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

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ABBREVIATIONS

ACR: American College of Rheumatology

AD: aqueous deficient

ARA: American Rheumatology Association

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

dcSSc: diffuse cutan systemic sclerois

DES: dry-eye syndrome

EPC: endothelial progenitor cell

EGF: epidermal growth factor

ELISA: enzyme-linked immunosorbent assay

EULAR: European League Against Rheumatism

IFN: interferon

IL: interleukin

ILD: interstitial lung disease

IP-10: interferon gamma-induced protein-10

lcSSc: limited cutan systemic sclerosis

MC: microcapillary tubes

MCP: monocyte chemoattractant protein

MGD: Meibomian gland dysfunction

MIG: monokine induced by gamma interferon

MMP: matrix metalloproteinase

mRNA: messenger ribonucleic acid

NST: nonstimulated tear

PAH: pulmonary arterial hypertension

PBS: phosphate buffer saline

PCR: polymerase chain reaction

PTF: precorneal tear film

RNA: ribonucleic acid

RNP: ribonucleoprotein

sIgA: secretory immunoglobulin A

SSc: systemic sclerosis

ST: stimulated tear

STS: Schirmer test strip

TGF-β: transforming growth factor-beta

TNF-α: tumor necrosis factor-alpha

TRIM: tripartite motif

VDBP: vitamin D binding protein

VEGF: vascular endothelial growth factor

WO: washout

1 INTRODUCTION

1.1 Systemic sclerosis

1.1.1 Etiology and epidemiology

Systemic sclerosis (SSc) is a chronic autoimmune connective tissue disorder which affects the skin and multiple organs, including the heart, lung, kidney, and gastrointestinal tract. Although the exact etiology is unknown, some predisposing factors, like environmental and infectious agents, tissue injury, and hypoxia or oxidative stress based on a susceptible genetic background may play a role in the development of the disease (1, 2). In a recent study Korman et al. reviewed the genetic and genomic aberrations that may be important in the development of SSc. Although the overall genetic burden is exiguous and only 2.6 % of patients' siblings develop SSc, the genetic predisposition is obvious from epidemiologic, familial and twin studies. SSc is associated with HLA loci, and a recent genome-wide association study identified 25 non-HLA loci associated with the disease (3). Prevalence and incidence of SSc vary widely around the world. Lower estimates of prevalence (<150 per million) and incidence (<10 per million per year) have been reported in northern Europe and Japan, while higher estimates of prevalence (276–443 per million) and incidence (14–21 per million per year) have been observed in southern Europe, North America and Australia (4). Female predominance is remarkable along with gender differences in disease activity and incidence. The overall female to male ratio ranges from 1:1 to 14:1 (5).

1.1.2 Symptoms, diagnosis and clinical progression of SSc

1.1.2.1 Skin and internal organ involvement in SSc

On the basis of the extent of their skin involvement, patients with SSc are divided into two subgroups: limited cutaneous systemic sclerosis (lcSSc) and diffuse cutaneous systemic sclerosis (dcSSc). In lcSSc, skin fibrosis is located in the fingers (so called sclerodactyly), distal extremities and face, in contrast to dcSSc, in which the trunk and proximal extremities are also affected. In patients with lcSSc, Raynaud phenomenon antedates skin and other organ manifestations in most cases by months to years, while patients with dcSSc have a more rapid disease progression with extensive skin changes and early development of visceral organ complications (6).

In addition to the two basic variants, some transitional forms and systemic sclerosis without skin lesions may occur (7). Approximately 90% of patients have lower esophageal stenosis as an internal organ complication, therefore they suffer from problems with swallowing and reflux. Pulmonary fibrosis occurs in over 40% of patients resulting in the development of circulatory and respiratory problems, like pulmonary arterial hypertension (PAH); while bronchial lesions may promote development of cancer (8).

Myocardial and pericardial fibrosis may result in severe heart failures. Myocardial fibrosis may cause conduction and rhythm disorders, while pericardial fibrosis commonly leads to inefficient cardiac performance. Lesions in renal vessels and parenchyma occur in approximately 10% of patients and may lead to malignant hypertension and renal cirrhosis with scleroderma renal crisis being the leading cause of patients' death (7-9). SSc-related organ complications are summarized in **Figure 1**. (10).

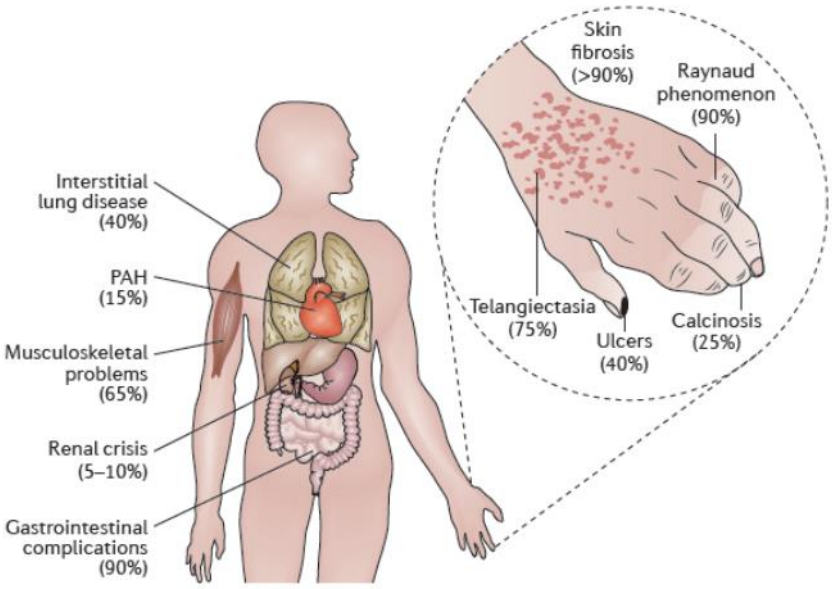


Figure 1. SSc-related organ complications and their frequency in patients
PAH: pulmonary arterial hypertension. Figure adapted from Allanore et al., 2015

1.1.2.2 Ophthalmological manifestations of SSc

There are only a few reports available concerning ophthalmological complications in the course of systemic sclerosis. These papers are mainly case reports; only few overall studies exist, and even these involve a small number of patients, since SSc is a rare disease (11, 12). Changes in the organ of vision are thought to be the consequences of systemic complications of scleroderma or adverse effects of the immunosuppressive treatment applied. Ocular symptoms may occur at any stage of the disease and may involve numerous ocular tissues. Their course can be clinically latent or very intensive. 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 (13). The most frequent ocular manifestation of SSc in our study 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, namely the water portion of the tear film. Furthermore, lipid layer disorder is caused by chronic blepharitis and Meibomian gland dysfunction (MGD), while increased evaporation of tears from the ocular surface is the consequence of restricted eyelid mobility and the consecutive reduced blinking

(12). DES was recently redefined as a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability and last but not least damage to the ocular surface (14). Increased osmolality of the tear film (15) and inflammation of the ocular surface (16) are the two major characteristic points of this ocular surface disease. The most important laboratory findings (16) are increased levels of several inflammatory cytokines are. Accordingly, tear cytokine levels are regarded 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).

1.1.3 Pathomechanism

1.1.3.1 Vasculopathy

Based on clinical and pathological evidence, the primary insult in the affected organs of SSc patients is directed at the blood vessels, resulting in tissue ischemia, fibrosis, and finally major organ malfunction. 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 confirmed that angiogenesis, the growth of new vessels from existing ones, and vasculogenesis, de novo formation of new vessels, are defective in SSc. One of the earliest clinical manifestations in the pathophysiology of SSc is an alteration of the peripheral microvasculature, confirmed by nailfold capillaroscopy results, which point to the fact that Raynaud's phenomenon and morphological changes may occur months or even years before the onset of fibrosis (17, 18). Furthermore, the severity of capillary injury correlates with the severity of internal organ involvement (19, 20). The loss of capillaries is evident along with the absence of visible new normal vessel formation. 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 are indicative of the above defective angiogenesis and abnormal vascular repair pathways (21).

1.1.3.2 Immune dysregulation

The abnormal immunological response in SSc is characterized by chronic mononuclear cell infiltration by cluster of differentiation (CD) 4+ T-lymphocytes and macrophages, dysregulated production of lymphokines and growth factors, and B-lymphocyte activation induced by autoantibodies (22). 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 (IL)-4 (23). IL-4 induces the production of transforming growth factor-β (TGF-β) and also the production of collagen by fibroblasts. It has been noted that T-cells in SSc skin lesions exhibit oligoclonal expansion suggesting an antigen-driven T-cell response. Earlier it was hypothesized that SSc is a graft versus host disease, and based on the theory of microchimerism, the antigens against which T-cells react are major histocompatibility complex antigens of offspring or maternal cells (24).

1.1.3.3 Fibrosis

Endothelial injury and dysfunction are initiated by direct or indirect stimuli, like free radicals or chemical and microbial agents. This injury is also initiated by the induction of immune activation and production of autoantibodies. The vascular injury activates platelet and coagulation pathways, which may cause vascular microthrombosis. On the basis of intimal hyperplasia in the small vessels vasculopathy occurs, and the consequential luminal narrowing results in tissue hypoxia and chronic ischemia. Hypoxia and ischemia-dependent release of vascular products activate resident fibroblasts, which perpetuate the vasculopathy by triggering vascular wall fibrosis (21) by promotion of a pro-fibrotic micro environment rich in extracellular matrix and growth factors, such as fibroblast growth factor and connective tissue growth factor (25).

