Systemic sclerosis: are anti-nuclear antibodies our guiding stars?

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PART I

The need for biomarker research

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Rituximab in early Systemic Sclerosis

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Abstract

Objectives
1) Hypothesis testing of the potency of rituximab in preventing fibrotic complications and 2) assessing acceptability and feasibility of rituximab in early Systemic Sclerosis.

Methods
A small, 24-month, randomized, double-blind, placebo-controlled, single-centre trial in Systemic Sclerosis patients diagnosed <2 years, was conducted. Patients received rituximab or placebo infusions at t= 0, t = 15 days and t = 6 months. Patients were clinically evaluated every three months, with lung function tests and HRCT every other visit. Skin biopsies were taken at baseline and month 3. Immunophenotyping of peripheral blood mononuclear cells was performed at every visit, except at month 9 and 18. Adverse events, course of skin and pulmonary involvement and B cell populations in skin and peripheral blood were evaluated.

Results
In total 16 patients (rituximab n=8, placebo n=8) were included. Twelve patients had diffuse cutaneous systemic sclerosis. Eighty-eight adverse events (rituximab n=53, placebo n=35, p=0.22) and 11 serious adverse events (rituximab n=7, placebo n=4, p=0.36) occurred. No unexpected rituximab related events were observed. Mean skin score over time did not differ between the groups. Over time, FVC and extent of lung involvement slightly improved with rituximab, but this difference was insignificant. In peripheral blood B cells depletion was demonstrated.

Conclusions
No unexpected safety issues were observed with rituximab in early Systemic Sclerosis. Although this small trial could not confirm or reject potential efficacy of rituximab in these patients, future placebo controlled trials are warranted, specifically in the subgroup of patients with pulmonary involvement.

Trial registration: www.clinicaltrialsregister.eu, EudraCT Number: 2008-07180-16

Introduction

Systemic sclerosis (SSc) is an autoimmune disease that is characterized by the triad of microvascular damage, dysregulation of innate and adaptive immunity, and generalized fibrosis in multiple organs(1) .

The pathogenesis of SSc is poorly understood and treatment is organ and symptom based. Current therapy targeting the dysregulated immune system, supported by clinical trial data, includes methotrexate for early skin involvement (2, 3), cyclophosphamide followed by mycophenolate mofetil or azathioprine for lung involvement (4-8) and autologous hematopoietic stem cell transplantation for severe, diffuse cutaneous Systemic Sclerosis (dcSSc) (9).

Experimental data suggested a key role for B cells in the pathogenesis of SSc(10). B cells seem to overexpress the stimulatory receptor CD19 and IL-10-producing regulatory B cells are decreased (11).

Previous observational open-label studies of anti-CD20 therapy (rituximab) and a nested-case control study in SSc showed potential efficacy for skin disease and stabilization of internal organ disease in dcSSc (12-21). Since natural disease course is variable and difficult to predict, these results are difficult to value and need to be replicated in randomized controlled trials (22).

We hypothesized that the window-of opportunity for rituximab (RTX) in SSc patients lies early in the disease course, when fibrotic complications are yet to develop. This hypothesis is based on observations in a study in mice in which B cell depletion with anti-CD20 was effective in prevention of skin fibrosis in new-born tight-skin mice while no benefit was observed in tight skin mice with established disease (23). Additionally, BAFF levels in these mice were elevated at 4 weeks after birth, while normalized at week 12 when skin fibrosis was established (24).

Based on these observations, we aimed to test the hypothesis that RTX can prevent development of severe fibrotic complications in early Systemic Sclerosis. Additionally, safety and feasibility of rituximab in early SSc is described, together with the influence of rituximab on immune cell subsets in peripheral blood and in skin tissue in SSc patients.

Methods

Trial design
The rituximab in early systemic sclerosis (RITIS) trial was designed as a 24-month, parallel, double-blind, placebo-controlled randomized trial. Randomization was performed in a 1:1 ratio by the Pharmacy of the Leiden University Medical Center (LUMC), Leiden, The Netherlands. Ethical approval was obtained from the Medical Ethical Committee (METC) of the LUMC and patients gave written informed consent. The study was monitored by a Data and Safety Monitoring Board until completion.

Patients
Between June 2010 and February 2014, patients with an established diagnosis of SSc according to the American Rheumatology Association (ARA) criteria (25) within the last 24 months before enrolment and aged between 18 and 70 years were included. Previous immunosuppressive therapy was allowed and continued throughout the trial. Patients with a history of deep tissue infection within 1 year prior to baseline, patients with chronic or recurrent infections and patients with a history of cancer were excluded.

Procedures
Patients received IV 1000mg rituximab (Mabthera©/Anti CD 20 mAb) or placebo (0.9% NaCl) on day 1 and day 15 as induction treatment. Consolidation treatment consisted of a single IV treatment with 1000mg rituximab or placebo (0.9% NaCl) at 6 months. Each infusion of rituximab was given together with methylprednisolone 100mg IV, oral paracetamol 1000mg and clemastine 2mg IV. Placebo treated patients received 1.6mL 0.9% NaCl together with oral paracetamol 1000mg and clemastine 2mg IV. Concomitant medications or other treatments deemed necessary for patients' supportive care and safety were allowed at the discretion of the treating physician. Patients, physicians and the observers performing the skin score were blinded for treatment allocation.

Data collection
Patients were seen every 3 months during the first year and every 6 months thereafter, for physical examination including the modified Rodnan skin score (mRSS), and assessing toxicity (National Cancer Institute, Common toxicity parameters (CTC))(26), urine analyses and laboratory testing(at t=0, 3,6, 12, and 24 months also including samples for immunophenotyping of peripheral blood) for a total follow-up of 24 months. Skin scores were assessed by an experienced research nurse (AV) and a research physician who was trained by AV (JM). In two-thirds of cases the skin score was assessed by AV and in one third by JM. Patients filled out the following

questionnaires at every visit: Short Form 36 (SF-36) (27, 28), EuroIQol-5D (EQ-5D) (29, 30) and Health Assessment Questionnaire Disability Index (HAQ-DI)(31, 32). Lung function tests including Forced Vital Capacity (FVC) and Diffusing capacity of the Lungs for carbon monoxide (DLCO), High-resolution computed tomography (HRCT) of the thorax and echocardiography were performed every 6 months. HRCT's were assessed using Goh criteria evaluating the extent of lung involvement at five levels: 1) origin of great vessels; 2) main carina; 3 pulmonary venous confluence; 4) halfway between the third and fifth section; 5) immediately above the right-diaphragm.(33) Scoring was performed consensus based, by 2 observers (AS and LK).

For histologic and immunohistochemical analysis of the skin, 4 mm skin biopsies were obtained from the dorsal side of the forearm, within 1 cm of each other, at baseline and at 3 months.

