Graves' Orbitopathy Results in Profound Changes in Tear Composition: a Study of Plasminogen Activator Inhibitor-1 (PAI-1) and Seven Cytokines

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Running title: Tear cytokines in Graves' orbitopathy

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ABSTRACT

Background: Secretion of cytokines and display of cytokine receptors have been reported in the orbital connective tissue in Graves' orbitopathy (GO). Lacrimal glands are putative autoimmune targets, and changes in tear film and ocular surface have also been described. Our aim was to characterize the cytokine profile of tears in Graves' patients with (GO) and without orbitopathy (GD).

Methods: Tear samples were collected from 54 eyes of GO patients (age 43.4 ±15.2 years), 18 eyes of GD patients (age 46.8±11.7 years), and 24 control eyes (age 38.6±13.8 years). Patients underwent ophthalmological examination including Clinical Activity Score (CAS). The level of interleukin (IL)-1β, IL-6, IL-13, IL-17A, IL-18, Tumor Necrosis Factor-α and RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted) as well as Plasminogen activator inhibitor-1 (PAI-1) were measured by multiplex bead array and release values were calculated.

Results: The release of IL-1β, IL-6, IL-13, IL-17A, IL-18, TNF-α and RANTES were significantly higher in GO patients compared to controls ($p<0.05$). There was a 2.5-fold increase of IL-6 release. No significant differences were found in cytokine release between the GO and GD groups. In the GO group, significant positive correlation was found between CAS and the release of IL-6 and PAI-1 into tears ($r=0.27$, $p<0.05$ and $r=0.24$, $p<0.05$, respectively). PAI-1 release was significantly higher in GO than in GD patients, and was increased in both the GD and GO groups compared to controls.

Conclusions: Impaired cytokine balance has been observed in tears of GO patients. Secretion of IL-6 into tears might be a useful indicator of disease activity in GO.

INTRODUCTION

Graves' orbitopathy (GO) is an autoimmune disorder characterized by enlargement of extraocular muscles and increased volume in orbital connective tissues (1). Although GO is the most frequent extrathyroidal manifestation of Graves' disease, Hashimoto's thyroiditis can also be accompanied with orbitopathy (2). GO may also precede or follow the hyperthyroid phase of Graves' disease without any clinical or biochemical evidence of thyroid dysfunction (3). Retrobulbar inflammation takes place in the orbital connective tissue and eye muscles; mononuclear cells, predominantly T lymphocytes and macrophages infiltrate the orbital tissue (4). Interaction between activated T lymphocytes and orbital fibroblasts results in the release of cytokines into orbital connective tissue (5). Increased expression of macrophage- derived cytokines Interleukin (IL)-1β, Tumor Necrosis Factor-α (TNFα) and IL-10 have been described in orbital tissues of GO patients (6). Several studies have also shown increased IL-6 levels in the sera of GO patients (7, 8). 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 (9). Orbital tissue remodeling in GO seems to be the result of cytokine dependent fibroblast activation and fibroblasts themselves also express high levels of T lymphocyte chemoattractants as RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted) and IL-16 (10). A change in cytokine profile accompanies the phases of the disease, with Th1 cytokines predominating in the active phase, and Th2 cytokines in the late, fibrotic phase (11).

Immune activity in orbitopathy is neither synonymous nor coincident with the clinical severity of eye disease (12). Several approaches have been proposed to evaluate disease activity in GO. Among them, Clinical Activity Score (CAS) (13) has been shown to be a useful method in everyday clinical practice.

Although the inflammation takes place in the retrobulbar space, changes in the anterior segment of the eye also appear in GO. Tears play an essential role in maintaining the homeostasis of the ocular surface. Cytokine release into tears is involved in the recruitment and activation of inflammatory cells. Alternations in the pattern of tear cytokine balance can be observed during the day (14) or by eye closure (15). Studies on tear cytokines have shown that many ocular or systemic diseases lead to changes in the cytokine profile of the tear film (16). Changes in cytokine levels in human tears can be caused by seasonal allergic conjunctivitis (17), cystic fibrosis (18) and even anti-glaucoma eyedrops (19). Sjögren's syndrome and keratoconjunctivtis sicca are also accompanied by alterations of cytokine balance in the tear fluid and conjunctival epithelium (20), and IL-6 and TNF-α levels are elevated in tears of patients with dry eye (21). Passive cigarette smoke exposure leads to alterations of the tear film and increased tear inflammatory cytokine levels (22) . Similar changes in tear composition of healthy smokers and patients with GO have been reported (23).

