
Universiteit
Leiden
The Netherlands

Innovative strategies to clinically characterize the human tear proteome : from fundamental exploration to ophthalmological relevance

Raus, P.P.M.

Citation
Raus, P. P. M. (2018, September 4). *Innovative strategies to clinically characterize the human tear proteome : from fundamental exploration to ophthalmological relevance*. Retrieved from https://hdl.handle.net/1887/65534

Version:	Not Applicable (or Unknown)
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/65534

Note: To cite this publication please use the final published version (if applicable).

Cover Page



Universiteit Leiden



The handle http://hdl.handle.net/1887/65534 holds various files of this Leiden University dissertation.

Author: Raus, P.P.M.
Title: Innovative strategies to clinically characterize the human tear proteome : from fundamental exploration to ophthalmological relevance
Issue Date: 2018-09-04

Universiteit Leiden

MIRO
Centrum voor ooglidchirurgie en esthetiek

Innovative strategies to clinically characterize the human tear proteome. From fundamental exploration to ophthalmological relevance.



Proefschrift ter verkrijging van de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties te verdedigen
op dinsdag 4 september 2018 klokke 13.45 uur

door

Peter Paul Magdalena RAUS, MD, FEBO
geboren te Mol (België) in 1957

Promotiecommissie:

Prof. Dr. Herman Spaink, Institute of Biology, Leiden University

Prof. Dr. Hubertus Irth, Leiden Advanced Centre for Drug Research, Leiden University

Prof. Dr. Thomas Hankemeier, Leiden Advanced Centre for Drug Research, Leiden University

Dr. Dennis Claessen, Institute for Biology, Leiden University

Prof. Dr. Gordon Laurie, Department of Cell Biology, Ophthalmology and Biomedical Engineering, University of Virginia, USA

Dr. Daniel Lopez-Ferrer, Thermo Fisher Scientific, San Jose, USA

Prof. Dr. Marie-José Tassignon, Department of Ophthalmology, Academic Hospital, University of Antwerp, Belgium

Dr. Gerrit Melles, Netherlands Institute for Innovative Ocular Surgery, Rotterdam

Promotores:

Prof. Dr. Peter D.E.M. Verhaert, Faculty of Health, Medicine and Life Sciences, Maastricht University

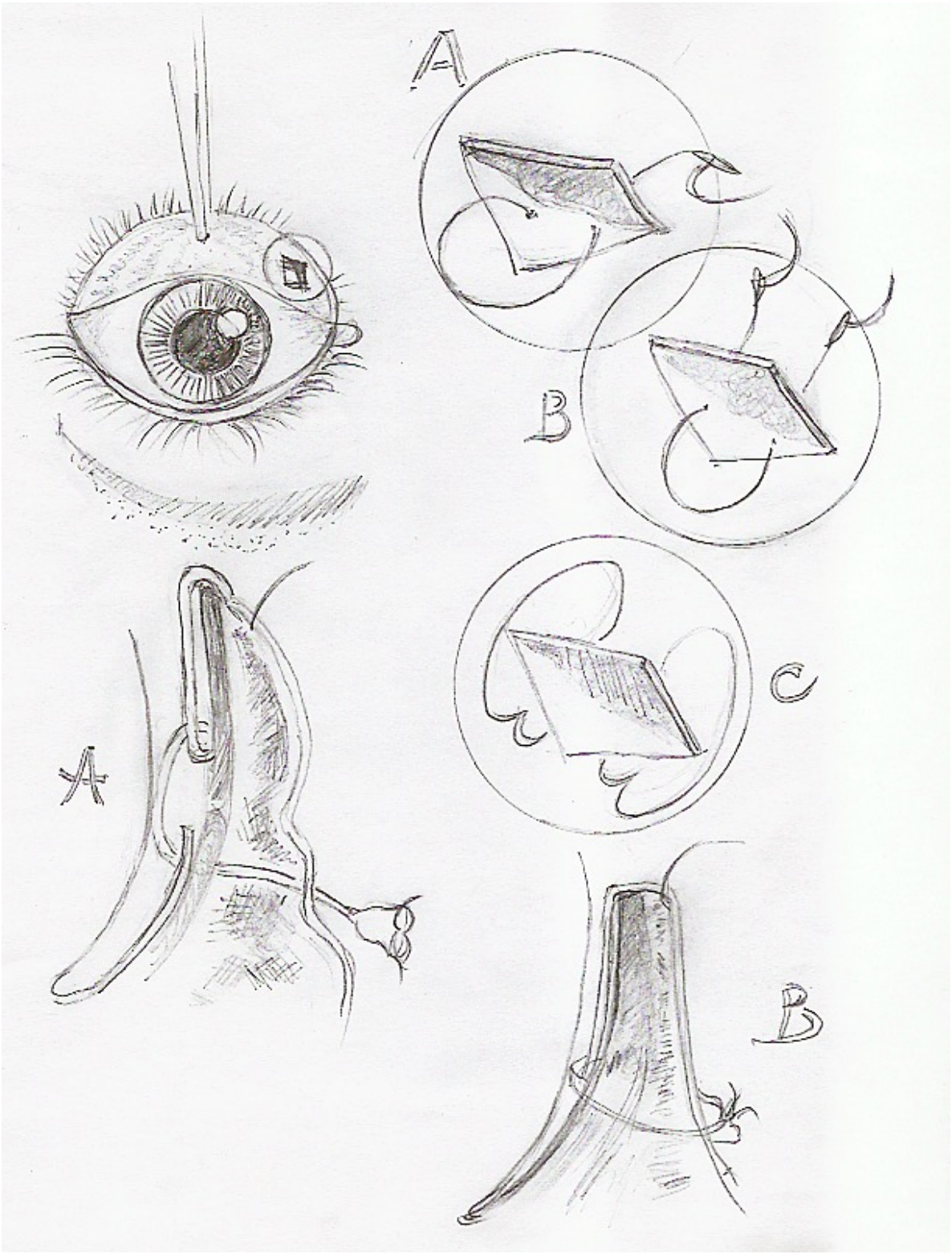
Prof. Dr. Johannes H. de Winde, Institute of Biology, Leiden University

Table of Contents

Preface vi
Voorwoord vii
Chapter 1: Introduction to DED: the vision of the patient, the ophthalmologist, and the analytical biotechnologist 1
1.0 General introduction. 1
1.1 Position of the patient. 1
1.2 Current state-of-the-art and clinical outlook: DED 'in the eyes of' the Ophthalmologist. 4
1.3 The perspective of the Analytical Biochemist/Biologist, i.c. a clinical (proteomics) biotechnologist. 11
1.4 Conclusion 14
Appendix 1.1 Triple classification 17
Chapter 2: Background for surgical intervention in DED 25
2.0 Introduction 25
2.1 Radiofrequency Surgery: 3500 years old and still young. 27
2.2 Upper lid blepharoplasty without cutting the orbicularis. 33
2.3 Transconjunctival lower lid blepharoplasty made easy by everting the eyelid. 39
2.4 Punctal Ectropion repair using the Raus-Garito clamp 43
Chapter 3: Innovative treatments for DED using RF surgery 49
3.0 Introduction 49
3.1 In office occlusion of the tear punctum using the CPO electrode (Current surgical DED treatment 1 51
3.2 Minor salivary gland transplantation (Current surgical DED treatment 2). 55
Appendix 3.1 The Chedly Punctal Occluder. A new treatment modality for Dry Eyes. 67
Chapter 4: First contact with Proteomics 73
4.0 Introduction: The age of Q-TOFs 73
4.1 Comparative GeLC MS/MS bottom-up proteomics analysis of tears and saliva on a quadrupole time-of-flight mass spectrometer. Towards tear protein analysis in the clinical context of dry eye disease 73
4.2 Supplemental data: list of all confidently identified proteins from section 4.1. 83
Chapter 5: Further exploration of Proteomics for clinical tear analysis. 93
5.0 Introduction: Entering the Orbitrap age 93
5.1 Bottom-up protein identifications from microliter quantities of individual human tear samples. Important steps towards clinical relevance 95
Chapter 6: Modern high-performance mass spectrometry approaches for future diagnostics in the ophthalmology clinic and beyond 101
6.0 Introduction 101
6.1 *Proteoformics* of the human tear: Modern orbitrap based mass spectrometers detect an unexpected multitude of proteoforms in (human) secretory fluids. 103
6.2 Rapid characterization of the proteoform landscape in human tear samples using top down mass spectrometry with multiple MS/MS strategies. 109
6.3 Clinically relevant profiling of proteoforms in human tear fluid using chip-based capillary electrophoresis coupled to mass spectrometry. 119
6.4 Evaluation of heat stabilization in human tear sample preparation for top-down proteomics. 125

Chapter 7: Conclusion & Perspectives. Analytical innovation in the ophthalmological practice - from novel diagnostics to new treatment and beyond. 131
Summary 137
Samenvatting 140
Dankwoord 143
Curriculum Vitae 145

"C'est tellement mystérieux, le pays des larmes."
[Le Petit Prince, Antoine de Saint-Exupéry]



Voor Linus, met dank aan Mevr. Mia Mertens

PREFACE

"Once upon a time a boy dreamed about becoming a plastic surgeon…," could be the first line of a fairy tale. In fact, it was the prelude of my medical career. My father, Bob Raus, was an ophthalmic surgeon, so to start studying medicine was a logical step in my education. Little by little, the idea arose to combine ophthalmology with plastic surgery. At the time, the surgical training of ophthalmologists was extremely limited, if not non-existing. Only after my residency (1986) I made my first steps in eyelid surgery, with my father as skillful guide. A true blood, sweat, and ***tears*** experience. At the occasion of the European Society of Ophthalmic Plastic and Reconstructive Society meeting (ESOPRS, Juan les Pins, France 1993) where I presented my first eyelid reconstruction, I met the late Dr. Stephen Bosniak who introduced me to the use of radio waves in surgery. He also taught me the transconjunctival blepharoplasty of the lower eyelid. Upon demand by Bosniak I joined the Editorial Board of the *International Journal Operative Techniques in Oculoplastic, Orbital and Reconstructive Surgery* in 1998. In this journal Prof. Juan Murube del Castillo (Universidad de Alcalá, Madrid) reported his transplantation of salivary glands for patients with very dry eyes. When lecturing in Manilla (17th congress of the Asia Pacific Academy of Ophthalmology, 1999), I met Murube in person and got convinced of the many advantages of his procedure. A few months later I performed my first transplantation operation on a patient with extremely dry eyes. The patient was so happy that she talked to the television news. Yet several of my colleagues argued that the surgery required additional medical evidence. I realized that, indeed, extra scientific proof would be helpful to support the surgery. Upon advice of my future promotors, we decided to evaluate whether an objective analysis of the protein composition of human tears and saliva would be valuable in this way. This is how we got introduced to the wonderful world of Proteomics.

