Evaluation of immunological activity in Graves’ orbitopathy

by Bernadett Ujhelyi M.D.

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The Examination takes place at the Department of Preventive Medicine, Medical and Health Science Center, University of Debrecen
25 April 2012.

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1. Introduction

Graves’ orbitopathy (GO) is an autoimmune disorder associated with different thyroid diseases. Although it has been first described almost two hundred years ago, the exact ethiopathogenesis is still obscure. GO is the most frequent extrathyroidal manifestation of Graves’ disease (GD) with a yearly incidence rate of 16/100 000 for females and 3/100 000 for males. Although women are more frequently affected, men usually develop more severe disease.

Smoking is one of the most important risk factors for GO. Not only smokers have higher risk for the disease but they are less likely to benefit from immunosuppressive treatment.

The pathogenesis of GO is a chronic retrobulbar autoimmune inflammation which takes place in the orbital connective tissue and eye muscles. Mononuclear cells, predominantly T lymphocytes and macrophages infiltrate the orbital tissue. The immunological process is mainly cellular; however, the autoantigen in the orbit has not been exactly identified. The inflammation takes place in the retrobulbar connective tissue, and fibroblasts have key role in the pathogenesis. Orbital tissue remodeling in GO is the result of cytokine dependent fibroblast activation. Activated fibroblasts express various cytokines, such as Interleukin (IL) -1 and IL-6 that enhance the prostaglandin E₂ and glycosaminoglycan (GAG) production of the fibroblasts. Fibroblasts themselves also express high levels of T lymphocyte kemoctines, such as RANTES (Regulated upon Activation, Normal T-cell Expressed and Secreted) and IL-16, and recruited lymphocytes augment the autoimmune process. Besides lymphocytes, macrophages also infiltrate the connective tissue of the orbit. Macrophage-derived cytokines play an important role in the pathogenesis of GO, and IL-1 and TNFα stimulate
Intercellular Adhesion Molecule 1 (ICAM-1) expression and glycosaminoglycan (GAG) production of orbital fibroblasts. The extracellular production of GAG, hyaluronic acid and collagen produced by fibroblast leads to increased osmotic pressure and consequential edema. The proliferation of the fibroblasts and the edematous swelling together results in the increase of the volume of the retrobulbar connective tissue; leading to specific signs and symptoms of Graves’ orbitopathy.

During the natural course of GO the retrobulbar autoimmune inflammation gradually decreases, the immunological process becomes inactive ending in a late, fibrotic phase. The initial phase of immunologically active and progressive disease usually lasts for 6 to 24 months. This active phase is often accompanied with only mild ocular symptoms. The active phase is followed by a plateau phase for 1 to 3 years, and the disease usually becomes inactive after 5 years. In the fibrotic stage proptosis and lid retraction may persist and can cause severe eye signs indistinguishable from active orbital inflammation, mimicking presumed enhanced immunological activity. Activity and severity are neither coincident nor synonymous in GO, as the immune process precedes the development of ocular signs and symptoms.

Even mild manifestation of GO has serious impact on the patients’ quality of life either in physical or in psychosocial aspects. From a clinical standpoint, it is important to predict if the patient will benefit from immunosuppression (corticosteroid treatment and/or retrobulbar irradiation) or, in the case of an immunologically inactive orbit, will only suffer from the side effects of otherwise ineffective immunosuppressive measures. Patients with inactive disease require a completely different treatment course, including rehabilitative surgery.
Several methods to evaluate disease activity in GO have been proposed; none has yet become generally accepted for routine use in clinical practice. The Clinical Activity Score (CAS) has been shown to be a useful method which is based on the ophthalmological findings of four out of the five classic inflammation signs (pain, swelling, hyperemia and functional impairment). A patient is considered as active if CAS is equal to or exceeds 4 out of the total score of 10. Simplicity makes CAS appropriate for everyday use. However it is important to admit, that while the major site of the autoimmune process is in the retrobulbar connective tissue, the CAS approach is indirect in this respect. Five of the 10 CAS scores are judged based on changes of the anterior segment, and two merely on patients’ complaint. For this reason CAS often “overestimates” disease activity.

The prolongation of the T2 relaxation time on magnetic resonance (MR) scans is a reliable marker of disease activity in the muscles, however, other orbital components, including fatty connective tissue, are not well graded for inflammation by MR. It is based on the detection of the proceeding edema, not the inflammation itself, however it provides exact images on the anatomical structure which is essential for planning a decompression surgery. Orbital uptake of $^{111}$In octreotide in GO patients detected by scintigraphy is a sensitive method to estimate immunologic disease activity. Lymphocytes that migrate into the inflammation site express somatostatin receptors on their surface. Octreoscan shows the inflammation precisely, but high cost is a serious drawback for everyday use. Recently we have reported single photon emission computed imaging of the orbits using $^{99m}$Tc-labelled diethylenetriamine-pentaacetic-acid (DTPA). The $^{99m}$Tc-DTPA complex (molecular weight 492 Da), administered intravenously, marks the high capillarization of inflammation sites leaving the vascular bed
through damaged capillary walls. It ‘leaks out’ into the interstitial fluid and
binds to polypeptides present in the extracellular fluid at inflammation sites.
It has been shown that DTPA findings are comparable to Octreotide in
identifying inflammation in GO at a substantially lower cost.

