Association of circulating miR-29b with microvascular dysfunction and digital ulcers in patients with systemic sclerosis: a single-centre pilot study

F. Tambaro, C. Pellicano, A. Gigante, C. Gallicchio, E. Rosato, M. Muscaritoli

Department of Translational and Precision Medicine, Sapienza University of Rome, Italy.

Abstract

Objective

Systemic sclerosis is a rare autoimmune disorder marked by vasculopathy, immune dysregulation, and progressive fibrosis. Digital ulcers (DUs) reflect severe microvascular dysfunction. This pilot, single-centre, observational study investigated whether circulating miR-29b is associated with DU occurrence, recurrence, and microvascular impairment.

Methods

Twenty-nine patients with systemic sclerosis and 17 healthy controls (HC) were evaluated. Clinical data, including DU history and recurrence, were collected. Cutaneous blood perfusion was assessed by laser speckle contrast analysis (LASCA), and proximal-distal gradient was calculated as perfusion difference between distal and proximal finger regions. Nailfold videocapillaroscopy classified microangiopathy as early, active, or late, while renal resistive index (RRI) served as an additional systemic vascular marker. Circulating miR-29b was quantified by RT-qPCR.

Results

Different modulation of miR-29b were observed between SSc and C ($p<0.001$). Patients with previous or recurrent DUs ($p=0.024$) and those with proximal-distal gradient <30 perfusion units showed significantly reduced miR-29b levels ($p=0.028$). Lower expression also characterised patients with RRI > 0.70 ($p=0.03$) and a late pattern ($p<0.01$). MiR-29b inversely correlated with disease activity ($r=–0.472$, $p<0.01$), fibrosis extent ($r=–0.471$, $p<0.01$), Cochin hand function score ($r=–0.454$, $p<0.01$), and RRI ($r=–0.506$, $p<0.01$), while showing a positive correlation with disease duration ($r=0.333$, $p<0.05$). In exploratory multivariable Cox regression analysis, lower miR-29b remained an independently associated with DU recurrence ($p<0.05$).

Conclusion

Overall, reduced circulating miR-29b was associated with advanced digital vasculopathy. Combined evaluation with LASCA-derived proximal-distal gradient and NVC pattern could may provide exploratory information for microvascular dysfunction and stratify patients at high risk of vascular complications.

Key words

systemic sclerosis, microvascular dysfunction, digital ulcers, microRNA-29b, laser speckle contrast analysis

*Federica Tambaro, PhD**
*Chiara Pellicano, MD**
Antonietta Gigante, MD
Carmen Gallicchio, MD
Edoardo Rosato, MD
Maurizio Muscaritoli, MD

**Contributed equally.*

Please address correspondence to:
Maurizio Muscaritoli
Dipartimento di Medicina Traslazionale e di Precisione,
Sapienza Università di Roma,
Piazzale Aldo Moro 5,
00185 Roma, Italy.
E-mail: maurizio.muscaritoli@uniroma1.it

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Introduction

Systemic sclerosis (SSc) is a rare autoimmune connective tissue disease characterised by progressive fibrosis affecting the skin and multiple internal organs, severe and diffuse vasculopathy with antibody production (1). Its pathogenesis is driven by endothelial dysfunction, immune activation, and fibroblast-mediated accumulation of extracellular matrix (ECM) (2). Raynaud's phenomenon (RP) often represents the earliest manifestation of SSc-related microvascular dysfunction, preceding irreversible damage to the digital circulation (3). In contrast, digital ulcers (DUs) are a debilitating vascular complication arising from advanced ischaemic injury and reflect systemic vasculopathy that may involve micro- and macrovascular compartments (4). DUs occur in approximately 30% of SSc patients annually and are associated with higher disease burden, reduced quality of life, and increased risk of systemic complications (5). Endothelial injury is considered an early and possibly initiating event in SSc (6), underscoring the need for reliable tools to detect and monitor microvascular damage. Despite advances in pathophysiological understanding, effective disease-modifying therapies remain limited (7, 8).

Non-invasive assessment of digital perfusion is central to evaluating SSc-related vasculopathy. Nailfold videocapillaroscopy (NVC), recognised as the gold standard for assessing structural microangiopathy, allows direct visualisation of capillary abnormalities and staging of microvascular damage (9). Complementing NVC, laser speckle contrast analysis (LASCA), an evolution of laser Doppler perfusion imaging (LDPI) (10, 11), provides high-resolution quantification of skin blood flow and is reproducible and sensitive to dynamic microcirculatory changes (12). The proximal-distal gradient (PDG), a LASCA-derived perfusion index, quantifies the perfusion difference between the fingertips and the hand dorsum. In healthy subjects, PDG is typically present, whereas in SSc it is often reduced or absent. Reduced PDG values have been associated with severe microangiopathy, DUs, major vascular complications, and increased 5-year mortality (13). The renal resistive index (RRI), a Doppler-derived parameter reflecting intrarenal vascular stiffness, has emerged as a marker of systemic microvascular involvement in SSc, correlating with DUs, NVC patterns, and subclinical renal dysfunction (14, 15).

