Survival, risk assessment and causes of death in a series of 439 patients with systemic sclerosis

Ph.D. thesis

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CONTENT

ABREVIATIONS

ACA	anticentromere antibody
ACR	American College of Rheumatology
anti-RNAP3	anti-RNA polymerase III antibody
anti-Scl70	antitopoisomerase I antibody
anti-topo I	antitopoisomerase I antibody
ACEi	angiotension convertase inhibitor
AUC	area under the curve
BMI	body mass index
BSA	body surface area
CI	confidence interval
CRP	C reactive protein
dcSSc	diffuse cutaneous systemic sclerosis
DIP	distal interphalangeal
DLCO	diffusing capacity of carbon monoxide
DMARD	disease modifying antirheumatic drugs
ECG	electrocardiogram
ELISA	enzime linked immunsorbent assay
EScSG-AI	European Scleroderma Study Group Activity Index
ESR	erythrocyte sedimentation rate
EULAR	European Legue Against Rheumatism
EUSTAR	European League Against Rheumatism Scleroderma Research And Trials
HAQ-DI	Health Assessment Questionnaire Disability Index
FVC	forced vital capacity
Gal-3	Galectin-3

GAVE	gastric vascular ectasia
HR	hazard ratio
HRCT	high resolution computer tomograph
ILD	interstitial lung disease
LVEF	left ventricular ejection fraction
lcSSc	limited cutaneous systemic sclerosis
MCP	metacarpophalangeal
MED	Minimal Essential Data
NT-proBNP	N terminal pro brain natriuretic peptide
NYHA	New York Heart Assosciations
OR	odds ratio
PAH	pulmonary arterial hypertension
PF	pulmonary fibrosis
PH	pulmonary hypertension
PIP	proximal interphalangeal
QoL	quality of life
RHC	right heart catheterization
ROC	reciever operating charachteristics
RP	Raynaud Phenomenon
SRC	scleroderma renal crisis
SSc	systemic sclerosis

1. INTRODUCTION

1.1. Predictors of outcome and mortality in SSc

Systemic sclerosis (SSc) is a systemic autoimmune disease characterized by macro- and microvascular lesions, fibrosis and inflammation of several organs. It is a chronic, disabling disease evolving a strong impact on both survival and quality of life (QoL). As a hallmark of SSc, the skin involvement is characterized by thickening, hardening and tightening of the skin. Besides the skin fibrosis, multiple organ manifestations including heart, lung, gastrointestinal, renal and musculoskeletal involvement are also present in this particular disorder.

Involvement of the internal organs including the lungs, heart and kidneys are the major determinants of the outcome of the disease. The clinical presentation of SSc is highly variable, therefore risk factor identification of either increased mortality or development of severe organ involvement is essential. Risk prediction consists of a careful assessment of different clinical features, internal organ involvement, autoantibodies, capillaroscopic findings and identification of certain comorbidities including arterial hypertension or obesity. Risk stratification is to be performed at patient level to improve both the outcome and QoL of the particular case.

There have been several attempts to make composite indices for risk prediction [1-3]. Previous studies showed that older age at onset [1-24], male gender [2-4,7,11,13,14,17,18,22,25-30], diffuse cutaneous SSc subset [1,3-5,7,8,12-14,16,17,21-23, 25-28,31,32] interstitial pulmonary involvement (ILD) [3,4,9,10,12-14,16-18,20,24,25,27,28,32-34], decreased forced vital capacity (FVC) and/or diffusing capacity of carbon monoxide (DLCO) [1-3,5-8,10,19,21-24,29,33,35], pulmonary arterial hypertension (PAH) [4,5,7,13,15,21,22,24,25,27,28,36], cardiac [3-5,7,13,15,21,22,24,25,27,28,36] and renal involvement [3,4,9,12,13,16-24,27,29,34,36,37] were associated with poor outcome. Certain

gastrointestinal manifestations [18,23] including esophageal involvement [12,29], malabsorption [23] and malnutrition [30,36] could also be associated with poor prognosis. Electrocardiogram (ECG) abnormalities [1,6,32,35] including high number of ventricular ectopic beats [33] and decreased ejection fraction [3,22] as well as pigmentation disturbances [12,23], elevated erythrocyte sedimentation rate (ESR) [1,2,6,10,12,14,15,23,29,37], low hemoglobin and/or hematocrit concentration [6,10,23,29], and hypoalbuminemia [29] were also found to predict poor outcome. Furthermore, presence of anti-topoisomerase [4-6,14,20,22,23] autoantibody (anti-Scl70) was associated with a poor survival, conversely anti-centromere antibody (ACA) [4,12,23,25,27,35] was associated with a favourable outcome.

The presence of contractures [3,10,12] is also a risk factor for poor outcome. Synovitis may appear in all disease stages, thus it is characteristically appear in the early stage of the disease. The frequency of synovitis is higher in patients with the diffuse cutaneous subset compared to the limited cutaneous subtype, but only in early disease. [38,39]. Arthritis-related pain is closely associated with SSc patients' health related quality of life [40]. Arthritis can be detected most often in the metacarpophalangeal joints (MCP), wrists, knees, distal interphalangeal joints (DIP), and proximal interphalangeal joints (PIP), respectively [41]. Arthralgia and hand stiffness were among the four highest rated symptoms in terms of frequency and impact on daily activities in the Canadian National Survey [42], more often appearing in patients with diffuse cutaneous SSc (dcSSc) than with limited cutaneous SSc (lcSSc) [42]. Moreover, Skare et al. reported that pain and stiffness were the symptoms that most affected musculoskeletal function [43]. Synovitis often ends up with the appearance of joint contractures, which is one of the major contributor to disability and impaired qulity of life in patients with SSc. They are often present in both subsets, however, the prevalence of joint contractures is higher in dcSSc than is lcSSc. Moreover, diffuse cutaneous subset is an independent predictor of the progression of flexion contractures. Though the development of

contractures is relatively slow and gradual, it is often present in the early stage of the disease [44-47]. Other musculoskeletal symptoms including muscle weakness and the presence of tendon friction rubs were also found to be poor prognostic signs [3,24].

The onset of SSc is usually in the forties, therefore the typical cases are middle aged or elderly women. In these particular age groups the presence of certain comorbidities may also substantially influence the outcome of the disease. Arterial hypertension [22,31,33], and coexistent malignancy [13,16,23] have also been identified as poor prognostic factors of SSc. Our previous study has also confirmed that a coexistent malignancy is an independent predictor of mortality [23].

1.2. Associations between SSc related antibodies and clinical manifestations

One of the important elements of risk assessment is to define subsets within SSc with well defined susceptibility to develop certain organ involvement. The clinical presentation and outcome is well characterized in patients having one of the three major SSc-specific autoantibodies including, Presence of ACA was found to be protective against interstitial lung involvement [48] and was associated with Raynaud Phenomenon (RP) associated alterations including puffy hands, arthralgia and skin sclerosis [49], whereas anti-Scl70 was associated with digital ulcers and the development of ILD [48,49]. Anti-RNA Polymerase III (Anti-RNAP3) is the third “classic” autoantibody presented in SSc in 4-25% [49-51] and it is frequently associated with PAH [48], scleroderma renal crisis (SRC) [49,52,53], gastric vascular ectasia (GAVE) [51] and tumor development [54,55]. Associations with joint pain, puffy hands and a trend for vascular involvement was identified in connection with RNAP3 [55]. These three particular scleroderma specific autoantibodies are almost always mutually exclusive.

There is some debate is about relevance of anti-RNAP3, and a remarkable geographic variation of its prevalence is also observed [56-59]. There is an unmet need to better

characterize the anti-RNAP3 antibody associated clinical manifestations including the appearance/frequency of coexistent malignancies [54,57,60-63]. To achieve this particular goal large multicentre studies should be performed because of the small number of patient with anti-RNAP3+ cases in the individual databases.

1.3. Role of autopsies in evaulatin of casuses of death in SSc

Evaluation of the subclinical manifestations, in particular heart and interstitial lung involvement is pivotal in risk assessment [64], but the value of autopsy still remains important, because in many cases only autopsy can reveal or verify organ involvements [64,66]. Furthermore, some inconsistency have been described between clinical and autopsy findings [64,67,68] with discrepancy rates for cause of death up to 63% [68] and for main diagnosis up to 33.6% [67]. Unfortunately, autopsies are performed less frequently [64], therefore the only approach to analyse autopsy findings is to perform large database-based multicenter studies.

1.4. Cardiac biomarkers as prognostic markers of mortality in SSc

Besides the routine clinical laboratory investigations discussed above, there is also an unmet need for introducing new serum biomarkers that are useful in the risk assessment of the different organ manifestations and prediction of disease outcome. Regarding the heart involvement, elevated level high sensitivity troponin and B-type natriuretic peptides (NT-proBNP) are associated with poor outcome [33,69]. The diagnostic and prognostic value of NT-proBNP is well established in SSc [70-72].

Recent clinical studies suggest that galectin-3 (Gal-3) may be related to the developmental process of skin and organ sclerosis as well as to the aberrant activation of angiogenesis in SSc, but the available data are inconsistent [73-75]. Evidences indicate that galectin-3 activates a variety of profibrotic factors, promotes fibroblast proliferation and transformation, and mediates collagen production [73]. Another important aspect of galectin-3 is to exert a

potent proangiogenic effect in accord with other proangiogenic factors, such as vascular endothelial growth factor and basic fibroblast growth factor [76]. Galectin-3 is a beta-galactoside-binding member of the lectin family also playing an important role in cell proliferation, adhesion, differentiation and apoptosis.

2. AIMS

2.1 Our major aim was to analyze the survival, causes of death and risk factors affecting mortality in a large series of patients with SSc followed up in a university tertiary care center in Hungary. Our further aim was also to investigate whether there is an improvement in the survival in the last decades compared to the previous Hungarian and international follow up studies. Furthermore, we also aimed to compare autopsy results of SSc patitens with our clinical finding in the framework of a European League Against Rheumatism Scleroderma Research and Trials (EUSTAR) study group organized for this particular purpose.

2.2 Our second aim was to identify new risk factors of poor survival in our cohort. We focused on the musculoskeletal system related risk factors and comorbidities.

2.3 We aimed to analyze the characteristics of anti-RNA polymerase III antibody positive patients with systemic sclerosis in the framework of the EUSTAR registry with a special focus on the risk of cancer development. Comparison between our local cohort cases and EUSTAR registry based patients was also performed.

2.4 Our further aim was to identify new serum biomarkers that are useful in risk assessment. We focused on the evaluation of serum galectin-3 levels. Our major question was if besides increased serum NT-proBNP levels, increased galectin-3 level is also a predictor of mortality in SSc.

3. PATIENTS AND METHODS

3.1. Clinical investigation of patients

3.1.1. Analysis of causes of death, risk factors of mortality in 439 SSc patients in a tertiary care center

A large follow up study on consecutive SSc patients was carried out. Cases attending our tertiary care at least two times between 1995 and 2015 were included. The diagnosis of SSc based on American College of Rheumatology (ACR) preliminary classification criteria [77] was found in 469 cases, and only 30 patients were considered lost to follow up (6.4%).

Baseline data were collected prospectively in our database, thus certain items were retrospectively evaluated. Patients lost to follow up were defined as patients who did not appear in the center for at least twelve months after their last visit. In order to clarify the cause of the patients' absence in the follow up, telephone calls were made and letters of inquiry were sent to patients and if possible to the local GP was also contacted. University medical records of the particular patients lost to follow up were also checked.

Causes of death were defined based on last discharge papers, consultation with the patients' GP and autopsy results (available in 20 out of 106 deceased patients). Besides the all cause mortality rate, SSc - related death was also defined as a cause of death clearly explained by major organ manifestation(s) of disease and/or the adverse events of the therapy. Causes of death due to coexistent malignancies and organ manifestations caused by scleroderma overlap syndromes were also evaluated. Each cause of death was extensively discussed, and a final agreement of the three investigators (GN, GK, LC) was achieved.

Interstitial lung disease was documented in case of fibrosis detected by high resolution computer tomography (HRCT) and concurrent decreased forced vital capacity (FVC<80%). Extensive pulmonary fibrosis was recorded in case of either the fibrosis affected the upper

and middle area of the lungs on HRCT and/or FVC<50% or honeycombing was detected by HRCT.

Cardiac involvement was recorded if the patient had at least one of the following conditions: decreased left ventricular ejection fraction <50% (EF), elevated right ventricular pressure detected by echocardiography (>40 mmHg if it was not wxplained by severe pulmonary involvement or other non-cardiac causes), left ventricular relaxation disorder (defined by the cardiologist evaluation based on E/A ratio), abnormal electrocardiogram (arrhythmia, conduction disturbances, brady- or tachycardia; heart rate consistently <60/min or higher than 85/min confirmed by cardiologist that it is heart manifestation related). Primary pulmonary arterial hypertension was recorded if elevated mean pulmonary arterial hypertension was verified by right heart catheterization (RHC). Patients having elevated right ventricular pressure on echocardiography were referred to RHC by the cardiologist based on a standard protocol [78]. The overwhelming majority of patients was examined by the same cardiologist team [79].

Esophageal involvement was documented if the patient had dysphagia and/or barium-swallowing x-ray showed dysmotility and/or strictures/dilatation.

Scleroderma renal crisis (including the normotensive form) was defined by the agreement of two experts which was based on the available definition [80].

Sicca complaints were recorded if the patient was complaining about xerostomia and/or xerophtalmia and it was confirmed with at least one functional test.

Small joint contractures were recorded if range of motion was less than 75% of normal in the metacarpophalangeal and proximal interphalangeal joints evaluated by rheumatologists of our tertiary care center.

Low body weight was defined as the patient's body mass index (BMI) was less than 18, normal if BMI was between 18-25, and higher if it was higher than 25.

Systemic arterial hypertension was documented if hypertension was diagnosed previously or diagnosis of hypertension was established simultaneously with SSc.

Coexistent malignancy was documented in cases when time between malignancy and SSc onset was maximum 5 years.

Abnormal nailfold capillaroscopic pattern was registered in presence of giant capillaries, hemorrhage, capillary loss or signs of neoangiogenesis [81].

Anemia was recorded in case if hematocrit <33%. Hypoalbuminemia was recorded if serum albumin levels were less than 35g/l. Elevated erythrocyte sedimentation rate (ESR) was recorded if higher than >30mm/h. Elevated C-reactive protein (CRP) level was recorded if higher than >5 mg/l.

Antinuclear (ANA-Ease ELISA Kit GD74, Alva, United Kingdom), anti-centromere (Orgentec, ORG 633, Mainz, Germany) and anti-Scl-70 (Orgentec, ORG 212-24, Mainz, Germany) antibodies were detected by enzyme-linkedimmunosorbent assay (ELISA) method. Anti-polymerase III antibodies were detected by immunoblot technique (Euroimmune, DL 1532-1601 G, Mountain Lakes, USA).

3.1.1.1. Study on comparison of clinical manifestations and autopsy findings in cases with SSc

European Scleroderma Trials and Research Group is a unique multinational database collecting prospectively and longitudinally SSc patients, throughout Europe. With this database selection bias can be minimalized and different races, subgroups can be investigated. For each item collected standard definitions are available, they are called the minimal essential dataset ("MEDS") [51,82]. Definition of MEDS variables [51,82] is similar to the definitions in our local database described above, and our tertiary care center continuously contribute with the follow up data of 250 cases to this particular database.

In July 2014, the EUSTAR database was reviewed for deceased SSc patients. All participating centres were invited to fill out a standardised autopsy questionnaire for patients who

underwent autopsy. The questionnaire assessed various organ systems as well as cause of death declared by pathologist and clinician and whether the death was related to SSc. Clinical information was obtained from the database and included disease manifestations of lung, heart, kidney, gastrointestinal, skin or musculoskeletal organ involvement.

Returned autopsy surveys were compared with the corresponding clinical data from the EUSTAR database. Dyspnea was defined as New York Heart Association (NYHA) Grade 3 or 4, arterial hypertension as blood pressure >140mmHg systolic or >90mmHg diastolic. Pulmonary fibrosis (PF) was diagnosed by CT. Lung restriction was defined as vital capacity <80%. Pulmonary hypertension (PH) (defined as systolic pulmonary artery pressure >40 mmHg), diastolic dysfunction and reduced left ventricular ejection fraction (LVEF) were diagnosed according to echocardiographic results.

Autopsy was performed in 16 EUSTAR cases and one patient met the inclusion criteria of this particular study, hence these particular data were sent for further analysis.

3.1.2. Clinical characterisation of patients with anti-RNA polymerase3 antibodies regarding tumor development

Data for this current study was extracted from the EUSTAR registry in March 31, 2014, when 11,399 patients from 118 centers fulfilling either the 1980 ACR or the 2013 ACR/EULAR classification criteria for SSc centers were recorded [77,83]. Finally, 4986 patients from the EUSTAR database with information on their anti-RNAP3 status were included. As a case-control study, 158 anti-RNAP3+ cases were evaluated. The participating particular centers were asked to provide data of the 199 local matched control cases. Some additional retrospective data, including malignancy history, were also queried in 13 participating EUSTAR centers including our tertiary care center.

Patients were included in this particular study when anti-RNAP3 antibody results were available during at least one visit. Patients were considered positive for anti-RNAP3 when the test was positive in at least one determination at the baseline or during the follow up. Patients

positive both for anti-RNAP3 and for other SSc-specific antibodies (ACA, anti-SCl-70) were excluded from comparisons between anti-RNAP3+ and anti-RNAP3– patients.

Our center contributed to this particular study with the data of 21 anti-RNAP3+ SSc patients (10.6% of all cases) and 21 control patients negative for anti-RNAP3. We performed the same analysis were performed in our particular subset of cases as in the entire EUSTAR cohort.

3.1.3. Study on galectin-3 and NT-pro-BNP levels on survival

The overall characterisation of the included 152 patients with SSc was the same as described above. Blood samples of consecutive patients were collected for storage between 1st January 2005 and 31st December 2008. Baseline clinical (body surface area-BSA, Rodnan skin score), laboratory (ESR, CRP, creatinine, hemoglobin), spirometric (FVC, DLCO in percent of the predicted value) and echocardiographic (ejection fraction, calculated right ventricular systolic pressure) data were collected at the same time.

Characterization of left ventricular diastolic function was based on mitral inflow pattern (E/A ratio), left ventricular wall thickness and left atrial size [84].

Follow-up time was defined in this study as the time between the date of blood sample collection and the date of death or the last clinical visit. During clinical visits detailed medical history was obtained from all subjects.

Significant coronary artery disease was defined as coronary artery stenosis >50% proved by invasive measurements or as history of previous myocardial infarction. The diagnosis of primary pulmonary arterial hypertension was based on results obtained by right heart catheterization (mean pulmonary artery pressure $\geq$ 25 mmHg and pulmonary capillary wedge pressure $\leq$ 15 mmHg). Patients with both transient and chronic atrial fibrillation were recorded.

