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ORIGINAL RESEARCH

Dysregulation of circulating collagen turnover markers in very early systemic sclerosis

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ABSTRACT

Objective Clinical observation suggests that vascular activation and autoimmunity precede remodelling of the extracellular matrix (ECM) in systemic sclerosis (SSc). We challenge this paradigm by hypothesising that ECM biomarkers are already disturbed in patients with very early SSc (veSSc) when fibrosis is not yet clinically detectable.

Methods 42 patients with veSSc, defined as the presence of Raynaud's phenomenon and at least one of puffy fingers, positive antinuclear antibodies or pathological nailfold capillaroscopy, not meeting the 2013 American College of Rheumatology/European Alliance of Associations for Rheumatology classification criteria for SSc, were compared with healthy controls (HCs, n=29). ECM degradation (BGM, C3M, C4M and C6M) and ECM formation biomarkers (PRO-C3, PRO-C4 and PRO-C5) were measured in serum using ELISAs. A cross-sectional analysis at baseline and a longitudinal analysis was performed.

Results Compared with HC, veSSc patients showed a strongly dysregulated turnover of type III and IV collagens (higher C3M, C4M, both p<0.0001 and PRO-C3, p=0.004, lower turnover ratios PRO-C3/C3M and PRO-C4/C4M, both p<0.0001). The biglycan degradation biomarker BGM was higher in veSSc than in HC (p=0.006), whereas the degradation biomarker for type VI collagen, C6M, was lower (p=0.002). In an ROC analysis, biomarkers of type III and IV collagen excellently distinguished between veSSc and HC: C3M, AUC=0.95, p<0.0001; C4M, AUC=0.97, p<0.0001; turnover ratios PRO-C3/C3M, AUC=0.80, p<0.0001; PRO-C4/C4M, AUC=0.97; p<0.0001.

Conclusion These findings indicate ECM remodelling as a very early phenomenon of SSc occurring in parallel with microvascular and autoimmune changes. Biomarkers of type III and IV collagens distinguished between veSSc patients and HC, indicating them as potential biomarkers for the detection of veSSc.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The current paradigm based on clinical observation postulates that vascular activation and autoimmunity precede remodelling of the extracellular matrix (ECM) in systemic sclerosis (SSc). However, data analysing ECM remodelling in very early prefibrotic stages of SSc are lacking.

WHAT THIS STUDY ADDS

⇒ This study reveals circulating ECM neoepitopes are disturbed in very early SSc (VeSSc). Thus, changes in ECM turnover are a very early phenomenon in patients with SSc, which is likely switched on in parallel with microvascular and autoimmune processes. Moreover, biomarkers of type III and IV collagens distinguished excellently between veSSc patients and healthy controls.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ These results challenge the current paradigm and bring a novel insight into ECM changes in veSSc, identifying collagen turnover markers as candidate biomarkers for the early diagnosis of veSSc patients.

INTRODUCTION

Systemic sclerosis (SSc) is a chronic, incurable connective tissue disease, associated with high morbidity and mortality. SSc is characterised by early microvasculopathy, inflammation, autoimmunity and increased extracellular matrix (ECM) deposition, leading to tissue remodelling and organ fibrosis.[1]

The spectrum of SSc spreads from very early SSc (veSSc, patients at risk of developing definite SSc) to advanced, life-threatening disease. Early diagnosis of patients with SSc is of utmost importance for an optimal management and prevention of morbidity.[2] However, because the onset of SSc is often insidious and heterogeneous, the early stages of the disease are still insufficiently understood and difficult to diagnose.

