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Original article

Circulating plasma microRNAs in systemic sclerosis-associated pulmonary arterial hypertension

Dirk M. Wuttge[1], Anting L. Carlsen[2,*,†], Gabriel Teku[3,*], Marie Wildt[1], Göran Rådegran[4,5], Mauno Vihinen [3], Niels H. H. Heegaard[2,6,7,†,‡] and Roger Hesselstrand[1,‡]

Abstract

Objectives. SSc-associated pulmonary arterial hypertension (SSc-APAH) is a late but devastating complication of SSc. Early identification of SSc-APAH may improve survival. We examined the role of circulating miRNAs in SSc-APAH.

Methods. Using quantitative RT-PCR the abundance of mature miRNAs in plasma was determined in 85 female patients with ACA-positive lcSSc. Twenty-two of the patients had SSc-APAH. Sixty-three SSc controls without PAH were matched for disease duration. Forty-six selected miRNA plasma levels were correlated with clinical data. Longitudinal samples were analysed from 14 SSc-APAH and 27 SSc patients.

Results. The disease duration was 12 years for the SSc-APAH patients and 12.7 years for the SSc controls. Plasma expression levels of 11 miRNAs were lower in patients with SSc-APAH. Four miRNAs displayed higher plasma levels in SSc-APAH patients compared with SSc controls. There was significant difference between groups for miR-20a-5p and miR-203a-3p when correcting for multiple comparisons ($P = 0.002$ for both). Receiver operating characteristics curve showed AUC = 0.69–0.83 for miR-21-5p and miR-20a-5p or their combination. miR-20a-5p and miR-203a-3p correlated inversely with NT-pro-Brain Natriuretic Protein levels ($r = -0.42$ and -0.47). Mixed effect model analysis could not identify any miRNAs as predictor of PAH development. However, miR-20a-5p plasma levels were lower in the longitudinal samples of SSc-APAH patients than in the SSc controls.

Conclusions. Our study links expression levels of the circulating plasma miRNAs, especially miR-20a-5p and miR-203a-3p, to the occurrence of SSc-APAH in female patients with ACA-positive lcSSc.

Key words: systemic sclerosis, pulmonary arterial hypertension, microRNA, NT-pro-Brain Natriuretic Protein

CLINICAL SCIENCE

Rheumatology key messages

- SSc-associated pulmonary arterial hypertension (SSc-APAH) is a devastating complication of SSc and early detection is warranted.
- Plasma miR-20a-5p and miR-203a-3p differ between SSc-APAH patients and closely matched SSc patient controls.
- Circulating miR-20a-5p may reflect the pathology of SSc-APAH.

[1]Department of Clinical Sciences Lund, Rheumatology, Lund University and Skåne University Hospital, Lund, Sweden, [2]Department of Autoimmunology & Biomarkers, Statens Serum Institute, Copenhagen S, Denmark, [3]Department of Experimental Medical Science, Protein Structure Bioinformatics, [4]Department of Clinical Sciences Lund, Cardiology, Lund University, [5]The Hemodynamic Lab, the Section for Heart Failure and Valvular Disease, VO Heart and Lung Medicine, Skåne University Hospital, Lund, Sweden, [6]Department of Clinical Biochemistry and Pharmacology, Odense University Hospital and [7]Institute of Clinical Research, Clinical Biochemistry, University of Southern Denmark, Odense, Denmark

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Correspondence to: Dirk M. Wuttge, Department of Clinical Science Lund, Rheumatology, Lund University and Skåne University Hospital, SE-221 85 Lund, Sweden. E-mail: dirk.wuttge@med.lu.se

*Shared second authorship.

†In memory of Niels H. H. Heegaard and Anting L. Carlsen.

‡Shared senior authorship.

Introduction

Pulmonary arterial hypertension (PAH) is a serious complication of SSc and affects 9–12% of the SSc patients [1]. SSc patients with ACAs have a high frequency of isolated vasculopathy, including PAH [2]. PAH development in SSc is also associated with age, occurrence of digital ulcers and telangiectasia, as well as decreasing diffusing capacity for carbon monoxide [1, 3, 4]. It is still difficult to predict which patients may develop PAH. PAH associated with SSc (SSc-APAH) has a poor prognosis [5]. Early identification of SSc patients at risk for development of SSc-APAH is therefore important since treatment may improve survival if started in due time [6, 7].

miRNAs are small RNA molecules that exert post-transcriptional regulation of protein expression [8]. Plasma miRNA measurements may be used as disease diagnostic and activity markers [9]. We have previously shown that expression profiles of certain circulating miRNAs are associated with SSc subtype and antibody status [10], as well as with the SSc diagnosis when compared with healthy controls and SLE patients [11]. Yet, it is not known whether circulating plasma miRNAs vary between SSc-APAH patients and SSc patients who have not developed SSc-APAH.