Severe pulmonary involvement was diagnosed when diffuse fibrosis or honeycombing was detected by HRCT and FVC <50% was measured by spirometry.

Analysis of galectin-3 levels was performed using Human Galectin-3 Platinum ELISA kit developed by eBioscience (San Diego, CA, USA). NT-proBNP was measured using electrochemiluminescence immunoassay on the Elecsys 2010 system (Roche Diagnostics, Mannheim, Germany).

3.2. Statistical Analysis

Regarding investigation of the series of 439 SSc patiens Kaplan-Meier curves and log-rank test were used to determine the survival and factors affecting the survival. Items found to be significant according to univariate analysis were further tested with Cox proportional hazard model to examine the independent prognostic factors. Comparisons between groups were performed using independent samples t-tests or Mann-Whitney test for continuous variables while chi square test for categorical variables.

Regarding the RNAP3 studies, the statistical analysis included chi-square test with Pearson correction or Fisher's exact test, and continuous variables using the Student t test, Mann-Whitney U test, or ANOVA, as appropriate. A multivariate logistic regression analysis (adjusted for sex, age, and disease duration) was also performed with calculation of OR estimates and 95% CI. Besides a priori potential confounders, variables associated with $p<0.05$ in univariable analysis were considered. Bonferroni correction for multiple comparison was applied.

Regarding the galectin-3 studies, clinical variables that correlate with galectin-3 level were determined using bivariate Pearson correlation. As first step, age correction was performed using partial correlation analysis. In a second step, age, gender and BSA were used as correcting factors. Since concentration of galectin-3 and NT-proBNP did not show normal distribution, logarithmic transformation was performed.

Relationship between logarithmic transformed galectin-3 and mortality was investigated by using Cox proportional hazards models (backward stepwise), including adjustment for age,

gender, and BSA. Finally, lnNTproBNP was also added to the model, given its known association with outcomes in systemic sclerosis. Hazard ratios (HR) were calculated with 95% confidence intervals (CI).

Receivers operating characteristic (ROC) curves were used to examine the performance of galectin-3 and NT-proBNP in predicting all-cause mortality. Area under the curve (AUC) was calculated from the ROC curve. Optimal cut-off value was chosen to maximize sensitivity and specificity. Kaplan-Meier survival curves were created and differences between groups were tested by Mantel-Cox log rank test.

Prognostic power of concordant versus discordant values for NT-proBNP and galectin-3 was evaluated, for this purpose, four groups were created defined by dividing each variable at the cut-off value obtained by ROC curve previously (low galectin-3/low NTproBNP, high galectin-3/low NT-proBNP, low galectin-3/high NT-proBNP, high galectin-3/high NT-proBNP). Kaplan-Meier survival curve was created and differences between groups were tested by log rank test.

For all our analysis, version 16.0 SPSS for Windows was used (Inc., Chicago, IL, USA).

3.3. Ethics

The study on prognostic analysis was approved by the Regional Ethics Committee (2939/2007). Analysis on the prognostic value of different heart related biomarkers was also approved by the Regional Ethics Committee (5338/2014), and analysis of patients enrolled to EUSTAR database was approved by the Hungarian National Ethics Committee (430/PI/2012 and 426/2013). Regarding all the follow up studies, written informed consent was obtained from patients according to the Declaration of Helsinki.

4. RESULTS

4.1. Survival and risk factor analysis

4.1.1. Major clinical findings of the follow up study

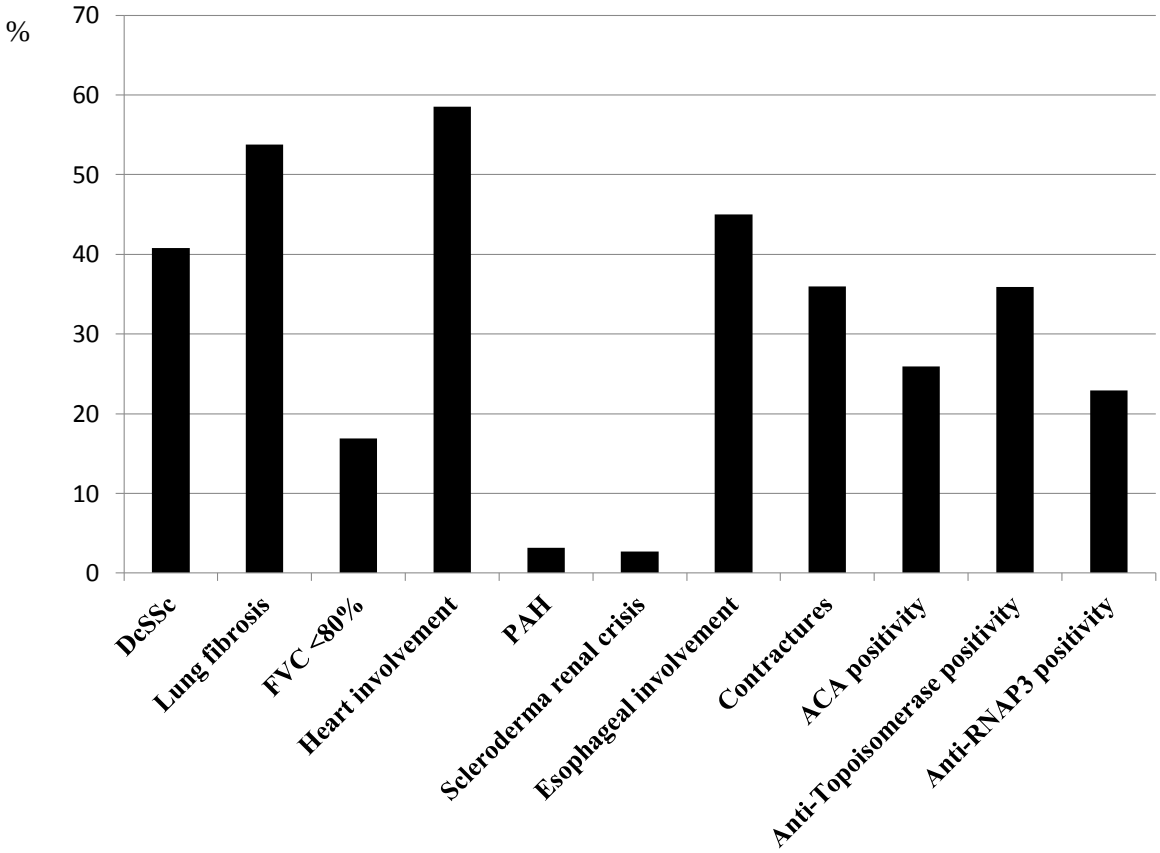
The median age at onset was 46 years. Diffuse cutaneous SSc (dcSSc) was present in 179 (40.8%) cases. Seventy-three (16.6%) male patients were enrolled. The main clinical characteristics are depicted in Table 1 and Figure 1.

Table 1: Baseline clinical features of 439 patients with systemic sclerosis

	N°/available (%)
N° of patients	439
Age at onset of Raynaud's phenomenon (median, lower; upper quartile) years	46 (34;55)
Age at the first non-Raynaud's phenomenon (median, lower; upper quartile) years	49 (40;58)
Mean follow up time from onset of Raynaud's phenomenon (years)	8.42±5.6
Time between onset of Raynaud's phenomenon and enrollment (median, lower;upper quartile) years	3 (0;4)
N° of male patients	73 (16.6)
N° of dcSSc patients	179 (40.8)
Extensive pulmonary interstitial involvement	103 (23.5)
Interstitial lung involvement	236 (53.8)
FVC<80%	72/427 (16.9)
DLCO<70%	216/418 (51.6)
Cardiac involvement	257/439 (58.5)
>40 mmHg right ventricular pressure on echocardiography	34/418 (8.1)
Ejection fraction<50%	9/418 (2.1)
Scleroderma renal crisis	12 (2.7)
Esophageal involvement	195/433 (45)
Pulmonary arterial hypertension	14/30 (3.2 of all patients)
Sicca complaints (26.4 – Schirmer's test was available in 85.6%; saliva measurements in 65.6% of the patients)	116
Small joint contractures	158 (36)
ACA positivity	113/437(25.9)
Anti-topoisomerase I positivity	157/437 (35.9)
Anti-RNA polymerase III positivity	31/135 (22.9)
Elevated ESR (>30mm/h)	127/422 (29.4)
Elevated CRP (>5mg/l)	219/424 (51.6)
Low hemoglobin level (male <137g/l; women <120g/l)	131/430 (30.5)
Low hematocrit level (<33%)	45/430 (10.5)
Azotemia (creatinine >100μmol/l)	45/421 (10.6)
Hypalbuminemia (<35g/l)	27/384 (7)
Coexistent malignancies	36 (8.2)
Concurrent or history of malignancies	54 (12.3)
History of arterial hypertension	175 (39.9)
Diabetes mellitus	26 (5.9)

For the details of clinical investigations, see Methods.

Figure 1: Organ-based manifestations of 439 patients with SSc



Coexistent malignancies were found in 36 cases, and the ratio of lcSSc to dcSSc patients was 22 to 14 (1.6:1). 29 female and 7 male patients had a coexistent malignancy. The mean time between onset of SSc and malignancy was 2.1 ±1.1 years. Twelve patients had anti-topoisomerase, 10 anti-centromere and 2 anti-RNAP3 antibodies, respectively. Out of the 29 female patients with coexistent malignancy, 11 had breast cancer, 5 cervical cancer, 1 colon adenocarcinoma, 4 hematologic malignancies, 2-2 lung and ovarian cancer, 1 endometrial carcinoma, 2 skin cancer (one melanoma and one non-melanoma skin cancer) and 1 multiple malignancy. In the 7 male patients with coexistent malignancies, 3 hematologic malignancies, 1 small cell lung cancer, 1 lung adenocarcinoma, 1 uroepithelial carcinoma and 1 prostate cancer were identified (Figure 2). No statistical difference was found in the major clinical features of patients with and without coexistent malignancies. Patients with coexistent

malignancy had significantly worse survival by univariate analysis compared to those without coexistent malignancy ($p<0.0001$) (Figure 3).

Figure 2: Distribution of different types of early malignancies

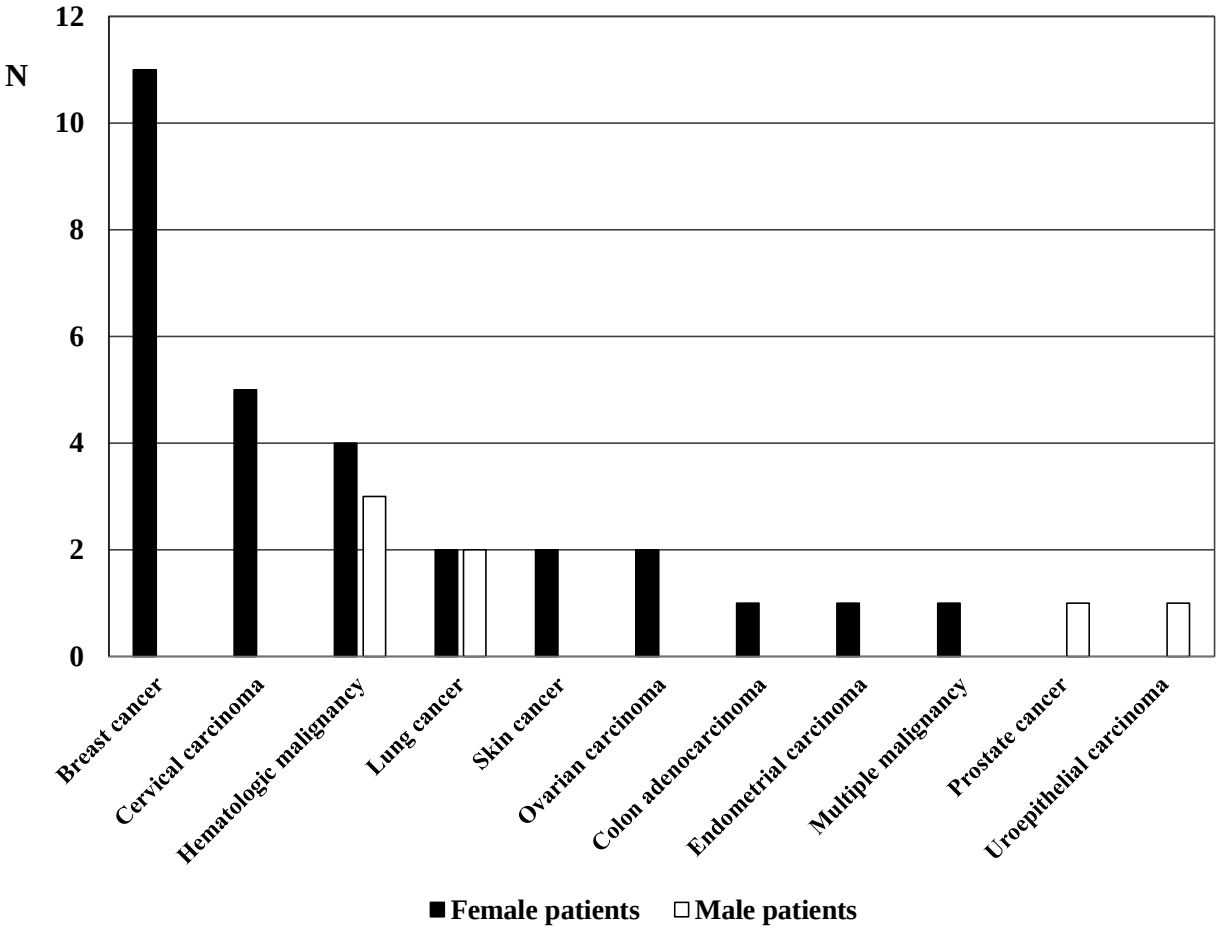
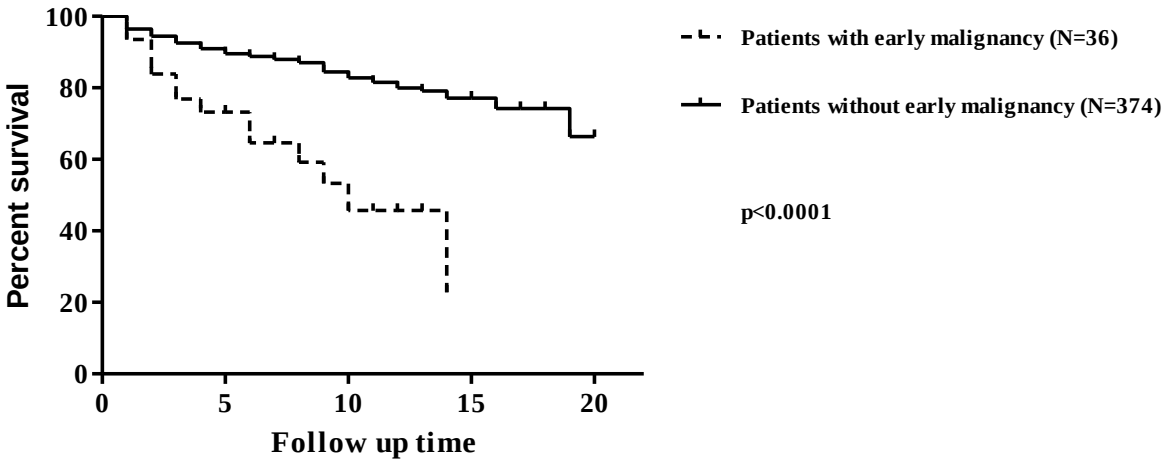


Figure 3: Survival of SSc patients with and without early malignancy



4.1.2. Mortality

The all-cause survival rate was 88.2% at 5 years, 80.8% at 10 years, 67.5% at 15 years and 31.6% at 20 years, respectively. When only the SSc related causes of death (clearly explained by major organ manifestation(s) of disease and/or the adverse events of the therapy) were taken, the survival rate showed 95.6% at 5 years, 87.5% at 10 years and 74.2% at 15 years, respectively. When fatal outcome caused by overlap syndrome (a coexistent another autoimmune disease's complication) and/or coexistent malignancy was added to the strictly SSc-related causes of death we found 88.1% survival at 5 years, 79.9% at 10 years, 73.6% at 15 years and 63.4% at 20 years (Figure 4). LcSSc patients had a significantly better survival compared to dcSSc cases, survival rate showed 92.1% at 5 years, 85.4% at 10 years and 82.1% at 15 years, respectively in lcSSc subgroup and showed 81.7% at 5 years, 70.3% at 10 years and 57.0% at 15 years, respectively in the dcSSc subgroup ($p<0.001$) (Figure5).

