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Title: European Multicentre Study validates Enhanced Liver Fibrosis Test as Biomarker of Fibrosis in Systemic Sclerosis

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Keywords: Systemic Sclerosis, scleroderma, enhanced liver fibrosis test, ELF test, fibrosis, biomarker, outcome measure.

Key messages:

- Second cohort multicentre study validates Enhanced Liver Fibrosis test and its components as biomarkers of fibrosis in Systemic Sclerosis
- Enhanced Liver Fibrosis score is an independent biomarker of skin and lung

fibrosis in Systemic Sclerosis

ABSTRACT

Objectives: To validate Enhanced Liver Fibrosis (ELF) test and its components - amino-terminal pro-peptide of procollagen-III (PIIINP), tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) and hyaluronic acid (HA) - as biomarkers of fibrosis in systemic sclerosis (SSc) in an independent, international, multicentre cohort.

Methods: Two hundred fifty-four SSc patients from six Rheumatology Centres were included. Sera were collected and stored according to EUSTAR biobanking recommendations and analysed through automated high throughput diagnostics. Statistical analysis was performed with SPSS software.

Results: Two hundred forty-seven SSc patients (mean age 55.7±13.9 years, 202 F) were analysed. ELF score, TIMP-1 and PIIINP levels were higher in males ($p=0.0197$, $p=0.0107$, $p=0.0108$ respectively) and in dcSSc ($p=0.001$, $p=0.0008$, $p<0.0001$ respectively). ELF score and the single markers significantly correlated with modified Rodnan skin score (mRSS) ($r=0.37$, $p< 0.0001$), disease activity and severity ($p< 0.0001$ for all markers, except for HA $p=0.0001$) and inversely with FVC% (TIMP-1, $r=-0.21$, $p=0.0012$; PIIINP, $r=-0.26$, $p=0.0001$), TLC% (ELF score, $r=- 0.20$, $p=0.0036$; TIMP-1, $r=-0.32$, $p<0.0001$; PIIINP, $r=-0.28$, $p<0.0001$), DLCO% ($p<0.0001$ for all markers, except for HA $p=0.0115$). Multivariate analysis indicated that age ($p<0.001$), mRSS ($p<0.001$) and DLCO% ($p=0.005$) were independently associated with ELF score.

Conclusion: Between the first and this validation studies the value of ELF score as independent marker of skin and lung involvement in SSc is confirmed in four hundred and fifty-seven patients. A longitudinal study is on-going to identify a SSc specific algorithm with predictive value for skin and lung progression.

INTRODUCTION

Systemic sclerosis (SSc) is characterised by tissue fibrosis whose pathogenesis is partially elucidated (1). A biomarker of overall fibrosis and fibrogenesis has been an unmet need for long time and would aid to fulfil the need of a molecular classification of patients with SSc, and their stratification for the level of profibrotic activity (2-4). For these purposes, we have recently proposed the Enhanced Liver Fibrosis (ELF) test, a serum test originally developed and validated on chronic liver fibrosis (CLF) diseases (5) and, more recently, shown to be a marker of overall fibrosis in SSc mainly reflecting skin and lung involvement (6). It is an algorithm including the serum concentrations of amino-terminal pro-peptide of procollagen type III (PIIINP), tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) and hyaluronic acid (HA), all markers known to be involved in the process of fibrogenesis and/or extracellular matrix remodeling (6). In a single centre cohort of two-hundred ten SSc patients, none of the three biomarkers was found significantly associated with any vascular manifestation of the disease (6). This study aimed to determine the value of ELF score and its single analytes in an independent multicentre cohort of SSc patients.