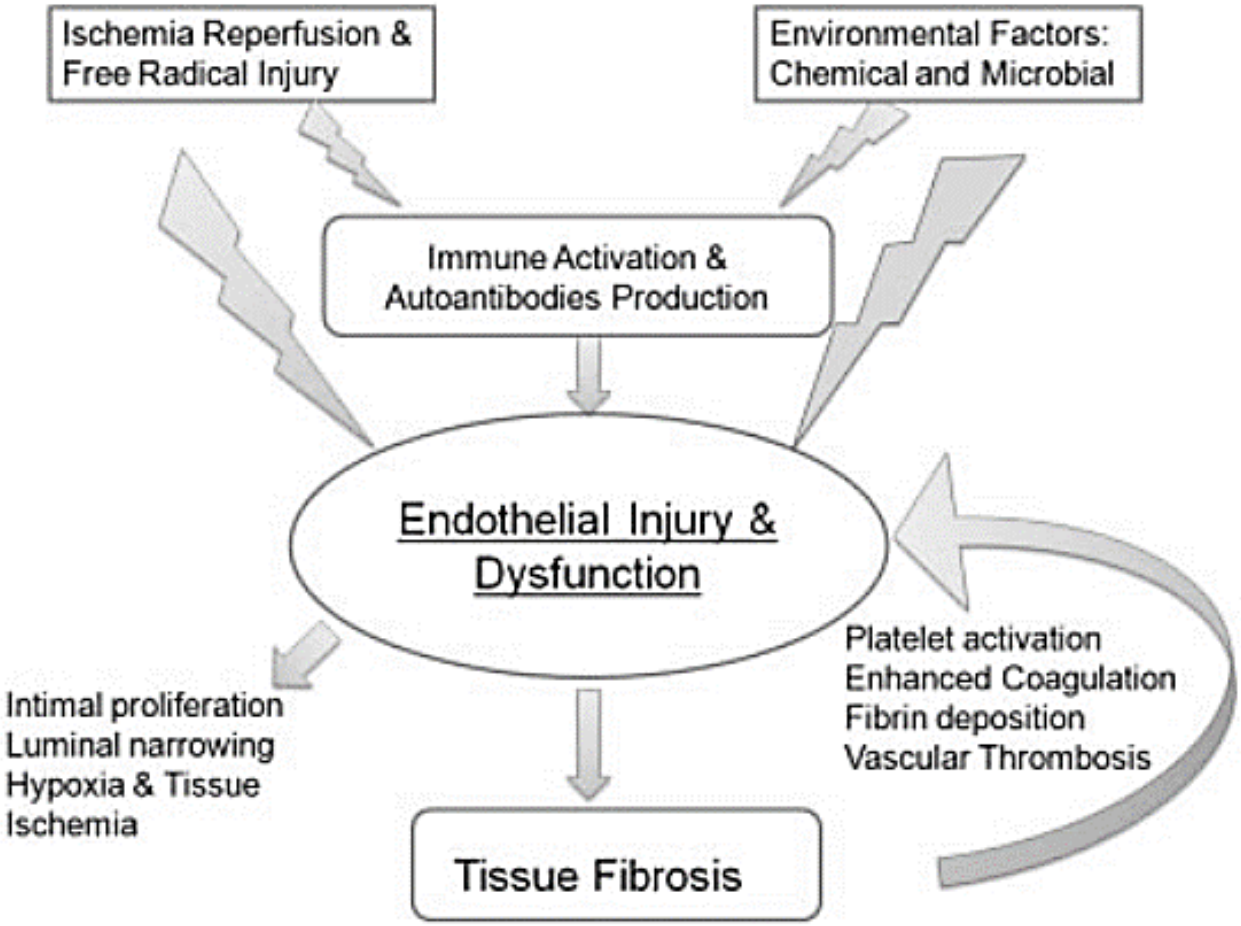


Figure 2. Pathogenesis of SSc
Figure adapted from Matucci-Cerinic et al., 2013

1.1.3.4 Molecular "players" in the pathomechanism of SSc

1.1.3.4.1 Vascular Endothelial Growth Factor, the major pro-angiogenic factor in SSc

While both pro- and antiangiogenic factors are overexpressed in SSc, there appears to be an imbalance in the ratio of these mediators, advancing inhibition of angiogenesis and progressive vascular lesions (21). The up-regulated pro- and antiangiogenic mediators in SSc are listed in **Table 1.**

Angiogenic	**Angiostatic**
VEGF	Endostatin
Basic fibroblast growth factor	IP-10/CXCL10
Platelet-derived growth factor	Angiostatin
IL-8/CXCL8	Angiopoietin 2
Stromal cell-derived factor 1/CXCL12	Pentraxin 3
IL-6	IL-4
CD44	Thrombospondin 1,2
Kallikrein	Platelet factor 4
Urokinase plasminogen activator receptor	MIG/CXCL9
MMP-9 and proMMP-1	Soluble endoglin

Table 1. The up-regulated pro- and antiangiogenic mediators in SSc
VEGF: vascular endothelial growth factor; IP-10: interferon gamma-induced protein; CXCL: chemokine (C-X-C motif) ligand; MMP: matrix metalloproteinase; CD: cluster of differentiation, MIG: monokine induced by interferon-gamma; MMP: matrix metalloproteinase
Table reproduced from Matucci-Cerinic et al., 2013

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 (26). 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 (27). 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 (28). VEGF has secondary influence in neovascular glaucoma (29) and hereditary retinal dystrophies (30). In developed countries these ocular conditions account for the majority of irreversible vision loss.

Since its discovery in the 1980s (31) VEGF has raised interest because of its central role in angiogenesis in a number of physiologic and pathologic processes, such as vascular development, wound healing, the female reproductive cycle, cancers, myocardial ischemia, rheumatoid arthritis and other autoimmune diseases (32, 33).

Although there is a lack of sufficient angiogenic response to chronic tissue ischemia in SSc that culminates in the loss of capillary vessels, the 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 (34). Later studies also confirmed that the expression of VEGF is increased in different cell types in the epidermis and dermis of patients with SSc (35).

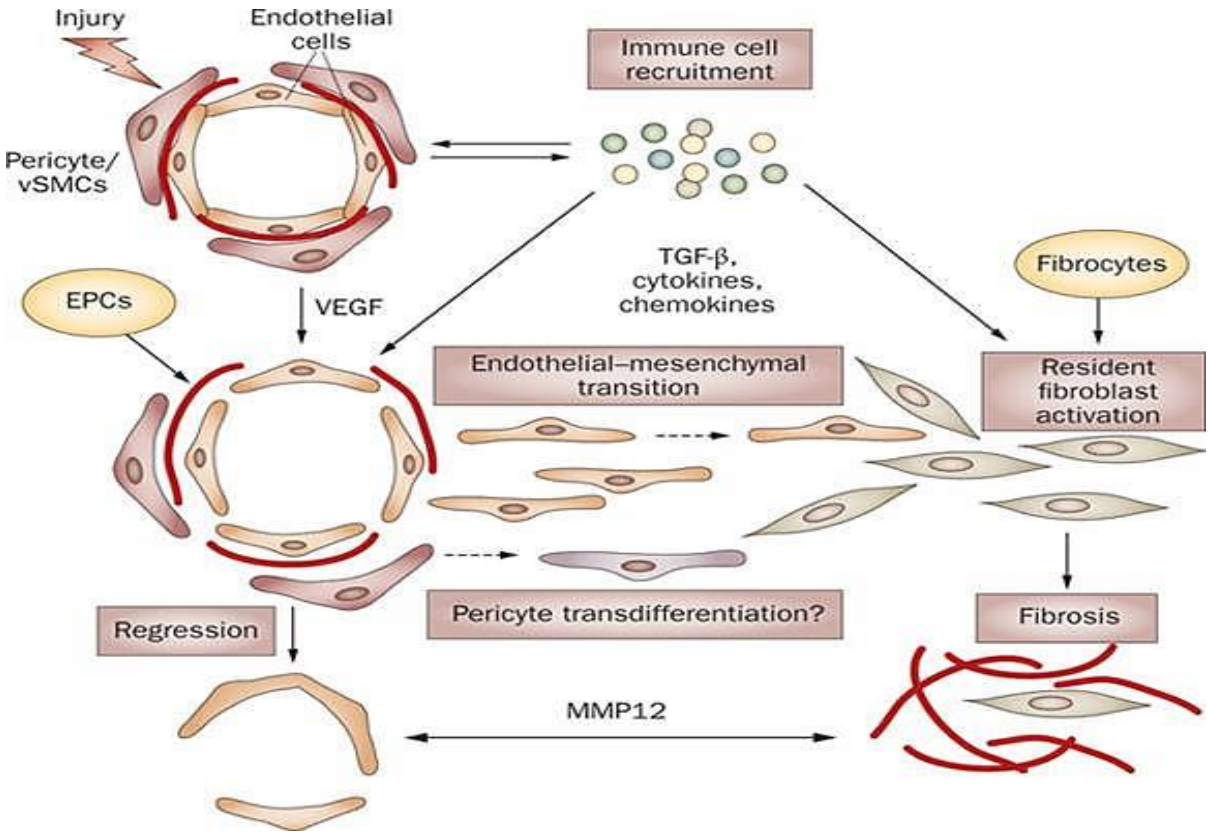


Figure 3. Vascular pathomechanism of SSc

EPCs: endothelial progenitor cells; MMP: matrix metalloproteinase; TGF-β: transforming growth factor-β; VEGF: vascular endothelial growth factor. Figure adapted from Trojanowska et al., 2010

High levels of VEGF and other proangiogenic mediators derived from the activated immune cells facilitate proliferation of endothelial cells and pericytes in an attempt to restore injured vessels. For reasons that are still not clearly understood this process fails, leading to vessel regression. In the presence of immune mediators endothelial cells could undergo endothelial–mesenchymal transition and enter the surrounding tissue, where they further differentiate into collagen-producing cells (e.g. into fibroblasts or myofibroblasts). In SSc, endothelial cells and pericytes are particularly prone to undergo these transitions. Activated resident fibroblasts and fibrocytes recruited from circulation to the injured tissue are likely to represent an additional source of collagen producing cells that contribute to fibrosis in SSc lesion (36).