Early studies reported abnormally high tear osmolarity and rapid tear film break up time in patients with GO (24). Alterations in tear film profile, low break up time and Rose Bengal staining in late GO indicate the presence of drying epithelial cells (25). Changes in tear protein profile by high-performance liquid chromatography (HPLC) have also been published, suggesting that GO has an effect on the lacrimal gland (26). Lacrimal gland enlargement was found in 22% of GO patients by orbital computed tomography (27). Expression of the TSH receptor on lacrimal gland acinar cells has also been reported. In GO Thyroid Stimulating Hormone (TSH) may bind to these cells causing lacrimal gland impairment (28). Also, lacrimal glands have been described as target organs for thyroid hormone (29). Probable involvement of the lacrimal gland as an area of immunological reaction in GO have been suggested based on octreotide scintigraphy (30, 31). Impression cytology findings also underline the ocular surface changes in GO (32). According to

proteomic analysis (biomarkers with a molecular weight between 3000 and 20 000 Da) the majority of tear proteins are downregulated in GO, with only few overexpressed proteins (33). Specific polymorphisms in proinflammatory cytokine genes (IL-12, Interferon γ (IFNγ), TNFα and IL-1β) are associated with susceptibility to GO among Graves' disease patients (34, 35).

Plasminogen activator inhibitor-1 (PAI-1), besides being the inhibitor of fibrinolysis, also plays an important role in regulation of vascular function and tissue remodeling (36, 37). TNFα is an agonist for PAI-1 expression and the role of this cytokine in PAI-1 gene activation is well established (38). Increased PAI-1 activity has been found in the serum of Graves' disease patients (39) .

In the present study, we investigated the cytokines IL-1β, IL-6, IL-13, IL-17A, IL-18, TNF-α and the chemokine RANTES, as well as PAI-1 in tear samples of patients with Graves' orbitopathy (GO), in patients with Graves' disease without orbitopathy (GD) and in healthy controls.

METHODS

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) according to standard criteria (40). 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. Clinical Activity Score (CAS)(13) was obtained in each case. 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. 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 were taken. The control group (C) consisted 24 eyes of twelve healthy volunteers (4 males, 8 females, age 38.6±13.8 yrs). All enrolled patients gave informed consent and the Institutional Review Board approved the study protocol in accordance with the 1989 Declaration of Helsinki.

Tear samples were obtained by 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. The amount of the tear sample collected (µl), and the collection time (sec) were recorded. The samples were frozen without centrifugation within 15 minutes of collection and stored at –70 ℃ until cytokine measurements were performed. Preliminary studies had demonstrated that centrifugation of the samples did not influence cytokine concentrations.

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 MedSystems GmbH, eBioscience Company, Vienna, Austria). Briefly, tear 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 150 µl 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 FlowCytomix^{TM} Pro 2.4 software. 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. Assay sensitivities 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.

Tears were collected from each eye for 2 minutes. For more precise evaluation we measured with a timer the exact collection time in seconds. The amount collected was also registered. The volume was calculated from the length of the tear column in the tube (100 mm length by 0.5 mm inner diameter capillary tubes were used) and was also checked when pipetting the samples for evaluation. We got the results of the flow cytometric assay in pg/µl for all cytokine concentrations. The result was multiplied with the volume of tear collected (µl) and divided by the collection time (sec).

$$\text{Release of cytokine} = \frac{\text{Concentration of cytokine in tear sample x Volume of tear collected}}{\text{Collection time}}$$

The 2 minutes cytokine release values (pg/2 min) were used for further statistical analysis.

Statistical analysis was carried out using the SAS for Windows 8.2 software. Hormone and cytokine levels in the three study groups (GO, GD and C) 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

Medians and upper and lower quartiles of both concentrations and release values of cytokines in each patient group are shown in Table 1. As release values reflect the real cytokine production, these were used for further analysis.

ANOVA analysis showed significant difference 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 regarding any cytokine tested, 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 (Fig. 1). 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 the control group's (Table 1).