Ocular surface changes in Graves’ orbitopathy have been the subject
of research for decades. Early studies by Gilbard et al reported abnormally
high tear osmolarity and rapid tear film break up time in patients with GO.
Alterations in tear film profile, low break up time and Rose Bengal staining
in late GO indicate the presence of drying epithelial cells. Changes in tear
protein profile have also been published by Khalil et al., suggesting that GO
has an effect on the lacrimal gland. According to proteomic analysis there are
changes in the tear protein composition in GO, although the identification of
these biomarkers are the subject of future research.

The lacrimal gland is a putative target in GO, supported by several
findings. Thyroid hormones have direct effect on the lacrimal gland, in both
functional and structural relation. Lacrimal acinar cells physiologically
express TSH receptor. In Graves’ disease autoantibodies might bind to the
TSH receptor, contribute to autoimmune lacrimal gland destruction. Probable
involvement of the lacrimal gland as an area of immunological reaction in
GO have been suggested based on Octreotide scintigraphy.
2. Aims

2.1. $^{99m}$Tc-DTPA SPECT in the evaluation of disease activity in GO

I. To verify the usefulness of $^{99m}$Tc-DTPA SPECT to predict the effectiveness of immunosuppressive therapy in GO
II. To evaluate the uptake of $^{99m}$Tc-DTPA radionuclide of the normal orbit by SPECT
III. To define the change in DTPA uptake which can be considered as real improvement, since biological systems have their inherent uncertainty resulted from the natural variation of the value
IV. To identify the orbital uptake value, above which patients are likely to benefit from corticosteroid treatment, to define a cut off value, above which GO can be considered as immunologically active.

2.2. Tear cytokine, chemokine and PAI-1 composition in GO and GD

I. To detect and observe possible differences in the cytokine, chemokine and PAI-1 composition of tears of patients with GO and GD without orbitopathy.
II. To evaluate possible relations between lacrimal secretory capacity and tear film cytokine balance
III. To examine the effect of smoking, as a risk factor for GO on the cytokine composition of the tear film
IV. To reveal possible connection between changes in tear film composition and disease activity in GO.
3. Patients and methods

Patients were enrolled from the outpatient clinic of the Department of Ophthalmology and the Department of Internal Medicine, Division of Endocrinology of the University of Debrecen Medical and Health Science Center. Patient enrollment was based on clinical protocols in accordance with international guidelines. All patients with ophthalmological or any other disease that would interfere with the results have been excluded. All enrolled patients gave informed consent and the Institutional Review Board approved the study protocol in accordance with the 1989 Declaration of Helsinki.

3.1. $^{99m}$Tc-DTPA SPECT in the evaluation of disease activity in GO

3.1.1. Patients

Our patient group consisted of 114 orbits of 57 patients with Graves’ orbitopathy (44 women and 13 men, mean age 52.74±10.70 years). The control group, for purposes of determining normal reference DTPA uptake values, consisted of 34 orbits of 17 patients (4 men, 13 women, mean age 48.12±11.99 years) who underwent forearm and hand SPECT using DTPA in order to examine microcapillary blood flow as part of their workup for Raynaud’s phenomenon. No control patient had any ophthalmological or endocrine disease, allergic rhinitis or sinusitis in the history was also an exclusion criteria.

3.1.2. Ophthalmological and endocrine evaluation and therapy

All patients had Graves’ disease. Consecutive patients with symptomatic newly diagnosed or relapsing GO and a CAS suggestive of an active autoimmune process (CAS≥4) were entered in the study if the attending
endocrinologist, as part of usual care, decided to start corticosteroid treatment. Patients underwent careful ophthalmologic examination (best corrected visual acuity, slit lamp examination, ophthalmoscopy, tonometry, Hertel exophthalmometry). Clinical Activity Score (CAS) was obtained in each case. A mean cumulative dose of $3193 \pm 2175$ mg methylprednisolone had been administered intravenously as immunosuppressive treatment. In addition to corticosteroids, 11 patients received orbital irradiation concurrently (10 times 2 Gy). All patients received local measures in the form of artificial tear drops and protective ointments.

3.1.3. $^{99m}\text{Tc} \text{ DTPA SPECT}$

For SPECT examination $^{99m}\text{Tc}$-DTPA (PromtCarry, Szeged, Hungary) was administered intravenously. After 20 minutes, a Nucline X-Ring four headed SPECT device (Mediso, Budapest, Hungary) was used for imaging. One hundred twenty-eight frames were acquired. Coronal and sagittal slice sets were generated perpendicularly to the transversal plane, covering the entire orbital area. To quantify DTPA accumulation in the orbits, regions of interest were drawn on the transversal slices, outlining areas corresponding to the right and left orbits. The sum of the six transaxial slices containing the entire orbital region was used in uptake calculations. For quantitative assessment of DTPA uptake, the sensitivity of the SPECT unit was precalibrated. The activity before and remaining activity in the syringe after intravenous injection was measured, and the time of injection. The method provides visual and numerical information on the orbits. SPECT was performed on two occasions in each GO case. First, it was performed before corticosteroid treatment was started and a second SPECT was performed 2 to 9 months (5.07±2.37 months) later. A single DTPA uptake was performed in controls.
None of the control individuals had known thyroid disease or relevant symptoms or signs, orbital or eye pathology, or seasonal rhinitis. They underwent forearm SPECT as part of their workup for Raynaud’s phenomenon and their orbital region was also evaluated in the same setting.