In this study, we analysed miRNA expression levels in plasma in ACA-positive female SSc patients that over two decades had developed SSc-APAH in our longitudinal SSc cohort. Our hypothesis was that SSc-APAH patients are characterized by specific miRNA profiles compared with SSc patients with otherwise comparable disease phenotype, but without development of PAH after similar disease duration.

Methods

Patients

The cohort of patients with SSc at the Rheumatology unit, Skåne University Hospital in Lund, Sweden, was searched retrospectively for ACA-positive female patients that fulfilled the criteria of SSc, either according the ACR [12] or the 2013 ACR/EULAR classification criteria [13], and were classified as having lcSSc according to the criteria of LeRoy *et al.* [14]. Between September 2003 and June 2016, 22 female patients with ACA-positive lcSSc, without significant pulmonary fibrosis, were diagnosed with SSc-APAH by right heart catheterization. Significant pulmonary fibrosis was defined as sign of fibrosis on high resolution CT [11] or on chest X-ray in combination with a vital capacity of $<70\%$. Sixty-three female ACA-positive patients with lcSSc, but without APAH and without significant pulmonary fibrosis, were identified to match 22 SSc-APAH patients regarding disease duration. For the cross-sectional analysis, we aimed to analyse blood samples at the time for PAH diagnosis and before the start of PAH treatment, if possible. Further, longitudinal samples could be identified for 14 SSc-APAH patients and 27 SSc controls. Two samples were analysed for all the 27 SSc patients. Two SSc-APAH patients had three samples analysed and the remaining SSc-APAH patients had two samples analysed. The longitudinal samples were identified by a person (M.W.) independent of the study, without knowledge on the individual miRNA data of the previously performed cross-sectional analysis. The regional ethics review board in Lund (Swedish Ethical Review Authority) approved the research protocol of this study (Dnr2010/32) and written informed consents were obtained from all patients according to the declaration of Helsinki.

Clinical data and risk class assessment

Clinical and routine paraclinical data were obtained under identical conditions and as close as possible to the blood sampling date, as previously described [15]. NT-pro-Brain Natriuretic Protein (NT-pro-BNP) was analysed at the local department of clinical chemistry using a sandwich electrochemiluminescence immunoassay (Cobas, NPU21571, Roche Diagnostics, Rotkreuz ZG, Switzerland), with detection based on a ruthenium derivate. Samples with NT-pro-BNP values <50 ng/l were considered negative and set to 50.

Evaluation of pulmonary hypertension

PAH was defined as a mean pulmonary arterial pressure (mPAP) ≥ 25 mmHg, pulmonary artery wedge pressure ≤ 15 mmHg and a pulmonary vascular resistance >3 Wood units, based on right hearth catheterization ($n = 22$) [5, 16]. In the remaining SSc patients, PAH was excluded by echocardiography [17], applying a maximal tricuspid regurgitation velocity ≤ 2.8 m/s [18], and thus a tricuspid gradient ≤ 36 mmHg (assuming a mean right arterial pressure ≤ 5 mmHg) calculated by the modified Bernoulli equation and without obvious right ventricle signs of PAH. If there was no regurgitation of the tricuspid valve to measure the tricuspid gradient, the systolic pulmonary arterial pressure was estimated to be normal and the value was set to 17 mmHg ($n = 18$). If SSc patients had not been examined by echocardiography at the time of sampling ($n = 18$), their records were screened to exclude later PAH development, especially when the diffusing capacity for carbon monoxide value was $\leq 70\%$.

Analysis of plasma miRNAs

Forty-six miRNAs were selected based on a literature search as previously described in the context of PAH, microvascular disease or SSc pathology-related pathways (supplementary Table S1, available at *Rheumatology* online). Plasma samples were collected during forenoon in EDTA—ontaining tubes and stored in aliquots at -70°C. None of the plasma samples had visible haemolysis. Total RNA was purified using Norgen Total RNA Purification Kit (Norgen Biotek Corp., Thorold, Ontario, Canada) and the panel of miRNAs was analysed after reverse transcription using stem–loop

primers, preamplification and qPCR using specific TaqMan miRNA assays from Applied Biosystems (Foster City, CA, USA). The qPCR in microfluidic platform from Fluidigm Corp (South San Francisco, CA, USA) allowed duplicate analysis of 46 miRNAs in one operation [9]. The 48 miRNA assays included 46 human target miRNAs and two *Caenorhabditis elegans* miRNAs (cel-miR-54 and -238) (supplementary Table S1, available at *Rheumatology* online). The mixture of the two synthetic cel-miRNAs was spiked into the lysis buffer for technical normalization [9]. For the RT-qPCR data average quantitation cycle values >35 were removed from the data set. The quantitation cycle values were then normalized using levels of spike-in controls and row means as described [9, 19].

