



Increased Expression of Growth Differentiation Factor-15 in Systemic Sclerosis-Associated Pulmonary Arterial Hypertension

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Background: Growth differentiation factor (GDF)-15 is a secreted member of the transforming growth factor- β cytokine superfamily. GDF-15 levels are elevated in the serum of patients with cardiovascular diseases. We hypothesized that GDF-15 levels would also be increased in the plasma and lung tissue of patients with systemic sclerosis-associated pulmonary arterial hypertension (SSc-PAH).

Methods: GDF-15 levels were measured in plasma in subjects with SSc-PAH ($n = 30$) and compared with subjects with systemic sclerosis (SSc) without pulmonary arterial hypertension (PAH) ($n = 24$). Patients with idiopathic PAH (IPAH) ($n = 44$) and normal individuals ($n = 13$) served as control subjects. Immunohistochemistry and immunofluorescence assay identified GDF-15 protein in lung tissue from patients with SSc-PAH and IPAH.

Results: Patients with SSc-PAH had significantly higher mean circulating levels of GDF-15 in plasma compared with patients with SSc without PAH (422.3 ± 369.5 pg/mL vs 108.1 ± 192.8 pg/mL, $P = .004$). GDF-15 levels correlated positively with estimated right ventricular systolic pressure on echocardiogram and plasma levels of the amino terminal propeptide form of brain natriuretic peptide. There was an inverse correlation between circulating GDF-15 and diffusing capacity of the lung for carbon monoxide (DLCO) and a positive correlation with the FVC to DLCO ratio on pulmonary function test. GDF-15 levels > 125 pg/mL were associated with reduced survival. GDF-15 protein expression was increased in lung tissue from patients with SSc-PAH.

Conclusions: GDF-15 may be a useful biomarker in PAH associated with SSc. Its presence in lung tissue may suggest a role in the pathology of the disease. *CHEST 2011; 139(5):994-1002*

Abbreviations: DLCO = diffusing capacity of the lung for carbon monoxide; ECHO = echocardiogram; ELISA = enzyme-linked immunosorbent assay; GDF = growth differentiation factor; IFA = immunofluorescence assay; IHC = immunohistochemistry; IPAH = idiopathic pulmonary arterial hypertension; mPAP = mean pulmonary artery pressure; NT-proBNP = amino terminal propeptide form of brain natriuretic peptide; PAH = pulmonary arterial hypertension; RHC = right-sided heart catheterization; RIA = radioimmunoassay; ROC = receiver operating characteristic; RVSP = right ventricular systolic pressure; SSc = systemic sclerosis; SSc-PAH = systemic sclerosis-associated pulmonary arterial hypertension; TR = tricuspid regurgitant

Growth differentiation factor (GDF)-15, a member of the transforming growth factor- β superfamily of cytokines, plays an important role in cell growth and differentiation. GDF-15 is normally expressed at high levels in the placenta¹⁻³ and at lower levels in the prostate, pancreas, and kidney.¹ Secreted GDF-15 functions in cell-to-cell signaling,⁴ signal transduction,^{5,6} and apoptosis regulation⁷⁻⁹ in a cell-type-specific manner.

Cardiac stress and tissue damage caused by acute coronary syndrome and chronic left-sided heart fail-

ure induce GDF-15 expression in heart tissue.^{10,11} Arteriosclerotic lesions of human carotid arteries express GDF-15, and this expression colocalizes to macrophages but not to smooth muscle or endothelial cells in atherosclerotic plaques.⁹ GDF-15 appears to have a protective effect on cultured cardiomyocytes^{10,11} and in murine models of ischemia and reperfusion injury.¹⁰ GDF-15 was identified as an independent predictor of long-term mortality in acute pulmonary embolism¹² and idiopathic pulmonary arterial

hypertension (IPAH)¹³ and was proposed as a potential biomarker in these diseases.^{12,13}

Pulmonary arterial hypertension (PAH) is associated with abnormal cellular proliferation in the precapillary pulmonary vasculature. Inflammation is believed to be a major pathogenic component in pulmonary vascular remodeling, with macrophages, lymphocytes, and dendritic cells identified in PAH vascular lesions.¹⁴

Approximately 10% to 16% of patients with systemic sclerosis (SSc) develop PAH,¹⁵ and nearly 30% of scleroderma-related deaths can be attributed to PAH.¹⁶ Patients with SSc-associated PAH (SSc-PAH) are less responsive to therapy¹⁷ and are believed to have a worse overall prognosis.¹⁸ Patients with SSc would benefit from biomarkers assessing risk for developing PAH and for disease management. We hypothesized that patients with SSc-PAH would have elevated serum levels of GDF-15 protein compared with patients with SSc without PAH and that there would be an increased expression of GDF-15 protein in the lung tissue of patients with SSc-PAH.