### 3.1.4. Special calculation method and statistical analysis

To determine normal reference DTPA uptake values, the 95th percentile value of the control group was calculated. CAS and DTPA uptake values before and after treatment were compared with paired t test in the GO group. Changes in DTPA uptake between the two SPECTs were calculated as $\Delta$DTPA$=\text{DTPA uptake before treatment} - \text{DTPA uptake after treatment}$. A positive $\Delta$DTPA value was considered suggestive of a decrease in the autoimmune process induced by corticosteroid therapy. An unchanged DTPA uptake or a negative $\Delta$DTPA was considered to indicate no change or progression of the immune process, respectively. The $\Delta$DTPA value was compared to the initial DTPA uptake in each orbit, the $\Delta$DTPA for each orbit was plotted against the pretreatment DTPA uptakes and linear regression analysis was performed on the values. As all measurements in biological systems have their inherent uncertainty, it was important to define what $\Delta$DTPA can be considered as real improvement instead of biological fluctuation. Using a linear regression model based on both the reference range of normal controls and the upper quartile of those GO patients who had an initial DTPA value in the control group’s reference range, the linear regression line identified $\Delta$DTPA=1.72; a change exceeding this value was considered a real improvement.
Correlation analysis was also performed for CAS vs. ΔCAS and ΔDTPA vs. ΔCAS.

For statistical analysis Kolmogorov-Smirnov test, paired t test, Spearman correlation analysis and linear regression analysis have been used. Analysis was carried out using the SAS for Windows 8.2 software with a significance level defined at p<0.05.

3.2. Tear cytokine, chemokine and PAI-1 composition in GO and GD

3.2.1. Patients

Tear samples were collected from 54 eyes of twenty seven patients with Graves’ orbitopathy (GO) (6 males, 21 females, age 43.4 ±15.2 years) and 18 eyes of nine Graves’ disease patients without orbitopathy (GD) (1 male, 8 females, age 46.8±11.7 years). The control group (C) consisted 24 eyes of twelve healthy volunteers (4 males, 8 females, age 38.6±13.8 years).

3.2.2. Ophthalmological and endocrine evaluation

Patients underwent careful detailed ophthalmological examination (slit lamp microscopy, corneal staining, Schirmer I test, tear film break up time (BUT), Hertel exophthalmometry, indirect ophthalmoscopy). Before tear collection, the anterior ocular status of each subject was carefully assessed: a slit-lamp under low illumination was used to avoid reflex tearing, while all other ophthalmological evaluations were performed after sample collection. All patients with any signs of corneal pathology have been excluded. All factors that are known to alter tear cytokine composition were exclusion criteria (corneal erosion, usage of eyedrops, especially antiglaucoma drops, uveitis or any chronic ophthalmological disease in history, previous operation on the conjunctiva or eyeball). Use of preservative free lubricant drops was allowed,
but patients must not instill the drop on the morning of sample collection. In
the control group decreased lacrimal secretory capacity or shortened tear film
break up time were also exclusion criteria. Clinical Activity Score (CAS) was
obtained in each GO case.
Thyroid status including serum hormone levels (TSH, free T4, free T3, TSH
receptor binding antibodies) were determined within a 5-day interval before
or after tear collection using electrochemiluminescence immunoassay (TSH,
FT3, FT4 assay by Elecsys/Cobas Roche Diagnostics GmbH, Mannheim,
Germany). History of smoking and any significant general or
ophthalmological disease was recorded.
3.2.3. Tear sample collection
Tear samples were obtained by sterile capillary flow with no nasal
stimulation or previous installation of drugs or vital dyes, by the same
examiner (BU). No anesthetic drops were instilled; samples were collected
non-traumatically from the inferior meniscus without touching the cornea,
conjunctiva or eyelids. None of the patients used any topical eye medications;
only non-preserved artificial tears were allowed which could not be instilled
on the morning of sample collection. Collected amount of the tear sample
(µl) and collection time (120 sec) were recorded and samples were frozen
without centrifugation within 15 minutes and stored at −70°C until further
evaluation.

3.2.4. Cytokine, chemokine and PAI-1 measurements
Levels of cytokines were measured by a multiplex bead array method.
Combined FlowCytomix™ Simplex Kits were used with an appropriate
FlowCytomix Basic Kit according to the manufacturer’s instructions (Bender
samples (in some cases diluted samples) or serial dilution of mixed cytokine standards were added to the wells of filter micro plates containing the fluorescent cytokine capture bead mixtures. Biotin conjugated anti-cytokine antibody mixtures were applied and the plates were incubated at room temperature for 2 hours protected from light on a microplate shaker. The filter plates were washed using a MultiScreen HTS Vacuum Manifold (Millipore, Billerica, MA, USA). Phycoerythrin conjugated streptavidin solution was added to the samples and were further incubated for 1 hour as described above. Plates were washed again, then 150μl sample buffer was added to the wells and sample data were acquired by multiparameter flow cytometric analysis with a FACS Array cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA, USA).

Data were analyzed with the BenderMedSystems FlowCytomixTM Pro 2.4 software. Assay sensitivities provided by manufacturer were 4.2 pg/ml for IL-1β, 1.2 pg/ml for IL-6, 4.5 pg/ml for IL-13, 2.5 pg/ml for IL-17A, 3.3 pg/ml for IL-18, 3.2 pg/ml TNF-α, 25 pg/ml for RANTES. and 13.5 pg/ml for PAI-1. During the preparation of the human cytokine standards, additional dilutions were applied to achieve higher sensitivity, and modified standard curves were generated during the analysis.