Positive correlation were found between the release of all tested cytokines and the secretion of PAI-1 in tears (IL-1β: $r=0.23$ $p=0.002$, IL- 6: $r=0.31$ $p=0.001$, IL- 13: $r=0.30$ $p=0.001$, IL-17A: $r=0.23$ $p=0.015$, IL-18: $r=0.24$ $p=0.012$, TNF-α: $r=0.29$ $p=0.002$ and RANTES: $r=0.23$ $p=0.014$).

We have found strong correlation between the release of all examined cytokines, 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: $r=0.89$, $p<0.001$).

Except for IL-17A, we found no correlation between tear cytokine concentrations and age; for IL-17A, the correlation was negative ($r= -0.21$, $p<0.05$). Also, a weak negative correlation was found between age and PAI-1 release ($r= -0.24$ $p<0.05$). ANOVA analysis showed no significant difference between the GO and GD groups regarding serum TSH, free thyroid hormone (fT4, fT3) and TSH receptor antibody levels.

We found significant difference between the left and right eyes in the release of all examined cytokines by both paired T test and Wilcoxon Matched Pairs Test ($p<0.05$ for all examined cytokines, and $p<0.001$ for Il-6, IL-18 and PAI-1).

Ophthalmological evaluation of GO patients showed the signs of ocular surface drying. However, no corneal pathology (fluorescein staining, erosion or ulceration) was present at the time of sample collection. 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, however, both were significantly lower than the control group. The mean BUT was 3.58 ± 2.35 sec in the GO group (range from 4 to 32 sec in healthy adults (41)). The CAS in the GO group was 3.82 ± 2.0.

Positive correlations were 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 were 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 were correlated with the lacrimal secretory capacity (Schirmer I test) and the BUT in the GO or GD group. We found a weak positive correlation between Schirmer I test and PAI-1 relese in the GO group (r=0.28 p=0.04) but not in the GD group.

In the GO group, a positive correlation was found between IL-6 release and the 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). Also positive correlation was found between PAI-1 release and the CAS (r=0.24, p=0.03).

Among the twenty-seven GO patients 10 were former or current smokers (cumulative number of cigarettes smoked 78309±53631 consuming an average of 14.73±6.61 cigarettes/day), while among the nine GD patients without orbitopathy 6 were former 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 former 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 differences were found in the release of any examined cytokine.

DISCUSSION

Tears play an essential role in maintaining the homeostasis of the ocular surface. Many ocular or systemic diseases have been shown to lead to changes in the cytokine profile of the tear film (16-21). In this study, we monitored the level of a selected set of cytokines that included TNF-α, IL-1β, IL-6, IL-18, IL-17A, IL-13, the chemokine RANTES and PAI-1 in tear samples of Graves' patients with and without orbitopathy.

The multiplex bead array, which was used in the present series of experiments, is a highly sensitive technique which enabled us to carry out parallel measurements of multiple cytokines by flow citometry in a small amount of tear. In contrast to previous results showing higher tissue concentrations of TNF-α, IL-1β and IL-6 (42) as well as serum concentrations of IL-6 (43) and PAI-1 in Graves' disease when compared to healthy controls, we found no significant difference between the GD and control groups in the release of tear cytokines. These findings indicate that tear cytokines are regulated differently, and the appearance of proinflammatory cytokines in the tears of patients with GO, but not in those with GD without orbitopathy, is not the result of the thyroid disease but the orbitopathy itself. Sources of cytokines and chemokines in tears may include the main and accessory lacrimal glands, the corneal and conjunctival fibroblasts and the immunovigilant cells normally present in the ocular surface (44). Previous studies have shown that the lacrimal gland is involved in GO and that it could also be an area of immunological reaction (26-31). Reports have been published on the early conjunctival involvement in Graves' disease, suggesting that the ocular surface impairment in GO is not only a consequence of mechanical events but ocular surface tissues are direct targets for autoantibodies in GD (45). Conjunctival and episcleral inflammation can occur before the classic signs of orbitopathy and the pathogenesis is assumed to be similar to that involved in the extraocular muscle inflammation and lymphocytic infiltration in GO (46).