MATERIALS AND METHODS

Subject Recruitment

The Institutional Review Board of the University of Colorado approved this study (COMIRB #06-0247). Written informed consent was obtained from subjects recruited from the Pulmonary Hypertension and Scleroderma clinics at the University of Colorado Denver. One hundred fifty-one subjects were screened; 40 were excluded after failing to meet entry criteria. PAH was confirmed by a resting mean pulmonary artery pressure (mPAP) > 25 mm Hg and a pulmonary capillary wedge pressure < 15 mm Hg by right-sided heart catheterization (RHC). Subjects with SSc without PAH were excluded if they had an estimated right ventricular systolic pressure (RVSP) > 35 mm Hg by echocardiography (ECHO) unless, when follow-up RHC data were available, the RHC results showed an mPAP < 25 mm Hg. Subjects with SSc without PAH were also excluded for abnormal second heart sound, evidence of tricuspid regurgitant (TR) murmur on physical examination, or diffusing capacity of the lung for carbon monoxide (DLCO) < 80% after correction for alveolar volume.¹⁹ SSc diagno-

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sis was determined by American College of Rheumatology criteria.²⁰ Normal subjects were self-identified as having neither PAH nor connective tissue disease. Lung tissues were autopsy samples obtained through a tissue bank maintained at the University of Colorado. Tissue sections were examined and characterized by a pathologist blinded to this study.

Subject Baseline Characteristics

The RHC, ECHO, and pulmonary function tests done closest to the blood draw were used. Other laboratory values were obtained within 1 month of the blood draw, and usually on the same day. Survival information was determined by review of medical records and was confirmed by review of the Social Security death index.

Echocardiography

The majority (76%) of ECHOs were performed at the University of Colorado Hospital using M-mode and two-dimensional ECHO with a Philips iE33 ultrasound machine (Philips Healthcare; Andover, Massachusetts). Continuous-wave Doppler signals were recorded with a Philips transducer (Philips Healthcare). RVSP was calculated from peak signal velocity from the TR signal using the modified Bernoulli equation. Right atrial pressure was estimated by evaluating the size and collapsibility of the inferior vena cava and was added to the calculated TR jet velocity to obtain the estimated pulmonary artery systolic pressure. The mean time from ECHO to the blood draw for this study in subjects with SSc-PAH was 91.7 days (\pm 41.9 SEM).

Right-Sided Heart Catheterization

The majority (84%) of RHCs were performed at the University of Colorado Hospital, using standard techniques. The right internal jugular vein or right femoral vein was used for venous access in all cases. Resting hemodynamics were measured in the supine position. The mean time from RHC to the blood draw for this study in subjects with SSc-PAH was 574.2 days (\pm 111.6 SEM).

Blood Collection

Peripheral whole blood was collected using a Vacutainer cell preparation tube containing sodium citrate (Becton, Dickinson and Co;

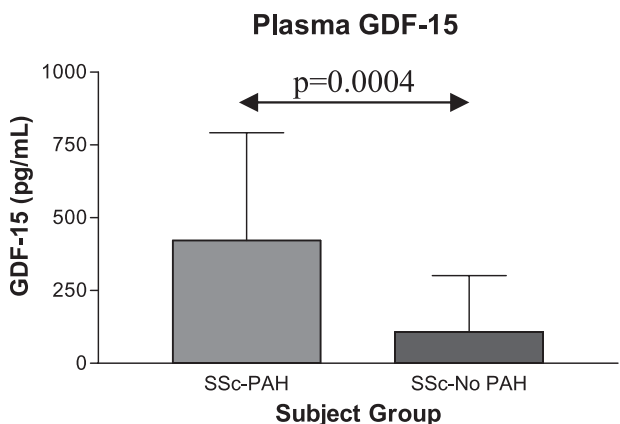


FIGURE 1. GDF-15 expression in plasma by enzyme-linked immunosorbent assay (ELISA). Plasma levels of GDF-15 are significantly higher in subjects with SSc-PAH (422.3 ± 369.5 pg/mL, $n = 30$) than in those with SSc-no PAH (108.1 ± 192.8 pg/mL, $n = 24$). GDF = growth differentiation factor; SSc-PAH = systemic sclerosis-associated pulmonary arterial hypertension; SSc-no PAH = systemic sclerosis without pulmonary arterial hypertension.

Table 1—Demographic and Clinical Baseline Characteristics of Subjects With SSc With and Without PAH

	SSc-PAH (n = 30)	SSc-No PAH (n = 24)	P Value
Age, y	61.0 ± 11.0	52.0 ± 9.7	.0029
Female sex	28 (93.3)	23 (95.8)	.6969
White race	26 (86.7)	18 (75.0)	.1113
Type 2 diabetes	3 (10.0)	1 (4.2)	.4256
AST, ^a U/L	25.3 ± 8.7 (16)	24.5 ± 9.0 (15)	.8226
Total bilirubin, ^a mg/dL	0.6 ± 0.2 (16)	0.7 ± 0.3 (15)	.0545
Creatinine, ^a mg/dL	1.1 ± 0.5 (19)	0.9 ± 0.1 (14)	.1246
Hemoglobin, ^a g/dL	13.1 ± 1.6 (17)	14.4 ± 1.0 (15)	.0083
NT-proBNP, ^a pmol/L	1130.0 ± 759.1 (29)	702.4 ± 346.0 (23)	.0158
GDF-15, pg/mL	422.3 ± 369.5	108.1 ± 192.8	.0004

Data are presented as No. (%) or mean ± SD, unless indicated otherwise. AST = aspartate transaminase; GDF = growth differentiation factor; NT-proBNP = amino terminal propeptide form of brain natriuretic peptide; PAH = pulmonary arterial hypertension; SSc = systemic sclerosis; SSc-PAH = systemic sclerosis-associated pulmonary arterial hypertension; SSc-no PAH = systemic sclerosis without pulmonary arterial hypertension. ^aValues were not available for all subjects. Data are presented as mean ± SD (number available).