3.2.5. **Special calculation method and statistical analysis**

We calculated a release value from the concentration results of the flow cytometric assay (pg/μl), the volume of tear collected (μl) and collection time (120 sec) for all examined parameters according to the function below.

\[
release = \frac{concentration\ in\ tears\ (pg/\mu l) \times tear\ amount\ collected(\mu l)}{collection\ time\ (min)}
\]
The 2 minutes cytokine release values (pg/2 min) were used for further statistical analysis. Hormone and cytokine levels in the three study groups (GO, GD and C) underwent logarithmic transformation, and as results became a Gaussian population, were compared by analysis of variance (ANOVA) with Duncan post hoc testing. Both cytokine levels and cytokine releases have been correlated with patient age, clinical parameters (CAS, Schirmer I test), smoking (number of cigarettes per day) by Spearman correlation analysis. Results from the two eyes of a patient were compared with paired t test and Wilcoxon Matched Pairs test. Analysis was carried out using the SAS for Windows 8.2 software with a significance level defined at $p<0.05$.

4. Results

4.1. $^{99m}$Tc-DTPA SPECT in the evaluation of disease activity in GO

4.1.1. Evaluation of the DTPA uptake of the healthy orbit

In the control group (patients with Raynaud syndrome), the DTPA uptake of the orbit was $7.9\pm2.6\text{MBq/cm}^3$. As normal reference range, we used the 5th and 95th percentiles of the DTPA uptake, which were $4.73\text{MBq/cm}^3$ and $12.28\text{MBq/cm}^3$ (CI 11.10-13.51), respectively. From these results we identify orbits with uptake value above $12.28\text{MBq/cm}^3$ as elevated, because inflammation with capillary leakage is probable in these cases (the lower value has no biological importance).
4.1.2.  **Response of orbits to immunosuppression in patients with GO by DTPA SPECT**

The mean DTPA uptake of the GO patients was lower after immunosuppressive treatment than before therapy (11.03±4.26MBq/cm$^3$ and 9.84±3.51MBq/cm$^3$, respectively, p<0.001).

The ΔDTPA for each orbit was calculated from the pretreatment and post treatment DTPA uptakes. There was a strong direct correlation between initial DTPA and ΔDTPA values (r=0.58, p<0.0001).

4.1.3.  **CAS evaluation; correlation between CAS and DTPA**

The baseline CAS before corticosteroid therapy was 4.93±0.87. This declined significantly after therapy (CAS 4.22±1.78, p=0.009). There was no correlation between the change in CAS (ΔCAS) and ΔDTPA (r=0.094).

4.1.4.  **Definition of “improvement” by DTPA SPECT**

To define the value of real improvement in DTPA uptake, independent from biological variability, a linear regression model has been used based on both the reference range of normal controls and the upper quartile of those GO patients who had an initial DTPA value in the control group’s reference range. The linear regression line f(x)=0.422x-3.46 identified ΔDTPA=1.72; a change exceeding this value was considered a real improvement. The mean ΔDTPA was 1.89±3.08 in the GO group.

4.1.5.  **DTPA SPECT uptake values as predictors of positive response to immunosuppression in GO**

Based on the upper normal reference value of the orbit (12.28MBq/cm$^3$) the GO group was divided into subgroups regarding to the initial DTPA uptake
(i.e. below or above the normal reference range). Among orbits with an initial DTPA uptake below 12.28MBq/cm$^3$, 27.5% had improved, while of those with an initial DTPA uptake above 12.28MBq/cm$^3$, 67.6% had improved. On the other hand, no orbit below an initial DTPA uptake of 6.1MBq/cm$^3$ had improved. It is important to remember that only patients with elevated CAS values (CAS≥4) were entered in the study. The positive predictive value of an initial DTPA >12.28MBq/cm$^3$ for a favorable treatment outcome was 76%, while a negative predictive value of a pretreatment DTPA ≤12.28MBq/cm$^3$ was 78%.

### 4.2. Tear cytokine, chemokine and PAI-1 composition in GO and GD

#### 4.2.1. Results of ophthalmological evaluation

Ophthalmological evaluation of the anterior segment of GO and GD patients by slit lamp we found signs of ocular surface drying, but no corneal pathology (fluorescein staining, erosion or ulceration) was present at the time of sample collection. It is important because these would have altered our results.

Schirmer I test showed a mean lacrimal secretory capacity of 13.94±10.07 mm in the GO group, 14.22±8.04 mm in the GD group and 19.37±9.17 mm in the C group; differences between the GO and GD patient groups were not statistically significant, both were significantly lower than the control group. CAS in the GO group was 3.82 ±2.0, according to definition CAS was not obtained in the other patients groups.
4.2.2. Cytokines, chemokines and PAI-1 in tears

4.2.2.1. Release and concentration values in the different patient groups
We found significant differences between the cytokine release values measured in the GO and C groups. The release of IL-1β, IL- 6, IL- 13, IL-17A, IL-18, TNF-α and RANTES were significantly higher in the GO group as compared to the control group (p<0.05). No significant difference was found between GD and group C in the release of the cytokines tested. Also, no statistically significant differences were found between the cytokine release into the tears of GO and GD patients, although release values tended to be higher in the GO group, i.e., the values of GD patients were intermediate between the GO and the control groups in the case of all cytokines. Release of PAI-1 into tears was significantly higher in the GO group compared to GD patients, and both GO and GD patients’ PAI-1 release values were significantly higher than that of the control group.

