Predictive value of vascular disease biomarkers for digital ulcers in systemic sclerosis patients

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ABSTRACT

Objective. *To investigate the role of endothelial dysfunction and angiogenesis vascular biomarkers as risk factors and their predictive value for digital ulcers in systemic sclerosis patients.*

Methods. *Endothelin-1 (ET-1), asymmetric dimethylarginine (ADMA), vascular endothelial growth factor (VEGF), endostatin and endoglin were measured in an observational prospective cohort of 77 SSc patients. The primary outcome was the occurrence of one or more new ischaemic digital ulcers during a planned 3-year follow-up.*

Results. *After the 3-year follow-up, 40 patients developed new digital ulcers. Logistic regression confirmed VEGF (HR 1.128, 95% CI 1.010–1.260, p=0.033) and ADMA (HR 0.995, 95% CI 0.991–0.998, p=0.006) as independent predictors of new digital ulcers. Patients with serum levels of ET-1>11.9pmol/ml (p<0.001) and VEGF<422.47 pg/ml (p=0.028) had significantly more DU in the 3-year follow-up. Although not significant, a trend towards increased serum levels of endoglin>4.215ng/ml (p=0.053) was associated to a new DU episode. No predictive serum value was found for ADMA (p=0.075) and endostatin (p=0.130).*

Conclusions. *Endothelial dysfunction and angiogenic vascular biomarkers have an important role in the underlying and in the progression of microvascular disease in systemic sclerosis. Increased serum levels of ET-1, ADMA and VEGF are strong predictors of severe microangiopathy complications, namely ischaemic digital ulcers.*

Introduction

Vascular disease is of fundamental importance in the pathogenesis of scleroderma from very early onset of the disease through late clinical complications (1). Vascular involvement is widespread with an extremely heterogeneous clinical expression, from Raynaud phenomenon to severe digital ulcers (DU) up to life threatening pulmonary arterial hypertension (2). Key issue is to understand the pathophysiology underling vascular dysfunction in order to explain why some patients with systemic sclerosis (SSc) progress to severe digital ischemia while others have no DU in the disease course.

Endothelial cell (EC) dysfunction has been postulated as a key and early inciting event in the disease process. Injured endothelium leads to an imbalance of microvascular tone control, favouring vasoconstriction, due to overproduction of vasoconstrictors by endothelial cells (such as potent vasoconstrictor ET-1) or to reduced endothelium-dependent vasodilation (3). Doubts persist about underproduction or impaired action of vasodilators produced by the endothelium, such as nitric oxide (NO) and prostacyclin (3).

Vascular remodelling following microvascular injury with intimal and medial thickening and adventitial fibrosis leads to progressive stenosis and vascular occlusion (4). It has been postulated that avascular areas are consequence of chronic hypoxia and that enlarged and bushy/ramified capillaries are a pro-angiogenic response not related to hypoxia but to the overexpression of VEGF (5) or as a consequence of an imbalance in angiogenic factors/angiostatic factors. Angiogenesis biomarkers have been extensively studied in SSc patients and investigated as possible putative biomarkers of organ involvement.

Our main objective was to analyse the role of vascular biomarkers of endothelial dysfunction and angiogenesis as risk factors for active DU or as predictors for new DU episodes in a 3-year follow-up.

Materials and methods

Patients

Seventy-seven SSc patients (72 women; mean age 52.95±12.6 years; range

14-79) attending Multidisciplinary Raynaud Clinics of the Clinical Immunology Unit at Centro Hospitalar do Porto in Portugal were followed in a prospective, longitudinal observational study between October 2011 and December 2014. All patients fullfield 2013 classification criteria for SSc of ACR/EULAR (6). Thirteen patients (16.9%) had diffuse SSc (dcSSc) and 64 (83.1%) had limited SSc (lcSSc) According to Leroy classification (7). Thirty-four healthy, sex/age matched, non-obese, without self-reported cardiovascular risk factors controls were invited to participate. No control subject was on any vasoactive medication.

Patients were divided into two groups: SSc-DU group - 38 patients with an active ulcer at baseline, with or without a past history of DU (34 women; mean age 52.7±14.8 years; range 14–75) and SSc-non-DU group - 39 patients with no DU in the course of disease until inclusion (38 women; mean age 53.2±10.3 years; range 30–79).

The local institutional health ethics committee approved this study and consent forms were signed by all participants, in accordance with the ethical standards of Helsinki Declaration.

Table I. Comparison of vascular biomarkers between groups at baseline.

