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Author: Raus, P.P.M.  
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Chapter 1: Introduction to DED:
the vision of the patient, the ophthalmologist, and the analytical biotechnologist.

1.0 General introduction.

Tears represent an essential component in the physiology of the eye. Quantity and quality of tear fluid are indispensable for an optimal eye function, and, thus, vision. Dry Eye Disease (DED), sometimes referred to as Dry Eye Syndrome, or less formally designated briefly as “Dry Eye”, is a frequently occurring ophthalmological condition, generally characterized by a reduced tear volume (quantity) and/or composition (quality).

In the daily practice of the ophthalmologist, each DED patient may represent a different clinical picture. DED symptoms vary dramatically and may evolve from a light irritation of the eye to a vision threatening pathology. Most of the many publications on DED therapy, however, are limited to symptomatic rather than causal treatment [Bhavsar et al., 2011]. Recent insights from individualized patient monitoring in our contemporary ocular surgery praxis by relevant tear analytics indicate that novel surgical DED treatments represent a highly effective alternative. We are convinced that a better understanding of the molecular causes and diversity of the disease etiology, will enable improved innovative diagnostics of the various subclasses of DED and hence a more precise therapy. Our work is aimed at novel DED diagnostics and improved treatment through clever application of innovative comparative analytics of human tears.

In this thesis, we describe our (co-)development of innovative surgical DED interventions, alongside with evaluations and implementation of novel mass spectrometry (MS) based protein analytics of human tear composition in healthy donors, as well as in DED patients.

As an introduction to our work, this First Chapter summarizes the positions and viewpoints of the three main stakeholders in the quest for innovation in DED diagnostics, analytics and treatment. The first part represents the viewpoint of an actual DED patient, describing the daily anxiety and problems encountered, and the hope for new and above all better diagnostics and treatment. Subsequently, we elaborate on definitions, physiology and current state of diagnostics and treatment from the viewpoint of the treating ophthalmologist. Finally, from the standpoint of the analytical biotechnologist we elaborate on new developments and possibilities of modern mass spectrometry as a promising tool for innovative approaches concerning DED.

1.1 Position of the patient.

Although not easily recognized by their surroundings, DED patients in most cases suffer from serious problems with eye functioning, severely impairing their everyday life. With current modern information technology prevailing, DED patients have access to and selectively read (parts of) the same scientific publications about novel DED treatments that are consulted by the best ophthalmologists worldwide. Several patient-oriented websites have become available, reporting on pathology, diagnostics and treatments (e.g. www.dryeyezone.com). Many patients subscribe to regularly appearing digital newsletters. Yet not all the information qualifies as peer-reviewed scientific reports, and/or are easily correctly interpreted by non-professionals. As a consequence, DED patients not seldom cultivate their own opinion
(prejudices?) and expectations (realistic or not) about their disease and treatment. Therefore, and regrettably, it is not an exception that patients are “shopping” between different physicians, in an attempt to secure the ‘most promising’ treatment in terms of efficacy and sustainability.

The following section is a representative, advocating, personal account from a contemporary DED patient.

Testimony of a DED patient, Mr. Zachary de Silva, Melbourne, Australia.

“My Dry Eye began in my early twenties caused by Meibomian gland dysfunction. Following the advice from a pharmacist I began to use artificial tears as the mainstay of treatment to control symptoms. While these provide temporary relief they never truly resolved the condition which continued to progress into severe symptoms which have become the norm, the control of which requires both lifestyle adjustments and advanced management techniques.

Compounded with a lack of help from specialists and being treated like a hypochondriac for many years, I decided to study this disease myself learning everything I could from scientific journals, articles and by communicating with leading researchers across the globe to understand the topic in a concerted effort to find solutions for all patients. My work in this field has galvanized the eye care community (particularly in Australia) to consider Dry Eye patients more seriously and manage/care for them more effectively. This is a common story amongst multiple patients that have contacted me over the years as they are often mistreated by their doctors and require psychological help in addition to their ophthalmic needs from a specialized Dry Eye clinic.

To this day I am actively researching and keep the eye care community up to date on all DED developments via an advocacy website (www.australiandryeye.webs.com) as a result of this growing and global public health issue. AustralianDryEye is recognized worldwide as a focal point for patients, specialists and scientists with an interest in dry eye as well as the general public to learn about the problem, new research and other latest news that I frequently disseminate in the latest news section. In addition, I have travelled twice to the United States to meet with leading scientific teams and clinics for further learning and am also consulted by local clinics (and patients) for advice and help.

Today, there is a plethora of research being conducted on Dry Eye around the world. Of the more promising projects include: ocular microbiome, lacritin (LACRT), lubricin, glycoproteins, atorvastatin, RNAi therapeutics and many others. My research compendium now accounts for over 50 active dry eye drug candidates in the development pipeline, with Restasis & Xiidra being the only two FDA approved prescription medications.

The digital age has caused an epidemic of Dry Eye across the developing globe. An estimated 300 million people around the world suffer with dry eye of varying severity. Dry Eye can occur from side effects of medications, laser eye surgery, pollution, poor diet, Sjögren’s syndrome and many other causes and it is the most common reason for a visit to an eye care professional, therefore the largest unmet need in ophthalmology. The potential market for newer generation Dry Eye diagnostics and treatments is vast and lucrative which is expected to reach $4.5 billion by 2020.
Current mainstay therapies such as artificial tears only offer transient relief and do not treat the causes of the diseased tear film / ocular surface thus are not a logical for patients. Both Restasis and Xiidra have known side effects including burning upon instillation and the latter frequently causing dysgeusia. In terms of topical therapy, there are no effective solutions available for Dry Eye that target the root cause of the issue for this cannot be achieved until further research is conducted to understand what exactly is contained in healthy human tears using newer technology; then comparing this to patients’ tear films. No existing product has a high success rate, Restasis studies demonstrate 15% improvement and Xiidra yet to be determined. These poor success rates can and must be improved with further research.

The rationale behind a proteomics analysis of clinically sampled tears is that comparative protein composition analysis of tears from diseased versus treated and/or healthy eyes, may yield medically relevant information regarding both the effectiveness of the treatment and the possible disease etiology. This could theoretically lead to new customized topical therapies which can be targeted at different types of dry eye rather than a one-size fits all approach. There is also the potential to develop newer diagnostic devices via further analysis of the tear film which is commercially interesting given the boom in interest in dry eye in recent times and epidemic of new patients each year in the digital age.

Dry Eye patients suffer from escalating anxiety and stress, debilitating physical pain, and an overall reduced quality of life. It affects ability to drive, use a computer, read, work outdoors, or do any fine detail work. This, in turn, affects the ability to support themselves in employment or even enjoy basic recreational and social activities, which has a profoundly negative impact on mental health. The impact of dry eye is real to each patient, not theoretical and it can be severely debilitating in many cases.

The potential of this new research project is immense as there are still many unanswered questions of the dry eye puzzle that can only be uncovered with further research. There is the possibility of categorizing patients via protein analysis which theoretically could lead to customized diagnostics and treatments for patients, both of which are commercially interesting due to the unique technology available to help shed further light on the puzzling tear film. I encourage and welcome sponsorship for this research from a pharmaceutical company or via crowdfunding as there is plenty of upside to the results that have been uncovered in preliminary tests thus far.

Despite being a challenging topic Dry Eye also provides a good opportunity for further research as there are still many unknowns about the condition. These types of studies are both important for both medicine and humanity in general as there are many positive outcomes that are possible upon completion of the analysis. My hope is that funding for this project will be sourced or approved from the correct channels to ensure the future of new research in this neglected area of ophthalmology.”
1.2 Current state-of-the-art and clinical outlook: DED ‘in the eyes of’ the Ophthalmologist.

