

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

**VASCULAR ENDOTHELIAL GROWTH FACTOR
AND CYTOKINE INVESTIGATIONS
IN TEARS OF PATIENTS WITH SYSTEMIC SCLEROSIS**

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UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF CLINICAL MEDICINE

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The Examination takes place at the Department of Gynecology, Faculty of Medicine,
University of Debrecen; 11 a.m., April 29, 2016

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ABBREVIATIONS

BCA: bicinchoninic acid

BSA: bovine serum albumin

CD: cluster of differentiation

CFD: complement factor D

CHI3L1: chitinase-3-like protein 1

CRP: C-reactive protein

CXCL: chemokine (C-X-C Motif) Ligand

DES: dry-eye syndrome

ELISA: enzyme-linked immunosorbent assay

IFN: interferon

IL: interleukin

IP-10: interferon gamma-induced protein-10

MC: microcapillary tubes

MCP: monocyte chemoattractant protein

MIG: monokine induced by gamma interferon

MMP: matrix metalloproteinase

mRNA: messenger ribonucleic acid

RNA: ribonucleic acid

RNP: ribonucleoprotein

sIgA: secretory immunoglobulin A

SSc: systemic sclerosis

STS: Schirmer test strip

TGF- β : transforming growth factor-beta

TNF- α : tumor necrosis factor-alpha

VDBP: vitamin D binding protein

VEGF: vascular endothelial growth factor

WO: washout

1 INTRODUCTION

1.1 Systemic sclerosis

With a prevalence of approximately 1 in 2,000 of the general population, SSc is a rare, multi-organ disease affecting the connective tissue of the skin and multiple visceral organs, such as the lungs, gastrointestinal tract, heart and kidneys. Among the different immune-mediated rheumatic diseases, SSc is one of the most incapacitating and life-threatening diseases. Apart from the clinically prominent thickening of the skin and the development of digital ulcers, the progressive deterioration of internal organs and polyarthritis result in high morbidity and mortality rates varying between 34 and 73% over 5 years.

The female predominance is remarkable along with gender differences in disease activity and incidence. The overall female to male ratio is of 1:1 to 14:1.

1.1.1 Ophthalmological manifestations of SSc

There are only few reports available concerning ophthalmological complications in the course of SSc. These papers are mainly case reports; only two overall studies exist, and even these involve only a small number of patients, since SSc is a rare disease. Ocular symptoms may occur at any stage of the disease and may involve numerous ocular tissues. The most prevalent clinical manifestations of soft tissue fibrosis and inflammation in patients with SSc include increased tonus and telangiectasia of the eyelid skin. The most commonly reported lesions are periorbital edema, palpebral ectropion and ciliary madarosis. In our study the most frequent ocular manifestation of SSc was dry eye syndrome (DES).

DES is a major healthcare problem because it affects the patient's quality of life. DES in SSc is believed to be caused by fibrosis-related impairment of lacrimal

gland secretion, while increased evaporation of tears from the ocular surface is the consequence of restricted eyelid mobility and the consecutive reduced blinking. The most important laboratory findings are increased levels of several inflammatory cytokines. Accordingly, tear cytokine levels are considered as potential markers of inflammation in DES.

In order to better understand the ophthalmological manifestations of SSc, first I will summarize the three pivotal sites in the pathomechanism of the disease, namely, widespread small vessel vasculopathy, immune dysregulation with autoantibody production, and progressive fibrosis.

1.1.2 Pathogenesis of SSc

The vasculopathy in SSc is systemic and progressive, which suggests that the obliteration of the microvasculature and associated structural disease is not normally repaired by either compensatory angiogenesis or vasculogenesis. It has been certified that angiogenesis, which means the growth of new vessels from existing ones, and vasculogenesis, which is de novo formation of new vessels, is defective in SSc. The hypoxic-ischemic state in SSc should be a trigger of neoangiogenesis, but instead of new cutaneous capillary formation, nailfold capillary examinations have shown substantial avascular areas, which suggest the abovementioned defective angiogenesis and abnormal vascular repair pathways.

