Fibroblast activities are associated with prolonged QTc and pulmonary arterial hypertension in patients with systemic sclerosis

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Abstract

Objective

In systemic sclerosis patients (SSc), we aimed at exploring the potential of serum biomarkers of fibrosis and immune-cell activity to detect subclinical heart involvement determined by electrocardiographic (ECG) alterations.

Methods

A panel of extracellular matrix (ECM) turnover biomarkers quantifying type III and VI collagen formation (PRO-C3 and PRO-C6), type IV collagen turnover (PRO-C4), MMP-degraded type III, IV, VI and VII collagen (C3M, C4M, C6M and C7M), human neutrophil elastase degraded elastin and calprotectin (ELA-HNE and CPa9-HNE), MMP-degraded C-reactive protein (CRPM), MMP-degraded and citrullinated vimentin (VICM) were measured by competitive ELISAs in serum from 102 well-characterised systemic sclerosis patients. Correlations to ECG-changes as well as clinical and paraclinical manifestations were explored.

Results

PRO-C3 and PRO-C6, biomarkers of fibroblast activation, were significantly increased in patients with prolonged QTc (>450ms) (p=0.043 and p=0.027, respectively), while no difference was detected for PRO-C4, C3M, C4M, C6M, and C7M. The ELA-HNE biomarker was significantly reduced in patients with prolonged QTc (>450ms). No difference was found for the CPa9-HNE, CRPM, VICM, and C4G. The PRO-C3 and PRO-C6 biomarkers were also significantly increased in the patients with pulmonal arterial hypertension (PAH) (p=0.041 and p=0.019, respectively), and increased with NYHA class (p=0.024 and p=0.045, respectively). In addition, C6M were significantly increased with NYHA class (p=0.021).

Conclusion

Patients with SSc and prolonged QTc, presence of PAH and high NYHA class presented an altered tissue turnover, particularly associated with increased fibroblast activation. Our study indicates that serum-based biomarkers could serve as convenient biomarkers of subtle cardiac disease in SSc but further studies are needed to confirm this.

Key words

systemic sclerosis, biomarkers, fibrosis, electrocardiogram, pulmonary arterial hypertension, prolonged QTc

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Introduction

Systemic sclerosis (SSc) is a severe systemic autoimmune disease dominated by vasculopathy and fibrosis of the skin and internal organs (1, 2). Cardiovascular involvement is a frequent and significant contributor to morbidity and mortality in SSc (1, 3-6). Cardiovascular manifestations include conduction abnormalities, arrythmias, ischaemic heart disease, pericarditis, heart failure, and peripheral vascular events as well as pulmonal arterial hypertension (PAH) (5). Myocardial disease seems to be a result of vasculopathy and fibrosis more than inflammation in SSc (7, 8). Myocardial involvement tends to develop silently and can be difficult to detect before overt clinical disease. However, asymptomatic myocardial involvement can be detected with cardiac MRI and echocardiography (9). Also, myocardial involvement can present early with subtle isolated conduction abnormalities such as the prolongation of the corrected QT (QTc) interval (10).

In SSc, we have previously shown that certain collagen and extracellular matrix (ECM) turnover biomarkers were elevated, and higher in the early more active pro-fibrotic phase of the disease and were associated with a higher modified Rodnan skin score (mRSS), a diffuse phenotype and having PAH (11-14). In a recent longitudinal study, type III and IV collagens were associated with progressive disease in the skin but not in the lungs (15). Particularly, the collagen type III and VI formation (PRO-C3 and PRO-C6) biomarkers, *i.e.* biomarkers of fibroblast activity, seemed most promising. These novel serum-based biomarkers have not yet been explored in SSc cardiac disease but hold the potential to conveniently detect early myocardial fibrosis, identify risk patients of clinical heart disease, and to help instruct treatment as well as monitor the disease progression.

The first step, and the objective of this study, is to measure a panel of ECM turnover biomarkers in SSc and explore associations to cardiac involvement detected by ECG and to PAH. In the panel, we included previously evaluated collagen degradation and formation biomarkers, but also included four new biomarkers that have not been explored in SSc. These biomarkers were human neutrophil elastase degraded elastin and calprotectin (ELA-HNE and CPa9-HNE), MMP-degraded C-reactive protein (CRPM), and MMP-degraded and citrullinated vimentin (VICM)).