Dry eye disease is the cause of an extremely important economic burden worldwide. Taking into account the growing number of people in the risk group of DED, the estimated total treatment cost per year has been calculated to increase to US $55.4 billion per year in the next 30 years... for the US alone [DEWS, 2007]. And this amount does not include the costs of sickness and work-loss.

DED today is still considered an incurable condition. Accordingly, there is little or no financial compensation for the time and effort the ophthalmologist spends with the patient to treat the disease. Ophthalmologists, therefore, are not lying when they complain about their lack of time and resources in their daily practice to make a correct (differential) diagnosis, let alone prescribe the correct treatment.

This obviously is particularly cumbersome for the DED patient, as complaints and clinical indicators are extremely varying, and diagnostic tests have a large variability. In addition, the specific complaints of the patient may not always match with the clinical symptoms, as observed by the ophthalmologist.

Up to 54.3% of the DED patients over 40 years of age visiting an ophthalmologist report dryness or associated uncomfortable factors [Suchi et al. 2015]. However, the result of even a simple refraction analysis can be extremely variable in DED, leading to unease and annoyance with both patient and physician.

To support the ophthalmologist in making a first subdivision in the heterogenic population of DED patients, one of the leading the pharmaceutical companies in ophthalmology (Allergan), has developed the ‘Ocular Surface Disease Index’ (OSDI) [Asiedu et al., 2007]. With the help of a questionnaire of 12 easy multiple choice questions the ophthalmologist or general practitioner (GP) calculates the patient’s OSDI score, which is a measure of the disability of his DED patient. It is an easy instrument to differentiate between healthy subjects and patients with normal, mild to moderate, or severe DED. In addition, it scales the effect of DED on vision-related eye function.

Definition of ‘Dry Eye Disease’: What is DED?

Due to the complexity of the multifaceted Dry Eye Disease (Fig.1.1) it has taken several decades for ophthalmologists to agree on a definition of DED, which could meet acceptance by the majority of colleagues working in this domain. The currently prevailing definition of DED is:

“A multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance and tear film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface.”

As such DED is defined by the Dry Eye Workshop (DEWS) [Lemp, 2007], a team of international experts who have labored over 3 years to compile an evidence-based review of the present knowledge of DED and of the current methods used to evaluate, diagnose, and manage the disorder.
DED is classified into two subgroups: the aqueous deficient and the evaporative form [Stern et al., 2004]. “Aqueous deficient” DED results from reduced lacrimal tear secretion and volume, whereas “evaporative” DED results from excessive water loss from the ocular surface in the presence of normal lacrimal secretory function.

The tear fluid, the various gland cells that secrete the different components constituting the tear, and the ocular surface are inseparably linked, and should be examined in combination in DED patients. In the context of tears and DED, this is termed the ‘lacrimal functional’ unit or LFU [Lemp, 2007; Stern et al., 2003] (Fig. 1.2).

**Tears and the ocular surface.**

DEWS [Lemp, 2007] defines the LFU as “the integrated functional unit comprising the lacrimal system, the ocular surface and its accessory glands and their neural interconnections”. As such
the LFU “is responsible for the maintenance of the tear film, for protection of the transparency of the cornea and of the health of the ocular surface”.

The Ocular Surface System (OSS) consists of the contiguous epithelia of the ocular surface, the cornea, conjunctiva and the tear compound producing glands. Whereas all these anatomical structures share the same embryological ectodermal origin, ophthalmologists discern 3 major gland types: the Meibomian glands in the eyelids, the goblet cells of the conjunctiva, and the (main and accessory) lacrimal glands. All components of the system are linked functionally, by continuity of the epithelia, by innervation, and by the endocrine, vascular, and immune system. The tear fluid thus contains information, which may tell the clinician much about the patient as a whole [Paulsen et al., 2003]. The Meibomian (or tarsal) glands are a special kind of sebaceous glands, which secrete their substance at the rim of the eyelids. This so-called ‘meibum’ prevents evaporation of the eye’s tear film [Qiao et al., 2013]. In addition, the eyelashes have associated glands, the glands of Moll (or ciliary glands; a kind of apocrine sweat glands), and the glands of Zeis (small sebaceous glands located on the edge of the eyelid at the basis of the eyelashes [Takahashi et al., 2013]. Strictly spoken, the ‘tear’ (lacrima) refers to the secretion product of the main lacrimal glands, and as such only one of the many components of what in clinical practice is called the ‘tear film’.

It makes sense to also include the nasolacrimal duct in this overview (Fig. 1.3). Indeed, the latter system controls and regulates tear outflow by adsorbing tear components through its cavernous vascular system.
Fig. 1.3. Drawing showing position of nasolacrimal duct and lacrimal gland around human eye [adapted from MAYO Foundation for Medical Education and Research, www.mayoclinic.org/diseases-conditions/dry-eyes/diagnosis-treatment/drc-20371869].

By doing so the nasolacrimal epithelium helps to maintain an appropriate tear level: a continuous and delicate balance between secretion and outflow [Stern et al., 2004].

The tear film.
The tear film is a tri-layered structure consisting of a mucous, an aqueous and a lipid component [Gipson, 2007] (Fig. 1.4).

- The mucous layer primarily consists of mucins secreted by the so-called goblet cells of the conjunctiva and transmembrane glycoproteins. It forms the interface between the hydrophobic cell membranes of the ocular cells and the hydrophilic (aqueous) tear film, protecting the underlying conjunctival and corneal epithelium from sheer force damage, drying, and bacterial
invasion. The thickness of the mucous layer is 0.8 micron [Stern et al., 2004]. Some studies consider the mucous and aqueous layer as one [Qiao and Yan, 2013]. However, we prefer and maintain the model of three separate layers in the tear film (Fig. 1.4). Indeed, DED as caused by a problem of mucins, is known to require a different approach and treatment in clinical practice.

- The **aqueous layer** is the thickest of the three layers (7 to 8 micron). It consists mainly of the secretions by the major and accessory tear glands, and consists of electrolytes and water-soluble proteins with a multitude of functions. These play an important role in protecting the eye against external threats: (micro)biological, chemical, or mechanical. The function of the aqueous layer, hence, goes far beyond a ‘simple’ wetting of the ocular surface.

- The **lipid layer** is mainly secreted by the Meibomian glands in the eyelids. With a thickness of a mere 0.1 micron, it acts as a thin shield to limit the tear film evaporation [Qiao and Yan, 2013].

Importantly, the tear film also helps to keep the eye surface clean and transparent, playing a crucial role in the refractive power of the eye. Instability of the tear film leads to cell hyperosmolarity, which is a key factor in the cascade of apoptosis and inflammatory reactions. This in turn increases the tear film instability, thus closing the circle (Fig. 1.1) [liu et al., 2009].

**Diagnosis of DED.**
Clinical assessment of DED starts with a routine eye examination that includes a complete anamnesis of the patient’s overall health. This is essential to make a correct differential diagnosis of the potential cause and the subtype of DED, and it must rule out other ocular pathologies.

Several tests can be used to measure the type and degree of DED. Overall, they can be divided into two main groups, evaluating tear quantity and quality.

Measuring the volume (quantity) of tears: The most commonly used test is the Schirmer test [Shapiro et al., 1979; Schirmer, 1903]. In this test, a blotting strip of paper is placed between the lower eyelid and the eyeball. After five minutes the length of the strip wetted by the patient’s tears is recorded. A representative result within normal limits is ≥15 mm. The test can be done with or without previous administration of a topical anesthetic into the eye before the filter paper to prevent irregular tear production due to the irritation from the paper. This is done to help ensure that only basal tear secretion is measured. Measuring the height of the tear meniscus has also been shown to be a useful alternative to obtain an idea about the volume of secreted tears [Mainstone et al., 1996].

