Title: European multicentre study validates ELF test as biomarker of fibrosis in systemic sclerosis

Giuseppina Abignano[1-3], Jelena Blagojevic[1,2,4], Lesley Anne Bissell[1,2], Raluca B Dumitru[1,2], Sookhoe Eng[1,2], Yannick Allanore[5], Jerome Avouac[5], Silvia Bosello[6], Christopher Denton[7], Oliver Distler[8], Gianfranco Ferraccioli[6], Suzana Jordan[8], Marco Matucci-Cerinic[4], Voon Ong[7], Michael Messenger[9], Michelle Hutchinson[1-2], Maya H Buch[1,2], Paul Emery[1,2], Francesco Del Galdo[1,2]

1. Leeds Institute of Rheumatic and Musculoskeletal Medicine, University of Leeds, Leeds, United Kingdom;

2. NIHR Leeds Biomedical Research Centre, Leeds Teaching Hospitals NHS Trust, Leeds, United Kingdom

3. Rheumatology Institute of Lucania (IReL), Rheumatology Department of Lucania, San Carlo Hospital of Potenza and Madonna delle Grazie Hospital of Matera, Potenza, Italy

4. Department of Experimental and Clinical Medicine, Division of Rheumatology, University of Florence, Florence, Italy

5. Department of Rheumatology, University of Paris Descartes, Paris, France

6. Division of Rheumatology, Fondazione Policlinico Universitario Agostino Gemelli, Rome, Italy

7. Department of Rheumatology, University College London, Royal Free Hospital, London, United Kingdom

8. Department of Rheumatology, University Hospital Zurich, Zurich, Switzerland

9. NIHR Diagnostic Evidence Co-operative, Leeds Teaching Hospitals NHS Trust, Leeds, United Kingdom

Funding: GA was funded by the "Fondazione Italiana per la Ricerca sull'Artrite (FIRA Onlus)"

Abstract

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

Methods: Two hundred fifty-four SSc patients from six European Rheumatology Centres were included in this study. Clinical data were collected at time of sampling. Serum samples were collected and stored according to EUSTAR biobanking recommendations. Sera were analysed employing a high-throughput in vitro diagnostic (Siemens Alpha-Centaur). Statistical analysis was performed with SPSS software for Mac V.24.0.

Results: Two hundred forty-seven SSc patients were analysed. Patients had a mean age 55.7 ± 13.9 years, and included 202 females and 80 patients with diffuse cutaneous SSc (dcSSc). ELF score, TIMP-1, PIIINP levels were higher in males and in dcSSc. ELF score and the single markers significantly correlated with the degree of skin involvement and inversely correlated with FVC%, TLC % and DLCO%. Concordantly, all markers significantly correlated with skin, lung and total Medsger's disease severity score. Multivariate analysis indicated that age, mRSS and DLCO% were independently associated with ELF score.

Conclusions: This study confirms in a second independent multicentre cohort the value of ELF score as independent marker of skin and lung involvement in SSc. A longitudinal study paired with analysis of large cohort of healthy controls is currently on going to

identify a SSc specific test with the highest predictive value for skin and lung progression independently of age and gender.

INTRODUCTION

The Enhanced Liver Fibrosis (ELF) test is a serum test originally developed and validated on chronic liver fibrosis (CLF) diseases (1) and, more recently, shown to be a marker of overall fibrosis in systemic sclerosis (SSc) mainly reflecting skin and lung involvement (2). 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 tissue remodeling (2). 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 (2). 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 locally in each participant centre. All patients fulfilled the 2013 ACR/EULAR classification criteria for SSc (3). Clinical data were collected at time of sampling and included a wide set of variables as previously described (2). Serum samples were collected and stored in each participant centre according to EUSTAR biobanking recommendations (4). 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 GraphPad Prism software V.6.0 and SPSS software for Mac V.24.0 (2). 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 cirrhosis. Ten patients had SSc in overlap with another connective tissue disease (three with Sjögren syndrome, four with dermatomyositis/polymyositis, one with systemic lupus erythematosus), rheumatoid arthritis (one patient), MPO positive vasculitis (one patient). One patient fulfilled SSc classification criteria being diagnosed with mixed connective tissue disease. To investigate whether liver disease could influence the ELF score, we first analysed the difference between viral hepatitis infection group (n=8), 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 not known 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 cirrhosis was influencing the fibrotic score. We therefore excluded from the subsequent analyses the seven patients with diagnosis of PBC. Table 1 shows the clinical characteristics of the multicentre cohort of two hundred forty-seven SSc patients. They are quite similar to those

of the original cohort in which the ELF test has been originally tested in the context of SSc (2). 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 (5) and severity (6). 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.