In 2006, we performed our first comparative protein analysis of tears and saliva. Samples were analyzed in the Department of Biotechnology of Delft University of Technology (TUDelft). With the available protein and peptide analytics in those days, we employed conventional SDS PAGE based bottom-up proteomics analyses to identify around 200 different proteins in the tear and the labial saliva of the same individual. More than a third of the identified proteins were found to overlap between tears and saliva, with salivary lacritin being one of the most striking. The name 'lacritin' refers to the Latin term 'lacrima' ('tear'). Work by Prof. Gordon Laurie (Univ. of Virigina, USA) had established that lacritin is a secretagogue, promoting the secretion of the various components of the human tear by the three different main glands. We validated our salivary lacritin identification using immunohistochemistry with Laurie's anti-lacritin, indicating that labial salivary glands indeed produce immunoreactive lacritin.

As it is not unlikely that also other proteins in tears and/or saliva have their relevance in the treatment of dry eye disease, we decided to follow the rapid technological developments in the discipline of Proteomics, where ever increasing analytical performance is combined with innovative quantitative protein analytical protocols, including peptidomics and top-down proteomics. We are convinced that the comparative analyses of tear protein and peptide composition of healthy subjects and patients will pave the way for the discovery of novel (panels of) biomarkers to better understand the pathophysiology, as well as to monitor the treatment efficacy in individual patients suffering from ophthalmological and, by extension, also from other diseases.

Peter Raus, MD [Geel (Belgium), April 2018]

VOORWOORD

Reeds als kind doomde ik ervan om plastisch chirurg te worden. Als zoon van Bob Raus, oogarts met een speciale interesse in ooglidchirurgie, groeide het plan om oogheelkunde te combineren met plastische chirurgie.
Het heelkundig aspect van de vorming tot oftalmoloog was destijds echter minimaal tot onbestaande. Het was dan ook nà mijn erkenning als oogarts (in 1986) dat ik mijn eerste bescheiden stappen zette in de ooglidchirurgie met mijn vader als bekwame gids.
Op het congres van de European Society of Ophthalmic, Plastic and Reconstructive Surgery (ESOPRS; Juan les Pins (France) 1993), waar ik mijn eerste ooglid-reconstructie presenteerde, leerde ik wijlen Dr. Stephen Bosniak uit New York kennen. Hij maakte me wegwijs in de chirurgie met hoogfrequente radiogolven, en leerde me ook de transconjunctivale blepharoplastie van het onderste ooglid. Op vraag van Bosniak vervoegde ik in 1998 de redactie van *Operative Techniques in Oculoplastic, Orbital and Reconstructive Surgery*. Zo leerde ik het werk kennen van Prof. Juan Murube del Castillo (Universidad de Alcalá, Madrid) die in dit tijdschrift zijn transplantatie publiceerde van lipspeekselklieren bij patiënten met extreem droge ogen. Ter gelegenheid van het 17de congres van de Asia Pacific Academy of Ophthalmology (Manila, 1999) ontmoette ik Murube in persoon en ik geraakte snel overtuigd van de vele voordelen van zijn chirurgie. Korte tijd later opereerde ik mijn eerste patiënte met deze techniek. De media pikten dit op, wat voor de nodige controverse zorgde in Belgische oogheelkundige middens. Het was me duidelijk dat bijkomende wetenschappelijke argumenten konden helpen om de waarde van de techniek van Murube te bevestigen, zoals een objectieve analyse van de biochemische samenstelling van traanvocht vóór en na een 'Murube-behandeling'.
In overleg met onze toekomstige promotoren bedachten we dat het een goed idee kon zijn de samenstelling van tranen (en speeksel) te analyseren voor wat betreft de aanwezige eiwitten. Dit was voor ons de introductie tot de boeiende wereld van Proteomics. In 2006 vergeleken we zo de eiwitten die konden worden geïdentificeerd in tranen en in speeksel met behulp van de toen aan de TUDelft beschikbare analytische infrastructuur. Van de om en bij de 200 verschillende proteïnen die we identificeerden in tranen en speeksel bleken meer dan 1/3 gemeenschappelijk. Vooral het in speeksel teruggevonden lacritine (de naam verwijst naar lacrima, Latijn voor traan) trok onze aandacht, omdat van dit eiwit gekend was (werk van Prof. Gordon Laurie aan de University of Virginia (US)), dat het een van de belangrijkste eiwitcomponenten van gezonde tranen betreft, en dat het m.n. de secretie van de diverse componenten in de menselijke traan (drie verschillende klieren) stimuleert. Met een antilichaam dat we kregen van Laurie valideerden we onze massaspectrometrische lacritine-identificatie immunohistochemisch, door aan te tonen dat lipspeekselkliertjes van één onzer patiënten effectief immunoreactief lacritine-materiaal aanmaken. Omdat mogelijk ook andere eiwitten van belang kunnen zijn om de effectiviteit van een lipspeekselklier-autotransplantatie te verklaren, beslisten we de ontwikkelingen binnen de snel evoluerende discipline van Proteomics op de voet te blijven volgen. Zo leerden we steeds gevoeligere en performantere instrumenten kennen, en zagen we de kans om ook nieuwe technieken te evalueren voor hun actuele dan wel toekomstige toepasbaarheid om proteinen- (en peptiden-) profielen klinisch te karakteriseren. Vergelijkende top-down analyses van tranen van gezonde donoren en patiënten (droge-ogen-lijders, maar ook andere) kunnen, in de (nabije) toekomst leiden naar biomarkers die inzicht verschaffen in de physiopathologie dan wel therapeutische follow-up van individuele patiënten.

Dr. Peter Raus [Geel, April 2018]

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.

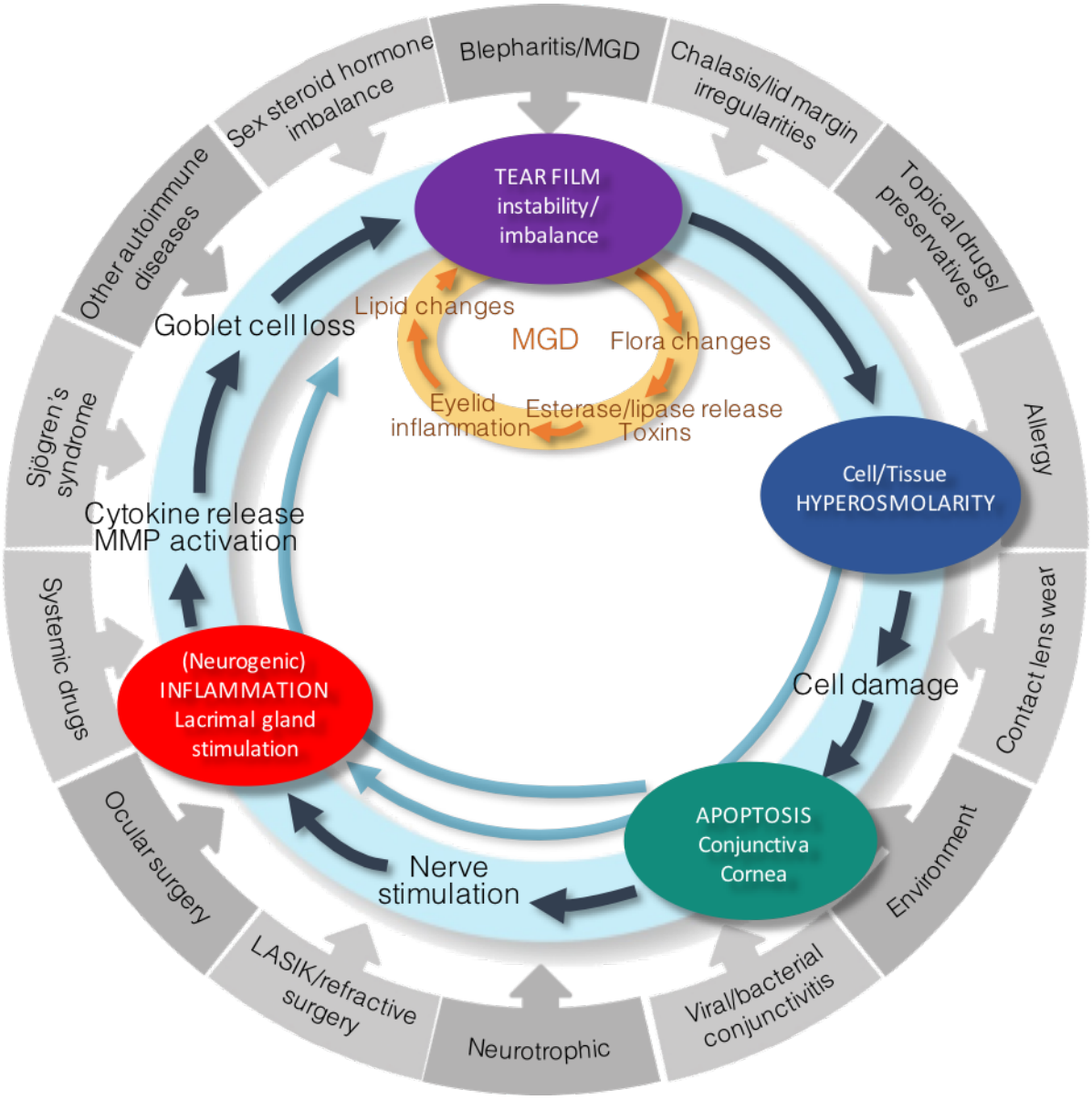


Fig. 1.1 The so-called vicious circle of DED [adapted from Baudouin et al., 2013]. LASIK, laser-assisted *in situ* keratomileusis (laser refractive surgery); MGD, Meibomian gland dysfunction; MMP, matrix metalloproteinase.

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*".