Methods

Study population

One-hundred and two patients, fulfilling the 2013 ACR/EULAR criteria for SSc, were recruited from the outpatient clinic from December 2016 to September 2018 at the Department of Rheumatology, Odense University Hospital, Denmark (16). Detailed methodology and selection of patients for this study have been described elsewhere (17). Briefly, patients were ≥18 years of age diagnosed with SSc at study visit, and participants completed a questionnaire concerning co-morbidities (confirmed by medical records review), current medication, family history of cardiovascular (CV) events, heart symptoms, and smoking habits. The modified Rodnan skin score was evaluated at the visit. Unfortunately, information on the cutaneous phenotype had not been noted (limited vs diffuse). Systolic and diastolic blood pressures and standard 12-lead electrocardiogram (ECG) were measured. Additionally, the recent (within 3 months) pulmonary function test (FVC% and DLCO%) was noted. A previous ICD-10 diagnosis of PAH was noted. The details behind the PAH diagnosis were unavailable. We don't have echocardiography and cardiac MRI were not performed. The following concurrent therapies with potential QTc effects were noted: 1. antibiotics: clarithromycin, clindamycin, erythromycin, moxifloxacin, sulfamethoxazole/trimethoprim; 2. antiarrythmics: flecainide, propafenone (lc) class I, dronedarone, sotalol and amiodarone class III; 3. antidepressants: amitriptylin, clomipramine, fluoxetine, mianserin; 4. antipsychotics: chlorprothixene; 5. tyrosine kinase inhibitors; 6. opioids: methadone; 7. anti-fungal: fluconazole; 8. antihypertensives: indapamide; and 9. antimalarials: hydroxochloroquine. Whole blood was

sampled for all patients and processed according to standard operating procedures, and the serum was stored at -80°C until analysis.

The study was approved by the Regional Committees on Healthy Research Ethics for Southern Denmark, and Danish Data Protection Agency. Verbal and written informed consent according to the Declaration of Helsinki was obtained from all participants.

Biomarker measurements by enzyme-linked immunosorbent assay (ELISA)

A panel of ECM turnover biomarkers quantifying type III and VI collagen formation (PRO-C3 and PRO-C6), type IV collagen turnover (PRO-C4), MMP-degraded type III, IV, VI and VII collagen (C3M, C4M, C6M and C7M), human neutrophil elastase degraded elastin and calprotectin (ELA-HNE and CPa9-HNE), MMP-degraded C-reactive protein (CRPM), and MMP-degraded and citrullinated vimentin (VICM) were measured by competitive ELISAs in serum from all 102 patients in the study population. All biomarkers are validated to measure in human serum samples (18-29). The inter- and intra-assay coefficients of variation are <15% and 10% respectively for all assays. Samples below lower limit of quantification (LLOQ) were given the value of LLOQ.

ECG assessments

A standard ambulatory resting 12-lead ECG at 25 or 50 mm/s was recorded. ECGs were read by a trained medical student with supervision from three clinical cardiologists according to standard published criteria (17). PQ, QRS and QT durations given by the automated ECG algorithm were manually validated. Bazett's formula (QT duration divided by the square root of the RR interval) was used to estimate the corrected QT (QTc) interval. Prolonged intervals were defined as: I) PQ interval ≥220ms in the majority of beats in any of leads I, II, III, aVL or aVF, II) QRS duration ≥120 ms in any of leads I, II, III, aVL or aVF, and III) QTc duration ≥450ms in any leads of V2, V3 or aVR (17).

Statistical analysis

Differences between biomarker levels in SSc patients with or without PAH and ILD, together with abnormal heart rate (<50 or >100), PQ>220, QT>450, qRS>120 or QTc>450ms assessed by ECG were exploratively calculated by Mann-Whitney U-test while differences in NYHA stages was examined by a Kruskal Wallis test. Correlations between clinical variables and biomarkers were assessed by Spearman's correlation. *p*-values less than or equal to 0.05 were considered significant. Data analyses were performed using R studio version 4. 2.1 (R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org; 2020). Graphical illustrations were created using GraphPad Prism v. 9.00 for Windows (GraphPad Software, GraphPad Software, San Diego, California USA, www.graphpad.com).

