OTKA K-112939 Final report, PI: László Czirják

Investigation of biomarkers and B-cell gene expression in the development of disease activity and damage in systemic sclerosis

1. Analysis of certain gene products involved in B-cell activation and signaling

The role of B cells in SSc is not fully understood but their activation seems to be an early event in the pathogenesis as transcriptome profiling identified local Th1, Th2 and B-cell activation in early dcSSc skin biopsies, but established disease did not show similar prominent immune signature [1][2]. The classical activation of B cells downstream of BcR involves phosphatidylinositol-3 kinases (PI3K) signaling pathway [3]. PI3K pathway also integrates the effects of multiple co-stimulatory receptors including CD40 and IL-4 receptor (IL4R) mediating an alternate signaling [4]. Moreover, Toll-like receptor (TLR) and complement receptor (CR) signaling seem to be mediated also by PI3K [5]. The involvement of PI3K was observed in ischemia-reperfusion induced tissue damage, where a PI3K inhibitor reduced acute tubular necrosis, and kidney B-cell infiltration [6]. Furthermore the participation of PI3K pathway has also been shown in fibrogenesis in SSc [7][8]. Dermal fibroblasts of SSc patients showed hyperactivity of PI3K signaling [7] and inhibition of this pathway repressed TGFβ1-induced expression of type1-collagen in lung and dermal fibroblasts [8].

1.1. Innate signaling elements of PI3K pathway are upregulated in dcSSc B cells

We have identified several molecules in PI3K pathway which expression is significantly altered in early untreated diffuse cutaneous systemic sclerosis (dcSSc) compared to healthy controls (HC) using pooled cDNA samples (n=5 and n=5 respectively). The expression of TLR4, C3, osteopontin (SPP1), phospholipase C beta 1 (PLCB1), phospholipase C gamma 1 (PLCG1) and IL4R was highly upregulated, and CD180, which is a TLR-homolog, was highly downregulated (**Figure 1A**). We have also shown that immunosuppressive therapy did not change the upregulated mRNA for complement C3, TLR4, Phospholipase C Beta 1 and the downregulated expression of CD180, but SPP1, Phospholipase C Gamma 1 and IL4R mRNA expression fi

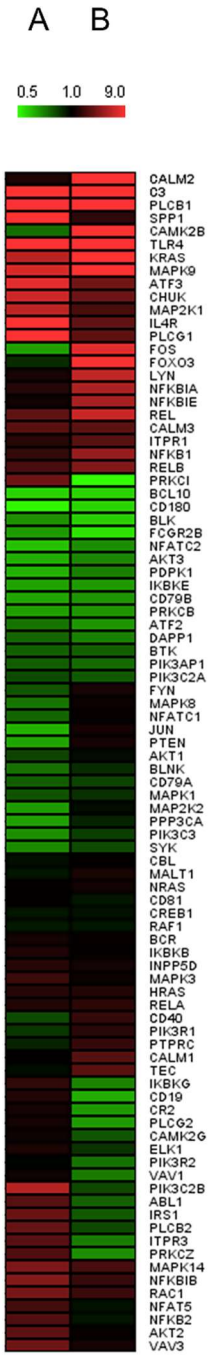


Figure 1. *Heatmap showing PI3K signaling pathway related gene expression in B cells from early dcSSc patients untreated and (B) under immunosuppressive therapy.*

Using individual patient samples from the above described pool, upregulated TLR4, C3 and downregulated CD180 mRNA were confirmed by qPCR and were also measured in 6 newly enrolled early dcSSc patients (**Figure 2**).

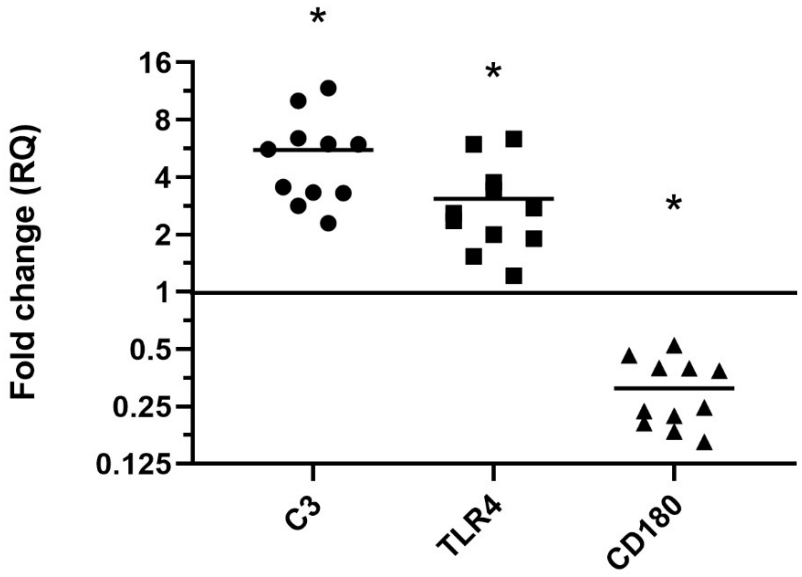


***Figure 2**. Gene expression of complement C3, TLR4 and CD180 in B cells of early untreated dcSSc patients compared to HCs. Gene expression was normalized to HCs and the horizontal line (value 1) represents the expression of control samples. * $p<0.05$*

Toll-like receptors (TLRs) are receptors of the innate immune system recognizing pathogen and damage associated patterns. TLR4 has been proven to be a dominant receptor in driving fibrogenesis in SSc as the expression of TLR4 and its endogenous ligands is elevated in skin biopsies of SSc patients, and induction of TLR4 signaling led to profibrotic responses in explanted fibroblasts [9]. CD180 is a TLR homolog, expressed on B cells, macrophages and little is known about its function. Ligation of anti-CD180 leads to B cell proliferation and enhanced activation via elevated CD86 expression [10]. Human B cells do not express TLR4 or only at low level, therefore the physiological function of TLR4 is not well known in human B cells, but it is believed that they gain novel roles under pathological conditions. The expression of TLR4 was found to be elevated in B cells of patients with systemic lupus erythematous (SLE) [11] and in inflammatory diseases [12]. CD180 is expressed on B cell, macrophages and dendritic cells [13], but little is known about its function in SSc. To date, it was only investigated as the potential inhibitor of TLR4 signaling in SSc fibroblasts [14]. TLR4 and CD180 is able to modulate TLR4 signaling [15] thus in the following experiments we focused on the functional analysis of TLR4 and CD180. The role of CD180 in B cells is not well characterized, but growing evidence suggests that it represents a new innate like pathway within the TLR family. Based on the literature and our current findings we hypothesized that CD180 may contribute to abnormal B cell activation in systemic sclerosis.

1.2. CD180 expression is decreased in B cells in early dcSSc patients

To investigate whether the downregulated mRNA expression of CD180 can be observed at protein level we analyzed the CD180 expression of B cells using flow cytometry and found that CD180 MFI is significantly decreased in B cells in early dcSSc patients (n=4) compared to HCs (n=4) (**Figure 3**).

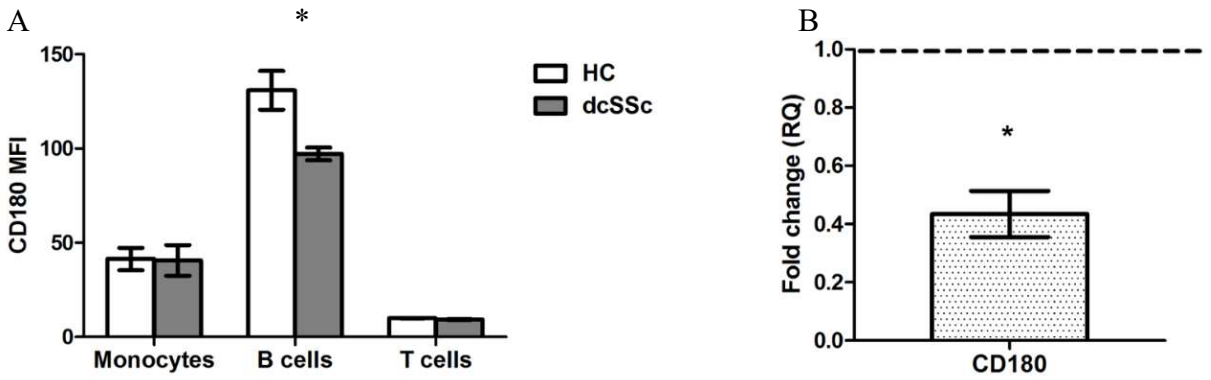


Figure 3. *Flow cytometric (A) and qPCR (B) analysis of CD180 expression in B cells of early dcSSc patients. Data are compared to HCs. * $p<0.05$*