Determining the quality of tears: With the use of special dyes in eye drops, the surface condition of the eye can be evaluated. E.g., after instillation of a Fluorescent dye, like fluorescein, or another colorant, like Rose Bengal, the staining patterns on the ocular surface (cornea and conjunctiva) can be assessed. In addition, the time it takes for tears to evaporate, the so-called tear break-up time (BUT) can be recorded [Savini et al., 2008]. To clinically measure BUT, fluorescein is administered and the patient is asked not to blink while the tear film is observed under a broad beam of cobalt blue illumination with a slit lamp. The BUT is recorded as the number of seconds that elapse between the last blink and the appearance of the first dry spot in the tear film. A DED patient typically has a BUT <10 sec.
As indicated in the definition of DED, the osmolarity of tears is an important parameter in making a correct diagnosis. An instrument like the Tearlab [Benelli et al., 2010] utilizes temperature-corrected impedance measurement to provide an indirect assessment of osmolarity. This osmolarity test employs a clinically usable test card held by an ‘Osmolarity Test Pen’, to collect nanoliter quantities of tear fluid directly from the eyelid margin [Sullivan et al., 2010, Benelli et al., 2010]. Although tear osmolarity per se is not conclusive in the daily practice, in conjunction with the fluorescein staining pattern described above, the TearLab readout is highly valued in DED diagnosis. However, since in both aqueous tear deficiency and evaporative DED the tear osmolarity is increased, this system does not make a differential diagnosis between those two subgroups.

DED in the doctor’s office.
The more patients studied, the more complex ‘Dry Eye’ appears to be. The list of possible causes/risk factors for DED continually gets longer:

- age: DED is more common in patients over 50 years;
- medications: antihistamine drugs, antidepressants, hormone replacement drugs, sleeping medication, drugs against Parkinson’s disease, antihypertensive agents, have all been reported to present a DED risk factor;
- topical drugs and the preservatives used therein;
- blepharitis and skin diseases like acne rosacea and atopic dermatitis;
- Sjögren’s syndrome and other autoimmune diseases;
- infectious keratitis and/or conjunctivitis;
- sex: women are more likely to develop DED, especially after the menopause;
- air quality: windy environments, especially in dry atmospheres (air conditioning, airplanes, ...);
- allergy;
- eyelid margin irregularities;
- neurotrophic causes;
- insufficient blinking during prolonged computer screen or TV watching;
- contact lenses; and
- refractive laser surgery, ...

When a diagnosis of suspected DED is made, the ‘Triple Classification of Dry Eye for practical and clinical use’ is typically employed. It was developed by an international group of ophthalmologists (including the author of this Thesis), specialized in DED [Murube et al., 2005; see Appendix 1.1]. ‘Triple Classification’ refers to the fact that the classification differentiates DED patients based on:
1. the etiopathogenesis,
2. the glands which are affected by the process, and
3. the severity of the clinical presentation which guides the ophthalmologist in his/her differential diagnosis.

This classification, although very complete and still a golden standard for ophthalmologists worldwide, is limited in that it only looks at DED from the point of view of the ophthalmologist in current daily practice. To broaden this clinical vision, and to explore new possibilities to treat
DED, we anticipate to additionally evaluate DED from an analytical bio(techno)logy/biochemistry viewpoint.

**Therapeutic options for DED patients.**

Whereas for the majority of cases no definite cure is available today, when taking into account the pathogenesis, clinical image, and severity of the problem, three different classes of options are currently considered as treatment for the DED patient: (1) increasing the tear (or tear components’) volume, (2) decreasing the loss of tears, and (3) improving the tear quality. Unfortunately, in most patients, none of these therapies addresses the actual underlying cause of the disease. Only incomplete and short-term relief is given, and, therefore, frequent treatment (reapplication) is required.

(1) Increasing the volume and/or the secretion of tears.

The easiest and most frequently used first treatment is the administration of a substitute for the biological tears, including artificial tears, gels or ointments. The choice of product to use will be determined after an evaluation of all above mentioned parameters. An evaporative DED patient will benefit from an artificial tear containing (more) lipids, whereas a severe DED patient may require a gel during the day and a protective ointment during the night.

In specific cases drugs are applied that increase the secretion of tears or components of normal tears. Examples include pilocarpine, rebamipide and diquafosol. Pilocarpine is a parasympathico-mimeticum that directly stimulates the glandular acini of the tear gland and, therefore, represents an effective option in treating severe DED. Because of the local side effects like miosis and temporarily myopia of the eye, an oral application is preferred [Kawakita et al., 2015]. Rebamipide increases the production of both membrane-associated and secreted-type mucins by the conjunctival goblet cells and corneal epithelial cells [Hirotaka et al., 2014]. Diquafosol ophthalmic solution 3% (available in Japan but repeatedly rejected by the US FDA) promotes both tear fluid and mucin secretion [Keating et al., 2015]. Meibomian glands can be stimulated to secrete with Intense Pulsed Light (IPL), thermal pulsation or intranasal tear neurostimulation [Craig et al., 2015; Lane et al., 2012; Pondelis, 2017].

In severe cases, surgical treatment of DED can be considered. Transplantation of labial or other salivary glands to the eyelids, obviously, is a more invasive procedure attempting to increase the tear volume. The technique was first described by prof. Juan Murube, who was motivated by the similarity between the composition of natural tears and saliva [Murube, 1998]. As described in Chapter 3.2 we co-developed a modified surgical technique together with Murube and Geerling. This has dramatically improved the quality of life of many patients. Based on our previous experience in eyelid surgery (described in Chapter 2), we were able to modify the original procedures by Murube to a much more patient-friendly procedure.

(2) Decreasing the loss of tears, and stimulating tear reabsorption.

The most logical way to decrease tear loss is to limit the tear outflow or to delay their evaporation. This can be achieved reversibly by putting an absorbable or non-absorbable plug in the tear *punctum* or tear duct [Yung et al., 2012]. A more permanent (virtually irreversible) way to limit or stop the outflow of tears through the tear ducts is to coagulate one or more tear *puncta*. This technique is discussed in Chapter 3.1.
It is appropriate to mention the sporadic administration of hyaluronic acid in artificial tear drops. Through its viscoelastic properties, hyaluronic acid attracts more water molecules, thereby delaying tear dehydration [Shimmura et al., 1995; Saeed et al., 2013]. To prevent the evaporation of tears, the emphasis is on the lipid layer of the tear film, which acts as a physical barrier to slow down the drying up of the tears. Also, a considerable physiological reabsorption of tear fluid across the mucosa of the nasolacrimal duct (see Fig. 1.3) during its passage has been described [Paulsen et al., 2003].

(3) **Improving the quality of tears.**

It may be clear that the above-mentioned tactics to increase the quantity of tears in the clinical practice have different and large effects on the composition, and thus the quality, of tears. Improving the quality of tears e.g. by making the tears more viscous, can contribute to keeping the tear on the ocular surface. Examples include the administration of Rebamipide or Diquafosol, or the use of artificial tears containing a mucimimetic component to smoothen the cornea and slow down tear evaporation, through increasing its mucin composition. The use of hyaluronic acid in tears (see above) may lead to increased tear stability, reduction of tear removal, and protective effects on the corneal epithelium.

Recently a bioactive fragment of the lacrimal glycoprotein lacritin, called Lacripep™ has entered clinical trials for its potential positive activating/stimulating effect on all tear component producing glands (see Fig. 1.4), including the goblet cells of the conjunctiva [Samudre et al., 2011; www.ClinicalTrials.gov, 2017]. We deem it highly interesting that (fragments) of endogenous protein compounds are currently entering clinical practice in attempts to increase tear quality and function. Indeed, this development strengthens our view that functional peptides (small proteins) will play an increasingly important role in future medical care [Uhlig et al. 2014], and certainly also in ophthalmology. This exactly underscores our decision to focus on proteomics-type research for innovative biochemical tear analysis.