METHODS

Patients and sera samples

Two hundred fifty-four SSc patients from six European Rheumatology centres were included in this study. Ethical approval was obtained by the Leeds Teaching Hospital Trust Committee (LTHT REC 10/H1306/88); clinical data and serum samples collection was approved by each local institutional ethics committee (Comitato Etico di Area Vasta Centro, Toscana, Azienda Ospedaliera Universitaria Careggi, Firenze; Comite de

protection des personnes Ile de France 3; Comitato Etico Fondazione Policlinico Universitario A. Gemelli, Università Cattolica del Sacro Cuore, Roma; Etikkommission Kanton Zürich; NRES Committee London-Hampstead). All patients fulfilled the 2013 ACR/EULAR classification criteria for SSc (7). Clinical data were collected at time of sampling and included a wide set of variables as previously described (6). Serum samples were collected and stored in each participant centre according to EUSTAR biobanking recommendations (8). The two hundred fifty-four sera were analysed employing a high-throughput in vitro diagnostic (Siemens Alpha-Centaur). Cut-off values of ELF test were applied in line with recommendations from Siemens Healthcare Diagnostics (<7.7 = no-mild fibrosis; ≥7.7 to <9.8 = moderate fibrosis; ≥9.8 to <12 = severe fibrosis/cirrhosis; ≥12 = cirrhosis).

Statistics

Statistical analysis was performed as previously described using SPSS software for Mac V.24.0 (6). Items found to show a significant correlation ($p < 0.05$) in univariate analysis were then tested in stepwise regression analysis to evaluate which baseline variables were independently associated with the ELF score.

RESULTS

Two hundred fifty-four SSc patients were originally included in this study. Seven patients had viral hepatitis positivity in absence of cirrhosis/liver fibrosis; one more patient, Hepatitis C Virus positive, had a history of liver transplantation. Fourteen patients were anti-mitochondrial antibody positive, seven of them had associated primary biliary cholangitis (PBC). Eleven patients had SSc in overlap with another rheumatic disease (three with Sjögren's syndrome, four with dermatomyositis/polymyositis, one with

systemic lupus erythematosus, one with rheumatoid arthritis, one with MPO positive vasculitis, one with mixed connective tissue disease). To investigate whether liver disease could influence the ELF score, we first analysed the difference between the infectious viral hepatitis group (n = 8), the PBC group (n = 7) and the rest of the cohort. Mean ELF score and age were not significantly different ($p > 0.05$) between the two disease groups and the rest of the cohort with unknown liver condition. However, based on the normal mRSS (= 0), absence of SSc-related fibrosis signs, also including chest HRCT scan, ELF score was unexpectedly high (10.89 and 10.85 respectively) in two lcSSc patients who had associated PBC, suggesting that cholangitis was influencing the fibrotic score. We therefore excluded from the subsequent analyses the seven patients with diagnosis of PBC.

Clinical characteristics of the multicentre cohort of two hundred forty-seven SSc patients are quite similar to those of the original cohort in which the ELF test has been originally tested in the context of SSc (6) (supplementary data). Two-hundred two (81.8%) were women, 80 (32.4%) were classified as having diffuse cutaneous SSc (dcSSc). One-hundred and four patients (42.1%) were positive for anticentromere antibody, and fifty-seven (23.1%) were antitopoisomerase-1 positive. As in the first study, this multicentre cohort of 247 SSc patients was heterogeneous with regard to organ involvement, disease activity (9) and severity (10). Total Medsger's severity score, sum of the nine severity scores, ranged between 1 and 19. The EScSG-AI ranged between 0 and 7.5. At the time of sampling, 110 patients (44.5%) were taking immunosuppressive/anti-rheumatic drugs including corticosteroids and/or cyclophosphamide, mycophenolate, azathioprine, methotrexate, hydroxychloroquine. Additional clinical details are reported in supplementary data.

The ELF score ranged from 6.2 to 12.1 with a mean of 8.9 (±1.1). Two hundred and nineteen (88.7%) patients had an abnormal ELF test (≥7.7). Distribution of age, mRSS, EScSG-AI and Medsger's total severity score among the four ELF reference ranges is shown in Figure 1. All four variables were significantly different across the first three groups ($p<0.05$) by ANOVA (Figure 1, A) and Kruskall–Wallis (Figure 1, B, C and D) and tests. After Dunn's multiple comparison post-test correction, mRSS and EScSG-AI showed no significant difference between <7.7 and 7.7–9.8 range groups, whereas Medsger's total severity score was not significantly different between 7.7–9.8 and 9.8–12 range groups ($p > 0.05$). Age remained significant between all three groups.