Figure 4: Kaplan-Meier curves for overall, SSc related and strictly SSc related mortality

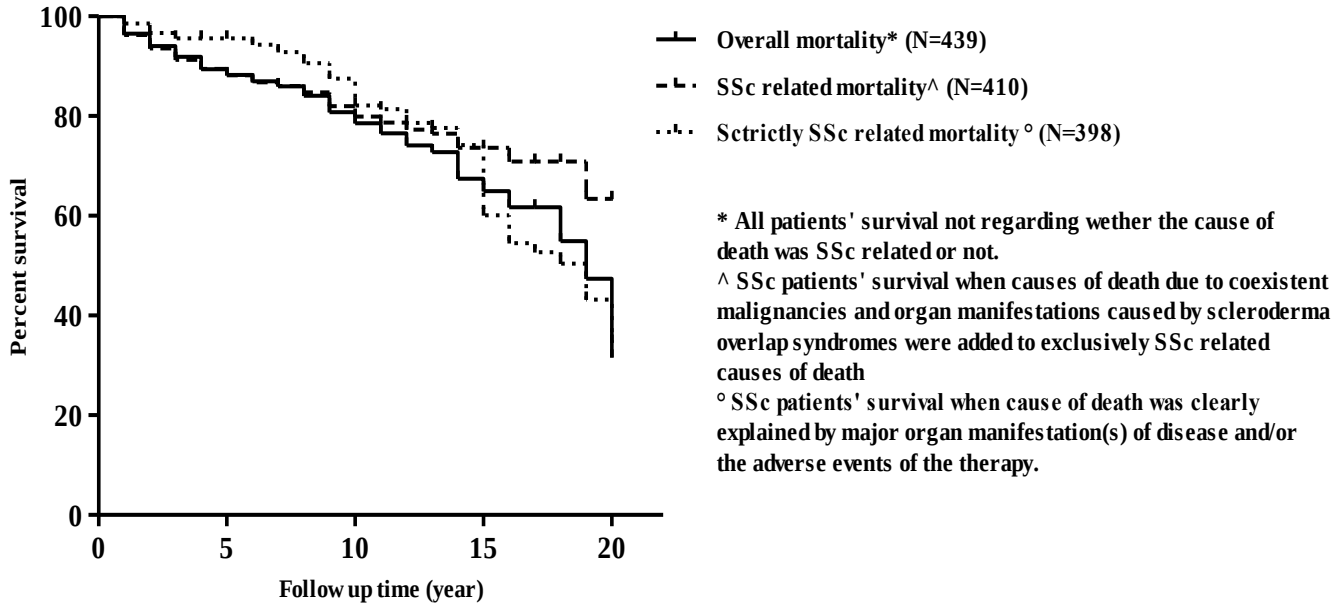
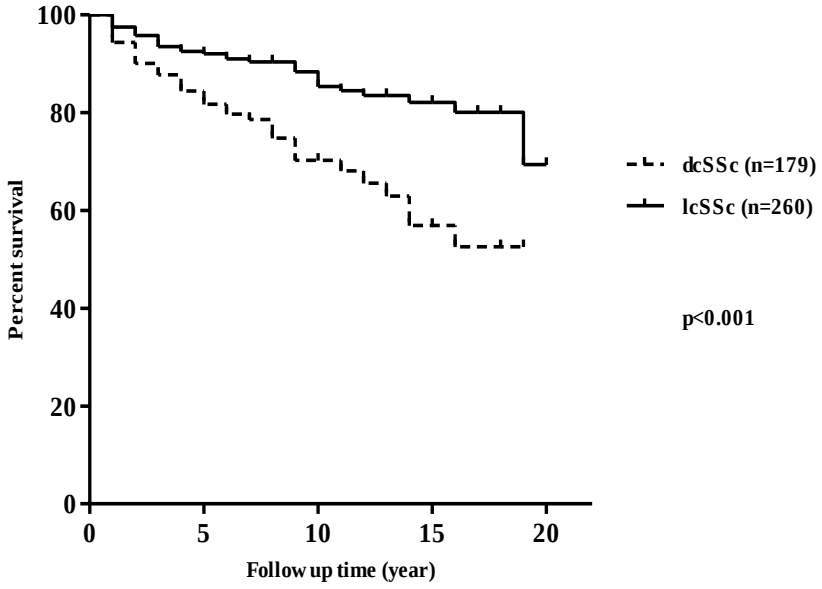


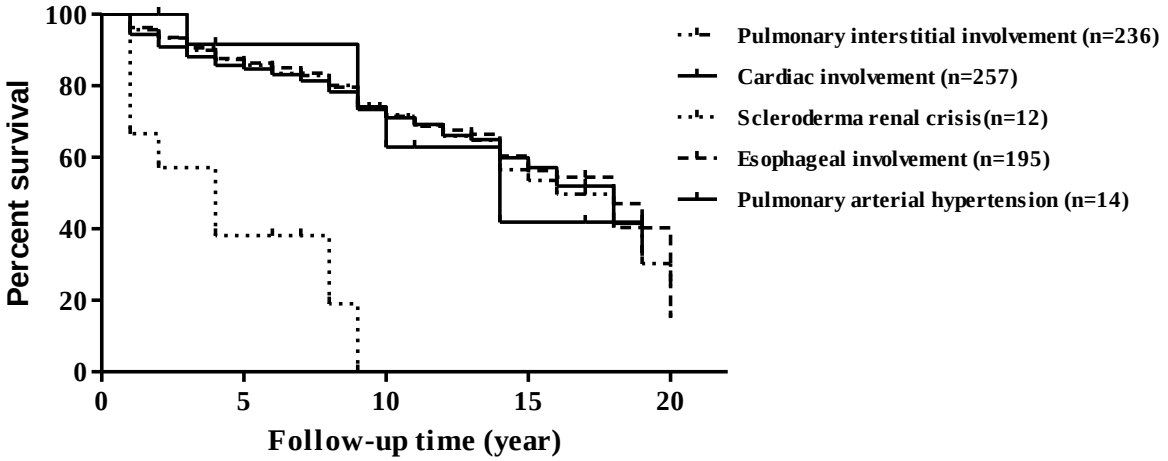
Figure 5: Survival in the two major SSc subsets



4.1.3. Univariate analysis

Univariate analysis showed that dcSSc, male gender, presence of small joint contractures, ILD, cardiac involvement, elevated right ventricular pressure on echocardiography, less than 50% ejection fraction, ECG abnormalities, esophageal involvement, scleroderma renal crisis, history of hypertension, anti-topoisomerase positivity, low hemoglobin, hematocrit and albumin levels, elevated ESR, coexistent and current or previously diagnosed malignancies were associated with poor prognosis. Conversely, the presence of anti-centromere antibodies and lack of giant capillaries, microhemorrhages neoangiogenesis or avascularity on the nailfold capillaroscopy showed a favourable outcome. In patients deceased of overlap syndrome and/or coexistent malignancy added to strictly SSc-related causes of death the same parameters made influence on survival by univariate analysis except for coexistent and previously diagnosed malignancies (Figure 6,7,8).

Figure 6: Survival of SSc patients with different internal organ involvement



For the definition of organ manifestations, see Methods.

Figure 7: Survival of SSc patients with different autoantibodies

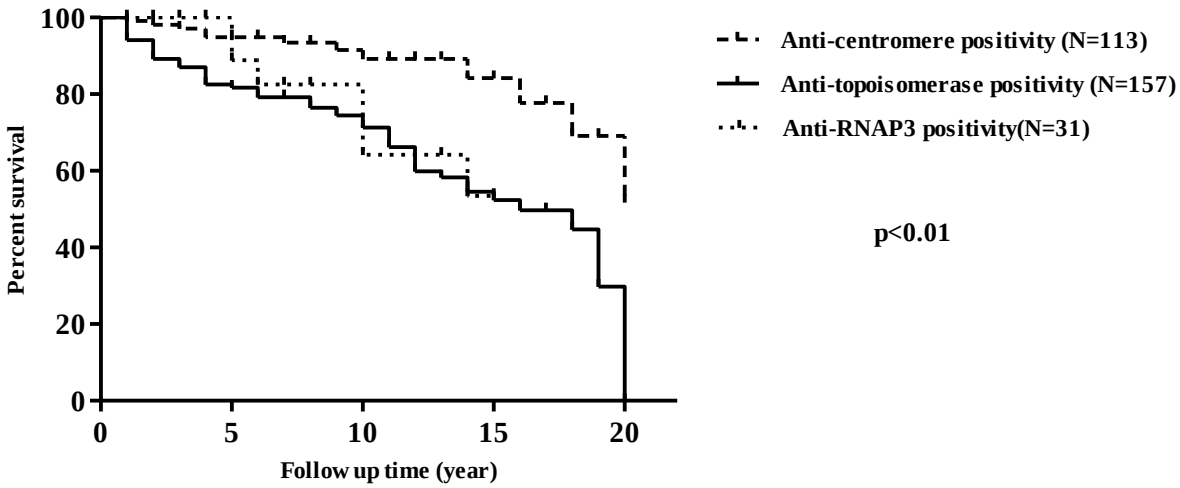
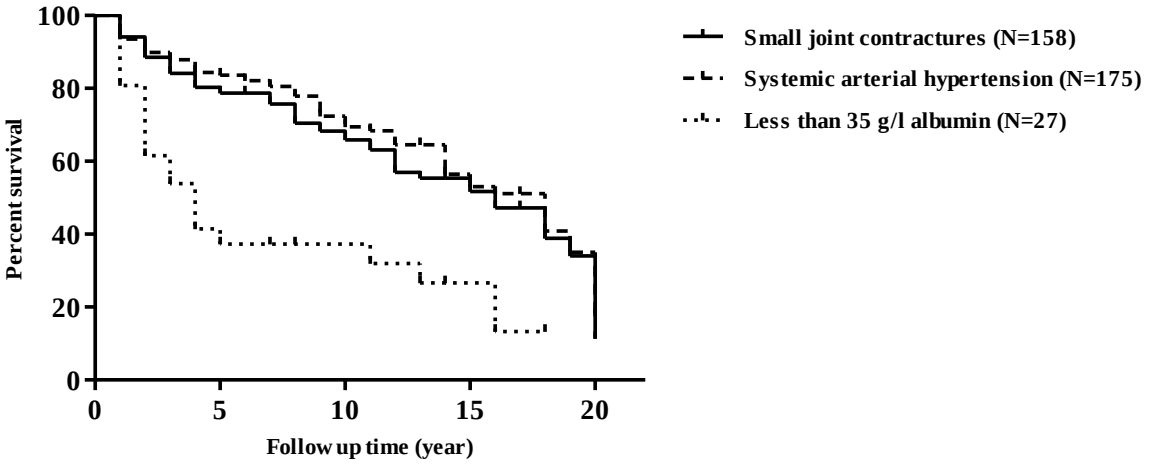


Figure 8: Survival of SSc patients with small joint contractures, hypoalbuminaemia and arterial hypertension



4.1.4. Multivariate analysis

Cox analysis showed that male gender, presence of topoisomerase I antibody, DLCO<70% and FVC<70%, presence of small joint contractures, more than 40 mmHg right ventricular pressure on echocardiography, ECG abnormalities, history of arterial hypertension, low hematocrit and albumin levels and presence of malignancies predict poor outcome.

Cox analysis, performed with the exclusion of patients died of SSc unrelated causes of death showed similar pattern, but arrhythmias, decreased FVC were no more independent predictors of mortality, while less than 50% of ejection fraction predicted worse outcome. Further exclusion of patients who died of paraneoplastic syndrome and overlap syndromes showed on multivariate analysis that abovementioned parameters with exception of malignancies and <70% DLCO and FVC predicted poor outcome (Table 2).

4.1.5. Analysis of cause of death

During the follow up period, 106 patients died, and in 77 (72.6%) cases the cause of SSc was SSc-related. After careful discussion, in ten patients multiple cause of death involving more than one organ was declared because according to the investigators' opinion the combination of at least two organ involvements was responsible for the fatal outcome. Sixteen patients'

death was attributed to cardiac involvement, 8 to PAH, 13 to ILD, 2 to scleroderma renal crisis, 6 to gastrointestinal involvement and 5 treatment-related infections. In two cases kidney and ILD, in two cases kidney and gastrointestinal involvement, and in two cases ILD and infection were the causes of death. In one particular case infection and PAH, in 1 cardiac involvement and ILD, in 1 gastrointestinal involvement and infection, in 1 cardiac involvement and infection caused the death of the patients (Figure 9).

Out of the 17 cases of death due malignancies 12 patients died of coexistent malignancy (onset of the tumor ±5 years) and 5 because of other tumors, but connection between the onset of malignancy and SSc was clear, mainly due to therapy used for SSc, predominantly lung cancers in patients with previous high dose cyclophosphamide therapy.

Figure 9: Causes of SSc related death in 439 patients with SSc

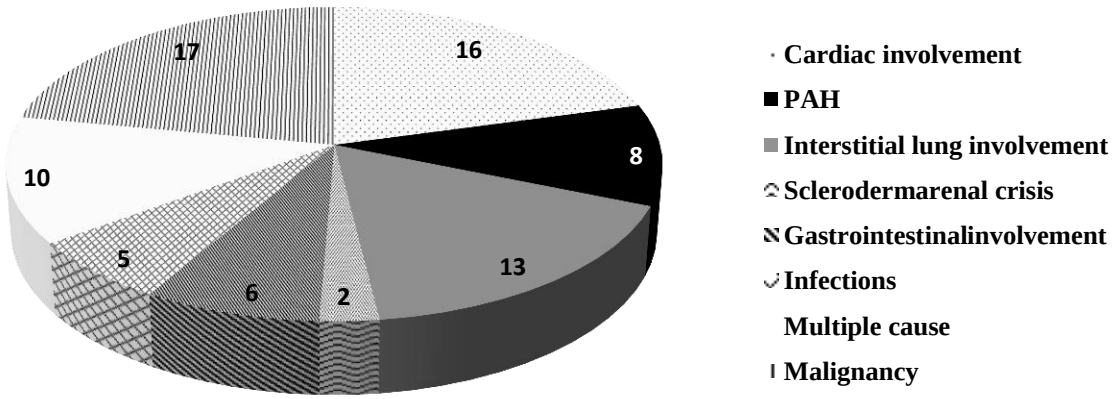


Table 2: Multivariate analysis of 439 patients with SSc

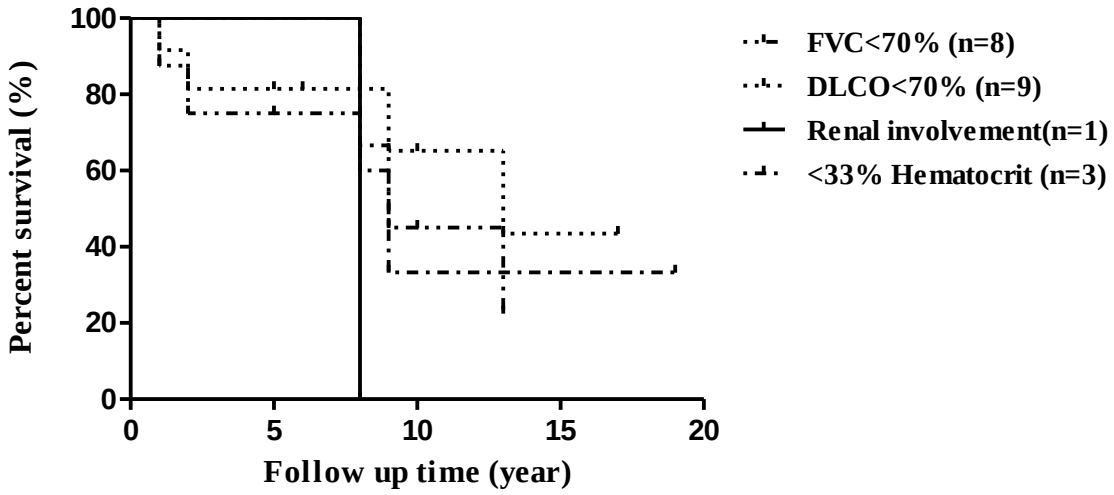
	Mortality risk for patients died of SSc related causes of death (excluding paraneoplasia and overlap syndromes)		**Mortality risk for patients died of SSc related causes of death, paraneoplasia and overlap syndromes**		**Overall mortality risk**	
(N°)	**HR (95% CI)**	**p**	**HR (95% CI)**	**p**	**HR (95% CI)**	**p**
Male gender (73)	3.877 (1.929-7.793)	<0.01	3.421 (1.858-6.301)	<0.001	3.256 1.883-5.631)	<0.001
Anti-topoisomerase I positivity (157)	1.730 (1.014-2.951)	<0.05	1.667 (1.023-2.714)	<0.05	2.158 (1.425-3.268)	<0.001
Small joint contractures ˜ (158)	2.758 (1.573-4.836)	<0.001	2.834 (1.721-4.665)	<0.001	1.834 (1.219-2.757)	<0.05
<70% FVC(72)					1.995 (1.146-3.470	<0.01
< 70% DLCO(216)					1.975 (1.281-3.045)	<0.01
< 50% DLCO(54)	2.680 (1.369-5.244)	<0.01	2.732 (1.597-4.673)			
> 40 mmHg right ventricular pressure on echocardiography (34)	4.974 (2.161-11.449)	<0.001	3.257 (1.515 -7.002)	<0.001	2.164 (1.219-3.842)	<0.01
< 50% ejection fraction (9)	4.468 (1.671-11.948)	<0.01	5.303 (2.065-13.618)	<0.01		
Brady- or tachycardia detected by ECG (81)	2.321 (1.262-4.268)	<0.01	3.738 (2.207-6.332)	<0.001	2.514 (1.577-4.007)	<0.001
Arrhythmia on ECG (39)	1.973 (1.048-3.715)	<0.05			1.675 (1.022-2.746)	<0.05
Arterial hypertension (175)	2.065 (1.220-3.495)	<0.01	2.063 (1.261-3.375)	<0.01	2.090 (1.390-3.143)	<0.01
Low hematocrit level (<33%) (45)	3.704 (2.046-6.704)	<0.01	2.728 (1.560-4.770)	<0.001	2.784 (1.749-4.430)	<0.01
Hypoalbuminemia (<35g/l) (27)	2.481(1.255-4.904)	<0.001	2.769 (1.428-5.370)	<0.01	2.299 (1.283-4.119)	<0.01
Concurrent or history of malignancies (54)			3.190 (1.792-5.679)	<0.001	2.956 (1.835-4.762)	<0.001

For details see methods, in page 7. N: patiets affected with the particular alteration.

4.1.6. Comparison of cases with early versus late onset of SSc

33 patients were followed up fulfilling our early onset SSc criteria, RP onset less than 20 year's old. The mean age at onset of RP was 15.2±4.3 years. Patients with an early onset of SSc had a significantly ($p<0.01$) more impaired FVC (less than both 80% and 50%), conduction disturbances detected by ECG, and low BMI (8/33 vs. 14/368; $p<0.001$) compared to cases with a disease onset between 21-64 years. Furthermore, significantly less early onset group patients had a history of PAH, malignancy and BMI>25 (Table 3). In this particular early onset subgroup, 6 (18.2%) out of the 33 patient died during the follow up, four of them had dcSSc. Kaplan-Meier analysis revealed that poor survival was associated with the presence of FVC<70% and DLCO<70%, renal involvement, elevated ESR, decreased hematocrit levels (Figure 10).

Figure 10: Factors associated with poor outcome in early onset SSc patients (n=33)



Out of the 439 examined patients 38 developed RP after the age of 65 (late onset SSc). The mean age at onset of RP was 69.9±3.9 years. When comparing clinical features of patients having late onset SSc we found significantly more patients having cardiac involvement,

elevated right ventricular pressure on echocardiography, ECG disturbances and elevated ESR compared to patients with RP developed between age 21 and 64. On contrary, normal BMI was less frequent compared to patients with RP developed between age 21 and 64 ($p<0.0001$) (Table 3).

Twenty-one (55.3%) out of the 38 patients died before the end of the 2015. Poor prognosis was associated with decreased hemoglobin and albumin levels and elevated CRP.

In the early onset group, the 5, 10, 15 years survival rates were 93%, 75% and 66%, respectively. In the elderly onset group the 5 and 10 years survival rates were 66% and 32%, respectively. Patients with RP onset between age 21 and 64 had 91% 5 years survival, 84% 10 years survival and 72% 15 years survival, respectively. Survival was significantly worse in patients with Raynaud's onset after age 65 compared to the other two patients groups ($p<0.001$) (Figure 11).

