



OPEN Natural killer cells are decreased in systemic sclerosis and have diagnostic value for pulmonary arterial hypertension incorporation

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The aim of this study was to investigate lymphocyte subsets, especially natural killer (NK) cells, in patients with systemic sclerosis (SSc) and evaluate the diagnostic value of NK cells in secondary pulmonary arterial hypertension (PAH). A total of 115 SSc patients and 100 age- and sex-matched health controls (HCs) were enrolled in this study. Flow cytometry was employed to quantify NK cells, while the association between NK cells and disease activity as well as PAH was investigated to further elucidate its diagnostic potential. The absolute count of NK (CD3-CD56+) cells significantly decreased in SSc patients. There was a negative correlation between the mRSS score and the injury index. The levels of cytokine exhibited significant elevation among SSc patients. Conversely, SSc-PAH patients demonstrated significantly elevated levels of CRP, UA, and BNP. Additionally, there was a significant reduction in the absolute level of NK cells. ROC curve analysis revealed that the optimal cut-off point for NK cells was 185 cells/ μ L, while for BNP it was 70.50 pg/mL and for UA it was 323.00 μ mol/L. Our study revealed a significant inverse correlation between peripheral blood NK cell levels and the incidence of complicated PAH in patients with SSc.

Keywords Systemic sclerosis, Pulmonary arterial hypertension, Natural killer cells, Pathogenesis

Systemic sclerosis (SSc) is a heterogeneous and multisystem autoimmune disorder characterized by chronic inflammation, endothelial dysfunction, and excessive collagen deposition and fibrosis¹. SSc can be classified into limited cutaneous systemic sclerosis (lcSSc), which is characterized by the distal elbow and knee skin thickening, involvement or absence of the face and neck, and often the presence of anti-centromere antibodies. Another disease subtype, diffuse cutaneous systemic sclerosis (dcSSc), is characterized by proximal skin thickening dominated by anti-topoisomerase I (Scl-70) antibodies and anti-RNA polymerase III antibodies². DcSSc involves organs earlier and more frequently. A small percentage of patients may exhibit clinical features of SSc without skin thickening (SSc sine scleroderma). The clinical manifestations of SSc encompass almost all organ systems, with common presentations including Raynaud's phenomenon, interstitial lung disease (ILD), renal crisis, etc¹. The global prevalence of SSc is approximately 176 cases per million people with an annual incidence rate of 14 cases per million people-years³. Among these cases, the incidence is higher in women; however, men experience a more severe prognosis. It is associated with significantly higher mortality rates and lower health-related quality of life (HRQoL)⁴. Pulmonary arterial hypertension (PAH) has emerged as the leading cause of death in SSc patients with a standardized mortality ratio of 5.27 and a median survival time of 3 years^{5,6}. It also contributes to increased healthcare utilization costs along with its associated economic burden⁵. Therefore, improving patient prognosis poses a major challenge in managing individuals with SSc necessitating early diagnosis for prompt initiation of treatment as well as rapid detection of treatment failure to facilitate immediate medication adjustments.

The etiology of SSc is multifactorial, involving genetic, environmental, immune, and endocrine factors. The interplay between activated endothelial cells, immune effectors, and fibroblasts plays a crucial role in the pathogenesis and clinical progression of this disease^{7,8}. Autoimmune dysregulation and abnormal inflammatory responses appear to run through all stages of the development of SSc. Lymphocyte characteristics in SSc patients

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indicate abnormal activation of T cell subsets⁹ and B cell subsets¹⁰, resulting in loss of immune tolerance and imbalanced subset proportions. Currently, studies on lymphoid cell numbers in SSc yield conflicting results; some show an increase in CD4+ T cells but a decrease in CD8+ T cells among patients with SSc¹¹, while others demonstrate a decrease in total T cell count¹². Natural killer (NK) cells are a heterogeneous population of innate lymphoid cells (ILCs) that primarily belong to classical innate immune cells. They possess the ability to rapidly eliminate target cells, such as host cells infected by viruses and tumors, without MHC restrictions. Additionally, they play a crucial role in the early stages of the body's anti-infection immune response^{13,14}. Moreover, NK cells exhibit robust cytokine and chemokine secretion functions, which not only regulate innate immune responses but also participate in the modulation of adaptive immune responses. Therefore, they are considered as a pivotal link between innate and adaptive immunity¹⁵. NK cells exist in the form of circulatory NK cells in peripheral blood, while in the form of tissue-resident NK cells in lymphoid tissues and non-lymphoid tissues¹⁶. The biological function of NK cells is mediated through the interplay between inhibitory and activating signals. The multifaceted functions of NK cells make them important initiators or contributors to various immune-mediated diseases, particularly rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), ankylosing spondylitis (AS), and other autoimmune-related disorders¹⁷. NK cell populations have shown alterations in some studies conducted on individuals with SSc. A reduction in the number of NK cells has been observed in the peripheral blood of patients with lcSSc¹². Conversely, an increase in NK cell count, along with altered cytokine production and impaired killing activity, has been noted in dcSSc patients¹⁸.

This study aimed to analyze the changes of T lymphocytes, NK cells, and cytokines in the peripheral blood of SSc patients while investigating their associations with organ involvement. The findings aim to provide novel insights into SSc pathogenesis as well as potential implications for cellular therapy.

Results

Patients' disposition and baseline characteristics

There were 96 female and 19 male patients; their average age was 56.58 ± 13.18 years. Their duration of illness and length of stay were 7.42 ± 8.25 years and 13.61 ± 5.67 days. Respectively, the disease type of most patients with SSc was dcSSc (77.39%), and 46.96% of patients had active disease status (EScSG score > 3). It is worth noting that most of the patients were positive for anti-nuclear antibodies (90.43%), among which the positive rates of anti-centromere antibodies, anti-Scl-70 antibodies, and anti-U1-SnRNP antibodies were 25.22%, 32.17%, and 20.37%. Furthermore, the most obvious organ involvement is interstitial lung disease, which occurs in more than half of patients (76 cases, 66.09%), followed by gastrointestinal involvement in 54 cases, SSc patients with pulmonary arterial hypertension in 32 cases, cardiovascular involvement in 19 cases, renal involvement and joint involvement affected 10 and 38 patient severally. The clinical characteristics of SSc patients are shown in Table 1.