Immunohistochemistry of skin tissue was performed on 4µm thick sections on polylysine-coated slides. After routine deparaffinization and rehydration, antigens were retrieved in a tissue microwave for 12 min at 98°C with a Target Retrieval Solution Tris/EDTA pH 9. Quenching of endogenous peroxidase activity was performed with 1% hydrogen peroxide in methanol for 10 minutes. Biopsies were incubated with: Haematoxylin and eosin staining (4085.9005 and 4085.9002; Klinipath; Duiven, Netherlands) (general histopathology assessment and mononuclear infiltration), PBS/1%BSA for 1hour with CD3 (1.41 µg/ml; M7254; DAKO) (T-cells), CD68 (0.12 µg/ml; M0814; DAKO) (macrophages), CD79a (1.875 µg/ml; M7050; DAKO) (B-cells including plasma cells). Human tonsil specimens were used as a positive control for antibodies.

Stained sections were coded and scored by three observers, who were unaware of clinical data and treatment regimen (AD, KQ, MB) with respect to the following points: histologic signs of scleroderma skin (such as presence and entrapment of adnexa), mononuclear infiltration (semi-quantitative scale), T cell infiltration (semi-quantitative scale), B cell infiltration (semi-quantitative scale) and macrophage infiltration (semi-quantitative scale). Semi-quantitative scoring for lymphocyte and macrophages was based on the scoring scale for lymphocytes proposed by Roumm et al. with '0' being a few scattered cells, '1' a maximum number of cells per collection of at least 10, '2' a maximum number of cells per collection between 10 and 50 and '3' a maximum number of cells per collection of at least 50(34). Median scores were used for analysis.

Peripheral blood mononuclear cells (PBMCs) were isolated from 50 mL of peripheral blood by Ficoll-Paque gradient centrifugation, incubated for 20 minutes at 4°C and subsequently stained with CD3 APC (clone SK7), CD4 FITC (clone RPA-T4), CD8 PE (clone RPA-T8), CD14 FITC (clone MSE2), CD16 PE (clone B73.1), CD19 PerCPcy5.5 and APC (clone Sj25C1), CD20 FITC (clone L27), CD27 PE (clone L128), CD38 PerCPcy5.5 (clone HIT2), CD56 PE (clone MY31), Polyclonal IgA FITC (DAKO), IgD FITC (clone IA6-

2), IgE Alexa Fluor 488 (gift from University of Antwerp), IgG FITC (clone G18-145), IgM APC and FITC (clone G20-127, all (except IgA and IgE) BD Biosciences. For isotype controls, IgG1 APC and PerCPcy5.5 (clone MOPC-21), IgG1 FITC and PE (clone X40), IgG2a FITC (clone X39), IgG2b FITC (clone 27-35), Rabbit immunoglobulin fraction (DAKO), Polyclonal Swine anti-Rabbit Immunoglobulins FITC (DAKO), all (Rabbit immunoglobulins) BD Biosciences, were used. In addition, B cells and plasmablasts were stained with CD20, IgA, IgD, IgE, IgG, IgM and appropriate isotype controls.

ELISPOT technique was used to detect functional antibody-secreting cells, with the use of goat anti–human IgG, IgA and IgM (Sanbio BV, Uden, The Netherlands) for coating (10 µg/ml in coating buffer, 100 µl/well) of ELISPOT plates (Millipore, The Netherlands). Plates were incubated overnight at 4°C, washed twice with PBS and blocked with 200 µl/well culture medium (IMDM + 10% FCS + 200 mM L-glutamine + 100 µg/ml penicillin/streptavidin) for 2 hours at 37°C in a 5% CO2 atmosphere. PBMCs were titrated on the ELISPOT plates in duplicate wells, and the plates were next incubated at 37°C in a 5% CO2 atmosphere overnight. The following day cells were discarded and washed from the plates with PBS/0.05% Tween 20 and tap water. Spots were visualised by detection with alkaline phosphatase-conjugated goat anti-human IgG, IgM or IgA (Biosource, USA) followed by substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma-Aldrich, St Louis, MO, USA) at 100 µl/well. Enzyme-linked immunosorbent spots (Elispots) were analysed using a stereomicroscope (Bioreader 5000; BIO-SYS GmbH, Karben, Germany.

Study end points

In the design of the trial the following parameters were defined as major clinical end points: treatment related mortality, toxicity and clinical efficacy of rituximab. Efficacy was defined as progression-free survival, with progression defined as any or a combination of the following changes relative to baseline at two consecutive evaluations: death, ≥ 10% drop in predicted FVC(33), ≥ 15% drop in predicted DLCO(33), ≥15% drop in left ventricular ejection fraction (LVEF), body weight(35), ≥ 30% drop in creatinine clearance(36), ≥ 30% increase in mRSS(37, 38), ≥ 0.5 point increase in HAQ-DI(38). The secondary end points defined were changes in mRSS (minimally important difference 3.2-5.3)(39), FVC, DLCO, HAQ-DI (minimally important difference 0.10-0.14)(39), left ventricular ejection fraction, creatinine clearance, SF 36, EuroQol 5 D, presence of interstitial lung disease as reported by HRCT thorax and skin biopsy scores.

However, unfortunately, the trial had major recruitment problems. In a time span of nearly 4 years, 17 patients had been included in the trial. Based on this low inclusion rate the METC advised to prematurely end inclusion and evaluate study outcome 1 year after inclusion of the last patient. As one patient showed early drop-out, n=16 patients (n=8 rituximab, and n= 8 placebo) had data available for analysis. All data collected by June 30th, 2015 were included in the analysis. Based on the small

sample size, we chose to focus on presentation of changes in mRSS, FVC, DLCO and extent of ILD as represented by Goh scores. Adverse events and serious adverse events and changes in HAQ-DI, LVEF, creatinine clearance, SF 36, EuroQol 5 D were assessed for both treatment groups. Immunologically, the influence of rituximab on mononuclear cell subsets in PBMC's and skin tissue was evaluated and described as planned.

Statistical analysis

As all patients participating in the trial also participated in the care program of the LUMC (40), including annual and comprehensive diagnostic evaluation with informed consent for use of data, missing data were imputed from clinical files when possible with a maximum time frame of 6 months between data collection and planned data collection according to the trial schedule. This way skin scores were available up till 24 months for all patients, pulmonary function tests for n=13/16 of patients at t=12 months and n=7/16 at t=24 months. HRCT images were available for scoring in n=15/16 at t=12 months and n=7/16 at t=24 months. Peripheral blood samples for PBMC assessment were available in n=15/18 at t=12 months and n=11/18 at t= 24 months.

Primary analyses included mean change over time over time in mRSS, percentage of predicted DLCO, percentage of predicted FVC, extent of ILD as represented by Goh scores and HAQ-DI, for both treatment groups. Additionally, mortality, treatment toxicity and efficacy according to pre-specified criteria were evaluated for both groups.

Ninety-five percent confidence intervals were computed where appropriate, with p-values less than 0.05 (2-sided) considered statistically significant. Binary variables were analysed by Fisher exact test.

