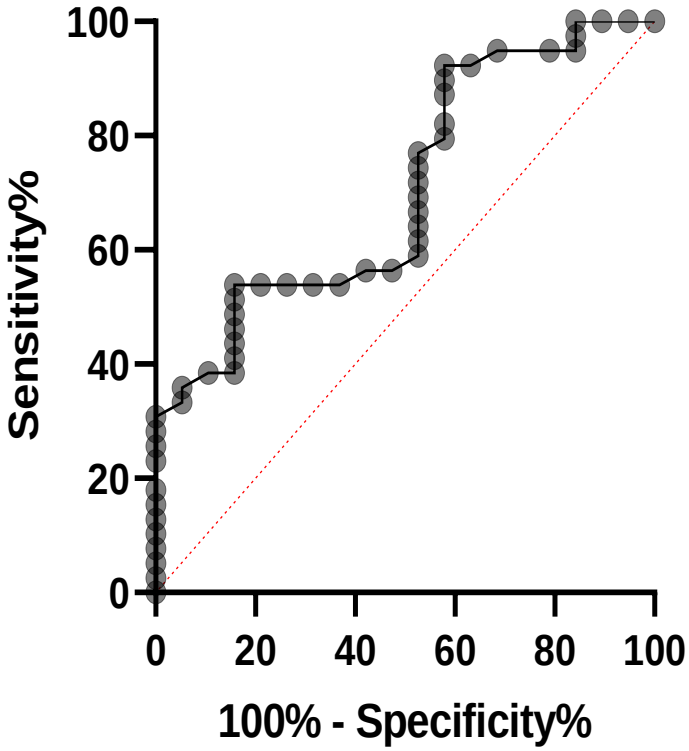


Figure 18. Receiver operating characteristic (ROC) analysis for CLT using DUs groups =1; DUs-digital ulcers; CLT- clot lysis time

4.6. Predictive markers for advanced digital vasculopathy

Over disease course, almost one-third of patients with history of DUs may experience association between advanced vasculopathy with recurrent ulcerations and reduced quality of life, higher rate of hospitalizations and progressive disease. In order to evaluate the potential pathophysiology pathway underlying progressive digital microvascular disease and to determine predictors for recurrent DUs, 39 patients with history of DUs were followed up for 18 months.

As presented in Figure 19. in the group of 39 patients with history of DUs that were included in the follow up, there were 20 (51%) cases with at least one new ischemic DU, with a median time to event of 7.5 (ranging 4–17) months. The majority of subjects experienced single (65%) while 35% had more than one DU during follow up period.

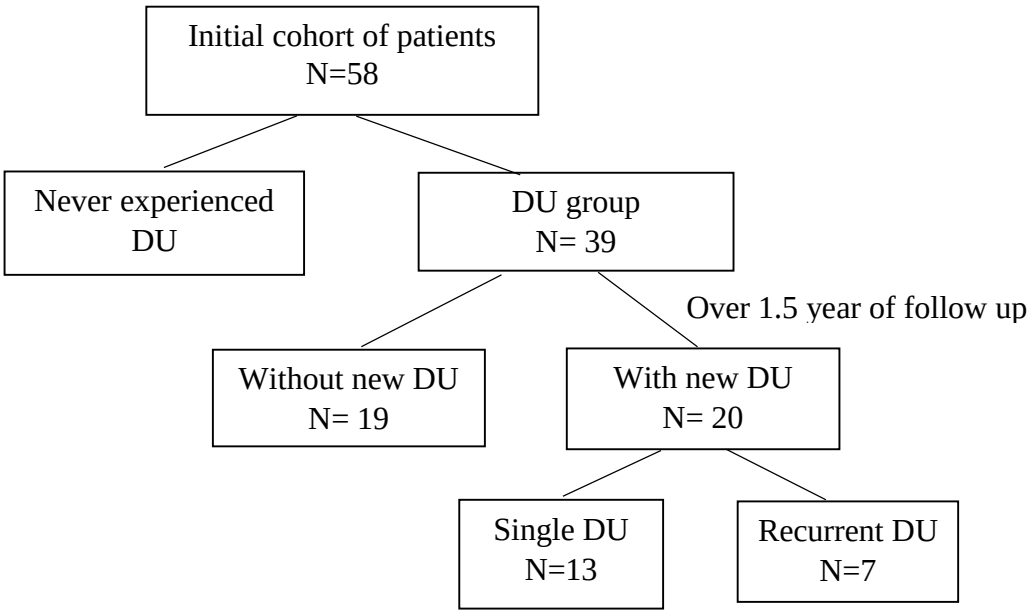


Figure 19. Flowchart- the stratification of the study cases regarding the history of digital ulcers and outcome set. DU- digital ulcer

Demographic baseline features of patients having new DUs during follow up and those without are tabulated in Table 52. As shown, no statistical difference was found in regard to age, gender, educational level and employment status between observed groups.

Table 52. Demographic features of patients with and without new DUs

Demographic variables	**With new DU(20)**	**Without new DU (19)**	**OR 95% (CI)**	**p-value**
Age, (years) [a]	52.3±11.2	53.5±10.4	1.0 (0.9-1.1)	0.990
Gender, n(%)				
Male	3(15)	2 (10)	1[b]	
Female	17 (85)	17 (90)	1.5 (0.2–10.1)	0.525
Education level, n (%)				
Elementary school	0	0		
High school	18 (90)	15(79)		
Higher education	2(10)	4(21)		0.407[c]
Employment status,n (%)				
Unemployed	6(30)	6 (32)		
Employed	9 (45)	5(26)		
Retirement	5 (25)	8(42)		0.404[d]

OR-odds ration; CI - confidence interval ; [a] Mean± SD; [b] reference ;[c] Fisher exact test; [d] X^2 test. DU-digital ulcer

Further on, there wasn't significant statistical association between smoking status, pack years, BMI, presence of obesity and arterial hypertension and new DUs onset (Table 53)

Table 53. Baseline lifestyle and comorbidities characteristics of patients with and without new DU onset

Lifestyle and comorbidities	With new DU(20)	Without new DU (19)	OR(95% CI)	p-value
Smoking, n (%)				
Never	14(70)	12(63)	1.00[d]	
Evera[a]	6(30)	7(37)	0.86(0.44-1.67)	0.651
Pack-years[b]	0(0-60)	0(0-30)	1.02(0.98-1.06)	0.481
BMI, (kg/m2)[c]	23.5±2.9	23.8±4.2	0.98(0.82-1.16)	0.821
Obesity, n (%)				
BMI<25	14(70)	11(58)	1.00[d]	
BMI≥25	6(30)	8(42)	0.59(0.16-2.21)	0.433
Arterial hypertension, n (%)				
No	9(45)	9(47)	1.00[d]	
Yes	11(55)	10 (53)	1.10(0.31-3.87)	0.882

[a] at lest of 60-day period any time prior to the study onset; [b] Median (Min-Max); [c] Mean ±SD; [d] reference group; OR-odds ratio; CI-confidence interval; DU-digital ulcers

4.6.1. The association of clinical features with onset of new DUs

As presented in Table 54, average age of patients with new DU at disease onset were 44.4 ± 10.6 with median disease duration at inclusion 5.5 (ranging 0-29) years. Those with dSSc subset had 5.6 fold higher risk while cases with sclerodactily had 5.1 fold increased risk of developing new DUs. The susceptibility of new DU was 1.38 fold times increased in individuals with mRSS >14, however the observed result has not reached wasn't statistical significance.

Table 54. Association of general clinical characteristics and skin manifestations with new DU onset

General clinical characteristics	With new DU[20]	Without new DU [19]	OR(95% CI)	p-value
Age at disease onset, (years)[a]	44.4± 10.6	45.9±11.1	0.97(0.93-1.05)	0.649
Disease duration, (years)[b]	5.4(0-29)	6.0 (0-24)	1.01(0.93-1.09)	0.894
Cutaneous subtype, n (%)				
Limited	8(42)	15(83)	1.00[c]	
Diffuse	12(60)	4(17)	5.6(1.45-23.34)	**0.017**
mRSS	13(4-31)	11(4-31)	1.01(0.92-1.01)	0.783
mRSS >14, n (%)	10(50)	8(42)	1.38(0.39-4.87)	0.621
Autoantibody status, n (%)				
Anti-centromere Ab	6(30)	7(37)	0.74(0.19-2.79)	0.651
Anti-topoisomerase I Ab	12(60)	7(37)	2.57(0.71-9.36)	0.152
Skin manifestations, n (%)				
Sclerodactily	17(85)	10(53)	5.1(1.11-23.37)	**0.036**

[a]Mean ±SD; [b] Median (Min-Max); [c] reference group; OR-odds ration; CI-confidence interval; mRSS- modified Rodnan skin score; DU-digital ulcer

There were no statistical associations between any of skeletal and cardiopulmonal organ involvement and the risk of new ischemic fingertip (Table 55). Of note, a trend of 3 fold higher risk had patients with ILD.

Table 55. Association of organ involvement characteristics with new DU onset

Organ involvement	With new DU(20)	Without new DU (19)	OR (95%CI)	p-value
Sceletal, n (%)				
Contracture	7(35)	6(32)	1.17(0.31-7.93)	0.821
Cardiopulmonal				
FVC % [a]	95.9±20.9	96.5±17.6	1.00(0.94-1.03)	0.934
FVC <80%,n(%)	4(20)	3(16)	1.33(0.26-6.94)	0.732
DLCO% [a]	63.6±13	66.6±20.1	1.00(0.95-1.02)	0.388
DLCO <70%	15(75)	11(58)	2.18(0.56-8.51)	0.261
FVC/DLCO %>1.6	9(45)	8(42)	1.13(0.32-3.99)	0.855
ILD, n(%)	14(70)	8(42)	3.01(0.86-12.09)	*0.084*
sPAP, (mmHg)[a]	30.1±5.4	30.1±7.2	1.00(0.91-1.11)	0.982

[a]Mean ±SD; OR-odds ratio; CI-confidence interval; DU-digital ulcers; FVC-forced vital capacity; DLCO- diffusing capacity for carbon monoxide;ILD- interstitial lung disease; sPAP- systolic pulmonary artery pressure

When analyzing peripheral vascular manifestations, individuals with either RP or active DUs at baseline were 4.02 and 5.20 times more prone of getting new DU. Around two-third of the cases experienced ≥ 3 DUs episodes before enrollment compared to group without new DUs and these patients exhibited trend of 3.71 higher risk for developing new DU episode over the follow up period (Table 56).

Table 56. Association of peripheral vascular manifestations with new DU onset

Peripheral vascular manifestations	With new DU(20)	Without new DU (19)	OR (95%CI)	p-value
RP present, n (%)	13(65)	6(32)	4.02(1.06-15.28)	**0.041**
Telangiectasia present, n(%)	11(55)	15(79)	0.33(0.80-1.34)	0.120
Pitting scars, n(%)	17(85)	15(79)	1.51(0.29-7.87)	0.624
Active digital ulcers, n(%)	13(65)	5(26)	5.20(1.32-20.54)	**0.019**
Acroosteolysis, n(%)	8(40)	4(21)	2.5(0.60-10.34)	0.206
DUs episodes≥3	13(72)	8(42)	3.71 (1.00–14.24)	*0.065*

OR-odds ratio; CI-confidence interval; DU-digital ulcers; RP-Raynaud phenomenon

The analysis of potential association between either NVC patterns; loss of capillaries or ramified/bushy capillaries and onset of new DU are presented on Figure 20-22. There was no significant difference in distribution of patients based on either NVC pattern, loss of capillaries or presence of ramified capillaries between two DU groups. It is worth noting that frequency of moderate (50%) and severe loss (45%) of capillaries was higher in cases in comparison to those without new event, thus cases with moderate loss exhibited around 12 11.67 fold higher risk OR=11.67, 95%CI 1.14-119.54, p=0.039) and those with sever loss of capillaries had 10.50 (OR=10.50, 95%CI 1.01-108.58, p=0.049) fold higher risk of getting new DUs when variable “no/few loss” was considered as referent.

