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PROGRAMA DE DOCTORADO EN CIENCIAS DE LA VISIÓN

TESIS DOCTORAL:

New Tools in Dry Eye Disease Research: a More Efficient Clinical Trial Design Using Controlled Environment and Molecular Biomarkers, and a New Clinical Questionnaire

"Nuevas Herramientas en Investigación en Síndrome de Ojo Seco: Un Diseño de Ensayo Clínico Más Eficiente Mediante Condiciones Ambientales Controladas y Biomarcadores Moleculares y un Nuevo Cuestionario Clínico"

Presentada por Francisco José Pinto Fraga para optar al grado de Doctor por la Universidad de Valladolid

Dirigida por:
Dra. Margarita Calonge Cano
Dra. Amalia Enríquez de Salamanca Aladro
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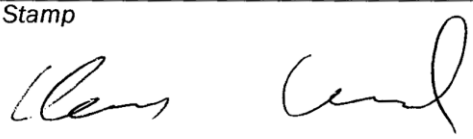
Firma y Sello/ *Signature & Stamp* 

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PROLOGUE

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Abbreviations

Throughout this manuscript, different abbreviations have been used, which are described below:

ACE	Adverse controlled environment	**BCVA**	Best corrected visual acuity
CCLR	Centre for Contact Lens Research	**CERLab**	Controlled Environment Research Laboratory
CI	Confidence interval	**CIC**	Conjunctival impression cytology
CsA	Cyclosporine A	**DED**	Dry eye disease
DEWS	Dry eye workshop	**ECS-Q**	Evaluation of change in symptoms-questionnaire
EDEV	Experimentally-induced dry eye in vitro	**EGF**	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay	**ETDRS**	Early Treatment for Diabetic Retinopathy Study
EVEIT	*Ex vivo* eye irritation test	**FC**	Fold-change
FDA	Food and Drug Administration	**FML group**	Fluorometholone group
GvHD	Graft versus host disease	**H**	Hour
HCE	Human corneal epithelia	**HLA-DR**	Human leukocyte antigen D-related
ICAM-1	Intercellular adhesion molecule 1	**ICC**	Intraclass coefficient correlation
ICES	Intelligently controlled environmental system	**IgA**	Immunoglobulin A
IL	Interleukin	**IOP**	Intraocular pressure
IFN	Interferon	**IP-10**	IFN-γ induced protein 10
LFA-1	Lymphocyte function-associated antigen 1	**LFU**	Lacrimal functional unit

M-DED	Moderate-DED group	***mb***	Millibar
MCP-1	Monocyte chemoattractant protein–1	**MDS**	Multidimensional scaling
MGD	Meibomian gland dysfunction	**NIBUT**	Non-invasive break-up time
OSDI	Ocular surface disease index	**PA**	Polyvinyl alcohol group
PM	Particular matter	**RA**	Receptor antagonist
RANTES	Regulated upon activation normal T-cell expressed and presumably secreted	**RH**	Relative humidity
ROS	Regression on order statistics	**RT-PCR**	Reverse transcription polymerase chain reaction
S-DED	Severe-DED group	**SANDE**	Symptoms assessment in dry eye
SD	Standard deviation	**SS**	Sjögren's syndrome
TBUT	Tear break-up time	**TGF-β**	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha	**USA**	United States of America
VAS	Visual analogue scales		

Organization

This Doctoral Thesis applies for the International-awarded Doctorate Degree. It has been performed at the University of Valladolid, under the regulations of the International Doctorate Committee. The joint requirements are as follows: the whole manuscript has been written in English and a general summary in Spanish, in which the objectives, methodology and summary of results are presented.

This Thesis report is organized in 8 sections as follows:

1. Prologue
2. Chapter 1: Introduction, Justification, Hypothesis, and Objectives
3. Chapter 2: New Design of Clinical Trials: Use of Controlled Environmental Chambers
4. Chapter 3: Tear Biomarkers
5. Chapter 4: New Questionnaire for Dry Eye Disease-Related Symptom Evaluation
6. Conclusions
7. Capítulo 5: Resumen de la tesis doctoral en español
8. Epilogue

Part of this Doctoral Thesis was developed during a 3-month research stay at the University of Cologne (Köln, Germany) in collaboration with the Department of Ophthalmology (Chairman: Claus Cursiefen) and under the direct supervision of Dr. Philipp Steven.

Summary

Objectives: The main aims of this doctoral thesis were 3: 1) to study the usefulness of a new two-step design of clinical trials using a controlled environment chamber to evaluate the safety and efficacy of new dry eye disease (DED) therapies; 2) to evaluate the effect of a common DED therapy on clinical symptoms and signs and tear inflammatory molecule levels at different time-points (i.e. pre- and post-treatment, pre- and post-adverse environmental condition [ACE; 23°C temperature, 5% relative humidity, 0.43 m/s localized airflow] exposure), identifying different biomarkers (disease severity, therapeutic efficacy, and disease activity); 3) to analyze why clinical symptoms usually fail at translating what patients feel and to develop a new and simpler questionnaire that can detect changes in DED-related symptoms between two time-points in an easier and yet more accurate way than the current questionnaires.

Methodology: To meet the first and second aims, a single-center, double-masked, randomized, vehicle-controlled, phase II clinical trial was conducted, assessing the efficacy of topical 0.1%-fluorometholone in moderate-to-severe DED patients for ameliorating the worsening of the ocular surface when exposed to an adverse environment. A total of 41 patients randomly received one drop 4 times daily of either topical 0.1%-fluorometholone (FML group) or polyvinyl alcohol (PA group) for 22 days. During the 4 visits of the study (V1, day 0, baseline / V2; day 21, pre-ACE exposure / V3, day 21, post-ACE exposure / V4, day 22, 24h post-ACE exposure) DED signs and symptoms were evaluated. Also, tear samples were collected at the beginning of each visit for further analysis. An immune bead-based array analyzed the concentrations of 18 molecules (EGF, IFN-γ, TNF-α, IL-1β, IL-1RA, IL-2, IL-4, IL-6, IL-8/CXCL8, IL-10, IL-12, IL-13, IL-17A, IP-10/CXCL10, MCP-1/CCL2, MIP-1α/CCL3, RANTES/CCL5 and MMP-9).

Multidimensional scaling (MDS) used molecule concentrations at V1 to determine the pattern of similarities among patients. A linear mixed effect model analyzed the influence of visits, treatment, and severity on changes in molecule concentrations. Multiple comparisons analysis was used to eliminate the effects multiple statistical tests.

Regarding the third aim, we carried out a prospective observational study in collaboration with the University of Cologne to perform a clinical-based evaluation of the Evaluation of Change in Symptoms-Questionnaire (ECS-Q), the new tool proposed. This study consisted on two visits (V1, baseline / V2, follow-up after therapy) in which DED-related signs and symptoms were evaluated, the latest ones using different questionnaires, OSDI, SANDE II, and ECS-Q. The new ECS-Q had 2 parts: ECS-Q1, that had three possible answers as to how to grade change after therapy in V2 (better, worse, equal), and ECS-Q2 that quantified the change observed.

Results: After 21-day treatment, the FML group had greater improvements in corneal and conjunctival staining, hyperemia, and tear break-up time than did the PA group. After ACE, the percentage of patients having a ≥1 grade increase in corneal staining was significantly higher in the PA group (63.1% vs 23.8%, respectively). Additionally, the FML group showed no significant changes in corneal staining, conjunctival staining, and hyperemia after the ACE exposure, while the PA group showed a significant worsening in corneal staining, conjunctival staining, and hyperemia. The FML group maintained V2 corneal staining scores, while the PA group did not recover the previous status 24h after the exposure. There were no adverse events.

Regarding the tear samples analysis, MDS divided patients into two groups based on differences in EGF, IFN-γ, IL-8/CXCL8, RANTES/CCL5, and MMP-9 levels. These groups had different clinical severities based on Schirmer test, conjunctival staining, and corneal staining, thus, these groups were named moderate and severe DED groups. Both groups presented significant different levels of EGF, IFN-γ, IL-2, IL-8/CXCL8, IL-10, IL-12, RANTES/CCL5, and MMP-9. IL-1RA, IL-2, and TNF-

α were differentially affected by time, depending on the treatment. Between V2-V3, there were significant changes in EGF, IL-1RA, IL-2, IL-8/CXCL8, IL-13, IP-10/CXCL10, TNF-α, and MMP-9. The strongest biomarker candidates were IFN-γ, IL-2, IL-8, IL-12, RANTES/CCL5, and MMP-9 as DED severity biomarkers; IL-2 as a therapeutic (FML) biomarker; and EGF as a DED activity biomarkers.

Finally, 36 DED patients were included in the observational study. Between V1 and V2, DED-related symptoms decreased with all the questionnaires used (OSDI, SANDE II, and ECS-Q). Some (5.5%) patients did not adequately complete SANDE II questionnaire, while all patient correctly interpreted ECS-Q.

Patients responding "better" in ECS-Q presented a significantly lower corneal staining that those responding "worse". Moreover, these patients had a significantly lower SANDE II score than those who responded "equal" or "worse". Finally, a poor concordance between OSDI and ECS-Q was observed, while between ECS-Q and SANDE II the concordance was moderate.

Conclusion: The new two-step clinical trial design proposed is useful to evaluate the efficacy of new DED therapies, both in the traditional way but also in the protective effect after an inflammatory desiccating stress. FML can be used as a positive control in future trials evaluating the effectiveness of new therapies for DED using this new clinical trial design. The following molecules (IFN-γ, IL-2, IL-12, RANTES/CCL5, and MMP-9) allow to classify patients according to their DED severity in an objective way. Thus, these biomarkers also can be useful to better select target patients for clinical trials. IL-2 is proposed as the strongest therapeutic biomarker for fluormetholone therapy that could be used as an objective therapeutic evaluation end-points in future clinical trials with that drug. EGF is proposed as a biomarker that could provide a better definition of DED disease activity. Due to its simplicity, the new questionnaire developed can be a useful tool for the evaluation of the patient´s perception regarding the evolution of their DED symptoms.

Sumario

Objetivos: Los objetivos principales de esta tesis doctoral fueron 3: 1) estudiar la utilidad de un nuevo diseño de ensayos clínicos en dos fases utilizando una cámara de ambiente controlado para evaluar la seguridad y la eficacia de las nuevas terapias para el síndrome de ojo seco (SOS); 2) evaluar en diferentes momentos (es decir, pre- y post-tratamiento, pre- y post-ambiente controlado adverso [ACA; temperatura de 23°C, humedad relativa del 5%, flujo de aire de 0,43 m/s) el efecto de un tratamiento común para el SOS sobre los síntomas y los signos clínicos, así como sobre los niveles de moléculas inflamatorias, identificando diferentes biomarcadores (severidad de la enfermedad, eficacia terapéutica y actividad de la patología); 3) analizar por qué la evaluación de la sintomatología no suele reflejar lo que los pacientes sienten, y desarrollar un cuestionario nuevo y más simple que pueda detectar cambios en los síntomas relacionados con SOS entre dos momentos de tiempo de una manera más sencilla y precisa que los cuestionarios actuales.

Metodología: Para cumplir con los objetivos primero y segundo, se realizó un ensayo clínico fase II, doble enmascaramiento, aleatorizado, y controlado por vehículo, que evaluó la eficacia de la fluorometolona tópica al 0.1% en la reducción del empeoramiento de la superficie ocular de pacientes con SOS moderado y/o severo cuando éstos se exponen a un ambiente adverso. Un total de 41 pacientes fueron asignados aleatoriamente al grupo de estudio (grupo FML; fluorometolona tópica al 0.1%) o al grupo control (grupo PA; alcohol polivinílico). Los participantes se administraron 1 gota del tratamiento asignado en cada ojo, 4 veces al día durante 22 días. En cada una de las 4 visitas del estudio (V1, día 0, visita inicial / V2, día 21, pre-ACA / V3, día 21, post-ACA / V4, día 22, 24h post-ACA) se evaluaron los signos clínicos y los síntomas, y se recogieron muestras de

lágrima para su posterior análisis. Este análisis se realizó mediante técnica de Luminex, analizando las concentraciones de 18 moléculas (EGF, IFN-γ, TNF-α, IL-1β, IL-1RA, IL-2, IL-4, IL-6, IL-8 / CXCL8, IL IL-12, IL-13, IL-17A, IP-10 / CXCL10, MCP-1 / CCL2, MIP-1α / CCL3, RANTES / CCL5 y MMP-9). Se realizó un escalamiento multidimensional (MDS) utilizando las concentraciones de las moléculas en V1 para determinar el patrón de similitudes entre los pacientes. Mediante un modelo de efecto mixto lineal se analizó la influencia de las visitas, el tratamiento y la severidad en los cambios en las concentraciones de las moléculas evaluadas. Finalmente, se llevó a cabo un análisis de comparaciones múltiples.

En cuanto al tercer objetivo, se realizó un estudio prospectivo observacional en colaboración con la Universidad de Colonia para realizar una evaluación clínica de un nuevo cuestionario "Evaluación de Cambio en Síntomas-Cuestionario" (ECS-Q). Este estudio consistió en dos visitas (V1, basal / V2, seguimiento tras terapia) en las que se evaluaron los signos y síntomas relacionados con SOS. Para ello se utilizaron diferentes cuestionarios, OSDI, SANDE II y ECS-Q. El nuevo ECS-Q tenía dos partes: ECS-Q1, que tenía tres respuestas posibles sobre el grado de cambio después de la terapia en V2 (mejor, peor, igual) y ECS-Q2 que cuantificaba el cambio observado.

Resultados: Después de 21 días de tratamiento, el grupo de FML tuvo reducciones mayores en tinción corneal y conjuntival, e hiperemia que el grupo PA, así como un aumento del tiempo de ruptura lagrima. Después de la exposición a ACA, el porcentaje de pacientes con un aumento ≥1 en la tinción corneal fue significativamente mayor en el grupo PA que en el grupo FML (63,1% vs 23,8%, respectivamente). Además, tras esta exposición el grupo FML no mostró cambios significativos en tinción corneal, conjuntival e hiperemia, mientras que el grupo PA mostró un empeoramiento significativo de los tres parámetros. El grupo FML mantuvo las puntuaciones de tinción corneal de V2, mientras que el grupo PA no recuperó el estado anterior 24 horas después de la exposición. No hubo eventos adversos.

En cuanto al análisis de las muestras de lágrimas, el análisis MDS dividió a los pacientes en dos grupos basándose en las diferencias en los niveles de EGF, IFN-γ, IL-8 / CXCL8, RANTES / CCL5 y MMP-9. Estos grupos tenían diferente gravedad clínica basada en los resultados del test de Schirmer, la tinción conjuntival y la tinción corneal, por lo tanto, estos grupos fueron denominados como SOS moderado y SOS grave. Ambos grupos presentaron niveles significativamente diferentes de EGF, IFN-γ, IL-2, IL-8 / CXCL8, IL-10, IL-12, RANTES / CCL5 y MMP-9. IL-1RA, IL-2 y TNF-α fueron afectados diferencialmente por el tiempo, dependiendo del tratamiento. Entre V2-V3, hubo cambios significativos en EGF, IL-1RA, IL-2, IL-8 / CXCL8, IL-13, IP-10 / CXCL10, TNF-α y MMP-9. Los candidatos a biomarcadores más fuertes fueron IFN-γ, IL-2, IL-8, IL-12, RANTES / CCL5 y MMP-9 como biomarcadores de severidad de SOS; IL-2 como biomarcador terapéutico (FML); Y EGF como biomarcadores de la actividad del SOS.

Finalmente, en el estudio observacional realizado con Colonia se incluyeron 36 pacientes de SOS. Entre V1 y V2, la sintomatología de SOS disminuyó con todos los cuestionarios utilizados (OSDI, SANDE II, y ECS-Q). Algunos pacientes (5,5%) no completaron adecuadamente el cuestionario SANDE II, mientras que todos los pacientes interpretaron correctamente ECS-Q. Los pacientes que respondieron "mejor" en ECS-Q presentaron una tinción corneal significativamente menor que aquellos que respondieron "peor". Además, estos pacientes tenían una puntuación SANDE II significativamente menor que aquellos que respondieron "igual" o "peor". Por último, se observó una pobre concordancia entre OSDI y ECS-Q, mientras que entre ECS-Q y SANDE II la concordancia fue moderada.

Conclusión: El nuevo diseño en dos fases de ensayo clínico propuesto es útil para evaluar la eficacia de las nuevas terapias de SOS, tanto de manera tradicional como estudiando el efecto protector del tratamiento frente a un estrés desecante inflamatorio. El FML se puede utilizar como un control positivo en ensayos futuros que evalúen la efectividad de nuevas terapias para SOS usando este nuevo diseño de ensayo clínico. Las siguientes moléculas (IFN-γ, IL-2, IL-12, RANTES / CCL5 y MMP-9) permiten clasificar a los pacientes de acuerdo con su severidad de SOS

de una manera objetiva. Por lo tanto, estos biomarcadores también pueden ser útiles para seleccionar mejor a los pacientes diana para ensayos clínicos. IL-2 se propone como el biomarcador terapéutico más fuerte para la terapia utilizada, y podría utilizarse como variable objetiva final durante la evaluación de la efectividad terapéutica en futuros ensayos clínicos con este fármaco. EGF se propone como un biomarcador que podría proporcionar una mejor definición de la actividad del SOS. Debido a su simplicidad, el nuevo cuestionario desarrollado puede ser una herramienta útil para la evaluación de la percepción del paciente con respecto a la evolución de sus síntomas de SOS.

INTRODUCTION, JUSTIFICATION, HYPOTHESIS, and OBJECTIVES

Chapter 1: Introduction, Justification, Hypothesis, and Objectives

This chapter explains the concept of the Lacrimal Functional Unit (LFU) and the definition, etiology, and diagnosis of Dry Eye Disease (DED), as well as its management and treatment. Additionally, it shows the relevance and influence of the environment in DED, reviewing the concepts of outdoor and indoor environment conditions, as well as their effects on the LFU. Furthermore, the most relevant studies regarding the effect of adverse environment conditions in both experimental DED (i.e. animal models, *ex vivo* models and *in vitro* models) and clinical research are presented.

Finally, knowing that DED is an inflammatory process, the impact of the disease in the expression of molecules in tears of DED patients, as well as the use of biomarkers in this pathology, are revised in the last section of this chapter.

1. Dry Eye Disease (DED)

1.1. Lacrimal Functional Unit (LFU)

The ocular surface was defined by Thoft et al.[1] in 1978 as an integrated unit comprising the cornea, the conjunctiva, the sclera-corneal limbus, and the overlying tear film *(Figure 1)*. These components are interconnected through a continuous epithelium with no breaks between regions, and neuroanatomically connected with the nervous, vascular, immune, and endocrine systems.

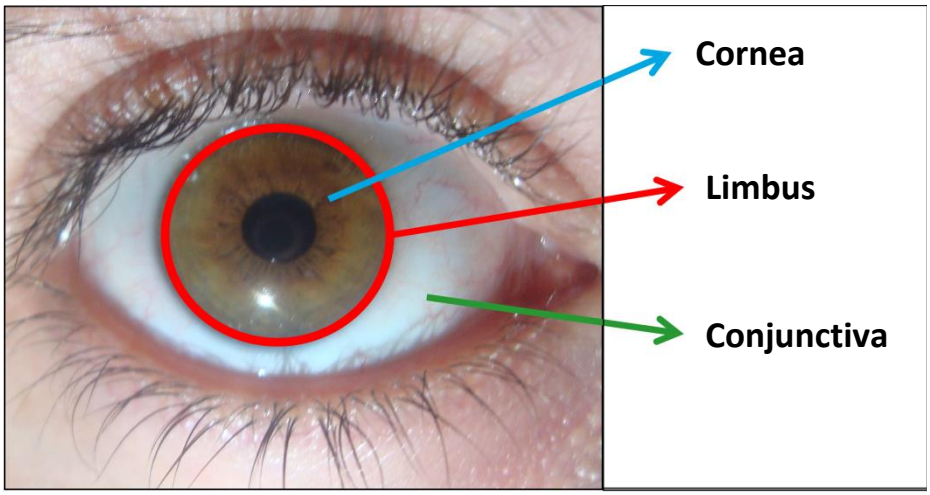


Figure 1. Ocular surface. Anatomical location of the conjunctiva, limbus and cornea on the ocular surface[2]

The concept of LFU was introduced by Stern et al.[3] in 1998, overcoming the concept of ocular surface previously explained. The LFU is composed of the cornea, conjunctiva, limbus, tear film, accessory lacrimal glands, Meibomian glands, main lacrimal gland, and the interconnecting innervation (V nerve, VII nerve, and autonomous system) that integrates the activities of each component *(Figure 2)*.[2–4] The role of the LFU is to secrete a precise tear film composition that maintains a homeostatic environment around the epithelial cells of the ocular surface.

Putting together the concept of LFU and the fact of DED as an inflammatory immune disorder, Stern et al.[3] proposed that "DED is the result of a localized inflammatory response mediated by the immune system affecting the LFU".[3]

Therefore, the alteration of any of the components of the LFU can alter the homeostasis of this unit as a whole. If this disturbance is not neutralized it will soon lead to DED.[5]

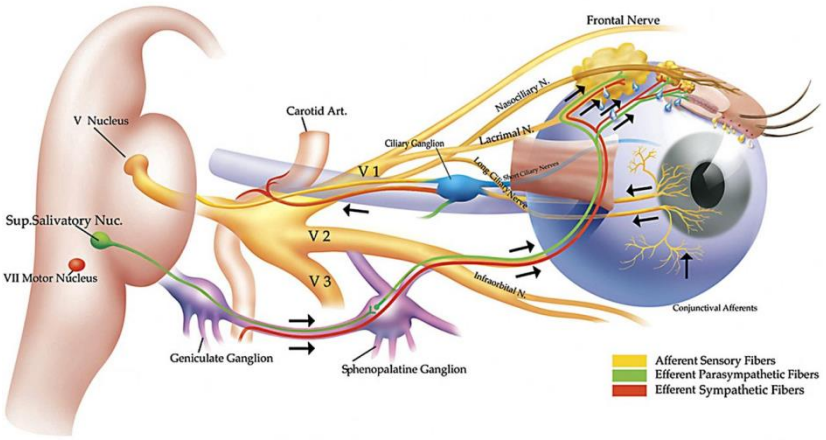


Figure 2. Lacrimal Functional Unit (LFU). *From Pflugfelder SC, Beuerman RW, Stern ME, eds. Dry Eye and Ocular Surface Disorders. New York, NY: Marcel Dekker, Inc.; 2004.*[2]

1.2. DED Definition

The definition and concept of DED have suffered multiple modifications along the years as understanding of the disease process has evolved. There are several difficulties to establish a widely-accepted definition of this pathology, mainly due to the lack of a complete understanding of all the events that contribute to the pathogenesis of the disease. Therefore, different terms have been proposed to refer to DED, like keratoconjunctivitis sicca,[6] keratitis sicca,[7] filamentary keratitis,[8] dry eye syndrome,[9] dry eye,[10,11], tear deficiency,[6,12] or dysfunctional tear syndrome.[13]

Historically, it was considered that DED was due to either insufficient production or impaired stability of tears. In 1903, the concept of tear deficiency was first proposed by O. Schirmer, who developed the Schirmer test to measure

tear production.[14] In 1933, H. Sjögren used the Latin term "keratoconjunctivitis sicca" referring to the DED or dry inflammation of the cornea and conjunctiva observed in Sjögren syndrome (SS), mentioning the triad of dry eye, dry mouth and joint pain.[15] For many years afterward, dry eye was considered to be equivalent to keratoconjunctivitis sicca, focusing only in the deficiency or disruption of the aqueous layer of the tear film.[6]

Later, in the period when the tear film structure was based in a 3-layer model, the classification of DED was based on the alteration of one or more of these layers.[16] Currently, the 3-layer model has been replaced. The new model used to explain the tear film structure claims that this is composed of a mucus-aqueous gel covered by a lipid layer.[17,18] This gel is secreted by the lacrimal glands and the epithelial cells and goblet cells located in the ocular surface, and is anchored to glycocalix by chemical bonds.

In 1995, the National Eye Institute of the United States of America (USA) defined DED as "a disorder of the tear film due to tear deficiency or excessive tear evaporation which causes damage to the interpalpebral ocular surface and is associated with symptoms of ocular discomfort".[19] The most important contribution of this definition was to consider that DED cannot only be caused by an aqueous production deficiency, but also by an excessive evaporation of the tear film. Thus, pathologies such as Meibomian gland dysfunction (MGD) or blepharitis are directly related with some DED categories.

This definition was an important step to get consensus among researches, but unfortunately, it continued focusing on tear deficiency as the most relevant factor in DED. Moreover, the concept "disease" was not included in the definition; thus, this definition could have induced the ophthalmological community to understand that DED was not a disease, but just a nuisance. On the other hand, the attempt to generate a global definition induced several problems. The assumption that the damage of the ocular surface was only interpalpebral, or the idea that DED necessarily entails the presence of symptoms, are examples of these problems.

Some years later, in 2006, after a Delphi panel approach "dysfunctional tear syndrome" was proposed as a new term to define the disease, concluding that treatment strategies should rely on symptoms and signs rather than tests.[13] This panel defined the clinical signs to be considered in assessing the severity of dysfunctional tear syndrome, upon which a severity-based treatment algorithm was suggested.[13]

A bigger step in the definition of DED was given in 2007, when the first International Dry Eye Workshop (DEWS) defined this pathology as "a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort,[20,21] visual disturbance,[22–25] and tear film instability,[26,27] with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film[28–31] and inflammation[32–34] of the ocular surface".[35] The new definition emphasized symptoms and global mechanisms, and recognized the multifactorial nature of DED. Moreover, inflammation and osmolarity were highlighted as potential risk factors for DED. According to this definition, symptoms of dry eye (including visual disturbances) should be present for the diagnosis of DED. In addition, the report states that to definitively confirm this diagnosis is necessary to observe a reduction of tear volume and the presence of ocular surface damage, observed by vital dyes.

A second International DEWS is working since May 2015 and will generate a report by 2017, which will revise the definition and diagnostic guidelines for DED. Unfortunately, this second DEWS will be missing the input of important experts in the field who profoundly disagree with DEWS policies.

1.3. DED Classification

The DEWS committee proposed in 2007 a new DED classification[35] based on both the "triple classification" generated from reports presented at the 14th Congress of the European Society of Ophthalmology[36,37] and the report of the Delphi panel.[13] From the "triple classification",[37] published in 2005, the DEWS committee took many conceptual aspects from the classification criteria based on three separate schemes; one based on etiopathogenesis, one based on the glands

and tissues targeted in DED, and one based on disease severity. From the Delphi Panel[13] the committee adopted a severity grading scheme *(Table 1)*.

Table 1. Dry eye disease (DED) severity grading scheme. *Modified from: The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop (2007).*[35]

DED severity level	***1***	***2***	***3***	***4***
Discomfort:				
- Severity	+	++	+++	+++ / D
- Frequency	Episodic	Both	Frequent/Constant	Constant
- Environmental stress needing?	Yes	Both	No	No
Visual symptoms	- / E	E Annoying and/or activity limiting	C or chronic Annoying and/or activity limiting	C / D
Conjunctival injection	- / +	- / +	- / +	+ / ++
Corneal staining	- / +	Variable	Marked central	N/A
Corneal/tear signs	- / +	+ debris, ↓meniscus	Filamentary keratitis, mucus clumping, ↑ tear debris	Filamentary keratitis, mucus clumping, ↑ tear debris, ulceration
Lid/Meibomian glands	MGD (E)	MGD (E)	MGD (F)	Trichiasis, keratinization, symblepharon
Tear film break-up time	Variable	≤ 10	≤ 5	Immediate
Schirmer score (mm/5 min)	Variable	≤ 10	≤ 5	≤ 2

- = Not present; + = Mild; ++ = Moderate; +++ = Severe; D = Disabling; E = Episodic; F =Frequent; C =Constant; MGD = Meibomian gland dysfunction

According to the etiopathogenic classification proposed by the DEWS report *(Figure 3)*, the major classes of DED are aqueous-deficient DED and evaporative DED.[35] It is important to recognize that both types of DED may coexist. In both cases, there is an alteration of the quality and/or the composition of the tear film, which leads to a reduction of the tear stability and an increase of its osmolarity.

Aqueous-deficient DED implies that the pathology is due to a failure in the lacrimal tear secretion.[38,39] This category of DED has been sub-divided in two subclasses, SS and non-SS-DED. In the first case, the lacrimal and salivary glands are targeted by an autoimmune systemic process. These structures are infiltrated by activated T-cells, which cause acinar and ductular cell death and hypo-secretion of tears and saliva. In the second case, there are no systemic autoimmune features. The non-SS-DED can be caused by several circumstances: deficiencies in the main lacrimal gland or the secondary lacrimal glands, obstruction of the lacrimal gland ducts or hyposecretion of reflex tears, all caused by multiple reasons.

Evaporative DED is due to an excessive water loss in the tear film from the exposed ocular surface in the presence of normal tear secretion. Two sub-categories have been described to distinguish those causes that are dependent on intrinsic conditions, due to intrinsic disease affecting lid structures or dynamics of the lids and ocular surface (e.g. MGD, low blink rate, etc.) from those that arise from extrinsic influences, where ocular surface disease occurs due to some extrinsic exposure (e.g. contact lens wear, allergic conjunctivitis, etc.).[35]

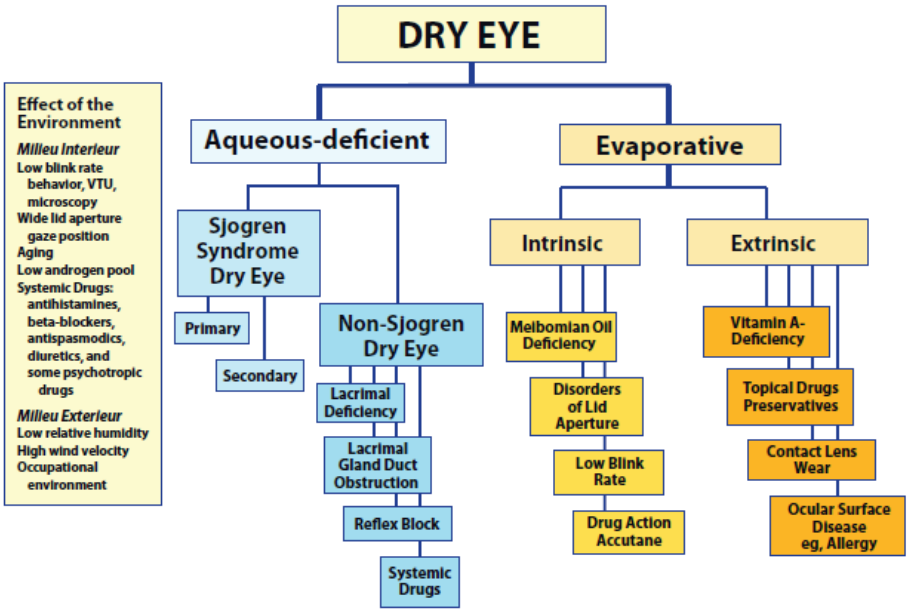


Figure 3. Major etiological causes of DED. *From The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop (2007).*[35]

Additionally, DED can be categorized as episodic or chronic. Episodic DED occurs when environmental or visual tasks with reduced blinking overwhelm the stability of the tear and produce symptomatic dry eye. Chronic DED, although aggravated by the same environmental conditions, persists continuously with symptoms and possible damage to the ocular surface.[40]

1.4. DED Epidemiology

DED is one of the most common ocular morbidities, affecting between 5.5% and 35% of the population.[41–44] The large differences observed in the prevalence of DED are due to variations in studied populations, geographical differences, differences in the diagnosis methodology, and even variations in the definition of the disease. Twenty-five percent of patients who visit ophthalmic clinics report symptoms of dry eye, making it a growing public health problem and one of the most common condition seen by eye care practitioners.[45] There are big differences between countries in the prevalence of DED. For example, while the

prevalence of DED in Australia is approximately 7.4%,[46] in Taiwan the prevalence increases to 33.7%;[47] but is common to find intermediate values of prevalence of DED, as the 25% reported in Canada[48] or the 33% observed in Japan.[49] In 2009 Viso et al.[50] reported a 11% prevalence of DED in Spain, observing that DED was more frequent in women than in men (11.9% and 9.0%, respectively). Regarding the prevalence of DED in Valladolid, city where this Thesis has been developed, Fuentes-Páez et al.[51] observed a higher prevalence than in the study of Viso et al., reporting a prevalence of 24.2%, with values of 27.6% and 19,6% in women and men, respectively.

It is important to mention that the prevalence and incidence of DED may be under-reported, as patients may fail to recognize the symptoms of DED or do not report the problem to a physician. In any case, the combined data from large population-based epidemiological studies indicates that DED is significantly most common in women that in men.[41,42] Finally, it has been reported that this condition presents an increased prevalence in patients with autoimmune diseases,[52] which affect approximately 8% of the population, of whom 78% are women.[53] DED prevalence is also increased in postmenopausal women[54] and elderly people.[43,47]

This disease represents a big burden in public healthcare, owing to the impact of DED on quality of life of patients by affecting visual-related tasks, social and physical functioning, and workplace productivity.[55–57] It is estimated that patients with moderate-to-severe DED have a quality of life similar to patients with moderate-to-severe angina or who undergo hospital dialysis.[58,59]

As previously explained, several etiopathogenic mechanisms usually interact to generate changes and anomalies in the LFU, eventually leading to tear film instability and ocular surface damage. Around 10% of patients with DED have a solely aqueous-deficient disorder. Hyper-evaporative disorders (mostly caused by dysfunction of the Meibomian glands) and mixed hyper-evaporative/aqueous-deficient forms account for more than 80% of cases.[60]

It is fully accepted that DED is a multifactorial disease. For this reason, there are several risk factors for this condition.[61] Some of the most relevant are summarized in *Table 2*.[35,62] The prevalence of DED is on the rise because of the global increase in elderly population, and due to the increase incidence of some of the risk factors associated to the disease.

Table 2. Major risk factors for dry eye disease (DED). *Modified from The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop (2007).*[35]

High level of evidence	*Moderate level of evidence*	*Low level of evidence*
• Age • Female sex • Postmenopausal estrogen therapy • Antihistamines • Collagen vascular disease • Corneal refractive surgery • Irradiation • Hematopoietic stem cell transplantation • Vitamin A deficiency • Hepatitis C • Androgen insufficiency	• Medications such as tricyclic antidepressants, selective serotonin inhibitors, diuretics, beta blockers • Diabetes mellitus • HIV/HTLV1 infection • Systemic chemotherapy • Cataract surgery • Keratoplasty • Isotretinoin • Low air humidity • Sarcoidosis • Ovarian dysfunction	• Smoking • Hispanic ethnicity • Anticholinergic drugs such as anxiolytics, antipsychotics • Alcohol • Menopause • Botulinum toxin injection • Acne • Gout • Oral contraceptives • Pregnancy

HIV = Human immunodeficiency virus; HTLV1 = human T cell leukemia virus type 1.

1.5. DED Diagnosis

A correct diagnosis of DED is necessary to distinguish it from other conditions such as infections or allergies, which can present similar clinical signs. This is not an easy task due to several factors previously explained.[63] An incorrect diagnosis of the pathology can lead to the use of treatments that worsen DED. The DEWS report described the ocular surface and tear parameters to consider when diagnosing DED.[64]

Traditionally, combinations of diagnostic tests have been used to assess symptoms and clinical signs.[64,65] This is necessary because the poor repeatability of many of the current diagnostic tests, as well as the low sensitivity and specificity of these tests. The DEWS committee published a diagnostic guideline[35] suggesting the following sequence of diagnosis tests:

a) Patient history/symptoms questionnaires.

b) Tear film break-up time (TBUT).

c) Ocular surface staining with fluorescein/Lissamine green.

d) Schirmer test with or without anesthesia.

e) Examination of the eyelid margins and Meibomian glands.

Following, some of the most relevant tests for DED diagnosis are detailed.

1.5.1. Symptoms Evaluation

Questionnaires are tools commonly used for the diagnosis of DED. These tools evaluate different aspects of DED symptomatology, including severity, effect on daily activities, quality of life, etc. By themselves, questionnaires are not a good method to establish the origin of eye problems, because the same symptoms can be caused by a variety of disorders of the ocular surface or the tear film.[66] Moreover, it is incorrect to assume that symptoms are the main feature of DED, because there is large evidence of the lack of correlation between signs and symptoms in this pathology.[67–70]

There are more than 18 questionnaires that have been used in randomized clinical trials, epidemiologic studies or prospective randomized studies *(Table 3)*.

Data from clinical trials show that the results from the tests currently available for the diagnosis of DED do not always correlate well with patient-reported symptoms, especially in mild-to-moderate disease.[69,71] In addition, low correlations have been found between different objective tests.

Table 3. Most used symptom-based questionnaires for dry eye disease (DED) diagnosis.

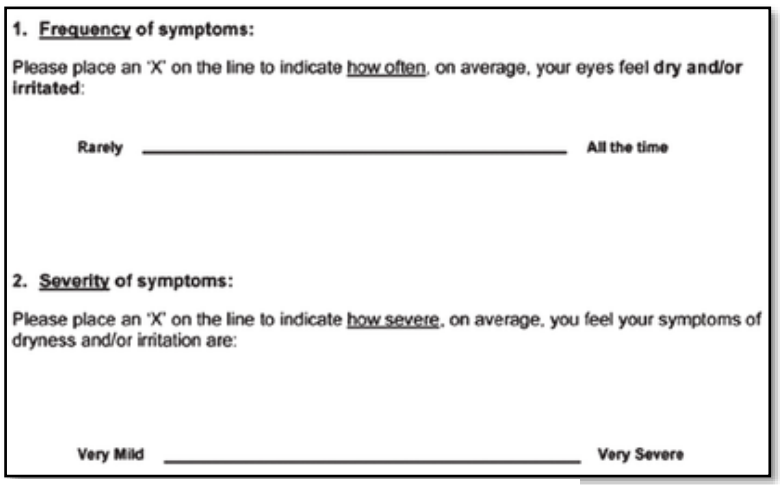
Questionnaire	*Questionnaire Summary*	*Year*
McMonnies[72,73]	14 items focusing on risk factors for DED; screening	1986
CANDEES[48]	13 items estimating the prevalence of symptoms in epidemiological studies	1997
Salisbury[68,74]	6 standardized items relating signs and symptoms of dry eye in the elderly	1997

DEEP[75]	19 items; screening	1998
NEI-VFQ[76,77]	25 items: 2 ocular pain subscale questions	1998
Melbourne Visual Impairment Project[46]	Self-reported symptoms elicited by interviewer-administered questionnaire	1998
Japanese Dry Eye Awareness Questionnaire[49]	30 items relating to symptoms and knowledge DED	1999
OSDI[78]	12 items (0-4) assessing a range of ocular surface symptoms, their severity and impact on visual function in a one-week recall period	2000
Bjerrum Questionnaire[79]	3-part questionnaire which includes an ocular part with 14 questions	1996
DEQ[20,80]	23 main questions on prevalence, frequency, diurnal severity, etc.	2002
Women´s Health Study Questionnaire[54]	3 questions from 14-item original questionnaire	2003
Sicca Symptoms Inventory[81]	Inventory of both symptoms and signs of Sjögren's syndrome	2003
SPEED[82]	8 questions about severity (0-4) and frequency (0-3) of symptoms	2005
SANDE[83]	Evaluation of intensity and frequency of dryness in a 100mm-horizontal VAS	2007
OCI[84]	12 items measuring ocular surface irritation	2007
SESoD[85]	3-item questionnaire to evaluate patient's perception of ocular discomfort related to dryness	2008
DEQ-5[86]	Subset of DEQ items that discriminate across self-assessed severity and various diagnoses of dry eye	2010
IDEEL[87]	57 questions evaluating dry eye symptom bother, impact on daily life and treatment satisfaction	2011
DEQS[88]	15 items; an overall summary scale and 2 multi-item subscales: impact on daily life and bothersome ocular symptoms	2013

CANDEES = Canadian Dry Eye Epidemiology Study; DED = Dry Eye Disease; DEEP = Dry Eye Epidemiology Projects; DEQ = Dry Eye Questionnaire; DEQS=Dry Eye Related Quality of Live; IDEEL = Impact of Dry Eye on Everyday Life; NEI-VFQ = National Eye Institute-Visual Function Questionnaire; OSDI = Ocular Surface Disease Index; SANDE = Symptom Assessment in Dry Eye; SESoD = Subjective Evaluation of Symptom of Dryness; SPEED = Standard Patient Evaluation of Eye Dryness questionnaire; VAS=Visual Analogue Scale.

Currently, OSDI[78] and SANDE[83] questionnaires are two of the tools most frequently used in DED studies and clinical trials to evaluate symptoms. Furthermore, OSDI questionnaire has undergone psychometric testing and has been accepted by the US FDA for its use in clinical trials.[78,89] This tool, introduced in 1997 by the Outcomes Research Group (Allergan Inc., Irvine, CA), consists of 12 items that assess symptoms and functional limitations over the preceding week. Each item has the same five-category Likert-type response option, and each of the three subscales has its own question type. Final OSDI scores range from 0 to 100.

SANDE I *(Figure 4)* comprises of two questions that quantify both severity and frequency of DED-related symptoms using two VASs. The one used for the measurement of symptoms frequency ranges from "rarely" to "all of the time" (left and right extremes of the VAS, respectively), while the one used to measure symptoms severity ranges from "very mild" to "very severe" (left and right extremes of the VAS, respectively). Due to its design, this tool evaluates symptoms in a particular moment. On the other hand, SANDE II *(Figure 5)* is a tool designed to evaluate changes of symptoms. It consists of a VAS scale with a mark in the middle of the line representing "no changes" in relation to the previous visit. Patients are asked to place a mark in the line indicating how severe/frequent the symptoms of dryness and irritation are now compared to the last visit. Marks on


1. **Frequency of symptoms:**

Please place an 'X' on the line to indicate how often, on average, your eyes feel **dry and/or irritated**:

Rarely _____ **All the time**

2. **Severity of symptoms:**

Please place an 'X' on the line to indicate how severe, on average, you feel your symptoms of dryness and/or irritation are:

Very Mild _____ **Very Severe**

Figure 4. Symptoms assessment in dry eye (SANDE) I.

the left side of the middle point indicate a decrease of frequency/severity while marks on the right side indicate an increase. The farer from the middle, the higher the change is.

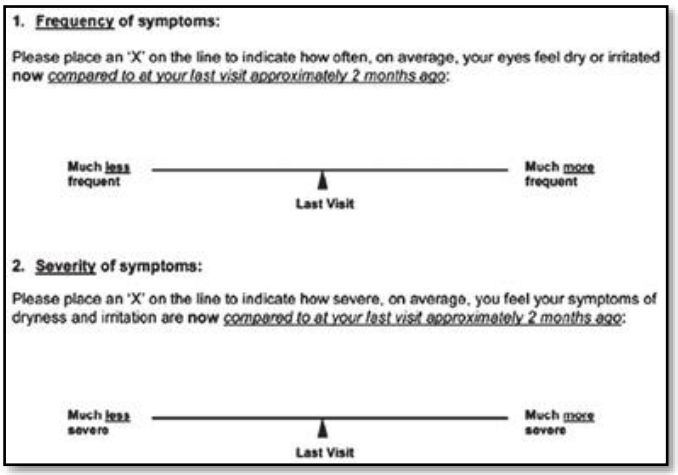


Figure 5. Symptoms assessment in dry eye (SANDE) II.

Additionally, reviewing the latest clinical trials and studies in the field of DED it is possible to observe that VAS has been used in an isolated way in most of them, encouraging patients to evaluate the intensity of a specific symptom or group of symptoms in the VAS scale *(Figure 6)*.

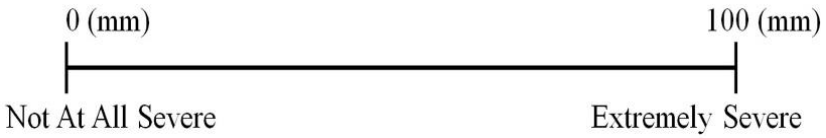


Figure 6. Visual Analogue Scale (VAS).

It is paradoxical to observe that VAS is widely used, in spite of the existence of much more complex tools for symptoms evaluation. This fact may result from the lack of consensus on the appropriateness of the existing questionnaires, or from the attempt to simplify the evaluation of symptoms as much as possible. The problem associated with the use of VAS is that patients usually do not remember their previous answer, so it is not possible to evaluate the changes in symptoms between two independent measures.

1.5.2. Tear Flow Evaluation

This group includes all the tests used to evaluate the volume, secretion and drainage of the tear film.

Tear film meniscus: Tear meniscus refers to the tears accumulated at the junction of the bulbar conjunctiva and inferior lid margins *(Figure 7)*. It contains 75-90% of tear volume; thus, the evaluation of the tear film meniscus, that can be performed by different methods, is a non-invasive technique that provides information about the amount of tears present on the eye.[90]

Tear meniscus characteristics such as height, width, radius of curvature, and cross sectional area have been reported to be useful in the diagnosis of DED. According to Lamberts et al.,[67] the tear meniscus height is normal when its value is between 0.1 and 0.3 mm, being associated with DED values below 0.1 mm.[91] There are studies that claim for this test has big diagnostic capacity (92% sensitivity, 90% specificity) taking a cut-off point of 0.164 mm.[92] However, the evaluation of this test is subjected to variability; thus, the range of normality depends on the technique used to perform the measurement.[93]

The simplest technique to evaluate the tear meniscus height is using a slip-lamp equipped with a graded ocular, but is important to mention that an excessive or prolonged use of illumination should be avoided to prevent reflex tearing. The second technique is to compare the tear meniscus height with the illuminated slit width by setting the slit horizontally in alignment with the lower lid margin, altering the slit width until it appears to match the height of the tear prism.[94] More complex techniques to the measure of the tear meniscus have been described, as the specular or reflective meniscometry,[95] the use of magnified optical sections of the tear meniscus and its analysis by specific software,[96] the use of optical coherence tomography (OCT),[97,98] or the utilization of new tools such as the Keratograph.[99] Moreover, video-meniscometry has been also described.[76] This technique allows the recording of images of the tear meniscus and analyse the changes produced along time, or during the development of other tests.[100]

However, the Fourier Domain-OCT technique provides the best reproducibility results.[101]

Not only the objective parameters of tear meniscus are useful. The observation of the meniscus profile is also extremely helpful. A regular tear meniscus is typically observed in a healthy eye while a meniscus with a scalloped edge is often associated with a dry eye.

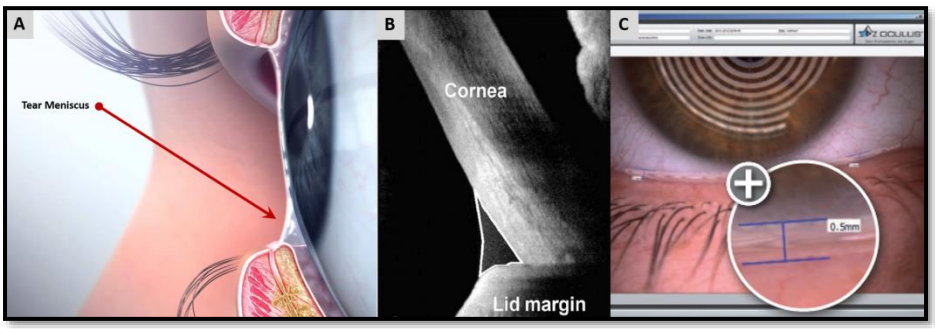


Figure 7. Anatomical location of the tear meniscus (A) and two evaluation methods, Optical coherence tomography (OCT) (B) and Oculus Pentacam®. *Modified from www.peterivins.co.uk.*

Tear Film Secretion: Schirmer test, described by Otto Schirmer in 1903,[14] continues being nowadays the main technique used to measure this parameter and it is considered one of the main ophthalmological tests for the diagnosis of DED.[64] There are some variations of this test, such as the Jones basal secretion test and the Schirmer II.

In the traditional version of the Schirmer test (Schirmer I), a filter paper strip (35 × 5 mm) is placed in the conjunctival sac of the temporal third of the lower eyelid and after 5 minutes, with the patient's eyes closed, the wetting of the strip is measured. Schirmer test evaluates the total amount of secretion (basal and reflex), because the strip insertion itself can cause reflex tearing. For this technique, the cut-off value varies between 5 and 15 mm, depending on the authors.[102,103] It has been reported that using 5 mm as the cut-off criterion, the sensitivity and specificity of the test were 47.2% and 100%, respectively.[14]

If the same technique is performed after the instillation of topical anesthesia the test is renamed as Schirmer II test.[104] In this case, the cut-off value is usually 5

mm, and provides a sensitivity of 60.9% in groups of patients with SS-DED, and 37.5% in groups of patients with other DED etiology; its specificity is 83.6%. Test results are about 40% lower than in Schirmer I, because theoretically this test measures only the basal secretion, without reflex tearing. This variant is also subjected to marked inter- and intra-individual fluctuations.

Jones basal test is performed after the instillation of topical anesthesia and with stimulation of the nasal mucosa, which causes reflex tear secretion. The reading is performed after 5 minutes and the cut-off value in this case is 15 mm.[105,106]

All the modalities of the Schirmer test should be done with the eyes-closed to prevent the interference of environmental factors.[107]

Tear Film Stability: As previously explained, tear hypo-secretion, as well as its hyper-evaporation, can alter the structure of the tear film.

TBUT evaluates the stability of the tear film. This method was described by Norm;[108] it is determined after the instillation of unpreserved fluorescein drops. After a complete blink, the time to the first break-up of the tear film is measured using a slit-lamp with a cobalt blue filter. To reduce the variability of the test it is recommended to perform three measures, taking as final value its average. The normal range lies between 20 and 30 seconds; values below 10 seconds are considered abnormal. Taking this value as a cut-off value the test presented good sensitivity (77.8%) but low specificity (38.9%).[109] For this reason, 7 mm has been selected as cut-off point by some authors.[110,111] The great disadvantage of this technique is that the instillation of the fluorescein causes tear film instability. It has been proposed to instil a controlled amount of fluorescein to reduce as much as possible tear disruption, thus obtaining more repeatable results.[112]

To avoid the disadvantages related to the instillation of fluorescein, Mengher et al.[113] described the non-invasive break-up time (NIBUT). It consists on the projection of an image on the surface of the cornea and the measurement of the time between a complete blink and the first deformation of the image *(Figure 8)*. The cut-off value for a normal NIBUT range between 10 and 15 seconds.[114] Using

10 seconds as cut-off value this test presented a 82% of sensitivity and a 86% of specificity for diagnosis of DED. [113]According to Wang et al.,[115] using a more restrictive cut-off value (5 seconds) the test presented better values of sensitivity and specificity (95.9% and 90.8%, respectively).