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 (5, 6) and were shown to stimulate ICAM-1 expression and GAG production by orbital fibroblasts (9). 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 chemoattractants such as RANTES (10) which may explain the increased release of RANTES in tears of patients with GO. A strong correlation between the releases of IL-1β and RANTES as well as IL-1β and IL-18 was also demonstrated in our study. Increased IL-18 serum levels in both GO and GD individuals have also been observed by others (47). 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.

As we found a more than twofold IL-6 release in the tears of the GO group as compared to controls, we theorize that elevated IL-6 release in tears might be an indicator of disease activity. This assumption is further supported by the fact that in the GO group, we found a positive correlation between IL-6 release and the CAS. A positive correlation was also found between IL-6 release and the degree of eyeball protrusion.

Others found serum IL-6 levels to be elevated in hyperthyroid GD and GO (43). However, the serum levels are influenced by additional factors, such as thyroid status and therapy, which limits its use as an activity indicator in GO (8). Elevation of IL-6 tear concentration has also been described in dry eye and dysfunctional tear syndrome (48). Although GO is accompanied by dry eye, including the tear deficient form (non-Sjögren type dry eye) (24) and the evaporative form due to exophthalmos (25), GO is not associated with Sjögren's syndrome. IL-6 levels in tears of Sjögren's syndrome patients is higher than in non-Sjögren type tear deficient dry eye (21). To evaluate lacrimal secretory capacity we performed the Schirmer I test. In both the GO and GD groups tear secretion was diminished. Our findings correspond to the results of Gurdal et al. (49) who found significantly lower Schirmer test readings among Graves' disease patients with and without orbitopathy than in controls. The authors suggested that ocular surface damage and accompanying surface inflammation may precede the development of classic GO symptoms (49). However, elevated

proinflammatory cytokine secretion into tears may also be the consequence of ocular surface inflammation, even when inflammation cannot be detected with clinical examination methods, and thus considered as an early sign for ocular involvement in GD. We found a positive correlation between Schirmer I test and all the cytokine releases tested, includig IL-6, in the control group. However, neither a positive or a negative correlation between Schirmer I test and cytokine releases was detected in the GO and GD groups. This important finding supports the notion that increased cytokine releases are not the result of decreased lacrimal secretory capacity. 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.

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 together with eotaxin-1 (50). About 30% of patients with Graves' disease have increased concentrations of IgE in their sera, and in one third of patients with hyperthyroid Graves' disease, Th2 cells are stimulated and secrete excess amounts of IL-13 (51). The elevated release of IL-13 in tears of patients with GO might be related to high serum IgE, or may be simply an indicator of ocular surface reparation of 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.

We believe that, to some extent, the ocular surface including the conjunctiva, is involved in the autoimmune process in patients with Graves' disease. Cytokine release values of the GO group were higher only when compared to the tears of healthy controls, while cytokine relases of the GD group did not differ from either the GO or the control group. However, the release values tended to be highest in the GO group, followed by the GD and C groups (Fig. 1). 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 is common in the orbits: extraocular muscle enlargement have been found in nearly 70% of adult patients with Graves' hyperthyroidism by orbital imaging (52). 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 was higher in both the GO and GD groups than in group C (Table 1). Others have reported higher PAI-1 concentrations in the plasma of GD patients compared to controls (39). In our series, the only examined protein that showed a statistically increased release in GO patients compared to GD patients was PAI-1. The tissue remodeling function of PAI-1 (36, 37) may account for the difference between the GD and GO groups in favour of the latter. The role of PAI-1 in normal control tears is the maintenance of ocular surface integrity (53). 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 (38). Both IL-6 and PAI-1 releases correlated with CAS in our patients. PAI-1 release in tears may either be an indicator of disease activity in GO or simply the result of anterior segment changes in GO, which are also represented in CAS. However, unlike IL-6 release, PAI-I does not seem to be a practical marker of disease activity in GO.

Passive cigarette smoke exposure leads to alterations in the tear film and increase in inflammatory cytokine levels (22). Similar changes in the tear composition of healthy smokers and patients with GO have been reported (23). We found no correlation between tear

cytokine levels or releases and smoking history. Previously, Salvi at al. described similar findings. These being the lack of smoking-induced changes in serum IL-6, TNF-α and IL-1β concentrations (8).