Results

Demographics

Patient demographics and clinical characteristics are summarised in Table I. Mean age was 59.5 years, 76% were female and with a median disease duration of 4.3 years. Forty-one, 11 and 4 patients were anti-centromere, -topoisomerase I or -RNA-polymerase III positive, respectively, in line with previous Danish SSc studies (30, 31). Unfortunately, we did not have information on the cutaneous phenotype (limited/diffuse).

At the time of ECG acquisition none had an arrythmia. Twenty-one had a prolonged QTc as the main conduction abnormality. Two of these received treatment with potential QTc effects (one in citalopram and one in sulfamethoxazole/trimethoprim). Eight had been diagnosed with PAH which is less than expected given the high proportion of anti-centromere positive patients. Five had a history of ischemic heart disease. None suffered from heart failure. Eleven had interstitial lung disease (ILD). Twenty-two were categorized as NYHA class II-IV.

Correlations between the ECM biomarkers

Some of the biomarkers are highly intercorrelated (highlighted RED and YELLOW, $r>0.5$, $p\leq0.0001$, Table II). C3M, C4M and PRO-C4 are highly intercorrelated ($r>0.8$, $p<0.0001$). PRO-C3 and PRO-C6 are also correlated ($r=0.525$, $p<0.0001$), while only PRO-C6 is correlated to C6M ($r=0.391$, $p=0.0001$). C6M is also correlated to CPa9-HNE ($r=0.527$, $p<0.0001$). Notably, ELA-HNE and VICM are weakly positively correlated ($r=0.268$, $p=0.0106$) and both inversely correlated to PRO-C3 ($r=-0.356$, $p=0.0006$) and PRO-C6 ($r=-0.258$, $p=0.014$).

Table I. Patient demographics and clinical characteristics.

	SSc patients (n=102)
Age at sampling years, mean (SD)	59.5 (12.4)
Female, n (%)	78 (76%)
Age at diagnosis, years (SD)	53.1 (13.6)
Disease duration, years, median (range)	4.3 (0–29.3)
HAQ (0–3), median (range)	0.1 (0–2)
SSc specific antibodies	
Anti-centromere, n (%)	41 (40%)
Anti-topoisomerase I, n (%)	11 (11%)
Anti-RNA-polymerase III, n (%)	4 (4%)
Modified Rodnan skin score, mean (range)	4 (0–16)
CRP, mg/l, median (range)	2.1 (0.6–40)
Interstitial lung disease	11 (11%)
Forced vital capacity, mean (SD)	106 (26)
Diffusing capacity, mean (SD)	67 21)
Cardiac symptoms	
Dyspnoea (NYHA II–IV) n (%)	22 (22%)
Angina	0 (0%)
Smoking	
Current smoker, n (%)	20 (20%)
Former smoker, n (%)	29 (29%)
Never smoker, n (%)	52 (51%)
Family history of CV events, n (%)	18 (19%)
BMI, kg/m2, mean (SD)	24.7 (4.4)
Hypertension, n (%)	66 (65%)
Diabetes, n (%)	2 (2%)
History of AMI, n (%)	5 (5%)
PAH, n (%)	8 (8%)
ECG	
Heart rate, bpm, mean (SD)	71 (14)
PQ (ms), mean (SD)	160 (23)
>220 ms	1 (1)
QRS (ms), median (range)	90 (68–126)
>120 ms n (SD?)	3 (3)
QTc (ms), mean (SD)	433 (23)
>450 ms	21 (22)

Values are expressed as n (%) unless stated otherwise.

SSc: systemic sclerosis; NYHA: New York Heart Association; HAQ: health assessment questionnaire; BMI: body mass index; AMI: acute myocardial infarction; PAH: pulmonary arterial hypertension; ECG: electrocardiogram.

Table II. Correlation analysis between the ECM biomarkers (r and *p*-values).

