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Clinical science

Biomarker analysis from the phase 2b randomized placebo-controlled trial of riociguat in early diffuse cutaneous systemic sclerosis

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Abstract

Objective: To examine disease and target engagement biomarkers in the RISE-SSc trial of riociguat in early diffuse cutaneous systemic sclerosis and their potential to predict the response to treatment.

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Methods: Patients were randomized to riociguat ($n=60$) or placebo ($n=61$) for 52 weeks. Skin biopsies and plasma/serum samples were obtained at baseline and week 14. Plasma cyclic guanosine monophosphate (cGMP) was assessed using radio-immunoassay. α-Smooth muscle actin (αSMA) and skin thickness were determined by immunohistochemistry, mRNA markers of fibrosis by qRT-PCR in skin biopsies, and serum CXC motif chemokine ligand 4 (CXCL-4) and soluble platelet endothelial cell adhesion molecule-1 (sPECAM-1) by enzyme-linked immunosorbent assay.

Results: By week 14, cGMP increased by 94 (78)% with riociguat and 10 (39)% with placebo ($P<0.001$, riociguat *vs* placebo). Serum sPECAM-1 and CXCL-4 decreased with riociguat *vs* placebo ($P=0.004$ and $P=0.008$, respectively). There were no differences in skin collagen markers between the two groups. Higher baseline serum sPECAM-1 or the detection of αSMA-positive cells in baseline skin biopsies was associated with a larger reduction of modified Rodnan skin score from baseline at week 52 with riociguat *vs* placebo (interaction *P*-values 0.004 and 0.02, respectively).

Conclusion: Plasma cGMP increased with riociguat, suggesting engagement with the nitric oxide–soluble guanylate cyclase–cGMP pathway. Riociguat was associated with a significant reduction in sPECAM-1 (an angiogenic biomarker) *vs* placebo. Elevated sPECAM-1 and the presence of αSMA-positive skin cells may help to identify patients who could benefit from riociguat in terms of skin fibrosis.

Trial registration: Clinicaltrials.gov, NCT02283762.

Keywords: biomarkers, diffuse cutaneous systemic sclerosis, riociguat, soluble guanylate cyclase stimulators.

Rheumatology key messages

- Lower baseline serum PECAM-1 or absent αSMA-positive skin cells predicted greater mRSS decline with placebo.
- Higher serum PECAM-1 or αSMA-positive cells predicted greater mRSS reductions with riociguat *vs* placebo.
- These markers may identify progressors in early disease and patients who could benefit from riociguat.

Introduction

Systemic sclerosis (SSc) is a severe and debilitating autoimmune connective tissue disease. It is characterized by fibrosis, inflammation, microvascular injury and systemic organ manifestations including pulmonary arterial hypertension (PAH), interstitial lung disease, renal dysfunction and failure, diffuse gastrointestinal disease, and myocardial involvement [1–4]. The nitric oxide (NO)–soluble guanylate cyclase (sGC)–cyclic guanosine monophosphate (cGMP) pathway plays an important role in tissue homeostasis through various mechanisms including antifibrotic and anti-inflammatory effects [5, 6]. In preclinical *in vitro* and *in vivo* studies, the soluble guanylate cyclase stimulator riociguat exhibited anti-inflammatory, antifibrotic and antiproliferative effects mediated partly by the attenuation of TGF-β signalling [5–7]. The phase 3 PATENT trial of riociguat in PAH [8, 9] included a subgroup with PAH associated with SSc, in whom riociguat prevented the decline in functional capacity and was well tolerated [10]. In addition, riociguat improved digital blood flow in some patients with Raynaud's phenomenon in a single-dose pilot study [11]. These observations suggested that riociguat may reduce tissue fibrosis in SSc, and led to the investigation of riociguat in the phase 2b RIociguat Safety and Efficacy in patients with early diffuse cutaneous Systemic Sclerosis (RISE-SSc) study [12]. Treatment with riociguat for 52 weeks did not significantly improve the primary end point (modified Rodnan skin score [mRSS]) *vs* placebo; however, a numerical decrease in mRSS was seen with riociguat ($P=0.08$ *vs* placebo) and analyses of secondary and exploratory endpoints showed potential efficacy [12].

Prognostic biomarkers help to identify patients who are at high risk for certain disease outcomes, such as organ involvement or death in patients with diffuse cutaneous systemic sclerosis (dcSSc), irrespective of treatment. Predictive biomarkers allow physicians to predict response to treatment [13]. Both types of biomarkers may help to inform clinical decision-making and efforts have been made to identify predictive parameters for disease progression in dcSSc [14–16]. Target engagement biomarkers confirm delivery of the drug and indicate that it is acting on its target. This report describes the pre-specified exploratory biomarker analysis from RISE-SSc. The objectives were to examine the effects of riociguat on its target pharmacological pathway, to investigate the prognostic value of biomarkers in the placebo group (who did not receive targeted treatments for SSc other than rescue therapy at investigator discretion from week 26), and to investigate whether biomarkers could predict the effects of riociguat on skin fibrosis, measures of disease activity and progression of lung disease.

Methods

Study design

RISE-SSc (Clinicaltrials.gov NCT02283762) was a randomized, double-blind, placebo-controlled, phase 2b study of riociguat in patients fulfilling American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification criteria for SSc [17], with dcSSc according to LeRoy and Medsager [18] and mRSS of 10–22 units (Supplementary Fig. S1, available at *Rheumatology* online) [12]. Patients were randomized 1:1 to receive riociguat or placebo up to 2.5 mg—maximum three times daily. From week 26, rescue therapy was permitted at the investigator's discretion. The primary end point was the change in mRSS from baseline to week 52 with riociguat *vs* placebo [12].