The presence of Raynaud's phenomenon, puffy fingers, positive antinuclear antibodies (ANAs) and signs of microvasculopathy by capillaroscopy are acknowledged as 'red flags'

for the presence of veSSc.[3–5] In a recent analysis, we could show that among patients with veSSc, only a minority have an early disease with short disease duration while in others it appears to be a long-standing very mild form of the disease. No clinical parameters could be identified differentiating the two subgroups of veSSc.[6]

There is a high unmet need to better understand the pathogenic processes underlying the veSSc phenotype. The current paradigm is that vascular injury and endothelial cell activation, together with an activation of the immune system, are the initiating events in the pathogenesis of SSc. This paradigm is supported by clinical observations: Raynaud syndrome, typical capillaroscopy changes and autoantibodies are hallmarks of veSSc. In this concept, activation of fibroblasts and dysregulation of the ECM metabolism are later events induced by the inflammatory and microvascular changes. Indeed, clinically detectable fibrosis of the skin and internal organs is often absent in patients with veSSc. However, the development of tissue fibrosis is a longer process, and molecular activation of fibroblasts and disturbance of ECM synthesis and degradation might be ongoing for a long time before organ fibrosis becomes clinically detectable.[7 8]

One possibility to assess ECM turnover and activation is to measure ECM neoepitopes. Circulating ECM neoepitopes resulting from synthesis and degradation of collagens can be measured in serum and reflect the ECM turnover. Previously, we have shown that ECM neoepitopes are dysregulated in SSc and that turnover markers of type III and IV collagens are predictive of progression of fibrosis in patients with established SSc.[9]

In this study, we wanted to challenge the current paradigm of the pathogenesis of SSc with vascular and autoimmune changes occurring first, followed by ECM remodelling.[8] We hypothesised that ECM remodelling while not yet clinically detectable, occurs in parallel with vascular and autoimmune changes. To address this hypothesis, we measured circulating neoepitopes in patients with veSSc, which show vascular manifestations and/or autoimmune features, but do not yet have skin and organ fibrosis present.

Patients and methods

We included patients with veSSc, defined as the presence of Raynaud's phenomenon and at least one other possible manifestation of SSc (puffy fingers, positive ANAs, pathological capillaroscopy), but who did not meet the 2013 American College of Rheumatology/European Alliance of Associations for Rheumatology (ACR/EULAR) classification criteria for SSc[10] and did not have any clinical evidence for skin fibrosis (modified Rodnan skin score of 0/51 points) and interstitial lung disease (ILD, by high-resolution CT of the chest). Patients with a baseline visit to our centre from 2011 to 2014 and serum samples at baseline were included. Medical data and biobanking samples were collected and processed by European Scleroderma Trials and Research standards.[11 12]

Table 1 Baseline characteristics of the cohort of patients with very early systemic sclerosis (N=42)

Parameter	Frequency
Age (years, mean±SEM)	50.6±2.2
Sex	
Female (n, %)	37 (88.1)
Male (n, %)	5 (11.9)
Disease duration since first Raynaud symptom at baseline (mean±SEM; years)	6.7±1.3
Follow-up time (years; median, min-max)	4.3 (4.6, 0.5–10.2)
Raynaud phenomenon (n, %)	42/42 (100.0)
ANA positive (n, %)	39/42 (92.9)
ANA positive without specific antibodies (n, %)	13/42 (31.0)
ACA positive (n, %)	21/42 (50.0)
Anti-Scl-70 positive (n, %)	0/42 (0.0)
Anti-RNA Pol III positive (n, %)	3/42 (7.1)
Anti-U1-RNP positive (n, %)	2/42 (4.8)
CRP elevation (n, %)	2/42 (4.8)
ESR elevation (>25 mm/1 hour)	1/42 (2.4)
CK elevation (n, %)	5/42 (11.9)
Proteinuria (n, %)	0/42 (0.0)
ILD on HRCT (n, %)	0/42 (0.0)
FVC<80% predicted (n, %)	3/42 (7.1)
DLCO SB<80% predicted (n, %)	7/42 (17.1)
Dyspnoea NYHA I-II (n, %)	40/42 (97.6)
Dyspnoea NYHA III-IV (n, %)	1/42 (2.4)
Pulmonary hypertension on echocardiography (n, %)	0/42 (0.0)
Conduction blocks	6/42 (14.3)
Diastolic dysfunction	7/41 (16.7)
Pericardial effusion	1/42 (2.4)
SSc-specific nailfold capillaroscopy (n, %)	20/42 (47.6)
Oesophageal symptoms (n, %)	14/42 (33.3)
Renal crisis (n, %)	0/42 (0.0)
Telangiectasia (n, %)	5/42 (11.9)
Digital ulcers (ever) (n, %)	0/42 (0.0)
Pitting scars (ever) (n, %)	0/42 (0.0)
Puffy fingers (ever) (n, %)	9/42 (21.4)
mRSS (median, min-max)	0 (0–0)
Joint synovitis (n, %)	8/42 (19.0)
Tendon friction rubs (n, %)	0/42 (0.0)
Calcinosis (n, %)	0/42 (0.0)