Variables	DU (n=38)	Non-DU (n=39)	Control (n=40)	*p*-value
Endoglin ng/ml Median (Q_1-Q_3)	3.01 (1.46-7.02)	1.88 (0.84-3.28)	0.28 (0.15-0.71)	<0.001*,a
Endostatin ng/ml Median (Q_1-Q_3)	0.695 (0.26-1.73)	0.429 (0.16-0.77)	0.565 (0.35-0.77)	0.164a
VEGF pg/ml Median (Q_1-Q_3)	245.06 (158.68-347.33)	422.47 (269.26-847.97)	178.03 (101.27-222.10)	<0.001*,a
ET-1 pmol/ml Median (Q_1-Q_3)	16.13 (10.97-21.17)	8.8 (5.89-12.68)	2.48 (0.00-5.60)	<0.001*,a
ADMA umol/L Median (Q_1-Q_3)	0.515 (0.45-0.63)	0.45 (0.41-0.51)	0.38 (0.32-0.43)	<0.001*,a

DU: digital ulcer; VEGF: vascular endothelial growth factor.
[a] Kruskal Wallis; *Statistical significance for a level of 5%.

Biomarkers

Fasting venous blood samples were collected into serum and sodium heparin tubes (Vacuette, Greiner-Bio-One, Austria). Serum was allowed to clot at room temperature and then separated from cells within 60 minutes and stored at -70 ºC until analysis.

ET-1 assessment (pmol/ml): Plasma was centrifuged immediately in a refrigerated centrifuge and stored at -70ºC until analysis for endothelin. Plasma endothelin was measured using a RIA assay, (Euro-Diagnostics AG, Sweden).

ADMA assessment (umol/L): Serum was allowed to clot at room temperature and then separated from cells within 60 minutes and stored at -70ºC before analysis for ADMA. Serum ADMA was measured using enzyme-linked immunosorbent assay, (Immunodiagnostik AG, Germany).

VEGF assessment (pg/ml): Serum VEGF was measured using enzyme-linked immunosorbent assay, (IBL International GMBH, Germany).

Endoglin and Endostatin assessment (ng/ml): Serum endoglin and endostatin were measured using enzyme-linked immunosorbent assay, (Uscn, Life Science Inc., Wuhan).

Table II. Logistic regression of vascular biomarkers as baseline.

	Simple				Adjusted			
	B	HR	CI	*p*-value	B	HR	CI	*p*-value
VEGF pg/ml	-0.004	0.996	0.994-0.999	0.007*	-0.006	0.994	0.989-0.998	0.009*
ET1 pmol/ml	0.194	1.214	1.096-1.344	<0.001*	0.153	1.165	1.041-1.304	0.008*
ADMA umol/L	9.533	1.381E4	41.229-4.625E6	0.001	10.378	3.216E4	10.109-1.023E8	0.012*
Endoglin ng/ml	0.197	1.218	1.021-1.452	0.028*	0.098	1.103	0.959-1.270	0.170
Endostatin ng/ml	0.089	1.093	0.909-1.315	0.342	0.205	1.228	0.9251.631	0.156

ET-1: endothelin-1; ADMA: asymmetric dimethylarginine; HR: hazard ratio; CI: confidence interval. *statistical significance for a level of 5%.

Table III. Logistic regression of vascular biomarkers as predictive mediators for new digital ulcers episode in the 3-year follow-up.

	Simple				Adjusted			
	B	HR	CI	*p*-value	B	HR	CI	*p*-value
VEGF pg/ml	0.169	1.184	1.067-1.313	0.001*	0.120	1.128	1.010-1.260	0.033*
ET1 pmol/ml	5.155	173.334	0.91-3.301E4	0.054	4.558	95.436	0.079-1.154E5	0.079
ADMA umol/L	0.004	0.996	0.993-0.999	0.002*	0.005	0.995	0.991-0.998	0.006*
Endoglin ng/ml	0.152	1.164	0.98-1.381	0.083	0.061	1.063	0.929-1.216	0.929
Endostatin ng/ml	0.155	1.168	0.913-1.493	0.216	0.293	1.341	0.972-1.851	0.972

ET-1: endothelin-1; ADMA: asymmetric dimethylarginine; HR: hazard ratio; CI: confidence interval. *statistical significance for a level of 5%.