### 1.3 The perspective of the Analytical Biochemist/Biologist, *i.e.* a clinical (proteomics) biotechnologist.

In this thesis, we refer to *Analytical Biotechnology* as the modern suite of techniques which enable the analysis of biomolecules present in a biological (biomedical, clinical, ...) sample. As such, the analytical biochemist/biotechnologist of today has countless new and powerful tools to study DED, as well as other medical conditions.

Because of the large variety of biomolecules, analytical biotechnology is a very broad discipline. Considering the basics of biology, it can focus on the genes (the DNA) in a system under investigation, on its transcripts (the (m)RNA), its gene products (the proteins), or even on the enzymatic products resulting from the proteins’ activities (the metabolites). The latter predominantly are small molecules, derived from amino acids, lipids, carbohydrates, or a combination of these. Whereas biochemists in the past were not seldom focusing on a single (class of) biomolecule, today the trend is to assess the data of the simultaneous analyses of many relevant biomolecules together. This so-called ‘Systems Biology’ approach is often referred to as ‘multi-omics’. As such it combines *genomics, transcriptomics, proteomics* as well as *metabolomics* (including *glycomics* and *lipidomics*) data [Lauwen et al., 2017]. In theory, each of those technologies can be employed to study DED (and any other disease), and to try and assess the effects of different disease treatments. All of those may claim to enable the
discovery of specific DED biomarkers, which may help to understand the pathophysiology of DED. Yet, a routine combined analysis of all of those analytical levels is unrealistic in the clinical practice.

It is evident that those modern analytical technologies imply a significant cost. Especially in (modern) medicine, an -omics approaches that should never be forgotten is economics. Thus, a full-blown Systems Biology approach is not a realistic option in the daily clinical practice is not realistic.

Therefore, the analytical biotechnologist is faced with the challenge to select and target the most relevant biomolecules to target with the best performant analysis protocols, using the best instrumentation available at present.

In the context of this thesis we considered three criteria to be crucial in selecting the optimal analytical approach at stake:

1. compatibility with clinical sampling (i.e. consistent with routine daily sampling in a clinical setting)
2. providing relevant information with regard to the disease biology addressed (i.e. on molecules as close to the disease mechanism as possible)
3. future translatability into clinical use (i.e. ease of use while maintaining quality sampling and scaling-up)

Based on the vision that it is the proteome which bridges the gap between the genes and cellular behavior, we opted to focus our efforts on a specific protein subset of the “eyeome” [https://www.hupo.org/Human-Eye-Proteome-Project]. Less indirectly than genomics, transcriptomics or metabolomics, the discipline of proteomics studies the actual gene products, as effective outcomes of the translation of the messenger RNAs which have been transcribed from specific genes in a specific physiological, developmental, pathological condition. Indeed, the proteins are the major effectors in the (regulation of) functions of any living cell, tissue or organ. Many, if not all, diseases manifest themselves at the level of the proteins. Specifically aiming at expanding our understanding of the pathogenesis of the wide varieties of DED, we set out to study those eye-proteins which are clinically the most easily accessible while least invasively sampled from the patient, namely the proteins of the tears themselves.

**Bottom-up proteomics in DED**

We set out with the working hypothesis that DED, and by extrapolation the effect of a DED treatment, would be reflected in an altered protein composition in a patient’s tear. Our initial proteomics experiments addressed the question whether the technology would be sensitive enough to enable protein identification in samples from one single patient. A first successful pilot experiment was run in 2007 (described in Chapter 4). This paved the way for follow-up experiments through which the clinical success of our published DED treatment of labial salivary gland transplantation could be monitored and confirmed through the analysis of tear proteins. Traditional “bottom-up” proteomics confirmed that a considerable overlap exists in the proteins identified in both the tears and the saliva of the same individual. Bottom-up proteomics, also known as shotgun proteomics derives the identity of the proteins in a biological sample from the mass spectra of enzymatic fragments, generated in vitro by a sequence specific protease, typically trypsin [Schrader et al., 2014].
Our initial results were obtained with a relatively primitive proteomics workflow, employing gel electrophoresis as protein separation technology, followed by trypsin digestion and nano-electrospray quadrupole time-of-flight (Q-TOF) mass spectrometry of the tryptic fragments (Chapter 4). In those early days data were acquired on a limited sample set; these, however, perfectly matched with data published later (references in Chapter 5) by various dedicated groups involved in substantially larger studies on tear protein analyses employing sample pools of large cohorts of patients and healthy donors.

From 2010, we obtained access to a high performance linear ion trap-orbitrap hybrid mass spectrometer. With this, we performed highly sensitive bottom-up proteomics on clinical samples. In the meantime, we had tested various different systems by which sufficient amounts of tear fluid (especially from DED patients) could be collected. Classically, tear collection used glass capillaries or porous polyester rods [Jones et al., 1997]. In our hands, the most elegant, and easiest to standardize, method appeared to be absorption on a Schirmer filter paper strip. Such collection involves less mechanical irritation than glass capillaries resulting in less discomfort for the patient [Stuchell et al., 1984]. We experienced that manipulation, storage (refrigerated or otherwise), as well as instant heat stabilization treatments, are much easier to accomplish with tear samples (Chapter 6, section 6.4) dried on a Schirmer paper strip, than when collected as tiny drops stored in capillaries or in small size polypropylene tubes. In brief, we showed that replicate proteomics measurements can be done on sample sizes compatible with single individual collections in the clinic (Chapter 5). This has opened up an analytical avenue to monitor tear protein variations within individual patients, at different times prior to as well as during treatment/therapy. The trends in protein variations of biomarker candidates proposed in the literature were nicely confirmed at the level of our individual patient/donor measurements (Chapter 5).

**From bottom-up to top-down proteomics**

To get around the limitations in the interpretation of bottom-up proteomics results, we set up an experiment to evaluate the performance of the latest state-of-the-art mass spectrometry instrumentation with our optimized tear sampling method, for “top-down proteomics”. In this approach, no trypsin (or alternative protease) is used prior to protein identification. As a result, several different species of a protein (now commonly called proteoforms) can be readily identified, whereas in “bottom-up” approaches a positive protein identification basically means no more than the identification of the genes which were translated, without a direct link to the actual protein species/proteoforms present in the sample.

Comparative bottom-up and top-down analyses of the very same samples illustrated the state-of-the-art of the technology. As expected, top-down analyses “identified” significantly less proteins (expressed protein genes). Approximately a quarter to a third of the bottom-up identifiable expressed genes are identified in a top-down experiment. However, the latter confirms the presence of multiple proteoforms of the large majority of the proteins in tears. This layer of information is almost totally missed/ignored in bottom-up experiments. Different (modified) forms of a protein are known to potentially have different biological activities [see e.g. Tran et al., 2016], and may thus play different roles in the physiology of the natural tear film. Hence, we decided to focus on the possibilities and opportunities offered by modern proteoform analyses for future implementation in ophthalmology diagnostics and treatment. We are thankful and proud to have been able to obtain access to the latest software and hardware developments through a highly appreciated collaboration with the research group of
Prof. N. Kelleher at Northwestern University (Chicago, IL, USA) and with the team of Dr. D. Lopez-Ferrer at Thermo Fisher Scientific (San Jose, CA, USA). Our results of a set of carefully planned exploratory experiments are described in Chapter 6.

1.4 Conclusion.

The continued dialogue between the ophthalmologist, the analytical biotechnologist and the patient is essential to create new insights into the complex pathology of DED. With all considerations, trials and experiments of the previous years and with the results presented in this thesis we conclude (Chapter 7) that we are indeed getting closer to an effective implementation of modern protein analytics in studying and monitoring DED at the level of the individual patient.