Selection of biomarkers

Variable selection was performed applying the stability selection approach. Only markers that were selected in at least 20% (of 1000 repetitions) were further assessed; markers that did not show prognostic or predictive potential were excluded. The biomarkers selected are summarized in Table 1.

cGMP was selected as a marker of activation of the NO–sGC–cGMP pathway by riociguat [7]. Asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) are endogenous inhibitors of NO [19], and ADMA levels are elevated in diffuse SSc [20]. Extracellular signal-related kinase (ERK) has been implicated in tissue fibrosis [21] and

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Table 1. Summary of biomarkers evaluated

Biomarker	Method
Markers of NO–sGC–cGMP system engagement/activity	
Plasma cGMP	RIA
Plasma ADMA and SDMA	HPLC–MS
p-ERK, p-VASP	IHC, skin biopsy
Thrombospondin-1	RT-qPCR, skin biopsy
Inflammatory markers	
Serum hsCRP	ITA
Serum sE-selectin, CXCL-4, sPECAM	ELISA
Components of extracellular matrix	
Collagen 1A1, collagen 1A2, collagen 3A1, fibronectin, cartilage oligomeric matrix protein,	RT-qPCR, skin biopsy
Autoantibodies	
Anti-Scl-70	Multiplex bead-based fluorescence immunoassay
Anti-RNA polymerase III	Semi-quantitative ELISA
αSMA	IHC, skin biopsy
Skin thickness	Histology/light microscopy, skin biopsy

ADMA: asymmetric dimethylarginine; αSMA: α-smooth muscle actin; anti-scl-70: anti-topoisomerase I; cGMP: cyclic guanosine monophosphate; CXCL-4: CXC motif chemokine ligand 4; IHC: immunohistochemistry; ITA: immunoturbidimetry assay; NO: nitric oxide; p-ERK: phosphorylated extracellular signal-related kinase; p-VASP: phosphorylated vasodilator-stimulated phosphoprotein; RIA, radio-immunoassay; RT-qPCR: reverse transcription–quantitative real-time PCR; SDMA: symmetric dimethylarginine; sE-selectin: soluble E-selectin; sGC: soluble guanylate cyclase; sPECAM: soluble platelet endothelial cell adhesion molecule-1.

contractile activity in scleroderma fibroblasts [22], and TGF-β stimulates phosphorylation of ERK in dermal fibroblasts [21]. Vasodilator-stimulated phosphoprotein (VASP) is present in vascular smooth muscle cells, endothelial cells, and fibroblasts and is a substrate for cGMP-dependent protein kinases [23]. sGC stimulators have been shown to increase phosphorylation of VASP [23]. ADMA, SDMA, phosphorylated ERK (p-ERK), and phosphorylated VASP (p-VASP) were assessed as indicators of NO–sGC–cGMP pathway activation and TGF-β signalling. High-sensitivity C-reactive protein (hsCRP), soluble E-selectin (sE-selectin), soluble platelet endothelial cell adhesion molecule-1 (sPECAM-1, also referred to as CD31) and CXC motif chemokine ligand 4 (CXCL-4) are inflammatory markers that are elevated in patients with SSc and are associated with increased disease activity or progression [16, 24–27]. Collagen 1A1, collagen 1A2, collagen 3A1, fibronectin and cartilage oligomeric protein are components of extracellular matrix [3, 28]. Thrombospondin-1 is a mediator of TGF-β-mediated cell contractility in SSc [29]. Anti-Scl-70 (anti-topoisomerase) and anti-RNA polymerase III autoantibodies are included in the diagnostic criteria for SSc [17] and are associated with internal organ involvement and progressive skin disease [14–16, 24–26]. α-Smooth muscle actin (αSMA) is a marker of fibroblast cell proliferation, myofibroblast deposition and contractile force generation [30]. Myofibroblasts detected by αSMA immunofluorescence are present in fibrotic skin samples from patients with scleroderma but not in healthy skin or atrophic dcSSc skin [31]. Skin thickness was assessed as this is a characteristic feature of early dcSSc [1, 2].

Biopsy techniques and biomarker analyses

Skin biopsies and plasma or serum samples were obtained for biomarker assessment on day 0 and week 14. Techniques for specimen collection and biomarker measurement and interpretation are provided in the Supplementary Data S1, available at *Rheumatology* online (pp. 1–5). Anti-Scl-70 antibodies were assessed semi-quantitatively using a multiplex bead-based fluorescence immunoassay (FIDIS Connective 10, Theradiag, Croissy Beaubourg, France). Anti-RNA polymerase III antibodies were assessed with a semi-quantitative enzyme-linked immunosorbent assay (QUANTA Lite, INOVA Diagnostics, San Diego, CA, USA). Details of both antibody tests are provided in the Supplementary Data S1, available at *Rheumatology* online (p. 5). Staining for αSMA to detect myofibroblasts in skin biopsies has been used in other studies in SSc [32–34].

Statistical analysis

All biomarkers and their absolute changes from baseline were summarized descriptively by assigned treatment group and visit.