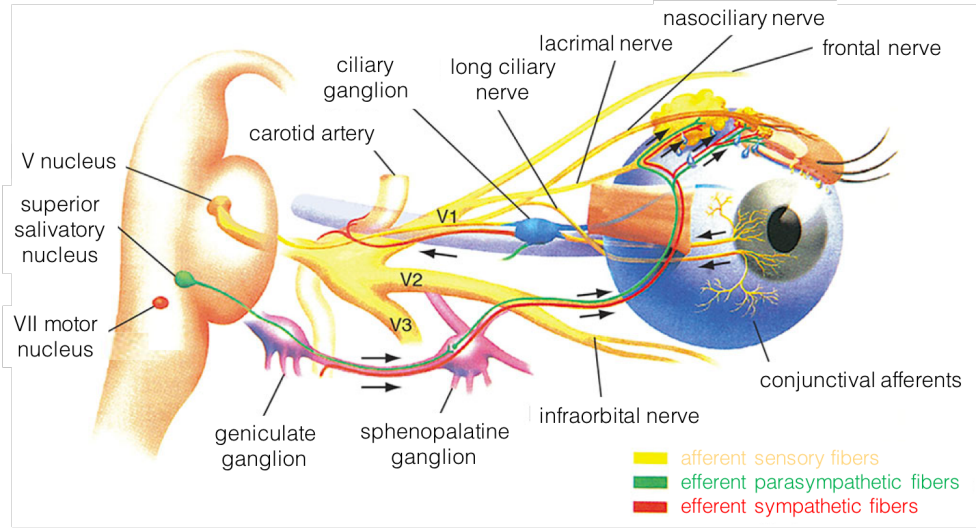


Fig. 1.2 Scheme illustrating eye and integrated LFU [adapted from Beuerman et al., 2003].

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.

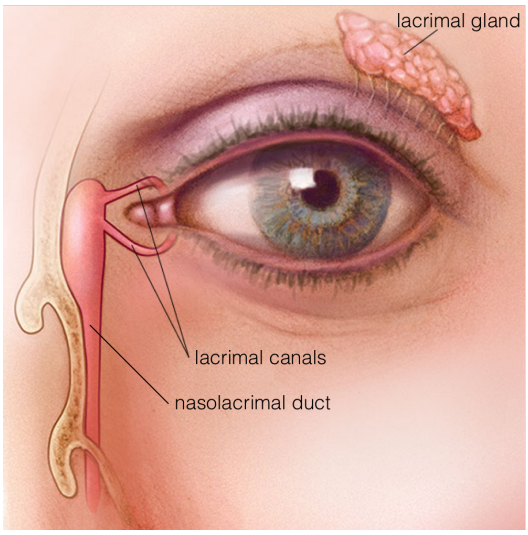


Fig. 1.3. Drawing showing position of nasolacrimal duct and lacrimal gland around human eye [adapted from MAYO Foundation for Medical Education and Research, www.mayoclinic.org/diseases-conditions/dry-eyes/diagnosis-treatment/drc-20371869].

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).

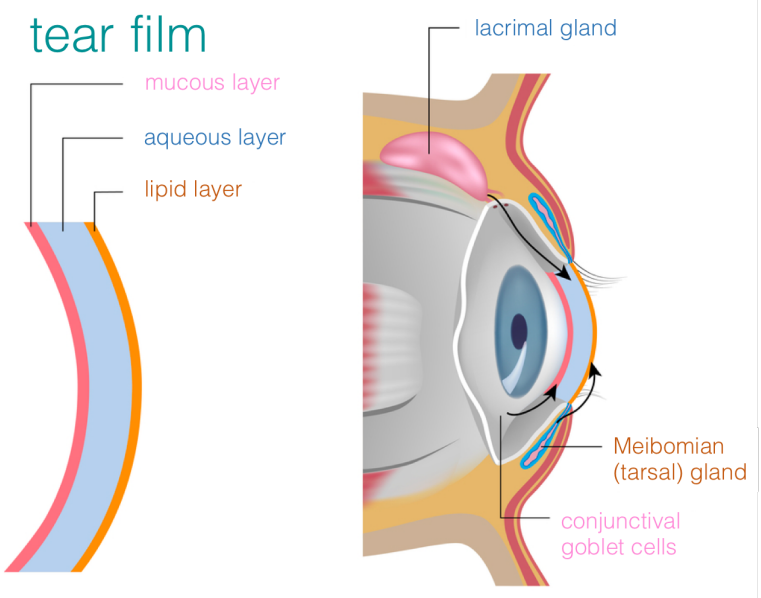


Fig. 1.4 Scheme of tear film and its producing glands [adapted from Bannenberg, 2015].

- 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 hyper-osmolarity, 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 myopisation 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 mucomimetic 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.c.* 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:

Asiedu K, Kyei S, Mensah S, Ocansey S, Abu L, Kyere E (2007) Ocular surface disease index (OSDI) versus the standard patient evaluation of eye dryness (SPEED): A study of a nonclinical sample. Cornea 35: 175-180.

Bannenberg G (2015) Visual function. Oral omega-3 long-chain PUFA intake to improve comfort of people with Dry Eye. PUFA Newsletter 20: 25-29.

Barbosa-Breda J, Himmelreich U, Ghesquière B, Rocha-Sousa A, Stalmans I (2017) Clinical metabolomics and glaucoma. Ophthalmic Res. DOI: 10.1159/000479158

Baudouin C, Aragona P, Messmer EM, Tomlinson A, Calonge M, Boboridis KG, Akova YA, Geerling G, Labetoulle M, Rolando M (2013) Role of hyperosmolarity in the pathogenesis and management of Dry Eye Disease: Proceedings of the OCEAN Group Meeting. Ocul. Surf. 11: 246-258.

Benelli U, Nardi M, Posarelli C, Albert TC (2010) Tear osmolarity measurement using the TearLab™ Osmolarity System in the assessment of dry eye treatment effectiveness. Contact Lens Anterior Eye 33: 61-67.

Beuerman RW, Mircheff A, Pflugfelder S, Stern M (2004) The lacrimal functional unit. In: Dry Eye and ocular surface disorders. (Pflugfelder SC, Beuerman RW, Stern ME, Eds.) CRC Press, New York, pp. 11-39.

Bhavsar AS, Samir G. Bhavsar SG, Jain SM (2011) A review on recent advances in dry eye: Pathogenesis and management. Oman J Ophthalmol. 4: 50-56.

Carrilho E, Garcia CD (2014) Instrumentation for capillary electrophoresis and microchip electrophoresis. Electrophoresis 35: 2067.

Craig JP, Chen YH, Turnbull PR (2015) Prospective trial of intense pulsed light for the treatment of meibomian gland dysfunction. Invest. Ophthalmo.Vis. Sci. 12: 1965-1970.

Davidson Kuonen VJ (2004) The tear film and ocular mucins. Vet. Ophthalmol. 7: 71-77.
DEWS (2007) The epidemiology of dry eye disease: Report of the Epidemiology Subcommittee of the International Dry Eye WorkShop. Ocul. Surf. 5: 93-107.
Geerling G, Sieg P (2008) Transplantation of the major salivary glands, Dev. Ophthalmol. 41: 255-268.
Gipson IK, Hori Y, Argüeso P (2004) Character of ocular surface mucins and their alteration in dry eye disease. Ocul. Surf. 2: 131-148
Gipson IK (2007) The Ocular Surface: The Challenge to Enable and Protect Vision. Invest. Ophthalmol. Vis. Sci. 48: 4390-4398.
Jones DT, Monroy D, Pflugfelder SC (1997) A novel method of tear collection: comparison of glass capillary micropipettes with porous polyester rods. Cornea. 16: 450-458.
Kashima T, Itakura H, Akiyama H, Kishi S (2014) Rebamipide ophthalmic suspension for the treatment of dry eye syndrome: a critical appraisal. Clin. Ophthalmol. 8: 1003-1010.
Kawakita T, Shimmura S, Tsubota K (2015) Effect of oral Pilocarpine in treating severe dry eye in patients with Sjögren syndrome. Asia Pac. J. Ophthalmol. 4: 101-105.
Keating GM (2015) Diquafosol ophthalmic solution 3 %: a review of its use in dry eye. Drugs 75: 911-922.
Lane SS, DuBiner HB, Epstein RJ, Ernest PH, Greiner JV, Hardten DR, Holland EJ, Lemp MA, McDonald JE 2nd, Silbert DI, Blackie CA, Stevens CA, Bedi R (2012) A new system, the LipiFlow, for the treatment of meibomian gland dysfunction. Cornea 31: 396-404.
Lauwen S, de Jong EK, Lefeber DJ, den Hollander AI (2017) Omics biomarkers in ophthalmology. Invest. Ophthalmol. Vis. Sci. 58: BIO88-BIO98.
Lemp MA (2007) The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop (2007). Ocul. Surf. 5: 75-92.
Liu H, Begley C, Chen M, Bradley A, Bonanno J, McNamara NA, Nelson JD, Simpson T (2009), A link between tear instability and hyperosmolarity in dry eye. Invest. Ophthalmol. Vis. Sci. 503: 671-3679.
Mainstone JC, Bruce AS, Golding TR (1996) Tear meniscus measurement in the diagnosis of dry eye. Curr. Eye Res. 15: 653-661.
McKenzie RW, Jumblatt JE, Jumblatt MM (2000) Quantification of MUC2 and MUC5AC transcripts in human conjunctiva. Invest. Ophthalmol. Vis. Sci. 41: 703-708.
Murube J. (1998) Labial salivary gland transplantation in severe dry eye, Oculoplast. Orbital Reconstr. Surg. 1: 104-110.
Murube J, Németh J, Höh H, Kaynak-Hekimhan P, Horwath-Winter J, Agarwal A, Baudouin C, Benítez del Castillo JM, Cervenka S, ChenZhuo L, Ducasse A, Durán J, Holly F, Javate R, Nepp J, Paulsen F, Rahimi A, Raus P, Shalaby O, Sieg P, Soriano H, Spinelli D, Ugurbas SH, Van Setten G (2005) The triple classification of dry eye for practical clinical use. Eur. J. Ophthalmol. 15: 660-667.
Nasir Saeed N, Qazi ZA, Butt NH, Siddiqi A, Maheshwary N, Athar Khan M (2013) Effectiveness of sodium hyaluronate eye gel in patients with dry eye disease: a multi-centre, open label, uncontrolled study. Pak. J. Med. Sci. 29: 1055-1058.
Paulsen FP, Schaudig U, Thale AB (2003) Drainage of tears: impact on the ocular surface and lacrimal system, Ocul Surf. 1:180-191.
Pondelis N, et al. Intranasal neurostimulator induces morphological changes in meibomian glands in patients with dry eye disease. Presentation at the Annual ARVO Meeting (Association for Research in Vision and Ophthalmology) May 7-11, 2017; Baltimore (MD, USA).