1.3. Analysis of signaling pathways in B cells after anti-CD180 antibody stimulation

We evaluated the phosphorylation of transcription factors involved in PI3K and mTOR signaling using flow cytometry to investigate which pathways could be activated upon anti-CD180 stimulation or by ligation of TLR4. We tried to stimulate TLR4 in B cells using its prototypical ligand LPS and endogenous TLR4 ligands HMGB1 and tenascin-C. HMGB1 and tenascin C are both damage associated endogenous ligands and induction of TLR4 signaling leads to profibrotic responses in explanted fibroblasts [9]. The TLR4 ligands failed to induce any activation or phosphorylation of transcription factors via TLR4. Therefore, we focused on anti-CD180 antibody stimulation. We also used the combination of anti-CD180 and anti-IgG/M (anti-Ig). The phosphorylation of Akt and S6 seemed to be mainly driven by anti-CD180+anti-Ig stimulation in SSc. The effect of anti-CD180+anti-Ig and anti-CD180 stimulation alone on the phosphorylation of Akt and S6 showed no significant difference in HC. Following anti-CD180 antibody stimulation the positivity of the two investigated phosphoproteins was lower in SSc (n=5) compared to HC (n=3) (**Figure 4**).

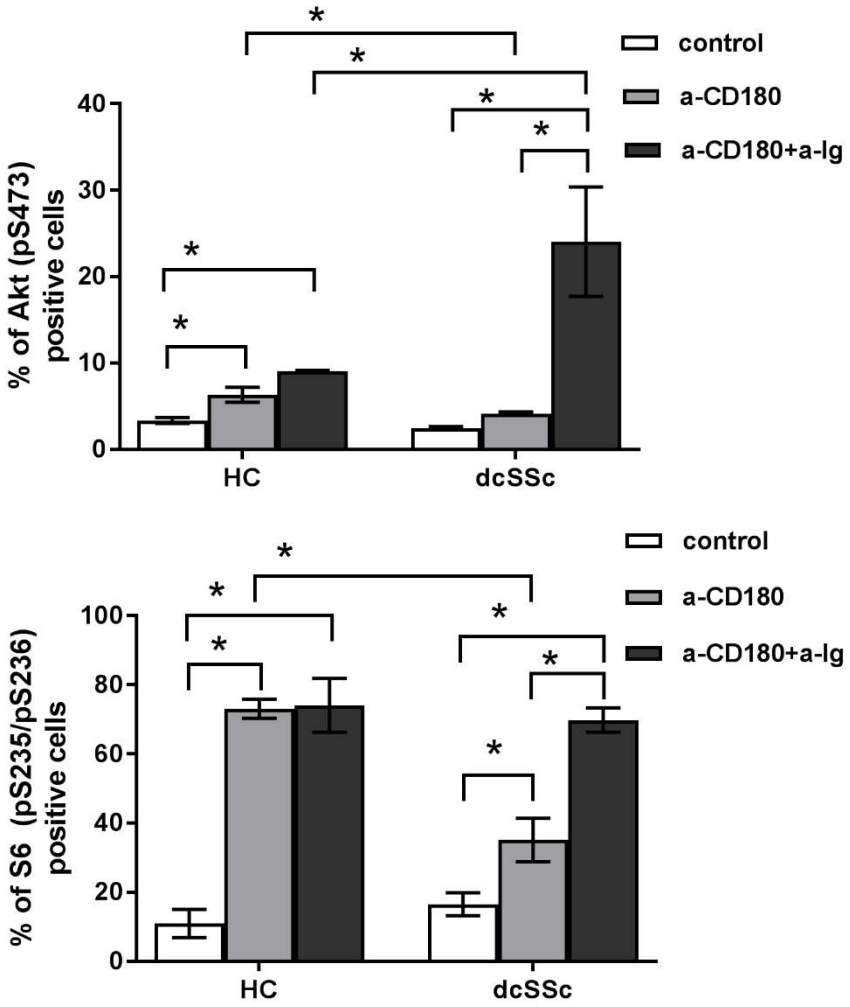


Figure 4. *Phosphorylation of Akt and S6 molecules in purified B cells in SSc and in HC following stimulation with anti-CD180 antibody and anti-CD180+anti-Ig. * $p<0.05$*

1.4. CD180 expression is reduced both at mRNA and protein level in B cells after anti-CD180 antibody stimulation

To further investigate the role of CD180 in phenotypical and functional alterations of B cells, we used tonsillar B lymphocytes as a model. The CD180-negative B cells were described as highly activated cells in SLE [16], and stimulation via CD180 is known to activate B cells [17]. Furthermore, TLR ligands were reported to downregulate the mRNA expression of CD180 molecule [18], thus we hypothesized that the decreased CD180 expression of dcSSc B cells could be a result of activation through TLRs. We measured the expression of CD180 at protein level using flow cytometry and mRNA level with qPCR, and found that following anti-CD180 ligation the MFI and mRNA level of CD180 significantly decreased and was not altered by the co-treatment with CpG (n=4) (**Figure 5**).

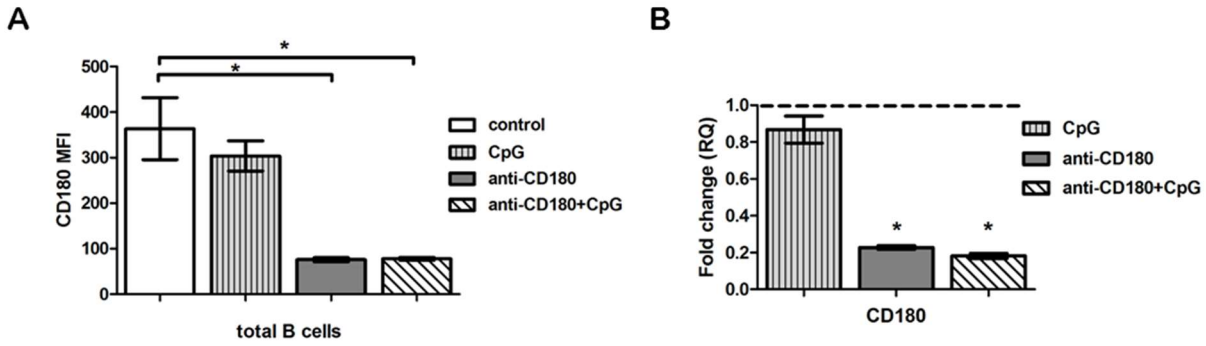


Figure 5. *Effect of stimulation via CD180 on CD180 protein (A) and mRNA (B) expression of B cells. * $p<0.05$*

1.5. The ratio of CD180 positive cells is the highest in NS B cells, and they show the strongest activation following anti-CD180 antibody stimulation

We investigated the expression of CD180 in four B cell subsets with flow cytometry: $CD27^+IgD^+$ non-switched memory (NS) B cells, $CD27^+IgD^-$ switched memory (S) B cells, $CD27^-IgD^+$ naive B cells (N) and $CD27^-IgD^-$ double negative (DN) B cells (n=4). We found that the percentage of CD180 positive cells was significantly higher in NS B cells compared to all other subsets. After anti-CD180 stimulation the frequency of $CD180^+$ cells was significantly decreased in all four B cell subsets. To study the influence of other TLR ligands we used CpG (TLR9 ligand). Addition of CpG did not alter the ratio of CD180+ B cell subpopulations (**Figure 6**).