Patients in the early onset SSc subgroup had significantly longer disease duration at enrollment compared to the late onset counterparts (6.69±8.08 years vs. 0.71±1.6 years, $p<0.05$)

Figure 11: Survival of SSc patients with different age of onset of Raynaud's Phenomenon

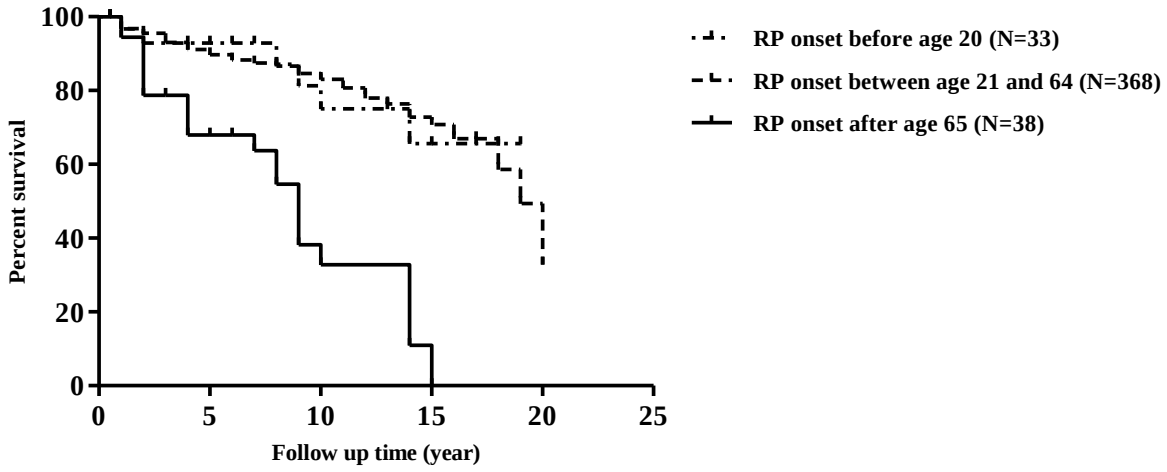


Table 3: Comparison of clinical features of patients with early and late onset of SSc

	First RP[a] before the age of 20 yr N°=33 (%)	**First RP[a] between the age of 21-64 yrs N°=368 (%)**	**First RP[a] after the age of 65 N°=38 (%)**	**RP[a] onset >65 years vs. RP onset between 21-64 years p (Fisher exact test)**	**RP[a] onset <20 years vs. RP onset between 21-64 yrs p (Fisher exact test**
Male	8 (24.2)	57 (15.5)	8 (21.1)	NS[b]	NS
dcSSc	15 (45.4)	150 (40.8)	14 (36.9)	NS	NS
ACA	5 (15.2)	95 (25.8)	13 (34.2)	NS	NS
Anti-topoisomerase antibody	13 (39.4)	127 (34.5)	17 (44.7)	NS	NS
Anti-RNAP3 antibody	2/11 (18.2)	28/119 (23.5)	1/5 (20)	NS	NS
Extensive pulmonary interstitial involvement[†]	8 (24.2)	84 (22.8)	11 (29)	NS	NS
Pulmonary interstitial involvement[††]	14 (42.4)	202 (54.9)	20 (52.6)	NS	NS
FVC<80%	12 (36.4)	58 (15.7)	2 (5.3)	NS	**<0.01**
FVC<70%	8 (24.2)	22 (6)	1 (2.6)	NS	**<0.001**
FVC<50%	3 (9.1)	5 (1.4)	0 (0)	NS	**<0.01**
Cardiac involvement[#]	21 (63.6)	203	33 (86.8)	**<0.0001**	NS
Right ventricular pressure>40 mmHg on echocardiography	3 (9.1)	24 (6.5)	7 (18.4)	**<0.05**	NS
Conduction disturbance on ECG	9 (27.3)	52 (14.1)	6 (15.8)	NS	**<0.05**
Arrhythmia on ECG	2 (6.1)	29 (7.9)	8 (21.1)	**<0.05**	NS
History of arterial hypertension*	5 (15.2)	150 (40.1)	20 (52.6)	NS	**<0.01**
Scleroderma renal crisis^	1 (3)	11 (3)	0 (0)	NS	NS
Esophageal involvement[§]	18 (54.6)	160 (43.5)	17 (44.7)	NS	NS
BMI<18	8 (24.2)	14 (3.8)	1 (2.6)	NS	**<0.001**
BMI 18-25	21 (63.6)	163 (44.3)	1 (2.6)	**<0.0001**	**<0.05**
BMI >25	4 (12.1)	181 (46.9)	16 (42.1)	NS	**<0.001**
Elevated ESR (>30mm/h)	8 (24.2)	100 (27.2)	19 (50)	**<0.01**	NS
Low hematocrit level (<33%)	4 (12.1)	36 (9.8)	5 (13.2)	NS	NS
Hypalbuminemia (<35g/l)	0 (0)	23 (6.3)	4 (10.5)	NS	NS
Concurrent or history of malignancies	0 (0)	46 (12.5)	8 (21.1)	NS	**<0.05**
Coexistent malignancies	0 (0)	31 (8.42)	5 (16.1)	NS	NS

For details, see Methods.

4.1.7. Comparing autopsy findings to clinincal presentation

The entire EUSTAR investigation consisted of 11 patients' data who deceased between 2007 and 2014. Six patients were female and five male. Eight had dcSSc and lcSSc. Regarding this particular investigation one patients' data were sent for further EUSTAR analysis from our tertiary care center.

The EUSTAR study showed that the cause of death defined by pathologist and clinician were identical in nine out of eleven cases. Seven deaths were defined as SSc-related by the clinician, whereas only five deaths were classified as SSc-related by the pathologist after autopsy. In two cases, no statement of pathologists was given and in one case there was a dissent. SSc-related causes of death reported by the pathologist were cardiopulmonary insufficiency, myocardial ischaemia, subacute diffuse alveolar damage, bilateral bronchopneumonia in combination with myocardial ischaemia and usual interstitial pneumonia. The four non-SSc-related deaths were postinterventional cardiac tamponade, cerebral bleeding, amyotrophic lateral sclerosis with ventilatory failure and respiratory insufficiency secondary to cardiac failure.

Regarding our single case, the pathological and clinical cause of death was not identical in this case. By clinicians endocarditis was determined, but by pathologist cardiorespiratory insufficiency, however both agreed it was SSc unrelated.

4.2. Clinical associations of cases with anti- RNAP3 antibodies based on the analysis of the EUSTAR database

4.2.1. Clinical associations of patients with anti-RNAP3 antibodies

In the EUSTAR database, information about the presence/absence of anti-RNAP3 antibodies was available in 4986 patients with SSc. These particular cases did not differ for those particular patients for whom information on anti-RNAP3 status was not available. Among the 4986 evaulated cases, 223 (4.5%) were found to have anti-RNAP3 antibody. Among these

particular anti-RNAP3+ patients, 47 were also positive for another SSc-specific major antibody in at least 1 visit (15 for ACA, 30 for anti-topo I, and 2 for both). These cases were excluded from further analysis. Finally, 176 anti-RNAP3+ and 4763 anti-RNAP3– patients with SSc were compared.

In our local registry 317 cases were followed up. Out of these particular cases, 21 patients fit the enrolment criteria of anti-RNAP3+. The same number of controls fitting the control criteria were also collected (see methods). Among the 21controls 4 patients were simultaneously ACA (19.0%) and 3 (14.3%) anti-topo I positive, none of them had both antibodies simultaneously.

Regarding the entire EUSTAR investigation, in univariable analysis, positivity for anti-RNAP3 was associated with male sex ($p < 0.0001$), arterial hypertension ($p = 0.03$), diffuse cutaneous involvement ($p < 0.0001$), renal crisis ($p < 0.0001$), and joint contractures ($p < 0.0001$) (Table 4). In the multivariable model (adjusted for sex, age at disease onset, and disease duration; Table 4), anti-RNAP3 positivity was independently associated with scleroderma renal crisis ($p < 0.0001$) and diffuse cutaneous skin involvement ($p < 0.0001$).

Table 4: Results of the univariable and multivariable analysis (adjusted on sex, age at disease onset, and disease duration) comparing anti-RNAP3– and anti-RNAP3+ patients at the last visit in the EUSTAR database (n = 4939). /Values are no./no. available data (%) unless otherwise specified./ [85]

Characteristics	**Univariable Analysis**			**Multivariable Analysis**		
	Anti-RNAP3–	**Anti-RNAP3+**	**p**	**Available Data, n (%)**	**OR (95% CI)**	**p**
Age at disease onset, yrs, mean (SD), (n available)	46.5 (14.2) (3946)	46.4 (13.1) (140)	0.981	4086 (82.7)		0.461
Disease duration, months, mean (SD), (n available)	138.6 (102.6) (3943)	131.0(125.0) (140)	0.391	4083 (82.7)		0.843
Male	684/4763 (14.4)	45/176 (25.6)	<**0.0001**	4939 (100)		0.306
Ethnicity						
White	3425/3605 (95.0)	134/144 (93.1)	0.295	3749(75.9)		
Asian	34/3605 (1.3)	3/144 (2.1)	0.175	3749 (75.9)		
Black	47/3605 (1.3)	1/144 (0.7)	0.524	3749 (75.9)		
Others	99/3605 (2.7)	6/144 (4.2)	0.311	3749 (75.9)		
Raynaud Phenomenon	4242/4546 (93.3)	157/165(95.2)	0.351	4711 (95,4)		
Esophagal symptoms	2721/4612 (59,0)	99/167 (59.3)	0.942	4779 (96.8)		
Stomach symptoms	1026/4536 (22.6)	42/163 (25.8)	0.346	4699 (95.1)		
Intestinal symptoms	1132/4603 (24.6)	50/167 (29.9)	0.116	4770(96.6)		
Arterial hypertension	1040/4600 (22.6)	50/168 (29.8)	**0.030**	4768 (96.5)		0.508
Scleroderma renal crisis	59/4608 (‘1.3)	21/169 (12.4)	<**0.0001**	4777(96.7)	7.06 (3.77-12.2)	<**0.0001**
Dyspnea significant	481/3999 (12.0)	18/150(12.0)	0.992	4149 (84.0)		
Diffuse cutaneous subtype	1289/4573 (28.2)	98/169 (58.0)	<**0.0001**	4742 (96.0)	2.35 (1.58-3-49)	<**0.0001**
Scleroderma/puffy fingers	1759/4144(42.4)	27/101(26.7)	0.739	4245 (85.9)		
Active digital ulcer	583/4523 (12.9)	17/166 (10.2)	0.316	4689 (94.9)		
Joint synovitis	563/4560 (12.3)	13/167 (7.8)	0.077	4727 (95.7)		
Joint contractures	1346/4489 (30.0)	74/160 (46.3)	<**0.0001**	4659 (94.3)		0.104
Tendon friction rubs	259/4479 (17.8)	13/158 (8.2)	0.199	4637 (93.9)		
Muscle weakness	802/4499 (17.8)	30/160 (18.8)	0.764	4659 (94.3)		
Muscle atrophy	417 /4494 (9.3)	18/159 (11.3)	0.385	4653 (94.2)		
Conduction blocks	462/3810 (12.1)	14/118 (11.9)	0.932	3928 (79.5)		
Elevated sPAP, by echocardiography	628/3882 (16.2)	18/133 (13.5)	0.415	4015 (81.3)		
Lung fibrosis on plain radiograph	1046/3346 (31.3)	42/112 (37.5)	0.162	3458 (70.0)		
Lung fibrosis on HRCT	1175/2520 (46.6)	41/74 (55.4)	0.136	2594 (52.5)		

In our local single center cohort of 21 positive and 21 control SSc cases, no clinical features showed correlations with anti-RNAP3, by univariable analysis hence multivariable analysis was not performed (Table 5).

Table 5: Results of the univariable analysis comparing anti-RNAP3– and anti-RNAP3+ patients at the last visit in our local tertiary care center (n = 42).

Characteristics	**Univariable Analysis**		
	Anti-RNAP3– (n=21)	**Anti-RNAP3+(n=21)**	**p**
Raynaud Phenomenon	21/21	21/21	NA
Oesophageal symptoms	15/21	18/21	0.2269
Stomach symptoms	6/21	8/21	0.3295
Intestinal symptoms	9/21	13/21	0.1771
Arterial hypertension	7/21	13/21	0.0607
Scleroderma renal crisis	0/21	0/21	NA
Dyspnoea significant	3/19	2/21	0.4506
Diffuse cutaneous subtype	13/21	13/21	NA
Scleroderma/puffy fingers	9/18	12/21	0.4506
Active digital ulcer	2/19	1/20	0.4802
Joint synovitis	2/20	2/21	0.6782
Joint contractures	16/21	17/21	0.5000
Tendon friction rubs	2/20	1/21	0.5179
Muscle weakness	6/21	8/21	0.3295
Muscle atrophy	6/21	3/21	0.3218
Conduction blocks	1/20	2/21	0.2858
Elevated sPAP, ECHO	3/8	1/10	0.2059
Lung fibrosis on plain radiograph	9/18	7/15	0.5631
Lung fibrosis on HRCT	0/2	1/1	NA

4.2.2. Association of anti-RNAP3 with cancer based on the case-control study

Thirteen EUSTAR centers participated in the case-control study, collecting retrospective data from 158 anti-RNAP3+ SSc cases and 199 anti-RNAP3– local SSc controls, matched for sex, disease duration, cutaneous subset, and age at disease onset. Among controls, 48% were anti-topo I+ and 22% ACA+. The interval between diagnosis and the last visit available was shorter in anti-RNAP3+ cases than in controls [median (interquartile range) 77 (38–132) months vs 100 (52– 155), p = 0.008].

There was no difference in the number of deaths and their causes between cases and controls (data not shown). Cumulative survival was not different between these 2 particular groups [at

5 years after SSc diagnosis, anti-RNAP3+ 91.6% (SE 2.3) vs anti-RNAP3– 94.4% (SE 1.7); at 10 years, anti-RNAP3+ 87.0% (SE 3.2) vs anti-RNAP3– 84.0% (SE 3.2), log-rank test p = 0.72].

Regarding our local anti-RNAP3+ and the local control cases, there was no difference in the number of death (p=0.092). In the anti-RNAP3+ group 5 patient deceased, causes of death were PAH in two cases, lung cancer in one case, pericardial tamponade in one case and in one case ILD. In the control group only one patient died, its cause of death was lung cancer. The 5 year survival was 94.18% in the anti-RNAP3 + group and 95.24 % in the anti-RNAP3- group, respectively showing no statistical difference (log rank p=0.2425) (Figure 12 and 13.).

Figure 12: Survival of anti-RNAP3 positive and negative patients in our tertiary care center

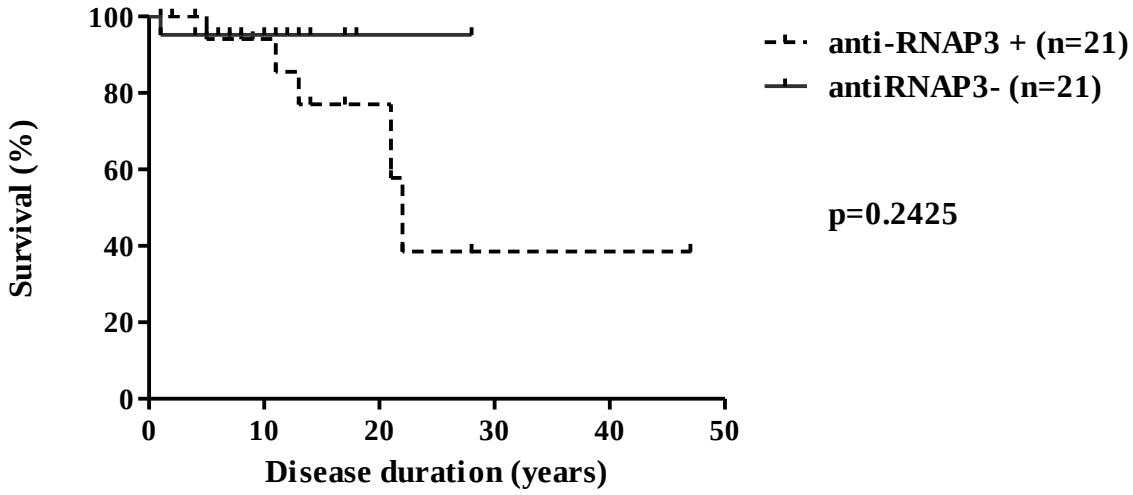
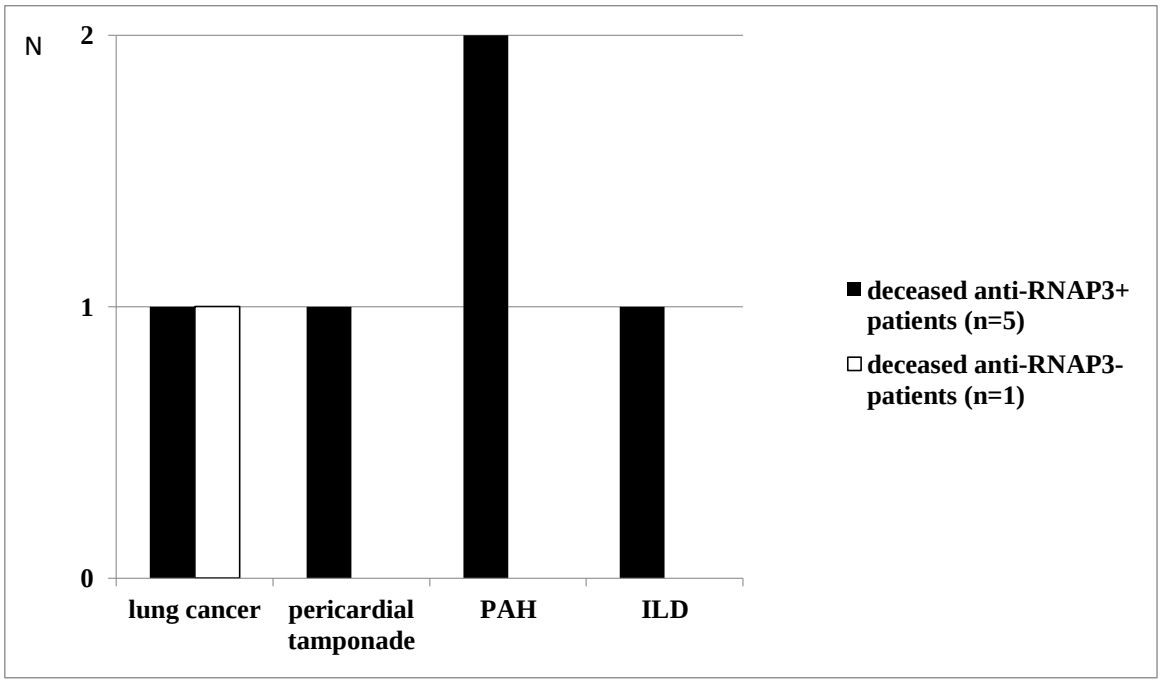


Figure 13: Causes of death among patients enrolled to case control study of anti-RNAP3 positive and negative patients



Regarding the total EUSTAR investigation, in multivariable analyses, anti-RNAP3 positivity was negatively associated with gastroesophageal reflux disease ($p = 0.003$), but was positively associated with renal crisis ($p = 0.0005$) and GAVE ($p = 0.0009$; Table 6). No difference was observed between the 2 groups for peak of mRSS. However, Kaplan-Meier analysis showed that the time to reach the peak of mRSS was shorter in anti-RNAP3+ patients than in matched controls ($p = 0.013$). In particular, the peak of mRSS was reached within 1 or 2 years in 71% and 87% of anti-RNAP3+ patients, respectively, as compared to 62% and 74% of controls.

