Serum miR-21 and miR-29a expression in systemic sclerosis patients

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Abstract

Objective

The aim of our study was to evaluate the expression levels of miR-21 and miR-29a in the serum of systemic sclerosis (SSc) patients and to determine their correlation with clinical and immunological parameters.

Methods

34 patients fulfilling the ACR/EULAR 2013 classification criteria for SSc were included in the study. miR-21 and miR-29a expression levels in the serum were determined by PCR (SYBR Green technology). $2^{-\Delta\Delta Ct}$ method was used for analysis. 14 healthy donors were used as controls (HCs).

Results

Expression levels of miR-21 were upregulated in the serum of 17 (50.0%) of the patients. The expression of miR-29a was downregulated in 15 (44.12%) of the SSc patients. Receiver operating characteristic (ROC) curve analysis was conducted in order to evaluate the diagnostic accuracy of the expression levels of the studied miRNAs in the serum. Area under the curve (AUC) for miR-21 was 0.634 (95% CI=0.479-0.790), p=0.147 with 64.7% sensitivity and 64.3% specificity. AUC for miR-29a was 0.605 (95% CI=0.420-0.790), with 64.3% sensitivity and 52.9% specificity but without statistical significance (p=0.257). The multimarker analysis of the ROC curves for both miRNAs showed AUC=0.714 (95% CI=0.569-0.860), p=0.021 with 79.4% sensitivity and 42.9% specificity. Levels of miR-29a correlated with the levels of miR-21 in the serum (with Spearman correlation coefficient 0.517, p=0.00017) and with the presence of anti-Scl70 antibodies in the serum (with Spearman correlation coefficient 0.438, p=0.010).

Conclusion

Our data showed a deregulation of miR-21 and miR-29a in the serum of patients with SSc which could suggests their potential role in the disease pathogenesis. Further analysis with higher number of patients is needed to confirm if these miRNAs could be used in the clinical practice as diagnostic biomarkers as well as biomarkers for both disease activity and progression.

Key words

miRNAs, systemic sclerosis, serum, biomarkers

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Introduction

Systemic sclerosis (SSc) is a chronic autoimmune connective tissue disorder characterised by vasculopathy, immunological alterations and excessive synthesis of extracellular matrix (ECM) proteins. These three main features underlie the pathological process leading to fibrosis of the skin and internal organs to which extend the disease is divided into two subtypes of clinical picture and course: a limited SSc (lSSc) and a diffuse SSc (dSSc) (1, 2).

Although the aetiology of SSc is still unknown studies have shown that the accumulation of collagen and other ECM proteins in the tissues is a result of fibroblast hyperactivity thus increased biosynthesis as well as of reduced degradation of these components. In the recent years of great interest is the potential biological significance of epigenetic changes such as altered micro-ribonucleic acid (microRNA, miRNA) expression in the disease pathogenesis (3, 4). miRNAs comprise a class of endogenous short noncoding RNA molecules that negatively regulate the gene expression on posttranscriptional level by targeting the messenger RNAs (mRNAs) (5). Functional studies have shown that miRNAs regulate critical fibrosis-related signalling pathways and molecules related to fibroblast hyperactivity and abnormal synthesis of ECM proteins as well as SSc-related genes thus playing as important role in the pathogenesis of fibrosis (6). miRNAs have been shown to directly or indirectly participate in the fibrotic process by targeting the transforming growth factor (TGF)/Smad3 canonical signalling pathway and the connective tissue growth factor (CTGF), by affecting the epithelial-to-mesenchymal transition and inducing myofibroblast proliferation and resistance to apoptosis (7-10).

Two of the most studied miRNAs in the pathogenesis of SSc are miR-21 and miR-29, having pro- and anti-fibrotic effects, respectively.