In any case, some authors consider that the interpretation of the results obtained with these methods is very limited, because its poor reproducibility and its variability.[112]

Finally, NIBUT can be automatically measured by a software that performs an automatized analysis of 10-seconds videokeratoscopic sequence (Tear Stability Analysis System).[116] This technique reaches a specificity similar to that of BUT, but with a sensitivity of 97.5% for the diagnosis of DED.[117]

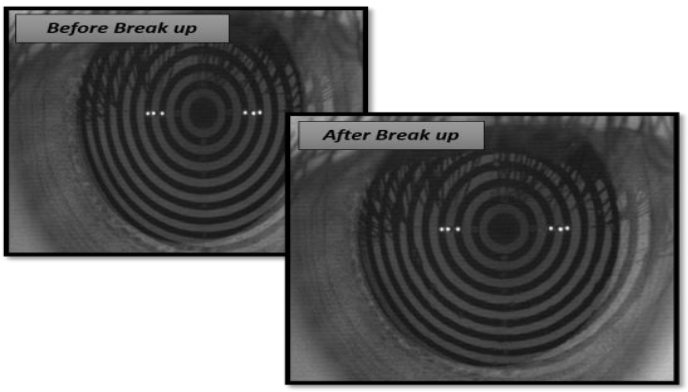


Figure 8. Tear stability measured with Non-Invasive Break-Up Time (NIBUT). *Provided by IOBA.*

1.5.3. Tear Osmolarity Evaluation

Osmolarity is defined as the quantity of dissolved solutes per volume unit of solvent. The measurement of tear film osmolarity is an indirect indicator of tear dynamics. If there is a high tear evaporation, or a low tear secretion, high osmolarity values will be found, due to the reduction in the solvent quantity; on the other hand, the osmolarity will decrease if there is a high tear secretion or a decrease in tear drain.[118]

Currently, there is a portable osmometer suitable for tear osmolarity analysis in routine clinical practice. The TearLab™ osmometer automatically collects by

microcapillarity a small tear sample from the lower tear meniscus and analyzes the osmolarity directly by measuring the impedance of the sample.[119]

The range for normal tear osmolarity is between 300 and 310 mOsm/L.[28] Values above 312 mOsm/L can be compatible with the diagnosis of DED,[120] although Tomlinson et al.[28] established a value of 315.6 mOsm/L as cut-off point between healthy subjects and DED patients. Using 312 mOsm/L as a cut-off value several authors obtained different sensitivities and specificities of the test, due to the differences in the studied groups *(Table 4)*. Because of the paucity of data and partially conflicting results, this technique is not part of the standard diagnostic repertoire yet.

Table 4. Sensitivity and specificity of osmolarity as a diagnostic test using 312 mOsm/L as cut-off value.

Author	*Sensitivity (%)*	*Specificity (%)*
Farris et al.[121]	95	94
Tomlinson et al.[122]	66	84
Gilbard et al.[120]	94.7	93.7

1.5.4. Ocular Surface Evaluation

The ocular surface is commonly examined using the slit-lamp and vital dyes. The usual dyes in clinical practice are fluorescein, Rose Bengal and Lissamine green. Fluorescein is the first-choice dye for DED diagnosis.[123] It is a hydro-soluble colorant that stains both the tear film and epithelial erosions in the conjunctiva and cornea.[124] To evaluate fluorescein staining, 2µL of 2% fluorescein should be instilled. The evaluation should be performed 2 minutes after the instillation using the slit-lamp and a cobalt blue filter. The contrast of the image can be improved by placing a yellow filter in front of the observation system.[125]

Rose Bengal stains those areas of the cornea or the conjunctiva that present a lack of membrane-associated mucins.[126] This test presents poor sensitivity and specificity.[68] Also, it is toxic to the corneal epithelium, producing

irritation and itchy.[127] For these reasons, this vital dye is less commonly used than fluorescein.

Lissamine green was introduced by Norm in 1973.[128] It is used at 1% concentration and stains mucin and degenerate epithelial cells.[128] The pattern of staining is similar to Rose Bengal, but with the advantage that it produces no discomfort or irritation after instillation, allowing greater contrast for observation of blood vessels and hemorrhages.[129]

For all dyes, the intensity of staining and the dye distribution pattern are assessed semi-quantitatively. Several indices are available for the assessment of staining. The most commonly used in clinical practice are the Van Bijsterveld scale,[130] the Oxford system,[131] the standardized version of the NEI / Industry Workshop,[19] and the CCLRU[132] and Efron scales.[133] However, it is desirable to keep the same graduation scale for clinical follow-up, since they cannot be used interchangeably.[134]

1.5.5. Blinking, Eyelids, and Meibomian Glands Evaluation

Blinking is essential to distribute the tear fluid over the ocular surface, and to support secretion from the Meibomian glands. Its observation, and a thorough slit-lamp examination of the eyelids and lashes, may highlight abnormalities associated with an evaporative DED. The normal blink rate should be regular, approximately 10-12 blinks per minute.[135] Blink rates are known to decrease with tasks of increasing difficulty,[136–138] as well as in states of fatigue,[139] which promotes evaporation of tear fluid. On the other hand, increased blink rates, reduced interval between blinks (from about 6 seconds to 2.6 seconds), as well as incomplete blinking, are changes that have been reported in DED patients.[140–143]

A detailed examination of the eyelid margin is necessary to collect information about its inflammation or any dysfunction of the Meibomian glands with associated hyperevaporative disorder *(Figure 9)*. Eyelashes, eyelid margins, and

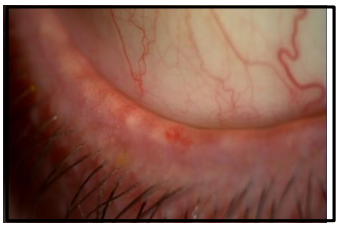


Figure 9. Meibomian Glands. *Image from IOBA*

Meibomian gland orifices can be examined using the slit-lamp; other technologies such as non-contact infrared meibography allows the direct visualization of the Meibomian glands.[144] Healthy Meibomian glands produce a clear lipid secretion, which contributes to the superficial tear film layer. In MGD, the lid margins are often inflamed and the gland orifices may be obstructed or reduced in number. Digital expression of the glands may release a cloudy, opaque, semi-solid or waxy substance, depending on MGD severity.

1.5.6. Visual Function Evaluation

Is well known the important role of tear film in the optical quality of the human eye.[145] Thus, its instability or disruption, generated by the changes on tear film structure, can cause altered vision in subjects with DED, which may be transient (improve with blinking) or more stable over time.[146,147] In addition to the usual clinical tests used to assess visual function (visual acuity and contrast sensitivity), different techniques have been described to aid in the analysis and understanding of impaired visual function in these patients, as well as to the objective determination of the visual disability that produces the pathology.

1.6. DED Therapies

The difficulty of the diagnosis of DED, summed to the multifactorial nature of the pathology, makes the management of dry eye patients and its treatment a very complex task, mainly in moderate-to-severe DED.[148] Currently, DED management includes the use of tear substitutes (i.e. artificial tears, lubricants, ointments), punctual plugs, autologous serum derivatives, and anti-inflammatory therapy (i.e. topical cyclosporine, oral tetracyclines, and topical steroids). It also includes environmental strategies (e.g. avoiding desiccating environments and visual display terminal use, humidifiers use, etc.), lifestyle changes (e.g. diet rich in Omega-3 fatty acid, eyelid hygiene, etc.) or even surgical procedures to increase tear retention. According to the recommendations of the Dry Eye Workshop, treatments should be adjusted to the severity of the pathology[149] *(Table 5)* and must maintain a balance between efficacy, safety, and patient convenience.[150]

Table 5. Treatment recommendations by severity level of dry eye disease. *Modified from: The definition and classification of dry eye disease: report of the Management and Therapy Subcommittee of the International Dry Eye WorkShop (2007).*[149]

Level 1	Level 2	Level 3	Level 4
• Education • Environmental/dietary modifications • Elimination of offending systemic medications • Artificial tear substitutes, gels/ointments • Eyelid therapy	*If level 1 treatments are inadequate, add:* • Anti-inflammatories • Tetracyclines (for meibomitis, rosacea) • Punctal plugs • Secretagogues • Moisture chamber spectacles	*If level 2 treatments are inadequate, add:* • Autologus serum • Contact lenses • Permanent punctal occlusion	*If level 3 treatments are inadequate, add:* • Systemic anti-inflammatory agents • Surgery

Below, some of the most common treatments used in the management of DED that are already available in the market are detailed. Information on steroids will be expanded as it is the subject of this thesis.

1.6.1. Education and Environmental Strategies

Patient education is an important step for the correct management of DED. It is necessary to explain to the patients that DED is a chronic disease and that treatment is long term and may be slow to take effect. As previously explained, there are several risk factors that can cause DED or its worsening, thus some changes in patient´s lifestyle can generate a reduction in DED signs and symptoms. Patients should try to avoid factors such as desiccating environments, smoke, use of visual display terminals during long periods, etc.[149,150]

Moreover, it has been reported that omega-3 plays a role in DED management by reducing inflammation at the ocular surface.[151,152] Thus, a higher dietary intake of omega-3 fatty acids with lower dietary ratio of omega-6 to omega-3 reduces the risk associated with DED symptoms.[153] The utilization of

topical or systemic omega-3 supplements has been recommended for moderate DED patients.[149]

Finally, DED is commonly associated with chronic posterior blepharitis (MGD or meibomitis).[154] MGD is indeed the most frequent cause of DED, through the induction of tear film instability. Eyelid hygiene, of which many variations exist,[155,156] removes bacteria from the eyelid margin and is a widely used treatment in the management of both blepharitis and MGD.[154] Manual expression of the Meibomian glands has been shown to increase lipid layer thickness and tear film stability in normal subjects.[157] A small non-randomized study with MGD patients showed that a daily regimen of eyelid scrubbing increased significantly lipid layer thickness and reduced DED symptoms.[158]

1.6.2. Artificial Tear Substitutes, Gels and Ointments

Artificial tears or tear supplements can potentially improve the quality of life of DED patients by keeping the ocular surface lubricated and relieving patient's discomfort. Small randomized studies have shown that artificial tears increase tear film stability, reduce ocular surface stress and improve contrast sensitivity and the optical quality of the ocular surface.[159–161] There is a large number of artificial tears on the market mainly based on polyvinyl alcohol, povidone, hydroxypropyl guar, cellulose derivatives, and hyaluronic acid. Their compositions vary substantially, so it is necessary to take it into account when choosing the most appropriate for each patient. Two important characteristics of an ocular lubricant are its viscosity and its viscoelasticity, because the hydration capability of an artificial tear and its correct distribution throughout the ocular surface are dependent on these parameters. Depending on the severity of disease, it will be necessary to choose between a whole range of substances, from low-viscosity preparations to high viscosity gels and/or ointments.[149] Also, the composition in electrolytes, osmolarity and viscosity will give the differences in the time of permanence in the eye and interference with vision. In any case, artificial tears cannot replace the cytokines and growth factors present in normal tears. Additionally, as they do not have direct anti-inflammatory effect, their efficacy

decrease as the DED severity increase.[150] In most DED cases, except for the very mild ones, artificial tears should be combined with other more powerful treatments, such as oral omega-3 essential fatty acid supplements, topical mucin secretagogues, topical short-term steroids, and/or topical cyclosporine, to combat underlying inflammation and restore the tear film in patients with DED. Finally, it is important to mention that tear supplements usually contain preservatives which can irritate the eye and additionally exacerbate symptoms.[162] Thus, patients requiring tear supplements more than 4 times a day (grade ≥ 2) should use preservative-free artificial tears.

1.6.3. Autologous Serum

As previously explained, artificial tears lack the biologically active components found in natural tears that are critical to the maintenance of the tear film. The composition of blood serum resembles that of tears with some variations (more vitamin A, lysozyme, transforming growth factor-β (TGF-β) and fibronectin, and less immunoglobulin A (IgA), epithelial growth factor (EGF) and vitamin C).[163–165] Autologous serum eyedrops are produced by separating the liquid and cellular components of the patient's blood. It contains multitude of growth factors, fibronectin, vitamin A, cytokines, and anti-inflammatory substances.

In 1975, autologous serum eye drops were applied for DED for the first time.[166] Since then, they have become increasingly popular for treating ocular surface diseases, being one of them DED. Typically, autologous serum eye drops are administrated to DED patients in a 20% concentration. It has been reported that at this concentration they produced reduction of symptoms, improvement of TBUT test, and reduction in Rose Bengal staining score in DED patients.[167] Higher concentrations (between 50% and 100%) have also been used.[167–172] This treatment is used particularly in severe cases of DED, but its efficacy is under discussion.[173] More recently autologous serum enriched with growth factors is also used for severe DED.[174]

1.6.4. Punctal Plugs

Punctal plugs are small collagen or silicone devices used to generate a temporary occlusion of tear ducts *(Figure 10)*. The objective is to reduce the drainage of tears and increase lubrication on the ocular surface.[175,176]

Its effectiveness has been reported in patients with severe aqueous-deficient DED.[177–179] In a retrospective study, punctal plugs led to an improvement in subjective symptoms in 73.9% of patients, with a significant reduction in surface staining.[179] Punctal plugs relieve DED-related symptoms in patients with SS, filamentary keratitis, chronic Stevens-Johnson syndrome, trachoma, neurotrophic and diabetic keratopathy, keratitis sicca, and dry eye after refractive surgery.[180] The most frequent complication of this therapy is the loss of the plug, while obstruction or inflammations of the lacrimal ducts as well as active blepharitis are the most common contraindications for the application of punctal plugs.[179,181]

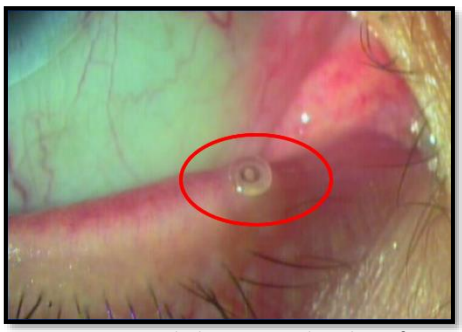


Figure 10. Punctal plug inserted in the inferior punctum. *Image from IOBA*

Since delayed tear drainage leads to the persistence of toxic and inflammatory factors on the ocular surface, punctal plugs should be applied after the inflammation has been brought under control,[182] or under the use of concomitant anti-inflammatory treatment.[183] Sainz de la Maza et al.[182] reported a higher efficacy of the punctal occlusion after steroids therapy. In any case, large, randomized and controlled studies are necessary to evaluate this therapy.[176]

1.6.5. Cyclosporine A

Cyclosporine A (CsA) is an immunomodulatory compound with anti-inflammatory properties approved by the USA Food and Drug Administration (FDA) for treatment of moderate-to-severe DED. The potential of CsA for treating

DED was initially recognized in dogs that develop spontaneous keratoconjunctivitis sicca,[184] and later scientifically demonstrated in dogs[185,186] and humans.[89,187–189] The immunomodulating effects of CsA are achieved by the inhibition of the calcineurin–phosphatase pathway by complex formation with cyclophilin, which reduces the transcription of T-cell-activating cytokines such as interleukin (IL)-2 or IL-4.[190] CsA increases goblet cell density[191] and inhibits apoptosis in conjunctival epithelia which may be particularly relevant to its therapeutic effect in the management of DED.[192,193] Moreover, topical application of CsA increases production of tear fluid,[89,187,194] possibly via inhibition of T-cell activation and down-regulation of inflammatory cytokines in the conjunctiva and lacrimal gland,[195,196] or via local release of parasympathetic neuro transmitters.[197]

Different studies have reported the efficacy of CsA in the treatment of DED, as well as its tolerability.[89,187,193,198–203] Treatment with CsA led to improvement in keratopathy, increased Schirmer test values, reduced symptoms (blurry vision, ocular dryness, foreign body sensation, and tearing), and generated a reduction in the use of artificial tears.[89,204,205] These clinical improvements were associated with a reduction in inflammatory cells and inflammatory markers on the ocular surface,[188,206] and an increase in the number of goblet cells in the conjunctiva.[204]

Unlike other treatments, such as corticosteroids, CsA can be used as a long-term therapy for DED because it is not associated with either significant systemic adverse events or local side effects. Commercially available 0.05% and 0.1% topical cyclosporine or compounded preparations are frequently utilized for treatment of various inflammatory ocular surface disorders, including DED.[207]

1.6.6. Oral Tetracyclines

Tetracyclines have anti-inflammatory and antibacterial properties that may make them useful for the management of chronic inflammatory diseases. Oral tetracyclines have been used off-label to treat DED, primarily DED associated with ocular rosacea.[208–211] It has been suggested that reduction of bacterial flora may decrease the breakdown of Meibomian lipids; however, tetracyclines are used in DED primarily for their anti-inflammatory actions. It has been reported a decrease

in the matrix metalloproteinase activity, and in the production of proinflammatory cytokines such as IL-1 and tumor necrosis factor-alpha (TNF-α).[212–215]

Several small, randomized clinical trials, mostly in ocular rosacea, provide limited evidence of its efficacy. Doxycycline, a semi-synthetic tetracycline derivative, has been shown to effectively inhibit Matrix Metalloproteinase (MMP)-9 in a wide variety of mouse and human cells including prostate epithelium, epidermal keratinocytes, and the aortic endothelium.[216–219]

Yoo et al.[220] compared 2 different doxycycline doses (200 mg and 20 mg/day) in patients who had chronic MGD and who had not responded to lid hygiene and topical therapy for more than 2 months. Both the high and the low dose group had statistically significant improvement in TBUT after treatment. This implies that low dose doxycycline (20 mg twice a day) therapy may be effective in patients with chronic MGD.[220,221]

1.6.7. Lifitegrast

Lifitegrast is a small-molecule integrin antagonist that has been recently approved by the FDA (5% lifitegrast ophthalmic solution) as a treatment for DED.[222] This molecule targets the inflammation associated with DED by blocking the binding of integrin lymphocyte function-associated antigen 1 (LFA-1) to intercellular adhesion molecule 1 (ICAM-1), which is its cognate ligand.[223] It has been demonstrated that DED patients present infiltration of T-cells in the conjunctiva, as well as an increased expression of ICAM-1 in lacrimal and conjunctival epithelial cells. Moreover, it has been recognized that the binding LFA-1/ICAM-1 is crucial in the T-cell recruitment, activation and inflammatory cytokine release.[224] Taking together these facts, it is not surprising the potential efficacy of this treatment breaking the inflammatory process in DED patients.[225]

Currently, there are 5 clinical trials that have studied the efficacy and safety of 5% lifitegrast ophthalmic solution in the treatment of DED patients.[226–230] Semba et al.[226] reported improvements in corneal staining and OSDI score in the group treated with lifitegrast compared to placebo, as well as its safety after 84 days of treatment. The efficacy of this therapy and its safety continue to be evaluated in

different studies: OPUS-1 (lifitegrast vs placebo during 84 days of treatment; reduction in symptoms, corneal and conjunctival staining; safe and well tolerated),[227] OPUS-2 (lifitegrast vs placebo during 84 days of treatment; symptoms reduction; safe and well tolerated),[228] OPUS-3 (lifitegrast vs placebo during 84 days of treatment; symptoms reduction; safe and well tolerated),[229] and SONATA (lifitegrast vs placebo during 1 year of treatment; safe and well tolerated).[230]

1.6.8. Topical Corticosteroids

Corticosteroids are among the most effective agents used for the treatment of non-infectious inflammatory diseases, especially in those mediated by the immune system. This therapy may be used for patients who have corneal disease and have persistent symptoms despite extensive use of artificial tears.[149] The benefit of topical corticosteroids in the treatment of DED has already been demonstrated, although it is still off-label. In any case, several clinical studies[182,231–238] and clinical trials[239–243] have reported the effectiveness of topical corticosteroids in the treatment of DED signs and symptoms, as well as its safety in a short-term treatment period (*Table 6*). There is a possibility of long-term side effects, but there are no studies in this regard, as they are not used as such. To reduce this potential danger and increase the benefit/risk ratio the so-called soft steroids can be used, such as loteprednol or rimexolone, which have less intraocular activity[244,245] and, therefore, less possibilities of raising intraocular pressure (IOP), which is the main adverse effect derived from corticosteroids use.[246]

Table 6. Studies and clinical trials published using topical corticosteroids for dry eye disease (DED).

Author	Type of study	Treatment	Duration	Main Results in the Corticosteroids Group
Marsh et al.[236]	Retrospective study	Methylprednisolone	14 d	↓symptoms; ↓corneal staining
Sainz de la Maza et al.[182]	Prospective study (2 groups)	Punctal occlusion (with and without prior treatment with Methylprednisolone)	14 d	↓symptoms; ↓corneal staining No adverse events
Avunduk et al.[239]	Clinical trial (3 groups)	Artificial tears Artificial tears + NSAID Fluorometholone + Artificial tears	30 d	↓corneal staining; ↓conjunctival staining; ↑goblet cells; ↓HLA-DR+ cells
Pflugfelder et al.[240]	Clinical trial (2 groups)	Loteprednol etabonate vs vehicle	28 d	Improvement after 2 weeks; No changes in IOP
Lee et al.[241]	Clinical trial (contralateral eye as control)	Prednisolone vs hyaluronic acid	28 d	↓symptoms; No changes in TBUT or Schirmer ↓nerve grow factor
Yang et al.[233]	Not masked cross-design study	Fluorometholone	30 d	↓symptoms; ↑TBUT; ↑Schirmer; ↓corneal staining
Hong et al.[237]	Not masked study	Methylprednisolone	14 d	↓symptoms; ↑TBUT; ↑Schirmer; ↑goblet cells
Jonisch et al.[238]	Retrospective study	Dexamethasone	4 m - 60 m	↓symptoms No changes in IOP; Good safety
Byun et al.[234]	Clinical trial (2 groups)	Methylprednisolone* + Cyclosporine A Cyclosporine A	21 d* + 69 d	Faster amelioration with a short-term use (3 weeks) of corticosteroid prior cyclosporine A ↓symptoms; ↓corneal staining; ↓IL-6 and IL-8
Aragona et al.[242]	Clinical trial (2 groups)	P-pyrrolidone + Clobetasone butyrate P-pyrrolidone + Placebo	30 d	↓symptoms; ↓corneal and conjunctival staining; ↓HLA-DR+ cells; No changes in IOP
Lee et al.[232]	Prospective study	Methylprednisolone + Sodium hyaluronate	56 d	↑TBUT; ↓corneal and conjunctival staining; ↓osmolarity ↓IL-1β; ↓IL-8
Sheppard et al.[243]	Clinical trial (2 groups)	Loteprednol etabonate* + Cyclosporine A Artificial tears* + Cyclosporine A	14d* + 42d	Faster and higher amelioration with a short-term use (2 weeks) of corticosteroid prior to cyclosporine A
Moore et al.[235]	Single-group crossover clinical trial	Dexamethasone vs artificial tears	14 d	↓corneal staining and eye irritation after low humidity exposure than with artificial tears. ↓HLA-DR+ cells

**Induction therapy; d = days; HLA-DR = human leukocyte antigen D related; IL = Interleukin; IOP = Intraocular pressure; m = month; NSAID = Non-steroidal anti-inflammatory drug; P-pyrrolidone = Polyvinylpyrrolidone; TBUT = Tear Break-Up Time*

*** Pinto-Fraga et al, published in 2016 is not mentioned here as it is a publication derived from this Thesis.*

Methylprednisolone

In 1999, Marsh et al.[236] published a retrospective study with 21 patients that presented severe DED associated to SS. These patients were symptomatic despite being under therapy. Patients were treated with topical methylprednisolone 3-4 times daily during 2 weeks. A moderate-to-complete symptomatic improvement and a significant decrease in corneal staining were observed, as well as a complete resolution of filamentary keratitis.[236] These authors also used this therapy in smaller doses but for a longer time, reaching a better long-term control of these patients. But, after 3 months, some adverse events were observed, related to a long-term steroid use (e.g. ocular hypertension, posterior sub-capsular cataract).[236]

One year later, a prospective and randomized trial[182] compared the effect of punctal plug occlusion with and without prior anti-inflammatory therapy (topical methylprednisolone) for 2-weeks prior occlusion; groups A and B respectively. After two months, 80% of patients from group A and 33% of patients from group B showed complete symptomatic resolution. Corneal staining was negative in 80% and 60% of the patients, respectively. No complications related to steroid use were observed.[182]

Another a study evaluated the recurrence of SS-DED in 106 patients treated with topical 1% methylprednisolone.[237] The therapy was safe and effective, improving symptoms, TBUT, Schirmer test and increasing the number of goblet cells observed in impression cytology.[237]

In 2012, Byun et al.[234] performed a clinical trial in Korea comparing the efficacy of topical cyclosporine 0.05% and combined treatment with 1% methylprednisolone acetate for the treatment of moderate-to-severe DED. This study compared two groups of moderate-to-severe DED patients: group a) 21 patients treated with 1% topical methylprednisolone during the first 3 weeks and with topical CsA only thereafter; group b) 23 patients treated with CsA only. This study demonstrated the safety and effectiveness of topical CsA. There was an improvement in symptoms and signs, as well as a decrease in IL-6 and IL-8 levels.

Additionally, short-term use of a topical steroid had the benefit of providing faster symptom relief and improvement of ocular signs.

Recently, a prospective study reported that a short-term treatment (8 weeks) with topical 1% methylprednisolone not only improved clinical outcomes (TBUT and corneal and conjunctival staining), but also decreased tear osmolarity and cytokine levels (IL-1β and IL-8).[232]

Loteprednol Etabonate

The efficacy of loteprednol etabonate have also been evaluated. Pflugfelder et al.[240] published a randomized, double-masked, multicenter, placebo-controlled trial in 64 patients with DED presenting delayed tear clearance. One group was treated 4 times daily for 4 weeks with 0.5% loteprednol etabonate, a modified steroid intended to cause less intraocular pressure elevations while the other group received the vehicle of the study treatment. In the loteprednol-treated group, the subpopulation of patients with the most notable inflammatory signs showed a significant amelioration after 2 weeks of treatment, which was retained 2 weeks after treatment, without intraocular pressure changes.[240] In 2014, Sheppard et al.[243] evaluated the effect of loteprednol etabonate before the initiation of topical CsA therapy in patients with mild-to-moderate DED. The study design was like the previously published by Byun et al.[234] The results showed that loteprednol etabonate induction therapy 2 weeks before the initiation of long-term CsA treatment provided a more rapid relief of DED signs and symptoms with greater efficacy than CsA and artificial tears alone.[243]

Prednisolone

A randomized, comparative trial[241] explored the treatment of 41 DED patients (not SS-related) using 0.1% prednisolone in one eye and 1% hyaluronic acid in the contralateral eye. Although it is known that these designs are not advisable, due to one eye is poor control of the contralateral, the steroid achieved a decrease in symptoms and nerve growth factor levels.[241]

Dexamethasone

In 2010, a retrospective study[238] was conducted to evaluate the short-term safety and efficacy of topical preservative-free dexamethasone 0.01% for the treatment of different disease. From the complete sample (31 patients) 19 suffered DED and were treated with the corticosteroid 4 times daily between 4 and 60 months. In the 87% of the patients an improvement in symptoms was observed (65% moderate or complete, 22% mild). The treatment was well tolerated, without elevations of IOP greater than 5 mmHg. Additionally, Moore et al.[235] reported the absence of ocular surface worsening in topical dexamethasone-treated DED patients exposed to low humidity environment.

Clobetasone Butyrate

Aragona et al.[242] studied the effects of a low administration rate and low concentration (0.1%) of clobetasone butyrate eyedrops in patients with SS. This clinical trial compared two different groups: group 1 received 2% polyvinylpyrrolidone eyedrops + placebo, while group 2 received 2% polyvinylpyrrolidone + 0.1% clobetasone butyrate. Both groups showed amelioration in signs, symptoms and reduction in human leukocyte antigen D related (HLA-DR) expression, but for all these parameters, the improvement was significantly greater in group 2. No adverse events were observed.

Fluorometholone

A randomized clinical trial[239] conducted at the University of Louisiana (USA) and published in 2003 compared the efficacy of 3 different therapies for the treatment of DED patients: group 1, topical artificial tear substitute; group 2, topical artificial tear substitute plus nonsteroidal anti-inflammatory drug (NSAID); and group 3, topical artificial tear substitute plus topical 0.1% fluorometholone. This trial demonstrated that the addition of topical steroids to artificial tears had a clearly beneficial effect in the improvement of subjective and objective parameters (less corneal and conjunctival staining and greater number of goblet cells). These effects were associated with the reduction of inflammation markers of conjunctival epithelial cells (decreased HLA-DR positive cells).[239] In a non-

masked cross-designed study[233] (patients received tears first and then the corticosteroid), 0.1% fluorometholone was tested against tears with hyaluronic acid. The 30 moderate-to-severe DED patients received one drop of corticosteroid 4 times daily for 1 month. Patients that were not sensitive to artificial tears presented an improvement in subjective and objective tests (symptoms, TBUT, corneal staining and Schirmer) after 1-month treatment with fluorometholone.[233]

2. Influence of Environmental Conditions in DED

As previously explained, epidemiological studies have shown that the incidence of DED is increasing.[41,43,247] One of the most important factors contributing to the prevalence increase of DED is the growing proportion of population that is continuously exposed to the so-called adverse environments or desiccating stress conditions. It is well known that adverse environmental conditions, such as excessive heat, wind or low relative humidity (RH), cause symptoms and signs of DED.[248] These environments exist in many parts of the world, especially in regions with desert or semi-desert climates, and not only in extreme areas. Some studies have demonstrated that environmental conditions found in different regional areas are directly related with the incidence of DED.[110,249] A study developed by Um et al.[249] analyzed over 16,000 people. This study found a higher incidence of DED in metropolitan cities and in areas where humidity was lower, sunshine duration was longer, and concentration of air pollutants (sulphur dioxide) was higher. Besides, Tesón et al.[110] analyzed the consistency of DED tests in normal individuals living in two geographic locations with different climates (continental: Valladolid, Spain vs. Atlantic: Braga, Portugal). Significant differences were observed between both samples on TBUT, corneal staining and conjunctival staining. This study provided evidence to support that DED test outcomes used to evaluate ocular surface integrity and tear stability are climate-dependent.

On the other hand, these conditions can be also found indoor. Millions of people are currently staying longer within artificially-created environments such as office buildings, shopping malls, air-conditioned vehicles, and households. These environments are characterized by low humidity and draftiness, conditions that cause tear film alterations that usually worsen DED. For many DED patients these conditions are unbearable.[250] In addition, the number of users of visual display terminals (including tablets and smart phones) and the amount of time spent using them have also increased dramatically.[251] These information technology devices reduce blink rate, causing tear film evaporation that can further worsen DED signs and symptoms.[252,253]

For a better understanding, the most important environmental factors to which the LFU is exposed, both outdoor (climate-related conditions) and indoor (controlled environment conditions), as well as their implications and effects are detailed below.

2.1. Outdoor Environment Conditions

The LFU is continuously exposed to the environmental conditions, both indoor (controlled environmental conditions) and outdoor. It has been previous reported that the environmental factors producing the highest impact over the LFU are RH, temperature and air flow.[41,250,254] Excessive dryness, high temperatures and/or windy conditions, would accelerate the normal rate of tear film evaporation by affecting its lipid layer, thus accelerating tear loss and consequently altering the cycle of tear production, distribution, and elimination, and ultimately altering the quality of the tear film and its function (e.g. optical, protective, etc.).[255] This fact, in a non-predisposed individual, can be compensated by producing more tear volume and/or blinking more often, but in a genetically-prone individual it might trigger DED or exacerbate pre-existing DED. The compromise of the lipid layer will decrease tear film stability and will eventually compromise ocular surface integrity, generating corneal and conjunctival staining.[256] This point has been previously demonstrated when healthy volunteers and DED patients were exposed to adverse environmental conditions.[257]

2.1.1. Relative Humidity (RH)

RH is defined as the amount of water vapor in the air, expressed as a percentage of the maximum amount that the air could hold at the given temperature. Thus, the closer the RH value is to 100%, the more humid the environment is.

RH is probably the most essential factor affecting the LFU. It has been demonstrated an inverse correlation between the environmental RH and the tear film evaporation. To evaluate the importance of RH on the tear film, some researchers have investigated its effects on tear film evaporation. More than 30 years ago, Rolando and Refojo[258] designed a tear evaporimeter consisting of

modified swimming goggles. During the study, subjects were fitted with these goggles so that the air over the test eye was conditioned to a desired RH (29.5%) and temperature (23°C) while subjects kept their eyes open to avoid blinking during 1 min. Under these conditions, they determined that the evaporation rate in normal eyes was 4.07 ± 0.40x10-7 g/cm^2/sec. This value increased in healthy eyes after topical anesthetic instillation and in subjects suffering from DED.[259]

Over the years, some other authors have used different types of evaporimeters. Different evaporation values have been found for normal individuals, but always higher in both tear-deficient and MGD-type DED.[260–262] It has been also demonstrated that low RH was the main causative factor increasing evaporation in both normal controls and DED patients, without differences between males and females.[263,264] McCulley et al.[263] demonstrated that the tear film evaporation rate in both controls and DED patients is much higher with 20-25% RH in comparison with 40-45% RH. Also, the evaporation rate under low RH conditions was higher in the DED group with MGD than in the non-disease group.[263] In parallel, these authors did not find differences between genders in tear evaporation rate. These results have been confirmed in more recent studies using controlled environmental chambers to maintain a tight control of the environmental parameters.[264]

A more recent study[265] measured transepidermal water loss from the ocular surface and surrounding periocular skin. It was observed that the water loss was significantly higher in both evaporative- and aqueous-deficient-DED patients compared with controls. This parameter was correlated positively with TBUT, corneal staining and symptoms, and negatively with Schirmer test values.[265]

Two more recent studies developed in controlled environmental chambers, where environmental parameters could be tightly controlled, confirmed the inverse correlation between RH and tear evaporation rate, as well as the higher evaporation rate in DED patients compared to normal subjects.[266,267] However, these differences were not observed under environments with a RH close to 70%. In a second study in the same environmental chamber comparing two environmental conditions (21°C, RH 40% vs 21°C, RH 5%), this group showed that

evaporation rate, tear film stability, ocular comfort, lipid layer thickness, and tear production were adversely affected by low RH (5%) after a 60-min exposure.[267] Therefore, the negative impact of climate and low humidity environment on tear film loss have been clearly demonstrated.

2.1.2. Temperature

Environmental temperature has a great influence on tear film evaporation and on the viscosity of lipid secretions from Meibomian glands. The main function of these lipid secretions is the maintenance of tear film stability and retarding evaporation.

The average corneal temperature is around 34°C with a broad range (26°C to 37°C) depending on the environmental temperature. A corneal temperature between 32°C-40°C is adequate to maintain the lipids contained in the Meibomian glands in a liquid state, allowing its correct production and secretion.[268] A temperature near 40°C alters those lipids, changing their properties and reducing their functionality, indicating that high temperatures tend to disrupt the tear film.[269] As RH and temperature are interdependent, some authors performed studies maintaining a constant RH at different temperatures. An in-vitro study demonstrated that in an environment with a normal RH (40%) and without air flow, an increase of 9°C (from 25°C to 34°C) in the environmental temperature generated a 3-fold increase in the evaporation rate of human tears.[255] Another study in an environmental chamber corroborated that an increase in ambient temperature, at a constant 50% RH, correlated with an increase in tear film evaporation.[267]

2.1.3. Wind / Air Flow

Airflow, or air speed, is another factor that can alter tear film stability, affecting the LFU status. It has been reported that under a constant temperature (25°C), the presence of a moderate air flow induced a 10-fold increase of the tear evaporation rate in an in-vitro model.[255] Wyon et al.[270] showed that exposing tear film to high air velocity (1.0 m/s) for 30 minutes caused a decrease in TBUT in

healthy eyes; however, exposure to air velocity of 0.5 m/s for 30 minutes showed no significant negative impact.

Koh et al.[271] evaluated the effect of shorter airflow exposure (5 minutes) at higher velocity (1.5±0.5 m/s) provoked a decrease in tear meniscus in normal subjects and evaporative DED patients.

2.1.4. Pollutants

Another environmental factor producing high impact in the population is air pollution, despite the fact that there is not an appropriate way to measure this factor.[272] To evaluate the effect of air pollutants, indoor studies have been widely performed, as mentioned below; these designs allow to maintain a tight control of the variables. However, outdoor studies have been also carried out.[273]

Correlations between the exposure to air pollution and ocular surface irritation, hyperemia, tearing, and dry eye sensation have been studied for many years.[274] Several studies have identified some pollution-induced ocular alterations in both clinical sings, such as increase of the blinking rate or tear film instability,[275–277] and tear molecular expression, such as a decrease of IL-5 and IL-10 due to the exposure to high levels of PM2.5. This suggests a possible modulatory action of ambient air pollution on ocular surface immune response.[278] Moreover, a positive association between exposure to air pollution and goblet-cell hyperplasia in human conjunctiva has been reported.[279] As a result of these effects, the LFU might be compromised because of conjunctival and corneal damage that can result in altered corneal epithelial barrier function. In fact, a study reported an increase in the levels of ocular symptoms, eye redness and irritation, and lower TBUT in persons travelling through highly polluted areas of the metropolis of Delhi, India compared with other volunteers that were not daily exposed to metropolitan pollution.[273] A late study reported that increased levels of air pollution reduced tear film osmolarity and conjunctival goblet cell density.[280] Finally, another study mentioned earlier suggested that the concentration of sulfur dioxide was associated with DED, while those of nitrogen dioxide, ozone, carbon monoxide, and PM10 were not.[249]

2.1.5. Ultraviolet Light

Ultraviolet radiation is a common risk factor to ocular surface health in people exposed for long periods without an adequate protection (e.g. outdoor workers). It has been suggested an association between DED and longer sunshine duration or excessive acute exposure to ultraviolet light resulting in acute tear film instability and a transient DED;[249] this assumption however is controversial.[281,282] On the other hand, it is well known the influence of ultraviolet radiation in development in the pathogenesis of pterygium, a pathology that can eventually result in tear film instability and cause DED-related signs and symptoms.[283,284]

2.2. Indoor Environmental Conditions

In developed countries, people presently spend a high percentage of their time in air-conditioned indoor environments such as office buildings, households, shopping malls, vehicles, etc. These environments are commonly referred to as "buildings' microclimate" and share low humidity, air draftiness and, in cold seasons, high temperature. These factors can cause tear film alterations that may elicit and/or worsen DED[250] and, as explained above, can have the same impact described for outdoor environments.

The most important of all those factors seems to be humidity. In fact, low RH (5-30%) has been long associated with an increase in the prevalence of DED-related symptoms.[285–289] In an interesting cross-over study,[288] office workers exposed to low RH (20-30%) had higher dryness symptom score than when they were exposed to a more humid environment (30-40%). Another study[287] carried out in hospital employees in Scandinavia demonstrated that raising RH to 40-50% for 4 months decreased the sensation of eye dryness in 73% of individuals, while this decrease in symptoms was reported in only 23% of individuals exposed to non-humidified conditions (25-35% RH).[287]

Regarding the effect of temperature, it has been reported that temperatures above 22°C caused dryness symptoms, in both humidified and non-humidified conditions.[285] Another cross-over study[290] in public office buildings showed that a 1°C decrease in room temperature (within 22°C and 26°C) was associated with a 19% decrease in the mean value for eye irritation severity,[290] which may be

associated with changes observed in tear film with higher temperatures, such as reduction in TBUT or tear layer thickness.[254]

Indoor air pollutants, specifically the concentration of volatile organic compounds, are becoming an important problem due to the continued increase in outdoor-air contamination, which directly affects indoor environments. There are several elements that can be evaluated to study indoor air pollution, such as particulate matter (PM) mass concentrations (PM10, PM2.5, PM1), ultrafine particles, or gas phase pollutants (NO_2, O_3, CO, CO_2). CO_2 is the reference parameter for indoor air quality, because high CO_2 concentrations indicate poor ventilation conditions and the possible accumulation of other indoor air pollutants.[291]

When considering the influence of indoor environment on eye symptomatology, not only the factors shared with outdoor conditions (humidity, temperature, airflow, pollutants) matter, but also illumination, use of visual display units (e.g. computers, tablets, smart phones) and other visual activities.

2.2.1. Computer Vision Syndrome (CVS)

The term "computer vision syndrome" (CVS) is defined by the American Optometric Association as a complex of eye and vision problems related to the activities that stress the near vision and that are experienced by 50-70% of people in relation to or during the use of computers.[292] It is estimated that this syndrome affects nearly 60 million people globally, and that one million new cases occur each year.[293] In any case, the prevalence of eye problems associated with CVS varies depending on the sample tested and the methods employed.[292,294] Thomson et al.[294] reported that up to 90% of computer users may experience DED symptoms after a prolonged use, while Hayes et al.[295] estimated a prevalence ranging from 75% to 90% among a similar population.

Video display terminal viewing alters blinking patterns, reducing blink rate and amplitude, increases the amount and time of interpalpebral ocular surface exposure, and generates an increase in the percentage of incomplete blinks.[296] The resulting increase in tear film evaporation can further worsen an already established DED or even provoke initial DED-related symptoms in a susceptible

person.[297–299] Its symptomatology includes DED-related symptoms (dryness, irritation, eye strain/fatigue, blurred vision, redness, burning, excessive tearing), double vision, headache, light/glare sensitivity, slowness in changing focus and changes in color perception.[300]

There are several factors that can affect the response of visual displays users, such as screen type and the distance from it, luminance, screen refresh rate, time of use, personal factors, as well as those previously explained (i.e. humidity, temperature and pollutants).[290,301–304] Tear film stability is strongly influenced by the position of the display. Vertical gaze position results in substantial increase of blink frequency during their use,[305] in addition to a significant reduction of TBUT.[306] Regarding the personal factors associated with CVS, one of the most relevant is gender,[304] probably related to the use of make-up, which has been associated with increase of eye irritation,[307] or to the age-dependent decrease of tear secretion among women after the 4th decade.[308,309] Also, medications, smoking habit,[310] or contact lens use are relevant factors to take into account.

2.2.2. Sick Building Syndrome

As previously stated, the general population in the most industrialized countries spend 80-90% of their time in indoor environments.[311] Consequently, the importance of healthy indoor environmental conditions has become a relevant issue. Still public health authorities do not seem to sufficiently regulate indoor environmental conditions. According to the World Health Organization,[312] the sick building syndrome can be defined as those situations in which building occupants experience acute health and comfort effects that appear to be linked to the time spent in a building, but no specific illness or cause can be identified. This syndrome is characterized by the presence of symptoms such as headaches, fatigue, shortness of breath, lack of concentration, eye and skin irritation, dryness, upper respiratory problems, tiredness, difficulty in concentrating, etc.[313] The initiation of these problems could be due to an improper air quality, insufficient ventilation, air pollutants, biological agents in the air, building materials, noise contamination, lighting, inadequate temperature and humidity control, or a combination of them.[314] The majority of respondents report that the symptoms

tend to decrease in severity, or even completely disappear when away from the office building.[315]

Eye problems are, upon a recent publication,[314] the most prevalent problem related with sick building syndrome, with 18.7% of individuals referring eye dryness. It is clear that low RH, high temperature and air pollutants, as well as the use of video display units for long periods of time generate an increase of tear evaporation, increasing the exposure the ocular surface.[313,316] Due to the high impact of the sick building syndrome, the American Society of Heating, Refrigerating and Air-Conditioning Engineers recommended comfort limits for indoor environments: a 30-70% RH range, a 20-24°C and 23-26°C of temperature range in winter and summer, respectively, and an airflow ≤ 0.15 m/s at head level, to avoid an increase of tear evaporation. These values are not significantly different respect to those recommended in other countries, like Japan (40-70% RH, 17-28°C),[289] Canada (40-60% RH, 20-24°C),[286] or Spain (30-70% RH, 17-27°C).

3. Recreation of Environmental Conditions in Experimental DED

As it has been previously explained, adverse environment conditions (i.e. low humidity, high temperatures, airflow) can cause tear film alterations, contributing to the apparition and worsening of DED. For this reason, these conditions are extensively used in experimental models of DED to create or aggravate the pathology. These models have greatly contributed to elucidate the pathology of DED and to test new therapies. Along the scientific literature, it is possible to find multiple attempts to study the importance of these environmental factors in the pre-clinical stage of the research process. It is important to emphasize the need for caution when extrapolating basic scientific findings or therapeutic activity from the animal, *ex vivo*, and *in vitro* models to human disease.[317]

Below, a review of the most important *in vitro* studies, *ex vivo* studies and animal models that have used adverse environmental conditions to generate and study DED is offered.

3.1. *In vitro* Models of DED

The appearance of *in vitro* 3-D models of human corneal epithelium allowed generating advances in the creation of *in vitro* DED models.[318–320] These models offer general advantages over *in vivo* studies, as these easy-to-handle models of human origin resemble human epithelial physiology better than conventional monolayer models. These models use some of the conjunctival cell lines that have been described: e.g. Chang conjunctival cell line,[321] Wong-Kilbourne derivative of Chang conjunctival epithelial cells, IOBA-normal human conjunctival (NHC) cell line,[322] and the ConjEp-1/p53DD/cdk4R/TERT cell line.[323]

Meloni et al.[324] established an experimentally-induced dry eye *in vitro* (EDEV) model on a human corneal epithelia (HCE) model. The reconstructed HCE model consisted of immortalized human corneal epithelial cells cultured on an inert, permeable polycarbonate filter of 0.5 cm^2 for 5 days at the air-liquid interface in a supplemented chemically defined medium. The overall morphology of the model was similar to that of HCE. To generate the *in vitro* DED model, HCE were

exposed to controlled environmental conditions (RH<40%, 40±5°C, 5% CO_2). Cell viability, histology and mRNA expression of selected genes were studied at 24h, 48h, and 72h. The EDEV-HCE model is characterized by an increase in MMP-9 and TNF-α expression (inflammatory molecules), as well as in mucin (MUC)-4 (lubricating and clearing function and its role as a surface-associated mucin in providing barrier function to corneal and conjunctival epithelia)[325] and defensin β-2 (role in antimicrobial protection and in ocular surface damage in subjects with DED).[326] These results agree with previous studies.[327–329] Moreover, a gradual breakdown and disruption of epithelial integrity following EDEV induction, as well as a reduction in EDEV-HCE tissue thickness were observed over the 72h period. However, EDEV-HCE tissue viability and morphology was fairly well preserved, as indicated by the viability assay and histomorphologic analysis.

In a second part of that study, some commercial tear substitutes were tested for their efficiency and capability to reduce and prevent desiccation of HCE cells in the EDEV model. The artificial tears were applied to tissues previously maintained for 24h under EDEV conditions. Tissues were then followed for further 24h to assess tears impact over the selected biomarkers and the microvilli structure. The application of artificial tears promoted an increase of MUC-4 gene expression, associated to a positive adaptive and defense mechanism to fight the atrophy, as well as a decrease of MMP-9 and defensin β-2.

Meloni et al.[324] claimed that the EDEV-HCE model reproduces the morphological and molecular modifications to the corneal epithelium in the DED, being suitable to test products activity in preclinical trials in both counteracting the induction of dryness conditions or restoring the homeostasis in an established EDEV-HCE model.

Recently, Barabino et al.[330] have used the *in vitro* EDEV-HCE model[324] explained above to evaluate the potential effects of T-LysYal®, a new supramolecular system,[331] on damaged corneal epithelial cells in DED. The protocol followed and environmental conditions used were the same previously detailed. This study concludes that there is the possibility of a new class of agents denominated ocular surface modulators that can restore corneal cells damaged

by dry eye conditions. On the other hand, researches from our institution are taking great steps in the construction of a three-dimensional model of human conjunctiva,[332,333] which will allow studying the effect of different factors (i.e. environmental conditions, inflammatory stress, etc.) over this structure.

3.2. *Ex vivo* Models of DED

Ex vivo models offer several advantages over both *in vitro* research (e.g. use of real tissues and not only cell cultures) and animal models (e.g. lower cost and the reduction of animals use in experimentation).

A porcine dry eye *ex vivo* model was developed by using corneas from recently enucleated eyes and exposing them to airflow, with temperature and RH kept constant.[334] Three different groups were studied. Experimental groups A and B were composed by 6 corneas exposed to air flow at room temperature (20–25°C) and constant RH (55–65%) during 4h and 6h, respectively. Group C (exposure control group) was composed by other 6 corneas exposed at the same conditions previously explained, but in this group lacrimation/blinking was simulated by wetting the corneas with Dulbecco Phosphate-Buffered Saline solution. Finally, control-baseline group was composed by 9 corneas, studying its viability within 5 minutes of enucleation. Trypan-blue exclusion technique showed significant differences between the exposure control group and the experimental groups A and B in the damage observed in both central and peripheral corneal regions.

Based on these results, this group generated a novel porcine dry eye model that used airflow to generate different grades of DED.[335,336] The consistency of this model has been studied by Chan et al.,[321] confirming its viability and validity. The porcine dry eye model was applied to compare the efficacy of different artificial tears in protecting the cornea against simulated and controlled severe desiccation induced by dry eye condition,[335] and to investigate the effect of blink rates in dry eye condition.[337]

One inherent problem of this model is the limited time-frame in which the model is useful, due to the increasing mortality rate of cells caused by the reduction in their metabolic capabilities in the external environment, as well as the inability to evaluate healing of epithelial defects. To address these limitations,

the "*ex vivo* eye irritation test (EVEIT)"[338] was modified and integrated into a model for corneal DED.[339] The EVEIT is an *ex vivo* model for the study, evaluation, and grading of eye irritation and corrosion in chemical toxicology, including the evaluation of recovery after chemical or mechanical trauma.[338] In this model, each isolated rabbit cornea, with about 2mm of adherent sclera, is placed in a special corneal culture chamber clamped between the upper and lower parts of the perfusion chamber. Then, the chamber is filled with perfusion medium, which is constantly replenished.[338] On the other hand, in the model proposed by Spöler et al.,[339] the bulbus is fixed by a plastic ring, with the cornea face up, allowing the immersion of the posterior half of the bulbus into a temperature-controlled water bath. This bath is used to maintain the corneal surface temperature constant at 32°C. Lacrimation is simulated by applying single drops of Ringer's solution at a defined interval onto the corneal surface using a cannula connected to a perfusion pump. The humidity of the ambient air can be reduced and flow rate modified (without air flow, 2 L/min or 4 L/min). Corneal drying is studied by measuring corneal layer thicknesses and scattering properties of the stroma by OCT, which authors considered sensitive indicators of environmental stress leading to irritation of the ocular surface. This model has been used in several studies to evaluate the efficacy of different artificial tears,[340,341] as well as its usefulness as an alternative test method for serious eye damage and irritation.[342]

3.3. Animal Models of DED

Several different animal models of dry eye have been developed along the years, including surgical removal of the tear-producing glands, ocular surface desiccation by mechanical inhibition of blinking, pharmacologic inhibition of tear secretion, etc.

