The diagnostic utility of miRNA21 in systemic sclerosis

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Key words: scleroderma, systemic, microRNAs, miR-21, biomarkers, epigenetics.

Summary

Objective. Systemic sclerosis (SSc) is a multisystem autoimmune disease of heterogeneous pathogenesis, including vascular, immunologic, genetic, epigenetic, and environmental factors. Progressive fibrosis is the hallmark of SSc. Intense research has been conducted to unveil new tools for early diagnosis and management, thus reducing morbidity and mortality. miR-21 has recently been considered to play an important role in the fibrosis of SSc. The objective of this study was to evaluate miR-21 levels in SSc patients and study its correlation to the extent of skin fibrosis and association with various clinical characteristics.

Methods. A total of 25 patients with SSc who fulfilled the American College of Rheumatology/European Alliance of Associations for Rheumatology 2013 classification criteria, as well as 25 controls, were enrolled in a cross-sectional study. The extent of skin fibrosis was evaluated using the modified Rodnan skin score, and disease severity was assessed using the Medsger severity score. The levels of miR-21 were measured by quantitative real-time polymerase chain reaction. The 2-ΔΔCt method was used for analysis. SSc patients affected by diabetes mellitus, hypertension, renal impairment, heart disease, malignancy, other autoimmune diseases, or a history of serious acute infection within 6 weeks were excluded.

Results. There was a high statistically significant difference in miR-21 levels between cases and controls ($p<0.001$). At a cut-off level of 2.55, miR21 could discriminate between SSc patients and controls with sensitivity 92% and specificity 100%. There was no significant correlation between miR-21 levels and the degree of skin fibrosis. There was a significant positive association between miR-21 levels and the presence of arthritis in SSc patients ($p=0.007$).

Conclusions. miR-21 was suggested as a robust diagnostic biomarker in SSc with superiority over the traditionally utilized antibodies. Additionally, due to its association with arthritis, it is supposed to play a proinflammatory role in addition to its pronounced profibrotic effects. Interestingly, the profibrotic miR-21 may not reflect the extent of skin fibrosis.

Introduction

Systemic sclerosis (SSc) exhibits a spectrum of variable and heterogeneous clinical presentations. Progressive skin fibrosis is the hallmark of SSc, followed by Raynaud's phenomenon and visceral organ involvement (1). The current classification categorizes SSc based on the extent of skin tightness into limited, diffuse, and sine scleroderma, which is not satisfactory due to significant overlap in the clinical characteristics across these subtypes, including pulmonary hypertension, interstitial lung disease, joint or renal involvement. Over the past decade, physicians have encountered substantial challenges in the diagnosis and management of SSc patients. The classification criteria were revised in 2013, achieving a sensitivity of 0.91 and a specificity of 0.92 (2). However, its reliance on specific clinical features may lead to misclassification of atypical cases. Furthermore, the 2013 classification criteria for SSc have limited utility in accurately identifying certain SSc subsets and distinguishing them from SSc-mimicking conditions, thereby increasing the likelihood of misclassification due to overlapping clinical characteristics. There was an emerging proposal to sort SSc patients according to the underlying pathogenesis into inflammatory, fibrotic, and near normal (3). However, whatever the underlying pathogenic process, all pathways ultimately culminate in fibrosis. Currently, no validated diagnostic test exists to definitely confirm or rule out SSc. The antibodies utilized for the classification of SSc patients exhibit limited sensitivity and specificity and often present with overlapping clinical associations: anti-scleroderma 70 (sensitivity 40%, specificity 83%), anti-centromere antibodies (sensitivity 57%, specificity 83%), and anti-RNA polymerase III antibodies (sensitivity 20%, specificity 98.8%) (4, 5). A meta-analysis uncovered inadequacy of the conventional clinical evaluation and standard laboratory tests as outcome measures in therapeutic trials for SSc (6). In contrast with other connective tissue diseases, *e.g.*, rheumatoid arthritis or systemic lupus erythematosus (SLE), SSc lacks a reliable activity score and well-defined therapeutic targets. Early SSc is rather more challenging regarding diagnosis, follow-up, or the possibility of reversing underlying inflammation. It has recently been proposed that SSc is a vascular disease with an initial endothelial injury that leads to endothelial cell activation, proliferation, and angiogenesis formation. Abnormally proliferating endothelial cells