Beyond imaging, additional circulating biomarkers may refine risk stratification and identify patients at higher risk of vascular complications. Circulating microRNAs (miRNAs), small non-coding RNAs that regulate gene expression post-transcriptionally (16), have emerged as promising diagnostic, prognostic, and therapeutic biomarkers (17). Dysregulation of specific miRNAs has been linked to fibrosis, immune activation, endothelial-to-mesenchymal transition, and vascular remodelling, all central processes in SSc pathogenesis (18). Among these, miR-29b is particularly interesting due to its established role in ECM remodelling (19). MiR-29b directly targets multiple ECM-related genes (*e.g.* COL1A1, COL3A1), and its downregulation promotes collagen accumulation through the TGF-β/SMAD pathway (19-21). TGF-β/SMAD signalling is a central profibrotic pathway that promotes fibroblast activation, myofibroblast differentiation, and ECM deposition (22). Within this profibrotic program, reduced miR-29b expression may favour sustained ECM accumulation and tissue remodelling. Consistently, we previously observed an inverse correlation between circulating TGF-β1 and miR-29b levels in SSc patients, suggesting possible regulatory feedback in which increased TGF-β1 may suppress the antifibrotic activity of miR-29b and amplify TGF-β-driven extracellular matrix remodelling (23). However, its association with digital ischaemia and vascular instability in SSc remains insufficiently defined.

This study aimed to investigate whether circulating miR-29b levels are associated with complementary clinical and functional markers of vascular involvement in SSc, including history and recurrence of DUs, LASCA-derived PDG, and RRI, as well as disease activity and severity indicators.

Materials and methods

Study design and patient characteristics

This was a single-centre observational pilot study, evaluating the relationship between circulating miR-29b levels, DUs, and microvascular dysfunction in SSc.

Twenty-nine patients with SSc who met the 2013 ACR/EULAR classification criteria (24) were consecutively enrolled at our SSc referral centre. All patients underwent baseline clinical assessment, nailfold videocapillaroscopy, laser speckle contrast analysis, renal Doppler ultrasound, and blood sampling for circulating miR-29b quantification. Patients were subsequently followed for 12 months for the occurrence of recurrent DUs, defined as the occurrence of at least three DU episodes during the 12-month follow-up period.

Disease duration was calculated from the first non-Raynaud's phenomenon symptom. At enrolment, no patients had received immunosuppressive therapy in the previous six months or corticosteroids at doses equivalent to prednisone ≥10 mg/day. Ongoing vasoactive therapies, including calcium channel blockers and intravenous iloprost, were recorded. Calcium channel blockers were discontinued 72 hours before renal microcirculation assessment, and patients receiving iloprost underwent renal Doppler ultrasound examination the day before the next scheduled infusion. Concomitant vasodilatory, vasoactive, and immunomodulatory treatments, as well as major comorbidities, were systematically collected and considered in the statistical analyses as potential confounding factors.

Exclusion criteria were age <18 years, oncologic diseases, smokers, hypertension, diabetes, pulmonary arterial hypertension, chronic kidney disease, and heart failure.

The study was approved by the Local Ethics Committee (approval no. IRB 0304), and all patients provided written informed consent in accordance with the Declaration of Helsinki.

Clinical assessment and sample collection

Comprehensive clinical, anthropometric, and biochemical parameters were recorded for all participants.

Based on the extent of skin involvement, patients were classified as having either limited cutaneous (lcSSc) or diffuse cutaneous (dcSSc) disease (25). Skin thickness was assessed using the modified Rodnan skin score (mRSS) (26). Global disease activity and severity were quantified using the Disease Activity Index (DAI) (27) and the Disease Severity Scale (DSS) (28), respectively. Hand functional disability was assessed using the Cochin Hand Function Scale (29).

Nailfold videocapillaroscopy (NVC) was performed at enrolment by an experienced rheumatologist with expertise in capillaroscopic assessment, using a videocapillaroscope equipped with a ×500 magnification optical probe (Pinnacle Studio Version 8 software) (Supplementary Fig. S1). It was performed in all patients at the distal phalanges of the second, third, and fourth fingers of both hands, after 20 minutes of acclimatisation in a room at a controlled temperature of 24±0.4°C. Capillaroscopic images were classified according to the validated Cutolo criteria into early, active, or late scleroderma patterns based on the presence and distribution of giant capillaries, capillary loss, microhaemorrhages, and abnormal capillary morphology (30, 31). Moreover, images were reviewed by the same experienced investigators according to the EULAR standardised definitions and assessment recommendations (30). In addition to qualitative pattern classification (early, active, and late scleroderma patterns), capillary density was quantitatively assessed as the number of capillaries per linear millimetre in the distal row (30) and was used as a continuous variable in subsequent analyses.

The assessor was blinded to circulating miR-29b levels and vascular outcome measures at the time of NVC evaluation.

Fasting peripheral blood was collected in EDTA tubes and processed within 2 hours. Plasma samples were separated by centrifugation at 3000 rpm for 15 minutes at 4°C, then aliquoted and stored at -80°C until further analysis.

Peripheral and renal microcirculation assessment

Peripheral digital perfusion was evaluated using laser speckle contrast analysis (LASCA, Pericam PSI System, Perimed AB, Sweden), performed at room temperature under standardised resting conditions (Suppl. Fig. S2).

The PDG was calculated to assess the relative perfusion of the fingertips compared with the dorsum of the hand. Impaired perfusion was defined as PDG ≤30 perfusion units (pU). This cutoff was selected based on previous evidence showing that PDG values ≤30 pU identify SSc patients with more severe peripheral vascular impairment and increased risk of DUs, major vascular complications, and mortality (13).

Renal Doppler ultrasound was performed to assess blood velocity in the interlobar arteries at three positions (mesorenal, superior, and inferior) (Suppl. Fig. S3). RRI was measured as the difference between the peak systolic and end-diastolic blood velocities divided by the peak systolic velocity. An RRI ≥0.70 was considered pathological (32).