Franklin Lakes, New Jersey). RBCs were removed by centrifugation at 1,800g in an Eppendorf 5810R centrifuge (Westbury, New York). Plasma was collected and stored at -80°C.

GDF-15 Enzyme-Linked Immunosorbent Assay and Brain Natriuretic Peptide Fragment Enzyme Immunoassay

The GDF-15 Duoset enzyme-linked immunosorbent assay (ELISA) Development kit (R&D Systems; Rochester, Minnesota)

was used to determine plasma levels of GDF-15, following R&D's protocol. The BNP Fragment enzyme immunoassay (Biomedica; Alpco Diagnostics; Salem, New Hampshire) was used to detect plasma levels of the amino terminal propeptide form of brain natriuretic peptide (NT-proBNP), following Biomedica's protocol. Plates were read on a VersaMax microplate reader (Molecular Devices; Sunnyvale, California) at 450 nm, with wavelength correction of 540 nm for GDF-15 and 620 nm for NT-proBNP. Results were analyzed using SoftMax Pro v3.1.2 analysis software (MDS Analytical Technologies; Sunnyvale, California).

Immunohistochemistry

Slides of formalin-fixed, paraffin-embedded tissue sections were obtained from a tissue bank maintained at the University of Colorado and were identified by a pathologist as SSc-PAH, SSc without PAH, IPAH, and controls. These tissues were from a cohort separate from the subjects whose serum was used in the ELISA. Antigen retrieval was via the heat-induced epitope retrieval method in pH = 6.0 citrate buffer (Vector Laboratories; Burlingame, California). Rabbit polyclonal α-GDF-15 (Prestige Antibodies, Sigma-Aldrich Co; St. Louis, Missouri) or normal rabbit IgG isotype (Vector Laboratories), and horseradish peroxidase-conjugated goat α-rabbit IgG (Vector Laboratories) were used. Slides were developed with diaminobenzidine (Dako North America, Inc; Carpinteria, California) and counterstained in hematoxylin (Dako North America, Inc).

Immunofluorescence

Slides were blocked with 3% hydrogen peroxide (Sigma-Aldrich Co) and incubated successively with avidin and biotin (Vector Laboratories). The same antibodies for GDF-15 and isotype control used in the immunohistochemistry (IHC) were used in the immunofluorescence assay (IFA). Biotinylated goat α-rabbit IgG antibody was applied, followed by incubation in ready-to-use

Table 2—Baseline and Clinical Comparisons of Subjects With SSc-PAH and Idiopathic PAH

Feature	SSc-PAH (n = 30)	IPAH (n = 44)	P Value
Age, y	61.0 ± 11.0	51.0 ± 13.6	.0013
ECHO			
Estimated RVSP, ^a mm Hg	66.4 ± 25.1	82.3 ± 28.3 (41)	.0164
Right-sided catheterization			
Mean PAP, mm Hg	43.4 ± 15.1	52.7 ± 14.0	.0085
Mean RAP, ^a mm Hg	5.7 ± 5.7 (28)	6.8 ± 4.1 (43)	.3305
Mean PCWP, mm Hg	7.2 ± 3.7	8.3 ± 5.0	.3228
PVR, Wood units	9.3 ± 5.5	12.5 ± 7.6	.0471
Cardiac output, ^a L/min	4.6 ± 1.8 (29)	4.2 ± 1.7	.3606
Pulmonary function test ^{a,b}			
FVC	71.6 ± 13.7 (30)	83.6 ± 19.0 (28)	.0075
Adjusted DLCO ^{a,b}	53.7 ± 24.7 (23)	81.3 ± 17.4 (27)	<.0001
NT-proBNP, ^a pmol/L	1130.0 ± 759.1 (29)	663.4 ± 507.0 (40)	.0003
GDF-15, pg/mL	422.3 ± 369.5	173.0 ± 193.0	.0004
Medications ^c			
Prostanoids	5 (16.7)	19 (43.2)	...
Endothelin receptor antagonist	19 (63.3)	17 (38.6)	...
Phosphodiesterase inhibitor	7 (23.3)	18 (40.9)	...
Calcium channel blocker	16 (53.3)	17 (38.6)	...

Data are presented as No. (%) or mean ± SD, unless indicated otherwise. DLCO = diffusing capacity of the lung for carbon monoxide; ECHO = echocardiogram; IPAH = idiopathic pulmonary arterial hypertension; PAP = pulmonary artery pressure; PCWP = pulmonary capillary wedge pressure; PVR = pulmonary vascular resistance; RAP = right atrial pressure; RVSP = right ventricular systolic pressure. See Table 1 for expansion of other abbreviations.