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 Kruskall–Wallis (figure 1B, C and D) and ANOVA (figure 1A) 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 2 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).

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=0.34, p<0.0001), with PIIINP, among its components, showing the most significant correlation (r=0.30, p<0.0001 for both) (Table 2). 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 (6) and activity (5) (p<0.0001 for the first three markers, p=0.0001 for the fourth one) confirming results of the original study (2).

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 out 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= - 0199, 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); 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 that the ELF score and its components are markers of fibrosis in SSc patients and are independently associated with skin and lung involvement. 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 (7, 8).

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. 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. Future studies will need to address all these aspects.

REFERENCES

1. Rosenberg WM, Voelker M, Thiel R, et al. Serum markers detect the presence of liver fibrosis: a cohort study. Gastroenterology 2004;127:1704-13.
2. Abignano G, Cuomo G, Buch MH, et al. The enhanced liver fibrosis test: a clinical grade, validated serum test, biomarker of overall fibrosis in systemic sclerosis. Ann Rheum Dis 2014;73:420

3. van den Hoogen F, Khanna D, Fransen J, et al. 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/European League against Rheumatism collaborative initiative. Ann Rheum Dis 2013;72:1747-55.
4. Beyer C, Distler JH, Allanore Y, et al. EUSTAR biobanking: recommendations for the collection, storage and distribution of biospecimens in scleroderma research.
5. Valentini G, Della Rossa A, Bombardieri S, et al. European multicentre study to define disease activity criteria for systemic sclerosis. II. Identification of disease activity variables and development of preliminary activity indexes. Ann Rheum Dis 2001;60:592–8.
6. Medsger TA Jr, Bombardieri S, Czirjak L, et al. Assessment of disease severity and prognosis. Clin Exp Rheumatol 2003;21:S42–6.
7. Aytekin M, Comhair SA, de la Motte C, Bandyopadhyay SK, Farver CF, Hascall VC, Erzurum SC, Dweik RA (2008) High levels of hyaluronan in idiopathic pulmonary arterial hypertension. Am J Physiol Lung Cell Mol Physiol 295:L789–L799.
8. Fenster BE, Lasalvia L, Schroeder JD, Smyser J, Silveira LJ, Buckner JK, Brown KK. Galectin-3 levels are associated with right ventricular functional and morphologic changes in pulmonary arterial hypertension. Heart Vessels 2016;31:939-46.

Table 1. 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%)
Modified Rodnan skin score, 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	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 % of patients with the specific feature among the total patients with available data; D diffuse cutaneous SSc; L limited cutaneous SSc; VE very early SSc; ANA antinuclear antibodies; ACA anti-centromere antibodies; HRCT high resolution computed tomography; FVC forced vital capacity; DLCO diffusion lung capacity of carbon monoxide; SV supra-ventricular; V ventricular; EScSG-AI European Scleroderma Study Group-Activity Index;

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 artery hypertension; RP, Raynaud's phenomenon; SSc, systemic sclerosis.

Table 2. Correlation coefficient (r) between ELF score, PIIINP, TIMP-1, HA serum levels and clinical variables