The immune dysregulation in SSc is characterized by chronic mononuclear cell infiltration by cluster of differentiation 4+ (CD4+) T-lymphocytes and macrophages, dysregulated production of lymphokines and growth factors, and B-lymphocyte activation induced by autoantibodies, which include anti-U3-ribonucleoprotein (RNP)/fibrillarin, anti-Th/To, and anti-RNA-polymerase I and II and anti-U11/U12 RNP. These immunological abnormalities and the fibrosis

observed in SSc could be linked together by a mainly Th-2-driven immune response with production of interleukin-4 (IL-4).

Excessive synthesis of fibroblasts and deposition of extracellular matrix (ECM) proteins, such as collagen type I, eventuate in fibrosis, terminally resulting in the disruption of the affected tissue's architecture. In fibrotic pathologies like SSc, resident fibroblasts persist and promote a pro-fibrotic micro environment rich in ECM and growth factors, such as fibroblast growth factor and connective tissue growth factor.

1.1.2.1 Vascular endothelial growth factor, the major pro-angiogenic factor in SSc

Vascular endothelial growth factor (VEGF) is one of the most important pro-angiogenic factors which plays a key role in the formation of new blood vessels. VEGF takes part in various steps of angiogenesis, including initial vasodilation, endothelial cell permeability, perivascular matrix remodelling, and induction of proliferation and migration of endothelial cells. The influence of VEGF in ophthalmic diseases is profound. It has been associated with a large number of retinal diseases and conditions like age-related macular degeneration and diabetic retinopathy, retinopathy of prematurity, sickle cell retinopathy and retinal vascular occlusion. VEGF has secondary influence in neovascular glaucoma and hereditary retinal dystrophies.

Although there is a lack of sufficient angiogenic response to chronic tissue ischemia in SSc that culminates in the loss of capillary vessels, VEGF expression has paradoxically been shown to be upregulated in this disease. Distler et al. found that serum levels of VEGF are markedly elevated in patients with SSc compared to healthy controls, furthermore they detected significant differences in serum VEGF levels according to the disease subset and the presence of anti-Scl-70

antibody. Later studies also confirmed that the expression of VEGF is increased in different cell types in the epidermis and dermis of patients with SSc.

1.1.2.2 Inflammatory cytokines and chemokines in SSc

Patients with SSc have an increased number of circulating monocytes, macrophages and T cells. Production of soluble mediators, including cytokines, by these cells may promote endothelial damage and fibrosis in SSc. Among the various chemokines, monocyte chemoattractant protein (MCP)-1 plays the most critical role in tissue fibrosis that occurs in SSc. Cytokines produced by macrophages, including IL-1, tumor necrosis factor (TNF)- α , interferon (IFN)- γ , IL-6, transforming growth factor (TGF)- β and platelet derived growth factor can regulate inflammation and tissue fibrosis. T cells are also important molecular players in the disease process through activation of macrophages and the direct release of inflammatory and pro-fibrogenic cytokines. Thus, the secreted cytokines from infiltrating leukocytes are likely to be involved in the development of tissue fibrosis in the course of SSc, since they stimulate collagen synthesis via fibroblasts.

1.1.3 Treatment

There is no single standardized approach to the treatment of patients with SSc as yet. General immuno-suppression and complication-specific therapies are the current tools of the management of SSc. More sensitive and more specific biomarkers could help in the assessment of optimal therapeutic approaches.

The ophthalmological manifestations in patients with SSc are often underestimated and not or not correctly treated. In order to better understand the

ocular features and also to use this body fluid as a potential tool for monitoring these important biomarkers, we have turned our attention to tear investigation.