	C3M	C4M	PRO-C4	C6M	CPa9-HNE	PRO-C6	VICM	PRO-C3	ELA-HNE
C3M	1	0.93 $p<0.0001$	0.91 $p<0.0001$	0.26 $p=0.012$	0.082 $p=0.44$	-0.10 $p=0.33$	0.20 $p=0.056$	-0.14 $p=0.18$	0.078 $p=0.47$
C4M		1	0.89 $p<0.0001$	0.26 $p=0.014$	0.127 $p=0.23$	-0.085 $p=0.42$	0.14 $p=0.18$	-0.048 $p=0.65$	0.063 $p=0.56$
PRO-C4			1	0.26 $p=0.014$	0.047 $p=0.66$	-0.13 $p=0.21$	0.26 $p=0.012$	-0.13 $p=0.22$	0.16 $p=0.12$
C6M				1	0.53 $p<0.0001$	0.39 $p=0.0001$	0.062 $p=0.56$	0.25 $p=0.017$	-0.044 $p=0.68$
CPa9-HNE					1	0.12 $p=0.25$	0.18 $p=0.098$	0.033 $p=0.76$	0.17 $p=0.10$
PRO-C6						1	-0.22 $p=0.035$	0.53 $p<0.0001$	-0.26 $p=0.014$
VICM							1	-0.19 $p=0.078$	0.26 $p=0.011$
PRO-C3								1	-0.36 $p=0.0006$
ELA-HNE									1

ECM turnover biomarkers quantifying type III and VI collagen formation (PRO-C3 and PRO-C6), type IV collagen turnover (PRO-C4), MMP-degraded type III, IV, VI and VII collagen (C3M, C4M, C6M and C7M), human neutrophil elastase degraded elastin and calprotectin (ELA-HNE and CPa9-HNE), MMP-degraded C-reactive protein (CRPM), and MMP-degraded and citrullinated vimentin (VICM).

Associations between ECM biomarkersand demographical parameters

VICM was negatively associated with age (r= -0.223, $p=0.024$), while CPa9-HNE and ELA-HNE were both positively correlated to BMI (CPa9-HNE: r=0.21, $p=0.034$; ELA-HNE: r=0.241, $p=0.015$). PRO-C4, C3M, C4M, C6M, C7M, CPa9-HNE and ELA-HNE were all correlated to CRP (PRO-C4: r=0.23, $p=0.020$; C3M: r=0.265, $p=0.007$; C4M: r=0.283, $p=0.004$; C6M: r=0.367, $p=0.0001$; C7M: r=0.404, $p<0.001$; CPa9-HNE: r=0.45, $p<0.0001$; ELA-HNE: r=0.37, $p=0.0001$).

Extracellular matrix turnover in SSc patients is increased in patients with prolonged QTc

The PRO-C3 and PRO-C6 biomarkers, describing type III and VI collagen formation respectively, were significantly increased in patients with prolonged QTc (>450ms) ($p=0.043$ and $p=0.027$, respectively) (Fig. 1A and 1B), while no difference was detected for PRO-C4, C3M, C4M, C6M, and C7M (all $p>0.05$, data not shown). The ELA-HNE biomarker describing human neutrophil elastase degraded elastin was significantly reduced in patients with prolonged QTc (>450ms) ($p=0.019$) (Fig. 1C). No difference was found for the CPa9-HNE, CRPM, VICM, and C4G (all $p>0.05$, data not shown). As mentioned above both VICM and ELA-HNE were negatively correlated to PRO-C3 and PRO-C6, however, VICM was not decreased in patients with prolonged QTc. It is difficult to determine if the associations to prolonged QTc are independent.