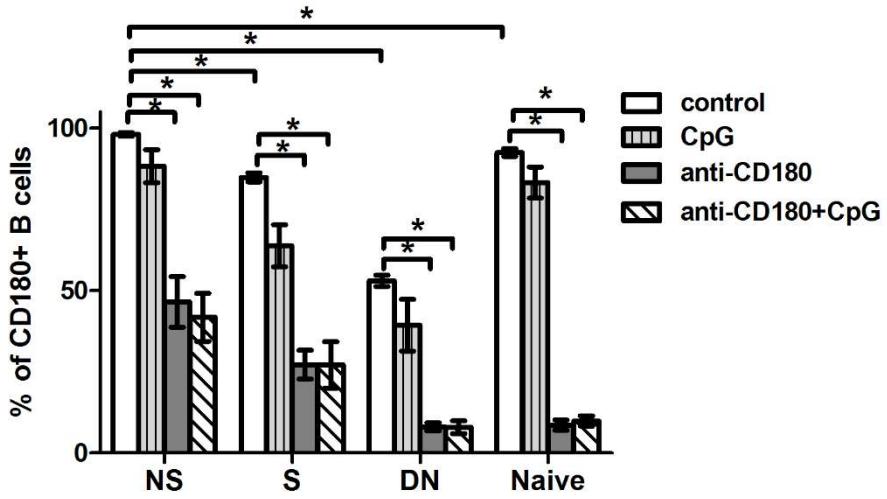


Figure 6. *Effect of stimulation via CD180 on the percentage of CD180+ cells in B cell subsets * $p<0.05$*

We also examined the activation of the four B cell subsets after anti-CD180, CpG, and anti-CD180 + CpG stimulation by detecting CD69, an early activation marker (n=4). Upon anti-CD180 stimulation, the frequency of $CD69^+$ cells was increased in all investigated subsets compared to CpG stimulation, with the highest ratio in NS B cells (**Figure 7**). This result is consistent with our observation that NS B cells show the highest ratio of CD180 positive cells.

CpG, when used together with anti-CD180 antibody stimulation, alters the percentage of $CD69^+$ cells only in naïve B cell subset compared to anti-CD180 stimulation alone.

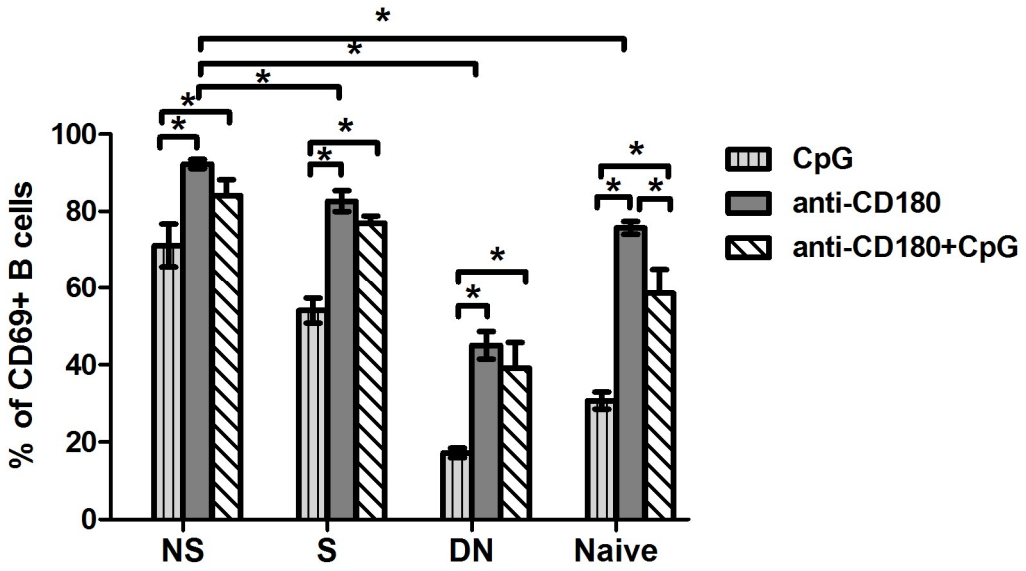


***Figure** 7. Effect of stimulation via CD180 on the percentage of* CD69+ cells in B cell subsets ** p<0.05*

1.6. Activation via CD180 induced the elevation of IL-6 production which was further enhanced by the addition of CpG

The production of scleroderma-specific autoantibodies, and secretion of pro-inflammatory and pro-fibrotic cytokines by B cells is a well-described result that reflects immune dysregulation affecting B cells in systemic sclerosis. To test whether the anti-CD180 stimulation influences the production of IL-6 and IL-10, we measured the concentration of these cytokines in the supernatant of stimulated B cells using ELISA (n=4). Anti-CD180 stimulation alone significantly increased the concentration of IL-6, while supplementing anti-CD180 antibody with CpG, significantly augmented the production of both IL-6 and IL-10 **(Figure 8)**.

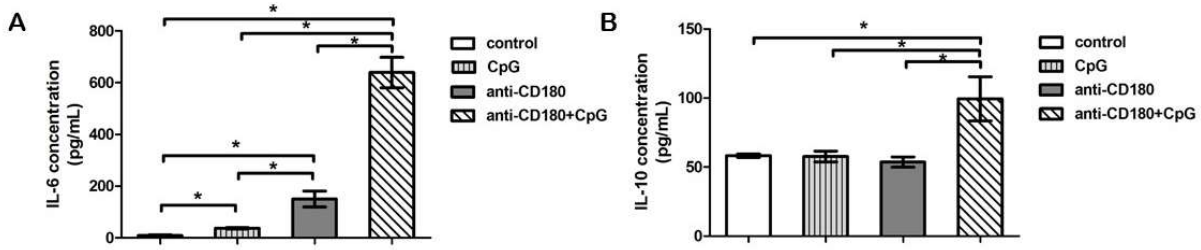


***Figure 8**. Detection of IL-6 (A) and IL-10 cytokines (B) in the supernatant of tonsillar B cells stimulated with CpG, anti-CD180 antibody or anti-CD180 + CpG or left unstimulated * p<0.05*

1.7. CD180 stimulation induces natural autoantibody production

Since CD180 stimulation resulted in the most pronounced activation of NS memory B cells, resembling the B1 B cell population, which includes cells responsible for natural IgM autoantibody production[19], we investigated the effect of CD180 ligation on the production of natural autoantibodies. We measured the anti-CS IgM autoantibody and the anti-topoisomerase I F4 fragment (anti-topo I) antibody in the supernatant of stimulated and B cells using ELISA (n=3) as previously described [20,21] (**Figure 9**). Anti-CD180 itself significantly raised only the level of anti-topo I IgM antibodies. However, anti-CD180 and CpG co-treatment significantly enhanced the production of both anti-CS and anti- topoisomerase I IgM antibodies. Antinuclear antibodies (ANA), anti-dsDNA, and anti-nucleosome antibodies were not detectable in the supernatant of B cells under the investigated conditions.

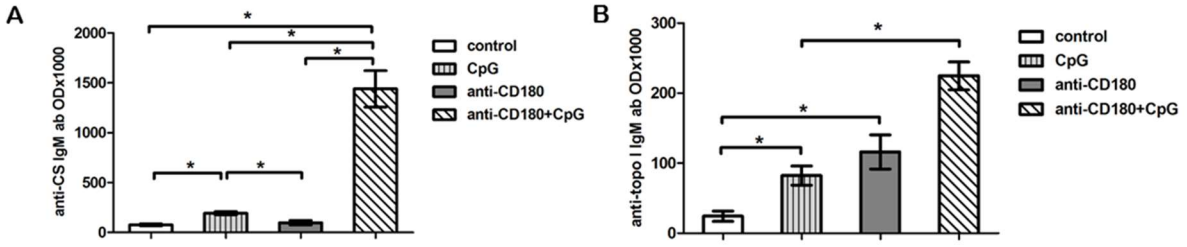


Figure 9. *Anti-citrate synthase IgM and (A) anti-DNA topoisomerase I IgM (B) production of B cells stimulated with CpG, anti-CD180 antibody or anti-CD180 + CpG or left unstimulated * $p<0.05$*

Overall, to find possible associations between B-cell dysfunction and SSc pathogenesis we were, to our best knowledge, the first to investigate the expression of 92 PI3K signaling –related genes in peripheral blood B cells of a clinically homogenous group of dcSSc patients. We found, that innate signals related molecules TLR4 and C3 are upregulated accompanied by decreased expression of CD180. We observed the same expression pattern of TLR4, CD180 and C3 genes in early dcSSc patients on immunosuppressive treatment. We also showed that the pattern of activated signaling pathways in B cells upon anti-CD180 antibody stimulation is different in early dcSSc and in HC. We were the first to investigate the distribution of CD180 molecule in human B cell subpopulations defined by CD27 and IgD labeling and found that the frequency of CD180+ cells was the highest among NS cells in tonsillar B cells and the NS subset showed the strongest activation after anti-CD180 antibody stimulation. Activation via CD180 induced the elevation of IL-6 production and the anti-DNA topoisomerase I IgM natural autoantibody secretion which was further increased by the addition of CpG. Gaining new insights into B-cell activation via CD180 in SSc could help the better understanding of the pathogenesis and may aid in finding new therapeutic targets.