Clinical data	SSc patients
Age at disease onset (years), mean \pm SD	56.58 \pm 13.18
The hospitalization time (days), mean \pm SD	13.61 \pm 5.67
The course of the disease (years), mean \pm SD	7.42 \pm 8.25
Sex-male, n (%)	19 (16.52%)
Sex-female, n (%)	96 (83.48%)
Anti-nuclear antibodies, n (%)	104(90.43%)
Anti-centromere antibodies, n (%)	29(25.22%)
Anti-Scl-70 antibodies, n (%)	37(32.17%)
Anti-U1-SnRNP antibodies, n (%)	22(20.37%)
No of organs affected, n (%)	14(12.17%)
Organ involvement, n (%)	101(87.83%)
Cardiovascular diseases, n (%)	19(16.52%)
Interstitial lung disease, n (%)	76 (66.09%)
Pulmonary arterial hypertension, n (%)	32(27.83%)
Gastrointestinal involvement, n (%)	54(46.96%)
Renal involvement, n (%)	10(8.70%)
Joint/tendon involvement, n (%)	38(33.04%)
Raynaud's phenomenon, n (%)	100(86.96%)
Diffuse cutaneous SSc (dcSSc), n (%)	89(77.39%)
Limited cutaneous SSc (lcSSc), n (%)	26(22.61%)
Active patients with disease activity index > 3 EScSG-AI, n. (%)	54(46.96%)

Table 1. Clinical findings of systemic sclerosis (SSc) cohort (n. 115 patients). Among those with active disease. Results are given as mean \pm SD, M (P25, P75) or percentage. EScSG-AI: European Scleroderma Study Group Activity Index.

Comparison of general clinical characteristics between 115 cases and 100 health controls

There were no statistically significant differences in age and gender distribution between SSc patients and HCs [56.58 ± 13.18 vs. 56.06 ± 7.01, $p=0.970$; 96 (83.48%) vs. 83 (83.48%), $p=0.768$]. Certain clinically common indicators, such as ESR and IgG, which reflect disease activity, were significantly higher in the SSc group. [ESR:20.00(10.00,33.00) vs. 11.5(7.00,17.00) mm/h, $p<0.001$; IgG: 13.19(10.75,16.63) vs. 10.80(10.15,12.78) g/L, $p=0.013$]. Notably, our results indicated a reduction among patients with SSc[279.50(223.925,322.40) vs. 311.50(273.00,361.50) $\mu\text{mol/L}$, $p<0.05$].

Subsequently, we compared the absolute and percentage levels of serum lymphocytes between SSc patients and HCs. Initially, the absolute count of T (CD3+) lymphocytes in SSc patients was significantly lower compared to HCs [1135.00 (671.47, 1452.00) vs. 1260.00 (1024.37, 1471.88) cell/ μL , $p=0.008$]; however, the percentage was increased [74.06 (65.82, 79.25) vs. 72.17 (65.59, 75.96) %, $p=0.034$] (Fig. 1A and B). Furthermore, detailed typing analysis of T lymphocytes in SSc patients revealed a significant reduction in the absolute count of Th cells (CD3+CD4+) [619.29 (323.78, 873.28) vs. 690.50 (574.85 vs. 908.49) cell/ μL , $p=0.012$]. Nevertheless, the percentage of Ts remained higher [30.00(21.80, 34.65) vs. 23.02 (20.00, 30.63) %, $p=0.005$]. Notably, both the number and percentage of NK cells (CD3-CD56+) were considerably lower in SSc patients[129.27 (68.52, 194.00) vs. 253.54 (164.00, 353.41) cell/ μL ; 9.42 (6.10, 12.50) vs. 14.85 (10.47, 20.21) %; $p<0.001$]. Furthermore, cytokine analysis indicated significantly elevated levels among SSc patients particularly for IL-2, IL-6, IL-10, and IFN- γ (as shown in Fig. 1C).

To further elucidate the relationships, we conducted multivariate analysis (as shown in Table 2) and found that the level of CD3-CD56+ NK cells was negatively associated with the occurrence of SSc (OR=0.989, 95% CI=0.980–0.999, $p=0.026$). Additionally, IL-2 and IL-6 levels were positive in our cohort. We also examined the correlation between NK cells and various parameters. However, no significant correlation was observed between NK cells and cytokine levels in our study (IL-2: $r = -0.094$, $p=0.354$; IL-6: $r = -0.138$, $p=0.170$). NK% was negatively correlated with mRSS score ($r = -0.289$, $p=0.003$), and the absolute value of CD3-CD56+ NK cells was similarly negatively correlated with damage index frequency scores (SCTC-DI) ($r = -0.184$, $p=0.045$).

The levels of peripheral blood CD3-CD56 + NK cells demonstrated a significant reduction in SSc patients with PAH

This study did not observe significant variations in both the absolute count and percentage of CD3-CD56+ NK cells in the peripheral blood among SSc patients with different subtypes and varying disease activity levels. Therefore, our focus was directed towards SSc cases involving vital organ complications, particularly those with PAH. We were divided into two groups: patients with SSc-associated PAH (SSc-PAH) and those without PAH (SSc-non-PAH).

Clinical characteristics in SSc-PAH and SSc-non-PAH

There were no significant differences in gender and age between SSc-PAH and SSc-non-PAH patients. However, the duration of the disease was relatively longer, and the frequency of combined cardiac and renal involvement was higher [10(30.30%) vs.9(10.98%), $p=0.012$; 6(18.18%) vs. 4(4.88%), $p=0.020$]. There were no statistically significant differences in antibody analysis. In the SSc-PAH group, we found significantly higher scores for certain scoring systems such as SCTC-DI and EScSG-AI. Several clinical markers including ESR (Fig. 2A), CRP (Fig. 2B), PLT (Fig. 2C), UA (Fig. 2D), B-type natriuretic peptide (BNP), prothrombin time, international normalized ratio, and D-dimer were also observed to be significantly elevated in PAH patients when compared to those without PAH (Table 3).

The decrease of CD3-CD56 + NK cells was more obvious in SSc-PAH patients

Our findings revealed that the absolute level of CD3-CD56+ NK cells was significantly lower in SSc-PAH compared to SSc-non-PAH, as well as HCs [90.00 (47.00,159.86), vs. 151.33 (85.05, 204.21), vs. 253.54 (164.00, 353.42), $p<0.05$] (Fig. 2E). Similar results were observed when analyzing the percentage of CD3-CD56+ NK cells between these groups (Fig. 2F). Furthermore, we investigated cytokine levels among the three groups and found that both SSc-PAH and SSc-non-PAH patients exhibited higher levels of IL-6, IL-10, and IFN compared to HCs; however, no significant difference was observed between the two patient groups (As shown in Fig. 2G). The results demonstrated that NK cells, UA, and BNP were independent risk factors for PAH in SSc by multivariate analysis to eliminate the confounding factors (Table 4). Specifically, the level of NK cells exhibited a negative correlation with PAH (OR=0.983, 95% CI=0.968–0.998, $p=0.023$). In contrast, both UA and BNP showed positive correlations [UA: OR=1.018, 95% CI=1.006–1.030, $p=0.004$; BNP: OR=1.008, 95% CI=1.002–1.015, $p=0.014$].