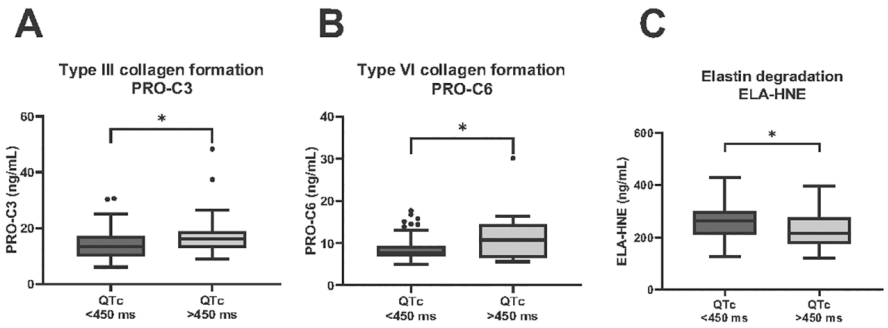

Fig. 1. Levels of ECM biomarkers in SSc patients with QTc<450 ms (n=81) *vs*. QTc>450ms (n=21). Serum levels of: **A**: type III collagen formation (PRO-C3); **B**: type VI collagen formation (PRO-C6); and **C**) elastin degraded by human neutrophil elastase (ELA-HNE).
Differences between groups were calculated by a non-parametric Mann-Whitney t-test. Significance threshold was set at $p<0.05$, and data are presented as Tukey Boxplots. Significance levels: *$p<0.05$.

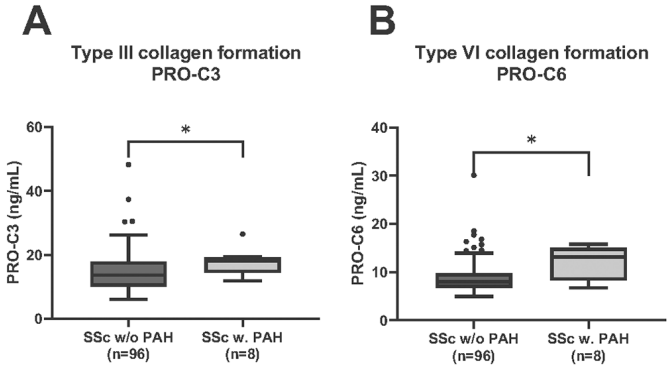

Fig. 2. Levels of ECM biomarkers in SSc patients without PAH (n=96) and with PAH (n=8). Serum levels of: **A**: type III collagen formation (PRO-C3); and **B**: type VI collagen formation (PRO-C6). Differences between groups were calculated by a non-parametric Mann-Whitney t-test. Significance threshold was set at $p<0.05$, and data are presented as Tukey Boxplots. Significance levels: *$p<0.05$.

Type III and VI collagen are increased in SSc patients with PAH and with NYHA stages

The PRO-C3 and PRO-C6 biomarkers were significantly increased in the patients with PAH ($p=0.041$ and $p=0.019$,

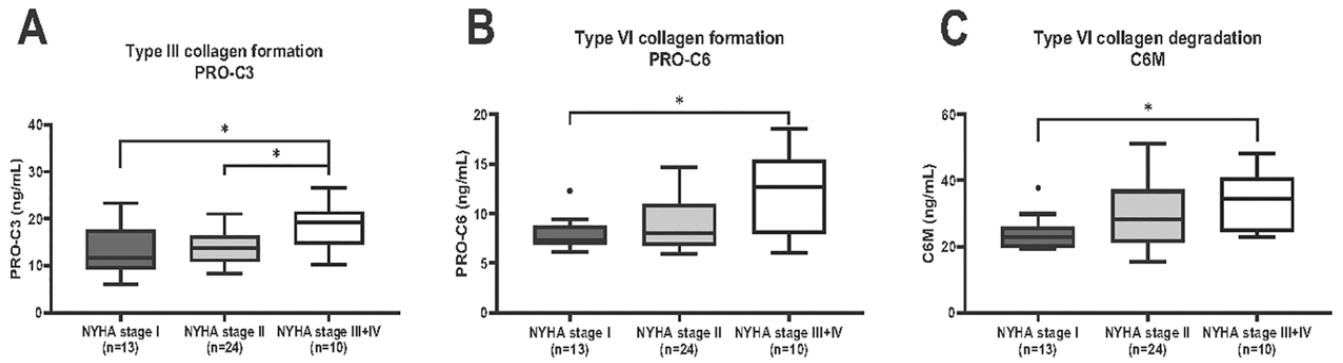

Fig. 3. Levels of ECM biomarkers in SSc patients divided into NYHA stage I (n=13), NYHA stage II (n=24) and NYHA stage III+IV (n=10). Serum levels of: **A**: type III collagen formation (PRO-C3); **B**: type VI collagen formation (PRO-C6); and **C**: type VI collagen degradation (C6M).
Differences between groups were calculated by a non-parametric Mann-Whitney t-test. Significance threshold was set at $p<0.05$, and data are presented as Tukey Boxplots. Significance levels: *$p<0.05$.