Data handling and statistical analyses

The demographic data are depicted as mean (S.D.) or *n* (%). miRNA values are shown as median and interquartile range. Mann–Whitney *U*-test was used when comparing numeric clinical variables between groups. Frequencies between groups were evaluated by two-tailed Fisher's exact test. NT-pro-BNP and CRP were both log10-transformed to obtain normal distribution [20]. Spearmans correlation (r_s) were used for correlation analysis of miRNAs and NT-pro-BNP. All residuals in multiple linear regression analyses were analysed for normal distribution and tested by Shapiro–Wilk test. W-values >0.93 were accepted as normally distributed. Partial correlations were used for age-adjusted correlations with NT-pro-BNP and for correlations of miRNA other factor in multiple linear regression analysis. The demographical statistics and clinical correlations were performed with STATISTICA v.12 (StatSoft, Tulsa, OK, USA).

The following analysis were performed with R, an open-source statistical software environment (http://www.r-project.org). We selected miRNAs from the cross-sectional data set based on Wilcoxon signed rank test, multicollinearity, and exhaustive backward model search and selection. The first step in the model selection was to select miRNAs with $P < 0.05$ from the Wilcoxon signed rank test. To avoid multicollinearity, in the second step we studied correlation and variance-inflation factors among miRNAs [21]. The variance-inflation factors quantify the degree of multicollinearity of each miRNA in the model. We then used an exhaustive backward model search of collinearity information to identify and select the most suitable model (https://CRAN.R-project.org/package=leaps).

To study the effects of miRNAs and time on the aetiology of SSc-APAH, we used mixed effect models with SSc-APAH as response, time in months and miRNAs as fixed effects, and random intercept with patients as grouping variable [22]. Student's *t*-test was used to analyse the longitudinal data for miRNA plasma levels in bulk. Generalized estimating equations (GEEs) were performed to estimate the differences in miRNA plasma levels in longitudinal samples between the two groups on a group level, by taking multiple (two or three) samples per individual into account. GEE was performed in SPSS Statistics 25 (IBM, Armonk, NY, USA). Probability values (*P*; two-sided) were considered significant when $P < 0.05$.

Results

Patient characteristics

Between September 2003 and June 2016, 22 female patients with ACA-positive SSc without significant pulmonary fibrosis were diagnosed with SSc-APAH by right heart catherization and compared with 63 matched female ACA-positive SSc controls (Table 1). The SSc-APAH patients were on average 4.5 years the SSc controls ($P = 0.048$) and had higher CRP levels ($P = 0.018$). Four SSc-APAH patients were taking endothelin receptor antagonist. One SSc control patient was treated with endothelin receptor antagonist to prevent digital ulcers ($P = 0.023$).

miRNA expression in plasma

Five miRNAs were excluded from analysis due to non-detectable expression (miR-196a-3p) or missing data (miR-424-5p, miR-200b-3p, miR-29b-3p and miR-206). Fifteen miRNAs were differentially regulated (Fig. 1). Let-7a-5p, let-7c-5p, let-7d-5p, miR-17-5p, miR-20a-5p, miR-140-5p, miR-191-5p, mir-199a/b-3p, miR-203a-3p and miR-223-3p levels were significantly lower in SSc-APAH patients compared with the SSc patients. miR-21-5p, miR-130a-3p, miR-150-5p, miR-155-5p and miR-486-3p displayed higher plasma levels in SSc-APAH patients compared with the SSc patients. miR-203a-3p and miR-20a-5p were significantly differentially expressed also after adjustment for multiple comparison (Table 2).

miRNA expression and confounding clinical factors

Mild interstitial lung disease (ILD) was present in six patients of the SSc-APAH group and nine patients in the control group ($P = 0.199$). None of the 15 miRNAs differed significantly between patients with SSc-APAH or SSc-APAH/ILD ($|P| \geq 0.083$) (supplementary Fig. S1, available at *Rheumatology* online).

Fourteen patients of the SSc-APAH group and 19 SSc controls had an increased CRP. One patient had on ongoing antibiotic treatment. None of the patients with high CRP values were outlies with regard of the plasma expression levels of the miRNAs. All patients were therefore included to retain power in the statistical analysis. miR-181b-5p, miR-199a/b, miR-203a-5p and miR-223-3p were correlated with CRP (supplementary Fig. S2, available at *Rheumatology* online).

Both miR-203a-3p and miR-20a-5p remained significantly differentially expressed between groups when analysed by multiple linear regression analysis with adjustment for age, skin score, vital capacity, CRP or use

TABLE 1 Clinical characteristics of the female ACA-positive lcSSc patients with or without pulmonary arterial hypertension