miR-29 family includes miR-29a, miR-29b-1, miR-29b-2 and miR29c and it is known as "master fibromiRNA" due to its pivotal role in the pathogenesis of fibrosis. It demonstrates anti-fibrotic effects and targets fibrosis-related genes, both structural ECM and enzymes, involved in tissue remodelling, including collagens, fibronectin, laminin, matrix metalloproteinase-2 (MMP-2). miR-29 is known to directly repress the expression of collagen type I, II and IV thus playing a key regulatory function in collagen expression in SSc (11). miR-29 inhibits the TGF-β1/Smad signalling pathway and suppresses the TGF-β1-induced pro-fibrotic process (12). Through targeting the TGF-β activated kinase 1 binding protein 1 (TAB 1) miR-29 represses the TAB1-mediated tissue inhibitor of metalloproteinases 1 (TIMP-1) production in dermal fibroblasts (13). The miR-29 family expression has been shown to be reduced in various types of tissue fibrosis and its levels inversely correlate with the severity of the fibrosis (14, 15). In addition, miR-29 family targets both pro- and anti-apoptotic Bcl-2 family members, thus functioning as tumour promotor or suppressor (16, 17). In dermal fibroblast from patients with SSc miR-29 has been shown to induce apoptosis via increasing the Bax:Bcl2 ratio (18). Additionally, miR-29 family directly or indirectly affects Mcl-1, an anti-apoptotic Bcl2 family member thus affecting cell survival and cell death (19).

miR-21 is a highly conserved miRNA with ubiquitous expression across most cell types and primary known for its role in oncogenesis. Following studies have shown that the increased expression of miR-21 promotes fibroblast proliferation and accumulation of ECM proteins thus playing role in the pathogenesis of fibrotic process. miR-21 is upregulated by TGF-β1 which in turn induces TGF-β1-related fibrogenesis in skin fibroblasts through targeting Smad7. The latter has been found to be a direct target of miR-21. The expression of miR-21 has been found to be increased in SSc skin tissues and fibroblasts (20).

The aim of our study was to evaluate the diagnostic value of serum miR-21 and miR-29a expression levels in SSc patients with regard to their use as diagnostic biomarkers in clinical practice.

Material and methods

Preparation of serum samples

Ethical approval for this study was granted by the ethics committee of the Medical University, Sofia. Informed consent was obtained from 48 participants from European ancestry (34 dSSc patients and 14 healthy controls (HCs), Table I). According to the ethics committee, HCs were over 18 years of age, with not known rheumatic conditions. SSc was diagnosed according to the American College of Rheumatology/European League against Rheumatism classification criteria for SSc from 2013 (21). In order to reduce the RNA degradation and to eliminate preanalytical variables all blood samples from patients and HCs after the blood draw stayed at room temperature for maximum of 2 hours before being processed in the laboratory where as soon as they arrived serum was isolated from blood tubes using the same parameters for centrifugation. Isolated serum from each patient and control was aliquoted in two cryotubes and stored at -80°C prior isolation. All samples were thawed only one time.

RNA isolation, cDNA synthesis and real-time PCR

Total RNA from the serum samples was extracted with miRNeasy Serum/Plasma kit (Qiagen®). The concentrations and quality of RNA samples were evaluated spectrophotometrically by NanoDrop. Equal concentrations of RNA samples (150 ng) were used in reverse transcription reactions for synthesis of copy DNA (cDNA) with miScript II RT kit (Qiagen®) using miScript HiSpec Buffer. Before use, every cDNA was diluted as recommended. Quantitative real-time polymerase chain reaction (PCR) was done on the Applied Biosystems 7900HT Fast Real-Time PCR System with miScript SYBR Green PCR kit (Qiagen®) and miScript Primer Assays (Qiagen®). cel-miR-39 exogenous and RNU6B endogenous controls were used as reference controls for normalisation. Relative changes of gene expression levels of studied miRNAs were calculated by the $2^{-\Delta\Delta Ct}$ method. HCs were used as calibrators in the analysis. Real-time experiments were performed in triplicates and the mean Ct values were calculated.

Table I. Clinical characteristics of the SSc patients included in the study.

