Supplemental Methods

Patients

Consecutive patients who were referred to the Northwestern Scleroderma Program (Northwestern University, Chicago, IL) from 2005–2015 and met American College of Rheumatology criteria for systemic sclerosis were enrolled in the Northwestern Scleroderma Patient Registry and Biorepository. All patients met the Leroy classification criteria for SSc (1). Patients who met American College of Rheumatology criteria for rheumatoid arthritis, inflammatory myopathies, systemic lupus erythematosus, scleroderma mimics (nephrogenic systemic fibrosis, eosinophilia-myalgia sydrome, paraneoplastic syndromes, scleredema, scleromyxedema) and localized scleroderma were excluded. Study of patients in the Northwestern Scleroderma Program was approved by the Northwestern University Institutional Review Board, and all patients provided written informed consent in accordance with the Declaration of Geneva.

198 patients were included in the adipokine biomarker study. These consisted of 116 lcSSc patients and 82 dcSSc patients. The differences seen between the lcSSc and dcSSc groups were largely expected with dcSSc patients having shorter disease duration (3.6 vs 13.2 years), more RNA polymerase III (43% vs 11%) and less anti-centromere antibodies (5% vs 31%) , higher burden of skin disease (MRSS 16.4 vs 4.7), lower FVC (75% vs 83% predicted), were more likely to be on immunomodulatory medication (60% vs 21%), and were less likely to have pulmonary arterial hypertension (4% vs 19%). In addition, dcSSc patients were less female (77% vs 88%).

Control individuals

33 healthy controls were included in the study. These individuals did not have a personal or family history of autoimmunity and were matched for age and BMI, but were more male (43% male vs 16% male in SSc, $p = 0.002$) than were SSc patients.

Clinical evaluation

All SSc patients were evaluated by one of three rheumatologists upon initial intake into the Northwestern Scleroderma Program. A detailed, standardized medical history was taken to evaluate SSc disease duration, defined as time between the onset of the first non-Raynaud symptom of SSc and the initial clinic visit, and disease complications. All patients underwent complete physical examination including documentation of age, gender, ethnicity, body mass index (BMI), disease duration (interval between first SSc-related non-Raynaud event and blood sampling), modified Rodnan skin score (MRSS) (0 to 51). Anti-nuclear autoantibodies were detected by indirect immunofluorescence, and antibodies against topoisomerase-1, centromere, and RNA polymerase III were assayed. Serum for research was collected for this study and was collected at enrollment in the registry and stored at -80C until assayed. The study sample consisted of 198 patients with SSc sub-classified based on criteria proposed by Leroy et al, all patients fulfilled the 2013 ACR/EULAR classification criteria(2).

Pulmonary function tests (PFT) and high-resolution computed tomography of the chest (HRCT) were performed at the initial study visit and repeated as clinically indicated. Interstitial lung disease was defined as the presence of any ground glass opacities, honeycombing, or fibrosis on high resolution computed tomography of the chest (3) and a forced vital capacity < 70% of predicted on pulmonary function testing.

All patients were screened for pulmonary hypertension with doppler echocardiography and pulmonary function testing. Any patient with unexplained dyspnea, signs and symptoms of right-sided heart failure, elevated PASP on echocardiography, or isolated decrease in diffusing capacity of carbon monoxide (DLCO) was referred for further evaluation by a pulmonary hypertension specialist, and cardiac catheterization was performed to determine if PAH was present. PAH was recorded as present if the patient carried a diagnosis of PAH at baseline (based on prior testing) or if PAH was diagnosed during initial clinical evaluation. The diagnosis of PAH required invasively documented mean pulmonary artery pressure > 25 mmHg, pulmonary capillary wedge pressure < 15 mmHg, and pulmonary vascular resistance > 3 Wood units (4).