Variables are presented either as mean±SM (if normal distribution) or median and IQR (if non-normal distribution); categorical variables are shown as frequencies/valid cases and percentages. Organ involvement was defined according to European Scleroderma Trials and Research standards.[12] Pulmonary hypertension is defined by echocardiography (systolic pulmonary arterial pressure ≥40 mm Hg) and judged by expert opinion.
ACA, anti-centromere antibodies; ANA, anti-nuclear antibodies; CK, creatine kinase; CRP, C reactive protein; DLCO, diffusing capacity of the lungs for carbon monoxide % predicted; ESR, erythrocyte sedimentation rate; FVC, forced vital capacity % predicted; HRCT, high-resolution CT; ILD, interstitial lung disease; mRSS, modified Rodnan skin score; N-number of patients, n-number of positive events; NYHA, New York Heart Association classification; SSc, systemic sclerosis.

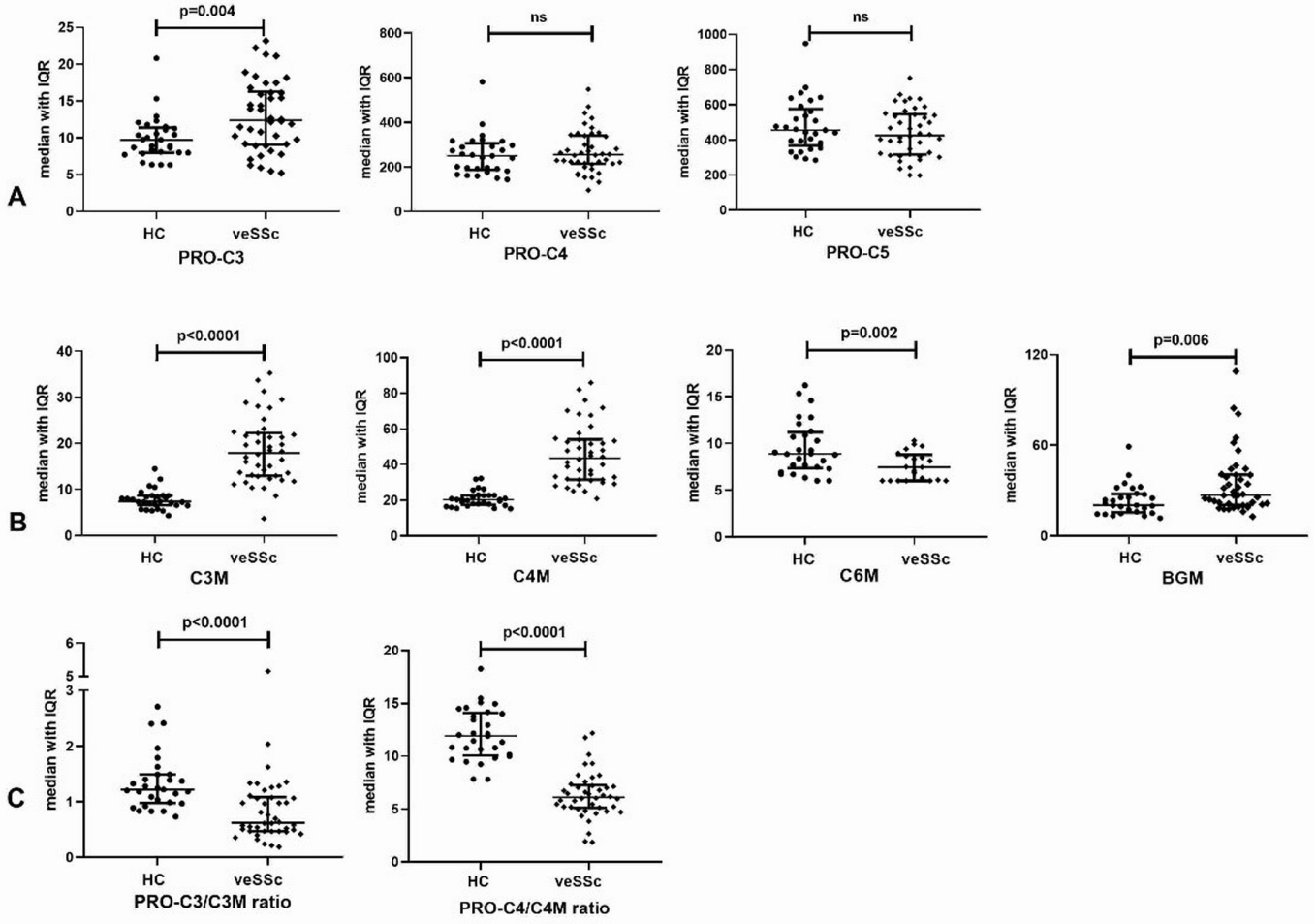

Figure 1 Levels of biomarkers in healthy controls (HCs) and patients with very early SSc (veSSc). (A) Formation biomarkers: PRO-C3-formation fragment of collagen type III; PRO-C4-amino terminal pro-peptide of procollagen type IV; PRO-C5-formation fragment of collagen type V; (B) Degradation biomarkers: C3M-degradation fragment of collagen III by MMP-9; C4M-degradation fragment of collagen type IV by MMPs; C6M-degradation fragment of collagen VI by MMP-2/9; BGM-degradation fragment of biglycan by MMP-9/12; (C) Turnover ratio of Pro-C3/C3M and P4NP/C4M2 in HC and veSSc. MMP-9, matrix metalloproteinase.

Healthy control (HC) volunteers from Zurich were included. This HC cohort has been described and results for ECM epitopes have been shown in a prior paper from our group.[9]

Candidate biomarkers (ECM neoepitopes) were selected by expert opinion (OD, RD, SJ, BM, MAK, A-CB-J, ASS and PJ) considering the most relevant collagens in fibrosis. These markers, generated by cleavage of procollagens or mature collagens in processes of formation or degradation, were measured by validated ELISA-assays in serum.[13–16] Measurements were performed in duplicate and were blinded. The formation markers were: PRO-C3 (the N-terminal propeptide of type III collagen), PRO-C4 (the N-terminal propeptide of type IV collagen) and PRO-C5 (the C-terminal propeptide of type V collagen). The degradation markers were C3M (peptide of type III collagen from degradation by matrix metalloproteinase (MMP)-9), C4M (peptide of type IV collagen from degradation by MMP-2, MMP-9 and MMP-12), C6M (peptide of type VI collagen from degradation by MMP-2 and MMP-9) and BGM (peptide of biglycan from degradation by MMP-2 and MMP-12).

In addition to the individual collagen neoepitopes, the ratios between the formation and degradation markers of collagens type III and IV were analysed as surrogate biomarkers of collagen turnover.