1.1.3.4.2 Inflammatory cytokines and chemokines in SSc

Patients with SSc have an increased number of circulating monocytes, macrophages and T cells (37, 38). Production of soluble mediators, including cytokines, by these cells may promote endothelial damage and fibrosis in SSc. Admittedly, many chemokines play a key role in the regulation of the migration and recruitment of specific leukocytes to regions of inflammation. In the course of SSc, dysfunctions of chemokines might explain the altered accumulation of effector leukocyte subsets in affected tissues (39).

Among the various chemokines, monocyte chemoattractant protein (MCP)-1 plays the most critical role in tissue fibrosis that occurs in SSc (40, 41). Cytokines produced by macrophages, including IL-1, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, IL-6, 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 by fibroblasts (42).

1.1.4 Treatment

SSc involves a wide panel of clinical features, which include vascular, immune and fibrotic manifestations. Clinical trials are limited by the lack of adequate outcome measures and also by the great variability of disease progression. 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 SSc management. 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 often 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 investigations.

1.2 Tear investigation

1.2.1 Tear biology: functions and pathological relations of the human tear

The tear film covering the ocular surface has several functions including the defense of the external surface of the eyeball thus constituting a mechanical and antimicrobial barrier. It lubricates the palpebral conjunctiva, i.e. the inner surface of the eyelids, and also the bulbar conjunctiva and the cornea by providing a smooth and reflective surface. By transporting oxygen and nutrients, the tear nourishes the avascular tissues of the cornea, and regulates its electrolyte composition and pH. Furthermore, it provides white blood cells with access to the cornea and conjunctiva and removes foreign substances from the ocular surface. Since tear film is also an optical refractive medium, its stability is pivotal in achieving appropriate vision (43-45).

The human tear is composed of three layers: the lipid, aqueous and mucin layers, but only two phases (43). The lipid component, which originates from the Meibomian glands of the tarsus, forms the superficial layer of the tear film. The aqueous component is primarily secreted by the lacrimal gland. This is the thickest layer, which contains water, electrolytes and diverse proteins, peptides and glycoproteins. Mucins, which are glycoproteins expressed by the epithelial tissues of mucous surfaces, protect tissues since they are antioxidants, provide lubrication, and inhibit bacterial adherence (46).

Under normal conditions, the precorneal tear film (PTF) flow in humans is around 0.5-2.2µl/min with a turnover rate of approximately 16% per minute (47). Its volume present in the human eye is 7–10 µl (48).

Its composition must be maintained quantitatively and qualitatively within particularly narrow limits to uphold a healthy homeostatic and functional visual system. Abnormalities of the tear film, affecting the constituents or the volume, can rapidly cause serious dysfunctions of the conjunctiva and eyelids and finally impairment of the cornea (46).

Irritating stimuli and diurnal patterns, environmental fluctuations and physiological status indicate reflex tear secretion via activation of the corneal nerves (49, 50). Early studies revealed differences between open and closed eye precorneal tears: tear protein levels increase during sleeping, which is explained by the lack of reflex tearing in the closed eye (51). Fullard and Synder demonstrated that nonstimulated (NST) and stimulated (ST) tears differ greatly in secretory IgA (sIgA) levels, which can be explained by the discrepancy of the secretion rate of

sIgA by the main lacrimal gland and the fluid secretion rate with increasing flow (52). This also proved to be true for "constitutive" proteins: their rate of secretion is determined by their rate of synthesis, and not by the rate of stimulation (53). In an experimental study on rats, Sullivan et al. demonstrated that the secretory component is a constitutively secreted lacrimal gland protein (54), which was found to be similar in humans (52).

Open and closed eye PTF differs in composition and origin. Open eye tear film is mostly the result of basal tear secretion, originating mainly from the accessory lacrimal glands. Reflex tear, which is derived from a neurologically inducible lacrimal or accessory gland secretion, is composed of the following proteins: lysozyme, lactoferrin, tear specific prealbumin, and a minor mixed alpha and beta globulin fraction. Reflex tearing is greatly reduced or ceases altogether upon eye closure hence tear is constitutively secreted with a decreased flow rate during sleep. In the closed eye environment sIgA and albumin are the main components of tear fluid with a markedly increased albumin level, which alludes to the subclinical inflammatory status of the closed eye (51).

Changes in tear composition are associated with many ocular diseases, such as DES, Sjögren's syndrome, diabetic retinopathy, glaucoma, MGD, autoimmune thyroid eye disease, pterygium, keratoconus, ocular rosacea, blepharitis, as well as various systemic diseases, such as inflammatory diseases and infections, diabetes mellitus, allergies, Parkinson's disease and certain types of cancer (breast, lung, prostate) (55-58). Despite the fact that some biochemical properties (e.g., pH, or osmolality) of tear and serum are similar, the protein composition, as well as the relative and absolute amount of the components is different. For example Vitamin A levels in tear are remarkably higher than in serum. Similarly, the fibronectin content of PTF is one order of magnitude higher than that of serum (59). Therefore tears should be considered a unique body fluid.

1.2.2 Major bioactive components of tears

Despite their small volume, tears are a remarkably complex biological fluid consisting of peptides, electrolytes, lipids, carbohydrates, salts and small bioactive molecules such as amino acids, nucleosides, vitamins, etc. (50, 60). Tear components are of different origin, they are derived from cellular debris, the main lacrimal gland and accessory gland secretions, ocular surface tissue products, serum exudates, and inflammatory and immune cells recruited into the

local environment (61). The amount and ratio of the various components have a definitive role in the physiological functioning of the surrounding tissues.

Normal tears have a total protein concentration of approximately 7 mg/ml (62, 63) and contain hundreds of different proteins, but the method of tear collection greatly influences the relative proportions of the proteins present in any individual tear sample - a fact that has been known since the early 1980s (64, 65). Many of the tear proteins play an important role in corneal wound healing, inflammatory processes, and corneal protection of various pathogens (66).

The most frequent proteins detected in tear samples include lactoferrin, lysozyme, sIgA, lipocalin, superoxide dismutase, cystatins, and α1-protease inhibitor. These proteins account for more than 90% of all tear proteins (67-69). In previous years, tear film protein profiles have been characterized using gel electrophoresis and Edman degradation (28-30). Later, sensitive immunoassay-based methods identified other proteins in mammals' tears including growth factors, neurotrophic factors, cytokines and cell adhesion molecules, matrix metalloproteinases, immunoglobulins and insulin (70-77).

Proteins may also play a role in the stabilization and organization of the tear lipid layer, which is located over the aqueous layer of the tear film. Part of this oily liquid is secreted from the Meibomian glands, which produce a mixture of hydrocarbons, wax esters, triglycerides, diesters, free sterols, sterol esters, free fatty acids, and polar lipids (46, 78). In addition, corneal or conjunctival epithelial cells are also a plausible source of polar lipids. The lipid layer has two main functions: to stabilize the tear film by reducing the overall surface free energy, and to control water evaporation from the surface (63). Since the lipid layer is in contact with the eyelid skin it acts as a barrier to the aqueous layer (62, 79) and also forms a watertight seal when the eyelids are closed (68).

1.2.3 How are markers of inflammation released into the tear fluid

Several cytokines, adhesion molecules and growth factors play a key role in inflammatory processes (80). Changes in the levels of these factors have already been assessed in serum (81). In some diseases these changes are also identified in tear samples, by the help of a less invasive sample collection method (82). Tears are secreted from the main and accessory lacrimal gland and cover the ocular surface. Tears drain into the canaliculi through the puncta after gas exchange between the air and the epithelium. They contain a wide spectrum of cytokines and growth factors secreted by the lacrimal glands. Some cytokines are locally produced and diffuse

into the tear film from the corneal and conjunctival epithelia. Furthermore, cytokines can also leak into the tear film from the conjunctival blood vessels (83).

1.2.4 Tear analysis in systemic and ocular diseases

Tears play an essential role in maintaining homeostasis of the ocular surface, therefore changes in the delicate equilibrium of their cytokine composition may lead to various pathophysiological conditions.