4.2.2.2. Relation between the release of examined markers
Strong correlation have been found between the release of all examined cytokines, for this evaluation we examined all tear samples as one population, as our purpose was to analyze the effect of cytokine release on each other, regardless to patient group. The correlation coefficient ranging between 0.66 and 0.97 (p<0.01 in all comparisons). The strongest correlations have been detected between the release of IL-1β and RANTES (r= 0.96 p<0.001), as well as IL-1β and the other members of the IL-1 family (IL-18: r=0.97, p<0.001, IL-17A: r=0.89, p<0.001). Positive correlation have been found between the release of all tested cytokines and the secretion of PAI-1 into tears (IL-1β: r=0.23 p=0.002, IL- 6:
4.2.2.3. The influence of age on tear cytokine release values

Except for IL-17A, we found no correlation between tear cytokine concentrations and age, for this evaluation we examined all tear samples as one population, as our purpose was to analyze the effect of age on cytokine release, regardless to patient group. For IL-17A, the correlation was negative (r= -0.21, p<0.05). Also, weak negative correlation have been found between age and PAI-1 release (r= -0.24 p<0.05).

4.2.2.4. Thyroid function

Patients’ sera were assayed for thyroid stimulating hormone, free thyroid hormone and TSH stimulating antibody levels (TSH, fT3, fT4, TRAb). No significant difference have been found in these parameters between patient groups GO and GD. This supports the hypothesis, that differences found in release values are not the result of the thyroid hormone status.

4.2.2.5. Lacrimal secretory capacity and tear cytokine composition

Positive correlations have been found between Schirmer I test and the release of all tested cytokines in the control group (IL-1 beta: r=0.53, p=0.002, IL-6: r=0.46, p=0.01, IL-13: r=0.50 p=0.005, IL-17A: r=0.54 p=0.002, IL-18: r=0.54 p=0.002, TNF alfa: r=0.52 p=0.003 and RANTES: r=0.56 p=0.001).

Similarly, positive correlation have been found between Schirmer I test and the release of PAI-1 in the control group (r= 0.40 p=0.002). However, none of the cytokine release values showed correlation with lacrimal secretory capacity in the GO or GD group. Weak positive correlation has been found between Schirmer I test and PAI-1 release in the GO group (r=0.28 p=0.04); there was no correlation in the GD group.
4.2.2.6. Smoking and cytokine release into tears

Among the 27 GO patients 10 were previous or current smokers (cumulative number of cigarettes smoked 78309±53631 with an average of 14.73±6.61 cigarettes/day), while among the 9 GD patients without orbitopathy 6 were previous or current smokers (cumulative number of cigarettes smoked 85166±101169 with an average of 11.67±10.81 cigarettes/day). In the control group, no patient was a current or previous smoker. We found no connection between tear cytokine levels or release values and smoking (by the number of cigarettes per day, or using digitomized variables yes/no). When the cytokine release values were analyzed in all patients irrespective of the presence of GO, as well as in controls, and were regrouped according to smoking history, no difference have been found in the release of any examined cytokine.

4.2.2.7. Correlation between immunological activity of GO and tear cytokine release

We found positive correlation between IL-6 release and CAS (r=0.27, p <0.05) as well as between IL-6 release and the degree of eyeball protrusion (mm in Hertel exophthalmometer) (r=0.34, p<0.05) in the GO group. Also positive correlation has been found between PAI-1 release and CAS (r=0.24, p=0.03).
5. Discussion

Graves’ orbitopathy (GO) is an autoimmune disorder associated with different thyroid diseases. In the initial phase autoimmune inflammation takes place in the orbital connective tissue (active phase), followed by the inactive phase when inflammation decreases and fibrotic transformation begins. From a clinical standpoint, it is essential to decide whether the immune process is active or inactive to decide on the appropriate therapeutic approach. Immunological activity and clinical signs and symptoms are not strictly connected to each other, neither their location nor timing. Disease activity and severity are neither coincident nor synonymous. Several methods have been established to evaluate disease activity, but all of them have certain limitations. For every day practice, the most widely used technique is Clinical Activity Score (CAS) evaluation. Main advantage of CAS is that it can be easily examined during a routine ophthalmological workup. However CAS often “overestimates” disease activity, as five out of the ten points are based on anterior segment changes, while the inflammation takes place in the orbital tissue.

The aim of the present research was to develop and assess new methods for the evaluation of disease activity in GO. Our studies were divided into two separate lines. First, a series of investigation was carried out with a recently introduced radionuclide $^{99m}$Tc labeled DTPA SPECT. In another series of experiments, our attention was focused on changes of tear composition in GO and GD.

Previously, Nuclear medicine techniques have been used in the evaluation of GO. $^{111}$In octreotide binds to the somatostatin receptor of lymphocytes that migrate into the inflammation site and can be detected by
scintigraphy (Octreoscan). Its main advantage, that it clearly marks the retrobulbar inflammation, is darkened by its high cost.