Characteristic	Overall (n = 85)	SSc-APAH (n = 22)	SSc (n = 63)	*P*-value
Age, years	66 (9.8)	70 (8.2	65 (10.1)	0.046
BMI, kg/m^2	26 (4.1)	25 (2.8	26 (4.5)	0.2
Disease characteristics				
Disease duration, years	12.7 (8.4)	12.0 (7.5)	12.9 (8.7)	0.8
Raynaud's duration, years	19.4 (13.4)	18.5 (10.1)	19.7 (14.5)	0.9
mRss, points	4.2 (4.8)	4.1 (6.7)	4.2 (3.8)	0.052
Telangiectasia, *n*	62 (73)	19 (86)	43 (68)	0.2
History of/current DU, *n*	15 (18)	4 (18)	11 (17)	1.0
Mild ILD, *n*	15 (18)	6 (27)	9 (14)	0.2
VC (% *P*)	97 (14)	90 (11)	99 (15)	0.006
DLCO (% *P*)	71 (24)	42 (10)	79 (20)	<0.001
VC/DLCO	1.5 (0.6)	2.2 (0.5)	1.3 (0.4)	<0.001
TG gradient, mmHg	32 (19)	56 (17)	21 (5.4)	<0.001
NT-pro-BNP, ng/L	867 (1552)	2062 (2274)	313 (482)	<0.001
NT-pro-BNP (log10)	2.48 (0.59)	2.98 (0.62)	2.25 (0.41)	<0.001
CRP, mg/L	5.6 (10.6)	8.5 (10.4)	4.6 (10.5)	0.018
Medication, *n*				
Prostacyclin analogue	3 (4)	0 0	3 (5)	0.6
Phosphodiesterase inhibitors	6 (7)	3 (14)	3 (5)	0.2
Endothelin receptor antagonists	5 (6)	4 (18)	1 (2)	0.015
Right heart catheterization	(*n* = 24)	(*n* = 22)	(*n* = 2)	
mPAP, mmHg	38 (12.0)	40 (10.2)	17 (10)	NA
PCWP, mmHg	7.2 (4.4)	7.2 (4.2)	6.5 (7.8)	NA
Mean RAP, mmHg	6.3 (5.9)	6.8 (6.0)	1.5 (3.5)	NA
CO, L/min	4.2 (1.2)	4.1 (1.2)	5.4 (0.1)	NA
CI, L/min/m^2	2.6 (0.9)	2.5 (0.9)	3.2 (0.1)	NA
SvO_2, %	61 (13)	60 (13)	75 (2)	NA
PVR, dyn. s/cm^5	8.4 (4.3)	9.0 (4.3)	2.0 (0.4)	NA

Data are shown as mean (S.D.) or as *n* (%). CCB: calcium channel blocker; CI: cardiac index; CO: cardiac output; DLCO: diffusing capacity for carbon monoxide; DU: digital ulcer; ILD: interstitial lung disease; mRss: modified Rodnan skin score; NA: not analysed; NT-pro-BNP: NT-pro-brain natriuretic peptide; mPAP: mean pulmonary arterial pressure; PCWP: pulmonary capillary wedge pressure; PVR: pulmonary vascular resistance; RAP: right arterial pressure; SvO_2: mean venous saturation; TG: tricuspid gradient; VC: vital capacity.

of endothelin receptor antagonists (supplementary Tables S2 and 3, available at *Rheumatology* online).

SSc-APAH prediction

To analyse whether the combination of several miRNAs could separate between the patients with or without SSc-APAH, an area under curve (AUC) analysis was performed. miR-20-5p and miR-21-5p discriminated between the groups at a moderate degree (AUC = 0.74, respectively, 0.69) (Fig. 2). The combination of the miR-20-5p and miR-21-5p distinguished between the groups with an AUC of 0.83. Similar results were obtained for the combination of miR-203-3p and miR-21-5p since the plasma expressions of miR-20-5p and miR-203-3p were highly correlated (r = 0.61). NT-pro-BNP values were available in 60 patients. The AUC for NT-pro-BNP was 0.80 (supplementary Fig. S3, available at *Rheumatology* online).