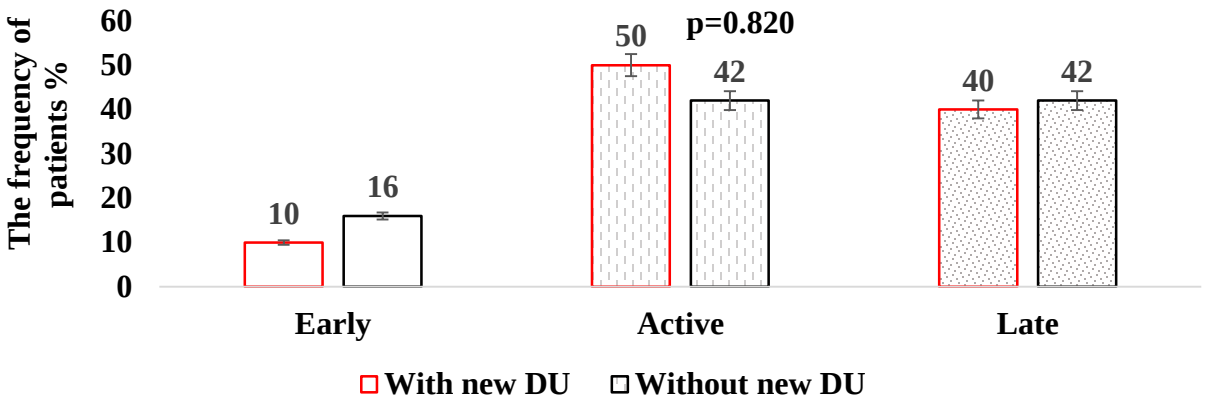


Figure 20. Distribution of 20 patients with and 19 without DUs based on NVC patterns. DU-digital ulcers; NVC- nailfold videocapillaroscopy

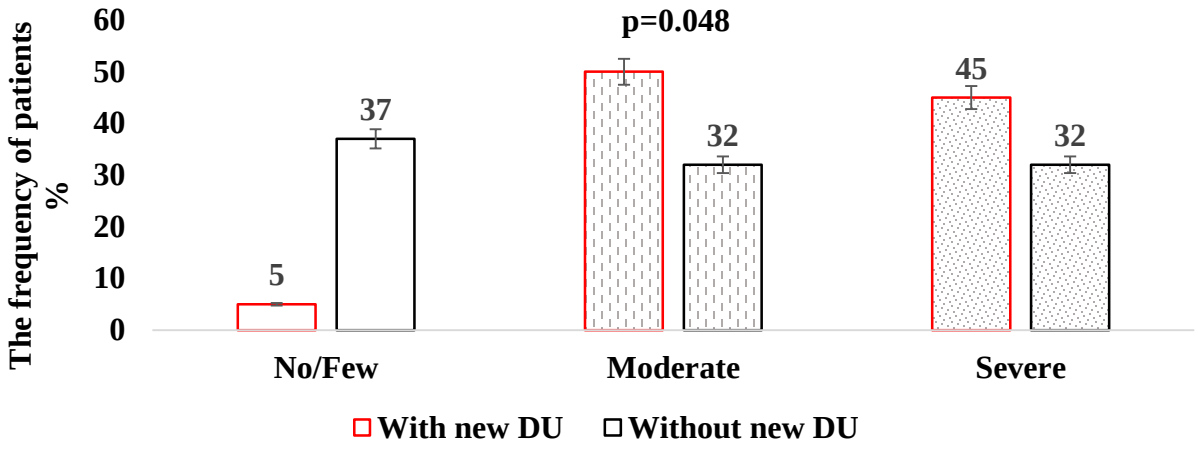


Figure 21. Distribution of 20 patients with and 19 without DUs based on loss of capillaries. DU-digital ulcers

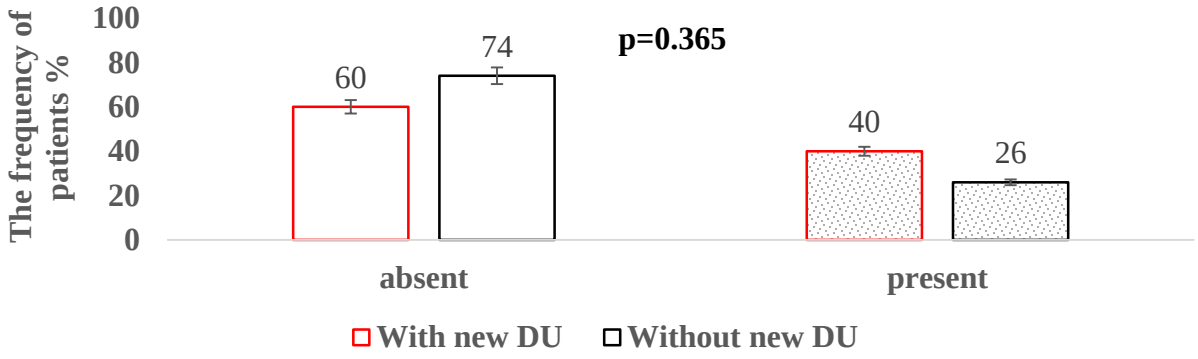


Figure 22. Distribution of 20 patients with and 19 without DUs according to present of ramyphed/bashy capillaries. DU-digital ulcers

4.6.2. The association of quality of life domains with onset of new DUs

Regarding quality of life SHAQ DI domains, higher values of arising and activity were associated with new DU onset but without achieving statistical significance. Observing VAS scale results, pain; RP and DU had higher impact on daily life among patients with new DU compared to those without; but only VAS RP was significantly associated with onset of new fingertip ulcer (Table 57).

Table 57. Difference between groups according to SHAD DI domains

SHAQ DI variable	With new DU(20)	Without new DU (19)	OR (95%CI)	p-value
Dressing	0(0-2)	0 (0-2)	1.05(0.43-2.55)	0.980
Arising	0(0-2)	0(0-1)	4.07(0.45-34.00)	0.195
Eating	1(0-3)	1(0-3)	1.14(0.56-2.35)	0.715
Walking	0(0-2)	0(0-2)	0.46(0.15-1.44)	0.181
Hygiene	0(0-2)	0(0-0)	/	0.999
Reaching	0(0-2)	0(0-2)	0.85(0.33-2.15)	0.845
Gripping	1(0-3)	1(0-3)	1.17(0.54-2.53)	0.695
Activity	0(0-2)	0(0-2)	1.79(0.69-4.56)	0.225
Overall	0.37(0-2.0)	0.37(0-1.50)	1.39 (0.33-5.93)	0.652
VAS scale, mm				
Pain	43(0-91)	29(0-72)	1.01(0.98-1.03)	0.543
RP	56(0-81)	22(0-58)	1.06(1.02-1.10)	**0.003**
DU	46.6(0-89)	0(0-89)	1.02(1.00-1.04)	*0.050*
Lung	0(0-90)	11(0-68)	0.99(0.97-1.02)	0.825
GIT	0(0-52)	0(0-46)	0.98(0.93-1.03)	0.477

Results are presented as Median (Min-Max); OR-odds ratio, CI-confidence interval; SHAQ-Scleroderma Health Assessment Questionnaire DU-digital ulcers; VAS- visual analog scale; RP-Raynaud phenomenon; GIT-gastrointestinal tract

Similarly to the observed results from SHAQ DI, the assessment of pain by EQ5D was not significantly worse in cases compared to subjects without new event (Table 58).

Table 58. Difference between groups according EQ5D domains

EQ5D domains	With new DU(20)	Without new DU (19)	OR (95%CI)	p-value
Mobility	1(1-2)	1(1-2)	0.19(0.02-1.96)	0.166
Self-care	1(1-2)	1(1-2)	2.13(0.34-13.24)	0.419
Usual activities	1(1-3)	1(1-2)	0.78(0.24-2.53)	0.680
Pain and discomfort	2(1-3)	2(1-2)	2.55(0.75-8.67)	0.135
Anxiety and depression	2(1-3)	2(1-3)	0.87(0.28-2.73)	0.811
Overall	0.69(0.08-1.00)	0.69(0.42-1)	0.37 (0.01-16.34)	0.604
VAS scale, mm				
Overall health	60(19-100)	61(35-93)	1.00(0.97-1.03)	0.940

Results are presented as Median (Min-Max); OR-odds ratio, CI-confidence interval; DU-digital ulcers; VAS- visual analog scale

4.6.3. The association of treatment modalities with onset of new DUs

As indicating in Table 59, there was no significant association between any treatment modalities and onset of new DU. However, it should be noted that frequency of ASA, previous treatment with cyclophosphamide and hyperbaric chamber were higher in patients with new DU in comparison with those without.

Table 59. Differences between group regarding therapeutic modalities

Treatment	With new DU(20)	Without new DU (19)	OR (95% CI)	p-value
I.Ongoing treatment, n (%)				
Glucocorticoids	6(30)	10(53)	0.38(0.10-1.43)	0.155
Metothrexate	4(20)	5(26)	0.70(0.16-3.13)	0.641
Chloroquine	2(10)	1(5)	2.00(0.17-24.07)	0.585
Azathioprine	2(10)	4(22)	0.39(0.06-2.44)	0.389
ACE inhibitor	5(25)	9(47)	0.37(0.90-1.44)	0.151
Calcium channel blocker	9(45)	10(53)	0.74(0.21-2.59)	0.634
Beta blockers	4(20)	4(21)	0.94(0.19-4.44)	0.935
ASA	9(45)	4(21)	3.07(0.75-12.59)	0.120
II. Previous treatment, n (%)				
Cyclophosphamide	10(51)	8(42)	1.37 (0.39-4.87)	0.621
HBO	10(50)	6(32)	2.17(0.59-7.90)	0.246

OR-odds ratio, CI-confidence interval; ACE-angiotensin converting enzyme; ASA - acetylsalicylic acid; DU-digital ulcers; HBO-hyperbaric oxygen therapy

Further, analyzing difference in the mean prednisolone dose or cumulative dose of cyclophosphamide between groups at baseline, statistical significance wasn't observed (Figure 23.).

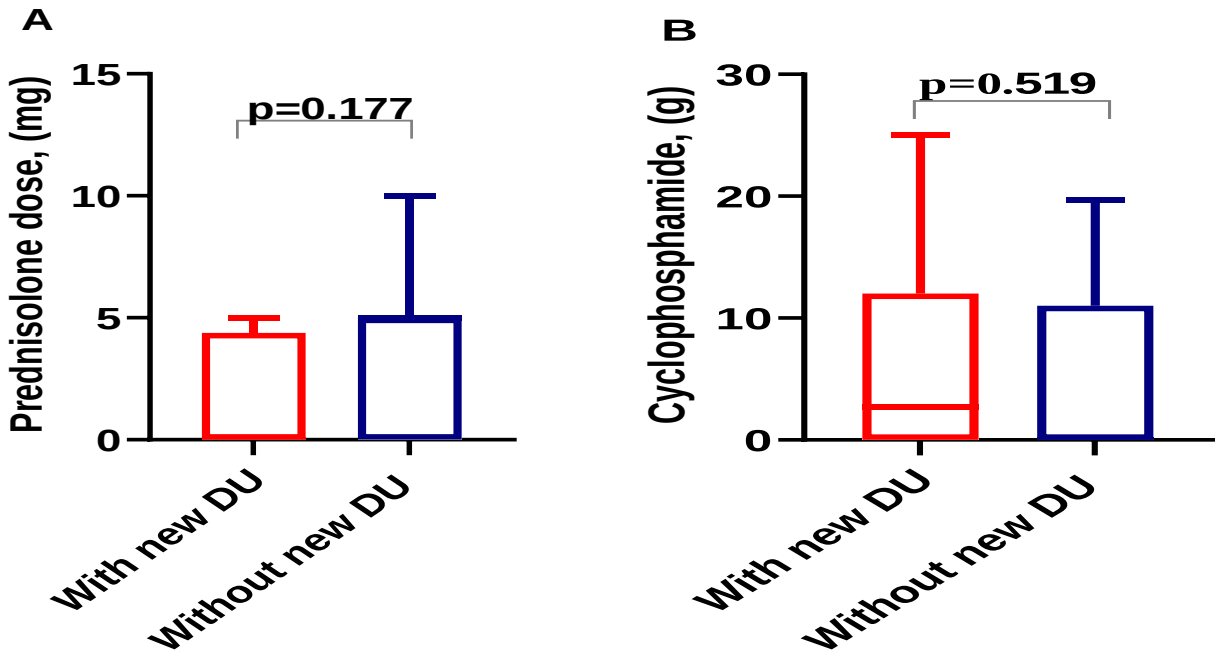


Figure 23. Differences between groups regarding doses: A. prednisolone, B. cyclophosphamide. DU-digital ulcer

It is important to note, that soon after enrollment to follow up study, 6 patients started cyclophosphamide therapy with similar frequencies between those with new DU (15%) and without (16%).