$^{99m}$Tc-labelled diethylenetriamine-pentaacetic-acid (DTPA) complex (molecular weight 492 Da), administered intravenously, marks the high capillarization of inflammation sites leaving the vascular bed through damaged capillary walls. It “leaks out” into the interstitial fluid and binds to polypeptides present in the extracellular fluid at inflammation sites. This is the background of high DTPA uptake in active GO. We have shown previously that results of Octreoscan and DTPA SPECT are comparable, but cost effectiveness is in favor of the latter.

Only patients with immunologically active disease respond to immunosuppressive therapy, while among patients in inactive phase there is no therapeutic effect. For this reason, if patients are enrolled by the standard criteria of active disease (CAS≥4), treated according to international guidelines and examined with the tested method (in this case DTPA SPECT) before and after therapy, those patients who are responders can be identified. Responders are the patients who can be considered as active at baseline.

DTPA uptake of the healthy orbit has not been established previously. Our first aim was, to define the uptake value, above which a result should be referred to as elevated. To do so, an ideal control group had to be found with no orbital or endocrine pathology. Forearm SPECT with $^{99m}$Tc labeled DTPA for the evaluation of Raynaud’s phenomenon is an accepted technique. For the control group we enrolled otherwise healthy patients with Raynaud’s phenomenon who underwent forearm-hand DTPA SPECT. During the scintigraphy of these patients, their orbital region has also been included in the field of imaging. The 95th percentile of the uptake of the control orbits was considered as the upper limit of the normal orbit.
(12.28MBq/cm³). Orbits above this value were, by definition, active and the ones below were inactive. To support this theory we designed and carried out a prospective study on patients with GO.

One hundred and fourteen orbits of 57 GO patients were enrolled into the study. ⁹⁹mTc-labelled DTPA SPECT was performed on two occasions in each case. First, it was performed before corticosteroid treatment was started. A second SPECT was performed 2 to 9 months (5.07±2.37 months) later. The main inclusion criteria was the evidence based indication of immunosuppressive treatment, so clinically active disease (CAS≥4). This explains that the mean DTPA uptake of the examined orbits was significantly lower after immunosuppressive treatment than before therapy.

As all measurements in biological systems have their inherent uncertainty, instead of using a single cut-off value, a “gray zone” needs to be defined, in which the change in DTPA uptake (ΔDTPA=DTPA uptake before treatment – DTPA uptake after treatment) may have resulted from natural variation of the value. To discriminate between those who did, and those who did not have a meaningful change in DTPA uptake after treatment we used a linear regression model based on the reference range of normal controls. There was a direct correlation between initial DTPA and ΔDTPA values. The regression function and the defined upper limit of the normal orbit defined the change ΔDTPA=1.72, exceeding this value was real improvement can be considered.

Regarding to the initial DTPA uptake value, 70% of the examined GO orbits was below the defined normal range (12.28 MBq/cm³). Complicating this finding is the fact that only patients with elevated CAS values (CAS≥4), also considered suggestive of an active autoimmune process, were entered in the study. Furthermore, 72.5% of these GO orbits
showed no improvement after immunosuppression by DTPA SPECT. According to the logic above, these GO orbits should be regarded as they were inactive at the time of their first evaluation, despite the fact that their CAS was equal or above 4. CAS is “overestimating” disease activity in these cases. Many inactive patients have severe subjective symptoms. It is a frequent finding that a patient cannot differentiate retrobulbar blunt pain from the sharp foreign body sensation and surface discomfort caused by ocular surface drying. As this “retrobulbar pain” is added to the CAS, it leads to false elevation of the final score by two points. Furthermore five out of ten points in CAS are based on ocular adnexal (eyelid) and anterior segment (conjunctiva, caruncule) findings. Hyperemia and swelling of these structures can be the result of congestion caused by extreme protrusion and increased orbital volume or pressure in inactive GO.

Our hypothesis is also supported by the finding that although a significant decrease in CAS was observed, we found no correlation between CAS and ΔCAS or between ΔDTPA and ΔCAS.

In summary, $^{99m}$Tc DTPA SPECT can predict the effectiveness of immunosuppressive therapy in GO, so it is a useful tool to evaluate disease activity. The positive predictive value of an initial DTPA >12.28MBq/cm³ for a favorable treatment outcome is 76%, while a negative predictive value of a pretreatment DTPA ≤12.28MBq/cm³ is 78%.

The second part of our series of investigations was focused on the alternation of the tear film in GO and GD, with special regards to cytokines, chemokines and plasminogen activator inhibitor -1 (PAI-1). Ocular surface changes in GO have been the field of interest for decades. First, increased tear film osmolarity had been observed. Consequential changes on the ocular
surface lead to decreased lacrimal secretory capacity and tear film break up time, resulting in corneal epithelial cell defect.

Tear film plays essential role in the maintenance of ocular surface homeostasis. Inflammation leads to the activation of lymphocytes and macrophages that secrete wide variety of cytokines and chemokines. Cytokine balance of the tear film can be altered by the course of the day or even by eye closure. Several ophthalmological and general conditions have been reported to effect the cytokine composition of tears. Smoking, one of the most important risk factor of GO, also increase the proinflammatory cytokine level in tears even in passive form. Similar changes have been reported in the composition of tears of patients with GO and healthy smokers.

Alternation in the protein composition of tears have been reported and the lacrimal gland is described as a putative target in GO. Proteomic findings support the changes in GO tear proteins, although the identification of these is the target of future research.