attract mononuclear cells, which produce many cytokines, autoantibodies, and growth factors. Transforming growth factor β (TGF-β) is the most important cytokine that mediates fibroblast differentiation into myofibroblast and proliferation and production of extracellular matrix (7). Numerous connections between fibrosis, autoimmunity, and vasculopathy have been proposed, including epigenetic phenomena that have been recently described as an intriguing contributor in the fibrotic process (8).

MicroRNAs (miRs) are short non-coding RNAs, one of the epigenetic phenomena that affect gene expression (9). They play a key role in many biological processes through the regulation of post-translational gene expression (10). Abnormal expression of miRs was reported in different autoimmune diseases as inflammatory bowel disease (11, 12), psoriasis (13), multiple sclerosis (14, 15), rheumatoid arthritis (16, 17), and SLE (18, 19).

In SSc, the expression levels of miRs are involved in the underlying mechanisms of fibrosis. Upregulation of certain miRs mostly inhibits antifibrotic genes, while downregulation of other miRs serves as inhibitory miRs for antifibrotic genes (20). miR-21 expression levels are found to be significantly elevated in SSc patients, suggesting its profibrotic role.

The role of miR-21 in the underlying mechanisms of fibrosis in SSc includes:

i. TGF-β/smad (drosophila mothers against decapentaplegic protein) signaling pathway: TGF-β, the main mediator of fibrosis, acts positively by activation of downstream genes (*Smad2*, *Smad3*), which control miR-21 maturation, and negatively by its inhibitor factor, *Smad7* (21, 22). *Smad7*, the recognized antagonist of the TGF-β profibrotic effect, is strongly induced by it through the feedback loop mechanism. TGF-β induces overexpression of miR-21 levels, which target *Smad7* directly. *Smad7* downregulation releases the feedback inhibition on TGF-β and leads to heightened TGF-β signaling, thus uncontrollable fibrosis and extracellular matrix deposition (23);
ii. B-cell lymphoma 2 (BCL-2): miR-21 controls the rate of apoptosis in fibroblasts by increasing the expression levels of BCL-2, which is considered to be an inhibitor of the apoptotic process. The lower susceptibility of fibroblasts to apoptosis is considered the main driver of the underlying fibrotic process in SSc (21);
iii. protein sprouty homolog 1 (SPRY1): miR-21 also inhibits SPRY1, a recognized antagonist of fibroblast proliferation. SPRY1 achieves its inhibitory role on fibroblasts by blocking extracellular receptor kinase (ERK) and mitogen-activated protein kinase (MAPK), two pathways implicated in fibroblast proliferation. miR-21 inhibits the ERK and MAPK pathways in an indirect manner, resulting in an increase in fibroblasts (24);
iv. the phosphatase and tensin homolog (*PTEN*) gene: miR-21 also mediates fibroblast differentiation through regulation of the expression of the *PTEN* gene. *PTEN* is a well-known inhibitor of epithelial to mesenchymal differentiation and is targeted and inhibited by miR-21 (25).

Experimental studies were conducted to thoroughly investigate miR-21 in SSc. Applying serum of SSc patients on normal fibroblast for 48 hours resulted in increased expression of miR-21 alongside fibrosis-related genes (26). Overexpression of miR-21 increased production of collagen I and promoted lung fibrosis (27, 28). Conversely, anti-miR-21 decreased dermal thickness, alleviated collagen deposition, and decreased the number of cytokine-producing cells in skin and lung tissues (28). These findings highlight that silencing miR-21 may have beneficial effects in mitigating fibrosis and associated inflammation in SSc.