Analyses were performed using SAS System v9.2 or later (SAS Institute, Cary, NC, USA) and R software v3.1.0 or later (R Foundation for Statistical Computing, Vienna, Austria). The analysis was conducted on the intention-to-treat population. As the primary end point of RISE-SSc did not reach the predefined $P < 0.05$ level, all *P*-values reported here should be considered nominal. *P*-values were not adjusted for multiplicity due to the exploratory nature of the analyses, do not imply statistical significance and are for information only. For this report, Spearman's correlation <0.3 between biomarkers or between biomarkers and endpoints is not generally shown because it would be of little scientific or clinical interest. Further details of statistical methods are described in the Supplementary Data S1, available at *Rheumatology* online (pp. 5–8).

Ethics statement

The study was performed in accordance with the Declaration of Helsinki and Good Clinical Practice. The study was approved by the University of Michigan Institutional Review Board and by the ethics committee or institutional review board of each participating site. All patients provided written informed consent.

Results

Study population and baseline biomarkers

The primary results of the double-blind phase of RISE-SSc have been published [12]. In total, 121 patients were randomized (riociguat, $n = 60$; placebo, $n = 61$). Mean (S.D.) mRSS was 16.8 (3.7) overall at baseline (riociguat, 16.9 [3.4]; placebo, 16.7 [4.1]) and 14.6 (6.6) and 15.7 (10.5) in the riociguat and placebo groups, respectively, at week 52. Baseline levels of biomarkers were generally similar between the two groups (Table 2). Three values considered outliers were removed from the data (i.e. set to 'missing') for all analyses of the marker in question because they would have disproportionately affected the results. Results for hsCRP were removed for two patients because of values at week 14 that were >14-fold and >290-fold greater than baseline (baseline, 1.8 and 0.3 mg/l; week 14, 25.9 and 88.3 mg/l, respectively). Results for CXCL-4 were removed for one patient because the level at week 14 was 3.8 mg/l, which was not considered credible. In addition, immunohistochemistry markers (baseline and week 14), ADMA (week 14) and SDMA (week 14)

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Table 2. Key baseline biomarker levels

Biomarker, mean (S.D.) [median; 10–90 percentile]	Placebo group ($n = 61$)	Riociguat group ($n = 60$)
Plasma cGMP, pmol/ml	7.4 (3.3) [6.5; 4.2–11.4]	7.2 (2.6) [6.8; 4.5–10.6]
Plasma ADMA, μg/l	109 (25) [110; 77–143]	112 (23) [109; 88–139]
Plasma SDMA, μg/l	108 (30) [101; 78–133]	102 (20) [99; 82–130]
Plasma p-ERK, %	57 (6) [58; 49–64]	57 (8) [58; 47–65]
Plasma p-VASP, %	59 (11) [60; 45–73]	59 (13) [61; 44–72]
Serum sPECAM-1, ng/ml	77.3 (17.1) [75.6; 56.6–101.1]	81.6 (22.2) [81.6; 55.0–107.0]
Serum CXCL-4, mg/l	8.8 (2.9) [9.0; 5.8–12.0]	9.2 (2.5) [9.4; 6.7–12.3]
Plasma hsCRP, mg/l	4.8 (6.7) [2.8; 0.5–11.4][c,d]	4.1 (6.7) [1.7; 0.3–10.1][c,e]
Serum sE-selectin, μg/l	45.9 (18.7) [43.7; 22.5–73.1]	46.1 (18.2) [44.2; 24.8–71.2]
αSMA, VAS[a]	19.3 (24.0) [5.0; 0.0–51.0]	17.0 (27.4) [2.0; 0.0–68.0]
Skin thickness, μm[b]	1708 (647) [1696; 966–2403]	1750 (504) [1749; 1275–2451]

[a] Measured with endpoints of 0 mm (no αSMA stain) and 100 mm (bright/diffuse αSMA stain) within each skin biopsy sample.
[b] Skin thickness was defined as the distance from the granular layer to the junction between the dermis and subcutaneous fat, assessed in biopsy specimens.
[c] Four values in the placebo arm and nine values in the riociguat arm were below the LLOQ, and were imputed at the LLOQ (0.3 mg/l).
[d] Maximum: 35.6 mg/l.
[e] Maximum: 40.8 mg/l. ADMA: asymmetric dimethylarginine; αSMA: α-smooth muscle actin; cGMP: cyclic guanosine monophosphate; CXCL-4: CXC motif chemokine ligand 4; hsCRP: high-sensitivity CRP; LLOQ, lower limit of quantification; p-ERK: phospho-extracellular signal-regulated kinase; p-VASP: phospho-vasodilator-stimulated phosphoprotein; SDMA: symmetric dimethylarginine; sE-selectin: soluble E-selectin; sPECAM-1: soluble platelet endothelial cell adhesion molecule-1; VAS: visual analogue scale.

were excluded due to withdrawal of informed consent by one patient.

Immunohistochemical αSMA status was unavailable for two patients in the placebo group and one patient in the riociguat group. Overall, 32% of patients with data available (riociguat, 34%; placebo, 31%) had no αSMA-positive cells, and of patients who were αSMA-negative, ≥95% were anti-RNA polymerase III-negative (assessed using immunofluorescence), while 23–37% of those who were αSMA-positive were anti-RNA polymerase III-positive (Supplementary Table S1, available at *Rheumatology* online). Patients who were αSMA-negative had lower baseline hsCRP levels than patients who were αSMA-positive (Supplementary Table S1, available at *Rheumatology* online).