3.3.1. Murine Models

Murine models are the most common DED models used in pre-clinical research. The first model that incorporated adverse environment conditions to generate the disease was published in 2002 by Dursun et al.[343] This is a murine model that uses adverse desiccating environmental stress (low RH and airflow) in

conjunction with pharmacological inhibition of tearing by systemic muscarinic acetylcholine blockade with scopolamine. To induce environmental stress, mice are placed in a blower hood with a flow rate of 90 m/min at 7 seconds, for 1h, 3 times per day for 4 days. Evaluations were performed pre-treatment and 1h, 4h, 12h, 24h, and 48h post-treatment. The animals developed clinical changes that recapitulate findings in human DED: lower tear production, corneal barrier disruption, decreased conjunctival goblet cell density, and inflammatory cell infiltration in the conjunctiva. Those changes were exacerbated by desiccating environmental stress. Other authors have used this model, with different variations, for multiple purposes *(Table 7)*. This model showed that the pathology was the result of local autoimmune inflammation in which T cells, B cells, antigen-presenting cells, and T regulatory cells were all involved *(Figure 11)*.[344] Additionally, it was demonstrated that pathology could be adoptively transferred to naïve animals;[345] and it has served to test multiple therapies.[346]

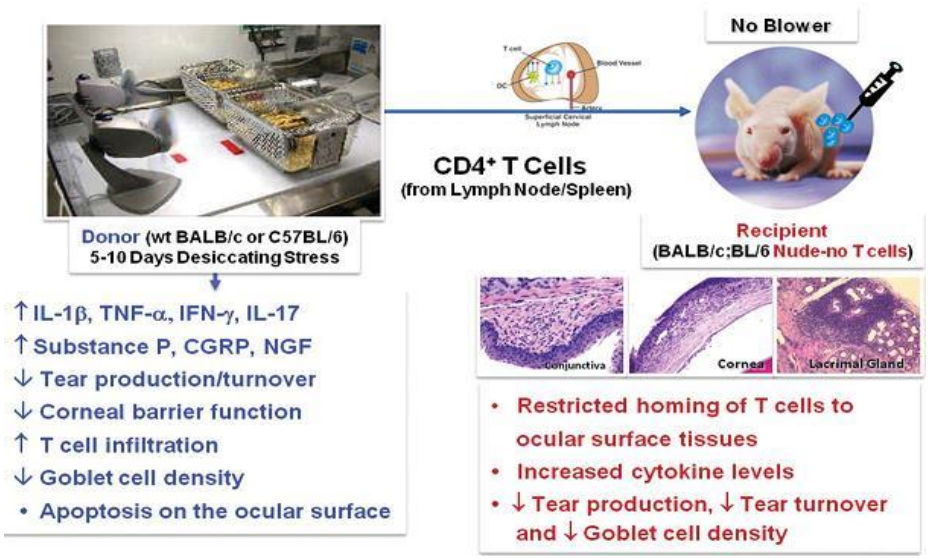


Figure 11. Experimental dry eye model and adoptive transfer model. Adapted from Stern ME, Schaumburg CS, Pflugfelder SC. Dry Eye as a Mucosal Autoimmune Disease. Int Rev Immunol. 2013;32(1):19-41.344

Table 7. Studies using the mouse dry eye disease (DED) model first published by Dursun et al.[343] in 2002.

Authors	RH (%)	T (°C)	Air flow	Exposure	Purpose	Groups
Yeh et al.[347]	50	18	Yes	12d	To evaluate the effect of EIDE on ocular apoptosis	*129SvEv/CD-1 white mice* a. * 5 mg/mL 3 times-day + 10h-day DS
Luo et al.[348]	<40	Not defined	Yes	4h and 1-3-5-10d	To evaluate whether EIDE activates MAPK pathways, JNK, ERK, and p38	*129SvEv/CD-1 white mice* a. † 1 µL 10^{-3}M b. † 1 µL 10^{-3}M + DS 4 h c. DS 4h d. * 2.5 mg/mL 4 times-day e. * 2.5 mg/mL 4 times-day + DS 4h
Pflugfelder et al.[349]	50	18	Yes	14d	To determine the pathogenic role of MMP9 in the corneal epithelial disease of DED	*129SvEv/CD-1 MMP-9 KO mice* a. * 2.5 mg/mL 4 times-day + DS 16h-day *Wild type mice* b. * 2.5 mg/mL 4 times-day + DS 16h-day
Stewart et al.[350]	20	Not defined	Yes	2d	To examine the changes in tear volume, sodium concentration, and osmolarity in response to EIDE	*BALB/c mice* a. * 2.5 mg/mL 4 times-day + DS 18h-day *C57BL/6J mice* b. * 2.5 mg/mL 4 times-day + DS 18h-day
Niederkorn et al.[351]	30-35	26	Yes	5 and 12d	To study if EIDE would break self-tolerance and induce the development of T cell-mediated inflammation directed against epitopes expressed on the ocular surface and the lacrimal gland	*BALB/c mice* a. * 2.5 mg/mL 4 times-day + DS 16h-day *T cell-deficient nude BALB/c mice* b. * 2.5 mg/mL 4 times-day + DS 16h-day *C57BL/6 mice* c. * 2.5 mg/mL 4 times-day + DS 16h-day *Control group* d. 50-75% RH, w/o air flow.
De Paiva et al.[346]	<40	Not defined	Yes	5d	To evaluate the mechanism of apical corneal epithelial barrier disruption in response to EIDE and the effects of two antiinflammatory agents	*C57BL/6 mice* a. Untreated controls w/o EIDE b. * 2.5 mg/mL 4 times-day + DS 18h-day

						c. * 2.5 mg/mL 4 times-day + DS 18h-day + 1µL/eye saline solution 4 times-day d. * 2.5 mg/mL 4 times-day + DS 18h-day + 1µL/eye 1% methylprednisolone 4 times-day e. * 2.5 mg/mL 4 times-day + DS 18h-day + 1µL/eye 0.025% doxycycline 4 times-day
Corrales et al.[352]	30-40	Not defined	Yes	2-5-10-12d	To determine the effects of EIDE on production of MMPs and their physiological inhibitors by the corneal epithelium	*BALB/c mice* a. * 2.5 mg/mL 4 times-day + DS 18h-day *C57BL/6 mice* b. * 2.5 mg/mL 4 times-day + DS 18h-day
De Paiva et al.[353]	<40	Not defined	Yes	5d	To investigate the effects of methylprednisolone and tetracycline doxycycline on MMP-9 production and the activation of MAPKs in the corneal epithelium in response to EIDE	*C57BL/6 mice* a. Untreated controls w/o EIDE b. * 2.5 mg/mL 4 times-day + DS 18h-day c. * 2.5 mg/mL 4 times-day + DS 18h-day + 1µL/eye balanced salt solution 4 times-day d. * 2.5 mg/mL 4 times-day + DS 18h-day + 1µL/eye 1% methylprednisolone 4 times-day e. * 2.5 mg/mL 4 times-day + DS 18h-day + 1µL/eye 0.025% doxycycline 4 times-day
Yoon et al.[354]	<40	Not defined	Yes	5 and 10d	To evaluate the effects of EIDE on the expression of chemokines and their receptors	a. Untreated controls w/o EIDE *C57BL/6 mice* b. * 2.5 mg/mL 4 times-day + DS 18h-day *BALB/c mice* c. * 2.5 mg/mL 4 times-day + DS 18h-day
Chen et al.[355]	15	21-23	10 L/min	14d	To investigate the differential effects of EIDE and mAChR inhibition on DED pathogenesis	*C57BL/6 mice* a. * 5 mg/mL 3 times-day + 10h-day DS b. ‡ + 10h-day DS c. Untreated controls w/o EIDE

* Subcutaneus injection of scopolamine; † Topical application of scopolamine; ‡ Extraorbital lacrimal gland excision.

d= days; DE = Dry eye; DS = Desiccating stress; EIDE = Experimental induced dry eye; ERK = extracellular-regulated kinases; h = hours; JNK = c-Jun N-terminal kinases; mAChR = systemic muscarinic acetylcholine receptor; MAPK = mitogen-activated protein kinase; MMP = matrix metalloproteinases; RH = Relative humidity; T = Temperature; w/o = without.

Later, the model proposed by Dursun et al.[343] was technically improved by constructing an environmental chamber where RH (range: 0%–100%, accuracy ±2%), temperature (range: 5–45°C, accuracy ±1°C), and airflow (0–50 L/min, accuracy ±5%) could be tightly regulated and monitored.[356,357] In this study BALB/c mice were placed into the chamber and exposed to controlled environmental conditions (<25% RH; air flow 15 L/min; 21–23°C) for 3, 7, 14, and 28 days, to determine the effect of these environmental conditions. Results showed as in the previous model, that exposure of normal mice to a low-humidity environment in a controlled environmental chamber can lead to significant alterations in tear secretion, goblet cell density, and acquisition of dry eye-related ocular surface signs.[356] In 2014, another research group used the methodology previously explained to establish and characterize the extraorbital lacrimal gland excision as a model of aqueous-deficient DED in mice.[358]

However, the environmental chamber proposed by Barabino et al.[356] is not able to perform a simultaneous control of different environmental factors. Moreover, it presents a relatively low efficiency and an unstable humidity. For these reason, Chen et al. established a novel murine model of DED using what they named as "intelligently controlled environmental system (ICES)".[357] This system introduced technical improvements, such as an adjustable temperature-dehumidifier, an intelligent dehumidifying device or a noise-free ventilator. In this study,[357] BALB/c mice were randomized to the experimental group (15% RH; air flow 2.1 m/s; 21-23°C) or the control group (60-80% RH; no air flow; 21-23°C). All conditions were constantly monitored. Both groups of animals were evaluated on 0, 3, 7, 14, 28, and 42 days. This study concluded that the biological and morphological changes of DED induced by the ICES in mice are like those found in humans. Other authors have used the ICES model in their investigations.[359,360]

3.3.2. Rat Models

These models are less commonly used than the previous ones. In 2005, Nakamura et al.[361] studied two groups of Sprague-Dawley rats, with and without jogging board treatment, and were compared under desiccating conditions (25%

RH; air flow 2-4 m/s; 23°C). Jogging treatment was introduced to reduce the blink rate of the rats, increasing the eye exposure to air flux. After 4h on the jogging board, the rats were returned to their cages for 30 minutes for food and water and again placed on the jogging board for 3.5h. For the remaining 16h, they were individually placed in cages. This series of treatments were repeated for up to 10 days. In this study, for the first time, a rat model of moderate dry eye was established using a persistent strain by jogging board treatment in combination with exposure to an evaporative environment, which induces disordered tear dynamics and abnormal blink frequency. It was observed a reduction of blink rate, as well as in Schirmer score and tear clearance test.

Another contribution was made by Kim et al.[362] This author evaluated whether topically applied PEP-1-FK506BP could ameliorate the symptoms of dry eye in a low humidity air flow-induced dry eye model. To generate the dry eye model, the central region of the corneal epithelium (0.4 mm^2) was scraped mechanically with an ophthalmic surgical blade under anesthesia. Then, rats were exposed to an adverse environmental condition (25-30% RH; air flow 2.4 m/s; 28 °C) for 5 h. In this study, Sprague-Dawley rats were divided in 4 groups: Group 1 (control), 5µL/eye saline solution in normal rats; Group 2, 5µL/eye saline solution in DED induced rats; Group 3, 0.1% sodium hyaluronate in dry eye induced rats; Group 4, PEP-1-FK506BP in dry eye induced rats. In the dry eye rat group, significantly decrease of the tear volumes were observed after exposure to dry air. In contrast, the group treated with PEP-1-FK506BP showed a marked increase in tear volume compared with the DED rat group. Regarding corneal staining, dry eye rats showed significantly increased corneal fluorescein staining compared with normal control rats, while the PEP-1-FK506BP-treated group showed significantly reduced staining compared with dry eye groups.

4. Recreation of Environment Conditions for the Clinical Study of DED: Controlled Environment Chambers

As mentioned, both adverse environments outdoor and indoor, affect the LFU, which eventually can cause or worsen DED.[61] To understand the way in which these situations affect the human body it is necessary to control environmental conditions such as humidity, temperature, airflow, atmospheric pressure and/or the presence of pollutants or allergens. With this purpose, controlled-environment laboratories, commonly referred to as environmental chambers, have been designed in several places of the world. These facilities can be defined as isolated rooms where some environmental conditions (depending on the design) can be tightly controlled and individuals can be exposed to some predetermined conditions. Thus, all clinical evaluation endpoints are measured without the variability of the external environment.

Environmental chambers have been used to assess the effectiveness of antiallergic drugs in subjects exposed to a controlled allergen charge,[363–365] or the effect of irritating factors such as cigarette smoke,[366] dust,[367] or indoor pollutants.[368] However, environmental chambers have been most used to evaluate the effect of environment in DED

To evaluate these effects in real conditions, environmental chambers have been used in numerous clinical studies, in which several different environmental factors have been studied: low humidity,[61,257,264,266,267,369–376] high or low temperature,[267,373] presence of airflow,[61,257,270,370–375] and low atmospheric pressure.[110] The published studies using controlled environmental laboratories or chambers for the study of DED are shown in *Table 8*.

The recreation of adverse environment conditions in these chambers allows researchers to simulate the sudden exacerbation of DED that patients suffer when they are exposed to the types of adverse environments noted above in their daily lives. This produces an increase of symptoms and conjunctival redness, as well as a decreased in ocular surface integrity (mainly more corneal staining), and tear

stability. Some normal asymptomatic individuals also manifest DED-related signs in these controlled adverse environments.

A modification of the environmental laboratories, in which the entire body is exposed, is to restrict exposure to the periocular area. A precedent of this can be found in the modified swimming goggles used to measure tear film evaporation, as previously explained.[258,259] But more recently, Alex et al.[370] studied the ocular surface of normal subjects and DED patients wearing modified laboratory goggles that expose their eyes to a controlled desiccating environment (21% RH and 2-5 L/min airflow) for 90 minutes. They found that the ocular surface staining increased similarly in controls and patients, except superior cornea, which staining was greater in patients; they also found that blink rate was higher in the DED group than in the normal group and that correlated to the baseline and the environmental-induced change in corneal fluorescein staining.[370]

With sufficient scientific evidence proving ocular surface worsening after controlled adverse environment exposure, the natural next step is to test therapies in these laboratories. The use of environmental chambers would have a double purpose: to study the potential effect of a therapy in the amelioration of the clinical signs worsened by desiccating stress, and to have clinical variables evaluated and clinical samples collected under the exact same environmental conditions (adverse or not). The therapeutic trials published under controlled environmental conditions range from artificial tears[377] or investigational drugs/delivery systems,[226,378,379] to topical steroids,[235] for which DED is still an off-label indication despite its generalized short-term use in this disease.

Table 8. Description of the published studies using controlled environmental laboratories (chambers) to study dry eye disease (DED). *Modified from: Calonge M, Jose Pinto-Fraga, González-García MJ, et al. Effects of the External Environment on Dry Eye Disease. International Ophthalmology Clinics, 2017;57(2):23-40.*

Controlled Environmental Facility	Environmental Conditions	Duration	Subjects (n)	Summary of Results	Year
Climate Chamber *Aarhus University, Aarhus, Denmark*[371]	RH: 10%, 30%, 50% and 70% T: 23ºC Airflow: 0.1 m/s	420 min	48 healthy subjects	Low RH decreases eye humidity perception	1973
Environmental Chamber *Electricity Council Research Centre, Capenhurst, Chester, UK*[373]	RH: 20%, 50% and 75% T: 23ºC - 28ºC Airflow: < 0.1 m/s	360 min	72 healthy subjects	High RH and high T increases eye humidity perception	1975
Climate Chamber *National Swedish Institute for Building Research, Gävle, Sweden*[270]	RH: not specified T: 21ºC -22ºC Airflow: 0.5, 0.67 and 1 m/s	30-45 min	41 healthy subjects	Airflow decreases TBUT, increases lacrimal flow and improves Ferning pattern	1987
Controlled Adverse Environment (CAE[SM]) *Ophthalmic Research Associates, Boston, MA, USA*[61,369]	RH: <10% T: 24.4ºC ± 3.4ºC Airflow: constant, not turbulent Visual task: TV and PC use	90 min	33 DED patients	CAE exposure decreases TBUT, palpebral fissure size, and increases corneal staining and redness	2005
				Tear film metric break-up area can detect changes in the ocular surface induced by a CAE[SM]	2012
Research Center for Human Environmental Adaptation *Kyushu University, Tokio, Japan*[376]	RH: 10%, 30%, 50% T: 25ºC Airflow: not specified	180 min	16 healthy subjects	Low RH increases dry eye perception	2006
Climate Chamber *International Centre for Indoor Environment and Energy, Technical University of Denmark, Lyngby, Denmark*[375]	RH: 28.4%, 16.0%, 11.2% and 6.8% T: 23.3ºC Airflow: 1.4, 3.3, 4.7 and 9.4 L/s	420 min	68 healthy subjects	No differences in eye dryness, visual acuity or Ferning test between environmental conditions	2007
Controlled Environment Research Laboratory (IOBA-CERLab) *Vision I+D, IOBA, University of Valladolid, Valladolid, Spain*[257,372,380]	*SIFC vs simulated standard conditions* RH: 5% vs 45% T: 23ºC Airflow: 0.43 m/s Barometric pressure: 750 vs 930 mb Visual task: TV watching	120 min	20 DED patients	Tear production and stability decrease and conjunctival hyperemia and corneal staining increase in simulated in-flight conditions. Tear MMP-9 and IL-6 increase and tear EGF decreases in simulated in-flight conditions.	2013
	RH: 5% T: 23ºC Airflow: 0.43 m/s Visual task: TV watching		20 healthy subjects 19 DED patients	Corneal staining increases and tear stability decreases in both groups; symptoms and hyperemia increase in the DED group. Tear MMP-9 increases and EGF decreases in both groups; IL-6 increases in healthy subjects.	2014
			14 SS-DED patients	CAE increases tear osmolarity, conjunctival hyperemia, and corneal staining.	2016
Controlled environment chamber *Glasgow Caledonian University, Glasgow, UK*[266,267]	RH: 5% and 40% T: 21ºC Airflow: not specified	60 min	12 healthy subjects	Low RH increases tear evaporation rate, and decreases NIBUT, lipid layer thickness, ocular comfort and tear production	2013
	RH: 5%, 40%, 70% T: 22ºC Airflow: not specified	10 min	10 healthy subjects 10 DED patients	Low RH provokes a difference in tear evaporation rate between both groups (higher in DED), no differences between groups at 70% RH	2013
	RH: 40% T: 5ºC, 10ºC, 15ºC, 20ºC, 25ºC Airflow: not specified	10 min	12 healthy subjects	High temperature increases tear evaporation rate. Low temperature decreases NIBUT and lipid layer thickness	2016

CAE = Controlled Adverse Environment; CER-Lab = Controlled Environment Research Laboratory; DED = Dry Eye Disease; EGF = Epithelial Growth Factor; IL-6 = Interleukin 6; MMP-9 = Matrix Metalloproteinase 9; NIBUT = Non-Invasive Break-Up Time; PC = Personal Computer; RH = Relative Humidity; SS =Sjögren's Syndrome; SIFC = Simulated In Flight Conditions; T = Temperature; TBUT = Tear Break-Up Time.

5. Biomarkers in DED

As previously explained, it is well known the lack of correlation between DED signs and symptoms.[67–70] This fact can make the adequate diagnosis, management, and evolution assessment of DED patients difficult. For these reasons, there is a need to find objective outcomes and endpoints that can be used in daily practice and/or in clinical trials.

The term "biomarker", was defined as a "cellular, biochemical or molecular alterations that are measurable in biological media such as human tissues, cells, or fluids."[381] More recently, a new definition has been proposed by the National Institutes of Health Biomarkers Definitions Working Group:[382] "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention". An even broader definition takes into account not just incidence and outcome of disease, but also the effects of treatments, interventions, and even unintended environmental exposure, such as to chemicals or nutrients.[383]

Biomarkers have been classified by Perera and Weinstein[384] based on the sequence of events from exposure to disease and, thus, its capability to provide insight into disease progression, prognosis, and response to therapy. Moreover, as it will be discussed in the Chapter 3, biomarkers can be used not only for screening or diagnosis, but also for disease severity grading, therapeutic effect assessment, and/or disease activity evaluation. Finally, biomarkers can also be used for reduction in disease heterogeneity in clinical trials or epidemiologic studies, or as objective endpoints in clinical trials.[385]

5.1. DED Biomarkers and Inflammation

As previously explained, DED is characterized by a chronic inflammation of the LFU, which contributes to tear instability, ocular surface damage and ocular discomfort.[35,386] It is well known that DED generates a rise of the inflammatory activity, increasing the expression of immune activation or adhesion molecules, matrix metalloproteinases, inflammatory cytokines, chemokines and their

receptors, and CD4+ T cells, conjunctival/corneal tissues, cells parameter such as viability or proliferation, tear proteomics, etc. These changes have been reported in a high number of pre-clinical and clinical studies.

5.1.1. Molecular Biomarkers

Cytokines and Chemokines

Several authors have reported a significant increase of different inflammatory cytokines/chemokines [i.e. IL-1, IL-6, TNF-α, MMP-9, IL-17A, IL-1 receptor antagonist (RA), IL-8/CXCL8, IL-22, interferon (IFN)-γ, IFN- γ induced protein 10 (IP-10)/CXCL10, macrophage inflammatory protein (MIP)-1α/CCL3, MIP-1β/CCL4, regulated upon activation normal T-cell expressed and presumably secreted (RANTES)/CCL5, etc.], in tears and/or conjunctival epithelial samples from DED patients.[32,327,328,387–398] Other molecules such as EGF, IL-12 or IL-17A, among others, have been found to be significantly decreased in these patients.[32,327,399] In addition, it has been reported differences in the cytokine profiles of different types of DED (i.e. SS-DED, aqueous-deficient DED, evaporative-DED, etc.).[396,400,401]

Moreover, different studies have reported correlations between some inflammatory tear molecule levels (i.e. IL-6, IL-8/CXCL8, TNF-α, IL-1RA, etc.), with clinical parameters and/or disease severity,[327,387,395–397,399,401,402] corroborating the utility of tear analysis for this disease. Specifically, MMP-9 measurement in tears has already been proposed as a sensitive method for DED severity determination,[328,398] and a commercial point-of care device has already been developed (InflammaDry®, RPD, USA).[403] Sambursky et al.[403] reported that this device presents 85% of sensitivity and 94% of specificity.

As explained in previous sections, a great number of studies in animal DED models have demonstrated alterations in tear film composition, as well as in corneal and conjunctival gene expression. The animal studies in which environment conditions were altered (see previous section) are indeed helping to understand that some DED biomarkers can also be exploited to better understand the influence of environment in human DED. *Table 9* shows a compilation of some of the most relevant results obtained in the study of the relation between cytokines/chemokines and DED.

Table 9. Potential biomarkers (cytokines/chemokines) for DED. *Modified from: Enríquez-De-Salamanca A, Bonini S, Calonge M. Molecular and cellular biomarkers in dry eye disease and ocular allergy. Curr Opin Allergy Immunol. 2012;12:523-533.*

Biomarker	Study subjects	Sample type	Technique	Results
IL-1α IL-1α mRNA	DED[32,327,390,396,399,404] Controls[32,327,396,402,404]	Tear samples[327,390,399] CIC[32,404] Schirmer strips[396,402]	Luminex[327,399,402] ELISA[32,390] RT-PCR[32,404] Antibody microarray[396]	↑in DED[390,396,399] ↑in SS-DED[32] Osmolarity (+)[402] Schirmer (-)[327,402] Corneal staining (+)[327] Lissamine green (+)[327]
IL-1β IL-1β mRNA	DED[327,390,394,396,399] Controls[394,396,402]	Schirmer strips[396,402] Tear samples[327,390,394,399] CIC[394]	Luminex[327,394,399,402] Antibody microarray[396] ELISA[390] RT-PCR[390,394]	↑in DED[390,394,396,399] Schirmer (-)[327,396,402] Corneal staining (+)[327] Corneal staining (-)[396] Lissamnine green (+)[327] Lissamine green (-)[396] TBUT (-)[396]
IL-2	DED[394] Controls[394]	Tear samples[394]	Luminex[394]	↑in DED[394]
IL-5	DED[394] Controls[394]	Tear samples[394]	Luminex[394]	↑in DED[394]
IL-6 IL-6 mRNA	DED[327,328,387,391,394,399] SS-DED[389] Controls[327,387,391,394,396]	Tear samples[327,328,387,391,394,399] CIC[394] Schirmer strips[396,402]	Luminex[327,387,394,399,402] ELISA[32,33,389,391,396] RT-PCR[32] Antibody microarray[396] IHC[391]	↑in DED[327,328,389,391,394,396,399] ↑in SS-DED[32] vs Non-SS[391] Osmolarity (+)[402] Symptoms (+)[327] Schirmer (+)[32, 327,391] Schirmer (-)[327,387,402] Corneal staining (+)[32,387,327] Corneal staining (-)[327,396] Lissamine green (+)[327] Lissamine green (-)[396] TBUT (+)[391] TBUT (-)[396] Tear osmolarity (+)[402] Globet cells density (-)[32,391] IL-1α mRNA (-)[32] IL-8 mRNA (-)[32]
IL-8/CXCL8 IL-8 mRNA	DED[327,387,394,396,397] Controls[327,387,394,396,397]	Tear samples[327,387,394,397] CIC[394] Schirmer strips[396,402]	Luminex[325,387,394,397,402] RT-PCR[32] Antibody microarray[396]	↑in DED[327,394] ↑in SS-DED[32] Schirmer (-)[327,387,397,402] Corneal staining (+)[32,327] Corneal staining (-)[327,396] Lissamine green (+)[327,397] Lissamine green (-)[396] Globet cells density (-)[32] Differences according DED severity[397]
IL-10	DED[327,394] Controls[327,394]	Tear samples[327,394]	Luminex[327,394]	↑in DED[394] Schirmer (-)[327,402] Lissamine green (+)[327]
IL-12	DED[327,399] Controls[327]	Tear samples[327,399]	Luminex[327,399]	↓in DED[327,399]

IL-16	DED[399]	Tear samples[399]	Luminex[399]	↑in DED[399]
IL-17A	DED[393,399]	Tear samples[393,399]	Luminex[393,399]	↓in DED[393,399]
IL-1RA	DED[387,390,397] Controls[387,397]	Tear samples[387, 390,397]	Luminex[387,390,397]	↑in DED[387,390] Lissamine green (+)[397] Schirmer (-)[387,397] TBUT (-)[387] Differences according DED severity[397]
CXCL10/IP-10	DED[387] Controls[387]	Tear samples[387]	Luminex[387]	↑in DED[387] Schirmer (-)[387]
EGF	DED[32,327,387] Controls[327,387]	Tear samples[327,387] CIC[32]	Luminex[327,387] ELISA[32]	↓in SS-DED[32] ↓in SS-DED[327] Schirmer (+)[327] Corneal staining (-)[327] Lissamine green (-)[327,387]
Fractalkine/ CX3CL3	DED[387] Controls[387]	Tear samples[387]	Luminex[387]	↑in DED[387]
TNF-α TNF-α mRNA	DED[327,391,394,396] Controls[327,391,394,396,402]	Tear samples[327,394,396] CIC[391,394] Schirmer strips[396,402]	Luminex[327,394,399,402] RT-PCR[33,394] ELISA[391] Antibody microarray[396] Immuno[391]	↑in DED[327,328,391,394,396] ↑in SS-DED[32,391] Osmolarity (+)[402]
IFN-γ	DED[327,394,396] Controls[327,394,396]	Tear samples[327,394] Schirmer strips[396]	Luminex[327,394]	↑in DED[394,396] ↓in DED[399] Corneal staining (+)[327] Corneal staining (-)[396] Lissamine green (+)[327]
CCL3/MIP-1α	DED[327,388] Controls[327,388]	Tear Samples[327,388] Conj. Biopsy[388]	Luminex[327] ELISA[388] IHC[388]	↑in DED[327,388] ↑in SS-DED[388] Globet cell density (+)[388] Schirmer (-)[327] Corneal staining (+)[327] Lissamine green (+)[327]
CCL4/MIP-1β	DED[388] Controls[388]	Tear samples[388] Conj. Biopsy[388]	ELISA[388] IHC[388]	↑in DED[388] ↑in SS-DED[388] Globet cell density (+)[388]
CCL5/RANTES	DED[388] Controls[388]	Tear samples[388] Conj. Biopsy[388]	ELISA[388] IHC[388]	↑in DED[388] ↑in SS-DED[388] TBUT (+)[388] Schirmer (+)[387] Globet cell density (+)[387]
CCR5	DED[388] Controls[388]	Conj. Biopsy[388]	IHC[388]	↑in DED[388]
MMP-1	Controls[402]	Schirmer strips[402]	Luminex[402]	Schirmer (-)[402]
MMP-2	Controls[402]	Schirmer strips[402]	Luminex[402]	Osmolarity (+)[402] Schirmer (-)[402]
MMP-7	Controls[402]	Schirmer strips[402]	Luminex[402]	Schirmer (-)[402]
MMP-9 MMP-9 mRNA	DED[328,390,403] Controls[328,402,403]	Schirmer strips[402] Tear samples[328,390,403] CIC[328]	MMP-9 activity assay[328] RT-PCR[328]	↑in DED[328,390,403] Symptoms (+)[328] Corneal staining (+)[327]

			Luminex[390,402] InflammaDry[403]	TBUT (-)[328] Osmolarity (+)[402] Schirmer (-)[402]
MMP-10	Controls[402]	Schirmer strips[402]	Luminex[402]	Osmolarity (+)[402] Schirmer (-)[402]
MUC-1 MUC-1 mRNA	DED[406,407] Control[406,407]	CIC[406,407]	RT-PCR[406] ELISA[407]	↓mRNA in DED[405] ↑in DED[406]
MUC-2	DED[406] Control[406]	CIC[406]	RT-PCR[406]	↓mRNA in DED[406]
MUC-4	DED[406] Control[406]	CIC[406]	RT-PCR[406]	↓mRNA in DED[406]
MUC-5AC	DED[406,408] Control[406,408]	CIC[406,408]	RT-PCR[406,408]	↓mRNA in DED[406]
MUC-16 MUC-16 mRNA	DED[407] Control[407]	CIC[407]	RT-PCR[407] ELISA[407]	↑in DED[407] Corneal staining (+)[407] Symptoms (+)[407]
TFG-β mRNA	DED[32,328] Controls[32,328]	CIC[32,328]	RT-PCR[32,328]	↑in DED[328] ↑in SS-DED[32]

+ = positive correlation; - = negative correlation; CIC = Conjunctival impression cytology; Conj. = Conjunctival; DED = Dry eye disease; EGF = Epithelial growth factor; ELISA = Enzyme-linked immunosorbent assay; IFN = Interferon; IHC = Immunohistochemistry; IL = Interleukin; MIP = Macrophage inflammatory protein; MMP = Matrix metalloproteinase; mRNA = Messenger ribonucleic acid; MUC = Mucin; RANTES = Regulated upon activation normal T-cell expressed and presumably secreted; RT-PCR = Reverse transcription polymerase chain reaction; SS = Sjögren syndrome; TBUT = Tear break-up time; TFG = Transforming growth factor; TNF = Tumor necrosis factor.

Non-Cytokine Proteins

The major source of tear proteins is the secretory acinar cells of the lacrimal gland, including the primary proteins of the tear film: lysozyme, lactoferrin, and lipocalin. These proteins, which have anti-oxidant properties, are essential for the maintenance of tear film integrity and the regulation of its function as a natural defense barrier against microbial infections.[408] Their decrease in tear film is a relevant indicator of lacrimal gland dysfunction.[409]

Lysozyme values range from 1 to 3 mg/mL in normal tears depending on the assay technique used. The level of tear lysozyme was noted to increase with age till 40 and decrease thereafter,[410] so that any value should be interpreted according to age-matched populations. It is probably the earliest tear protein studied in conjunction with DED. Versura et al.[411] reported a decrease in the concentrations of lysozyme in idiopathic DED and SS compared to controls. However, a later study showed that lysozyme concentration did not differ between non-SS-DED, SS-DED or normal controls.[412]

Lactoferrin in tears provides anti-microbial efficacy by binding free iron, thus reducing the availability of iron necessary for microbial growth and survival.[413,414] Lactoferrin measurement has shown some disparity in ocular surface diseases, which demonstrates the relative lack of specificity of lactoferrin assays in tears. Some authors have pointed out the lack of specificity and the poor correlation with clinical tests of lactoferrin assay in mild-to-moderate DED.[415] Indeed, Bjerrum[416] proposed the ratio of albumin to lactoferrin as a diagnostic tool for discriminating SS in which an albumin: lactoferrin ratio greater than 2:1 was 67% sensitive and 100% specific.

Lipocalins represent the greatest group of lipid affinity proteins in tears; they can associate with a wide variety of lipids.[417] The binding between lipocalin-lipid increases lipids solubility, promoting the formation of a homogeneous lipid layer, which reduces tear film evaporation and increase optical quality.[418] In addition, lipocalins are believed to serve as scavengers for the removal of wasted lipid molecules that would contaminate the system.[419]

Many other proteins have been identified in human tears.[420] However, the literature is inconsistent in the number of proteins in tears and their specific functions. The objective of proteomics is to correlate the changes of tear protein profiles with DED and potentially use them as diagnostic biomarkers. Changes in the tear protein profile have been shown to correlate with DED severity.[421]

Mucins

The tear film on the ocular surface epithelia is maintained by the mucins presented on its surface, as well as by membrane-associated mucins in the apical surface of the cell. MUC-1, -3A, -3B, -4, -12, -13, -15, -16, -17 and -20 are membrane associated and MUC-2, -5AC, -5B, -6, -7 and -19 have been classified as secreted mucins.[422]

The large gel-forming mucin MUC-5AC is expressed by conjunctival goblet cells. This mucin is the most important marker associated with goblet cells. The assessment of MUC5AC gene expression using Reverse Transcription polymerase chain reaction (RT-PCR) or MUC5AC protein expression by immunostaining techniques may be used to

identify and quantify goblet cells.[407] It has been observed lower levels of this mucin in DED patients than in controls.[423] Also, MUC5AC expression is reduced in SS-DED.[424]

Some cells of the lacrimal gland acini express the small soluble mucin MUC-7. The corneal and conjunctival epithelia express the membrane-associated mucins MUCs 1, 4, and 16. Glycosylation of MUC16 appears to be altered in non-SS-DED.[424]

Finally, it is important to mention that our research group proposed MUC-1 and MUC-4 as high sensitivity DED biomarkers.[405]

5.1.2. Cellular Biomarkers

Conjunctival impression cytology (CIC) enables collection of the cells located in the conjunctiva superficial layers and it therefore allows the analysis of the cells having reached their final differentiation. This technique can also be applied to the cornea. Three main populations of conjunctival cells can be found at this level: epithelial cells, goblet cells, and inflammatory cells.[425] Another technique that can be used to collect conjunctival cells is brush cytology. In this technique, a soft brush is used to obtain both superficial cells (as in CIC) and basal cells. It is possible to use different methods, such as immunocytochemistry, flow cytometry, enzyme-linked immunosorbent assay (ELISA), RT-PCR, etc. to analyze these samples.

As previously explained, it is possible to evaluate the percentage expression of different proteins observed in these samples. For example, expression of cytokines such as IL-1α, mature IL-1β, and IL-1RA are significantly greater in CIC from SS-DED patients than in those from controls.[390] Also, Jones et al.[404] and Pflugfelder et al.[32] observed a very high expression of mRNAs encoding IL-6 and IL-8, as well as human leukocyte antigen (HLA)-DR, ICAM-1, TNF-α, IL-1α, IL-1β, and TGF-β in the conjunctival epithelium of eyes with SS as compared with normal eyes using the RT-PCR method in CIC. These results, among others, are presented in *Table 9*.

Cell characteristics

Some cell parameters, such as conjunctival cell viability, cells proliferative capacity, number and types of cells collected, number and relative expression of intraepithelial lymphocyte subsets, presence of keratinized cells, squamous metaplasia, etc., have been suggested as potential disease biomarkers in DED.[426]

A decrease in the density of conjunctival goblet cells seems to be a constant finding in chronic DED.[427] By collecting CIC samples, it is possible to calculate goblet cell density. Furthermore, the application of flow cytometry for analyses of CIC or brush cytology specimens[428,429] allows obtaining a precise evaluation of these samples. It is possible to identify the presence of epithelial cells, containing snake-like chromatin or intracellular inclusions, and of non-epithelial cells in the superficial conjunctival layers (e.g., lymphocytes, dendritic cells, neutrophils, eosinophils). The analysis and grading of the characteristics of these cell populations (shape, number, density, and pathological modifications) provides valuable information concerning the status of the ocular surface.[430]

Regarding the use of cell viability as DED biomarker, Reinoso et al.[431] evaluated cell viability and cell cycle kinetics of the conjunctival epithelium of 23 patients with evaporative-type DED caused by MGD, as compared to 17 healthy controls. Conjunctival cells were collected from inferior fornix and tarsal conjunctiva by brush cytology before and after 2 months of treatment. Conjunctival cell viability and proliferative capacity, in addition to the number if intraepithelial lymphocytes were significantly decreased in DED patients.[431] DED treatment caused a significant decrease in lymphocytes and in the CD4/CD8 ratio. In a posterior study, this research group studied the topographical distribution of epithelial cells and intraepithelial lymphocytes in the human ocular mucosa, reporting that each topographical zone from normal human conjunctiva has a unique profile of immune cells phenotype, viability, and proliferative state that could be related to a differentiated regional functionality.[432]

HLA-DR

HLA-DR is a class II histocompatibility antigens that are normally expressed primarily on antigen-presenting cells, such as macrophages, B lymphocytes, and activated T cells, on which they play a major role in the initiation of the immune response to an antigen (pro-inflammatory marker).

HLA-DR expression is increased in DED patients, directly correlating with inflammation and disease severity.[33] Brignole et al.[433] demonstrated significantly upregulated expression of HLA-DR in conjunctival epithelial cells in patients suffering from autoimmune keratoconjunctivitis sicca such as SS as compared to non-immune DED syndromes.

The expression of HLA-DR is stimulated in response to inflammatory cytokines such as IFN-γ, TNFα, IL-6, and IL-1.[434,435] Brignole et al.[433] showed that CD40 and CD40 ligand were also upregulated in the ocular surface of patients with keratoconjunctivitis sicca and was positively correlated with HLA-DR.

A recent study by Yafawi et al. evaluated impression cytology samples utilizing the cellular surface biomarker HLA-DR as an ocular surface inflammatory biomarker by flow cytometry. They found that it was a sensitive, reliable, simple, non-invasive method for investigating ocular surface diseases such as allergic conjunctivitis and DED.[436] HLA-DR expression is probably the most relevant biomarker because of its high sensitivity depending on the degree of inflammatory reactions. This biomarker has already been used in clinical practice and validated in numerous multicenter clinical trials. Topical cyclosporin A effectively reduced HLA-DR expression in DED patients, significantly compared to the vehicle, which showed only a mild non-significant effect at the inflammatory level.[437] In a large randomized study, Brignole et al.[438] have recently shown the effects of oral supplementation with omegas-3 and -6 fatty acids on the reduction of HLA-DR inflammatory marker in conjunctival cells compared to placebo. Recently, another study have shown the effect of a 30-days treatment period with lubricant eyedrops in DED patients in the reduction of HLA-DR expression.[439]

HLA-DR-positive cells have been proposed as a biomarker of DED in clinical trials.[440] Both HLA-DR-positive cells and changes in tear cytokine expression in both humans[387,391,396,441,442] and animals,[393,443–446] can be used as minimally invasive objective biomarkers to classify disease severity, elucidate disease mechanisms, and assess treatments.[447]

Intercellular Adhesion Molecule (ICAM)-1

ICAM-1 is an intercellular adhesion molecule that is essential for communication between lymphocytes and other cells and for controlling leukocyte migration and adhesion to different target tissues in inflammatory processes. Like HLA-DR, ICAM-1 expression by epithelial cells was found higher in DED. Tsubota et al.[448] found a good correlation on brush cytology between HLA-DR expression and ICAM-1. In keratoconjunctivitis sicca, ICAM-1 is also upregulated on lymphocytes and/or vascular endothelial cells, resulting in lymphocytic diapedesis to the lacrimal and conjunctival tissue. ICAM-1 levels were shown to be positively correlated with disease progression and severity.[449] Like HLA-DR, ICAM-1 is upregulated by IFN-γ through a tyrosine kinase-dependent mechanism.[33]

Other analysis such as proteomics, tear lipidomics, tear metabolome, tear neuromediators evaluation, and tear glycans analysis have been proposed in the searching of DED biomarkers.[450,451]

5.2. Effect of Environment Conditions on DED Biomarkers

As previously mentioned, there are different studies in which the influence of environmental conditions on the molecular expression have been analyzed. These studies are not only important to establish a better understanding of the pathophysiology of human DED, but also to elucidate which molecules or genes have the potential to be used as diagnostic biomarkers,[452] inflammatory activity biomarkers[370,372,380] or therapeutic biomarkers.[453,454]

Several studies from our group, in which both healthy subjects and DED patients were exposed to different environmental conditions in our IOBA-CER-Lab have

demonstrated that tear levels of some molecules in both groups were significantly affected depending on RH and barometric pressure conditions.[110,257,372] Particularly, when exposed for 2h to low RH (5%), MMP-9 tear levels were significantly increased in DED patients.[110] This study also showed that these environmental conditions affected not only molecule tear levels in DED patients, but also in healthy subjects in whom tear levels of MMP-9 and IL-6 were significantly increased and EGF significantly decreased. These molecular results further confirmed the clinical data of acute DED exacerbation in DED patients and healthy subjects after exposure to an adverse environment. Additionally, under these environmental conditions (low humidity + air flow) SS-DED patients presented an increase in IL-1RA measured in tears.[372]

We also assessed the effect of environmental conditions consisting on low RH plus low barometric pressure (5%, 750 mb), resembling those in-flight conditions in an airplane cabin, into tear molecule levels of DED patients.[257] Our results confirmed that after 2h under these conditions, IL-6 and MMP-9 tear levels were significantly increased, and those of EGF significantly decreased.

It is therefore quite clear that environmental factors do affect not only clinical parameters but also molecular tear levels and/or conjunctival gene expression. For this reason and for standardization purposes, exposure to standard, normal controlled environmental conditions previously to sample collection is performed in several studies. This is the case of the studies by Cocho et al.[452,455] from our group, in which conjunctival gene expression and tear cytokine levels were studied in samples from ocular chronic graft versus host disease (GvHD)-DED patients who were kept under normal environmental conditions for 30 min before clinical evaluation and sample uptake.

So, equally to clinical data, and in order to obtain reproducible results and to further validate the use of tear molecule as biomarkers for DED, it becomes completely necessary to determine the effect of environmental conditions in molecule tear and conjunctival gene expression in humans.

5.3. Effect of DED Therapies on Biomarkers under Adverse Environment Conditions

While there is a large evidence on the anti-inflammatory effect of corticosteroids in ocular inflammation and in molecule expression, the literature is scarce regarding their effect on levels of these molecules in the tear film/ocular surface epithelium of DED patients exposed to adverse environments.[456,457] One of the few studies, published by Moore et al.,[235] showed that topical dexamethasone mitigated clinical signs of DED in response to a low RH environment generated using special goggles, with a parallel decrease in the expression of HLA-DR, a marker of inflammation. This study showed that after 90 min under low humidity conditions (18-25%) and 2-5 L/min air flow rate, HLA-DR transcripts in nasal bulbar conjunctiva were significantly increased. This HLA-DR expression change was significantly reduced after preservative-free 0.1% dexamethasone treatment, 4 times daily for 2 weeks, whereas it significantly increased after artificial tears treatment. This molecular result was in agreement with those changes in clinical parameters before and after desiccating stress exposure.

6. Justification

As previously explained, DED is a chronic multifactorial inflammatory disorder of the LFU in which several etiopathogenic mechanisms usually interact to develop into an anomalous LFU, eventually leading to tear film instability and ocular surface damage.[3] Most common symptoms are ocular discomfort, visual disturbance, photophobia, and chronic pain.[35] There are numerous risk factors for DED,[41] such as menopause, low (systemic or local) androgen levels, autoimmune diseases, transplant of allogeneic immune bone marrow–derived cells (provoking GvHD), central sensitization syndromes, long-term use of certain systemic or topical drugs/preservatives, exposure to chemical irritants, long-term abuse of contact lenses, alteration of corneal innervation, and prolonged exposure to adverse environments, among others. This Doctoral Thesis focuses on the effect of the external adverse environment as a risk factor for DED, and on how to use it in our favor to better understand this disease and to better evaluate potential therapies.

The Ocular Surface Group at IOBA, University of Valladolid, has been highly involved in the study of inflammatory diseases affecting the LFU, especially DED. In 2008, we published a first study using an environmental chamber located at the Architecture School of the University of Valladolid, in which it was possible to control RH and temperature. This study showed, among other things, that a 2h-exposure to an adverse environment condition induced an impairment of the LFU healthy subjects, provoking an increase in DED-related signs and symptoms.[458] It was part of the first Doctoral Thesis developed at IOBA in relation to the control of environmental conditions and its effect on the LFU (Dr. María Jesús González García, 2008).

In 2008, due to the good results obtained in the study mentioned above, a newer and better environmental chamber was constructed at IOBA, allowing us to tightly control environmental variables such as temperature, RH, barometric pressure, illumination and air flow, during the development of clinical studies and clinical trials. This environmental chamber is part of the so-called IOBA-Controlled

Environmental Research Laboratory (IOBA-CERLab) and it will be described in detail in Chapter 2.

On the other hand, our research group is actively involved in the search for biomarkers to find objective outcomes and endpoints that can be used in daily practice and/or in clinical trials. In this line, our research group has developed several studies under the supervision of Dr. Enríquez-de-Salamanca.[257,372,380,387,450–453,455,459–462] The use of controlled environmental conditions in these studies provides the possibility to obtain tear samples and/or cell material from ocular surface cytologies removed under the exact same environmental conditions (adverse or not) and at the same time of clinical evaluation. Thus, it is possible to study in the IOBA-CERLab if variables such as tear protein expression, conjunctival/corneal gene expression, and others, are affected by environmental conditions similarly as clinical variables, allowing the search for new molecular potential biomarkers of DED.

From 2008 to present, IOBA-CERLab environmental chamber has been used to develop some studies evaluating the hypothesis that adverse environment conditions (i.e. desiccating stress) can cause a deterioration of the LFU in both DED patients and healthy subjects, as evaluated by the alteration of DED diagnostic tests (signs, symptoms, and tear molecules).[110,257,372,380] These studies conformed the second Doctoral Thesis developed at IOBA-CERLab in this research line (Dr. Marisa Tesón Yudego, 2013). Three different samples were included in these studies: healthy control subjects, mild-moderate DED patients, and severe DED patients. One of these studies[380] showed that DED patients suffered an increase in DED-related signs (TBUT and phenol red thread test decrease, corneal staining increase) after exposure to an adverse environment simulating in-flight conditions (23°C, 5% RH, localized air flow, and 750 mb of barometric pressure). A second study[257] confirmed these results. It was observed that both moderato-to-severe DED patients and healthy asymptomatic subjects experienced an acute exacerbation of DED signs (TBUT reduction, corneal staining increase) after facing controlled adverse environment conditions. This acute response resembles the sudden

worsening that DED patients experience in their daily lives. This study also showed an increase of MMP-9 tear levels in both groups after the adverse environment exposure, as well as a decrease of EGF levels, and an increase of IL-6 in the tears of healthy subjects. Finally, a third study[372] evaluated the response of the LFU of DED-SS patients exposed to two simulated daily life environmental conditions. Again, it was observed that even a short exposure to a desiccating environment produced a significant deterioration of the LFU (tear osmolarity, conjunctival hyperemia, and corneal staining increases), increasing the inflammatory activity (IL-1RA, IL-6, IL-8, and MMP-9 levels increase).

These studies confirmed and validated the usefulness of the CERLab environmental chamber to increase the inflammatory activity observed in eyes of both DED patients and healthy subjects by the recreation of an adverse environment condition. The exposure to the desiccating stress generates an exacerbation of DED signs that mimics those acute episodes of worsening that DED patients suffer in their daily lives. However, the clinical symptom questionnaires failed to show the impairment that patients casually verbalized when they were coming out of the chamber.

Continuing with this research line, the natural next step is to test if the worsening process (increase of DED signs, DED-related symptoms, and molecular LFU inflammation) generated under adverse environment conditions can be mitigated by using some treatment. If this goal could be achieved, we would have a positive control that would be useful for conducting efficacy studies on new DED therapies. And even more importantly, we would have a new two-step clinical trial design to offer pharmaceutical companies: the traditional one and the one in which a therapeutic candidate would have to show its potential efficacy on an environmentally-induced inflammation of the LFU. Additionally, we anticipate that, once again, clinical questionnaires will fail at showing what patients feel, so it is well justified to make efforts to develop a new simpler tool that reflects a change in symptomatology more accurately.

7. Hypothesis and Objectives

Hypothesis

Our research group has previously demonstrated that exposure to adverse environmental conditions in the IOBA-CERLab (Controlled Environment Research Laboratory) elicits an inflammatory reaction in the Lacrimal Functional Unit (LFU) of Dry Eye Disease (DED) patients. This is seen by increased clinical signs and tear levels of inflammatory molecules. The chamber, however, failed to show significant differences in symptoms.

The hypothesis of this Doctoral Thesis establishes that this environmentally-induced inflammatory exacerbation of the LFU can be reduced, clinically and molecularly, by pretreating DED patients with a well-known anti-inflammatory topical agent. This study serves as a proof-of-concept that the chamber adverse conditions will be useful in testing candidate therapeutics. By using tear concentration of multiple molecules at different time-points, we also believe that we can determine several types of DED biomarkers that could be, at least, surrogate biomarkers in future clinical trials

Additionally, we also hypothesize, given that clinical symptoms invariably fail to show differences, this therapy will also fail to show any symptomatic improvement. A goal of this work will be to develop a new simpler questionnaire that can better assess whether DED patients improve or not under a certain therapy.