Statistical analysis

Normally distributed data are shown as mean±SD, non-normally distributed data as median and IQR. The biomarker graphics display the SE of the mean (SEM). Differences between groups were compared by the Mann-Whitney U test and correlations by Spearman's test. Categorical variables were compared by χ^2 or Fisher's exact test. Bonferroni correction for multiple tests was applied. Biomarkers were investigated for sensitivity and specificity by Receiver Operating Characteristic (ROC) curve. A predictive analysis using Cox logistic regression was also performed. Missing data were reported as observed.

Table 2 Comparison between biomarker levels between HC and veSSc and diagnostic accuracy for veSSc

Biomarker	Median serum level in HC (ng/mL)	Median serum level in veSSc patients (ng/mL)	Compared distribution between HC and veSSc patients*, p value	Accuracy for distinguishing veSSc from HC†, AUC (95%CI), p value	Optimal cut-off (ng/mL)§ (sensitivity, specificity)
C4M	20.4	42.9	<0.001	0.967 (0.932 to 1.002), <0.001	27.5 (91%, 93%)
PRO-C4	249.8	253.1	0.531	0.532 (0.406 to 0.683), 0.532	–
PRO-C4/C4M	11.9	6.0	<0.001	0.972 (0.941 to 1.004), <0.001	9.4 (93%, 90%)
C3M	7.4	17.6	<0.001	0.950 (0.894 to 1.005), <0.001	10.9 (91%, 93%)
PRO-C3	9.7	12.5	0.004	0.701 (0.578 to 0.825), 0.001	12.1 (55%, 86%)
PRO-C3/C3M	1.2	0.6	<0.001	0.794 (0.691 to 0.897), <0.001	0.8 (60%, 97%)
C6M‡	8.9	7.3	0.002	0.732 (0.591 to 0.873), 0.001	8.7 (76%, 57%)
BGM	20.3	27.0	0.006	0.695 (0.571 to 0.819), 0.002	17.6 (95%, 35%)
PRO-C5	455.1	420.2	0.231	0.415 (0.281 to 0.550), 0.219	–

C4M (peptide of type IV collagen from degradation by matrix metalloproteinases (MMP-9)), PRO-C4 (the N-terminal propeptide of type IV collagen), C3M (peptide of type III collagen from degradation by MMP-9), PRO-C3 (the N-terminal propeptide of type III collagen), C6M (peptide of type VI collagen from degradation by MMP-2 and MMP-9), BGM (peptide of biglycan from degradation by MMP-2 and MMP-12), PRO-C5 (the C-terminal propeptide of type V collagen), AUC (area under the curve).
*Mann-Whitney.
†ROC (Receiver Operating Characteristic) analysis.
‡C6M was tested in 28/29 HC and 21/42 veSSc patients (limited sample volume); all the other markers were tested in all patients.
§Calculated according to the Youden Index for the markers that showed a significant difference between HC and veSSc.
HC, healthy control; veSSc, very early systemic sclerosis.

The analyses and graphical illustration were performed using IBM SPSS Statistics V.29.0 and GraphPad Prism V.8.

RESULTS

Cohort description

We analysed 42 patients with veSSc and 29 HC. 22 out of 29 (75.9%) HC were female with a mean age±SEM of 53.9±3 years,[9] showing a similar distribution to the veSSc patients (88% female, mean age±SEM of 50.6±2.2 years, table 1). As expected, the clinical findings were very mild, and none of the patients had skin fibrosis or ILD, as defined by the inclusion/exclusion criteria. The most frequent characteristics of the veSSc patients were Raynaud phenomenon (100%), puffy fingers (21.4%), joint synovitis (19.0%), ANA positivity (92.2%) and abnormal capillaroscopy (50.0%). The detailed cohort description is presented in table 1.