Subjects underwent echocardiography as part of their initial clinical evaluation (within 6 months of initial clinic visit). Echocardiograms were performed by sonographers blinded to all clinical and laboratory data. Patients underwent comprehensive two-dimensional echocardiography with

Doppler and tissue Doppler imaging according to published guidelines(5, 6) using a Philips ie33 or Sonos 7500 echocardiographic machine. For the purposes of this study, a single experienced echocardiographer who was blinded to clinical and laboratory data, made quantitative measurements on all echocardiograms according to a systematic, pre-established research protocol (7). LV ejection fraction was calculated from the biplane method of discs (5). LV mass was calculated from the 2D area-length method. LV diastolic dysfunction was assessed using tissue doppler E' velocities at the lateral mitral annulus and stratified based on age (8). Specifically, patients were classified as having diastolic dysfunction if they had E' velocity <9 cm/s (patients under 65) or <8 cm/s (patients over 65). Right ventricular (RV) measurements, including tricuspid annular plane systolic excursion (TAPSE), were obtained as described previously (9). PASP was calculated from tricuspid regurgitant (TR) jet peak velocity (to determine TR gradient) with the addition of estimated right atrial pressure (5).

Subjects underwent echocardiography as part of their initial clinical evaluation within 6 months of serum collection. Echocardiograms were performed by sonographers blinded to all clinical and laboratory data. Patients underwent comprehensive two-dimensional echocardiography with Doppler and tissue Doppler imaging according to published guidelines(5) using a Philips ie33 or Sonos 7500 echocardiographic machine. For the purposes of this study, a single experienced echocardiographer who was blinded to clinical and laboratory data, made quantitative measurements on all echocardiograms according to a systematic, pre-established research protocol . LV ejection fraction was calculated from the biplane method of discs. LV mass was calculated from the 2D area-length method. LV diastolic dysfunction was assessed using tissue doppler E' velocities at the lateral mitral annulus and stratified based on age. Right ventricular (RV) measurements, including tricuspid annular plane systolic excursion (TAPSE), were obtained as described previously. PASP was calculated from tricuspid regurgitant (TR) jet peak velocity (to determine TR gradient) with the addition of estimated right atrial pressure.

Serum collection and storage

After blood collection, sera were separated by centrifugation within 2 hours and were subsequently stored at −80°C until assayed (range 0-6 years) and were not subject to repeated freeze-thaw cycles.

Comparison of adipsin and BNP as markers of SSc-PAH

Sensitivity versus the false-positive frequency (1-specificity) for predicting pulmonary hypertension (echo PASP >35) with adipsin levels was analyzed with a receiver-operated characteristic (ROC) curve. The predictive accuracy was assessed by using the area under the curve (AUC) to determine the optimal cut-off level. Analysis of serum adipsin values demonstrated an area under the curve of 0.725 (0.627-0.823, $p<0.001$) for detecting echo PASP > 35 and AUC of 0.652 (0.446-0.771, $p = 0.02$) for RHC-defined PAH compared to AUCs of 0.505 (0.407-0.612, $p = 0.857$ for echo PASP>35) and 0.618 (0.525-0.761, $p = 0.08$ for RHC-defined PAH) for BNP. The specificity in this cohort for high adipsin to detect elevated echo PASP was 77% and RHC-proven PAH was 85% while elevated BNP (according to reference lab cutoff) of >100 demonstrated a specificity of 81% for echo pulmonary pressure of >35 and 57% for RHC-proven PAH, and compared to a BNP cutoff of 65 (reported previously as predictive of PAH (10)in SSc) which demonstrated. NT-pro-BNP which has been more widely described as an SSc-PAH biomarker(11, 12) was not clinically assessed.

Genetic analyses

Genetic analysis was performed on a subset of SSc patients from a previous genome-wide association study(13) who underwent echocardiograms and had both genetic and echocardiographic data available in dbGAP. SNPs from the GWAS within the CFD gene or in strong linkage disequilibrium (HapMap CEU D' > 0.8) with SNPs in the CFD gene were included and were assessed in patients with echocardiographic evidence of PAH (PASP>40) or normal echocardiograms (PASP<40). All SNPs were tested for departures from Hardy-Weinberg equilibrium expectations by a χ^2 goodness-of-fit test using PLINK. To test each SNP for association with SSc-PAH, we computed the overall allelic test of association using chi squared statistic calculated from two by two tables. SNPs found to be associated with PAH were assessed for presence of genotype/tissue expression eQTLs using the GTeX portal (http://www.gtexportal.org/).