Objectives

The following objectives are established as a way to demonstrate the protective effect of an anti-inflammatory agent used in DED, topical fluorometholone. This will be evaluated by measuring the mitigation of inflammatory exacerbation provoked in DED patients after 2h of controlled adverse environment in the IOBA-CERLab:

Objective 1:

To design a two-step protocol for therapeutic clinical trials in DED: the traditional normal environmental clinical trial followed by the environmentally-challenged.

Objective 2

To evaluate the clinical efficacy of this therapy in this new clinical trial format.

Objective 3

To evaluate the changes in tear molecule concentration under this therapy at the different time-points during and after exposure to controlled environment conditions.

Objective 4

To transform those molecule variations with time into three kinds of potential biomarkers: 1) disease severity, 2) inflammatory activity and 3) therapeutic efficacy.

Objective 5

To analyze why clinical symptoms usually fail at translating what patients feel and to develop and test a new clinical questionnaire.

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NEW DESIGN OF CLINICAL TRIALS: USE OF CONTROLLED ENVIRONMENTAL CHAMBERS

Chapter 2
New Design of Clinical Trials: Use of Controlled Environmental Chambers

As previously explained, environmental conditions have a big impact on the status of the ocular surface of DED and even normal patients.

The use of environmental chambers during clinical trials allows to introduce different environmental exposures or conditions, which will offer the following benefits:

- The evaluation of patients after they have remained under a stable and non-adverse environment will reduce or eliminate possible clinical alterations caused by exposure to adverse and/or different environmental conditions during the hours prior to evaluation (i.e. vehicle travelling, air-conditioning in waiting rooms, different outdoors conditions, etc.). In this way, it contributes to normalize as much as possible not only the clinical evaluation, but also the quality of a potential sample (i.e. tears, cells, etc.). Thus, the final intention is to have a more homogenous sample of patients, avoiding bias caused by external factors.

- The introduction of a controlled exposure of DED patients to adverse environmental conditions allows to simulate the acute episodes of worsening that they report to suffer in their daily lives. This fact provides the chance to observe and analyze (clinically, cellularly and/or molecularly) one of these acute episodes, which would not be possible in real life. Including an adverse environmental exposure allows to evaluate the magnitude of this worsening, as well as the capacity and effectiveness of new treatments or therapies to avoid such episodes.

Consequently, the main goals of the present clinical trial are two: firstly, to assess the clinical efficacy of a 3-week 0.1% fluorometholone therapy in DED

patients but, more importantly, to assess if this therapy could ameliorate the expected worsening of the LFU after exposure to a desiccating stress set in a controlled environmental laboratory. If successful, this therapy could be of use to help patients cope with these adverse environments. As an additional consequence, the design of this clinical trial can be helpful to ascertain if future therapies in DED will also prevent damage from desiccating stress.

1. Materials and Methods

A single randomized, double-masked, vehicle-controlled, parallel-group, Phase III clinical trial was performed to explore the safety and efficacy of topical 0.01% fluorometholone as a therapy for DED exacerbation provoked by exposure to an adverse controlled environment (ACE) in moderate-to-severe DED patients. This clinical trial was approved by the Comité Ético de la Universidad de Valladolid (Valladolid, Spain) and by the Spanish Regulatory Agency (Spanish Drugs and Health Products Administration, AEMPS, www.aemps.gob.es/en/home.htm) with EUDRA number 2013-002183-63 *(Annex I and II, respectively)*. The trial was registered at clinicaltrials.gov (Identifier: NCT02051023).

The study was sponsored by and conducted at the Instituto de Oftalmobiología Aplicada (IOBA; University of Valladolid, Valladolid, Spain) in accordance with the tenets of the Declaration of Helsinki and in compliance with Good Clinical Practices. Therefore, there was not any pharmaceutical company involved, not even in the purchase of the treatments.

1.1. Study Procedure

The study was performed in an environmental chamber within the Controlled Environmental Research Laboratory (IOBA-CERLab, University of Valladolid, Valladolid, Spain) to carefully control the exposure conditions *(Figure 12)*. This facility is composed of an exposure room, with a maximum capacity of 8 subjects, and an evaluation room *(Figure 13A-B)*. Temperature (range: 15-30°C, 1°C steps), and RH (range: 5–80%, 1% steps) can be tightly controlled in both rooms simultaneously. Additionally, airflow (blower exit velocity range: 0.60–3.60 m/s), illumination (range: 10–1000 lux, 1 lux steps), and atmospheric pressure [range: 930–450 millibar (mb), 1 mb steps] can be controlled in the exposure room. Using

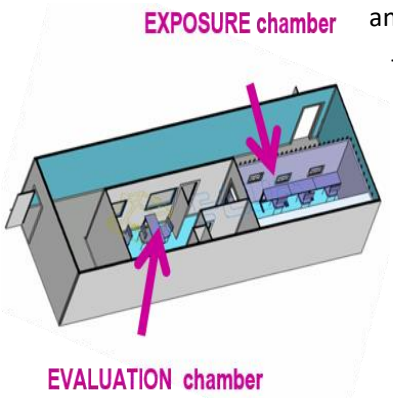


Figure 12. IOBA-CERLab scheme.

a control display located outside of the chamber *(Figure 13C)*, environmental conditions inside the laboratory can be monitored throughout the entire duration of the experiment and recorded in 5 min intervals. On the other hand, the evaluation room is completely equipped with the clinical ophthalmic instruments needed to evaluate the LFU *(Table 10)*.

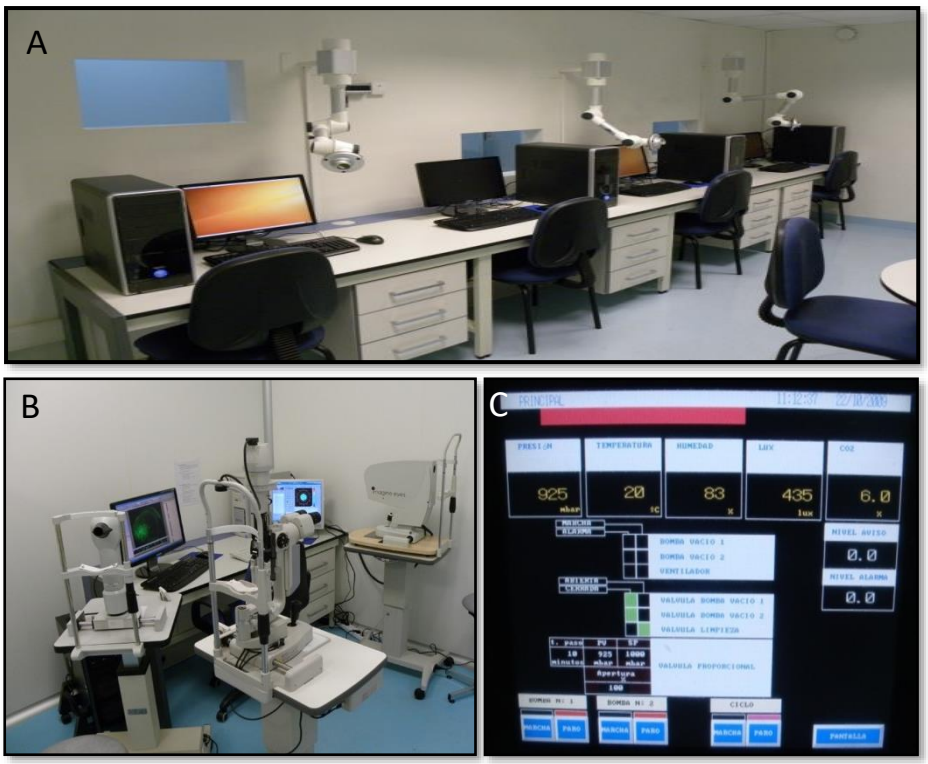


Figure 13. Detail of IOBA-CERLab exposure room (A), evaluation room (B), and control display (C). *Images from IOBA.*

Table 10. Equipment of the evaluation room.

Parameter	Measurement	Instrument
Visual Acuity	Digital LogMAR ETDRS chart	Topcon CC-100XP
Corneal topography	Computerized corneal topographer	Medmont E300
Aberrometry	Hartmann-Shack wavefront aberrometer	IRX3, Imagine eyes
Biomicroscopy	Video slit-lamp system	Topcon SL-8z
Tear stability	Tearscope®	Keeler
Tear osmolarity	Impedance osmometer	Tearlab
	Freezing-point osmometer	Fiske 3100
Corneal sensitivity	Gas esthesiomether	Belmonte noncontact gas esthesiometer

Additionally, the IOBA-CERLab has two auxiliary laboratories for sample analyses: the Ocular Pathology Laboratory and the Molecular Biology Laboratory, both located outside the chamber, but in the same building.

The clinical trial consisted of four visits and a total duration of 22 days. *Figure 14* shows the clinical trial design and the environmental conditions used, while *Table 11* presents the evaluations performed at each visit.

The first 30 min of visits 1, 2, and 4 (V1, V2, and V4) were within a normal controlled environment, 23°C of temperature, 50% RH, and with no localized airflow. These periods were used to evaluate all patients (tear samples collection included) under the exact same normalized environmental conditions. During visit 3 (V3) patients were exposed to an adverse environment for 2h, to study the response of the ocular surface to desiccating stress and the possible protective effect provided by the study treatment. The adverse controlled environment consisted of an environment of 23°C temperature, 5% RH, and airflow at 0.43 m/s localized to the face.

The design of this clinical trial has then two different parts; from V1 to V2 corresponds to the prototypical clinical trial, in which drug efficacy is sought. From V2 to V3 corresponds to the controlled inflammatory-challenge clinical trial.

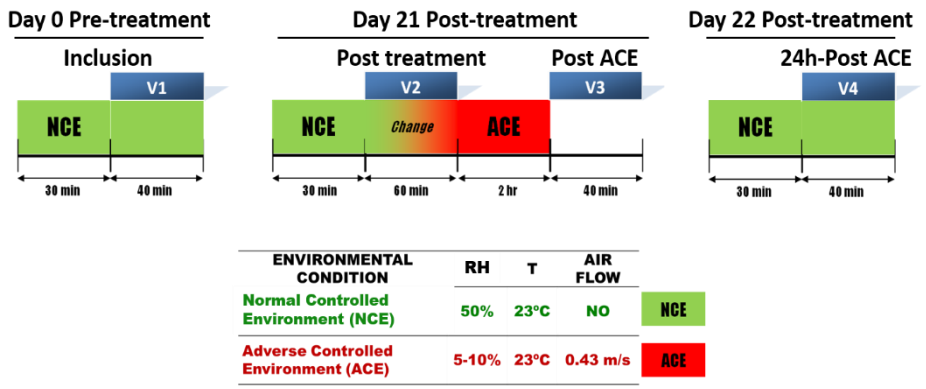


Figure 14. Clinical trial flowchart. ACE = Adverse controlled environment; NCE = Normal controlled environment; RH = Relative humidity; T = Temperature; V = Visit.

During V1 *(Day 0; Figure 14)* the informed consent was obtained *(Annex III)*. After spending 30 min under normal controlled conditions, the inclusion/exclusion criteria were checked and baseline data were recorded. At the end of V1, the included patients received treatment medication consisting of either 0.1% fluorometholone ophthalmic suspension (FML group; FML®, Allergan Inc., Irvine, CA, USA) in a vehicle of polyvinyl alcohol (PA), or only the vehicle (PA group; Liquifilm Tears™ eye drops, Allergan Inc.). The assignment to the FML group or to the PA control group of each patient was randomized, and the patients were instructed to use the assigned treatment until the end of the study (22 days).

Table 11. Visits schedule and test sequence.

	Visit 1	**Visit 2**	**Visit 3**	**Visit 4**
	Day 0	Day 21	Day21	Day 22
Procedure	*Baseline*	*Pre-ACE*	*Post-ACE*	*24 h post-ACE*
Written informed consent	X			
Inclusion/exclusion criteria	X			
Clinical history	X			
Tear samples collection	X	X	X	X
OSDI questionnaire	X			
SANDE I questionnaire (A)	X	X	X	X
BCVA, high and low contrast (B)	X			X
Tear osmolarity (C)	X	X		
Treatment satisfaction (C)		X		X
Tear Break-up time (C)	X	X	X	X
Fluorescein corneal staining (A)	X	X	X	X
Lissamine green conjunctival staining (C)	X	X	X	X
Biomicroscopy with slit-lamp (B, C)	X	X	X	X
Unanesthetized Schirmer test (C)	X			X
Intraocular pressure (B)	X			X
Fundus evaluation and optic cup/disk ratio (B)	X			X
Adverse events record (B)		X	X	X
Pregnancy test	x			x
Study medication delivery/collection	Delivery			Collection

A = Primary outcome; B = Safety outcome; C = Secondary outcome.
ACE = Adverse controlled environment; BCVA = Best corrected visual acuity; IOP = Intraocular pressure; OSDI = Ocular surface disease index; SANDE = Symptom assessment in dry eye

At Day 21, V2 and V3 were performed. During V2, the patients were first exposed to normal controlled conditions for 30 min and then evaluated. After completion of the V2 evaluation, the patients began V3 in which they were exposed for 2h to the adverse controlled environment. When the 2h exposure was completed, clinical evaluations were again performed, then finalizing V3 *(Table 11)*. The final visit (Day 22, V4), was performed 24h after exposure to the adverse environment. During V4, patients were exposed to the normal controlled environment for 30 min and then evaluated. At the end of V4, all treatments were collected and the study concluded.

1.2. Drug Masking and Administration

Liquifilm® was selected as an ideal control treatment because it is the vehicle used in FML® *(Table 12)*. As can be observed, there are two small differences in the composition of both treatments: the presence of Polysorbate 80 in FML®, and a difference of 0.001% in the concentration of benzalkonium chloride. In our opinion, these differences are not relevant, in any case this point will be later discussed.

Table 12. Composition of FML® and Liquifilm® (Allergan Inc., Irvine, CA, USA)

FML®	**LIQUIFILM®**
Fluorometholone: 1 mg/mL	----
Polyvinyl alcohol: 14 mg/mL	Polyvinyl alcohol: 14 mg/mL
Benzalkonium chloride: 0.04 mg/mL	Benzalkonium chloride: 0.05 mg/mL
Edetate disodium	Edetate disodium
Sodium phosphate monobasic and dibasic	Sodium phosphate monobasic
Sodium chloride	Sodium chloride
Polysorbate 80	----
Sodium hydroxide	Sodium hydroxide/Hydrochloric acid
Purified water	Purified water

Treatments were supplied in commercial bottles. The original labels of these bottles were removed and bottles were re-labeled with the study number and a coded lot number to avoid potential identification of the treatment by site-personnel or patients *(Figure 15)*. The bottles were placed into sealed opaque envelopes and solely identified by the treatment codes after randomization.

These codes were maintained at the IOBA´s statistical unit. Treatment was not delivered or collected by any investigator that performed evaluations during the clinical trial, and none of them were involved in the masking process described above.

Figure 15. Drugs masking; Left: FML® (0.1% fluorometholone), Right: Liquifilm Tears™ (Polyvinyl Alcohol).

All patients were instructed to use the treatment with a dose regimen of 1 drop, 4 times a day in both eyes while awake, during the entire 22-day duration of the study. This dose regimen is within the recommended range for FML® and Liquifilm Tears™. Patients were allowed to use their artificial tears throughout the duration of the study only if they considered it necessary. During the follow-up, they were asked if they had used them and how often. Importantly, all patients were instructed not to use any eyedrop (i.e., study medication, artificial tears) in the 4h preceding each visit. Adherence to this protocol was specifically asked before beginning each visit.

1.3. Patient Selection

Patients were required to be 18 years of age or older, and have a previous diagnosis of moderate-to-severe DED, equivalent to grades 2 to 4 according the "Dry Eye Severity Scheme" published in the 2007 Dry Eye Workshop Study report.[1] Inclusion criteria were corneal fluorescein staining score ≥1 (Oxford scale) in both eyes, TBUT ≤7 seconds in both eyes, an unanesthetized Schirmer test ≤10 mm/5 min in both eyes, and an Ocular Surface Disease Index (OSDI) score >12 points.[2] Importantly, the patient had to express a worsening of DED-related symptoms when exposed to adverse environmental conditions during their daily life and the

use of artificial tears prior to beginning the study. Patients were accepted into the study if they were taking other topical or systemic treatment if it had begun at least 3 months before inclusion and the dosage was to be maintained throughout the whole study. Finally, patients had to have a best corrected visual acuity (BCVA) ≤ 0.1 logMAR in each eye.

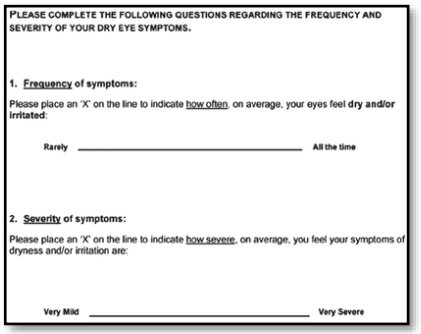
Patients were excluded if they had known sensitivity or intolerance to any of the treatments used in the study, history of ocular infection or severe ocular inflammation (other than DED-related) 6 months before inclusion in the study, any active ocular disease (different from DED), any uncontrolled severe systemic disease that may have affected the eye (except SS), any ocular surgery or trauma that could affect corneal sensitivity and/or normal tear distribution in the 6 previous months or any ocular or systemic surgery or procedure planned during the study duration that could affect outcomes, occlusion of the lacrimal puncta either surgically or with plugs within three months prior to study, contact lenses wear within 3 months prior the study or during the study, or use of any topical medication except for DED. Other exclusion criteria included initiation, discontinuation or change of dosage of antihistaminic, cholinergic agents, beta-blocking agents, antidepressants or any systemic medication with possible effects over the tear film, history of glaucoma or intraocular pressure > 22 mmHg in any measurements 2 months prior to baseline, optic disk/cup ratio > 0.6 mm, pregnancy, lactation, or not use of adequate contraception methods. Also, patients were excluded if undergoing topical CsA eye drops within 3 months prior to the inclusion and/or topical corticosteroid eye drops within 1 month prior to the inclusion.

1.4. Clinical Assessment

All examinations were performed in both eyes. Only data from the eye randomly selected were computed for this study. The examinations were performed in the sequence outlined below and followed the established schedule *(Table 11)*.

OSDI questionnaire:[2] This test *(Annex IV)* was used to determine if candidates complied with the inclusion criteria. This questionnaire evaluates symptoms of ocular discomfort and dryness using 12 questions. The total score can range from 0 (no symptoms) to 100 (maximum severity). OSDI > 12 points is related with DED.[3]

Symptoms Assessment In Dry Eye (SANDE) questionnaire:[4] We used the SANDE I version *(Figure 16)*, in which patients were asked to place a mark on two given 100-mm horizontal Visual Analogue Scales (VAS) to depict the extent of their DED-related symptoms separately in terms of frequency and severity.


PLEASE COMPLETE THE FOLLOWING QUESTIONS REGARDING THE FREQUENCY AND SEVERITY OF YOUR DRY EYE SYMPTOMS.

1. Frequency of symptoms:

Please place an 'X' on the line to indicate how often, on average, your eyes feel dry and/or irritated:

Rarely _____ All the time

2. Severity of symptoms:

Please place an 'X' on the line to indicate how severe, on average, you feel your symptoms of dryness and/or irritation are:

Very Mild _____ Very Severe

Figure 16. SANDE I questionnaire.

Best Corrected Visual Acuity: This was one of the five safety outcome measures. It was measured using the standard Early Treatment for Diabetes Retinopathy Study (ETDRS) chart. High contrast (100%) and low contrast BCVA (10%) were measured. Patient with a BCVA > 0.1 logMar were not included.

Tear osmolarity: The TearLab Osmolarity System[5] (TearLab Cooperation, San Diego, CA, USA) was used to measure tear osmolarity *(Figure 17A)*. Abnormal values were considered > 300 mOsm/L *(Figure 17B)*.

Figure 17. TearLab osmolarity system (A) and Tear osmolarity scale (B).

Treatment satisfaction: To study the satisfaction that patients experienced with treatments, patients were required to mark with a vertical line on a 100-mm horizontal VAS. The extreme left of the line (0) represented "complete absence of satisfaction", while the extreme right (100) represented "highest level possible of satisfaction".

TBUT:[6] It was defined as the time between the last of a complete blink and the appearance of the first dry spot *(Figure 18)*. This test was performed after instillation of 5µL of 2% sodium fluorescein using a micropipette. The procedure was repeated 3 times and the mean value was recorded.

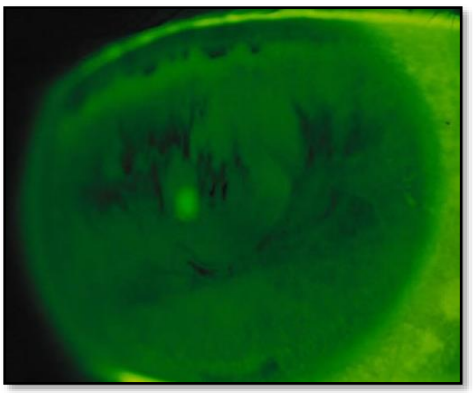


Figure 18. Dry spots (black zones) during tear break-up time evaluation (TBUT).

Fluorescein corneal staining:[7] Corneal staining was evaluated 2 min after instillation with a micropipette of 5 µL of 2% sodium fluorescein *(Figure 19)*. The evaluation was performed using a cobalt blue filter (Topcon Corp., Tokyo, Japan) and a yellow Wratten #12 filter (Eastman Kodak, Rochester, NY, USA). Grading was performed with both the Oxford global scheme (0–5 score, *Figure 20 left*)[8] and a modified Centre for Contact Lens Research (CCLR, University of Waterloo, Waterloo, Ontario, Canada; *Figure 20 right*) staining score.[9] At each corneal zone (superior, inferior, central, nasal and temporal) staining was evaluated according to the grade (from 0-4) and to the extension (from 0-100%). The modified CCLR final score was calculated for each area by multiplying the grade by the percentage of staining.

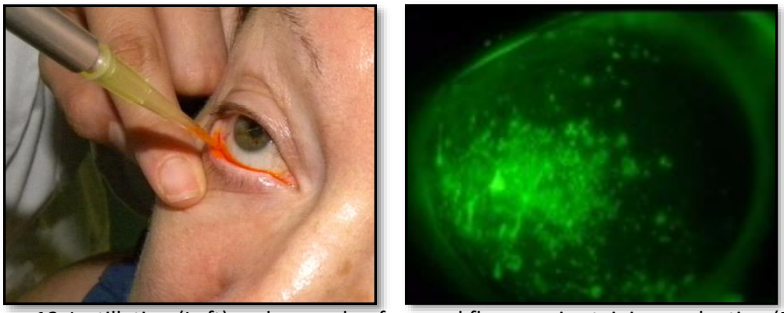


Figure 19. Instillation (Left) and example of corneal fluorescein staining evaluation (Right). *Images from IOBA*

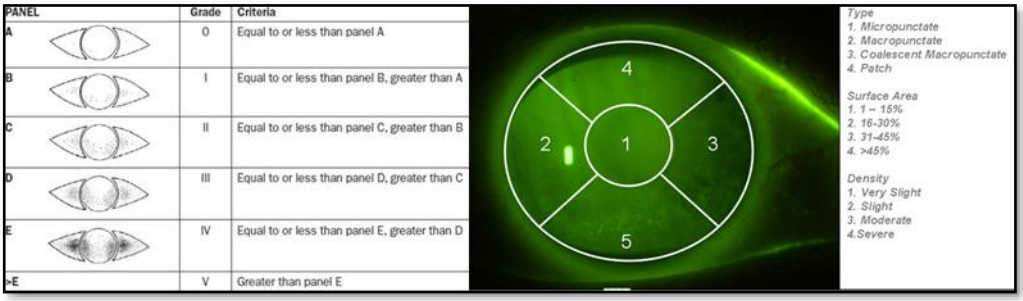


Figure 20. Fluorescein staining grading scales used in the clinical trial. Oxford scale (Left) and CCLR scale (Right)

Lissamine green conjunctival staining:[10] Lissamine green strips (GreenGlo; HUB Pharmaceuticals, LLC, Rancho Cucamonga, CA, USA) were wet with 25 µL sodium chloride and were applied gently into the eye *(Figure 21)*. One minute later, staining was evaluated according to the Oxford scheme *(Figure 20 left)*.[8]

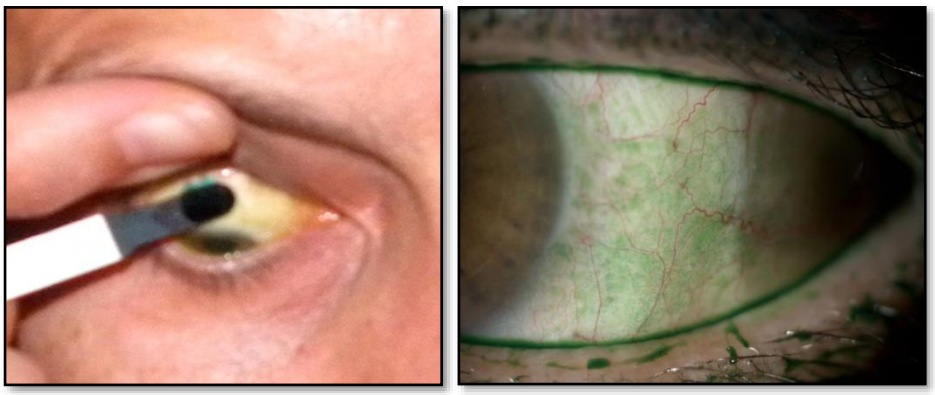


Figure 21. Lissamine green conjunctival staining instillation (Left) and evaluation (Right). *Images from IOBA.*

Slit-lamp biomicroscopy: Ocular surface and anterior segment findings were evaluated using the Efron scale *(Annex V)*.[11]

Schirmer test without anesthesia:[12] One Schirmer sterile strip (Tearflo; HUB Pharmaceuticals, LLC) was placed in the lateral canthus of the inferior lid margin. Patients maintained their eyes closed during 5 min and then the length of wetting was measured. Schirmer ≤10 mm/5 min was selected as cut-off value.

Intraocular pressure (IOP): IOP was evaluated as a safety outcome. It was performed using the Perkins tonometer (Perkins MK 2; HS Clemens Clarke International, Essex, UK). IOP normal values ranged between 12-22 mm Hg.

Figure 22. Slit-lamp.

Fundus evaluation and optic cup/disk ratio: These safety outcomes were evaluated by indirect ophthalmoscopy under pharmacological mydriasis. Any cup-disk ratio of 0.7 or more, any vertical cup-disk ratio larger than the horizontal cup-disk ratio, and any disparity between the direct ophthalmoscope estimation was carefully evaluated.[13]

1.5. Outcome Measurement Endpoints

1.5.1. Primary Efficacy Outcome Measures

Efficacy outcomes were evaluated as changes in corneal fluorescein staining and symptoms. For fluorescein staining, the change was defined as the percentage of patients with an increase ≥1 point in corneal staining between V2 and V3, i.e., before and after 2h of desiccating stress in patients treated for 21 days. Staining was measured again at V4 on day 22 to measure the recovery from the 2 h of desiccating stress exposure during V3. For symptoms, the change was recorded in the percentage of patients with a reduction of ≥20 points in SANDE score between V2 and V3 and again between V3 and V4.

1.5.2. Secondary Efficacy Outcome Measures

All other evaluations were considered as efficacy secondary variables, except those included as safety measures *(Table 11)*. They were evaluated between V1 and V2, V2 and V3, and V3 and V4. Differences between different visits in the same group and between both groups at the same visit were analyzed.

1.5.3. Safety Outcome Measures

The safety of both treatments was assessed by recording the nature, severity, and duration of all adverse events and their relationship to the study medications. Four additional safety endpoints were included in the trial *(Table 11)*: changes in BCVA, fundus evaluation and optic cup/disk ratio, anterior segment anomalies (especially corneal epithelial problems or signs of infection), and IOP.

1.6. Sample Size and Statistical Analysis

The generation of random allocation sequence and the statistical analyses were carried out in the IOBA Statistical Unit under the supervision of a PhD licensed statistician, Dr. Itziar Fernández, using the R-statistical package (version 3.1.1). Statistical significance was defined as $p \leq 0.05$. Random allocation sequence was generated using blocked randomization of size 2. The sample size was estimated to detect a 1-point difference in the main efficacy variable (corneal fluorescein staining, Oxford scale), at a significance level of $p \leq 0.05$, with a statistical power of 0.9, and with an estimate of a 10% loss of the sample size.

Mean and median values were used for expressing quantitative variables, while only median values were used for qualitative ones. In addition, 95% confidence intervals (95%CI) were always constructed. The normality of distribution assumption was checked by Shapiro-Wilk test. Differences between the means of treatment groups were tested by Student's t-test, or the non-parametric alternative, Mann-Whitney U test. Chi-squared test was used to compare percentages between treatment groups, except when there were sparse data, in which case the Fisher's exact test was used. Equality of means between two visits was checked by Student's t-test for paired samples or Wilcoxon's

signed-rank test when the differences between pairs were not normally distributed.

The percentage of change *(Table 13)* was calculated for each variable to evaluate three effects: 21-day treatment effect, treatment effect after adverse environment exposure, and treatment effect in the recovery phase (24h post-adverse environment exposure). These effects were defined as relative changes between V1 and V2, V2 and V3, and V3 and V4, respectively. To take into account the minimum and maximum boundary values of each variable, the rate of change per individual was calculated as the relative difference between the two subsequent measurements with respect to the maximum change over the considered time period. Results were reported as the mean and 95%CI of the percentage of change, and Student's t-test was used to determine if the average change differed significantly from 0 (one-sample t-test). It was also used to determine if the treatment groups were different in average changes (two independent samples t-test).

Table 13. Percentage change (ΔY) analysis.

Quantitative variables	**Qualitative variables**
$\Delta Y = \frac{Y_f - Y_i}{Y_i} \cdot 100$	$if \begin{cases} Y_{post} > Y_{pre} \rightarrow \Delta Y = \frac{Y_{post} - Y_{pre}}{Y_{max} - Y_{pre}} \cdot 100 \\ Y_{post} = Y_{pre} \rightarrow \Delta Y = 0 \\ Y_{post} < Y_{pre} \rightarrow \Delta Y = \frac{Y_f - Y_i}{Y_i - Y_{min}} \cdot 100 \end{cases}$

Y_{post}: Final value; Y_{pre}: Initial value; Y_{min}: Minimum value; Y_{max} : Maximum value.

2. Results

2.1. Patient and Baseline Characteristics

The study DED population included 21 patients (17 females, 4 males) in the FML group (59.0 years, 95%CI: 55.2–62.8) and 19 (17 females, 2 males) in the PA group (60.3 years, 95%CI: 56.0–64.7). As one patient dropped from the study due to work-related issues, one more individual was recruited and thus a total of 41 patients were eventually included in the study, but only data from 40 were analyzed *(Figure 23)*.

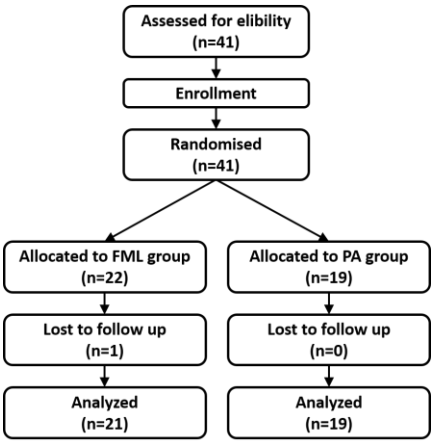


Figure 23. Flow-chart of patients inclusion in the clinical trial.

There were no significant differences in age or gender distribution between the two groups (p=0.37 and p=0.66, respectively). Enrollment started in March 2014 and finished in October 2014 when all patients were recruited, the last follow-up visit was performed in November 2014. For baseline clinical characteristics, there were no statistical differences (p≥0.06) between groups in corneal and conjunctival staining, hyperemia, TBUT, unanesthetized Schirmer test, BCVA, tear osmolarity, and IOP *(Table 14)*.

The use of artificial tears by patients was required for inclusion. The most frequently used artificial tear compounds were carmellose sodium (20), sodium hyaluronate (7), carbomer (4), hypromellose (3), polyethylene glycol (3), polyvinyl alcohol (2), and trehalose (1). Only 3 patients had to use their artificial tears during the study, in each case less than 4 times a day.

Table 14. Clinical signs and symptoms.

		FML group (n=21)		**PA group** (n=19)		
Visit	**Variable**	***Mean (median)***	***95%CI***	***Mean (median)***	***95%CI***	***P value****
V1	OSDI (unit)	56.5 (52.5)	49.1–64.0	53.8 (54.5)	45.9–61.6	0.59
	SANDE I. Frequency (unit)	8.33 (8.10)	7.50–9.16	6.86 (7.0)	5.56–8.17	0.06
	SANDE I. Severity (unit)	7.29 (7.0)	6.39–8.17	6.06 (6.6)	4.86–7.25	0.08
	Tear osmolarity (mosm/Kg)	338.4 (331)	326.6–350.2	329.5 (328)	317.3–341.8	0.28
	Corneal staining (unit)	2.33 (3)	1.94–2.72	1.95 (2)	1.57–2.33	0.18
	Conjunctival staining (unit)	2.14 (2)	1.75–2.53	1.74 (2)	1.29–2.18	0.19
	Conjunctival Hyperemia (unit)	1.86 (2)	1.42–2.29	1.58 (1)	1.20–2.96	0.32
	TBUT (seconds)	3.1 (3.2)	2.4–3.8	3.4 (3.2)	2.7–4.2	0.46
	Schirmer test (mm)	5.2 (5)	4.1–6.3	5.5 (6)	4.1–6.9	0.65
	VA high contrast (logMAR)	0.10 (0.06)	0.02–0.19	0.07 (0.08)	0.02–0.12	0.97
	VA low contrast (logMAR)	0.53 (0.54)	0.43–0.64	0.53 (0.5)	0.44–0.62	0.93
	IOP (mmHg)	15.2 (15)	14.5–15.9	16.3 (16)	15.4–17.2	0.05
	Cup/Disk ratio	0.37 (0.35)	0.34–0.4	0.37 (0.35)	0.33–0.41	0.94
V2	SANDE I. Frequency (unit)	4.09 (4.0)	2.95–5.23	4.52 (4.9)	2.97–6.07	0.64
	SANDE I. Severity(unit)	4.07 (4.0)	2.68–5.46	4.65 (5.0)	2.96–6.34	0.57
	Tear osmolarity (mosm/Kg)	338.5 (335)	331.6–345.4	336.9 (334)	326.7–347.1	0.77
	Corneal staining (unit)	0.86 (1)	0.47–1.25	1.95 (2)	1.57–2.33	**0.0008**
	Conjunctival staining (unit)	0.95 (1)	0.54–1.37	1.68 (2)	1.29–2.08	**0.01**
	Conjunctival hyperemia (unit)	0.71 (1)	0.41–1.02	1.95 (2)	1.63–2.26	**<0.0001**
	TBUT (seconds)	3.3 (3.3)	2.5–4.0	2.9 (3.1)	2.5–3.4	0.45
	Satisfaction (unit)	73.9 (80)	63.3–84.4	47.5 (35)	31.4–63.9	**0.03**†
V3	SANDE I. Frequency (unit)	4.18 (5.0)	2.57–5.80	3.84 (3.0)	1.95–5.72	0.83
	SANDE I. Severity (unit)	3.99 (3.9)	2.43–5.54	3.71 (3.0)	1.93–5.48	0.83
	Corneal staining (unit)	1.05 (1)	0.59–1.51	2.58 (3)	2.17–2.98	**0.0001**
	Conjunctival staining (unit)	1.19 (1)	0.75–1.63	2.47 (3)	2.07–2.88	**0.0005**
	Conjunctival hyperemia (unit)	1.14 (1)	0.71–1.58	2.84 (3)	2.62–3.07	**<0.0001**
	TBUT (seconds)	2.9 (2.2)	2.1–3.8	2.7 (2.4)	2.1–3.3	0.96
V4	SANDE I. Frequency (unit)	2.98 (2.0)	1.65–4.31	3.92 (4.0)	2.54–5.29	0.34
	SANDE I. Severity (unit)	2.54† (2.0)	1.40–3.69	3.68 (4.0)	2.31–5.05	0.22
	Corneal staining (unit)	0.90 (1)	0.42–1.39	2.37† (2)	2.06–2.68	**0.0001**
	Conjunctival staining (unit)	0.90 (1)	0.48–1.33	2.00 (2)	1.63–2.37	**0.0012**
	Conjunctival hyperemia (unit)	0.95 (1)	0.64–1.27	2.27† (2)	2.01–2.52	**<0.0001**
	TBUT (seconds)	3.3 (2.3)	2.3–4.3	2.8 (2.7)	2.2–3.3	0.88
	Schirmer test (mm)	5.6 (6)	4.2–7.0	5 (6)	3.8–6.2	0.39
	BCVA high contrast (logMAR)	0.06 (0.02)	-0.02–0.14	0.06 (0.1)	0–0.11	0.33
	BCVA low contrast (logMAR)	0.47 (0.46)	0.36–0.58	0.54 (0.54)	0.46–0.62	0.27
	IOP (mmHg)	15.4 (16)	14.8–16.1	16.5 (16)	15.6–17.3	0.10
	Cup/Disk ratio	0.38 (0.4)	0.35–0.41	0.38 (0.4)	0.34–0.42	0.85
	Satisfaction (unit)	79.8 (80)	73.1–86.4	46.4 (40)	31.8–61.0	**0.0002**†

FML = Fluorometholone; PA = Polyvinyl alcohol; CI = Confidence interval; IOP = Intraocular pressure; OSDI = Ocular surface disease index; SANDE = Symptom assessment in dry eye; TBUT = Tear break up time; BCVA = Visual acuity.
* Between-group comparison. Student´s t-test or Mann Whitney U test.
† p<0.05. Between-visits comparison (V2 vs V4). Student´s t-test or Wilconxon's test.
Bold values indicate statistical significance.

2.2. Efficacy Analysis

There was no difference in corneal staining between the two groups at V1 (2.33 [95% CI:1.94/2.72] vs 1.95 [95% CI:1.57/2.33], FML group and PA group respectively; p=0.18, *Figure 24*), but there was significantly greater corneal staining in the PA group at V2 (0.86 [95% CI:0.47/1.25] vs 1.95 [95% CI:1.57/2.33]; p=0.0008), V3 (1.05 [95% CI:0.59/1.51] vs 2.58 [95% CI:2.17/2.98]; p=0.0001), and V4 (0.90 [95% CI:0.42/1.39] vs 2.37 [95% CI:2.06/2.68]; p=0.0001).

The variation in corneal staining throughout the whole study is detailed in *Figure 24*. After 21 days of treatment, there was a significant (p<0.0001) decrease in corneal staining in the FML group (V2-V1= -1.47 [95% CI: -1.89/-1.05]). In contrast, there was no significant change in the PA group (V2-V1= 0.00 [95% CI: -0.14/0.14]). The adverse environment exposure provoked a significant (p=0.0001) increase in corneal staining in the PA group (V3-V2: 0.79 [95% CI:0.45/1.12]), but not (p=0.77) in the FML group (V3-V2: 0.19 [95% CI: -0.02/0.40]). After 24 h, the FML group maintained its low levels of unaffected corneal staining whereas the worsening experienced in the PA group did not recover V2 levels.

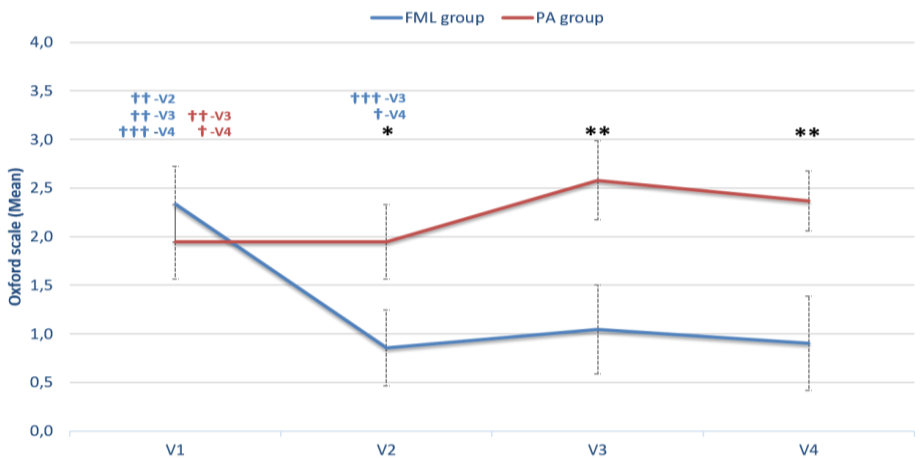


Figure 24. Corneal staining evolution. *: Comparison between groups for each visit (Mann-Whitney U test), *P<0.05 and **P≤0.001. †: Comparison between visits within each group (Student t test plus Bonferroni correction): †P<0.05, ††P<0.001, and †††P≤0.0001. Values are mean and 95% confidence interval. FML group = topical 0.1% fluorometholone ophthalmic suspension group; PA group = topical polyvinyl alcohol group; V1 (visit 1) = baseline data; V2 = visit 2, 21-day treatment, data before adverse controlled environment exposure; V3 = visit 3, 21-day treatment, data after adverse controlled environment exposure; V4 = visit 4, 22-day treatment, data 24h after adverse controlled environment exposure.

Regarding the evaluation of the treatment efficacy, it was analyzed using corneal fluorescein staining and symptoms outcomes as main efficacy variable, as described above *(Figure 25)*. After 21 days of treatment and the 2h exposure to the adverse controlled environment (V3), a significantly (p=0.03) higher percentage of patients had a ≥1 grade increase in corneal staining in the PA group (63.1%, 95%CI: 38.6–82.7) compared to the FML group (23.8%, 95%CI: 9.1–47.5). The percentage of patients with a ≥1 grade increase in corneal staining at V4 was not different (p=0.23) for the FML group (14.3%, 95%CI: 3.7–37.3) and the PA group (0.0%, 95%CI: 0.0–20.9). On the other hand, there was no significant difference between the FML and the PA groups for the percentage of patients with a reduction ≥20 points in SANDE I questionnaire after the 2h exposure to the adverse environment (FML group: 61.9%, 95%CI: 38.6–81.0 vs PA group: 57.9%, 95%CI: 33.9–78.8; p=1.0). Additionally, 24h after exposure to the adverse environment there was no difference in the percentage of patients with symptom reduction ≥20 points (FML group: 66.7%, 95% CI: 43.1–84.5 vs PA group: 68.4%, 95%CI: 43.5–86.4; p=1.0).

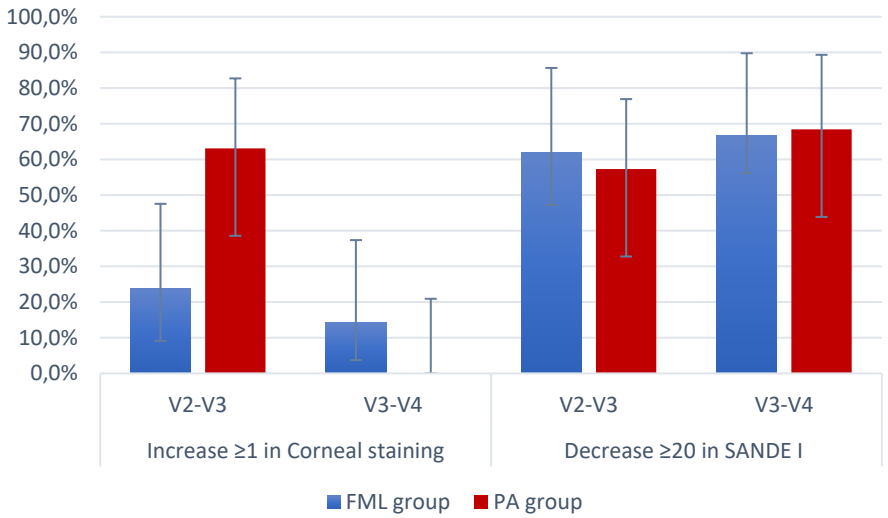


Figure 25. Main efficacy variables. Corneal staining increase ≥1, Symptoms Assessment iN Dry Eye (SANDE) score decrease ≥20.

2.3. Safety Analysis

No adverse events or treatment-related adverse reactions were observed throughout the study. Slit-lamp anterior segment and funduscopy examination at the end of the treatment period revealed no treatment-related abnormalities. In particular, there were no significant changes in IOP and no signs of corneal epithelial healing-related problems or secondary infections as potential side effects from steroid use *(Table 14)*.

For the FML group, at the end of the study there was significant improvement in high and low contrast BCVAs (V1 vs V4; p=0.03 and p=0.01 respectively; *Table 14*). In contrast, for the PA group, there was no change (V1 vs V4; high contrast, p=0.88; low contrast, p=0.69; *Table 14*). However, there were no significant differences between groups for these variables at any visit (p≥0.27, *Table 14*).

2.4. Secondary Outcome Measures

Secondary variables were analyzed as the mean change from the previous visit except for treatment satisfaction. To facilitate understanding, we grouped the results by effects (V1 vs V2: 21-day treatment efficacy, V2 vs V3: 2h of desiccating stress after 21 days of treatment, V3 vs V4: recovery from the V3 desiccating stress after 22 days of treatment). Differences between different visits of the same group and between both groups at the same visit were analyzed. All data are exposed in *Table 15* in the original magnitude.

Efficacy of 21-day treatment (V1-V2): There were significant differences (p≤0.03) in the percent change between both groups for corneal staining, conjunctival staining, hyperemia, and TBUT *(Figure 26)*. For the FML group, there was a significant improvement (p≤0.0001) in scores for SANDE I, corneal and conjunctival staining, and hyperemia. In contrast, patients included in the PA group had a significant worsening (p≤0.04) of conjunctival hyperemia and TBUT and a decrease in SANDE I frequency of symptoms score. *Table 15* shows a more detailed analysis of corneal staining changes using the modified CCLR scale.

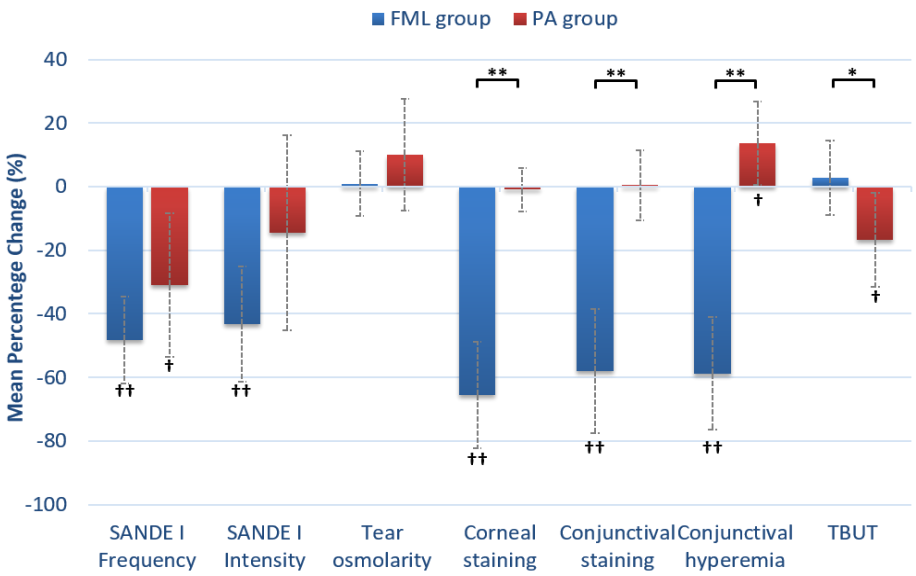


Figure 26. Percentage change between baseline (visit 1) and 21-day treatment (visit 2). Student t test for 2 independent samples was used to compare changes between treatment groups: *P<0.05 and **P≤0.0001. Student t test for single samples was used to determine if the changes differ significantly from 0: †P<0.05 and †††P≤0.0001. FML group = topical 0.1% fluorometholone ophthalmic suspension group; PA group = topical polyvinyl alcohol group; SANDE = Symptom Assessment in Dry Eye; TBUT = tear film breakup time. Bars = 95% confidence intervals.

Table 15. Mean percentage change in corneal staining using the modified CCLR scale.

		FML group (n=21)			**PA group** (n=19)			
Comparisons	**Area**	***Mean (%)***	***95%CI***	***P-value****	***Mean (%)***	***95%CI***	***P-value****	***P-value†***
Efficacy of 21-day Treatment (V1-V2)	Superior	-16.67	-33.29 – -0.05	**0.05**	0	0 – 0	----	----
	Inferior	-64.29	-83.29 – -45.28	**<0.0001**	-7.11	-22.87 – 8.66	0.35	**<0.0001**
	Central	-44.15	-67.79 – -20.5	**0.0009**	-23.03	-43.53 – -2.52	**0.03**	**0.04**
	Nasal	-32.13	-83.09 – -41.18	**<0.0001**	-18.34	-38.34 – 1.66	0.07	**0.003**
	Temporal	-58.83	-79.31 – -38.35	**<0.0001**	-28.38	-50.55 – -6.21	**0.01**	**0.04**
Efficacy of 21-day Treatment after ACE Exposure (V2-V3)	Superior	0	0 – 0	----	0.66	-0.29 – 1.61	0.16	----
	Inferior	-3.19	-21.03 – 14.66	0.71	35.5	16.79 – 54.22	**0.0009**	**0.003**
	Central	-0.23	-6.51 – 6.06	0.94	5.93	1.08 – 10.79	**0.02**	0.11
	Nasal	-5.5	-20.37 – 9.38	0.45	-5.18	-21.46 – 11.11	0.51	0.97
	Temporal	-5.73	-16.17 – 4.7	0.26	8.04	-3.02 – 19.11	0.14	0.06
Efficacy of 22-day Treatment in Recovery after ACE Exposure (V3-V4)	Superior	-4.76	-14.7 – 5.17	0.33	0.35	-0.39 – 1.09	0.33	0.31
	Inferior	-11.31	-30.15 – 7.53	0.22	7.93	-5.24 – 21.1	0.22	0.09
	Central	-11.01	-23.14 – 1.12	0.07	-0.5	-13.04 – 12.03	0.93	0.21
	Nasal	-7.82	-25.33 – 9.7	0.36	-23.07	-46.23 – 0.09	**0.05**	0.27
	Temporal	-14.52	-29.88 – 0.84	0.06	-8.37	-27.69 – 10.95	0.37	0.60

ACE = Adverse controlled environment; CCLR = Centre for contact lens research; CI = Confidence interval; FML = Fluorometholone; PA = Polyvinyl alcohol; V1 = Initial visit; V2 = After 21 days of FML® or LIQUIFILM® treatment; V3 = 2h after adverse controlled environment (ACE) on Day 21; V4 = 24h after ACE; * Within-group comparison. Student´s t-test for single samples; † Between-group comparison. Student´s t-test for two independent samples.