Emerging questions have been raised about whether the contribution of miR21 to SSc pathogenesis can be translated into a meaningful tool for diagnosis, monitoring, or predicting response to treatment. Moreover, it raises the possibility of miR-21 being utilized as a parameter to assess patient eligibility for antifibrotic therapies. This study aimed to investigate the potential of miR-21 to differentiate between SSc patients and healthy individuals, determine the cut-off levels for diagnosis, sensitivity, and specificity, and explore its associations with various clinical presentations. Additionally, we intended to assess the clinical implications of the profibrotic properties of miR21, which has been demonstrated in previous bench studies, as compared to the modified Rodnan skin score (mRSS).

Materials and Methods

A total of 25 Egyptian patients with SSc who fulfilled the American College of Rheumatology/European League Against Rheumatism criteria for the classification of SSc (2013), as well as 25 age and sex matched healthy Egyptian controls, were enrolled in this study. All patients were recruited from the outpatient Rheumatology and Clinical Immunology Clinic of the Internal Medicine Department, as well as the inpatient Internal Medicine Department of Kasr Al-Ainy Hospital, Cairo University and the outpatient clinic, Newgiza University (NGU). SSc patients affected by diabetes mellitus, hypertension, renal impairment, coronary heart disease, malignant tumors, other autoimmune diseases, or a history of serious acute infection within 6 weeks of admission were excluded from the study. Patients were subjected to thorough history taking, complete physical examination with special emphasis on SSc findings (*e.g.*, digital ulcers, pitting scars, calcinosis, tightening of the skin) as well as laboratory investigations including ESR, complete blood count, liver, kidney function tests, and immunological profile (anti-nuclear antibody by immunofluorescence, antiscleroderma-70). Early SSc was defined as 0-3 years since diagnosis (2).

Ethical committee approval

Ethical approval was obtained from the Internal Medicine Departments, Faculty of Medicine, New Giza University (N-17-2023) and Cairo University. All individual participants included in the study gave written informed consent. All procedures carried out in this study involving human subjects were in line with the institutional and/or national research committee's ethical standards and with the Helsinki Declaration of 1964 and its subsequent amendments or equivalent ethical standards.

Assessment of skin fibrosis

The degree of skin involvement was evaluated using the modified mRSS (range 0-51) (29). Assessment of skin thickness was done by one operator with 2 years of experience using the two thumbs to form a skin fold between advancing lateral thumb borders. Scoring of each cutaneous area was done out of 17 areas.

Assessment of disease severity

Disease severity was assessed using the Medsger severity score (30). Significant peripheral vascular disease was defined by a Medsger score ≥2 (0 = no Raynaud's or presence of Raynaud's not requiring vasodilators; 1 = Raynaud's requiring vasodilators; 2 = digital pitting scars; 3 = digital tip ulcers; 4 = digital gangrene). Significant skin involvement was defined by a Medsger score ≥2 (0=mRSS=0; 1=mRSS=1-14; 2=mRSS=15-29; 3=mRSS=30-39; 4=mRSS≥40).

MicroRNA extraction

Detection of miR-21 by quantitative real-time polymerase chain reaction (QRT-PCR): venous blood (5 mL) was taken from the patient and control and divided into two parts; a portion of the blood (3 mL) was allowed to clot and then centrifuged at 8000 xg for 5 minutes to separate the serum, which was kept frozen at -80°C till used for miR analysis. The second portion of the blood (2 mL) was collected in ethylenediaminetetraacetic acid vacutainer tubes and stored at -80°C until the DNA extraction for genotyping.

miRs were extracted from the serum samples using the mirvana kit USA. miRs additive (100 µL) was added to the samples (200 µL) and mixed well by inverting the tube several times. The mixture was left on ice for 10 minutes. In this step, chloroform equal to the initial volume of lysate was used. The samples were vortexed for 30-60 seconds to mix, then centrifuged at room temperature for 5 minutes at maximum speed (10,000 xg) to separate the aqueous and organic phases. After centrifugation, the interphase was compacted. The aqueous (upper) phase was carefully removed without disturbing the lower phase and was then transferred to a fresh tube. At the end of this procedure, RNA was eluted in nuclease-free water.