Leonardi et al. investigated multiple mediators, such as cytokines, matrix metalloproteases, angiogenic and growth factors in tears of patients with vernal keratoconjunctivitis. These analyses identified in tears of patients previously unreported factors including matrix metalloproteinase (MMP)-3 and MMP-10 and multiple proteases, growth factors and cytokines, which may all be instrumental in the pathogenesis of conjunctival inflammation. Tear collection is a noninvasive method for collecting human biofluid, however, it has a limitation regarding the quantity of obtainable sample (84).

In 2012 Zakaria et al. identified different factors in human tears that are involved in the development and maintenance of corneal neovascularization. Concentrations of the pro-angiogenic factors like IL-6, IL-8, VEGF, MCP-1 and Fas Ligand were determined in blood and tear samples simultaneously. These results indicate 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 (83).

1.2.5 Tear sampling methods

Precorneal tears as a biological fluid are very easily accessible with non- or very low-invasive methods at a relatively low cost. Tears not only lubricate the ocular surface carrying secreted molecules from corneal epithelial cells and tissues producing tear components, but also represent the whole physiological status of the body. Due to the very limited amount of samples and the relative instability of the components sample collection is a critical step in tear research and diagnostics.

Although tear analysis is of increasing interest in ophthalmology, no studies have investigated tears of patients with SSc as yet, possibly because of the technical challenge posed by the extremely small sample volumes available (85).

1.2.5.1 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 their composition on the other (85). Tear sampling is definitely a major challenge and has a most significant influence on the precision and reproducibility of the analytical results. Basically it can be performed in two ways; directly and indirectly.

1.2.5.1.1 Direct sampling methods

In direct sampling methods microcapillary tubes (86) or micropipettes are used for sampling. This requires previous stimulation or instillation of different volumes of saline (100–200 µl) into the cul-de-sac and collecting after appropriate mixing. This procedure causes dilution and may not permit collection of samples from specific sites of the ocular surface (87). Kalsow et al. investigated the tear cytokine response to multipurpose solutions in contact lens (59) wearing. Prior to contact lens removal, NST tears were collected from each eye from the inferior tear meniscus between the 6 o'clock and lateral canthus positions using a 10 µl flame-polished glass micropipette. Following collection, a 5.5 µl tear volume was immediately transferred to a sterile 0.2 ml tube containing 49.5 µl of storage solution to produce a 1:10 tear dilution for immediate storage at -80°C (88).

Guyette et al. compared low-abundance biomarker levels in capillary-collected NST tears and washout (WO) tears of aqueous-deficient and normal patients. 10-microliter polished micropipettes were used to collect tears from the inferior marginal strip, taking special care to minimize ocular surface contact. Tear collection rate was continuously monitored. Individual NST tear samples were collected in 10-minute aliquots, each being immediately transferred to a sterile polymerase chain reaction (PCR) tube. An equal volume of assay buffer was added and the sample was stored at -86 °C. A total of at least 6.5 µl NST tears was collected from each study participant, and each 10-minute aliquot was stored without delay in a separate PCR tube. Prior to WO tear sample collection 10 µl sterile physiologic saline solution was added to the lower conjunctiva by a digital pipette. The patient was instructed to gently close the eyes and avoid any eye movements for one minute. Tears were then collected using the same method as for NST samples, but a shorter collection time of 5 minutes per aliquot was used to make up the 6.5 µl minimum volume requirement. Tear collection volume and time were continuously monitored to measure the tear collection rate (89).

There have been several research projects in dry eye syndrome and nowadays the emphasis in dry eye research has shifted toward the role of inflammation in the anterior surface of the eye (90). Since inflammatory mediators originating from various ocular surface sources and the main lacrimal gland do not constitute a totally homogenous mix, the way the tears are collected will influence the resulting biomarker profile. NST tear samples from the inferior marginal strip cover a broader spectrum of the sources, whereas ST tear samples contain a higher proportion of the lacrimal gland secretion (45). Explicit protein profile differences between NST and ST tears demonstrate that these two sample types are not equivalent (52, 91). Although NST tears represent specifically the inflammatory status of the ocular surface, the volume of NST tears is limited, especially in aqueous-deficient dry eye. Even though tear sampling frequently makes use of capillaries as they are less irritating and the resulting sample is an exact representative concentration of molecules, the main limitation of the method is the volume of sample (2–3 µl) to be gained (47).

One way to increase the available tear sample volume is to add fluid (e.g., sterile saline) to the eye prior to sample collection, effectively ‘‘washing out’’ ocular surface molecules (92, 93). In an experimental dry eye study, Luo et al. collected tears from mice with tear fluid washing (94). Tear fluid washings were collected by a previously reported method by Song XJ et al. (95). Briefly, 1.5 µl of phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) was instilled into the conjunctival sac. The tear fluid and buffer were collected with a 10 µl volume glass capillary tube by capillary action from the tear meniscus in the lateral

canthus. The 2 µl sample of tear washings was pooled from both eyes of each mouse and was stored at -80°C until zymography and enzyme linked immunosorbent assay (ELISA) were performed.

Validity of the WO method depends on the extent to which it changes the NST tear biomarker profile. By determining tear sIgA, inducement of reflex tearing is easily detected because tear sIgA levels decrease with reflex tear flow rate (91). Markoulli et al. found equal tear sIgA–total tear ratios in WO and NST tears, which suggests that WO tear samples do not significantly induce reflex tearing (93). Guyette's study evaluated WO tear collection as a replacement for microcapillary NST tear collection and applied this to compare biomarker levels between aqueous deficient dry eye (AD) and non-AD patients (52, 89, 96).

1.2.5.1.2 Indirect methods

An indirect method means that collection of PTF is carried out 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 (97).

Acera et al. analyzed the inflammatory markers in the PTF of patients with ocular surface disease. 10 µl of tear samples was collected by a Weck cell sponge (98). The concentrations of IL-1β, IL-6 and pro-MMP-9 were measured by ELISA, and the MMP-9 activity was evaluated by gelatin zymography.

Inic-Kanada et al. compared ophthalmic sponges and extraction buffers for quantifying cytokine profiles in tears using Luminex technology. They found that Luminex detection of cytokine/chemokine profiles of tears collected with Merocel sponges may be useful in clinical studies, for instance to assess cytokine profiles evaluation in ocular surface diseases (99).

Samples obtained from the Schirmer test procedure have a higher mucus, lipid, and cellular content than microcapillary (MC) samples (100). STS also suffers incomplete, non-uniform elution of proteins from the filter matrix (97). Although micropipette and STS collection provide different biomarker profiles for a given donor, the correctly applied micropipette method is more consistent (101). STS is widely applied as the volume of sample collected with this method is larger compared to other methods, but it can cause reflexive tearing due to irritation, which increases the volume of the samples, therefore aggravates the detection of the investigated tear component(s) e.g., drug levels (87).

In comparative studies the tears of the same patient are collected using several collection methods to determine the same biomarkers from the different tear samples.

Green-Church et al. collected tears using small volume (1–5 μl) Drummond glass MC tubes with 1.6X slit-lamp magnification. Non-reflex tears were collected from the inferior tear prism without contact with the lower lid until a total of 5 μl had been collected. During a separate visit, tear collection was performed by placing an STS over the lower lid. The lid was canthus. The subject was instructed to close his/her eyes for the 5-minute test duration; the wet length was not recorded but was observed to be within normal ranges in all cases. The STS was then placed in 1.6 ml amber Eppendorf tube at 4°C until analysis (101).

Lee et al. used two collection techniques for the comparative analysis of polymerase chain reaction assay for herpes simplex virus 1 detection (102). Tear samples were collected from the lower fornix using STS for five minutes, a method adopted in a previous study of Satpathy et al. (103). The other collection method they used was micropipetting tears, after irrigating 100 μl saline in the lower fornix; a method that was described in a previous study of Markoulli et al. (93), who validated the "flush" tear collection technique as a viable alternative to basal and reflex tear collection.

Author, date	Objects of examination	Biomarkers	Tear collection method(s)	Reference
Kalsow et al. *(2013)*	humans *(contact lens wearers)*	cytokines	NST (10 µl glass micropipette)	(88)
Guyette et al. *(2013)*	humans *(AD and non-AD patients)*	low-abundance biomarkers	NST (capillary collected) WO (10 µl polished micropipette)	(89)
Luo et al. *(2004)*	mice *(129SvEv/CD-1)*	IL-1β, TNF-α, MMP-9	WO	(94)
Song XJ et al. *(2003)*	mice *($NRTN^{+/+}$ and $NRTN^{-/-}$)*	IL-1β, MMP-9	WO	(95)
Markoulli et al. *(2011)*	humans *(healthy subjects)*	sIgA	NST and WO	(93)
Acera et al. *(2008)*	humans *(patients with ocular surface disease)*	inflammatory markers (IL-1β, IL-6, MMP-9)	Weck cell sponge	(98)
Inic-Kanada et al. *(2012)*	humans *(healthy subjects)*	cytokines	ophthalmic sponges (Merocel, Pro-ophta, Weck-Cel)	(99)
Green-Church et al. *(2008)*	humans *(healthy subjects)*	tear proteome	glass MC	(101)
Lee et al. *(2013)* **Satpathy et al. *(2011)***	humans *(patients with herpes keratitis)*	herpes simplex virus 1	ST (Schirmer strips) WO (micropipette)	(102) (103)

Table 2. Tear collection methods of different authors

AD: aqueous deficient; MC: microcapillary; MMP: matris metalloproteinase; IL: interleukin; NST: nonstimulated tear; sIgA: secretory immunoglobulin A; ST: stimulated tear; TNF-α: tumor necrosis factor-α; WO: washout

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 (104, 105). 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 group was 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, 75 µl, L 75 mm, Hischmann 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 a stopwatch.