1.2 Tear investigations

1.2.1 Tear collection methods and their application in practice

Quantitative determination of tear proteins is of increasing interest in ophthalmology, but a technical problem still remains due to small tear sample volumes available on the one hand and the complexity of its composition on the other. Tear sampling is definitely a major challenge and has significant influence on the precision and reproducibility of the analytical results. Basically it can be performed in two ways; directly and indirectly.

In direct tear sampling methods, microcapillary tubes or micropipettes are used for sampling and this frequently requires prior stimulation or instillation of different volumes of saline (100–200 μ l) into the cul-de-sac and collection after appropriate mixing. This procedure causes dilution and may not permit collection of samples from specific sites of the ocular surface.

In indirect tear sampling methods, collection of precorneal tear is carried out by using absorbing supports such as Schirmer test strips (STS), filter paper disks, cellulose sponges and polyester rods. STS collection is the most commonly used method among them.

2 AIMS

The aims of our studies were the following:

1. To select an appropriate sampling method to investigate VEGF and cytokines in tears of SSc patients.
2. To detect VEGF in tears of SSc patients.
3. To compare VEGF levels in tears of patients with SSc to those in healthy controls.
4. To determine a wider panel of cytokines and chemokines that have a role in immunopathogenesis and inflammatory processes in tears of patients with SSc.
5. To compare the levels of these mediators in tears of these patients and controls, and to select the most significantly differing ones for further investigations.
6. To determine the selected mediators with the help of a more sensitive and specific laboratory method in tears of both patients and controls.

3 PATIENTS AND METHODS

3.1 Patients and healthy controls

In the first study 43 patients with SSc (40 female and 3 men) and 27 healthy controls were included. In the second study we enrolled 9 patients and 12 controls. Mean (SD) age of the patients was 61.85 (48-74) years. SSc was diagnosed based on the corresponding international criteria. Patients were enrolled from the outpatient clinic at the Department of Rheumatology. They went through ophthalmological examination and basal tear sample collection at the Department of Ophthalmology. None of the patients had secondary Sjögren's syndrome. The healthy control groups were composed of age- and gender matched volunteers with no history of any autoimmune or ocular disorder. Patients did not take immunosuppressive medications at the time of the tear sampling.

Written informed consent was obtained from all patients and controls. Study protocol was approved by the local bioethics committee and followed the tenets of the declaration of Helsinki.

3.2 Tear sample collection

Unstimulated, open eye tear samples were gently collected from the inferior temporal meniscus of both eyes, using glass capillary tubes (Haematokritkapillare, 75uL, L 75 mm, Hirschmann Laborgerate, Germany), minimizing irritation of the ocular surface or lid margin as much as possible.

In the course of the first study samples were collected between 11 am and 16 pm by the same physician. Tear-secretion velocity was counted by dividing the volume of collected sample with time of secretion. Volume was calculated from the lengths of the fluid column in the capillary tube, measured with a vernier

caliper, and from the known diameter of the tube. Time of tear collection was measured with stopwatch.

In the course of the second study tear collection was performed between 9 and 11 o'clock.

Tears were transferred into low binding capacity Eppendorf tubes by the help of a steril syringe and a needle, carried on dry ice to the laboratory and stored at -80°C until assessment. The samples were obtained from both eyes of each individual, and were pooled due to the small volume available.

3.3 Quantification of total protein and VEGF levels in tear samples of patients with SSc

First, as a point of reference for VEGF, total tear protein concentrations were determined using the Microplate method of the bicinchoninic acid (BCA) Protein Assay Kit (Pierce Biotechnology, Rockford, USA) adapted to a 384 well microplate due to the small sample amounts. The kit is a two-component, high-precision, detergent-compatible assay. Total protein concentration determination based on colour intensity measurement, proportional to the peptide bound and the reagent set given product of the protein. The reaction absorbs visible light (A562 nm).

For the quantitative determination of VEGF in tear fluid we used a human VEGF immunoassay kit by Quantikine (R&D Systems, Minneapolis, MN USA). This assay employs the quantitative sandwich enzyme immunoassay technique.