RNA isolation and real-time PCR

Total RNA was extracted from plasma using the RNeasy Serum/Plasma Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. 3 μL of cel-miR-39-3p synthetic miRNA spike-in control (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) were added after the addition of lysis reagent to monitor RNA extraction efficiency. Plasma samples were visually inspected for haemolysis and spectrophotometrically assessed by measuring absorbance at 414 nm. Samples showing evidence of haemolysis were excluded from the analysis. RNA concentration was determined using a Multiskan Sky spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). cDNA synthesis was performed from 10 ng of total RNA using the TaqMan Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Quantitative real-time PCR was carried out us-

ing TaqMan Fast Advanced Master Mix (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) and Step-OnePlus Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). The TaqMan probe for hsa-miR-29b-3p was used for quantfication; hsa-miR-16-5p was used as endogenous control (assay ID A25576, Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). The raw Ct values of miR-16-5p showed no significant differences between SSc patients and healthy controls (17.65±1.39 *vs*. 16.90±1.83, p=0.123), supporting its suitability as an endogenous reference in this cohort. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. All samples were analysed in duplicate. Assay reliability was monitored through duplicate concordance and cel-miR-39-3p spike-in amplification.

Statistical analysis

This was a pilot, exploratory study and no formal sample size calculation was performed, all patients were enrolled consecutively in our scleroderma unit. All statistical analyses were considered exploratory. Data are presented as the mean ± standard deviation (SD) or standard error of the mean (SEM), and as the median with interquartile range (IQR), depending on the data distribution. Group comparisons were performed using Student's t-test or one-way ANOVA for normally distributed variables, and Mann-Whitney or Kruskal-Wallis tests for non-normally distributed variables. For multiple pairwise post-hoc comparisons following significant Kruskal-Wallis tests, Bonferroni correction was applied to control for type I error. For comparisons among the three NVC categories (early, active, and late), three pairwise comparisons were performed and adjusted accordingly. Unadjusted and Bonferroni-adjusted p-values are reported where appropriate. The correlation between miR-29b levels and clinical variables was analysed using Spearman's rank correlation coefficient. Cox proportional hazards regression models were employed to assess the association between miR-29b and digital ulcer recurrence. For time-to-event analysis, baseline was defined as the date of clinical assessment and blood sampling for miR-29b measurement. Time-to-event was calculated from baseline to the first documented recurrent DU episode. Patients who did not experience recurrent DUs during follow-up were censored at 12 months or at the last available follow-up visit. Hazard ratios (HR) with 95% confidence intervals (CI) were reported for univariable and multivariable models. To explore whether the association between circulating miR-29b levels and recurrent DUs was independent of established markers of disease severity, an additional logistic regression analysis was performed. Recurrent DUs were entered as the dependent variable, while circulating miR-29b levels, dcSSc, and late NVC pattern were included as covariates. Odds ratios (ORs) with 95% CI were calculated. A p-value <0.05 was considered statistically significant. Analyses were performed using IBM® SPSS Statistics version 26.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.0 (San Diego, California, USA).

Table I. Demographic and clinical characteristics of 29 systemic sclerosis patients enrolled.

	Patients with SSc (n=29)	Control group (n=17)
Age, years	55 (45;66)	56 (44;63)
Female/Male	26 (89.7) / 3 (10.3)	14 (82.4) / 3 (17.6)
Disease duration, years	11 (6;20)	–
mRSS	13 (6;18)	–
SSc specific antibodies		
Scl70	14 (48.3)	
ACA	9 (31)	
None	6 (20.7)	
NVC		–
Early	3 (10.3)	
Active	11 (37.9)	
Late	15 (51.7)	–
DAI	1.6 (1.1;4)	–
DSS	5 (4;6)	–
DUs history	15 (51.7)	–
Recurrent DUs	10 (34.5)	–
Cochin	12 (1;26)	–
PDG, pU	0.36 (-7.49;30.36)	–
PDG ≤ 30 pU	21 (72.4)	–
RRI	0.70 (0.66;0.74)	–
RRI > 0.70	12 (41.4)	–
miR-29b	0.30 (0.20;0.40)	0.77 (0.45;1.19)

All results are expressed as median and interquartile range (IQR) or absolute frequency and percentage (%). SSc: systemic sclerosis; mRSS: modified Rodnan skin score; ACA: anticentromere; NVC: nailfold videocapillaroscopy; DAI: disease activity index; DSS: disease severity scale; DUs: digital ulcers; PDG: proximal-distal gradient; RRI: renal resistive index.

Results

Patients characteristics

Twenty-nine consecutive SSc patients and 17 healthy controls were enrolled in this study. Patients with SSc (89.7% female) presented with a median age of 55 (45;66) years, whereas median age of controls (82.4% female) was 56 (44;63) years. Demographic and clinical characteristics are summarised in Table I.

The median disease duration was 11 (6;20) years. The median DAI was 1.6 (1.1;4), and the DSS had a median value of 5 (4;6). Skin involvement, assessed by mRSS, showed a median value of 13 (6;18). Based on NVC patterns, 51.7% of patients were classified as "late", 37.9% as "active", and 10.3% as "early". Regarding autoantibody profiles, 14 (48.3%) patients were positive for anti-Scl-70 antibodies, 9 (31%) for anticentromere antibodies (ACA), and 6 (20.7%) tested negative for both. A history of DUs was present in 15 (51.7%) patients, and recurrent DUs occurred in 10 (34.5%). The Cochin score assessed the functional disability of the hand, with a median value of 12 (1;26). Assessment of hand perfusion using LASCA showed a PDG of 0.36 pU

(-7.49; 30.36), and 21 patients (72.4%) had PDG ≤30 pU, indicating impaired distal perfusion. The RRI had a median value of 0.70 (0.66;0.74), with 12 patients (41.4%) showing values >0.70.

miR-29b expression reflects disease severity and microangiopathy progression

We first assessed different modulation of plasma levels of miR-29b between patients with SSc (n=29) and control group by RT-qPCR (Fig. 1). Significant lower levels of miR-29b were observed in the group of patients with SSc compared to controls (Fig. 1A). We next assessed whether circulating miR-29b levels reflect the extent of microangiopathy and overall disease severity and analysed their association with NVC patterns and clinical indices.