The procedure of microRNA purification

The previously prepared sample was inserted into the spin cartridge in a collection tube. It was centrifuged at 12,000 xg for 1 minute. Ethanol (100%) was added to the flow and mixed well by vortex. 700 µL was transferred to another spin cartridge in a collection tube and centrifuged at 12,000 xg for 1 minute, so the small RNA molecules bind to the spin cartridge, and the flow-through was discarded. The spin cartridge was washed with 500 µL ethanol wash buffer, centrifuged at 12,000 xg for 1 minute, and the flow-through was discarded. The wash step was repeated once with 500 µL wash buffer. The collection tube was discarded, and the spin cartridge was placed in a wash tube supplied with the kit. The spin cartridge was centrifuged at maximum speed for 1 minute to remove any residual wash buffer. It was then placed in a 1.7 mL clean recovery tube supplied with the kit. The miR was eluted with 50 µL sterile RNase-free water supplied with the kit. The cartridge was incubated at room temperature for 1 minute, then centrifuged at maximum speed for 1 minute to elute miR. The recovery tube contained small RNA molecules, and the spin cartridge was discarded. The purified miR was stored at -80°C.

Assessment of quality and concentration of isolated RNA

The nanodrop spectrophotometer was used to measure the absorbance of isolated RNA at 260 nm, 280 nm, and 230 nm. Absorbent at 260 nm was used to measure the amount of nucleic acid in the sample. Aromatic amino acids absorb light at 280 nm, so absorbance measurements were used at that wavelength to estimate the amount of protein in the sample. Measurements at 230 nm were used to quantify other contaminants that may be present in the samples, such as guanidine thiocyanate, which is commonly used in nucleic acid purification kits. To estimate the purity of nucleic acids, the ratio of the absorbance contributed by the nucleic acid to the absorbance of the contaminants was calculated. The acceptable purity ratio is 1.8-2.2, while the requirements of A260/A230 are generally >1.7.

Detection and quantification of the amplified microRNA using quantitative real-time polymerase chain reaction

Quantification using the TaqMan MicroRNA Assays (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) was done by two-step reverse transcription (RT)-PCR. In the RT step, cDNA is reverse transcribed from total RNA samples using specific miR primers from the TaqMan MicroRNA Assays and reagents from the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). In the PCR step, PCR products were amplified from cDNA samples using the TaqMan MicroRNA Assay together with the TaqMan Universal PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). Each TaqMan assay included one tube of miR-specific RT primer and one tube of a mix of MicroRNA-specific forward PCR primer, specific reverse PCR primer, and MicroRNA-specific TaqMan MGB probes. The TaqMan MGB probes contained a reporter dye (FAM™ dye) linked to the 5'end of the probe, an amin or groove binder, and a non-fluorescent quencher at the 3'end of the probe.

The process of synthesis of single-stranded cDNA from total RNA samples using the TaqMan MicroRNA RT Kit involved preparing the RT master mix, preparing the RT reaction plate, and performing RT.

Polymerase chain reaction amplification

During the target amplification step, the AmpliTaq Gold DNA polymerase (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) amplifies the target cDNA synthesized from the RNA sample, using the sequence-specific primers from the TaqMan Assay Plates. Performing the PCR step required preparing the reaction plate, setting the plate document, and running the plate.

Polymerase chain reaction

Amplification of cDNA was carried out on a step-one real-time PCR System (Applied Biosystems, Waltham, MA, USA). It was held at 50°C for 2 minutes with an initial step of enzyme activation at 94°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing, and extension at 60°C for 60 seconds.