To assess the influence on clinical efficacy analyses of patients included under protocol violation, analyses were repeated excluding these patients. Inter-observer agreement of skin biopsy scoring was evaluated using Fleiss kappa (41). Statistical analyses were performed using IBM SPSS Statistics 23 and GraphPad Prism 6.

Results

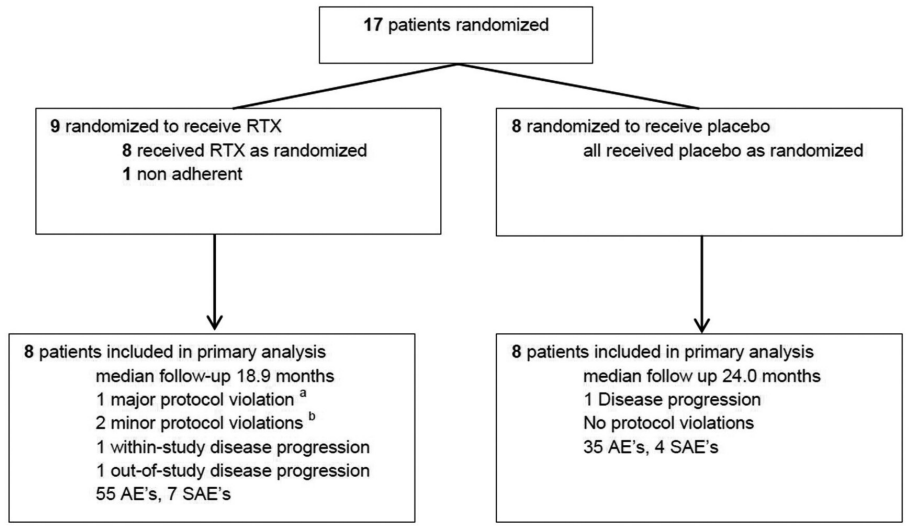
Patients and treatment

From April 2010 to February 2014, 17 patients were included, of which 9 patients were randomized to rituximab and 8 were randomized to placebo (Fig. 1). All patients included fulfilled ARA criteria as well as ACR/EULAR 2013 SSc criteria (42). Two patients were included with a time since diagnosis of > 24 months: one rituximab patient (time since diagnosis 3.5 years, time since non-Raynaud 3.5 years and time since Raynaud 5.3 years) and one placebo patient (time since diagnosis 4.2 years,

time since non-Raynaud 4.2 years and time since Raynaud 4.9 years). One patient (placebo group) died due to disease progression, after drop-out at 6 months because of active disease. One patient did not start the allocated treatment based on active, severe disease with rapid progression of skin score and myocarditis/pericarditis, for which the treating physician judged the chance for placebo as possibly life threatening. Baseline characteristics of this patient did not differ from other patients included.

Baseline characteristics of the 16 patients included for analysis (rituximab n=8, placebo n=8) were similar between the 2 groups, though there was a minor difference in disease duration, with slightly longer disease duration in the placebo group (Table 1). The median follow-up of patients was 19.1 months (IQR 17.6 – 24.4). According to Goh criteria, mean extent of lung involvement at baseline was 9.5%±11.0 for RTX and 6.9% ±10.8 for placebo (p=0.65).

Figure 1 Flow of RITIS (Rituximab in Scleroderma) trial



RTX; rituximab, AE; adverse event, SAE; serious adverse event

[a] One patient refused to have a treatment at 6 months

[b] Two patients incidentally received a dose of verum methylprednisolone instead of placebo methylprednisolone together with the RTX/placebo infusion

Previous immunosuppressive therapy included prednisone (RTX n=2, placebo n=1), methotrexate (RTX n=3, placebo n=0) and azathioprine (RTX n=2, placebo n=0). At start of the trial use of immunosuppressive medication included prednisone (RTX n=2, placebo n=0), methotrexate (RTX n=5, placebo n=3), plaquenil (RTX n=1, placebo n=1), mycophenolate mofetil (RTX=1, placebo n=1). During the trial background immunosuppressive treatment was changed in 2 patients in the rituximab group: methotrexate was stopped at the 18 month visit in both cases (n=1: pregnancy; n=1: pancytopenia).

Table 1 Baseline characteristics of study patients

Characteristic	**RTX group** (n = 8)	**placebo group** (n = 8)	**p-value**
Demographic			
age, mean (yr.)	44.5±5.6	36.6±4.3	0.21 [a]
female sex (% of patients, n)	87.5 (7)	87.5 (7)	1 [b]
caucasians (% of patients, n)	75 (6)	62.5 (5)	0.58 [b]
Disease specific			
dcSSc (% of patients, n)	87.5 (7)	62.5 (5)	0.57 [b]
duration of scleroderma (yr.)			
since diagnosis, (median, range)	0.9 (0.7-3.5)	1.3 (0.2-4.2)	0.44 [a]
since onset first Raynaud symptom (median, range)	2.3 (0.7-5.3)	4.3 (0.7-16.1)	0.13 [a]
since onset first non-Raynaud symptom (median, range)	1.2 (0.6-3.5)	2.4 (0.7-4.2)	0.25 [a]
Skin and musculoskeletal			
modified Rodnan Skin Score (mean±SE)	16.4±4.4	14.0±3.8	0.88 [a]
Heart and Lungs			
LVEF (mean±SE)	61.1±4.2	62.0±4.6	0.96 [a]
FVC (% of predicted)	97.9±6.6	92.0±6.1	0.67 [a]
DLCO (% of predicted)	67.1±4.2	72.3±6.0	0.34 [a]
Total extent of lung disease on HRCT (mean %)	9.5±11.0	6.9±10.8	0.65 [a]
Extent ground glass (mean %)	8.3±9.4	5.4±8.0	0.44 [a]
Extent reticular pattern (mean %)	4.0±8.7	3.9±7.1	1 [a]
Function and Quality of Life			
HAQ-DI (mean±SE)	1.39±0.27	1.31±0.32	0.65 [a]