Efficacy of Adverse Controlled Environment Exposure after 21-day treatment (V2-V3): Changes from V2 to V3 were analyzed to determine the potential protective effect of FML® when patients were exposed to adverse environmental conditions. For the FML group, there were no significant changes in corneal staining, conjunctival staining, and hyperemia *(Figure 27)*. In contrast, for the PA group there was significant worsening (p≤0.009) in these variables and in TBUT.

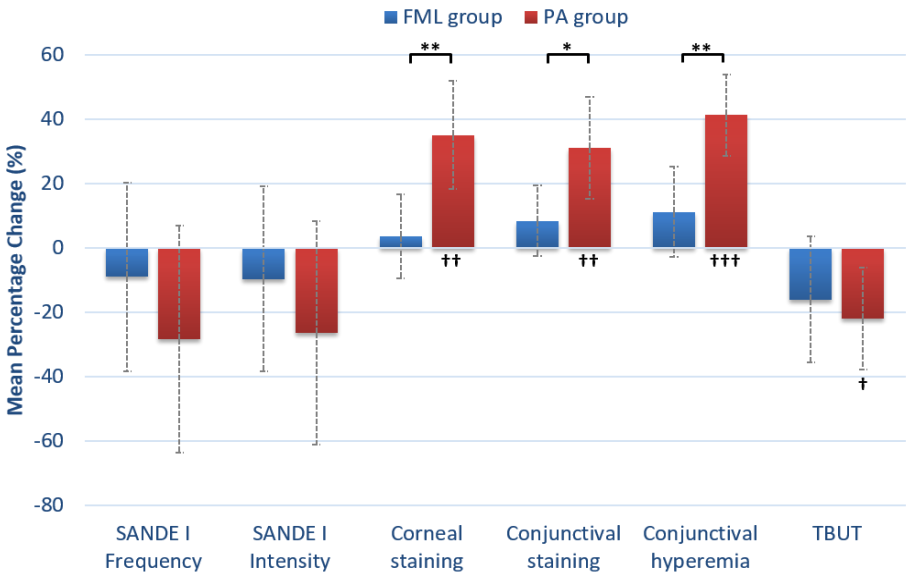


Figure 27. Percentage change between before exposure to an adverse controlled environment (ACE; visit 2) and after exposure to an ACE (visit 3) after 21 days of treatment. Student t test for 2 independent samples was used to compare changes between treatment groups: *P<0.05 and **P≤0.01. Student t test for single samples was used to determine if the changes differ significantly from 0: †P<0.01, ††P<0.001 and †††P≤0.0001. FML group = topical 0.1% fluorometholone ophthalmic suspension group; PA group = topical polyvinyl alcohol group; SANDE = Symptom Assessment in Dry Eye; TBUT = tear film breakup time. Bars = 95% confidence intervals.

Efficacy of 22-day Treatment in Recovery after Adverse Controlled Environment Exposure (V3-V4): Patients were evaluated 24h after adverse environment exposure to determine if there were any differences between the two groups in the recovery from changes provoked by desiccating stress *(Figure 28*). There were no significant differences (p≥0.19) in the mean changes between groups for any variable measured. For the FML group, in which there were no significant impairments under adverse environment exposure, there were significant (p≤0.03) decreases in terms of mean percentage change, for symptoms and conjunctival staining. For the PA group, there were significant (p≤0.04) mean percentage reduction for corneal and conjunctival staining and hyperemia. Overall, as *Figure 24* shows, corneal integrity in the FML group did not worsen under environmental stress and remained constant during the 24 h recovery period (1.05 [95% CI:0.59/1.51] vs 0.90 [95% CI:0.42/1.39, respectively, p=0.77), meaning that there was no difference between before the adverse exposure (V2) and 24 h later (V4). In contrast, the corneal integrity in the PA group worsened under environment stress (1.95 [95% CI:1.57/2.332.58] vs 2.58 [95% CI:2.17/2.98], V2 and V3, respectively; p=0.0001) and remained equally during the ensuing 24h recovery period as compared to V2 (V4: 2.37 [95% CI:2.06/2.68], p= 0.44), meaning that corneal staining did not recover the pre-exposure levels.

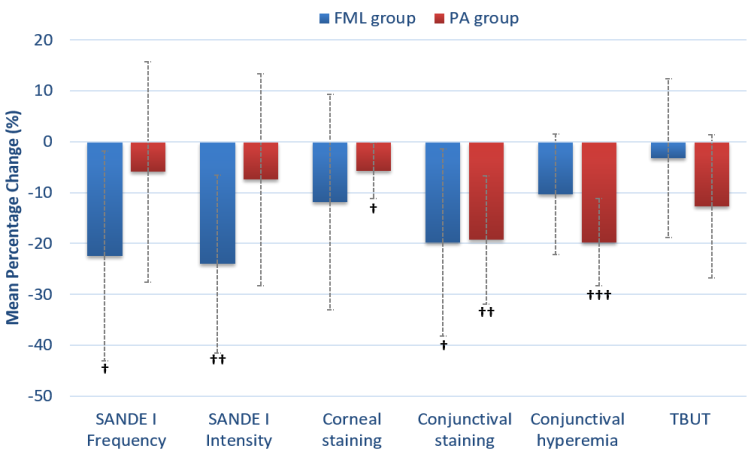


Figure 28. Percentage change between before exposure to an adverse controlled environment (ACE) after 21 days of treatment (V3) and 24h after exposure to an ACE (visit 4). Student t test for single samples was used to determine if the changes differ significantly from 0: †P<0.05, ††P<0.01 and †††P≤0.0001. FML group = topical 0.1% fluorometholone ophthalmic suspension group; PA group = topical polyvinyl alcohol group; SANDE = Symptom Assessment in Dry Eye; TBUT = tear film breakup time. Bars = 95% confidence intervals.

2.5. Treatment Satisfaction

We evaluated satisfaction of the patients at V2 and V4 *(Figure 29)*. After 21 days of therapy, the FML group expressed significantly higher satisfaction than the PA group (V2: 73.8±23.1 units vs 47.5±34.0 units, respectively; p=0.03). At V4, 24h after the adverse environment exposure, the FML group again expressed greater satisfaction than did the PA group (FML group, 79.7±14.6 units vs PA group, 46.4±30.3 units, respectively; p=0.0002). In the FML group the satisfaction increased significantly (mean value: 33.8%; 95% CI: 13.5–54.1, p=0.002), while in the PA group it decreased slightly (mean value: 6.1%; 95% CI: -32.4–20.3, p=0.63). Changes observed in the satisfaction scores were significantly (p=0.01) different between groups.

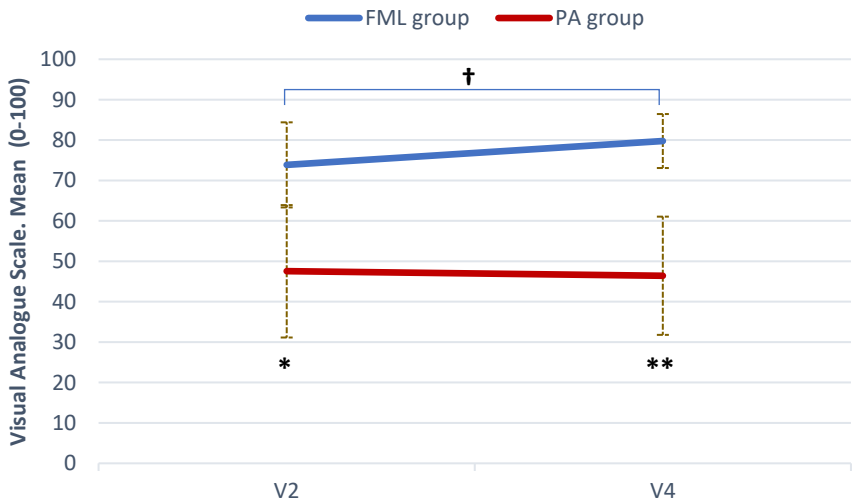


Figure 29. Treatment satisfaction levels referred by patients at V2 and V4. Student t test for 2 independent samples was used to compare both treatment groups: *P<0.05 and **P≤0.001. Student t test for single samples was used to determine if the changes differ significantly from 0: †P<0.01. FML group = topical 0.1% fluorometholone ophthalmic suspension group; PA group = topical polyvinyl alcohol group.

3. Discussion

This clinical trial evaluated the efficacy of topical 0.1% fluorometholone in DED patients after 21 days of treatment and in the prevention of the exacerbation of DED signs and symptoms that patients suffer when they are exposed to adverse environmental desiccating stress.[14–16] In this study, we compared the responses of DED patients treated for 21 days with 0.1% fluorometholone or PA artificial tears prior to exposure to the adverse environment. We found that patients treated with 0.1% fluorometholone maintained the status of their ocular surface after adverse environment exposure while those treated with PA artificial tears had noticeable impairment.

Some studies have evaluated the efficacy of corticosteroids in both short-term and long-term treatment of DED.[17–30] Our findings confirm the efficacy of topical corticosteroids as a short-term (≤4 weeks) DED treatment as previously shown by other research groups.[17,18,20,22,23,25,26,30] It is remarkable that, unlike our trial, the vast majority of the studies that evaluated corticosteroids for DED lack the rigorous design of a randomized double-masked vehicle-controlled clinical trial (2 retrospective[17,24] and 6 prospective[18,21,23,26,28,30]), which might be the state-of-the-art study that properly demonstrates drug efficacy. In fact, out of the 14,[17–30] only 6 are clinical trials.[19,20,22,25,27,29] One of them[29] (out of the 6) is not similar to our study because it reports not only the effect of loteprednol etabonate 0.5% on DED, but the combination of this steroid with topical cyclosporine 0.05%. Another one[22] used one eye as study group and the other one as control group, which is not a desirable design. Three more studies[20,25,27] followed adequate clinical trial designs, but they evaluated different drugs[20,27] (Loteprednol etabonate vs vehicle; dexamethasone phosphate vs placebo; polyvinylpyrrolidone + clobetasone butyrate vs polyvinylpyrrolidone + placebo, respectively), or different via of administration (i.e. ocular iontophoresis).[25] Finally, the most similar one[19] to our design and drug, is single-masked (not double-masked as ours), and reported that FML® is better than NSAID therapy. However more importantly, we demonstrated the protective effect

of this therapy for coping with adverse conditions in the daily lives of these patients, which is the main novelty of our study.

Corticosteroids are among the most effective agents used to treat noninfectious inflammatory diseases, especially those mediated by the immune system. They reduce cellular infiltration, inhibit chemotaxis, and restore the appropriate vascular permeability.[31] Corticosteroids also reduce or suppress capillary dilation, fibroblast proliferation, and collagen deposition. They stabilize intra- and extra-cellular membranes, increase the synthesis of lipocortins that block phospholipase A2, and inhibit histamine synthesis in mast cells. Inhibition of phospholipase A2 prevents the conversion of phospholipids to arachidonic acid, a critical step in the inflammatory cascade. Corticosteroids also increase the enzyme histaminase and modulate transcription factors present in mast cell nuclei.[31]

As previously explained, the benefit of corticosteroids in the treatment of DED and in the improvement of both signs and symptoms has been demonstrated in several studies,[17–30] and our clinical data are consistent with these reports. Although prolonged use of corticosteroids has been associated with IOP elevation and cataract formation[17] (reason why cyclosporine is preferred for long-term treatment) some clinical studies have suggested that application of topical corticosteroid for short-term (≤4 weeks) use is safe.[23,24] For this reason a 21-day therapy was conducted in this clinical trial. FML® was selected as the study treatment because it has been previously shown to be effective in DED therapy.[19,21] Moreover, 0.1% fluorometholone penetrates the ocular tissues less than other corticosteroids,[32] which minimizes the potential complications of this therapy.

In our study, after 21-days of treatment, 0.1% fluorometholone reduced corneal and conjunctival staining and hyperemia, while no obvious effects were observed with PA artificial tears. The clinical improvement in corneal staining was in concordance with previous studies assessing corticosteroids.[17–19,21,27,28,30] These results could explain the increase in BCVA that we also found because there is a positive correlation between corneal epithelial damage and visual acuity.[33] We also observed an improvement in conjunctival staining as earlier reported.[19] As reported

by others,[21] we found that 0.1% fluorometholone decreased conjunctival hyperemia, which is associated with the degree of inflammation.[34] In contrast, polyvinyl alcohol artificial tears had no effect on hyperemia. Both FML® and Liquifilm Tears™ contain benzalkonium chloride as a preservative but in small concentrations (0.004% an 0.005%, respectively). Even though small concentrations of BAK have shown negative effects in *in vitro* experiments,[35] there is a huge difference between testing conditions *in vitro* and in clinical settings. For *in vitro* research, BAK is in direct contact with cell membranes, where it stays for the testing period without any fluid movement or clearance. In the clinical setting, BAK never comes in direct contact with cell membranes due to the thick mucus layer, the aqueous layer with hundreds of proteins, and the lipid layer of the tear film. Additionally, the tear film is in constant movement, and tear film clearance removes topically applied substances quite efficiently.

Supporting the above, Okahara et al.[36] have elegantly demonstrated that topical ocular repeated application of BAK-containing vehicle for up to 52 weeks, up to 8 times/day, or at concentrations up to 0.01%, in monkeys and rabbit eyes caused no clinical changes suggestive of irritation, allergy, or corneal damage and no histopathologically detectable changes in eyelid, eyeball, lacrimal gland, or nasal cavity. This *in vivo* study, with eyes similar to human eyes, also demonstrated that a 0.001% difference in BAK, as the existing between the treatments used in this clinical trial, is irrelevant.

Our research group[14,16] and other authors[15] have previously demonstrated that heathy volunteers and patients with DED can suffer DED-related signs and symptoms exacerbations when exposed to adverse controlled conditions. The adverse environmental condition used in this clinical trial (23°C, 5% RH, localized airflow 0.43 m/s) causes deterioration in the state of the ocular surface in both healthy subjects and patients with DED.[14] This exposure simulates the low relative humidity and airflow to which population could be exposed in their daily lives (e.g., artificially air conditioned buildings, vehicle travelling, flights, etc.). The ocular surface deterioration observed in the PA group reinforces the fact that DED patients

can experience exacerbations under desiccating conditions, and that tear substitutes are not sufficient to protect the ocular surface in these adverse situations. In contrast, there were no significant changes in the FML group after adverse environment exposure, confirming the appropriateness of 0.1% fluorometholone 21-day treatment for preserving the ocular surface.

Our findings agree with outcomes observed by Moore et al.,[30] who reported the absence of ocular surface worsening in topical dexamethasone-treated DED patients exposed to low humidity.[30] Thus, we have shown that this topical 0.1% fluorometholone therapy could be a suitable prophylactic approach for DED patients who expect to experience an adverse environmental condition. This therapy could safely prepare them to better cope with any desiccating stress scenario. Additionally, we believe that the methodology followed in this clinical trial could also be suitable for rapidly testing the efficacy, safety, and potential protective effects of current or new DED therapies.

To assess the recovery of DED patients after an adverse condition, we included in this clinical trial a visit, V4, scheduled 24h after exposing the patients to desiccating conditions. We observed statistical significant improvements in the FML and PA group *(Figure 28)* for some clinical variables in terms of percentage change, however, improvements should be placed in perspective from a clinical standpoint. As in the FML group, the corneal staining had not worsened significantly under adverse conditions (i.e. patients remained stable despite they should have suffered an ocular surface worsening[14–16]), an improvement would have been unrealistic *(Figure 24)*. We observed a very positive effect as the lack of impairment was maintained 24h later. On the contrary, the significant degradation of the corneal staining as a result of the 2-h exposure to the desiccating conditions in the PA group *(Figure 24)* was clinically maintained after 24h (Table 14: corneal staining scores: V4= 2.37 vs V3= 2.58), and moreover, corneal staining values did not return to pre-exposure levels (V2*, Figure 24)*.

The present study has several limitations. First, the majority of the patients in the trial were women (85.4%). This was expected because DED is more prevalent in

women, as has been explained in chapter 1. Moreover, the difference in gender ratio increased with DED severity as Sjögren's syndrome is between 10 and 20 times more frequent in women than men,[37] and this study included a large number of severe DED patients. Despite this gender imbalance, we did not see any evidence to indicate a gender difference in the response to 0.1% fluorometholone or polyvinyl alcohol artificial tears, and to our knowledge, none has been published. Second, 0.1% fluorometholone was the only topical corticosteroid that we studied. We chose it because of its low rate of ocular penetration[32] and the associated good benefit-to-risk ratio. Future studies may include other topical corticosteroids that are also known to be efficacious in DED therapy.[17,20,22,24,27,29] The third limitation is that we recruited only 41 DED patients in this clinical trial. Because of the small size of the study population, this could be considered as a pilot study. However, it is large enough as detailed in the statistical section to provide evidence of the effectiveness of 0.1% fluorometholone in protecting the ocular surface against adverse environments that DED patients are expected to undergo.

In conclusion, the present clinical trial shows that three-week topical 0.1% fluorometholone is a safe and effective therapy for DED patients to reduce ocular surface signs after a 21-day treatment. Importantly, we found that 0.1% fluorometholone therapy can also prevent ocular surface worsening in DED patients exposed to desiccating stress. Thus, this treatment could be administered occasionally to such patients expecting to undergo adverse environments during their daily life (e.g., office buildings, shopping centers, movie theatres, air-conditioned vehicles, etc.). The treatments could be used to avoid the exacerbation periods that they might suffer from time-to-time. Finally, the study design that we have followed could also be suitable to test the efficacy and safety of new DED therapies. Our new methodology improves the experimental designs usually carried out in conventional multicenter DED clinical trials by exposing the patients to tightly-controlled environmental conditions as is recommended.[38]

4. References

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TEAR BIOMARKERS

Chapter 3
Tear Biomarkers

It is well known that environmental conditions have a big impact on the status of the ocular surface, overall in DED patients. As explained in Chapter 1, several studies have evaluated the effect of environmental conditions, as well as the effect of DED treatments, on tear inflammatory molecules and LFU. On the other hand, there is a need to find objective outcomes, such as molecular biomarkers, that could be used in clinical trials or daily practice to establish in a more reliable way parameters like the efficacy and effectiveness of a therapy, the severity of the pathology, the activity of the disease, etc.

Consequently, this chapter presents the results of a biomarkers sub-analysis of the global clinical trial previously explained (see Chapter 2). This sub-analysis pursued two main objectives:

- The first aim of this sub-analysis was to study the effect of 0.1% fluorometholone therapy on tear inflammatory molecule levels in DED patients after a 21-day treatment period and after undergoing an acute controlled environmental adverse desiccating stress exposure.
- The second aim was to use that information to identify three types of potential tear biomarkers of DED: disease severity, therapeutic efficacy (FML in this study), and disease activity.

1. Materials and Methods

1.1. Tear sample collection

As *Table 11* (Chapter 2) shows, non-stimulated tear samples were collected at each visit from all participants included in the study: baseline (V1), pre-ACE after 21days of treatment (V2), post-2-h-ACE after 21 days of treatment (V3), and 24 h post-ACE after 22-days of treatment (V4). Tears were collected immediately after the environmental exposures and before any other evaluation to avoid possible alterations induced by other tests.

Tear samples (1 µL) were collected from the external canthus of one randomly selected eye. Each tear sample was collected with a glass microcapillary tube (Drummond Scientific, Broomall, PA, USA) in a non-traumatic manner, avoiding reflex tearing as much as possible. Samples were diluted 1/10 in a 0.5 mL microtube (Sarstedt AG&Co, Nümbrecht, Germany) containing ice-cold Cytokine Assay Buffer (Milliplex, EMD Millipore Corp, Billerica MA, VA, USA) and frozen at -80°C until analysis.

1.2. Tear Inflammatory Molecules Analysis

An immune-bead based array using LuminexTM x-MAP® multiplexing bead technology was used to analyze 18 molecules in the tear samples with a Luminex IS-100 instrument (Luminex Corporation, Austin, TX, USA).[1,2] This assay consists of an immune-based assay on a series of a mix of microspheres (beads) containing fluorochromes of differing intensity embedded within the bead, giving a unique signal, which can be detected in a Luminex equipment, to each group of beads with specific recognition molecule attached. The multiplex system enables the detection and quantification of multiple analytes (proteins and peptides, or nucleic acids) in a single and small sample volume.[3]

The concentrations of EGF, IFN-γ, TNF-α, IL-1β, IL-1RA, IL-2, IL-4, IL-6, IL-8/CXCL8, IL-10, IL-12, IL-13, IL-17A, IP-10/CXCL10, monocyte chemoattractant protein–1 (MCP-1)/CCL2, MIP-1α/CCL3, RANTES/CCL5, and MMP-9 were measured simultaneously in an 18-plex assay (HCYTO-60K SPR 591 18X-Milliplex,

Millipore Corp.). Samples were analyzed following the manufacturer's protocol. Briefly, 10 µl of the 1/10 diluted sample were incubated under agitation overnight at 4°C with beads coated with antibodies specific for each molecule. After washing, the beads were incubated with biotinylated human antibodies for 1h, followed by incubation with streptavidin-phycoerythrin for 30 min at room temperature. Standard curves of known concentrations of recombinant human cytokines/chemokines and other inflammatory molecules were used to convert fluorescence units detected in the Luminex to concentration units (pg/mL). Data were stored and analyzed with the BeadViewTM Software (Upstate-Millipore Corporation, Watford, UK).

The minimum detectable concentrations (in pg/mL) provider by kit manufacturer for the molecules analyzed is shown in *Table 16*.

Table 16. Minimum detectable concentrations (in pg/mL) for the molecules analyzed during the study.

Molecule	Minimun detectable	Molecule	Minimun detectable
EGF	2.7	IL-12	0.4
IFN- γ	0.1	IL-13	1.3
IL-1β	0.4	IP-10	1.2
IL-1RA	2.9	RANTES	1
IL-2	0.3	TNF-α	0.1
IL-4	4.5	MMP-9	10
IL-6	0.3	MCP-1	1.9
IL-8	0.2	IL-17A	0.2
IL-10	0.3	MIP-1α	2.9

EGF = Epidermal growth factor; IFN = Interferon; IL = interleukin; IP = Induced protein; MCP = Monocyte chemoattractant protein; MIP = Macrophage inflammatory protein; MMP = Metalloproteinase; RA = Receptor antagonist; RANTES = Regulated upon activation normal T-cell expressed and presumably secreted; TNF = Tumor necrosis factor.

1.3. Definition of the Type of Tear Biomarkers

We defined three different types of tear biomarkers: 1) disease severity, 2) therapeutic efficacy, and 3) disease activity.

1) Disease severity biomarkers were defined as tear molecules that allowed differentiation between moderate and severe DED patients.

2) Therapeutic efficacy biomarkers (of topical FML in this specific trial) were defined as tear molecules that allowed differentiation between FML group and PA group patients and/or molecules that varied in time depending on the treatment received.

3) Disease activity biomarkers were defined as tear molecules that changed concentration significantly between V2 (before entering the environmental chamber) and V3 (2h after ACE) after 21 days of treatment with FML or PA.

1.4. Statistical Analysis

The statistical analyses were carried out under the supervision of a PhD licensed statistician (Dra. Itziar Fernández), using the R statistical package version 3.1.1 (R Core Team. Foundation for Statistical Computing, Vienna, Austria. URL: https://www.R-project.org/). Statistical significance was set at $p \leq 0.05$. Inflammatory molecule concentrations were analyzed as base-2 log-transformed variables. Levels below the limit of detection were imputed using the robust regression on order statistics (robust ROS) method introduced by Helsel,[4] and implemented in the non-detects and data analysis (NADA) R package.[5] However, molecules that showed low percentages of detection in the samples (<25%) were not further analyzed.

Mean and median values were used to describe quantitative variables, while only median values were used for ordinal ones. Also, 95% CI were constructed where appropriate.

To analyse the pattern of similarities among patients based on their tear inflammatory molecules levels at baseline (V1), a multidimensional scaling (MDS) procedure was used. Differences in tear molecule levels and clinical parameters

among the groups generated by the MDS (MDS-groups) were tested by Student's t-test or the non-parametric alternative, Mann-Whitney U test when the normally assumption was not valid. Normality of data and homogeneity of variances were checked by the Shapiro-Wilk test and the Brown-Forsythe test, respectively. When the results were normally distributed, but there was significant heterogeneity of variance, the Welch's t-test was used. To assess the association between two qualitative variables, the Chi-square test or the Fisher's exact test with small expected frequencies were used.

Linear mixed effects models were used to evaluate the effect of time, MDS group, treatment, and interactions on the changes of each inflammatory molecule. The effects were quantified using fold-change (FC), along with the 95% CI. FC, denoted by $FC_{A:B}$, was defined as the ratio of the average raw expression level of the corresponding inflammatory molecule in a group, condition, or visit (A) against the average raw expression level in a different group, condition, or visit (B). On the FC scale, the "up changes" are in the interval 1 to infinity, and the "down changes" are in the 0 to 1 interval. Finally, a free step-down re-sampling approach was used to control the family-wise error rate[6] and to compute the adjusted p-values for multiple comparisons.

2. Results

As previously explained in Chapter 2, forty-one patients were initially included in the study, but one was discontinued because of his inability to attend one of the visits *(Figure 23)*. For the remaining 40 patients, the mean age was 59.6 years (95% CI: 56.9-62.4 years). Twenty-one patients (17 females, 4 males) were randomly included in the FML group and 19 patients (17 females, 2 males) in the PA group. There were no significant differences in age or sex in the distribution between the two treatment groups.

2.1. Inflammatory Molecule Concentrations in Tears

The concentration of each inflammatory molecule is shown in *Table 17*. Based on their very low percentage of detection, neither IL-17A (V1: 17.5%; V2: 10.0%; V3: 10.0%; V4: 10.3%) nor MIP-1α/CCL3 (V1: 15.0%; V2: 15.0%; V3: 7.5%; V4: 12.8%) were further analyzed.

Table 17. Complete dataset of molecule concentrations during the clinical trial.

Visit	Molecule	FML group						PA group						Total					
		n	%	Mean (pg/ml)	SD	95% CI (Mean)		n	%	Mean (pg/m)l	SD	95% CI (Mean)		n	%	Mean (pg/ml)	SD	95% CI (Mean)	
						Lower	Upper					Lower	Upper					Lower	Upper
V1	EGF	21	100	1809.85	1585.04	1088.35	2531.35	19	100	1654.79	1238.82	1057.7	2251.88	40	100	1736.19	1415.22	1283.59	2188.8
	IFN-γ	9	42.86	23.03	23.73	12.23	33.83	11	57.89	51	75.04	14.83	87.17	20	50	36.32	55.57	18.54	54.09
	IL-1β	10	47.62	25.87	71.06	-6.47	58.22	8	42.11	27.49	37.42	9.46	45.53	18	45	26.64	56.89	8.45	44.84
	IL-1RA	21	100	2988.87	4374.85	997.47	4980.28	18	94.74	4651.75	10945.62	-623.87	9927.37	39	97.5	3778.74	8112.8	1184.14	6373.34
	IL-2	3	14.29	12.27	13.74	6.02	18.53	11	57.89	43.14	51.28	18.43	67.86	14	35	26.94	39.43	14.33	39.55
	IL-4	10	47.62	98.65	140.37	34.76	162.55	9	47.37	139.61	176.3	54.63	224.58	19	47.5	118.11	157.73	67.66	168.55
	IL-6	18	85.71	56.94	84.05	18.68	95.2	15	78.95	125.94	324.37	-30.41	282.28	33	82.5	89.71	231.09	15.81	163.62
	IL-8	21	100	960.71	1886.59	101.95	1819.48	19	100	3513.97	11309.96	-1937.26	8965.19	40	100	2173.51	7907.62	-355.47	4702.49
	IL-10	14	66.67	31.52	31.68	17.1	45.94	15	78.95	73.85	100.54	25.39	122.3	29	72.5	51.62	75.09	27.61	75.64
	IL-12	17	80.95	77.75	50.93	54.56	100.93	11	57.89	126.93	125.42	66.48	187.39	28	70	101.11	95.97	70.42	131.8
	IL-13	9	42.86	24.05	23.32	13.44	34.67	14	73.68	52.94	68.3	20.02	85.86	23	57.5	37.78	51.43	21.33	54.22
	IP-10	21	100	24201.05	18361.03	15843.21	32558.89	18	94.74	23627.4	25477.94	11347.42	35907.37	39	97.5	23928.56	21738.59	16976.22	30880.9
	RANTES	18	85.71	58.08	37.33	41.09	75.07	16	84.21	81.37	92.3	36.88	125.86	34	85	69.14	69.18	47.02	91.26
	TNF-α	16	76.19	22.15	11.13	17.08	27.21	14	73.68	35.45	30.17	20.91	49.99	30	75	28.46	23	21.11	35.82
	MMP-9	19	90.48	18704.99	33086.73	3644.09	33765.89	17	89.47	29120.61	78371.03	-8653.02	66894.23	36	90	23652.41	58514.32	4938.62	42366.2
	MCP-1	19	90.48	822.95	726.07	492.45	1153.45	19	100	1442.18	1964.25	495.45	2388.92	38	95	1117.09	1466	648.24	1585.94
V2	EGF	21	100	2182.95	1883.45	1325.62	3040.29	18	94.74	1474.31	970.8	1006.4	1942.22	39	97.5	1846.35	1543.56	1352.69	2340
	IFN-γ	11	52.38	26.74	24.23	15.7	37.77	11	57.89	71.28	136.7	5.39	137.16	22	55	47.89	97.12	16.83	78.95
	IL-1β	6	28.57	9.12	9.39	4.84	13.39	8	42.11	24.04	41.16	4.21	43.88	14	35	16.21	29.73	6.7	25.72
	IL-1RA	20	95.24	3550.55	5616.22	994.08	6107.03	18	94.74	4783.3	8673.66	602.73	8963.87	38	95	4136.11	7161.47	1845.76	6426.46
	IL-2	7	33.33	16.3	18.93	7.69	24.92	7	36.84	44.91	95.5	-1.12	90.93	14	35	29.89	67.84	8.19	51.58
	IL-4	8	38.1	136.3	307.71	-3.77	276.37	9	47.37	282.88	572.15	7.11	558.64	17	42.5	205.92	452.93	61.07	350.78

	IL-6	16	76.19	41.44	33.05	26.4	56.48	15	78.95	105.02	163.47	26.23	183.81	31	77.5	71.64	118.01	33.9	109.38
	IL-8	21	100	713.97	1509.82	26.71	1401.23	19	100	1098.83	2414.33	-64.84	2262.5	40	100	896.78	1974.13	265.42	1528.13
	IL-10	14	66.67	31.91	25.52	20.29	43.53	11	57.89	75.43	175.82	-9.31	160.18	25	62.5	52.58	122.82	13.3	91.86
	IL-12	15	71.43	85.25	60.45	57.74	112.77	11	57.89	151.7	177.71	66.05	237.36	26	65	116.82	132.59	74.41	159.22
	IL-13	14	66.67	43.43	29.46	30.02	56.83	12	63.16	64.78	97.7	17.69	111.87	26	65	53.57	70.48	31.03	76.11
	IP-10	21	100	22798.57	20464.56	13483.21	32113.93	19	100	19913.37	20153.2	10199.84	29626.9	40	100	21428.1	20108.51	14997.09	27859.11
	RANTES	19	90.48	49.63	28.73	36.55	62.71	14	73.68	92.91	169.32	11.3	174.52	33	82.5	70.19	118.89	32.17	108.21
	TNF-α	20	95.24	23.03	9.26	18.82	27.24	13	68.42	37.18	45.85	15.09	59.28	33	82.5	29.75	32.64	19.31	40.19
	MMP-9	20	95.24	15615.26	30068.47	1928.26	29302.26	16	84.21	24684.08	67707.8	-7950.04	57318.19	36	90	19922.95	50995.44	3613.82	36232.08
	MCP-1	20	95.24	1309.42	1847.48	468.46	2150.38	17	89.47	1701.49	2730.79	385.29	3017.69	37	92.5	1495.65	2287.24	764.16	2227.15
V3	EGF	21	100	1080.23	916.39	663.09	1497.36	18	94.74	1037.15	828.1	638.02	1436.28	39	97.5	1059.77	864.65	783.24	1336.3
	IFN-γ	9	42.86	28.9	33.41	13.7	44.11	10	52.63	42.63	59.63	13.89	71.37	19	47.5	35.42	47.56	20.21	50.63
	IL-1β	7	33.33	8.29	7.82	4.73	11.85	6	31.58	15.49	25.83	3.05	27.94	13	32.5	11.71	18.77	5.71	17.72
	IL-1RA	21	100	1506.6	2468.84	382.79	2630.4	17	89.47	5073.46	12482.25	-942.79	11089.71	38	95	3200.86	8848.19	371.07	6030.64
	IL-2	9	42.86	28.38	25.59	16.73	40.03	7	36.84	34.12	42.28	13.74	54.5	16	40	31.11	34.2	20.17	42.04
	IL-4	5	23.81	51.4	85.38	12.54	90.26	8	42.11	116.73	236.33	2.82	230.63	13	32.5	82.43	174.95	26.48	138.38
	IL-6	16	76.19	50.02	93.87	7.3	92.75	15	78.95	89.54	175.56	4.92	174.15	31	77.5	68.79	138.36	24.54	113.04
	IL-8	21	100	546.03	1345.39	-66.39	1158.44	19	100	1513.22	4961.52	-878.16	3904.59	40	100	1005.44	3539.64	-126.59	2137.47
	IL-10	14	66.67	41.3	34.18	25.74	56.85	11	57.89	56.83	79.16	18.67	94.98	25	62.5	48.67	59.6	29.61	67.74
	IL-12	16	76.19	85.06	65.84	55.09	115.03	13	68.42	114.39	114.23	59.34	169.45	29	72.5	98.99	92.01	69.57	128.42
	IL-13	14	66.67	35.47	31.52	21.12	49.81	8	42.11	49.11	74.22	13.34	84.88	22	55	41.95	55.67	24.14	59.75
	IP-10	21	100	17919.19	16974.5	10192.49	25645.89	17	89.47	17151.22	21434.05	6820.33	27482.1	38	95	17554.4	18972.38	11486.74	23622.06
	RANTES	16	76.19	51.23	45.28	30.62	71.84	15	78.95	74.76	65.97	42.97	106.56	31	77.5	62.41	56.58	44.31	80.5
	TNF-α	11	52.38	16.98	13.31	10.92	23.04	16	84.21	32.61	33.99	16.23	48.99	27	67.5	24.4	26.2	16.02	32.78
	MMP-9	20	95.24	5750.77	14942.26	-1050.87	12552.4	15	78.95	13124.02	33858.69	-3195.34	29443.39	35	87.5	9253.06	25642.06	1052.33	17453.79
	MCP-1	20	95.24	811.05	1101.1	309.84	1312.27	18	94.74	1477.69	2150.17	441.34	2514.03	38	95	1127.7	1693.88	585.98	1669.43

V4	EGF	20	100	2304	2327.65	1214.63	3393.37	19	100	1930.63	1743.21	1090.43	2770.83	39	100	2122.1	2045.52	1459.02	2785.18
	IFN-γ	9	45	24.51	24.66	12.97	36.05	8	42.11	48.28	57.92	20.37	76.2	17	43.59	36.09	45.14	21.46	50.73
	IL-1β	6	30	8.54	9.74	3.98	13.11	6	31.58	11.8	17.75	3.24	20.36	12	30.77	10.13	14.12	5.55	14.71
	IL-1RA	19	95	4127.5	7197.93	758.77	7496.23	19	100	2737.99	4753.55	446.86	5029.13	38	97.44	3450.56	6091.27	1.476	5425.12
	IL-2	9	45	22.63	15.19	15.52	29.73	7	36.84	35.42	45.11	13.67	57.16	16	41.03	28.86	33.48	18	39.71
	IL-4	7	35	26.93	43.85	6.41	47.45	4	21.05	82.03	181.84	-5.62	169.67	11	28.21	53.77	131.92	11.01	96.54
	IL-6	17	85	58.85	72.75	24.81	92.9	15	78.95	60.99	77.69	23.55	98.44	32	82.05	59.9	74.2	35.84	83.95
	IL-8	20	100	1146.26	2332.92	54.42	2238.09	19	100	807.56	1382.72	141.11	1474.01	39	100	981.25	1912.15	361.4	1601.1
	IL-10	12	60	29.05	26.67	16.56	41.53	13	68.42	46.56	67.27	14.13	78.98	25	64.1	37.58	50.77	21.12	54.04
	IL-12	12	60	70.71	60.86	42.22	99.19	15	78.95	97.61	102.81	48.05	147.16	27	69.23	83.81	83.93	56.61	111.02
	IL-13	13	65	32.51	22.67	21.9	43.13	10	52.63	40.69	46.93	18.07	63.31	23	58.97	36.5	36.3	24.73	48.26
	IP-10	20	100	24197	18892.93	15354.84	33039.16	19	100	19882.21	18051.57	11181.63	28582.79	39	100	22094.92	18373.88	16138.8	28051.05
	RANTES	16	80	51.11	26.59	38.67	63.56	15	78.95	70.42	68.06	37.62	103.22	31	79.49	60.52	51.41	43.85	77.18
	TNF-α	16	80	24.4	11.19	19.16	29.64	15	78.95	25.33	20.1	15.64	35.02	31	79.49	24.85	15.94	19.68	30.02
	MMP-9	18	90	13548.17	29115.99	-78.53	27174.87	17	89.47	12926.9	21464.72	2581.24	23272.56	35	89.74	13245.5	25341.9	5030.61	21460.39
	MCP-1	18	90	1750.85	2737.56	469.63	3032.07	19	100	1158.98	1253.98	554.58	1763.38	37	94.87	1462.5	2140.51	768.63	2156.38

CI = confidence interval; EGF = epidermal growth factor; FML = fluorometholone; IFN = interferon; IL = interleukin; IP = induced protein; MCP = monocyte chemoattractant protein; MIP = macrophage inflammatory protein; MMP = metalloproteinase; PA = polyvinyl alcohol; RA = receptor antagonist; RANTES = regulated upon activation normal T-cell expressed and presumably secreted; SD = standard deviation; TNF = tumor necrosis factor.

2.2. Disease Severity Biomarkers

A MDS methodology was used to study if there was a homogeneous distribution of the patients based on their tear molecule levels at baseline (V1). After performing this analysis, the total sample could be divided into two groups of patients (named "A" and "B", n= 19 and n= 21, respectively), which showed statistically significant (Student T-test, p≤0.013) differences at V1 in the tear levels of 5 inflammatory molecules: EGF, IFN-γ, IL-8/CXCL8, RANTES/CCL5 and MMP-9 *(Table 18)*. Particularly, Group "B" had significantly higher levels of IL-8/CXCL8 and MMP-9 and lower levels of EGF, IFN-γ and RANTES/CCL5 than Group "A".

To assess the relevance of the obtained 2 clusters, and their relation to DED severity, clinical data *(Table 18)* from "A" and "B" groups were compared. There were significant differences between the two groups for fluorescein corneal staining, conjunctival lissamine green staining, and Schirmer test values. Based on the differences in inflammatory molecules expression between the groups, Group A was designated as the "moderate-DED group" (M-DED) and Group B was designated as the "severe-DED group" (S-DED). Consequently, in addition to the comparisons between the FML and PA groups, we also compared clinical variables in the moderate and severe disease groups *(Table 19)*. There were no demographic differences between the two categories of groups, and treatment groups were balanced by the MDS groups.

Linear mixed effect models analysis revealed a significant effect of the "severity group" on EGF ($FC_{M\text{-}DED:S\text{-}DED}$=1.67 [95%CI: 1.03, 2.7], p=0.0377); IFN-γ ($FC_{M\text{-}DED:S\text{-}DED}$=2.63 [95%CI: 1.45, 4.79], p=0.0022; IL-2 ($FC_{M\text{-}DED:S\text{-}DED}$=2.01 [95%CI: 1.27, 3.19], p=0.0038); IL-8/CXCL8 ($FC_{M\text{-}DED:S\text{-}DED}$=0.5 [95%CI: 0.25, 0.98], p=0.0444); IL-10 ($FC_{M\text{-}DED:S\text{-}DED}$=1.74 [95%CI: 1.03, 2.93], p=0.0384); IL-12 ($FC_{M\text{-}DED:S\text{-}DED}$=1.86 [95%CI: 1.19, 2.89], p=0.0076); RANTES/CCL5 ($FC_{M\text{-}DED:S\text{-}DED}$=1.81 [95%CI: 1.18, 2.77], p=0.0076), and MMP-9 ($FC_{M\text{-}DED:S\text{-}DED}$=0.14 [95%CI: 0.04, 0.43], p=0.0013). The levels of MMP-9 and IL-8/CXCL8 were higher on the S-DED group than on the M-DED group, while the levels of EGF, IFN-γ, IL-2, IL-10, IL-12 and RANTES/CCL5 were lower on the S-DED group than on the M-DED group *(Figure 30)*.

Table 18. Statistically significant differences at baseline (V1) in clinical variables and molecule levels (pg/ml) between the groups generated according the multidimensional scaling (MDS) methodology.

Molecules	**Group A; M-DED** (n=21)		**Group B; S-DED** (n=19)		**Between Groups**
	Mean (Median)	**95% CI**	**Mean (Median)**	**95% CI**	**P value**
EGF	2158.3 *(1710)*	1473.0 – 2843.5	1269.7 *(924)*	702.7 – 1836.7	**0.0070**
IFN-γ	50.4 *(27.5)*	19.6 – 81.3	20.7 *(8.2)*	4.7 – 36.7	**0.0036**
IL-1β	16.4 *(4.8)*	5.63 – 27.21	37.9 *(7.7)*	0.21 – 75.68	0.3942
IL-1RA	1472.1 *(531)*	587.98 – 2356.15	6328.2 *(2340)*	932.04 – 11724.4	0.1549
IL-2	35.5 *(16.6)*	12.61 – 58.48	17.4 *(10.1)*	8.23 – 26.62	0.1981
IL-4	107.5 *(37.1)*	36.65 – 178.45	129.8 *(52.3)*	51.15 – 208.49	0.3838
IL-6	45.5 *(34.9)*	25.01 – 66.06	138.5 *(33.8)*	-20.33 – 297.42	0.2692
IL-8/CXCL8	217 *(143)*	144.6 – 289.4	4335.9 *(540)*	-1075.4 – 9747.4	**0.0130**
IL-10	67.5 *(42.6)*	27.44 – 107.5	34.1 *(20.1)*	7.68 – 60.54	0.0814
IL-12	119.3 *(80.9)*	71.19 – 167.41	81.0 *(56.9)*	41.47 – 120.54	0.0867
IL-13	44.8 *(22.9)*	17.24 – 72.33	30.0 *(13)*	11.1 – 48.95	0.5188
IP-10/CXCL10	22914.5 *(17700)*	16599.42 – 29229.63	25049.3 *(12900)*	11352.92 – 38745.77	0.1433
RANTES/CCL5	88.5 *(57.9)*	52.3 – 124.8	47.7 *(30.3)*	24.1 – 71.3	**0.0030**
TNF-α	31.3 *(24.8)*	20.75 – 41.9	25.3 *(15.1)*	14.25 – 36.36	0.1743
MMP-9	2428.7 *(225)*	-593.0 – 5450.4	47110.2 *(11700)*	8965.8 – 85254.6	**<0.0001**
MCP-1/CCL2	1201.6 *(839)*	610.1 – 1793.04	1023.7 *(584)*	222.54 – 1824.88	0.0612
Clinical variables					
SANDE I frequency (units)	68.62 *(70)*	55.71 – 81.53	85 *(80)*	78.49 – 91.51	0.0933
SANDE I severity (units)	67.81 *(66)*	55.59 – 80.03	66.16 *(70)*	57.56 – 74.76	0.8186
OSDI (units)	51.86 *(47.91)*	44.22 – 59.51	58.95 *(57.5)*	51.78 – 66.11	0.1679
Tear osmolarity (mOsm/kg)	332.71 *(333)*	322.99 – 342.44	335.79 *(331)*	321.13 – 350.45	0.7114
Corneal staining (units)	*(1)*	1 – 2	*(3)*	3 – 3	**<0.0001**
Conjunctival staining (units)	*(2)*	1 – 2	*(3)*	2 – 3	**0.0022**
Conjunctival hyperemia (units)	*(2)*	1 – 2	*(2)*	1 – 3	0.9546
TBUT (seconds)	3.43 *(2.76)*	2.65 – 4.21	3.08 *(3.22)*	2.42 – 3.74	0.4741
Schirmer test (mm)	6.48 *(7)*	2.09 – 5.53	4.05 *(4)*	2.7 – 2.75	**0.0028**

Boldface indicates statistical significance.
CI = Confidence interval; M-DED = Moderate dry eye disease group; OSDI = Ocular surface disease index; S-DED = Severe dry eye disease group; SANDE = Symptom assessment in dry eye; TBUT = tear break up time.
95%CI is provided for the mean in case of quantitative variables, and for the median in case of ordinal variables.

Table 19. Distribution, demographics and baseline clinical characteristics of the patients included in each treatment and severity groups.

Variable	Severity		FML group		PA group		Between Groups
			Mean (median)	95% CI	Mean (median)	95% CI	p-value
MDS group size (n; %)	All		21; 52.5	36.3–68.2	19; 47.5	31.8–63.7	0.7392
	M-DED		10; 47.6	26.4–69.7	11; 52.4	30.3–73.6	
	S-DED		11; 57.9	33.8–78.9	8; 42.1	21.1–66.0	
Age (years)	All		59.0	55.5–62.5	60.0	56.0–64.0	0.3708
	M-DED		60.2	55.4–65.0	63.7	61.5–65.9	0.417
	S-DED		57.9	52.6–63.2	56.0	48.2–63.8	0.7723
Gender (%)	All	Male	4; 19.0	-0.2–38.3	2; 10.5	-19.5–40.6	0.6642
		Female	17; 80.9	76.4–85.5	17; 89.5	85.9–93.0	
	M-DED	Male	3; 30.0	0.1–59.9	0; 0.0	0.0–0.0	0.0902
		Female	7; 70.0	57.2–82.8	10; 100	100.0–100.0	
	S-DED	Male	1; 9.1	-47.3–65.5	2; 22.3	-18.5–63.1	0.5459
		Female	10; 90.9	85.3–96.5	7; 77.7	66.0–89.4	
OSDI (units)	All		56.5 *(52.5)*	49.1–64.0	53.8 *(54.5)*	45.9–61.6	0.5900
	M-DED		58.6 *(57.7)*	45.8–71.4	45.5 *(41.1)*	34.9–56.3	0.0931
	S-DED		54.6 *(50.0)*	44.3–64.9	62.8 *(65.9)*	52.9–72.7	0.2190
SANDE I. Frequency (units)	All		8.3 *(8.1)*	7.5–9.2	6.9 *(7.0)*	5.6–8.2	0.0651
	M-DED		7.8 *(8.5)*	6.1–9.5	5.5 *(5.0)*	3.5–7.4	0.0765
	S-DED		8.8 *(8.1)*	8.2–9.5	8.4 *(8.6)*	7.0–9.8	0.7201
SANDE I. Severity (units)	All		7.3 *(7.0)*	6.4–8.2	6.1 *(6.6)*	4.9–7.2	0.0882
	M-DED		7.6 *(7.5)*	5.9–9.3	5.3 *(5.5)*	3.6–7.1	0.1020
	S-DED		7.0 *(7.0)*	5.9–8.0	6.8 *(7.9)*	5.0–8.7	0.8801
Corneal staining (units)	All		*(3.0)*	2–3	*(2.0)*	1–3	0.1816
	M-DED		*(1.5)*	1–2	*(1.0)*	1–2	0.3347
	S-DED		*(3.0)*	3–3	*(3.0)*	3–3	0.1450
Conjunctival staining (units)	All		*(2.0)*	1–3	*(2.0)*	1–2	0.1999
	M-DED		*(2.0)*	1–2	*(1.5)*	1–2	0.4505
	S-DED		*(3.0)*	1–3	*(2.0)*	1–3	0.3589
Osmolarity (mOsm/Kg)	All		338.4 *(331)*	326.6–350.2	329.5 *(328)*	317.3–341.8	0.2834
	M-DED		335.3 *(330)*	317.8–352.7	324.6 *(319)*	312.3–336.8	0.2711
	S-DED		341.2 *(331)*	322.2–360.1	335.0 *(333)*	309.9–360.1	0.6543
Conjunctival hyperemia (units)	All		*(2.0)*	1–3	*(1.0)*	1–2	0.3259
	M-DED		*(2.0)*	1–3	*(1.0)*	1–2	0.0710
	S-DED		*(1.0)*	0–3	*(2.0)*	1–3	0.7795
TBUT (seconds)	All		3.1 *(3.2)*	2.4–3.8	3.4 (3.2)	2.7–4.2	0.4693
	M-DED		3.8 *(3.8)*	2.7–4.8	3.9 (3.5)	2.6–5.2	0.8739
	S-DED		2.4 *(2.3)*	1.6–3.3	2.9 (3.1)	1.9–3.9	0.3992
Schirmer test (mm)	All		5.2 *(5.0)*	4.1–6.3	5.5 (6.0)	4.1–6.9	0.6527
	M-DED		6.1 *(6.0)*	4.8–7.3	6.8 (7.0)	5.1–8.4	0.4575
	S-DED		4.3 *(4.0)*	2.5–6.2	4.0 (3.0)	1.7–6.2	0.7800
VA high contrast (logMAR)	All		0.10 *(0.10)*	0.02–0.19	0.07 *(0.08)*	0.02–0.12	0.9783
	M-DED		0.09 *(0.05)*	-0.05–0.23	0.04 *(0.08)*	-0.04–0.12	0.4687
	S-DED		0.11 *(0.06)*	-0.01–0.24	0.09 *(0.06)*	0.02–0.16	0.9079
VA low contrast (logMAR)	All		0.53 *(0.54)*	0.43–0.64	0.53 *(0.50)*	0.44–0.62	0.9364
	M-DED		0.48 *(0.44)*	0.30–0.67	0.58 *(0.55)*	0.44–0.72	0.3626
	S-DED		0.58 *(0.63)*	0.45–0.71	0.47 *(0.38)*	0.35–0.60	0.2143
IOP (mmHg)	All		15.2 *(15.0)*	14.5–15.9	16.3 *(16.0)*	15.4–17.2	0.0540
	M-DED		14.9 *(15.0)*	13.9–15.8	16.3 *(16.0)*	15.1–17.5	0.0934
	S-DED		15.4 *(16.0)*	14.3–16.5	16.2 *(16.0)*	14.6–17.8	0.3707
Cup/Disk ratio	All		0.37 *(0.30)*	0.34–0.40	0.37 *(0.35)*	0.33–0.41	0.9443
	M-DED		0.35 *(0.30)*	0.30–0.40	0.36 *(0.35)*	0.31–0.41	0.6881
	S-DED		0.39 *(0.40)*	0.34–0.43	0.38 *(0.40)*	0.31–0.46	0.9378

Boldface indicates statistical significance.
CI=Confidence interval; FML=Fluorometholone; IOP=Intraocular pressure; M-DED=Moderate dry eye disease group; OSDI=Ocular surface disease index; PA=Polyvinyl Alcohol; S-DED=Severe dry eye disease group; SANDE=Symptom assessment in dry eye; TBUT=tear break up time; VA=Visual acuity. 95%CI is provided for the mean in case of quantitative variables and for the median in case of ordinal variables.