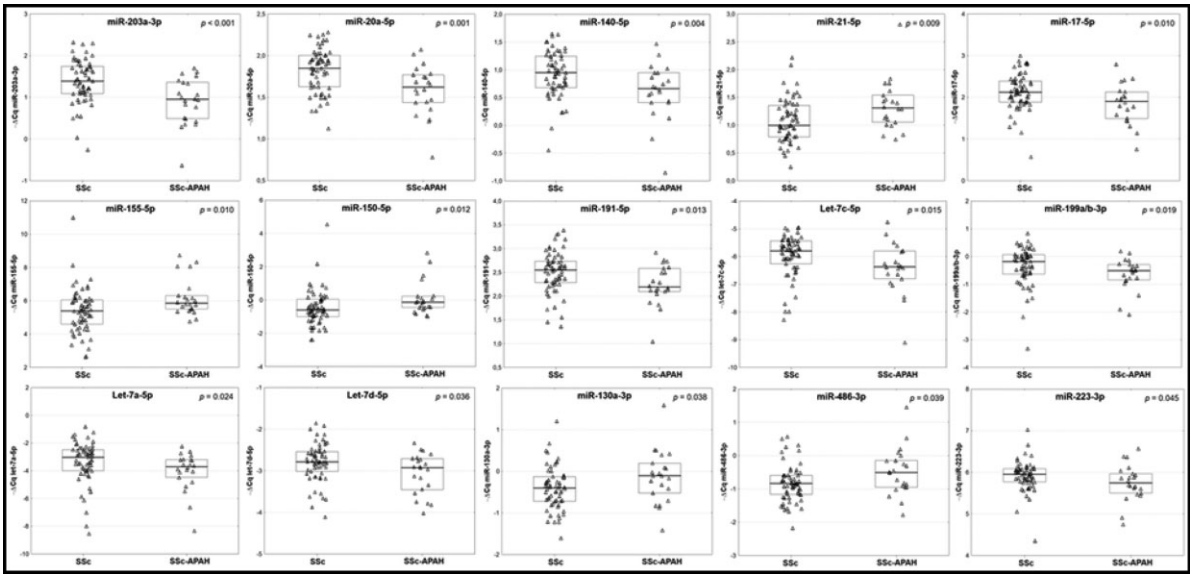
Correlation to NT-pro-BNP

Plasma levels of miR-20a-5p and miR-203a-3p showed an inverse correlation with NT-pro-BNP levels (Fig. 3). Both the miRNAs remained significantly inversely correlated with NT-pro-BNP after adjustment for age (r = −0.39, *P* = 0.002 for miR-20a-5p; and r = −0.39, *P* = 0.003 for miR-203a-3p). Also, miR-17-5p showed an inverse correlation with NT-pro-BNP levels (supplementary Fig. S4, available at *Rheumatology* online).

Analysis of longitudinal plasma samples

PAH may develop gradually in SSc. Longitudinal samples (n = 84) obtained in our real-life setting were therefore analysed from 14 SSc-APAH patients and 27 SSc controls. The mean (min–max) time period of the blood samples was 76 (8–244) months before the last sample. Analysis of the cohort showed reduced plasma levels of miR-20a-5p in the patients who later developed SSc-APAH (Fig. 4A and supplementary Fig. S5, available

FIG. 1 miRNA expressions in the plasma of SSc patients with APAH or without



Displayed are −ΔCq values of miRNAs in plasma of patients with SSc or with SSc-APAH. Shown are the raw data (triangles), median (line) and interquartile range (box). SSc-APAH: SSc-associated pulmonary arterial hypertension; Cq: quantitation cycle.

TABLE 2 Differential expression of plasma miRNAs in SSc-APAH patients compared with SSc patient controls

miRNA	Fold change	*P*-value	*P*-value (FDR adjusted)
miR-203a-3p	0.46 ↓	<0.001	0.022
miR-20a-5p	0.24 ↓	0.001	0.022
miR-140-5p	0.30 ↓	0.004	0.058
miR-21-5p	−0.25 ↑	0.009	0.068
miR-17-5p	0.30 ↓	0.010	0.068
miR-155-5p	−0.67 ↑	0.010	0.068
miR-150-5p	−0.47 ↑	0.012	0.068
miR-191-5p	0.26 ↓	0.013	0.068
let-7c-5p	0.49 ↓	0.015	0.070
miR-199a/b-5p	0.32 ↓	0.019	0.082
let-7a-5p	0.66 ↓	0.024	0.095
let-7d-5p	0.24 ↓	0.036	0.120
miR-130a-3p	−0.29 ↑	0.038	0.120
miR-486-3p	−0.33 ↑	0.039	0.120
miR-223-3p	0.17 ↓	0.045	0.130

miRs are ranked according to significance levels after FDR adjustment. ↓: miR expression levels is lower in SSc-APAH than in SSc controls. ↑: miR expression levels is higher in SSc-APAH than in SSc controls. FDR: false-discovery rate to adjust for multiple comparison; SSc-APAH: SSc-associated pulmonary arterial hypertension.

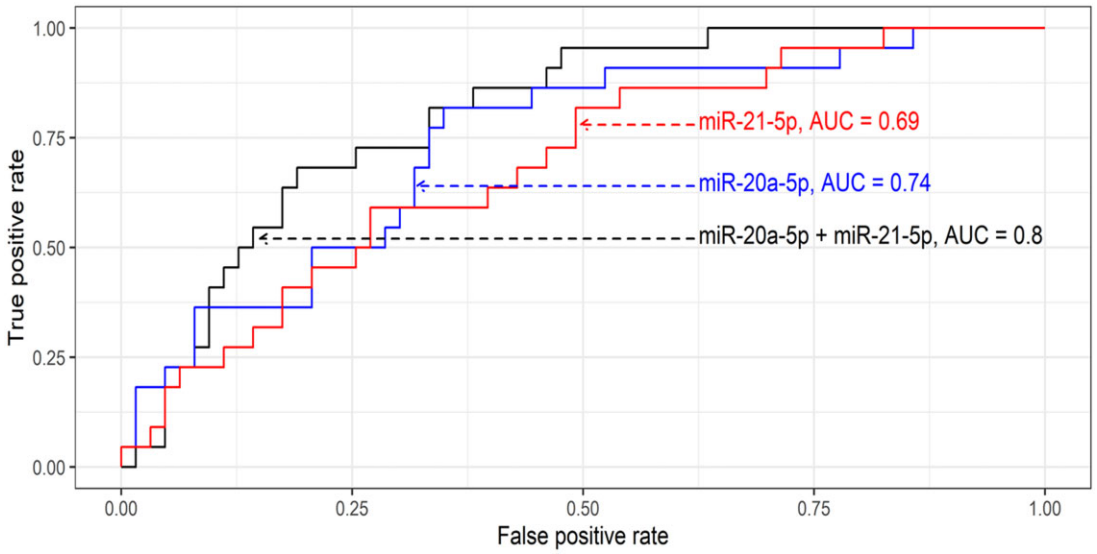
at *Rheumatology* online). The data did not show correlation to time (data not shown). Application of mixed effect models did not identify any miRNAs useful for clinical prediction probably due to individual fluctuation of the miRNAs. Taking into account repeated sample measures with GEE analysis, the SSc-APAH patients nevertheless had lower plasma miR-20a-5p levels compared with the SSc controls ($P = 0.043$) (Fig. 4B). In addition, plasma levels of miR-20a-5p also showed an inverse correlation with NT-pro-BNP levels in 41 samples that had accompanying NT-pro-BNP measurements ($r_s = -0.38$, $P = 0.013$; supplementary Fig. S6, available at *Rheumatology* online).