CD3-CD56 + NK cells, BNP, and uric acid can assist in the diagnosis of SSc-PAH

To assess the diagnostic value of these indicators, ROC curve analysis was performed (Fig. 3). We determined that the optimal cutoff point for CD3-CD56+ NK cells was 185 cell/ μL [sensitivity: 37.5(95%CI=27.69–48.45%), selectivity: 90.91(95%CI=76.43–96.86%), Youden's Index=0.284]; BNP was 70.50 pg/mL [sensitivity: 64.29(95%CI=38.76–83.66%), selectivity: 83.33(95%CI=60.78–94.16%), Youden's Index=0.476]. UA was 323.00 $\mu\text{mol/L}$ [sensitivity: 85.51 (95%CI=75.34–91.93%), selectivity: 44.83(95%CI=28.41–62.45%), Youden's Index=0.303]. To further investigate whether there were changes in PAN prevalence at this optimal cutoff point for these indicators, we conducted a Chi-square analysis based on the grouping according to the cutoff points. Interestingly, SSc patients with lower CD3-CD56+ NK cells values exhibited a higher prevalence of PAH (Fig. 4A), and BNP levels showed a significant increase in PAH prevalence (Fig. 4B). Notably, UA levels had a significant negative association with PAH, specifically, lower UA levels were less likely to be associated with PAH (Fig. 4C).

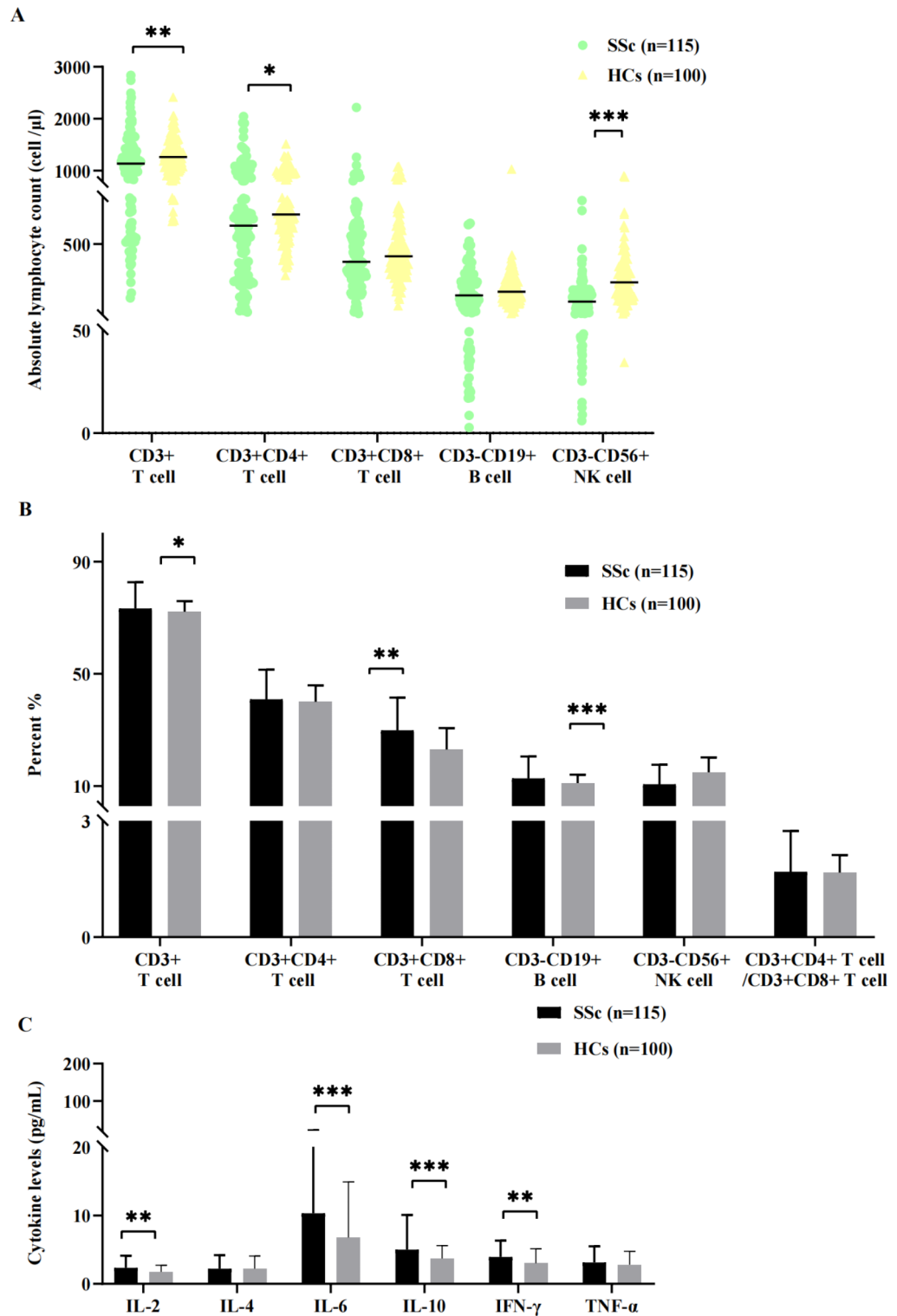


Fig. 1. Lymphocyte subsets of SSc patients and HCs (A) The absolute counts of lymphocytes, T (CD3+), Th (CD3 + CD4+), Ts (CD3 + CD8+), B (CD3-CD19+), and CD3-CD56 + NK (CD3-CD56 +) cells in SSc patients and HCs. (B) The percentage of lymphocytes T (CD3+), Th (CD3 + CD4+), Ts (CD3 + CD8+), B (CD3-CD19+) and NK (CD3-CD56 +) cells between SSc patients and HCs. (C) The serum levels of cytokines (IL-2, IL-4, IL-6, IL-10, IFN- γ , TNF- α) in SSc were significantly increased. HC, Healthy controls; SSc, systemic sclerosis; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by Mann-Whitney's U-test.

Variables	Beta	OR (95% CI)	p Values
CD3 + T cells	0.002	1.002 (0.998–1.005)	0.318
CD3 + T cells (%)	-0.012	0.989 (0.870–1.123)	0.860
CD3 + CD4 + Th cells	-0.003	0.997 (0.991–1.002)	0.179
CD3-CD56 + NK cells	-0.011	0.989 (0.980–0.999)	0.026
ESR	0.156	1.168 (1.058–1.290)	0.002
IL-2	0.824	2.281 (1.041–4.995)	0.039
IL-4	0.003	1.003 (0.925–1.088)	0.937
IL-6	0.150	1.162 (1.036–1.304)	0.010
IFN- γ	-0.059	0.943 (0.714–1.244)	0.677
Constant	-1.182	0.307	0.813

Table 2. Risk factors of SSc upon logistic multivariate regression. OR: Odds Ratio; CI: Confidence Interval; Th cells: T Helper cells; NK cells: Natural Killer cells; ESR: Erythrocyte sedimentation rate; IL-2: Interleukin-2; IL-4: Interleukin-4; IL-6: Interleukin-6; IFN- γ : Interferon- γ ; Bold values mean statistical significance.