Table 6: Univariable analysis comparing anti-RNAP3+ patients with anti-RNAP3– SSc controls, matched for sex, disease duration, cutaneous subset, and age at disease onset [85]

Characteristics	Anti-RNAP3+	Anti – RNAP3-	p	OR (95% CI)	Anti RNAP-Anti-TopoI +	p[a]	Anti-RNAP3-ACA+	p[a]
Age at disease onset, yrs., mean (SD) (n available)	50.7 (13.9) (144)	48.5 (13.2) (192)	0.145		48.3 (12.6) (61)	0.255	38.4 (13.0) 30	0.400
Male	34/150 (22.7)	47/195 (24.1)	0.755		19/65 (29)	0.307	1/30 (3)	**0.011**
White	36/39 (92.3)	80/84 (95.2)	0.678		44/47 (94)	1.000	19/19 (100)	0.544
Country of origin								
Italy	43/158	54/199	0.115					
Swiss	22	38						
Sweden	21	34						
Hungary	21	21						
France	27	28						
Others	24	24						
Diffuse cutaneous subtype	74/121 (61.2)	114/168 (67.9)	0.239		55/64 (86)	<0.0001	9/29 (31)	**0.006**
Peak mRSS (0/51 to 51/52) (n available)	21.3 (12.0) (95)	18.6 (10.6) (157)	0.131		22.3 (8.7) (52)	0.594	9.9 (7.0) (21)	**<0.001**
Gastroesophageal reflux disease	100/157 (63.7)	155/199 (77.9)	**0.003**	0.50 (0.30-0.82)	52/65 (80)	0.018	24/30 (80)	0.095
Anorectal incontinence	4/158 (2.5)	5/199 (2.5)	1.000		0/65	0.325	2/30 (7)	0.245
SIBO requiring therapy	6/157 (3.8)	13/199 (6.5)	0.344		2/65 (3)	1.000	3/30 (10)	0.159
Primer biliary cirrhosis	3/154 (1.9)	2/198 (1.0)	0.657		0/65 (0)	0.557	0/30 (0)	1.000
GAVE	13/157 (8.3)	2/197 (1.0)	**0.0009**	8.80 (1.85-57.4)	0/65 (0)	0.012	0/30 (0)	0.133
Scleroderma renal crisis	19/158 (12.0)	5/199 (2.5)	**0.0005**	5.30 (1.81-16.6)	2/65 (3)	0.043	0/30 (0)	**0.047**
Death	25/158 (15.8)	31/198 (15.7)	0.966		13/65 (20)	0.441	1/30 (3)	0.085
Malignancies	28/158 (17.7)	18/199 (9.0)	**0.015**	2.17 (1.15-4.08)	4/65 (6)	0.034	2/30 (7)	0.176
Malignancies synchronous, -6/+12 mos	11/158 (7.0)	2/199 (1.0)	**0.004**	7.38 (1.61-33.8)	0/65 (0)	0.004	0/30 (0)	0.079
Malignancies nonsynchronous	17/58 (10.7)	16/199 (8.0)	0.486		4/65 (6)	0.327	2/30 (7)	0.745
Malignancies synchronous ±2 yrs[b]	14/155 (9.0)	5/199 (2.5)	**0.007**	3.85 (1.36-10.9)	1/61 (2)	0.073	0/30 (0)	0.131
Malignancies non-synchronous ≤2>+2 yrs[b]	1	13/199 (6.5)	0.423		3/61 (5)	0.120	2/30 (7)	1.00
Solid tumors	22/158 (13.9)	12/199 (6.0)	**0.012**	2.52 (1.21-5.27)	44/65 (6)	0.113	1/30 (3)	0.133
Solid tumors synchronous±2 yrs[b]	13/155	4/199 (2.0)	**0.010**	4.46 (1.32-16.6)	1/61 (2)	0.120	0/30 (0)	0.132
Breast cancer	11/158 (7.0)	4/199 (2.0)	**0.030**	3.65 (1.14-11.7)	1/65 (2)	0.187	1/30 (3)	0.694
Breast cancer synchronous, ±2 yrs[b]	7/155 (4.5)	0/199 (0.0)	**0.003**	20.2 (1.41-355)	0/61 (0)	0.195	0/30 (0)	0.600
Solid tumors other than breast cancer synchronous ±2 yrs[b]	6/155 (3.9)	4/199 (2.0)	0.344		1/61 (2)	0.676	0/30 (0)	0.591
Hematologic malignancies including leukemia	1/158 (0.6)	2/199 (1.0)	0.702		0/61 (0)	1.000	0/30 (0)	1.000
Nonmelanoma skin cancer	3/158 (1.9)	4/199 (2.0)	0.940		0/61 (0)	0.558	0/30 (0)	0.504
Melanoma	2/158	0/199 (0.0)	0.195		0/61 (0)	1.000	0/30 (0)	1.000

[a] Compared with anti-RNAP3+ patients. [b] Patients with < 2 years of follow up were excluded.

The overall rate of malignancies was higher in anti- RNAP3+ patients than in controls (17.7% vs 9.0%, Table 6). Cancers appearing simultaneously with the onset of SSc were more frequently found, either between 6 months before and 12 months after SSc onset (7.0% vs 1.0%, p = 0.004), or 2 years before and after SSc onset (9.0% vs 2.5%, p = 0.007). The risk of cancer was not increased beyond 2 years following the diagnosis of SSc (Table 10). Malignancy was diagnosed after the SSc diagnosis within the first 2 years in 9 patients. The frequency of solid tumors in anti-RNAP3+ patients was higher than in controls (p = 0.012), particularly for breast cancers (p = 0.03). Notably, the diagnosis of breast cancer was simultaneous (± 2 yrs) to the onset of SSc in 7 out of 155 anti- RNAP3+ patients and in none of 199 anti-RNAP3– matched SSc controls (p = 0.003). The percentage of women with breast cancer diagnosis synchronous to the onset of SSc was 6.0% (95% CI 3.0–12.0).

In our local cohort, only three patients had malignancies, none of them had an early onset of synchronous, coexistent malignancy (Table 7).

Table 7: Univariable analysis comparing anti-RNAP3+ patients with SSc (cases) and anti-RNAP3– SSc controls, matched for sex, disease duration, cutaneous subset, and age at disease onset at local center

Characteristics	**Anti-RNAP3+**	**Anti – RNAP3-**	**p**
Age at disease onset, yrs., mean (SD) (n available)	41.86±10.28	46.90±8.01	0.987
Male	5/21 (23.8)	5/21 (23.8)	0.6407
Diffuse cutaneous subtype	13/21 (61.9)	13/21 (61.9)	0.6423
Peak mRSS (0/51 to 51/52) (n available)	19.5 (10) (47.6)	1856 (14) (66.7)	0.820
Gastroesophageal reflux disease	16/21	16/21	0.6407
Anorectal incontinence	0/21 (0.0)	1/21(4.8)	0.500
SIBO requiring therapy	1/21(4.8)	5/21 (23.8)	0.092
Primer biliary cirrhosis	1/21(4.8)	1/21(4.8)	0.756
GAVE	1/21(4.8)	0/21 (0.0)	0.500
Scleroderma renal crisis	0/21 (0.0)	0/21 (0.0)	NA
Death	5/21 (23.8)	1/21(4.8)	0.092
Malignancies	1/21(4.8)	2/21(9.5)	0.500
Malignancies synchronous, -6/+12 mos	0/21 (0.0)	0/21 (0.0)	NA
Malignancies nonsynchronous	1/21(4.8)	2/21(9.5)	0.500

The 14 anti-RNAP3+ patients with malignancies synchronous to SSc onset had an older mean age at SSc onset ($p < 0.001$) and an increased proportion of diffuse cutaneous involvement ($p = 0.008$) compared to the other 144 investigated anti-RNAP3+ patients (Table 8). In fact, in patients with dcSSc, the prevalence of synchronous malignancies was higher among anti-RNAP3+ than anti-RNAP3– patients ($p = 0.001$), but no differences were observed when anti-RNAP3+ and anti-RNAP3– cases with lcSSc were compared. Furthermore, the prevalence of synchronous malignancies was higher among anti- RNAP3+ patients with diffuse than with limited cutaneous involvement ($p = 0.009$). A trend for increased proportion of men was also observed comparing patients with malignancies synchronous to SSc onset with other anti-RNAP+ patients ($p = 0.058$; Table 8). In particular, the risk of non-breast cancer synchronous with SSc was much higher in male than in female patients (43% vs 0.8%, $p < 0.001$, OR 95.2, 95% CI 10.2–890).

Table 8: Results of the univariable analysis comparing anti-RNAP3+ patients with or without synchronous cancer. Values are no./available data no. (%) unless otherwise specified. [85]

Characteristics	**Anti-RNAP3+ with Synchronous Cancer**	**Anti-RNAP3+ without Synchronous Cancer**	**p**	**OR (95% CI)**
Age at disease onset, yrs, mean (SD), (n available)	65.3 (10.0) (12)	49.3 (13.3) (128)	<0.001	
Male	6/14 (42.9)	28/136 (20.6)	0.058	
Diffuse cutaneous subtype	13/14 (92.9)	67/121 (55.4)	**0.008**	10.5 (1.33–82.6)
Peak mRSS, 0/51 to 51/51, mean (SD), (n available)	23.3 (13.7) (12)	21.0 (11.8) (83)	0.537	
Time to peak of mRSS, mos, mean (SD), (n available)	12.0 (14.7) (12)	14.5 (17.8) (83)	0.587	
Gastroesophageal reflux disease	8/14 (57.1)	92/143 (64.3)	0.593	
GAVE	3/14 (21.4)	10/143 (7.0)	0.061	
Scleroderma renal crisis	2/14(14.3)	17/044 (11.8)	0.785	

4.3. Effect of serum galectin-3 and NT-proBNP levels on the outcome of SSc

4.3.1. Overall characteristics of the 152 investigated patients

Baseline plasma samples were available for 152 SSc patients; they were enrolled into the study. Mean age of the study cohort was 55 years. 107 patients had limited cutaneous while 45 patients had diffuse cutaneous form of the disease. Preserved (≥55%), moderately reduced (35-54%) and severely reduced (<35%) EF was found in 138 (90.8%), 13 (8.6%) and 1 (0.6%) patients, respectively. In 11 patients PAH was diagnosed by right heart catheterization. Significant coronary artery disease was reported in the medical history of 23 patients. Six of them had acute myocardial infarction, but coronary angiography was not performed. Ten patients underwent percutaneous coronary intervention while in 2 cases coronary artery bypass surgery was performed. In 5 cases coronary intervention was not feasible. For patients with PAH, phosphodiesterase-5 inhibitor treatment was initiated then combined with endothelin receptor antagonists when it was necessary.

Baseline clinical data of the 152 SSc patients as well as detailed description of the further co-morbidities and cardiovascular medication is outlined in Table 9.

4.3.2. Correlations of galectin-3 and NT-proBNP with clinical variables

Both biomarkers showed positive correlation with age. NT-proBNP levels significantly correlated with right ventricular pressure and with PAH diagnosed by right hert catheterisation. Both galectin-3 and NT-proBNP levels correlated positively with the grade of left ventricular diastolic function as well as with the laboratory parameters of inflammation. A negative correlation was found between DLCO and both these particular biomarkers. Both biomarkers showed significant correlation with the survival, even after adjustments for age, gender and BSA. Further correlations of galectin-3 and NT-proBNP with clinical variables are depicted in Table 10.

Table 9: Characteristics of the SSc study population on the effect of serum galectin-3 and NT-proBNP levels on the outcome of SSc

	All patients (n=152)	**Galectin-3≤10.25 ng/ml (n=90)**	**Galectin-3>10.25 ng/ml (n=62)**		**p**
Age (years)	54.9±11.1	53.5±12.0	56.9±9.2		0.062
Female gender (%)	138 (90.8%)	80 (88.9%)	58 (93.5%)		0.329
BSA (m^2)	1.72±0.19	1.73±0.18	1.70±0.20		0.267
DcSSc (%)	45 (29.6%)	22 (24.4%)	23 (37.1%)		0.093
Duration of the disease (years)	6.5±5.3	5.6±3.9	7.8±6.7		0.099
ACA (%)	29 (19.1%)	15 (16.7%)	14 (22.6%)		0.379
Anti-Scl70 antibody (%)	62 (40.8%)	35 (38.9%)	27 (43.5%)		0.566
Death (%)	35 (23%)	11 (12.2%)	24 (38.7%)		**0.000**
Echocardiographic parameters (baseline)					
Ejection fraction (%)	61.5±7.1	61.9±6.5	60.9±8.0		0.373
TR derived right ventricular pressure (mmHg)	30.7±6.2	30.4±5.6	31.2±7.1		0.441
Grade of left ventricular diastolic function - Normal - Impaired relaxation - Pseudonormal	18 (11.8%) 85 (56%) 49 (32.2%)	15 (16.7%) 53 (58.9%) 22 (24.4%)	3 (4.8%) 32 (51.6%) 27 (43.5%)		**0.012**
Laboratory and pulmonary function (baseline)					
Galectin-3 (ng/ml)	10.34±4.69	7.33±1.80	14.70±4.10		**0.000**
NT-proBNP (pg/ml)	217.3±370.8	176.4±266.3	276.5±480.4		**0.047**
ESR (mm/h)	23.6±20.3	20.0±15.9	28.8±24.6		0.053
CRP (mg/l)	12.6±19.6	9.4±13.6	17.3±25.4		0.077
Creatinine (µmol/l)	84.1±45.6	79.0±17.0	91.5±67.8		0.297
Hemoglobin (g/l)	126.3±14.6	126.8±12.9	125.6±16.8		0.640
FVC (%)	96.5±19.9	98.2±19.4	94.0±20.6		0.211
DLCO (%)	64.9±17.4	68.3±15.6	59.8±18.8		**0.003**
Co-morbidities (end of follow up)					
Hypertension (%)	117 (77%)	64 (71.1%)	53 (85.5%)		**0.039**
Coronary artery disease (%)	23 (15.1%)	9 (10.0%)	14 (22.6%)		**0.033**
Pulmonary arterial hypertension (%)	11 (7.2%)	7 (7.8%)	4 (6.5%)		0.756
Atrial fibrillation (%)	23 (15.1%)	8 (8.9%)	15 (24.2%)		**0.010**
Medication (end of follow up)					
Ca-channel blockers (%)	100 (65.8%)	52 (57.8%)	48 (77.4%)		**0.012**
ACE inhibitors (%)	59 (38.8%)	33 (36.7%)	26 (41.9%)		0.512
Loop diuretics (%)	68 (44.7%)	38 (42.2%)	30 (48.4%)		0.453

Table 10: Correlations of Galectin-3 (ln) and NT-proBNP (ln) with clinical variables

	Correlations of Galectin-3 (ng/ml)			**Correlations of NT-proBNP (pg/ml)**		
	Pearson bivariate	**Partial, corrected for age**	**Partial, corrected for age, gender, BSA**	**Pearson bivariate**	**Partial, corrected for age**	**Partial, corrected for age, gender, BSA**
Age (years)	0.233 **p=0.004**			0.298 **p=0.000**		
Female gender	0.136 p=0.094	0.086 p=0.293		0.225 **p=0.005**	0.166 **p=0.041**	
BSA (m^2)	-0.111 p=0.173	-0.096 p=0.241		-0.313 **p=0.000**	-0.305 **p=0.000**	
NT-proBNP (pg/ml)	0.188 **p=0.020**	0.128 p=0.117	0.102 p=0.217			
DcSSc	0.136 p=0.094	0.178 **p=0.029**	0.196 **p=0.017**	0.106 p=0.194	0.159 p=0.052	0.180 **p=0.028**
Duration of the disease (yrs)	0.206 **p=0.012**	0.178 **P=0.031**	0.161 p=0.053	0.001 p=0.994	-0.050 p=0.547	-0.118 p=0.158
Rodnan skin-score	-0.029 p=0.784	0.048 p=0.643	0.057 p=0.587	0.007 p=0.943	0.094 p=0.369	0.148 p=0.158
Ejection fraction (%)	-0.097 p=0.235	-0.050 p=0.538	-0.046 p=0.578	-0.129 p=0.112	-0.072 p=0.382	-0.057 p=0.488
TR derived right ventricular pressure (mmHg)	0.031 p=0.709	0.006 p=0.941	-0.023 p=0.779	0.307 **p=0.000**	0.277 **p=0.001**	0.252 **p=0.002**
Left ventricular diastolic function	0.261 **p=0.002**	0.200 **p=0.020**	0.193 **p=0.026**	0.295 **p=0.000**	0.201 **p=0.020**	0.194 **p=0.025**
ESR (mm/h)	0.224 **p=0.006**	0.189 **p=0.021**	0.172 **p=0.036**	0.370 **p=0.000**	0.334 **p=0.000**	0.284 **p=0.000**
CRP (mg/l)	0.229 **p=0.005**	0.214 **p=0.009**	0.200 **p=0.015**	0.260 **p=0.001**	0.245 **p=0.003**	0.204 **p=0.013**
Creatinine (µmol/l)	0.081 p=0.325	0.059 p=0.472	0.062 p=0.456	0.324 **p=0.000**	0.309 **p=0.000**	0.331 **p=0.000**
Hemoglobin (g/l)	-0.069 p=0.398	-0.054 p=0.513	-0.017 p=0.835	-0.174 **p=0.033**	-0.159 p=0.051	-0.066 p=0.423
FVC (%)	-0.075 p=0.365	-0.106 p=0.199	-0.117 p=0.161	-0.130 p=0.115	-0.179 **p=0.030**	-0.204 **p=0.014**
DLCO (%)	-0.249 **p=0.002**	-0.229 **p=0.005**	-0.228 **p=0.006**	-0.331 **p=0.000**	-0.310 **p=0.000**	-0.306 **p=0.000**
Hypertension	0.184 **p=0.023**	0.151 p=0.065	0.155 p=0.059	0.187 **p=0.021**	0.144 p=0.078	0.156 p=0.057
Coronary arterial disease	0.190 **p=0.019**	0.137 p=0.094	0.150 p=0.067	0.054 p=0.505	-0.027 p=0.741	-0.005 p=0.949
PAH	-0.037 p=0.650	-0.039 p=0.637	-0.053 p=0.518	0.187 **p=0.021**	0.195 **p=0.017**	0.166 **p=0.043**
Atrial fibrillation	0.184 **p=0.023**	0.125 p=0.128	0.140 p=0.088	0.277 **p=0.001**	0.207 **p=0.011**	0.264 **p=0.001**
Death	0.291 **p=0.000**	0.243 **p=0.003**	0.247 **p=0.002**	0.326 **p=0.000**	0.266 **p=0.001**	0.238 **p=0.004**

4.3.3. All-cause mortality

During the follow-up time of 7.2±2.3 years, 35 SSc patients (23%) died. The leading cause of death was cardiovascular (in 16 cases), PAH (4), pulmonary fibrosis (4), malignancy (3), traumatic injury (3), renal (1), gastrointestinal (1), infection (1) and not known (2).