4.6.4. The association of standard laboratory parameters with new DUs development

Interestingly, although all inflammatory markers were elevated in cases with new DUs compared to those without, statisticaly significant difference has not been noticed (Table 60).

Table 60. The association of standard laboratory parameters with new DUs

Laboratory parameters	**With new DU(20)**	**Without new DU (19)**	**OR 95%CI**	**p-value**
Markers of renal function				
Urea, mmol/L[a]	5.4±1.9	4.7±1.3	1.32(0.87-1.99)	0.197
Creatinine, ηmol/L[a]	76.6±9.5	73.5±13.1	1.03(0.97-1.08)	0.392
Lipid panel				
Cholesterol, mmol/L[a]	6.7±1.7	6.5±1.4	1.11(0.73-1.69)	0.616
Triglycerides, mmol/L[a]	1.5±0.6	1.8±0.7	0.40(0.14-1.18)	0.196
Inflammatory markers				
CRP, (mg/l) [b]	6(0.25-11)	4.8(0.24-16.4)	0.99(0.86-1.14)	0.912
ESR,(mm/hr) [b]	19(5-66)	18(4-58)	1.01(0.70-1.01)	0.692
Fibrinogen, g/L[a]	5.3±2.0	4.4±1.8	1.27(0.89-1.79)	0.174
Complement level				
C3, g/L[a]	1.3±0.3	1.3±0.3	1.14(0.13-10.36)	0.902
C4, g/L[a]	0.2 ± 0.1	0.2 ± 0.1	49.11(10^{-3} -1.9x10^{6})	0.471

[a] Mean ± SD; [b] Median (Min-Max); OR-odds ratio; CI-confidence interval;CRP -C reactive protein; ESR - erythrocyte sedimentation rate; DU-digital ulcers

4.6.5. The association of endothelial dysfunction markers and haemostatic parameters with new DUs onset

Similarly with the obtained results regarding inflammatory markers, no significant association, in terms of new DU risk occurrence was observed when markers of endothelial dysfunction were assessed. Nevertheless higher serum levels of ICAM1, E selectin, P selectin and VWF:Ag were noted in cases with outcome of interest, with a trend of significancy for P selectin, as presented in Table 61.

Table 61. The association of vascular biomarkers with new DU onset

Vascular biomarkers	**With new DU(20)**	**Without new DU (19)**	**OR (95%CI)**	**p-value**
ICAM1, ng/ml[a]	32.5±8.6	27.9±8.4	1.07 (0.98-1.15)	0.104
VCAM1, ng/ml[a]	41.1±9.8	41.3±16.8	0.94 (0.95-1.05)	0.965
E selectin, ng/ml[a]	5.6±2.2	5.4±2.1	1.04 (0.77-1.40)	0.783
P selectin, ng/ml[b]	7.7 (0-24.81)	5.9(0.87-11.11)	1.16(0.98-1.36)	*0.082*
VWF:Ag[b]	1.7(1.13-7.56)	1.8 (0.98-2.51)	1.8 (0.66-4.84)	0.253

[a] Mean ± SD; [b] Median (Min-Max); OR - odds ratio; CI- confidence interval; ICAM-1: intercellular adhesion molecule 1; VCAM-1: vascular cell adhesion molecule 1; VWF:Ag: von Willebrand factor antigen; DU-digital ulcer

Results of global haemostatic assays (CAT and OHP) and turbidity features of fibrin clot formation are summarized in Table 62. Patients with new DU showed trend of ETP elevation, significantly increased OHP values and decreased OFP. Furthermore, occurrence of new DU was associated with borderline higher Cmax and significantly increased CLT values.

Table 62. The association of haemostatic parameters with new DU onset

Haemostatic assays				
CAT assay	**With new DU(20)**	**Without new DU (19)**	**OR (95%CI)**	**p-value**
ETP, (nM/min)	2134.7±426.1	1899.2±353.7	1.002(1.00-1.003)	0.077
Lag time, (min)	3.1±0.5	3.3±0.5	0.38(0.09-1.55)	0.178
Peak, (nM)	347.5±79.5	303.9±92.6	1.00(0.99-1.01)	0.125
Time to peak,(min)	5.9±1.4	6.5±1.2	0.72(0.43-1.19)	0.197
OHP assay				
OHP, (Abs-sum)	184.8±70.4	115.5±48.8	1.02(1.01-1.04)	**0.009**
OCP, (Abs-sum)	330.0 ±65.9	306.1±42.3	1.01(0.99-1.02)	0.188
OFP, (%)	43.9±18.2	62.8±12.1	0.93(0.88-0.98)	**0.005**
Turbidity assay				
Coagulation				
Lag phase clot, (min)	4.9±1.0	5.2±0.8	0.71(0.34-1.49)	0.364
Cmax, (Abs)	1.2±0.3	1.1±0.2	22.73(0.91-566.89)	0.052
Fibrinolysis				
Lag phase lysis, (min)	5.4±2.0	6.0±1.9	0.84(0.61-1.17)	0.298
CLT, (min)	44.1±9.6	31.4±6.8	1.18(1.14-1.30)	**0.001**

Data are presented as Mean±SD DU-digital ulcer; OR-odds ration; CI-confidance interval; CAT- Calibrated automated thrombogram assay; ETP-endogenous thrombin generation; OHP- overall haemostasis potential; OCP - overall coagulation potential; OFP-overall fibrinolysis potential; Cmax Abs- clot maximum absorbance; CLT-clot lysis time

Kaplan-Meier analysis was done in order to explore relation between time to event and prolonged CLT (Figure 24.). As indicating, cases with prolonged CLT >35.4 minutes developed new DUs notably earlier than those with shorter CLT ($p < 0.001$).

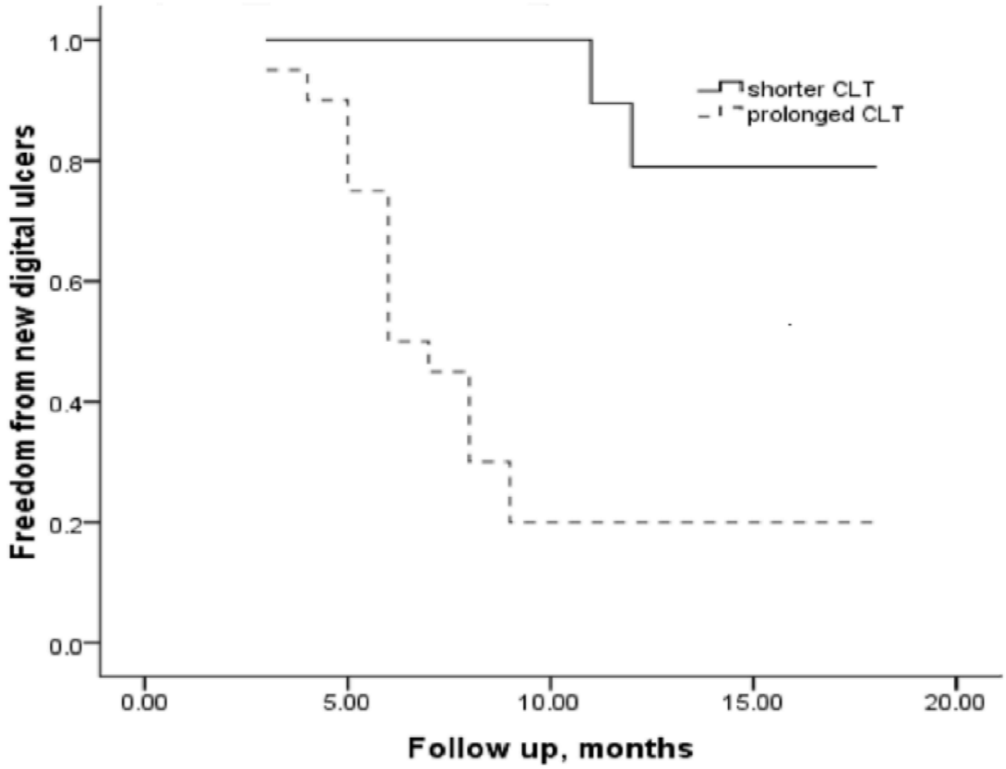


Figure 24. Kaplan-Meier analyses- onset of new DUs in a 1.5-year follow-up of 39 patients with SSc with history of DUs. The curve is presented for short (≤ 35.4 min) or prolonged (> 35.4 min) CLT. CLT: clot lysis time; DU: digital ulcer; SSc: systemic sclerosis

4.6.6. Correlation between fibrin clot properties with different disease features in patients with new DU

Since our results revealed remarkably prolonged fibrinolysis and trend of denser clots in patients with new DU, further we assessed which variables might be associated with Cmax and CLT among cases. First correlation analysis between either CLT or Cmax with clinical features of disease (Table 63) showed trend of moderate positive correlation between CLT with sPAP and interestingly invers correlation with mRSS, while Cmax confirmed strong invers association with DLCO%.

Table 63. Correlation of CLT and Cmax with clinical characteristics within patients with new DU onset

		Clinical characteristics					
Haemostatic parameters		**Age at disease onset (years)**	**Disease duration (years)**	**FVC (%)**	**DLCO (%)**	**sPAP (mmHg)**	**mRSS**
CLT, (min)	r	0.157	-0.180	0.196	-0.178	**0.432**	**0.453**
	p	0.510	0.447	0.408	0.453	**0.057**	**0.045**
Cmax, (Abs)	r	-0.150	0.102	-0.323	**-0.521**	0.220	0.071
	p	0.528	0.668	0.165	**0.019**	0.352	0.767

r-correlation coefficient; p-p value; FVC-forced vital capacity; DLCO- diffusing capacity for carbon monoxide; ILD- interstitial lung disease; sPAP- systolic pulmonary artery pressure, mRSS-modified Rodnan skin score; Cmax Abs- clot maximum absorbance; CLT-clot lysis time

The results of correlation between either Cmax or CLT with inflammatory markers are summarized in Table 64. except the strong positive correlation between Cmax and CRP levels, no other significant correlation was found.

Table 64**.** Correlation of CLT and Cmax with inflammatory markers within patients with new DU onset

		Inflammatory markers		
Haemostatic parameters		**ESR, mm/hr**	**CRP, mg/l**	**Fibrinogen, g/L**
CLT, (min)	r	-0.034	0.108	0.104
	p	0.887	0.652	0.662
Cmax, (Abs)	r	0.292	**0.514**	0.282
	p	0.212	**0.021**	0.229

r-correlation coefficient, p-p value; CRP -C reactive protein; ESR - erythrocyte sedimentation rate; Cmax Abs- clot maximum absorbance; CLT-clot lysis time

As it's shown in Table 65., patients treated with the higher cumulative dose of CYP had higher Cmax, addressing that more progressive disease was related with more procoagulant clot features.

Table 65. Correlation of CLT and Cmax with therapy doses within patients with new DU onset

Haemostatic parameters		**Prednisolone dose, (mg)**	**Cyclophosfamide cumulative dose, (g)**
CLT, (min)	r	-0.131	0.213
	p	0.805	0.554
Cmax, (Abs)	r	0.393	**0.762**
	p	0.441	**0.010**

r-correlation coefficient; Cmax Abs- clot maximum absorbance; CLT-clot lysis time

Further on, correlation between either CLT or Cmax with other components of CAT assay (ETP, ETP lag time, peak of thrombin generation and time to peak) was performed (Figure 25.). As expected Cmax showed significant strong positive association with ETP and trend of moderate correlation with CLT, while CLT exhibited strong positive correlation with ETP and moderate with peak of thrombin generation.