To determine if adipsin (CFD) expression differed in SSc and SSc-PAH, data from three previous microarray studies were assessed, two in PBMCs (GSE19617and GSE22356) and one in lung tissue from patients undergoing lung transplantation (GSE48149)(14-16). Adipsin expression values for each individual sample were normalized to the mean of the controls and groups relevant SSc were plotted as boxplots of relative gene expression.

Statistical analysis

Continuous variables are described as mean ± standard deviation (SD), and categorical variables as frequencies and percentages ± standard error. The distributions of all variables were examined. Serum levels of adiponectin, adipsin, leptin, visfatin, and resistin were non-normally distributed (based on the Kolmogorov–Smirnov test) and thus non-parametric tests were used to assess differences between groups. The high/low cutoff value/threshold for adipsin was ascertained by taking the mean + 2 standard deviations from the control group.

Baseline demographic and clinical characteristics amongst subsets of SSc patients were compared with χ^2 or Fisher Exact test for discrete variables, and the Mann-Whitney test for continuous variables. In addition, χ^2 tests were used to assess associations between high/low adipsin levels and discretized PAH measures including FVC<70, DLCO<50, PASP >35, and TAPSE>1.6 which have previously been reported as relevant cutoff values in SSc(11, 17-20).

Differences in adipokine levels were compared among dcSSc, lcSSc with low adipsin, and lcSSc with high adipsin using ANOVA and were corrected for age, sex, BMI, and disease duration using ANCOVA. Univariate and multivariate logistic regression analyses were used to assess adipokine correlates with MRSS, radiographic ILD, PFT measures, and echo parameters as dichotomous variables. Relevant known confounders including age, sex, BMI, race, and disease duration were included in multivariate analyses.

Genetic association tests were performed to test each CFD region SNP for association with SSc-PAH. We computed the overall allelic test of association using chi squared statistic calculated from two by two tables.

All statistical tests were two-sided with a statistical significance defined as $P < 0.05$. R (R Foundation for Statistical Computing, Vienna, Austria), SPSS 23.0 (IBM Corp, Armonk, NY, USA) and GraphPad (GraphPad Software, La Jolla, CA, USA) were used for analysis.

Supplemental figure legends

Supplemental figure 1. Adipsin levels are not associated with skin or lung fibrosis in SSc. A. Serum adipsin levels were significantly negatively correlated with MRSS strata. B. However, when high/low modified Rodnan skin score (MRSS) status was considered, there was no difference in lcSSc patients with high/low adipsin levels and the only difference noted was between dcSSc and lcSSc. C. Adipsin levels were not different in SSc or its subgroups based on the presence or absence of radiographically defined interstitial lung disease (ILD). D. No differences in forced vital capacity (FVC) were seen between dcSSc and lcSSc patients with high and low adipsin levels.

Supplemental figure 2. Receiver operator curves showing the sensitivity and specificity of adipsin (panels A and C) and B natriuretic peptide (BNP, panels B and D) for detection of pulmonary arterial hypertension by either echocardiography (PASP>35, top panels) or by right heart catheterization (mean pulmonary artery pressure > 25 mmHg, pulmonary capillary wedge pressure < 15 mmHg, and pulmonary vascular resistance > 3 Wood units, bottom panels). Note that adipsin has increased area under the curve for detection by both echo (0.725 vs 0.509) and RHC (0.652 vs 0.618) defined PAH and is significantly different than a random distribution ($p<0.001$ for echo and $p=0.02$ for RHC) whereas BNP's ROC curves are not statistically significant.

Supplemental figure 3. Adipsin expression is elevated in peripheral blood mononuclear cells in SSc patients with PAH relative to patients with PAH in a second cohort of patients. Clinical data including disease subtype and presence/absence of interstitial lung disease was not available for this dataset. Data derived from GEO accession GSE22356.

