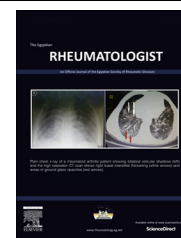




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ORIGINAL ARTICLE

Serum vascular endothelial growth factor (VEGF), soluble VEGF receptor-1 (sVEGFR-1) and sVEGFR-2 in systemic sclerosis patients: Relation to clinical manifestations and capillaroscopy findings

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KEYWORDS

Systemic sclerosis;
 Vascular endothelial growth factor;
 Angiogenesis;
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Abstract *Introduction:* The role of angiogenesis in the pathogenesis of systemic sclerosis (SSc) is well known. The imbalance between vascular endothelial growth factor (VEGF) and their anti-angiogenic soluble receptors (sVEGFR-1 and VEGFR-2) has been proposed as a possible cause of microangiopathy.

Aim of the work: To determine the levels of VEGF, sVEGFR-1 and sVEGFR-2 and the VEGF/sVEGFR1 and VEGF/sVEGFR2 ratios in SSc patients and to study their relation with clinical manifestations and capillaroscopy findings.

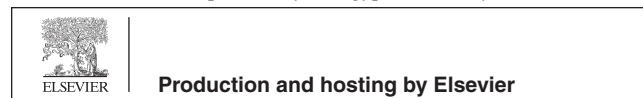
Patients and methods: The study included 44 SSc patients and 44 controls. The sclerosis severity was assessed by the modified Rodnan skin score (mRss) and capillaroscopy performed in patients. Serum VEGF, sVEGFR-1 and sVEGFR-2 were measured in patients and control.

Results: SSc patients had a mean age of 40.7 ± 12.8 years, M:F (1:9) and disease duration was 56.2 ± 60.6 months. 27 patients (61.4%) had diffuse-SSc and 17 (38.6%) limited. The mean VEGF was significantly higher (363.4 ± 133.9 pg/ml) and sVEGFR-2 lower (2039.6 ± 109 pg/ml) in

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patients compared to control (93.9 ± 25.2 pg/ml and 2366 ± 116.5 pg/ml; $p = 0.05$ and $p = 0.04$, respectively). Serum levels of sVEGFR-2 in patients with early, active and nonspecific scleroderma pattern of capillaroscopy was higher in comparison to patients with late scleroderma pattern ($p = 0.05$). There were no significant differences in the studied parameters between those patients with and without digital ulcerations and interstitial pulmonary fibrosis. A significant correlation was found between mRss and VEGF ($p = 0.04$).

Conclusion: An overproduction of VEGF, a potent angiogenic molecule or down regulated production of its natural inhibitors (sVEGFR-2) might be involved in the development of vasculopathy in SSc patients.

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1. Introduction

Systemic sclerosis (SSc) is a connective tissue disorder that affects the skin and internal organs [1]. Microvasculopathy in the finger vessels leads to Raynaud's phenomenon that can result in digital ischemia [2,3]. Excessive synthesis and deposition of collagen in the extra-cellular matrix are involved in the pathophysiology of scleroderma [4]. The vascular injury in SSc is not an inflammatory process, characterized by a vasculopathy in the absence of vasculitis [5,6]. An increased risk of peripheral arterial disease has been reported in SSc [7]. In a previous study [8], fibroblast growth factor-23 had no obvious role in the pathogenesis or clinical manifestations of Iranian SSc patients. Endothelial cell injury is an early and main event in patients with SSc [9]. High serum level of Von Willebrand factor [10], endothelin-1 (ET-1) [11], and circulating dead endothelial cells [12] are involved in endothelial injury and in the pathogenesis of SSc [6,13].

Higher level of ET-1 has been reported in patients with scleroderma renal crisis, pulmonary hypertension and Raynaud's phenomenon [14,15]. The vascular endothelial growth factor (VEGF) is a potent angiogenic peptide and main regulator of blood vessels by a variety of cell types [16]. Hypoxia and tissue ischemia lead to expression of angiogenic growth factors, e.g. VEGF [17–19]. Because of its intimate association with both normal and abnormal angiogenesis, VEGF has become an attractive target for both proangiogenic and antiangiogenic therapy [20–23]. VEGFs mediate angiogenic signals to the vascular endothelium via high affinity receptor tyrosine kinases, designated soluble VEGF receptors-1 [soluble fms-like tyrosine kinase (sFlt)-1] (sVEGFR-1), soluble VEGF receptor-2 [Flk1/KDR] (sVEGFR-2), and VEGFR-3 (Flt4) [24].