The aim of our study was to observe alternations of the human tear film in active and inactive GO, and GD without orbitopathy with special attention to various cytokines (TNF-α, IL-1β, IL-6, IL-18, IL-17A and IL-13), chemokine RANTES and PAI-1.

Multiplex bead array technique has been used for cytokine detection, which allows to measure several markers in one sample by flow cytometry. A protein, which is secreted into a fluid, can be more precisely described by its secretion rate (release) than its concentration. It is especially important in the manner of tears as lacrimal secretory capacity shows wide variety among individuals. Protein release into tears in 2 minutes (pg/2 min) is a parameter used by different authors previously. We also used this unit for our series of investigation.
Elevated tissue concentration of TNF-α, IL-1β and IL-6 have already been described in GD, as has the serum concentration of IL-6. However we did not find significantly elevated release into tears in GD. These findings suggest that the following increase of cytokine release into tears is not the result of Graves’ disease alone, but the orbitopathy itself. Various cells and tissues are responsible for cytokine and chemokine secretion: lacrimal gland, minor lacrimal glands of the conjunctiva, fibroblasts and immunocompetent cells migrating to the ocular surface can secrete these markers. Lacrimal gland, as a putative target of inflammation in GO has already been verified. Early inflammation of the conjunctiva or episclera can appear before the development of the classic findings in GO. Recently, ocular surface inflammation is regarded as manifestation of the autoimmune process itself in GO, instead of the classical theory that it is the consequence of exophthalmos. Ocular surface inflammation can be the only presenting clinical sign in GD, well before the development of the classic findings of GO.

In our study, we found a significant increase of cytokine release of TNF-α, IL-1β, IL-6, IL-18, IL-17A, IL-13 and RANTES in the tears of the GO patient group as compared to controls.

The importance of the macrophage-derived cytokines TNF-α and IL-1β in GO have been described and were shown to stimulate ICAM-1 expression and GAG production by orbital fibroblasts. High release of TNF-α and IL-1β into tears of patients with GO may indicate their presence not only in the retrobulbar connective tissue, but also in the lacrimal gland and ocular surface. IL-1β-activated fibroblasts express high levels of T cell chemokines such as RANTES which may explain the increased release of RANTES in tears of patients with GO. Increased IL-18 serum levels in both GO and GD
individuals have also been observed. In our study, however, elevated release of IL-18 could be measured only in the tears of GO patients, but not in the GD or the control group.

We found a more than twofold IL-6 release in the tears of the GO group as compared to controls, and also a positive correlation between IL-6 release and CAS. We theorize that elevated IL-6 release in tears might be an indicator of disease activity. Increased IL-6 in GO is not novelty, others found serum IL-6 levels to be elevated in hyperthyroid GD and GO. Elevation of IL-6 tear concentration has also been described in Sjögren’s syndrome. Although GO is accompanied by dry eye, including the tear deficient form (non-Sjögren type dry eye) and the evaporative form due to exophthalmos, it is not associated with Sjögren’s syndrome. IL-6 level in tears of Sjögren’s syndrome patients is higher than in non-Sjögren type tear deficient dry eye. To evaluate lacrimal secretory capacity we performed Schirmer test that showed diminished tear secretion in both the GO and GD groups. Our findings correspond with the results of others who found significantly lower Schirmer test readings among Graves’ disease patients with and without orbitopathy than in controls.

The following important findings support the notion that increased cytokine releases are not the result of decreased lacrimal secretory capacity. We found a positive correlation between Schirmer test and all the cytokine releases tested, including IL-6, in the control group. However, neither positive nor negative correlation between Schirmer test and cytokine releases has been detected in the GO and GD groups. Instead, cytokine release and lacrimal secretory capacity are two distinct mechanisms. The lacrimal glands’ tear secretory function is damaged in GO and GD, while cytokine release is elevated as part of the immune process. This supports that elevated IL-6
release into tears is the result of the autoimmune inflammation, and its correlation with CAS suggests that it is a humoral indicator of disease activity.

IL-13 is a Th2 cytokine that plays a role in IgE-mediated immunity. In chronic ocular inflammation, where keratopathy is present, an increased level of IL-13 can be observed. About 30% of patients with Graves’ disease have increased concentrations of IgE in their sera. The elevated release of IL-13 in tears of patients with GO might be related to high serum IgE. Although it is more likely that it indicates the ocular surface reparation due to clinically non-detectable exposure keratopathy. The latter assumption is supported by the lack of fluorescein staining during slit lamp examination in any of our patients. As previously described, ocular surface pathology needs to be considered in GO patients even without detectable signs, as the autoimmune process itself also affects the anterior segment of the eye.

Cytokine release values of the GO group were higher when compared to the tears of healthy controls regarding to all examined markers (TNF-α, IL-1β, IL-6, IL-18, IL-17A, IL-13 and RANTES), while cytokine releases of the GD group did not differ from either the GO or the control group statistically. However, the release values tended to be highest in the GO group, followed by the GD and C groups. We assume that this might be the result of the clinically non-detectable orbital involvement of patients in the GD group. We speculate that there are GD patients who fail to present with clinically detectable orbitopathy, although their orbital connective tissues and lacrimal glands are already affected. Subclinical eye involvement in GD might remain silent but may proceed to manifest GO resulting in continuous changes in the orbital structures that are represented in tears. Thus, tear cytokine release in these GD patients tends to be higher than in
controls but remains below the values of GO patients, representing the possible manifestation of a subclinical disease.