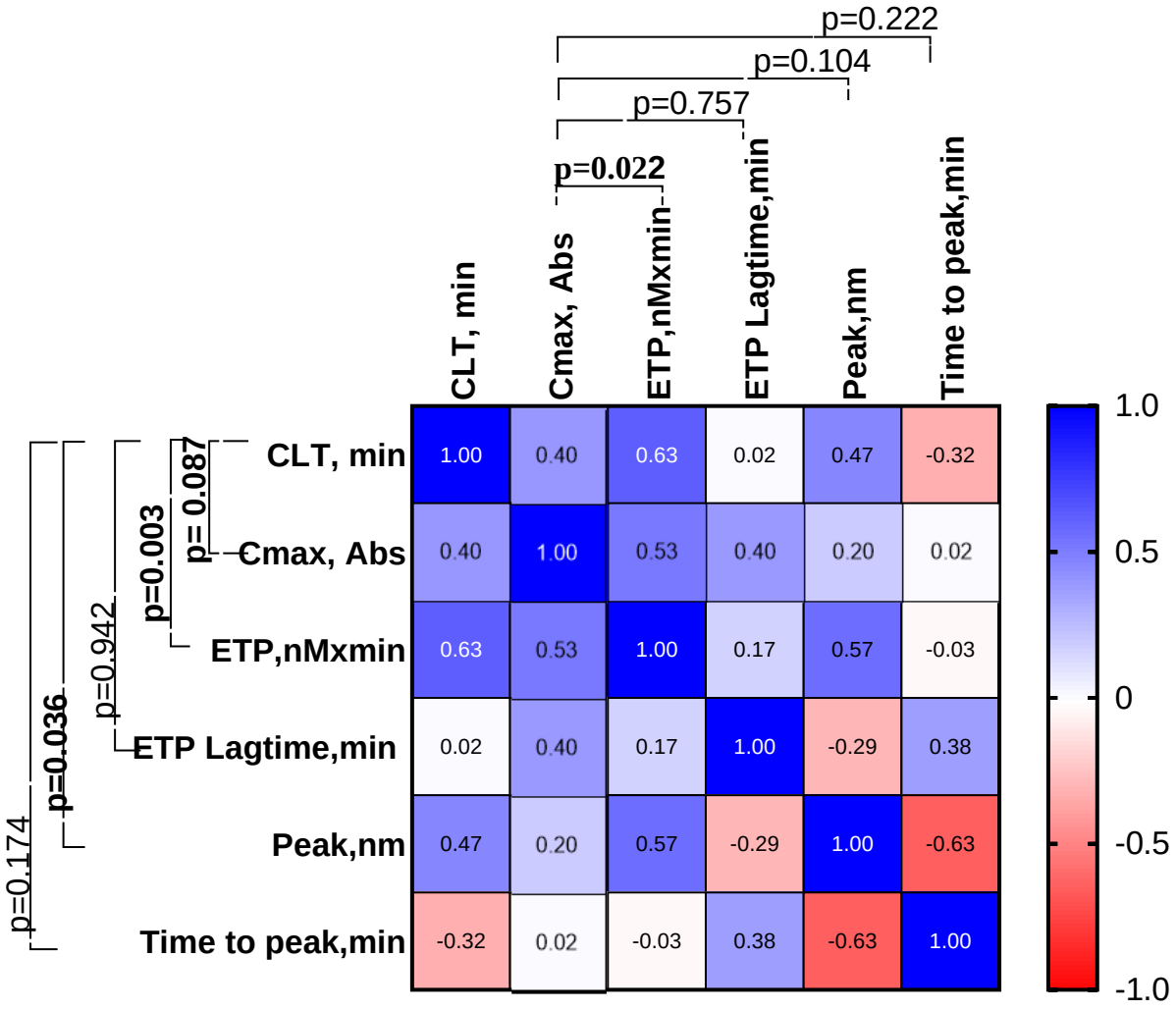


Figure 25. Correlation matrix between either Cmax or CLT with CAT assay parameters within patients with new DUs. DUs-digital ulcers; Cmax Abs- clot maximum absorbance; CLT-clot lysis time; CAT-Calibrated automated thrombogram assay; ETP-endogenous thrombin generation

In term to explore is there any impact of endothelial dysfunction on coagulation disturbance and diminished fibrinolysis within patients with new DU onset, correlation between fibrin clot parameters and the detected serum levels of investigated vascular markers was done (Figure 26.). No statistical correlation has been observed between Cmax and any of explored variables, on the other hand CLT remained strong related with ICAM1). Interestingly, only ICAM1 and E selectin (r=0.550, p=0.012) were significantly correlated when interanalysis was done between all vascular markers.

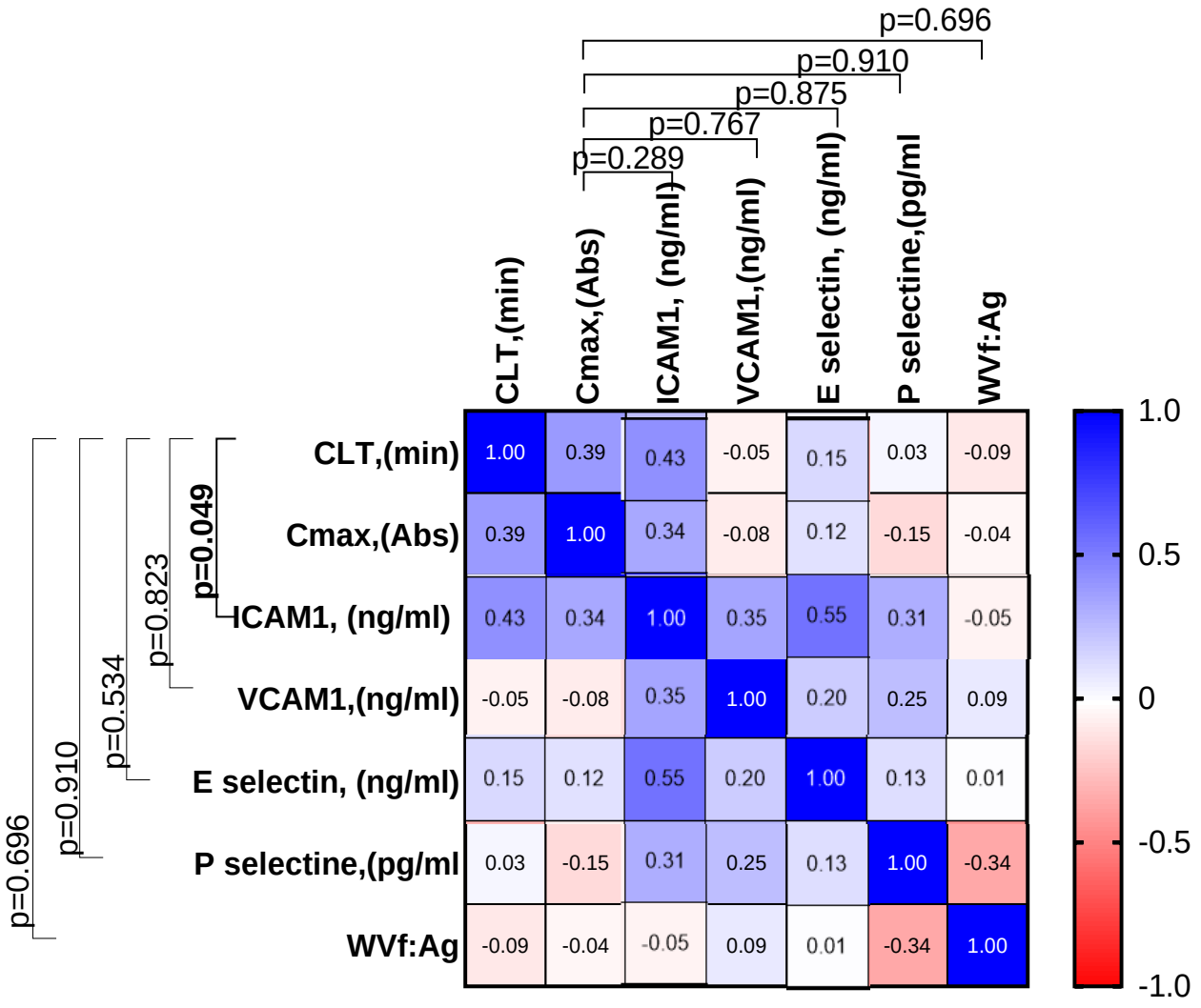


Figure 26. Correlation matrix between either Cmax or CLT with vascular biomarkers within patients with new DUs. DUs-digital ulcers. Cmax Abs- clot maximum absorbance; CLT-clot lysis time; ICAM-1: intercellular adhesion molecule 1; VCAM-1: vascular cell adhesion molecule 1; vWF:Ag: von Willebrand factor antigen; DU-digital ulcer

4.6.7. Risk factors for new digital ulcer onset

Since our study focused on the risk factors for new digital ulcer onset, multivariate forward-stepwise logistic regression was run (Table 66). Domain of SHAQ DI, VAS Raynaud (OR 1.07, 95% CI 1.015–1.123, p=0.012) and CLT (OR 1.19, 95%CI 1.06–1.33, p=0.003) were independently predictive for new DUs development. Intercept only model showed AIC 56.15. In contrary, when all variables from univariate logistic regression analysis shown in Table entered into multivariate regression AIC was 45.85, while our final forward stepwise approach exhibited AIC of 34.89 showing the best goodness of fit. Further, power of explained variation presented with Nagelkerke s R squared was 64.6%. Final model had negative predictive power of 80% and positive predictive power of 84.2%.

Table 66**.** Factors associated with onset of new digital ulcer in Systemic sclerosis patients.

	Univariate logistic regression		**Multivariate logistic regression**	
Variable	**OR (95% CI)**	**p**	**OR (95% CI)**	**p**
Age at disease onset	1 (0.9-1.1)	0.6		
VAS Raynaud, mm	1.1 (1.02-1.2)	0.003	1.1(1.02-1.1)	**0.012**
VAS digital ulcers, mm	1.02 (1-1.04)	0.05		
ICAM1, ng/Ml	1.1 (1-1.2)	0.11		
P selectin, pg/mL	1.16(0.98-1.36)	0.082		
ETP, nM/min	1.002(1.00-1.003)	0.077		
CLT,min	1.2 (1.1-1.3)	0.001	1.2(1.1-1.3)	**0.003**

OR-odds ration; CI-confidence interval; VAS-visual analog scale; ICAM1- Intercellular Adhesion Molecule; ETP-endogenous thrombin generation; CLT-clot lysis time

ROC curve showed AUC 0.919, 95% CI 0.839-0.999, $p < 0.001$, confirming that CLT and VAS RP had strong predictive value on new DU onset (Figure 27.).

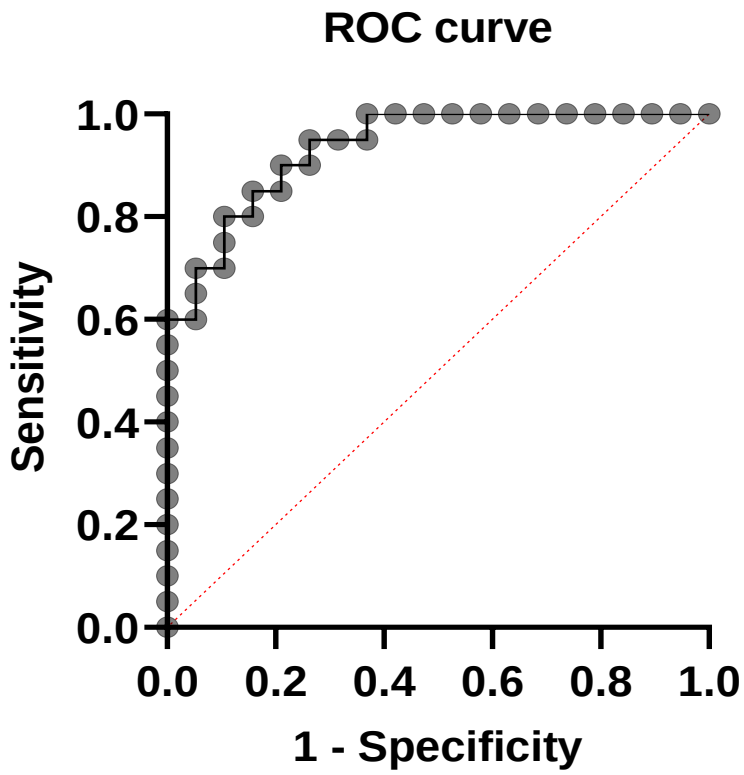


Figure 27. ROC analysis of final predictive model

Ultimately, the difference in haemostatic disturbance was explored in regard to the number of DU episodes during the follow up period (Figure 28.). As expected, patients with recurrent DUs had significantly higher OHP, decreased OFP and prolonged CLT values compared to those without new DU. Slightly higher OHP was found in the group with episodic DU, compared to non DU group, while longer CLT levels were found in cases with either recurrent versus episodic events or in those with episodic versus non DU. Namely, those results provided one more evidence that impaired fibrinolysis underlay progressive vasculopathy.