Expression of VEGF and its receptors is increased in the skin of SSc patients [25,26]. Soluble Flt-1 (sFlt-1) is an endogenous inhibitor of VEGF given that it is a variant form of VEGF receptor 1 (VEGFR-1) that lacks the transmembrane and intracellular domains of VEGFR-1 yet binds to VEGF with the same affinity as VEGFR-1 and therefore blocks VEGF function. It is an endogenous anti-VEGF that may cause microangiopathy [27] and is associated with endothelial dysfunction in chronic kidney diseases (CKD) [28]. They are expressed in atherosclerotic and restenotic lesions as well as in post-injury rat aortic intimal thickening but not in normal tunica media [10–13]. In another study, the increase in soluble Flt-1 was associated with a particularly high risk of severe fetal growth restriction [29]. The recently discovered sVEGFR-2 has shown potent antiangiogenic effects [30]. We need to know the

levels and ratios of these angiogenic and antiangiogenic factors (VEGF/sVEGFR-1 and VEGF/sVEGFR-2) in different diseases with angiogenic disturbance.

In this study, we determined the serum levels of VEGF as an angiogenic factor and sVEGFR-1 and 2 (as anti-angiogenic factors) in SSc patients compared with healthy controls as a risk factor in the pathogenesis of scleroderma. Also, the correlation of these factors with the main disease manifestations including lung and heart involvement and digital ulcers as well as to the capillaroscopy findings has been evaluated.

2. Patients and methods

In this case-control study, 44 Iranian patients who fulfilled the American College of Rheumatology criteria for PSS [31] were recruited and grouped into patients with limited or diffuse skin involvement according to a proposed classification system [32]. Patients were referred to the scleroderma clinic of Hafez Hospital of Shiraz University of Medical Sciences, Iran, from April 2011 to April 2012. Forty four age and sex matched healthy controls without any rheumatic diseases were also included. Patients were above 16 years and selected through random sampling. All patients signed an informed consent before inclusion in the study. The study was approved by the Shiraz University of Medical Sciences research ethics committee and was in accordance to the 1964 Helsinki declaration and its later amendments. Cases with overlap syndrome, impaired renal function ($GFR < 60$ ml/min/1.73 m²), smokers, diabetes mellitus, active infection and receiving immunosuppressives as cyclophosphamide, azathioprine, mycophenolate mofetil and methotrexate were excluded; patients using prednisolone ≥ 7.5 mg were also excluded from the study.

The demographic and clinical data were collected, pulmonary involvement assessed by high resolution lung CT scan (HRCT), skin sclerosis severity assessed by the modified Rodnan skin score (mRss) [33], vascular involvement by digital tip ulcerations and pulmonary artery hypertension (PAH) defined by echocardiography; also, if pulmonary artery pressure was more than 40 mm Hg, it was confirmed by cardiac catheterization.

Capillaroscopy: It was performed by stereomicroscope (Euromex ST, 1740, $\times 250$ power and video camera Cnex D.C. 5000, 5 megapixels, Holland). Immersion oil was applied on nailfold bed to improve the resolution. Eight fingers of the two hands excluding the thumbs were assessed. The fingers with thick nailfolds and ulcerated ones were not studied. Data including distribution, shape of capillaries,

the largest diameter of arterial or venous side (dilated loops: irregular or homogeneous increase of capillary diameter ≥ 20 and $< 50 \mu\text{m}$; giant capillaries: homogeneously enlarged loop with a diameter $\geq 50 \mu\text{m}$), capillary length, mean capillary density, avascular area, microhemorrhages and neoangiogenesis or ramified capillaries (branching, bushy, interconnected capillaries, originating from a single capillary) were recorded in a form. The whole findings were defined as normal, specific changes for early, active, and late scleroderma pattern or non-specific changes [2]. The available capillaroscopic results of the patients that were done before and after the time of blood sampling were reviewed and considered. Even patients with late SSc pattern by capillaroscopy were not receiving any previous medications.