2. Evaluation of clinical relevance of different B cell subsets

The B cell compartment in peripheral blood of SSc patients was reported to contain an elevated number of naïve B cells and a decreased number of memory cells compared to HCs [22]. According to our results the ratio of $CD27^{-}IgD^{+}$ naive B cells was higher, the proportion of memory B cells, more markedly the ratio of NS B cells was decreased in SSc patients compared to HCs.

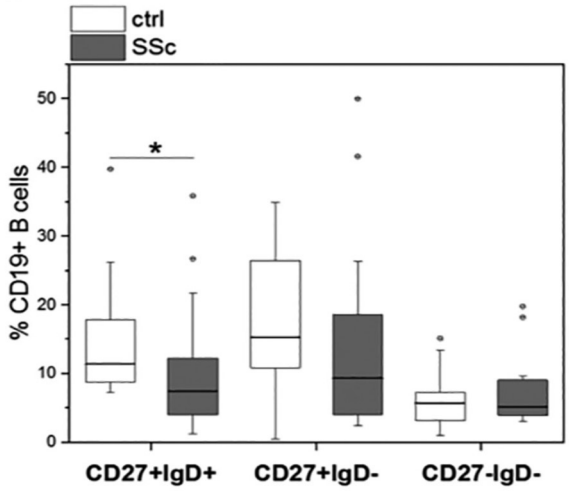
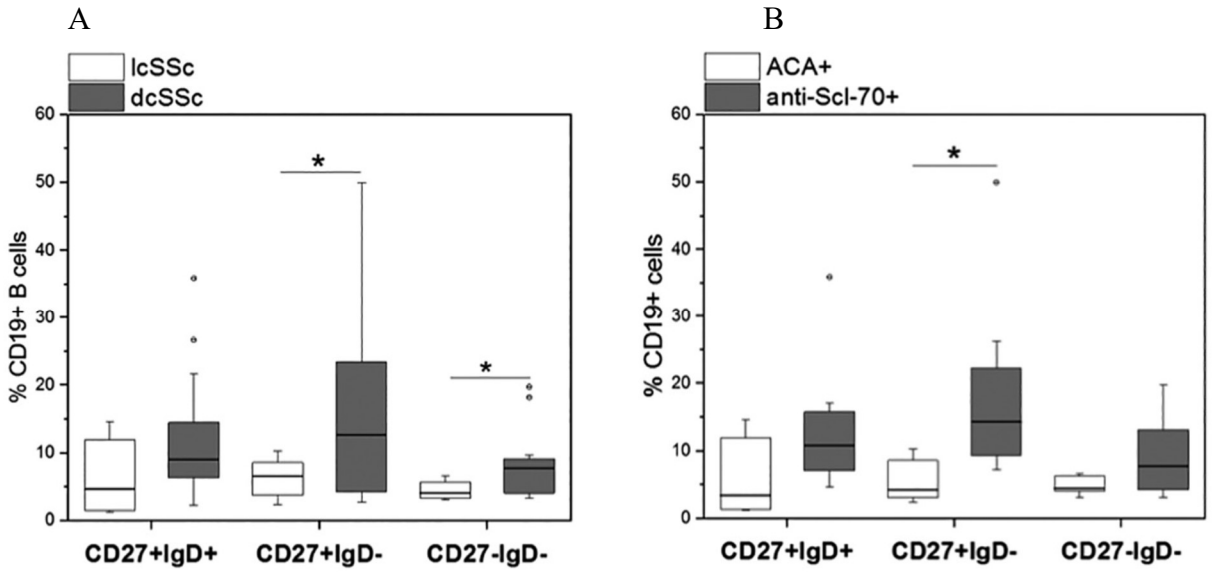


Figure 10. *Elevated naive and diminished memory (CD27+) B cells and decreased NS B cells are shown as percentage of CD19+ total peripheral blood B cells in SSc compared to HC.* * *p<0.05*

Among SSc patients the ratio of S and $CD95^+$ memory B cells was higher in dcSSc. Patients with pulmonary fibrosis showed increased proportions of S B cells and their ratio of $CD95^+$ memory B cells was also higher. The S B cells was also elevated in anti-Scl-70 antibody positive group compared to anti-centromere antibody (ACA) positive patients (**Figure 11**).



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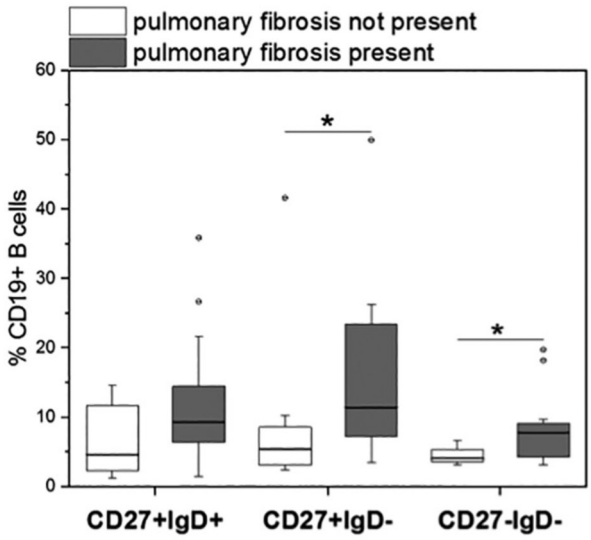

Figure 11. *Alteration in the distribution of NS, S and DN B cell subsets in SSc subgroups: dcSSc – lcSSc (A), anti-Scl-70 antibody - ACA positivity (B), patients with and without pulmonary fibrosis (C). * p<0.05*

Phenotypic analysis of peripheral blood B cell subsets showed significant differences not just between healthy controls and SSc patients but also among subgroups of SSc. The found alterations of the various B cell subsets were associated with the diffuse form of the disease, postivity for anti-Scl-70 antibody and with the presence of pulmonary fibrosis. The reduction of NS B cells could result in the decreased proportion of memory B cells in SSc leading to an imbalance between the tolerogenic and activated memory B cell types. According to our results detailed flow cytometric analysis of memory B cell subsets could contribute to better distinction between the two SSc subtypes, evaluation of disease severity and prediction of as elevated percentage of switched and activated CD95+DN B cells may serve as a biomarker for dcSSc.

3. New disease activity and damage-related serum biomarkers

3.1. Investigations of potential markers of disease activity and damage

Disease activity assessment is crucial in defining the appropriate therapy and to monitor the efficacy of treatment in systemic sclerosis.

We evaluated 77 patients (50 diffuse /dcSSc/ and 27 limited cutaneous SSc /lcSSc/ patients) from a single tertiary clinical center. Cohort enrichment was performed to increase the number of patients with early disease and dcSSc. Seventy-two patients were re-evaluated after one year. Nine patients had overlap syndromes: rheumatoid arthritis (n=3), Sjögren syndrome (n=2), polymyositis (n=2), and mixed connective tissue disease (n=2). The overall disease activity was evaluated using both composite indices (EUSTAR disease activity index from 2003 /EUSTAR-AI-2003/, 9.5 point and 12 point disease activity developed by our research group /PECS-AI-9.5point, PECS-AI-12point/, and revised EUSTAR disease activity index from 2017 /EUSTAR-AI-2017) and the global evaluation of physician of disease activity (PGA), based on the blinded evaluation of a single physician [1-3]. In addition to the minimal essential data from the EUSTAR database we also performed detailed assessment of the musculoskeletal involvement evaluating measures of hand function, Disease Activity Score in 28 joints (DAS28) scores, and the Clinical Disease Activity Index (CDAI) [4].

Three times more patients with active disease were identified by the EUSTAR-AI-2017 compared to the EUSTAR-AI-2003 at baseline investigation (n=37, 48.7%, vs. n=11, 14.3%). Two patients (18%) with active disease based on the EUSTAR-AI-2003 were missed by the EUSTAR-AI-2017. PECS-AI-9.5point index identified 15 patients (19.5%) with active disease (cut-off >2.75 points). Active disease was equally frequent in dcSSc and lcSSc patients based on EUSTAR-AI-2003, but was more frequent in dcSSc patients based on the EUSTAR-AI-2017 in the whole cohort, and also after excluding overlap cases.