Table 1 shows correlation of ELF and its analytes with clinical characteristics. ELF score, TIMP-1, PIIINP levels were higher in males than in females ($p = 0.0197$, $p = 0.0107$, $p = 0.0108$ respectively), in dcSSc than in limited cutaneous SSc (lcSSc) patients ($p = 0.001$, $p = 0.0008$, $p < 0.0001$ respectively) and showed no correlation with disease duration ($p > 0.05$). Furthermore, no statistically significant difference was found between patients with and without digital ulcers and among different videocapillaroscopy patterns ($p > 0.05$).

Regarding the therapy at the time of sampling, ELF score, TIMP-1 and HA levels were not significantly different ($p > 0.05$) between patients that were or were not on immunosuppressive/antirheumatic treatment. By contrast PIIINP level was significantly higher in the group of patients on immunosuppressive/antirheumatic therapy [median (range) = 7.19 (1.1 - 34.63) vs 5.83 (0.91 - 21.27), $p = 0.0098$].

TIMP-1, PIIINP, HA and ELF score as markers of skin and lung fibrosis

Confirming results of the previous study, ELF score significantly correlated with the degree of skin involvement as assessed by modified Rodnan skin score (mRSS) ($r = 0.37$, $p <$

0.0001) and skin severity according to Medsger's severity scale ($r = 0.31$, $p < 0.0001$), with PIIINP, among its components, showing the most significant correlation ($r = 0.30$, $p < 0.0001$ for both) (Table 1). TIMP-1 and ELF score were significantly higher in patients with flexion contractures ($p = 0.0012$ and $p = 0.04$). With respect to lung involvement all markers were significantly higher in patients with dyspnoea (TIMP-1 and ELF score $p < 0.0001$; PIIINP = 0.0004; HA $p = 0.0005$) and correlated with NYHA class severity ($p < 0.0001$ for all). TIMP-1 and PIIINP levels were higher in patients with lung fibrosis assessed by chest high resolution computed tomography (HRCT) scan ($p = 0.0047$ and $p = 0.0308$ respectively). All markers inversely correlated with DLCO% ($p < 0.0001$ for all, except for HA $p = 0.0115$). TIMP-1 and PIIINP inversely correlated with FVC% ($r = -0.21$, $p = 0.0012$; $r = -0.26$, $p = 0.0001$ respectively) and TLC% ($r = -0.32$, $p < 0.0001$; $r = -0.28$, $p < 0.0001$ respectively). ELF score inversely correlated with TLC% ($r = - 0.20$, $p = 0.0036$). HA and, subsequently, ELF score, were significantly higher in patients with PAH ($p = 0.0001$ and $p = 0.0005$ respectively). Significant correlation was found between ELF score, TIMP-1, PIIINP, HA and total disease severity (10) and activity (9) ($p < 0.0001$ for the first three markers, $p = 0.0001$ for the fourth one) confirming results of the original study (6).

Independent associations of ELF score, PIIINP, TIMP, HA

Clinical variables found statistically significant in univariate analysis were included in multiple regression analysis. When ELF score was set as the dependent variable a model including age (standardized coefficient beta = 0.482, $p < 0.001$), mRSS (standardized coefficient beta = 0.279, $p < 0.001$), and DLCO % (standardized coefficient beta = - 0.199, $p = 0.005$) as predictors was obtained. Stepwise regression modelling with each one of the

remaining markers of ELF test as the outcome variable showed that TIMP-1 was independently associated with gender (standardized coefficient beta = 0.291, p = 0.001), FVC% (standardized coefficient beta = - 0.273, p = 0.002), age (standardized coefficient beta=0.243, p=0.003), ESR (standardized coefficient beta = 0.204, p = 0.012); PIIINP was independently associated with mRSS (standardized coefficient beta = 0.409, p < 0.001) and DLCO% (standardized coefficient beta = -0.23, p = 0.008); HA was independently associated with age (standardized coefficient beta = 0.445, p < 0.001) and mRSS (standardized coefficient beta = 0.244, p < 0.001).