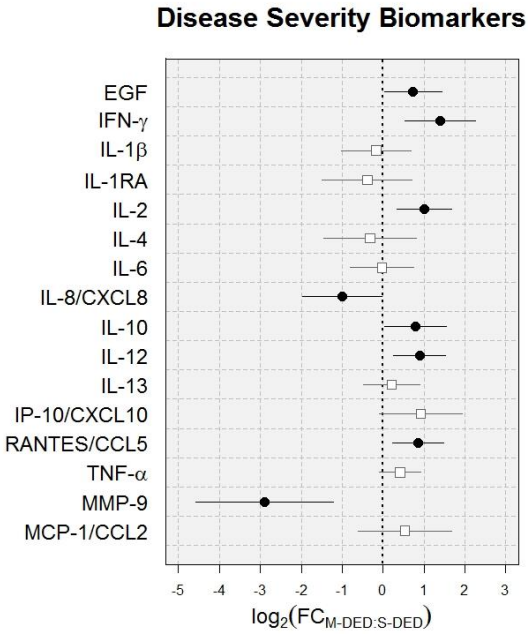

Figure 30. Disease severity tear biomarkers. The x-axis is the base-2 logarithmic fold change (log2 $FC_{M\text{-}DED:S\text{-}DED}$) in inflammatory molecule concentrations between M-DED and S-DED groups. Black circles, significant difference; white squares, insignificant difference. The 95%CIs for log2 $FC_{M\text{-}DED:S\text{-}DED}$ are plotted as horizontal lines. The vertical bold dotted line represents the no change value. Positive values (right of the vertical dotted line) mean higher concentrations in the M-DED group than in the S-DED group, while negative values (left of the vertical dotted line) mean lower concentrations in the M-DED group than the S-DED group. A 95%CI line (horizontal line) touching the vertical dotted line means no change. Therefore, EGF, IFN-γ, IL-2, IL-10, IL-12, and RANTES/CCL5 had significantly higher levels in M-DED tears than S-DED. MMP-9 and IL-8/CXCL8 had higher values in S-DED than in M-DED. CI=confidence interval; FC=fold change; M-DED=moderate dry eye disease group; S-DED=severe dry eye disease group.

2.3. Therapeutic Biomarkers

Linear mixed effect models analysis showed that the interaction between time and treatment group (FML or PA) significantly affected the tear levels of IL-1RA (p=0.0244), IL-2 (p=0.0103), and TNF-α (p=0.0153) *(Figure 31, Table 20)*. Each of the molecules varied significantly with time, depending on the treatment. For the PA group, there was a decrease in IL-2 (p=0.0076, *Figure 31A*). Also, there was a decrease in IL-1RA between V2 and V4 (p=0.031) *(Figure 31F)*. For all other periods, there were no significant changes in IL-1RA, IL-2, or TNF-α.

For the FML group, after the treatment (V1-V2 period), there were no significant changes in the concentration of any of the inflammatory molecules. However, the concentrations of IL-1RA and IL-2 were higher (p=0.0324 and p=0.0022, respectively) at the end of the study compared to baseline (period V1-V4, *Figure 31E*). FML-treated patients also had lower levels of TNF-α after ACE exposure (V3) compared to the rest of visits (period V1-V3, *Figure 31D*, p=0.0044; period V2-V3, *Figure 31B*, p=0.0006; and period V3-V4, *Figure 31C*, p=0.0002). In addition, the concentration of IL-1RA was higher at the end of the study compared to V3 (period V3-V4, *Figure 27C*, p=0.0003), while the concentration of IL-2 was higher at V3 compared to baseline (period V1-V3*, Figure 31D*, p=0.0014).

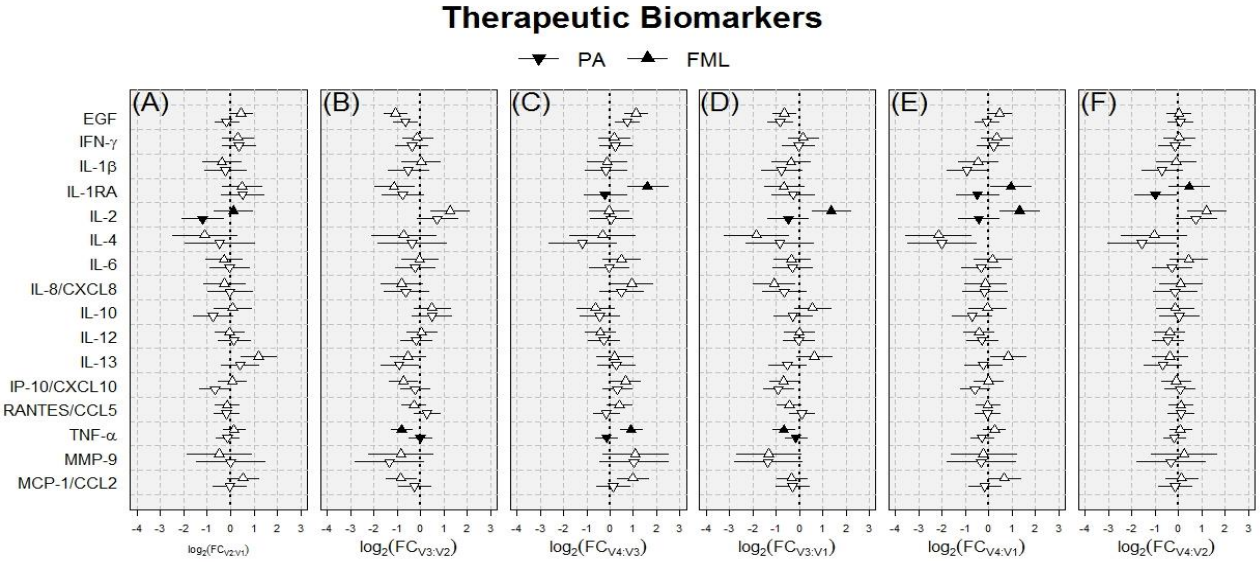

Figure 31. Therapeutic biomarkers. The x-axis is the base-2 logarithmic fold change (log2 $FC_{A:B}$) in inflammatory molecule concentrations between visit A (e.g., V1) and visit B (e.g., V2). Black triangles, significant difference between the FML and PA groups; white triangles, insignificant difference between the FML and PA groups. 95%CIs for log2 $FC_{A:B}$ are plotted as horizontal lines. The vertical bold dotted line represents the no change value. Positive values (right of the vertical dotted line) mean higher concentrations in visit A than visit B, while negative values (left of the vertical dotted line) mean lower concentrations in visit A than visit B. The 95%CI line (horizontal line) touching the vertical dotted line means no change. For IL-2, TNF-α, and IL-1RA, the log$FC_{A:B}$ point plotted outside the 95%CI line of the other treatment group indicated a significantly different interaction between time and treatment group. CI=confidence interval; FC=fold change; FML=fluorometholone group; PA=polyvinyl alcohol group; V1=Baseline data; V2=21-day treatment, data before adverse controlled environment exposure; V3=21-day treatment, data after adverse controlled environment exposure; V4=22-day treatment, data 24h after adverse controlled environment exposure.

Table 20. Differential effect of time on molecules in each treatment group (FML or PA), according the linear mixed effect models.

Molecule	Treatment Group	$FC_{V2:V1}$ (95% CI)	$FC_{V3:V2}$ (95% CI)	$FC_{V4:V3}$ (95% CI)	$FC_{V3:V1}$ (95% CI)	$FC_{V4:V1}$ (95% CI)	$FC_{V4:V2}$ (95% CI)	Time*Treatment effect p-value	Adjusted p value
EGF	FML	1.34 (0.96,1.88)	0.47 (0.34,0.67)	2.15 (1.52,3.03)	0.64 (0.45,0.89)	1.37 (0.97,1.93)	1.02 (0.72,1.44)	0.2904	0.766
	PA	0.89 (0.62,1.27)	0.64 (0.44,0.91)	1.67 (1.16,2.39)	0.57 (0.39,0.81)	0.94 (0.66,1.35)	1.06 (0.74,1.52)		
IFN-γ	FML	1.24 (0.79,1.94)	0.91 (0.58,1.42)	1.13 (0.71,1.78)	1.12 (0.72,1.76)	1.27 (0.8,2)	1.02 (0.65,1.62)	0.9531	0.954
	PA	1.27 (0.79,2.05)	0.77 (0.48,1.24)	1.17 (0.73,1.89)	0.98 (0.6,1.58)	1.14 (0.71,1.85)	0.9 (0.56,1.46)		
IL-1β	FML	0.77 (0.43,1.37)	1.01 (0.57,1.8)	0.93 (0.52,1.66)	0.78 (0.44,1.39)	0.72 (0.4,1.3)	0.94 (0.52,1.69)	0.7039	0.868
	PA	0.85 (0.46,1.56)	0.69 (0.38,1.28)	0.89 (0.48,1.64)	0.59 (0.32,1.08)	0.52 (0.28,0.97)	0.62 (0.34,1.14)		
IL-1RA	FML	1.39 (0.77,2.5)	**0.45 (0.25,0.81)**	**3.05 (1.68,5.55)**	0.63 (0.35,1.13)	**1.92 (1.06,3.49)**	1.38 (0.76,2.51)	**0.0244**	0.283
	PA	1.42 (0.76,2.65)	0.58 (0.31,1.09)	0.86 (0.46,1.61)	0.83 (0.44,1.54)	0.71 (0.38,1.33)	**0.5 (0.27,0.94)**		
IL-2	FML	1.08 (0.61,1.9)	**2.39 (1.35,4.22)**	0.97 (0.54,1.74)	**2.57 (1.45,4.54)**	**2.5 (1.4,4.46)**	**2.32 (1.3,4.14)**	**0.0103**	**0.047**
	PA	**0.44 (0.24,0.8)**	1.64 (0.89,3.01)	1.04 (0.57,1.9)	0.71 (0.39,1.31)	0.74 (0.4,1.36)	1.7 (0.93,3.11)		
IL-4	FML	0.46 (0.18,1.19)	0.61 (0.23,1.58)	0.8 (0.3,2.11)	0.28 (0.11,0.72)	0.22 (0.09,0.59)	0.49 (0.19,1.29)	0.7441	0.995
	PA	0.72 (0.26,1.98)	0.77 (0.28,2.12)	0.44 (0.16,1.21)	0.56 (0.2,1.53)	0.24 (0.09,0.67)	0.34 (0.12,0.94)		
IL-6	FML	0.82 (0.47,1.42)	0.97 (0.56,1.68)	1.41 (0.81,2.46)	0.8 (0.46,1.37)	1.12 (0.64,1.96)	1.37 (0.79,2.39)	0.6498	0.985
	PA	0.96 (0.54,1.71)	0.85 (0.48,1.52)	0.98 (0.55,1.75)	0.81 (0.46,1.45)	0.8 (0.45,1.42)	0.83 (0.47,1.49)		
IL-8/CXCL8	FML	0.83 (0.45,1.55)	0.57 (0.31,1.05)	1.9 (1.01,3.55)	0.47 (0.26,0.88)	0.9 (0.48,1.68)	1.08 (0.57,2.01)	0.8924	0.971
	PA	0.98 (0.51,1.89)	0.65 (0.34,1.25)	1.39 (0.72,2.68)	0.64 (0.33,1.22)	0.89 (0.46,1.71)	0.9 (0.47,1.74)		
IL-10	FML	1.05 (0.61,1.82)	1.4 (0.81,2.43)	0.65 (0.38,1.14)	1.48 (0.85,2.55)	0.97 (0.55,1.69)	0.92 (0.53,1.61)	0.4269	0.932
	PA	0.59 (0.33,1.05)	1.4 (0.78,2.51)	0.74 (0.41,1.32)	0.83 (0.46,1.48)	0.61 (0.34,1.09)	1.04 (0.58,1.86)		
IL-12	FML	0.96 (0.62,1.48)	1.04 (0.67,1.6)	0.75 (0.48,1.16)	0.99 (0.64,1.53)	0.74 (0.48,1.16)	0.78 (0.5,1.2)	0.9507	0.997
	PA	1.11 (0.7,1.76)	0.88 (0.55,1.39)	0.83 (0.53,1.32)	0.97 (0.61,1.55)	0.81 (0.51,1.29)	0.73 (0.46,1.16)		
IL-13	FML	2.27 (1.35,3.82)	0.68 (0.41,1.15)	1.15 (0.67,1.95)	1.55 (0.92,2.62)	1.78 (1.05,3.03)	0.78 (0.46,1.33)	0.1530	0.741
	PA	1.33 (0.76,2.31)	0.53 (0.3,0.92)	1.21 (0.69,2.1)	0.7 (0.4,1.22)	0.85 (0.49,1.47)	0.64 (0.37,1.11)		
IP-10/CXCL10	FML	1.04 (0.69,1.58)	0.6 (0.39,0.91)	1.58 (1.03,2.41)	0.62 (0.41,0.95)	0.98 (0.64,1.5)	0.94 (0.62,1.44)	0.3468	0.904
	PA	0.63 (0.4,0.98)	0.85 (0.55,1.33)	1.24 (0.8,1.93)	0.53 (0.34,0.83)	0.66 (0.43,1.03)	1.06 (0.68,1.65)		
RANTES/CCL5	FML	0.9 (0.63,1.28)	0.82 (0.57,1.17)	1.32 (0.92,1.9)	0.74 (0.51,1.05)	0.97 (0.67,1.4)	1.08 (0.75,1.56)	0.3698	0.837
	PA	0.88 (0.6,1.29)	1.21 (0.83,1.77)	0.89 (0.61,1.31)	1.07 (0.73,1.56)	0.96 (0.65,1.4)	1.08 (0.74,1.58)		

TNF-α	FML	1.1 (0.8,1.52)	**0.57 (0.41,0.78)**	**1.88 (1.36,2.59)**	**0.63 (0.46,0.86)**	1.18 (0.85,1.63)	1.07 (0.77,1.47)	**0.0153**	0.252
	PA	0.91 (0.65,1.27)	1 (0.71,1.39)	0.9 (0.64,1.26)	0.9 (0.65,1.27)	0.81 (0.58,1.14)	0.89 (0.64,1.25)		
MMP-9	FML	0.72 (0.28,1.86)	0.56 (0.21,1.44)	2.13 (0.81,5.6)	0.4 (0.15,1.04)	0.85 (0.32,2.24)	1.18 (0.45,3.12)	0.9457	0.99
	PA	0.99 (0.36,2.73)	0.4 (0.14,1.09)	2.04 (0.74,5.62)	0.39 (0.14,1.08)	0.8 (0.29,2.21)	0.81 (0.29,2.23)		
MCP-1/CCL2	FML	1.43 (0.91,2.26)	0.56 (0.35,0.88)	1.98 (1.24,3.16)	0.8 (0.51,1.27)	1.59 (1,2.53)	1.11 (0.7,1.77)	0.2180	0.756
	PA	0.98 (0.6,1.59)	0.83 (0.51,1.35)	1.09 (0.67,1.77)	0.81 (0.5,1.32)	0.89 (0.54,1.44)	0.91 (0.56,1.48)		

Boldface indicates statistical significance.
CI = Confidence interval; FC = Fold-change. Relative expression of the corresponding inflammatory molecule level comparison between visits; FML = Fluorometholone; PA = Polyvinyl Alcohol.

2.4. Disease Activity Biomarkers

The linear mixed effects analysis showed a significant global effect of the time between V2 and V3 (ACE effect) on several molecules *(Figure 32, Table 21)*. There was a significant increase in IL-2 (p=0.0015) and significant decrease in EGF (p<0.001), IL-1RA (p=0.0025), IL-8/CXCL8 (p=0.0295), IL-13 (0.41–0.88; p=0.009), IP-10/CXCL10 (p=0.0296), TNF-α (p=0.0163), and MMP-9 (p=0.0338).

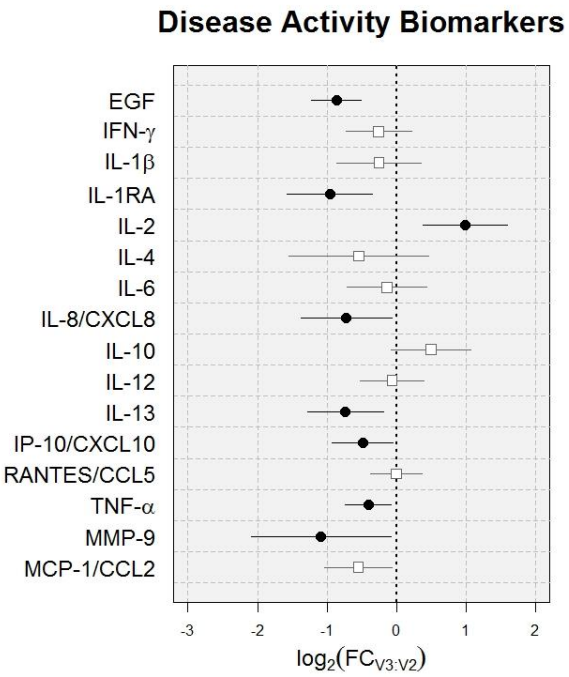

Figure 32. Disease activity biomarkers. The x-axis is the base-2 logarithmic fold change (log2FCV3:V2) in inflammatory molecule concentrations between visit 3 (V3) and visit 2 V2. Black circles, significant difference between V2 and V3; white squares, insignificant difference. The 95% CIs for log2 FCV3:V2 are plotted as horizontal lines. The vertical bold dotted line represents the no change value. Positive values (right of the vertical dotted line) mean higher concentration at V3 compared to V2, while negative values (left of the vertical dotted line) mean lower concentration at V3 compared to V2. A 95% CI line (horizontal line) touching the vertical dotted line means no change. Therefore, EGF, IL-1RA, IL-8/CXCL8, IL-13, IP-10/CXC10, TNF-α, and MMP-9 showed significant decreases after the adverse environment exposure, while IL-2 showed a significant increase in this period.
CI=confidence interval; FC=fold change; V2=21-day treatment, data before adverse controlled environment exposure; V3=21-day treatment, data after adverse controlled environment exposure.

Table 21. Global effect of time on tear molecule levels, according the linear mixed effects models.

Molecule	$FC_{V2:V1}$ (95% CI)	$FC_{V3:V2}$ (95% CI)	$FC_{V4:V3}$ (95% CI	$FC_{V3:V1}$ (95% CI)	$FC_{V4:V1}$ (95% CI)	$FC_{V4:V2}$ (95% CI)	Global time effect p-value	Adjusted p value
EGF	1.09 (0.85,1.4)	**0.55 (0.43,0.7)**	**1.89 (1.47,2.43)**	**0.6 (0.47,0.77)**	1.13 (0.88,1.46)	1.04 (0.81,1.33)	**<0.0001**	**0.007**
IFN-γ	1.25 (0.9,1.74)	0.84 (0.6,1.16)	1.15 (0.82,1.6)	1.05 (0.75,1.45)	1.2 (0.86,1.68)	0.96 (0.69,1.34)	0.4704	0.951
IL-1β	0.81 (0.53,1.23)	0.84 (0.55,1.28)	0.91 (0.59,1.39)	0.68 (0.45,1.03)	0.61 (0.4,0.94)	0.76 (0.5,1.16)	0.1162	0.674
IL-1RA	1.41 (0.92,2.16)	**0.51 (0.33,0.79)**	**1.62 (1.06,2.5)**	0.72 (0.47,1.11)	1.17 (0.76,1.8)	0.83 (0.54,1.28)	**0.0194**	0.238
IL-2	0.69 (0.45,1.04)	**1.98 (1.3,3)**	1 (0.66,1.53)	1.35 (0.89,2.05)	1.36 (0.89,2.07)	**1.99 (1.31,3.02)**	**0.0037**	0.087
IL-4	0.58 (0.29,1.15)	0.68 (0.34,1.37)	0.59 (0.3,1.2)	**0.39 (0.2,0.79)**	**0.23 (0.12,0.47)**	**0.41 (0.2,0.82)**	**0.0008**	**0.048**
IL-6	0.89 (0.59,1.32)	0.91 (0.61,1.35)	1.18 (0.79,1.76)	0.8 (0.54,1.2)	0.95 (0.63,1.41)	1.07 (0.71,1.6)	0.7315	0.971
IL-8/CXCL8	0.9 (0.58,1.42)	**0.61 (0.39,0.95)**	**1.63 (1.03,2.56)**	**0.55 (0.35,0.86)**	0.89 (0.57,1.4)	0.99 (0.63,1.55)	**0.0424**	0.392
IL-10	0.79 (0.53,1.17)	1.4 (0.94,2.09)	0.7 (0.46,1.04)	1.1 (0.74,1.65)	0.77 (0.51,1.15)	0.98 (0.65,1.46)	0.2036	0.818
IL-12	1.03 (0.75,1.41)	0.95 (0.7,1.31)	0.79 (0.57,1.09)	0.98 (0.72,1.35)	0.78 (0.57,1.07)	0.75 (0.55,1.04)	0.2838	0.894
IL-13	**1.74 (1.19,2.54)**	**0.6 (0.41,0.88)**	1.18 (0.8,1.73)	1.04 (0.71,1.52)	1.23 (0.84,1.8)	0.71 (0.48,1.04)	**0.0200**	0.32
IP-10/CXCL10	0.81 (0.6,1.1)	**0.71 (0.53,0.97)**	**1.4 (1.03,1.9)**	**0.58 (0.43,0.78)**	0.81 (0.59,1.1)	1 (0.73,1.36)	**0.0058**	0.155
RANTES/CCL5	0.89 (0.69,1.15)	1 (0.77,1.29)	1.09 (0.84,1.41)	0.89 (0.68,1.15)	0.96 (0.74,1.25)	1.08 (0.83,1.41)	0.7451	0.943
TNF-α	1 (0.8,1.26)	**0.75 (0.6,0.95)**	**1.3 (1.03,1.64)**	**0.75 (0.6,0.95)**	0.98 (0.78,1.24)	0.98 (0.77,1.23)	**0.0425**	0.332
MMP-9	0.84 (0.42,1.69)	**0.47 (0.23,0.94)**	**2.08 (1.03,4.2)**	**0.4 (0.2,0.8)**	0.83 (0.41,1.67)	0.98 (0.49,1.98)	**0.0466**	0.35
MCP-1/CCL2	1.18 (0.85,1.65)	0.68 (0.49,0.95)	1.47 (1.05,2.06)	0.81 (0.58,1.13)	1.19 (0.85,1.66)	1 (0.72,1.41)	0.0794	0.528

Boldface indicates statistical significance.
CI = Confidence interval; FC = Fold-change. Relative expression of the corresponding inflammatory molecule level comparison between visits.

2.5. Best Potential Biomarkers after Multiple Comparisons Analysis

We performed a multiple comparisons analysis to develop a definitive tear biomarker panel that would eliminate possible false positive values that can arise due to the use of multiple statistical comparisons. The strongest tear biomarker candidates useful as indicators of disease severity were IFN-γ (p-adjusted value [p-adj]=0.006), IL-2 (p-adj=0.01), IL-12 (p-adj=0.027), RANTES/CCL5 (p-adj=0.019), and MMP-9 (p-adj=0.002). The strongest candidate useful as a FML therapeutic biomarker was IL-2 (p-adj=0.047), and the best candidate to serve as a disease activity biomarker was EGF (p-adj=0.007).

3. Discussion

Currently there is a need to standardize the methods for diagnosis of DED and for understanding its evolution and activity. Additionally, the methods for evaluating treatment efficacy in the daily practice and in research studies or clinical trials should be standardized. The use of tear inflammatory molecules as objective endpoints can help in developing and determining the efficiency of new treatments in a more accurate way by eliminating the bias induced by the variables that are currently used. In this study, we employed a specific clinical trial design to identify several potential DED biomarkers of severity, therapeutic efficacy, and disease activity. These markers could be used as objective endpoints in clinical trials of new treatments or therapeutic actions for DED.

At present, the classification of DED patients according to disease severity is performed based on signs and symptoms,[7] as has been explained in previous chapters. These classification criteria have clear disadvantages, mainly due to the lack of correlation between both the signs and symptoms,[8] which can generate misclassification. The MDS analysis that we used to study the homogeneity of the patients based on their tear molecule levels at baseline, compared with the clinical results observed, showed clear differences in EGF, IFN-γ, IL-8/CXCL8, RANTES/CCL5, and MMP-9 tear levels in M-DED and S-DED patients. Additionally, the linear mixed effect models performed to evaluate the effect of the different factors involved in the study, i.e., time, MDS group, treatment, and factor interactions, showed a significant effect of the MDS group factor in IL-2, IL-10, and IL-12. Thus, the levels and/or changes during the trial of these eight molecules (EGF, IFN-γ, IL-8/CXCL8, RANTES/CCL5, MMP-9, IL-2, IL-10, and IL-12) were dependent on the severity of the DED. Based on the results obtained after the multiple comparisons analysis, MMP-9, IFN- γ, RANTES/CCL5, IL-2, and IL-12, were selected as the strongest disease severity biomarkers.

As our clinical trial, several studies reported elevated levels of MMP-9 in DED,[9–12] that correlate with DED severity.[11] Thus this inflammatory molecule had already been proposed as a potential biomarker to grade DED severity, and a Point of Care

device has been commercialized to measure MMP-9 activity.[11,13] In contrast to MMP-9, we found lower levels of IFN-γ and RANTES/CCL5 in S-DED than in M-DED. Regarding IFN-γ, our results agree with the lower IFN-γ tear levels observed by Na et al.[14] in severe DED patients. In contrast, other authors have reported higher levels of this molecule in DED patients than in controls.[15,16] This lack of agreement also exists observing the correlations of IFN-γ with clinical tests. For example, both a positive[16] and negative[17] correlation between IFN-γ and corneal fluorescein staining have been reported. Further studies are necessary to elucidate this controversy. For RANTES/CCL5, tear levels were higher in both M-DED and S-DED patients than previously reported in healthy controls,[18] which agrees with results from other authors.[17,19] The higher levels of RANTES/CCL5 in the M-DED group compared to the S-DED group could be due to the higher levels found in evaporative DED compared to non-evaporative DED.[17] In our study, the origin of DED was not taken into account. The inclusion of different DED etiologies, (i.e., tear deficient-, evaporative-, and SS-DED), would have generated more variables that could have resulted in a lack of correlation between RANTES/CCL5 and DED severity levels. Regarding IL-12, our finding (lower levels in M-DED than S-DED) is in accordance with the results reported by Na et al.[14] Finally, Massignale et al.[15] observed higher levels of IL-2 in DED patients than in healthy subjects. This result agrees with the increase of this molecule that we have found in S-DED patients.

On the other hand, subsequently the multiple comparisons analysis we also identified IL-2 as a potential therapeutic biomarker. After 21 days of treatment, the level of IL-2 decreased in the PA group, but not in the FML group. IL-2 is involved in the regulation of T cell maturation and function, and high levels of IL-2 contribute to the termination of persistent immune responses.[20] The decrease in IL-2 levels in PA-treated patients is thus consistent with the increase of signs and symptoms of this group when exposed to the ACE.[21] In contrast, there was an increase of this molecule in the FML group under ACE conditions. In our previously reported clinical trial, we found that FML-treated patients maintained the status of their ocular surface after ACE exposure, in contrast to the noticeable

impairment of those treated with PA.[21] Thus, our findings that IL-2 levels are maintained and do not decrease in the tears of FML-treated patients exposed to the ACE suggests a mechanism by which the signs and symptoms are mitigated after ACE exposure. These findings confirmed the efficacy of topical corticosteroid as a short-term (≤4 weeks) DED treatment as previously shown by other research groups.[22,23] More importantly, our results demonstrated the protective effect of this therapy for coping with adverse conditions in the daily lives of these patients, which was the main novelty of this study.

Finally, with our clinical trial design and setting, we have identified EGF as a disease activity biomarker. The tear EGF levels significantly decreased for both the PA- and the FML-treatment groups between V2 and V3, during the adverse environmental desiccating stress exposure. This result agrees with previous studies by our group[9,24] and others.[17] While the decrease in EGF levels was associated with the desiccating stress suffered by the patients, it was independent of the clinical worsening, which occurred in the PA-treated group, but not in the FML-treated group.

In addition to the molecules above proposed and discussed as the strongest candidates to be used as different biomarkers, our statistical study also revealed several interesting changes in the concentrations of other molecules, such as IL-8/CXCL8, IL-1RA, TNF-α, IL-13, and IP-10/CXCL10. These changes may provide relevant and important information.

The concentration of IL-8/CXCL8 was correlated with DED severity, in agreement with several studies that have reported increased levels of this chemokine in tears from DED patients.[16,17,25–27] It has been proposed as one of the major mediators of the inflammatory response.[28]

Also, after adverse environmental desiccating stress exposure, IL-1RA decreased in the FML group but not in the PA group. IL-1RA is an endogenous inhibitor of IL-1 and impedes the activities of the pro-inflammatory forms of IL-1 by competitively binding to the type 1 IL-1 receptor.[29] As previously reported by Solomon et al.,[30] some of the IL-1RA found on the ocular surface of patients with

DED may be derived from inflammatory cells infiltrating the conjunctival epithelium. For this reason, the decrease in the level of this cytokine in the FML group after ACE exposure could be due to the effectiveness of the treatment in the reduction of the inflammation, resulting in the lack of stimuli necessary to induce the secretion of this cytokine. Moreover, Huang et al. have reported a direct relationship between the concentration of this tear molecule and the severity of the DED and corneal staining.[31] Furthermore, Amparo et al.[32] observed that a topical IL-1 receptor antagonist reduced symptoms and corneal epitheliopathy in patients with DED. Both results agree with the clinical results obtained and published by our group.[21]

TNF-α responded similarly to IL-1RA, with a significant decrease in the FML group and no changes in the PA group after the ACE exposure. TNF-α is a potent pro-inflammatory cytokine that is increased in DED tears.[16,27,33] It is rapidly secreted in large quantities by macrophages in response to inflammatory stimuli, so the decrease of this molecule found between V2 and V3 (after ACE exposure) in the FML group suggests that FML blocks the response of the patients against the desiccating stress. This agrees with the results previously observed in an animal model; Zhu et al. evaluated the effect of topical artificial tears, FML, 0.1% nepafenac, and 0.4% ketorolac on inflammatory cytokine expression by the ocular surface in a botulinum toxin B-induced murine dry eye model.[34] In this study, after a one-week treatment, topical FML significantly decreased the levels of TNF-α and IL-1β proteins in the corneal and conjunctival epithelia while topical artificial tears, 0.1% nepafenac, and 0.4% ketorolac had no effects.[34] Furthermore, high levels of IL-13, similar to those found by us in patients after 21 days of FML-treatment, inhibit the production of TNF-α,[35] which further agrees with our results here and in our clinical study data.[21]

The decrease observed in IL-13 tear levels in the PA group during the V2-V3 period could be related to an increase in the inflammation in this group, as IL-13 has an inhibitory effect on inflammatory cytokine production.[36] Following this line of argument, it is possible to attribute a protective effect to the increase of IL-13 found after the 21-days treatment period in the FML group.

Furthermore, the significant decrease in IP-10/CXCL10 level in the FML group after ACE exposure (but not in the PA group) would be related to a protective effect of FML therapy, as IP-10/CXCL10 is a potent chemoattractant for activated Th1 and natural killer cells. Particularly, this molecule seems to play an important role in DED as it is increased in the corneal and conjunctival epithelium of a DED animal model,[34] as well as in tears of Sjögren[35] and evaporative DED patients.[20]

The present study has several limitations. First, 40 DED patients participated in the clinical trial. Because of the small size of the study population, this could be considered as a pilot study. However, it was large enough to provide a proof of concept that allowed us to identify different molecules as potential tear biomarkers of DED. The validity of these markers should be confirmed in studies with larger sample sizes. On the other hand, our use of the restrictive multiple comparisons analysis increased the likelihood that the selected potential biomarkers are valid indices of DED severity, therapeutic efficacy, and disease activity. Because of the exploratory nature of this study, the lack of statistical significance before multiple comparisons analysis was not a sufficient criterion to categorically eliminate other potential biomarkers. Regarding the potential therapeutic biomarkers, our results are valid only for the specific therapy tested, i.e., FML. Other treatments could result in the same or different indicators of therapeutic activity, and each must be independently tested. Nevertheless, our results now allow the use of FML as a positive control in future studies or clinical trials.

In conclusion, our clinical trial design allowed identification of potential biomarkers of disease severity, therapeutic effect, and disease activity in tears of DED patients. The severity biomarkers could be useful generate a better classification of patients and a better selection of target patients for clinical trials. The therapeutic biomarkers could provide new and objective therapeutic evaluation end-points in clinical trials. Finally, the activity biomarkers could provide a better definition of DED disease activity.

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NEW QUESTIONNAIRE FOR DRY EYE DISEASE-RELATED SYMPTOM EVALUATION

Chapter 4
New Questionnaire for DED-Related Symptom Evaluation

This chapter presents the results of a clinical study that evaluated the efficacy of a new questionnaire for the detection of long-term changes (3 months) in DED-related symptoms.

This study was conducted at University of Cologne (Köln, Germany) in collaboration with the Department of Ophthalmology from this institution.

To develop this study, the pre-doctoral student undertook a 3-month research stay at University of Cologne, in compliance with the rules to be eligible to the "International mention" for this Doctoral Thesis.

1. Justification

As explained in Chapter 2, we carried out a clinical trial to evaluate the efficacy of 0.1% topical fluorometholone (FML group) against its vehicle (PA group) in the protection of DED patients against adverse environmental conditions. We observed that topical fluorometholone was an effective short-term treatment for DED since significantly decreased corneal staining, conjunctival staining, and conjunctival hyperemia *(Table 14, Chapter 2)*. However, we were unable to show the same benefit on symptoms. In spite of obtaining a significant reduction in SANDE I scores (frequency and intensity) in the FML group, while only the SANDE I frequency score decreased in the PA group, there was no significant difference in symptoms between the FML and the PA groups after a 21-day treatment.

We noted a similar trend during the second part of our clinical trial design. After exposure to adverse environmental conditions (V2-V3) the FML group showed no worsening in any of the signs evaluated, while the PA group had a significant increase in corneal staining, conjunctival staining, and conjunctival hyperemia, along with a significant reduction in tear stability *(Table 14, Chapter 2)*. Regarding symptoms, however, there was no significant difference between the FML and the PA groups in the percentage of patients with a reduction ≥20 points in SANDE I after the 2h exposure to the adverse environment (FML group: 61.9%, 95%CI: 38.6–81.0 vs PA group: 57.9%, 95%CI: 33.9–78.8; p=1.0). In conclusion, we yet again witnessed the poor concordance between the evolution of DED signs and the changes observed in symptoms in our clinical trial, as so abundantly reported by other authors.[1–3]

Our perception of the problem is that tools like OSDI and SANDE I are better designed to evaluate symptoms in a specific time-frame, and not the change produced by a specific intervention (such as therapies, exposure to an adverse environment, etc.). Although there are some questionnaires designed to evaluate such changes (SANDE II, for instance) they seem not to be accurate enough, as will be explained below.

There is another important aspect that we have gathered after having interviewed a large number of DED patients. In previous studies,[4–6] patients used to spontaneously comment that they had felt worse during the 2h period of environmental exposure; and almost invariably, their questionnaires failed to show their own casual comments to us.

Likewise, in the present clinical trial, and when it had already completely concluded, patients either offered or were asked their opinion about how they had felt in general with whatever the treatment option they had been randomized to. This "opinion" (e.g. "I must have gotten the real drug, as I improved" or "I am sure I got the artificial tear as I did not improve at all") was not part of the clinical trial design, but it was "casually" collected in the patient clinical chart when they were later evaluated in the regular Dry Eye Clinic. When the randomization code was opened, we realized that invariably, patients have guessed correctly. We did not attempt to evaluate this aspect, as it was not part of the original clinical trial design. However, all clinicians in our group wondered why patients verbalized correctly their improvement or lack of it and yet they failed to say so in the self-answered questionnaires.

For this reason, it is possible that the bad results obtained during symptom evaluation, as well as the lack of concordance between signs and symptoms, could be due to the poor accuracy of questionnaires to reflect the change or the status observed in patients, and/or to the lack of understanding by patients about how to complete the questionnaires due to its complexity.

2. Materials and Methods

2.1. Phase I: Tool Design

We designed this simple questionnaire in the belief, as previously said, that the simpler, the shorter and the fewer words used, the better for patients understanding. In accordance to our knowledge and the previous experiences of our research group, it is necessary to use the easiest possible questionnaires to evaluate patient's subjective symptomatology, in order to avoid mistakes or misunderstandings. *Figure 33* shows the Evaluation of Change in Symptoms-Questionnaire (ECS-Q), the tool proposed for this study.

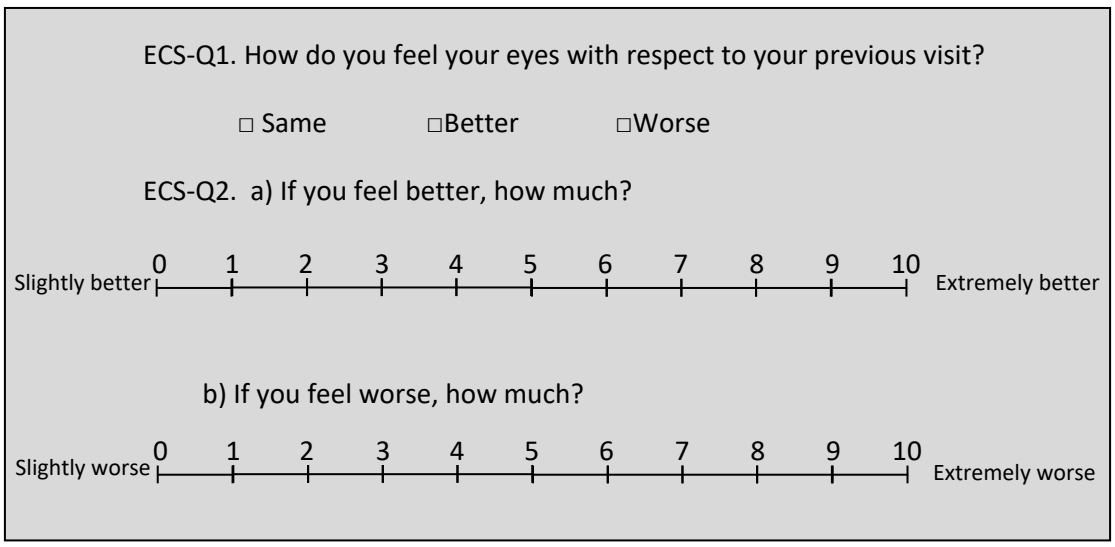

Figure 33. Evaluation of Change in Symptoms-Questionnaire (ECS-Q). New questionnaire developed in order to facilitate patient comprehension and to better evaluate the change in symptoms between two different moments.

It consists of two questions, ECS-Q1 and ECS-Q2. The last one is subdivided in two parts: a) in case the patient respond he/she feels better; b) in case the patient respond he/she feels worse.

ECS-Q1 evaluates, in the simplest possible way, the change in symptoms occurred with respect to a previous time. This question forces patients to reflect clearly and unambiguously their perception of the symptom´s changes experienced.

ECS-Q2 a) and b) evaluate the magnitude of the change in symptoms.

Amelioration and worsening are presented in different visual numerical scales to avoid the possible mistakes due to a misinterpretation of the questionnaire by patients.

As previously explained, this study was conducted at the University of Cologne. For this reason, ECS-Q was translated to German by a native German speaker. The ECS-Q version used for this study is shown in *Annex VI*.

2.2. Phase II: Tool Evaluation

This pilot study was designed as a prospective observational study to perform a clinical-based evaluation of the ECS-Q.

It was approved by the University Hospital of Cologne Ethics Committee (Köln, Germany; *Annex VII*) and conducted at the Department of Ophthalmology at the University Hospital of Cologne of the University of Cologne in accordance with the ethical principles of the Declaration of Helsinki and in compliance with Good Clinical Practices. Informed consent was obtained from each participant prior commencement of the study *(Annex VIII)*.

2.3. Patient Selection

The study included patients over 18 years of age with DED-related ocular symptoms. These patients were recruited at the University Hospital of Cologne under the supervision of Dr. Philipp Steven (MD; Ophthalmologist).

For inclusion in the study, participants should present an OSDI score ≥ 12 points. Exclusion criteria included the initiation, discontinuation or change, during 1 month after inclusion, in the dosage of any topical or systemic medication with possible effects over the tear film or DED. Finally, if the patient was under topical cyclosporine A or topical corticosteroids, its use should have been initiated 3 and 1 month prior inclusion, respectively.

2.4. Study Procedure

This pilot study consisted of two visits: baseline visit (V1) and one scheduled follow-up visit (V2). V1 took place during patient´s evaluation at the University Hospital of Cologne, after being diagnosed whit DED (any severity) and before receiving new treatments or changes in the therapies already used. V2 was scheduled when, according to the opinion of the ophthalmologist in charge (always the same), the prescribed therapy at V1 had had enough time to produce an effect according to its mechanism of action. A study flowchart is shown in *Figure 34*.

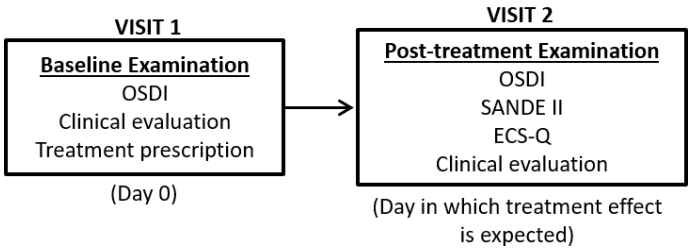

Figure 34. Pilot study flowchart. ECS-Q = Evaluation of change in symptoms-questionnaire; OSDI = Ocular surface disease index; SANDE = Symptoms assessment in dry eye.

Visit 1

Before performing any evaluation concerning the clinical study, the objectives and procedures were explained to the subjects. Then, each patient interested in participating in the study signed an informed consent form and then the inclusion and exclusion criteria were checked. After that, patients were asked about their ocular symptoms using the OSDI questionnaire to record baseline data. Other relevant clinical evaluations *(Table 22)* were performed to correctly phenotype all the participants. At the end of visit 1 patients were scheduled for the next visit.

Visit 2

During this visit, participants were asked again about their symptoms, using the OSDI questionnaire, SANDE II and the ECS-Q, in order to analyse symptoms changes from V1. The order for SANDE II and ECS-Q administration was randomly

chosen. Also, clinical data were collected *(Table 22)* to study the evolution of each patient and to compare it with the changes observed in the symptom scores. Patients were also asked about changes in treatments or therapies, use of artificial tears and frequency of its use, and changes in health status.

Table 22. Visits schedule and test sequence.

TEST	Visit 1	Visit 2
Written informed consent	X	
Inclusion and exclusion criteria	X	
Changes in treatments, therapies and/or health status		X
OSDI questionnaire	X	X
SANDE II questionnaire*		X
ECS-Q*		X
Best corrected visual acuity	X	X
Tear break-up time	X	X
Fluorescein corneal staining	X	X
Conjunctival hyperemia	X	X
Schirmer test with anesthesia	X	X

ECS-Q = Evaluation of change in symptoms-questionnaire; OSDI = Ocular surface disease index; SANDE = Symptoms assessment questionnaire in dry eye.

*The order of administration of these questionnaires was randomly determined for each patient.

2.5. Sample Size and Statistical Analysis

A PhD licensed statistician, Dr. Itziar Fernández, using the R-statistical package (version 3.3.2), carried out the statistical analyses. A sample size of 36 participants was calculated to detect a 20 mm change in VAS scale between V1 and V2 (assuming an SD of 30 mm) at α=0.05 bilateral and 1-β = 0.80.

Variables were described using different methods depending on its kind. Mean, SD, and median, were used to describe quantitative variables, while to

describe qualitative variables the percentage for each category was used. In addition, 95% CI were always constructed. Shapiro-Wilk test was used to check the distributions normality. Statistical significance was defined as p≤0.05

Equality of means between two visits, and/or between right and left eye, was checked by Student's t-test for paired samples, or Wilcoxon's signed-rank test when the differences between pairs were not normally distributed.

SANDE II and ECS-Q2 are measures of the change, thus the hypothesis that mean is 0 was contrasted. The ECS-Q2 ranged between 0-100 mm, but it was constructed from ECS-Q1. For this reason, and in order to evaluate changes with ECS-Q in the same way that SANDE II, ECS-Q2 was transformed as follows: value 0 for those patients who answered "equal" in ECS-Q1, positive values to those who responded "better" and negative values for those who felt "worst". Thus, the final score (ECS-Qt) ranges from -100 to 100.

For all variables, the percentage of change *(Table 23)* was calculated and defined as relative changes between V1 and V2. Results were reported as the mean and 95%CI of the percentage of change, and Student's t-test was used to determine if the average change differed significantly from 0 (one-sample t-test).

Table 23. Percentage change (ΔY) analysis.

Quantitative variables	**Qualitative variables**
$\Delta Y = \frac{Y_{V2} - Y_{V1}}{Y_{V1}} \cdot 100$	$if \quad \begin{cases} Y_{V2} > Y_{V1} \rightarrow \Delta Y = \frac{Y_{V2} - Y_{V1}}{Y_{max} - Y_{V1}} \cdot 100 \\ Y_{V2} = Y_{V1} \rightarrow \Delta Y = 0 \\ Y_{V2} < Y_{V1} \rightarrow \Delta Y = \frac{Y_{V2} - Y_{V1}}{Y_{V1} - Y_{min}} \cdot 100 \end{cases}$

Y_{V1}: initial value; Y_{V2}: final value; Y_{min}: minimum value; Y_{max}: maximum value.

To stablish relations between changes in the clinical variables and the questionnaires assessed, the Pearson correlation coefficient, or the non-parametric alternative, the Spearmen correlation coefficient, was used when the two variables were quantitative. When both variables were qualitative, contingency tables were used, calculating chi square test (or Fisher test if frequencies observed were low). Finally, if one variable was quantitative and the

other was qualitative Student-t test or 1 factor ANOVA was used, depending on the groups for the qualitative variable (2 or more). Alternatively, if normality was not assessed the non-parametric alternatives were used, Mann-Whitney U test or Krustall-Wallis ANOVA.

Finally, the intraclass coefficient correlation (ICC) was calculated to stablish the concordance between questionnaires. Also, a 95%CI for this parameter was constructed. The concordance measured with ICC was interpreted as follows: 0.31-0.5 poor, 0.51-0.7 moderate, 0.71-0.9 strong, >0.9 excellent.

3. Results

A total of 36 patients with DED-related symptoms with a mean age of 56.0 ± 14.5 years (range 25-85; 95%CI 51.1-61.0 years) were included in the study. The sample consisted of 21 women (mean age 55.6 ± 14.2 years, range 34-85) and 15 men (mean age 55.2 ± 14.2 years, range 25-85). All subjects attended the two visits of the study (baseline and follow-up; V1 and V2, respectively). The mean time interval between visits was 3.28 ± 0.6 months (median 3.2; 95%CI 3.08-3.48, range 2-4 months).

The diagnoses of the patients included in the study are presented in *Table 24*. It is important to mention that some patients had more than one disease or pathology.

Table 24. Diagnoses in patients included in the study at baseline visit (V1).

		n	%	95%CI	
				Inf	Sup
DED type	Evaporative DED	21	58.33	40.88	74.04
	Aqueous tear-deficient DED	10	27.78	14.79	45.43
Etiology of DED	Sjögren syndrome	4	11.11	3.62	27.00
	Chronic ocular GvHD	11	30.56	16.92	48.27
	Rosacea	7	19.44	8.80	36.57
Other ocular findings	Exoforia	2	5.56	0.97	20.01
	Ocular hypertension	2	5.56	0.97	20.01
	Cornea gutatta	1	2.78	0.14	16.26
	Ambliopia	2	5.56	0.97	20.01
	Atopic dermatitis	1	2.78	0.14	16.26

DED = Dry Eye disease; MGD = Meibomian gland dysfunction; GvHD = Graft vs host disease

As has been noted above, clinical examinations were performed at each visit. The complete dataset of the clinical variables is shown in *Table 25*.

Table 25. Complete dataset of the clinical variables measured during the study.

Test	Eye	Visit 1				Visit 2				P* between visits
		n	Mean (median)	95% CI		n	Mean (median)	95% CI		
				Inf	Sup			Inf	Sup	
BCVA	RE	36	0.8 *(0.8)*	0.7	0.9	36	0.81 *(0.8)*	0.71	0.91	0.6397
	LE	36	0.77 *(0.8)*	0.66	0.87	36	0.74 *(0.75)*	0.63	0.85	0.9552
	p†			0.2382			0.1119††			
Conjunctival hiperemia	RE	36	*(1)*	1	1	36	*(1)*	1	1	0.3583
	LE	36	*(1)*	1	1	36	*(1)*	1	1	1
	p†			1			0.0719			
Schirmer test	RE	35	8.17 *(6)*	5.35	10.99	33	8.52 *(6)*	5.52	11.51	0.9780
	LE	35	7.94 *(5)*	5.15	10.74	33	8.39 *(5)*	5.54	11.25	0.9779
	p†			0.5896			0.6484			
TBUT	RE	35	3.4 *(3)*	2.59	4.21	36	3.56 *(3)*	2.89	4.22	0.6301††
	LE	35	3.11 *(2)*	2.16	4.07	36	3.92 *(3)*	3.1	4.74	**0.0347**
	p†			0.4901			0.2396			
Corneal staining	RE	36	*(1)*	1	2	36	*(1)*	1	2	0.3583
	LE	36	*(2)*	1	3	36	*(1)*	1	2	1
	p†			**0.0318**			0.66			
OSDI (0-100)		35	52.06 *(51)*	44.71	59.41	35	44.89 *(43)*	37.71	52.07	**0.0021**
SANDE II (-50/50)				¥		34	-11.29 *(-10)*	-18.6	-3.98	**0.0035**¥
ECS-QT (-100/100)				¥		36	-25.28 *(-24.5)*	-39.75	-10.8	**0.0011**¥

*Comparison between visits, Wilcoxon test; †Comparison between eyes at each visit, Wilcoxon test; ††Student-t test for 2-paired samples.