Table 1 Baseline characteristics of study patients *(continued)*

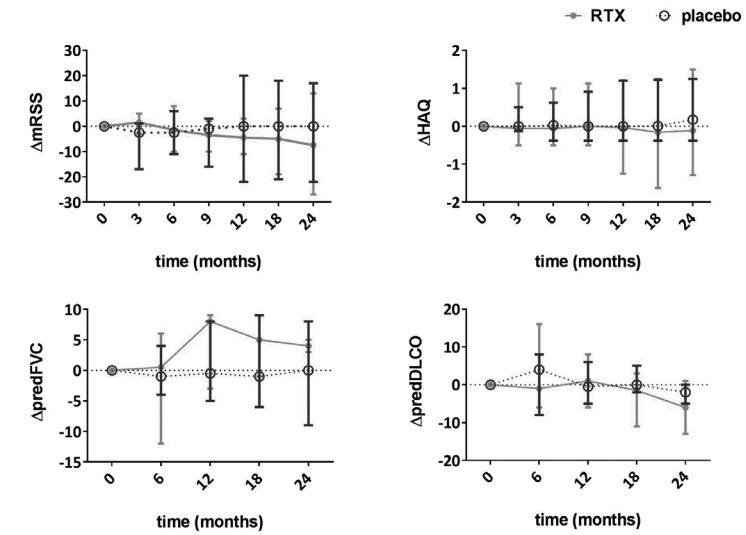
Characteristic	**RTX group** (n = 8)	**placebo group** (n = 8)	**p-value**
Therapy			
Previous immunosuppressive therapy* (% of patients)	50.0	12.5	0.28 [b]
months of use (median, range)	1.5 (0.0-36.0)	0.0 (0.0-9.0)	0.20 [a]
Immunosuppressive therapy** (% of patients)	87.5	62.5	0.57 [b]
months of use (median, range)	8.1(0.0-42.6)	3.2 (0.0-26.3)	0.33 [a]
Laboratory findings			
ANA-positive (% of patients)	100	87.5	1.00[b]
anti-topoisomerase I (% of patients)	12.5	50.0	0.28 [b]
anti-RNA polymerase III (% of patients)	25.0	0.0	0.47 [b]

dcSSc; diffuse cutaneous systemic sclerosis, RTX; rituximab [a] : Mann-Whitney [b] : Fisher's exact
*Previous immunosuppressive therapy included high-dose (> 15mg/day) prednisone (RTX n=2, placebo n=1), methotrexate (RTX n=3, placebo n=0) and azathioprine (RTX n=2, placebo n=0).
**Current immunosuppressive treatment included high-dose (> 15mg/day) prednisone (RTX n=2, placebo n=0), methotrexate (RTX n=5, placebo n=3), plaquenil (RTX n=1, placebo n=1), mycophenolate mofetil (RTX=1, placebo n=1).
*** Extent of lung disease in HRCT was scored according to Goh criteria (33) ; the extent was evaluated over five levels and averaged (1 origin of great vessels; 2 main carina; 3 pulmonary venous confluence; 4. halfway between the third and fifth section; 5.immediately above the right hemi-diaphragm)

Analysis of clinical disease parameters

Course of changes in mRSS, FVC, DLCO and HAQ are shown in Figure 2. There were no significant differences in change between baseline and 12-month follow-up of mRSS (placebo -1.8 vs. RTX -3.6, p=0.95), FVC (placebo+0.3 vs. RTX +4.7, p=0.43), DLCO (placebo -0.3 vs. +0.7, p=0.91) and HAQ (placebo +0.18 vs. RTX 0.0, p=0.94). Also, at 24-month follow-up, there were no significant differences in change from baseline in mRSS (placebo -1.9 vs. RTX -5.3, p=0.95), FVC (placebo -1.4 vs. RTX +4, p=0.65), DLCO (placebo -2.2, RTX -6.0, p=0.77) and HAQ (placebo 0.2313 vs. RTX -0.0675, p=0.94) results. Numerically, n=4/8 rituximab vs. n=2/8 in placebo improved >5 points in mRSS, there were no improvers in either FVC or DLCO (minimal important difference 10%) and n=1/8 in rituximab vs. n=0/8 in placebo improved in HAQ (minimal important difference 0.5 points) after one year.

Figure 2 Change in modified Rodnan Skin Score, Forced Vital Capacity, Diffusing Capacity of the Lung and Health Assesment Questionnaire during 24 month follow up in patients with systemic sclerosis treated with rituximab or placebo



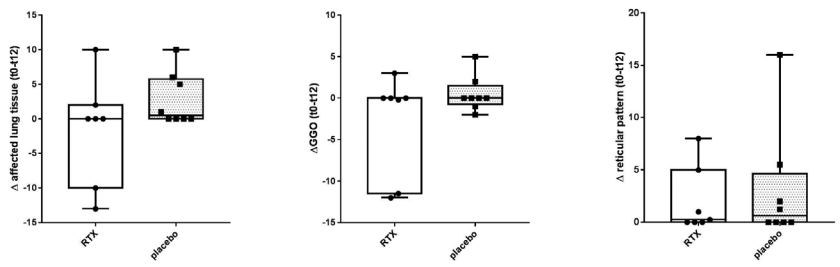
RTX; rituximab – mRSS; modified Rodnan Skin Score – predFVC; forced vital capacity, percentage of predicted – predDLCO; diffusing capacity of the lung; percentage of predicted - HAQ; Health Assessment Questionnaire - Dots indicate medians, error bars indicate ranges

Analysis of HRCT data according to Goh criteria showed a mean change in percentage of affected lung tissue between baseline and 12 months of -1.6% for rituximab and + 2.8% for placebo (p=0.28). Beneficial effects were explained by a decrease in ground glass opacities with rituximab treatment in two rituximab treated patients (Fig 3+4). Numerically, n=2/7 rituximab patients and n=0/8 placebo patients showed improvement on HRCT (-10% or more change in mean extent of lung involvement), n=4/7 in rituximab vs. n=7/8 in placebo had stable lung involvement on HRCT (between -10% and +10% change in mean extent of lung involvement) and n=1/7 in rituximab vs. n=1/8 in placebo had worsening lung involvement on HRCT (+10% or more change in extent of lung involvement).

Analysis of change in AUC showed no significant differences in the mRSS, FVC and HAQ-DI between the groups (Supp. Table S2). Within the first year, mean change from baseline to 12 months follow-up in mRSS was comparable between groups, with -1.4 for the rituximab and -2.7 for the control group (difference 1.3; 95%CI -3.4 to 6.2; p=0.55). For FVC and HAQ-DI differences in AUC between baseline and one year were also insignificant. For FVC there was a slight improvement with rituximab and a slight detoriation with placebo (mean change AUC baseline to 12 months follow-up 0.6 for RTX and -0.4 for placebo, p=0.59). Also during the second year, no significant differences were observed in AUC for mRSS, FVC and HAQ-DI (Supp. Table S2).

Efficacy analyses for the individual disease parameters were repeated excluding the two patients with disease duration > 24 months since diagnosis. These analyses did not show different results.