Qiao J, Yan X (2013) Emerging treatment options for meibomian gland dysfunction. Clin. Ophthalmol. 7: 1797-1803.
Rhodes M, Heinrich C, Featherstone H, Braus B, Manning S, Cripps PJ, Renwick P (2012) Parotid duct transposition in dogs: a retrospective review of 92 eyes from 1999 to 2009. Vet. Ophthalmol. 15: 213-222.
Samudre S, Lattanzio FA, Lossen V, Hosseini A, Sheppard JD, Mc Cown RL, Laurie GW, Williams PB (2011) Lacritin, a novel human tear glycoprotein, promotes sustained basal tearing and is well tolerated. Invest. Ophthalmol. Vis. Sci. 52: 6265-6270.
Savini G, Prabhawasat P, Kojima T, Grueterich M, Espana E, Goto E (2008) The challenge of dry eye diagnosis. Clin. Ophthalmol. 2: 31-55.
Shapiro A, Merin S (1979) Schirmer test and break up time of tear film in normal subjects. Am. J. Ophthalmol. 88: 752-757.
Shimmura S, Ono M, Shinozaki K, Toda I, Takamura E, Mashima Y, Tsubota K (1995) Sodium hyaluronate eyedrops in the treatment of dry eyes. Br. J. Ophthalmol. 79: 1007-1011.
Schirmer O (1903) Studien zur Physiologie und Pathologie der Tranen-Absonderung und Tranenabfuhr. Graefes Arch. Clin. Exp. Ophthalmol. 56: 197-291.
Schrader M, Schulz-Knappe P, Fricker LD (2014) Historical perspective of peptidomics. EuPA Open Proteomics 3: 171-182.
Spurr-Michaud S, Argüeso P, Gipson I (2007) Assay of mucins in human tear fluid. Exp. Eye Res. 84: 939-950.
Stern ME, Gao J, Siemasko KF, Beuerman RW, Pflugfelder SC (2004) The role of the lacrimal functional unit in the pathophysiology of dry eye. Exp. Eye Res. 78: 409-416.
Stuchell RN, Feldman JJ, Farris RL, Mandel ID (1984) The effect of collection technique on tear composition. Invest. Ophthalmol. Vis. Sci. 25: 374-377.
Suchi S, Harsha J (2015) Prevalence and associated factors of dry eye: Our experience in patients above 40 years of age at a Tertiary Care Center. Oman J. Ophthalmol. 8: 151-156.
Sullivan BD, Whitmer D, Nichols KK, Tomlinson A, Foulks GN, Geerling G, Pepose JS, Kosheleff V, Porreco A, Lemp MA (2010) An objective approach to dry eye disease severity. Invest. Ophthalmol. Vis. Sci. 51: 6125-6130.
Takahashi Y, Watanabe A, Matsuda H, Nakamura Y, Nakano T, Asamoto K, Ikeda H, Kakizaki H (2013) Anatomy of secretory glands in the eyelid and conjunctiva: a photographic review. Ophthalmol. Plast. Reconstr. Surg. 29: 215-219.
Tran DT, Cavett VJ, Dang VQ, Torres HL, Paegel BM (2016) Evolution of a mass spectrometry-grade protease with PTM-directed specificity. Proc. Natl. Acad. Sci. USA 113: 14686-14691.
Uhlig T, Kyprianou T, Martinelli FG, Oppici CA, Heiligers D, Hills D, Ribes Calvo X, Verhaert P (2014) The emergence of peptides in the pharmaceutical business: From exploration to exploitation. EuPA Open Proteomics 4: 58-69.
Vijmasi T, Chen FYT, Balasubbu S, Gallup M, McKown RL, Laurie GW, McNamara NA (2014) Topical administration of lacritin is a novel therapy for aqueous-deficient dry eye disease. Invest. Ophthalmol. Vis. Sci. 55: 5401-5409.
www.ClinicalTrials.gov (2017) Trial n° NCT03226444. Lacripep™ in subjects with dry eye associated with primary Sjögren's syndrome.
Yung YH, Toda I, Sakai C, Yoshida A, Tsubota K (2012) Punctal plugs for treatment of post-LASIK dry eye. Japan. J. Ophthalmol. 56: 208-213.

European Journal of Ophthalmology / Vol. 15 no. 6, 2005 / pp. 660-667

Appendix 1.1 Triple classification

The triple classification of dry eye for practical clinical use

J. MURUBE[1] (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)

[1]Department 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 affectation of the various dacryoglands 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, dysgenic, 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, lipo deficient, 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*

Accepted: Otober 3, 2005

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,

1120-6721/660-08$15.00/0

© Wichtig Editore, 2005

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, keratopathia filamentosa, 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, prognosis, 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. I). The first five groups of this etiopathogenetic decalogue generally, but not always, affect many exocrinic glands (lacrimal, salivary, nasal, vaginal, etc.) because the damage is usually produced in cellular structures common to exocrinic glands. The last five groups usually only affect the dacryoglands (aqueo-serous, lipid, mucinic), 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 exocrinic glands, and consequently there is presentation of a general dryness in the body, including the dacryoglands. 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 horizontality, 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, hypoovarism, 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.

Hormonal dry eye is generally multiexocrinic. Aquoserous 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, chlomethiazole), antiparkinsonians (biperiden, benztropine), diuretics (chlorthalidone, furosemide), vascular antihypertensives (chlorothiazide, clonidine), anticholinergics (atropine, metoclopramide), antihistaminics (dexchlorpheniramine, 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 (cocaine, 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 dacryoglands 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 exocrinic glands and ocular surface, such as the pluriglandular endocrine deficiencies or Schmidt's syndrome (thyroid and adrenal insufficiency).

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
4. Immunopathic	**9**. Neurologic
5. Hyponutritional	**10**. Tantalic

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 B_2, B_{12}, 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 γενεη, 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,

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 dacryoglands: 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 *flamma*, 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 dacryoglands (tuberculous, fungic) are at present very rare. Infection/inflammation of the lipid dacryoglands (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 dacryoglands and ocular surface may be mechanical (surgical or accidental), chemical, or radiation induced. This damage to dacryoglands 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 transplantation, 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 salivalis 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 lacrimale, 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 decompensation, 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 scurfy 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 decalogue 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 dacryoglands — aqueo-serous, lipid, and mucinic — 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 glycocalix, 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 epitheliopathy*** 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

Patient :.....

1) ETIOPATHOGENESIS

X	1. Age-related	*… 62 years*
X	2. Hormonal	*… menopause*
X	3. Pharmacologic	*… sleeping pills*
	4. Immunopathic	
	5. Hyponutritional	
	6. Dysgenic	
X	7. Infectious/inflammatory	*… chronic blepharitis*
	8. Traumatic	
	9. Neurologic	
	10. Tantalic	

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	
	Grade 1	*… dryness sensation, occasional ocular itching, vespertinal BIVA*
X	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

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

The affected dacryogland may be initially of one, two, or of all types. It depends on the type of etiology. In any case, all dacryoglands 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 dacryoglands. 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 dacryoglands and the epithelium of the ocular surface.

Ocular surface epitheliopathy is diagnosed by slit lamp-biomicroscopic 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-lalopalassia), 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 dacryoglands 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 clonic 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, hypolysozyme, 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, keratopathia punctata, keratopathia 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.

The authors have no commercial or proprietary interest in any aspect of the article.

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

REFERENCE

1. Murube J, Benítez del Castillo JM, ChenZhuo L, Berta A, Rolando M. The Madrid triple classification of dry eye. Arch Soc Españ Oftalmol 2003; 78: 587-94.

Chapter 2: Background for surgical intervention in DED.

2.0 Introduction

Moderate cases of DED, where the problem is not yet pathological, are typically treated by compensating for the lack of tear quality (substitution) and/or quantity (addition of artificial tear fluid).
In more severe cases where the patient fails to respond to the above forms of treatment, surgery is an option. Surgery for DED can have (a combination of) one out of 4 objectives [Kruse et al., 2006]:
- reduction of tear clearance (e.g. closure of tear puncta);
- addition of tears or tear components to the ocular surface (e.g. transplantation of salivary glands);
- decreasing the area of evaporation of tears (e.g. tarsorrhaphy, induction of ptosis);
- surgical correction of diseases that contribute to dry eyes (e.g. repair of eyelid pathology such as ectropion or trichiasis).

The surgical therapy of DED through autotransplantation of labial salivary glands to the eyelids, was originally described by Murube [1998]. Our most important modification of this technique was the use of Radiofrequency (RF) surgery and the use of a running subconjunctival polypropylene suture to fix the graft (Chapter 3.2).
Because of the similarity between low frequency electrosurgery (500 KHz; which causes significantly more tissue destruction) and RF surgery with high frequency radio waves (4.0 MHz), scientific evidence was needed to justify the modification of Murube's surgery, who already described excellent results.
In Chapter 2.1 the history of RF surgery is covered, and also the modifications that have led to the success of high frequency low temperature RF surgery and the explanation for the superior results of RF surgery as compared to electrosurgery or surgery with a blade.
The study of upper lid blepharoplasty without cutting the orbicularis muscle with RF surgery (Chapter 2.2) shows that with RF surgery the thin skin of the upper eyelid can be excised selectively. The same careful dissection is needed when dissecting the conjunctiva to prepare the recipient bed of the tarsal conjunctiva for the graft and also for the excision of the labial mucosa and underlying salivary glands. Excessive bleeding during prelevation of the graft with a blade jeopardizes the survival of the transplant. And too much coagulation as with electrosurgery can destroy the precious and delicate salivary glands.
Everting the eyelids during the transplantation surgery is similar to what is done in a transconjunctival lower lid blepharoplasty (Chapter 2.3) when a Desmarres lid retractor is used. Also in this type of lower lid blepharoplasty, a careful dissection of the tarsal conjunctiva is needed, avoiding damage to the underlying structures and limiting intra- and post-operative bleeding.
When performing transplantation of labial salivary glands, bleedings can delay or prevent vascularization and thus survival of the graft.

The use of the Raus-Garito clamp (Chapter 2.4) is another example of how RF surgery allows selective dissection of small and superficial layers of tissue and illustrates how everting the eyelid facilitates surgery of the conjunctiva by stretching the tissue.

We conclude that RF surgery may have added value to the transplantation of labial salivary glands to the eyelids.

References:

Kruse FE, Holbach LM (2006) Surgery of Dry Eye Disease. In: Dry Eye Disease, the clinician's guide to diagnosis and treatment (Asbell PA, Lemp MA, eds.), Thieme, New York, Stuttgart pp. 151.

Murube J (1998) Labial salivary gland transplantation in severe dry eye. Oculoplast. Orbital Reconstr. Surg. 1: 104-110.

2.1 Radiofrequency Surgery: 3500 years old and still young.

[P.P.M. Raus, P.D.E.M. Verhaert (2017) Orbit doi:10.1080/01676830.2017.1383469]

ORBIT
https://doi.org/10.1080/01676830.2017.1383469

Taylor & Francis
Taylor & Francis Group

REVIEW ARTICLE

Check for updates

Radiofrequency surgery: 3500 years old and still young

Peter P. M. Raus[a] and Peter D. E. M. Verhaert[b]

[a]Department of Ophthalmology, Miró Centrum voor Oogheelkunde en Esthetiek, Geel, Belgium; [b]ProteoFormiX, Janssen Pharmaceutica Campus, Beerse, Belgium

ABSTRACT

Purpose: To review the history of Radiofrequency surgery, delineate the actual situation and describe the applications in eyelid surgery.
Design: Review.
Methods: Review of literature, personal communication with several pioneers in the field, and own experience.
Conclusion: Radiofrequency surgery has evolved from rude burning to a sophisticated surgical technique.