^aValues were not available for all subjects. Values are then given as mean ± SD (number available).

^bData expressed as % predicted values adjusted for age, sex, and race.

^cMost subjects are on multiple therapies; thus, the total exceeds 100%.

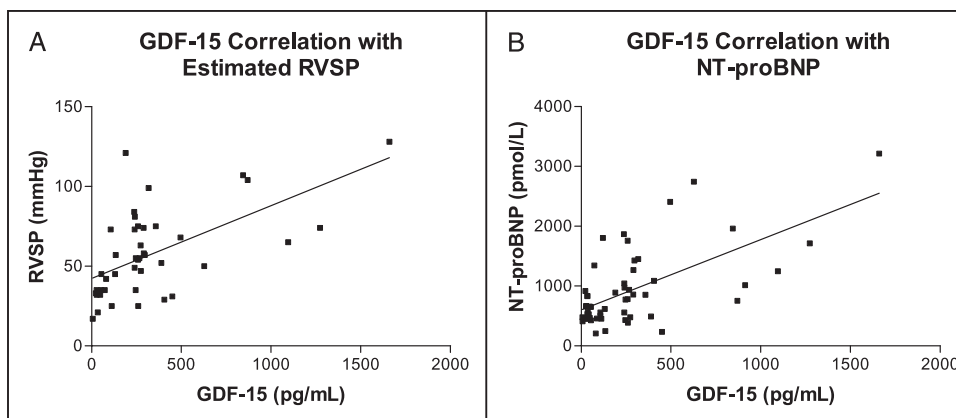


FIGURE 2. Echocardiogram and NT-proBNP correlations with GDF-15. A, A significant positive correlation exists between GDF-15 and estimated RVSP on echocardiogram (Spearman $r = 0.5557$, $P = .0001$) in subjects with SSc (systemic sclerosis) ($n = 43$). B, There is a significant correlation between GDF-15 and NT-proBNP plasma levels (Spearman $r = 0.4843$, $P = .0003$) in subjects with SSc ($n = 52$). NT-proBNP = amino terminal propeptide form of brain natriuretic peptide; RVSP = right ventricular systolic pressure. See Figure 1 legend for expansion of other abbreviations.

streptavidin-horseradish peroxidase (both from Vector Laboratories). Tyramide signal amplification reagent (Perkin-Elmer; Waltham, Massachusetts) was applied, followed by streptavidin-Texas Red conjugate (Invitrogen; Carlsbad, California). Slides were then incubated with α -CD68 mouse monoclonal (Dako North America, Inc) and Alexa Fluor 488-conjugated α -mouse (Invitrogen) antibodies. Analysis for IHC and IFA was performed using an Eclipse E800 microscope (Nikon Inc; Melville, New York) with a Photometrics Coolsnap HQ CCD camera (Roper Scientific; Tucson, Arizona) and NIS Elements AR 3.00 software (Nikon Inc).

Statistical Analysis

Data are presented as absolute numbers, percentages, and mean \pm SD. GDF-15 comparisons were performed by Student t test. Relations between GDF-15 and clinical variables were assessed by Spearman rank correlation coefficients. For comparison of the prognostic values of GDF-15 and NT-proBNP, we generated receiver operating characteristic (ROC) curves, the areas

under the curves (C statistic) were calculated, and clinically relevant threshold levels were chosen to optimize sensitivity and specificity. Levels of GDF-15 and NT-proBNP identified in the ROC curves were used to examine survival using Kaplan-Meier plots. Univariate and multivariate logistic regression models were used to determine the ability of GDF-15 treated as a continuous variable to classify PAH with the a priori selected covariants, age (years), serum hemoglobin (g/dL), and serum creatinine (mg/dL). A P value $< .05$ was considered statistically significant. Calculations were performed with JMP, version 7 (SAS Institute Inc; Cary, North Carolina) and GraphPad Prism, version 4.03 for Windows (GraphPad Software; San Diego, California).

RESULTS

GDF-15 levels were elevated in patients with SSc-PAH compared with patients with SSc without PAH (Fig 1), with patients with IPA

