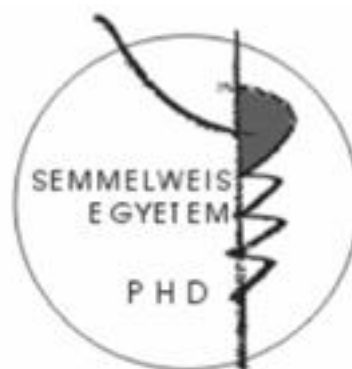


# Investigation of the tear film and conjunctival samples in patients with different grade of conjunctivochalasis

Doctoral thesis

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## Introduction

Conjunctivochalasis (CCh) is a redundant, loose, non –oedematous, bilateral, bulbar conjunctival folds interposed between the globe and the eyelids. Different grades of this condition lead to tear film instability and epiphora in severe cases. In the last century, severe CCh was found worthy of attention as a cause of marginal corneal ulcers and subconjunctival haemorrhage. Later moderate CCh was described as a reason of disorder of tear clearance with intermittent but frequent tearing, and occlusion of the inferior lacrimal punctum. Nowadays, mild CCh is taken into consideration also as possible reason of an unstable tear film and dry eye. The prevalence and incidence of CCh have been only partially studied. In the first two decades of life the prevalence of CCh is very low and increases steadily after the third decade, becoming a common condition in elderly people. Symptoms complained by patients vary according to the severity of the clinical picture. At the mild stage CCh may be asymptomatic or may induce significant changes of the tear film and epiphora in severe cases.

The main hypotheses have been referred about the pathogenesis of CCh are immune based inflammation of the conjunctiva and breakdown of elastic fibres in the subconjunctival tissue. These are the most important pathogenetic mechanism responsible for the development of CCh.

Ocular surface inflammation in conjunctivochalasis can be investigated by several methods. HLA-DR expression of conjunctival epithelial cells can be investigated by flow cytometry. HLA-DR molecule is expressed by the antigen presenting cells; however, in immune based ocular surface inflammation conjunctival epithelial cells also express HLA – DR on their surface.

Variation of the qualitative or quantitative parameters of the tear film or tear film instability leads to tear hyperosmolarity. Hyperosmolar stress causes cytokine release from the ocular surface cells and apoptosis. This vicious circle influences permanent ocular surface inflammation.

After conjunctival excision of patient with sever conjunctivochalasis, sings of the chronic inflammation can be investigated in conjunctival tissue samples. Conjunctival epithelial changes, lymphocytes, goblet cells and elastic degeneration can be observed.

## **Purpose**

### **1. Analysis of HLA-DR expression of conjunctival epithelial cells in different grade of conjunctivochalasis**

In our study the aim was to evaluate and compare HLA-DR expression of conjunctival epithelial cells in different grade of conjunctivochalasis by impression cytology samples of the upper bulbar conjunctiva.

### **2. Investigation of the tear film osmolarity in different grade of conjunctivochalasis**

The aim of our study was to evaluate and compare the tear film osmolarity as a sign of ocular surface inflammation in patients with different grade of conjunctivochalasis.

### **3. Histological analysis of conjunctival samples in severe conjunctivochalasis**

The aim of our case series was to evaluate the chronic inflammation markers in severe cases of conjunctivochalasis after conjunctival excision. We compared the inflammation signs and the subconjunctival elastosis in these samples.

## Methods

### **1. Analysis of HLA-DR expression of conjunctival epithelial cells in different grade of conjunctivochalasis**

Thirty subjects (mean age:  $57 \pm 21$  yrs) and seven healthy controls (mean age:  $48 \pm 16$  yrs) participated in the clinical examination. Based on the LIPCOF grade, the patients were divided into three groups. Patients with other source of dry eye and ocular surface disease and with previous ocular surgery in their clinical history were excluded. Furthermore, systemic diseases, which are able to influence inflammation of the ocular surface, were excluded. Patients were observed with slit lamp. Diagnostic tests were performed consecutively. At first, conjunctivochalasis was graded with LIPCOF test. After all vital staining of the ocular surface was performed with fluorescein installation and tear film break-up time was measured. Finally, lissamine-green staining and Schirmer test were also performed. Impression cytology was performed on all the three groups included in the study. Specimens were collected at least 15 min after instillation of the last staining eye drop to avoid any interference with immunofluorescence analysis. Samples were obtained after administration of topical anesthesia. Two separate polyethersulfone filters were applied on the upper bulbar conjunctiva of both eyes, and removed after 5 - 10 sec. All the samples were immediately stored in tubes containing 2 ml of cell culture medium (RPMI 1640) containing 10% of foetal calf serum (FCS) and kept at  $4^{\circ}\text{C}$  before laboratory evaluation. Immunofluorescence staining was performed to allow flow cytometry analysis using standard procedures. Two sets of antibodies and corresponding negative controls were used. Mouse-anti-human-cytokeratin19, clone K4.26, was purchased from Sigma-Aldrich; goatanti-mouse-IgG1 PE, mouse-anti-human-HLA-DR FITC and isotype control mouse IgG2a FITC were purchased from BD Bioscience. Cells were analyzed by flow cytometry using FACSCalibur and CELLQuestPro software (BD Bioscience). HLA-DR expression on conjunctival epithelial cells was analyzed by gating on CK19 positive cells.

