

Serum fatty acid profile in systemic sclerosis compared to rheumatoid arthritis and healthy controls

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Abstract

Background

Systemic sclerosis is a heterogeneous autoimmune disease associated with decreased life quality and expectancy. As fully curative treatment is not available, early and accurate risk stratification remains an unmet need. Lipids are involved in vascular and immune regulation – two key processes of systemic sclerosis pathogenesis. Therefore, this study aimed to characterise fatty acid profiles in systemic sclerosis and to assess their potential diagnostic and clinical relevance.

Methods

The study included 54 systemic sclerosis patients, 36 rheumatoid arthritis patients, and 43 healthy adults. Rheumatoid arthritis was included as an additional control group with autoimmune disease to help distinguish systemic sclerosis-specific fatty acid alterations from changes related more broadly to autoimmunity, inflammation, comorbidities, and long-term pharmacotherapy. Their clinical data and serum samples were collected. Fatty acid measurement was performed using gas chromatography-mass spectrometry. Differences between studied groups and subgroups of systemic sclerosis were assessed using appropriate univariate tests. Multivariate analyses included principal component analysis and partial least squares discriminant analysis. Diagnostic performance was evaluated using receiver operating characteristics.

Results

Patients with systemic sclerosis showed a global decrease in serum fatty acid concentrations compared with both rheumatoid arthritis patients and healthy controls. Several fatty acids and fatty acid classes demonstrated discriminatory potential, particularly in systemic sclerosis versus rheumatoid arthritis comparisons. The multivariate model discriminating systemic sclerosis from rheumatoid arthritis showed good classification performance, with an area under the curve of 0.87. Weaker discrimination was found for systemic sclerosis versus healthy control (area under the curve 0.68). Candidate biomarkers mainly included polyunsaturated and branched-chain fatty acids. Fatty acid alterations were more pronounced in active systemic sclerosis. Several potential biomarkers for disease activity were indicated, whereas no biomarkers of specific organ involvement were identified after correction for multiple testing.

Conclusions

Systemic sclerosis is characterised by a distinct fatty acid profile, different from healthy controls and rheumatoid arthritis. Our findings suggest that fatty acid profiling may provide candidate biomarkers for

systemic sclerosis, particularly in relation to disease activity. Further studies on larger independent groups are needed to validate the presented observations and clarify the mechanisms underlying fatty acid disturbances in systemic sclerosis.

Background

Systemic sclerosis (SSc) is a rare, complex autoimmune disease burdened with decreased life expectancy and severe disability. SSc patients present a vast spectrum of symptoms (1). Predominantly, skin fibrosis, Raynaud's phenomenon (RP), and internal organ involvement are observed. Currently, the incidence of SSc is growing. Although the exact aetiology of SSc remains elusive, it is confirmed that prominent pathomechanisms involve vasculopathy, inflammation, and tissue fibrosis. So far, no fully curative treatment has been proposed, and available therapies are associated with dire side effects. Therefore, early diagnosis and precise risk stratification is fundamental to maintain good quality of life, impede disease progression and prevent iatrogenic complications (2).

To address this issue, many branches of science have been involved, including metabolomics. This approach has already proven its potential in rheumatology. Analysis and simultaneous comparison of concentrations of multiple metabolites are more than a key to biomarker discovery (3). It also elucidates altered pathways that lead to disease development and may help to indicate new therapeutic targets.

In the past decades, the immunomodulatory role of lipids has been thoroughly studied, as well as their significance in vasculopathy. Therefore, their potential contribution to SSc development has been explored, with the focus on fatty acids (FA) and their derivatives (4). Modern research pointed at multiple aberrations that brought new light into SSc pathology. Some molecules (eg. alprostadil) have already proved therapeutic potential and have been implemented into clinical practice (5, 6). Despite advances, the need for new diagnostic and risk-scoring methods remains highly unmet.

Considering the above, we found it necessary to continue studies on lipid aberrations in SSc, we decided to investigate FA disturbances in this group of patients, by examining a holistic FA profile using gas chromatography-mass spectroscopy (GC-MS). Moreover, we found it necessary to compare SSc patients not only to HC, but also to those suffering from other connective tissue diseases, who may also be burdened with comorbidities and long and complex history of pharmacotherapy with an unknown impact on their metabolome. Rheumatoid arthritis was chosen as a disease control group because, although it shares with SSc an autoimmune background and chronic inflammatory component, it is characterized by a more overt systemic inflammatory phenotype, predominant musculoskeletal involvement, and a distinct clinical course, thereby enabling discrimination between metabolic alterations related to SSc-specific pathophysiology and those reflecting autoimmunity, inflammation, comorbidity burden, or long-term pharmacotherapy more broadly.

Here we present our study, which aimed to find potential biomarkers for SSc and lead to a better understanding of the pathology of the disease.

Materials and Methods

Patients

Three groups of patients were included in this study during 2021–2024. The inclusion criteria for the first studied group were: age over 18 years, and SSc diagnosis according to ACR/EULAR 2013 classification criteria (7). In total, 54 patients, with the mean age of 57.0 years (with a standard deviation of 14.2 years) were included. 44 (80%) of them were female. Disease activity was assessed using the EUSTAR activity index (8). To assess the quality of life and disability, patients were asked to fill out the HAQ-DI questionnaire (9, 10), the response rate was 72%. Moreover, past medical history and follow-up data were collected to screen progression and organ involvement. Follow-up was scheduled after 3 months, 6 months, and later according to patients' requirements (minimum 1 year, maximum 4 years of observation). The second group included adult patients diagnosed with RA according to 2010 ACR/EULAR classification criteria (11). In total, 36 patients with a mean age 63 years (SD 11.3 years) were included; 31 (86%) of them were female. The disease activity was measured using the DAS-28 score (12). In the two groups (SSc and RA), nutritional status was evaluated using body mass index (BMI), the SGA-7 scale (Subjective Global Assessment 7-point scale) (13), and serum albumin concentration. Risk of malnutrition was recognised when any of the following was confirmed: BMI lower than 18.5 kg/m², albumin < 34 g/L, or SGA-7 score lower than 5 points. Exclusion criteria for SSc and RA groups were age under 18 years, active infection or consent withdrawal.

The control group included 43 non-obese adult subjects in good general health. The mean age was 59.3 years (SD 14.4 years), and 32 (74%) of them were female. Exclusion criteria were: clinical evidence of any rheumatic, autoimmune, liver, kidney, neurological disease, infection, or consent withdrawal.