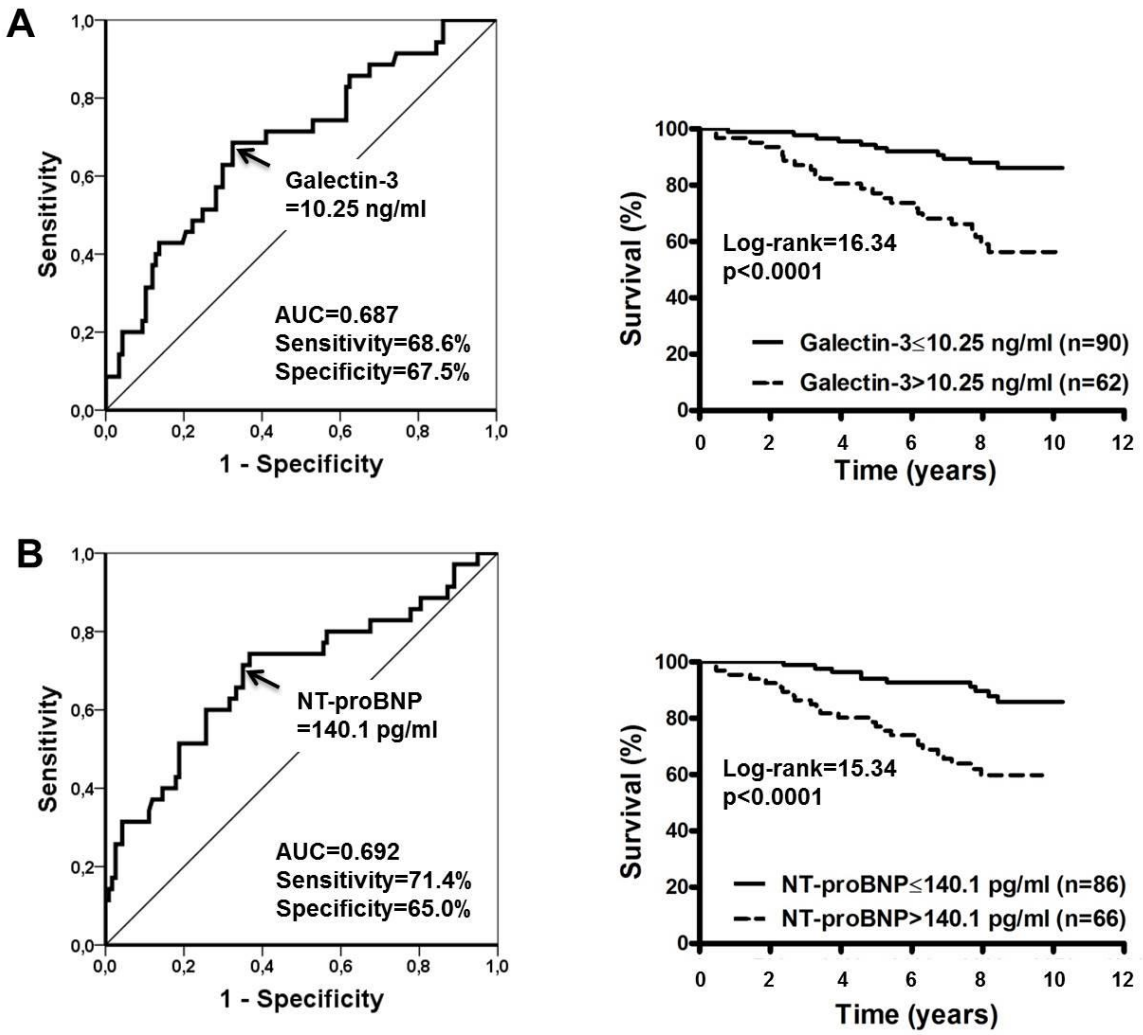
In univariate Cox regression analysis, both galectin-3 (HR=4.611; 95% CI of HR: 2.074-10.252; p=0.000) and NT-proBNP (HR=2.109; 95% CI of HR: 1.526-2.915; p=0.000) showed significant association with all-cause mortality. In multivariate Cox regression analysis galectin-3 remained an independent predictor of all-cause mortality after adjustment for age, gender and BSA. This independent association persisted following the inclusion of NTproBNP. Final multivariate regression model is reported in Table 11.

Table 11: All-cause mortality. Final model of Cox multivariate regression analysis

Variable	**HR (95% CI)**	**p**
Galectin-3 (ln)	2.780 (1.320-5.858)	0.007
Age (years)	1.053 (1.012-1.095)	0.011
BSA (m^2)	0.143 (0.021-0.952)	0.044
NT-proBNP (ln)	1.731 (1.209-2.478)	0.003
Variable removed from the equation: Gender (p=0.126)		

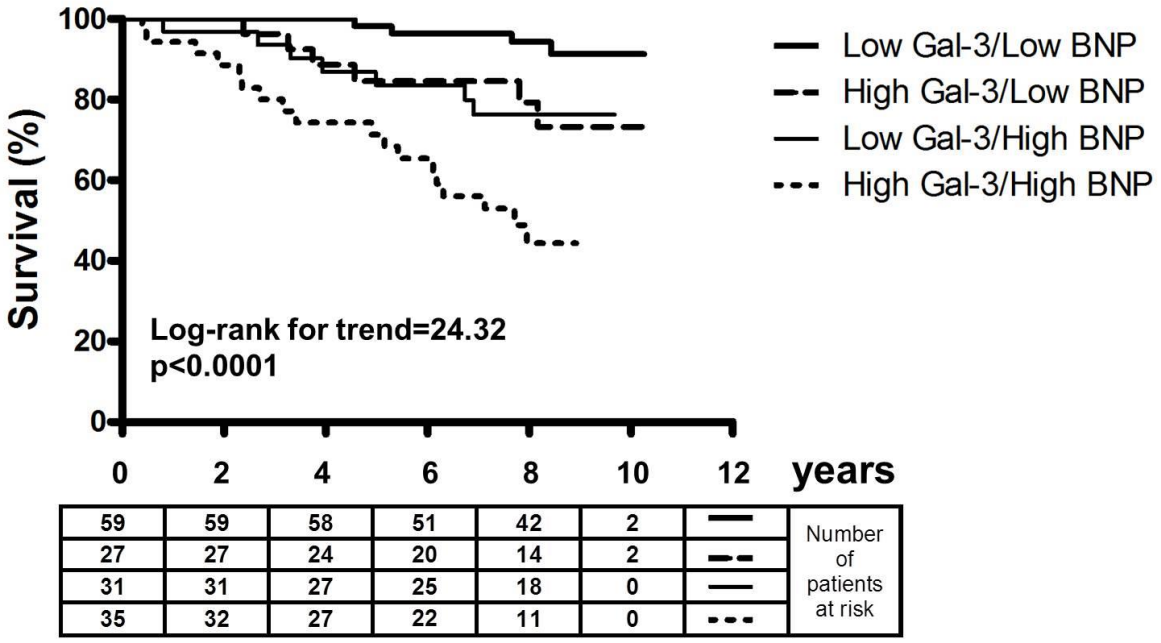
Using ROC analysis, galectin-3>10.25 ng/ml and NT-proBNP>140.1 pg/ml were the best predictors of the all-cause mortality. The AUC, sensitivity and specificity of both biomarkers as well as Kaplan-Meier cumulative survival curves demonstrating the predictive role of these cut-off values are presented in Figure 14. Baseline characteristics of our study cohort stratified by the cut-off galectin-3 level are shown in Table 9.

Figure 14: ROC curves and Kaplan–Meier survival curves demonstrating the diagnostic accuracy of galectin-3 (A) and NT-proBNP (B) in predicting all-cause mortality



When evaluated by comparing groups above and below the cut-off value for each biomarker, NTproBNP and galectin-3 were discordant for 58 subjects (38.2%), divided approximately equally between high galectin-3/low NTproBNP (n=27) and low galectin-3/high NTproBNP (n=31). Compared with the reference group of low galectin-3/low NTproBNP, high galectin-3/low NT-proBNP (HR: 4.884, p=0.024) and low galectin-3/high NT-proBNP (HR: 4.196, p=0.026) groups had similarly higher mortality rates while the highest mortality was observed in the high galectin-3+high NT-proBNP group (HR: 12.180, p<0.0001) (Figure 15).

Figure 15: All-cause mortality stratified by galectin-3 and NT-proBNP profile of the patients. (galectin-3≤ vs. >10.25 ng/ml; NT-proBNP≤ vs. >140.1 pg/ml)



4.3.4. SSc-related mortality

The leading cause of death was related to SSc in 21 patients (60% of all deaths). In univariate Cox regression analysis, both galectin-3 (HR=4.678; 95% CI of HR: 1.670-13.100; p=0.003) and NT-proBNP (HR=2.668; 95% CI of HR: 1.805-3.942; p=0.000) were significant predictors of the SSc-related mortality. In Cox multivariate regression analysis galectin-3 remained independent predictor of SSc-related mortality after adjustment for age, gender and BSA, and after the inclusion of NTproBNP. Final multivariate regression model is reported in Table 12.

Table 12: SSc-related mortality. Final model of Cox multivariable regression analysis

Variable	HR (95% CI)	p
Galectin-3 (ln)	2.770 (1.039-7.386)	0.042
BSA (m^2)	0.043 (0.003-0.523)	0.014
NT-proBNP (ln)	2.465 (1.615-3.763)	0.000
Variables removed from the equation: Age (p=0.113); Gender (p=0.187)		

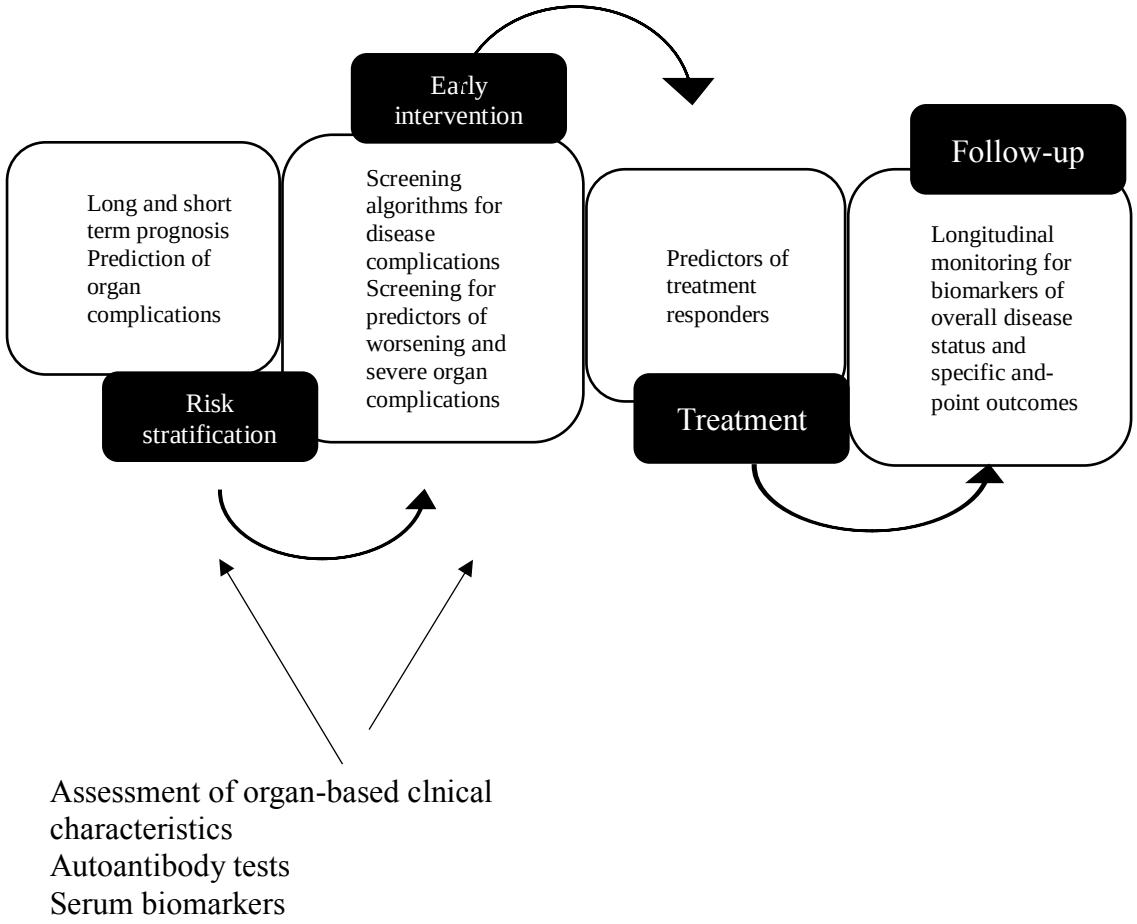
5. DISCUSSION

5.1. Survival, causes of death and factors affecting outcome

SSc is a multisystem disorder characterized by autoimmune phenomena, vasculopathy, and tissue fibrosis. This particular systemic autoimmune disease severely affects the survival and also the quality of life. In spite of the fact that there is no currently available DMARD (disease modifying antirheumatic drug) for the treatment of SSc, the outcome showed a gradual improvement in the last decades [5,9,14,16,17,19,24,25,28,30-32,86]. One of the major causes of this particular improvement is the overall progress in medical care, in particular in cardiology. The other fact that is important in the improvement is the early cytostatic treatment of extensive skin disease and lung involvement. The third important factor in the improved survival is the regular follow up and early treatment of the particular organ involvements.

In the management of patients with SSc, the basic question is the early risk assessment and the individualized treatment. Risk assessment and analysis of the outcome is important in patients with SSc because this particular disorder is characterized by high variability in clinical presentation. Risk assessment (consisting of assessment of internal organ involvement, autoantibody status, capillaroscopy) should be carried out in every patient and also identifying the features predicting possible worsening of certain internal organ involvement. Patient at high risk for developing severe internal organ complication(s) should be treated early and aggressively. This is also valid for the musculoskeletal involvement in particular for the joint involvement. Early and aggressive treatment of synovitis can substantially improve the quality of life of SSc patients. It is also necessary to reevaulte patients state of health time by time to provide the patient the best outcome as possible (Figure16).

Figure 16: Strategic aspects of follow up and management of patients with SSc



SSc is a rare disease, defining prognostic subsets therefore is difficult. It is clear that multicentre data collection to further improve risk assessment is important. Our patient database and prospective data collection may enable us to improve risk assessment.

The outcome/mortality of the different SSc subgroups is substantially different. Our major aim was to investigate the risk factors of mortality in a large series of patients followed up for a relatively long time period with low lost to follow up rate. The strength of our particular long term follow up study was that due to an extensive "tracing down work", the number of patients lost to follow up was low (6.4%). Furthermore, the Hungarian health care system is centralized for the treatment of patients with systemic autoimmune diseases including SSc,

therefore our study represents the overwhelming majority of patients in the South-West Hungarian district.

In accordance with previous studies we also showed that the leading cause of death was the cardiopulmonary manifestation of the disease [3,8,14,16,17,22,24,25,26,28,30,31,34,36,87,88]. The ratio of deceased patients is similar to our previous investigation; however the frequency of SSc unrelated causes of death was increased. This is in accordance with previous observations [14,86]. It is likely that patients nowadays more likely to survive SSc related complication due to aggresive and early therapeutic intervention and die due to an SSc unrelated disease (e.g. stroke, cancer, cardiopulmonary insfufficency) like their contemporaries. Causes of SSc reletad death also show changes over time in causes of SSc related death: scleroderma renal crisis as cause of death shows a decresing incidence, whereas pulmonary and cardiac involvement increases [14,53,87] (Figure 15).

An improvement of survival is clearly observed in cases with SSc, in the last decades. In 1971 Bennett found 50% 10 years survival rate [32], in 1991 Lee found 61% 10 year survival rate [9], and in the last decade the 10 year survival rate varied between 64-88% [5,14,16,17,25,27,28,31]. The survival rate we found was similar to those published in recent studies, and it was also substantially better than previous decades (Table 13). In some cases it is difficult to compare the different studies because both the time interval the studies were conducted and the area where the studies were performed are highly variable. Studies presented in Table 13 shows a variable picture, there are studies from worldwide, from 1947 to 2018, furthermore the items calculated are very different with different methods used. There are prospective and retrospective studies as well. Taking into account all these difficulties, it is clear that there is an overall improvement in survival. Some centers are reevaulating their data regarding survival [12,14,17,23]. These monocenter data also suggests

the improvement in survival. Reevaulation of monocenter data is also important, measuring data changes in a certain area is necessary to have at least a feedback for the center and measure the changes in disease charchteristic. Regarding our local data, the survival improved compared to our previous survival analysis [23]; in the abovementioned study the 10 years survival rate was 72.6% compared to 79.9% in the present study. This is possible due to shorter time between disease onset and enrollment or better therapeutic options (Figure 17).