Data analysis (normalization method)

It was done by relative quantification (Rq), which describes the change in the expression of the target gene relative to a reference group, such as a healthy control, using the 2-ΔΔCT method. The ΔΔCT is calculated by subtracting the ΔCT of the patient sample from a control. Fold change (FC) is calculated by raising 2 to the power of the negativeΔΔCT value, with the following equation [Eq. 1]:

$$\Delta CT = CT(\text{microRNA of interest}) - CT(\text{endogenous control})$$
$$\Delta\Delta CT = \Delta CT(\text{patient}) - \Delta CT(\text{control})$$
$$FC \text{ (or } Rq) = 2^{-\Delta\Delta CT} \qquad \text{[Eq. 1]}$$

Using the 2-ΔΔCT method, the data is presented as the fold change in miR expression normalized to an endogenous control and relative to healthy controls.

Statistical methodology

The data were coded and entered using the statistical package

for the Social Sciences (SPSS) version 25 (IBM Corp., Armonk, NY, USA). Comparisons between quantitative variables were done using the Mann-Whitney non-parametric test, as data were not normally distributed. A Chi-square (χ^2) test was used to compare the categorical data. The Exact test was used instead when the expected frequency was less than 5. Correlation between quantitative variables was performed using the Spearman correlation coefficient. A p-value less than 0.05 was considered statistically significant.

Results

A total of 25 patients with SSc and 25 age- and sex-matched healthy controls (p=0.28, p=0.22, respectively) were recruited in this study from Kasr Alainy hospital, Cairo University. Their clinical laboratory data and treatment are summarized in *Supplementary Table 1*. A total of 20 patients were females (80%). The participants in this study were 18 to 60 years old, with a mean age of 35.36±10.24 years. The duration of SSc ranged from 3 to 240 months, with a mean of 54.12±54.32 months. The control group mean age was 32.5±7.77 years; 15 participants were women (60%).

Levels of miR-21 in the cases and control groups

In this study, miR-21 levels were significantly higher in the SSc group compared to the control group (p<0.001). SSc patients exhibited miR-21 levels ranging from 1.90 to 17.50 with a mean of 7.67±4.59, while the control group had levels ranging from 0.73 to 2.40 with a mean of 1.27±0.46 (Figure 1). At a cut-off level of 2.55, miR21 could efficiently discriminate between SSc patients and the control group with an area under the curve of 0.99, sensitivity 92% and specificity 100% (Figure 2). There was no significant correlation between miR21 expression levels, and the degree of skin fibrosis as assessed by mRSS (p=0.845) (Figure 3). Notably, miR21 was significantly associated with the presence of arthritis in SSc patients (p=0.007) (Table 1). There was no significant correlation between miR21 levels and ESR (r=0.251, p=0.273).

There was no statistically significant correlation between miR21 levels, the age of the patients (p=0.94), or the duration of the disease (p=0.51). There was no statistically significant association between miR21 levels and smoking, Raynaud's phenomenon, telangiectasia, digital ulcers, pitting scars, presence of puffy hands at the beginning of their illness, dysphagia, or pres-

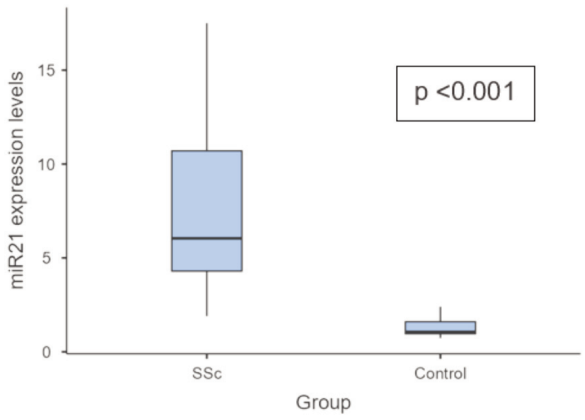

Figure 1. The box plot shows the difference in miR-21 expression levels between the systemic sclerosis and control groups. SSc, systemic sclerosis.