In all studied groups, dietary habits were assessed using the Food Frequency Questionnaire (FFQ-6) (14). The form enables a semiquantitative evaluation of the consumption of 62 product groups (averaged over the last 12 months), which has been validated for the Polish population (15). Questionnaire data were missing for 17 subjects (12.8%).

The study was conducted in accordance with the World Medical Association's Declaration of Helsinki and was approved by the Local Bioethics Committee at the Medical University of Gdańsk (decisions: NKBBN/527/2021, NKBBN/527–772/2021, and KB/527 – 162/2024). All participants provided written consent to participate in the project.

Fatty acids analysis

Venous blood samples (9 ml) were collected in the morning hours, after an overnight fast, into serum clot activator tubes. After 30 minutes of incubation at room temperature, the clot was removed, and the serum was centrifuged (5 minutes, 21°C, 5000 rpm). Then, samples were deep-frozen at -74°C.

Total lipids were extracted from serum samples with a chloroform-methanol mixture (2:1, v/v) according to Folch et al. (16) and, after drying under a nitrogen stream, were subjected to 3 h of hydrolysis with 0.5 M KOH at 90°C. After incubation, the mixtures were acidified with 6 M HCl. 1 mL of water was added, unesterified FAs were extracted thrice with 1 mL of n-hexane and the organic phase was evaporated under a nitrogen stream. The extracts were then derivatised into fatty acid methyl esters (FAME) with 10% boron trifluoride in methanol solution at 55°C for 1.5 h. Then, 1 mL of water was added, and the FAMEs were extracted with 3×1 mL n-hexane, dried under a nitrogen stream, and stored at – 20°C until analysis. The FAMEs were analysed with a GC-EI-MS QP-2020 NX (Shimadzu, Kyoto, Japan) with chromatographic separation on a Zebron ZB-5MSi capillary column, 30 m × 0.25 mm i.d. × 0.25 µm film thickness, (Phenomenex, Torrance, CA, USA). The samples were injected into dichloromethane. 1 µL of the sample was injected in split mode. The column temperature was set in a range of 60°C to 300°C (4°C/min), with helium as the carrier gas at the column head pressure of 60 kPa. The temperature of injection, ion source, and transfer line was 300°C, 200°C, and 300°C, respectively. The electron energy used for FAME ionisation was 70 eV; 19-methylarachidic acid was used as an internal standard. Full scan mode was used with a mass scan range of m/z 45 to 700. Accurate identification of the FA profile was possible based on FAME mixture standards (Larodan, Michigan, USA, and Merck, Darmstadt, Germany).

Statistical analysis

Statistical analyses were performed using Python 3.12 (SciPy 1.16.3, statsmodels 0.14.6, pingouin 0.6.1) and MetaboAnalyst 6.0. Normality was assessed using the Shapiro–Wilk test. Differences were considered statistically significant at $p < 0.05$. For two-group comparisons, Welch’s t-test or Mann–Whitney U test was applied as appropriate. For comparisons among three groups, Welch’s ANOVA was used due to its robustness to unequal variances and unbalanced sample sizes. Significant results were followed by Games–Howell post hoc tests. Correlations were assessed using Spearman’s rank coefficient. A p-value < 0.05 was considered statistically significant at the nominal level. To account for multiple testing in univariate analyses, p-values were adjusted using the Benjamini–Hochberg false discovery rate (FDR), and results with $q < 0.05$ were considered statistically significant after correction. Findings with nominal $p < 0.05$ that did not survive FDR correction were interpreted as exploratory. Prior to multivariate analysis, data were normalised by sum, log10 transformed, and autoscaled (mean-centred and divided by the standard deviation). Multivariate analyses included principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) with cross-validation and permutation testing. Diagnostic performance was evaluated using receiver operating characteristics (ROC) curves, with area under the curve (AUC) and 95% confidence interval (CI).

Results

Table 1a presents results of standard biochemical screening of examined groups. Most of the SSc and RA patients at the time of sample collection were treated with the immunosuppressive agents, which are presented in Table 1b.

Systemic sclerosis (SSc) and rheumatoid arthritis (RA) presented higher concentrations of creatinine and C-reactive protein (CRP) than healthy volunteers (control). Moreover, systemic sclerosis patients had lower level of total cholesterol comparing to other groups (Table 1a).

Table 1
Characteristics of the study groups

a. Standard screening			
	Control	Systemic sclerosis	Rheumatoid arthritis
CRP [mg/L]	1.14 ± 1.06	4.51 ± 6.67 ^a	12.6 ± 25.6 ^b
Glucose [mg/dL]	93.16 ± 9.51	88.6 ± 9.80	97.3 ± 18.2
ALT [U/L]	23.2 ± 10.1	21.4 ± 14.1	25.8 ± 16.8
Creatinine [mg/dL]	0.83 ± 0.18	0.92 ± 1.26 ^a	1.10 ± 0.10 ^b
Total cholesterol [mg/dL]	213.4 ± 38.6	186.8 ± 37.4 ^a	209.3 ± 47.5
HDL [mg/dL]	65.3 ± 18.9	57.3 ± 23.9	63.5 ± 16.2
LDL [mg/dL]	135.8 ± 45.7	117.9 ± 34.8	131.0 ± 41.0
Triglycerides [mg/dL]	100.7 ± 37.3	106.3 ± 45.7	104.0 ± 44.6
Albumin [g/L]	Not determined	40.06 ± 7.07	39.17 ± 4.91
BMI [kg/m ²]	Not determined	23.25 ± 3.94	25.44 ± 4.59
b. Immunosuppressive and immunomodulatory therapies			
		Systemic sclerosis	Rheumatoid arthritis
Methotrexate		12 patients, 22%	24 patients, 66.7%
Low-dose corticosteroids		12 patients, 22%	16 patients, 44.4%
Mycophenolate mofetil		11 patients, 20%	No patients
Tocilizumab		3 patients, 6%	1 patient, 2.8%
Rituximab		1 patient, 2%	No patients
Cyclophosphamide		1 patient, 2%	No patients
Leflunomide		No patients	2 patients, 5.6%
Antimalarial drugs		6 patients, 11%	6 patients, 16.7%
Cyclosporine		No patients	1 patient, 2.8%
Sulfasalazine		No patients	1 patient, 2.8%
c. Raynaud's phenomenon treatment in systemic sclerosis			
Calcium channel blockers	31 patients, 57%		