Figure 17: Changes in 10 year survival rate in Hungary

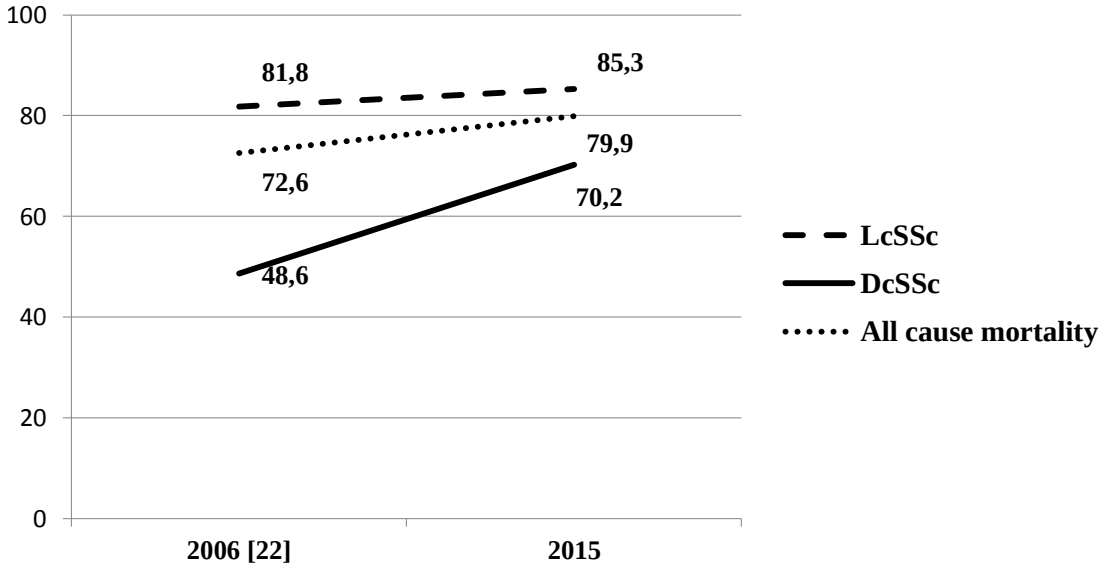
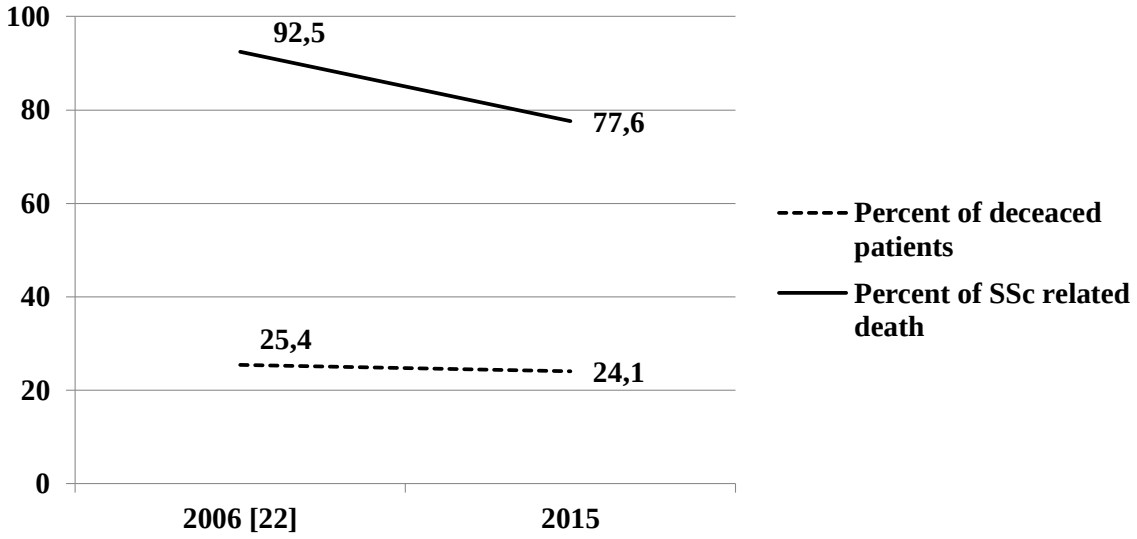


Figure 18: Causes of death changes in Hungary



Our results are in accordance with previous univariate analysis, we have also confirmed that dcSSc [1,3,7,8,12-14,16,17,22,23,25-28,31] male gender [3,7,11,13,17,18,22,23,25-28], pulmonary [3,9,10,12,14,16-18,23,27,34] and cardiac involvement [5,9,10,12,17,23,27,31,37], scleroderma renal crisis [3,9-14.17-19.22-24,27,34,37], elevated right ventricular pressure [3,14,22], decreased ejection fraction [22], ECG abnormalities [1,6,32], decreased DLCO [1,6,7,10,22] and FVC [5,7,8,10,19,22,23,29], esophageal involvement [12,29] were associated with poor survival. Furthermore, anti-topoisomerase I antibody positivity [6,14,22,23], scleroderma capillaroscopic pattern [13,19], anemia [6,10,12,23,29,37], elevated ESR [6,10,12,14,16,23,37] were poor prognostic signs. Conversely, anti-centromere antibody was associated with a favorable outcome [12,23,25,27]. We also confirmed, as in former studies, that low concentration of hemoglobin and/or low hematocrit [6,10,23,29] predict poor outcome. Most of the factors predicting poor outcome are included in different activity indices, hence higher activity may predict poor outcome [89].

Our study showed that lower than 35g/l concentration of serum albumin level was also associated with increased mortality risk, confirming previous findings [29] (Table 14). Most of the findings of our previous survival analysis studies [10,23] were confirmed except the presence of skin pigmentation abnormalities which was not associated with poor survival in our current investigation. In our current study, the time between disease onset and patient enrollment was shorter which may partially explain this particular difference between the current and earlier studies.

The impact of musculoskeletal manifestations in particular the joint involvement has not been previously extensively studied [3,12,28,29], although it is an important problem, because the prevalence of articular erosion in SSc has been estimated at between 5% and 40%, joint contractures have been reported to occur in up to 31% to 97% in previous studies [41,90,91]. Joint involvement often ends up with joint contractures which substantially influences the

quality of life of patients [43]. Contractures and deformities of the hands consist of decreased flexion of the MCP joint, decreased extension of the PIP joint, and decreased abduction of the thumb. Thus it was not clearly defined, the presence of osteoarticular involvement –definition not added- was found to be a risk factor of mortality in a large series of patients from Brazil [28]. Joint deformity was also significantly more frequently present among deceased patients in a univariate analysis in another American cohort [29]. Presence of contractures was also associated with increased mortality risk in another series [10]. Our earlier local study on a cohort independent from this particular study already indicated that presence of hand deformity with contractures is associated with a poor survival [12]. In this particular study, consecutive SSc cases were enrolled. These particular consecutive cases are those patients who regularly attend our department. In our new current study, we carefully traced down all the patients attending our department including those SSc cases whose adherence to therapy and compliance were highly variable. In our new investigation, we demonstrated that presence of small joint contractures is an independent poor prognostic marker of mortality in patients with SSc. Joint involvement appears early [92], and can cause a substantial damage [92]. This kind of damage and damage accrual is a risk factor for poor outcome.

Parallel to our work the data showing that presence of joint contractres is associated with greater mortality risk was confirmed by an EUSTAR analysis and the presence of joint contractures became a component of the risk prediction score of 3 year mortality of SSc [3].

Regarding comorbidities, univariate analysis of the EUSTAR database showed that patients with arterial hypertension had significantly worse outcome (HR=1.38, $p<0.007$) [22]. This was also confirmed in Canadian and American studies [31,35]. In our series, the presence of history of hypertension was strongly associated with mortality, the risk of mortality was twice compared to patients with no arterial hypertension.

Multivariate analysis of risk factors for outcome is presented in several studies (Table 14). There are numerous risk factors associated with increased mortality risk. Different studies are using different items investigated, and this is the possible the cause of the highly variable data. There are risk factors which are almost always investigated (lung, heart, kidney involvement, as well as sex or age at onset) and there are some which are rarely investigated (proteinuria, joint involvement). There are risk factors which are not always associated with increased mortality risk (male sex, subset). This can be explained with different methods used for the analysis, with the different number of patients investigated or with the area where the study was conducted, furthermore some studies have a special focus for example on disease subset or race. To avoid these particular disadvantages of the single center studies, multicentric evaluation is necessary [3,22].

It is well-known that patients with SSc have a higher risk for malignancies compared to the general population, as we have also demonstrated in our earlier study [23]. Some other studies also revealed a close temporal relationship between onset of SSc and tumors [93], especially in patients with anti-polymerase III antibodies [93]. It is supposed that genetic susceptibility, like mutations of POLR3A gene may contribute to in both SSc and cancer development [94]. Patients with anti-polymerase III and diffuse subtype were at higher risk for short SSc-cancer interval [93], but in accordance with our current and earlier study [23], malignancy can develop in other subsets including patients with lcSSc [93]. Sixty percent of patients with coexistent malignancies had lcSSc. In our series only 2 (5%) patients were anti-RNAP3+ and more than 20 (55.5%) ACA or anti-Scl70 positive. Similar to another series [93], we did not find any significant differences in the clinical features of patients with and without coexistent malignancies. The possible explanation for this particular lacking association is that we have a very low number of cases. Like in this particular EUSTAR study the appropriate approach is to use large multicenter study with the inclusion of a high number of patients.

Other studies revealed that presence of coexistent malignancies influence the survival [13,16,23], however not all of these particular studies made distinctions between early and/or late onset malignancies.

Regarding the type of malignancies, in a Spanish study the most common types of neoplasia were breast cancer- similar to our study and skin cancer [16], but clear distinction between paraneoplastic and non-paraneoplastic cases were not made. In our cohort cervical carcinoma showed the second highest prevalene, possible caused by the poor screening.[95] Our previous Hungarian study examining patients of two tertiary care centers [23], proved in a multiple regression analysis that presence of early malignancies were associated with additional risk [23]. In this particular study we demonstrated the increased risk for survival only by univariate analysis, probably due to the recent better diagnostic and therapeutic possibilities available.

Onset of the disease is usually in the forties of the patients [5,7,8,16,19,26,27], but SSc can develop in both younger [21,96-101] and elderly patients [21,102-105]. In the EUSTAR database 1.2% of cases accounted to early onset SSc [101]. Prognostic factors of the childhood SSc are rarely investigated because of low incidence, but previous studies showed pericarditis [98], heart failure [98], arrhythmias [98] were associated with poor survival. A multicenter study investigating the juvenile SSc revealed that gastrointestinal, pulmonary, cardiovascular, central nervous system and renal involvement were frequent among deceased patients [99]. Follow-up investigations of SSc starting in young or elderly patients are rarely published, mainly due to a low incidence of these particular cases. In previous studies the upper limit of early SSc varied between 16 to 30 years [21,96,98,101]. Based on our results we can confirm the already known fact that renal involvement [99] is associated with poor outcome in cases with early onset. Furthermore, in contrast to Martini's [98] work we found that decreased FVC as well as low hemoglobin and hematocrit concentrations are associated

with poor survival similarly to the adulthood disease. In the early onset SSc subgroup we found significantly more patients with decreased FVC, low body weight and conduction disturbances at enrollment compared to the "middle aged" group (onset of 21-64 years). Conduction disturbances were also more frequently present in this particular young age group in a Spanish series of patients, but it did not reach the statistically significant level [21]. A recent EUSTAR investigation showed that patients with young age at disease onset (<30 years when first non-Raynaud symptom appeared) had most significantly dcSSc, anti-Scl70 positivity, however decreased FVC did not reach the statistically significant level [106]. It can be explained with the inclusion criteria: in our study the first RP symptom should have been when less than 20 years old, and also territorial differences might exsist. Our overall conclusion is that a special focus on the appearance/presence of interstitial lung involvement should be paid during the follow up of the young SSc patients. Furthermore, the search for GI involvement and myositis may also be important in young cases. These two particular manifestations of the disease may be predominantly responsible for the decreased body weight of the young SSc cases. The potential early appearance of conduction disturbances on ECG requires regular cardiological search for the presence of heart involvement.

Late onset scleroderma is also rarely studied, previously 1.4-19% of prevalence was reported [21,102,104,105,107]. Survival in elderly patients is significantly lower compared to patients with not elderly onset SSc patients [105]. Generally lcSSc is more frequently observed in this particular patient subset [21,104,105,107], thus our previous series of SSc showed a more prevalent diffuse SSc subset [102], minor cases might not been reffered to the tertiary care center. Patients with elderly onset SSc patients have a higher risk for pulmonary hypertension [105,107], cardiac disease [104,107], pulmonary [102,103,105] and renal [104] involvement compared to patients with younger-age at disease onset. The definition of late onset SSc varies between wide ranges, 60-75 year was the cut off value previously [2,102-105,107]. The

late onset form seems to be a milder type of the disease; the limited form was more common among these patients as in other studies [21,103-107]. The higher prevalence of cardiac involvement in elderly patients may be explained by the high overall incidence of cardiac disease in the elderly population. Significantly higher rate of cardiac involvement (including ECG abnormalities) was also presented in other studies [21,104-107], hence we can claim our patients having late onset SSc are quite similar to patients in other regions, however assessment and follow up of elderly onset SSc patients should contain detailed cardiac involvement, not only due to their age, but due to their SSc as well. Also special attention is required to patients having low albumin level or anemia at disease onset as these alterations are associated with poor outcome.

Table 13: Demographic data and mortality rates reported in different large systemic sclerosis cohorts from 1971 to 2015*

First author	Year of publication	Patient enrollment	Country	N° of patients	Deceased patients %	Male %	dcSSc %	Age at onset	Age at diagnosis	Disease duration	Follow-up time	5 year survival %	10 year survival %	15year survival %	20 year survival %
Bennett et al.[32]	1971	1947-1970	English	67	38.8	16.4	NA	NA	46.2	NA	NA	73	50	NA	NA
Farmer et al.[37]	1960	1945-1952	English	271	48.7	26.6	NA	NA	42.9	NA	103.8	NA	NA	NA	NA
Lee et al.[9]	1991	1979-1990	Canadian	237	25.7	17.3	43	NA	43.3	3.8y	5.7y	3 year: 86%//6 year: 76%//9 year:61%			
Altman et al.[29]	1991	1983-1985	American	264	50	NA	NA	NA	NA	NA	5.2y	2 year: 80% 12 year 30%			
Czirják et al.[12]	1993	1982-1992	Hungarian	118	22,8	10.2	28	NA	NA	NA	5.8y	NA	NA	NA	NA
Hesselstrand et al.[26]	1998	1983-1995	Swedish	249	49	45	25	44.9	NA	10.4 y	5.8 y	86	69	NA	NA
Jacobsen et al.[11]	1998	1960-1996	Danish	344	46.51	19	34	NA	55	8.6 y	NA	81	71	55	42
Bryan et al.[1]	1999	1982-1991	English	280	27.14	23.2	47.4	45.7	NA	17 m	NA	NA	NA	NA	NA
Geirsson et al.[8]	2001	1982-1995	Swedish	100	30	33	34	42.4	NA	NA	7.7y	NA	NA	NA	NA
Ferri et al.[17]	2002	1955-1999	Italian	1012	27.6	11.4	44	NA	NA	NA	7.1y	NA	69.2	NA	45.5
Scussel-Lonzetti et al.[6]	2002	1984-1999	Canadian	309	21.3	14.3	9.4	NA	NA	NA	NA	NA	NA	NA	NA
Simeon et al.[19]	2003	1976-1996	Spanish	79	15.2	14.	28	44.2	48.8	4.5	NA	71	64	62	NA
Mayes et al.[18]	2003	1989-1998	American	706		16.3			46.1			77.9	55.1	37.4	26.8
Ionnadis et al.[20]	2005	NA	NA	1645	35.1	19.8	44.6		49.6		7y	NA	NA	NA	NA
Trad et al.[15]	2005	1980-2004	French	86	19.7	13.6	100		44.5		72.5m				
Czirják et al.[23]	2007	1983-2005	Hungarian	366	25.41	13.9	27.6	NA	NA	13.5 y	6y	84	72.6	NA	NA
Assasi et al.[35]	2009	2005-2008	American	250	14.9	16	57.4	48.8	NA	NA	6.2y	NA	NA	NA	NA
Hachulla et al.[7]	2009	2002-2003	French	546	8.6	15.9	27.5	46	47.8	NA	3.1y	NA	NA	NA	NA
Joven et al.[16]	2010	1980-2006	Spanish	204	NA	11	31	43	49	NA	8y	85	75	NA	55
Kim et al.[5]	2010	1972-2007	Korean	230	14.3	10.9	43.9	NA	43.7	NA	8.6y	85.4	80.1	NA	NA
Tyndall et al.[22]	2010	2004-2008	European	5860	5.2	19.4	35.6	NA	NA	NA	0.9y	NA	NA	NA	NA
Al-Dhaher et al.[31]	2010	1994-2004	Canadian	185	23	NA	37	NA	NA	9.1	NA	90	82	NA	NA
Hissaria et al. [13]	2011	1993-2007	Australian	736	42.11	19.9	19.3	46.7	NA	16,4y	NA	74	NA	NA	NA
Hashimoto et al. [27]	2011	1973-2008	Japanese	405	21.2	7.2	32.6	47	NA	14y	NA	NA	88	NA	77.4
Sampio-Barros et al. [28]	2012	1991-2010	Brazil	947	17.7	11.5	31	42.6	NA	12.6y	9.6y	90	84	NA	NA
Hoffmann et al.[25]	2013	1999-2009	Norwegian	312	14	NA	NA	47	54	9.9y	8.1y	95	86	NA	NA
Ferri et al.[14]	2014	2000-2011	Italian	821	9.1	9.1	12.5	53.7	NA	3.6y	4.5y	NA	80.7	NA	NA
Alba et al.[21]	2014	2006-2012	Spanish	1037	14.6	12	40	45	51	NA	5.2y	90.7	NA	NA	NA
Simeón-Aznar et al.[4]	2015	1970-2008	Spanish	879	15.7	14.8	27.6	NA	NA	NA	N	96	93	NA	83
Santosa et al. [36]	2016	2008-2013	Chinase	349	10	13.2	37.1	NA	46.2	NA	NA	NA	NA	NA	NA
Poormoghim et al [24]	2016	1998-2012	Iranian	220	14.5	12.7	40	38.9	NA	NA	6.64	92.6	82.3	NA	NA
Cruz-Domingez et al [30]	2017	2005-2015	Mexican	220	28	5.5	57.5	NA	NA	11.68	5.85	83	70	NA	NA
Elhai et al [3]	2017	2004-2015	European	11193	9.6	14	31	NA	NA	12.3	3y	3year: 89.3			
Ooi et al. [34]	2018	2005-2016	New-Zeland	132	15.2	NA	NA	NA	52	NA	NA	NA	NA	NA	

*Studies on SSc patients recruiting basic information on organ manifestation were selected. Studies investigating one specific organ involvement, subset or race were excluded [108].