We are not aware of any previous studies on cytokines and PAI-1 in tears of patients with Graves' disease. We are the first to show that there is a correlation between disease activity and IL-6 release in tears in GO. 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. Correlations between IL-6 release in tears and CAS as well as the degree of eyeball protrusion were also detected. We propose that high IL-6 release in tears may serve as a useful indicator of disease activity in GO.

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REFERENCES

1. Burch HB, Wartofsky L 1993 Graves ophthalmopathy - Current concepts regarding pathogenesis and management. Endocr Rev **14**:747-793.

2. Marcocci C, Bartalena L, Bogazzi F, Panicucci M, Pinchera A 1989 Studies on the occurence of ophthalmopathy in Graves-disease. Acta Endocrinol **120**:473-478.

3. Solomon DH, Chopra IJ, Chopra U, Smith FJ 1977 Identification of subgroups of euthyroid Graves ophthalmopathy. N Engl J Med **296**:181-186.

4. Weetman AP, Cohen S, Gatter KC, Fells P, Shine B 1989 Immunohistochemical analysis of the retrobulbar tissues in Graves Ophthalmopathy. Clin Exp Immunol **75**:222-227.

5. Natt N, Bahn RS 1997 Cytokines in the evolution of Graves' ophthalmopathy. Autoimmunity **26**:129-136.

6. Kumar S, Bahn RS 2003 Relative overexpression of macrophage-derived cytokines in orbital adipose tissue from patients with Graves' ophthalmopathy. J Clin Endocrinol Metab **88**:4246-4250.

7. Wakelkamp I, Gerding MN, Van Der Meer JWC, Prummel MF, Wiersinga WM 2000 Both Th1-and Th2-derived cytokines in serum are elevated in Graves' ophthalmopathy. Clin Exp Immunol **121**:453-457.

8. Salvi M, Pedrazzoni M, Girasole G, Giuliani N, Minelli R, Wall JR, Roti E 2000 Serum concentrations of proinflammatory cytokines in Graves' disease: effect of treatment, thyroid function, ophthalmopathy and cigarette smoking. Eur J Endocrinol **143**:197-202.

9. Cawood TJ, Moriarty P, O'Farrelly C, O'Shea D 2006 The effects of tumour necrosis factor-alpha and interleukin1 on an in vitro model of thyroid-associated ophthalmopathy; contrasting effects on adipogenesis. Eur J Endocrinol **155**:395-403.

10. Gianoukakis AG, Khadavi N, Smith TJ 2008 Cytokines, Graves' disease, and thyroid-associated ophthalmopathy. Thyroid **18**:953-958.

11. Xia N, Zhou SX, Liang YZ, Xiao CQ, Shen HL, Pan HL, Deng HM, Wang NZ, Li QDQ 2006 CD4(+) T cells and the Th1/Th2 imbalance are implicated in the pathogenesis of Graves' ophthalmopathy. Int J Mol Med **17**:911-916.

12. Wiersinga WM, Bartalena L 2002 Epidemiology and prevention of Graves' ophthalmopathy. Thyroid **12**:855-860.

13. Mourits MP, Prummel MF, Wiersinga WM, Koornneef L 1997 Clinical activity score as a guide in the management of patients with Graves' ophthalmopathy. Clin Endocrinol **47**:9-14.

14. Uchino E, Sonoda S, Kinukawa N, Sakamoto T 2006 Alteration pattern of tear cytokines during the course of a day: Diurnal rhythm analyzed by multicytokine assay. Cytokine **33**:36-40.

15. Uchino E, Sonoda S, Nakao K, Sakamoto T 2006 Alteration of tear cytokine balance by eye closure: analysis by multicytokine assay. Graefes Arch Clin Exp Ophthalmol **244**:747-749.

16. Fodor M, Facsko A, Rajnavolgyi E, Harsfalvi J, Bessenyei E, Kardos L, Berta A 2006 Enhanced release of IL-6 and IL-8 into tears in various anterior segment eye diseases. Ophthalmic Res **38**:182-188.

17. Leonardi A, Curnow SJ, Zhan H, Calder VL 2006 Multiple cytokines in human tear specimens in seasonal and chronic allergic eye disease and in conjunctival fibroblast cultures. Clin Exp Allergy **36**:777-784.