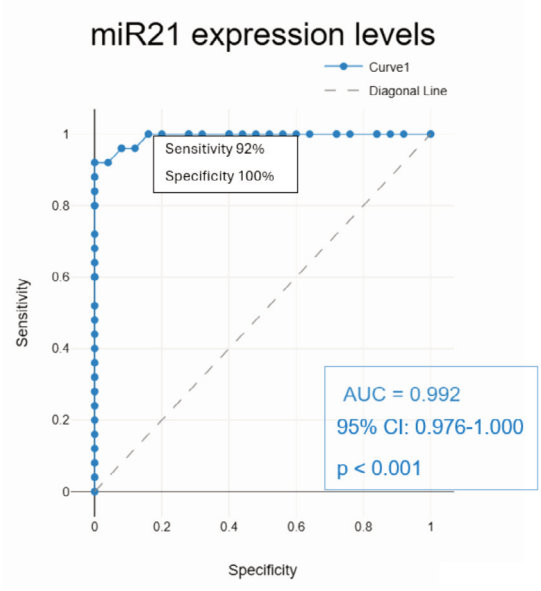

Figure 2. Receiver operating characteristic curve for the detection of systemic sclerosis using miR-21 expression levels. AUC, area under the curve; CI, confidence interval.

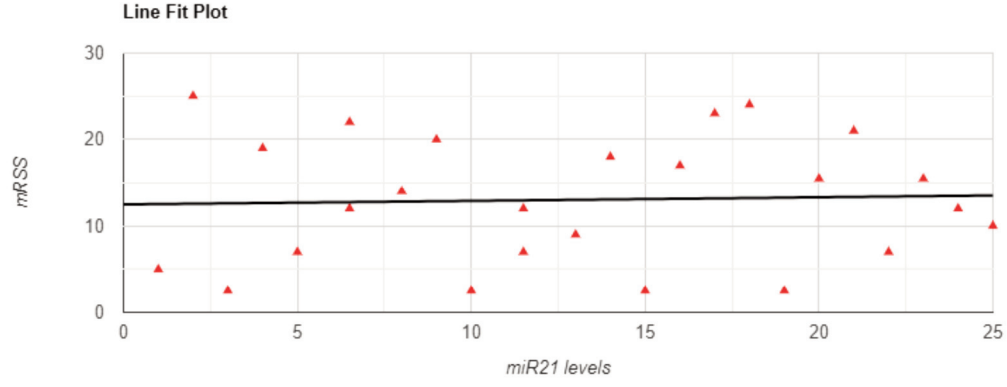

Figure 3. The line plot shows the correlation between miR-21 expression levels and modified Rodnan Skin Score (mRSS).

ence of interstitial lung disease (*Supplementary Table 2*). There was no statistically significant association between the levels of miR21 and all the following: mucocutaneous manifestations, significant peripheral vascular disease, or significant skin involvement by Medsger disease severity score, intake of steroids or vasodilator medications (*Supplementary Table 2*). SSc patients were divided into three subgroups: limited SSc, diffuse SSc, and sine scleroderma. No statistically significant differences were observed in miR-21 levels between them. Subsequently, patients were categorized based on disease duration as early-stage and late-stage. Similarly, miR-21 levels did not exhibit significant variance between these two categories.

Discussion and Conclusions

Epigenetic phenomena are recently getting the attention of researchers. miR-21 is one of the epigenetic phenomena that plays a pivotal role in the pathogenesis and fibrosis in SSc.

In this study, the authors reported that miR-21 levels were significantly higher in SSc patients compared to controls. This finding aligns with previous studies reporting upregulation in miR expression in serum, plasma, or skin samples of SSc patients (21, 26, 27, 31, 32). Wermuth *et al.* reported that miR-21 content of exosomes isolated from SSc patients was also significantly elevated compared to normal serum (33). A meta-analysis identified miR-21 as one of the most consistently differentially expressed miRs in SSc (20). Based on these findings, miR-21 was suggested as a robust diagnostic tool in SSc. Elevated miR-21 levels may help clinicians identify SSc in patients presenting with symptoms.