ARTICLE HISTORY
Received 16 January 2017
Accepted 19 September 2017

KEYWORDS
Radiofrequency surgery; electrosurgery

History: From cautery to radiosurgery

Already a few thousand years ago, wounded soldiers were treated by pouring boiling oil into the wound to arrest bleeding. This application of 'cautery' appears in ancient Indian medical writings like Atharva-Veda, Choraka, and Sushruta. The dates are not completely certain, but may be anywhere around 1500 BC. The same Indian writings report the use of a sort of cautery to destroy breast lesions.[1] Hippocrates himself (around 400 BC), advised to use cautery to destroy lesions, if other methods failed. The eruption of Mount Vesuvius in 79 AD was key to preserve a lot of surgical instruments of ancient Rome. They now give us an idea about the medical knowledge at that time (Figure 1).

In ancient China, already 2000 years ago, Moxa, a dried plant (*Artemisia argyi*), was used to heat and stimulate acupuncture points. The process is called *moxibustion* and this kind of therapy has been in use as adjunctive technique during acupuncture sessions ever since.[2] In so called 'direct moxibustion', a small, cone-shaped amount of the plant is put on an acupuncture point and then burned. This direct moxibustion is further divided into a scarring and a non-scarring subtype. With scarring moxibustion, the moxa is placed on an acupuncture point to burn until it causes blisters and scarring down to a deep second degree burn. With non-scarring moxibustion, the burning Moxa is removed before it actually scars the skin.[2]

The use of hot oil for coagulation to stop bleeders was used until in the 16th century. Ambroise Paré (1510–1590), a French barber surgeon and a pioneer in surgical techniques and battlefield medicine, still used boiling oil. Although he used a cauterizing iron to stop the bleeding when amputating limbs, for other wounds, he sometimes poured boiling oil in the wound, such as in cases of bullet wounds. Gunpowder was thought to be poisonous and the oil was hoped to destroy the poison. One day, Paré had to treat more patients than he had oil for, and he experimented with an old Roman recipe. He used a cocktail of oil of roses, egg white, and turpentine. The next morning the soldiers treated with hot oil went very bad and some had even died during the night. But the men treated with Roman ointment were well rested, their wounds calm and even beginning to heal. Then he continued with this approach to sealing wounds, rather than the usual method of cauterizing wounds.[3] In 1791 Luigi Galvani was the first to demonstrate that an electric current can activate muscles. As in a lot of other famous discoveries, the correlation between electricity and muscle contraction was discovered 'by accident'. The legend goes that Galvani was skinning a frog at a table, where he had been conducting experiments with static electricity by rubbing frog skin. When Galvani's assistant touched the exposed sciatic nerve of the frog with a metal scalpel that had picked up a charge, he saw sparks and the dead frog's leg kicked as if in life.[4] In the scientific literature, we find that already in 1780 Galvani discovered that two connected different metals

CONTACT Peter P. M. Raus Peter.raus@gmail.com Department of Ophthalmology, Miró Centrum voor Oogheelkunde en Esthetiek, Stationsstraat 130, 2440, Geel, Belgium.

Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/iorb.

© 2017 Taylor & Francis

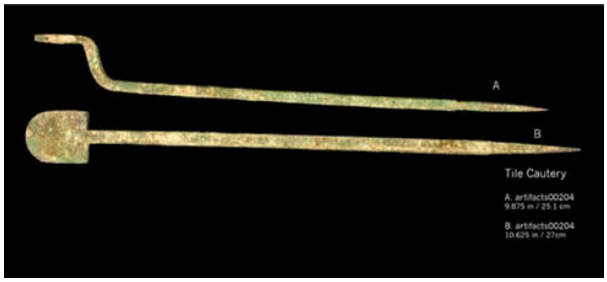


Figure 1. Cautery instruments from 79 AD. Courtesy of historical collections & services, Claude Moore health sciences library, University of Virginia.

(e.g. copper and zinc) at the same time touch two different parts of a frog leg's nerve, make the leg contract. He called this phenomenon "animal electricity".[5] The voltaic pile, invented by Alessandro Volta, a contemporary and friend of Galvani, consists of a pile of cells similar to the galvanic cell. However, Volta built it entirely out of non-biological material. Indeed, the Volta cell essentially consists of two different metals connected by a salt bridge, or individual half-cells separated by a porous membrane.

The first monography on the application of electrical current in surgical interventions was published in 1854 by Dr. Albrecht Theodor Middeldorf.[6(p.1824–1868)] Middeldorf used the term 'galvanocautery' that he described as a procedure in which especially constructed thin platinum wires were transformed into glowing heat by a galvanic current from a zinc-platinum-battery. Thanks to this procedure, it was possible to perform dissection and destruction of tissue and coagulation of bleeding vessels for hemostasis during surgery. About a century later, Grenet introduced his single-element battery source with flasks of varying volumetric capacity, from 1/3 liter to 3 liters. His invention was published in 1885 in the Journal 'Electrical Review'.[7] It was this type of battery that was used by Paul Broca for his so called "electric knife". He modulated the electric current to the blade and in this way, he was able to change the temperature of the blade. This was achieved by lifting the plates of the battery more or less out of the salt solution. It was Antoine Séré, a French military surgeon, who in 1862 developed the housing for this battery with changeable power and to connect it with a kind of pedal to facilitate its use.[8] Thanks to these modifications the temperature of the blade could be altered between 600 and 4500 degrees Centigrade. Modern diathermy with high-frequent alternating current was introduced in medicine by the German Dermatologist Dr. Franz Nagelschmidt from Berlin. In 1913 he published his



Figure 2. McIntosh diathermy with adjustable frequency and Tesla coils (From the collection of John Abele with kind permission of John Abele and Steven D Schwaitzberg).



Figure 3. Liebel-Flarsheim "Bovie" (From the collection of John Abele, with kind permission of John Abele and Steven D. Schwaitzberg.

'Lehrbuch der Diathermie'.[9] William T. Bovie introduced the "Bovie" electrosurgical device in the 1920's. In the beginning, its main use was primarily for coagulation.[10] At that time other devices used Tesla coils with exposed spark gap generators (Figure 2–3). Bovie was the first to develop an electrosurgical unit that provided not only coagulation, but also cutting settings. He was the son of William Bovie, a farmer physician. To avoid confusion with his father the insertion 'T' was added as surname prefix. What the 'T' stands for still is a well-kept secret. He discovered that electric current above certain frequencies could cut tissue without inducing muscular contraction. The first units were hand-held via a pistol-grip, allowing easy manipulation by hand activation. The electrodes were interchangeable.[11] Dr. Harvey Williams Cushing,

a neurosurgeon whose name is associated with Cushing's disease, popularized the electrosurgery machine. Cushing is known as "The Father of Neurosurgery." He incorporated electrosurgical techniques in neurosurgery. Cushing's crucial collaboration with William T. Bovie and his electrosurgical apparatus allowed him not only to operate in patients whose tumors had been previously described as 'inoperable'. In 1926, Cushing had been unsuccessful in removing a mass from a patient's head due to extensive bleeding. However, a few days later he did a re-intervention, using the Bovie unit. In his notes from that day he wrote, "…with Dr. Bovie's help I proceeded to take off most satisfactorily the remaining portion of tumor with practically none of the bleeding which was occasioned in the preceding operation."[12] Dr. Bovie sold the patent to the Liebel-Flarsheim Company symbolically for only one dollar. They began producing the unit under the Liebel-Flarsheim name. This was an electrosurgery unit that was marketed exclusively to U.S. hospitals and only in 1965 variations of the original Bovie were commercially sold (Figure 4). From that year on, the Hyfrecator and other low frequency-high temperature devices were being marketed to office practices for coagulation of bleeders (Figure 5). In Europe those units were called Diathermy. In order to increase sales, some of these companies claimed cutting ability and produced wire electrodes such as loops.

Irving Ellman, a dentist and electronic engineer at UCLA School of Dentistry, began to use electrosurgery devices in dentistry. At UCLA, he had a close friend John Flocken who was head of the Oral Surgery Department and Biomedical Engineering Department. The electrosurgery companies realized that, if they could convince dentists to use the electrosurgical



Figure 4. Liebel-Flarsheim short wave radiofrequency generator from the 1930s (From the collection of John Abele with kind permission of John Abele and Steven D. Schwaitzberg).



Figure 5. The Birtcher Crusader developed in the 1930s, a forerunner of the Hyfrecator (From the collection of John Abele, with kind permission of John Abele and Steven Schwaitzberg).

machines, their selling markets would increase exponentially. Indeed, at that time there were over 100 thousand dentists in the U.S., all in private offices and all did surgery. So several electrosurgical companies gave units to the dental schools to try on patients. UCLA School of Dentistry was one of them. Flocken discussed it with his friend Irving Ellman. Both knew that, if electrosurgery worked well in the oral cavity it would be a huge improvement to be able to gently cut gum tissue and to control bleeding in the mouth. However, as Flocken began to use the electrosurgery machines that were given to the dental clinic he noticed that these units burned the thin gingival tissue to a crisp.[13] In Flocken's words: 'These machines were a disaster in the dental cavity'. He wrote letters to other Universities cautioning them to not use these medical electrosurgery machines on gingival tissue and gave lectures showing the destroyed oral tissue after electrosurgical use. Other professors joined in warning the dental profession against its use. In less then 1 year the dental profession completely banned electrosurgical machines for dental use. The gingival tissue around the teeth is extremely thin, almost transparent. When it was burned it looked very ugly, when the patients smiled. Ellman asked his friend Flocken to send him the banned electrosurgical machine, so he could evaluate it and do further tests on this device. In the late 1960s Irving Ellman worked in the laboratory with a Variac. This electronic device allows one to vary the frequency for testing frequency purposes. He noticed that 4 MHz was the optimum frequency that produced the smoothest cutting effects on the tissue at the lowest temperature.[14] More tissue alteration was seen as he went beyond the 4 MHz. However, at that time it was not possible to build a device that would be fixed at 3.8

or 4 MHz. So the next best choice was to use the strong vacuum tube and design a circuit around the vacuum tube power source. Ellman also realized that the electrosurgical machines were too large and bulky to fit well in a small dental operatory. He took advantage of the new miniaturized electronic components. Small portable radios were becoming popular around that time. In 1973 Dr. Irving Ellman issued patents on a unit with a vacuum tube. It was the Dento-Surg Radiosurgical device. It was mainly a 90 Watts, 3.8 MHz device with three currents with a different waveform. In a further effort to get different results on tissue then he had discovered that by changing the waveforms and he could further reduce the temperature and improve the histological effect on the tissue:

- The Fully filtered waveform was developed by using a series of rectifiers. It gave 90% cutting and only 10% of coagulation.
- Fully rectified: 50% cutting and 50% coagulation
- Partially rectified: 10% cutting and 90% coagulation.