Collagen turnover markers are differently expressed between veSSc patients and HCs

Significantly higher levels of type III and type IV collagen markers were identified in veSSc patients compared with HC: PRO-C3 (p 0.004), C3M (p<0.001), C4M (p<0.001, figure 1A,B). A particularly strong difference was seen for the type III and IV collagen degradation markers C3M and C4M. The turnover ratios of PRO-C3/C3M (p<0.001) and PRO-C4/C4M (p<0.001) were, correspondingly, significantly lower in veSSc patients versus HC (figure 1C). Furthermore, BGM was higher in veSSc versus HC (p 0.006), and C6M was lower (p 0.002) (figure 1).

Collagen turnover markers can distinguish between veSSc patients and HCs

A very good discriminatory capacity between veSSc and HC was found for markers of type III and type IV collagen, especially for the degradation markers C3M (area under the curve, AUC=0.95, 95% CI (0.89 to 1.00), p<0.0001) and C4M (AUC 0.97, 95% CI (0.93 to 1.00), p<0.0001), as well as for the turnover ratio of type IV collagen, PRO-C4/C4M (AUC 0.97, 95% CI (0.94 to 1.00), p<0.0001), as shown in table 2 and online supplemental figure S1. Optimal cut-offs for the markers which showed a good discriminatory capacity were calculated using the Youden index (table 2).

Cross-sectional and longitudinal investigation of the relation between collagen turnover markers and clinical findings

First, we investigated for associations between the levels of collagen turnover markers and clinical variables representative of veSSc using a cross-sectional analysis at baseline. The following relevant demographic and clinical variables which were also representatively observed in our cohort were selected by expert opinion: age, disease duration since the first Raynaud symptoms (including subgroups by thresholds set at 3 years and 5 years, respectively), puffy fingers, ANAs, anticentromere antibodies, SSc specific capillaroscopy, the presence of giant capillaries on capillaroscopy and joint synovitis.

The results showed slightly higher values of PRO-C3 in patients with disease duration of 5 years or more (median titre 11.6 vs 15.8 ng/mL, p=0.046, Mann-Whitney). However, this result was not significant after Bonferroni

correction for multiple testing (online supplement). No further significant clinical associations or correlations were found (online supplemental tables S1 and S2).

To further investigate a potential predictive role of the collagen turnover markers, a longitudinal analysis was performed. Overall, 37/42 (88%) of patients had at least one documented follow-up visit. The median follow-up time was 4.6 years, with a minimum of 0.5 years and a maximum of 10.2 years. During the follow-up, a total of 14/37 (37.8%) of patients eventually met the 2013 ACR/EULAR classification criteria for SSc. Time to fulfilment of the classification criteria ranged from 0.5 to 7 years (median 2.7 years). Notably, the characteristics leading to fulfilment of the classification criteria were mostly mild additional clinical findings like telangiectasia, with or without puffy fingers (in 7/14 patients), sclerodactily in four other patients, pitting scars and telangiectasia in one patient, puffy fingers and anticentromere antibodies in another patient and, lastly, only one patient met the criteria due to development of organ involvement (pulmonary arterial hypertension). None of the patients who fulfilled the classification criteria at follow-up developed relevant skin fibrosis beyond sclerodactily or experienced the onset of ILD. Overall, in the entire cohort, only one patient developed ILD at follow-up but did not formally fulfil the 2013 ACR/EULAR classification criteria.

The baseline serum values of the ECM neoepitopes did not show significant differences between patients who met and who did not meet the SSc criteria at follow-up (online supplemental table S3). Furthermore, a univariable analysis of the biomarker levels at baseline using Cox regression with the outcome fulfilment of the classification criteria at follow-up did not show significant results (online supplemental table S3).

DISCUSSION

The results from this study indicate ECM remodelling as a very early phenomenon of SSc, preceeding clinical skin fibrosis and likely occurring in parallel with microvascular and autoimmune/inflammatory changes. Our data show that biomarkers of ECM turnover are already significantly elevated in the very early stages of the disease when no fibrosis is clinically detectable and the disease is clinically dominated by Raynaud's phenomenon, microvascular changes on nailfold capillaroscopy and autoimmune features such as autoantibodies.