Table 14: Risk ratio of different factors affecting survival in series of scleroderma by multivariate analysis*

First author	Male sex	dcSSc	PAH	Lung	Heart	Kidney	Low BMI	Anti-Scl70 +	Hyper-tension	Osteoarticular / joint involevemnt	Muscle weakness	TFR	Cancer	Malnut-ritiom	Age	Low hemoglobin level	Elevated ESR or CRP	Elevated right ventricular preasure	higher mRSS	Protein-uria	Digital ulcer
Lee et al.[9]	1.1			2.3	5	2.8									1.2						
Ferri et al.[17]	1	5.27		2.52		8.1									1.08						
Scussel-Lonzetti et al.[6]				4.3											1.036	2.37	ESR: 3.89				
Trad et al.[15]			4.09			4.10									1.057						
Czirják et al.[23]	1.13	2.37		3.38									3.2				3				
Hachulla et al.[7]			7.246												1.052						
Assasi et al.[35]				2.46			12.94		3.14						4.37						
Altman et al.[29]	1.47																				
Kim et al.[5]		2.5		2.8	4.2			3							1.7-7.4						
Tyndall et al.[22]			2.018	1.644											1.295				1.198	3.343	
Ioannidis et al.[20]	1.5			1.6	2.8	1.9		1.3							1.6						
Joven et al.[16]			2.2	2		4.5									1.2						
Hasimoto et al. [27]				2.21	1.77	1.35															
Sampaio-Barros et al.[28]	2,35			4.2		9.96				4.38									1.71		
Clements et al.[10]				6.09															3.69		
Hoffmann et al.[25]	2.1	2.8	8	3.1																	
Alba et al. [21]		2.22	1.89	1.79		6.16									1.05						
Frasen et al.[2]															1.03		ESR: 1.89			2.29	
Bryan et al.[1]																	ESR: 7.4			23.6	
Ferri et al.[14]	6.05			5.34		10.49									1.03			4.98			2.78
Simeón-Aznar[4]	1.115	2.700	2.69		3.187	6.448															
Santosa et al. [36]						2.5								8.8				5.1			
Poormoghin et al [24]				11.5						arthritis: 3.56		6.39			5.1						
Cruz-Domingez [30]	5.84													3.77							
Elhai et al [3]	1.34	1.25		1.26		1.48				contractures 1.28	1.34				1.86-3.63		CRP: 2.34			1.95	1.24

* The publications using multivariate regression analysis were selected.

The comparison of autopsy results and clinical findings showed that in several individuals myocardial fibrosis was only found only at autopsy, but was not recognized clinically. This is important as it shows that despite modern diagnostic tools, occult organ involvement in SSc is still often present, *e.g.* showing myocardial SSc involvement in 80–90% of patients [109], despite normal ECG and normal left ventricular systolic function eve when more sophisticated cardiac imaging procedures such as Doppler or MRI as well as biopsies heart are performed [110].

In the EUSTAR analysis aimed to investigate the autopsies performed in SSc patients a discordance was found between clinician and pathologist regarding the cause of death. Even if few cases were investigated, remarkable discrepancies up to 63% can be observed between the treating physician and pathologist for the cause of death are not uncommon in SSc patients [68]. One reason for this may be the fact that clinically silent vascular events in different location may occur in patients with SSc. There are difficulties these particular vascular events, because the access to the sophisticated, expensive new imaging techniques is limited. Clearly, there is an unmet need to improve the follow up recommendations for cardio-respiratory involvement of SSc [111,112].

Autopsies are rarely performed, but might help to educate the clinicians, who treat severe SSc to better understand the disease and improve patient care. In conclusion, more autopsies should be perfmed to identify subclinical alteration and it might be useful to clinically differ between probable, possible and definite SSc related deaths. As autopsies are rarely performed monocenter investigations are not sufficient, only multicentre data can provide powerful results.

5.2. Role of anti-RNAP3 in screning for early malignancies and certain internal organ manifestations

Besides the cardiorespiratory and renal involvement, the coexistence of a malignancy (paraneoplastic syndrome) also strongly influences the outcome of SSc. Anti-RNAP3 and anti-PmScl antibody positivity was found to be associated with an increased risk of cancer in patients with SSc [54,55,58-60,113-115]. Because organ complications, such as SRC or GAVE, usually developing early during the disease course [116-118], these particular features indicate that anti-RNAP3 identifies a SSc subset characterized by particularly rapid onset and progression.

In the large EUSTAR study the clinical associations of anti-RNAP3 in SSc were analyzed, taking advantage of the EUSTAR collaborative group, focusing particularly on the associations with malignancies. Previous reports described a more severe cutaneous involvement in anti-RNAP3+ than in anti-topo I+ patients [52,119-121] and a shorter interval between the appearance of Raynaud's phenomenon and the first non-Raynaud SSc symptom [122]. This particular study confirms that progression in skin thickening is particularly rapid in anti-RNAP3+ patients [54].

The identification of anti-RNAP3 as a marker of an SSc disease subset and an indicator of a concomitant onset of cancer was first described by a single-center American study in which anti-RNAP3 were evaluated both by immunoprecipitation and ELISA [54]. This was subsequently confirmed by several reports, whatever the geographic origin of the patients despite some differences in research design [58-60,96,115]. The OR for diagnosis of cancer in anti-RNAP3+ patients as compared to other patients with SSc, was calculated at 5.08 (95% CI 1.60–16.1) [60] and 5.83 (95% CI 3.1–10.9) [59] in an interval of 2 or 3 years around the onset of SSc, respectively. This particular study showed an OR of 7.38 (95% CI 1.61–33.8) within an interval between 6 months before and 12 months after SSc onset. Our center contributed to this large study with 1/5 of the all included cases. Our local results did not show the same risk because the number of patients in our local cohort was low. It is clear that the relevant approach of the investigation is a multicentre investigation, because individual centers are not able to include a sufficient number of patients into a study analysing a small subset of patients with an orphan disease.

In the EUSTAR cohort an increased overall prevalence of cancer in anti-RNAP3+ patients was also observed, thus with a low OR, similar to what is found in a previous study [59], but not in some others which included small numbers of cancer cases [53,60,115]. In years distant from the onset of SSc the EUSTAR investgation like others [59] did not observe any increased frequency of cancer in anti-RNAP3+ patients. Therefore, no available data thus far suggest that the risk of cancer is extended beyond an interval of a few years around SSc onset. The large majority of malignancies associated with the onset of anti-RNAP3+ SSc were solid cancers; in particular anti-RNAP3+ patients were more likely to develop breast cancer within 2 years as compared with matched SSc controls with an OR of

20.2. Although the CIs were wide, it is noteworthy that a similar OR of 19.0 as compared with ACA+ patients within a 3-year interval was previously reported [59].

Many previously published case series described a higher incidence of breast cancer in patients with SSc compared to the general population, and close temporal relationship with SSc onset was frequently reported [61-63]. A metaanalysis demonstrated the association of SSc with lung and hematological malignancies, but did not confirm the association with breast cancer [123]. However, the analysis excluded breast cancer cases diagnosed before SSc, which are fairly common in the years close to SSc onset in anti-RNAP3+ patients [60]. These particular data indicate that the mechanisms evolving the association of SSc with lung and hematological neoplasms may be the oxidative damage of DNA and the use of alkylating agents [50]. This is clearly different from those explaining the association with SSc and breast cancer. Indeed, genetic mutation in the POLR3A gene, encoding for RNAP3 polypeptide A, and humoral and cell-mediated immune response against this mutated antigen were demonstrated in anti-RNAP3+ patients, but not in patients with other SSc-specific antibodies and cancer [124]. These particular data suggest that an autoimmune response initiated by mutation in the autoantigen in the cancer cells may explain the onset of SSc as a paraneoplastic disease in these particular breast cancer associated cases [57], thus this putative mechanism of cancer-induced autoimmune response may be not limited to this particular type of malignancy.

In this particular EUSTAR study, patients with older age or diffuse cutaneous involvement were particularly at risk. These risk factors may help in clinical practice to institute appropriate cancer screening at SSc diagnosis in these individuals.

The association of anti-RNAP3 with GAVE [116,117], a complication associated with renal crisis, hypertension, reduced DLCO/alveolar volume, and telangiectasia was also confirmed [116,126-128], thus reinforcing the characterization of anti-RNAP3 as markers of an SSc subset particularly prone to microangiopathic complications. Accordingly, anti-RNAP3 was associated with increased risk of pulmonary hypertension (PH) [48]. This could not be confirmed, but the phenotyping of PH in the EUSTAR cohort (initially based on echocardiographic data) was only recently improved, and thus the

analysis could not yet include enough patients with proven diagnosis of PH. Further studies are therefore needed to clarify this important issue.

It is noteworthy that single-center observational studies on the relative risk of cancer simultaneous to the onset of SSc [59,60] were very similar to the results of this particular EUSTAR study. Anti-RNAP3 antibodies are associated with poor prognostic manifestations of SSc, including increased risk of malignancy diagnosis (in particular of breast cancer) close to the SSc onset.

In the EUSTAR series, the crude frequency of having a diagnosis of cancer within 2 years before or after SSc onset in anti-RNAP3+ patients was 11%. As in other series [55,59,60,115], around half of these particular malignancies were breast cancers. These results suggest the possible need of a cancer screening program for anti-RNAP3+ patients, similar to what is generally applied in dermatomyositis [57], considering that the number of patients needed to be screened to find synchronous malignancy would be relatively low. Furthermore, there is a need of a special focus on the screening for presence of breast cancer.

5.3. Galectin-3 and NT-pro-BNP as a maker of cardiac disease and poor outcome in SSc

Our other major aim was to describe new potential serum biomarkers which are useful in risk assessment and prediction of disease outcome. There is an unmet need for the development of additional serum biomarkers useful for prediction of disease outcome. The different forms of heart involvement are important determinants of outcome. Furthermore, heart involvement is patients with SSc is highly variable [51,70,71,84].

The diagnostic and prognostic value of NT-proBNP is already well established in SSc [70-72], and was also confirmed in our patient cohort. In univariate Cox regression analysis NT-proBNP was significant predictor of all-cause and disease related mortality with a HR of 2.

NT-proBNP is a neurohormone secreted mainly in the cardiac ventricles in response to volume expansion and pressure overload. In SSc, NT-proBNP allows the detection of several cardiac diseases including left or right heart failure and pulmonary arterial hypertension. Our results also indicate that besides NT-proBNP, another serum biomarker, galectin-3 is also an independent predictor of all-cause and disease-related mortality in SSc. Added to NT-proBNP, galectin-3 provides valuable

complementary prognostic information, mainly by reflecting mortality risk associated to organ sclerosis and inflammation.

Recent studies suggest that galectin-3 plays key role in the fibrogenesis in different organ systems including lung and heart muscle [73]. The potential association between serum galectin-3 levels and clinical characteristics of disease severity in SSc have already been investigated. Taniguchi et al. reported that serum galectin-3 levels were significantly decreased in early diffuse cutaneous SSc, but became higher during the course of the disease, compared with the control subjects. In this particular study, serum galectin-3 levels were higher in SSc patients with digital ulcers and/or elevated right ventricular systolic pressure than in those without these symptoms [75]. In addition, Koca et al. found, that serum galectin-3 levels were higher in SSc patients than in healthy controls, but did not show significant correlations with inflammatory markers or cytokines in SSc patients [74].

In our SSc population no correlation was found between galectin-3 levels and Rodnan skin score or pulmonary vascular involvement leading to pulmonary arterial hypertension. It might be explained with the fact that we observed lower levels of Gal-3 than others and the ratio of dcSSc patietns were low (25%). Our cardiac follow up is quite regular, performed by the same team mainly. Hence, early intervention and detection of PAH is possible.

At the same time, galectin-3 levels showed positive correlation with the duration of the disease. In addition, galectin-3 levels were higher in the diffuse cutaneous form of the disease, which may be the sign of the more extensive fibrosis in this form of SSc.

Galectin-3 is a regulatory molecule acting at various stages along the continuum from acute inflammation to chronic inflammation and tissue fibrogenesis.[129] In heart failure patients with reduced and preserved ejection fraction DeBoer et al. found significant correlation between galectin-3 and various pro-inflammatory cytokines, as well as CRP [130]. Likewise, in our SSc population galectin-3 levels showed significant correlation with the laboratory parameters of inflammation, such as erythrocyte sedimentation rate and CRP.

In contrast to the findings of Taniguchi et al., [75] we have found a significant negative correlation between DLCO and galectin-3 levels, suggesting, that galectin-3 may be a biomarker of interstitial

lung disease in SSc. This result is in line with the work of Nishi et al. reporting increased level of galectin-3 in bronchoalveolar lavage fluid from patients with idiopathic pulmonary fibrosis and pulmonary fibrosis associated with collagen vascular disease [130].

Myocardial fibrosis is a major component of left ventricular diastolic dysfunction and heart failure. In SSc patients, left ventricular diastolic dysfunction is highly prevalent and is associated with increased risk of mortality [131,132]. In our population, galectin-3 levels correlated positively with the grade of the left ventricular diastolic dysfunction. Similar finding were reported in the work of Shah at al., where higher levels of galectin-3 were related to Doppler indices of impaired myocardial relaxation and higher filling pressure in patients with heart failure [133]

It is still a question of debate whether galectin-3 plays a causative role in the pathogenesis of atrial fibrillation [134,135] or coronary artery disease [136]. In our population, galectin-3 levels showed significant correlation with both the presence of atrial fibrillation and coronary artery disease, but these correlations lost their significance after adjustment for age, not supporting a causal relationship. This is the first study investigating the long-term prognostic value of galectin-3 for the prediction of all-cause and disease-related mortality in SSc patients. In Cox multivariate regression analysis galectin-3 was proved to be independent predictor of the all-cause and disease-related mortality even after the inclusion of NT-proBNP. In an analysis comparing four groups defined by whether each biomarker was elevated above the cut-off values or not, patients with elevated levels of both biomarkers had significantly higher risk (HR: 12.180) of reaching the primary outcome compared to those with both biomarkers below the cut-off values. These results suggest, that galectin-3 reflect a different pathophysiologic axis than NT-proBNP.

Elevated galectin-3 levels in our population showed clear associations with the signs of the advanced organ fibrosis including left ventricular diastolic dysfunction (caused by myocardial patchy fibrosis), low DLCO levels (caused by predominantly lung vascular/interstitial disease), and with the laboratory parameters of inflammation, explaining the prognostic power of this biomarker. Nevertheless, high serum levels of galectin-3 has been described in a number of other conditions such as COPD, pneumonia, heart failure, sepsis and kidney disease, therefore it is still questionable, if galectin-3 plays

a specific role in the pathogenesis of SSc. Recent data suggests that galectin-3 is rather a general marker of disease severity and mortality [137]. Furhter validation studies are required to establish whether galectin-3 may be considered as a useful and simple biomarker for selecting patients with high mortality risk in SSc.

The mean galectin-3 level in our population tended to be lower than those in the recent heart failure studies [130,133,137-140]. One of the possible explanations of this phenomenon is that only less than a half of our patients had a clinically manifest heart failure (44.7% was treated with loop diuretics). On the other hand, a large number of galectin-3 immunoassays are available, and the measured concentrations may vary between assays.

Regarding the risk assessment and follow up of the patients with cardio-respiratory involvement serum NT-proBNP level is an excellent negative predictor, because its normal level indicates that signs of either left or rigth heart failure are not present. In case of and elevated NT-proBNP, the background heart/lung disease should be clarified. A further additional help in risk assessment is the detection of galection-3 level, which indicates the presence of significant heart involvement causing poor outcome.

6. SUMMARY

Systemic sclerosis is a multisystem disorder characterized by autoimmune phenomena, vasculopathy, and tissue fibrosis. This particular systemic autoimmune disease severely affects the survival and quality of life.

The mortality and the extent of disability of the different SSc subgroups is substantially different. Our major aim was to investigate the risk factors of mortality in a large series of patients followed up for a relatively long time period with low lost to follow up rate (6.4%). The Hungarian health care system is centralized for the treatment of SSc cases. Our study most probably represent the overwhelming majority of patients in the South-West Hungarian district.

Our current study showed that there was an overall improvement in survival of patients with SSc either compared to our earlier studies or international publications. The outcome showed a gradual improvement in the last decades [5,9,14,16,17,19,24,25,28,30-32,86] and we found the same in our cohort. One of the major causes of this particular improvement is the overall progress in medical care. In particular, SSc patients experienced a substantial benefit from the improvement in the early recognition and management of cardiac/lung disorders. The other fact that is important in the improvement is the introduction of early cytostatic treatment of extensive skin disease and lung involvement. The basic question is the early risk assessment and the individualized follow up of the patients with SSc. Risk assessment and analysis of the outcome is important, because this particular disorder is characterized by high variability in clinical presentation. Our patient database and prospective data collection enables us to improve risk assessment.

To best our knowledge we proved first in a multivariate analysis, besides the well known risk factors, that the presence of small joint contractures was associated with increased mortality risk. Furthermore, patients with a history of systemic arterial hypertension were also at higher risk for outcome. These two particular clinical findings can be used for an improved risk assessment in the future.

Besides the overall risk assessment, it is crucial to define patient subsets with different outcomes. We have confirmed the well-known fact that disease onset in elderly age causes a poor survival. Furthermore, we first described that patients with an onset of Raynaud’s syndrome before the age of

20 years are likely to have a lung involvement with a decreased FVC, therefore a special focus on the signs of early interstitial lung involvement is mandatory during the follow up.

However anti-Scl70 and ACA positive patients charachterize a wellknown subset, anti-RNAP3 positive cases also define a diffuse SSc patient subset with characteristic clinical findings. Earlier monocenter studies showed that these patients exhibited a relative risk of cancer appearing simultaneously to the onset of SSc. As a part of the large European multicentre study we have confirmed that the risk of the presence of simultaneous cancer was high in this particular subset of SSc cases. This multicenter investigation also proved that anti-RNAP3 was associated with GAVE and SRC development. Subanalysis of our small case cohort did not show the same results indicating that for risk assessment of a small subset of SSc patients large multicentre studies are required.

Beside clinical investigations it is important to define new serum biomarkers which could improve either risk assessment of a patient subset requiring special focus on one particular organ involvement. In our study galectin-3 levels showed positive correlation with the grade of left ventricular diastolic function as well as ESR and CRP. Negative correlation was found between galectin-3 and DLCO. Furthermore, the follow up study clearly indicated that galectin-3 is an independent predictor of all-cause and disease-related mortality in SSc. Added to NT-proBNP, galectin-3 provides complementary prognostic information, mainly by reflecting mortality risk associated to organ sclerosis and inflammation.

7. NEW RESULTS

Our current study showed that there was an overall improvement in survival of patients with SSc either compared to our earlier studies or international publications.

We have first described that the presence of small joint contractures at onset of SSc is associated with increased mortality risk; this is an independent risk factor for mortality in SSc. This particular finding can be used for risk assessment in patients with SSc.

Patients with hypoalbuminemia have a poor survival; it is an independent risk factor of mortality.

Patients with onset of Raynaud's syndrome before the age of 20 years are likely to have a decreased FVC. Young patients with SSc need a special focus on interstitial lung involvement during the follow up.

Anti-RNAP3 positive cases show a relative risk of cancer appearing simultaneously to the onset of SSc in the large European multicentre study. The same was not found in our small cohort. For this kind of risk assessment, a large multicenter study is the appropriate investigation.

Galectin-3 levels showed positive correlation with the grade of left ventricular diastolic function as well as ESR and CRP. Negative correlation was found between galectin-3 and DLCO.

Galectin-3 is an independent predictor of all-cause and disease-related mortality in SSc. Added to NT-proBNP, galectin-3 provides complementary prognostic information, mainly by reflecting mortality risk associated to organ sclerosis and inflammation.

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