Supplementary References

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Supplemental Table 1. Serum adipokine levels in SSc and Controls

1A. Adipokine Levels in SSc patients and healthy individuals

	Control (n=33)	SSc (n=198)	p-value (univariate)	p-value (multivariate)
Adiponectin (μg/mL)	5.73 ± 4.45	7.16 ± 7.32	0.5	0.88
Adipsin (μg/mL)	0.90 ± 0.29	1.18 ± 1.04	0.66	0.41
Leptin (ng/mL)	7.85 ± 9.54	15.38 ± 26.83	0.03	0.42
Resistin (ng/mL)	18.68 ± 6.31	18.39 ± 5.76	0.84	0.87
Visfatin (ng/mL)	5.53 ± 6.63	6.99 ± 10.75	**0.006**	0.47

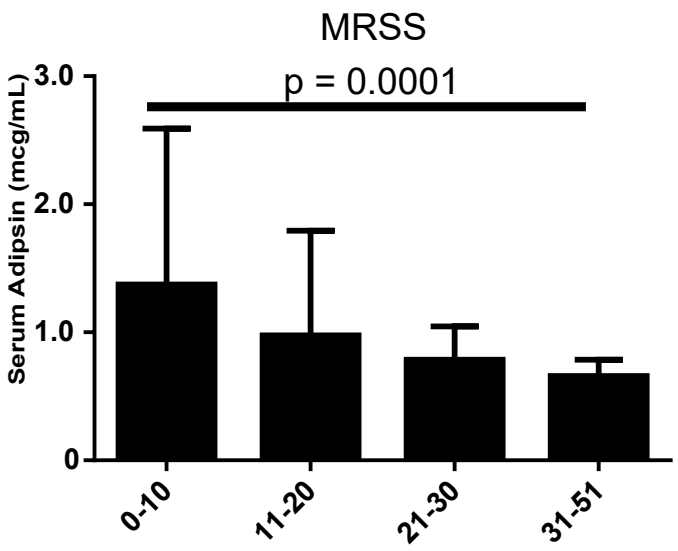
Serum levels of 5 adipokines were assessed, mean ± standard deviation (S.D.) is indicated for control individuals and SSc patients. Univariate ANOVA associations are shown as are p-values for ANCOVA which considered age, sex, and body mass index as covariates. Note that while leptin and visfatin levels were elevated in SSc relative to controls, these differences were no longer significant after correction for age, sex, and BMI. p-values were considered at the alpha = 0.01 level after a Bonferroni correction for five tests run simultaneously.

1B. Adipokine levels in lcSSc and dcSSc patients

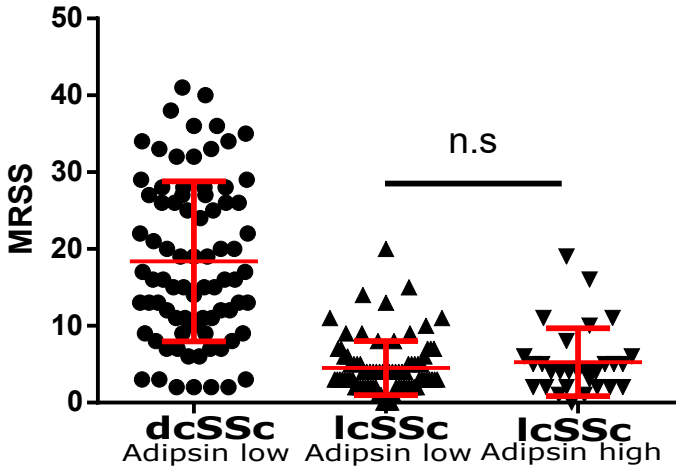
	lcSSc (n=116)	dcSSc (n=82)	p-value (univariate)	p-value (multivariate)
Adiponectin (μg/mL)	9.16 ± 8.09	3.41 ± 3.21	< **0.0001**	***0.003***
Adipsin (μg/mL)	1.51 ± 1.25	0.71 ± 0.22	< **0.0001**	***0.001***
Leptin (ng/mL)	20.38 ± 33.19	8.35 ± 10.09	**0.002**	0.07
Resistin (ng/mL)	19.60 ± 6.40	16.97 ± 4.52	**0.002**	0.22
Visfatin (ng/mL)	6.89 ± 8.34	7.15 ± 13.49	0.87	0.83

Serum levels of 5 adipokines were assessed, mean ± standard deviation (S.D) is indicated for lcSSc and dcSSc patients. Univariate ANOVA associations are shown as are p-values for ANCOVA which considered age, race, sex, disease duration, and body mass index as covariates. Note that only adipsin and adiponectin levels remain significantly different between groups after correction for clinical and demographic covariates. p-values were considered at the alpha = 0.01 level after a Bonferroni correction for five tests run simultaneously.