Characteristics	SSc	HCs
Number of patients	34	14
Gender (men/women)	3/31	6/8
Age in years (range) mean age	32 ÷ 72 (54.21 ± 11.68)	28 ÷ 72 (50.85 ± 14.27)
Disease duration in months, mean (min, max)	76.74 (1÷480)	n/a
ANA (+), number (%)	33 (97.06 %)	0 (0.0 %)
Anti-Scl70 Ab (+), number (%)	9 (26.47 %)	n/a
Interstitial pulmonary fibrosis, number (%)	14 (41.18 %)	n/a
Secondary Raynaud syndrome, number (%)	33 (97.05%)	n/a
Capillaroscopy – scleroderma pattern	33 (97.05%)	n/a
Capillaroscopy – early phase, number (%)	12 (35.29%)	n/a
Capillaroscopy – active phase, number (%)	10 (29.41%)	n/a
Capillaroscopy – late phase, number (%)	11 (32.35%)	n/a
Corticosteroids intake, number (%)	14 (41.18%)	n/a
DMARD intake, number (%)	32 (94.12 %)	n/a

Ab: antibody; ANA: antinuclear antibodies; anti-Scl70 Ab: anti-topoisomerase I antibodies; DMARD: disease modifying anti-rheumatic drug; HCs: healthy controls; n/a: not applicable; SSc: systemic sclerosis.

Laboratory and clinical measurements

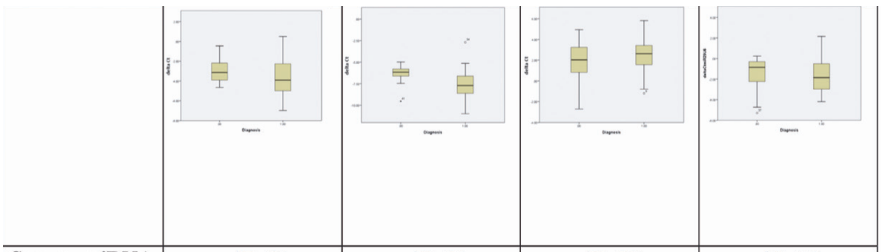
Immunological profile was determined by measurement of the titer of antinuclear antibodies (ANA) by ELISA method, anti-extractable nuclear antigen antibodies (ENA) and levels of complement components – C3 and C4. Testing for ENA was performed by using ANA immunoblot including IgG antibodies against 15 extractable nuclear antigens - U1 ribonucleoprotein/Smith protein (nRNP/Sm), Sm, Robert-Antigen 52 and 60/Sjögren's A (Ro60 and Ro52/SSA), Lane-Antigen/Sjögren's B (La/SSB), topoisomerase I (Scl70), PM/Scl, histidyl-transfer RNA synthetase (Jo-1), centromere protein B (CENP-B), proliferating cell nuclear antigen (PCNA), double-stranded DNA (dsDNA), Nucleosomes, Histones, Ribosomal P-protein, Mi-2 (Euroline 3, Euroimmun®, Germany). Laboratory activity was measured by elevated acute phase reactants (erythrocyte sedimentation rate - ESR, C-reactive protein - CRP). The frequency of concomitant Raynaud syndrome was determined and nailfold capillaroscopy by two independent examiners was performed in order to examine the morphological changes in the nailfold capillaries. The classification of "scleroderma"-type capillaroscopic changes which includes three stages – early, active and late, was used for the description and the analysis (22).

Statistical analysis

Statistical analysis was performed using SPSS Statistics v. 20.0. The Student's t-test and the Spearman's rank correlation coefficient were used for comparison and estimation of correlations between miRNA expression levels and clinico-pathological characteristics such as age, disease duration, immunological parameters, presence of secondary Raynaud syndrome, nailfold capillaroscopic pattern, presence of interstitial lung diseases as well as treatment with disease-modifying antirheumatic drugs (DMARDs). Receiver operating curve (ROC) analysis was performed for evaluation of specificity and sensitivity of serum miRNA expression levels for discriminating SSc patients from HCs. Diagnostic accuracy for combination of biomarkers was also determined by calculating weight coefficients for every biomarker obtaining the largest possible area under the curve (AUC) in ROC analysis. Calculation of coefficients was performed according to Pepe and Thomson (23). Two-tailed *p*-values were taken into account.