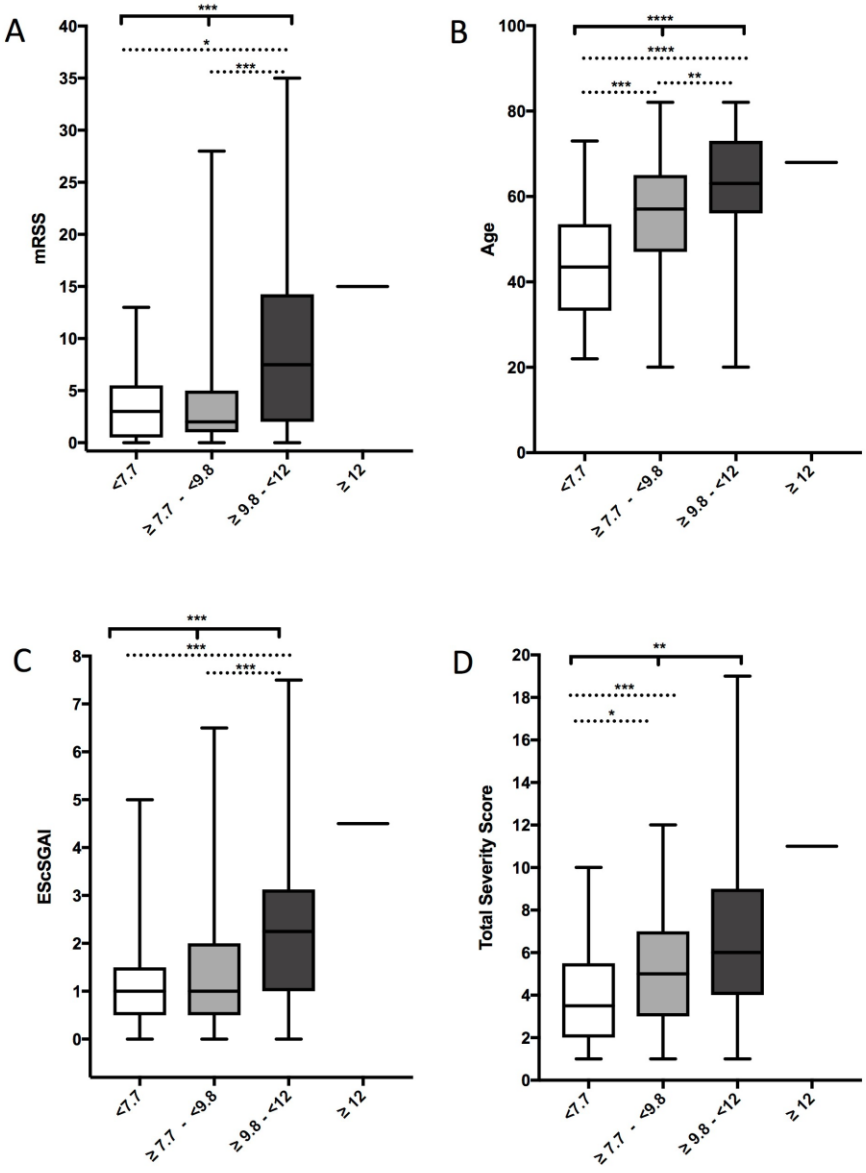
	ELF score	PIIINP (ng/mL)	TIMP-1 (ng/mL)	HA (ng/mL)
Serum values (median, range)	8.85, 6.22-12.2 r	6.44, 0.91-34.63 r	221, 19.09-595.4 r	36.34, 4.52-355.5 r
Age	0.40****	0.07	0.25****	0.51****
DD RP	0.02	-0.21**	-0.03	0.05
DD 1st non RP	0.10	-0.05	0.05	0.12
mRSS	0.37****	0.30****	0.20**	0.18**
Hb	-0.21***	-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. DLCO%, diffusion lung capacity of value; ELF, enhanced liver fibrosis; activity index; ESR, erythrocyte sedimentation rate; FVC, forced vital capacity; GI, gastrointestinal; HA, hyaluronic acid; HAQ-DI, health assessment

questionnaire– disability Index; mRSS, modified Rodnan skin score; PIIINP, propeptide of procollagen type III; TIMP-1, tissue inhibitor of matrix metalloproteinase-1.

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) ND 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 Dunn's (B, C and D) and Bonferroni's (A) multiple comparison post-test p values between groups. *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$.


A
mRSS
***
*
***
<7.7
≥ 7.7 - <9.8
≥ 9.8 - <12
≥ 12
B
Age
****
****
***
**
C
EScSGAI
***
***
***
D
Total Severity Score
**
***
*