We hope that through the research and results presented in this thesis, we may significantly contribute to improved, non-invasive and personalized diagnostics of DED, culminating into better and more effective treatment of the various forms of this disease. Moreover, we make a case for a comparable, modern proteomics-based approach toward improved diagnostics in other clinical conditions, leading the way to personalized diagnostics and treatment in a broader medical setting. By formulating suggestions to go forward, we hope that future clinical application of our approach and findings will not remain a dream for much longer.

References:


Appendix 1.1 Triple classification

The triple classification of dry eye for practical clinical use

J. MURUBE¹ (Spain), J. NÉMETH (Hungary), H. HÖH (Germany), P. KAYNAK-HEKIMHAN (Turkey),
J. HORWATH-WINTER (Austria), A. AGARWAL (India), C. BAUDOUIN (France),
J.M. BENÍTEZ DEL CASTILLO (Spain), S. CERVENKA (Czech Republic), L. CHENZHUO (China),
A. DUCASSE (France), J. DURÁN (Spain), F. HOLLY (USA), R. JAVATE (Philippines), J. NEPP (Austria),
F. PAULSEN (Germany), A. RAHIMI (Iran), P. RAUS (Belgium), O. SHALABY (Egypt),
P. SIEG (Germany), H. SORIANO (Argentina), D. SPINELLI (Italy), S.H. UGURBAS (Turkey),
G. VAN SETTEN (Sweden)

¹Department of Ophthalmology, University of Alcala, Madrid - Spain

ABSTRACT. Clinicians need a practical classification to face diagnosis, prognosis, and treatment. Dry eyes have many etiologies and pathogenesis, different affection of the various dacrtyoglands and ocular surface epithelium, and diverse grades of severity. The specialists in xero-dacryology must know these three parameters to evaluate any case of dry eye, and to establish an adequate treatment. To facilitate this, an open session in the VIII congress of the International Society of Dacryology and Dry Eye (Madrid, April, 2005) proposed modifying the Triple Classification of Dry Eye approved in the XIV congress of the European Society of Ophthalmology (Madrid, June 2003). The following classification has been established: first, a classification of the etiopathogenesis, distributed in 10 groups: age-related, hormonal, pharmacologic, immunopathic, hyponutritional, infectious/inflammatory, traumatic, neurologic, and tantalic; second, a classification of the affected glands and tissues, which under the acronym of ALMEN includes the aqueo-serous deficient, lipodeficient, mucin deficient, and epitheliopathic dry eyes, and the non-dacryologic affected exocrine glands (i.e., saliva, nasal secretion, tracheo-pharyngeal secretion); third, a classification of severity, in three grades: grade 1 or mild (symptoms without slit lamp signs), grade 2 or moderate (symptoms with reversible signs), and grade 3 or severe (symptoms with permanent signs). (Eur J Ophthalmol 2005; 15: 660-7)

KEY WORDS. Dry eye, Tears, Triple Classification

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INTRODUCTION

Dry eye is a lexicon in common use among scientists, patients, and the general population, which like any other word may have diverse, fluctuating, and changing meanings. The term dry eye is usually referred to a symptom, a sign, a syndrome, and many diseases. The scientific definition of the syndrome of the several diseases is that “dry eye is a disorder produced by the inadequate interrelation between lacrimal film and ocular surface epithelium, caused by quantitative and qualitative deficits in one or both of them. It can be produced by one or combined etiologic causes, affect one or several of the secretions of the glands serving the ocular surface, and produce secondary manifestations of different grades of severity.”

Dry eye is one of the most frequent ophthalmologic conditions. It can be produced by hundreds of causes. Dry eye diseases are almost always chronic, progressive,
and until the present incurable. Usually they produce mild or moderate manifestations, but in severe cases they provoke incapacitating discomfort and severe low vision. The prevalence of dry eye syndrome is not well established as it changes with sex, race, geography, epoch, socio-sanitary levels, age, and severity. By using the two last variables (severity and age) it is possible to calculate approximately that grade 1 is present in 1% of the population under 30 years, 20% between 30 and 60 years, and 100% over 60 years. Grade 2 is present in 0.1% of people under 30 years, 1% between 30 and 60 years, and 10% over 60 years. Grade 3 is present in 0.002% of persons under 30 years, 0.01% between 30 and 60 years, and 0.1% over 60 years. Women are usually more precociously affected than men.

The evolution of our knowledge on dry eye was at first slow, then it became quicker, and it is at present, vertiginous. The historical evolution can be divided into three eras: Hippocratic (from Hippocrates to the end of the 19th century), Sjögrenic (last years of the 19th century, and 20th century), and 21st century. The limits among these three periods are not precise and brusque, and the two connecting periods overlap in a progressive transition. These three periods correspond approximately with the knowledge and evidence of severe (classical xerophthalmia), moderate (keratitis punctata, keratoconjunctivitis, sicca), and mild dry eye (symptoms of dry eye without slit lamp signs).

During the transition years between Sjögrenic and 21st century periods many etiopathogenetic causes and combinations, glandular affectations, and severity manifestations of clinical combinations were discovered or became better known. It was evident that a classification for practical clinical use was necessary. Therefore, at the XIV Congress of the European Society of Ophthalmology, held in Madrid in June 2003, it was decided that one of the preferential tasks of xerodacryology was to make a classification for practical clinical use. It was presented, discussed, decided, and published as the Madrid Triple Classification of Dry Eye (1). Two years later the 8th Congress of the International Society of Dacryology and Dry Eye took place in Madrid, in April 2005, and it was decided to discuss and improve the previous classification. The results are reported in the present article.

When a clinician receives, examines, and determines the characteristics of any dry eye, he or she needs to know several characteristics throughout the anamnesis and examination in order to elaborate a diagnosis, prog-

nosis, and treatment. For practical clinical use, dry eye should be expressed by means of three parameters: etiopathogenesis, damaged exocrine glands and tissues, and severity.

Classification according to the etiopathogenesis

The many causes that can produce a clinical dry eye can be distributed for practical clinical use in 10 groups (Tab. 1). The first five groups of this etiopathogenetic decalogue generally, but not always, affect many exocrine glands (lacrimal, salivary, nasal, vaginal, etc.) because the damage is usually produced in cellular structures common to exocrine glands. The last five groups usually only affect the dacryoglands (aqueo-serous, lipid, mucinotic), or even only some of them, or even only those of one eye.

Age-related. With aging, all cellular structures of the body undergo a progressive apoptotic process. This also affects all exocrine glands, and consequently there is presentation of a general dryness in the body, including the lacrimal glands. The lacrimal secretion begins to diminish from the age of 30 years, but as there is an overabundance for the normal necessities, it is only noticed by people in situations of overexposure. The critical level between production and necessities is reached at about 45 years. Production decreases at about 60 years, when secretion becomes insufficient for the necessities of some normal situations. Many persons over 60 feel symptoms or signs of dry eye in circumstances such as late afternoon or at night when the circadian rhythm of tear production is lower, when working for a long time in front of a video display terminal (VDT) doing convergence in horizontal, when using contact lenses, or in drafts or dry environment, as tear evaporation leads to an increase in tear film osmolarity.

Age-related dry eye is usually multiexocrinic (eye, mouth, nose, tracheo-pharynx, vagina, etc.). As to the severity, it is usually grade 1, but frequently reaches grade 2.

Hormonal. Lacrimal secretion is influenced by some endocrine gland activity, the most important of which are androgens, estrogens, and prolactin. Dry eye is frequently a hormone-related condition in cases of aging, castration, antiandrogenic treatment, hypovarism, ovariectomy, climacteric, menopause, estrogenic contraceptives, and lactation. Other hormones, such as insulin and thyroxin/thyroxamine, have no consensus in the interpretation of their action on dry eye.
The triple classification of dry eye for practical clinical use

Hormonal dry eye is generally multiexocrinic. Aquouserous and lipid secretions are the most affected. Severity usually reaches grades 1 or 2.