Discussion

SSc is a severe autoimmune disease with heterogeneous multifactorial characteristics. The incidence, prevalence, disease activity, and prognosis vary among different races and nationalities, leading to diverse clinical manifestations⁷. PAH is a progressive destructive condition affecting small and medium pulmonary arteries, leading to pulmonary vascular remodeling¹⁹. If left untreated, this process can result in right ventricular dysfunction, heart failure, and ultimately death²⁰. SSc-PAH has been reported in 8–12% of patients with SSc and represents the leading cause of mortality among them²¹. In this study, 32 SSc patients were identified to have coexisting PAH, representing a prevalence rate of 27.83%. Previous studies have demonstrated that T cell-mediated cellular immunity along with B cell-mediated humoral immunity contribute to the immune damage involving dysregulation, characterized by dysregulation of multiple immune cell subsets²². Although the exact pathogenesis remains incompletely understood at present, autoimmune disorders consistently underlie the pathological processes associated with SSc. Thus, investigating the differences made by immune cells in SSc and SSc-PAH pathogenesis continues to be an active research area within autoimmune diseases.

This study confirmed the consistent involvement of immune dysfunction in the pathological process of SSc and SSc-PAH. The results demonstrated elevated levels of ESR and IgG in SSc patients, along with a high positive rate of antinuclear antibodies, suggesting severe immune dysfunction. Herein, we additionally report absolute and percentage changes in peripheral blood lymphocyte levels among SSc patients. The absolute counts of T cells and Th cells were decreased, whereas the percentages of T cells and Th cells were increased in SSc patients. Our analysis indicated that the changes of peripheral blood lymphocytes in SSc patients were mainly T cell subsets. These changes are attributed to the complex interactions among multiple immune cells in the pathogenesis of SSc, rather than being solely driven by a single cell type [7]. Additionally, different disease activity states or subtypes may yield varying results. Consequently, our study reflected a cohort of SSc patients with relatively long disease durations (7.42 ± 8.25 years) and a predominance of dcSSc. A cohort study investigating phenotypic abnormalities of T and NK cells revealed T cell lymphocytosis both pre-SSc and during active disease stages primarily within CD8+ and CD56+ T cell subsets while demonstrating evidence of decreased activated T cell numbers¹². These findings align with previous research highlighting the prominent role played by T cells as key contributors to SSc²³.

As a pivotal cytotoxic cell, NK cells play a unique role in the pathogenesis and progression of SSc, but the available data on NK cell alterations in SSc are scarce and contentious. To minimize the potential impact of glucocorticoids and immunosuppressive medications, we specifically enrolled treatment-naïve SSc patients. Our study confirmed that both the absolute count and percentage of NK cells in the peripheral blood of SSc patients are significantly reduced. The variations in NK cell numbers are influenced by multiple factors, including the complex etiology of SSc, the specific tissue microenvironment, the origin and phenotype of NK cells, and the experimental detection methods employed. Firstly, these differences may be attributed to the disease stage or involvement of different organs²⁴. Consistently, in dcSSc cases or advanced disease stages is a reduced frequency or percentage of NK cell presence^{25,26}. Conversely, studies have also observed an increased absolute number of NK cells among dcSSc patients compared to lcSSc patients¹⁸. Recently, Gumkowska-Sroka reported that cytometry analysis of 46 adult SSc patients exhibited reduced absolute counts of NK in the lymphocyte population, and the percentage of NK cells was significantly lower in SSc patients experiencing joint pain²⁷. In this study, absolute levels of NK cells were found to be significantly diminished in SSc-PAHs compared to SSc-non-PAHs and healthy controls; approximately one-third and half respectively. Furthermore, ACA-positive patients exhibited an increased number of NK cells according to an investigation on the effects of autoantibodies on immune cell subsets in SSc²⁸. Based on CD56 expression density on their surface, two distinct subpopulations can be identified: CD56^{bright} NK cells and CD56^{dim} NK cells. In human peripheral blood, CD56^{dim} NK cells constitute the majority (90–95%), while CD56^{bright} NK cells account for only 5–10%. Functionally speaking, CD56^{bright} NK cells are primarily involved in cytokine secretion with low cytotoxic activity; whereas CD56^{dim} NK cells function in killing function with limited cytokine production capabilities²⁹. Van der Kroef's³⁰ studies demonstrated a reduction in CD56^{hi} NK cells in SSc patients compared to healthy controls, which is consistent with phenotypic abnormalities previously observed by Almeida et al.¹². Studies have demonstrated that the frequency of CD56^{bright}