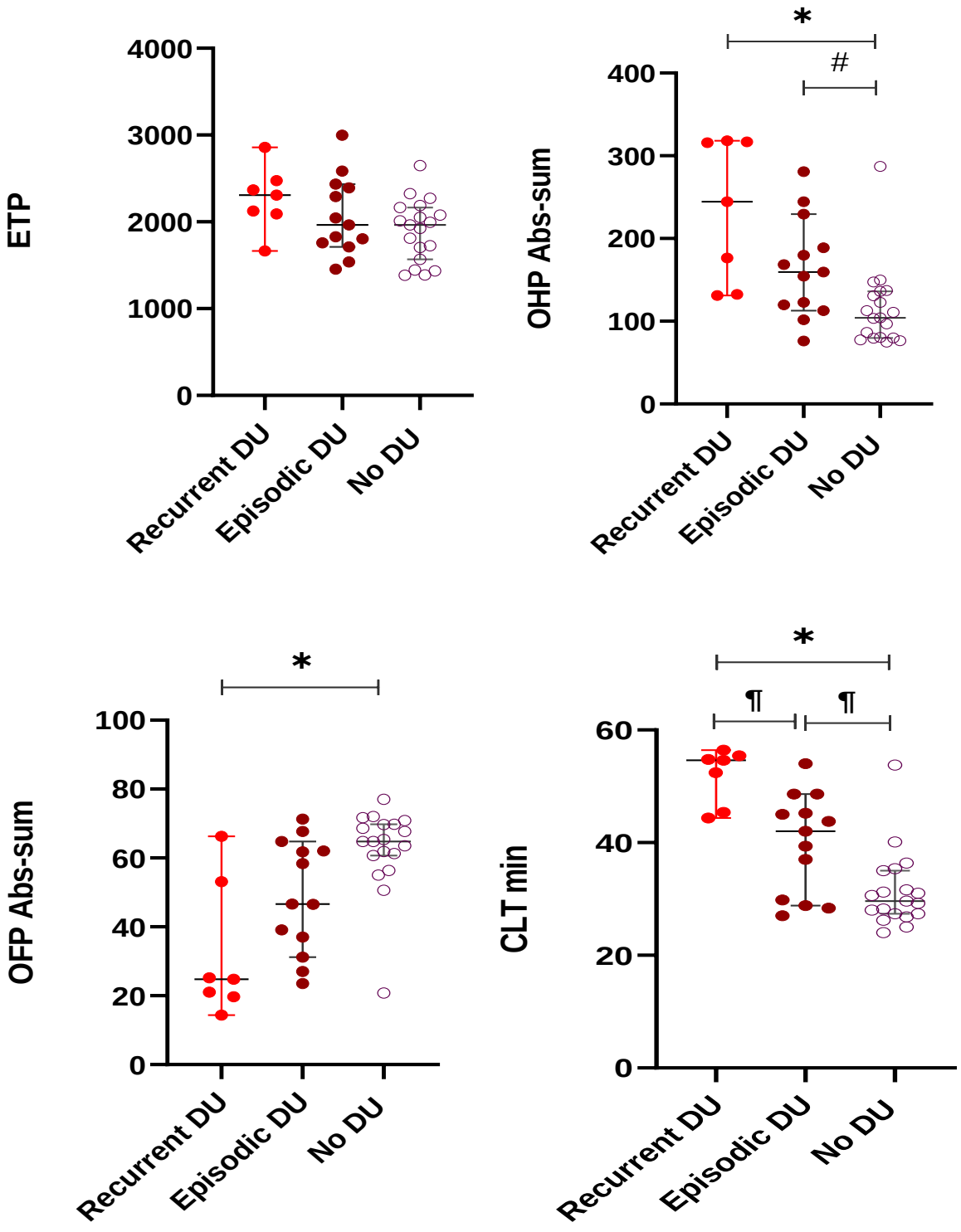


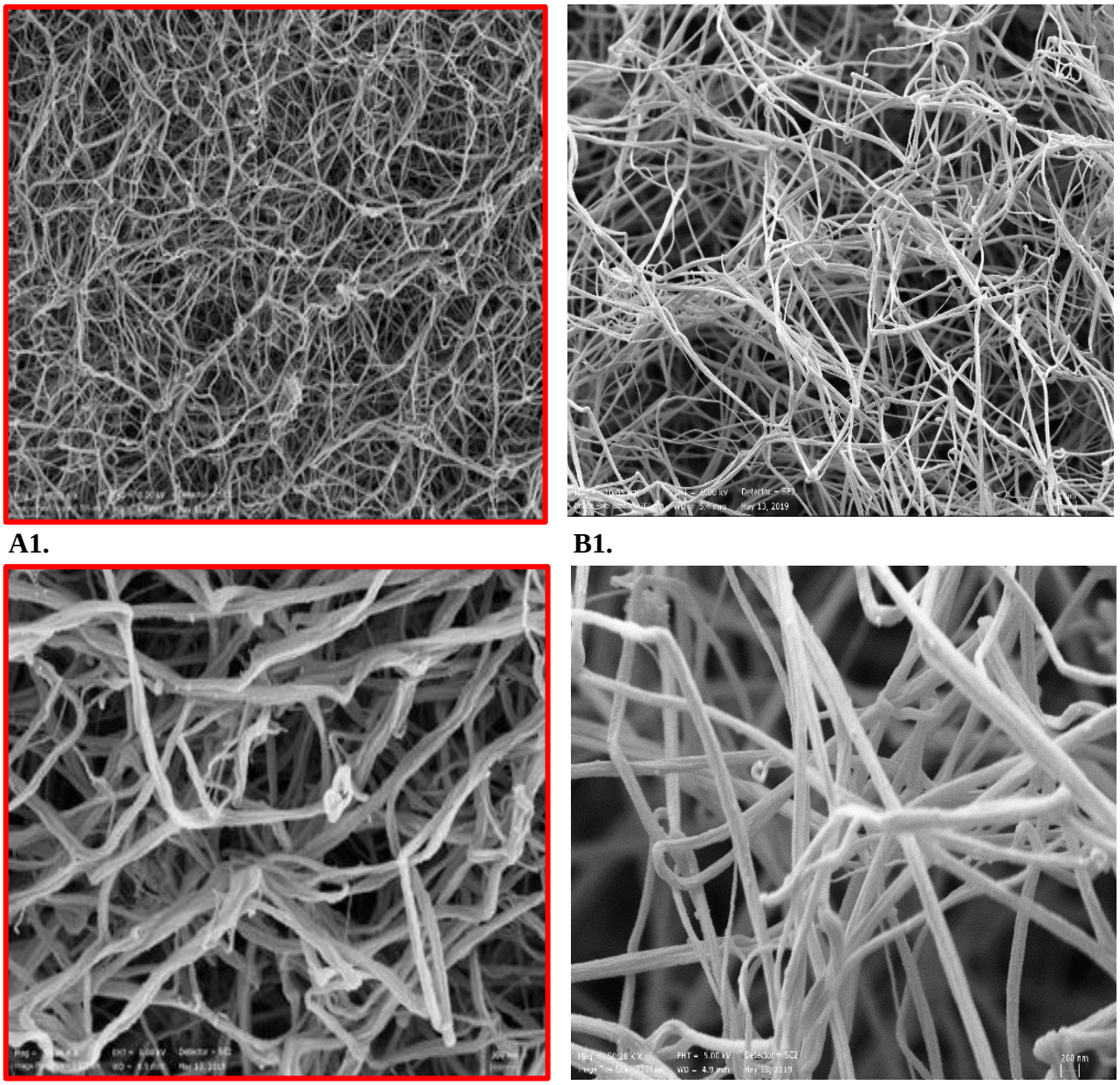
Figure 28. (A) ETP; (B) OHP; (C) OFP, and (D) CLT in patients with either recurrent; episodic DUs ornon DU over follow-up. * $p < 0.05$, # $p = 0.052$, ¶ $p = 0.07$. CLT: clot lysis time; DU: digital ulcer; ETP: endogenous thrombin generation; OFP: overall fibrinolytic potential; OHP: overall haemostatic potential.

4.7. Scanning electron microscopy

In order to confirm visually structure of fibrin clots, SEM was done and results are shown at Figure 29-30.

A.

B.



A1.

B1.

Figure 29. Fibrin clot structures visualized by SEM in A. patient with systemic sclerosis and B. healthy controls, using the magnification of 300nm (A.-B.) and 1 μm (A1. and B1.)

As presented on Figure 29, healthy controls had bigger pores with a lower density, the so called "looser" structure that is more susceptible for fibrinolysis in respect to SSc patients.

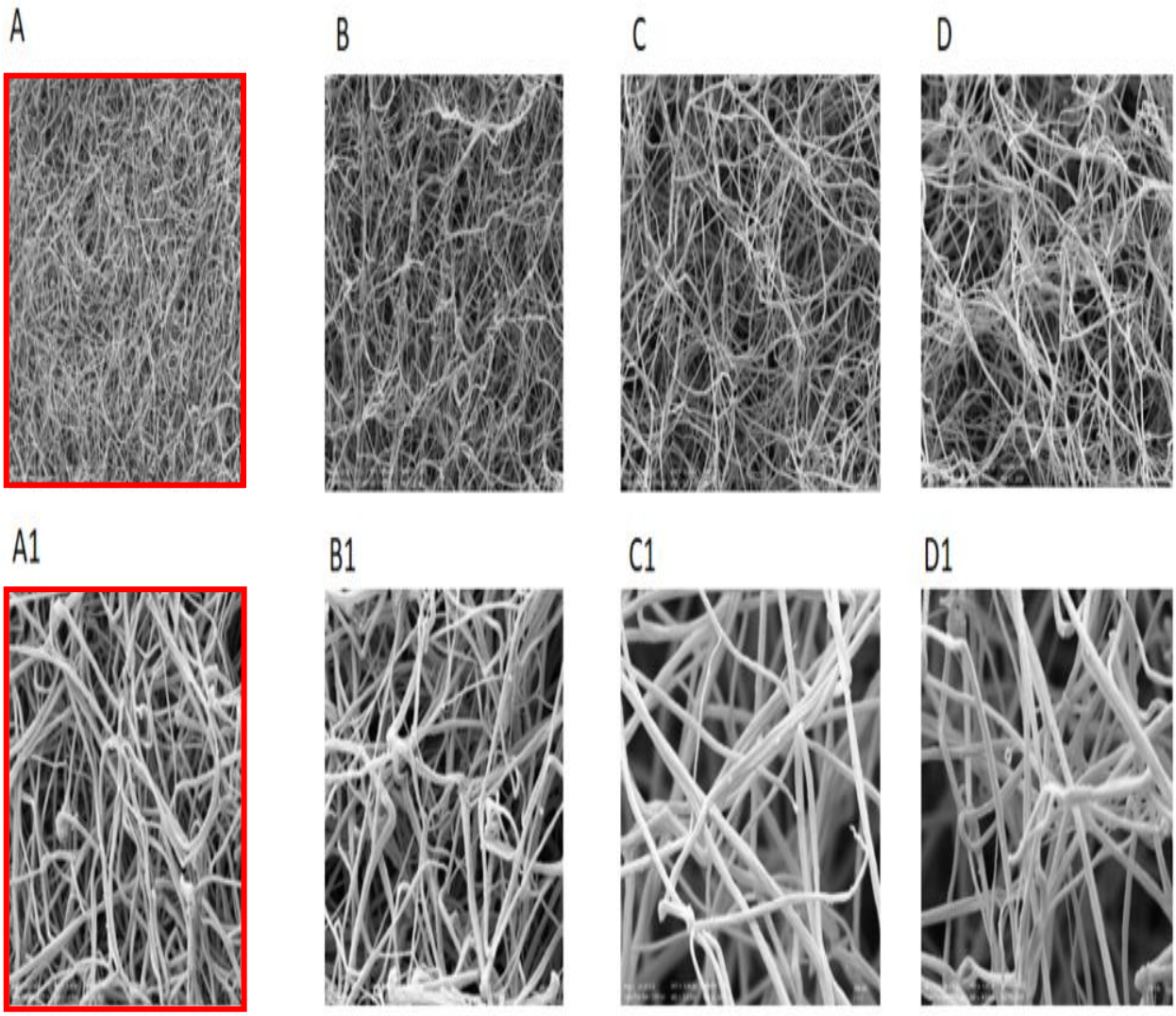


Figure 30. Comparative analysis of SEM fibrin clot structures from (A.-A1.) patient with new DU onset; (B.-B1.) patient without new DU over follow up; (C.-C1.) DU naïve patients and (D.-D1.) healthy controls, using the magnification of 300nm (A.-D.) and 1 μm (A1. - D1.)