The cut-off level of miR-21 in the recruited SSc patients was 2.55 with a specificity of 100% and a sensitivity of 92%. A recently calculated cut-off level of miR-21 in SSc was 2.54 with a sensitivity of 70.5% and a specificity of 100% (32). The similarity of the cut-off values underscores the strong consistencies of the diagnostic threshold of miR21 across different studies. The excellent performance of miR21 makes it superior compared to the traditionally used antibodies: anti-scleroderma 70 (sensitivity 40%, specificity 83%), anti-centromere antibodies (sensitivity 57%, specificity 83%), and anti-RNA polymerase III antibodies (sensitivity 20%, specificity 98.8%). This reinforces the reliability of miR21 as a biomarker for SSc. However, when studied in the context of SLE, the established cut-off level to discriminate patients from healthy individuals was determined to be greater than 2.67 with a specificity of 70% and sensitivity of 64.3% (34). The similarity between the cut-off value of miR-21 in SSc and SLE may be due to shared autoimmunity and a high degree of overlap between genetic risk factors for SSc and those for other autoimmune diseases, thus a possible overlap with epigenetic factors between autoimmune diseases (35).

We found no correlation between miR-21 levels and mRSS. This was in agreement with a previous report on 50 SSc patients (32). The unexpected lack of correlation between the profibrotic miR-21 and mRSS suggests that miR-21 may not directly reflect the extent of skin fibrosis. It may be potentially contributing to the underlying pathogenic mechanisms of fibrosis without necessarily correlating with clinical fibrosis. Another suggestion is the limitation of mRSS to capture the subtle changes of skin involvement in SSc, in addition to the encountered inaccuracy of mRSS in edematous or atrophic phases of SSc. There are rising efforts to standardize skin examination by ultrasonography and elastography in SSc (36), which may offer a more objective tool for assessment of skin fibrosis, *e.g.*, assessment of dermal and epidermal thickness, echogenicity, and degree of stiffness.

Notably, an association between miR-21 levels and the presence of arthritis was seen. This finding represents the first report linking miR-21 to inflammatory arthritis in SSc patients. A recent study demonstrated the correlation of miR21 expression to disease activity score in rheumatoid arthritis patients (37). Additionally, miR21 has been found to be associated with tenosynovitis in psoriatic arthritis patients (38). On the other hand, miR-21 levels were not associated with arthritis in SLE patients (39). miR-21 was found to be implicated in inflammatory arthritis by modulating the balance between regulatory T cells and T helper 17 cells (17). Additionally, inflammatory cytokines such as interleukin (IL)-6 and IL-21 can enhance miR-21 transcription by acting on signal transducer and activator of transcription 3 (40). Furthermore, miR-21 activates the NF-κB pathway, which in turn accelerates the proliferation of fibroblast-like synoviocytes (41). Advanced research work is required to thoroughly investigate the possible proinflammatory role of miR21 in SSc.

In this study, miR-21 expression levels were not associated with the presence of pitting scars, oral ulcers, malar rash, alopecia, digital ulcers, telangiectasia, or interstitial lung disease. These results align with those of Koba *et al*., where miR-21 levels were not significantly associated with clinical or laboratory parameters of SSc patients (42).

miR-21 was not previously studied in interstitial lung diseases associated with SSc. Most studies investigating miR-21 have utilized bleomycin-treated models to evaluate its profibrotic effects in comparison to idiopathic pulmonary fibrosis. A meta-analysis concluded that miR-21 was one of the overlapping upregulated miRs between SSc and idiopathic pulmonary fibrosis (43). These findings highlight the questionable reflection of miR-21 on the clinical presentation of SSc patients.

There was no significant difference in miR-21 expression levels between smokers and non-smokers. Several research groups have shown in both human and rat cell models that exposure to cigarette smoke resulted in dysregulation of miR expression. However, the exact mechanism is not fully understood (44, 45). One study suggested that high-dose cigarette smoking can affect miR-21 levels, yet the results in this study unveiled non-significant upregulation of miR-21 in high-dose cigarette smoking-exposed mice (46).