Results

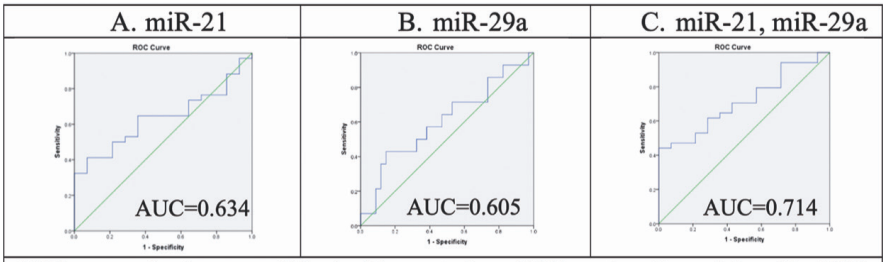
Relative miRNA expression levels in serum samples of SSc patients were obtained by using cel-miR-39 exogenous and RNU6B endogenous controls as reference gene for normalisation as de-



Serum miRNA expression in SSc patients *vs.* HCs	miR-21 exogenous control	miR-21 endogenous control	miR-29a exogenous control	miR-29a endogenous control
	Number of SSc patients (%)			
Without significant difference in expression	11 (32.35)	14 (41.18)	16 (47.06)	15 (44.12)
Decreased expression	6 (17.65)	2 (5.88)	15 (44.12)	9 (26.47)
Increased expression	17 (50.00)	18 (52.94)	3 (8.82)	10 (29.41)

HCs: healthy controls; miR: microribonucleic acid; SSc: systemic sclerosis.

Fig. 1. Serum miRNA expression in SSc patients compared to HCs. Box plots for normalised expression of the studied miRNAs in SSc patients and controls are shown in the upper part.



AUC: area under the curve; HCs: healthy controls; miRNAs: microribonucleic acids; ROC: receiver operating curve; SSc: systemic sclerosis.

Fig. 2. ROC curve analysis for determination the specificity and sensitivity of the studied miRNA in the serum for discriminating SSc patients from HCs.

Table II. Receiver operating curve (ROC) curve analysis by using miRNAs in serum to differentiate SSc patients from HCs.

Serum miRNA SSc vs HCs	AUC (95 % CI)	*p*-value	Se (%)	Sp (%)
miR-21	0.634 (0.479÷0.790)	0.147	64.70	64.30
miR-29a	0.605 (0.420÷0.790)	0.257	64.30	52.90
miR-21 and miR-29a	0.714 (0.569÷0.860)	0.021	79.40	42.90

AUC: area under the curve; HCs: healthy controls; miR: microribonucleic acid; Se: sensitivity; SSc: systemic sclerosis; Sp: specificity.

scribed in detail below and HCs as calibrator samples. Relative quantification (RQ) values were calculated by the $2^{-\Delta\Delta Ct}$ method for evaluation of the expression levels where RQ values between 0.500 and 1.999 mean no significant difference in expression, values ≤ 0.499 mean decreased expression and values ≥2.00 mean increased expression.

Expression level of miR-21 in serum

When using a cel-miR-39 exogenous control for normalisation levels of miR-21 were overexpressed in 50% of the SSc patients compared to the HCs (Fig. 1)

When an endogenous RNU6B control was used for normalisation, levels of miR-21 were overexpressed in 52.94 % of the SSc patients when compared to the HCs (Fig. 1).

The mean Ct value for RNU6B in the examined patients' samples was 35.52 and, in the HCs 34.07, while for cel-miR-39 the mean Ct value was 31.44 and 30.64 for the SSc and HCs samples, respectively. In some of the samples, Ct values of RNU6B were higher than 39, which means that RNU6B is at very low concentration in the serum and could not serve as a reliable normalisation control. The Ct values of cel-miR-39 were lower compared to these of RNU6B in all samples and in the followed analysis we used only cel-miR-39 as a reference control.

Expression level of miR-29a in serum

Levels of miR-29a were downregulated in 44.12% of the serum of SSc patients when compared to HCs when using cel-miR-39 as a reference control for normalisation (Fig. 1)

Diagnostic value of analysed miRNA for discriminating patients with SSc from HCs

The ROC curve analysis showed that the expression levels of miR-21 could not discriminate SSc patients from HCs with AUC=0.634 (95% CI = 0.479÷0.790, p=0.147), with 64.7% sensitivity and 64.3% specificity. The AUC for miR-29a was AUC=0.605 (95%CI 0.420÷0.790) with 64.3% sensitivity and 52.9% specificity but did not reach statistical significance (p=0.257). The diagnostic accuracy did improve when combination of the studied miRNAs was used in the multimarker ROC curve analysis AUC=0.714 (95%CI 0.569÷0.860, p=0.021), 79.4% sensitivity and 42.9% specificity (Fig. 2, Table II).