In our cohort, circulating miR-29b levels showed significant variation across NVC patterns (Fig. 1). In patients with a late NVC pattern, miR-29b levels were significantly lower compared with those with an active pattern (p<0.01) (Fig. 1B). Pairwise post-hoc analysis showed significantly lower miR-29b levels in patients with a late NVC pattern compared with those with an active pattern (unadjusted p=0.002; Bonferroni-adjusted p=0.006), whereas no significant differences were observed between the early and active or between the early and late patterns. We next considered skin involvement, and we observed that in patients with dcSSc, miR-29b expression was significantly lower compared to those with lcSSc (p=0.010) (Fig. 1C).

To further characterise the relationship between miR-29b and clinical phenotype, we analysed its correlation with continuous disease activity and severity indices. Correlation analyses showed significant inverse associations between miR-29b levels and mRSS (r=–0.471, p<0.01) (Fig. 2A), DAI (r=–0.472, p<0.01) (Fig. 2B), and DSS (r =–0.408, p<0.05) (Fig. 2C).

We then extended the analysis to parameters related to disease duration and perceived functional limitations. A significant positive correlation was found between miR-29b and disease duration (r=0.333, p<0.05) (Fig. 2D). Lastly, we

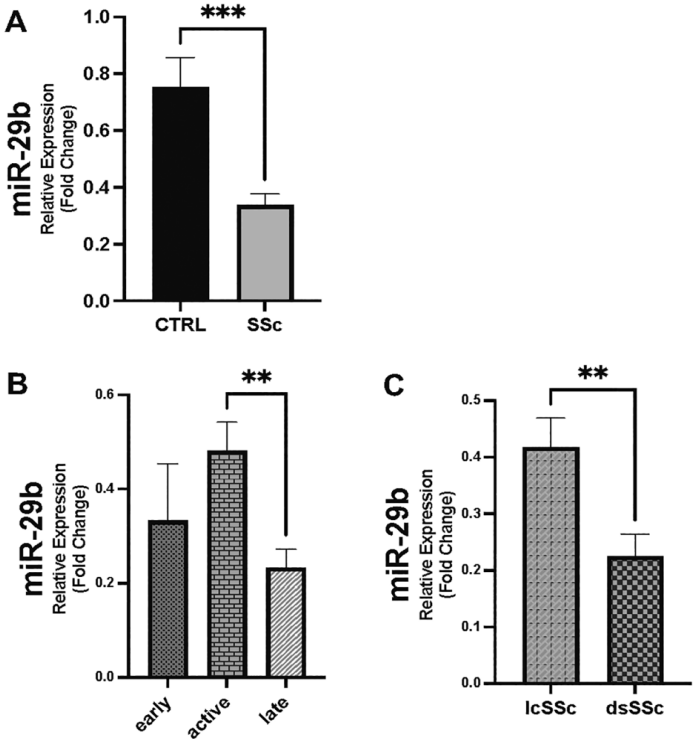

Fig. 1. miR-29b expression reflects disease severity and microangiopathy progression.
Circulating miRNA levels were analysed by quantitative real-time PCR from patients with systemic sclerosis (SSc) (n=29) in relation to NVC patterns and cutaneous subsets and in control group (n=17).
A. Data shown significant lower levels of miR-29b in patients with SSc [0.30 (0.20;0.40)] compared to controls [0.77 (0.45;1.19)] (p<0.001).
B. Data showed significant higher levels of miR-29b in SSc patients with an active pattern (n=11) [0.40 (0.40;0.70)] compared with those with a late pattern (n=15) [0.20 (0.10;0.30)] (unadjusted p=0.002; Bonferroni-adjusted p=0.006), whereas no significant differences were observed between the early and active or between the early and late patterns.
C. When patients were stratified by cutaneous subset, those with lcSSc (n=17) showed higher miR-29b levels [0.40 (IQR 0.20;0.50)] compared with those with dcSSc (n=12) [0.20 (0.10;0.30)] (p=0.010).
Data are expressed as fold change and are presented as median (IQR). Student's t-test or Mann-Whitney test and One-way ANOVA test or Kruskal-Wallis test were used for normally distributed data or non-normally distributed data, respectively.

examined the association between miR-29b levels and functional status: Cochin hand function score was inversely correlated with miR-29b (r=–0.454, p<0.01) (Fig. 2E).

Moreover, circulating miR-29b levels showed a strong positive correlation with capillary density (r=0.768, p<0.001), indicating that lower miR-29b expression was associated with more severe capillary rarefaction (Fig. 2F).

Reduced miR-29b levels are associated with DUs burden

To investigate the link between miR-29b and the vascular manifestations of SSc, we focused on its association with DUs history and recurrence (Fig. 3). We first compared miR-29b levels in relation to the history of DUs, and we observed significant downregulation in patients with a history of DUs compared to those without (p=0.032) (Fig. 3A). Likewise, patients who experienced recurrent DUs (n=10; 34.5%) had reduced miR-29b expression compared to patients without recurrence (n=19; 65.5%) (p=0.024) (Fig. 3B).