Changes in biomarkers of NO–sGC–cGMP pathway engagement

Percentage changes in biomarker levels from baseline to week 14 are shown in Fig. 1. Mean (S.D.) plasma cGMP (assessed by radio-immunoassay) increased from 7.22 (2.57) pmol/ml to 12.92 (5.24) pmol/ml with riociguat, and from 7.44 (3.34) pmol/ml to 7.50 (3.02) pmol/ml with placebo (mean [S.D.] increase of 94 [78]% and 10 [39]%, respectively; $P < 0.001$ riociguat *vs* placebo). There were no significant differences between treatment groups in changes in ADMA or SDMA (assessed by HPLC–MS), or in immunohistochemistry-assessed p-ERK or p-VASP, from baseline to week 14 (data not shown). Absolute changes in biomarkers are shown in Supplementary Table S2, available at *Rheumatology* online.

Changes in cGMP levels from baseline to week 14 correlated with changes in the riociguat area under the plasma concentration–time curve ($r = 0.489$; $P = 0.001$), maximum plasma concentration ($r = 0.485$; $P = 0.008$) and trough plasma concentration ($r = 0.496$; $P = 0.001$) (Fig. 2). Pharmacokinetic parameters for riociguat are shown in Supplementary Table S3, available at *Rheumatology* online.

Changes in biomarkers of disease activity

At week 14, sPECAM-1 and CXCL-4, assessed using ELISA, were reduced in the riociguat group compared with placebo (Fig. 1): mean (S.D.) change in sPECAM-1 was −11.91 (20.42)% in the riociguat group and 2.18 (27.59)% in the placebo group ($P = 0.004$) and mean (S.D.) change in CXCL-4 was −13.56 (27.36)% in the riociguat group and 5.74 (35.42)% in the placebo group ($P = 0.008$). Changes in immunoturbidimetry-assessed hsCRP or ELISA-assessed sE-selectin did not differ significantly between the riociguat and placebo groups (see Supplementary Data S1, available at *Rheumatology* online [p. 3 and Supplementary Table S2]).

mRNA markers of fibrosis (collagen 1A1, 1A2 and 3A1, cartilage oligomeric matrix protein, thrombospondin-1, and fibronectin), assessed in skin biopsies using reverse transcription–quantitative real-time PCR, were highly correlated with each other; however, the changes in these biomarkers did not differ significantly between treatment groups (data not shown). No significant changes in collagen 1A1, 1A2 or 3A1 were seen between baseline and week 14 with riociguat (mean fold-changes 1.27, 1.24 and 1.21, respectively) or placebo (mean fold-changes 0.96, 1.11 and 1.05, respectively).

Prognostic significance of biomarkers (data from placebo arm)

In the placebo arm, there were no correlations with $r > 0.3$ between biomarker values including cGMP at baseline or week 14 and the change in mRSS from baseline to week 52 (apart from change in cGMP; $r = 0.315$) (Supplementary Table S4, available at *Rheumatology* online). In the placebo arm, higher baseline sPECAM-1 was associated with an increase in mRSS from baseline to week 52 (interaction $P = 0.004$; correlation between baseline sPECAM-1 and change in mRSS: 0.275) (Fig 3). Other biomarkers showed no prognostic potential with respect to mRSS at week 52 in the placebo arm. Patients in the placebo arm who were αSMA-positive ($n = 35$) had numerically less improvement in mRSS at week 52 than those who were αSMA-negative ($n = 16$), with a prognostic effect of −3.82 (95% CI: −8.81, 1.18); $P = 0.131$.

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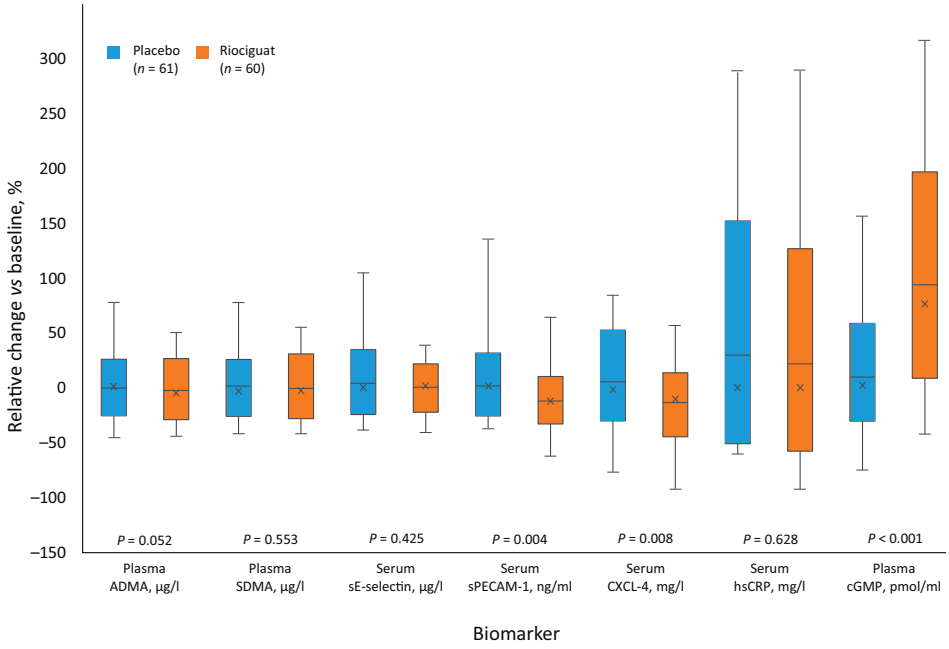