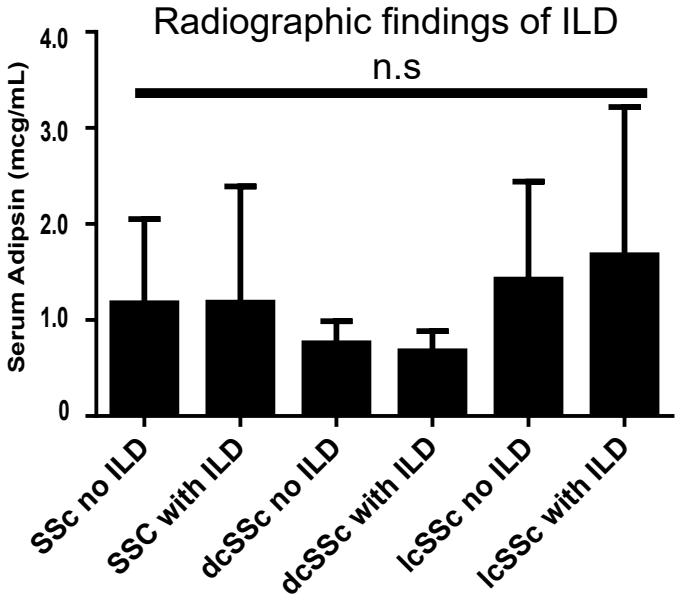
A.


MRSS
p = 0.0001
Serum Adipsin (mcg/mL)
3.0
2.0
1.0
0
0-10
11-20
21-30
31-51

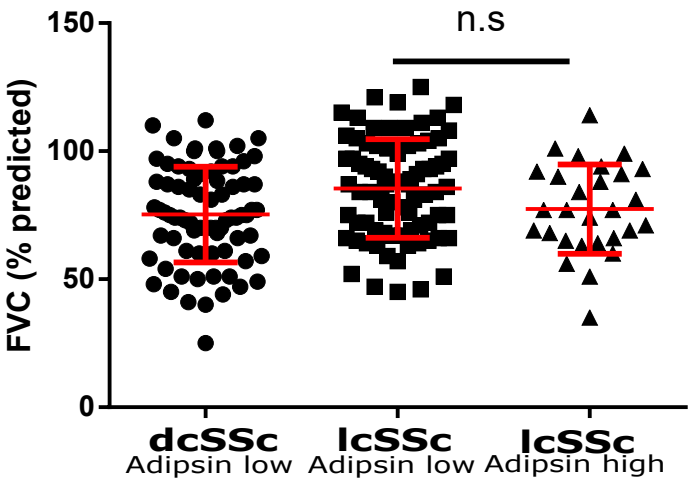
B.


MRSS
50
40
30
20
10
0
n.s
dcSSc
Adipsin low
lcSSc
Adipsin low
lcSSc
Adipsin high

C.


Radiographic findings of ILD
n.s
Serum Adipsin (mcg/mL)
4.0
3.0
2.0
1.0
0
SSc no ILD
SSC with ILD
dcSSc no ILD
dcSSc with ILD
lcSSc no ILD
lcSSc with ILD

D.