a. Standard screening			
	Control	Systemic sclerosis	Rheumatoid arthritis
Sildenafil		12 patients, 22%	
Alprostadil (intravenous)		9 patients, 17%	
Pentoxifylline		9 patients, 17%	
Sulodexide		5 patients, 9%	
d. Other drugs used by rheumatoid arthritis and systemic sclerosis patients			
vitamin D supplementation (38 patients), proton pump inhibitors (34 patients), folic acid (29 patients), levothyroxine (20 patients), acetylsalicylic acid (16 patients), calcium supplementation (16 patients), statins (15 patients), β -blockers (14 patients), angiotensin-converting enzyme inhibitors (13 patients), iron supplementation (10 patients), tramadol (10 patients), ramipril (9 patients), angiotensin II receptor blockers (8 patients), itopride (8 patients), ibandronic acid (5 patients), torasemide (5 patients), oral anticoagulants (5 patients), oral nutritional supplements (5 patients), paracetamol (5 patients), perindopril (4 patients), fluoxetine (4 patients), spironolactone (4 patients), trimethoprim/sulfamethoxazole (3 patients), citalopram/escitalopram (3 patients), enoxaparin (3 patients), metformin (3 patients), tamsulosin (3 patients), ipratropium bromide (2 patients), bilastine (2 patients), finasteride (2 patients), furosemide (2 patients), empagliflozin (2 patients), allopurinol (2 patients), nintedanib (2 patients), opipramol (2 patients), pregabalin (2 patients), denosumab (2 patients), trazodone (2 patients), montelukast (1 patient).			

In Table 1a data are presented as mean $\pm$ standard deviation. Values were considered statistically significant when $p < 0.05$. ^a significant difference between SSc and control, ^b significant difference between RA and control. No significant difference between SSc and RA were found.

The impact of used therapies on fatty acid profile was assessed for every drug which was used by 10 and more systemic sclerosis patients. Scarce significant differences were observed only for statin therapy. No other significant differences were found.

The dietary analysis of the FFQ-6 questionnaire showed scarce differences with limited significance (supplementary Table 1).

Characteristics of the studied systemic sclerosis patients

The median disease duration was 4 years (IQR 1;8.25 years), and the median diagnostic delay, defined as the time from the onset of non-RP symptoms to diagnosis, was 1 year (IQR 0;4 years). 35 patients were diagnosed with limited cutaneous systemic sclerosis (lcSSc) and 19 with diffuse cutaneous systemic sclerosis (dcSSc) according to LeRoy classification (17). The most common serotypes were positive for anti-topoisomerase I antibodies (anti-Scl-70) (26 patients) and anti-centromere antibodies (17 patients). Only 6 patients were positive for anti-polymerase III (RNAP III) antibodies. In 19 cases, SSc was classified as active (18). Patients were undergoing immunosuppressive and immunomodulatory therapies as presented in Table 1b. 15 patients reported a subjective feeling of symptom deterioration

within 30 days before sample collection. Progression of the disease was recognised as any non-RP symptom or organ complication deteriorated (e.g. new digital ulcers, expanding skin fibrosis, new interstitial changes in chest computed tomography), regardless of implemented therapy. 13 (24%) of patients presented progression within 3 months since sample collection; 13 patients presented progression and 4 patients died in longer follow-up. 1 person was lost to the 3-month follow-up, and 7 subjects were lost to follow-up longer than 3 months. Most of the patients presented with multiple organ involvement (Table 2). All of them suffered from Raynaud's phenomenon, implemented therapies are listed in Table 1c. Skin thickening was measured using modified Rodnan Skin Score, the median score was 3.5 points, and 7 patients had skin involvement assessed for 18 or more points.

Table 2
Organ involvement in the systemic sclerosis.

Organ involvement	Systemic sclerosis (n = 54)
Telangiectasias	39 patients (72%)
Digital ulcers (active)	13 patients (24%)
Digital ulcers (in medical history)	33 patients (61%)
Microstomia	36 patients (67%)
Acroosteolysis *	15 patients (28%)
Calcinosis *	14 patients (26%)
Arthralgia **	33 patients (61%)
Myalgia **	14 patients (26%)
Digestive tract symptoms (upper/lower part) **	41 (76%) / 6 (30%)
Interstitial lung disease (described in chest-CT)	29 patients (54%)
Pulmonary arterial hypertension (proved in right heart catheterisation)	6 patients (11%)
Heart involvement (confirmed in cardiac MRI)	8 patients (15%)
Renal crisis	1 patient (2%)

Please note that heart catheterisation, cardiac magnetic resonance imaging, and x-ray were not performed for every patient, unless there was a clinical indication for such procedures

*Clinically evident or visualised on X-ray

***Present at any time since the onset of the disease. The most common gastrointestinal symptoms included dysphagia or reflux disease (upper part), and diarrhoea (lower part).*

Arterial hypertension and thyroid diseases were the most common comorbidities in this group of patients. To proceed further, we compared profiles of patients who had a history of thyroid disease to those who never had hypo- or hyperthyroidism. No significant differences were found. In the evaluation of quality of life and disability, 24 subjects presented no or mild functional decline, 13 patients presented moderate deficits, and 2 severe impairments. Nutritional evaluation showed that in total 22 (40.7%) SSc patients were at risk of malnutrition (Supplementary Table 1a). Well-nourished patients with active SSc and malnourished patients with non-active SSc were underrepresented (both groups included only 3 patients). Therefore limited number of participants precluded meaningful statistical analysis, and malnourishment was regarded as a prominent feature of active SSc.

Clinical assessment of rheumatoid arthritis group

The median disease duration was 8.5 years (IQR 3;18 years). Most patients (97%) were seropositive for rheumatoid factor or/and anti-ACPA antibodies. 15 patients (41.7%) presented moderate or high activity (DAS-28 score > 3.2 points). In this group, arterial hypertension and thyroid diseases were also the most common comorbidities. Nutritional evaluation revealed that 5 patients (13.9%) were identified to be at risk of malnutrition (Supplementary Table 1a).

Characteristics of fatty acid profile

The targeted analysis of FAs revealed multiple differences among patients with SSc, RA, and HC (Table 3, Supplementary Table 2) – generally decreased concentrations of FAs were detected in SSc. Interestingly, the exact opposite tendency was observed in the RA group. The diagnostic potential of significantly aberrated FAs and groups of FAs was explored using ROC curves. Most of the FAs of significantly aberrated concentrations (in all groups) had an AUC higher than 0.7, which suggested predictive value (Table 3). Additionally, n-6/n-3 polyunsaturated fatty acid (PUFA) ratio was calculated and was found to be significantly decreased in RA group compared with both SSc and control subjects (p value respectively < 0.05 and < 0.001). There was no significant difference in n-6/n-3 PUFA ratio between SSc and control group (Supplementary Table 2).