This unit is distributed today by Ellman for Dentistry. This Dento Surg formed the basic design of the Surgitron (see below), a device still being sold today.

Several of the Dento Surg units were sent to UCLA for Dr. Flocken to evaluate in the dental clinic. He studied tissue shrinkage, tissue alteration, healing, postoperative pain, cosmetic effect, and many other tissue parameters. He compared everything to the scalpel and other gold standard taught tools for altering and removing gingival tissue.[13] In the same year, Jon Garito, began working with Ellman when he was in college (Figure 6). Garito had married Ricki Ellman, Irving Ellman's daughter in 1972. Jon Garito started law school, but found that he loved working at Ellman Dental Mfg., and continued combining work with law school. During the course of the next year, Irving Ellman was diagnosed with colon cancer and he passed away in 1975. Jon Garito stepped in and took over the business. In the same year, Garito went to UCLA to work with Dr. Flocken in the Oral Surgery department and the Biomedical department. He assisted Dr. Flocken with many experiments and studies and worked with biomedical engineering professors and graduate students. He also joined Dr. Flocken for most of his over 1000 lectures at every dental school in the U.S. Internationally he was invited to give lectures and workshops at congresses and in Universities. These continuing education courses included hands-on workshops, where calf mandibles would be used to simulate the oral cavity. Dr. Flocken designed a Radiosurgery hands-on pre-surgical workshop for dentists. His workshop syllabus was later changed to reflect the different medical specialties Radiosurgery applications. The basic pre-clinical steps of the hands-on workshop was kept the same and is still used today to teach doctors how to start using the Radiosurgery device.

At this same time, Dr. Arthur Goldstein from Monaco, introduced the name 'Dento-Surg Radiosurgery' to avoid confusion with the earlier tissue destroying electrosurgical machines[15] (Figure 7). In 1978, Jon Garito introduced the Radiosurgery unit to the Veterinary market. Dr. Bob Altman a Professor at University of Pennsylvania School of Veterinary Medicine began publishing articles on its use in small

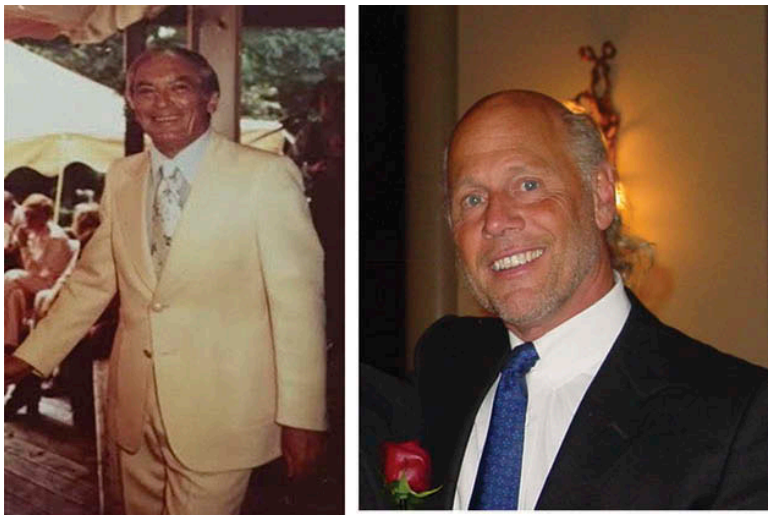


Figure 6. Dr. Irving Ellman (left) and Dr. Jon Garito (right) (courtesy of Dr. Jon Garito.



Figure 7. The Dento-Surg from the 1970s.



Figure 8. The surgitron FFPE from the 1980s.

animals and later large animals.[16] Dr. Garito designed a new circuit, which increased the power, making it more robust for the larger animals. The higher powered RF device was named Surgitron (Figure 8). In 1981 with the encouragement and support of Dr. Sheldon Pollack, head of Dermatology at Duke University, Radiosurgery entered the Dermatology market.[17] He had taken a Dental Radiosurgery course and then adapted the training manual to Dermatology. He used this Radiosurgery syllabus to teach courses at Duke University's Dermatology department.

The first use in Gynecology was in 1987. Sheldon Weinstein, gynecology professor at the University of Texas began performing cervical biopsies with excellent results and significant advantages over cryosurgery and lasers.[18] Around 1984, Dr. Garito met Stephen Bosniak, an oculoplastic surgeon in New York. He was the first Oculoplastic surgeon to use Radiosurgery in. 1985[19] Stephen Bosniak was a good



Figure 9. Stephen Bosniak and senior author Peter Raus after Radiofrequency surgery in New York in May 1996.

colleague, friend, and teacher of the senior author of this review[20] (Figure 9). ENT followed with the support of Klaus Vogt[21] and Mahmoud Moravej; plastic surgery with Randolf Waldman in. 1990[22] In 2000 Radiosurgery entered Family Practice with Jack Pfenninger, a professor of Family Practice at the University of Michigan, Podiatry with Walter Gursky, University of Connecticut and Plastic Surgery with Albert Hofmann in Germany.[14] Finally in 2005 Neurosurgery and Pediatric Neurosurgery followed with James Goodrich who separated the head-joined twins Carl and Clarence Aguirre, Steve Schneider and Anders Cohen.[23] In 1995, the first 4 MHz unit was developed as it had appeared to give better surgical results with a finer cut and less lateral heat spread in the tissues. Garito also discovered the importance of the specific material and size of the electrodes. So it was both the development of the optimum RF energy source and innovative RF electrode designs that worked in tandem to provide surgeons with the improved surgical outcomes. Garito patented over 150 Radiosurgery accessories as well. In 1997 the manufacturer Ellman switched from a vacuum tube to a solid state microprocessor. At that time an important competition was going on in Europe and many other countries. Europe had introduced the new regulations by the International Electrotechnical Commission (IEC). Up to that point, all countries relied on U.S. FDA regulations. So, with an FDA approval medical devices could be distributed internationally. However, the IEC regulations posed many technical problems for manufacturers. One was leakage current of the original vacuum tube unit. Other points of discussion were the type of connection plugs, the antenna plate and many others. These were mainly safety issues that were raised due to the problems that in the first place occurred in the OR's with the larger surgical devices.

However, rather than separate the large Electrosurgical Units from the smaller powered devices, all were grouped together. That is why the original RF unit had to be redesigned. That was the genesis of the Dual Frequency device. Because, there are also advantages to the lower frequency in coagulation of bleeders, not only the 4 MHz but also the 1.7 MHz frequency was included. The Dual Frequency device met the IEC regulations. Indeed, because sometimes a more coagulating current can be very useful to perform surgery in more vascularized areas, several RF machines offer 2 different types of current or different frequencies to work with. This Dual Frequency RF device invented by Dr. Jon Garito was granted a U.S. and International patents in 1999. The 4 MHz RF devices produce less tissue alteration then the 3.8 MHz RF tube devices. For this reason, it was ideal for the Neurosurgical and Spine procedures. The most recent modification is a 4 MHz, 500 KHz, portable wireless Radiowave device (patent pending). The most important advantages of this new unit can be:

- Reduction of the power of the unit,thanks to less loss of signal over the cable between the unit and the handpiece,
- no antenna plate needed,
- less interference with other electronic devices in the OR.[14]

Conclusion

The basics of Radiofrequency surgery go back to 3500 years ago. The technique has evolved from rude burning to a sophisticated surgical technique with further innovations expected for the near future.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

1. Shapiro AK, Shapiro E. The powerful placebo: From ancient priest to modern physician. *Bmj*. 1998;316:1396B. doi:10.1136/bmj.316.7141.1396b.
2. Yang L, Jiang H, Wang L, Ma H. Development and application of new temperature control moxibustion device. *Zhongguo Zhen Jiu*. 2015;35:745–747.
3. Ross JB, McLauglin MM, Paré A. A surgeon in the field. In: eds. *The Portable Renaissance Reader*. New York,: Viking Penguin; 1981:558–563.
4. Bresadola M. Medicine and science in the life of Luigi Galvani (1737-1798). *Brain Res Bull*. 1998;46:367–380. doi:10.1016/S0361-9230(98)00023-9.
5. Piccolino M. Luigi Galvani and animal electricity: Two centuries after the foundation of electrophysiology. *Trends Neurosci*. 1997;20:443–448. doi:10.1016/S0166-2236(97)01101-6.
6. Sachs M, Sudermann H. History of surgical instruments: 7. The first electrosurgical instruments: Galvanic cauterization and electric cutting snare. *Zentralbl Chir*. 1998;123:950–954.
7. Grenet E. The single-element battery source. *Elect Rev*. 1885; 6(18). New York, NY, United States. July 4:3. col. 1.
8. Segal A, Ferrandis JJ. Eugene de Séré (1828-1870), inventor of the electric lancet. *Hist Sci Med*. 1998;32:255–264.
9. Nagelschmidt F. *Lehrbuch Der Diathermie: Für Ärzte Und Studierende*. Berlin: Julius Springer; 1913.
10. Goldwyn RM. Bovie: The man and the machine. *Ann Plast Surg*. 1979;2:135–153. doi:10.1097/00000637-197902000-00008.
11. Schwaitzberg SD, Evolutions and revolutions in surgical energy. In: Feldman L, Fuchshuber P, Jones DB, eds. *The Sages Manual on the Fundamental Use of Surgical Energy*. Part I, 3–15. New York, Dordrecht, Heidelberg, London: Springer; 2012.
12. Voorhees JR, Cohen-Gadol AA, Laws ER, Spencer DD. Battling blood loss in neurosurgery: Harvey Cushing's embrace of electrosurgery. *J Neurosurg*. 2005;102:745–752. doi:10.3171/jns.2005.102.4.0745.
13. Flocken JE. Electrosurgical management of soft tissues and restorative dentistry. *Dent Clin North Am*. 1980;24:247–269.
14. Hofmann A, Wüstner M, Ciric B. Radiowave surgery casereport. *Int J Aesthetic Res Surg*. 1996;4:131–132.
15. Goldstein A. Radio microsurgery update: Troughing and impression taking. *Dent Today*. 2007;26:120–121.
16. Altman RB, Principles and applications of electrosurgery. In: *Assoc. Avian Vet. Annual Conference Practical Labs Proceedings and Manual*. Washington: District of Columbia United States; 1994;85–92.
17. Pollack SV, Carruthers A, Grekin RC. The history of electrosurgery. *Dermatol Surg*. 2000;26:904–908. doi:10.1046/j.1524-4725.2000.026010904.x.
18. Turner RJ, Cohen RA, Voet RL, Stephens SR, Weinstein SA. Analysis of tissue margins of cone biopsy specimens obtained with "cold knife," CO_2 and Nd: YAGlasers and a radiofrequency surgical unit. *Reprod Med*. 1992;37:607–610.
19. Bosniak S, Zilkha MC. Cosmetic radio-blepharoplasty. *Int J Aesthetic Res Surg*. 1995;3:53–56.
20. Raus P, Mertens E. Evaluation of radiosurgery as a cosmetic surgery technique. *Int J Aesthetic Res Surg*. 1997;5:96–100.
21. Vogt K, Enache R. Radiofrequency turbinotomy: Basic, practice and statistics. *Rom J Rhinol*. 2012;2:133–138.
22. Waldman SR. Cutaneous procedures: management of superficial skin lesions in a cosmetic surgery practice. In: Stucker FJ, eds. *Plastic and Reconstructive Surgery of the Head and Neck*. Part 13/Chapter 120. Philadelphia: BC Decker; 1991:595–598.
23. Personal communication by Dr. Jon Garito.