Pharmacologic. Some systemic medicines have a collateral exocrinic hyposecretory effect. Among them are antidepressants (fluoxetine, imipramine), anxiolytics (bromazepam, diazepam, clorazepate), sleeping pills (brotizolam, chloral hydrate, chlormethiazole), antiparkinsonians (biperiden, benztrapine), diuretics (chlorothalidone, furosemide), vascular antihypertensives (chlorothiazide, clonidine), anticholinergics (atropine, metoclopramide), antihistaminics (dexamethasone, cetirizine), and antiarrhythmics (disopyramide, mexiletine). Some of these drugs are mainly taken by elderly or menopausal people, or at night, when they can aggravate the multiexocrinic dryness. Systemic pharmacologic dry eye is generally multiexocrinic, and is usually grade 1 or 2 severity.

Some topical collyria and ointments produce damage of the corneal epithelium, conjunctiva, or lid margin. Among these are some preservatives (benzalkonium chloride, thiomersal, chlorobutanol, EDTA), anesthetics (caine, tetracaine, proparacaine, lidocaine), and vitamin A derivatives (topical or systemic isotretinoin). The damage is usually restricted to the ocular surface and related structures when the application is local. Drug-induced adverse effects are far from being restricted to only allergic reactions and the long-term use of eye drops has consistently been reported to induce inflammatory ocular surface changes, causing progressive ocular discomfort upon instillation, tear film instability, corneal surface impairment, and subconjunctival fibrosis.

Immunopathic. Some autoimmune diseases can produce eye dryness by damaging the dacroey glands and/or ocular surface. There are several main groups of immunologic dry eyes: 1) those preferentially affecting the glands, as occurs with what were known until recently as primary Sjögren syndromes, in which vasculitis by immunocomplex deposits and pseudolymphomas and lymphomas are sometimes associated; 2) those affecting the exocrine glands and the connective tissue as in rheumatoid arthritis, systemic lupus erythematosus, dermatomyositis, scleroderma, etc., as occurs with what were also known until recently as secondary Sjögren’s syndromes. Today it is preferable to use the expression “Sjögren syndrome associated with...,” in order not to confuse the many different varieties of Sjögren syndromes; 3) those where there is an autoimmune attack of the ectodermal and mesodermal tissues and the secondary destruction of non-attacked glands, as occurs in the mucus membrane pemphigoids, Lyell syndromes, Stevens-Johnson syndromes, and CREST syndrome; 4) those affecting other tissues, which secondarily can affect the exocrine glands and ocular surface, such as the pluriglandular endocrine deficiencies or Schmidt’s syndrome (thyroid and adrenal insufficiency).

Immunopathic dryness is usually multiexocrinic. It affects about 1% of the population. Sjögren’s syndromes frequently reach grades 1 and 2, and occasionally, grade 3. But immunopathic dry eyes of group 3 frequently reach grade 3, with permanent ocular surface damage and sometimes definitive decreased visual acuity.

Hyponutritional. Hypovitaminosis A was the most frequent cause of severe xerophthalmia for millennia. It produces multiexocrinic dryness and other ophthalmic manifestations such as Bitot’s spots in the conjunctival exposed trigoni, keratomalacia, blepharitis, and bad scotopic adaptation. It can be produced by severe hyponutrition or by a selective fat-free diet. It may also be due to the intestinal malabsorption associated with Crohn’s disease, chronic alcoholism, and intestinal resection.

Lack of omega-3 essential polyunsaturated fatty acids (alpha-linolenic acid, EPA, and DHA) available from dark oily fish such as salmon, sardine, and tuna produces dry eye through mechanisms that are currently being explored.

Other controversial deficiencies with respect to their influence in dry eye are vitamins B₂, B₁₂, and C.

Xerophthalmia by hypovitaminosis A when treated in time retrogrades without producing sequelae, but if not treated in time, it may produce corneal blindness or even corneal melting.

Dysgenetic. In the evolution of the medical language genetic and congenital (from Greek γενής, birth) were applied for centuries to conditions presented at birth. They were due to hereditary parenteral transmission, or to intrauterine exposure to infections, toxins, traumas,

| TABLE I - ETIOPATHOGENETIC CLASSIFICATION OF DRY EYE |
|---------------------|---------------------|
| Pan-exocrinic | Dacryo-exocrinic |
| 1. Age related | 6. Dysgenetic |
| 2. Hormonal | 7. Infectious/Inflammatory |
| 3. Pharmacologic | 8. Traumatic |
| 5. Hyponutritional | 10. Tantalic |
mechanical factors, or unknown causes. When genes were discovered and understood in the 20th century, genetic also took a second meaning: expressed by anomalous genes (even when developed in the phenotype not at birth but in childhood, puberty, or later). In the future terminology it seems that genetic and genic will be reserved for hereditary diseases (expressed at birth or later on), and that congenital and connatal will be the ones appearing at birth (by gene diseases or by accidental occasional damage to the embryo or fetus). As the future terminology is not yet precise, in this classification we consider dysgenetic to include all genetic and congenital diseases.

Dysgenetic dry eye can affect one or several types of dacrogylands: aqueo-serous (alacrima, dysplasia ectodermica anhydrotica), lipid (epicanthus-blepharophimosis syndrome, keratopathy-ichthyosis-deafness syndrome, first branchial arc syndromes, dysplasia ectodermica anhidrotica), and mucinic (aniridia, Bietti syndrome), or the ocular surface epithelium (Meesmann dystrophy, Fleischer cornea verticillata, Franceschetti-Cogan microcystic dystrophy).

**Infectious/inflammatory.** Infection (from Latin *inficere*, filled with noxious corruption or germs) is today applied to the contamination of the body by harmful organisms. Inflammation (from Latin *flammare*, flame) had a classical definition recorded by Celsus in the 1st century: *tumor, rubor, calor, et dolor* (swelling, redness, heat, and pain). Usually inflammation was due to infection. For the last two centuries the term inflammation has been applied to mild conditions without some of those signs. As it has recently been discovered that proinflammatory mediators promoting reaction and healing are present in any corporal tissue damage, the name of inflammation may be applied to new meanings, which can result in confusion.

Infection/inflammation of the aqueo-serous dacrogylands (tuberculous, fungic) are at present very rare. Infection/inflammation of the lipid dacrogylands (blepharitis), both posterior (meibomitis) or anterior, usually have a causal or secondary infectious component. The abundance of cholesterol esters in normal meibomian secretion makes a good culture medium for microorganisms such as *Staphylococcus aureus* that produces lipases that denature the meibomian secretion and increase the evaporation of the aqueo-serous phase of the tears. About 10% of the tear of the lacrimal basin evaporates, and this rate increases in patients with blepharitis due to the lipid layer insufficiency of their lacrimal film.

Mucoadenitis of the conjunctiva (conjunctivitis) is highlighted by trachoma, herpes zoster, herpes simplex, and adenoviruses.

At present, aqueo-serous dacryoadenitis is very infrequent, conjunctivitis is in decreasing prevalence, but blepharitis is very frequent.

**Traumatic.** The traumatic damage of dacrogylands and ocular surface may be mechanical (surgical or accidental), chemical, or radiation induced. This damage to dacrogylands is produced accidentally or with therapeutic aims, and may affect the aqueo-serous glands (surgical ablation, tumor radiation), the lipid glands (lid wound, Mustardé lid reconstruction, Webster operation for distichiasis), the mucinic glands (chemical caustication, thermic destruction, conjunctivectomy), and the corneal epithelium (abrasion, caustication, limbal destruction).

Severity of traumatic dry eyes varies, depending on the causes, the affected tissues, and the intensity of the destruction.

**Neurologic.** Lacrimal secretion is very dependent on nervous stimulation. Its influence may be separated into three types: hypothalamic and limbic influences, afferent neurodeprivation, and efferent neurodeprivation.