Serum levels of VEGF (Quantikine, R&D systems, USA), sVEGFR-1 (Quantikine, R&D systems, USA) and sVEGFR-2 (Adipo Bioscience, USA) were measured in all samples using ELISA kits, according to the manufacturer's recommendations. The sensitivity of the kits for VEGF, sVEGFR-1 and sVEGFR2 was 9.0 pg/ml, 3.5 pg/ml and 15 pg/ml, respectively.

As sVEGFR-1 could play a role in diabetic nephropathy and CKD, respectively, the estimated creatinine clearance rate (eCCr) was checked using Cockcroft–Gault formula to find the glomerular filtration rate (GFR) of patients [27,28].

Statistical analysis: Data were analyzed in SPSS, version 16, using descriptive, Chi-square test, *T*-test, Man Whitney test, and Pearson correlation. Results were presented as mean \pm SD (range) and as number (percentage). The standard error was considered to reduce the number digits of the values. The significance level was set as $p \leq 0.05$.

3. Results

The SSc patients had a mean age of 40.7 ± 12.8 years (range: 16–74 years) and had a M:F (1:9). The mean matched age of the control was 39.4 ± 11.76 years ($p > 0.05$) and they were 3 males and 41 females. 27 patients (61.4%) had diffuse SSc and 17 (38.6%) limited. Demographic features, clinical characteristics and capillaroscopic data are shown in Table 1. The disease duration was 56.2 ± 60.6 months (range: 2–240) being longer in those with diffuse SSc subtype (72 ± 71.8 months) compared to those with limited form (56.2 ± 60.6 months) ($p = 0.27$). In these patients, none had pulmonary hypertension or cardiac involvement. Capillaroscopic data were available for 18 patients.

Mean serum levels of VEGF, sVEGF-R1, sVEGF-R2 as well as the VEGF/sVEGFR1 and VEGF/sVEGFR2 ratios were compared between patients and controls (Table 2). Serum levels of VEGF, sVEGF-R1, and sVEGF-R2 were higher in patients with diffuse SSc compared to the limited subtype, but those differences were not significant ($p = 0.34$, $p = 0.54$ and $p = 0.8$, respectively). Levels were also comparable between each one of the disease subtypes and control. Of interest, the levels of VEGF and VEGF/sVEGF-R2 were significantly different between diffuse SSc and control ($p = 0.014$ and $p = 0.008$, respectively).

Mean serum levels of VEGF (444.4 pg/ml), sVEGF-R1 (1203.5 pg/ml) and sVEGF-R2 (2036.3 pg/ml) and the ratio of VEGF/sVEGFR1 (2.4) and VEGF/sVEGFR2 (0.22) in patients with active, early and nonspecific scleroderma pattern in their capillaroscopy were higher in comparison to patients

Table 1 Demographic features, clinical characteristics and capillaroscopic findings of the systemic sclerosis patients.

Characteristic mean \pm SD (range) or n (%)	SSc patients (n = 44)
Age (years)	40.7 \pm 12.8 (16–74)
Gender M:F	4:40 (1:9)
Disease duration (months)	56.2 \pm 60.6 (2–240)
<i>Subtypes:</i>	
-Limited	17 (38.6)
-Diffuse	27 (61.4)
mRss score (in 42 patients)	21.48 \pm 8.6 (4–41)
Raynaud's phenomenon	44 (100)
Digital pitting scars	44 (100)
Finger tip ulcers	9 (20)
Interstitial pulmonary fibrosis	4 (9)
<i>Capillaroscopy findings</i> (n = 18)	
-Normal	0 (0)
-Nonspecific change	3 (16.7)
-Early scleroderma pattern	1 (5.5)
-Active scleroderma pattern	11 (61.1)
-Late scleroderma pattern	3 (16.7)
-Dilated loops	18 (100)
-Giant loops	12 (67)
-Neoangiogenesis	10 (55)
-Microhemorrhages	13 (72)
Number of capillaries/mm	3–8

SSc, systemic sclerosis; mRss, modified Rodnan skin severity.

with late scleroderma pattern (256.5 pg/ml, 71.3 pg/ml, 955.8 pg/ml, 4.9 and 0.43 respectively) and these levels reached the significant level only for sVEGF-R2 ($p = 0.6$, $p = 0.2$, $p = 0.04$, $p = 0.5$ and $p = 0.4$, respectively) (Fig. 1).