respectively) (Fig. 2A and 2B), and increased with NYHA class (p=0.024 and p=0.045, respectively) (Fig. 3A and 3B). In addition, C6M (type VI collagen degradation) were significantly increased with NYHA class (p=0.021) (Fig. 3C). None of the other biomarkers were modulated with presence of PAH or worsening in NYHA stages (all $p>0.05$, data not shown). We observed a negative correlation to DLCO for PRO-C6, C6M and CPa9-HNE (PRO-C6: r= -0.223, p=0.025; C6M: r= -0.287, p=0.004; CPa9-HNE: r= -0.196, p=0.050), while no correlations were found to FVC or having ILD. We also observed a positive correlation to the modified Rodnan skin score for PRO-C6 and C4G (PRO-C6: r=0.215, p=0.030, and C4G: r=0.197, p=0.047).

Discussion

Myocardial dysfunction and pulmonal arterial hypertension develop discretely and are associated with a poor prognosis in SSc. In this study, SSc patients with prolonged QTc exhibited an altered tissue turnover by an increased level of PRO-C3 and PRO-C6, and a reduced level of ELA-HNE. Additionally, SSc patients with PAH, low DLCO and a NYHA class higher than II had increased PRO-C3 and PRO-C6 levels. PRO-C6 and C4G were also associated with higher skin scores. The observations regarding PAH and skin fibrosis are in line with previous observations. The main novel findings are the associations between specific fibrosis biomarkers and conduction abnormalities of the heart. PRO-C3 and PRO-C6, reflecting fibroblast activation, seem to hold the greatest potential as serum-based biomarkers of myocardial involvement in SSc. Since the differences are small and subject to uncertainty, validation studies are needed to reproduce the observations and support the potential direct link between fibroblast activities and disease manifestations in SSc.

The panel of biomarkers was chosen based on previous observations of increased ECM turnover indicating active fibrosis and more extensive disease in SSc (and other fibroinflammatory disorders) (11-13, 15). Despite inconsistencies across the clinical cohort studies, PRO-C6 and C6M and PRO-C3 (and PRO-C4) have been most promising (11-15). Juhl *et al*. showed that biomarkers of formation (PRO-C3, PRO-C4, PRO-C5 and PRO-C6) and degradation (C3M, C4M and C6M) were increased in early phases of diffuse SSc (12, 13). Kubo *et al*. found that C6M and PRO-C5 and PRO-C6 but not PRO-C3 were increased in SSc compared to healthy controls (14). PRO-C3 and PRO-C6 and C6M were associated with PAH, while PRO-C3 and PRO-C6 were correlated to higher skin scores. Dobrota *et al*. observed in two large independent cohorts that both PRO-C3 and PRO-C4 were associated with progressive disease, particularly in the skin (15). Most of the findings in the present study, particular skin fibrosis and PAH, fit with this.

In cardiovascular disease, the biomarkers have been investigated only in a few studies in ischemic heart disease, diabetes and liver cirrhosis exploring clinical and paraclinical correlates as well as CV outcomes and mortality. The focus of our study was on the ECM proteins as surrogate biomarkers of conduction abnormalities and arrythmias on resting ECG. In our cohort, none had an arrythmia on standard resting ECG at the time of inclusion. The main conduction abnormality was long QTc (22% of the patients). The prevalence is similar to other SSc cohorts reporting conduction abnormalities in 20-30% of the patients on resting ECG and higher prevalence of conduction abnormalities and arrythmias detected on 24-h Holter monitoring and in patients with functional impairment of the heart (32). QTc duration reflects the ventricular repolarisation period and is of clinical relevance as it increases the risk of serious ventricular arrhythmias and sudden death (17). It has been proposed, that the majority of conduction abnormalities are a consequence of damaged myocardium rather than specific damage to the proximal portion of the specialised conduction tissue (32, 33). Here, we observed that prolonged QTc was associated to increased fibroblast activity (PRO-C3 and PRO-C6). No other studies have investigated these biomarkers and ECG abnormalities. Our findings suggests that that they could serve as surrogate markers of subtle myocardial fibrosis and damage. In support of this notion, patients with liver cirrhosis had increased liver and cardiac extracellular volume (ECV) on MR, and ECV was highly significantly correlated to the levels of PRO-C3 and PRO-C6 in