Figure 1. Relative changes in serum and plasma biomarkers in riociguat and placebo groups from baseline to week 14. *P*-values were generated by exploratory *t*-test and are for information only. Vertical lines: maximum and minimum values; top of box: 90 percentile; ×: median change; horizontal lines: mean change; bottom of box:10 percentile. ADMA: asymmetric dimethylarginine; cGMP: cyclic guanosine monophosphate; CXCL-4: CXC motif chemokine ligand 4; hsCRP: high-sensitivity CRP; SDMA: symmetric dimethylarginine; sE-selectin: soluble E-selectin; sPECAM-1: soluble platelet endothelial cell adhesion molecule-1

Association of biomarker levels at baseline with effects of riociguat on mRSS at week 52

Higher baseline levels of sPECAM-1 were associated with a greater reduction of mRSS at week 52 with riociguat *vs* placebo (interaction $P = 0.004$) (Fig. 3). Baseline levels of hsCRP, CXCL-4, p-VASP or fold-change of fibronectin had no clear predictive value, showing interaction $P > 0.05$ ($P = 0.06$ for hsCRP). With the exception of CXCL-4 (correlation 0.377) there were no correlations >0.3 between baseline biomarkers (including cGMP) and the change in mRSS from baseline to week 52 in the riociguat group (Supplementary Table S5, available at *Rheumatology* online).

Associations of αSMA-positive cells at baseline with effects of riociguat on mRSS at week 52

In patients showing no αSMA-positive cells, the treatment difference between riociguat and placebo for mRSS at week 52 was 3.76 (95% CI: −1.11, 8.64); in patients with αSMA-positive cells the difference was −3.59 (95% CI: −6.74, 0.44) (interaction $P = 0.02$) (Fig. 4A). Progression of mRSS (increase of 4–22 units) to week 52 was observed in 40% of patients with αSMA-positive cells at baseline in the placebo arm and in 8% of such patients in the riociguat arm (Fig. 4B).

Among patients with αSMA-positive cells, the treatment difference of change in mRSS for riociguat compared with placebo was −4.90 (95% CI: −10.04, 0.23) in those with baseline mRSS 17–22 units, and 0.75 (95% CI: −2.89, 4.4) if baseline mRSS was 10 to <17 units. These observations suggest a reduction in mRSS with riociguat in patients with αSMA-positive cells and higher mRSS at baseline. The effect of αSMA-positive cells at baseline on the response to riociguat was seen in patients who were also positive for anti-RNA polymerase III or anti-Scl-70 with a treatment difference of −5.6 (95% CI: −9.28, 1.91; interaction $P = 0.005$ for baseline αSMA cell status), but not seen in those who were also both anti-RNA polymerase III and anti-Scl-70-negative (treatment difference: 0.42; 95% CI: −4.96, 5.82). Analysis of the change in mRSS at week 52 in relation to changes in αSMA-positive cell counts at week 14 categorized into quartiles showed no clear association (interaction $P = 0.186$; Supplementary Table S6, available at *Rheumatology* online).

Association of changes in biomarkers at week 14 with effects of riociguat on mRSS at week 52

There was no clear evidence of an association between the change in mRSS at week 52 and changes in αSMA-positive cell counts at week 14 categorized into quartiles (see Supplementary Table S6, available at *Rheumatology* online). Changes from baseline to week 14 in other biomarkers including cGMP had no clear relationship with change in mRSS (all correlations <0.3; Supplementary Table S5, available at *Rheumatology* online). Fold-changes of mRNA for collagen 1A1, cartilage oligomeric matrix protein and fibronectin showed correlations of 0.339, 0.306 and 0.358, respectively, with change from baseline to week 52 in mRSS in the riociguat group. Correlations for other mRNA fold-changes were <0.3.

Associations of baseline levels, or changes in biomarker levels at week 14, with effects of riociguat on other endpoints

Baseline levels of biomarkers or their changes from baseline to week 14 showed no clear relationship with changes from baseline to week 52 in forced vital capacity (FVC) % predicted, carbon monoxide diffusing capacity (DL_{CO}) %

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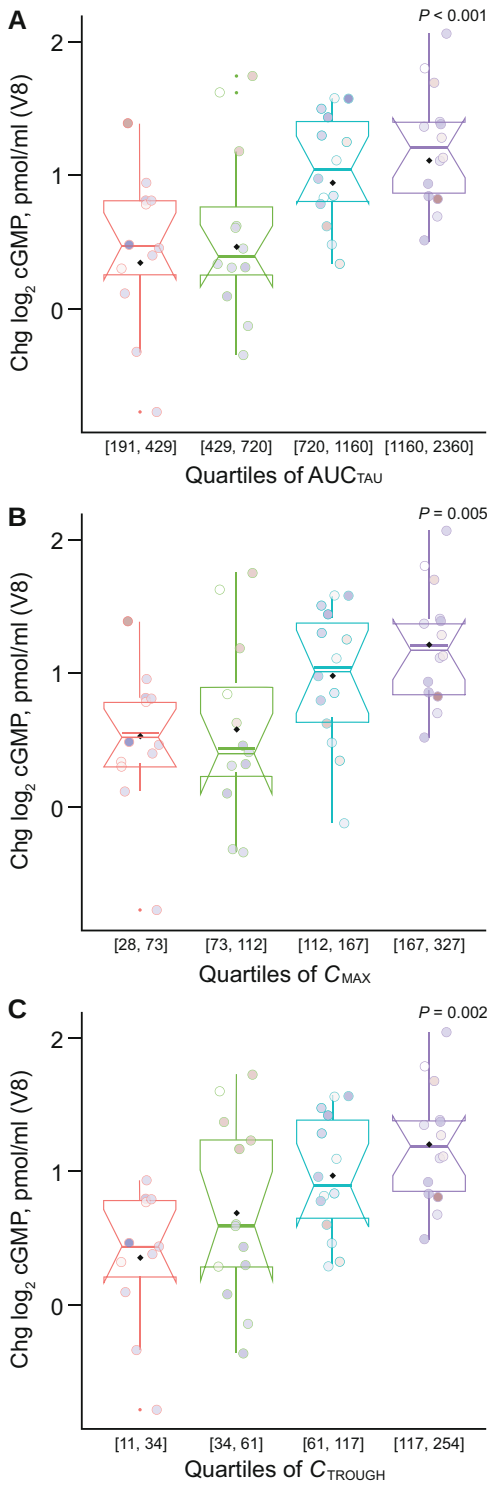