Patients with active disease based on the EUSTAR-AI-2003 had more frequently rheumatoid factor (6/9, vs. 12/45, p=0.047), and DLCO<70% (11/11, vs. 36/65, p<0.01). Active disease based on the EUSTAR-AI-2017 was associated with current cyclophosphamide treatment (9/37, vs.2/39, p=0.023), and diabetes mellitus (7/30, vs. 0/39, p<0.01). The PGA correlated moderately at both baseline and one year follow-up examination with the EUSTAR-AI-2003 (rho: 0.519, and rho: 0.692, respectively, p<0.001), the EUSTAR-AI-2017 (rho: 0.401, and rho: 0.429, respectively, p<0.001), and the PECS-AI-9.5point (rho: 0.425, and rho: 0.593, respectively, p<0.001).

CDAI correlated significantly with the EUSTAR-AI-2003 (rho: 0.345, and rho: 0.283, respectively, p<0.05) and the PECS-AI-9.5point (rho: 0.363, and rho: 0.324, respectively, p<0.05) at both the baseline and one-year follow-up investigations, but showed no consistent correlation to the EUSTAR-AI-2017 or PGA.

The two validated disease activity indices identify different patient groups. Joint involvement is potentially underrepresented in the EUSTAR-AI-2017. Active disease is also present in lcSSc and should be assessed regularly in these patients.

We also aimed to further assess promising biomarkers (vascular endothelial growth factor /VEGF/, von Willebrand factor /vWF/ and soluble P-selectin glycoprotein ligand-1 /sPSGL-1/) which we have previously found as possible disease activity markers, and to test whether these biomarkers can be integrated into the EUSTAR-AI-2017, the 9.5-point activity index and the 12-point activity index to improve their sensitivity to detect active disease.

We have also measured the levels of other biomarkers (resistin, myeloperoxidase /MPO/, COMP, hyaluronan, soluble vascular cell adhesion molecule /sVCAM-1/, YKL-40, procollagen I N-terminal propeptide /PINP/, N-terminal procollagen III propeptide /PIIINP/, angiopoietin-2 /Ang-2/, tissue inhibitor of metalloproteinase-1 /TIMP-1/, CC chemokine ligand 18 /CCL18/, matrix metalloproteinase-12 /MMP12/, Krebs von den Lungen-6 /KL6/, Surfactant Associated Protein D /SPD/, fetuin a, pentraxin-3 /PTX-3/, alpha-2-macroglobulin) related either to inflammation, altered collagen metabolism, endothelial cell activation, altered alveolar cell function, or damage of the cartilage.

Out of the investigated biomarkers, the levels of YKL-40 (rho=0.322, p=0.005), sPSGL-1 (rho=0.258, p=0.027), and pentraxin-3 (rho = 0.275, p=0.019) correlated at both baseline and one-year follow-up examination with the EUSTAR-AI-2017 **(unpublished data)**.

We further aim to make different subset analyses (based on antibody profile, internal organ involvement, etc) to test the significance of these laboratory data regarding disease activity assessment and we also aim to test the sensivity to change of these biomarkers based on the changes of the investigated disease activity indices.

3.2 Evaluation of biomarkers related to musculoskeletal involvement-evaulation of cartilage oligomeric matrix protein (COMP) and Human Cartilage Glikoprotein 39 (YKL-40)

COMP or thrombospondin-5 is an extracellular glycoprotein belonging to the thrombospondin gene family that forms a disulphide-linked pentamer and is predominantly found in cartilage, tendons and ligaments. It binds to type I, type II, type IX collagen fibres, fibronectin and stabilizes the collagen fibre network [5,6].

We have measured the serum levels of COMP and YKL-40 in 77 SSc patients, 39 with RA, 20 with primary Raynaud's syndrome and 28 healthy volunteers as controls. All participants were clinically assessed using the Hand Mobility in Scleroderma Index (HAMIS), DAS28, simplified disease activity index (SDAI) and CDAI. Furthermore, patients filled questionnaires related to disability, hand function and quality of life: Health Assessment Questionnaire (HAQ), Cochin Hand Function Score (CHFS), quick Disability of the Arms, Shoulders and Hands (qDASH), and Short Form Health Survey (SF36) questionnaire.

There was a significant difference ($p < 0.001$) between the COMP level of SSc patients and the COMP level of control groups, the highest values were shown by the SSc group (**Figure 12**).

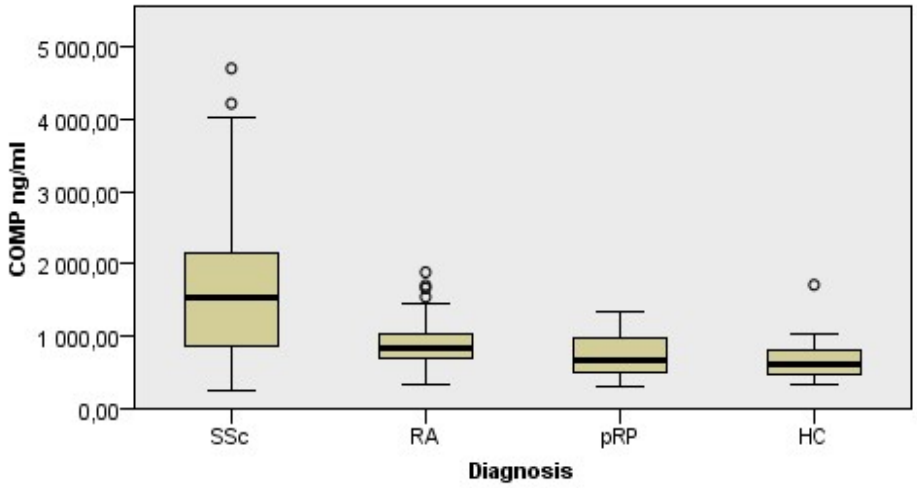

Figure 12. *Levels of Cartilage oligomeric matrix protein in examined groups ($p < 0.001$)*

YKL-40 was significantly higher in SSc patients compared to the healthy control and primary Raynaud group ($p < 0.001$). The YKL-40 levels in the RA group were higher compared to the SSc group (**Figure 13**).

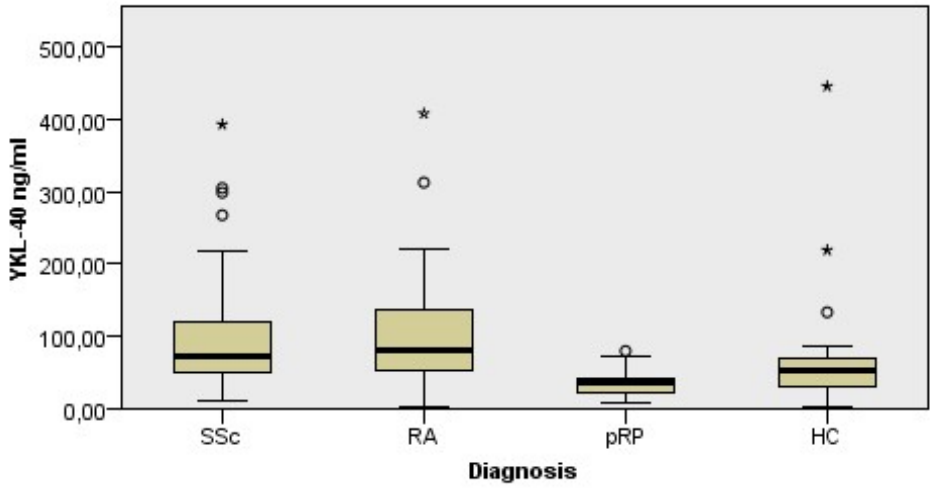

Figure 13. *Levels of Human Cartilage Glikoprotein 39 in examined groups ($p < 0.001$)*

COMP serum level showed significant consistent correlations with morning stiffness duration, and DAS28 (ESR) in SSc. YKL-40 levels showed significant correlations in SSc with morning stiffness duration, SDAI, HAMIS, qDASH, SF36 physical component score (SF-36 PCS), HAQ, CHFS, CRP and contracture count (**Table 1**.)