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

Tear samples were transferred into low binding capacity Eppendorf tubes by the help of a sterile 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 bicinchonicid 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 is based on color intensity measurement, proportional to the peptide bond and the reagent set given product of the protein. The reaction absorbs visible light (A562 nm).

The initial step in the determination of total tear protein was the preparation of diluted Bovine Serum Albumin (BSA) standards using the suggested volumes of diluent.

After the preparation of BCA working reagent we pipetted 25 µL of each standard or unknown replicate sample into the wells of an ELISA microplate (Greiner 384 Flat bottom Transparent Polystyrol) then added 200 µL of the working reagent to each well and mixed plate thoroughly on a plate shaker for 30 seconds. Covering the plate and incubating at 37°C for 30 minutes were the next steps. After cooling the plate to room temperature we measured the absorbance at 562 nm on Infinite 200 M plate reader (Tecan, Austria).

Standard curve was prepared by plotting the average Blank-corrected 562 nm measurement for each BSA standard versus its concentration in µg/mL. We used the standard curve to determine the protein concentrations of each unknown sample.

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. First we prepared the VEGF standard with 1.0 mL of calibrator diluent, which resulted in a stock solution of 2000 pg/mL (2000 pg/vial of recombinant VEGF165 in a buffered protein base with preservatives, lyophilized). VEGF standards were prepared using prolypropylene tubes. 100 µL of calibrator

diluent was pipetted into each tube, then dilution series were produced using the stock solution. Tubes were mixed thoroughly before the next transfer. The undiluted standard served as the high standard (2000 pg/mL) and the calibrator diluent served as the zero standard (0 pg/mL).

100 µl of assay diluent was pipetted into each well of the VEGF Microplate (384 well polystyrene microplate coated with a mouse monoclonal antibody against VEGF) adding 100 µl of the standards, the controls and the tear samples. Two different human serum samples were used as controls. Due to the small amounts, 10 µl was taken from each tear sample and was diluted with 90 µl of NaCl, reaching the final volume of 100 µl. The plate was covered with adhesive strips, and was incubated for 2 hours at room temperature, while any present VEGF was bound by the immobilized antibody. After any unbound substances were washed away, an enzyme-linked polyclonal antibody specific for VEGF was added to the dry wells, and then the covered plate was incubated for 2 hours at room temperature again. Following a 3-times-wash with wash buffer to remove any unbound antibody-enzyme reagent, substrate solution was added to the wells, then the covered plate was incubated for 25 minutes at room temperature. Color developed in proportion to the amount of VEGF bound in the initial step. The color development was stopped through pipetting 2 N sulfuric acid as stop dilution to each well. The intensity of the color was measured within 30 minutes with Tecan absorbance reader at 450 nm and also at 540 nm for wavelength correction. Finally a standard curve was prepared and concentrations were calculated.

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

In the course of our second study two types of measurements were taken.

First, a cytokine array was performed, which is a rapid, sensitive, and economic tool that simultaneously detects cytokine differences between samples. Using this array the relative levels of 102 human soluble proteins could be determined without performing numerous immunoassays.

We brought all reagents to room temperature before use. The capture and control antibodies were spotted in duplicate on nitrocellulose membranes.

To remove cells, cellular debris and contaminant particles tear samples were centrifuged (10 minutes, 15.000 revolutions per minute, 4ºC) prior to use. Samples were prepared by diluting 50 µl of the tear samples to a final volume of 1.5 mL with an array buffer, followed by

incubation overnight with the Proteome Profiler Human XL Cytokine Array (R&D Systems). The membrane was washed with wash buffer to remove unbound material followed by incubation with a cocktail of biotinylated detection antibodies. Streptavidin – horseradish peroxidase and chemiluminescent detection reagents were applied, then membranes were placed in an autoradiography film cassette. Membranes were exposed to X-ray film for 10 minutes multiple times. A signal was produced at each capture spot corresponding to the amount of protein bound, accordingly the positive signals seen on developed film were identified by placing the transparency overlay template on the array image and aligning it with the pairs of reference spots in three corners of each array.

The pixel densities on developed X-ray films were analyzed using a transmission mode scanner and ImageJ software.

Alternatively, the absolute levels of MCP-1, complement factor D (CFD), IP-10 and C-reactive protein (CRP) were determined from diluted tear samples (MCP-1, CFD and CRP: 1:10; IP-10: 1:40) by Human Luminex Performance Assays (R&D Systems).

After preparing all reagents, working standards, and samples according to the manufacturer's instructions, the filter-bottomed microplate was pre-wetted by filling each well with 100 µL of wash buffer. Then, by the help of a vacuum manifold the liquid was removed through the filter at the bottom of the plate.

Analyte-specific antibodies were pre-coated onto color-coded microparticles. Quantity of 50 µl of the microparticles, standards and diluted tear samples were pipetted into wells and the immobilized antibodies bound the analytes of interest. After washing away any unbound substances, a biotinylated antibody cocktail specific to the analytes of interest was added to each well. Following a wash to remove any unbound biotinylated antibody, streptavidin-phycoerythrin conjugate, which binds to the biotinylated antibody, was added to each well. A final wash was done to remove unbound streptavidin-phycoerythrin and the microparticles were resuspended in buffer and read within 90 minutes by Bio-Plex 200 Systems (Bio-Rad) workstation.

3.5 Statistical analysis

Prism 5 statistical software (GraphPad Software Inc.) was used for the statistical analyses. Comparison of values was carried out by two-tailed Mann-Whitney U test. All values are shown as the mean ± 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 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 ± SD.

4 RESULTS

4.1 VEGF in tear samples of patients with SSc

The average tear secretion velocity in patients was 4.53 µl/min, median 3.8 µl/min (1.5-25.6). Duration of tear sample collection from patients varied between 20 and 313 seconds, until the minimally required 5 µl 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 µg/µl (1.8-12.3) and the average concentration of VEGF was 4.9 pg/µl (3.5-8.1) in the case of basal tear secretion (**Figure. 4.)**.

Control tears contained an average of 4.132 µg/µl (0.1-14.1) protein and 6.15 pg/µl (3.84-12.3) VEGF (**Figure 5.**).

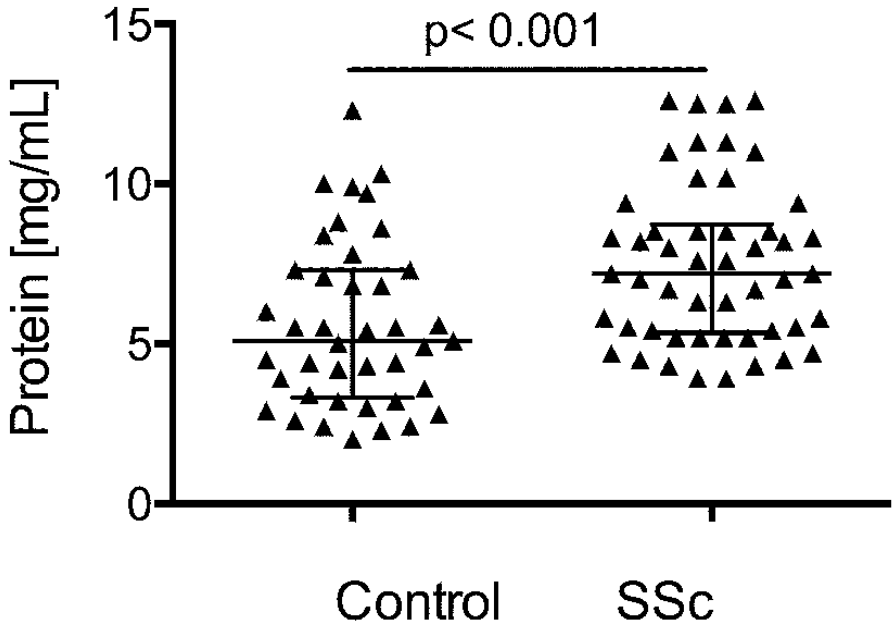


Figure 4. Total protein concentrations (mg/mL) in tears of healthy controls (left) and patients with SSc (right)

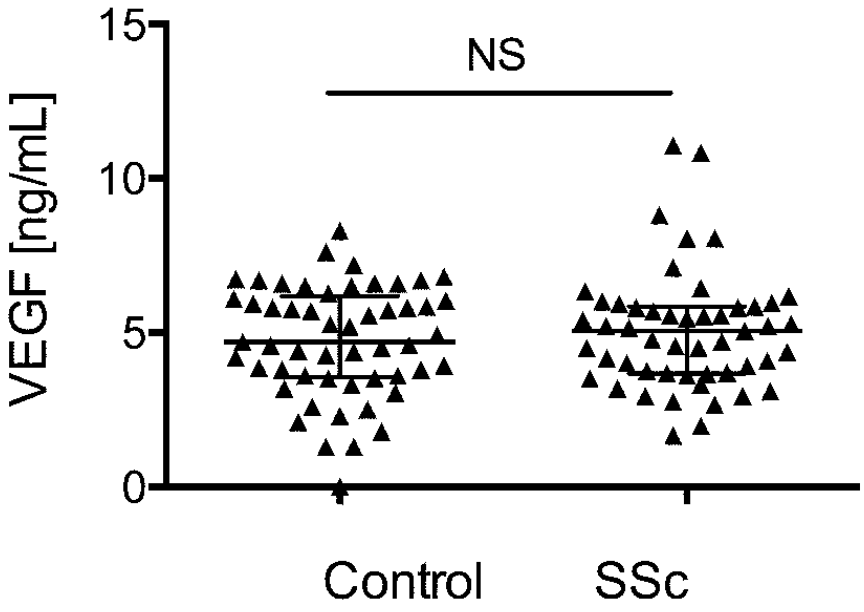


Figure 5. VEGF concentrations (ng/mL) in tears of healthy controls (left) and patients with SSc (right). NS: non-significant

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

4.2.1 Cytokine array results

Non-stimulated tear cytokine profiles of the control group and patients with SSc were analyzed by cytokine array detecting 102 different cytokines (**Figure 6. a, b**). Array results revealed a shift in cytokine profile characterized by predominance of inflammatory mediators. The following 9 ou 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 calledCXCL-10), Monocyte Chemoattractant Protein-1 (MCP-1), Monokine induced by gamma interferon (MIG), Matrix metallopeptidase 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 (**Figure 6. c**).