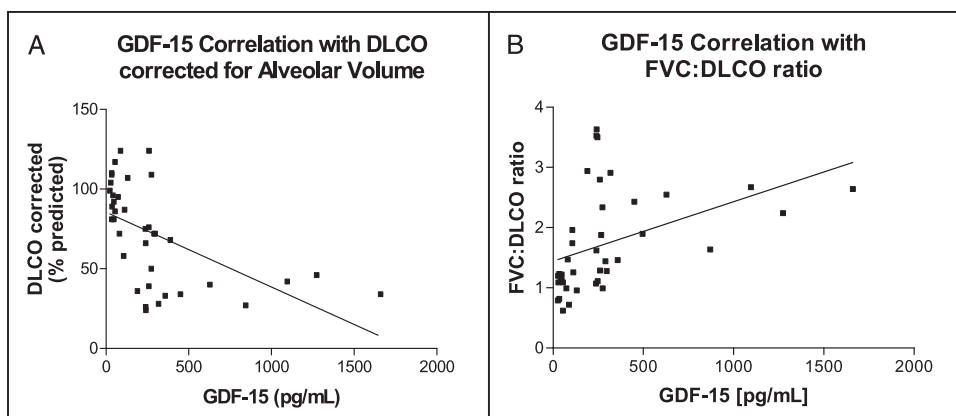


FIGURE 3. DLCO correlations with GDF-15. A, There is a significant negative correlation between plasma levels of GDF-15 and DLCO (% predicted value, corrected for alveolar volume) in subjects with SSc (Spearman $r = -0.6510$, $P < .0001$, $n = 38$). B, The FVC to DLCO ratio has a significant positive correlation with plasma GDF-15 levels in subjects with SSc (Spearman $r = 0.5967$, $P < .0001$, $n = 40$). DLCO = diffusing capacity of the lung for carbon monoxide. See Figure 1 and 2 legends for expansion of other abbreviations.

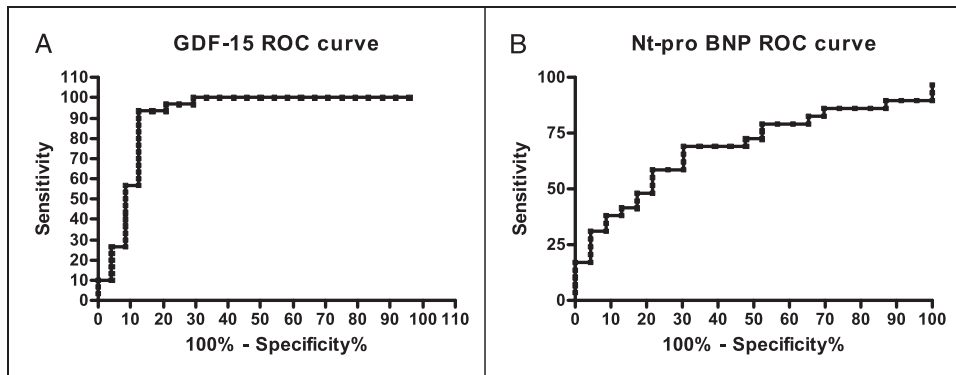


FIGURE 4. ROC curves were generated to determine clinically relevant plasma levels of GDF-15 and NT-proBNP, optimizing sensitivity and specificity. A, An ROC curve for GDF-15 (area under the curve [AUC] = 0.91, $P < .0001$). B, An ROC curve for NT-proBNP (AUC = 0.69, $P = .023$). ROC = receiver operating characteristic. See Figure 1 and 2 legends for expansion of other abbreviations.

(173.0 ± 193.0 pg/mL, $P = .0003$), and with control subjects (66.1 ± 46.3 pg/mL, $P = .0013$). Subjects with SSc were compared with respect to age, sex, race/ethnicity, and clinical laboratory values (Table 1). Pulmonary and hemodynamic features and PAH-specific medications were compared between subjects with SSc-PAH and subjects with IPAH (Table 2). Subjects with IPAH had more severe PAH than those with SSc-PAH in terms of hemodynamics (Table 2). Significant differences existed between subjects with PAH in FVC, DLCO, plasma NT-proBNP level, and age (Table 2). We found a significant positive correlation between GDF-15 levels and estimated RVSP on ECHO in subjects with SSc (Fig 2A), but GDF-15 level did not correlate with other invasively measured cardiac hemodynamics.

Plasma NT-proBNP levels correlated significantly with levels of GDF-15 in subjects with SSc (Fig 2B). NT-proBNP levels were significantly higher in patients

with SSC-PAH than in those with SSc without PAH (Table 1) and in those with IPAH (Table 2).

There was a significant negative correlation between GDF-15 plasma levels and DLCO in subjects with SSc (Fig 3A). A significant positive correlation existed between GDF-15 plasma levels and the FVC to DLCO ratio in subjects with SSc (Fig 3B).

In subjects with SSc, an ROC curve was generated for the diagnosis of PAH using GDF-15 plasma values. The area under the curve was 0.91, with a P value of $< .0001$ (Fig 4A). A diagnostically relevant value for GDF-15 of 125 pg/mL was derived from the ROC curve, with 93% sensitivity and 88% specificity for the diagnosis of PAH. A Kaplan-Meier survival curve using 125 pg/mL as a cutoff showed survival probabilities of 86.3%, 67.7%, and 58.1% after 1, 2, and 3 years of follow-up, respectively, whereas those with a plasma level < 125 pg/mL were all alive at follow-up (Fig 5A). Mean time to follow-up was 30.0 ± 9.3 months, with