18. Mrugacz M, Kaczmarski M, Bakunowicz-Lazarczyk A, Zelazowska B, Wysocka J, Minarowska A 2006 IL-8 and IFN-gamma in tear fluid of patients with cystic fibrosis. J Interferon Cytokine Res **26**:71-75.

19. Malvitte L, Montange T, Vejux A, Baudouin C, Bron AM, Creuzot-Garcher C, Lizard G 2007 Measurement of inflammatory cytokines by multicytokine assay in tears of patients with glaucoma topically treated with chronic drugs. Br J Ophthalmol **91**:29-32.

20. Pflugfelder SC, Jones D, Ji ZH, Afonso A, Monroy D 1999 Altered cytokine balance in the tear fluid and conjunctiva of patients with Sjogren's syndrome keratoconjunctivitis sicca. Curr Eye Res **19**:201-211.

21. Yoon KC, Jeong IY, Park YG, Yang SY 2007 Interleukin-6 and tumor necrosis factor-alpha levels in tears of patients with dry eye syndrome. Cornea **26**:431-437.

22. Rummenie VT, Matsumoto Y, Dogru M, Wang Y, Hu YQ, Ward SK, Igarashi A, Wakamatsu T, Ibrahim O, Goto E, Luyten G, Inoue H, Saito I, Shimazaki J, Tsubota K 2008 Tear cytokine and ocular surface alterations following brief passive cigarette smoke exposure. Cytokine **43**:200-208.

23. Baker GRC, Morton M, Rajapaska RS, Bullock M, Gullu S, Mazzi B, Ludgate M 2006 Altered tear composition in smokers and patients with graves ophthalmopathy. Arch of Ophthalmol **124**:1451-1456.

24. Gilbard JP, Farris RL 1983 Ocular surface drying and tear film osmolarity in thyroid eye disease. Acta Ophthalmol **61**:108-116.

25. Khurana AK, Sunder S, Ahluwalia BK, Malhotra KC 1992 Tear film profile in Graves ophthalmopathy. Acta Ophthalmol **70**:346-349.

26. Khalil HA, Dekeizer RJW, Kijlstra A 1988 Analysis of tear proteins in Graves ophthalmopathy by high-performance liquid-chromatography. Am J Ophthalmol **106**:186-190.

27. Chang TC, Huang KM, Chang TJ, Lin SL 1990 Correlation of orbital computed-tomography amd antibodies in patients with hyperthyroid Graves-disease. Clin Endocrinol **32**:551-558.

28. Eckstein AK, Finkenrath A, Heiligenhaus A, Renzing-Kohler K, Esser J, Kruger C, Quadbeck B, Steuhl KP, Gieseler RK 2004 Dry eye syndrome in thyroid-associated ophthalmopathy: lacrimal expression of TSH receptor suggests involvement of TSHR-specific autoantibodies. Acta Ophthalmol Scand **82**:291-297.

29. Dias AC, Modulo CM, Jorge AG, Braz AM, Jordao AA, Bertazolli R, de Paula JS, Rocha EM 2007 Influence of thyroid hormone on thyroid hormone receptor beta-1 expression and lacrimal gland and ocular surface morphology. Invest OphthalmolVis Sci **48**:3038-3042.

30. Moncayo R, Baldissera I, Decristoforo C, Kendler D, Donnemiller E 1997 Evaluation of immunological mechanisms mediating thyroid-associated ophthalmopathy by radionuclide imaging using the somatostatin analog In-111-octreotide. Thyroid **7**:21-29.

31. Kainz H, Bale R, Donnemiller E, Gabriel M, Kovacs P, Decristoforo C, Moncayo R 2003 Image fusion analysis of Tc-99m-HYNIC-octreotide scintigraphy and CT/MRI in patients with thyroid-associated orbitopathy: the importance of the lacrimal gland. Eur J Nucl Med Mol Imaging **30**:1155-1159.

32. Cavusoglu T, Nurozler AB, Titiz C, Ustun H, Aral Y, Ozer E, Kasim R, Tezel S 2007 Tear function and ocular surface changes in multinodular goiter patients. Ophthalmologica **221**:264-268.