We performed exploratory Cox regression analyses to evaluate the association between baseline miR-29b levels and DU recurrence (Table II). In univariable analysis, low miR-29b expression was significantly associated with DU recurrence (p<0.05). In multivariable analysis, the association between lower miR-29b levels and DU recurrence remained statistically significant (p<0.05) (Table II).

To address the potential overlap between recurrent DUs, dcSSc and late NVC pattern, we performed an additional logis-

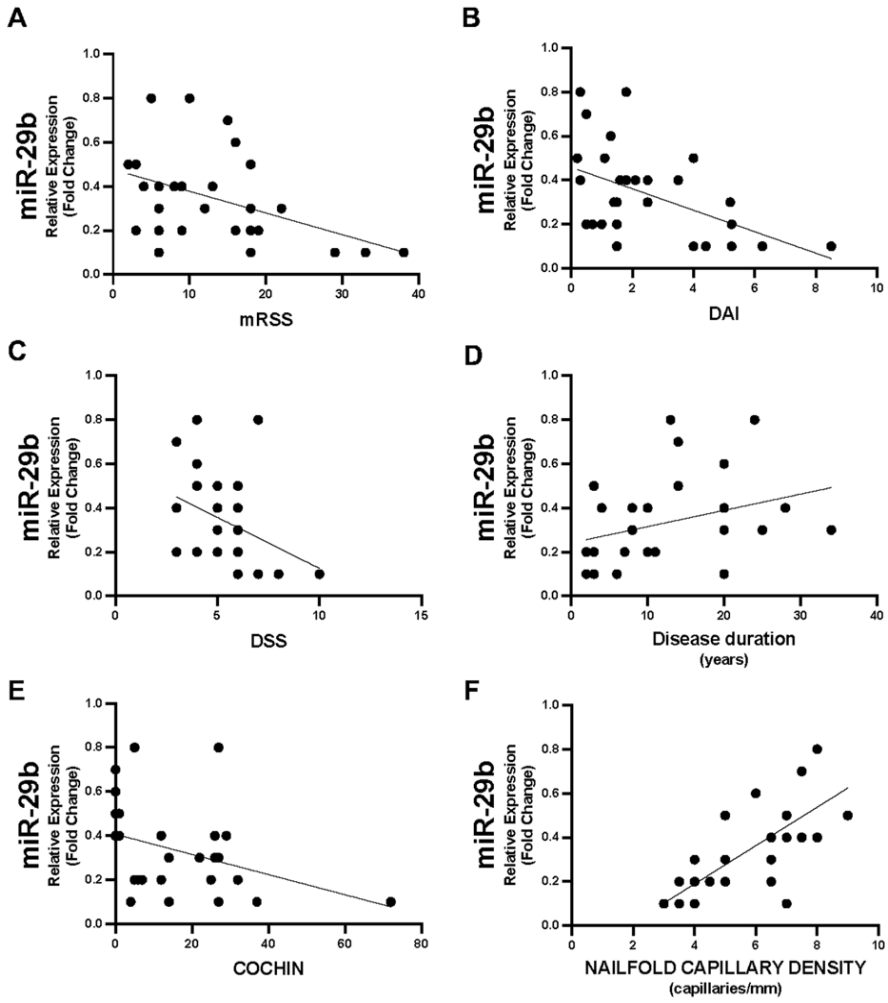

Fig. 2. miR-29b expression reflects disease severity and microangiopathy progression.
Spearman's correlation analyses between circulating miR-29b levels and indices of disease activity, severity, and functional status in SSc.
A. A significant inverse correlation was observed between miR-29b levels and mRSS (r=–0.471, p<0.01). **B.** miR-29b levels were inversely correlated with DAI (r=–0.472, p<0.01). **C.** A significant negative correlation was also found with DSS (r=–0.408, p<0.05). **D.** A positive correlation emerged between miR-29b and disease duration (r=0.333, p<0.05). **E.** miR-29b levels inversely correlated with Cochin hand function score (r=–0.454, p<0.01). **F.** A positive correlation emerged between miR-29b and quantitative capillary density (r=0.768, p<0.001).
mRSS: modified Rodnan skin score; DAI: disease activity index; DSS: disease severity scale.

tic regression analysis including circulating miR-29b levels, dcSSc, and late NVC pattern in the same model (Suppl. Table S1). In univariable analyses, lower miR-29b levels were associated with recurrent DUs (p<0.05). However, after adjustment for dcSSc and late NVC pattern, the association was attenuated and no longer statistically significant. Similarly, neither dcSSc nor late NVC pattern independently reached statistical significance in the multivariable model, likely reflecting the substantial overlap among these markers of disease severity and the limited sample size of this pilot study.

Circulating miR-29b levels are reduced in patients with peripheral and systemic vascular impairment

Given the central role of microvascular dysfunction in DU pathogenesis, we assessed functional perfusion using LASCA-derived PDG and the association between miR-29b levels and peripheral microvascular function. We previously showed that low PDG predicts DUs, major vascular complications, and 5-year mortality (13).

Patients with impaired peripheral perfusion (PDG ≤ 30 pU) showed significantly lower levels of circulating miR-29b compared to those with preserved perfusion (PDG >30 pU) (p=0.028) (Fig. 4A).

To further assess systemic microvascular involvement, we examined miR-29b expression in relation to renal vascular resistance, measured by RRI. In SSc, RRI is a validated marker for detecting subclinical kidney involvement and systemic vascular stiffness (15).

miR-29b levels were significantly lower in patients with elevated RRI (>0.70) with respect to patients with normal RRI values (p=0.030) (Fig. 4B).