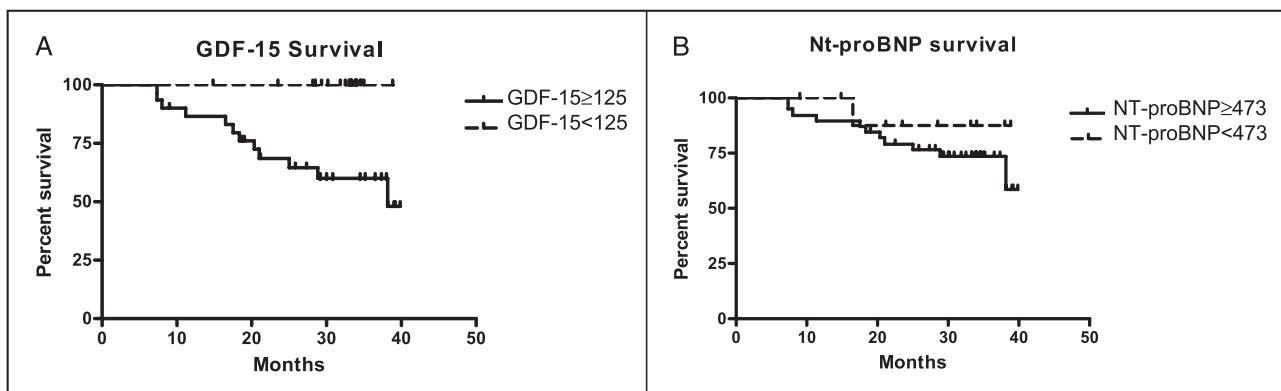


FIGURE 5. A, A Kaplan-Meier survival curve for dichotomous level of GDF-15 (125 pg/mL). B, A Kaplan-Meier survival curve for the dichotomous level of NT-proBNP (473 pmol/L) in subjects with SSc. Elevation of GDF-15 plasma levels above 125 pg/mL was associated with increased mortality in subjects with SSc ($n = 54$, $P = .0021$). There was no statistically significant difference in mortality between patients with NT-proBNP plasma levels above 473 pmol/L ($n = 52$, $P = .3813$). See Figure 1 and 2 legends for expansion of abbreviations.