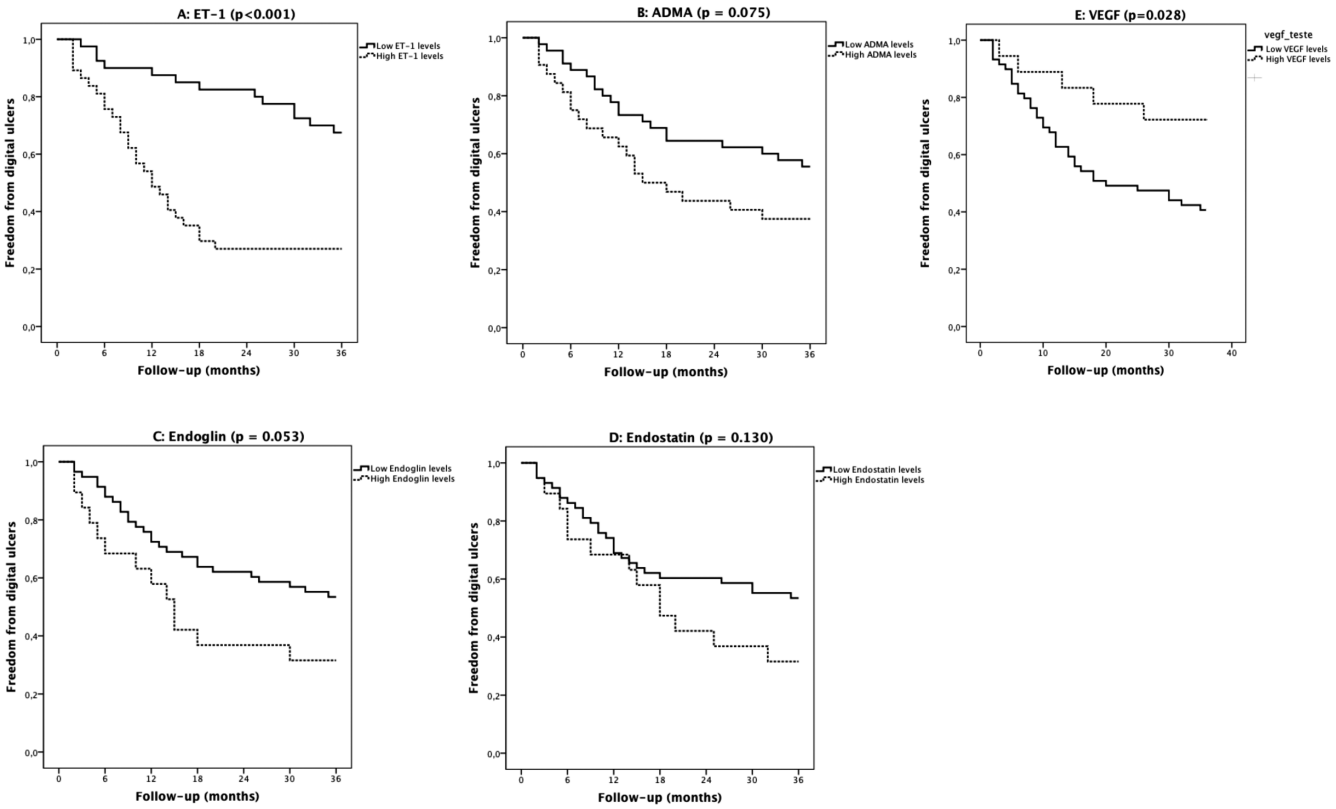

Fig. 1 (A-E). Kaplan-Meier analyses of freedom from new digital ulcers in 36 months follow-up of 77 systemic sclerosis patients. Curves are shown for: **A**: Low ET-1 serum levels (≤11.9 pmol/ml) or high levels (>11.9 pmol/ml); **B**. ADMA low serum levels (≤0.49 umol/l) or high levels (>0.49 umol/l); **C**: low Endoglin serum levels (≤4.215 ng/ml) or high levels (>4.215 ng/ml); **D:** low Endostatin serum levels (≤1.246 ng/ml) or high levels (>1.246 ng/ml); **E:** low VEGF serum levels (≤ 422.47 pg/ml) or high levels (>422.47 pg/ml).
ET-1; endothelin-1; ADMA: asymmetric dimethylarginine; VEGF: vascular endothelial growth factor. Statistical significance for a level of p<0.05.

Statistical analysis

For comparison of normally distributed variables, we used unpaired or paired two-sided student's *t*-test or analysis of variance (Anova). In these cases, data were described by mean ± standard deviation (SD). Normal distribution was tested by Q-Q plots. In non-normally distributed variables, we used non-parametric tests: Mann-Whitney and Kruskal Wallis tests and data were described by median followed by the interquartile interval (Q_1-Q_3), where Q_1 and Q_3 represent the first and the third quartiles, respectively. For comparison of categorical variables, we used Chi-square or Fisher's exact probability test. Predictors of digital ulcers were evaluated by univariate and multivariate logistic regression. We applied survival analysis to determine the probability of freedom from new DU and evaluated the effects of the biomarkers in that probability using the Kaplan-Meier method and the Cox regression. Values of $p \leq 0.05$ were considered as significant. Data were analysed using the SPSS software (v. 22.0, SPSS, Chicago, IL).

Results

Baseline digital ulcers

The demographic and clinical baseline characteristics of the 77 SSc patients are described in supplementary data. (Available online - supplementary data: Table I). All biomarkers had significant differences between groups and controls (Table I).