There was no significant correlation between the serum levels of VEGF with that of sVEGF-R1 and sVEGF-R2 in the control ($r = 0.09$, $p = 0.56$; $r = 0.15$, $p = 0.32$ respectively). There was a significant correlation between serum VEGF and sVEGF-R1 in SSc patients ($r = 0.43$, $p = 0.004$). But no correlation was seen between VEGF and sVEGF-R2 in patients ($p = 0.09$, $r = 0.26$). There was no correlation between all the studied parameters and disease duration or with the GFR (checked in 23 patients) ($p > 0.05$).

sVEGF-R1 and sVEGF-R2 serum levels in patients with digital tip ulceration were lower ($p = 0.54$ and $p = 0.8$ respectively) while VEGF levels were higher ($p = 0.34$) compared to those without although the difference was not significant (Table 3). Levels of VEGF, sVEGF-R1, sVEGF-R2 and the ratio of VEGF/sVEGFR1 and VEGF/sVEGFR2 were not significantly different between patients with and without interstitial pulmonary fibrosis ($p = 0.52$, $p = 0.7$, $p = 0.7$, $p = 0.12$, and $p = 0.74$, respectively) (Table 4). There was no association between the presence of dilated loops, giant loops, neoangiogenesis, microhemorrhages, avascular areas or the number of capillaries in each millimeter of their nail bed with the VEGF, sVEGF-R1 and sVEGF-R2 ($p > 0.05$). There was a significant correlation between the mRss and levels of VEGF ($r = 0.31$, $p = 0.04$). sVEGF-R1 and sVEGF-R2 did not show a significant correlation with the skin severity score ($r = 0.15$, $p = 0.36$, and $r = 0.01$, $p = 0.96$, respectively).

Table 2 Serum levels of VEGF, sVEGF-R1, sVEGF-R2 and their ratios in systemic sclerosis patients and control.

Mean \pm SE (range)	VEGF (pg/ml)	sVEGF-R1 (pg/ml)	sVEGF-R2 (pg/ml)	VEGF/VEGF-R1	VEGF/VEGF-R2
SSc patients	363.4 \pm 133.9 (0–4647.9)	816.5 \pm 356.6 (0–13072.4)	2039.6 \pm 109 (518.2–3488.4)	1.7 \pm 0.3 (0–7.6)	0.18 \pm 0.1 (0–1.75)
Diffuse	514.4 \pm 1167 (0–4647.9)	1177.7 \pm 2970 (0–13072.4)	2066.2 \pm 761 (699.2–3488.4)	1.89 \pm 2.4 (0–7.6)	0.23 \pm 0.4 (0–1.75)
Limited	181.6 \pm 310 (0–1181.5)	473.9 \pm 1521 (0–5758.2)	1926.2 \pm 795 (518.2–2963.9)	1.48 \pm 1.7 (0–5.5)	0.12 \pm 0.15 (0–0.49)
Control	93.9 \pm 25.2 (1–884.66)	1025.6 \pm 900.9 (1–39728.8)	2366 \pm 116.5 (80.79–4267.14)	34.7 \pm 20.4 (0–884.7)	0.08 \pm 0.04 (0–1.62)
<i>P</i> -value ¹	0.05	0.9	0.04	0.61	0.38
<i>P</i> -value ²	0.014	0.85	0.1	0.49	0.008
<i>P</i> -value ³	0.4	0.42	0.17	0.19	0.36

SSc, systemic sclerosis; VEGF, vascular endothelial growth factor; sVEGF-R1, soluble VEGF receptor-1; sVEGF-R2, soluble VEGF receptor-2. *P*-value 1: between SSc patients and control. *P*-value 2: between diffuse SSc patients and control. *P*-value 3: between limited SSc patients and control. Bold values are significant at $p \leq 0.05$.