Clinical and laboratory parameters and serum miRNA expression levels

Serum levels of miR-21 in SSc patients correlated with haemoglobin levels (Spearman correlation coefficient was 0.448, p=0.008), the presence of thrombocytosis or thrombocytopenia (Spearman correlation coefficient was 0.463, p=0.006). Serum levels of miR-29a correlated with the serum levels of miR-21 (Spearman correlation co-

efficient was 0.517, p=0.00017), and the presence of anti-Scl70 antibodies (Spearman correlation coefficient was 0.438, p=0.010). We didn't find a correlation between serum levels of miR-21 and miR-29a with the disease duration, organ involvement, capillaroscopic pattern or treatment with DMARDs. There was a positive correlation between the capillaroscopic pattern and the disease duration (Spearman correlation coefficient was 0.447, p=0.008) and the presence of pulmonary fibrosis (Spearman correlation coefficient was 0.514, p=0.002).

Discussion

SSc is an autoimmune disease with an unknown pathogenesis and limited therapeutic options. The disease leads to skin and internal organ fibrosis, vasculopathy and immunological alterations related to poor prognosis and high mortality risk. Thus, biomarkers related to early diagnosis, disease progression and therapeutic response prediction are needed (24-26). Due to their stability and no known postprocessing modifications as well as the simple and reliable detection miRNA could serve as promising novel non-invasive biomarkers in the clinical practice. Extracellular miRNAs in serum and plasma as a reliable source of miRNAs have generated much interest in the analysis of miRNA expression as a potential non-invasive biomarker in variety of diseases including SSc (27). Several studies have found a deregulated expression of miRNAs in the circulation as well as in disease-specific tissues in SSc patients compared to HCs proving the usefulness of miRNA as potential biomarkers for diagnosis and severity in the clinical practice as well as potential future therapeutic targets. miRNA alterations have been reported to be related to SSc itself, to disease manifestations or to immunological alterations (28-30).

To evaluate the diagnostic potential of miRNAs in SSc we analysed expression values of two chosen miRNAs in serum of patients with SSc patients and HCs. Systemic expression levels of miR-21 and miR-29a in SSc was analysed in regard to their use as diagnostic biomarkers in the clinical practice. miR-21 and miR-29a was selected among others due to their participation in the fibrotic process and the literature data about their expression profiles in SSc patients. miR-21 plays a role in fibrogenesis and thus its expression in tissues and cells related to SSc is of great interest. Altered expression of miR-29 has been linked in several studies to tissue fibrosis and thus miR-29 is one of the most studied miRNA in SSc (13, 20).

Although the increased and the decreased serum expression of miR-21 and miR-29a in SSc patients compared to HCs, respectively, couldn't reach statistical significance in our study we found that the combination of the two studied miRNAs increases the diagnostic accuracy in comparison to their separate use for distinguishing SSc patients from controls.

Altered expression of miR-21-5p has previously been reported in the circulation or tissue specific lesions of SSc patients. Dirk et al. detected elevated levels of miR-21 in the plasma of patients with SSc-associated pulmonary arterial hypertension (SSc-APAH) compared to SSc patients without PAH. In addition, the combination of miR-20-5p or miR-203a-3b with miR-21-5b has been found to have the strongest differentiation accuracy between the patients' groups (29). Similar to our study, the multimarker analysis showed better diagnostic accuracy than a single miRNA expression profile. Zhou *et al*. found increased levels of miR-21 in skin tissues from SSc (31). Maurer *et al*. found a significant downregulation of miR-29a in SSc fibroblasts and skin tissues compared to HCs (14). Similar to that, miR-21 was upregulated and miR-29a was downregulated in dermal fibroblasts of patients with diffuse cutaneous SSc (dcSSc) and TGF-β treated fibroblasts which correlated with the expression alterations of the both miRNAs in our study (32). Additionally, miR-29a downregulation has been reported in hair samples from SSc patients compared to the control group and healthy controls and Kawashita *et al*. found a significant correlation between the reduced serum levels of miR-29a in SSc patients and the higher right ventricular systolic pressure compared to patients with normal miR-29a levels (33, 34). In contrast Wajda *et al*. found that the serum expression of miR-29a was significantly higher only in lSSc patient when compared to HCs (35). A possible reason for the different miRNA expression among the patients and different studies could be due to the heterogeneity of the analysed group in regard to disease subtype, limited or diffuse SSc, disease duration, severity, autoimmunity and ongoing treatment.