Figure 2. Changes in plasma cGMP to week 14 in riociguat arm by week 14 riociguat concentration quartiles. (A) AUC_{TAU}, (B) C_{MAX}, and (C) C_{TROUGH}. AUC_{TAU}: area under the plasma concentration–time curve. cGMP: cyclic guanosine monophosphate; Chg: change; C_{MAX}: maximum plasma concentration; C_{TROUGH}: trough plasma concentration; V8: study visit 8 (week 14). Circles show values for individual patients. Values in parentheses on *x*-axes are the upper and lower limits of each quartile. Shading of data points is for visualization only. *P*-values were generated by exploratory *t*-test and are for information only.

predicted, HAQ-DI, digital ulcer burden or (except for two assessments of sPECAM-1) Raynaud's disease assessments (see Supplementary Data S1, available at *Rheumatology* online, pp. 9–10). The baseline level of cGMP or its change from baseline to week 14 also had no clear relationship with these endpoints (correlations <0.3).

Discussion

This analysis examined several biomarkers in patients with early treatment-naïve dcSSc treated with riociguat or placebo in RISE-SSc [12]. Elevation of plasma cGMP with riociguat indicated engagement with the NO–sGC–cGMP pathway and correlated with pharmacokinetic variables of riociguat. By contrast, changes in ADMA, SDMA, p-ERK and p-VASP were similar between riociguat and placebo. This may be due to these molecules being further downstream than cGMP, and thus less direct measures of engagement [35].

In RISE-SSc, despite recruitment of a very early progressive SSc population, many patients had αSMA-negative skin biopsies (vascular and glandular tissue were excluded from the counts). Patients with negative αSMA counts have been observed previously, reflecting the heterogeneity of myofibroblast activation in SSc [33, 34]. In addition, extracellular matrix deposition in early SSc may be due to other cell–cell interactions such as endothelial-to-mesenchymal transition [36].

In our analysis, higher baseline serum sPECAM-1 levels were associated with an increase in mRSS from baseline to week 52 (Fig. 3). PECAM-1 is involved in the transmigration of leucocytes into tissues [26, 37]. Compared with controls, serum levels of sPECAM-1 are significantly elevated in patients with dcSSc or limited SSc (lSSc), and significantly more so in the latter [26]. Elevated serum sPECAM-1 was associated with lSSc of relatively early onset and with lower frequency and severity of pulmonary fibrosis, suggesting that sPECAM-1 elevation may protect against development of skin sclerosis and pulmonary fibrosis in SSc [26]. Studies in PECAM-1-deficient animal models suggest that PECAM-1 has a protective action [27], and transition of endothelial cells from patients with SSc toward a mesenchymal phenotype is associated with reduced PECAM-1 expression [38]. It is unclear why elevated serum sPECAM-1 was associated with progression of skin fibrosis in our study but it may reflect a compensatory response to disease activity. Also, serum measurements might not reflect intracellular levels or expression in specific tissues.

αSMA positivity at baseline was associated with a greater effect of riociguat on mRSS at week 52, the primary end point of RISE-SSc. Anti-Scl-70 or anti-RNA polymerase III antibodies are associated with poor outcomes in SSc [14, 39]. In subgroup analyses, the greater change in mRSS at week 52 with riociguat in patients with *vs* without αSMA-positive cells at baseline was only apparent in those who were also anti-RNA polymerase III- or anti-Scl-70-positive. Thus, the presence of anti-RNA polymerase III antibodies may have been the driver for the effect of riociguat in αSMA-positive patients. Small patient numbers (only one patient was anti-RNA polymerase III-positive and αSMA-negative) preclude further analysis. The current results should be viewed in terms of signal detection, and any subgroup results should be confirmed by further analyses. We were unsurprised to see a lack of association between change in αSMA-positive cells at week 14 and change in DL_{CO} % predicted or FVC % predicted given the known dissociation of skin and lung progression in dcSSc [40, 41]. However, our results indicate that the