### **2. Investigation of the tear film osmolarity in different grade of conjunctivochalasis**

Forty subjects (mean age:  $59.1 \pm 18.6$  years) were divided into four groups based on their lid-parallel conjunctival fold (LIPCOF) grade. Patients with local or systemic conditions which may influence the osmolarity of tear were excluded (including dry eye and ocular surface diseases in history, previous ocular surgery in their clinical history or systemic diseases which may lead to ocular surface inflammation). Furthermore, use of any ophthalmic

eye drops or ointment was avoided for a minimum of 5 days before collecting samples. Tear samples were collected from the right eyes of patients in order to measure tear osmolarity by TearLab System. Thereafter, classical dry eye tests have been performed. Tear osmolarity test was the first to do – before slit lamp examination – by TearLab to minimize reflex tearing. Tear fluid was collected from the tear meniscus adjacent to the temporal canthus without touching the cornea and ocular surface to avoid any reflex tearing. One single measurement was done on each patient, because repeated collecting of tear could stimulate reflex tearing. Conjunctivochalasis was graded with LIPCOF test according to the description of H $\ddot{o}$ h et al. After all vital staining of the ocular surface was performed with fluorescein staining and tear film BUT was measured. Finally, lissamine-green staining and Schirmer test were performed.

### **3. Histological analysis of conjunctival samples in severe conjunctivochalasis**

In our case series 10 samples of 6 patients (4 female, mean age: 68 years and 2 male, mean age: 53 years) with severe conjunctivochalasis underwent conjunctival excision. All of these patients were examined and operated by the Tenets of Declaration of Helsinki. Patients with local or systemic inflammatory conditions were excluded (including ocular surface diseases in history, previous ocular surgery in their clinical history or systemic diseases which may lead to ocular surface inflammation). Before the operation all patients were examined with slit lamp and conjunctivochalasis was graded by LIPCOF test. Conjunctival excision was performed at least 2 mm far from the limbal area in the region of the folds. Wound closure was performed by suturing or tissue glue (Tissucol Duo®, Baxter, Wien). Samples were collected and fixated by overnight formalin buffer solution. After bedding of paraffin every samples were observed by microscope after histological staining.

## Results

### **1. HLA-DR expression of conjunctival epithelial cells in different grade of conjunctivochalasis**

No difference in age and gender was found among the groups, except for the severe cases where participants were predominantly female. Patients with severe CCh had a higher rate of HLA-DR positive conjunctival epithelial cells. This difference between the patients with severe CCh and normal controls was statistically significant (Kruskal–Wallis test,  $p = 0.0011$ ). However, mild and moderate study groups showed similar expression of HLA-DR to normal subjects. Positive correlation was found between fluorescein staining and conjunctival HLA-DR expression (Kendall-tau–b test,  $r = 0.36$ ,  $p = 0.009$ ) and between lissamin–green staining and HLA-DR expression (Kendall-tau–b–test,  $r = 0.30$ ,  $p = 0.027$ ). Significant differentiation was found between expression of HLA-DR and Schirmer test (Mann-Whitney test,  $p=0.009$ ). Positive correlation was found between HLA\_DR expression and BUT (Kendall-tau-b test,  $r = 0,46$ ,  $p = 0.0001$ ) if patients were analysed non-parametric correlation, but no relation was found (Mann-Whitney-test,  $p=0.45$ ) if patients were analysed as subgroups.

### **2. Tear film osmolarity in different grade of conjunctivochalasis**

No difference in age and gender was found among the groups. Difference of tear osmolarity between normal controls and severe groups was statistically significant (Kruskal–Wallis test,  $p = 0.0007$ ). However, mild and moderate study groups showed similar osmolarity to normal subjects. A significant increase of osmolarity was found in patients with abnormal BUT (Mann – Whitney – U test,  $p = 0.018$ ) and in patients with positive lissamine-green staining (Mann-Whitney-U test,  $p = 0.023$ ). No association could be elicited between the results of Schirmer test when compared to those of fluorescein staining or osmolarity.

### **3. Histological alteration in severe cases of conjunctivochalasis**

Conjunctival epithelial hypertrophy and keratosis were observed all of the conjunctival samples except one. Infiltration of polymorphonuclear leucocytes was observed in two samples of one patient (HE staining). Chronic inflammatory infiltration was observed all of the samples except them, who have acute inflammation (HE staining). Goblet cell density was

decreased in three samples (PAS staining). Subconjunctival elastosis was found all of the samples (Hart-Van Gieson staining).

## **Conclusion and new results**

### **1. HLA-DR expression of conjunctival epithelial cells in different grade of conjunctivochalasis**

In our study, conjunctival cytological samples were collected from the upper, bulbar conjunctiva and they were analysed by flow cytometry to evaluate HLA-DR expression of the conjunctival epithelial cells. In our cases, patients with severe CCh, HLA –DR was over expressed in the conjunctival epithelial cells relative to normal eyes and mild or moderate cases. Conjunctivochalasis was graded one of the grading system which differs from fine alteration of the conjunctiva, the LIPCOF test. Using CK 19 gating, conjunctival epithelial cells were investigated separately by flow cytometry. In severe cases of conjunctivochalasis, expression of HLA-DR by conjunctival epithelial cells were higher compared the normal controls and both of mild and moderate cases.

### **2. Tear film osmolarity in different grade of conjunctivochalasis**

In our study, tear film osmolarity were measured in different grade of conjunctivochalasis with TearLab System, which is a „lab-on-a-chip” osmometer. Our results confirm that patients with severe CCh have a higher tear osmolarity relative to normal eyes and mild or moderate cases of CCh.

### **3. Histological alteration in severe cases of conjunctivochalasis**

Immune based inflammation of the conjunctiva and degeneration of the elastic fibers in subconjunctival tissue were evaluated in our case – series. Patients with severe CCh were operated and redundant conjunctival tissue was excised. Histopathological samples were analysed after staining. In our cases, inflammatory cells (lymphocytes, plasma cells), lymphangiectasia and reduced goblet cell density were evaluated most of cases. However, elastic fiber degeneration was detected all of our conjunctival samples.



## Publications

### Publications on the subject of the thesis

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