When considered as a continuous variable, RRI showed a significant inverse correlation with miR-29b expression (r=–0.506, p<0.01) (Fig. 4C).

Discussion

SSc is a rare disease causing fibrosis and vascular damage characterised by endothelial dysfunction, leading to complications like DUs, pulmonary hypertension, and scleroderma renal crisis (33, 34). Although important advances have been achieved through vasoactive and immunomodulatory therapies, no curative treatment is currently available, and vascular complications such as DUs remain a major source of morbidity (35). Understanding molecular pathways linking fibrosis and vascular injury is therefore crucial.

Vascular assessment in SSc relies mainly on non-invasive imaging techniques, each capturing different aspects of the disease. LASCA is a reproducible method for quantifying skin blood perfusion and detecting dynamic microvascular changes (36). The LASCA-derived PDG reflects perfusion differences between fingertips and the dorsum of the hand. In healthy subjects, PDG is positive, whereas in SSc it is often reduced or absent (37), with lower values associated with severe microangiopathy, DUs, major vascular events and mortality (13). Although imaging techniques improve structural and functional assessment (38), they do not reflect underlying molecular mechanisms. This has stimulated interest in circulating biomarkers that may complement vascular imaging. miRNAs, a class of noncoding RNAs regulating gene expression post-transcriptionally (39), have emerged as promising candidates. In SSc, several

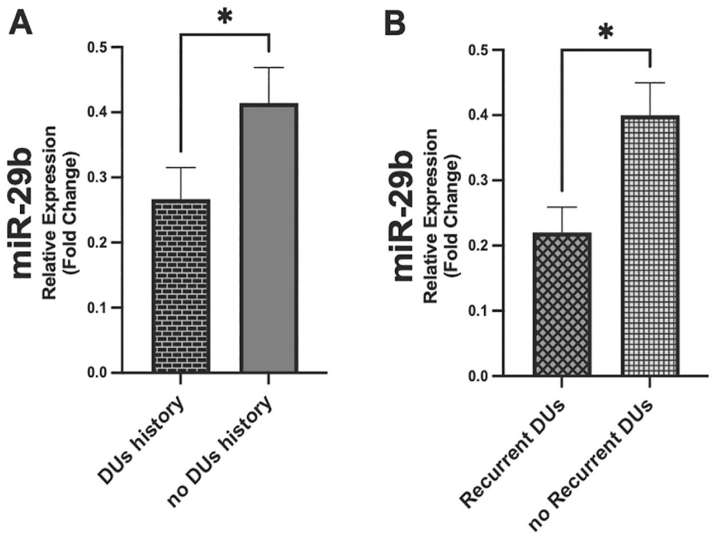

Fig. 3. Reduced miR-29b levels are associated with DUs burden.
Circulating miR-29b levels were analysed in relation to DUs history and recurrence SSc.
A. Data showed significantly lower levels of miR-29b in patients with a history of DUs (n=15) [0.20 (0.20;0.40)] compared to those without (n=14) [0.40 (0.20;0.50)] (p=0.032).
B. Data from patients who experienced DU recurrence (n = 10) [0.20 (0.10;0.30)] showed significantly lower levels of miR-29b compared with those without recurrence (n=19) [0.40 (0.30;0.50)] (p=0.024).
Data are expressed as fold change and are presented as median (IQR). Student's t-test or Mann-Whitney test was used for normally distributed data or non-normally distributed data, respectively.

Table II. Univariable and multivariable Cox regression analysis for the development of recurrent digital ulcers (DUs) in 29 systemic sclerosis (SSc) patients.

	Univariable analysis		Multivariable analysis	
Variables	HR (95% CI)	*p*	HR (95% CI)	*p*
miR-29b	0.002 (0.000;0.292)	*<0.05*	0.000 (0.000;0.318)	*<0.05*
late	5.634 (1.182;26.868)	*<0.05*	1.141 (0.157;8.262)	*>0.05*
Scl70	4.512 (0.956;21.295)	*<0.05*	5.870 (0.877;39.284)	*>0.05*

SSc: systemic sclerosis; DUs: digital ulcers; HR: hazard ratio; CI: confidence intervals.

miRNAs are implicated in fibrosis, angiogenesis, and immune dysregulation (40). Among these, miR-29b is particularly relevant for its dual role in ECM regulation and vascular remodelling (41). Downregulation of miR-29b has been consistently observed in the skin, fibroblasts, and circulation of SSc patients (19, 21, 23). Studies have shown that miR-29b directly targets multiple ECM genes (*e.g.*, COL1A1, COL3A1), and its expression is negatively regulated by TGF-β1 (42-44). This regulatory interaction is particularly relevant in SSc, where TGF-β signalling represents a central driver of fibroblast activation, myofibroblast differentiation, and extracellular matrix accumulation (45). In SSc skin biopsies and dermal fibroblasts, reduced miR-29b expression has been associated with COL1A1 upregulation, while stimulation of healthy dermal fibroblasts with recombinant TGF-β was shown to decrease miR-29b and increase COL1A1 expression, supporting a link between TGF-β signalling, miR-29b downregulation, and collagen accumulation (20). Beyond fibrosis, miR-29b dysregulation has also been implicated in vascular dysfunction in other conditions, where reduced miR-29b promotes extracellular matrix accumulation, vascular stiffening, and endothelial dysfunction (46-49), suggesting a broader role in vascular homeostasis.