3.4 Membrane array and multiplex bead analysis of tear cytokines in SSc

To remove cells, cellular debris and contaminant particles tear samples were centrifuged (10 minutes, 15.000 rpm, 4°C) prior to use.

Tear samples of controls and patients were used for cytokine profiling. The relative levels of 102 different cytokines were determined by Proteome Profiler Human XL Cytokine Array Kit (R&D Systems) using 50 µl samples according to the manufacturer's instructions. The pixel density in each spot of the array was determined by ImageJ software.

Alternatively, the absolute levels of MCP-1, CFD, IP-10 and CRP were determined from diluted tear samples (CFD, MCP-1 and CRP: 1:10; IP-10: 1:40) by Human Luminex Performance Assays (R&D Systems) according to the manufacturer's instructions. The measurement was run on Bio-Plex 200 Systems (Bio-Rad) workstation.

3.5 Statistical analysis

Prism 5 statistical software (GraphPad Software Inc.), was used for statistical analyses. Comparison of values was carried out by two-tailed Mann-Whitney U test. All values are shown as the mean \pm SD. P values less than 0.05 were considered statistically significant.

Cytokine array data are representative of 4 control and 4 SSc samples. Integrated density values obtained from densitometry were corrected with background levels, then they were normalized to positive control spots and total protein content of the samples.

In case of Luminex measurement, values represent the mean of 9 control and 12 SSc samples. Concentration values calculated by Bio-Plex Manager software were normalized to total protein content of the samples. The data are expressed as mean \pm SD.

4 RESULTS

4.1 Vascular endothelial growth factor in tear samples of patients with systemic sclerosis

The average tear secretion velocity in patients was 4.53 $\mu\text{l}/\text{min}$, median 3.8 $\mu\text{l}/\text{min}$ (1.5-25.6).

Duration of tear sample collection from patients varied between 20 and 313 seconds, until 5 μl , the minimally required volume was reached.

The average collected tear fluid volume was 10.4 μl (1.6-31.2) in patients and 15.63 μl (3.68-34.5) in controls.

In tear samples of patients with SSc the average total protein level was 6.9 $\mu\text{g}/\mu\text{l}$ (1.8-12.3) and the average concentration of VEGF was 4.9 $\text{pg}/\mu\text{l}$ (3.5-8.1) in the case of basal tear secretion.

Control tears contained on average 4.132 $\mu\text{g}/\mu\text{l}$ (0.1-14.1) protein and 6.15 $\text{pg}/\mu\text{l}$ (3.84-12.3) VEGF.

4.2 Membrane array and multiplex bead analysis of tear cytokines in systemic sclerosis

4.2.1 Cytokine array results

Non-stimulated tear cytokine profiles of the control groups and patients with SSc were analyzed by cytokine array detecting 102 different cytokines. Array results revealed shifted cytokine profile characterized by predominance of inflammatory mediators. The following 9 out of the 102 analyzed molecules were significantly increased in tears of patients with SSc: Complement factor D (CFD), Chitinase-3-like protein 1 (CHI3L1), C-reactive protein (CRP), Epidermal growth factor

(EGF), Interferon- γ -inducible protein 10 (IP-10, also called CXCL-10), Monocyte Chemoattractant Protein-1 (MCP-1), Monokine induced by gamma interferon (MIG), Matrix metalloproteinase 9 (MMP-9), Vitamin D-binding protein (VDBP).

Integrated density values were normalized to positive control spots and total protein content of the samples. Cytokine array data are representative of 4 control and 4 SSc samples.