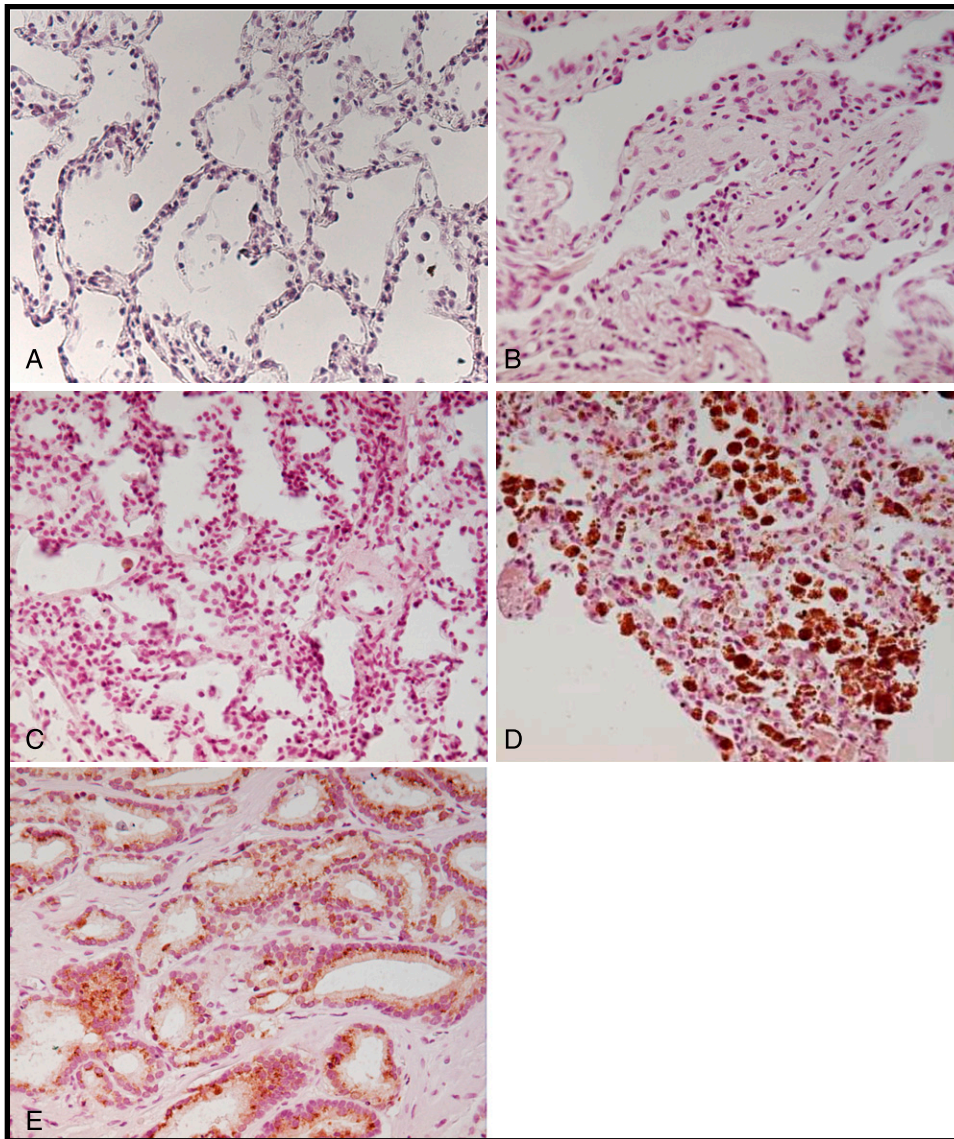


FIGURE 6. Immunohistochemistry for GDF-15 in lung tissue stained with anti-GDF-15 antibody. A, Normal lung tissue. B, SSc-no PAH. C, Idiopathic pulmonary arterial hypertension. D, SSc-PAH. E, Prostate cancer tissue (positive control). (A-E, original magnification $\times 20$). See Figure 1 legend for expansion of the abbreviations.

a maximum follow-up of 39.9 months and a minimum of 7.3 months.

A ROC curve was generated for NT-proBNP with an area under the curve of 0.69 and a P value of .0023 (Fig 4B). A diagnostically relevant value for NT-proBNP of 473 pmol/L was derived from the ROC curve, with 86% sensitivity and 30% specificity. No statistically significant difference existed in survival between the SSc groups using the 473 pmol/L value by Kaplan-Meier (Fig 5B).

The relation of GDF-15 plasma levels to the diagnosis of SSc-PAH was statistically significant in a univariate logistic model for odds of PAH when GDF-15 was treated as a continuous variable (OR, 1.09; 95% CI, 1.03-1.15; $P = .002$). The OR of 1.09 was

expressed per 10-U increase in GDF-15 levels, meaning a 9% increase in the odds of PAH for every 10-U increase in GDF-15. Adjusting for covariants of age, serum hemoglobin, and serum creatinine reduced the odds of PAH to 3% for every 10-U increase in GDF-15 and was not statistically significant (OR, 1.03; 95% CI, 0.96-1.10; $P = .4$).

GDF-15 protein levels were increased in the lung tissue of patients with SSc-PAH compared with patients with SSc without PAH or with IPAH by IHC (Fig 6). Lung tissue from patients with IPAH had significantly fewer cells stain positive for GDF-15. Normal lung tissue samples did not express GDF-15. IFA demonstrated colocalization of GDF-15-positive cells with a monocyte/macrophage marker, CD68 (Fig 7).

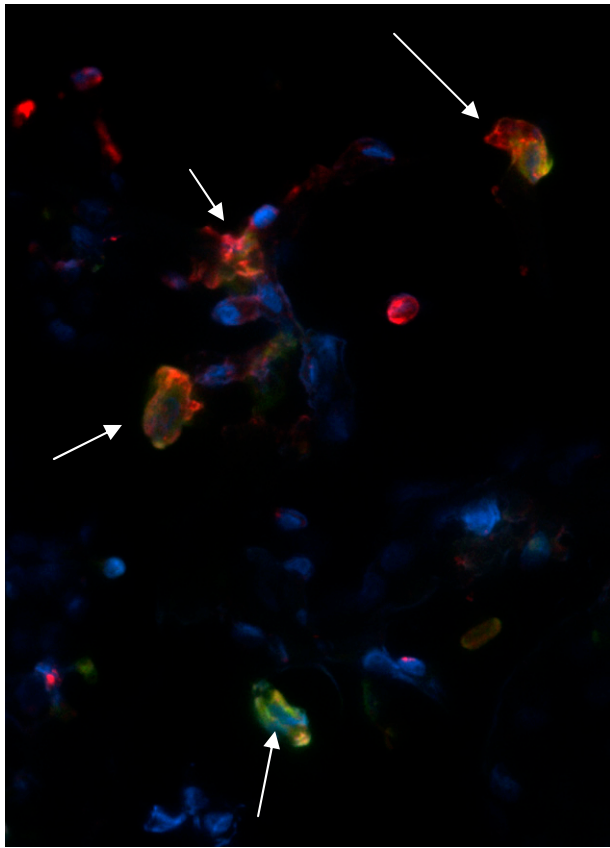


FIGURE 7. Immunofluorescence staining for GDF-15 and CD68 in lung tissue. Lung tissue from a subject with SSc-PAH was stained with both GDF-15 (red) and CD68 (green), a monocyte/macrophage marker, to demonstrate colocalization (arrows). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (blue). See Figure 1 legend for expansion of abbreviations.

DISCUSSION

PAH is a common complication of SSc and is associated with poor prognosis.²¹ SSc-PAH is generally diagnosed at a late stage of disease, when treatment may be less effective.^{17,21,22} There is a need for non-invasive biomarkers to help identify and risk stratify this group. Nickel et al¹³ previously reported elevation of plasma GDF-15 in subjects with IPAH by radioimmunoassay (RIA). We confirmed elevated plasma concentrations of GDF-15 in our PAH population using a simple ELISA. Notably, GDF-15 concentrations were significantly higher in subjects with SSc-PAH than in subjects with IPAH, even though the IPAH group appeared to have more severe disease based on hemodynamics.