Altered miRNA expression in SSc have been found to correlated with immunological parameters. Plasma levels of miR-29a differ among patients with limited SSc and presence of anticentromere antibodies (ACA) compared to anti-U1 ribonucleoprotein (RNP) antibodies (36). We also aimed to study the correlations between miRNA expression levels in the serum with clinical parameters in SSc and found a good correlation between the serum expression level of miR-29a with the presence of anti-Scl70 antibodies as well as with the serum levels of miR-21.

According to the literature there are different miRNAs that could be used as endogenous or exogenous reference controls (37-39). In our study we tested two miRNAs as reference controls and found out that for expression analysis of miR-21 and miR-29a in the serum cel-miR-39 is a more reliable normalisation control.

This study has some limitations. miRNAs expression profile in the serum has been evaluated in a limited number of patients which could interfere with the interpretation of the results. Second, evaluating the expression of specific miRNAs at a specific disease state might miss the dynamically modulated miRNAs and be a limitation for conclusions regarding correlations between miRNA expression and disease valuables like disease duration or treatment response. This indicates the need of larger studies to confirm our results in the clinical practice.

Conclusion

Both clinical and experimental studies have shown that the altered miRNA

expression in the circulation or tissues is related to the immune activation and development of vasculopathy and fibrosis in SSc. We tested two miRNAs with pro- and anti-fibrotic properties, respectively, and found out that their combined multimarker analysis could serve as a potential biomarker to differentiate SSc patients from HCs in the clinical practice. Larger studies including patients with both limited and diffuse SSc as well as on different disease stages are needed to confirm the diagnostic utility of the studied miRNAs in SSc.