Discussion

The present study shows that (**i**) 15 miRNAs were differentially expressed in plasma of SSc patients with SSc-APAH compared with closely matched SSc controls without PAH; (ii**)** plasma levels of miR-20-5p or miR-203a-3p combined with miR-21-5p showed the strongest discrimination between the patient groups; (iii**)** miR-20a-5p and miR-203a-3p correlated inversely with serum NT-pro-BNP; and (iv**)** miR-20a-5 levels may be lower in SSc-APAH patients before clinical diagnosis of SSc-APAH.

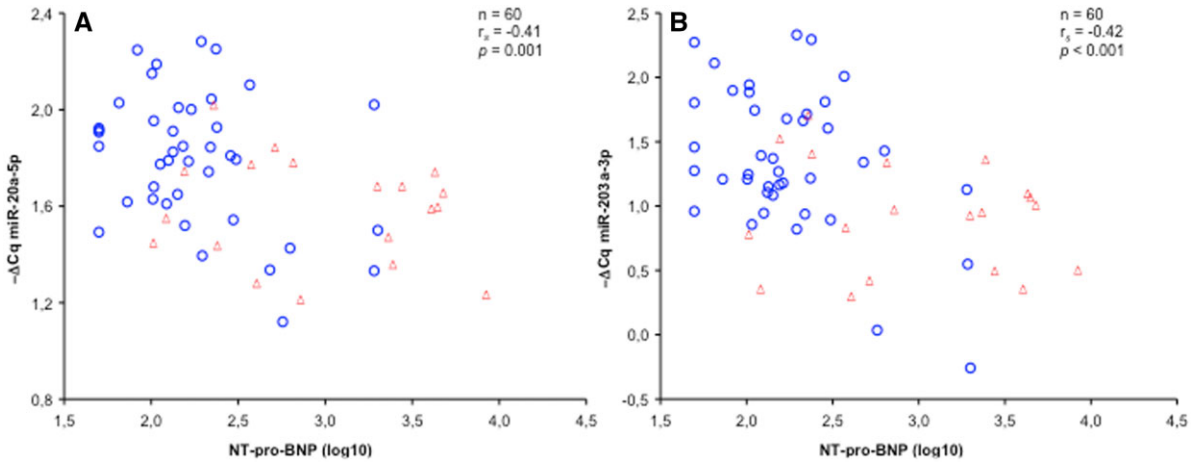
miR-20a and miR-17 are part of the miR-17–miR-92 cluster that has been found to be associated with PAH [23, 24]. Inhibition of these two miRNAs led to pronounced apoptosis under hypoxic conditions [25] and may be p53-signalling mediated. In contrast, IL-6- and STAT-3-mediated increased expression of the miRNAs' led to downregulation of BMP receptor type II, which is a pathogenic hallmark of pulmonary hypertension [26]. The reduced plasma expression levels of miR-17-5p and miR-20a-5p in SSc-APAH patients in our study may

Fig. 2 AUC analysis of plasma miRNA levels to predict pulmonary arterial hypertension in SSc patients



AUC values are depicted for miR-20a-5p (blue), miR-21-5p (red), combination of the miR-20a-5p and miR-21-5p (black). AUC: area under the curve.