the circulation. ECV, native T1 and particular late gadolinium enhancement in cMR are considered measures of myocardial fibrosis (34). Interestingly in SSc, native T1 and ECV on cMR were increased in both patients with very early signs of SSc (the "VEDOSS" cohort) and in established SSc, while global longitudinal strain (GLS), as a marker of subtle functional systolic impairment of the left ventricle, were only increased in established SSc (9). GLS, native T1 and ECV were all associated with mortality. Thus, ECV and native T1 seemed more sensitive in detecting early myocardial involvement than GLS. In a small cohort of women with angina pectoris, however, EVC was associated with C5M but not PRO-C3 or PRO-C6 on cMR (35). Unfortunately, we did not have cMR or echocardiography data on our cohort.

We also observed that ELA-HNE biomarker describing human neutrophil elastase degraded elastin was significantly reduced in patients with prolonged QTc. This biomarker has not been measured in SSc previously, and was previously upregulated in patients with idiopathic pulmonary fibrosis and lung cancer (20). Neutrophils and the release of neutrophil extracellular traps has previously been associated to SSc patients with vascular complications, meaning we hypothesised that the ELA-HNE biomarker would be higher in patients with prolonged QTc (36). Nevertheless, in our study we saw the levels being reduced with prolonged QTc, which may be due to functional defect of neutrophils in SSc patients (37). The functional defects of neutrophils in SSc were described by Impellizzieri *et al*. and highlight an impairment of NET formation. The ELA-HNE biomarker is released upon neutrophil activation, nevertheless if neutrophils are defect or impaired, as a cause of prolonged QTc, one may speculate this downregulation is caused by the defect of neutrophils.

PRO-C6 have also been associated with cardiovascular outcomes. In a large study of patients with atherosclerosis, PRO-C6 was associated with cardiovascular events (ischaemic), heart and all-cause mortality (38). In type I diabetes, PRO-C6 was also independently associated with mortality while association to heart failure and CV events was not significant after adjustment (39). In a large randomised clinical trial of canagliflozin in type 2 diabetes (CANVAS), levels of PRO-C6 was independently associated with heart failure, CV death and overall mortality (40).

In the present study, PRO-C6 and C4G were associated with higher skin scores. C4G is a novel finding, but is well inline with the disease pathogenesis where T-cells play an active role as the TH2 cells are activated and produce profibrotic cytokines, which are increased in skin lesions of SSc patients. However, we found no associations with having ILD or FVC predicted values, only to DLCO. In the aforementioned SSc studies and also in studies of idiopathic lung fibrosis (IPF), PRO-C6 were associated with ILD (41-43). Maybe the lack of associations here were due to the fact that only 10% had ILD in the cohort, and that the ILD most likely is heterogenous in terms of type, severity and phase (stable/progressive). Unfortunately, we had no detailed information on ILD. Regarding the DLCO, the values were disproportionally reduced compared to FVC (normal values) suggesting that the DLCO reductions and associations were related to PAH rather than lung fibrosis.

The panel of biomarkers was chosen based on previous observations in SSc and fibroinflammatory disorders. We also included four novel biomarkers (VICM, CRPM, CPa9-HNE and C4G), based on their potential relationship with SSc pathology, that had never been explored in SSc. Since there were no non-SSc control groups we cannot conclude whether the levels were altered in SSc.

Conclusion

Patients with SSc and prolonged QTc, PAH and high NYHA-class presented an altered tissue turnover associated with increased fibroblast activity. Our study indicates that serum-based profibrotic biomarkers may serve as biomarkers of these manifestations and warrant further studies in cardiac disease in SSc.

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