	COMP (ng/ml)				YKL-40 (ng/ml)			
	SSc				SSc			
	baseline		follow-up		baseline		follow-up	
Parameter	Rho	P-value	Rho	P-value	Rho	P-value	Rho	P-value
Age	-.179	.154	-.307**	.009	.072	.538	.052	.687
Disease duration	.051	.662	-.083	.494	-.045	.701	.197	.124
Morning stiffness duration	**.404****	**.000**	**.261***	**.028**	**.278***	**.016**	**.277***	**.029**
DAS28 CRP	.199	.088	.182	.128	.281*	.015	.201	.117
DAS28 ESR	**.236***	**.042**	**.235***	**.048**	.292*	.011	.213	.097
SDAI	.235*	.043	.189	.115	**.248***	**.032**	**.256***	**.045**
CDAI	.259*	.025	.192	.108	.247*	.033	.232	.070
HAMIS	.061	.606	-.050	.680	**.343****	**.003**	**.265***	**.037**
qDASH	.247*	.032	.033	.787	**.234***	**.044**	**.411****	**.001**
SF36 PCS	-.325**	.005	-.113	.350	**-.276***	**.018**	**-.413****	**.001**
HAQ	.215	.066	-.006	.962	**.275***	**.018**	**.330****	**.009**
CHFS	.185	.111	.020	.871	**.262***	**.023**	**.349****	**.005**
Contracture count	.162	.168	-.060	.619	**.420****	**.000**	**.260***	**.041**
CRP	-.021	.856	-.027	.820	**.310****	**.007**	**.260***	**.041**
ESR	.121	.301	.130	.282	.308**	.007	.204	.112

Table 1. *Correlation between serum COMP and musculoskeletal involvement specific parameters in SSc*

In conclusion COMP might be a promising biomarker of joint-activity in SSc, furthermore YKL-40 correlates with the parameters of musculoskeletal-activity and musculoskeletal-damage **(unpublished data).**

4. Musculoskeletal composite index for the evaluation of damage

4.1 Assessment of musculoskeletal involvement in systemic sclerosis

The musculoskeletal involvement in SSc dramatically influences the patients' functional status, working capacity and health related quality of life, however at present this type of organ involvement is poorly studied and there are no instruments – composite indices or biomarkers – available, which quantify and summon the musculoskeletal involvement of systemic sclerosis.

Musculoskeletal (MSK) symptoms in patients with SSc include articular involvement (arthralgia, synovitis, contractures), which is often an early phenomenon and significantly contributes to the disability. Predominantly the hands are affected. Consensus in outcome measures of articular involvement is missing. The HAQ-DI, CHFS, HAMIS, and DAS28 may be used for the assessment of different aspects of joint involvement. There is an unmet need for therapies confirmed by randomized controlled clinical trials (RCTs) to treat both synovitis and non-inflammatory joint involvement. The few rehabilitation studies that have been conducted have shown some promising efficacy.

Muscle involvement may be an early symptom. The presence of clinically meaningful muscle involvement often heralds an unfavourable prognosis. The SSc-specific fibrinous tenosynovitis (tendon-friction rubs /TFRs/) is a frequent finding in SSc. Patients with TFR are at increased risk of developing renal, vascular, cardiac and gastrointestinal involvement and have reduced survival rates.

Our research group has recently published an extensive review on the musculoskeletal involvement in SSc and suggested further research agenda in this field [7].

4.2. Validation of disease activity indices in joint involvement in systemic sclerosis

We have validated four different musculoskeletal disease activity indices (DAIs) in SSc [4]. The articular DAIs were calculated according to the original formulas (DAS28 ESR, DAS28-CRP, CDAI and Simple Disease Activity Index /SDAI/). All participants filled out a set of fully validated questionnaires on hand function, global function and quality of life (HAQ, CHFS, the qDASH, and the SF-36 questionnaire).

The OMERACT filter was used to assess the validity of the DAIs including feasibility, truth and discrimination.

In the SSc group, the wrists, MCPs and PIPs were affected most often, while knee, elbow and DIP involvement was much less frequent. There was no significant difference in the number of tender DIPs and the number of swollen DIPs between RA and SSc patients (**Figure 14**).

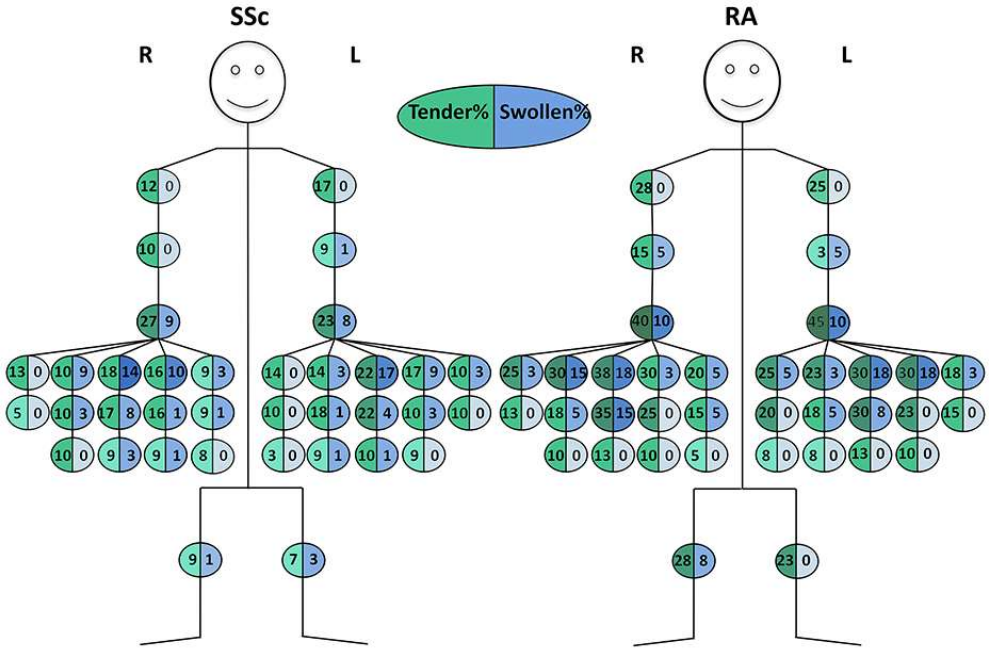

Figure 14. *Percentage of patients with tenderness and swelling in each examined joint in the investigated patients with systemic sclerosis (n=77) and rheumatoid arthritis (n=40)*

R: right, L: left, SSc: systemic sclerosis, RA: rheumatoid arthritis. All values account for prevalence in percentages in the examined cohort. Darker shade represents higher percentage.

DAS28-ESR, DAS28-CRP, SDAI and CDAI showed significant correlation with the EUSTAR-AI-2003, HAQ-DI, CHFS, and the physical component of SF36 ($p<0.001$). All four indices discriminated patients with SSc from RA, RP and healthy controls, respectively ($p<0.01$). With the exception of DAS28-CRP, the other three indices also discriminated between subgroups of SSc based on value of EUSTAR-AI-2003 (≤ 3 and >3) ($p<0.05$). All four DAIs showed a good inter- and intraobserver reliability based on repeated measures of two independent investigators ($p<0.001$) [4].

4.3. Medium term effect of complex physiotherapy in SSc

Joint damage leading to contractures in both the small hand joints and large joints is a frequently observed characteristic of SSc, as our research group has previously demonstrated [8]. There is a lack of data on the long-term efficacy of intensive hand physiotherapy in SSc.

We published the results of our study where we used a combined, extensive treatment approach (including thermal and mud baths, whirlpool therapy, soft tissue massages in combination with stretching hand exercise and ergotherapy) and we evaluated the long-term (six month) effectiveness [9].

Fifty-three patients with SSc consented to participate. By the end of the three-week extensive physical therapy, the health-related functional parameters showed a significant improvement globally. The HAQ-DI showed a significant improvement exclusively in the group receiving physical therapy for the hands ($p<0.05$). Measures of hand anatomic impairment Hand

Anatomic Index (HAI), delta finger-to-palm (delta-FTP) showed only a short-term significant improvement in both groups.

Regarding hand-function, the DASH and HAQ-DI improved significantly ($p<0.05$), furthermore the Cochin test showed some improvement in the interventional group, but the changes were not statistically significant in any of the groups.

After six months, only the hand-focused physiotherapy group showed a sustained improvement in HAQ-DI, DASH, VAS-pain and VAS-Raynaud ($p<0.05$).

Between the lcSSc and the dcSSc groups no significant differences in the results of any tests have been found neither at the end of the three-week investigation period nor after six months follow-up, both in the interventional and the control group.