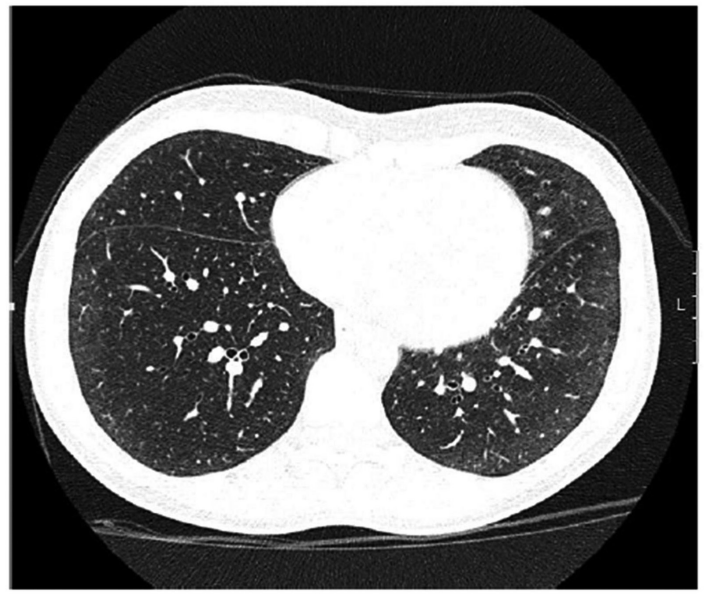
Figure 3 Change in mean extent of lung tissue and lung tissue with ground glass or reticular pattern involvement from baseline to 12 months follow-up in patients with Systemic Sclerosis



RTX; rituximab – t0;baseline – t12;12 months follow-up
vertical axis represent differences in Goh scores between baseline and 12 months for (from left to right): 1) mean extent of affected lung tissue, 2) GGO; mean extend of ground glass opacities, 3) mean extend of affected lung tissue with reticular pattern.

At 12 months a non-significant trend in favour of rituximab was observed (mean change in lung involvement: -1.6% RTX group vs. +2.8% placebo group [p=0.28]). The beneficial effect in the rituximab group was explained by a decrease of ground glass opacities.

Figure 4 Improvement of lung involvement as evaluated by HRCT of a patient treated with rituximab



HRCT High Resolution Computed Tomography; inspiratory scan, halfway between pulmonary venous confluence and right hemi-diaphragm

Upper HRCT: baseline HRCT before rituximab use, lower HRCT: 12 months after initial gift of rituximab

Recorded parameters reflecting vascular complications did not differ between the treatment arms: no patients in the trial had impaired kidney function and eGFR rates within study arms were comparable (mean eGFR at T=0: placebo 118.5 ml/min/1.73m2, RTX 106.8 ml/min/1.73m2) and were stable throughout the trial. Also LVEF (as measured by echocardiography) remained stable throughout the trial in all participants (mean LVEF at T=0: placebo 62.0%, RTX 60.2%, at T=24: placebo 60.2%, RTX 65.5%). Digital ulcers occurred both in the placebo (n=3) and the rituximab group (n=3).

In the analyses SF-36 scores and EQ-5D scores no differences were seen (data not shown).

Two patients in the placebo group showed disease progression during follow-up according to pre-specified criteria, including the patient that died after drop-out. In the rituximab group one patient showed disease progression. Apart from the patient that died, study disease progression was based on a ≥30% increased mRSS relative to baseline at the 12-month (placebo, n=1) and 18-month visits (RTX, n=1) in both cases. Including the patient that died after drop-out, there was no difference in progression free survival between groups (Log Rank (Mantel-Cox) p=0.674). Also after excluding the two patients with disease duration > 24 months there was no significant difference in progression free survival between the groups.

Safety and toxicity

No patients died during the study. One patient (placebo group) died due to disease progression, after drop-out at 6 months because of active disease. This patient eventually died at 23 months due to scleroderma renal crisis after autologous hematopoietic stem cell transplantation.

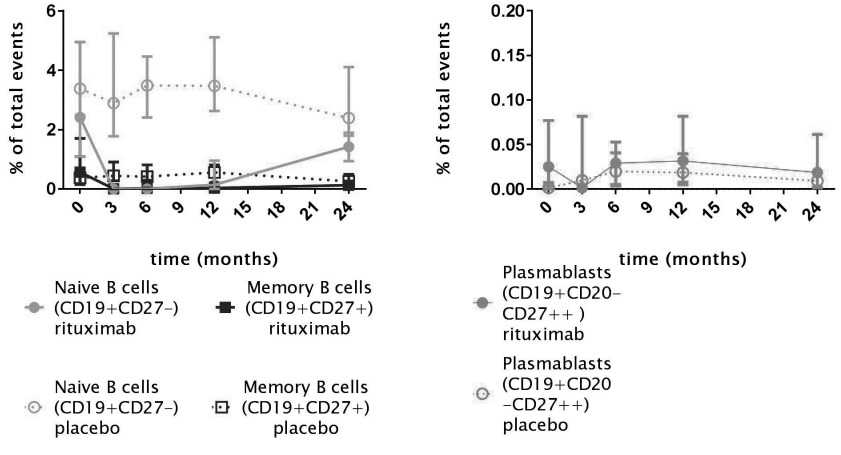
A total of 88 adverse events (AE's) occurred during the study: 52 in the rituximab group (6 grade 3 AE's, 2 grade 4 AE's) and 36 in the control group (7 grade 3 AE's, 0 grade 4 AE's)(p=0.22)(Supp. Table S1). There were 7 serious adverse events (SAE's) in the rituximab group and 4 in the placebo group (p=0.36). Serious adverse events in the rituximab group were a breast carcinoma (18 months after 1st gift of RTX), abnormal cervical histology leading to hysterectomy (6 months after first gift of RTX; medical history of this patient mentioned abnormal cervical histology also before inclusion in the trial), an anaemia due to severe menstruation (7 months after 1st gift of RTX), a pancytopenia (12 months after 1st gift of RTX) and 3 events related to digital ulcers (n=2 at 1 month after 1st gift of RTX, the other at 7 months). Serious adverse events in the placebo group included severe weight loss which required treatment by percutaneous endoscopic gastrostomy placement (17 months after first gift of RTX) and 3 events related to digital ulcers (1, 14 and 18 months after initial RTX). There were more grade 1 AE's in the rituximab group due to mild infusion reactions (system organ class type: immune system). A clear causal relation between adverse events and treatment with rituximab could not be established, except for mild infusion reactions.

Immunophenotyping of peripheral blood mononuclear cells

At baseline there were no differences seen in proportions of macrophages (CD14+), NK cells (CD56+), T helper cells (CD3+/CD4+), cytotoxic T cells (CD3+/CD8+) or B cells (CD19+) between placebo and rituximab group (Data not shown). Three months after initial anti-CD20 treatment significant depletion B cells was seen and simultaneously a decline in T cells was observed. Counter wise the proportion of macrophages increased.

When observing the different subsets of B cells during the study, as shown in Figure 5, naïve and memory B cells were depleted 3 months after the first gift of rituximab. In 5 of 8 patients with rituximab a reduction of CD19+CD20-CD27++ plasmablasts was seen. Reduction of plasmablasts was significant within the rituximab group when compared to baseline, but insignificant when compared to the placebo at the same time point. When assessing depletion of immunoglobulin expressing (IgG, IgA, IgM and IgD) naïve (CD19+CD27-) and memory (CD19+CD27+) subsets, as a positive control, all subsets were depleted (Data not shown). At time of consolidation treatment (month 6), repopulation of naïve B cells, memory B cells and plasmablasts was present. However, throughout the complete follow-up period repopulation of naïve B cells and memory B cells was incomplete in the rituximab group (Figure 5).