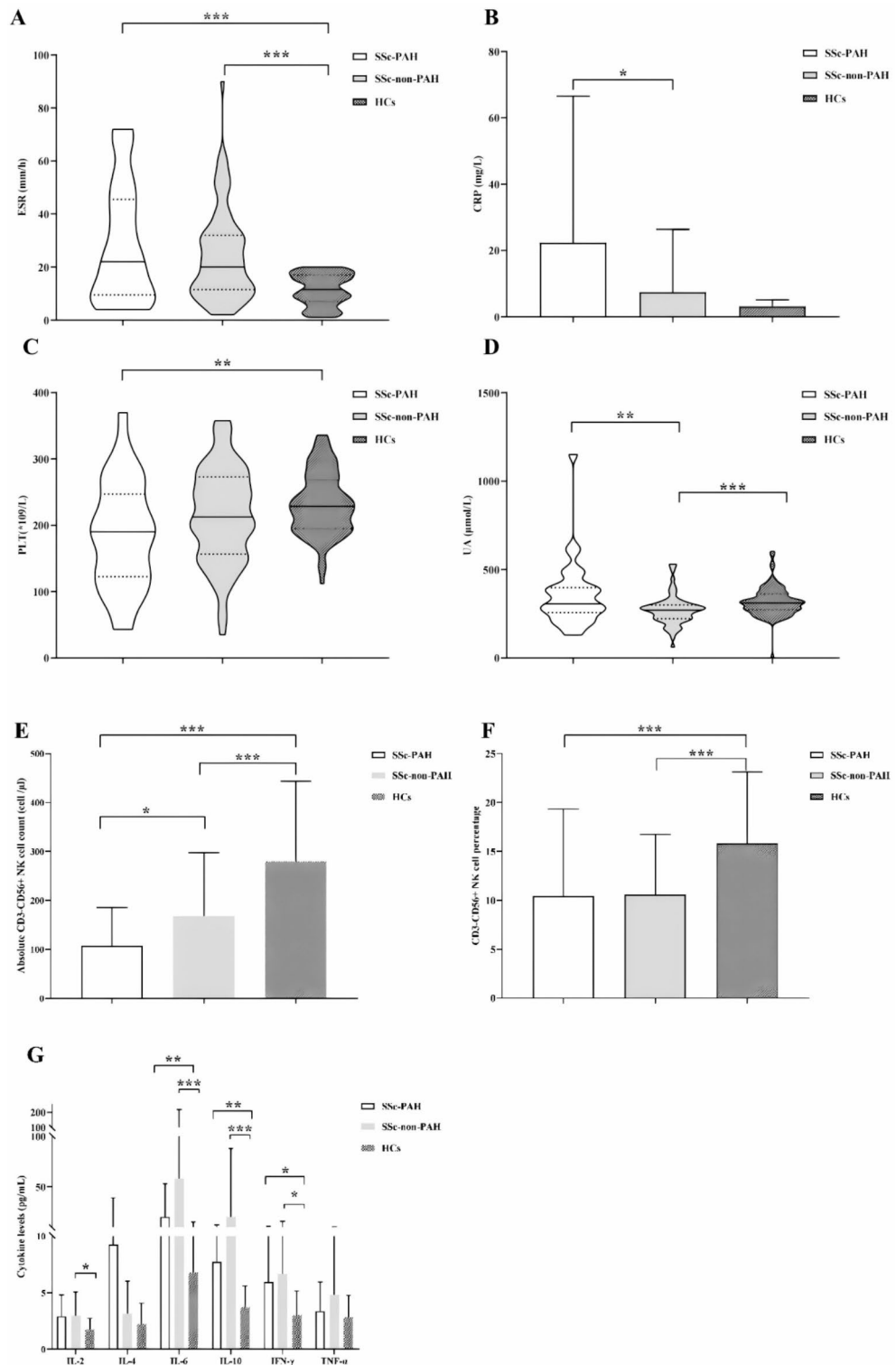


Fig. 2. Clinical characteristics in SSc-PAH, SSc-non-PAH, and HCs (A) The level of ESR between the three groups; (B) (CD3-CD19+) and NK (CD3-CD56+) cells in SSc patients and HCs. B. The level of CRP between the three groups. (C) The level of PLT between the three groups; (D) The level of UA between the three groups; (E) The absolute counts of NK (CD3-CD56+) cells between the three groups; (F) The percentage of lymphocytes NK (CD3-CD56+) cells between the three groups. (G) The serum levels of cytokines (IL-2, IL-4, IL-6, IL-10, IFN- γ , TNF- α) between the three groups; Healthy controls; SSc, systemic sclerosis; * $p < 0.05$; ** $p < 0.01$; *** < 0.001 by Kruskal-Wallis H Test.

	SSc-PAH(n=33)	SSc-non-PAH(n=82)	p Value
Sex-female, n(%)	27(81.81%)	69(84.14%)	0.761
The hospitalization time (days), mean ± SD	14.94 ± 8.42	13.07 ± 4.22	0.590
Age at disease onset (years), mean ± SD	54.64 ± 13.88	54.56 ± 12.93	0.530
The course of the disease (years), mean ± SD	11.87 ± 9.56	5.62 ± 6.96	< 0.001
Anti-nuclear antibodies, n (%)	33(100%)	72(87.80%)	0.083
Anti-centromere antibodies, n (%)	9(27.27%)	21((25.61%)	0.854
Anti-Scl-70 antibodies, n (%)	8(24.24%)	29(35.37%)	0.248
Cardiovascular diseases, n (%)	10(30.30%)	9(10.98%)	0.012
Interstitial lung disease, n (%)	24 (72.73%)	52(63.41%)	0.340
Gastrointestinal involvement, n (%)	19(57.58%)	35(42.68%)	0.148
Renal involvement, n (%)	6(18.18%)	4(4.88%)	0.022
Raynaud's phenomenon, n (%)	32(96.97%)	68(82.97%)	0.086
dcSSc, n (%)	27(81.82%)	62(75.61%)	0.472
lcSSc, n (%)	6(18.18%)	20(24.39%)	0.472
mRSS, M (P25, P75)	10.00(6.00,16.50)	8.00(5.00,14.00)	0.204
SCTC-DI, M (P25, P75)	5.00(4.00,8.00)	2.00(1.00,3.00)	< 0.001
EScSG-AI, M (P25, P75)	5.00(3.50, 9.00)	3.00(3.00,4.00)	< 0.001
BNP, M (P25, P75)	170.00(80.00,552.50)	54.00(30.75,305.50)	0.048
Prothrombin time	11.60(11.00,12.32)	11.20(10.70,11.70)	0.014
International normalized ratio	1.07(1.02,1.14)	1.040(0.99,1.0825)	0.017
D-dimer	190.00(112.00,669.50)	134.00(68.50,240.50)	0.005

Table 3. Demographic, clinical data of SSc patients with and without pulmonary arterial hypertension (PAH). *Results are given as mean ± SD, M (P25, P75) or percentage; dcSSc: diffuse cutaneous SSc; lcSSc: limited cutaneous SSc; mRSS: modified Rodnan Skin Score; SCTC-DI: Scleroderma Clinical Trials Consortium Damage Index; EScSG-AI: European Scleroderma Study Group Activity Index; BNP: B-type natriuretic peptide; Bold values mean statistical significance.

Variables	Beta	OR (95% CI)	p Values
CD3-CD56 + NK cells	-0.017	0.983 (0.968–0.998)	0.023
UA	0.018	1.018 (1.006–1.030)	0.004
The course of the disease	0.213	1.237 (1.051–1.457)	0.010
ESR	-0.008	0.992 (0.943–1.043)	0.747
International normalized ratio	0.608	1.838 (0.269–12.547)	0.535
CRP	0.002	1.002 (0.972–1.033)	0.915
PLT	-0.011	0.989 (0.975–1.004)	0.164
BNP	0.008	1.008(1.002–1.015)	0.014
D-dimer	-0.028	0.973 (0.937–1.010)	0.146
Constant	-2.16	0.115	0.35

Table 4. Risk factors of SSc-PAH upon logistic multivariate regression. OR: Odds Ratio; CI: Confidence Interval; NK cells: Natural Killer cells; UA: Uric acid; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; PLT: platelets; BNP: B-type natriuretic peptide; Bold values mean statistical significance.

NK cell subsets parallel to the progression of SSc increases linearly, suggesting an aggravating role of NK cells in the pathogenesis of SSc³¹. This finding provides valuable insights, and we will proceed to verify the phenotyping of peripheral blood NK cells in SSc in subsequent experiments.

In addition to the abnormalities mentioned in NK cell expression and surface receptors in SSc, evidence of aberrant NK cell function was also observed. Horikawa et al. reported an increase in the production of IFN- γ , IL-5, and IL-10 by non-stimulated NK cells in SSc patients, while stimulated NK cells showed a decrease in IFN- γ production¹⁸. The natural cytotoxic activity of NK cells was impaired in SSc patients, with reduced secretion of granzyme B. Our analysis demonstrated significantly elevated levels of IL-2, IL-6, IL-10, and IFN- γ in SSc patients. The correlation analysis revealed no significant association between NK cells and cytokines. Multivariate analysis identified NK cells, IL-2, and IL-6 as independent factors. The relationship between NK cells and IFN- γ warrants further investigation through cellular-level experiments. We speculate that the decrease of NK cells may be related to the regulation of T and B cells in SSc. The diminished presence of NK cells weakens their regulatory effect on adaptive immune responses leading to an imbalance between innate and adaptive