2.2 Upper lid blepharoplasty without cutting the orbicularis.

[N. Van Meer, T. Backaert, P. Verhaert, P. Raus (2017) Int. J. Ophthalmol. Eye Res. 5: 308-312]

OPEN ACCESS http://scidoc.org/IJOES.php

SciDoc Publishers
Infer, Interpret & Inspire Science

International Journal of Ophthalmology & Eye Science (IJOES)
ISSN 2332-290X

Upper Lid Blepharoplasty Without Cutting the *Orbicularis* Muscle

Research Article

Van Meer N[1], Backaert T[2], Verhaert P[3], Raus P[4*]

[1] Universitair Ziekenhuis VUB, Jette, Belgium.
[2] A.Z. St. Rembert, Torhout, Belgium.
[3] Pathology Unity, General Hospital Herentals (AZ Herentals), Herentals, and ProteoFormiX, Beerse, Belgium.
[4] Miró Centrum Voor oogheelkunde en Esthetiek, Geel, Belgium.

Abstract

We have compared the aesthetic outcomes of upper blepharoplasty with or without resection of the preseptal *orbicularis oculi* muscle. In addition we evaluated MRI to visualize the differences in the upper eyelids before and after surgery.
An interventional randomized double-blind left right study was conducted in 10 patients with upper eyelid dermatochalazis. One side was randomly chosen for incision of the preseptal *orbicularis oculi* muscle and direct coagulation or resection of fat pockets.
The *orbicularis oculi* muscle of the contralateral side was not incised. Only a soft coagulation was performed on the muscle to induce a septal contraction and push the orbital fat back. All interventions were performed with an Ellman Dual frequency high frequency low temperature unit. Digital photos were taken before and after surgery and patients were asked to fill in a questionnaire to evaluate the surgery itself as well as the aesthetic outcome.
In an attempt to objectify these findings, NMR imaging of the orbits was performed before and 6 weeks after surgery.
Symptoms scoring was similar in both groups. At 6 weeks post-operation, no significant differences were observed between left and right eye.
In conclusion, patients with dermatochalazis but without prominent fat prolapse in the upper eyelid, did not exhibit significant differences after cutting or not the *orbicularis* muscle to excise orbital fat. Even in cases with moderate fat prolapse, the fat can be pushed back by soft coagulation of the *orbicularis* and underlying orbital septum. When the preseptal *orbicularis oculi* muscle is preserved, the risk of hematoma and orbital cellulitis can be limited.

Introduction

According to the American Society for Aesthetic Plastic Surgery, 161, 389 cosmetic eyelid surgery procedures were performed in the United States in 2013 (www. Surgery.org). This makes blepharoplasty the third most popular elective cosmetic procedure, behind liposuction (363, 912) and breast augmentation (313, 327), but ahead of abdominoplasty (160, 077) and rhinoplasty (147, 996).

For the age group of 51 years and older, blepharoplasty even was one of the top three procedures, the other two being liposuction and facelift.

Blepharoplasty can be performed by many operative approaches and an almost uncountable number of different techniques have been published [1, 9, 13, 14, 18].

Pathogenesis of Dermatochalasis

With age the mesodermal content of the body shrinks. This results in the skin becoming too large, and the appearance of wrinkles and redundant skin folds [3, 4, 18].

In the upper eyelids atrophy of mesodermal structures implies loss of adnexal structural support by the tarsus, the canthal tendons, and *orbicularis* muscle. This, in combination with a thinning eyelid skin [7], leads to orbital fat prolapse and eyelid malposition [5, 10, 11]. The desinsertion or attenuation of the *levator* muscle aponeurosis may cause involutional (aponeurotic) ptosis [19].

Involutional changes in the orbital septa cause downward migration of the brow fat, leading to a brow ptosis. It is not uncommon that a brow ptosis is misdiagnosed as upper eyelid dermatochalazis. The potential complication that arises if a blepharoplasty is

***Corresponding Author**:
Dr. Peter Raus,
Miró Centrum voor oogheelkunde en Esthetiek, Geel, Belgium.
E-mail: peter.raus@gmail.com

Received: March 25, 2017
Accepted: April 17, 2017
Published: April 22, 2017

Citation: Van Meer N, Backaert T, Verhaert P, Raus P (2017) Upper Lid Blepharoplasty Without Cutting the *Orbicularis* Muscle. *Int J Ophthalmol Eye Res.* 5(4), 308-312.

Copyright: Raus P© 2017. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution and reproduction in any medium, provided the original author and source are credited.

performed on a patient with significant brow ptosis is that the brow is pulled further downward to the lashes and a shortage of the anterior lamella remains. This can lead to a *lagophthalmos* or lid lag and even the need for a future skin graft [6]. In the eyelid itself anterior migration of the preaponeurotic fat pads results in pseudoptosis.

Age dependent histological changes in the *orbicularis oculi* muscleare minimal [2, 8]. That is why in patients without prominent fat pockets, an upper lid blepharoplasty, limited to a simple cutaneous resection often yields satisfactory aesthetic results. In those patients the *orbicularis* muscle does not need to be incised, but can be fully preserved to maintain eyelid 'fullness' and prevent a hollow aspect of the upper lid, particularly in younger patients. Indeed, fullness of the upper eyelid is a sign of youth. The most important question with aesthetically disturbing eyelid fat pockets is whether this is due to a herniation of a normal fat pocket or by an age-related expansion of orbital fat [22]. Upper eyelid fat pockets can mostly be pushed back by a soft coagulation of the *orbicularis* and underlying orbital septum [13].

Two upper eyelid fat pockets can classically be distinguished: a nasal one and one in the middle of the eyelid. In the lateral part of the upper lateral quadrant of the orbit, just below the orbital rim lies the palpebral part of the lacrimal gland. Care has to be taken not to damage this gland; as this could lead to an irreversible and very difficult to treat dry eye. That is why, even when fat resection in the upper eyelid appears to be mandatory, we advice to always limit the incision of the *orbicularis* muscle and the orbital septum to the nasal two thirds. In 2004 Persichetti et al., described a third fat compartment in the upper eyelid in 21.3% of the examined upper eyelids (47 patients who underwent upper blepharoplasty and 11 fresh cadavers; [15, 20]). In spite of this high incidence, but given the high similarity de visu of palpebral fat and glandular structures and the risk of causing a terrible dry eye, we prefer not to explore the lateral third of the suborbicular space to look for this extra fat pocket [21].

Blepharoplasty Step by Step

Marking the Skin to be Excised

Preoperative markings should be made with a skin marker before injecting the local anaesthetic and with the patient sitting upright in neutral gaze with the brow properly positioned.

The lower limit of excision should be along the eyelid crease. In cases of a double crease the surgeon should mark the lower one to avoid a double lid crease postoperatively.

The lateral extent of the marking should be limited by an imaginary line from the lateral end of the brow to the lateral canthus. The extent of excision should be at least 10 mm from the inferior border of the natural brow, making a pattern of skin excision with an acute angle laterally and an obtuse angle nasally. We want to emphasize that many women epilate their brows and that the visible inferior border of the brow might be quite a bit higher than the natural border, so surgeons should take care of that.

A skin pinch test can confirm the preoperative markings. Alternatively the patient can be asked to look up. In this position the lower and upper demarcation lines should 'almost' join.

For all our surgery we used the Ellman Dual Freqency high frequency low temperature device.

Skin Resection

For the skin resection a fine wire electrode is used.

In a first step the border of the skin fragment to be excised is in cised very superficially while care is taken not to hit the *orbicularis* muscle. Then, with an assistant nurse pulling the eyelid upwards, a superificial excision of only skin is performed. Bleeding vessels can be coagulated by indirect coagulation via a surgical forceps grasping the vessel, touching the forceps with the electrode and activating the unit. Alternatively the RF electrode can also be used for contact coagulation (i.e. in direct contact with the tissue).

Indirect coagulation has our personal preference. When a bleeding vessel is encountered the surgeon can immediatley grasp the 'bleeder' and use the indirect coagulation without the need to change electrode, waveform, or power. Indeed, even in the cutting mode of the RF unit, the current has 10% of cutting power that is accumulated when not moving the electrode through the tissue.

The surgeon will then switch to a ball electrode and with the unit in coagulation mode, a soft coagulation of the *orbicularis oculi* muscle is achieved inducing limited fibrosis of the muscle and a septal contraction. Attempts should be made to avoid to coagulate tissues close to the wound edges because this might delay the healing time due to clogged feeding capillaries.

In the majority of cases no fat resection is needed. The soft coagulation of the *oribicularis* muscle and underlying orbital septum will be enough to push back the orbital fat pockets. The local fibrosis, induced by the coagulation will consolidate the position of the fat pockets after the surgery. In these cases the skin can already be sutured with a 6/0 monofilament suture material. I personally prefer to separate sutures starting with one in the middle and then suturing the skin in the temporal to nasal direction.

Even when some fat resection appears to be mandatory to achieve an acceptable result, a soft coagulation of the *orbicularis* musclewith a ball electrode is still preferred before making an incision in the *orbicularis* at about 14 mm above the lid margin. This high incision gives direct acces to the fat pockets with out the risk of damaging the underlying levator muscle or aponeurosis. For the incision in the *orbicularis* we use a thicker electrode to limit the bleeding. This incision is limited to the nasal two thirds of the eyelid to avoid damage to the lacrimal gland. The fat is carefully disected and directly resected without using a clamp but with the unit in a cut-coagulation mode.