¥ This test measures symptoms change, so it was only assessed at V2 to evaluate changes respect to V1. ¥Indicates that mean change is significantly different from zero, Student-t test for 2-independent samples.

BCVA = Best corrected visual acuity; ECS-Q = Evaluation of Change in Symptoms-Questionnaire; LE = Left eye; OSDI = Ocular surface disease index; RE = Right eye; SANDE = Symptoms assessment in dry eye; TBUT = Tear break-up time;

3.1. OSDI

At baseline, symptom-severity data collected by the OSDI questionnaire ranged between 18 and 100, with a mean score of 52.06 ± 21.4 (95%CI 44.71-59.41). Based on the OSDI scores,[7] the severity of symptoms reported by the 36 patients included was as follows: 3 patients (8.33%) reported mild symptoms (OSDI score, 13-22), 3 patients (8.33%) reported moderate symptoms (OSDI score 23-33), and 30 patients (83.33%) presented severe symptoms (OSDI score >33).

At the follow-up visit (V2), a mean score of 44.89 ± 20.9 (95%CI 37.71-52.07) was observed for OSDI questionnaire, which was significantly lower than the observed at V1 (Saphiro-Wilk test, p=0.0021). This change represents an average reduction of 11% in OSDI score between V1 and V2 (Student-t test, p=0.0132).

The number of mild-DED patients did not change (n=3, 8.57%), while it was observed a reduction in the number of severe-DED patients (n=28, 68.87%) that changed to the moderate-DED group (n=8, 22.86%).

3.2. SANDE II

As previously explained, SANDE II was designed to evaluate changes between two moments. For this reason, this questionnaire was only used at V2. At this visit, it was observed a SANDE II average significantly lower than 0 (Student-t test, p=0.0035), which indicates a significant reduction of the DED-related symptoms measured with this questionnaire.

It is important to note that two patients (5.5%) did not complete the SANDE II questionnaire correctly.

3.3. ECS-Q

The new questionnaire proposed for the evaluation of the subjective perception of the patients is composed by two questions. For this reason, results from ECS-Q are presented separately; 1) those from the first question (ECS-Q1; "equal", "worse", "better"), and 2) those from the final score of the proposed tool (ECS-Qt; -100 – 100).

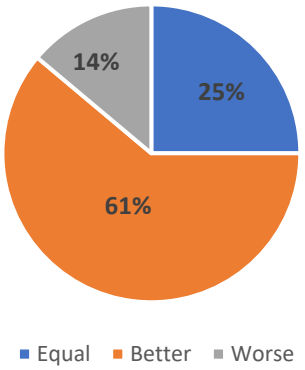

Figure 35. Percentage of patients that felt "better", "worse" or "equal" at visit 2 with respect to baseline (visit 1).

As *Figure 35* shows, analyzing the responses to ECS-Q1 it is possible to observe that most of the patients reported feeling better (22 patients, 61.11%, 95%CI 43.52 – 76.37), while just few patients reported feeling worse (5 patients, 13.89%, 95%CI 5.22 – 30.28). Finally, there were some patients that did not describe changes in their status (9 patients, 25%, 95%CI 12.72 – 42.54).

Regarding the final score (ECS-Qt) of the proposed questionnaire, it is important to remember that this score is constructed from ECS-Q1 and ECS-Q2, as explained in the section 4.5 of this Chapter, and ranges between -100 (maximum amelioration; ECS-Q1 = "better" and ECS-Q2 = 100) and 100 (maximum worsening; ECS-Q1 = "worse" and ECS-Q2 = 100), representing 0 no variation in symptoms (ECS-Q1 = "equal").

An ECS-Qt average significantly lower than 0 (Student-t test, p=0.0011), was observed, which indicates a significant reduction of the DED-related symptoms measured with this tool.

Finally, it is important to mention that no patient misinterpreted the ECS-Q.

3.4. Correlations and Questionnaires Concordance

Some clinical variables significantly change between V1 and V2. The average score of corneal staining presented a significant decrease (↓23%; Student-t test,

p=0.005), while a significant increase was shown in TBUT (↑26%; Student-t test, p=0.0066).

A negative relation was observed between OSDI score and Schirmer test from right eye (Pearson correlation coefficient = -0.3864, p=0.0264).

Some interesting results were found studying the correlation between corneal staining and ECS-Q1. A significant difference in corneal staining was observed from right eye, as well as from the mean from the two eyes, between the groups generated whit ECS-Q1 (Fisher test, p=0.0077 and p=0.0307, respectively). Patients that answered "better" in ECS-Q1 presented a significantly lower corneal staining in the right eye (Student-T test, p=0.0007) and in the mean of both eyes (Student-T test, p=0.0102) than those who responded "worse".

A similar result was found for SANDE II score. Groups "better", "equal" and "worse" at ECS-Q1 presented significantly different SANDE II scores at V2 (Fisher test, p=0.0002). It was observed that subjects that reported to feel "better" had a significantly lower score in SANDE II than those that felt "equal" or "worse" at ECS-Q1 (p=0.0009 and p=0.0001, respectively).

On the other hand, as *Figure 36* shows, we found a positive relation between the Q2 score from ECS-Qt and OSDI (Pearson correlation coefficient = 0.3528, p=0.0377), and between ECS-Qt and SANDE II (Pearson correlation coefficient = 0.6812, p<0.0001).

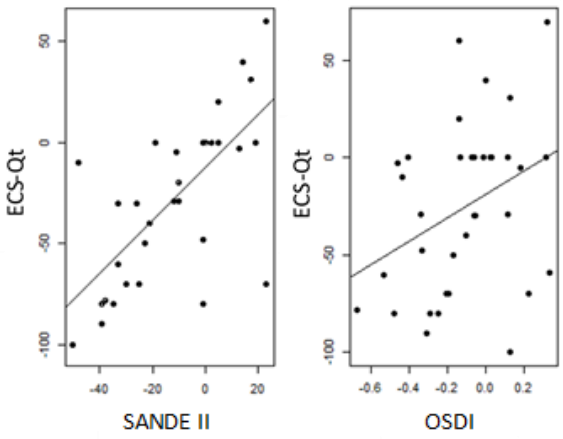

Figure 36. Scatter plots to stablish correlations between the Transformed Study Tool Q2 score and both SANDE II (Left), and OSDI (Right). Line represents the best adjustment.

Finally, to study the concordance between the questionnaires evaluated, ICC was calculated, observing that the accord between OSDI and ECS-Qt is poor (0.365 95%CI 0.045-0.619, p=0.0135), while between SANDE II and ECS-Qt this correlation is moderate (0.687 95%CI 0.461-0.83, p<0.0001).

4. Discussion

Questionnaires are widely used for the evaluation of DED related symptoms; however, there is a clear lack of consensus about which one is the best tool to accomplish this purpose. As previously explained, our research group recently developed a clinical trial that evaluated the efficacy of topical 0.1% fluorometholone in the treatment of DED patients, as well as the potential protective effect of this therapy against desiccating stress generated in a controlled environmental chamber (IOBA-CERLab, University of Valladolid, Valladolid, Spain).[8] Results from clinical signs confirmed the effectiveness of the treatment evaluated in the amelioration of clinical signs after 21-days treatment. Moreover, the protective effect of the therapy when patients faced adverse environmental conditions was also confirmed analyzing clinical variables. Nevertheless, results from this clinical trial were not as consistent when symptoms changes were analyzed. From our point of view, the lack of agreement between the results of signs and symptoms was due to the poor capacity of the questionnaires used to accurately reflect the patient's status, either because of a lack of precision of the tools, or because of misinterpretations or mistakes introduced by patients. In addition, according to our clinical experience, the questionnaires do not reflect what patients express when they are asked directly about their feelings or perceptions regarding their status or evolution. For these reasons, a new evaluation tool has been proposed.

In this study, we compared the capacity of OSDI questionnaire, SANDE II questionnaire, and the ECS-Q, in evaluating symptoms changes in a group of DED patients after a 3-month treatment (V1 vs V2). Results from these questionnaires were compared with the changes observed in clinical signs. Between V1 and V2 there was a corneal staining reduction of 23%, as well as a significant increase in TBUT. These results seem to confirm the efficacy of the prescribed treatments, thus a reduction of symptoms, or at least a positive perception of patients regarding their status or evolution would be expected.

All evaluated questionnaires showed an amelioration of symptoms after treatments. OSDI score was significantly lower at V2 than at V1, while SANDE II presented a negative mean at this visit, which also reflects an improvement. These results were also established with the two questions (ECS-Q1 and ECS-Q2) of the ECS-Q. The results/answers from the first question (ECS-Q1) showed that most of the patients felt "better" at V2 in comparison with V1. Moreover, the answers/results to the second question (ECS-Q2) confirmed this perception, presenting a mean score significantly lower than 0, which indicates improvement. However, although these results seem to indicate that all these questionnaires evaluated the evolution of patients in the same way, a deeper analysis shows that this is not the case.

Firstly, one important point is the number of misinterpretations produced by each questionnaire. All patients answered correctly both OSDI and the new tool (ECS-Q1 and ECS-Q2), but there were two patients (5%) that misunderstood SANDE II. In addition, it is necessary to note that the mean age of the patients included in the study was 56.05 ± 14.53 years. This fact is important because one could have expected a higher number of mistakes if older people had composed the sample. This result could be due to the fact that the ECS-Q is easier to understand than the SANDE II. This fact could contribute to reduce the variability of the responses and/or the loss of information during symptoms evaluation.

Secondly, the comparison of the three questionnaires among them, as well as the comparison of these questionnaires with the clinical variables, yielded two interesting results. On the one hand, the group of patients that answered "better" in the ECS-Q1 of the ECS-Q showed significantly lower corneal staining than those who responded "equal". It is interesting to note that there were no differences in corneal staining between patients who felt "better" and those who felt "worse". This fact indicates that patient´s feelings regarding their status or evolution are not directly related with corneal staining, or at least this is not the determining factor in the subjective perception of the patient's condition. This result agrees with the already described poor correlation between DED signs and symptoms.

On the other hand, the group of patients that answered “better” in the ECS-Q1 of the ECS-Q presented significantly lower SANDE II score than those who responded “equal” or “worse”. The interesting point here is that there were no differences in SANDE II score between the groups “equal” and “worse”. This result implies that SANDE II questionnaire did not accurately separate patients that did not change from those that suffered a worsening, while the ECS-Q did.

Finally, the study of the concordance of the questionnaires shows that OSDI, which is an adequate tool to classify patients according to their symptoms, it is not good enough evaluating changes between two moments.

This study presents several limitations. First, this is an observational study. For this reason, the conclusion obtained must be confirmed with an experimental study. Moreover, although the number of patients included fit the calculated sample size, it would be desirable to increase this sample size, thus increasing the statistical power of the study. Finally, in future studies it would be necessary to control a number of factors that may influence the way in which patients respond to questionnaires, such as age, education level, etc…

In conclusion, the new tool proposed could be a useful questionnaire due to its simplicity for the evaluation of the subjective patient´s perception regarding their evolution and/or status.

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CONCLUSIONS

Conclusions

1. This novel two-step clinical trial design is effective in evaluating the efficacy of new dry eye therapies. This is demonstrated in a traditional standard protocol but additionally with the use of an ocular surface stress (adverse environment) where therapies are assessed by their protective effect. This finding should be confirmed with other therapies.

2. When used as directed, topical 0.1% fluorometholone is a safe and effective therapy for dry eye patients, reducing ocular surface signs after 21-days of treatment.

3. A three-week course of topical 0.1% fluorometholone can also prevent progressive impact on the lacrimal functional unit in dry eye patients exposed to desiccating stress for 2h in a controlled environment chamber.

4. This topical therapy can then be used a positive control in future clinical trials evaluating the effectiveness of new therapies for dry eye syndrome using the new two-step clinical trial design proposed.

5. The following tear inflammatory molecules can be used as potential DED severity biomarkers: IFN-γ, IL-2, IL-12, RANTES/CCL5, and MMP-9. They have allowed classifying patients according to their disease severity in an objective way. Thus, these biomarkers can be useful for a better selection of target patients for clinical trials.

6. EGF is proposed as the best disease activity biomarker. “Activity” is utilized here as a term for the disease inflammatory response as seen in the adverse environmental chamber.

7. When topical fluorometholone is used under our protocol design, lL-2 is the best therapeutic biomarker. It is important to understand that other drugs, even under the same protocol and schedule, will most likely have other different therapeutic biomarkers depending upon their mechanism of action.

8. Due to its simplicity, the new symptom questionnaire developed can be a useful tool for the evaluation of the subjective patient´s perception regarding their evolution and/or status concerning DED symptoms. This questionnaire, however, needs further validation with a larger number of patients and different DED conditions and severity.

9. For all these reasons, we can confirm that this new clinical protocol design utilized in this proof-of-concept two-step clinical trial can be useful to not only show clinical results but also in the identification of disease specific biomarkers. These biomarkers could be used in the next natural step, multicenter clinical trials. By obtaining good clinical results and potentially useful biomarkers, pharmaceutical companies will be able to make better informed decisions regarding proceeding to larger, more costly multicenter clinical trials. Alternatively, companies will save a great deal of resources by not going into multicenter trials if their candidate therapy does not show a clear signal in our two-step design.

Resumen en español

Resumen

Este apartado presenta un resumen en español de la tesis doctoral, cumpliendo así con los requisitos establecidos por la Universidad de Valladolid para la defensa de la tesis con mención internacional.

1. Introducción

El Síndrome de Ojo Seco (SOS) es uno de los trastornos oculares más frecuentes en la población adulta. Algunos autores han reportado que entre el 5,5% y el 33,7% de la población se ve afectada por esta patología. Las diferencias en los porcentajes calculados se deben a diferencias en los criterios utilizados para el diagnóstico del SOS, así como las diferentes características de los grupos de población estudiados o la inclusión/exclusión en dichos estudios de algunas etiologías de SOS. Por otra parte, la prevalencia de SOS aumenta con la edad. Este factor, unido al continuo envejecimiento de la población, hace que el número de personas afectadas por esta patología esté en constante incremento. Otro factor importante que contribuye al aumento de la prevalencia de SOS, y ciertamente lo convierte en un problema más grave, es la creciente proporción de la población que está expuesta a los llamados ambientes adversos. Actualmente, nuestra sociedad pasa gran parte de su tiempo dentro de espacios en los que las condiciones ambientales están artificialmente creadas, tales como edificios de oficinas, centros comerciales, vehículos con aire acondicionado, e incluso hogares. Estos entornos se caracterizan por la baja humedad, las altas temperaturas y la presencia de flujos de aire. Todos estos factores contribuyen a generar una alteración de la película lagrimal, lo cual puede iniciar o empeorar el SOS. Para muchos pacientes con SOS estas condiciones son insoportables. Además, el número de usuarios de terminales tales como tablets, teléfonos inteligentes, etc. y la cantidad de tiempo dedicado a usarlos también han aumentado dramáticamente. El uso de dichos dispositivos reduce la tasa de parpadeo, causando un incremento en la velocidad de evaporación de la película lagrimal, lo cual puede empeorar aún más los signos y síntomas de SOS.

Actualmente, el manejo del SOS incluye el uso de lágrimas artificiales, derivados de suero autólogo, terapias antiinflamatorias (es decir, ciclosporina tópica, tetraciclinas orales y esteroides tópicos). También incluye estrategias ambientales (es decir, evitar los entornos de desecación y el uso prolongado de los terminales mencionados anteriormente) o incluso la cirugía, dependiendo del

nivel de severidad del SOS. La terapia antiinflamatoria está indicada a partir del nivel de gravedad 2, incluyendo la prescripción de esteroides tópicos. Diversos estudios han demostrado su eficacia y seguridad en el tratamiento del SOS, sin embargo, hasta donde nosotros conocemos no existen informes que aborden el posible efecto protector de la fluorometolona tópica en pacientes con SOS cuando éstos están expuestos a condiciones ambientales adversas similares a las que frecuentemente se enfrentan en su día a día. Estas condiciones tienen un impacto adverso sobre la llamada unidad funcional lagrimal, el cual se manifiesta produciéndose alteraciones de la superficie ocular y aumentando la actividad inflamatoria medida en lágrima. Por otro lado, si bien existe una gran evidencia sobre el efecto antiinflamatorio de los corticosteroides sobre la expresión de citoquinas, quimiocinas y otras moléculas inflamatorias, la literatura científica es escasa con respecto al efecto de estos tratamientos en los niveles de moléculas disueltas en la película lagrimal de pacientes con SOS expuestos a ambientes adversos. Moore et al. mostraron que la dexametasona tópica mitiga los signos clínicos de SOS en respuesta a un ambiente de baja humedad, con una disminución paralela en la expresión de HLA-DR, un marcador de inflamación.

En la actualidad, el diagnóstico y la clasificación de los pacientes con SOS se basa en la evaluación de los signos y síntomas clínicos, lo que puede generar errores de clasificación debido a la falta de correlación entre ellos y la variación inter-observador al evaluar los resultados. Por lo tanto, existe la necesidad de estandarizar la evaluación de los tratamientos y el seguimiento de los pacientes, tanto en la práctica diaria como en los ensayos clínicos, eliminando tanto como sea posible el sesgo asociado con las variables clínicas actualmente utilizadas. El uso de biomarcadores moleculares permitiría a los investigadores y médicos evaluar la eficacia de nuevos fármacos y / o la actividad de la enfermedad de una manera más fiable. La capacidad de recolectar muestras de lágrimas de forma fácil y eficiente y la disponibilidad de técnicas que miden muchas moléculas simultáneamente en estas muestras son grandes ventajas que permiten la búsqueda de diferentes tipos de biomarcadores en lágrimas.

2. Hipótesis

Nuestro grupo de investigación ha demostrado previamente que la exposición a condiciones ambientales adversas en el IOBA-CERLab provoca una reacción inflamatoria en la unidad funcional lagrimal (LFU) de los pacientes con Síndrome de Ojo Seco (SOS). Este hecho se confirma gracias al empeoramiento de los signos clínicos y el aumento de los niveles en lágrima de moléculas inflamatorias observado tras dicha exposición. Sin embargo, la tras la exposición a condiciones ambientales adversas en el IOBA-CERLab, no se han encontrado diferencias significativas en cuanto a la sintomatología de SOS.

La hipótesis de esta Tesis Doctoral establece que esta exacerbación inflamatoria en la LFU, inducida por la exposición a condiciones ambientales adversas, puede reducirse, tanto clínicamente como molecularmente, mediante el tratamiento previo de los pacientes con SOS con un agente antiinflamatorio tópico. Este estudio sirve como prueba de concepto de que las condiciones ambientales adversas de la cámara de ambiente controlado serán útiles en el estudio de la eficacia de nuevas terapias y tratamientos para el SOS. Además, midiendo y analizando en diferentes momentos la concentración en lágrima de múltiples moléculas, creemos que podemos determinar varios tipos de biomarcadores de SOS que podrían ser, al menos, biomarcadores de sustitución en futuros ensayos clínicos.

Además, dado que en los estudios previos realizados por nuestro grupo en el IOBA-CERLab los síntomas clínicos invariablemente no han mostrado diferencias, creemos que en este ensayo clínico podríamos de igual manera no hallar ninguna mejora sintomática. Por lo tanto, un objetivo de este trabajo será desarrollar un nuevo cuestionario más simple que pueda evaluar mejor si los pacientes con SOS mejoran o no su sintomatología bajo una determinada terapia.

3. Objetivos

Objetivo 1: Diseñar un protocolo de dos pasos para realizar ensayos clínicos terapéuticos en SOS: la fase tradicional seguida de la fase de exposición ambiental adversa.

Objetivo 2: Evaluar, utilizando el nuevo diseño de ensayo clínico, la eficacia de una terapia habitualmente utilizada en el tratamiento del SOS.

Objetivo 3: Evaluar en varios momentos del ensayo clínico, después de que los pacientes hayan sido tratados, los niveles en lágrima de diferentes moléculas y evaluar los cambios en dichas concentraciones durante y después de la exposición a condiciones ambientales controladas,

Objetivo 4: Transformar las variaciones de las moléculas analizadas en tres tipos de biomarcadores potenciales: 1) severidad de la enfermedad, 2) actividad inflamatoria y 3) eficacia terapéutica.

Objetivo 5: Analizar por qué las herramientas de evaluación de los síntomas clínicos suelen no reflejar adecuadamente la percepción de los pacientes y desarrollar y probar un nuevo cuestionario clínico para evaluar dicho parámetro.

4. Nuevo diseño de ensayos clínico y biomarcadores en lágrima (Capítulos 2 y 3)

4.1. Justificación

Las condiciones ambientales tienen un gran impacto en el estado de la superficie ocular de cualquier persona, pero este impacto se acrecienta cuando se trata de pacientes con SOS.

Los ensayos clínicos son el tipo de estudio utilizado para la evaluación de nuevos tratamientos y terapias para el SOS, y éstos pueden verse afectados por las condiciones ambientales. Por ello, el uso de cámaras de ambiente controlado durante el desarrollo de dichos ensayos clínicos ofrece los siguientes beneficios:

- En primer lugar, permiten exponer a los pacientes a condiciones ambientales estables y no adversas. Por lo tanto, se reducirán o eliminarán las posibles alteraciones en el estado de la superficie ocular de los pacientes, causadas por exposiciones ambientales adversas durante las horas previas a su evaluación (aire acondicionado en vehículos, diferentes condiciones ambientales exteriores, climatización de salas de espera, etc.). Con la utilización de cámaras de ambiente controlado se contribuye a normalizar tanto la evaluación clínica, como la calidad de las muestras que habitualmente se toman para investigación en SOS (lágrimas, células, etc.). Por lo tanto, la intención final es conseguir una muestra más homogénea de pacientes, evitando el sesgo causado por factores externos.
- La utilización de una cámara de ambiente controlado permite exponer a los pacientes a condiciones ambientales adversas, pudiéndose así simular los episodios agudos de empeoramiento que los pacientes refieren sufrir en su día a día cuando se enfrentan a dichas condiciones. De esta forma se puede observar y analizar uno de estos episodios tanto clínicamente como celular y/o molecularmente, lo cual no es posible en la vida real. Además, realizando esta exposición controlada se pueden estudiar los cambios sufridos por los pacientes y evaluar la eficacia de diferentes tratamientos en la protección de los pacientes con SOS frente a dichas condiciones ambientales adversas.

En consecuencia, los objetivos principales del presente ensayo clínico son dos: en primer lugar, evaluar la eficacia clínica de un tratamiento con fluorometolona al 0,1% de 3 semanas en pacientes con SOS, y, en segundo lugar, evaluar si esta terapia podría mejorar el previsible empeoramiento de la unidad funcional lagrimal de dichos pacientes debido a la exposición a condiciones ambientales adversas. Si se obtienen resultados satisfactorios, esta terapia podría ser útil para ayudar a los pacientes a enfrentarse a estos ambientes adversos sin sufrir un empeoramiento en su estado. Adicionalmente, el diseño de este ensayo clínico puede ser útil como control positivo para evaluar si futuras terapias son capaces de proteger a los pacientes con SOS frente a los daños causados por un estrés desecativo.

4.2. Material y Métodos

Se realizó un ensayo clínico Fase III, randomizado, doble-ciego y controlado con vehículo, para comprobar la siguiente hipótesis: la fluorometolona al 0,1% aplicada a nivel tópico ocular y utilizada durante 21 días (4 veces al día) es más eficaz que las lágrimas artificiales utilizadas con la misma pauta, para la disminución del empeoramiento producido por la exposición a ambientes adversos consistentes en baja humedad y flujo de aire (recreado en una cámara de ambiente controlado -CERLab-) en pacientes afectados por SOS moderado o severo.

Este ensayo clínico fue aprobado por el Comité Ético de la Universidad de Valladolid (Valladolid, España) y por la Agencia Española de Medicamentos y Productos Sanitarios (AEMPS, www.aemps.gob.es/en/home.htm; EUDRA 2013-002183-63, Anexo 1). El ensayo fue además registrado en la web clinicaltrials.gov (Identificador: NCT02051023).

El ensayo fue esponsorizado y conducido en el Instituto de Oftalmobiología Aplicada de la Universidad de Valladolid (IOBA, Valladolid, España) de acuerdo con la declaración de Helsinki y las Normas de Buena Práctica Clínica. En ningún momento existieron intereses comerciales detrás de este ensayo clínico.

4.2.1. Descripción del ensayo clínico

En el estudio se incluyeron pacientes mayores de 18 años con SOS moderado y severo (grados 2 a 4 según la clasificación DEWS 2017).1 Los criterios de inclusión fueron: tinción corneal con fluoresceína ≥1 (escala Oxford) en ambos ojos, tiempo de ruptura lagrimal ≤7 segundos en ambos ojos, test de Schirmer sin anestesia ≤10 mm/5 min en ambos ojos, y puntuación en cuestionario OSDI >12 puntos. Además, los pacientes debían referir sufrir un empeoramiento al enfrentarse a condiciones ambientales adversas. Los pacientes fueron excluidos en caso de padecer intolerancia o sensibilidad a cualquiera de los tratamientos utilizados, historia reciente de cirugía, trauma, infección o inflamación ocular así como glaucoma o alteraciones retinianas, enfermedad sistémica con posible afectación ocular (excepto Síndrome de Sjögren), uso de lentes de contacto, oclusión de las vías lagrimales, utilización de tratamientos tópicos oculares (excepto para SOS) o tratamientos sistémicos que pudieran afectar a los resultados del estudio (antihistamínicos, anticolinérgicos, antidepresivos, etc.), embarazo o lactancia. Finalmente, en caso de que los pacientes estuvieran siendo tratados con ciclosporina A y/o corticoesteroides tópicos, dichos tratamientos debían haberse retirado 3 y 1 meses antes de la inclusión, respectivamente.

El protocolo de ensayo clínico consistió en 4 visitas desarrolladas en un periodo de 22 días, tal y como se muestra en la *Figura 1* (día 0: V1; día 21: V2 y V3; día 22 V4). Durante el ensayo clínico se utilizaron dos condiciones ambientales diferentes. En la *Tabla 1* pueden observarse las pruebas realizadas durante cada una de las visitas del estudio, así como el orden de realización de las mismas. Todas las pruebas se realizaron en ambos ojos, pero solo los datos de uno de ellos, seleccionado aleatoriamente, se utilizaron en el análisis estadístico.

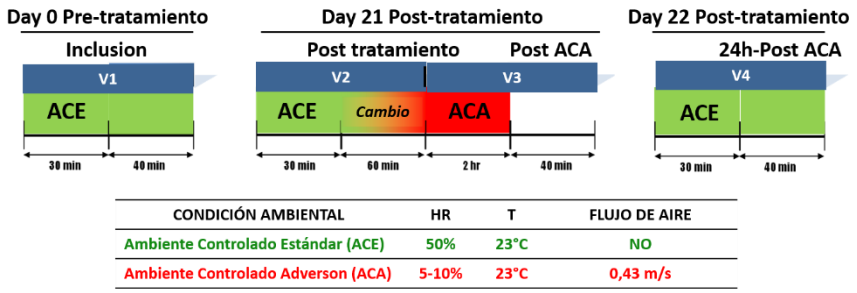

Figura 1. Diagrama de flujo del ensayo clínico.

Tabla 1. Secuencia de test realizados en cada una de las visitas del estudio.

Procedure	**Visita 1** Día 0 *Visita Basal*	**Visita 2** Día 21 *Pre-ACA*	**Visita 3** Día 21 *Post-ACA*	**Visita 4** Día 22 *24h post-ACA*
Consentimiento informado	X			
Criterios de inclusión/exclusión	X			
Historia clínica	X			
Recogida de muestras de lágrima	X	X	X	X
Cuestionario OSDI	X			
Cuestionario SANDE I (A)	X	X	X	X
AV, alto y bajo contraste (B)	X			X
Osmoalridad lagrimal (C)	X	X		
Satisfacción con el tratamiento (C)		X		X
Tiempo de rupture lagrimal (C)	X	X	X	X
Tinción corneal con fluoresceina (A)	X	X	X	X
Tinción cojuntival con verde de Lisamina (C)	X	X	X	X
Biomicroscopía (B, C)	X	X	X	X
Test de Schirmer sin anestesia (C)	X			X
Presión intraocular (B)	X			X
Evaluación de fondo de ojo (B)	X			X
Recogida de acontecimientos adversos (B)		X	X	X
Test de embarazo	x			x
Entrega/recogida tratamientos de estudio	Entrega			Recogida

A = Variable primaria; B = Variable de seguridad; C = Variable secundaria.
ACA = Ambiente controlado adverso; AV = Agudeza visual; OSDI = Ocular surface disease index; SANDE = Symptom assessment in dry eye

Durante los 30 primeros minutos de V1, V2 y V4 los pacientes fueron expuestos a un ambiente controlado de normalización (ACN, temperatura 23°C, HR 50%, sin flujo de aire), con la intención de "normalizar" a los pacientes, evaluándolos a todos los bajo las mismas condiciones ambientales. En V3 los pacientes fueron expuestos durante 2h a una condición ambientales controlada adversa (temperatura 23°C, HR 5%, flujo de aire de 0.43 m/s enfocado hacia la cara). Esta exposición fue utilizada para evaluar la respuesta de la superficie ocular de los pacientes frente a un estrés desecativo, así como para estudiar el posible efecto protector del tratamiento de estudio (fluorometolona tópica al 0.1%).

Por lo tanto, el nuevo diseño de ensayo clínico tiene dos partes claramente diferenciadas: el periodo V1-V2, correspondiente al ensayo clínico "tradicional" en el que se evalúa la eficacia terapéutica del tratamiento estudiado, y el periodo V2-V3, correspondiente a la fase de estrés desecativo/inflamatorio controlado, en la que se evalúa el efecto protector del tratamiento. Al finalizar V1 los pacientes incluidos en el estudio recibieron los tratamientos correspondientes según randomización. El grupo de estudio recibió fluorometolona tópica al 0.1% en un vehículo basado en alcohol polivinílico (grupo FML; FML®, Allergan Inc., Irvine, CA, USA), mientras que el grupo control recibió únicamente el vehículo anteriormente mencionado (grupo PA; Liquifilm Tears™, Allergan Inc.). Los pacientes fueron instruidos acerca de la utilización del tratamiento (1 gota en cada ojo, 4 veces al día durante un periodo de 22 días).

Liquifilm® fue seleccionado como tratamiento control debido a que es el vehículo utilizado en FML®, el tratamiento de estudio. Ambos tratamientos fueron convenientemente enmascarados para asegurar el doble ciego.

4.2.2. Análisis de los niveles de citoquinas en lágrima

Como se ha mostrado en la *Tabla 1*, en cada visita se recogieron muestras de lágrima basal de todos los pacientes incluidos en el estudio. Las muestras (1 µl) fueron recogidas del menisco lagrimal inferior con un microcapilar (Drummond Scientific Co, Broomall, PA, USA) tratando de evitar el reflejo lagrimal tanto como

fue posible. Las muestras se diluyeron 1/10 en un microtubo de 0,5 ml con Cytokine Assay Buffer, y se congelaron a -80ºC hasta su análisis.

Posteriormente, se realizó un análisis multianalito usando la tecnología LuminexTM x-MAP® en la plataforma Luminex IS-100 (Luminex Corporation, Austin, TX, USA). Mediante tecnología multiplex (HCYTO-60K SPR 591 18X-Milliplex, Millipore Corp) se midieron las concentraciones de 18 moléculas: factor de crecimiento epidérmico (EGF), interferón gamma (IFN-γ), factor de necrosis tumoral (TNF-α), interluquina (IL)-1β, IL-1RA, IL-2, IL-4, IL-6, IL-8/CXCL8, IL-10, IL-12, IL-13, IL-17A, IP-10/CXCL10, proteína quimiotáctica de monocitos-1 (MCP-1)/CCL2, proteína inflamatoria de macrófagos (MIP)-1α/CCL3, RANTES/CCL5, y metaloproteinasa 9 (MMP-9).

Finalmente, se definieron tres tipos de biomarcadores: biomarcadores de severidad, biomarcadores de eficacia terapéutica y biomarcadores de actividad.

- Los biomarcadores de la severidad se definieron como aquellas moléculas que permitían la diferenciación entre los pacientes con SOS moderada y severo.

- Los biomarcadores de eficacia terapéutica se definieron como aquellas moléculas que permitían la diferenciación entre el grupo FML y el grupo PA y/o aquellas moléculas que variaron en el tiempo de manera significativamente distinta dependiendo del tratamiento recibido.

- Los biomarcadores de actividad de la enfermedad se definieron como aquellas moléculas que sufrieron cambios significativos en su concentración entre V2 y V3 (pre- post-ACA, respectivamente) después de 21 días de tratamiento con FML o PA.

4.3. Resultados más relevantes

Para simplificar su compresión, los resultados han sido divididos en dos secciones. Por un lado aquellos correspondientes a la parte clínica y por otro lado los obtenidos tras el análisis de las moléculas evaluadas.

4.3.1. Fase clínica

1) Inclusión:

a. Se incluyeron 21 pacientes en el grupo de estudio (grupo FML, 17 mujeres y 4 hombres) y 19 en el grupo control (grupo PA, 17 mujeres y 2 hombres).

2) Variable principal de eficacia:

a. En V1 no se encontraron diferencias significativas entre grupos de tratamiento ni en edad, ni en género, ni en ninguno de los parámetros clínicos evaluados.
b. Tras 21 días de tratamiento (V1-V2) el grupo FML presentó una reducción significativa de la tinción corneal (variable primaria de eficacia), mientras que el grupo PA no mostró cambios en dicha variable.
c. Tras 2h de exposición a condiciones ambientales adversas (V2-V3) el grupo FML no experimentó cambios en la tinción corneal, mientras que el grupo PA sufrió un incremento significativo en dicha variable. En este mismo periodo se observó que en el porcentaje de pacientes con un aumento ≥ 1 punto en la tinción corneal fue significativamente mayor en el grupo PA que en el grupo FML.
d. En V2, V3 y V4 el grupo PA presentó una tinción corneal significativamente mayor que el grupo FML.

3) Variable secundaria de eficacia:

a. *Eficacia del tratamiento tras 21 días de utilización (V1-V2).* En el grupo FML se observaron reducciones significativas en SANDE I, tinción corneal, tinción conjuntival, e hiperemia. En el grupo PA se produjo un aumento de la hiperemia y una reducción del tiempo de ruptura lagrimal. Comparando ambos grupos de tratamiento se observaron diferencias significativas en la variable cambio para tinción corneal, tinción conjuntival, hiperemia y tiempo de ruptura lagrimal.
b. *Eficacia del tratamiento el día 21 tras 2h de exposición a condiciones ambientales adversas (V2-V3).* En el grupo FML no se produjeron cambios

significativos en ninguna de las variables evaluadas. En el grupo PA se produjo un aumento de la tinción corneal, la tinción conjuntival y la hiperemia, así como una reducción del tiempo de ruptura lagrimal. Comparando ambos grupos de tratamiento se observaron diferencias significativas en la variable cambio para tinción corneal, tinción conjuntival, e hiperemia.

c. *Eficacia del tratamiento el día 22 tras 24h desde la exposición a condiciones ambientales adversas (V3-V4).* En este periodo se observó una reducción de SANDE I y tinción conjuntival en el grupo FML, mientras que el grupo PA se produjo una reducción en tinción corneal, tinción conjuntival, e hiperemia conjuntival.
d. En cuanto a la satisfacción de los pacientes con el tratamiento, se observó que en V2 y V4 los niveles de satisfacción fueron significativamente más altos en el grupo FML que en el grupo PA. Entre estas dos visitas se produjo un aumento de la satisfacción en el grupo FML.

4) Variables de seguridad:

a. No se produjeron acontecimientos adversos o reacciones adversas durante el estudio.
b. En el grupo FML se observó un aumento significativo en la agudeza visual de alto contraste y de bajo contraste.

4.3.2. Biomarcadores en lágrima

1) Concentración de las moléculas medidas en lágrima:

a. Debido al bajo porcentaje de detección IL-17A y MIP-1α/CCL3 no fueron incluidas en el análisis.

2) Biomarcadores de severidad:

a. Se establecieron dos grupos de pacientes con diferencias significativas en los niveles de EGF, IFN-γ, IL-8/CXCL8, RANTES/CCL5 y MMP-9 (Grupos A y B)

b. Se compararon las diferencias existentes a nivel clínico entre los grupos generados, observándose diferencias significativas en tinción corneal, tinción conjuntival (mayores en el grupo B en ambos casos) y test de Schirmer (menor en el grupo B). Por lo tanto, los grupos A y B pasaron a denominarse grupo-Moderado (M) y grupo-Severo (S).
c. El modelo lineal de efectos mixtos mostró que los grupo-M y el grupo-S presentaban en V1 niveles significativamente diferentes de EGF, IFN-γ, IL-2, IL-8/CXCL8, IL-10, IL-12, RANTES/CCL5 y MMP-9. El grupo-S tenía niveles más altos de MMP-9 e IL-8/CXCL8 que el grupo-M, mientras que los niveles de EGF, IFN-γ, IL-2, IL-10, IL-12 y RANTES/CCL5 eran más bajos.
d. Tras el análisis de comparaciones múltiples se determinó que IFN-γ, IL-2, IL-12, RANTES/CCL5 y MMP-9 eran los candidatos más fuertes para ser utilizados como biomarcador de severidad.

3) Biomarcadores de eficacia terapéutica:

a. El modelo lineal de efectos mixtos mostró que la interacción entre tiempo y grupo de tratamiento (FML o PA) afectaba significativamente a los niveles de IL-1RA, IL-2 y TNF-α.
b. Tras el análisis de comparaciones múltiples se determinó que IL-2 era el candidato más fuerte para ser utilizados como biomarcador de eficacia terapéutica.

4) Biomarcadores de actividad de la patología:

a. El modelo lineal de efectos mixtos mostró un efecto global significativo de la variable tiempo entre V2 y V3 en varias moléculas.
b. En dicho periodo se observó un incremento de IL-2, mientras que se produjo una disminución en la concentración de EGF, IL-1RA, IL-8/CXCL8, IL-13, IP-10,CXCL10, TNF-α, y MMP-9.
c. Tras el análisis de comparaciones múltiples se determinó que EGF era el candidato más fuerte para ser utilizados como biomarcador de actividad de la patología.

5. Nuevo cuestionario para la evaluación de la sintomatología en SOS (Capítulo 4)

En este apartado aborda todo lo referente al trabajo realizado en la Universidad de Colonia, en el que se estudió la utilidad de un nuevo cuestionario para la evaluación de la sintomatología en SOS.

Para desarrollar dicho estudio el doctorando realizó una estancia de investigación de 3 meses de duración en la Universidad de Colonia, Alemania, lo cual permite que esta Tesis doctoral pueda ser considerada para obtener la mención internacional.

5.1. Justificación

Para evaluar los síntomas relacionados con SOS se utilizan diferentes cuestionarios, sin embargo, no existe un consenso claro acerca de cuál es el más adecuado por lo que actualmente hay un gran número de diseños existentes.

De acuerdo con la experiencia de nuestro grupo de investigación estas herramientas fallan a la hora de reflejar la percepción de los pacientes, siendo esto causado por dos motivos principales: la falta de precisión de los cuestionarios y la complejidad de los mismos, lo que dificulta a los pacientes el interpretar estas herramientas y dar respuestas adecuadas.

5.2. Material y Métodos

5.2.1. Diseño del nuevo cuestionario

Un gran número de pacientes, principalmente los de edad avanzada, encuentran dificultades para expresar adecuadamente en las herramientas de medición más comúnmente utilizadas sus percepciones subjetivas sobre sus síntomas. De acuerdo con nuestro conocimiento y las experiencias anteriores de nuestro grupo de investigación, es necesario simplificar lo máximo posible dichos cuestionarios, con el fin de evitar errores o malentendidos por parte de los pacientes.

Con esta idea en mente se diseñó el Cuestionario de Evaluación de la Cambios en la Sintomatología (ECS-Q), herramienta que se presenta en la Figura 2. El cuestionario

propuesto consta de dos preguntas, ECS-Q1 y ECS-Q2, la cual a su vez se subdivide en dos partes: a) en caso de que el paciente refiera una mejoría; b) en caso de que el paciente refiera un empeoramiento.

ECS-Q1 evalúa, de la manera más simple posible, el cambio sufrido en los con respecto a un momento anterior. Esta pregunta obliga a los pacientes a reflejar clara e inequívocamente su percepción de los cambios experimentados en su sintomatología.

ECS-Q2 a) y b) evalúan la magnitud del dicho cambio.

La mejoría y el empeoramiento se presentan en diferentes escalas numéricas visuales para evitar los posibles errores debidos a una mala interpretación del cuestionario por parte de los pacientes. Como se explicó anteriormente, este estudio se llevó a cabo en la Universidad de Colonia. Por esta razón, ECS-Q fue traducido al alemán por un nativo.

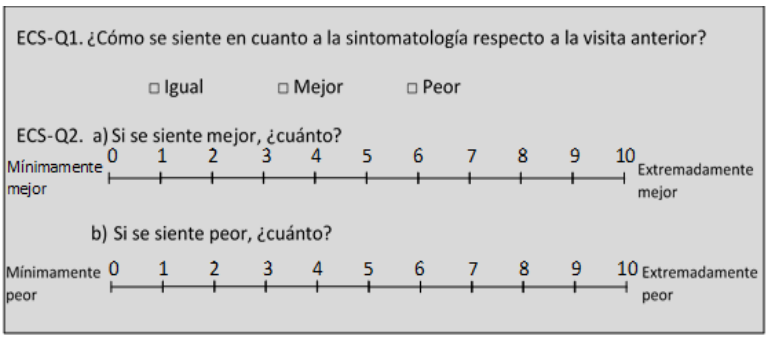

Figura 2. Versión en español del Cuestionario de Evaluación de la Cambios en la Sintomatología (ECS-Q).

5.2.2. Desarrollo del estudio piloto

Este estudio piloto fue diseñado como un estudio observacional prospectivo para realizar una evaluación clínica del ECS-Q. Fue aprobado por el Comité de Ética del Hospital Universitario de Colonia (Colonia, Alemania) y realizado en el Departamento de Oftalmología del Hospital Universitario de Colonia de la Universidad de Colonia, de conformidad con los principios éticos de la Declaración de Helsinki y las Normas de Buena Práctica Clínicas. Se obtuvo el consentimiento informado de cada participante antes del inicio del estudio.

En el estudio se incluyeron pacientes mayores de 18 años con sintomatología relacionada con SOS. Estos pacientes fueron reclutados en el Hospital

Universitario de Colonia bajo la supervisión del Dr. Steven (MD, oftalmólogo). Los participantes debían tener una puntuación en el cuestionario OSDI ≥ 12 puntos. Los criterios de exclusión incluyeron la iniciación, interrupción o cambio, durante 1 mes después de la inclusión, en la dosificación de cualquier medicación tópica o sistémica con posibles efectos sobre la película lagrimal o el SOS. Además, si el paciente estaba bajo tratamiento tópico con ciclosporina A o con corticosteroides tópicos, su uso debería haberse iniciado 3 y 1 mes antes de la inclusión, respectivamente. Este estudio piloto consistió en dos visitas: visita de inclusión (V1) y visita de seguimiento (V2). V1 tuvo lugar durante la evaluación del paciente en el Hospital Universitario de Colonia, después de ser diagnosticado con SOS (cualquier gravedad) y antes de recibir nuevos tratamientos. V2 fue programado cuando, según la opinión del oftalmólogo encargado, la terapia prescrita en V1 hubiera tenido tiempo suficiente para producir un efecto sobre el paciente. La *Tabla 2* muestra las pruebas realizadas en cada visita y la secuencia de realización de las mismas.

Tabla 2. Secuencia de test realizados en cada una de las visitas del estudio.

TEST	Visita 1	Visita 2
Consentimiento informado	X	
Criterios de inclusión y exclusión	X	
Recogida de cambios en tratamientos, terapias y/o cambios de salud		X
Cuestionario OSDI	X	X
Cuestionario SANDE II*		X
Cuestionario ECS-Q*		X
AV mejor corregida	X	X
Tiempo de ruptura lagrimal	X	X
Tinción corneal con fluoresceína	X	X
Hiperemia conjuntival	X	X
Test de Schirmer con anesthesia	X	X

AV = Agudeza visual; ECS-Q = Cuestionario de evaluación de cambios en la sintomatología; OSDI = Ocular surface disease index; SANDE = Symptoms assessment questionnaire in dry eye.

*El orden decumplimentación de estos cuestionarios fue designado al azar para cada paciente.

5.3. Resultados más relevantes

1) Inclusión:

a. Se incluyeron 36 pacientes con sintomatología relacionada con SOS (21 mujeres y 15 hombres).

b. La media de edad de los pacientes incluidos fue de 56.0 ± 14.5 años, no existiendo diferencias significativas entre hombres y mujeres. El tiempo medio entre V1 y V2 fue de 3.28 ± 0.6 meses.

2) Resultados del cuestionario OSDI:

a. En V1 se obtuvo una media de OSDI de 52.06 ± 21.4 puntos. Basándonos en los resultados de dicho cuestionario, y en la escala de clasificación de severidad comúnmente utilizada, se clasificaron a los pacientes de la siguiente forma: 3 pacientes SOS leve (OSDI 13-22), 3 pacientes SOS moderado (OSDI 23-33), 30 pacientes SOS severo (OSDI>33).

b. En V2 la puntuación media del cuestionario OSDI fue de 44.89 ± 20.9 puntos, siendo significativamente más baja que en V1. Además, 5 pacientes clasificados en V1 como SOS severo pasaron al grupo de SOS moderado.

3) Resultados del cuestionario SANDE II:

a. En V2 se observó una puntuación media de SANDE II significativamente menor que 0, lo que implica una reducción significativa de la sintomatología medida con este cuestionario.

b. Un 5.5% de los pacientes no completaron este cuestionario correctamente

4) Resultados del cuestionario ECS-Q:

a. La pregunta ECS-Q1 mostró que en V2 un 61.11% de los pacientes refirió encontrarse mejor, mientras que sólo un 13.89% refirió sentirse peor. El 25% de los pacientes manifestó sentirse igual.
b. Se observó que en V2 la puntuación final del ECS-Q fue significativamente menor que 0, lo que muestra una reducción significativa de la sintomatología medida con este cuestionario.
c. Ningún paciente cometió errores durante la cumplimentación de este cuestionario.

5) Análisis de correlaciones y acuerdo de los cuestionarios:

a. Entre V1 y V2 se observó una reducción significativa de la tinción corneal, así como un aumento del tiempo de ruptura lagrimal.
b. Los pacientes que respondieron “mejor” en ECS-Q1 mostraron en V2 una tinción corneal significativamente menor que los que respondieron “peor”.
c. Los pacientes que respondieron “mejor” en ECS-Q1 mostraron en V2 una puntuación en SANDE II significativamente menor que los que respondieron “igual” o “peor”.
d. Se encontró una correlación directa entre la puntuación final del ECS-Q y los cuestionarios SANDE II y OSDI, siendo la primera más fuerte que la segunda.
e. La concordancia entre OSDI y ECS-Q es pobre, mientras que entre SANDE II y ECS-Q es moderada.

6. Conclusiones

1. El Nuevo diseño de ensayo clínico con dos fases propuesto es útil en la evaluación de la eficacia de nuevas terapias de ojo seco. Este punto ha sido demostrado tanto en la fase tradicional de ensayos clínicos como mediante la exposición de los pacientes a condiciones ambientales adversas, bajo las cuales el tratamiento en estudio ha demostrado su efecto protector. Estos resultados deben ser confirmados bajo el uso de otros tratamientos.

2. La fluorometolona tópica al 0.1% es un tratamiento seguro y eficaz para el síndrome de ojo seco, reduciendo los signos clínicos en la superficie ocular tras 21 días de tratamiento.

3. La utilización durante 3 semanas de flurometolona tópica al 0.1% puede prevenir el impacto sobre la unidad funcional lagrimal de pacientes con ojo síndrome de ojo seco provocado por la exposición a condiciones ambientales adversas durante 2 h en una cámara de ambiente controlado.

4. Esta terapia puede utilizarse como control positivo en ensayos clínicos futuros que evalúen la efectividad de nuevos tratamientos para el síndrome del ojo seco usando el nuevo diseño de ensayo clínico de dos pasos propuesto.

5. Las siguientes moléculas inflamatorias disueltas en lágrima pueden usarse como potenciales biomarcadores de severidad del síndrome de ojo seco: IFN-γ, IL-2, IL-12, RANTES / CCL5 y MMP-9. Estas moléculas han permitido clasificar a los pacientes de acuerdo con la gravedad de su enfermedad de una manera objetiva. Por lo tanto, estos biomarcadores pueden ser útiles para una mejor selección de pacientes diana para ensayos clínicos.

6. EGF se propone como el mejor biomarcador de la actividad de la enfermedad. La "actividad" se utiliza aquí como un término para la respuesta inflamatoria de la enfermedad, tal y como se ve en la cámara ambiental.

7. Bajo nuestro diseño de protocolo, lL-2 muestra ser el mejor biomarcador terapéutico utilizando fluorometolona tópica como tratamiento. Es importante entender que otros fármacos, incluso bajo el mismo protocolo y programación, probablemente tendrán otros biomarcadores terapéuticos diferentes dependiendo de su mecanismo de acción.

8. Debido a su simplicidad, el nuevo cuestionario de síntomas desarrollado puede ser una herramienta útil para la evaluación de la percepción subjetiva del paciente en cuanto a su evolución y / o estado en relación con los síntomas de síndrome de ojo seco. Este cuestionario, sin embargo, necesita una validación con un mayor número de pacientes y diferente gravedad y etiología de la patología.