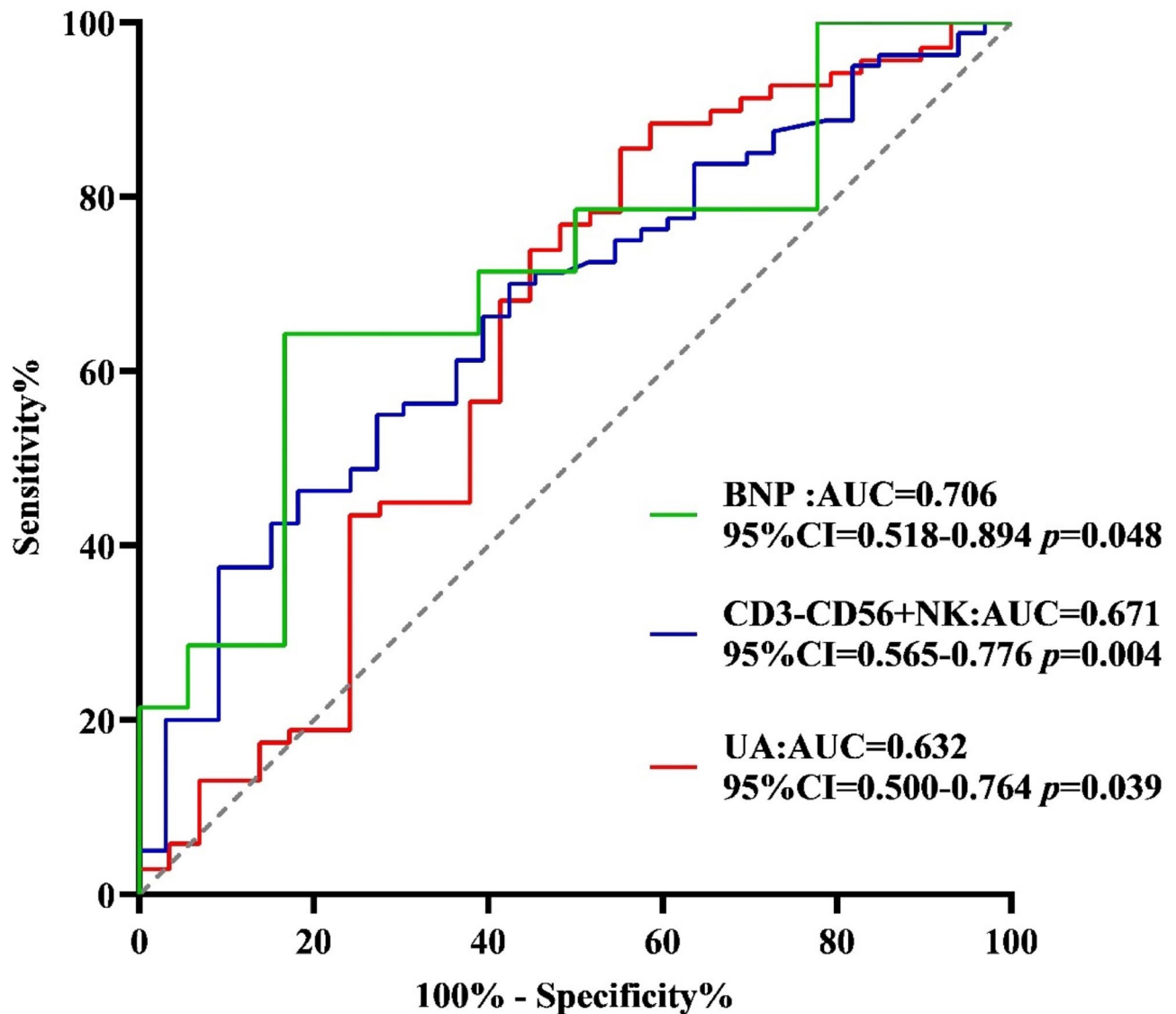


Fig. 3. ROC curve analysis in the presence of PAH NK cells: Natural Killer cells; UA: Uric acid; BNP: B-type natriuretic peptide; Bold values mean statistical significance.

immunity. Moreover, weakened inhibition against autoantibodies results in excessive autoantibody formation in SSc patients. Immune modulation involving NK cells plays a crucial role in the pathogenesis and progression of SSc. Interestingly, our findings revealed that the decreased absolute count was significantly correlated with the mRSS score and SCTC-DI, indicating an inverse relationship between the percentage of NK cells and disease severity. However, Theodoros Ioannis Papadimitriou's study proposed a depletion process, where cytotoxic cells undergo functional changes as their numbers decrease. They observed a large number of activated NK cells in SSc skin³², which was not inconsistent with our results. We speculate that the reduction in peripheral blood NK cells in SSc patients may serve as a predictor of more severe skin involvement. Taken together, these results collectively imply a crucial role for NK cells in the pathogenesis and progression of SSc.

No significant differences in the absolute counts and percentages of CD3-CD56+NK cells were observed among SSc patients with varying subtypes or levels of disease activity within this study cohort. However, a notable decrease in NK cells was evident in SSc patients with PAH. Consequently, our focus was on evaluating the changes in related indicators specifically in SSc patients with PAH. Right cardiac catheterization (RHC) is the gold standard test for definitively diagnosing PAH³³. Despite its widespread acceptance, there are currently no internationally recognized clinical guidelines regarding best practices for conducting RHC³⁴. Due to the invasive nature of hemodynamic monitoring, it is imperative to develop a convenient biomarker that can accurately assess disease severity and effectively predict poor patient outcomes, enabling early treatment and achieving clinical remission. Several studies have reported significantly increased serum uric acid levels in SSc-PAH patients, which negatively correlate with the 6-minute walk test³⁵ and linearly correlate with pulmonary artery pressure³⁶. In this study, we also observed significantly higher serum uric acid levels in SSc-PAH patients compared to non-SSc-PAH patients. Further ROC curve analysis revealed that a serum uric acid level > 323.00