**Hypothalamic and limbic influences.** Hypothalamus determines a circadian production of tear secretion that is at its maximum at morning and noon, diminishes at sunset, and reaches its minimum at night and when sleeping.

Limbic influences such as anxiety, tiredness, psychic influences, and somnolence diminish the basal tear secretion.

**Afferent neurodeprivation.** The afferent means of the reflex stimulation of tear secretion is due to lid and eye friction, environmental temperature, and intermittent changes of corneal thermometry when blinking, retinal light activation, trigeminal activity, etc. Therefore, ocular surface anesthesia due to herpetic keratitis, topical anesthetics abuse, corneal refractive surgery, corneal transplant, and pre- and post-semilunar trigeminal damage diminishes lacrimal secretion.

Contact lenses, mainly hydrophilic and semipermeables, restrict the external stimuli. Lamellar refractive surgeries (mechanical keratotomy, femtosecond laser-assisted *in situ* keratomileusis, etc.) produce a moderate dry eye that is partially recoverable. The discomfort of eye dryness in persons with altered rapid eye movement (REM) sleep becomes worse, maybe because the objective of REM sleep is to stimulate tear secretion during prolonged sleep.
**Efferent neurodeprivation.** The efferent means of the reflex stimulation of tear secretion may be damaged in the pontobulbar nuclei (nucleus salivaris superior and lacrimalis) and their connections, nervus intermediarius and nervus facialis pregeniculi, nervus intermedius Wrisbergi, ganglion geniculi, nervus petrosus superficialis major, nervus canalis pterygoidei sive vidianus, ganglion pterygopalatinum Meckeli, nervus zygomaticus, ramus communicans cum nervo lacrimalis, and nervus lacrimalis. This can be produced by different causes, such as trauma, tumors, and botulinic toxin infiltration, and may have various collateral manifestations such as neurotrophic keratitis, pregeniculate facial palsy, neuralgia, and crocodile tears.

**Tantalic.** Tantalos, son of Zeus and Pluto, offended the Olympic gods, and therefore was condemned to live in the Tartarus lake, but when he tried to drink, the water drew back. So, despite living in water, he suffered from thirst and dryness. Therefore, tantalic dry eyes are those that, despite having enough tears, have a dry ocular surface. There are three types of tantalic dry eyes: lid-eye incongruency, epitheliopathic, and evaporation.

**Lid-eye incongruency.** Lids cannot create, maintain, and reshape the lacrimal film onto the ocular surface because of lid palsy, ectropion, lagophthalmos, lid coloboma, exophthalmos, local protrusion by pterygium or dermoid cyst, blepharochalasis, conjunctivochalasis, antimongoloid lid fissure, or half-opened eye sleep.

**Epitheliopathic.** Corneal and conjunctival epithelium is hydrophobic. They need to increase their critical surface tension with a healthy surface covered with the appropriate mucins to make them dacryophilic, so that tear spreads over them forming a lacrimal film. Therefore, epithelial dystrophies, limbal deficiency, corneal conjunctivalization, keratitis-ichthyosis-deafness syndrome, rare cases of diabetic or hypoparathyroid endocrine keratitis, corneal caustications, corneal thesaurismosis, endothelial decomposition, and many other causes can produce a tantalic dry eye.

**Evaporation** due to environmental circumstances (and not to the patient's condition). Among these are excessive air conditioning, fans, electric fans, open car window, wind, running without spectacles, polluted air, or dry air.

The “Stingy taxi driver syndrome” includes dry eye and scurvy hair; it is caused by the taxi driver lowering his window for coolness, and the back passenger receives the draft on his/her head.

As an addendum to the decologue of etiologic groups, it must be explained that most of the dry eye conditions are multicausal, and sometimes the aggressiveness of one of them puts it in a prevalent position in diagnosis, clinical severity, and treatment. Each cause has its own evolutive characteristics: self-limited, permanent, progressive. Most causes will last for life. Only some of them are reversible in the present state of medicine, such as most pharmacologic causes, and incipient hyponutritional ones.

When the underlying etiopathogenesis is identified, it can sometimes be classified in different decalogic groups if etiology and pathogenesis are applied; for instance, traumatic damage of the pregeniculate nervus facialis is a traumatic and a neurodeprivative dry eye. Therefore, for descriptive purposes they may be classified in both groups.

Each classification of the Triple Classification of Dry Eye for a patient must be written in the patient’s chart or the sheet for the Triple Classification of Dry Eye for the Clinical Record, which can be attached to the patient’s chart, as seen in Table II.

**Classification according to the damaged glands and tissues (“ALMEN” classification)**

From a clinical point of view, and in order to establish a treatment, the etiologic classification must be completed with the evaluation of the participation of the different parts that form the lacrimal basin, i.e., the anatomic space between the ocular surface, posterior surface of the lids, and lid rim, where the mixture of the lacrimal sea is poured. These components of the tear may be simplified as produced by three basic types of dacryoglobins — aqueo-serous, lipid, and mucin — with an important component of the epithelium, mainly the corneal one. The affected parts of the lacrimal basin may be summarized in this histopathologic classification expressed with the acronym ALMEN, in which the A indicates the aqueo-serous deficiency; L, the lipid deficiency; M, the mucin deficiency; E, the epithelial deficiency; and N, the non-dacryologic exocrinic deficiencies.

The **aqueo-serous deficiency** is basically produced by the damage of the main and accessory lacrimal glands. The aqueo-serous production may be measured by the Schirmer test, tear clearance, volumetry of the lacrimal menisci (cisterna and rivi lacrimales), lactoferrin, and other tests of controversial value but increasing efficacy. Some of these tests such as BUT (breakup time) or osmolarime-
try do not establish the aqueo-deficiency but only a tear film deficiency in which other deficiencies, such as lipid or mucinic, can participate or be the primary cause. In any case, the objective of the triple classification is not to establish the present suitable tests to determine the deficiency, but to establish the necessity to define the dryness as aqueo-serous deficient or not.

The lipo-deficiency is mainly due to the abnormality of the meibomian lipid glands, and to a lesser extent of the Zeis' glands, the pilosebaceous gland of the eyelashes, and the fatty component of the Moll's glands, which participate in the anterior antievaporative lipid phase of the lacrimal film. The deficiency is at present deduced from the presence of a blepharitis, and the interferometry of the lipid layer, but more and more osmolarimetry, new methods of interferometry, reflective meniscometry, evaporation test, humidometry, lipid analysis, BUT, and others do more exact determinations.

The mucin deficiency is produced mainly by the damage of the goblet cells of the conjunctiva, and the epithelial glycolcalix, and also by lacrimal gland participation. The most practical determination is not only by impression cytology of the ocular surface, BUT, and vital staining of the ocular surface epithelium, but also by the tear crystallization or ferning test, retraction of the lacunar sulci and lower fornix conjunctival folds, and laboratory determination of mucin MUC5AC. Some of these tests may be effective to determine the presence of a dry eye, but are not so specific in determining the type of ALMEN deficiency.