Name of the cytokines and chemokines	Normalized density - patients with SSc	Normalized density - healthy controls	Significance of the difference (p)
CFD	50.35 (23.17-53.76)	22.33 (18.39-24.75)	0.002072
CHI3L1	94.41 (31.9-95.98)	31.06 (20.37-45.85)	0.000000
CRP	25.98 (15.28-53.16)	4.55 (4.35-4.66)	0.018250
EGF	53.42 (34.86-70.23)	34.04 (20.42-47.61)	0.032818
IP-10	123.42 (93.81-152.35)	21.99 (12.12-29.01)	0.000000
MCP-1	19.93 (5.38-42.44)	1.72 (1.44-2.27)	0.044726
MIG	22.85 (5.6-64.14)	3.58 (3.29-3.88)	0.033787
MMP-9	49.10 (4.24-129.04)	12.74 (10.29-17.56)	0.000068
VDBP	31.35 (11.87-64.68)	10.18 (8.3-13.84)	0.019733

Normalized densities of cytokines and chemokines in patients with SSc and healthy controls

Mean total protein values did not differ significantly in tears of patients and controls. Mean total protein value was 40.9239 µg/ml in tears of patients with SSc and 42.536 µg/ml in tears of healthy controls ($p=0.863604$).

4.2.2 Multiplex cytokine bead assay results

With the more sensitive and more specific Luminex bead assay 4 chosen molecules were determined in tears of 9 healthy controls and 12 patients with SSc. Based on the Luminex bead results mean CRP levels were 103.44 (3.57-359.02) µg/mg protein in tears of patients with SSc and 7.41 (0.87-18.03) µg/mg protein in tears of healthy controls.

Mean IP-10 levels were 564.78 (252.62-1107.2) µg/mg protein in tears of patients with SSc and 196.118 (101.66-514.37) µg/mg protein in tears of healthy controls. Mean MCP-1 levels were 2626.83 (457.84-5619.4) µg/mg protein in tears of patients with SSc and 661.27 (397.87-1171.4) µg/mg protein in tears of healthy controls.

Mean CFD levels were 15.27 (5.00-35.28) µg/mg protein in tears of patients with SSc and 23.31 (5.18-106.63) µg/mg protein in tears of healthy controls.

Except in the case of CFD ($p=0.34224$) all results were significant at $p=0.0138773$ for CRP, $p=0.00115$ for IP-10 and $p=0.1187$ for MCP-1, respectively.

Values represent the mean (\pm SD) of the 9 control and 12 patient samples, which are the fold change of normalized cytokine levels.

The difference between total protein values of control and SSc tear samples was not significant ($p=0.37263$). Mean total protein was 818.46 (779.94-1162.4) µg/mL in tears of patients and 872.46 (771.78-1359.5) µg/mL in tears of controls. Based on both the cytokine array and the multiplex bead assay results, concentrations of IP-10 showed the most significant difference in tears of patients and controls.

5 DISCUSSION

Although the exact pathogenesis of SSc is still unclear, ischemia–reperfusion usually antedates the development of skin sclerosis. Thus, endothelial cell injury caused by ischemia–reperfusion may trigger inflammatory cell infiltration and subsequent cytokine production that leads to tissue fibrosis. In this process different chemokines may play an important role as they mediate leukocyte chemotaxis and activate and induce an interaction between leukocytes and fibroblasts.

The diagnosis of SSc is based on clinical signs, assessment of autoantibodies (specifically anti-centromere, anti-topoisomerase 1 and anti-ribonucleotide acid (RNA) polymerase III antibodies) and results of skin biopsy.

As an alternative diagnostic and progression monitoring method, the determination of serum cytokines is becoming prevalent. A few studies have revealed correlation between the increased serum MCP-1, IL-6, IP-10 and TNF- α levels and the clinical manifestation/severity of SSc.

Although ocular manifestations in systemic autoimmune diseases have significant debilitating effects, tear analysis has been lacking from the investigations repertoire. Since tear represents the local homeostasis of the ocular surface better than serum, this makes tear an ideal sample for assessing ocular pathology in the disease. There are two possible ways for cytokines to appear in the precorneal tear film. Some of them are locally produced and diffuse into the tear film from the corneal and conjunctival epithelia, other cytokines leak into the tear film from the conjunctival blood vessels. Tear investigation is a challenging research field due to the fact that while sample collection is noninvasive, it has an insurmountable limitation namely the quantity of the sample obtainable.