References

1. PATTANAIK D, BROWN M, POSTLETHWAITE BC, POSTLETHWAITE AE: Pathogenesis of systemic sclerosis. *Front Immunol* 2015; 6: 272. https://doi.org/10.3389/fimmu.2015.00272
2. HO YY, LAGARES D, TAGER AM, KAPOOR M: Fibrosis--a lethal component of systemic sclerosis. *Nat Rev Rheumatol* 2014; 10(7): 390-402. https://doi.org/10.1038/nrrheum.2014.53
3. LUO Y, WANG Y, WANG Q, XIAO R, LU Q: Systemic sclerosis: genetics and epigenetics. *J Autoimmun* 2013; 41: 161-7. https://doi.org/10.1016/j.jaut.2013.01.012
4. YU J, TANG R, DING K: Epigenetic modifications in the pathogenesis of systemic sclerosis. *Int J Gen Med* 2022; 15: 3155-66. https://doi.org/10.2147/ijgm.S356877
5. SHUMNALIEVA R, KACHAKOVA D, SHOUMNALIEVA-IVANOVA V, MITEVA P, KANEVA R, MONOV S: Whole peripheral blood miR-146a and miR-155 expression levels in Systemic lupus erythematosus patients. *Acta Reumatol Port* 2018; 43(3): 217-225.
6. ZHU H, LUO H, ZUO X: MicroRNAs: their involvement in fibrosis pathogenesis and use as diagnostic biomarkers in scleroderma. *Exp Mol Med* 2013; 45(9): e41. https://doi.org/10.1038/emm.2013.71
7. BABALOLA O, MAMALIS A, LEVTOV H, JAGDEO J: The role of microRNAs in skin fibrosis. *Arch Dermatol Res* 2013; 305: 763-76. https://doi.org/10.1007/s00403-013-1410-1
8. LIU G, FRIGGERI A, YANG Y *et al*.: miR-21 mediates fibrogenic activation of pulmonary fibroblasts and lung fibrosis. *J Exp Med* 2010; 207: 1589-97. https://doi.org/10.1084/jem.20100035
9. DUISTERS RF, TIJSEN AJ, SCHROEN B *et al*.: miR-133 and miR-30 regulate connective tissue growth factor: implications for a role of microRNAs in myocardial matrix remodeling. *Circ Res* 2009; 104: 170-8. https://doi.org/10.1161/circresaha.108.182535
10. DI BATTISTA M, BARSOTTI S, ORLANDI M *et al*.: One year in review 2021: systemic sclerosis. *Clin Exp Rheumatol* 2021; 39 (Suppl. 131): S3-12. https://doi.org/10.55563/clinexprheumatol/izadb8
11. HARMANCI D, ERKAN EP, KOCAK A, AKDOGAN GG: Role of the microRNA-29 family in fibrotic skin diseases. *Biomed Rep* 2017; 6(6): 599-604. https://doi.org/10.3892/br.2017.900
12. LI J, CEN B, CHEN S, HE Y: MicroRNA-29b inhibits TGF-β1-induced fibrosis via regulation of the TGF-β1/Smad pathway in primary human endometrial stromal cells. *Mol Med Rep* 2016; 13(5): 4229-37. https://doi.org/10.3892/mmr.2016.5062
13. CIECHOMSKA M, O'REILLY S, SUWARA M, BOGUNIA-KUBIK K, VAN LAAR JM: MiR-29a reduces TIMP-1 production by dermal fibroblasts via targeting TGF-β activated kinase 1 binding protein 1, implications for systemic sclerosis. *PLoS One* 2014; 9(12): e115596. https://doi.org/10.1371/journal.pone.0115596
14. MAURER B, STANCZYK J, JUNGEL A *et al*.: MicroRNA-29, a key regulator of collagen expression in systemic sclerosis. *Arthritis Rheum* 2010; 62: 1733-43. https://doi.org/10.1002/art.27443
15. VETTORI S, GAY S, DISTLER O: Role of microRNAs in fibrosis. *Open Rheumatol J* 2012; 6: 130-9. https://doi.org/10.2174/1874312901206010130
16. KOLE AJ, SWAHARI V, HAMMOND SM, DESHMUKH M: miR-29b is activated during neuronal maturation and targets BH3-only genes to restrict apoptosis. *Genes Dev* 2011; 25: 125-30. https://doi.org/10.1101/gad.1975411
17. PANDI G, NAKKA VP, DHARAP A, ROOPRA A, VEMUGANTI R: MicroRNA miR-29c down-regulation leading to de-repression of its target DNA methyltransferase 3a promotes ischemic brain damage. *PLoS One* 2013; 8: e58039. https://doi.org/10.1371/journal.pone.0058039
18. JAFARINEJAD-FARSANGI S, FARAZMAND A, MAHMOUDI M *et al*.: MicroRNA-29a induces apoptosis via increasing the Bax:Bcl-2 ratio in dermal fibroblasts of patients with systemic sclerosis. *Autoimmunity* 2015; 48(6): 369-78. https://doi.org/10.3109/08916934.2015.1030616
19. MOTT JL, KOBAYASHI S, BRONK SF, GORES GJ: mir-29 regulates Mcl-1 protein expression and apoptosis. *Oncogene* 2007; 26(42): 6133-40. https://doi.org/10.1038/sj.onc.1210436
20. HUANG Y, HE Y, LI J: MicroRNA-21: a central regulator of fibrotic diseases via various targets. *Curr Pharm Des* 2015; 21(17): 2236-42. https://doi.org/10.2174/1381612820666141226095701