When comparing fibrin clots between different DU groups and controls (Figure 30.), it has been observed that patients with new DU onset during follow up had a denser fibrin clot structure with smaller pores less prone to fibrinolysis compared to either those without new DUs over follow up, DU naïve or control subjects. Thus our results testify fibrin clots alteration across DU groups with the worse fibrinolysis impairment within patients with advanced recurrent vasculopathy.

5 DISCUSSION

There is a growing body of evidence that vasculopathy is the hallmark of SSc [34,35]. In line with previous studies [33,57,71], we observed highly elevated levels of reliable biomarkers of endothelial dysfunction (ICAM1, VCAM1, E selectin, and VWF Ag) in plasma from SSc patients compared to controls. In addition, within the SSc cohort, mutual significant positive connection of examined adhesion molecules was found along with their association to both E selectin and VWF Ag, confirming the coexistence of ECs activation and injury in SSc pathogenesis. Even though the concentration of P selectin was higher in plasma in SSc cases, a significant difference compared to control values was not observed in our study. P selectin may be released from both activated ECs and platelets either in soluble form or expressed on the surface of MPs. Recently, platelet MPs have been found dominantly elevated in SSc and inversely associated with serum P selectin levels, suggesting that potentialy measuring endothelial MPs bearing P selectin would be a more sensitive marker of endothelial dysfunction [179–181].

Novel data revealed an increased risk of CVD [182] and venous thromboembolic events (VTE) [183] in SSc patients, indicating that SSc may be considered a prothrombotic life-threatening disease. Thus, the evaluation of coagulation/fibrinolysis imbalance is imposed as a clinical imperative for the early treatment of patients at risk.

Many studies have demonstrated enhanced coagulation among SSc patients, mostly measuring plasma or serum levels of coagulation cascade components, including thrombin-antithrombin, fragments 1+2, VWF Ag, a supranormal form of VWF, fibrinogen, and factor VIII: C [71,77,79]. On the other hand, data regarding fibrinolysis are inconsistent. Almost 50 years ago, American dermatologists could not observe impaired fibrinolytic activity of the blood from a series of SSc patients. However, using the fibrin slide technique, decreased fibrinolytic activity was found at the site of arterial thrombosis in these patients [184]. Later on, normal cutaneous and plasma fibrinolytic activity, assessed by euglobulin lysis time, was described in 11 SSc patients [185], and *Herrick et al.* have confirmed intact plasma fibrinolysis measured by PAI1 and tPA levels among 26 SSc patients [79]. Soon after, *Ames et al.* also tested 26 SSc patients and claimed that a hypofibrinolysis exists in SSc based on the finding of impaired tPA release and elevated PAI1 levels [77]. Lastly, *Cerinic et al.* demonstrated that altered coagulation was not followed by potent clot lysis since decreased D dimer was observed [71].

Most of the abovementioned studies used single coagulation/fibrinolytic factors and/or inhibitors for assessing complex haemostasis, thus only obtaining partial information about the whole process. To cross that issue, we used three global haemostatic assays providing a comprehensive overview of the haemostatic process. CAT assay includes information regarding the total quantity and the kinetics of thrombin generation over time, showing good sensitivity for detecting hypo/ hypercoagulability plasma state [186]. Additionally, the novelties of our study are OHP and turbidity assays, assessing not only the coagulation side of haemostasis but also fibrinolysis by measuring the rate of fibrin formation/degradation and fibrin clot properties [187].

Our results demonstrated unfavourable pronounced coagulation in SSc patients compared to controls, characterised by enhanced *in vitro* thrombin generation (increased ETP, peak of thrombin generation, shorter time to peak), faster fibrin clot formation (shorter lag time in turbidity coagulation assay) with prothrombotic properties (increased clot density). On the other hand, our results support the hypothesis

of hypofibrinolysis in SSc since decreased OFP and prolonged CLT were observed among patients. In this study, we have applied broad exclusion criteria in order to avoid the impact of other diseases on haemostasis and endothelial function. Thus, we believe that our results point towards association between SSc per se with coagulation/fibrinolysis imbalance. Moreover, our results were adjusted for well-known confounders influencing fibrin clot features such as ageing, pack years and BMI [178,188,189].

To date, only *Kuszmiersz et al.* have evaluated CAT assay in SSc, showing increased parameters of thrombin generation (ETP, PT), the association between ETP and markers of endothelial dysfunction and inflammation (CRP, fibrinogen), suggesting multifaceted regulation of prothrombotic changes in SSc [78]. A novel finding of our study is faster thrombin generation (shorter time to pick and, to less extent, lag time) in the SSc cohort, confirming a prothrombotic state. In contrast, previously mentioned authors have observed a delay in lag time. A possible explanation for this discrepancy may be notably higher fibrinogen concentrations and thereby potentialy higher inflammatory burden in patients from our cohort. Moreover, almost one third of our patients were newly diagnosed SSc cases within one year of inclusion, with the highest fibrinogen values observed within the cohort.

To extend this hypothesis, we noticed the minimum disease duration of two years in patients from their cohort. Thus, we can speculate that faster thrombin generation in our cohort could result from augmented inflammation mainly observed during the early phase of the disease. Possibly due to sample size, a statistically significant difference was not confirmed in any of the CAT assay parameters and inflammatory markers when comparing groups with recent disease onset to others. Thus, further studies with a larger number of patients with newly diagnosed SSc are warranted to confirm our results. Drawing results from other studies showing increased thrombin generation in patients with CVD [190], deep venous thrombosis [191], and even with VTE in anti-neutrophil cytoplasmic antibody-associated (ANCA) vasculitis [192], we may expect that SSc patients with altered thrombin generation could also be at risk of prothrombotic condition.

To the best of our knowledge, the present study is the first to analyse OHP supported by turbidity assay in SSc. It has been reported that coagulation/fibrinolysis imbalance observed by OHP/turbidity assays underlays other autoimmune systemic diseases, including SLE [193], ANCA vasculitis [192] and RA [194]. Furthermore, procoagulant fibrin clot properties with increased Cmax and longer CLT have been found in acute coronary syndrome. Additionally, prolonged CLT has been identified as a risk for arterial thrombosis in APS patients [195] and VTE in primary RP [196]. In summary, it may project that SSc patients with the same clot properties could be prone to thrombotic events.

The presence of prothrombotic clot phenotype may be associated with disease progression and even mortality, since the EUSTAR activity index score was found to be predictive for disease progression and the onset of severe organ involvement, while high disease per se was a risk factor for death [197,198]. Our data showed activation of coagulation and diminished fibrinolysis in SSc patients with the active disease compared to healthy controls as well as more altered fibrin clot properties in the active compared to the inactive stage of the disease. These results imply an independent effect of active disease on developing a procoagulant state among SSc patients. This is further supported by the finding that fibrin clot density may distinguish active disease cases with good predictive value. Thus, our results show that complex interplay between altered inflammation, vasculopathy, and fibrosis may drive procoagulant clot feature genesis.

From all observed SSc clinical features, only ILD was considerably associated with impairment of both haemostatic processes, addressing their critical role in the pathogenesis of lung fibrosis. Namely, haemostatic disturbance followed by excessive fibrin deposition and upregulation of TF expression was observed in alveolar space among SSc ILD patients, contributing further to the enchanted circle of events with extensive fibrosis development [199–201]. Increasing evidence supports the close association between an impaired coagulation system and lung fibrosis onset, including SSc ILD [202].

Eventually, we noticed that in the whole SSc cohort and within patients without ILD, increasing clot density correlated to the decreased values of markers of disease severity (FVC% and DLCO%) suggesting that procoagulant fibrin clot phenotype may be linked with not only ILD pathogenesis but also with worse prognosis. In fact, decreased FVC% and DLCO% were found predictive for ILD onset [203]. A prospective EUSTAR study including 5,860 SSc patients reported that PFT findings of restricted lung function and the decline of DLCO% were independent risk factors for mortality [29]. A nationwide Norwegian SSc cohort study observed that mortality risk starts to increase with decreasing FVC% from even normal values (FVC<100%)[204]. Even though a progressive course of ILD is not as typical in SSc as in idiopathic pulmonary fibrosis, identifying prognostic biomarkers would be of great importance for optimal treatment [205]. So far, the only biomarker in clinical use shown to be prognostic for progressive ILD is CRP [206,207]. We found within ILD patients a clear inverse association between thrombin generation and FVC% on the one hand and positive relation of ETP with CRP on the other, implying that thrombin could have an essential role in ILD progression. Our finding is novel but not surprising since thrombin has been considered a key player in SSc ILD pathogenesis, mediating inflammation, inducing generation of profibrotic molecules like TGF β via PAR1 stimulation, promoting myofibroblast differentiation, and affecting the production of specific autoantibodies in SSc [202,208]. Furthermore, we also observed a positive association between ETP and VWF only among patients with ILD. Thus, enhanced thrombin generation could be a novel risk factor for thromboembolic events in patients with ILD since previously VWF Ag has been found elevated in SSc patients with increased D dimer, which was associated with thrombotic events [73]. Thus, our results may identify ILD patients at risk of progression and thromboembolism who would benefit mostly from direct thrombin inhibitor therapy, which has already been shown to have a positive effect in vitro on ILD [209,210]. Recently, dabigatran has proven to be a safe and well-tolerated therapy in patients with SSc ILD, impacting BAL thrombin activity [211].

Our patients with progressive phenotype-like dSSc subtype, contractures/signs of late NVC pattern had modestly denser fibrin clots. Additionally, a global marker of fibrinolysis, CLT, was found prolonged in patients with sclerodactyly, pitting scars, or DUs. All mentioned clinical characteristics are related to poor prognosis, confirming that a procoagulant state may contribute to a worse outcome. These results further point towards close relation between progressive vasculopathy, chronic hypoxia, hypercoagulable state, and fibrosis in SSc pathogenesis. Recently, it has been shown that activation of hypoxia-inducible factor 1 signalling pathway stimulates thrombus formation targeting TF and PAI1 [212,213].

Besides evidence that diminished fibrinolysis underlays peripheral vascular manifestations in our cohort, prolonged CLT was found in cases with a higher risk of PAH. Thus, prothrombotic fibrin network, less prone to lysis, may be associated with severe proliferative vasculopathy of life importance.

Fibrin clot structure is mainly determined by fibrinogen and thrombin [46]. Our results revealed that, apart from fibrinogen, ICAM1 independently impacted the formation of denser clots in the SSc cohort.

There are several potential mechanisms by which ICAM1 may affect prothrombotic clot genesis: [214] , platelet activation [215], upregulation of TF production [216], and mediating NETosis [217]. Since denser fibrin clots were associated with severe loss of capillaries in our study, we cannot exclude that ICAM1 contribution to clot structure may partially be secondary due to hypoxia [218].

Our research mainly focused on analysing haemostasis disturbance and endothelial dysfunction in SSc patients with the most common visible presentation of peripheral microvascular disease. Almost 40% of patients could experience their first DUs within the first year, and even 73% of patients within 5 years of disease onset. Moreover, irrespective of new therapeutic modalities for DUs, 1-2/3 of patients with DUs still have recurrent ulcers linked with a higher disease burden [142–144]. From our cohort with a median disease duration of 4.5 years, 67% of patients had DUs, while 87% of them have experienced at least 2 episodes of active ulcers over the course of the disease. Over 1.5 years of follow-up, 51% got new DUs and 65% were episodic. None of our patients with digital vasculopathy has ever been treated with therapy for either DU prevention and healing or RP, except with CCB recommended by EULAR 2017 [142]. Therefore, we believe that our results mirror the natural course of SSc digital vasculopathy.