Normalized density of CFD was 50.35 (23.17-53.76) in tears of patients with SSc and 22.33 (18.39-24.75) in tears of healthy controls (p=0.002072), normalized density of CHI3L1 was 94.41 (31.9-95.98) in tears of patients with SSc and 31.06 (20.37-45.85) in tears of healthy controls (p=0.000000), normalized density of CRP was 25.98 (15.28-53.16) in tears of patients with SSc and 4.55 (4.35-4.66) in tears of healthy controls (p=0.018250), normalized density of EGF was 53.42 (34.86-70.23) in tears of patients with SSc and 34.04 (20.42-47.61) in tears of healthy controls (p=0.032818), normalized density of IP-10 was 123.42 (93.81-152.35) in tears of patients with SSc and 21.99 (12.12-29.01) in tears of healthy controls (p=0.000000), normalized density of MCP-1 was 19.93 (5.38-42.44) in tears of patients with SSc and 1.72 (1.44-2.27) in tears of healthy controls (p=0.044726), normalized density of MIG was 22.85 (5.6-64.14) in tears of patients with SSc and 3.58 (3.29-3.88) in tears of healthy controls (p=0.033787), normalized density of MMP-9 was 49.10 (4.24-129.04) in tears of patients with SSc and 12.74 (10.29-17.56) in tears of healthy controls (p=0.000068), normalized density of VDBP was 31.35 (11.87-64.68) in tears of patients with SSc and 10.18 (8.3-13.84) in tears of healthy controls (p=0.019733), respectively. (**Table 3.**)

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

Table 3. 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 (**Figure 6. d**). 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

4 chosen molecules were determined in tears of 9 healthy controls and 12 patients with SSc with the more sensitive and more specific Luminex bead assay.

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.

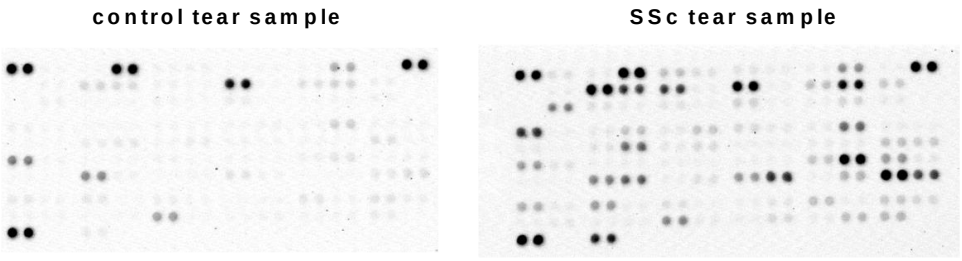
Mean CFD levels were 15.27 (5.00-35.28) µg/mg protein in tears of patients with SSc and 23.31 (5.18-106.63) µg/mg protein in tears of healthy controls.

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

Values represent the mean (± SD) of the 9 control and 12 patient samples, which are the fold change of normalized cytokine levels (**Figure 7. a, b**).

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 (**Figure 7. c**).

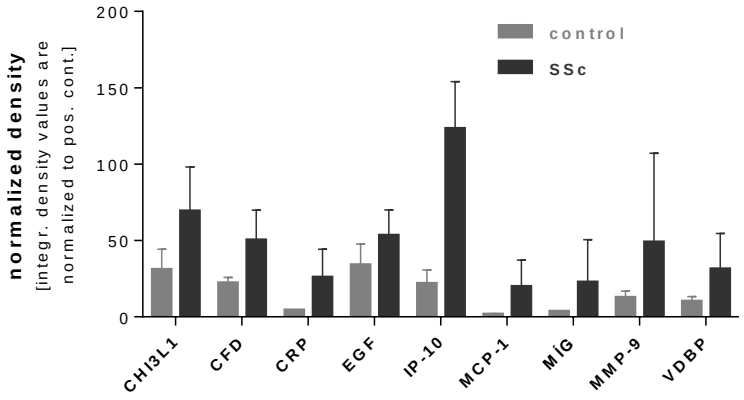
a



b

	1-2	3-4	5-6	7-8	9-10	11-12	13-14	15-16	17-18	19-20	21-22	23-24
A	+ control	ADPN	ACAN	ANG	ANGPT1	ANGPT2	BAFF	BDNF	C5/C5a	CD14	CD30	+ control
B	-	CD40L	CHI3L1	CFD	CRP	Cripto-1	CST3	Dkk-1	CD26	EGF	CD147	-
C	-	CXCL5	CD105	CD95L	FGF-2	FGF-7	FGF-19	FLT3LG	G-CSF	GDF-15	GM-CSF	-
D	CXCL1	GH	HGF	ICAM-1	IFN-γ	IGFBP-2	IGFBP-3	IL-1α	IL-1β	IL-1ra	IL-2	IL-3
E	IL-4	IL-5	IL-6	IL-8	IL-10	IL-11	IL-12p70	IL-13	IL-15	IL-16	IL-17A	IL-18BPa
F	IL-19	IL-22	IL-23	IL-24	IL-27	IL-31	IL-32α/β/γ	IL-33	IL-34	IP-10	CXCL11	KLK3
G	Leptin	LIF	LCN2	MCP-1	MCP-3	M-CSF	MIF	MÍG	MIP-1α/β	MIP-3α	MIP-3β	MMP-9
H	MPO	OPN	PDGF-AA	PDGF-AB/BB	PTX3	CXCL4	RAGE	CCL5	RBP4	RLN2	Resistin	CXCL12
I	PAI-I	SHBG	IL-1 R4	CCL17	TFF3	CD71	TGF-α	TSP-1	TNF-α	UPAR	VEGF	-
J	+ control	-	VDBP	-	-	-	-	-	-	-	-	- control

c



d

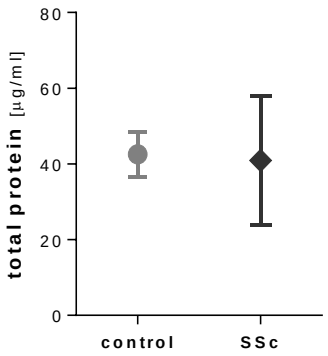


Figure 6. Tear cytokine profile of the control group and patients with SSc

Non-stimulated tear samples of healthy controls and patients with SSc were analyzed by cytokine array detecting 102 different cytokines (a, b), which revealed a shifted cytokine profile characterized by predominance of inflammatory mediators (c). The results represent the fold change of tear cytokine levels normalized to total protein contents (d).