Fig. 3 Correlations between plasma miR-20a-5p and miR-203a-3p expressions and serum NT-pro-BNP



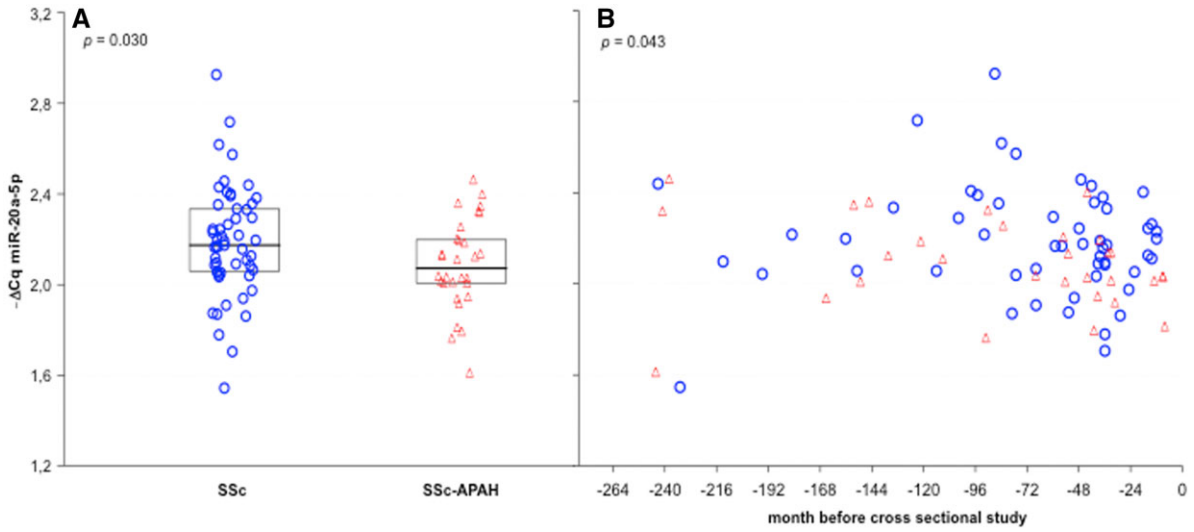
Displayed are correlation between −ΔCq values of miRNA miR-20a-5p (**A**) and miR-203a-3p (**B**) in plasma and serum NT-pro-BNP levels (log10 transformed). SSc patients with APAH ($n = 19$) are depicted as red triangles and SSc patients without APAH ($n = 41$) are shown as blue circles. NT-pro-BNP: NT-pro-Brain Natriuretic Protein; Cq: quantitation cycle; APAH: associated pulmonary arterial hypertension.

thus suggest a hypoxia-induced mechanism of PAH development in ACA-positive lcSSc rather than an IL-6-driven mechanism. Neither miR-17-5p nor miR-20a-5p correlated with CRP levels (data not shown).

miR-203a-3p has not been studied in human pulmonary hypertension or SSc-APAH to any great extent. Hypoxia resulted in downregulation of miR-203a in smooth muscle cells in a hypoxia-induced rat model of pulmonary hypertension [27] and in a hepatocyte a model for sleep apnoea-induced insulin resistance [28]. miR-203a may play an important SNAI-2-mediated inhibitory role in TGF-β-mediated epithelial mesenchymal transition, thereby inhibiting the development of fibrosis [29]. Low miR-203a-3p plasma levels in SSc-APAH patients may result both from TGF-β activation and hypoxia in SSc-APAH.

miR-21-5p levels were upregulated in SSc-PAH patients compared with SSc control patients. Increased miR-21 levels have previously been detected in skin lesions of SSc patients when compared with healthy controls [30]. miR-21 expression has been studied extensively in cardiac failure, and elevated plasma levels

FIG. 4 Plasma expression levels of miR-20a-5p over time in SSc patients with APAH or SSc controls



Samples from patients that develop SSc-APAH later are depicted as red triangles ($n = 14$, SSc-APAH). Samples from patients that did not develop SSc-APAH are shown as blue circles ($n = 27$, SSc). Two to three samples/patient were analysed. (**A**) Student's *t*-test analysis of the bulk of all longitudinal samples for the two groups are shown. (**B**) The longitudinal distribution of the samples is shown in relation of the time before the last sample (of the cross-sectional analysis) was taken. Expression levels of miR-20a-5p were analysed by linear generalized estimating equations taking in to account the repeated samples of the individuals. SSc-APAH: SSc-associated pulmonary arterial hypertension; Cq: quantitation cycle.

may be used as a marker of right ventricular failure [31]. Interestingly, the upregulated expression of miR-21 occurs in pulmonary tissue in patients with idiopathic PAH and in rodent models of pulmonary hypertension [32]. The increased miR-21 plasma levels may therefore reflect both cellular vessel pathology and cardiac-related physiological events in SSc-APAH patients. miR-21-5p is upregulated by TGF-β and may have profibrotic activity through downregulation of SMAD-7 [33]. miR-21 is also upregulated in pulmonary vascular cells by hypoxia [34]. Finally, miR-21 may control multiple target genes for pulmonary hypertension, such as BMP receptor 2 and hypoxic reprogramming, suggesting pleiotrophic effects in PAH [23].