DISCUSSION

This is the first study assessing the value of the ELF score in a multicentre SSc patients cohort. The data presented in this study confirm in a second independent multicentre cohort the results of our original study (6) and indicate that the ELF score and its components are markers of fibrosis in SSc patients and are independently associated with skin and lung involvement. Indeed, ELF score and the single markers were significantly higher in patients with dcSSc, severe skin involvement, fibrosis on chest HRCT scan, abnormal pulmonary function and DLCO. The higher PIIINP level of patients on immunosuppressive therapy is consistent with published data and may reflect a higher level of ECM turnover (11). By contrast, ELF and the single markers showed no difference in patients with digital ulcers or with specific videocapillaroscopy patterns compared to patients without signs of severe vasculopathy. The significant correlation of HA and, subsequently, of ELF score, with PAH, not found in the first study, might reflect the role of HA in pulmonary vascular remodeling (12, 13).

As in the first study, here we analysed, and subsequently validated, ELF test in a cohort of SSc patients. Merging the two cohort studies results, these data are now confirmed in four hundred and fifty-seven patients enrolled in seven different SSc centres. The significant correlation with age in both studies is driven by HA and warrants a large healthy controls cohort assessment of ELF score in order to develop a new algorithm corrected by age. Furthermore, the score was originally developed on CLF diseases and a new score, SSc-specific, is needed based on the weight and statistical significance of the single biomarkers in this condition. Indeed, the ELF score brackets have been determined as a best fit with the semiquantitative scoring of fibrosis in liver biopsies (5). The lack of significant difference in mRSS between the first two brackets reflects the need for a design of a SSc specific score. Limitation of this study is the cross-sectional nature that was not able to assess the sensitivity to change of these biomarkers and their predictive value for progression of skin and lung fibrosis. Studies addressing these aspects are currently on going.

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Conflicts of interest statement: Prof. Dr. O. Distler had consultancy relationship and/or has received research funding from AnaMar, Bayer, Boehringer Ingelheim, Catenion, CSL Behring, ChemomAb, Roche, GSK, Inventiva, Italfarmaco, Lilly, medac, Medscape, Mitsubishi Tanabe Pharma, MSD, Novartis,Pfizer, Sanofi, and UCB in the area of potential treatments of

scleroderma and its complications. In addition, Prof. Distler has a patent mir-29 for the treatment of systemic sclerosis licensed. The real or perceived potential conflicts listed above are accurately stated. The other authors declare no conflicts of interest.

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Table 1. Correlation coefficient (r) between ELF score, PIIINP, TIMP-1, HA serum levels and clinical variables

	ELF score	PIIINP (ng/mL)	TIMP-1 (ng/mL)	HA (ng/mL)
Serum values (median, range)	8.85, 6.22-12.12	6.44, 0.91-34.63	221, 19.09-595.4	36.34, 4.52-355.5
	r	r	r	r
Age	0.40****	0.07	0.25****	0.51****
DD RP	0.02	-0.21**	-0.03	0.05
DD 1st non RP	0.10	-0.05	0.05	0.12
mRSS	0.37****	0.30****	0.20**	0.18**
Hb	-0.21***	-0.08	-0.006	-0.22***
ESR	0.31****	0.12	0.26****	0.28****
CRP	0.18**	0.27****	0.21**	0.19**
FVC%	-0.13*	-0.26****	-0.21**	0.01
TLC%	-0.21**	-0.28****	-0.32****	-0.08
DLCO%	-0.24****	-0.31****	-0.30****	-0.17*
Sev_general	0.31****	0.11	0.12	0.24***
Sev_vascular	0.07	0.07	0.16*	0.02
Sev_skin_	0.31****	0.30****	0.25****	0.19**
Sev_joint/tendon	0.15*	0.13*	0.17**	0.12
Sev_muscle	0.15*	0.15*	0.02	0.08
Sev_GI	0.03	-0.07	0.04	0.05
Sev_lung	0.24***	0.28****	0.3****	0.17**
Sev_heart	0.17**	0.14*	0.15*	0.13*
Sev_kidney	0.07	0.03	0.14*	-0.03
Sev_total	0.34****	0.27****	0.35****	0.25***
EScSG-AI	0.33****	0.29****	0.30****	0.25***

*p<0.05. **p<0.01. ***p<0.001. ****p<0.0001.