The corneo-conjunctival epithelopathy is sometimes primary, but is more frequently secondary to the other glandular deficiencies. Primary epitheliopathies with respect to dry eye are those in which a corneal problem not related with the tear production alters the epithelium and causes problems in the formation of the tear film. Examples are Meesmann epithelial dystrophy, amio-

### TABLE II - CLASSIFICATION OF DRY EYE FOR CLINICAL USE

<table>
<thead>
<tr>
<th>Patient:</th>
<th>Etiopathogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1)</td>
<td>Age-related</td>
</tr>
<tr>
<td>X</td>
<td>1. Age-related</td>
</tr>
<tr>
<td>X</td>
<td>2. Hormonal</td>
</tr>
<tr>
<td>X</td>
<td>3. Pharmacologic</td>
</tr>
<tr>
<td>4.</td>
<td>Immunopathic</td>
</tr>
<tr>
<td>5.</td>
<td>Hypo-nutritional</td>
</tr>
<tr>
<td>6.</td>
<td>Dysgenic</td>
</tr>
<tr>
<td>X</td>
<td>7. Infectious/inflammatory</td>
</tr>
<tr>
<td>8.</td>
<td>Traumatic</td>
</tr>
<tr>
<td>9.</td>
<td>Neurologic</td>
</tr>
<tr>
<td>10.</td>
<td>Tantalic</td>
</tr>
</tbody>
</table>

2) AFFECTED GLANDS or ALMEN

| X | Aquo-serous deficiency | ... Schirmer 5 mm, BUT 6", osmolarity 320 mOsm/L, muramidase < 0.7 g/L |
| X | Lipodeficiency | ... blepharitis, marmoreal interferometry, 318 mOsm/L |
| X | Mucodeficiency | ... ferning Rolando III, BUT 6", low impression cytology |
| X | Epitheliopathy | ... BUT 8", fluorescein +, Bengal rose 1-1-1 |
| X | Non-lacrimal affected exocrine glands | ... mouth, vagina |

3) SEVERITY

| Grade 1-minus | ... dryness sensation, occasional ocular itching, vespertinal BIVA |
| Grade 1 | |
| Grade 2 | ... conjunctival redness, cornea punctata |
| Grade 3 | |
| Grade 3-plus | |

An example of the application of this Triple Classification to a patient, taken from the most frequent profile of patients with dry eye seen in an outpatient department. The sheet is filled with the collected tests and results. Each accurate or speculative identified etiopathogenesis, affected glandular system, and grade of severity is marked with a cross or mark.
The triple classification of dry eye for practical clinical use

darone thesaurismosis, stromal mucopolysaccharide deposits, Fuchs endo-epitheliopathy, and corneal endothelium decompensation. Secondary epitheliopathies produced by dry eye are those in which an aqueo-serous, lipid, or mucinc deficiency due to any dysfunction of the dacrocyglands damages the normal corneal epithelium, increasing the problem of the eye dryness.

The affected dacrocygland may be initially of one, two, or of all types. It depends on the type of etiology. In any case, all dacrocyglands and the lacrimal basin are usually finally implicated in a vicious circle that with different intensities affects all of them. For instance, an age-related dry eye produces directly and with a certain synchrony a general affection of all dacrocyglands. But the extirpation or radiation of the main lacrimal gland initially only affects the aqueo-serous secretion, and little by little secondarily affects all other ocular surface dacrocyglands and the epithelium of the ocular surface.

Ocular surface epitheliopathy is diagnosed by slit lamp-biimicroscopic signs, short BUT, punctate vital staining, cellular or secretory filaments, laboratory histopathologic tests such as impression cytology, or biochemical tests such as low mucins MUC1, MUC4, MUC16, or aquaporin AQP5. There is a steady advance in examination techniques.

The non-ocular exocrine glands deficiencies are an important orientation about etiology because they can indicate if it belongs to one of the multiexocrinic conditions, such as age-related, hormonal, pharmacologic, or autoimmune. The more bothersome organs because of their objective or subjective dryness manifestation are as follows:

**Mouth**: oral cavity and lip dryness sensation, thirst, frequent linguo-labial humidification movements, dense saliva, bad breath (halitosis), taste dysfunction (dysgeusia), expulsion of saliva drops when speaking (sialo-laloplasia), fungal stomatitis.

**Nose**: dryness sensation, dry nasal mucus, itching, impairment of the sense of smell (dysosmia, anosmia).

**Throat**: dryness sensation, thirst, need to clear the throat when talking, dense phlegm, dense sputum, hoarseness, change of voice or raucousness (dysphonia).

**Skin**: cutaneous dryness, axillary itching.

**Vagina**: pruritus, itching, painful coitus (dyspareunia), vaginitis sicca.

**Seminal glands**: dense ejaculation.

**Ear**: itching of the outer ear, earwax plugs.

These pluri-exocrinic manifestations are not usually of synchronic presentation, and do not all reach the same clinical level. Frequently, the dryness of an exocrinic system does not usually correspond with the subjective sensation of the patient. So, a similar dryness in several exocrinic body glands is usually first noticed in the eyes and mouth. Throat, nose, and vagina occupy an intermediate position, followed by skin and tracheo-bronchial tract. Dryness of ear, seminal glands, and intestinal tract are not usually noticed.

**Classification according to the severity**

The clinical symptoms and signs of the many millions of patients with dry eye can present with thousands of combinations related to etiologies, affected types of dacrocyglands and ocular surface, and severity of the damage. In order to do a practical classification of severity capable of satisfying the clinician it has been decided to classify them into only three grades, attending the bases of the clinical examination, i.e., symptoms and signs: grade 1 or mild (symptoms without slit lamp signs); grade 2 or moderate (symptoms with reversible signs); or grade 3 or severe (symptoms with permanent signs).

**Grade 1 or mild.** Patients in this grade frequently have symptoms of ocular surface dryness in normal environmental circumstances: dryness sensation, itching, ocular tiredness, photophobia, photoinduced cough, momentarily blurry vision that improves with repeated blinking (BIVA: blinking-improved visual acuity), and fissural cionic blepharospasm.

No signs related to these symptoms can be seen when fentobiomicroscopically examined at the slit lamp. Fentobiomicroscopy is the basic or gold standard ocular surface examination used and interpreted by ophthalmologists. With any symptom there is always a sign that sometimes in present day medicine could be inferred or detected with analytical, electrophysiologic, or invasive tests, such as hyperosmolarity, hypolypoosyme, or inflammatory cytokines. These non-slit lamp signs are excluded from grade 1 of this classification, which is done for practical clinical use.

In this grade 1 or mild dry eye, a previous initial period can be introduced with the name of grade 1-minus when these symptoms appear only under overexposure that in the same conditions do not produce symptoms in normal persons, i.e., wind, fan, open car window, air conditioning, polluted air, low environmental humidity, contact lens wear, or physical corporal tiredness. Usually, in this stage of grade 1-minus the patient is not aware that he or she has an incipient dry eye.
Grade 2 or moderate. The patient, besides more or less evident symptoms, has reversible slit lamp signs, such as epithelial erosion, keratopathy punctata, keratopathy filamentosa, short BUT, hyperemia of the exposed conjunctival trigoni, secretion sleep of the ocular surface, or marginal blepharitis.

Grade 3 or severe. The patient, besides the symptoms of ocular dryness, has signs that have evolved to permanent sequelae, such as corneal ulcers, nephelions and leukomas of the cornea, corneal neovascularization, squamous epithelial metaplasia, or retraction of the conjunctival folds of the lower cul-de-sac or of the lacunar sulci (the first between the nasal conjunctival trigonus and the plica semilunari, and the second between the plica semilunaris and the caruncula).

In this grade 3 or severe dry eye a grade 3-plus may be introduced when the keratinization, scarring, and lesions of the central cornea permanently reduce the visual acuity. The present one is a clinical classification, and the clinical situation and incapacity of the patient is very different from when the lesions are in the periphery or do not diminish the visual acuity than when they are in the center and do diminish visual acuity. For this reason grade 3 has been enriched with this more incapacitating grade 3-plus.

COMMENTS

To find a consensus in a medicine that is continuously changing and progressing is not easy. This classification has been made not only by collecting many opinions, but also by producing a classification of dry eye conditions for practical clinical use that allows diagnosis, prognosis, and treatment of patients with dry eye.

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Reprint requests to:
Prof. Juan Murube, MD, PhD
University of Alcala
Moralzarzal Street, 43
28034 Madrid, Spain
murubejuan@terra.es

REFERENCE