Table 1. Association of miRNA21 expression levels and the presence of arthritis in systemic sclerosis patients.

Arthritis	miR21 expression levels					
	N	Mean	Median	SD	SE	p
Yes	6	12.0	10.8	2.54	1.04	0.007*
No	19	6.30	4.70	4.25	0.975	

N, number; SD, standard deviation; SE, standard error; *p<0.05 is significant

Gender was unequally represented in the enrolled patients because SSc is predominantly represented in females (47). The female predilection in SSc is referred to as DNA hypomethylation through reactivation of genes by hypomethylation of typically methylated X chromosomes of female SSc patients. In this study, there was no relation between the miR-21 levels and gender among SSc patients. This could be explained as miR-21 is a non-X-linked miR. Hence, miR-21 might not show gender bias in SSc patients.

We also reported no significant association between miR-21 expression levels and age in SSc patients, in accordance with another study (48).

In agreement with the previous report, there was no difference between miRNA 21 levels in SSc patients who were on steroid therapy or vasodilators and those who were not (48). However, a possible connection has been raised between miRNA21 and corticosteroids: miRNA21 expression levels in RA patients who were on corticosteroids were found to be lower (49). This could be explained by the fact that corticosteroids inhibit NF-κB, and its absence blocks the expression of miRNA21.

The strength of this study is that it shows that miR-21 demonstrates superior sensitivity and specificity compared to the current traditional biomarkers. In addition, the novel unveiled association of miR21 with arthritis in SSc implicates its possible additional inflammatory contribution to the pathophysiology of the disease for further confirmatory studies.

The limitation of this study was the challenge in recruiting patients with SSc, which has a notably low prevalence of 1.66 per 100,000 individuals in the Arab region and 17.6 per 100,000 individuals globally, resulting in a small sample size that limits generalization of the findings (50, 51). Another limitation was the cross-sectional nature of the study, which limited the ability to draw conclusions about the prognostic value of miR-21. Longitudinal prospective studies are needed to better understand the relationship between miR-21 and disease progression over time. More objective assessment tools, such as musculoskeletal and skin ultrasound, should be utilized in comparison to miR-21 levels, rather than relying solely on clinical evaluations and the mRSS, for a more comprehensive analysis of the clinical implications of miR-21. More studies are needed to clarify the association between arthritis and miR-21 in different autoimmune diseases (SSc, SLE, rheumatoid arthritis, and ankylosing spondylitis).

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Contributions: GGM contributed to the conception of the idea, data collection, and drafting of the preliminary manuscript. MMF supervised the work. LAR performed the data analysis. MTH supervised the study and critically revised the final manuscript. All authors read and approved the final version of the manuscript.

Conflict of interest: the authors have declared no conflict of interest.

Ethics approval and consent to participate: this work was accepted by the local ethical committee of the Department of Internal Medicine, School of Medicine, Cairo University. All procedures carried out in this study involving human subjects were in line with the institutional and/or national research committee's ethical standards and with the Helsinki Declaration of 1964 and its subsequent amendments or equivalent ethical standards.

Informed consent: all individual participants included in the study gave their written informed consent.

Patient consent for publication: all participants provided written informed consent prior to enrolment in the study. Consent for publication of anonymized data was also obtained from all participants.

Availability of data and materials: the data that support the findings of this study are available from the corresponding author upon reasonable request.

Funding: none.

Acknowledgments: the authors would like to thank all participants in this study.

Received: 11 July 2024.
Accepted: 10 April 2025.
Early access: 25 September 2025.

Reumatismo 2025; 77:1773
doi:10.4081/reumatismo.2025.1773

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Online supplementary material:

Supplementary Table 1. Pattern of clinical characteristics of patients with systemic sclerosis.
Supplementary Table 2. Association of miRNA21 with the clinical characteristics of systemic sclerosis patients.