In Table 3 we present potential lipid biomarkers significantly differing between groups in univariate analysis after false discovery rate correction. Fatty acids presented in bold font had AUC > 0.7 in univariate ROC analysis. FAs and FA groups distinguishing SSc from the control were detected. All fatty acids that differentiated SSc from the control were also among those that distinguished SSc from RA. The FAs specific for SSc vs RA in univariate comparison were C14:1, iso C15, anteiso C17, ARA, ETA, EDA, AdA, C25, and C28. Among the FAs distinguishing the RA group from the control group, good discriminative potential showed: C15, iso C17, EPA, DHA, and C25.

Table 3. Significant differences of serum fatty acids concentrations between systemic sclerosis, rheumatoid arthritis, and healthy controls.

SSc vs control	RA vs control	SSc vs RA
C14, anteiso C15, C15, iso C16 , C16, iso C17, C17, ALA, LA , C18, DGLA, C20, C22, C24, SFA, n-6PUFA, total PUFA, ECFA, OCFA, iso BCFA , anteiso BCFA, total BCFA, VLSCFA, total FAs	C15, C16:1, iso C17, EPA , C20:1, DHA, DPAn3, C25, n-3PUFA, n-6/n-3 PUFA ratio	C14:1, C14, iso C15, anteiso C15, C15, iso C16, C16:1, C16, iso C17, anteiso C17, C17, ALA, LA, C18, ARA, EPA, ETA, DGLA, EDA, C20:1, C20, DHA, AdA, DPAn3, C22, C24, C25, C28, SFA, n-PUFA, n-3 PUFA, total PUFA, n-6/n-3 PUFA ratio, ECFA, OCFA, iso BCFA, anteiso BCFA, total BCFA, VLSCFA, total FAs

Fatty acids presented in bold font had AUC > 0.7 in univariate ROC analysis. Green font indicates decreased concentration and red stands for elevated concentration.

SSc - systemic sclerosis, RA - rheumatoid arthritis, AdA - adrenic acid, ALA - α -linolenic acid, ARA - arachidonic acid, BCFA - branched-chain fatty acids, DGLA - dihomo- γ -linolenic acid, DHA - docosahexaenoic acid, DPA - docosapentaenoic acid, ECFA - even-chain fatty acids, EDA - eicosadienoic acid, EPA - eicosapentaenoic acid, ETA - eicosatetraenoic acid, LA - linoleic acid, OCFA - odd-chain fatty acids, PUFA - polyunsaturated fatty acids, SFA - saturated fatty acids, VLSCFA - very-long-chain saturated fatty acids, AUC - area under the curve, ROC - receiver operating characteristics.

To explore differences using a holistic approach and to assess potential clustering patterns a principal component analysis was performed (Figure 1). Most of the variability in the dataset (PC1: 23.1% and PC2: 11.8%) was associated with a broad dispersion of the results within SSc, while the RA group had the most consistent lipid profile. The PCA suggests that n-3PUFA and BCFA contributed most to noted differences. PCA models for pairwise comparisons (SSc vs control and SSc vs RA) were also performed plots are presented in Supplementary Figures S1 and S2.

Figure 1. Principal component analysis of fatty acid profiles in systemic sclerosis, rheumatoid arthritis, and healthy controls.

PC - principal component, SSc - systemic sclerosis, RA - rheumatoid arthritis, EDA - eicosadienoic acid, DPA - docosapentaenoic acid. The first two principal components explained 34.9% of total variance (PC1: 23.1%; PC2: 11.8%). Each point represents an individual sample, and ellipses indicate group dispersion at the 95% confidence. Loading vectors illustrate the contribution of individual fatty acids to the sample distribution

Further, partial least squares discriminant analysis(PLS-DA) was performed to explore clustering patterns and identify discriminative variables (SSc vs HC and SSc vs RA). The SSc vs HC model showed modest class separation (Figure 2a, Supplementary Figure 3). Despite statistical significance, major overlap between SSc and HC groups indicated weak discriminative capacity, in contrast to SSc vs RA model, which demonstrated better group separation (Figure 2b, Supplementary Figure 4). The most

important variables contributing to discrimination (VIP scores) were: C18:1, ALA, LA, anteiso C15, C14 (SSc vs HC), and C16, C15, C14:1, anteiso C15, C14 (SSc vs RA) (Figures 2c and 2d), all of which were decreased in SSc. To quantify classification potential, multivariate ROC testing based on PLS-DA models was performed. As increasing the number of latent variables (LVs) did not improve classification performance significantly, two-component models were selected for further analysis. SSc vs HC model reached an AUC of 0.68 (95% CI 0.53–0.78) (Figure 2e, Supplementary Figure 5), confirming weak discriminatory potential. In the predictive analysis, sensitivity and specificity values remained moderate (Supplementary Figure 6). In contrast, SSc vs RA model showed an AUC of 0.87 (95% CI 0.75–0.94), confirming good discriminatory ability (Figure 2f, Supplementary Figure 7). In the predictive view, sensitivity and specificity were balanced and reached approximately 77%, consistent with the predictive accuracy observed across the selected model configuration (Supplementary Figure 8). Finally, feature selection frequency analysis was performed, and results are presented in Figures 2g and 2h.

Figure 2. Multivariate analysis of systemic sclerosis vs healthy control and SSc vs rheumatoid arthritis models.

- a.** PLS-DA score plot for SSc vs HC comparison. Cross-validation (CV) indicated four optimal components, predictive performance was limited (accuracy = 0.72, $R^2 = 0.55$; $Q^2 = 0.21$), permutation testing (1000 permutations) confirmed statistical significance ($p < 0.001$) (Supplementary Figure 3).
- b.** PLS-DA score plot for SSc vs RA. CV indicated three components as optimal, predictive performance was moderate (accuracy = 0.83, $R^2 = 0.61$; $Q^2 = 0.40$), permutation testing (1000 permutations) confirmed statistical significance ($p < 0.001$) (Supplementary Figure 4).
- c and d.** VIP scores for SSc vs HC and SSc vs RA models, respectively, indicating fatty acids most important for group discrimination.
- e.** ROC curve for the SSc vs HC model, showing limited classification performance (AUC = 0.68; 95% CI 0.53–0.78).
- f.** ROC curve for the SSc vs RA model, showing limited classification performance (AUC = 0.87; 95% CI 0.75–0.94).
- g and h.** Feature selection frequency plots presenting the most frequently selected FAs in classification models for SSc vs HC and SSc vs RA comparisons.