Figure 5 Naïve B cells (CD19+CD27 -), memory B cells (CD19+CD27+) and plasmablasts (PB CD27++) levels in patients with systemic sclerosis



Indicated points resemble medians; Rituximab: T0, n= 8; T3 n=8; T6 n=7; T12 n=6; T24 n=4; Placebo: T0, n=8; T3, n=8; T6 n=8; T12 n=7; T24 n=7
B cell depletion after rituximab treatment is seen in all B cell subsets
Total events are defined as the number of detected cells by the flow cytometer.

Skin biopsies
Skin biopsies were performed in 15 patients (RTX n=7, placebo n=8) at baseline and in 14 patients (RTX n=7, placebo n=7) at 3 months (Supp. Table S3). For 3 patients in the placebo group, and for 1 patient in the rituximab group, skin was clinically unaffected at the site of biopsy Inter-observer agreement of histologic skin score evaluated by Fleiss kappa was κ =0.49 for T cells, κ=0.32 for B cells and κ=0.63 for macrophages.

There were no significant differences found in immune cell presence in skin neither between groups, nor within groups over time. At baseline there was a trend towards more mononuclear infiltration in the placebo group, based on the presence of more T cells. Over time, presence of T cells in the rituximab group increased at 3 months compared to baseline. Presence of other immune cells was stable over time (Supp. Table S3.).

B cells were rarely present in skin tissue, only 1 patient in the placebo group that showed a collection of >10 cells, but less than 50 cells at baseline. Scattered B cells (range 2-7 per biopsy) were seen in 5 out of 15 biopsies at baseline (RTX n=3, placebo n=2). Over time, there were no changes in the presence of B cells in skin of rituximab treated patients, 3 months after initial gift, with B cells present in 4 out of 7 biopsies. This was identical to the number of placebo patients with B cell presence in skin at three months (4 out of 7).

Discussion

This small randomized, placebo controlled trial cannot reject nor confirm the hypothesis of RTX preventing fibrotic complications. No major safety issues were observed with rituximab in the subset of early SSc patients. Immunologically, rituximab achieved its presumed biological effect: a depletion of circulating B cells up to minimal counts, but with persistence of antigen secreting cells and incomplete depletion of the CD27++ plasmablast compartment. No change in the small number of cells from the B cell lineage present in skin tissue was observed with rituximab treatment. Over time, small, non-significant differences in FVC, extent of pulmonary involvement and HAQ-DI in favour of the rituximab group were found. Further research must confirm the credibility of these findings. A larger scale RCT in patients with proven pulmonary involvement therefore seems plausible and feasible.

Unfortunately, the trial had recruitment problems resulting in premature termination of inclusion. Moreover, patients in the control group experienced an unexpected favourable disease course, which complicates firm conclusions about efficacy of rituximab in preventing fibrotic complications. This study aimed to include patients with early dcSSc. Indeed, our placebo group included patients of which the majority

had dcSSc at baseline (63%), and 4 of 8 patients were either ATA or RNApIII positive. Both these antibodies are associated with more severe disease course (43, 44). Despite these characteristics reflecting high risk, early dcSSc, 75% of patients in the placebo group had favourable outcome after 2 years.

There is a small insignificant difference in disease duration between the rituximab and the placebo group, with the placebo group having a longer disease duration. It has been shown that with longer disease duration, chances to improve spontaneously slightly increase(45) which might partially explain the beneficial disease course in placebo. However, excluding the two patients with the longest disease duration did not change our results.

Several case reports, open-label studies and a nested case-control study thus far reported a potential beneficial effect of rituximab on pulmonary function, skin fibrosis and functional impairment in SSc (12-21, 46-49). Our observations are in line with the study from Lafyatis (15), who treated 15 patients with early SSc with rituximab and did not find a clear beneficial effect on skin fibrosis and pulmonary function at 6 and 12 months of follow-up. Various explanations can account for the difference between previous open-label studies and our findings. As these studies did not include a placebo group, part of the observed efficacy might reflect natural disease course. In addition, most open-label studies included patients with longer disease duration (13, 14, 50) and thus possibly selected an immunologically different subgroup of SSc patients. In comparison to the open label studies of Smith and Lafyatis (15, 17), who both also included patients with early disease, mean skin scores were lower in our population, which complicates the possibility of demonstrating clear clinical efficacy on skin involvement. When analysing only the patients with dcSSc at baseline, with rituximab n=3/7 showed a decrease in mRSS >5, versus n=2/5 within the placebo group. On the other hand, it is known that patients with more skin fibrosis at baseline are more likely to regress even without therapy over the next year (51).

Immunophenotyping of peripheral blood showed almost complete B cell depletion, which is in line with previous studies (15, 17). It is known that during treatment with rituximab, plasmablasts and plasma cells can persist (52). Besides confirming this with ELISPOT, thereby showing persistence of IgA, IgG and IgM antigen secreting cells after treatment with rituximab, this is also demonstrated by the incomplete depletion of the CD27++ plasmablast compartment (Supp Figure S1). Other studies found CD20-positive B cells in skin biopsies in approximately half of patients at baseline and depletion in most of these cases (12, 13, 17). To overcome possible interference of rituximab treatment with detection of CD20-positive B cells in skin we chose to use CD79a staining on skin, which also stains plasmablasts and plasma cells that lack CD20, while in previous studies CD20 staining was used. Based on morphology, the persisting B-cells in our samples could reflect unaffected long-lived

B cell populations. The exact nature and the relevance of these persistent B cells for development and persistence of skin fibrosis remains to be determined, and might be relevant in determining subsets of SSc patients with high likelihood of responding to rituximab.

Remarkably, 2 out of 8 rituximab treated patients showed evident improvement of the extent of ground glass opacities in HRCT at 1 year follow-up versus none of the placebo treated patients. Radiologic improvement of CTD-associated and RA-ILD after treatment with rituximab has also been described by other authors(53, 54). Out of interest, and to possibly guide future research in the field we compared different B cell subsets in baseline PBMC's between rituximab patients with pulmonary improvement and those without. Rituximab treated patients with pulmonary improvement both had higher counts of naïve B cells (CD19+,CD27-) counts of naïve B cells (CD19+, CD27-) (mean CD19+CD27+ of n=2 non-improvers under RTX 1.8% of total events vs. n=6 improvers under RTX 5.6% of total events, p=0.003), while other subsets were comparable. We speculate that this subgroup of patients, possibly reflecting those with very early and active inflammatory pulmonary involvement might be the subset of patients most likely to benefit from B cell depleting therapy. However, these observations obviously await replication.