Significantly elevated markers of EC activation (ICAM1, VCAM1, E selectin) and enhanced coagulation (increased ETP, Cmax) were noticed only in patients with a history of DUs compared to controls, which is indicative of the master role of endothelial injury in the pathogenesis of digital vasculopathy [33,115]. The evidence that a hypercoagulable state is also crucial for DU pathogenesis is in line with previously observed microthrombosis inside vascular lumen, documented by biopsies from the digits of SSc patients [219]. Several pathways have been described in the literature that may contribute to the pathogenesis of prothrombotic state underlying DUs. MPs exposing phosphatidylserine, TF or VWF may be implicated in the coagulation cascade activation, thrombin generation, and platelet aggregation. Higher levels of endothelial MPs were reported in patients with a history of DUs [148,179]. Furthermore, anti-annexin V antibodies associated with prothrombotic conditions, such as foetal loss and thrombotic complications in SLE, were also elevated within SSc patients suffering from DUs [220,221]. CD40 ligands have been reported to be related to DUs, and their role in microvascular thrombosis was previously proven by an experimental study [150,222].

We are the first to assess the relation of SSc DUs with the global fibrinolysis marker. We report notably prolonged CLT in patients with a history of DUs compared to DU naïve, as well as in patients with new DU onset during the follow-up period, compared to cases without, especially in cases with recurrent DUs. Furthermore, CLT showed good discriminatory features in identifying patients with the aforementioned digital complications. Therefore, our results imply that hypofibrinolysis may underlay DU pathogenesis and the progressive stage of microangiopathy. To some extent, those results could be expected since *Zuk et al*. have recently reported an independent association between prolonged CLT with primary RP [196], as well as with the risk for VTE development in this group of patients. Our results are supported by SEM analysis of fibrin clots. Namely, the most densely packed fibrin fibres were visualised in patients with new DUs over follow-up compared to patients without new ulcers, DU naïve patients, and healthy controls. It is well known that fibrin clot structure directly impacts fibrinolysis rate [223]. Thus, denser clots composed of extensively branched fibres with tiny pores are more rigid, with reduced permeability, and less liable to lysis [224].

Patients with either a history of DUs or with new DU onset during follow-up compared to those without did not differ regarding CRP levels and smoking status. Interestingly, when CLT was adjusted for

fibrinogen and ETP, it remained positively associated with either exhibiting DUs over the SSc course, or new DU onset over 1.5 years, suggesting that other pathways could have an impact on diminished fibrinolysis and fibrin structure. Widely recognised as an important regulator of fibrinolysis is EPCR, showing anticoagulant effect on inactivating factors Va and VIIIa. The critical enzyme of fibrinolysis, plasmin, is regulated by the plasmin α2 plasmin inhibitor complex, influenced by EPCR. Previously, reduced EPCR levels were observed in SSc patients with DUs, which might have contributed to the hypofibrinolysis [168]. Another possible explanation for diminished fibrinolysis may be impaired TFPI levels specifically in SSc patients with peripheral vascular manifestations, RP and telangiectasia, addressing the role of the extrinsic coagulation cascade in vasculopathy genesis [225].

Our study is the first to show a clear relation between ICAM1 serum levels and plasma CLT values within the whole SSc cohort, patients with a history of DUs and even those with new DU onset in the longitudinal study. Even after adjustment for either fibrinogen or ETP, this association persisted at the study group level and among cases with DUs. At the same time, ETP was the primary determinant of CLT in patients with recurrent DUs. Experimental data support the implication of ICAM1 in fibrin clot formation. Thrombin directly promotes ICAM1 expression on ECs independently of cytokine status, and one of the ICAM1 ligands is fibrinogen, enhancing leucocyte-EC interaction [226,227].

Furthermore, ICAM1 is considered a key regulator of leucocyte recruitment, cellular adhesion and transmigration into subendothelium. Consequently, ICAM1 promotes inflammation and vascular leaking upon EC activation, which could be enhanced by PAI1 [55,228]. It has already been noticed that activated ECs may contribute to the development of compact clots resistant to lysis, suggesting the presence of abnormal clot structure as a link between inflammation and thrombosis [229]. In addition, in patients with primary RP, CLT was also independently influenced by EC activation/injury marker WVF. Taking our findings together, the formation of altered fibrin clots underlying digital vasculopathy seems to be highly impacted by endothelial injury.

In our study, none of the recruited patients had chronic non-healing DUs, inflamed active DUs, osteomyelitis, or lower-limb ulcers at baseline. As expected, the presence of active DUs was significantly related to new DU onset. Nevertheless, there was no difference observed in the levels of investigated vascular markers, parameters of global assays and inflammatory markers between patients with active and past DUs, implying that the complex ulcer healing process has not impacted the development of abnormal clot structure in our cohort. Notably, some other clinical characteristics reflecting progressive microangiopathy and related per se to prolonged CLT, such as severe loss of capillaries and sclerodactyly, were more common in cases with new DU development. Therefore, we believe that detected breakdown of the fibrinolytic system is essential for development of advanced digital vasculopathy.

So far, there is no consensus regarding anticoagulant/antiplatelet therapy in SSc patients with DUs, while they are often prescribed in daily clinical practice. Very new data from one of the most extensive EUSTAR analyses including 3710 SSc cases, revealed platelet inhibitors as the main therapeutic predictor for the absence of DUs at the next visit. At the same time, oral anticoagulants did not show a positive effect. Notably, one of the study limitations were unknown reasons for these therapeutic modalities, drug doses, DU origin, lack of tracking severity and total numbers of DUs between visits, making it challenging to evaluate the effect of the antiplatelet drugs on DU pathophysiology [230]. On the contrary, we have not observed a significant difference regarding the treatment with ASA between either patients with/without DU history or absent/present new DU onset over follow-up. However, since

many SSc patients have upper gastrointestinal system impairment with a higher risk of digestive haemorrhages and the fact that there is a lack of RCT testing anticoagulant/antiplatelet therapy in SSc generally, defining patients for these targeted therapies would be incremental. Therefore, our finding of altered clot properties in patients with active disease, dSSc, ILD, sclerodactyly, late NVC pattern, pitting scars, and DUs may potentialy help rheumatologists in decision-making regarding the use of antiplatelet therapy and in defining SSc cohorts at risk for evaluating drug efficacy in RCT. Interestingly, daily use of ASA at the dose of 75mg/day did not show an impact on fibrin clot properties in our SSc cohort. Our results may mirror previous reports showing that antiplatelet drugs may have a dose-dependent impact on thrombosis development and fibrin clots permeability. Namely, it has been observed that clopidogrel (75mg) inhibits only platelet activation without influencing plasma serotonin levels which are pivotal for microthrombi and fibrosis development [74]. On the other hand, only higher doses of ASA (320mg) have shown a beneficial effect on fibrin network permeability [231]. Large RCTs are needed to demonstrate the safety and efficacy of these therapy modalities in SSc patients, mainly focusing on DUs.

The beneficial effects of immunosuppressive therapies, including glucocorticoids and cyclophosphamide on endothelial function and haemostatic balance, have been under debate [232,233]. Almost 70 years ago, the link between GCs and atherosclerosis was observed. Later, it was demonstrated that dose-dependent effect of GCs on increased CVD risk and mortality, ameliorating with doses higher than 7.5mg per day. In inflammatory diseases, such as SSC, to some extent, GCs may have dual action via promoting oxidative stress underlying the genesis of CVD or suppressing inflammation, improving endothelial and epithelial function [234,235]. Similarly, Cyp exhibits a pleiotropic beneficial effect on SSc pathogenesis through improving microvascular remodelling, impacting both angiogenesis and neovascularisation, contributing to the improvement of NVC changes. Furthermore, Cyp mediates lung fibrosis by suppressing autoimmune inflammation, promoting vascular repair, and positively influencing EndoMT and interstitial lung fibroblast. Today, Cyp is one of the recommended therapeutic options for SSc ILD by EULAR [236]. However, in vivo studies suggested that Cyp may up-regulate TF activity and increase thrombin generation, promoting a prothrombotic state [237]. Our observation that patients on GCs had increased thrombin generation while higher cumulative doses of Cyp were related to denser fibrin clots may support the hypothesis of their procoagulant effect but could also be a reflection of the severity of the disease, since the majority of patients treated with these drugs had progressive disease related to altered clot properties. Interestingly, it has been demonstrated that co-treatment with dabigatran and Cyp synergistically prevents thrombotic events by decreasing the level of MPs expressing TF and platelet activation and inhibiting tumour progression and metastasis development by suppressing TGF-β [238]. Hence, since the slightly positive impact on thrombin profile was observed among patients with former Cyp therapy along with the previously mentioned link between ETP and ILD progression, we can speculate that adding direct thrombin inhibitors to Cyp could contribute to a similar beneficial effect as previously demonstrated on tumour model.

Since SSc is a chronic disease, survival analysis is no longer supposed to be the only endpoint, instead improving, restoring or preserving the quality of life should be one of the main goals in clinical practice. Our HRQL data from the cross-sectional study mirror those of other studies [239,240], addressing that the presence of DUs significantly reduces the quality of life, affecting hand function, bodily pain and emotional status. We demonstrated that DUs only impacted the EQ5D overall score without influencing the total SHAQ DI. A validation study for SHAQ DI was the first to assess the impact of DUs, reporting that presence of DUs wasn't related to the total HAQ score, supporting the implementation of VAS for

specific SSc features that were not captured by standard HAQ [10,241]. SSc characteristics related to the presence of DUs in our cohort, like shorter disease duration and presence of lung disease, were reported as negative predictors for quality of life assessed by SF36 and EQ5D [162], suggesting that they may have an impact on patients perception in our study too. Our findings are in a line with previous findings [124], showing that gripping, eating and dressing, rather than mobility, were diminished in patients with DUs, even though the statistical difference was not observed. Recently, hand functional impairment, which was evaluated by the fingertip to palm distance showed a noteworthy link with worsening of EQ5D over time. Therefore, our results further indicate the importance of assessing hand function regarding DUs in order for therapy-specific programmes to be developed, as previously emphasised [242] and for improving quality of life. Steen V et al. reported that increased pain levels assessed by VAS pain SHAQ DI were presented in patients with progressive digital vasculopathy associated with gangrene or amputation [10], implying that higher pain levels may be a red alert for complication development. Thus, close monitoring of SSc DUs patients with a higher PR pain burden should be implemented in daily clinical practice.

The trend of higher anxiety and depression was observed among our DU patients assessed by EQ5D sub-analysis. An SSc-specific PRO instrument for assessing emotional distress has not yet been developed. Data from both cross-sectional and prospective studies are in line with our results demonstrating that DUs in SSc were associated with higher depression and anxiety measured by the Beck Depression questionnaire and the Hospital Anxiety Scale [239,243]. One of the recommended therapy modalities for RP severity belongs to antidepressants (Fluoxetine), thus RCT assessing its effect on emotional status too should be created among SSc patients with manifested vasculopathy.

To date, there is no information in the literature on whether patients can foresee DUs. Thus, we explored the predictive value of quality-of-life domains for new DU occurrence over 1.5 years of follow-up. The novelty of our study is that new DU episodes may be predicted independently by the severity of RP reported by patients using the VAS RP scale of the SHAQ DI questionnaire. Furthermore, the severity of the VAS DU scale also showed a positive association with experiencing new DUs while in the multivariate regression model, which included, among other variables, VAS RP, the significance of predictive value was lost. To some extent, this finding is not surprising, since it is well known that RP may underlay critical ischemia and DU development, even leading to severe complications like necrosis and amputation [244]. Of note is that the presence of RP at the baseline was associated with new DU occurrence in our study, implying that the severity of vasospasm among patients who already have digital vasculopathy may be critical for progressive angiopathy development. Recently, a prospective study including 492 SSc patients revealed that the presence of DUs and VAS RP were independently associated with deterioration of either physical (PCS) or mental health components from SF36 and EQ5D over time [162]. Moreover, a large longitudinal study with 1,085 SSc subjects reported that increasing DU severity, defined by the number of new DUs, was related to higher PCS worsening and shorter survival [240]. Thus, the subjective perception of the impact of RP severity on daily life may be related not only to progressive angiopathy development but also to a further worsening of quality of life and mortality, which needs to be examined in other studies. Our results support the implementation of PROs in daily clinical practice for easy assessment of broader aspects of SSc severity especially related to digital vasculopathy, allowing in time identification of patients at risk for poor prognosis.