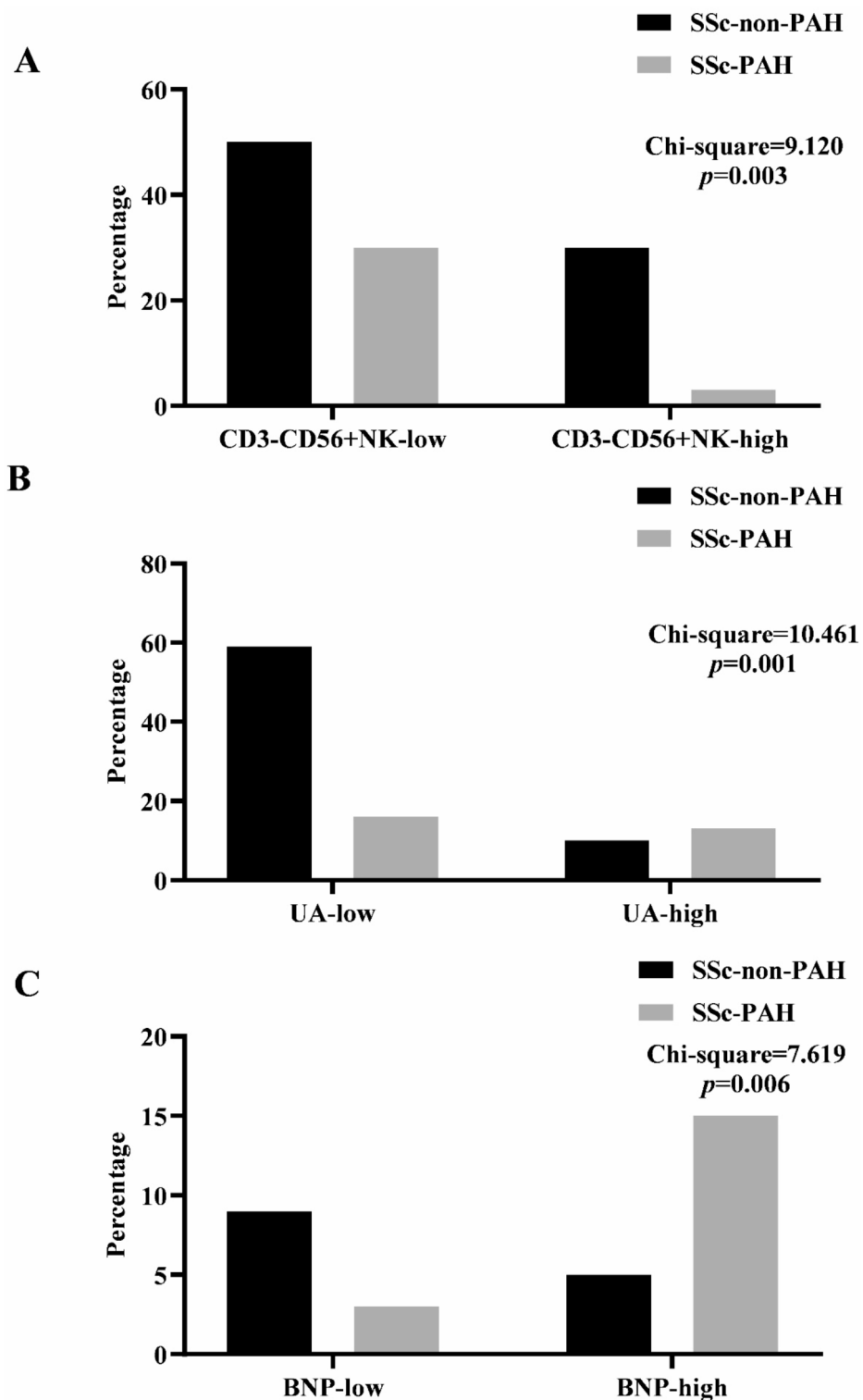


Fig. 4. A Chi-square analysis based on the grouping according to the cutoff points (A). Prediction rates of PAH by CD3–CD56+NK cells. (B) Prediction rates of PAH by UA. (C). Prediction rates of PAH by BNP. NK cells: Natural Killer cells; UA: Uric acid; BNP: B-type natriuretic peptide.

$\mu\text{mol/L}$ had a sensitivity of 85.51% and selectivity of 44.83%. Studies have demonstrated that elevated blood uric acid activates inflammatory cells by releasing free radicals through xanthine oxidase, exacerbating vascular endothelial damage and tissue hypoxia³⁷. Based on these findings, we believe that blood uric acid levels can serve as an effective indicator for predicting the occurrence of PAH in SSc patients and hold certain significance for clinically monitoring pulmonary artery pressure and evaluating disease progression. BNP and its N-terminal

segment (NT-proBNP) are clinically available tests that have been demonstrated to be elevated in patients with congestive heart failure of multiple etiology³⁸. Although baseline levels of BNP or NT-proBNP have not been shown to predict SSc-PAH incidence, they are useful for monitoring disease severity³⁹. A meta-analysis evaluating the diagnostic value of natriuretic peptides in SSc-PAH indicated that BNP/NT-proBNP had some diagnostic value in PAH⁴⁰. In our study, we found that BNP was significantly higher in SSc-PAH patients than those with non-SSc-PAH, and the AUC value assessed for BNP was 0.706, indicating good predictive value. As previously mentioned, we observed a more pronounced decline in NK cell levels among SSc-PAH patients. Furthermore, we conducted an in-depth analysis to determine the diagnostic efficiency of NK cells in identifying SSc-PAH patients. We concluded that the optimal cut-off point for CD3-CD56+ NK cells was 185 cells/ μ L below which SSc patients are more likely to have concurrent PAH. There is limited research on the pathogenesis of NK cells in PAH, and further basic research is needed to elucidate the reasons behind their significant decrease. Furthermore, we assessed the potential of combining uric acid with BNP, NK cells, or their combination to diagnose SSc-PAH. Regrettably, no considerable efficacy was observed when these three groups were combined or when any two groups were combined ($p=0.773$).

In our study, we enrolled 115 SSc patients and, for the first time, integrated vascular-related factors with immune factors to evaluate the activity and severity of SSc disease, particularly SSc-PAH, using commonly used clinical indicators. Although there are alternative biochemical markers available for predicting the development of SSc with PAH, measuring serum UA and BNP provides a straightforward simple and widely accessible approach in routine medical practice that can be repeated experimentally. However, several limitations exist in this study. Firstly, it is based on single-center clinical data; therefore, long-term multi-center observations involving larger sample sizes are required to validate these findings. Secondly, the retrospective design may introduce potential biases; thus high-quality large-scale prospective studies with long-term follow-up are warranted to further assess safety and efficacy. Furthermore, there is a need for further investigation into the specific phenotypes, subgroups, and functional abnormalities of NK cells in SSc patients. Additionally, this study did not clearly classify the etiology of PAH; hence further investigations should focus on its classification for better understanding. In future research endeavors, we aim to collect clinical data from idiopathic PAH cases to explore unique biomarkers specific to SSc-PAH.

In conclusion, SSc patients exhibit intricate immune dysfunction primarily characterized by lymphocyte subset disorders. The reduction in peripheral blood NK cell count is closely associated with disease severity, while the decrease in NK cell count often correlates with the damage observed in SSc-PAH patients. Furthermore, we have also determined that abnormal levels of UA and BNP are evident in SSc-PAH patients. Therefore, assessing these indicators can aid in evaluating disease activity and pulmonary hypertension incidence in SSc. Although they possess clinical value for diagnosing PAH, their independent diagnostic utility for SSc-PAH remains suboptimal. Encouraging more comprehensive screening for SSc-PAH is necessary to confirm the diagnostic efficacy of novel markers and establish appropriate thresholds to enhance accuracy. Consequently, early intervention should be implemented to improve clinical prognosis before irreversible pathophysiological changes occur.