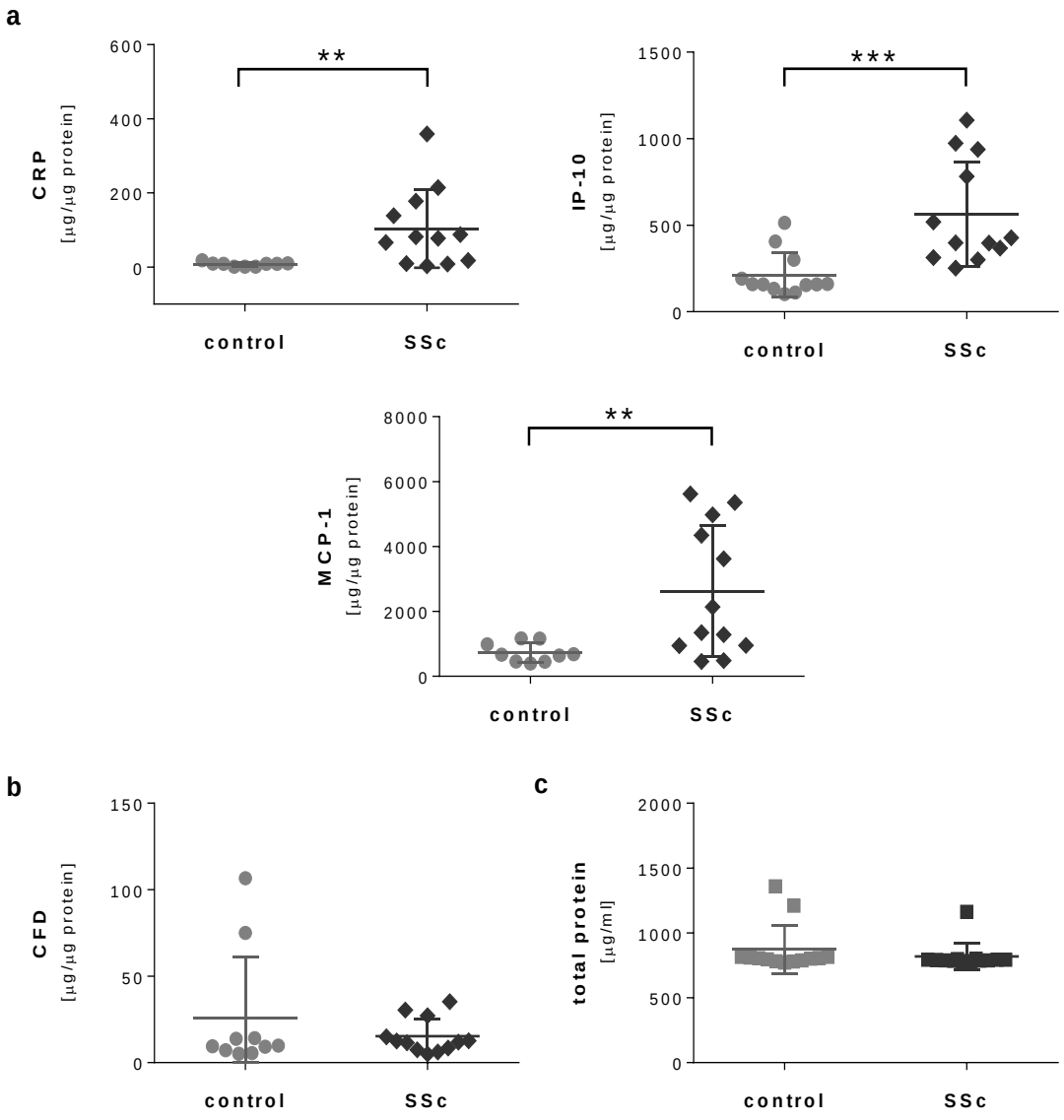


Figure 7. SSc is accompanied by CRP, IP-10 and MCP-1 inflammatory cytokine accumulation in tears

Non-stimulated tear samples of healthy controls and patients were tested for MCP-1, CRP, IP-10 and CFD by bead-based Luminex assays (a, b). Total protein concentrations of patients and controls (c). Cytokine levels normalized to total protein content represent the mean (± SD) of control and SSc samples. ** P<0.01; *** P<0.001

CRP, C-reactive protein, IP-10, interferon gamma-induced protein-10, MCP-1, monocyte chemoattractant protein-1, CFD, complement factor D

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.

Collaborations with the Department of Rheumatology and the laboratories of the Clinical Research Center and of the Department of Biochemistry were organized by the supervisor.
Reviewing the literature and collection of tear samples were accomplished by the author.
Adjustment of the Microplate method for determination of total protein concentrations in tear fluid was carried out by the author.
Experiments for the first study were performed by the author (≈50%).
Experiments for the second study were performed by the author (≈50%).
Experimental data were interpreted by the author (≈50%).

5 DISCUSSION

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 (106).

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 (107).

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.

Autoantibodies are present in more than 95% of patients, and are also associated with distinctive clinical subsets, specific patterns of organ involvement, and different prognostic features. Moreover, several other autoantibodies can be detected in patients with SSc, which include anti-U3-ribonucleoprotein (RNP)/fibrillarin, anti-Th/To, and anti-RNA-polymerase I and II and anti-U11/U12 RNP (2). Autoantibodies that are reported in other autoimmune rheumatic diseases, like anti-PM/Scl, anti-Ku, anti-U1-RNP, anti-SS-A/Ro60, and anti-NOR 90 can also be present, but they are considered less specific for SSc (108). **Table 4.** represents the frequency of autoantibodies in SSc (109).

Autoantibody	% Frequency in SSc
Anti-centromere	20-38
Anti-topoisomerase I	15-42
Anti-RNA polymerase III	5-31
Anti-U3RNP (fibrillarin)	4-10
Anti Th/To	1-13
Anti U11/U12 RNP	3.2
Anti-U1 RNP	2-14
Anti-PM/Scl	4-11
Anti-Ku	2-4
Anti-hUBF (NOR 90)	<15
Anti-Ro52/TRIM21	15-20

Table 4. Frequency of autoantibodies in SSc
RNA, ribonucleic acid; RNP, ribonucleoproteine; TRIM, tripartite motif.
Table reproduced from Kayser and Fritzler, 2015

For the diagnosis of SSc in the patients of our study group the international ’Criteria for the classification of early systemic sclerosis’ by Leroy and Medsger were applied (104). This classification system includes Raynaud's phenomenon, autoantibodies, nailfold capillaroscopy and skin fibrosis. Earlier, in the 1980’s, the American Rheumatology Association (ARA) criteria ’Preliminary criteria for the classification of systemic sclerosis (scleroderma)’ had four main items: scleroderma proximal to the metacarpophalangeal joints, sclerodactyly, digital pitting scars and bilateral basilar pulmonary fibrosis (110). The main limitation of the 1980 ARA classification was its development in longstanding SSc. In 2013 the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) established a committee to provide a joint proposal for new classification criteria for SSc. Van den Hoogen et al. created new classification criteria for SSc, which was principally meant to include SSc patients in studies, not for SSc diagnosis. The purpose of classification criteria is to involve patients with similar clinical characteristics for research.

The ACR-EULAR classification criteria for SSc performed better than the 1980 ARA criteria for SSc, due to its higher sensitivity and specificity, therefore it should allow for more patients to be classified correctly as having SSc. The newly developed classification system includes disease manifestations of the three hallmarks of SSc, namely fibrosis of the skin and/or internal organs, production of autoantibodies, and vasculopathy. The four items of the 1980 ARA are also included, as well as the items of the 2001 proposal for classification of SSc by Leroy and Medsger. The ACR-EULAR criteria include only one sufficient criterion: skin thickening of the fingers extending proximal to the metacarpophalangeal joints, which is also a part of the 1980 criteria. If the sufficient criterion is not fulfilled, then the point system is applied. Patients with 9 points or more are also classified as having SSc, extending the classification borders (111). The ACR-EULAR point system is summarized in **Table 5.**

Item	Sub-item	Weight or score
Skin thickening of the fingers of both hands extending proximal to the metacarpophalangeal joints		9
Skin thickening of the fingers (only count the highest score)	Puffy fingers	2
	Sclerodactyly of the fingers (distal to the metacarpophalangeal joints but proximal to the interphalangeal joints)	4
Fingertip lesions (only count the highest score)	Digital tip ulcers	2
	Fingertip pitting scars	3
Telangiectasia		2
Abnormal nailfold capillaries		2
Lung involvement	PAH and/or interstitial lung disease	2
Raynaud phenomenon		3
Scleroderma-related autoantibodies	Any of centromere-, topoisomerase I- and RNA polymerase III-specific antibodies	3

Table 5. ACR-EULAR criteria for classification of SSc

ACR, American College of Rheumatology; EULAR, European League Against Rheumatism; PAH, pulmonary arterial hypertension. Table reproduced from Van den Hoogen et al., 2013

Classification criteria are not synonymous with diagnostic criteria; rather they reflect the list of criteria that are used for diagnosis (112) as well. However, classification criteria are generally more standardized and less inclusive than clinical diagnosis. Determination of serum cytokines is becoming increasingly prevalent as an alternative diagnostic and progression monitoring method. A few studies have revealed a correlation between increased serum MCP-1, IL-6, IP-10 and TNF-α level and clinical manifestation/severity of SSc (106, 113).

Many ocular manifestations of SSc have been described, including conjunctival telangiectasia, DES and filamentous keratitis (113).

Although ocular manifestations in systemic autoimmune diseases have significant debilitating effects, tear analysis has been lacking from the investigations repertoire. Since tear represent the local homeostasis of the ocular surface better than serum, this makes tears ideal 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 (83). 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 (84).

Previously tear investigation studies have been performed in different ocular and systemic disorders (83, 114, 115). 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 (83). 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. In order to transfer the tear fluid from the microcapillary tube to the collection tube we applied a sterile syringe and a needle. This tear sampling method proved to be suitable for our experiments on tear cytokines.

5.1 VEGF in tear samples of patients with SSc

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

They calculated the average tear fluid secretion in healthy controls, which was 8.1 µl/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 (116).

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. VEGF in the tears of patients with SSc decreased by 20%, which can be explained also by the decreased tear secretion of patients.

The question why contrary to our expectations VEGF levels are not higherin patients with SSc than in the healthy group needs further investigation.

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

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 cyokines, 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.

CHI3L1, a protein which takes part in the processes of inflammation and tissue remodeling, has not been previously described in relation to the pathomechanism of SSc. We have found elevated levels of CHI3L1 in patients with SSc. This result correlates well with the fact that inflammation and tissue injury caused by hypoxia and oxidative stress are always present in the course of SSc.