9. De acuerdo a los resultados obtenidos en esta prueba de concepto, el nuevo diseño de protocolo de ensayo clínico utilizado puede ser útil no sólo para mostrar los resultados clínicos, sino para la identificación de biomarcadores específicos de la enfermedad. Estos biomarcadores podrían ser utilizados en ensayos clínicos multicéntricos. Mediante la obtención de buenos resultados clínicos y biomarcadores potencialmente útiles, las compañías farmacéuticas podrán tomar mejores decisiones con respecto a los ensayos clínicos multicéntricos, más grandes y costosos. Como alternativa, las empresas ahorrarán una gran cantidad de recursos al no participar en ensayos multicéntricos si su terapia candidata no muestra una señal clara en nuestro diseño de dos pasos.

EPILOGUE

Limitations and Future Studies

This work has some limitations that are addressed in this section.

- Forty-one DED patients participated in this clinical trial. Because of the small size of the study population, this could be considered as a pilot study. However, it is large enough, as detailed in the statistical section, to provide evidence of the effectiveness of 0.1% fluorometholone in protecting the ocular surface against adverse environments. Also, this sample size is large enough to provide a proof of concept that allowed us to identify different molecules as potential tear biomarkers of DED.

- Most the patients included in the clinical trial were women. This was expected because DED is more prevalent in women, as explained in Chapter 1. Moreover, the difference in gender ratio increased with DED severity as Sjögren's syndrome is between 10 and 20 times more frequent in women than men, and this study included many severe DED patients. Despite this gender imbalance, we did not see any evidence to indicate a gender difference in the response to 0.1% fluorometholone or polyvinyl alcohol artificial tears, and to our knowledge, none has been published.

- Fluorometholone was the only topical corticosteroid that we studied. We chose it because of its low rate of ocular penetration and the associated good benefit-to-risk ratio. Future studies may include other topical corticosteroids that are also known to be efficacious in DED therapy.

- The validity of the biomarkers proposed should be confirmed in studies with larger sample sizes. On the other hand, our use of the restrictive multiple

comparisons analysis increased the likelihood that the selected potential biomarkers are valid indices of DED severity, therapeutic efficacy, and disease activity. Because of the exploratory nature of this study, the lack of statistical significance before multiple comparisons analysis was not a sufficient criterion to categorically eliminate other potential biomarkers.

- Concerning the potential therapeutic biomarkers, our results are valid only for the specific therapy tested, i.e., FML®. Other treatments could result in the same or different indicators of therapeutic activity, and each must be independently tested. Nevertheless, our results now allow the use of FML® as a positive control in future studies or clinical trials.

- Regarding the prospective observational study developed to evaluate the ECS-Q tool, further studies are necessary to confirm the results obtained. It is necessary to increase the sample size of this study, but also to develop it in other countries, because results could be different dependent on the characteristics of the population included.

- The observational study evaluated the ECS-Q capability in detection of long-term changes in DED-related symptoms (3 months). It could be interesting to check if this tool is also useful in the evaluation of short-term changes.

Scientific Dissemination

The following publications used data coming from the work carried out for this doctoral thesis:

Indexed original publications:

- **Pinto-Fraga J**, López-Miguel A, González-García MJ, Fernández I, López-De la Rosa A, Enríquez-De-Salamanca A, Stern M, Calonge M. Topical Fluorometholone Protects the Ocular Surface of Dry Eye Patients from Desiccating Stress. Randomized Controlled Clinical Trial. *Ophthalmology*. 2016;123(1):141-53. Impact Factor 2015: 6.75 (#2-Q1)

- Calonge M, **Pinto-Fraga J**, González-García MJ, Enríquez-de-Salamanca A, López-de la Rosa A, Fernández I, López-Miguel A. Effects of the External Environment on Dry Eye Disease. Int Ophthalmol Clin. 2017 ;57(2):23-40. Requested review.

- Calonge M, **Pinto-Fraga J,** Enríquez-De-Salamanca A, Fernández I, González-García MJ, López-Miguel A, López-De la Rosa A, Beuerman R, Calder V, Stern M. Tear cytokine biomarkers in dry eye patients subjected to environmental stress and treated with topical 0.1% fluorometholone. *Invest Ophthalmol Vis Sci*. 2016;57. Impact Factor 2015: 3.427 (#6-Q1) [Poster Abstract]

Original articles in progress for publication:

- **Pinto-Fraga J**, Enríquez-de-Salamanca A, Calonge M, González-García MJ, López-Miguel A, López-de la Rosa A, García-Vázquez C, Calder V, Stern M,

Fernández I. Severity, Therapeutic, and Activity Tear Biomarkers in Dry Eye Disease. Submitted to Invest Ophthalmol Vis Sci.

- **Pinto-Fraga J**, González-García MJ, Enríquez-de-Salamanca A, Calonge M, Steven P. Development of a New Questionnaire to Evaluate Changes in DED-Related Symptoms. Under elaboration.

Oral communications in International and National Congresses:

- *Dry Eye Disease Biomarkers in Clinical Studies*. "Ophthalmology Exchange Meeting. University College of London (UCL)". March 2017, London, United Kingdom.
- García-Vázquez C, **Pinto-Fraga J**, Enríquez-de-Salamanca A, Fernández I, González-García MJ, Calonge M. *Medida de biomarcadores en pacientes con síndrome de ojo seco (SOS), expuestos a condiciones ambientales adversas y tratados con fluorometolona tópica al 0.1%.* "XXIX Congreso Asociación Española de Técnicos de Laboratorio (AETEL)". May 2016, Salamanca, Spain.
- **Pinto-Fraga J**, López-Miguel A, González-García MJ, Fernández I, López-De la Rosa A, Enríquez-De-Salamanca A, Stern M, Calonge M. *La fluorometolona tópica al 0.1% protege la superficie ocular de los pacientes con síndrome de ojo seco frente a condiciones ambientales adversas.* "XXIV International Congress in Optometry, Contactology and Ophthalmic Optics". April 2016, Madrid, Spain.
- Calonge M, González-García MJ, Enríquez-De-Salamanca A, Fernández I, **Pinto-Fraga J**, Tesón M, Martín-Montañez V, Stern M, López-Miguel A. *Environmental factors in ocular surface disease.* "European Association for Vision and Eye Research. EVER 2015". October 2015, Nize, France.
- **Pinto-Fraga J**, López-Miguel A, González-García MJ, Fernández I, López-De la Rosa A, Enríquez-De-Salamanca A, Stern M, Calonge M. *Topical steroids*

protects the lacrimal functional unit of dry eye disease patients from dessiccating stress. "International Symposium on Ocular Pharmacology and Therapeutics. ISOPT 2015". July 2015, Berlin, Germany.

- **Pinto-Fraga J**, López-Miguel A, González-García MJ, Fernández I, López-De la Rosa A, Enríquez-De-Salamanca A, Stern M, Calonge M. *Desarrollo de nuevos tratamientos para síndrome de ojo seco: propuesta de un modelo acelerado de ensayo clínico. "*IOBA´s day". May 2014, Valladolid, Spain.

Poster communications in International Congress:

- Calonge M, **Pinto-Fraga J,** Enríquez-De-Salamanca A, Fernández I, González-García MJ, López-Miguel A, López-De la Rosa A, Beuerman R, Calder V, Stern M. *Tear cytokine biomarkers in dry eye patients subjected to environmental stress and treated with topical 0.1% fluorometholone.* "*The Association for Research in Vision and Ophthalmology (ARVO 2016)."* May 2016, Seattle, USA.

Others:

- Part of this Thesis was presented to participate in the contest "3MT-EsDUVa", in which the PhD predoctoral student Jose Pinto-Fraga was finalist. October 2016. Valladolid, Spain.
- The predoctoral student Jose Pinto-Fraga delivered a lecture on "New tools in dry eye research" at the end of his 3-month stay at the Department of Ophthalmology at the University Hospital of Cologne. August 2016. Köln, Germany.

Annex

I. Comité Ético de la Universidad de Valladolid Approval
II. Spanish Drugs and Health Products Administration (AEMPS) Approval
III. Patient information sheet and informed consent for the clinical trial 2013-002183-63.
IV. OSDI questionnaire
V. Efron scale
VI. ECS-Q version used during the study
VII. University of Cologne Ethics Committee Approval
VIII. Patient information sheet and informed consent for the observational prospective study evaluating the ECS-Q

ANNEX I. Comité Ético Universidad de Valladolid Approval

DICTAMEN DEL COMITÉ ÉTICO DE INVESTIGACIÓN CLÍNICA

D. F. Javier Álvarez González, Secretario del COMITÉ ÉTICO DE INVESTIGACIÓN CLÍNICA ÁREA DE SALUD VALLADOLID – ESTE (CEIC-VA-ESTE-HCUV)

CERTIFICA

En la reunión del CEIC del Área de Valladolid – Este de 31 de Octubre de 2013, se procedió a la evaluación del siguiente Ensayo Clínico:

Código en el HCUV CASVE	TÍTULO	IP + Promotor
13-170	"ENSAYO CLÍNICO FASE III DOBLE ENMASCARADO, ALEATORIZADO Y CONTROLADO PARA EVALUAR LA SEGURIDAD Y EFICACIA DE FLUOROMETOLONA 0,1% (FML® SUSPENSIÓN OFTÁLMICA) DURANTE 3 SEMANAS EN LA EXARCEBACIÓN INFLAMATORIA PROVOCADA POR LA EXPOSICIÓN A AMBIENTES ADVERSOS EN PACIENTES CON DÍNDROME DE OJO SECO (SOS)/ QUERATOCONJUNTIVITIS SECA (QCS) EN UNA CÁMARA DE AMBIENTE CONTROLADO." Código: IOBA-CERLab 003-2013 Nº EudraCT: 2013-002183-63 Protocolo Versión 3.0 del 30 de octubre de 2013. Hoja de Información al Paciente y Consentimiento Informado Versión 3.0 del 30 de octubre de 2013.	Dra. MARGARITA CALONGE CANO IOBA

Considera que:

- El ensayo se plantea siguiendo los requisitos del Real Decreto 223/2001, de 6 de febrero y las normas que lo desarrollan, y su realización es pertinente.
- Se cumplen los requisitos necesarios de idoneidad del protocolo en relación con los objetivos del estudio y están justificados los riesgos y molestias previsibles para el sujeto.
- Son adecuados tanto el procedimiento para obtener el consentimiento informado como la compensación prevista para los sujetos por daños que pudieran derivarse de su participación en el ensayo.
- El alcance de las compensaciones económicas previstas no interfiere con el respeto a los postulados éticos.
- La capacidad del investigador y sus colaboradores, y las instalaciones y medios disponibles, tal y como ha sido informado, son apropiados para llevar a cabo el estudio.

Este CEIC actuando como comité de referencia, emite un **DICTAMEN FAVORABLE**.

Este CEIC acepta que dicho ensayo sea realizado en Centro Instituto de Oftalmología Aplicada (IOBA) por el Investigador Principal Dª. Margarita Calonge Cano.

Junta de Castilla y León

COMITÉ ÉTICO DE INVESTIGACIÓN CLÍNICA - VALLADOLID ESTE 1

El CEIC del Hospital Clínico Universitario de Valladolid a fecha 31 de octubre de 2013 (resolución de la Dirección General de Salud Pública de la Consejería de Sanidad de la Junta de Castilla y León por la que se renueva la acreditación del CEIC del Área Este de Valladolid, 5 de julio de 2013) estaba compuesto por:

Dª Mª Paz de la Torre Pardo - Presidenta
D. José Luis González Martínez-Zárate
Dª Belén Cantón Álvarez
D. Luis María Arribas Gómez,
Dª Ana López González
Dª Hortensia Marcos Sánchez
D. Luis Orejón Sanz
Dª Ana Mª Ruiz San Pedro
D. Vicente Molina Rodríguez
Dª Rafaela de las Heras Vicente
D. Manuel Castanedo Allende
D. Jesús Francisco Bermejo Martín
D. Vicente Roig Figueroa
D. F Javier Álvarez González –Secretario

Los miembros del CEIC del Hospital Clínico Universitario de Valladolid que asistieron a la reunión del 31 de octubre son:

Dª Mª Paz de la Torre Pardo – Presidenta
D. José Luis González Martínez-Zárate
Dª Belén Cantón Álvarez
D. Manuel Castanedo Allende
Dª Ana López González
Dª Hortensia Marcos Sánchez
D. Luis Orejón Sanz
Dª Ana Mª Ruiz San Pedro
D. Vicente Molina Rodríguez
D. F Javier Álvarez González –Secretario
Asistentes no miembros:
Dª. María Ana Prado Prieto

Lo firmo en Valladolid, a 31 de octubre de 2013

Prof. F. Javier Álvarez.
Secretario CEIC Área de Valladolid – Este
(CEIC-VA-ESTE-HCUV)
Farmacología
Facultad de Medicina,
Universidad de Valladolid,
c/ Ramón y Cajal 7,
47005 Valladolid
alvarez@med.uva.es;jalvarezgo@saludcastillayleon.es; tel: 983 423077

Junta de Castilla y León

ANNEX II. AEMPS Approval

DEPARTAMENTO
DE MEDICAMENTOS
DE USO HUMANO
Área de Ensayos Clínicos

DESTINATARIO

Meditrial
C/Méndez Álvaro,18
28045 Madrid (España)

REFERENCIA: MUH/AEC

ASUNTO: RESOLUCIÓN DE AUTORIZACIÓN DEL ENSAYO CLINICO Nº EUDRACT 2013-002183-63

Adjunto se remite la resolución sobre el ensayo clínico titulado **Ensayo clínico fase III doble enmascarado, aleatorizado y controlado para evaluar la seguridad y eficacia de fluorometolona 0,1% (FML® suspensión oftálmica) durante 3 semanas en la exacerbación inflamatoria provocada por la exposición a ambientes adversos en pacientes con Síndrome de Ojo Seco (SOS)/Queratoconjuntivitis Seca (QCS) en una cámara de ambiente controlado**, Nº EudraCT: **2013-002183-63**.

El promotor o solicitante nombrado por éste deberá notificar la fecha de inicio del ensayo en España, remitir la información pertinente o solicitar autorización a la Agencia Española de Medicamentos y Productos Sanitarios, según proceda y de acuerdo con lo que establece el Real Decreto 223/2004, de las modificaciones relevantes a la documentación del ensayo, informes de seguimiento, sospechas de reacciones adversas graves e inesperadas, finalización del ensayo y demás circunstancias que establezca la legislación vigente.

Firmado digitalmente por: Agencia Española de Medicamentos y Productos Sanitarios
Fecha de la firma: 12/11/2013
Localizador: FXR27PY7B5

Puede comprobar la autenticidad del documento en la aplicación Localizador de la Web de la AEMPS

CORREO ELECTRÓNICO
smhaem@aemps.es

C/ CAMPEZO, 1 - EDIFICIO 8
28022 MADRID
Tel.: 918225073
Fax: 918225043

Referencia: MUH/CLIN

ASUNTO: RESOLUCIÓN DE AUTORIZACIÓN DEL ENSAYO CLINICO Nº EUDRACT 2013-002183-63

DESTINATARIO: IOBA
Paseo de Belén 17
47011 Valladolid (España)

Vista la solicitud formulada por **Meditrial** para la realización del ensayo clínico número **2013-002183-63**, titulado **Ensayo clínico fase III doble enmascarado, aleatorizado y controlado para evaluar la seguridad y eficacia de fluorometolona 0,1% (FML® suspensión oftálmica) durante 3 semanas en la exacerbación inflamatoria provocada por la exposición a ambientes adversos en pacientes con Síndrome de Ojo Seco (SOS)/Queratoconjuntivitis Seca (QCS) en una cámara de ambiente controlado**, código de protocolo del promotor **IOBA-CERLab-003-2013**, cuyo promotor es **IOBA** se emite resolución a tenor de los siguientes:

ANTECEDENTES DE HECHO

PRIMERO: Con fecha **21/05/2013**, solicita la autorización de este ensayo clínico.
SEGUNDO: Con fecha 28/05/2013 se solicitaron aclaraciones que fueron adecuadamente respondidas.

A estos antecedentes de hecho les es de aplicación los siguientes:

FUNDAMENTOS DE DERECHO

Único.- Son de aplicación al presente procedimiento la Ley 30/1992, de 26 de noviembre, de Régimen Jurídico de las Administraciones Públicas y del Procedimiento Administrativo Común, modificada por la Ley 4/1999, de 13 de enero; la Ley 12/2000, de 29 de diciembre de medidas fiscales, administrativas y de orden social; Ley 29/2006, de 26 de julio, de Garantías y Uso Racional de Medicamentos y Productos Sanitarios; el Real Decreto 223/2004 de 6 de febrero, por el que se regulan los ensayos clínicos con medicamentos; el Real Decreto 1275/2011, de 16 de septiembre, por el que se crea la Agencia estatal «Agencia Española de Medicamentos y Productos Sanitarios» y se

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Página 2 de 3

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aprueba su Estatuto, y demás normas aplicables.

Así, del expediente se deduce que se cumplen los requisitos establecidos para su autorización de acuerdo con el artículo 22 del Real Decreto 223/2004.

Por todo lo anteriormente expuesto la Directora de la Agencia de Medicamentos y Productos Sanitarios en el ejercicio de sus competencias **RESUELVE:**

1º.- AUTORIZAR la realización de este ensayo clínico con número EudraCT 2013-002183-63

OBSERVACIONES

El promotor deberá comunicar al CEIC de referencia, la versión del protocolo autorizada por la AEMPS.

Contra esta Resolución, que pone fin a la vía administrativa, puede interponerse potestativamente Recurso de Reposición ante el/la Director/a de la Agencia Española de Medicamentos y Productos Sanitarios en el plazo de un mes, conforme a lo dispuesto en el artículo 116 y 117 de la Ley 30/1992, de 26 de noviembre, de Régimen Jurídico de las Administraciones públicas y del Procedimiento Administrativo Común, o interponerse Recurso Contencioso-Administrativo ante el Juzgado Central de lo Contencioso-Administrativo de Madrid, en el plazo de dos meses a contar desde el día siguiente a la recepción de la presente notificación, conforme a lo dispuesto en la Ley Reguladora de la Jurisdicción Contencioso-Administrativa de 13 de julio de 1998, y sin perjuicio de cualquier otro recurso que pudiera interponerse.

LA DIRECTORA DE LA AGENCIA ESPAÑOLA
DE MEDICAMENTOS Y PRODUCTOS SANITARIOS

Dª. Belén Crespo Sánchez-Eznarriaga

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Fecha de la firma: 12/11/2013
Localizador: FXR27PY7B5

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ANNEX III. HOJA DE INFORMACIÓN AL PACIENTE

Título del Ensayo: Ensayo clínico fase III doble enmascarado, aleatorizado y controlado para evaluar la seguridad y eficacia de fluorometolona 0,1% (FML® suspensión oftálmica) durante 3 semanas en la exacerbación inflamatoria provocada por la exposición a ambientes adversos en pacientes con Síndrome de Ojo Seco (SOS)/Queratoconjuntivitis Seca (QCS) en una cámara de ambiente controlado

Nº de estudio: EUDRA CT 2013-002183-63. IOBA-CERLab 003-2011

Coordinador: IOBA (Instituto de Oftalmobiología Aplicada), Universidad de Valladolid

Investigador Principal: Dra. Margarita Calonge Cano

PROPÓSITO DEL ENSAYO

Se le ha invitado a participar en un ensayo clínico que se llevará a cabo en el IOBA para evaluar, bajo condiciones ambientales controladas, un medicamento para disminuir el empeoramiento que los pacientes con Síndrome de Ojo Seco (SOS) sufren en condiciones ambientales adversas.

CONDICIONES DEL ESTUDIO

Se estima que su participación en este estudio tenga una duración total de 22 días, durante los cuales tendrá que acudir 3 veces al IOBA para realizar un total de 4 visitas, con una duración estimada de 1-2 hr el primer y cuarto día y de 3-4 horas el segundo día.

Visita 1: Si decide participar, en su primera visita de estudio (visita de inclusión) se le examinará para saber si puede formar parte del estudio. Las pruebas se realizarán después de que usted haya permanecido durante 30 minutos en una sala con unas condiciones de temperatura y humedad controladas (23ºC y 50%). Posteriormente, se recogerá la siguiente información sobre usted:

- Se hablará sobre el estudio, el investigador responderá todas las preguntas que tenga sobre este estudio o sobre el consentimiento informado y se le pedirá que firme este formulario de consentimiento antes de iniciar su participación en el estudio.
- Se le preguntará sobre su actual estado de salud, general y ocular y se le pedirá que cumplimente unos cuestionarios sobre sus síntomas oculares.
- Se evaluará la lágrima y la superficie de su ojo.

- Se le tomará la visión, se medirá la tensión ocular y se le explorará el fondo del ojo.

- Si es mujer en edad fértil, se le facilitará un test de embarazo antes de la primera visita del estudio para que se pueda valorar el resultado antes de ser incluida en el estudio.

Se utilizarán dos productos ya comercializados y ampliamente utilizados para el tratamiento de SOS. Usted será aleatorizado para recibir uno de los dos productos y se le entregará al final de la visita. Deberá usarlo 4 veces al día hasta la última visita, es decir, durante 22 días. Deberá devolver los envases en esta última visita.

Ni usted ni el evaluador sabrán en ningún momento el tipo de medicamento que está utilizando. Si lleva algún otro tratamiento para su síndrome de ojo seco, este no se le cambiará a lo largo del estudio, incluido el uso de lágrimas artificiales.

Visita 2: Se realizará 21 días después de la visita 1. Tras permanecer 30 minutos en condiciones ambientales de normalización se le evaluarán los síntomas, la superficie de su ojo y se tomará una muestra de su lágrima con un capilar.

Visita 3: Se realizará el mismo día que la visita 2 y a continuación de ésta. Tras permanecer durante 2 horas bajo condiciones ambientales "adversas" (23ºC, 10% de humedad y flujo de aire), se le evaluarán los síntomas, la superficie de su ojo y se tomará una muestra de su lágrima con un capilar. Se le citará para el día siguiente y se le pedirá que siga utilizando el tratamiento durante ese día.

Visita 4: Se realizará 24 horas después de la visita 3. Tras permanecer 30 minutos en condiciones ambientales de normalización se le evaluarán los síntomas, la superficie de su ojo y se tomará una muestra de su lágrima con un capilar. Se recogerá el tratamiento sobrante. Además, se le tomará la visión, se medirá la tensión ocular y se le explorará el fondo del ojo al finalizar la consulta. Si es mujer en edad fértil, se le pedirá repetir el test de embarazo realizado en la primera visita del estudio.

USTED DEBERÁ INSTILAR LA ULTIMA GOTA DEL MEDICAMENTO 2 HORAS ANTES DE LA HORA EN QUE HAYA SIDO CITADO EN CADA VISITA

Ninguno de los procedimientos que se le van a realizar durante las visitas es invasivo o resulta doloroso, únicamente puede sentir una ligera molestia o picor

al poner los colirios necesarios para evaluar sus ojos. Para la toma de la tensión ocular que se realizará en la primera y última visita, se le instilará anestésico tópico previamente al la toma mediante tonometría por aplanación, tal y como está protocolizado.

Todas las muestras recogidas serán utilizadas exclusivamente para la realización de las medidas descritas en el protocolo de este estudio. Cualquier resto de muestra que pudiera quedar al finalizar las pruebas necesarias será destruido.

Si usted o el personal del estudio creen necesario realizar otra visita entre las visitas programadas, se programará otra cita con usted. Si el personal investigador lo estimara necesario se podrán precisar pruebas adicionales.

SUS RESPONSABILIDADES

Usted deberá usar las soluciones que se le proporcionen durante el estudio de forma diaria durante 4 veces al día, además de su tratamiento habitual para el síndrome de ojo seco.

Deberá acudir a todas las visitas del estudio y avisar al centro, tan pronto como pueda, si no pudiera acudir a alguna de estas visitas. Se le pedirá que comunique cualquier cambio en su medicación (con o sin prescripción médica) y comunicar al personal investigador del estudio cualquier cambio que usted experimente. Deberá responder a todas las preguntas de los cuestionarios sinceramente.

RIESGOS Y MOLESTIAS RAZONABLEMENTE PREVISIBLES PARA EL PARTICIPANTE

Los dos tratamientos que se va a usar durante el estudio están comercializados y evaluados, no previéndose ninguna complicación en el tiempo de duración del ensayo.

CONFIDENCIALIDAD

Puede publicarse un informe de los resultados de este estudio o enviarse a las autoridades sanitarias pertinentes, pero su nombre no aparecerá en estos documentos. De acuerdo a la Ley Orgánica
15/1999, su confidencialidad será debidamente respetada si la información es transferida a otros países. Su nombre puede ser revelado a las autoridades sanitarias gubernamentales como la AEMPS (Agencia Española de Medicamentos y Productos Sanitarios) o a los Comités Éticos de Investigación Clínica (CEIC) en caso de que necesiten inspeccionar sus archivos médicos. Se

tomarán las medidas oportunas para mantener la confidencialidad de los archivos médicos y de la información personal.

COMPENSACIÓN

Usted no tendrá que pagar nada por cualquiera de las visitas realizadas durante el estudio. Todos los productos necesarios para realizar el estudio se le facilitarán sin coste económico alguno.

El promotor le compensará los posibles gastos en los que incurra (dietas y desplazamiento) en caso justificado por la asistencia a las visitas del estudio. Dicha compensación la percibirá al completar en tiempo y forma todas las visitas requeridas y será reembolsada tras la presentación de las facturas correspondientes.

PERSONA DE CONTACTO

Se le anima a que consulte con el personal encargado del estudio cualquier duda que tenga debiendo recibir respuestas satisfactorias a todas sus preguntas. Si durante el estudio experimenta algún cambio en su salud o en la medicación, o si tiene alguna pregunta adicional, deberá ponerse en contacto con:

Equipo Investigador:

Dra. Margarita Calonge Cano Número de teléfono: 983184750/33

Dra. Mª Jesús González García Número de Teléfono: 983184756

PARTICIPACIÓN

Usted dispondrá del tiempo suficiente para decidir sobre su participación en el estudio.

Su participación en este estudio es totalmente voluntaria. Usted puede rechazar participar o puede abandonar el estudio en cualquier momento, por cualquier motivo, sin que pierda ninguno de los derechos o beneficios a los que por otro lado tiene derecho. Si usted decide no participar en el estudio, o si decide abandonar el estudio antes de su finalización, su atención habitual en este centro no se verá perjudicada en modo alguno. El personal clínico del estudio puede retirarle del estudio en cualquier momento si lo considera necesario. El IOBA puede suspender este estudio

en cualquier momento si tiene razones para determinar qué es lo adecuado.

INFORMACIÓN NUEVA

El personal del estudio le informará a usted o a su representante legalmente autorizado sobre cualquier información nueva acerca de los productos del estudio que pudiera conocerse durante el transcurso de esta investigación y que pudiera influenciar su voluntad de participar en el estudio.

Se le entregará una copia firmada y fechada de este formulario de consentimiento para sus propios archivos antes de su participación en el estudio.

CONSENTIMIENTO INFORMADO POR ESCRITO

Título del Ensayo: Ensayo clínico fase III doble enmascarado, aleatorizado y controlado para evaluar la seguridad y eficacia de fluorometolona 0,1% (FML® suspensión oftálmica) durante 3 semanas en la exacerbación inflamatoria provocada por la exposición a ambientes adversos en pacientes con Síndrome de Ojo Seco (SOS)/Queratoconjuntivitis Seca (QCS) en una cámara de ambiente controlado

Nº de estudio: EUDRA CT 2013-002183-63. IOBA-CERLab 003-2011
Coordinador: IOBA (Instituto de Oftalmobiología Aplicada), Universidad de Valladolid
Investigador Principal: Dra. Margarita Calonge Cano

Al firmar abajo, yo declaro que:

1) He leído, o me han leído, y entiendo completamente el contenido del formulario de información adjunto, Versión 2.0 de 30 de agosto de 2013.

2) He tenido la oportunidad de preguntar y obtener respuestas satisfactorias a cada una de mis preguntas.

1) Acepto de forma voluntaria participar en este estudio de investigación y sé que puedo retirarme en cualquier momento sin que se vea afectada la continuidad de mi tratamiento.

2) Personal del equipo investigador: _____, Dirección: IOBA, Valladolid; tfn 983 184750; me ha explicado la información para el paciente y el formulario de consentimiento y comprendo lo que implica la investigación.

3) He comprendido completamente que los representantes del patrocinador, el Comité Ético Independiente o los representantes de las autoridades regulatorias pueden examinar mis registros médicos donde aparece mi nombre para verificar la exactitud de la información obtenida y entiendo que estas personas tendrán el deber de manejar esta información con confidencialidad utilizándola solamente con un objetivo legítimo para la salud pública.

4) Acepto comunicar al personal clínico del estudio todos los efectos secundarios u otros cambios en mi salud y todos los cambios de mi tratamiento médico

5) Se me entregará una copia firmada y fechada de este formulario de consentimiento para mis propios archivos

Nombre del paciente

Fecha Firma

Nombre del investigador principal

Fecha Firma

Nombre de la persona que explicó el consentimiento

Fecha Firma

ANNEX IV. OSDI QUESTIONNAIRE

CUESTIONARIO DE SINTOMATOLOGÍA OSDI

¿Ha experimentado alguno de los siguientes síntomas durante la última semana?

	Todo el tiempo	La mayoría del tiempo	La mitad del tiempo	Algunas veces	Nunca
1. Ojos sensibles a la luz	4	3	2	1	0
2. Sensación de arenillas	4	3	2	1	0
3. Dolor o irritación ocular	4	3	2	1	0
4. Visión borrosa	4	3	2	1	0
5. Mala visión	4	3	2	1	0

¿Ha tenido problemas oculares que hayan impedido realizar algunas de las siguientes actividades la última semana?

	Todo el tiempo	La mayoría del tiempo	La mitad del tiempo	Algunas veces	Nunca
6. Leer	4	3	2	1	0
7. Conducir por la noche	4	3	2	1	0
8. Uso de ordenadores	4	3	2	1	0
9. Ver la televisión	4	3	2	1	0

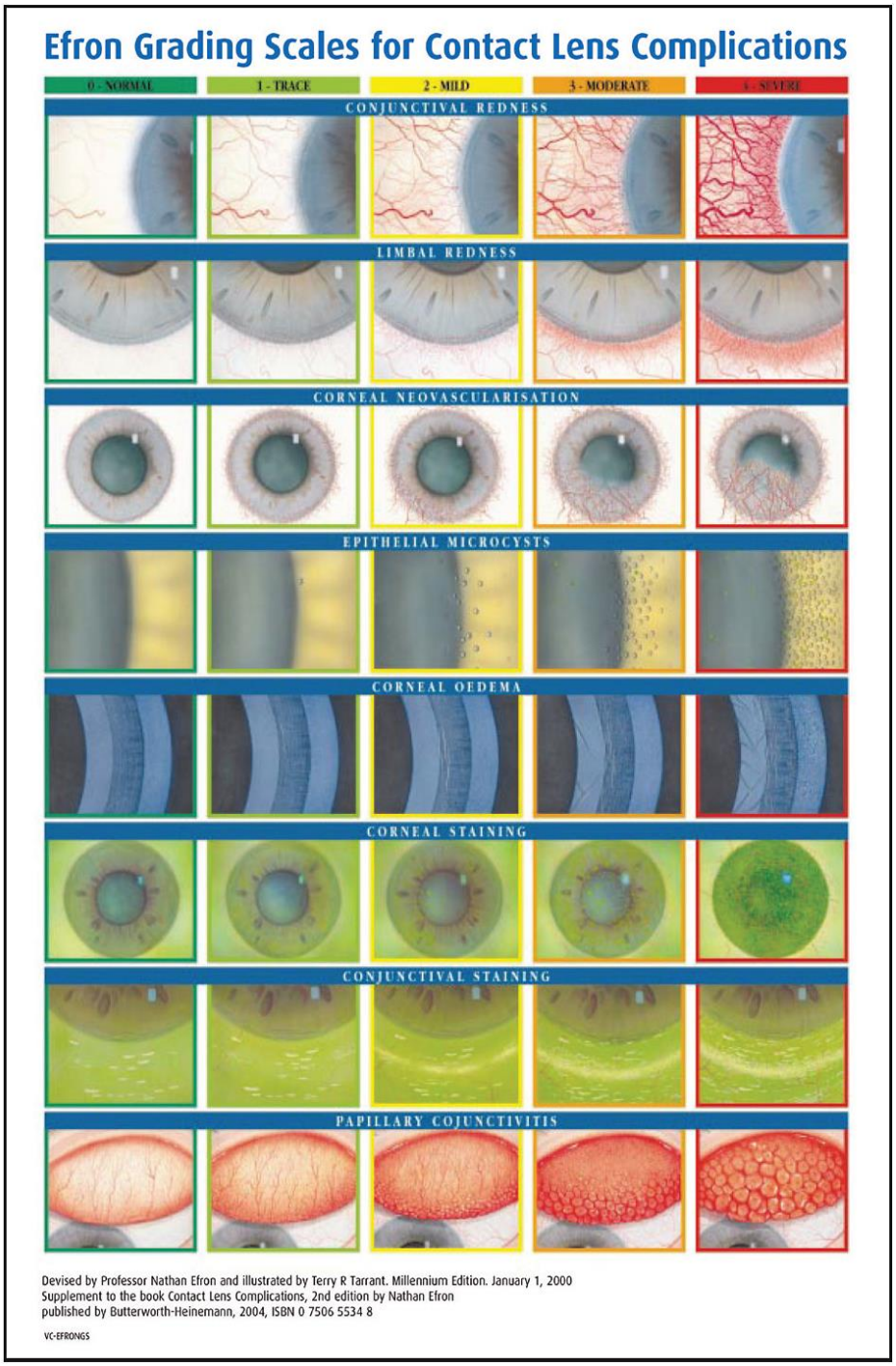
¿Ha sentido los ojos molestos en alguna de las siguientes situaciones durante la última semana?

	Todo el tiempo	La mayoría del tiempo	La mitad del tiempo	Algunas veces	Nunca
10. Con viento	4	3	2	1	0
11. Lugares muy secos	4	3	2	1	0
12. Con aire acondicionado	4	3	2	1	0

TOTAL TEST OSDI:

Normal: 0-12 puntos, Leve: 13-22 puntos, Moderado: 23-32 puntos, Severo: 33-100 puntos

ANNEX V. EFRON SCALE



ANNEX VI. ECS-Q EXAMPLE

Gruppe A, Teilnehmer 11 Datum: 02.12.16

Gruppe B, Teilnehmer____

1. Wie fühlen sich Ihre Augen im Vergleich zur letzten Untersuchung an?
   ☒ Gleich ☐Besser ☐Schlechter

2. a) Wenn Sie sich **besser** fühlen, wieviel?

Wenig besser 0 1 2 3 4 5 6 7 8 9 10 Sehr viel besser

b) Wenn Sie sich **schlechter** fühlen, wieviel?

Wenig schlechter 0 1 2 3 4 5 6 7 8 9 10 Sehr viel schlechter

ANNEX VII. University of Cologne Ethics Committee Approval

Universität zu Köln

Geschäftsstelle Ethikkommission • Universität zu Köln • 50931 Köln

Herrn
PD Dr. Philipp Steven
Klinik und Poliklinik für Allgemeine
Augenheilkunde
Uniklinik Köln

- im Hause -

Per Fax:
0221 478 97836

Köln, 07.06.2016

Unser Zeichen: 16-145

Development of a new tool to detect changes in dry eye related symptoms and evaluation of its usefulness and effectiveness in a pilot study

Sehr geehrter Herr Dr. Steven,

die Ethikkommission der Medizinischen Fakultät der Universität zu Köln hat sich in ihrer Sitzung vom 02.06.2016 mit Ihrem Antrag befasst. Hierbei wurde Ihnen Gelegenheit zu mündlichen Erläuterungen gegeben. Die Beratung erfolgte nach § 15 Abs. 1 der Berufsordnung der Nordrheinischen Ärztinnen und Ärzte in Verbindung mit § 2 Abs. 1 der Satzung für die Ethikkommission der Medizinischen Fakultät der Universität zu Köln.

Der Antrag wird zustimmend bewertet. Die zustimmende Bewertung wird an den Eintritt der folgenden Bedingungen geknüpft:

1) Die Information und Einwilligungserklärung ist unter Berücksichtigung folgender Punkte zu überarbeiten und erneut vorzulegen:

   a) Die Anrede ist weniger direkt zu halten. Erst nachdem die angesprochene Person ggf. ihr Einverständnis erteilt, wird sie zum Teilnehmer.

   b) Über die Dauer der Teilnahme (Gesamtdauer, Dauer der einzelnen Untersuchungen) ist zu informieren (vgl. Abschnitt 4.8.10 s) ICH/GCP).

   c) In der Einwilligungserklärung sollte nochmals der Name und Sitz der für die Datenverarbeitung Verantwortlichen wiederholt werden (vgl. § 4 Abs. 1 (4) GDSG NW, Art. 10 a) 1995/46/EG).

Medizinische Fakultät der Universität zu Köln

Geschäftsstelle der Ethikkommission

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BIC BFSWDE31

Schreiben der Ethikkommission vom 07.06.2016
Antragsnummer 16-145

Begründung

Die Unterlagen, einschließlich des Studienplans und der Modalitäten für die Auswahl der Studienteilnehmer entsprechen dem Stand der wissenschaftlichen Erkenntnisse. Die vorhersehbaren Risiken und Nachteile der Studie sind gegenüber dem Nutzen für die Person, bei der sie durchgeführt werden soll, und der voraussichtlichen Bedeutung der Ergebnisse für die Heilkunde ärztlich vertretbar.

Hinweise

Die Ethikkommission bittet um Kenntlichmachung und optische Hervorhebung der nach den Anregungen des Votums der Ethikkommission geänderten Passagen bei erneuter Vorlage. Die Dokumente sind in einfacher Ausfertigung in Papierfassung und auf elektronischem Datenträger (z. B. CD) einzureichen.

Wir dürfen Sie darum bitten, die Ethikkommission unverzüglich von sämtlichen nachträglichen Änderungen im Studienplan (abgesehen von rein formellen) zu unterrichten, da sie eine erneute Beratung erforderlich machen.

Die Ethikkommission bittet darum über alle Vorkommnisse und Änderungen des wissenschaftlichen Erkenntnisstandes, die während der Studie bekannt werden und die Sicherheit der Teilnehmer oder die Durchführung der Studie beeinträchtigen könnten, unverzüglich informiert zu werden. Diese Information soll nur in Verbindung erfolgen mit einer Stellungnahme des Studienleiters, ob aus seiner Sicht das Nutzen-/Risiko-Verhältnis des Vorhabens verändert ist.

Die Ethikkommission bittet um Unterrichtung über den Beginn der Studie sowie ferner über einen möglichen frühzeitigen Abbruch der Studie. Wir bitten um Übersendung eines jährlichen Zwischenberichtes (vgl. Artikel 23 der Deklaration von Helsinki in der Fassung von 2013). Nach Abschluss des Projektes bitten wir um Übersendung eines Schlussberichtes.

Die Ethikkommission der Medizinischen Fakultät der Universität zu Köln setzt sich zusammen und arbeitet gemäß den nationalen gesetzlichen Bestimmungen. Hierbei werden die Grundsätze, wie sie in der „Note for Guidance on Good Clinical Practice" (CPMP/ICH/135/95) niedergelegt sind, berücksichtigt.

Schreiben der Ethikkommission vom 07.06.2016
Antragsnummer 16-145

Entsprechend der Funktion der Ethikkommission betrifft diese Stellungnahme nur die ethische Beurteilung der Konzeption, der vorgesehenen Methoden, Durchführung und Überwachung des betreffenden Projektes sowie der beabsichtigten Patientenaufklärung. Die ärztliche und juristische Verantwortung verbleibt jedoch uneingeschränkt beim Projektleiter und seinen Mitarbeitern, so dass alle zivil- oder haftungsrechtlichen Folgen, die sich ergeben könnten, von dieser Seite zu tragen sind.

Mit freundlichen Grüßen

Prof. Dr. Uwe Fuhr

Dipl.-Ges.-Ök. Christine Grimm

Liste der Beschluss fassenden Kommissionsmitglieder

Herr Prof. Dr. Frank Berthold

Herr Prof. Dr. Uwe Fuhr (Vorsitz)

Herr Prof. Dr. Kai Hübel

Frau Prof. Dr. Barbara Krug

Herr Dr. Dr. Frank Pluisch

Herr Dr. Kourosh Zarghooni

ANNEX VIII. Patient information sheet and informed consent for the observational prospective study evaluating the ECS-Q

UNIKLINIK KÖLN | Zentrum für Augenheilkunde

Information über die Teilnahme an der Studie

Entwicklung eines neuen Fragebogens zur Erfassung von Symptomen des Trockenen Auges

Sehr geehrte Dame, sehr geehrter Herr,

wir freuen uns, dass Sie an unserer Forschung zum Trockenen Auge interessiert sind. Im Folgenden möchten wir Sie über die Ziele und den Verlauf der oben genannten Studie informieren und Ihnen erklären, warum Ihre Mitarbeit im Falle einer Studienteilnahme wichtig ist. Die Studie wird zu Forschungszwecken durchgeführt.

Wir bitten Sie, diese Information sorgfältig zu lesen und anschließend zu entscheiden, ob Sie an dieser Studie teilnehmen möchten. Der Übersichtlichkeit halber wird im Folgenden auf die geschlechtsbedingte Unterscheidung „Patient / Patientin“ verzichtet.

Ziel der Studie

Unter der Leitung von PD Dr. med. Philipp Steven aus der **Klinik für Allgemeine Augenheilkunde** beschäftigen wir uns mit dem Beschwerdebild des sogenannten Trockenen Auges.

Im Rahmen dieser Studie möchten wir einen neuen Fragebogen testen und evaluieren, um so besser die Änderungen von Symptomen beim Trockenen Auge erfassen zu können. In dieser Studie sollen keine Therapieverfahren getestet werden, sondern nur Daten durch etablierte diagnostische Verfahren gesammelt und ausgewertet werden.

Die Ethikkommission der Medizinischen Fakultät zu Köln hat das vorliegende Forschungsvorhaben beraten und zustimmend bewertet.

Untersuchungsmethoden, Ausschlusskriterien und mögliche Nebenwirkungen

Innerhalb der nächsten 6 Monate sollen insgesamt 36 Patienten mit Trockenem Auge und 36 Probanden, die in trockenen klimatischen Bedingungen beruflich tätig sind, in die geplante Studie eingeschlossen werden.

An dieser Studie können alle erwachsenen Patienten/Probanden teilnehmen, die an einem Trockenen Auge leiden und ihr schriftliches Einverständnis zur Teilnahme an der Studie geben.

Nicht teilnehmen können Patienten/Probanden mit *mangelhaften deutschen Sprachkenntnissen*, und fehlender Einwilligungserklärung.

Da die Untersuchungen, die in der Studie durchgeführt werden allesamt Routineuntersuchungen sind, sind keine Risiken vorhanden.

Verlauf der Studie

Patienten der Spezialsprechstunde Trockenes Auge oder entsprechende Probanden werden mit standardisierten Methoden untersucht und füllen einen bereits etablierten und den neuen Studienfragebogen aus.

Bei einer zweiten Untersuchung in der Spezialsprechstunde nach Beginn oder Änderung der Augentherapie oder bei den Probanden am Arbeitsplatz nach längerer Exposition gegenüber der trockenen Umgebungsluft werden die Fragebögen ein zweites Mal ausgefüllt.

Aufklärung und Einwilligung

Nach der mündlichen Aufklärung durch den durchführenden Arzt und dem Durchlesen der Studienteilnehmerinformation geben die Teilnehmer freiwillig ihre schriftliche Einwilligung zur Teilnahme an der Studie.

Untersuchungen

Ärztliche Anamnese, Sehtest, Schirmertest (Messung der Tränenmenge), Tränenfilmaufrisszeit, Anfärbung der Hornhaut mit Fluoreszein, Anfärbung der Bindehaut mit Lissamingrün Spaltlampenuntersuchung, OSDI (Ocular Surface Disease Index)

Die Dauer der Untersuchungen insgesamt beträgt ungefähr 20 Minuten.

Abwägung von Nutzen und Risiko der Studie

Als Teilnehmer haben Sie von dieser Studie keinen direkten Nutzen. Man erhofft sich jedoch einen Nutzen für die Wissenschaft und die zukünftige Behandlung von Patienten mit **Trockenem Auge.**

Datenverarbeitung und Datenschutz

Im Rahmen der Studie werden Ihre Daten/Krankheitsdaten einschließlich der Daten über Geschlecht, Alter, Gewicht und Körpergröße pseudonymisiert, das heißt ohne Namensnennung, sondern nur codiert durch z. B. eine Nummer, aufgezeichnet. Eine Zuordnung ist nur über eine beim Studienarzt hinterlegte Identifikationsliste möglich. Nach Beendigung der Studie werden alle Daten nach den derzeit gültigen Vorschriften entsprechend gespeichert und archiviert.

Die Bearbeitung der erhobenen Daten erfolgt in Verantwortung von PD Dr. Steven, Zentrum für Augenheilkunde, Uniklinik Köln. Sie haben das Recht, Einsicht in Ihre Daten zu nehmen, die während der Studie erhoben werden. Sollten Sie dabei Fehler in Ihren Daten feststellen, so haben Sie das Recht, diese durch den Studienarzt korrigieren zu lassen.

Im Falle der Veröffentlichung von Studienergebnissen bleibt die Vertraulichkeit Ihrer persönlichen Daten ebenfalls gewährleistet. Einsicht in Ihre, beim Studienarzt vorliegenden personenbezogenen Daten, nimmt unter Umständen die zuständige Ethikkommission.

Ihre Daten werden über einen Zeitraum von 10 Jahren in einem sicheren System gespeichert und im Anschluss gelöscht, sofern gesetzliche Gründe nicht eine längere Speicherung vorschreiben.

Freiwilligkeit und Rücktritt von der Teilnahme

Die Teilnahme an dem Forschungsvorhaben ist ganz und gar freiwillig. Sie können jederzeit und ohne Angabe von Gründen Iihr Einverständnis zur Teilnahme zurücknehmen, ohne dass Ihnen hieraus irgendwelche Nachteile entstehen.

Probanden-/Patientenversicherung

Eine Probandenversicherung besteht für die vorliegende Studie nicht. Auch besteht keine Versicherung für Zwischenfälle, die Ihnen auf dem Weg zur Untersuchung oder nach dem Ende der Untersuchung auf Ihrem Rückweg widerfahren.

Mögliche Gründe für ein vorzeitiges Studienende

Es erfolgt eine kontinuierliche Datenauswertung. Die Teilnahme an der Studie wird abgebrochen:

- Auf Wunsch des Patienten
- Die gesamte Studie wird abgebrochen, wenn nicht ausreichend viele Patienten für diese Studie gewonnen werden können.

Aufwandsentschädigung

Eine Aufwandsentschädigung wird Ihnen für Ihre Teilnahme an der Untersuchung nicht gezahlt. Es entstehen Ihnen jedoch auch keinerlei Kosten durch die Teilnahme an der Studie.

Haben Sie weitere Fragen?

Sollten Sie noch weitere Fragen zum Ablauf der Studie, zum Datenschutz, zu Ihren Rechten, usw. haben, wenden Sie sich bitte an einen der Prüfarzte.

Information über neue Erkenntnisse

Ihr Prüfarzt wird Sie in einer angemessenen Frist auch über jede weitere wichtige, während der Studie bekannt werdende Information in Kenntnis setzen, die Ihre Einwilligung zur weiteren Teilnahme beeinflussen könnte.

Adresse und Telefonnummer des Studienzentrums

Zentrum für Augenheilkunde, AG Augenoberfläche
Leitung: Priv.-Doz. Dr. Philipp Steven
trockenes-Auge@uk-koeln.de
Tel.: 0221 478 4313

Einwilligungserklärung

- Ich habe die Probandeninformation gelesen und Ziel, Ablauf und Durchführung der Studie verstanden. Ich wurde mündlich über Wesen, Bedeutung, Tragweite und Risiken der geplanten Studienteilnahme informiert. Mir wurde ausreichend Gelegenheit gegeben, alle offenen Fragen mit meinem Prüfarzt zu klären. Ich habe jederzeit das Recht, weitere Informationen zur Studie zu erfragen.
- Ich erkläre mich freiwillig bereit, an der Studie teilzunehmen.
- Ich bestätige, vollständige und wahrheitsgemäße Angaben zu meiner Krankengeschichte, meinem Gesundheitszustand, zur Einnahme von Arzneimitteln sowie weiteren Fragen im Zusammenhang mit der Studie gemacht zu haben.
- Ich habe jederzeit das Recht, ohne Angabe von Gründen von der Studie zurückzutreten, ohne dass für mich Nachteile in der medizinischen Behandlung daraus entstehen.
- **Ich wurde darüber informiert, dass meine Daten in pseudonymisierter Form gespeichert, weitergegeben und analysiert werden.**

Einwilligungserklärung zum Datenschutz:

Bei dieser wissenschaftlichen Studie werden personenbezogene Daten und medizinische Befunde über Sie erhoben. Die Bearbeitung der

erhobenen Daten erfolgt in Verantwortung von PD Dr. Steven, Zentrum für Augenheilkunde, Uniklinik Köln. Die Speicherung, Weitergabe und Auswertung dieser Daten erfolgt gemäß gesetzlichen Bestimmungen und setzt vor Teilnahme an der Studie die folgende freiwillige Einwilligung voraus:

1. Ich erkläre mich damit einverstanden, dass meine Daten nach Beendigung oder Abbruch der Studie bis zu zehn Jahre aufbewahrt werden. Danach werden meine personenbezogenen Daten gelöscht, soweit nicht gesetzliche Aufbewahrungsfristen entgegenstehen.

2. Ich bin darüber aufgeklärt worden, dass ich jederzeit die Teilnahme an der Studie beenden kann. Dies schließt auch das Recht ein, meine Einwilligung in die Datenverarbeitung zu widerrufen. In diesem Falle wird der Personenbezug zu den Daten gelöscht.

Ich habe die vollständige Probandeninformation zur Studie sowie ein unterschriebenes Exemplar dieser Einwilligungserklärung erhalten.

_____

Vor- und Nachname des Studienteilnehmers

_____ _____

Ort und Datum (persönlich auszufüllen) Unterschrift des Studienteilnehmers

Mit meiner Unterschrift bestätige ich, dass ich diesem Probanden Natur, Ziel und mögliche Komplikationen dieser Studie erklärt habe, und dass ich ihm eine Kopie dieser Einwilligungserklärung ausgehändigt habe. Nach körperlicher und psychischer Verfassung war der Proband in der Lage, Wesen, Bedeutung und Tragweite der Studie einzusehen und seinen Willen hiernach zu bestimmen.

_____

Vor- und Nachname des Studienarztes

_____

Ort und Datum (persönlich auszufüllen) Unterschrift des Studienarztes