In the present study, we investigated whether circulating miR-29b levels may reflect the extent of vascular injury in SSc.

In line with previous studies (23, 50), circulating miR-29b levels exhibited significant reduction between patients with SSc and controls.

We next analysed circulating miR-29b levels in association with structural microangiopathy assessed by NVC. In our cohort, NVC allowed staging of structural capillary loss, providing the framework for interpreting molecular associations. In patients with more advanced ("late") NVC patterns, characterised by severe capillary loss, miR-29b showed the lowest levels compared to the "active" stage. The observed association between circulating miR-29b levels and reduced capillary density supports a link between miR-29b and the severity of microvascular damage in SSc, extending the information provided by qualitative NVC pattern classification alone. We also observed that miR-29b was reduced in patients with diffuse cutaneous SSc compared to those with limited cutaneous disease. Our findings suggest that lower miR-29b levels are associated with more severe structural microangiopathy and greater skin involvement. This is in line with prior evidence showing that progressive capillary loss parallels increased fibrotic burden and worse prognosis in SSc (51). However, we cannot exclude that this association is partly shaped by the broader vascular and fibrotic burden of dcSSc, in which extensive skin fibrosis, microvascular damage, and inflammatory activity frequently coexist.

We further analysed miR-29b expression in relation to indices of disease activity, severity, and functional status. Our data showed inverse correlations with mRSS, DAI, and DSS, indicating that lower miR-29b levels are associated with greater microvascular and fibrotic burden. We also observed a positive correlation with disease duration, suggesting that miR-29b levels may be higher in patients with long-standing SSc than in those with more recent disease. This pattern may indicate that miR-29b downregulation is more pronounced during active or progressive phases, whereas relative stabilisation in long-standing disease might partially attenuate this reduction, although long-term therapeutic exposure and differences in residual disease activity may also contribute to this association. This temporal dissociation was paralleled by an inverse association between miR-29b

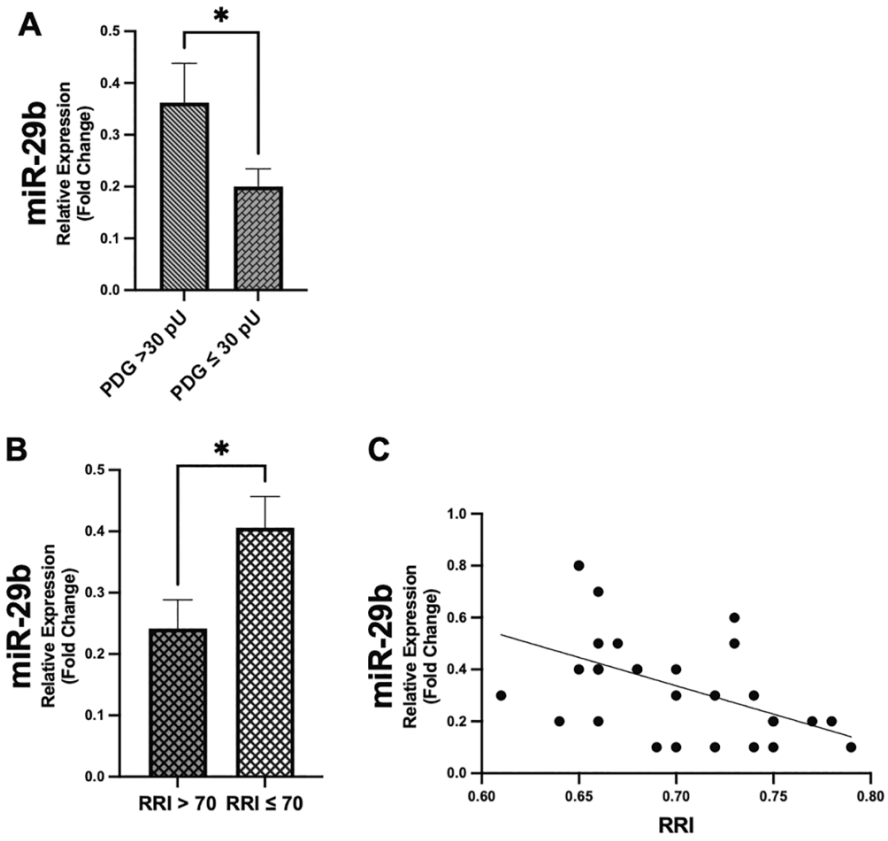

Fig. 4. Circulating miR-29b levels are reduced in patients with peripheral and systemic vascular impairment.

Circulating miR-29b levels were analysed according to peripheral microvascular perfusion and systemic vascular stiffness.

A. Data showed that in patients with impaired PDG (≤30 pU) (n=21) [0.20 (0.20;0.40)] miR-29b levels were significantly lower compared to those with preserved PDG (>30 pU) (n=8) [0.45 (0.35;0.75)] (p=0.028).

B. Patients with elevated RRI (>0.70) (n=12) [0.20 (0.10;0.30)] showed significant downregulation of miR-29b levels compared with patients with RRI ≤0.70 (n=17) [0.40 (0.30; 0.50)] (p=0.030).

C. Data showed a significant inverse correlation between RRI and circulating miR-29b levels (Spearman's r=–0.506, p<0.01).

Data are expressed as fold change and are presented as median (IQR). Student's t-test or Mann-Whitney test was used for normally distributed data or non-normally distributed data, respectively.