SSc - systemic sclerosis, HC - healthy control, RA - rheumatoid arthritis, PLS-DA - partial least squares discriminant analysis, ROC - receiver operating characteristics, VIP - variable importance in projection, AUC - area under the curve, CI - confidence interval, CV - cross-validation, ALA - α -linolenic acid, LA - linoleic acid, EPA - eicosapentaenoic acid, DHA - docosahexaenoic acid, FA - fatty acid

Activity of systemic sclerosis

Next, univariate analysis of serum FA concentrations in active, non-active SSc and HC was performed, which confirmed that FA aberrations correlate with the activity of SSc (Table 4, Supplementary Table 3).

Table 4 presents potential lipid biomarkers significantly differing between groups in univariate analysisafter false discovery rate correction (FDR). FAs presented in bold font had AUC > 0.7 in univariate receiver operating characteristics (ROC) analysis. Active SSc generally presented lower FA concentrations, and differed significantly from healthy controls in multiple FAs and FA groups, whereas non-active disease presented scarce differences (good discriminative potential had only ALA and C22, or SFA, n-6 PUFA, ECFA and OCFA. There were significant differences between active and non-active disease, although no marker was specific for this comparison.

Table 4. Significant fatty acid (FA) concentration differences depending on the activity of systemic sclerosis (SSc).

Active SSc vs HC	Non-active SSc vs HC	Active SSc vs non-active SSc
C14:1, C14, iso C15, anteiso C15, C15, iso C16, C16, iso C17, anteiso C17, C17, ALA, LA, C18, ARA, DGLA, EDA, C20, DPAn6, DHA, AdA, DPAn3, C22, C24, C26, C28, SFA, n-6PUFA, n-3PUFA, total PUFA, n-6/n-3 PUFA ratio, ECFA, OCFA, iso BCFA, anteiso BCFA, total BCFA, VLSCFA, total FAs	C16, C17, ALA, LA, C22, SFA, n-6PUFA, n-6/n-3 PUFA ratio, ECFA, OCFA, total FAs	Iso C15, anteiso C15, C15, iso C16, iso C17, anteiso C17, C17, LA, C18, ARA, EDA, DPAn3, DHA, DPAn6, C22, C24, C26, n-6PUFA, total PUFA, OCFA, iso BCFA, anteiso BCFA, total BCFA, VLSCFA

Fatty acids presented in bold font had AUC > 0.7 in univariate ROC analysis. Green font indicates decreased concentration and red stands for elevated concentration.

AdA - adrenic acid, ALA - α-linolenic acid, ARA - arachidonic acid, BCFA - branched-chain fatty acids, DGLA - dihomo-γ-linolenic acid, DHA - docosahexaenoic acid, DPA - docosapentaenoic acid, ECFA - even-chain fatty acids, EDA - eicosadienoic acid, LA - linoleic acid, OCFA - odd-chain fatty acids, PUFA - polyunsaturated fatty acids, SFA - saturated fatty acids, VLSCFA - very-long-chain saturated fatty acids, AUC - area under the curve, ROC - receiver operating characteristics.

To further explore the diagnostic potential, ROC analysis of significantly aberrated FAs (active vs non-active disease) was performed. The strongest activity biomarkers were: iso C17, iso BCFA, total BCFA, total PUFA, n-6 PUFA reached AUC > 0.8 (Supplementary Figure 9).

Clinical subtypes of systemic sclerosis

A comparison between lcSSc and dcSSc revealed that C16, C18:1, anteiso C19, C28, SFA, ECFA and total FAs had differential concentrations in univariate analysis (Supplementary Table 4). Among them, three FAs (C16, C18:1 and anteiso C19) demonstrated moderate discriminatory performance in ROC analysis (AUC > 0.7, supplementary Figure 10). Consistently, no significant differences were observed in n-6/n-3 PUFA ratio between those two clinical subtypes. The multivariate models did not present clear group

separation (Supplementary Figure 11). The strongest differentiation was observed for dcSSc vs control (PC1 + PC2 = 35.1%, $p = 0.017$), although predictive performance remained limited ($Q^2 \approx 0.12$). LcSSc vs HC showed subtle separation of borderline significance ($Q^2 \approx 0.18$; $p = 0.051$), and no meaningful discrimination between LcSSc and dcSSc was found. Nevertheless, given the limited sample size, particularly in the dcSSc subgroup, these findings should be interpreted as exploratory and require external validation.

Serotypes of systemic sclerosis

Although FA alterations were evident in both anti-centromere and anti-Scl-70 positive patients, no individual FA differentiated between these subgroups, suggesting a shared lipidomic phenotype across immunological subsets of systemic sclerosis.

It is known that the presence of anti-centromere antibodies is strictly correlated with LcSSc type, whereas anti-Scl-70 patients may present a broader spectrum of symptoms (mainly associated with dcSSc type) (Cavazzana, 2022, Żebryk 2023). To explore further, we tested for differences in FA concentrations between LcSSc and dcSSc in patients positive for Scl-70. The nominal significant difference was reached for isoC14, C16, C18:1, anteiso C19, C19:1, and MUFA, but none remained significant after correction (Supplementary Table 5).

Organ involvement

We next explored potential metabolomic biomarkers associated with specific clinical manifestations and organ involvement in systemic sclerosis.

As the data did not follow a normal distribution, associations between FA

concentrations and modified Rodnan Skin Score were evaluated using Spearman's rank correlation analysis. C18:1 and MUFA showed nominal significance ($p < 0.05$), but no statistically significant correlations were identified after adjustment for multiple testing, and this observation should be interpreted as exploratory (Supplementary Table 6).

Furthermore, no differences in fatty acid profiles were observed with respect to interstitial lung disease (ILD), acroosteolysis, telangiectasias, arthralgia, myalgia, active digital ulcers, or gastrointestinal involvement. Analyses of cardiac involvement, renal involvement, and pulmonary arterial hypertension (PAH) were not performed due to insufficient sample sizes in these subgroups.

Disease progression

Next, we studied the profiles of patients who presented with disease progression. Progressive SSc tends to have lower FAs concentrations. Although some fatty acids showed nominal significance ($p < 0.05$), none remained significant after controlling for FDR, indicating that these findings should be interpreted as exploratory (Supplementary Table 7)

Discussion

The main achievement of this work is creation of an FA profile for systemic sclerosis, its comparison with healthy controls and another rheumatological condition (rheumatoid arthritis), together with the identification of potential diagnostic and prognostic biomarkers.