By univariate logistic regression analysis there were significant differences between DU and non-DU groups regarding disease subset dcSSc (p<0.001), increased serum levels of ET-1 (HR 1.214, 95% CI 1.096–1.344, p<0.001), ADMA (HR 1.381E4, 95% CI 41.229–4.625E4, p=0.001) and endoglin (HR 1.218, 95% CI 1.021–1.452, p=0.028) as well as reduced levels of VEGF (HR 0.996, 95% CI 0.994–0.999, p=0.007). Multivariate logistic regression analyses confirmed ET-1 (HR 1.165, 95% CI 1.041–1.304, p=0.008), ADMA (HR 3.216E4, 95% CI 10.109–1.023E8, p=0.012), and VEGF (HR 0.994, 95% CI 0.989–0.998, p=0.009) as independent risk factors for active ulcers (Table II).

Main outcome: new digital ulcer episode in 3-year follow-up

In the 3-year follow-up, 40(51.95%) patients presented new ischaemic digital ulcers. By univariate analysis, history of at least one DU before enrolment (p<0.001), dcSSc (p=0.048), increased ADMA (HR 0.996, 95% CI 0.993-0.999, p=0.002) and low serum VEGF levels (HR 1.184, 95% CI 1.067–1.313, p<0.001) were identified as risk factors for the occurrence of at least one new DU. Although not significant increased serum ET-1 levels (HR 173.334, 95% CI 0.91–3.301E4, p=0.054) were also associated to new DU episodes. Multivariate logistic regression confirmed VEGF (HR 1.128, 95% CI 1.010–1.260,

p=0.033) and ADMA (HR 0.995, 95% CI 0.991–0.998, p=0.006) as independent predictors of new DU (Table III).

We determined serum cut-off levels (Q_2) of the vascular biomarkers to further evaluate their prediction value for new DU in the 3-year follow-up. Kaplan-Meyer analysis of freedom of DU are shown in Figure 1. Patients with serum levels of ET-1 >11.9pmol/ml (p<0.001) and VEGF <422.47 pg/ml (p=0.028) had significantly more DU. Although not significant, a trend towards increased serum levels of Endoglin >4.215ng/ml (p=0.053 was associated to a new DU episode. No predictive value was found for ADMA >0.49umol/l (p=0.075) and Endostatin >1.246ng/ml (p=0.130).

Discussion

The present study demonstrated that increased circulating serum levels of ET-1 and ADMA as biomarkers of endothelium dysfunction as well as low angiogenic mediator VEGF were independent risk factors of active digital ulcers. Analysing the prediction value of these vascular biomarkers only ADMA and VEGF were identified as independent predictors of new DU episodes.

Endothelin-1, apart from being one of the strongest endogenous vasoconstrictor mediators, has also been recognised as a potent mitogen, and there is experimental evidence to suggest that ET-1 contributes to the vascular remodeling and organ damage in different clinical conditions (8).

Endogenous inhibitor of endothelial NO-synthase, ADMA, has emerged as a promising biomarker of endothelial dysfunction in cardiovascular diseases (9). A reduction in NO amplifies vasoconstrictor episodes and promotes pathological changes in vascular system such as thrombotic and inflammatory signalling and vascular remodelling (10). Our results suggest increased serum levels of ADMA as a risk factor for active ulcers as well as predictive biomarker for new DU.

Reduced VEGF levels suggest that ineffective angiogenesis may contribute to the avascular areas largely responsible for the ischaemic territory underlying digital ulcers. Other explanation is that a prolonged, uncontrolled and chronic overexpression of VEGF in SSc may have a deleterious effect on the vascular network resulting in a chaotic vascular morphology with reduced blood flow in the newly formed vessels (5, 11). The present data confirm previous reports regarding serum ET-1 (8, 12) and VEGF (4, 13, 14) and their association to DU.

Regarding angiostatic biomarkers, we found an association between active ulcers and increased levels of endoglin, similar to Wipff *et al*. (15) but no predictive value for new DU episode. Conflicting results have been reported regarding endostatin in SSc patients (4, 13, 16). We failed to show any connection or predictive value of endostatin and DU.

Our study has some limitations, small patient sample all recruited from the same centre and limited by number of patients with active DU at time of enrolment. Additional studies, with larger cohorts are needed to validate these predictive factors, enabling further understanding of the progression of vascular damage and angiogenesis in the aetiology of DU.

Conclusion

Our study confirmed the relationship and predictive value of endothelial dysfunction and angiogenic biomarkers with DU.

Overproduction of the potent vasoconstrictor ET-1, increased ADMA concentration down-regulating the production of NO-synthase with consequent abnormal regulation of NO production and impaired angiogenesis due to low VEGF values underlie microvascular disease pathways. Angiostatic factors although increased in patients with DU had no predictor value for new fingertip ulcers.

The analysis of these vascular mediators associated to SSc vasculopathy are particularly interesting as they might help to identify new therapeutic targets in order to prevent further vascular injury.

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