DD, disease duration; DLCO, diffusion lung capacity of carbon monoxide; ELF, enhanced liver fibrosis; ESR, erythrocyte sedimentation rate; EScSG-AI, European Scleroderma Study Group–activity index; FVC, forced vital capacity; GI, gastrointestinal; HA, hyaluronic acid; mRss, modified Rodnan skin score; PIIINP, propeptide of procollagen type III; RP, Raynaud's phenomenon; Sev, severity; TIMP-1, tissue inhibitor of matrix metalloproteinase-1.

Figure title.

Figure 1. Distribution of clinical parameters among the Enhanced Liver Fibrosis reference ranges.

Figure legends.

Figure 1. (A–D) Distribution of age (A), modified Rodnan skin score (mRSS) (B), European Scleroderma Study Group–activity index (EScSG-AI) (C) and Medsger's total severity score (D) among the four Enhanced Liver Fibrosis reference ranges. Box plots with upper and lower bars showing minimum and maximum values. Upper, middle and lower lines in the box show 75th, 50th (median) and 25th centiles, respectively. Statistical analysis included analysis of variance (A) and Kruskal–Wallis (B, C and D) tests across the first three groups (p values indicated by continuous lines) as the fourth group (>12) comprised only one patient and it was therefore not statistically evaluable. Dotted lines show significant Bonferroni's (A) and Dunn's (B, C and D) multiple comparison post-test p values between groups. *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$.

Supplementary Data

Table. Clinical features of the 247 SSc patients	
Gender (F/M)	202/45
Age, mean (S.D.), years	55.7 (13.9)
Disease duration from RP, mean (S.D.), years	14.5 (13.7)
Disease duration from first non RP, mean (S.D.), years	9.2 (8.8)
Disease subset (D/L)	80/167
ANA +	244 (98.8%)
ACA +	104 (42.1%)
Anti-topoisomerase I +	57 (23.1%)
mRss, median (range)	3 (0-35)
Raynaud's Phenomenon	247 (100%)
Digital ulcers	50 (20.4%)
Telangectasias	148 (66.7%)
Synovitis	26 (10.6%)
Flexion contractures	79 (32.2%)
Tendon friction rubs	16 (6.5%)
Proximal muscle weakness	16 (6.5%)
Serum CK elevation	15 (6.5%)
Reflux/dysphagia	158 (64.5%)
Early satiety/vomiting	69 (28.3%)
Diarrhoea/constipation/bloating	59 (24.2%)
Dyspnoea	124 (52.5%)
Chest X-ray fibrosis	41 (24.4%)
Chest HRCT fibrosis	70 (39.1%)
Restrictive defect (FVC, DLCO)	47 (20.3%)
Pulmonary hypertension (Doppler Echo)	20 (7.8%)
Confirmed PAH (by RHC)	18 (7.3%)
Palpitations	40 (16.3%)
Conduction defects	8 (3.5%)
SV arrhythmias	3 (1.5%)
V arrhythmias	4 (1.8%)
Diastolic dysfunction	90 (37.7%)
Reduced ejection fraction	9 (3.8%)
Arterial hypertension	42 (17.1%)
Renal crisis	2 (0.8%)
SSc capillary pattern	153 (92.2%)
EScSG-AI	1 (0-7.5)
Sev_general	0 (0-3)
Sev_peripheral vascular	1 (1-3)
Sev_skin	1 (0-3)
Sev_joint/tendon	0 (0-4)
Sev_muscle	0 (0-3)
Sev_GI tract	1 (0-3)
Sev_lung	1 (0-4)

Sev_heart	0 (0-3)
Sev_kidney	0 (0-2)
Sev_total	5 (1-19)
Immunosuppressive/anti-rheumatic therapy	110 (44.5%)

Number in parenthesis refers to percentage of patients with the specific feature among the total patients with the available test results. ANA, antinuclear antibodies; ACA, anti-centromere antibodies; CK, creatine kinase; D, diffuse cutaneous systemic sclerosis; DLCO, diffusion lung capacity of carbon monoxide; EScSG-AI, European Scleroderma Study Group–activity index; FVC, forced vital capacity; GI, gastrointestinal; HRCT, high resolution computed tomography; L, limited cutaneous systemic sclerosis; mRSS, modified Rodnan skin score; PAH, pulmonary arterial *hypertension; RP, Raynaud's phenomenon;* Sev, Severity; SSc, systemic sclerosis.