FVC (% predicted)
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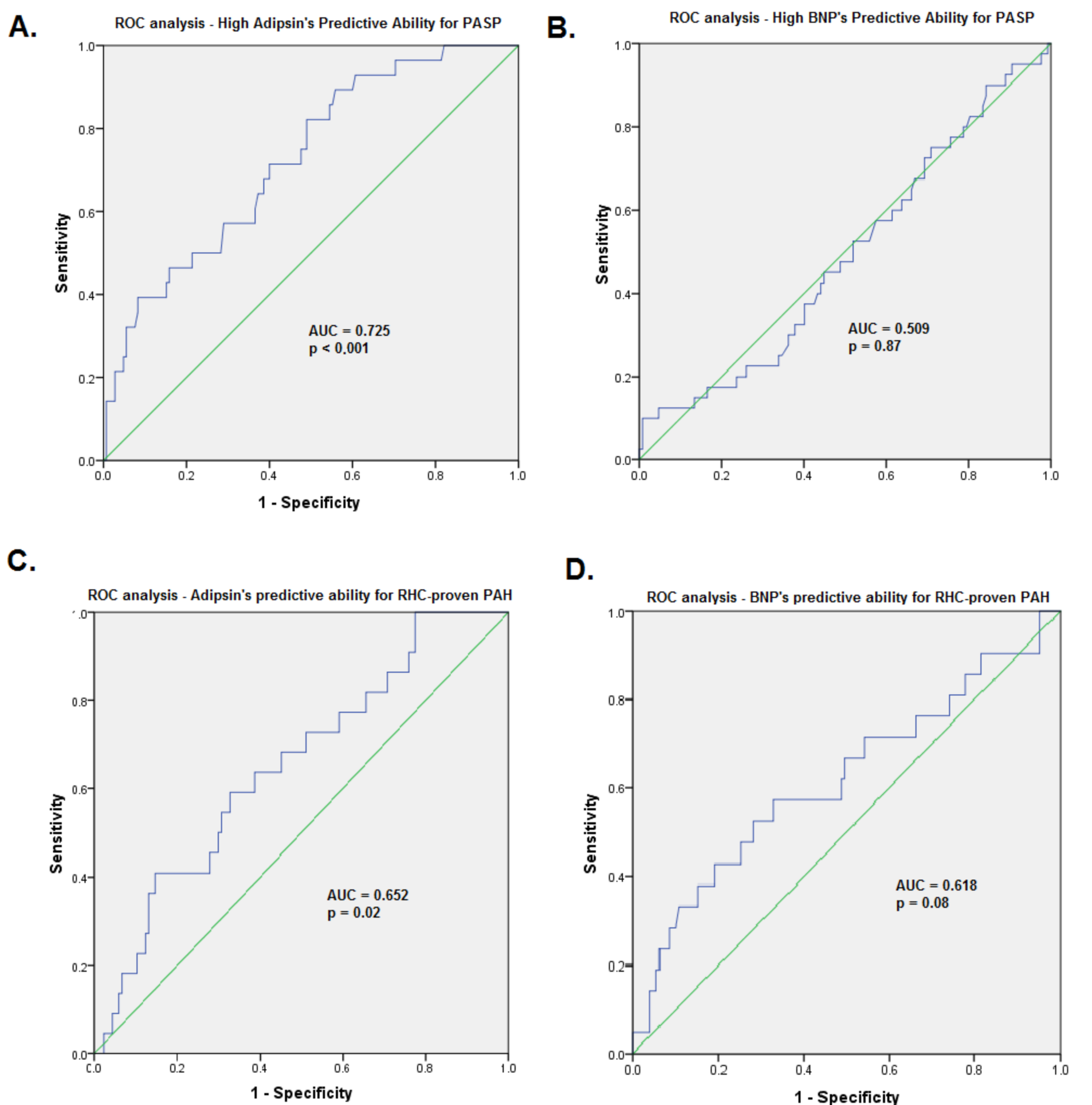
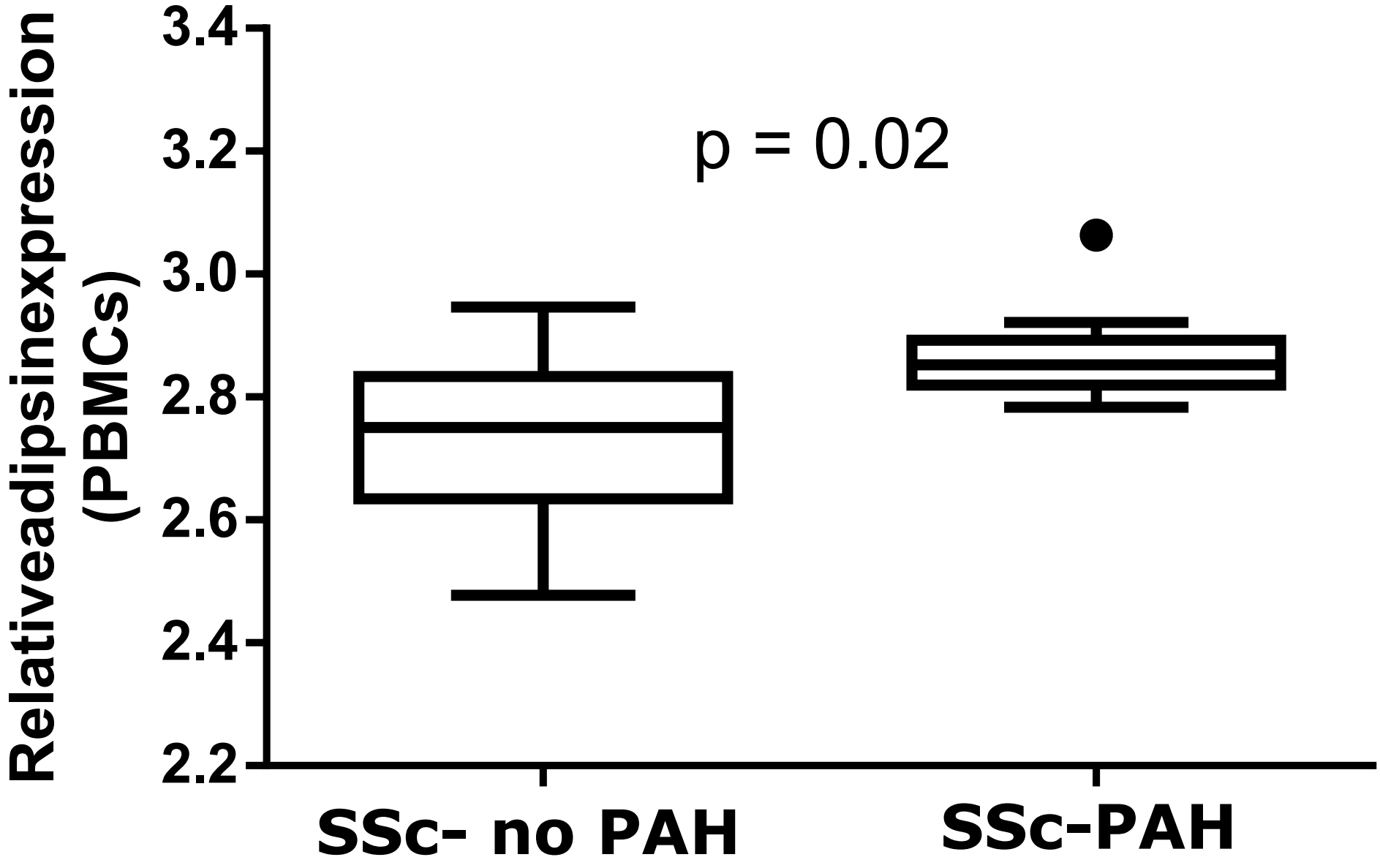
100
50
0
n.s
dcSSc
Adipsin low
lcSSc
Adipsin low
lcSSc
Adipsin high


A.
ROC analysis - High Adipsin's Predictive Ability for PASP
Sensitivity
1 - Specificity
AUC = 0.725
p < 0.001
B.
ROC analysis - High BNP's Predictive Ability for PASP
Sensitivity
AUC = 0.509
p = 0.87
C.
ROC analysis - Adipsin's predictive ability for RHC-proven PAH
Sensitivity
1 - Specificity
AUC = 0.652
p = 0.02
D.
ROC analysis - BNP's predictive ability for RHC-proven PAH
Sensitivity
1 - Specificity
AUC = 0.618
p = 0.08


Relativeadipsinexpression
(PBMCs)
3.4
3.2
3.0
2.8
2.6
2.4
2.2
p = 0.02
SSc- no PAH
SSc-PAH