PAI-1 release was significantly higher in the GO group than in the GD group, and it was the only marker with higher release in both the GO and GD groups than in group C. The tissue remodeling function of PAI-1 might explain its role in the pathogenesis and may account for the difference between the GD and GO groups in favor of the latter. Strong positive correlations have been found between the release of all examined cytokines and the release of PAI-1 in tears, supporting the previously described role of cytokines in PAI-1 gene activation. Similarly to IL-6 and PAI-1 releases correlated with CAS in our patients. The role of PAI-1 in normal tears is the maintenance of ocular surface integrity. At this point PAI-1 does not seem to be a practical marker of disease activity in GO. PAI-1 release in tears may either be an indicator of disease activity in GO or more likely the result of anterior segment changes in GO, which are also represented in CAS. Elevated release found in the GD group might be the result of the previously described “subclinical” disease.

Smoking is a known risk factor of GO. Even passive cigarette smoke exposure leads to alterations in the tear film and increase in inflammatory cytokine levels. Similar changes in the tear composition of healthy smokers and patients with GO have been reported. However we have not found any correlation between tear cytokine levels or releases and smoking history. Previously, Salvi et al. described similar findings on the lack of smoking-induced changes in serum IL-6, TNF-α and IL-1β concentrations.

Our findings demonstrate that Graves’ orbitopathy results in changes in the cytokine profile of the tear film. We detected an elevation of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6, IL-18, IL-17A, IL-13 and
RANTES in the tears of patients with GO. Elevated release of PAI-1 in GD tears, and a similar tendency in other examined markers refer to “subclinical” orbital involvement in Graves’ disease. Correlations between IL-6 release in tears and CAS was also detected. We are not aware of any previous studies on cytokines in GO and GD tear film. We propose that high IL-6 release in tears may serve as a useful indicator of disease activity in GO.

In the thesis the author presents two possible new techniques for GO activity evaluation. In the first part a series of investigations is presented using orbital DTPA SPECT which has been shown to be appropriate in the estimation of GO activity. DTPA SPECT recently became available for clinical practice. In the second part cytokine and PAI-1 studies are presented on tears of patients with GO and GD. With the development of molecular biology techniques, tear film analysis is becoming more simple and accessible. However, the introduction of these tests into everyday routine needs further investigations.
6. Summary of new results

6.1. $^{99m}$Tc-DTPA SPECT in the evaluation of disease activity in GO

I. $^{99m}$Tc-DTPA SPECT is able to predict the effectiveness of immunosuppressive therapy in GO

II. The upper normal limit of the DTPA uptake of the healthy orbit has been defined at 12.28 MBq/cm$^3$.

III. The orbital uptake value, above which patients are likely to benefit from corticosteroid treatment, so above which GO can be considered as immunologically active has been defined.

6.2. Tear cytokine, chemokine and PAI-1 composition in GO and GD

I. Alternations of the tear film in GO has been verified, as the release of various cytokines and a chemokine increases.

II. Release of mediators into tears is also elevated in GD, regarding to PAI-1 this increase has also been found significant. This supports the theory of subclinical orbital involvement in GD, without any ophthalmological signs and symptoms.

III. Lacrimal secretory capacity has no proven effect on the tear film composition of cytokines in GO; it is the result of the autoimmune process of the lacrimal gland and ocular surface.

IV. Correlation has been found between immunological activity (CAS) and the release of IL-6 into tears in GO. We assume that IL.6 release is a humoral indicator of disease activity.
List of publications related to the dissertation

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   IF: 4.327 (2010)

   DOI: http://dx.doi.org/10.1089/thy.2008.0298
   IF: 2.602

List of other publications


   *Szemészeti* 144, 111-114, 2007.

8. Szűcs-Farkas, Z., Tóth, J., Kollár, J., Galuska, L., Burman, K.D., Boda, J., Leővey, A., Varga, J., 
   DOI: http://dx.doi.org/10.1089/thy.2005.15.145 
   IF: 2.175

Total IF: 9.104
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The Candidate’s publication data submitted to the Publication Database of the University of Debrecen have been validated by Kerezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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7. List of other publications

7.1. List of cited abstracts related to the thesis


7.2. List of posters related to the thesis


Ujhelyi B, Varga Zs, Balázs E, Nagy EV, Berta A. Proinflammatory cytokines in tears of patients with Graves’ orbitopathy. Association for Research in Vision and Ophthalmology (ARVO) 2009 Annual Meeting, Fort Lauderdale, Florida USA 2009

7.3. List of oral presentations related to the thesis


_Ujhelyi B, Varga Zs, Balazs E, Nagy EV, Berta A_. Cytokines in tears of patients with Graves’ orbitopathy. Annual Congress of the Hungarian Ophthalmological Society, Budapest, Hungary, 2009


_Bodor M, Szabados L, Galuska L, Cseke B, Gazdag A, Ujhelyi B, Berta E, Nagy EV_.

_Ujhelyi B, Varga Zs, Balázs E, Nagy EV, Berta A_. Cytokines in tears of patients with Graves’ orbitopathy.
Annual Congress of the Hungarian Ophthalmological Society, HARVO Symposium Szeged, 2010

7.4. List of other presentations