In fact, different pathways may lead to vascular dysfunction processes in SSc, such as direct vascular damage or pro-inflammatory responses. Studies in different diseases have shown functional links between activated complement molecules and these pathways. CFD, a serine protease, also known as adipsin, plays a key role in these processes (117, 118). CFD is the rate

limiting enzyme in the activation cascade of the alternative pathway and its level in blood is quite low. Our cytokine array results showed increased CFD levels, which confirms the role of the complement system in the ocular pathology of SSc.

Levels of EGF were also elevated in tear samples of patients with SSc. EGF is a growth factor that stimulates cell growth, proliferation, and differentiation (119). Elevation of EGF may be explained by the above processes of vasculopathy. The next molecule, which appeared to be higher in patients' tears is matrix metallopeptidase-9 (MMP-9). As a protease of the MMP family, it is involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, angiogenesis, bone development, wound healing, cell migration, as well as in pathological processes, such as intracerebral hemorrhage, arthritis and metastasis (120-122). In a study of Kim et al. serum MMP-9 concentrations were found to be elevated in patients with SSc correlating well with skin scores (123). Their results suggest that increased MMP-9 concentrations may be due to their overproduction by dermal fibroblasts and also that the enhanced production of MMP-9 may contribute to fibrogenic remodelling during the progression of skin sclerosis in SSc. Our results of tear cytokin array are parallel with the finding that MMP-9 is increased in the course of SSc.

In a previous study expression of antiangiogenic chemokines and their receptors were determined in the sera and skin of patients with SSc (124). Based on their results MIG and its receptor are elevated in serum and highly expressed in the skin of patients with SSc. We have also found increased levels of MIG in tear samples of patients, which confirms the fact that dysregulated angiogenesis is an important feature in the pathomechanism of SSc. The next protein that appeared to be higher is VDBP, which belongs to the albumin gene family. VDBP is a multifunctional protein found in plasma, ascitic fluid, and cerebrospinal fluid and on the surface of many cell types. It binds to vitamin D and its plasma metabolites and transports them to target tissues (125). Others have measured significant quantities of VDBP-actin complexes in the plasma following injury (126). The presence of tissue injury is likely to be the explanation of our results, namely the elevated levels of VDBP in the tears of patients with SSc.

Based on our results of multiplex bead assay the three molecules that showed significant differences in tears of patients and controls were IP-10, MCP-1 and CRP. Previous studies have already demonstrated elevated levels of these markers in the sera of patients with SSc.

General markers of inflammation, such as CRP, are expected to be higher in a disease like SSc. In earlier trials CRP appeared to be elevated in the sera of patients with SSc, and was associated with poor survival. Therefore, it may be a useful indicator of disease activity and severity in SSc (127, 128).

Another inflammatory chemokine, IP-10, also called CXCL-10, has often been investigated in SSc studies (107, 124, 129). IP-10 has an angiostatic function as it suppresses neovascularization, furthermore, it is involved in immune regulation (130).

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 (131) and also in autoimmune diseases like rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, autoimmune thyroid diseases, type 1 diabetes mellitus, Addison's disease, and SSc (107, 132-134). CXCL10 is secreted by CD4+, CD8+, natural killer and natural killer T cells, and is dependent on interferon-γ. CXCL10 can also be secreted by several other cell types, including endothelial cells, fibroblasts, keratinocytes, thyrocytes, preadipocytes, etc. Detecting a high level of CXCL10 in peripheral fluids is therefore a marker of host immune response (128), which correlates well with our results of cytokine bead assay measurements.

Finally, MCP-1, which is a key participant of the fibrotic processes in SSc, also appeared to be higher in patients' tears. MCP-1, which recruits monocytes, memory T cells, and dendritic cells to the sites of inflammation, is produced by either tissue injury or infection (135). It is known as one of the most pathogenic chemokines during the development of inflammation and fibrosis in SSc (40). 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 (136). Hasegawa et al. have previously shown that serum MCP-1 levels are elevated when skin and lung are affected in patients with SSc (137). It has also been reported that cultured dermal fibroblasts from patients with SSc show augmented expressions of MCP-1 mRNA and protein (138).

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 the 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, hence it could help us choose the appropriate treatment, in particular 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 (107, 131) 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 SUMMARY

Systemic sclerosis is a chronic autoimmune disorder, characterized by widespread small vessel vasculopathy, immune dysregulation with production of autoantibodies, and progressive fibrosis. There are only few reports available concerning ophthalmological complications in the course of SSc, although ocular manifestations, e.g. DES occurs frequently and decreases the quality of life of these patients.

VEGF, the major pro-angiogenic factor plays a key role in the pathomechanism of SSc. Although elevated levels of VEGF in sera have already been demonstrated, VEGF analysis in tears of patients with SSc has not been performed in previous studies. Beside the determination of VEGF, our aim was to measure a wide panel of cytokines and chemokines with a semiquantitative method in tears of patients with SSc compared to healthy controls and to assess the most significant molecules with a more sensitive and specific quantitative method.

Tear collection method has a critical impact on the effectiveness of assay and on the quality of the results. Accordingly, after reviewing the literature on methodology, we have chosen the microcapillary method for the collection of nonstimulated tear samples.

VEGF in the tears of patients with SSc was decreased by 20%, compared to healthy controls. The reason why the VEGF levels are not elevated in the tears of patients with SSc needs further investigations, as does the simultaneous analysis of the sera of the same patients.

The cytokine array results revealed a shift in the cytokine profile characterized by predominance of inflammatory mediators. Our current data depict a group of cytokines and chemokines, which play a significant role in ocular pathology of SSc; furthermore, they might function as excellent candidates for future therapeutic targets in SSc with ocular manifestations.

8 ÖSSZEFOGLALÁS

Az SSc egy krónikus autoimmun betegség, kiterjedt kisér-vaszkulopátiával, immun diszregulációval, autoantitest termelődéssel és progresszív fibrózissal.

Kevés tanulmány foglalkozik az SSc szemészeti manifesztációival, habár ezek a betegek gyakran szenvednek az életminőségüket kedvezőtlenül befolyásoló szemészeti tünetektől, pl. száraz szem szindrómában.

A VEGF, mint a fő pro-angiogén faktor, kulcsfontosságú szerepet játszik az SSc pathomechanizmusában. Bár a VEGF emelkedett szintjét szérumban már kimutatták, SSc-s betegek esetében korábban könnyvizsgálatot még nem végeztek ennek tanulmányozására. A VEGF mennyiségi kimutatásán kívül szerettük volna meghatározni ezeknek a betegeknek a könnymintáiban citokinek és kemokinek széles skáláját egy szemikvantitatív módszerrel, majd az egészséges kontroll személyek könnymintáihoz képest szignifikáns különbséget mutató molekulákat egy nagyobb szenzitivitású és specificitású kvantitatív módszerrel tovább vizsgálni.

A könnymintavétel módja meghatározó lehet a vizsgálat hatékonyságának és az eredmények minőségének szempontjából, ezért az irodalmi adatok áttekintése után a kapillárissal történő könnymintavételi módszert választottuk a stimulálás nélküli könnyminta gyűjtéséhez.

Az SSc-ben szenvedő betegek könnymintáiban mintegy 20%-kal alacsonyabb VEGF szintet mértünk az egészséges kontrollokhoz viszonyítva. Annak magyarázata, hogy miért nem emelkedett a VEGF mennyisége SSc-s betegek könnymintáiban további vizsgálatokat igényel, például egyidejű szérum analízist ugyanazon betegek esetén, akiktől könnymintát gyűjtöttünk.

Citokin array eredményeink a gyulladásos folyamatok irányába eltolt citokin profilt mutattak SSc-ben szenvedő betegek könnyében. Vizsgálataink alapján a citokineknek és a kemokineknek egy olyan csoportját írtuk le, amelyek fontos szerepet játszanak az SSc szemészeti folyamatainak pathogenezisében, továbbá kiváló célpontok lehetnek a jövőben a szemészeti manifesztációkkal bíró SSc kezelésében.

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10 APPENDIX

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Subject: Ph.D. List of Publications

Candidate: Anikó Rentka
Neptun ID: KEMU6K
Doctoral School: Doctoral School of Clinical Medicine

List of publications related to the dissertation

1. **Rentka, A.**, Hársfalvi, J., Szűcs, G., Szekanecz, Z., Szodoray, P., Köröskényi, K., Kemény-Beke, Á.: Membrane array and multiplex bead analysis of tear cytokines in systemic sclerosis. *Immunol. Res. Epub ahead of print (2015)*
DOI: http://dx.doi.org/10.1007/s12026-015-8763-9
IF:3.098 (2014)

2. **Rentka, A.**, Hársfalvi, J., Berta, A., Köröskényi, K., Szekanecz, Z., Szűcs, G., Szodoray, P., Kemény-Beke, Á.: Vascular Endothelial Growth Factor in Tear Samples of Patients with Systemic Sclerosis.
Mediat. Inflamm. 2015 (Article ID 573681), 2015.
DOI: http://dx.doi.org/10.1155/2015/573681
IF:3.236 (2014)

Total IF of journals (all publications): 6,334
Total IF of journals (publications related to the dissertation): 6,334

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

19 February, 2016

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11 KEYWORDS

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

KULCSSZAVAK

Szisztémás sclerosis, száraz szem szindróma, könny, vaszkuláris endotheliális növekedési faktor, citokin, könnymintavétel, összfehérje, enzyme-linked immunosorbent assay, citokin array, multiplex bead assay

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