In this targeted lipidomic study, univariate analysis demonstrated that SSc is associated with broad reduction of FA concentrations compared with RA patients and HC. Therefore, multivariate assessment was performed. PCA did not show clear separation between SSc, RA and control. SSc patients demonstrated greater intragroup variability which may correspond to its known clinical heterogeneity. RA presented the most homogenous group. The loadings were primarily driven by PUFAs and BCFAs, which can potentially be linked to inflammatory processes and gut microbiome disturbances. The PLS-DA model of SSc and HC profiles showed limited performance, as we speculate was due to the diversity of SSc patients. On the other hand, multivariate analyses of SSc vs RA showed better classification potential, which strengthens the concept of distinct underlying pathologies that may require specific therapeutic approaches and highlights the need for further studies including patients with other connective tissue diseases, which may lead to improvements in rheumatological diagnostics.

To investigate specific biomarkers univariate analyses with receiver operating characteristics were performed. SSc vs HC comparisons detected multiple significant differences and several metabolites of moderate diagnostic potential (anteiso C15, C15, iso C16, C17, ALA, LA, C20, C22, C24 and SFA, n-6 PUFA, total PUFA, ECFA, OCFA, iso BCFA, VLSCFA, total FAs). Potential biomarkers for RA vs HC were distinct from SSc vs HC (C15, iso C17, EPA, DHA, C25, n-3 PUFA, and n-6/n-3 PUFA ratio). In accordance with results from PLS-DA (SSc and RA), a vast panel of FA showed differentiating potential in SSc vs RA ROC tests.

FA alterations were present in both active and non-active SSc. In our study, FA depletion was more pronounced in patients with active SSc. Further, some metabolites (mainly PUFA and BCFA) showed discriminatory active vs non-active disease performance in ROC analysis suggesting potential exploratory biomarker value. Differences between clinical subtypes (lcSSc vs dcSSc) were modest, and this aspect should be explored in further research. In our study lcSSc patients had more pronounced alterations than dcSSc patients. No association with disease progression, serotype or organ involvement remained significant after correction for multiple testing, therefore cautious interpretation and further study on bigger groups are needed.

Although available research on FA disturbances in SSc remains limited, we compared our results to metabolomic research performed so far. Studies based on serum lipidomics using the LC-MS technique align with our findings, reporting decreased concentrations of multiple FAs (18, 19). Our observation of elevation of certain FA concentrations in RA serum is also in accordance with metabolomic studies on RA (20).

Observed generalised FA depletion in SSc may reflect combined enhanced lipid metabolism and reduced supply. Circulating free FAs derive from adipose tissue lipolysis, dietary intake (including contributions from gut microbiota), and endogenous synthesis. Various gastro-intestinal complications, including gut microbial imbalance, are highly prevalent in SSc, and significantly hamper adequate intake of essential fatty acids, including BCFA and PUFA (21, 22). Therefore, malnutrition is commonly observed (23). Similarly, the diagnosis of RA also predisposes to nutritional deficits, which are also associated with disease activity (24). In both our studied groups, malnutrition was significantly present (40.7% of SSc and 13.9% of RA patients), yet we detected opposite lipid aberrations. Independent of nutritional status, atrophy of adipose tissue is a characteristic feature of SSc, and growing data suggest that lipolysis can enhance tissue fibrosis (25). However, the observed depletion of serum FA contrasts with the expected increase associated with enhanced adipose tissue lipolysis, suggesting more complex alterations in FA metabolism in SSc. Therefore the correlation of atrophy of adipose tissue and serum FA profile in SSc patients remains to be determined.

FAs are also used in multiple signalling pathways, including vascular and immune system regulation – the two major pathomechanisms of SSc. Reduced concentrations of some LCFAs, especially ARA, may result from the overproduction of their derivatives (prostaglandins and thromboxanes) as a reaction to ischaemia after RP. Their imbalance in SSc has been studied (26, 27). This phenomenon may partially correspond to our findings of reduced FAs concentrations observed in lcSSc patients, a subgroup characterised by more severe RP, a higher incidence of PAH type 1, and a greater prevalence of other typically vascular complications, such as telangiectasias. Furthermore, certain lipid biomarkers associated with PAH, including fatty acyl esters and hydroxy FAs, selected eicosanoids and steroid hormones, have been presented (28). This research demonstrated the potential of lipids not only to differentiate SSc patients suffering from PAH and those without this complication, but also to distinguish SSc-PAH from idiopathic PAH. In our study patients with diagnosed PAH were underrepresented, precluding statistical analysis.

The second substantial pathomechanism of SSc is autoimmunity (29). Lipids can partially modulate the autoaggression at multiple stages (30, 31). In contrast to their well-characterised derivatives like leukotrienes (32), the role of FAs themselves is still being explored (33). Notably, FA classes most specific for active SSc according to our results (PUFA and BCFA), were implicated in the immune response via T-lymphocytes and macrophages (34, 35). The general FA depletion detected in our cohort may reflect a link between FAs and immune dysregulation in SSc. Further study is needed to explore whether observed patterns are rather primary phenomena disturbing the immune system or secondary events reflecting compensatory mechanisms. Interestingly, there was no difference between specific serotypes, which supports the hypothesis that, although specific, production of antibodies in SSc is likely a secondary event (36).

Major limitation of our study is that the research did not focus specifically on organ involvement - screening and evaluation were performed according to the current standard of care and differed among patients. Therefore, even though we report no biomarkers for specific complications, these data should

be interpreted with caution. Another limitation of our study is the diversity of the studied patients, clinical subtypes, organ involvement, significant comorbidity they suffered from and therapies they were undergoing. Due to the rarity of SSc and the urge for immediate implementation of treatment, the recruitment of a group consisting only of untreated patients is beyond a single-centre study. For the very same reason patients were included regardless of disease duration. We did not perform measurements of oxylipins, and other pro- and anti-inflammatory FA derivatives, but we find it important to investigate this in future studies on bigger groups of patients. As this is single-centre study, and SSc is a rare condition, groups of patients were limited.

Nevertheless, we believe that our findings provide new insight into SSc pathology that will help to understand its diversity. Although it is still distant from clinical practice, implementing FAs as biomarkers could lead to improvement in the standard of rheumatological care. Moreover, further research is needed to determine whether modulation of SSc lipid metabolism (through dietary, pharmacological or microbiome targeted intervention) can influence the course of the disease.

Conclusions

In conclusion, SSc is characterised by a global FA depletion, distinct from RA and HC. These aberrations are associated with disease activity and likely correspond to systemic metabolic changes that integrate vascular, immunomodulatory, and fibrotic pathways, as well as consequences of intestinal microbiome alterations. In this light, lipidomics emerges as a promising but exploratory tool for biomarker discovery, and further studies including a multiomic approach, other rheumatological conditions and larger patient cohorts are needed.