In conclusion, we performed a double blind placebo controlled trial in patients with early SSc and show in-depth analysis of B cells in peripheral blood and skin tissue. Although given the small sample size and the unexpected favourable disease course in the placebo group no firm conclusions on clinical efficacy of rituximab in early SSc can be drawn, our data show that a larger RCT in early SSc with proven pulmonary involvement might be worthwhile. In addition, inclusion of peripheral blood and skin tissue analyses is also warranted in future trials to determine the nature, role and relevance of persisting B-cells in skin, and persisting plasmablasts and plasma cells in peripheral blood. Analysis as presented herein might help to identify a subset of SSc patients most likely to benefit from B cell depleting therapies.

Supporting information

Supplementary data is available at the website of RMD Open or can be obtained by contacting the first author

References

1. Allanore Y, Simms R, Distler O, Trojanowska M, Pope J, Denton CP, et al. Systemic sclerosis. Nature Reviews Disease Primers. 2015:15002.
2. Johnson SR, Feldman BM, Pope JE, Tomlinson GA. Shifting our thinking about uncommon disease trials: the case of methotrexate in scleroderma. The Journal of rheumatology. 2009;36(2):323-9.
3. Pope JE, Bellamy N, Seibold JR, Baron M, Ellman M, Carette S, et al. A randomized, controlled trial of methotrexate versus placebo in early diffuse scleroderma. Arthritis Rheum. 2001;44(6):1351-8.
4. Tashkin DP, Elashoff R, Clements PJ, Goldin J, Roth MD, Furst DE, et al. Cyclophosphamide versus placebo in scleroderma lung disease. The New England journal of medicine. 2006;354(25):2655-66.
5. Hoyles RK, Ellis RW, Wellsbury J, Lees B, Newlands P, Goh NS, et al. A multicenter, prospective, randomized, double-blind, placebo-controlled trial of corticosteroids and intravenous cyclophosphamide followed by oral azathioprine for the treatment of pulmonary fibrosis in scleroderma. Arthritis Rheum. 2006;54(12):3962-70.
6. Derk CT, Grace E, Shenin M, Naik M, Schulz S, Xiong W. A prospective open-label study of mycophenolate mofetil for the treatment of diffuse systemic sclerosis. Rheumatology (Oxford, England). 2009;48(12):1595-9.
7. Gerbino AJ, Goss CH, Molitor JA. Effect of mycophenolate mofetil on pulmonary function in scleroderma-associated interstitial lung disease. Chest. 2008;133(2):455-60.
8. Nagaraja V, Denton CP, Khanna D. Old medications and new targeted therapies in systemic sclerosis. Rheumatology (Oxford, England). 2015;54(11):1944-53.
9. van Laar JM, Farge D, Sont JK, Naraghi K, Marjanovic Z, Larghero J, et al. Autologous hematopoietic stem cell transplantation vs intravenous pulse cyclophosphamide in diffuse cutaneous systemic sclerosis: a randomized clinical trial. Jama. 2014;311(24):2490-8.
10. Sakkas LI, Bogdanos DP. Systemic sclerosis: New evidence re-enforces the role of B cells. Autoimmun Rev. 2016;15(2):155-61.
11. Mavropoulos A, Simopoulou T, Varna A, Liaskos C, Katsiari CG, Bogdanos DP, et al. Breg Cells Are Numerically Decreased and Functionally Impaired in Patients With Systemic Sclerosis. Arthritis & rheumatology (Hoboken, NJ). 2016;68(2):494-504.
12. Bosello S, De Santis M, Lama G, Spano C, Angelucci C, Tolusso B, et al. B cell depletion in diffuse progressive systemic sclerosis: safety, skin score modification and IL-6 modulation in an up to thirty-six months follow-up open-label trial. Arthritis Res Ther. 2010;12(2):R54.
13. Daoussis D, Liossis SN, Tsamandas AC, Kalogeropoulou C, Kazantzi A, Sirinian C, et al. Experience with rituximab in scleroderma: results from a 1-year, proof-of-principle study. Rheumatology (Oxford, England). 2010;49(2):271-80.
14. Jordan S, Distler JH, Maurer B, Huscher D, van Laar JM, Allanore Y, et al. Effects and safety of rituximab in systemic sclerosis: an analysis from the European Scleroderma Trial and Research (EUSTAR) group. Annals of the rheumatic diseases. 2015;74(6):1188-94.
15. Lafyatis R, Kissin E, York M, Farina G, Viger K, Fritzler MJ, et al. B cell depletion with rituximab in patients with diffuse cutaneous systemic sclerosis. Arthritis Rheum. 2009;60(2):578-83.

16. Smith V, Piette Y, van Praet JT, Decuman S, Deschepper E, Elewaut D, et al. Two-year results of an open pilot study of a 2-treatment course with rituximab in patients with early systemic sclerosis with diffuse skin involvement. The Journal of rheumatology. 2013;40(1):52-7.
17. Smith V, Van Praet JT, Vandooren B, Van der Cruyssen B, Naeyaert JM, Decuman S, et al. Rituximab in diffuse cutaneous systemic sclerosis: an open-label clinical and histopathological study. Annals of the rheumatic diseases. 2010;69(1):193-7.
18. Moazedi-Fuerst FC, Kielhauser SM, Brickmann K, Hermann J, Lutfi A, Meilinger M, et al. Rituximab for systemic sclerosis: arrest of pulmonary disease progression in five cases. Results of a lower dosage and shorter interval regimen. Scandinavian journal of rheumatology. 2014;43(3):257-8.
19. Bosello SL, De Luca G, Rucco M, Berardi G, Falcione M, Danza FM, et al. Long-term efficacy of B cell depletion therapy on lung and skin involvement in diffuse systemic sclerosis. Semin Arthritis Rheum. 2015;44(4):428-36.
20. Daoussis D, Liossis SN, Tsamandas AC, Kalogeropoulou C, Paliogianni F, Sirinian C, et al. Effect of long-term treatment with rituximab on pulmonary function and skin fibrosis in patients with diffuse systemic sclerosis. Clin Exp Rheumatol. 2012;30(2 Suppl 71):S17-22.
21. Moazedi-Fuerst FC, Kielhauser SM, Bodo K, Graninger WB. Dosage of rituximab in systemic sclerosis: 2-year results of five cases. Clinical and experimental dermatology. 2015;40(2):211-2.
22. Mendoza FA, Keyes-Elstein LL, Jimenez SA. Systemic sclerosis disease modification clinical trials design: quo vadis? Arthritis Care Res (Hoboken). 2012;64(7):945-54.
23. Hasegawa M, Hamaguchi Y, Yanaba K, Bouaziz JD, Uchida J, Fujimoto M, et al. B-lymphocyte depletion reduces skin fibrosis and autoimmunity in the tight-skin mouse model for systemic sclerosis. The American journal of pathology. 2006;169(3):954-66.
24. Matsushita T, Fujimoto M, Hasegawa M, Matsushita Y, Komura K, Ogawa F, et al. BAFF antagonist attenuates the development of skin fibrosis in tight-skin mice. Journal of Investigative Dermatology. 2007;127(12):2772-80.
25. Preliminary criteria for the classification of systemic sclerosis (scleroderma). Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Arthritis Rheum. 1980;23(5):581-90.
26. Institute' NC. Common Terminology Criteria for Adverse Events (CTCAE). USDEPARTMENT OF HEALTH AND HUMAN SERVICES.
27. Ware JJS, K.; Kosinki, M. Health survey manual and interpretation guide.1994.
28. Newnham EA, Harwood KE, Page AC. Evaluating the clinical significance of responses by psychiatric inpatients to the mental health subscales of the SF-36. Journal of Affective Disorders. 2007;98(1):91-7.
29. Dolan P. Modeling valuations for EuroQol health states. Medical care. 1997;35(11):1095-108.
30. Lamers L, Stalmeier P, McDonnell J, Krabbe P, Van Busschbach J. Kwaliteit van leven meten in economische evaluaties: het Nederlands EQ-5D-tarief. Nederlands tijdschrift voor geneeskunde. 2005;149(28):1574-8.
31. Fries JF, Spitz P, Kraines RG, Holman HR. Measurement of patient outcome in arthritis. Arthritis & Rheumatism. 1980;23(2):137-45.