It is remarkable that in particular ECM degradation markers were differentiating controls from veSSc patients. This could indicate that fibrosis is not yet clinically apparent in these patients because the increased synthesis of ECM by activated fibroblasts is successfully counterbalanced by an increase in ECM degradation. Indeed, ECM synthesis markers such as PRO-C3 were elevated in veSSc patients in our study but not to the same extent as degradation markers. This hypothesis needs to be tested by tissue studies. While circulating ECM epitopes might be good biomarkers for the disease and potentially useful to monitor its progression,[17] they do not necessarily reflect the detailed molecular changes occurring in the tissues.

Also, different cell types might contribute to the generation of circulating ECM epitopes. For example, type III collagen, which is most abundant in soft connective tissue and regulates the early phases of wound healing, is produced by interstitial fibroblasts. Dysregulation of type IV collagen turnover, which is a biomarker of vascular and basement membrane remodelling, may also be connected to the microvasculopathy through disruption of the endothelium.

The excellent discriminatory capacity of the ECM epitopes (eg, AUC 0.97% for PRO-C4/C4M) differentiating veSSc patients from HCs raises the question, of whether they could be included in diagnostic algorithms. While the current results are promising and provide a strong signal, this has to be tested in further prospective studies. These studies need to include larger patient numbers and additional controls relevant to the differential diagnosis of veSSc. In addition, the relative weight and additive value to other established clinical markers need to be assessed.

Our study has several strengths and limitations. It is to our knowledge the first study assessing ECM remodelling in patients with veSSc without clinically detectable fibrosis and it is potentially changing current paradigms. Limitations include the moderate sample size. However, given the overall limited data available for veSSc, the strong and clear differences observed and the consistency with our previous findings in established SSc,[9] we consider the results to bring valuable information on the role of ECM neoepitopes in the incipient stages of the disease. We could not detect significant findings in the predictive analysis in our study. However, this could be explained by the low frequency of progression in our veSSc cohort, in which patients progressing to definite SSc did not develop major organ fibrosis. Assumptions on the mechanistic associations of the identified dysregulated circulatory collagen turnover markers are beyond the scope of this study and need to be addressed by further analyses.

In conclusion, this study shows for the first time data supporting the dysregulation of ECM in patients with veSSc. It might have implications for the diagnostic workup of patients with veSSc and for therapeutic considerations. Further studies analysing ECM remodelling and its relation to vascular and immunological changes in different cohorts of patients with veSSc, as well as 'relevant' controls with primary Raynaud's, are needed, in order to confirm and further analyse the complex interplay between these processes in incipient disease stages. If the hypothesis raised by our current study is confirmed, this could indicate a paradigm change in the understanding and approach to veSSc.

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Contributors OD is the guarantor and accepts full responsibility for the work and the conduct of the study, has access to the data and controls the decision to publish. The authors as listed on the title page of the manuscript have all made substantial contributions which qualifies them as authors. All authors contributed to critical revisions and approved the final version of the manuscript. RD: design of the study, acquisition of data, analysis, interpretation of data, drafting and revising the article. SJ: design of the study, analysis, interpretation of data, drafting and revising the article. PJ: design of the study, acquisition of data, analysis, interpretation of data, drafting and revising the article. NDP: acquisition of data, interpretation of data, revising the article. BM, MB and CM: acquisition of data, analysis, interpretation of data, revising the article. A-CB-J, MAK and ASS: design of the study, analysis, interpretation of data, revising the article. OD: design of the study, acquisition of data, analysis, interpretation of data, drafting and revising the article.

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Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by the ethic committee of the Canton of Zurich (KEK-ZH), Switzerland (Nr._2016-01515, EK839). Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available on reasonable request. Anonymised data might be available from OD at the Department of Rheumatology, University Hospital Zurich, University of Zurich, Switzerland on reasonable request.

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