In conclusion the long-term, parallel improvement in HAQ-DI and DASH reflects the beneficial effect of the complex physical therapy program on hand function in patients with SSc. Some additional benefits including analgesic effect and improved Raynaud's symptoms have also been observed [9].

4.4. Contractures as poor prognostic factors

We also aimed to test the prognostic significance of musculoskeletal involvement in our SSc patient cohort, as one of the potential risk factors for poor prognosis. We have retrospectively examined data of all SSc patients with at least two visits at our tertiary clinic between 1995 and 2015. Diagnosis of SSc was set up in 469 cases, and only 30 patients were considered lost to follow up - patients who did not appear in the center for twelve months after their last visit. Causes of death were defined based on last discharge papers, consultation with the patients' GP and autopsy results. Each cause of death was extensively discussed, and a final agreement of the investigators was achieved.

The all-cause mortality 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 were taken, the survival rate showed 95.6% at 5 years, 87.5% at 10 years and 74.2% at 15 years, respectively. Patients with coexistent malignancy had significantly worse survival by univariate analysis compared to those without coexistent malignancy ($p<0.001$) [10].

Univariate analysis showed that besides the well-known poor prognostic factors (dcSSc, male gender, ILD, cardiac involvement, elevated right ventricular pressure on echocardiography, less than 50% ejection fraction and ECG abnormalities, anti-topoisomerase positivity, esophageal involvement, scleroderma renal crisis, low hemoglobin, hematocrit and albumin levels, elevated ESR, coexistent and current or previously diagnosed malignancies) the presence of small joint contractures was also associated with poor prognosis. Conversely, the presence of anti-centromere antibodies and lack of SSc capillary pattern showed a favourable outcome.

Multivariate Cox analysis confirmed male gender, presence of topoisomerase I antibody, DLCO 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 as predictors of poor outcome (**Table 2).** In addition to well-known factors predicting poor outcome in SSc, the presence of small joint contractures was a newly identified independent risk factor of mortality. Our data also confirmed a recent finding showing that history of arterial hypertension was also a poor prognostic factor [10].

	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	
	HR (95% CI)	p	HR (95% CI)	p	HR (95% CI)	p
Male gender	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	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	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					1.995 (1.146-3.470	<0.01
< 70% DLCO					1.975 (1.281-3.045)	<0.01
< 50% DLCO	2.680(1.369-5.244)	<0.01	2.732 (1.597-4.673)			
> 40 mmHg right ventricular pressure on echocardiography	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	4.468 (1.671-11.948)	<0.01	5.303 (2.065-13.618)	<0.01		
Brady- or tachycardia detected by ECG	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	1.973 (1.048-3.715)	<0.05			1.675 (1.022-2.746)	<0.05
Arterial hypertension	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%)	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)	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			3.190 (1.792-5.679)	<0.001	2.956 (1.835-4.762)	<0.001

***Table 2**: Multivariate analysis of mortality risk factors in 469 SSc patients*

5. Prognostic value of biomarkers in the evaluation of activity and damage in cardio-respiratory system

5.1. Prognostic significance of galectin-3

Galectin-3 is a beta-galactoside-binding lectin that may be related to tissue sclerosis or aberrant activation of angiogenesis in systemic sclerosis (SSc). The aim of our study was to determine the associations between galectin-3 levels and patient characteristics, as well as to investigate the long term prognostic value of galectin-3 in a large cohort of SSc patients. We have published our results on serum galectine-3 levels which is an independent prognostic marker of overall survival in cases with SSc [11].

Galectin-3 levels showed positive correlation with the grade of left ventricular diastolic function ($r=0.193$; $p=0.026$), erythrocyte sedimentation rate ($r=0.172$; $p=0.036$) and serum level of C-reactive protein ($r=0.200$; $p=0.015$) while negative correlation with diffusing capacity for carbon monoxide ($r=-0.228$; $p=0.006$), in age, gender and BSA adjusted analyses.

Both galectin-3 and NT-proBNP showed positive correlation with age. NT-proBNP levels significantly correlated with right ventricular pressure and with the diagnosis of PAH. Both biomarkers correlated positively with the grade of left ventricular diastolic function as well as with the laboratory parameters of inflammation. Negative correlation was found between DLCO and both biomarkers. During the follow-up of 7.2±2.3years, 35 SSc patients (23%) died.

In multivariate Cox regression analyses adjusted for age, gender, BSA, creatinine and NT-proBNP levels, galectin-3 was an independent predictor both of the all-cause mortality (HR: 2.780, 95% CI: 1.320-5.858, $p=0.007$) and cardiovascular mortality (HR: 3.346, 95% CI: 1.118-10.012, $p=0.031$). Using receiver-operating characteristic analysis, galectin-3>10.25ng/ml was found to be the best predictor of the all-cause mortality**.**

Our results suggest that galectin-3 is an independent predictor of all-cause and cardiovascular mortality in SSc. Validation studies are required to establish whether galectin-3 may be proposed as simple biomarker for identifying patients with high mortality risk in SSc [11].

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. The results suggest, that galectin-3 reflect a different pathophysiologic axis than NT-proBNP.

5.2. KL-6 as useful biomarker in SSc related damage and activity

KL-6 is a parameter of lung damage, and it is very useful (negative) prognostic marker in patient survival. Lung involvement in an important prognostic factor in survival, while musculoskeletal manifestation of SSc dramatically influence the patients' health related quality of life.

We measured KL-6 levels in 77 SSc patients, 40 with RA, 20 with primary Raynaud's syndrome and 20 healthy volunteers as controls twice, at baseline and one year later.

Our baseline data showed that KL-6 correlated with different activity and potential damage parameters, both related to global disease activity and also the musculoskeletal involvement:

1. a, disease activity parameters:

- 9.5 point activity index: r= 0.254, p= 0.038
- DLCO%: r= 0.362, p= 0.003
- DAS28: r= 0.361, p= 0.003
- SDAI: r=0.363, p=0.003
- CDAI: r= 0.369, p= 0.002,
- SF36 physical activity: r= 0.310, p= 0.011

2. b, damage parameters:
    - Cochin: r= 0.261, p= 0.033
    - SF36 general health: r= 0.312, p= 0.010
    - SF36 RP (role physical): r= 0.446, p< 0.0001
    - Mouth Handicap Scale (MHIS): r= 0.308, p= 0.011

It is very important, that the HAQ, which is related both to disease activity and damage, was also correlated with KL-6:, r= 0.317, p= 0.009.

KL-6 serum levels were higher in the SSc cohort compared to the group of primary RP patients and healthy volunteers ($p<0.01$ and $p<0.05$, respectively) at the first measurement.

In the second year, the serum titer of KL-6 was significant higher than at baseline (**Figure 15**), possibly reflecting the worsening of physical health.

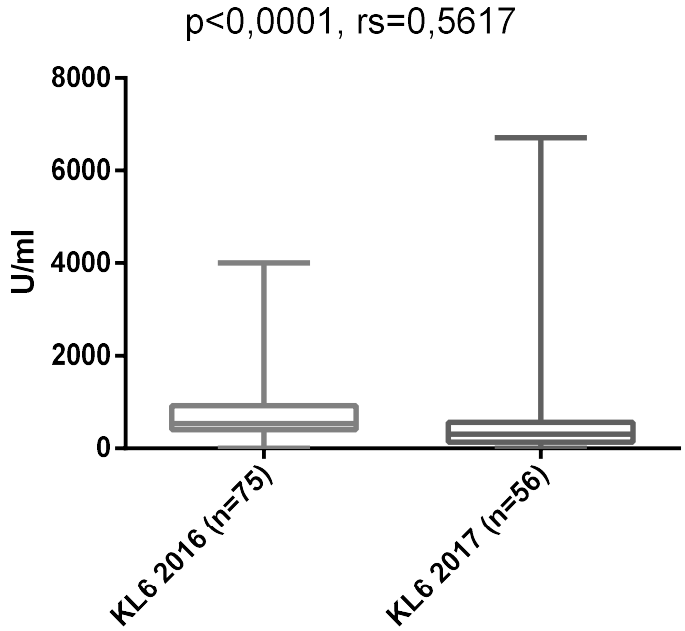

Figure 15. *KL-6 titers in first and second study year in 77 SSc patients*

One year follow-up KL-6 results showed deterioration in:

- patients with worsening in heart-lung status based on questionnaires: p= 0.048,
- patients with pulmonary fibrosis on HRCT: p= 0.039,
- patients with diastolic dysfunction: p= 0.001,
- patients with ventricular arrhythmias: p= 0.025.