Abbreviations

SSc
systemic sclerosis
lcSSc
limited cutaneous systemic sclerosis
dcSSc
diffuse cutaneous systemic sclerosis
RA
rheumatoid arthritis
HC
healthy control
RP
Raynaud's phenomenon
PAH
pulmonary arterial hypertension
ILD

interstitial lung disease
HAQ
DI-Health Assessment Questionnaire Disability Index
DAS
28-Disease Activity Score (28 joints)
BMI
body mass index
SGA
7-Subjective Global Assessment
FFQ
Food Frequency Questionnaire
CT
computed tomography
MRI
magnetic resonance imaging
FA
fatty acid
FAME
fatty acid methyl ester
GC
MS-gas chromatography-mass spectroscopy
LC
MS-liquid chromatography-mass spectroscopy
AdA
adrenic acid
ALA
 α -linolenic acid
ARA
arachidonic acid
BCFA
branched-chain fatty acids
DGLA
dihomo- γ -linolenic acid
DHA
docosahexaenoic acid
DPA
docosapentaenoic acid
ECFA
even-chain fatty acids
EDA

eicosadienoic acid
EPA
eicosapentaenoic acid
ETA
eicosatetraenoic acid
LA
linoleic acid
MUFA
monounsaturated fatty acids
MU
VLCFA-monounsaturated very-long-chain fatty acids
OCFA
odd-chain fatty acids
PUFA
polyunsaturated fatty acids
SFA
saturated fatty acids
VLSCFA
very-long-chain saturated fatty acids
VLOCFA
very-long-chain odd-chain fatty acids
SD
standard deviation
FDR
false discovery rate
IQR
interquartile range
BH
Benjamini-Hochberg correction
NS
non-significant
PCA
principal component analysis
PLS
DA-partial least squares discriminant analysis
ROC
receiver operating characteristics
AUC
area under the curve
CI

confidence interval
VIP
variable importance in projection
CV
cross-validation
LV
latent variable

Declarations

Acknowledgements & declarations

We would like to thank all coworkers from our departments.

Ethics approval and consent to participate

Our research was conducted in accordance with the World Medical Association's Declaration of Helsinki principles and was approved by the Local Bioethics Committee at the Medical University of Gdańsk (decisions: NKBBN/527/2021, NKBBN/527–772/2021, and KB/527 – 162/2024). Patients were recruited at the Department of Rheumatology, Clinical Immunology, Geriatrics and Internal Medicine of University Clinical Centre in Gdańsk, and all subjects provided written consent to participate in the project.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Author Contribution

ZG designed the project, examined patients and collected clinical data, participated in sample collection, performed statistical analysis, and drafted the manuscript. ŻS, ZZ designed the project, examined patients and collected clinical data, revised the manuscript. AW examined patients and collected clinical

data, revised the manuscript. AM designed the project, performed biochemical analysis, and revised the manuscript. MC and TŚ revised the manuscript. All authors reviewed the manuscript.

Data Availability

The datasets analysed during the current study are available from the corresponding author on reasonable request.

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Figures

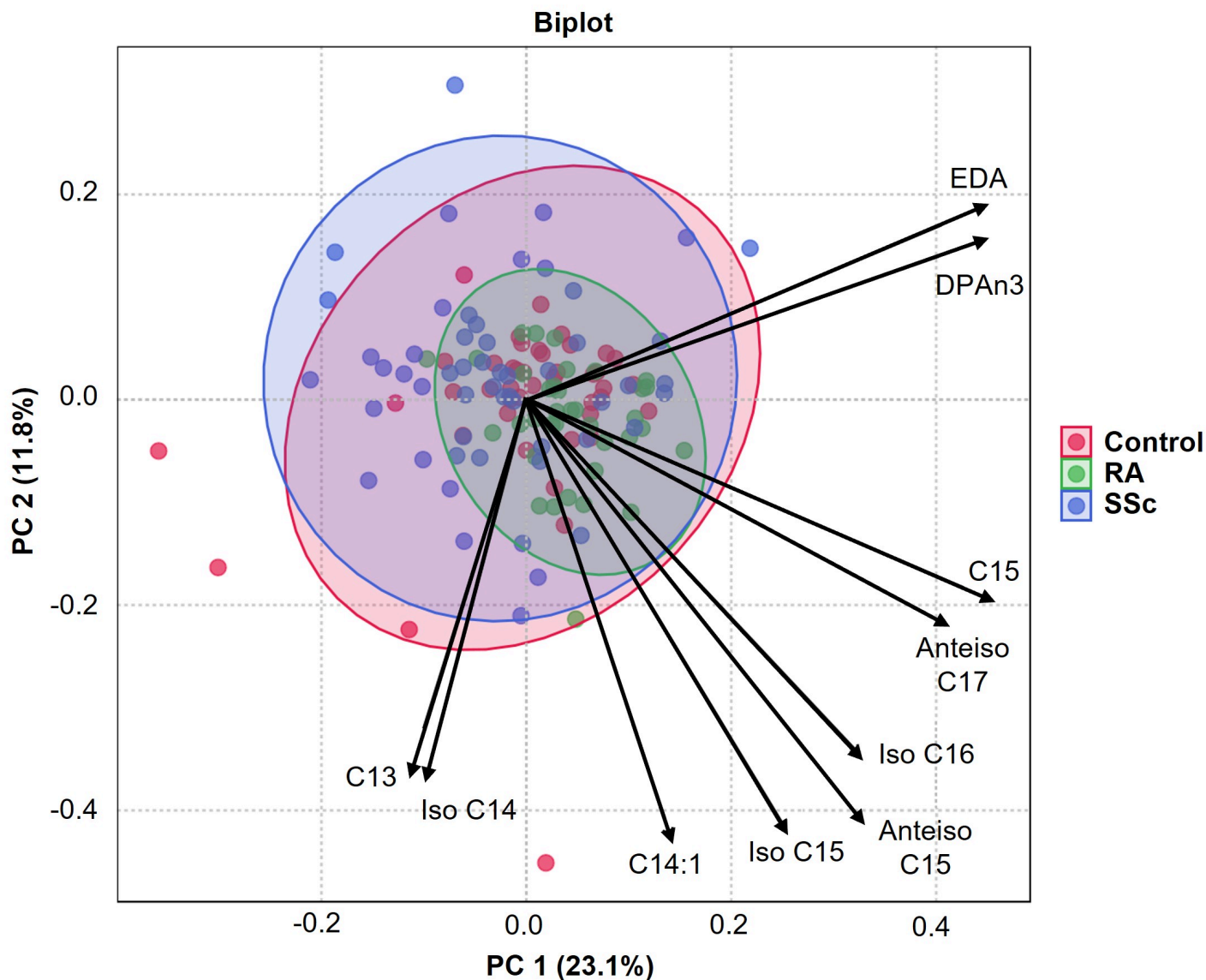


Figure 1

Principal component analysis of fatty acid profiles in systemic sclerosis, rheumatoid arthritis, and healthy controls.