21. VAN DEN HOOGEN F, KHANNA D, FRANSEN J *et al*.: 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/European League against Rheumatism collaborative initiative. *Arthritis Rheum* 2013; 65(11): 2737-47. https://doi.org/10.1002/art.38098
22. LAMBOVA SN, MÜLLER-LADNER U: Nailfold capillaroscopy in systemic sclerosis - state of the art: The evolving knowledge about capillaroscopic abnormalities in systemic sclerosis. *J Scleroderma Relat Disord* 2019; 4(3): 200-11. https://doi.org/10.1177/2397198319833486
23. PEPE MS, THOMPSON ML: Combining diagnostic test results to increase accuracy. *Biostatistics* 2000; 1(2): 123-40. https://doi.org/10.1093/biostatistics/1.2.123
24. LEPRI G, ORLANDI M, DI BATTISTA M *et al*.: Systemic sclerosis: one year in review 2022. *Clin Exp Rheumatol* 2022; 40(10): 1911-20. https://doi.org/10.55563/clinexprheumatol/3401fl
25. CASTRO SV, JIMENEZ SA: Biomarkers in systemic sclerosis. *Biomark Med* 2010; 4(1): 133-147. https://doi.org/10.2217/bmm.09.79
26. UTSUNOMIYA A, OYAMA N, HASEGAWA M: Potential biomarkers in systemic sclerosis: a literature review and update. *J Clin Med* 2020; 9(11): 3388. https://doi.org/10.3390/jcm9113388
27. TERRINONI A, CALABRESE C, BASSO D *et al*.: The circulating miRNAs as diagnostic and prognostic markers. *Clin Chem Lab Med* 2019; 57(7): 932-53. https://doi.org/10.1515/cclm-2018-0838
28. CIECHOMSKA M, ZARECKI P, MERDAS M *et al*.: The role of microRNA-5196 in the pathogenesis of systemic sclerosis. *Eur J Clin Invest* 2017; 47(8): 555-64. https://doi.org/10.1111/eci.12776
29. WUTTGE DM, CARLSEN AL, TEKU G *et al*.: Circulating plasma microRNAs in systemic sclerosis-associated pulmonary arterial hypertension. *Rheumatology* (Oxford) 2022; 61(1): 309-18. https://doi.org/10.1093/rheumatology/keab300
30. RUSEK M, MICHALSKA-JAKUBUS M, KOWAL M, BELTOWSKI J, KRASOWSKA D: A Novel miRNA-4484 is up-regulated on microarray and associated with increased MMP-21 expression in serum of systemic sclerosis patients. *Sci Rep* 2019; 9(1): 14264. https://doi.org/10.1038/s41598-019-50695-y
31. ZHOU B, ZUO XX, LI YS *et al*.: Integration of microRNA and mRNA expression profiles in the skin of systemic sclerosis patients. *Sci Rep* 2017; 7: 42899. https://doi.org/10.1038/srep42899
32. JAFARINEJAD-FARSANGI S, GHARIBDOOST F, FARAZMAND A *et al*.: MicroRNA-21 and microRNA-29a modulate the expression of collagen in dermal fibroblasts of patients with systemic sclerosis. *Autoimmunity* 2019; 52(3): 108-16. https://doi.org/10.1080/08916934.2019.1621856
33. TAKEMOTO R, JINNIN M, WANG Z *et al*.: Hair miR-29a levels are decreased in patients with scleroderma. *Exp Dermatol* 2013; 22(12): 832-3 https://doi.org/10.1111/exd.12245
34. KAWASHITA Y, JINNIN M, MAKINO T *et al*.: Circulating miR-29a levels in patients with scleroderma spectrum disorder. *J Dermatol Sci* 2011; 61(1): 67-9. https://doi.org/ 10.1016/j.jdermsci.2010.11.007
35. WAJDA A, WALCZYK M, DUDEK E *et al*.: Serum microRNAs in systemic sclerosis, associations with digital vasculopathy and lung involvement. *Int J Mol Sci* 2022; 23(18): 10731. https://doi.org/10.3390/ijms231810731
36. WUTTGE DM, CARLSEN AL, TEKU G *et al*.: Specific autoantibody profiles and disease subgroups correlate with circulating microRNA in systemic sclerosis. *Rheumatology*

(Oxford) 2015; 54(11): 2100-7. https://doi.org/10.1093/rheumatology/kev234
37. FARALDI M, GOMARASCA M, SANSONI V, PEREGO S, BANFI G, LOMBARDI G: Normalization strategies differently affect circulating miRNA profile associated with the training status. *Sci Rep* 2019; 9(1): 1584. https://doi.org/10.1038/s41598-019-38505-x
38. SCHWARZENBACH H, DA SILVA AM, CALIN G, PANTEL K: Data normalization strategies for microRNA quantification. *Clin Chem* 2015; 61(11): 1333-42. https://doi.org/10.1373/clinchem.2015.239459
39. LI Y, HUANG J, HU C *et al*.: MicroRNA-320a: an important regulator in the fibrotic process in interstitial lung disease of systemic sclerosis. *Arthritis Res Ther* 2021; 23(1): 21. https://doi.org/10.1186/s13075-020-02411-9