Our study has some limitations. Measuring serum levels of endothelial injury markers is less sensitive than assessing their tissue expression or detecting expression of these molecules on the surface of endothelial MPs. Furthermore, the control cohort was relatively small, and markers of EC injury were tested only among 23 of 46 individuals, so the inclusion of a larger control group would be more suitable. Only Caucasians were enrolled, restricting the extrapolation of our results to other ethnicities. Although longitudinal cohort study design was principally used for assessing the predictive risk for new DU onset, our study could profit by including a larger cohort and a longer tracking period. Concerning data obtained for ILD, prospective studies are needed to confirm the role of in vitro thrombin generation in progression of lung fibrosis. Prospective studies are also required to evaluate the link between procoagulant clot properties and thrombotic events in SSc.

Furthermore, we used a nested cohort study. In contrast, a prospective longitudinal study following patients from the time of diagnosis, ultimately therapy-naïve, would allow assessment of the relation between tested parameters and the event of interest along with evaluating the efficacy of drugs. A relatively low number of SSc cases within the follow-up group did not let us include clinical categorical variables in multivariable analysis and test their impact on new DU occurrences. We decided to exclude DU-naïve cases from the prospective study since selection bias may impact the results. Lastly, the measurement of examined variables has not been repeated at the time of new DU onset nor at the time of DUs wholly cured.

6 CONCLUSIONS

1. Endothelial dysfunction is characterized by the coexistence of ECs activity and injury

    - Within the SSc cohort, a positive correlation was found between ICAM1 and VCAM 1
    - Positive correlation was observed between all examined adhesion molecules and both E selectin and VWF Ag

2. Our study confirmed that vasculopathy have essential role in SSc pathogenesis

    - Serum levels of endothelial activity and injury biomarkers, including ICAM1, VCAM1, E selectin and VWF Ag, were significantly elevated in SSc patients compared to HC

3. Enhanced coagulation and impaired fibrinolysis may have a critical role in SSc pathogenesis

    - Both parameters of thrombin generation in vitro (ETP, peak to thrombin generation) were significantly elevated in SSc patients compared to HC
    - SSc cases exhibited 5 fold higher ETP
    - Significantly faster thrombin generation was found in SSc patients compared to HC
    - OHP was increased in the SSc cohort
    - OFP was significantly decreased in SSc patients
    - Faster coagulation with denser fibrin clot formation was noticed in SSc patients
    - SSc patients had 2.71 fold higher risk of denser fibrin clot formation
    - Significantly longer CLT was observed in SSc cases
    - SSc patients had a 3.06 fold higher risk of prolonged clot lysis

4. The procoagulant state is associated with the active/very active SSc

    - Enhanced in vitro thrombin generation, denser fibrin clot and longer clot resolution were presented in active SSc cases with respect to HC
    - Modestly altered fibrin clot was found in active/very active patients compared to cases with inactive/moderate active disease
    - ROC analysis revealed that Cmax showed good discriminatory ability in recognizing active/very active disease

5. An altered fibrin clot may be suggestive of the presence of SSc clinical features with a poor prognosis

    - With each increase in Cmax, the risk for ILD increased 16.25 time
    - Cmax was significantly higher in patients with contractures or NVC signs of late pattern (severe loss of capillaries, ramified capillaries)
    - CLT was modestly prolonged in patients with sclerodactily and pitting scars

6. Increased endothelial activity and coagulation could be considered key points in DU pathogenesis

    - Biomarkers of endothelial activation (ICAM1,VCAM1 and E selectin) were significantly elevated in patients with a history of DUs compared to HC
    - Significantly pronounced ETP and Cmax were observed within DUs cases with respect to HC

7. Impaired fibrinolysis itself may be involved in digital vasculopathy genesis and could be a new predictive marker of advanced digital vasculopathy

    - Prolonged CLT was independently associated with the history of DUs
    - CLT may discriminate with high probability patients with a history of DUs
    - Prolonged CLT could independently predict new DUs onset
    - Fibrin clot structure from cases with new episode DU onset was denser with smaller pores less prone to fibrinolysis compared to either clot from a patient without new DU over follow-up, DU naïve or control subjects

8. Hypofibrinolysis is associated with a higher burden of digital vasculopathy

    - CLT was significantly prolonged in patients with recurrent DUs in respect to those without new DUs over follow up
    - Cases with prolonged CLT >35.4 minutes developed new DUs notably earlier than those with shorter CLT over follow up

9. ICAM1 could be considered an important determinant of CLT

    - ICAM1 was independently associated with CLT either within the whole SSc cohort or patients with a history of DUs
    - Positive correlation was found between CLT and ICAM1 in cases with new DU episodes over follow up

10. DUs have a great impact on patient's quality of life

    - EQ5D utility index was significantly decreased in patients with a history of DUs in respect to naive cases
    - Intensity of anxiety and depression along with pain were more pronounced in patients with a history of DUs

11. VAS RP could serve as a new predictive marker of novel DUs episode in SSc patients with established digital vasculopathy

    - VAS RP was independently associated with new DUs onset over follow - up period

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LIST OF ABREVIATION

ACA:	Anticentromere antibodies
ACR :	American College of Rheumatology
Ag :	Antigen
ANA :	Antinuclear antibodies
ANCA:	Anti:neutrophil cytoplasmic antibody
ANOVA :	Analysis of variance
Anti Topo I :	Antitopoisomerase I antibody
CAT:	Calibrated automated thrombogram
CCB:	Calcium channel blockers
CI :	Confidence intervals
CLT:	Clot lysis time
COPD :	Chronic obstructive lung disease
CRP :	C:reactive protein
Cyp:	Cyclophosphamide
DLCO:	Diffusing capacity of the lungs for carbon monoxide
DPS :	Digital pitting scars
dSSc:	Diffuse cutaneous systemic sclerosis
DUs:	Digital ulcers
ECM:	Extracellular matrix
ECs	Endothelial cells
ELISA :	Enzyme linked immunosorbent assays
EPCR :	Endothelial protein C receptor
EPCs:	Endothelial progenitor cells
EQ5D:	EuroQol 5:dimensions
ERA :	Endothelin receptor antagonists
ESR :	Erythrocyte sedimentation rate
ET1 :	Endothelin 1
ETP :	Endogenous thrombin potential
EULAR :	European Alliance of Associations for Rheumatology
EUSTAR:	The EULAR Scleroderma Trials and Research Group
FVC:	Forced vital capacity
GIT	Gastrointestinal tract
HAQ DI:	Health Assessment Questionnaire Disability Index
HC:	Healthy controls
HRCT :	High:resolution computed tomography
HRQoL :	Health:related quality of life
ICAM1 :	Intercellular adhesion molecule 1
IL :	Interleukin
ILD:	Interstitial lung disease
IR:	The Institute of Rheumatology
KI :	Karolinska institutet
lSSc:	Limited cutaneous systemic sclerosis
MCP:	Metacarpophalangeal joint
mRSS:	Modified Rodnan skin score
MS:	Musculoskeletal

NVC:	Nailfold videocapillaroscopy
OCP:	Overall coagulation potential
OFP :	Overall fibrinolysis potential
OHP :	Overall haemostatic potential
OR :	Odds ratio
PAH:	Pulmonary arterial hypertension
PAR :	Protease activated receptor
PDE5 :	Phosphodiesterase type 5
PFTs:	Pulmonary functional tests
PIP:	Proximal interphalangeal joint
PK :	Peak thrombin
pmp:	Per million people
PPP :	Platelet poor plasma
PROs:	Patient reports outcomes
RA:	Rheumatoid arthritis
ROC:	Receiver:operating characteristic
RP:	Raynaud's phenomenon
SEM :	Scanning electron microscopy
SF36 :	Short Form Health Survey
SHAQ:DI:	Scleroderma Health Assessment Questionnaire Disability Index
SLE :	Systemic lupus erythematous
sPAP :	Systolic pulmonary artery pressure
SRC:	Scleroderma renal crisis
SSc:	Systemic sclerosis
t:PA :	Tissue plasminogen activator
TF :	Tissue factor
TFPI :	Tissue factor pathway inhibitor
TGFβ:	Transforming growth factor beta
TNFα :	Tumor necrosis factor alpha
TP:	Time to peak
u:PA :	Urokinase type plasminogen activator
u:PAR:	Urokinase: type plasminogen activator receptor
VAS:	Visual analog scale
VCAM1 :	Vascular cell adhesion molecule 1
VEDOSS :	Very early diagnosis of systemic sclerosis
VEGF:	Vascular endothelial growth factor
VTE:	Venous thromboembolic events
VWF:	Von Willebrand factor

BIOGRAPHY

Jelena Čolić obtained MD degree at Faculty of Medicine, University of Belgrade in 2010, with average grade 9.42. Over the course of her studies, she was founded by Scholarships of honors. In 2008, she joined the research group headed by Assistant Professor Danijela Vuković at Institute of Pathophysiology with a main focus of assessing oxidative stress in atherosclerosis. In 2011, she started working at Faculty of Medicine, University of Belgrade as a Research assistant on project No 41022 entitled „Acute coronary syndrome: researching vulnerability (plaque, blood, and myocardium), the optimal treatment and determination of prognostic factors“, supported by the Serbian Ministry of Education and Science, headed by Academician Professor Miodrag Ostojić. Since 2013, MD Čolić has been working at the Institute of Rheumatology in Belgrade. In 2013 she started PhD studies at the Faculty of Medicine in Belgrade-Epidemiology module and her residency in Internal Medicine (completed in 2020). In 2014, she finished Specialist Academic Studies, Cardiology module supervised by Professor Dragan Simić. MD Čolić spent 14 months over 2018-2022 at the Karolinska Institutet (KI), Stockholm (Sweden) supervised by Associated Professor Aleksandra Antović. She finished two Phd courses regarding endothelial function and biostatistics at KI. In 2019 she won the prize for the best poster presentation on the King Gustaf V:s 80-year Foundation Rheuma retreat symposium. In 2020, she started subspecialization in Rheumatology. She completed EULAR Online Course on Systemic sclerosis (2022).

MD Čolić is a present EMEUNET Country Liaison for Serbia, Member of Initial Board of PhD Students Association at the Faculty of Medicine in Belgrade (2016 - present) and their representative at the Scientific council at the same Faculty (2021-present).

образац изјаве о ауторству

Изјава о ауторству

Име и презиме аутора Јелена Чолић

Број индекса ЕП-11/14

Изјављујем

да је докторска дисертација под насловом

The association of global hemostasis and endothelial injury markers with the risk of new digital ulcer onset in systemic sclerosis: prospective cohort study

- резултат сопственог истраживачког рада;
- да дисертација у целини ни у деловима није била предложена за стицање друге дипломе према студијским програмима других високошколских установа;
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У Београду, 29.11.2022

образац изјаве о истоветности штампане и електронске верзије докторског рада

Изјава о истоветности штампане и електронске верзије докторског рада

Име и презиме аутора Јелена Чолић

Број индекса ЕП-11/14

Студијски програм Епидемиологија

Наслов рада "The association of global hemostasis and endothelial injury markers with the risk of new digital ulcer onset in systemic sclerosis: prospective cohort study "

Ментор Проф. др Мирјана Шефик Букилица

Изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла ради похрањивања у **Дигиталном репозиторијуму Универзитета у Београду.**

Дозвољавам да се објаве моји лични подаци везани за добијање академског назива доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

Потпис аутора

У Београду, 29.11.2022

образац изјаве о коришћењу

Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић“ да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

The association of global hemostasis and endothelial injury markers with the risk of new digital ulcer onset in systemic sclerosis: prospective cohort study

која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигиталном репозиторијуму Универзитета у Београду и доступну у отвореном приступу могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

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