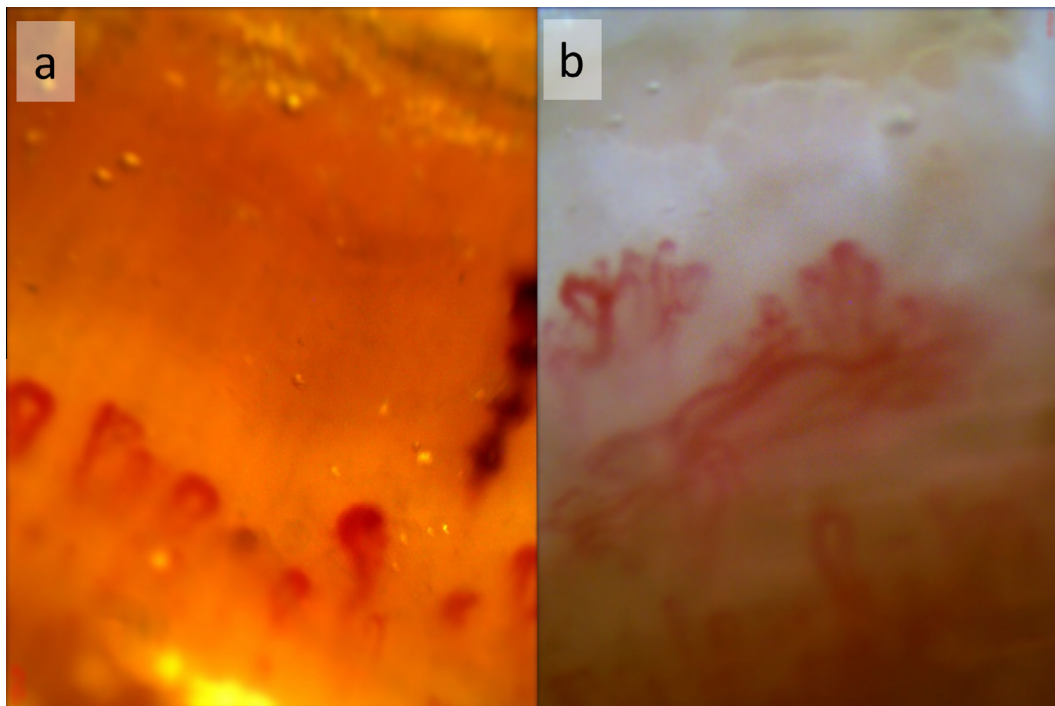


Figure 1 (a) Active scleroderma pattern of nailfold capillaroscopy showing multiple giant loops and microhemorrhages without neoangiogenesis, (b) late scleroderma pattern of capillaroscopy showing decreased number of capillaries with ramification and neoangiogenesis.

4. Discussion

The results of the present study indicating higher VEGF levels in the sera of SSc patients compared to healthy controls are in agreement with previous studies [17,19,25,26]. Accordingly, overproduction of VEGF as an angiogenic factor in SSc might play an important role in pathophysiology of the disease. Unlike VEGF, the levels of sVEGF-R2 (as an anti-angiogenic factor) in the sera of SSc patients were significantly lower than those in healthy controls. Therefore, failure to produce sufficient amount of sVEGF-R2 might involve in SSc pathogenesis. Consequently, administration of sVEGF-R2 might be considered as a new approach for treatment of SSc patients. In disharmony to another study [34] that reported lower concentrations of sVEGFR-1 in SSc patients compared

to controls, we did not find any significant difference. A possible reason for this difference could be that they included 659 SSc patients and 511 controls. Moreover, in concordance with another study [35], the results of the present work showed a tendency to increase in the VEGF/sVEGF-R2 ratio in patients compared to controls. It is thus suggested that high levels of VEGF and lower levels of sVEGF-R2 predispose to SSc. On the other hand, higher levels of VEGF were reported in diffuse SSc patients compared to limited ones [19]. In agreement, the results of the present study also showed a tendency to higher levels of all the above mentioned factors in diffuse compared to limited SSc patients.

In the present study, the possible association of VEGF or its soluble receptors with different clinical manifestations was also investigated. In the present study, due to the limited

Table 3 Serum levels of VEGF, sVEGF-R1, sVEGF-R2 and their ratios in systemic sclerosis patients with and without digital tip ulcers.