Previously tear investigation studies have been performed in different ocular and systemic disorders. Leonardi et al. assessed multiple mediators, such as cytokines,

matrix metalloproteases, angiogenic and growth factors in tears of patients with vernal keratoconjunctivitis. These analyses identified previously unreported factors in tears of patients, including MMP-3 and MMP-10 and multiple proteases, growth factors and cytokines, which may all be instrumental in the pathogenesis of conjunctival inflammation. Different molecules were identified in human tear samples that were involved in the development and maintenance of corneal neovascularization. Concentrations of the pro-angiogenic cytokines such as IL-6, IL-8, VEGF, MCP-1 and Fas Ligand were determined in blood and tear samples using flow cytometry-based multiplex assay. Their results show that the concentration of pro-angiogenic cytokines in human tears are significantly higher compared to their concentrations in serum, with highest levels found in basal tears. These findings lend further support to the importance of our current studies. After reviewing the literature on direct and indirect tear sampling methods in various ocular and systemic disorders, we have chosen the microcapillary method for tear sampling in patients with SSc, since it is safely applicable for the collection of non-stimulated tears. For transferring the tear fluid from the microcapillary tube to the collection tube we applied a steril syringe and a needle. This tear sampling method proved to be suitable for our experiments on tear cytokines.

5.1 Vascular endothelial growth factor in tear samples of patients with systemic sclerosis

VEGF is a component of normal tear fluid. Vesaluoma determined VEGF concentrations in healthy tears. The median VEGF concentration was 5 pg/ μ l (4-11), which corresponds with our results, as control tears contained an average of 6.15 pg/ μ l (3.84-12.3) VEGF.

They calculated the average tear fluid secretion in healthy controls, which was 8.1 $\mu\text{l}/\text{min}$ (0.7-20.8), using the same tear collecting method as we did in our study. Results show that patients with SSc have significantly decreased tear secretion that could be explained by DES, which is a probable sequel of the disease or of the side effects of the therapeutic drugs.

Tear secretion velocity was lower by 67% in patients with SSc than in healthy controls. The difference was significant ($p < 0.01$). The reason for this sign could be explained by the pathophysiology of the disease, namely fibrotic processes of the lacrimal gland.

Total protein values in patients with SSc were higher by 42% than in healthy controls. This may indicate that total protein production – or simply protein concentration, since patients with SSc have a decreased tear secretion velocity – is only increased because of the smaller tear volume. Though VEGF in the tear of patients with SSc decreased by 20%, it can be explained also by the decreased tear secretion of patients.

The question why the VEGF levels are not higher, as we expected, in patients with SSc than in the healthy group needs further investigation.

5.2 Membrane array and multiplex bead analysis of tear cytokines in systemic sclerosis

Based on our cytokine array results, nine cytokines and chemokines had significantly higher levels in tears of patients with SSc. This screening method was performed for the assortment of 102 cytokines, selecting the most relevant ones in the pathogenesis of SSc for further experiments. All molecules which appeared to be significantly higher in tears of patients are molecular players of the immune responses and inflammatory processes, which confirms the presence of

ocular surface inflammation in patients with SSc possibly as a consequence of DES.

Based on our results of multiplex cytokine assay the following three molecules showed significant differences between the tear samples obtained from patients and healthy individuals.

Interferon- γ -inducible protein 10 (IP-10, also called CXCL-10), which is a 10 kDa protein, is functionally categorized as an 'inflammatory' chemokine. Moreover, it has an angiostatic function due to suppressing neovascularization, and it also takes part in the immune regulation. Recent reports have shown that the serum and/or the tissue expressions of IP-10 are increased in various bacterial, viral, fungal and protozoal infections and also in autoimmune diseases like rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, autoimmune thyroid diseases, type 1 diabetes mellitus, Addison's disease, and last but not least in SSc.