Plasma GDF-15 levels correlated well with other indicators of PAH in subjects with SSc, including a significant positive correlation with estimated RVSP on ECHO, plasma levels of NT-proBNP, and FVC to DLCO ratio, and a significant inverse correlation with DLCO.²³ NT-proBNP has been proposed as a potential biomarker for PAH in patients with SSc.²⁴ We

found the plasma levels of GDF-15 were better able to identify SSc-PAH and predicted mortality with greater reliability than NT-proBNP.

We did not find significant correlations between GDF-15 and invasively measured hemodynamic parameters such as mPAP and cardiac output. We hypothesized that this was due to the length of time between RHC and the blood draw in this study. Blood samples were drawn during clinic visits, and a significant time lag existed between the blood draw and the most recent RHC in many cases.

Several weaknesses are associated with this study. The SSc-PAH population was older than the population with SSc but no PAH (mean 61.0 ± 11.0 years vs 52.0 ± 9.7 years, $P = .0029$) and the IPAH cohort (51.0 ± 13.6 years, $P = .0013$). Therefore, age could have influenced our findings. We note, however, that this discrepancy mirrors the age difference found between SSc populations with and without PAH and may be a difficult confounder to avoid. Another weakness was the time difference between RHC and measurement of GDF-15 levels. However, ECHO results were much closer to the blood draw, and estimated RVSP correlated significantly with GDF-15 levels in plasma. Few patients diagnosed with SSc without PAH underwent RHC to definitively confirm the absence of PAH. However, we carefully excluded PAH in this cohort by setting stringent requirements for ECHO, DLCO, and physical examination. Furthermore, we followed this patient population prospectively for a mean of 2.8 years, confirming the lack of clinical evidence of PAH. SSc-PAH is a progressive disease and we feel confident that our SSc without PAH cohort were, in fact, true PAH-negative at the time of the blood draw. Almost all patients were on multiple PAH treatments (Table 2). Although we cannot exclude treatment as a possible cause of differences in plasma GDF-15, no individual treatment correlated with GDF-15 levels, including prostacyclins ($P = .31$). GDF-15 measurements were taken at one specific point in time. Future studies could examine GDF-15 levels over multiple time points, or by assessing changes with therapy. We did not exclude subjects with interstitial lung disease in this study, which could have been a potential confounder. Finally, Mathai et al²⁵ recently reported that levels of NT-proBNP predicted survival in subjects with SSc-PAH, but not in those with IPAH. We did not find the same predictive strength for NT-proBNP in our study, possibly because of the larger number of subjects in the Mathai study.

We demonstrated that GDF-15 can be detected in plasma using a commercial ELISA kit, eliminating the requirement for the more technically difficult RIA. Although levels found in the ELISA were significantly lower overall than those found by RIA, the

decrease was proportional in all subject groups and we were able to confirm previous correlations made with RIA results.^{13,26,27} We demonstrated, to our knowledge for the first time, that circulating GDF-15 levels are significantly higher in patients with SSc-PAH than in patients with SSc without PAH.

By IHC, we showed that GDF-15 is detectable in lung tissue from patients with SSc and demonstrated colocalization of GDF-15 with a macrophage marker by IFA. To our knowledge, this is the first time that evidence of GDF-15 protein has been found in lung tissue.

Elevated GDF-15 levels can be found in a variety of tissues when exposed to stress or injury. Inflammatory cells in the lungs of patients with PAH and SSc-PAH could secrete GDF-15, causing an antiapoptotic phenotype and contributing to the proliferation of cells in the pulmonary vasculature characteristic of PAH.

CONCLUSIONS

We propose that circulating GDF-15 levels could serve as a valuable biomarker in patients with SSc-PAH. GDF-15 could be useful in risk stratification and might serve to predict disease in patients with SSc in whom PAH is clinically unrecognized, especially in combination with changes in ECHO or DLCO, or combined with NT-proBNP levels. However, the application of GDF-15 in earlier diagnosis of PAH in SSc will require further investigation.

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Author Contributions: Ms Meadows and Dr Bull had full access to all the data and take responsibility for the integrity of the data and the accuracy of the data analysis.

Ms Meadows: contributed to the conception and design of the study, acquisition of the data, analysis and interpretation of the data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, and statistical analysis.

Dr Risbano: contributed to research subject consent, analysis and interpretation of the data, statistical analysis, and drafting of the manuscript.

Ms Zhang: contributed to technical assistance, analysis and interpretation of the data, and critical revision of the manuscript for important intellectual content.

Dr Geraci: contributed to the conception and design of the study; critical revision of the manuscript for important intellectual content; and administrative, technical, or material support.

Dr Tudor: contributed to the analysis and interpretation of the data; critical revision of the manuscript for important intellectual content; and administrative, technical, or material support.

Dr Collier: contributed to the conception and design of the study, research subject consent, and critical revision of the manuscript for important intellectual content.

Dr Bull: contributed to the conception and design of the study; analysis and interpretation of the data; research subject consent; writing of the manuscript; statistical analysis; obtaining of funding; administrative, technical, or material support; supervision of the study; and approval of the final manuscript.

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