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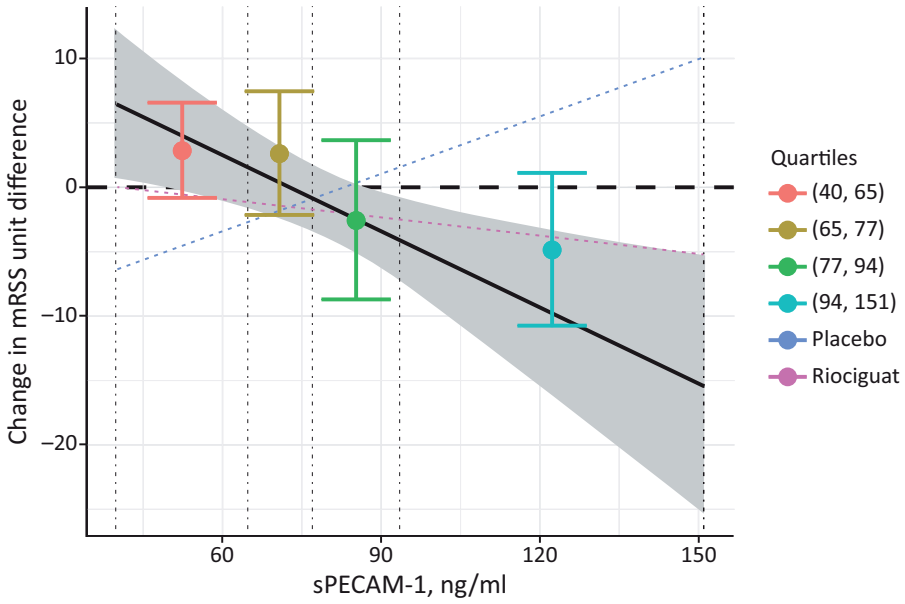

	Change in mRSS from baseline to Week 52		
	Placebo (n = 51)	Riociguat (n = 56)	Difference
sPECAM-1 concentration (ng/ml)			
40.00	−6.40 (−10.46, −1.94)	0.03 (−3.85, 3.91)	6.43 (0.74, 12.11)
76.60	−0.96 (−3.14, 1.22)	−1.71 (−3.77, 0.36)	−0.75 (−3.28, 1.78)
150.90	10.09 (1.63, 18.55)	−5.22 (−11.08, 0.64)	−15.31 (−25.23, −5.40)

Data are means (95% CI)
Interaction P-value 0.004

Figure 3. Relationship between baseline sPECAM-1 quartiles and treatment difference between riociguat and placebo (change in mRSS at week 52). Vertical dashed lines indicate quartiles of sPECAM concentration; grey shading indicates 95% CI of the linear regression line. Interaction P-value obtained from an analysis of covariance model adjusting for baseline mRSS and region, treatment, continuous sPECAM, and interaction between treatment and sPECAM. mRSS: modified Rodnan skin score; sPECAM: soluble platelet endothelial cell adhesion molecule

presence of αSMA in early SSc has value as a biomarker for progression of skin fibrosis.

CXCL-4 is elevated in SSc, correlating with the presence and progression of complications such as lung fibrosis and PAH, and was therefore evaluated as a marker of dcSSc progression [42]. CXCL-4 mediates fibrosis by transforming endothelial and stromal cells into myofibroblasts with excessive collagen production, whereas absence or blockade of CXCL-4 diminishes tissue fibrosis in numerous models [43]. Levels of CRP correlate with the severity of lung, skin and joint involvement in SSc, and increased levels are associated with shorter survival [44, 45]. In RISE-SSc, at week 14, treatment with riociguat was associated with a decrease in sPECAM-1 and CXCL-4, but not other biomarkers, including hsCRP, which increased. The explanation for the differing responses between biomarkers is unclear, but week 14 may have been too early to observe effects for the other biomarkers. The mechanism of the decrease in CXCL-4 with riociguat is unclear; inhibition of platelet activation appears unlikely since effects of riociguat on platelets have been seen only at concentrations far exceeding those seen in therapy [46]. CXCL-4 is present in platelet granules and is released upon platelet activation. In the current study, the CXCL-4 assay was performed on platelet-poor plasma (see Supplementary Data, available at *Rheumatology* online [p. 3] for details) to avoid overestimation of 'physiological' CXCL-4 levels due to CXCL-4 release from platelets. However, removal of platelets may have been incomplete, and therefore the CXCL-4 results reported here should be viewed with caution.

TGF-β is an important mediator of the fibrotic process in SSc; it promotes endothelial cell activation, differentiation toward mesenchymal cells, and the expression of mesenchymal markers such as αSMA [21, 36, 38]. Riociguat inhibits TGF-β1 signalling [5–7] and could potentially provide benefits in dcSSc by inhibiting endothelial-to-mesenchymal transition. This effect might be expected to be most marked in patients with active endothelial-to-mesenchymal cell transition, and in our study patients with αSMA-positive cells obtained the greatest benefit from riociguat. In the present study, riociguat had no effect on skin collagen markers as measured by fold-change or on αSMA staining, so may not be able to reverse more advanced fibrosis despite its potential effect on TGF-β signalling. There are many mediators of skin fibrosis in SSc and most of these were not significantly changed by riociguat compared with placebo. Injured endothelial cells in SSc produce low levels of NO and endothelial NO synthase [47]; stimulation of the NO–sGC–cGMP pathway by riociguat could therefore improve vascular function. Elevation of CXCL-4 also appears to play a role in peripheral vasculopathy in SSc [48]; reduction of this biomarker by riociguat could be another potential mechanism of benefit.