Mean ± SE (range)	Systemic sclerosis patients (n = 44)				
	VEGF (pg/ml)	VEGF-R1 (pg/ml)	VEGF-R2 (pg/ml)	VEGF/VEGF-R1	VEGF/VEGF-R2
<i>Digital ulcer</i>					
With (n = 9)	674.4 ± 500.9 (1–4647.9)	522.9 ± 478.5 (0–4349.8)	1876.02 ± 347.4 (518.2–3488.4)	15.1 ± 12.4 (0.01–113.9)	0.3 ± 0.14 (0–1.3)
Without (n = 35)	312.2 ± 129.01 (1–3812.2)	1011.6 ± 502.6 (1–13072.4)	2085.3 ± 121.5 (699.2–3362.7)	25.4 ± 16.4 (0–453.6)	0.16 ± 0.06 (0–1.75)
<i>p</i>	0.34	0.54	0.8	0.5	0.32

SSc, systemic sclerosis; VEGF, vascular endothelial growth factor; sVEGF-R1, soluble VEGF receptor-1; sVEGF-R2, soluble VEGF receptor-2.

Table 4 Serum levels of VEGF, sVEGF-R1, sVEGF-R2 and their ratios in systemic sclerosis patients with and without interstitial pulmonary fibrosis.

Mean ± SE (range)	Systemic sclerosis patients (n = 44)				
	VEGF (pg/ml)	VEGF-R1 (pg/ml)	VEGF-R2 (pg/ml)	VEGF/VEGF-R1	VEGF/VEGF-R2
<i>IPF</i>					
With (n = 4)	190.4 ± 63.9 (75.8–370.2)	2227.3 ± 110.4 (1916.2–2423)	79.2 ± 12.4 (55.6–110)	2.7 ± 1.3 (1.19–6.65)	0.08 ± 0.02 (0.03–0.17)
Without (n = 40)	380.6 ± 465 (0–4647.9)	2020.8 ± 377 (518.2–3488.4)	890.1 ± 1235.5 (0–13072.4)	1.4 ± 1 (0–7.58)	0.1 ± 0.1 (0–1.76)
<i>p</i>	0.52	0.68	0.67	0.12	0.74

SSc, systemic sclerosis, IPF, interstitial pulmonary fibrosis; VEGF, vascular endothelial growth factor; sVEGF-R1, soluble VEGF receptor-1; sVEGF-R2, soluble VEGF receptor-2.

number of patients with interstitial pulmonary fibrosis ($n = 4$), we could not find any significant association with the studied factors. However, in a previous study, a significant association was found between VEGF and interstitial lung disease [36]. Of interest, a significant correlation was only detected between the levels of VEGF and skin score (mRss). Hence, VEGF might be involved in the induction of pathologic changes of skin in SSc. Lower levels of sVEGF-R2 were seen in patients with late scleroderma pattern of capillaroscopy compared to those with early, active and nonspecific patterns. Given the fact that higher neoangiogenesis and ramification and lower number of capillaries are well-known characteristics of the late pattern of capillaroscopy [2], lower levels of anti-angiogenic sVEGF-R2 in these patients are expectable. Although when all components of capillaroscopy were separately checked, there was no significant association among neoangiogenesis, microhemorrhages, giant loops, avascular area and lower number of capillaries with all factors. Similar to the present findings, in a study done on 44 scleroderma patients no association was found between scleroderma patterns of nailfold capillaroscopy and VEGF-A level [36].

In the current patients, and not in the control, we found a significant association between levels of VEGF and sVEGF-R1 but not with sVEGF-R2 that may again show the significance of low sVEGF-R2 in these patients. Overall, the results of the present study suggest the important possible role of VEGF or its soluble receptors (VEGF-R1 and sVEGF-R2) especially sVEGF-R2 in the pathogenesis and clinical manifestations of SSc. However, replication of this study with higher

number of patients without previous history of medications is recommended.

In conclusion, SSc patients, VEGF, a very potent angiogenic molecule, is overexpressed while the levels of sVEGF-R2, an anti-angiogenic factor decreased. Our results suggest that these factors might be involved in the induction of vasculopathy and may be neoangiogenesis in patients with SSc.

Conflicts of interest

None.

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