Serum KL-6 levels showed significant correlation with the 28 tender joint count, the 28 swollen joint count (rho: 0.246 $p<0.05$), the DAS28 (ESR) score (rho: 0.361 $p<0.01$), the DAS28 (CRP)

score (rho: 0.330 $p<0.01$) the HAQ (rho: 0.317 $p<0.05$) and CHFT (rho: 0.261 $p<0.05$), but did not correlate with delta-FTP, HAI.

There was a significant difference in the KL-6 serum levels of SSc subgroup with and without tender and/or swollen joints ($p<0.05$). There was no significant difference in the KL-6 serum levels between the diffuse and limited cutaneous SSc subgroups.

Our preliminary results support our hypothesis about the applicability of KL-6 as biomarker in determination of disease progression during the disease course. KL-6 might be also a promising biomarker of inflammatory joint involvement in SSc **(unpublished data).**

5.3. Evaluation of microangiopathy by nailfold video capillaroscopy in patients with connective tissue diseases

Raynaud's phenomenon is the first symptom in the majority of patients with systemic sclerosis and it is probably one of the driving events that leads to the development of SSc. However, the clinical significance and characteristics of microangiopathy is relatively understudied in the literature.

Therefore our aim was to characterize capillary density and capillary morphology by nailfold video capillaroscopy (NVC) in different CTDs with a special focus on the presence/absence of Raynaud's phenomenon (RP) in SSc and overlap syndromes. We examined 296 patients with systemic sclerosis, systemic lupus erythematosus, idiopathic inflammatory myopathies (IIM), Sjogren's syndrome (SS), antiphospholipid syndrome (APS), rheumatoid arthritis, systemic vasculitis and undifferentiated connective tissue disease (UCTD) by NVC. Control groups consisted of 25 healthy controls (HC) and 22 primary RP cases.

The mean capillary density was significantly decreased in SSc, SLE, SS, IIM and APS either compared to HC or PRP cases. Mean microangiopathy evolution score (MES) was higher in SSc, SLE, SS, IIM and APS compared to both HC and PRP cases. SSc, SLE, SS patients had a significantly higher giant capillary number compared to either HC or primary RP control cases.

Average hemorrhage score was significantly higher in SSc and SS but not in SLE, RA, IIM and APS compared to HC. Except RA, all investigated CTDs showed significantly higher capillary loss score compared to either PRP controls or HC.

SSc capillary pattern was present in 75.3% of all SSc cases, most commonly SSc late pattern was observed (58.43%). SSc pattern was present in other CTD cases too (15.4-40.7%), most frequently SSc late pattern was present. SSc active and early pattern were rarely seen in other CTDs than SSc, predominantly in cases with RP **(Figure 16).**

Regarding clinical associations, SSc patients with a DLCO<70% had significantly more giant capillaries, avascularity, late SSc pattern, increased MES, and lower capillary density compared to cases with DLCO>70%, but in the other CTDs decreased DLCO was not associated with similar pronounced capillary damage. Therefore abnormal capillaroscopic pattern could be more prevalent in either lung or heart involvement and should influence the clinician to screen for these internal organ involvements more attentively **(unpublished data).**

The examination of association of capillaroscopic patterns and biomarker levels reflecting endothelial cell activation and damage (e.g, VEGF, VCAM-1, vWF) with changes on nailfold videocapillaroscopy in SSc are currently in progress.

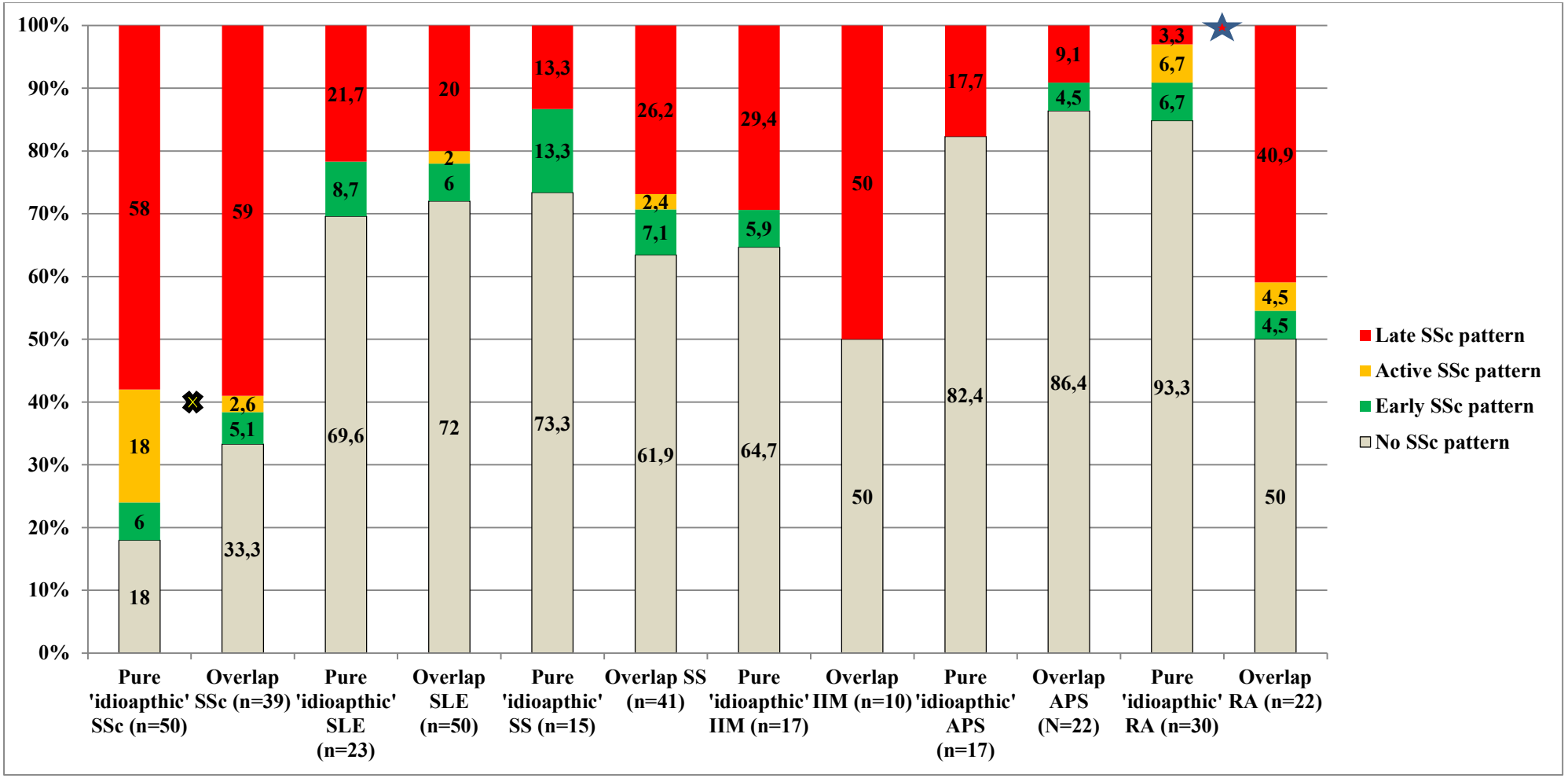

***Figure 16**: Distribution of SSc pattern in pure 'idiopathic' and overlap connective tissue diseases*

×: p <0.05: Comparison of SSc active pattern in pure idiopathic systemic sclerosis and overlap systemic sclerosis, * p<0.01: comparison of SSc late pattern in pure'idiopathic' rheumathoid arthritis and overlap rheumathoid arthritis. SSc: systemic sclerosis, SLE: systemic lupus erythemathodes, Ray: cases with Raynaud's Phenomenon, NR: Cases without Raynaud's phenomenon, SS: Sjogren's syndrome, APS: Antiphospholipid syndrome, RA: Rhemathoid arthritis, IIM: idiopathic inflammatory myosistis

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Judit Rapp, Vivien Telek, Tunde Minier, László Czirják, Tímea Berki, Diana Simon: Analysis of phosphatidylinositol 3-kinase pathway in B cell activation of systemic sclerosis patients, Annual European Congress of Rheumatology EULAR 2019, Madrid, Spain, 12-15 June 2019

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