PDG: proximal-distal gradient; RRI: renal resistive index; pU: perfusion unit.

levels and hand disability, as assessed by the Cochin score. Our findings are consistent with previous reports showing that greater microangiopathy severity and/or skin fibrosis are associated with worse hand function in SSc (29). While we cannot distinguish whether this relationship reflects ischaemic damage, fibrotic contracture, or both, our results align with the hypothesis that miR-29b may be a potential player in the mechanisms linking fibrosis, vascular damage, and functional impairment.

In our cohort, both DU history and recurrence were associated with reduced miR-29b levels, and exploratory Cox regression models showed that lower baseline miR-29b levels were associated with DU recurrence. These findings suggest that miR-29b may add molecular information to established clinical and capillaroscopic predictors of DU risk. Given the frequent coexistence of DU recurrence with diffuse cutaneous involvement and advanced NVC patterns, these results should be interpreted as part of an integrated vascular-fibrotic disease profile. The association between reduced miR-29b levels and recurrent DUs was attenuated after adjustment for dcSSc and late NVC pattern. This finding suggests that miR-29b may primarily reflect the broader vascular-fibrotic phenotype of SSc, characterised by advanced microangiopathy and more severe disease manifestations, rather than representing a fully independent predictor of ulcer recurrence. In SSc, DUs represent a clinical model of chronic ischaemic wounds, where impaired tissue perfusion and persistent hypoxia lead to delayed healing (52).

Our results align with previous evidence showing that miR-29b regulates wound healing through ECM deposition, angiogenesis, and inflammation (50), and that its downregulation may impair repair processes in SSc patients with severe microangiopathy. Reduced miR-29b levels may therefore reflect vascular injury and could be involved in pathways related to defective DUs healing and recurrence.

Given the established role of miR-29b in ECM regulation, it is conceivable that persistent miR-29b downregulation may interact with pathways regulating cellular stress responses and endothelial turnover, potentially favouring chronic hypoxia and delayed healing. To further support this interpretation, experimental evidence from *in vivo* ischaemia models demonstrated that acute upregulation of miR-29b exacerbates oxidative stress and apoptosis via PI3K/Akt/Bax signalling. In contrast, its inhibition mitigates injury (53). Although this model reflects acute neurological ischaemia rather than chronic SSc vasculopathy, it highlights the ability of miR-29b to modulate oxidative and apoptotic pathways that are also active in SSc-related hypoxia (54). These data raise the possibility that miR-29b may exert context-dependent effects depending on the chronicity and nature of vascular stress.

We further extended our investigation to examine whether miR-29b may reflects dynamic functional microvascular impairment using LASCA-derived PDG.

Our findings showed that patients with reduced PDG had lower miR-29b levels than those with preserved perfusion. This supports the hypothesis that miR-29b reduction parallels impaired peripheral perfusion and more severe microangiopathy.

Along this line, evidence from other vascular diseases, such as diabetic retinopathy, shows that miR-29b can modulate the vascular endothelial growth factor (VEGF) axis and angiogenic responses (55). In this context, our results sug-

gest that reduced miR-29b in SSc may not only reflect vascular rarefaction but also potentially contribute to defective VEGF-driven angiogenesis, thereby potentially perpetuating chronic ischaemia and impaired vascular repair.

To extend our investigation beyond microvascular alterations, we assessed renal vasculopathy using Doppler-based RRI, a non-invasive marker of intrarenal vascular resistance (56). In our cohort, high RRI was associated with reduced miR-29b, and RRI inversely correlated with miR-29b as a continuous variable. While our data cannot establish causality, the inverse relationship between RRI and miR-29b may reflect that this miRNA may capture peripheral microvascular damage and systemic macrovascular stiffening. We therefore propose that in SSc, miR-29b may capture not only peripheral microvascular damage but also systemic microvascular dysfunction at both peripheral and systemic levels. Whether this dual role reflects a pathogenic mechanism or a secondary adaptation remains to be clarified.

Our study has some limitations. The relatively small sample size and single-centre design limit statistical power, particularly for multivariable analyses, and prevents robust subgroup exploration. Additionally, comparisons across NVC capillaroscopic stages should be considered exploratory. Accordingly, multivariable results should be interpreted with caution and require confirmation in larger, adequately powered cohorts. The predominantly limited observational design precludes causal inference and limits temporal interpretation of the observed associations. Furthermore, although concomitant treatments were recorded and considered in the statistical analyses, their potential residual influence on vascular and molecular outcomes cannot be completely excluded. Moreover, the limited number of control group, and the lack of tissue-level or functional experiments to confirm mechanistic links remain relevant limitations. Future studies integrating *in vitro* and longitudinal approaches will be necessary to establish the mechanistic role of miR-29b and its value as a biomarker or therapeutic target in SSc-related vascular disease.

Conclusion

Our study shows that circulating miR-29b is consistently reduced in SSc patients with severe structural and functional microangiopathy, DUs, impaired fingertip perfusion and increased vascular resistance. Lower miR-29b levels were also associated with DUs recurrence.

These observations expand current knowledge of the potential role of microRNAs in SSc and suggest that miR-29b may integrate components of fibrotic and vascular disease. Given the close interplay between vascular injury and fibrosis, miR-29b downregulation may reflect a combined fibrotic-microangiopathic phenotype, potentially linking peripheral microvascular damage to systemic vascular alterations. However, these findings should be interpreted cautiously given the exploratory design, limited sample size, and observational nature of the study. Whether this dysregulation represents a driver of disease progression or a secondary consequence of ongoing tissue injury remains to be clarified.

Future longitudinal and mechanistic studies are warranted to validate these associations, define causality, and determine whether modulation of miR-29b expression may attenuate the vascular-fibrotic trajectory of SSc.

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