33. Okrojek R, Grus FH, Matheis N, Kahaly GJ 2009 Proteomics in Autoimmune Thyroid Eye Disease. Horm Metab Res **41**:465-470.

34. Anvari M, Khalilzadeh O, Esteghamati A, Esfahani SA, Rashidi A, Etemadi A, Mahmoudi M, Amirzargar AA 2010 Genetic susceptibility to Graves' ophthalmopathy: the role of polymorphisms in proinflammatory cytokine genes. Eye **24**:1058-1063.

35. Liu YH, Chen RH, Wu HH, Liao WL, Chen WC, Tsai YH, Tsai CH, Wan L, Tsai FJ 2010 Association of Interleukin-1 beta (IL1B) Polymorphisms with Graves' Ophthalmopathy in Taiwan Chinese Patients. Invest Ophthalmol Vis Sci **51**:6238-6246.

36. Fay WP 2004 Plasminogen activator inhibitor 1, fibrin, and the vascular response to injury. Trends Cardiovas Med **14**:196-202.

37. Gils A, Declerck PJ 2004 Plasminogen activator inhibitor-1. Curr Med Chem **11**:2323-2334.

38. Hou BD, Eren M, Painter CA, Covington JW, Dixon JD, Schoenhard JA, Vaughan DE 2004 Tumor necrosis factor alpha activates the human plasminogen activator inhibitor-1 gene through a distal nuclear factor kappa B site. J Biol Chem **279**:18127-18136.

39. Wahrenberg H, Wennlund A, Hoffstedt J 2002 Increased adipose tissue secretion of interleukin-6, but not of leptin, plasminogen activator inhibitor-1 or tumour necrosis factor alpha, in Graves' hyperthyroidism. Eur J Endocrinol **146**:607-611.

40. Wiersinga WM, Perros P, Kahaly GJ, Mourits MP, Baldeschi L, Boboridis K, Boschi A, Dickinson AJ, Kendall-Taylor P, Krassas GE, Lane CM, Lazarus JH, Marcocci C, Marino M, Nardi M, Neoh C, Orgiazzi J, Pinchera A, Pitz S, Prummel MF, Sartini MS, Stahl M, von Arx G, European Grp Graves Orbitopathy EU 2006 Clinical assessment of patients with Graves' orbitopathy: the European Group on Graves' Orbitopathy recommendations to generalists, specialists and clinical researchers. Eur J Endocrinol **155**:387-389.

41. Paschides CA, Kitsios G, Karakostas KX, Psillas C, Moutsopoulos HM 1989 Evaluation of tear break-up time, Schirmers-I test and Rose-Bengal staining as confirmatory tests for keratoconjunctivitis sicca. Clin Exp Rheumatol **7**:155-157.

42. Feldmann M, Brennan FM, Chantry D, Haworth C, Turner M, Katsikis P, Londei M, Abney E, Buchan G, Barrett K, Corcoran A, Kissonerghis M, Zheng R, Grubeckloebenstein B, Barkley D, Chu CQ, Field M, Maini RN 1991 Cytokine assays - role in evaluation of the pathogenesis of autoimmunity. Immunol Rev **119**:105-123.

43. Salvi M, Girasole G, Pedrazzoni M, Passeri M, Giuliani N, Minelli R, Braverman LE, Roti E 1996 Increased serum concentrations of interleukin-6 (IL-6) and soluble IL-6 receptor in patients with Graves' disease. J Clin Endocrinol Metab **81**:2976-2979.

44. Carreno E, Enriquez-de-Salamanca A, Teson M, Garcia-Vazquez C, Stern ME, Whitcup SM, Calonge M 2010 Cytokine and chemokine levels in tears from healthy subjects. Acta Ophthalmol **88**:e250-e258.

45. Versura P, Campos EC 2010 The ocular surface in thyroid diseases. Curr Opin Allergy Clin Immunol **10**:486-492.

46. Gupta A, Sadeghi PB, Akpek EK 2009 Occult Thyroid Eye Disease in Patients Presenting with Dry Eye Symptoms. Am J Ophthalmol **147**:919-923.

47. Mysliwiec J, Kretowski A, Stepien A, Mironczuk K, Kinalska I 2003 Interleukin 18 and transforming growth factor beta 1 in the serum of patients with Graves' ophthalmopathy treated with corticosteroids. Int Immunopharmacol **3**:549-552.