C-reactive protein (CRP) is an acute-phase reactant that is a major marker of infection and inflammation. CRP has been examined in sera of patients with SSc and found to be elevated, and associated with poor survival. Therefore, it may be a useful indicator of disease activity and severity in SSc.

Monocyte Chemoattractant Protein-1 (MCP-1) is one of the most pathogenic chemokines during the development of inflammation and fibrosis in SSc. MCP-1 is not only a chemoattractant molecule for monocytes and T cells, it also induces Th2 cell polarization and stimulates collagen production by fibroblasts. Hasegawa et al. have previously shown that serum MCP-1 levels are elevated when skin and lung is affected in SSc patients. It has also been reported that cultured dermal fibroblasts from patients with SSc show augmented expressions of MCP-1 mRNA and protein.

Of the last three molecules IP-10 and MCP-1 are the two whose molecular characteristics make them potential candidates for therapies against the

pathological consequences of diseases such as SSc. Monitoring these factors in tears of patients with SSc can be a non-invasive alternative instead of serum investigation. Furthermore, in patients with ocular manifestations, such as DES, tear analysis is far more informative, provides information of the ocular surface, therefore it could help us choose the appropriate treatment, particularly artificial tears or anti-inflammatory eye drops. Further studies are needed to understand the signaling pathways regulating IP-10 and MCP-1, with the aim of developing new interventions against autoimmune diseases mediated by these chemokines as well as inventing novel therapeutic possibilities for the ocular manifestations of SSc.

6 NOVEL FINDINGS

1. After reviewing the literature of tear sampling techniques we labored the adequate tear sampling methods and collected tears with capillary system from SSc patients in order to investigate VEGF molecule and cytokines.
2. We were the first to demonstrate the presence and concentration of VEGF, an element that plays an important vascular role in the pathogenesis of SSc, with the help of a method that is based on a quantitative sandwich immunoassay technique.
3. By the help of our survey, which is based on a quantitative sandwich immunoassay technique, we verified a level of VEGF reduced by 20% concentration in tears of SSc patients compared to healthy controls.
4. We were the first to perform a wide cytokine profile in tears of SSc patients using an array that monitors of 102 cytokines simultaneously.
5. Based on our cytokine array results, we revealed that 9 out of the 102 cytokines and chemokines had significantly higher levels in tears of patients with SSc. All of them are molecular players of the immune responses and the inflammatory processes. These findings legitimate the existence of ocular surface inflammations which are quite frequent in patients with SSc. In addition, they are in accordance with former study results regarding the pathomechanism of SSc.
6. We were the first to demonstrate in tear samples, using a highly sensitive and specific multiplex bead assay, the increased levels of IP-10, MCP-1 and CRP in tear samples of patients with SSc. Previous studies have already demonstrated the elevated levels of these biomarkers in sera of these patients, therefore tear analysis is to be raised as a possible choice in relation to the diagnostic, prognostic, and maybe therapeutic aspects of SSc.

7 KEY WORDS

Systemic sclerosis, dry eye syndrome, tear, vascular endothelial growth factor, cytokine, tear sampling, total protein, enzyme-linked immunosorbent assay, cytokine array, multiplex bead assay

8 APPENDIX



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Candidate: Anikó Rentka

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Doctoral School: Doctoral School of Clinical Medicine

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Ph.D. List of Publications

List of publications related to the dissertation

1. **Rentka, A.**, Hársfalvi, J., Szűcs, G., Szekanez, Z., Szodoray, P., Köröskényi, K., Kemény-Beke, Á.: Membrane array and multiplex bead analysis of tear cytokines in systemic sclerosis.
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2. **Rentka, A.**, Hársfalvi, J., Berta, A., Köröskényi, K., Szekanez, Z., Szűcs, G., Szodoray, P., Kemény-Beke, Á.: Vascular Endothelial Growth Factor in Tear Samples of Patients with Systemic Sclerosis.
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