Several limitations of this study should be considered. Biomarkers were sampled at baseline and week 14; additional sampling would have been valuable, as would analysis of

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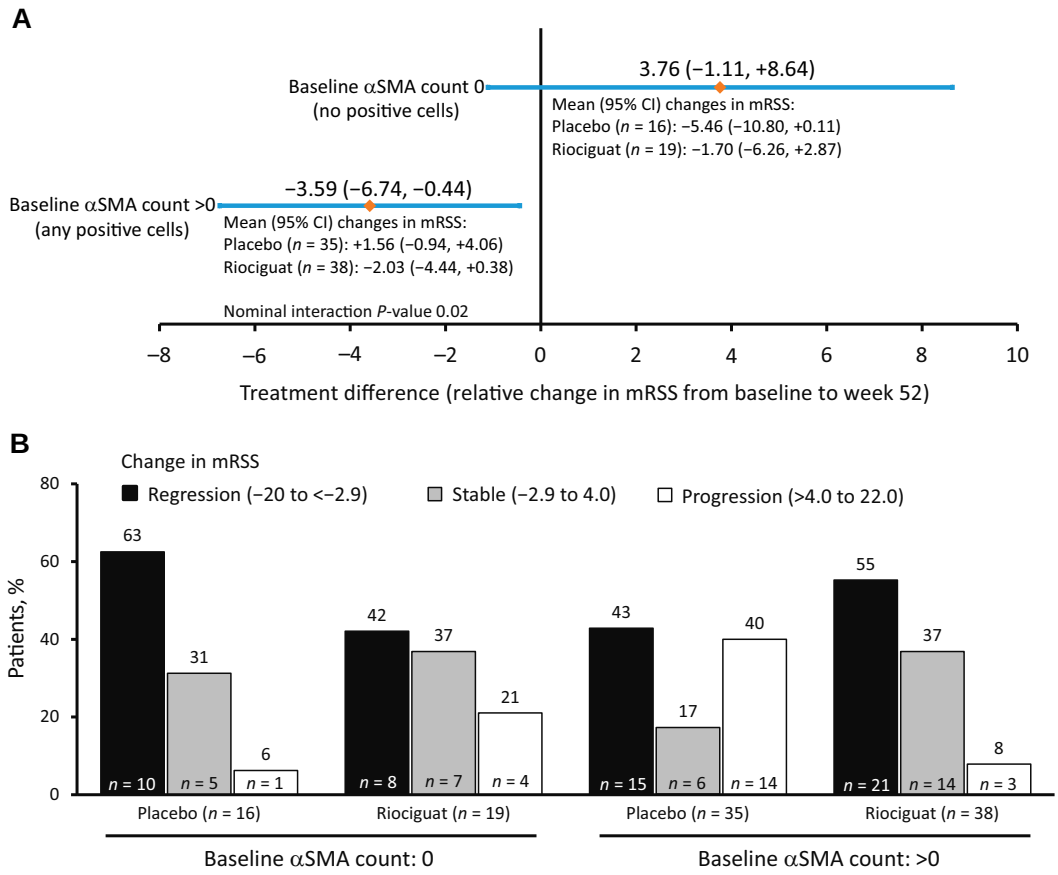

Figure 4. Treatment difference between placebo and riociguat (**A**) and changes in mRSS (**B**) by baseline αSMA status. Interaction *P*-value obtained from an analysis of covariance model adjusting for baseline mRSS and region, treatment, continuous αSMA, and interaction between treatment and αSMA. αSMA: α-smooth muscle actin; mRSS: modified Rodnan skin score

mRSS at other time points. Assessment of αSMA by two reviewers using a 0–100 visual analogue scale has been reported in blinded studies [33, 34], but this technique may have contributed to some of the variation observed. Imputing values for data below or above the limit of quantification may introduce systematic bias. Our analyses did not control for baseline disease severity. Another limitation is the lack of a larger effect of riociguat on mRSS in the main study, although the current results suggest that riociguat may influence mRSS progression in patients with rapidly progressive disease. While RISE-SSc was successful in part in selecting patients at greater risk of skin fibrosis progression [12], our results may reflect low disease activity in some patients. When considering the subgroups according to anti-Scl-70 and anti-RNA polymerase III status, it is important to bear in mind that there is a lack of standardization in clinical care, particularly with regard to Scl-70, but this consideration has limited reference to the current study, in which autoantibodies were assessed in a standardized laboratory. The results might not be generalizable to patients outside the study population (e.g. advanced dcSSc or lSSc).

Overall, αSMA positivity status was most consistently associated with clinical endpoints. The close association with anti-RNA polymerase III-positive status might drive the effects of riociguat in patients with an αSMA-positive cell count at baseline. A nominally significant decrease of CXCL-4 and sPECAM-1 from baseline to week 14 suggests anti-inflammatory properties of riociguat in patients with dcSSc and our findings suggest patients with increased circulating sPECAM-1 at baseline may have a greater response to riociguat. Further research is warranted to clarify the relative importance of biomarkers in dcSSc to aid clinical decision-making. The long-term open-label extension phase of the RISE-SSc trial has recently been reported [49].

Supplementary material

Supplementary material is available at *Rheumatology* online.

Data availability

Availability of the data underlying this publication will be determined according to Bayer's commitment to the European Federation of Pharmaceutical Industries and Associations and Pharmaceutical Research and Manufacturers of America principles for responsible clinical trial data sharing, pertaining to scope, time point and process of data access. Bayer commits to sharing upon request from qualified scientific and medical researchers, patient-level clinical trial data, study-level clinical trial data and protocols from clinical trials in patients for medicines and indications approved in the US and European Union as necessary for performing legitimate research. This commitment applies to data on new medicines and indications that have been approved by the European Union and US regulatory agencies on or after 1 January 2014. Interested researchers can use www.clinicalstudydatare

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quest.com to request access to anonymized patient-level data and supporting documents from clinical studies to perform further research that can help advance medical science or improve patient care. Information on the Bayer criteria for listing studies and other relevant information is provided in the study sponsors section of the portal. Data access will be granted to anonymized patient-level data, protocols and clinical study reports after approval by an independent scientific review panel. Bayer is not involved in the decisions made by the independent review panel. Bayer will take all necessary measures to ensure that patient privacy is safeguarded.

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