48. Lam H, Bleiden L, De Paiva CS, Farley W, Stern ME, Pflugfelder SC 2009 Tear Cytokine Profiles in Dysfunctional Tear Syndrome. Am J Ophthalmol **147**:198-205.

49. Gurdal C, Sarac O, Genc I, Kirimlioglu H, Takmaz T, Can I 2011 Ocular Surface and Dry Eye in Graves' Disease. Curr Eye Res **36**:8-13.

50. Cook EB 2004 Tear cytokines in acute and chronic ocular allergic inflammation. Curr Opin Allergy Clin Immunol **4**:441-445.

51. Yamada T, Sato A, Komiya I, Nishimori T, Ito Y, Terao A, Eto S, Tanaka Y 2000 An elevation of serum immunoglobulin E provides a new aspect of hyperthyroid Graves' disease. J Clin Endocrinol Metab **85**:2775-2778.

52. Enzmann DR, Donaldson SS, Kriss JP 1979 Appearance of Graves-disease on orbital computed-tomography. J Comput Assist Tomogr **3**:815-819.

53. Wang ZY, Sosne G, Kurpakus-Wheater M 2005 Plasminogen activator inhibitor-1 (PAI-1) stimulates human corneal epithelial cell adhesion and migration in vitro. Exp Eye Res **80**:1-8.

Table 1. Concentrations and release values of tear cytokines and PAI-1

		Release			Concentration		
		Median	**Lower quartile**	**Upper quartile**	**Median**	**Lower quartile**	**Upper quartile**
IL-1 β	C	9,58	4,47	14,04	701,02	403,99	974,41
	GD	16,13	5,58	19,51	797,83	384,2	1124,57
	GO	20,67*	6,04	32,32	1083,54	585,47	1263,51
IL-6	C	1,61	0,72	2,01	119,7	66,38	152,12
	GD	2,62	1,13	3,98	133,03	90,5	166,99
	GO	4,07*	0,92	6,65	204,19	100,95	243,56
IL-13	C	5,25	2,63	7,19	424,45	220,14	515,85
	GD	7,73	3,38	10,63	444,34	298,83	545,33
	GO	9,31*	4,29	13,38	547,28	317,01	692,33
IL-17A	C	3,39	0,32	4,97	179,21	70,27	258,44
	GD	5,52	0,67	7,13	257,57	78,05	299,68
	GO	8,39*	1,23	12,22	386,34	95,71	455,27
TNF-α	C	3,97	1,42	6,18	264,31	168,34	307,62
	GD	6,58	2,09	8,98	332,89	167,21	418,03
	GO	8,58*	2,11	12,44	444,31	178,06	535,31
RANTES	C	6,4	1,38	9,69	379,41	246,44	479,59
	GD	9,74	2,83	15,08	496,96	267,57	663,63
	GO	12,34*	3,69	19,97	648,81	322,94	740,32
IL-18	C	29,96	9,91	49,77	1855,66	1158,36	2581,87
	GD	50,83	14,67	70,82	2480,56	1213,82	3553,84
	GO	66,28*	15,79	100,87	3411,37	1722,58	3913,63
PAI-1	C	6,87	3,025	9,9	366,67	227,89	687,5
	GD	9,81*	1,93	15,95	463,89*	210,07	623,33
	GO	11,73**	1,38	22	555,32**	265,83	673,75

Table and figure legends:

Table 1. Concentrations and release values of tear cytokines and PAI-1

The concentrations and release values are expressed as pg/ml and pg/2min, respectively. Values marked with * are significantly different form control ($p<0.05$). ** shows that PAI release and concentration are significantly higher in the GO group compared to the GD group. Values were compared by analysis of variance (ANOVA) with Duncan post hoc testing.
GO - Graves' orbitopathy, GD – Graves' disease without orbitopathy, C - Controls

Figure 1. Median of tear cytokine and PAI-1 release values into tears (pg/2min)

Cytokine releases marked with *are significantly different form controls ($p<0.05$). GD patients were intermediate between the GO and the control groups in the case of all cytokines. This also applies to IL-18 (not shown in the figure).

Figure 1. Median of tear cytokine and PAI-1 release values into tears (pg/2min)