PC - principal component, SSc - systemic sclerosis, RA - rheumatoid arthritis, EDA - eicosadienoic acid, DPA - docosapentaenoic acid. The first two principal components explained 34.9% of total variance (PC1: 23.1%; PC2: 11.8%). Each point represents an individual sample, and ellipses indicate group dispersion at the 95% confidence. Loading vectors illustrate the contribution of individual fatty acids to the sample distribution

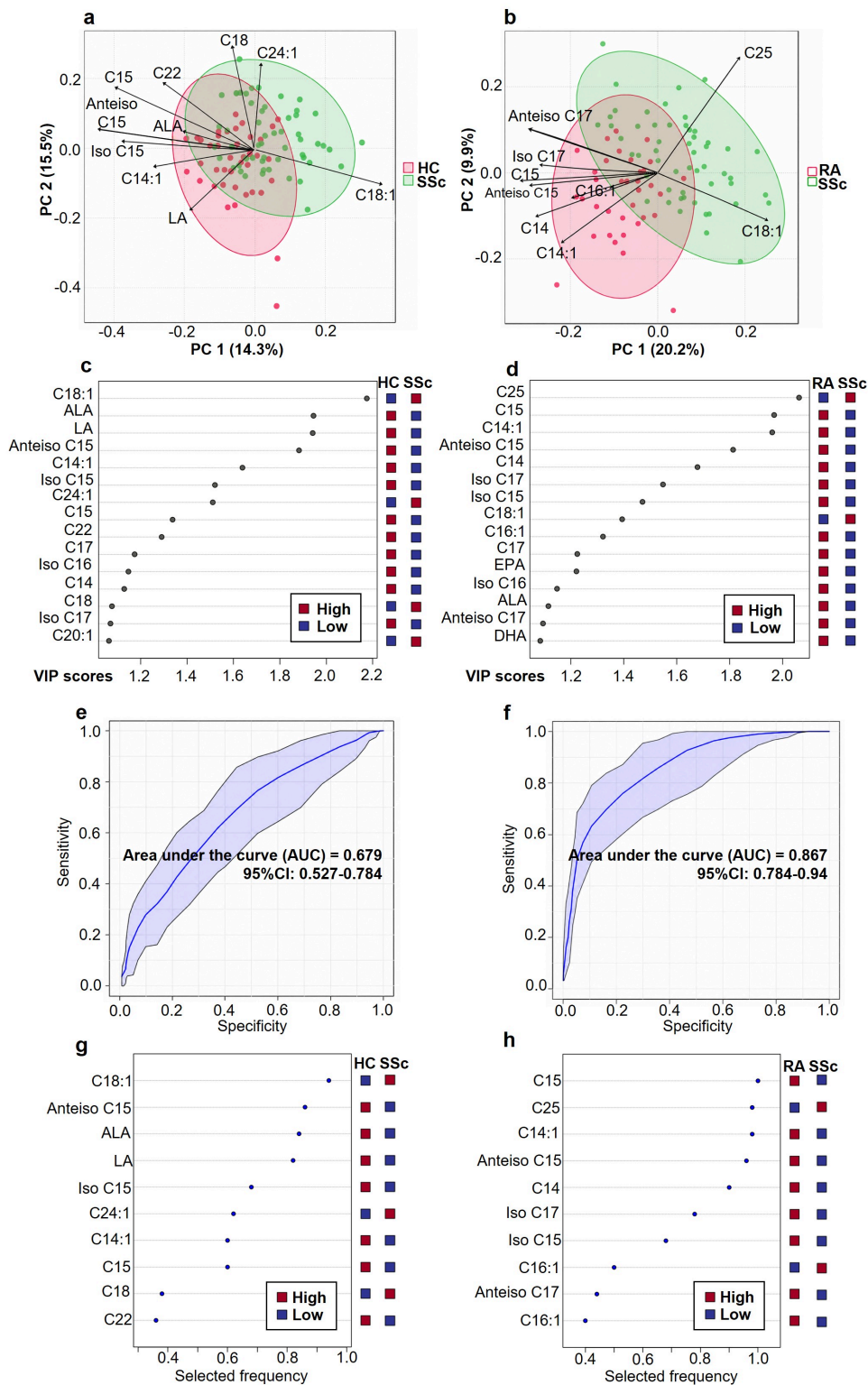


Figure 2

Multivariate analysis of systemic sclerosis vs healthy control and SSc vs rheumatoid arthritis models.

a. PLS-DA score plot for SSc vs HC comparison. Cross-validation (CV) indicated four optimal components, predictive performance was limited (accuracy = 0.72, $R^2 = 0.55$; $Q^2 = 0.21$), permutation testing (1000 permutations) confirmed statistical significance ($p < 0.001$) (Supplementary Figure 3).

b. PLS-DA score plot for SSc vs RA. CV indicated three components as optimal, predictive performance was moderate (accuracy = 0.83, $R^2 = 0.61$; $Q^2 = 0.40$), permutation testing (1000 permutations) confirmed statistical significance ($p < 0.001$) (Supplementary Figure 4).

c and d. VIP scores for SSc vs HC and SSc vs RA models, respectively, indicating fatty acids most important for group discrimination.

e. ROC curve for the SSc vs HC model, showing limited classification performance (AUC = 0.68; 95% CI 0.53–0.78).

f. ROC curve for the SSc vs RA model, showing limited classification performance (AUC = 0.87; 95% CI 0.75–0.94).

g and h. Feature selection frequency plots presenting the most frequently selected FAs in classification models for SSc vs HC and SSc vs RA comparisons.

SSc - systemic sclerosis, HC - healthy control, RA - rheumatoid arthritis, PLS-DA - partial least squares discriminant analysis, ROC - receiver operating characteristics, VIP - variable importance in projection, AUC - area under the curve, CI - confidence interval, CV - cross-validation, ALA - α -linolenic acid, LA - linoleic acid, EPA - eicosapentaenoic acid, DHA - docosahexaenoic acid, FA - fatty acid

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