32. Clements PJ, Wong WK, Hurwitz EL, Furst DE, Mayes M, White B, et al. The Disability Index of the Health Assessment Questionnaire is a predictor and correlate of outcome in the high-dose versus low-dose penicillamine in systemic sclerosis trial. Arthritis & Rheumatism. 2001;44(3):653-61.
33. Goh NS, Desai SR, Veeraraghavan S, Hansell DM, Copley SJ, Maher TM, et al. Interstitial lung disease in systemic sclerosis: a simple staging system. American journal of respiratory and critical care medicine. 2008;177(11):1248-54.
34. Roumm AD, Whiteside TL, Medsger TA, Jr., Rodnan GP. Lymphocytes in the skin of patients with progressive systemic sclerosis. Quantification, subtyping, and clinical correlations. Arthritis Rheum. 1984;27(6):645-53.
35. Baron M, Bernier P, Côté L, Delegge M, Falovitch G, Friedman G, et al. Screening and management for malnutrition and related gastro-intestinal disorders in systemic sclerosis: recommendations of a North American expert panel. Clin Exp Rheumatol. 2010;28(Suppl 58):S42-S6.
36. Penn H, Howie A, Kingdon E, Bunn C, Stratton R, Black C, et al. Scleroderma renal crisis: patient characteristics and long-term outcomes. Qjm. 2007;100(8):485-94.
37. Clements P, Lachenbruch P, Siebold J, White B, Weiner S, Martin R, et al. Inter and intraobserver variability of total skin thickness score (modified Rodnan TSS) in systemic sclerosis. The Journal of rheumatology. 1995;22(7):1281-5.
38. Gazi H, Pope JE, Clements P, Medsger TA, Martin RW, Merkel PA, et al. Outcome measurements in scleroderma: results from a delphi exercise. The Journal of rheumatology. 2007;34(3):501-9.
39. Khanna D, Furst DE, Hays RD, Park GS, Wong WK, Seibold JR, et al. Minimally important difference in diffuse systemic sclerosis: results from the D-penicillamine study. Annals of the rheumatic diseases. 2006;65(10):1325-9.
40. Meijs J, Schouffoer AA, Ajmone Marsan N, Kroft LJ, Stijnen T, Ninaber MK, et al. Therapeutic and diagnostic outcomes of a standardised, comprehensive care pathway for patients with systemic sclerosis. RMD open. 2016;2(1):e000159.
41. Fleiss JL. Measuring nominal scale agreement among many raters. Psychological bulletin. 1971;76(5):378.
42. van den Hoogen F, Khanna D, Fransen J, Johnson SR, Baron M, Tyndall A, et al. 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/ European League against Rheumatism collaborative initiative. Arthritis Rheum. 2013;65(11):2737-47.
43. Graf SW, Hakendorf P, Lester S, Patterson K, Walker JG, Smith MD, et al. South Australian Scleroderma Register: autoantibodies as predictive biomarkers of phenotype and outcome. Int J Rheum Dis. 2012;15(1):102-9.
44. Nihtyanova S, Tang E, Coghlan J, Wells A, Black C, Denton C. Improved survival in systemic sclerosis is associated with better ascertainment of internal organ disease: a retrospective cohort study. Qjm. 2010;103(2):109-15.
45. Steen VD, Medsger TA. Severe organ involvement in systemic sclerosis with diffuse scleroderma. Arthritis & Rheumatism. 2000;43(11):2437-44.
46. Daoussis D, Liossis SN, Tsamandas AC, Kalogeropoulou C, Kazantzi A, Korfiatis P, et al. Is there a role for B-cell depletion as therapy for scleroderma? A case report and review of the literature. Semin Arthritis Rheum. 2010;40(2):127-36.

47. Giuggioli D, Lumetti F, Colaci M, Fallahi P, Antonelli A, Ferri C. Rituximab in the treatment of patients with systemic sclerosis. Our experience and review of the literature. Autoimmun Rev. 2015;14(11):1072-8.
48. McGonagle D, Tan AL, Madden J, Rawstron AC, Rehman A, Emery P, et al. Successful treatment of resistant scleroderma-associated interstitial lung disease with rituximab. Rheumatology (Oxford, England). 2008;47(4):552-3.
49. Haroon M, McLaughlin P, Henry M, Harney S. Cyclophosphamide-refractory scleroderma-associated interstitial lung disease: remarkable clinical and radiological response to a single course of rituximab combined with high-dose corticosteroids. Therapeutic advances in respiratory disease. 2011;5(5):299-304.
50. Giuggioli D, Lumetti F, Colaci M, Fallahi P, Antonelli A, Ferri C. Rituximab in the treatment of patients with systemic sclerosis. Our experience and review of the literature. Autoimmun Rev. 2015;14(11):1072-8.
51. Dobrota R, Maurer B, Graf N, Jordan S, Mihai C, Kowal-Bielecka O, et al. Prediction of improvement in skin fibrosis in diffuse cutaneous systemic sclerosis: a EUSTAR analysis. Annals of the rheumatic diseases. 2016.
52. Mei HE, Frolich D, Giesecke C, Loddenkemper C, Reiter K, Schmidt S, et al. Steady-state generation of mucosal IgA+ plasmablasts is not abrogated by B-cell depletion therapy with rituximab. Blood. 2010;116(24):5181-90.
53. Sharp C, McCabe M, Dodds N, Edey A, Mayers L, Adamali H, et al. Rituximab in autoimmune connective tissue disease–associated interstitial lung disease. Rheumatology. 2016:kew195.
54. Md Yusof M, Kabia A, Darby M, Lettieri G, Beirne P, Vital E, et al. Effect of rituximab on the progression of rheumatoid arthritis-related interstitial lung disease: 10 years' experience at a single centre. Rheumatology (Oxford, England). 2017.