Materials and methods

Patients and controls

We evaluated 115 SSc patients fulfilling the 2013 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR classification criteria⁴¹, who visited the Rheumatology and Immunology Department of Shanxi Bethune Hospital from January 2018 to December 2023, and enrolled in the study were initial treatment and aged between 18 and 80 years. The patients were excluded from this study if they suffered from malignant disease, had a history of malignancy, had recently experienced a clinically significant infection, or had any other connective tissue disease. Additionally, 100 healthy controls (HCs) were included, and subjects who were free from any diseases and not taking any medication at the time of enrollment were included. They visited our physical examination center during the same period, had no rheumatological immunology and family history, and matched SSc patients for age and sex. Each participant signed an informed consent, and peripheral blood and serum samples were collected. Clinical and laboratory indicators as well as autoantibodies were also tested using fresh blood samples. Our study has been approved by the Ethics Committee of Shanxi Bethune Hospital (batch number: NO.LYLL-2024-003/PJ03), and all participants have signed informed consent. The methods employed in this study were conducted in strict adherence to the relevant guidelines and regulations, as well as in accordance with the principles outlined in the Declaration of Helsinki.

Clinical measurements

We collected clinical, anthropometric, and laboratory characteristics of all patients, including duration of disease, time of onset of Raynaud's phenomenon, and skin involvement as determined by the modified Rodnan Skin Score (mRSS)⁴². Blood samples and skin assessments were collected concurrently.

Moreover, the presence of ILD was ascertained by a high-resolution computed tomography scan (HRCT, GE DiscoveryRT) of the chest, which has four features: single ground glass lesions, ground glass lesions, and reticular nodular lesions (mixed pattern), single reticular nodular lesions (reticular-nodular), and honeycomb changes. As invasive right heart catheterization is the gold standard for diagnosing PAH, our diagnostic criteria based on Doppler echocardiography are as follows: pulmonary systolic blood pressure > 35 mmHg or mean pulmonary blood pressure > 25 mmHg at rest or \geq 30 mmHg during activity⁴³. The presence of esophageal dysfunction was defined as the manifestation of acid reflux symptoms such as heartburn or the identification of reflux esophagitis through gastrointestinal endoscopy. Cardiovascular diseases were evaluated using color Doppler echocardiography. The diagnosis of hypertension with renal involvement was established when the blood pressure exceeded 140/90 mmHg (except essential hypertension), and impaired kidney function was defined as a serum creatinine concentration increase of more than 30% from baseline at any point during the

study. Additionally, the presence of joint contracture was also documented. The disease activity according to the EScSG (European Scleroderma Study Group) disease activity index⁴⁴. Furthermore, we employed the SCTC-DI scoring system, a validated tool utilized for quantifying organ damage in SSc individuals⁴⁵.

Laboratory studies

The levels of C3 and C4 complement, total serum immunoglobulin titers, and erythrocyte sedimentation rate (ESR) were quantified using nephelometry. After a 1–2 h incubation period, the serum was separated from 4 ml venous blood and stored at -20 °C. Serum cytokine levels [interleukin-2 (IL-2), IL-4, IL-6, IL-10, tumor necrosis factor- α (TNF- α), interferon- γ (IFN- γ),] in SSc were measured by flow cytometry using a cytometric bead array (CBA) kit purchased from Jiangsu Sage Biotechnology Co., Ltd. (Jiangsu, China). The measurements were performed according to the manufacturer's instructions with results expressed as pg/ml.

Two PB samples (4.5 ml each) were collected in tubes containing tri-potassium ethylenediaminetetraacetic acid (EDTA-K3) for lymphocyte immunophenotyping or without anticoagulant for serum cytokine measurement. The peripheral blood of patients and healthy volunteers was used to isolate PBMC and PBS, which were then adjusted to a density of $0.1-2 \times 10^6/100\mu\text{l}$ to prepare single-cell suspensions. Surface antibodies (CD45 for lymphocytes, CD3 for T lymphocytes, CD4 for Th lymphocytes, CD8 for Ts lymphocytes, CD19 for B lymphocytes, and CD56 for NK cells) were added followed by gentle swirling at room temperature away from light for 15 min (Supplementary Table S1). After adding 1 ml of PBS solution, the samples were centrifuged at 300 g for 5 min. The supernatant was discarded and PBS (200–500ul) was added before testing on the machine. Flow cytometry analysis (Calibur) was performed using gated lymphocytes differentiated based on forward angular scattered light relative to lateral angular scattered light (side scatter). By utilizing a combination of two parameters, forward scatter (FSC) for size differentiation and side scatter (SSC) for particle differentiation, the removal of debris is facilitated, distinct cell populations are discerned, and lymphocyte populations are delineated. CD4 was used to distinguish CD4+ T cells from the SSC gate; 10,000 cells from the gate were taken. CD8 was used to distinguish CD8+ T cells from the SSC gate; 10,000 cells from the gate were taken. Subsequently, the percentage of CD3-CD56+ NK cells was analyzed and their absolute count was determined (see Supplementary Fig. S1 online). The relative percentages were obtained and analyzed using CellQuest software. The absolute number of cells in each subgroup was calculated using the following equation: absolute cell number = percentage of positive cells in each subset \times the absolute number of cells (cells/ μl) whole blood. The relative percentages obtained were further analyzed using CellQuest software while BD Multitest software automatically measured the absolute numbers of T lymphocyte subsets. All immunofluorescent antibodies used in this study were purchased from BD Biosciences.

Statistical analysis

The statistical analyses were performed using SPSS software version 26.0 (IBM, Armonk, NY, USA). Dichotomous variables were presented as percentages and absolute frequencies, while continuous features were expressed as mean \pm standard deviation (SD) or median with interquartile range [M (P25, P75)]. Chi-square goodness-of-fit test was used for count data analysis. An Independent sample t-test was employed to compare between two groups. For non-normally distributed data, Mann-Whitney's U-test was conducted to compare differences between groups, and Kruskal-Wallis H test was used for comparisons among multiple groups. Spearman's rank correlation coefficient was utilized for correlation analysis. Logistic regression analysis examined the association between outcome variables "SSc" and "SSc-PAH" with variables separately. A multivariate adjusted regression model was established to identify confounding factors based on a significance level of $p < 0.05$. The diagnostic efficacy was assessed utilizing the receiver operating characteristic (ROC) curve. All reported P values were two-sided and not adjusted for multiple testing. The significance level was set at $P < 0.05$.

Data availability

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation. If data from this study is requested, it should be directed to the corresponding author of this study.

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Author contributions

RH-G performed the data analyses and wrote the manuscript. MM-Z, YN-G, YT-H, YL-J participated in the collection of samples and clinical data. JF-G, YL-Y and LY-M participated in the study design and revising of the manuscript. K-X provided intellectual input and supervision throughout the study and made a substantial contribution to manuscript drafting. All authors contributed to the article and approved the submitted version.

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Declarations

Competing interests

The authors declare no competing interests.

Ethics approval and consent to participate

The studies involving human participants were reviewed and approved by the Ethics Committee of Shanxi Bethune Hospital. Written informed consent was obtained from all patients/participants before their inclusion in this study.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-025-89238-z>.

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