NT-pro-BNP is a serum marker for increased mechanical cardiac stress and may have prognostic value for the survival of SSc-APAH patients [20, 35, 36]. NT-pro-BNP reflects hemodynamic stress in the heart [37] and is also associated with hypoxia [38]. miR-20a-5p and miR-203a-3p and NT-pro-BNP levels were inversely correlated and may reflect heart failure. Lower levels of circulating plasma miR-17-5p, miR-20a-5p and miR-106b-5p were associated with decreased survival of heart failure [39]. Gene ontology analysis indicated that these miRNAs were related to gene expression pathways in heart failure, such as TGF-β-, apoptotic- and hypoxia-related signalling pathways. These signalling pathways are also central for pathogenesis of SSc [40] and for development of PAH [41]. The relation of miR-203a with NT-pro-BNP and heart failure deserves further research.

To identify PAH-related differences in miRNA plasma levels we retrospectively matched patients with SSc-APAH to SSc controls. We are aware of the continuing disease development of APAH in SSc and that vascular changes may occur in pulmonary vessels in patients with normal haemodynamic values [42]. It is also possible that patients with subclinical SSc-APAH may be present in the SSc control group. We may therefore have missed some differentially displayed miRNAs. Several miRNAs that previously have been associated with pulmonary hypertension were not statistically significantly expressed between the groups after adjusting for multiple comparisons. These miRNAs may still have biological relevance for the pathophysiology of SSc-APAH. It remains to be determined whether the changes in miRNA plasma levels were due to secondary changes to APAH-related haemodynamic changes in heart, due to hypoxia or due to cellular expression changes in vessel or circulating cells caused by SSc disease pathology.

Early identification of patients at risk for SSc-APAH might help to prevent development of vascular complications as treatments initiated in due time can prevent vessel remodelling [43]. We therefore analysed whether changes in miRNA plasma levels occur prior to the clinical debut of SSc-APAH. We analysed plasma samples obtained during follow-up and before SSc-APAH

diagnosis. SSc-APAH is a late complication that occurs 5–20 years after the disease onset in 8–12% of SSc patients [44]. As plasma samples are seldomly collected over this long period of time, the number of available plasma samples was limited. The statistical analysis was further complicated since the plasma samples were taken at different time points (see Fig. 4). We therefore searched for statistical methods that could provide an indication of whether plasma miRNA expression predicts clinical onset of SSc-APAH. Unfortunately, the predictive power was not sufficient to answer the question. Another explanation may be that plasma concentrations of circulating miRNA displayed individual fluctuations despite the fact that samples were taken during forenoon, which should avoid the diurnal variation [45]. miRNAs are expressed in different cell types, which might contribute to the fluctuations. This calls for clarifying studies to validate these miRNAs as applicable biomarkers for SSc-APAH such as NT-pro-BNP. Our results, however, indicate that miR-20a-5 plasma levels were lower in SSc-APAH patients compared with SSc controls in the longitudinal samples as detected in the cross-sectional study and correlated to NT-pro-BNP in both study settings.

Our study has some limitations inherent for any cross-sectional design. In this single centre setting, the blood samples were collected during an extended time to obtain enough samples for matching in regard to phenotype and disease duration. Thirdly, we have not been able to age-match the patients exactly. However, the age difference is in line with data from other SSc-APAH cohorts [46] and relevant analyses were adjusted for age. Fourthly, the study does not give any information on circulating plasma miRNA expression in healthy controls. The focus for this study was the clinical challenge to find useful biomarker for early identification of SSc-APAH patients, which may lead to improved survival, in line with other studies [46]. We cannot draw any far-reaching conclusions about differences in plasma expression levels of miRNA between SSc-APAH and SSc-APAH with ILD since we tried to exclude ILD to get as pure information about SSc-APAH as possible. Further, our approach to study candidate miRNAs may have led to us missing other important miRNAs that could be used as biomarkers to differentiate between SSc patients with SSc-APAH and without. Also, we may have missed significant differences in miRNA plasma expression due to over matching. On the other hand, the obtained significances are likely be due to complications in SSc-APAH. Finally, our SSc controls may have included patients that are developing SSc-APAH, as new thresholds for the diagnosis of early PAH have been suggested recently [47]. Thus, the control group may have been diluted with early PAH patients; however, this limitation should not affect the relevance of the obtained differentially displayed miRNAs. Our data should therefore provide support for associations of the detected miRNAs with SSc-APAH in ACA-positive SSc female patients.

Conclusions

To our knowledge, this is the first study that links expression levels of specific circulating plasma miRNAs to SSc-APAH. Circulating miRNAs may reflect the state of APAH or be involved in the pathogenesis of SSc-APAH. To reduce the mortality among SSc-APAH patients, SSc patients eligible for early treatment disease-course modifying drugs should be identified. Prospective longitudinal multicentre studies that collect plasma samples annually during one to two decades from disease onset would be valuable for evaluation of plasma miRNA levels as clinical biomarkers for identification of these patients.

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Data availability statement

The data underlying this article will be shared on reasonable request to the corresponding author.

Supplementary data

Supplementary data are available at *Rheumatology* online.

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