The *orbicularis* is sutured with a few Vicryl 6/0 sutures.

To compare the outcome of upper lid blepharoplasty with or without incision of the *orbicularis* + fat resection we performed a bilateral upper blepharoplasty in 10 patients with a fat resection on one side and without fat resection on the other eye.

The side with or without fat resection was randomly chosen and the patient was not aware of this choice.

After standardized excess skin marking, one side was randomly chosen to remove skin and a slice of *orbicularis* muscle with a RF electrode and excision of prolapsed medial and central orbital fat pads. The skin of the contralateral upper eyelid was also excised with radio frequency using the needle electrode and the underlying *orbicularis* muscle and orbital septum was shrunk with radio frequency using a ball electrode and switching the unit from a pure cut to a coagulation setting.

The *orbicularis*, when incised, was closed with 3 to 4 Vicryl 6/0 sutures. The skin was sutured with separate Prolene 6/0 sutures that were removed after 6 days.

All patients had an ophthalmic examination before, 1 and 6 weeks after surgery. All patients had standardized digital photographs before and 6 weeks after surgery.

All patients had standardized orbital magnetic resonance imaging before and 6 weeks after surgery.

All patients were asked to compete a patient satisfaction questionnaire after 6 weeks.

They were asked to quote on the following parameters.

Pain: a scale from 0 (no pain) to 4 (severe pain)
Swelling: a scale from 0 (no swelling) to 4 (severe swelling)
Eye irritation: a scale from 0 (no irritation) to 4 (severe irritation)
Difficulty with closing the eyes in the first week after surgery 0 = no 1 = yes.
Aesthetic outcome: scale from 1 (not good) to 10 (perfect).
Preference between left or right eye, without knowing the technique used for which eye.

The purpose of MR imaging was to ascertain whether MR imaging could show the effect of the proposed blepharoplasty technique, compared with the original technique (with excision of orbital fat) and whether we could detect a difference in the localization of the *septum orbitale* [16, 17].

Our patients underwent bilateral orbital high-resolution MR imaging on a 3T system pre- and postoperatively using T1-imaging. We look for differences in fat quantity pre-and postseptal, and evaluate the position of the septum, comparing both eyes and thus both techniques.

Results

Originally 10 patients were included in our study but one patient was lost for follow-up so the respective data are not included.

These included 8 female and 1 male patient participated. The average age was 57.3 (spread between 51-70).

In 3 cases the left eye was operated without *orbicularis* incision; of the 6 other patients the right eye.

On the pain scale (from 0 [no pain] to 4 [severe pain]), 4 patients recorded "0" and 5 patients "1".

For the parameter 'swelling' (a scale from "0" [no swelling] to "4" [severe swelling]), 2 patients reported "0", 3 patients scored "1", and 3 others noted "2".

Of all patients, 1 reported a swelling of "2" at the side without *orbicularis* incision and "3" on the eyelid where the *orbicularis* was opened with fat resection.

For the parameter 'bruising' (hematoma) 8 patients reported the same bruising on both sides, whereas 3 patients recorded no bruising at all, 3 patients reports limited bruising (value "1") and 2 patients value "2".

Only 1 patient reported a limited bruising on the side where the *orbicularis* was incised.

For the parameter "irritation", 7 patients scored "0" (did not experience any irritation at all), 1 reported score "1" and 1 patient scored "2".

Only 2 patients reported some difficulty in closing the eyes (score "1") in the first week after surgery.

All patients were happy with the aesthetic outcome of the surgery with no preference for left or right eye with a minimum score of 8/10 (4 patients), 9/10 (2 patients) and 10/10 (3 patients) (Figures 1-2).

Unfortunately only in one case the septum was clearly depicted on both pre-and post op scans. In this case a difference in the position between the septum pre- and postoperative could be shown even in the eye where the *orbicularis* was not incised and only coagulated (Figure 3). In the other cases the septum could not be visualized in one or both examinations due to small motion artefacts, or a slightly different eye orientation, making a good comparison impossible. As the septum is a very thin structure, results are very dependent on the cooperation of the patient and on the patient's ability to lie perfectly still for 5-10 min. without moving his/her eyes. No significant change was observed in the amount of retroseptal fat but the expected volume change is very limited.
So, high-resolution MR imaging showed a different septum position comparing pre- and postoperative scans using our technique in one patient. An exact depiction of the septum orbitale remains very much conditional on the cooperation of the patient.

Due to motion artifacts and the very subtle changes no volume difference could be seen.

Discussion

Upper lid blepharoplasty is a common surgical intervention and many different techniques can be employed to achieve a good functional and aesthetic outcome. Although only 9 patients (18 eyelids) were included in the present study, our results suggest that in most cases surgery can be limited to an appropriate skin resection and soft coagulation of the *orbicularis* muscle and underlying orbital septum. Also, omitting the incision of the *orbicularis* muscle limits the risk of superficial and deep bleeding and orbital cellulitis.

Patients' acceptance is excellent thanks to the good aesthetic out-

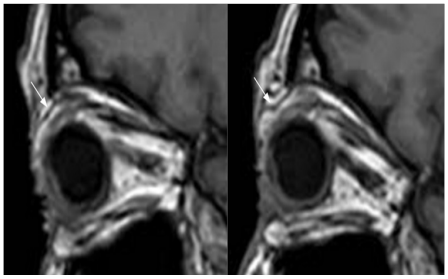
Figure 1. Patient X before Surgery.



Figure 2. Same Patient after Surgery; Right Eye without Cutting *Orbicularis* Muscle.



Figure 3. MRI of Patient X: Left Image before and Right Image 6 Weeks after Surgery. Arrow Indicates Orbital Septum.



come and the fast recovery.

Conclusion

Upper lid blepharoplasty without cutting the *orbicularis* muscle appears to be an excellent technique for patients with dermatochalasis with no or only moderate orbital fat prolapse. Unfortunately MRI of the orbit is not appropriate to visualize the orbital septum and other imaging techniques should be explored if a visualization of surgical results is needed.

References

[1]. Pfeiffer MJ (2006) Update on upper lid blepharoplasty. Oculoplastics and Orbit. Springer Science & Business media.

[2]. Salvi SM, Akhtar S, Currie Z (2006) Ageing changes in the eye. Postgraduate Medical J. 82(971): 581-587.

[3]. De Backer CM, Putterman AM, Zhou L, Holck DE, Dutton JJ (1998) Age-related changes in type-I collagen synthesis in human eyelid skin. Ophthal Plast Reconstr Surg. 14(1): 13-16.

[4]. Krutmann J, Béhar-Cohen F, Baillet G, de Ayguavives T, Reme C, et al., (2014) Towards standardization of UV eye protection: what can be learned from photodermatology? Photodermatol Photoimmunol Photomed. 30(2-3): 128-136.

[5]. Jacobs LC, Liu F, Bleyen I, Gunn DA, Hofman A, et al., (2014) Intrinsic and extrinsic risk factors for sagging eyelids. J Am Assoc Dermatol. 150(8): 836-843.

[6]. Keyvan N (2008) Complications in Dermatologic Surgery. Elsevier Health Services. 286.

[7]. Hwang K, Kim DJ, Kim SK (2006) Does the upper eyelid skin become thinner with age? J Craniofac Surg. 17(3): 474-476.

[8]. Manners RM, Weller RO (1994) Histochemical staining of *orbicularis oculi* muscle in ectropion and entropion. Eye. 8(3): 332-335.

[9]. Damasceno RW, Cariello AJ, Cardoso EB, Viana GA, Osaki MH (2011) Upper blepharoplasty with or without resection of the *orbicularis oculi* muscle: randomized double-blind left-right study. Ophthal Plast Reconstr Surg. 27(3): 195-197.

[10]. Goldberg RA, McCann JD, Fiaschetti D, Ben Simon GJ (2005) What causes eyelid bags? Analysis of 114 consecutive patients. Plast Reconstr Surg. 15(5): 1395-1402.

[11]. Feher J, Olah Z (2008) Age related changes of the human eye. Age-Related changes of the eyelid. Springer. 9-33.

[12]. Stricker M, Pujo J, Simon E (2009) Controversies regarding the orbital septum, Ann Chir Plast Esthet. 54(6): 551-566.

[13]. Van der Lei B, Timmerman IS, Cromheecke M, Hofer SO (2007) Bipolar coagulation-assisted orbital (BICO) septoblepharoplasty: a retrospective analysis of a new fat-saving upper-eyelid blepharoplasty technique. Ann Plast Surg. 59(3): 263-267.

[14]. Meyer DR, Linberg JV, Wobig JL, McCormick SA (1991) Anatomy of the orbital septum and associated eyelid connective tissues. Implications for ptosis surgery. Ophthal Plast Reconstr Surg. 7(2): 104-113.

[15]. Persichetti P, Di Lella F, Delfino S, Scuderi N (2004) Adipose compartments of the uper eyelid: anatomy applied to blepharoplasty. Plast Reconstr Surg. 113(1): 373-378.

[16]. Al-BakriM, Rasmussen AK, Thomsen C, Toft PB (2014) Orbital Volumetry in Graves' Orbitopathy: Muscle and Fat Involvement in relation to Dysthyroid Optic Neuropathy. ISRN Ophthalmol. 2014: 435276.

[17]. Darcy SJ, Miller TA, Goldberg RA, Villablanca JP, Demer JL, et al., (2008) Magnetic resonance imaging characterization of orbital changes with age and associated contributions to lower eyelid prominence. Plast Reconstr Surg. 122(3): 921-929.

[18]. MoraxS, Herdan ML (1990) The aging eyelid. Schweiz Rundsch Med. Prax. 79(48): 1506-1511.

[19]. Shore JW, McCord CD Jr (1984) Anatomic changes in involutional Blepharoptosis. Am.J Ophthalmol. 98(1): 21-27.
[20]. Malhotra R, Mahadevan V, Leatherbarrow B, Barrett AW (2015) The Post-Levator Aponeuros is Fat Pad. Ophthal Plast Reconstr Surg. 31(4): 313-317.
[21]. Schaudig U, Schumacher S (2012) Simple rules for orientation in transcutaneous ptosis surgery: a contribution to the surgical anatomy or: how not to get lost in the upper eyelid. Klin Monbl Augenheilkd. 229(1): 31-34.
[22]. Pessa JE, Desvigne LD, Lambros VS, Nimerick J, Sugunan B, et al., (1999) Changes in ocular globe-to-orbital rim position with age: Implications for aesthetic blepharoplasty of the lower eyelids. Aesthetic Plast Surg. 23(5): 337-342.

2.3 Transconjunctival lower lid blepharoplasty made easy by everting the eyelid.

[P.P.M. Raus (2017) Int. J. Ophthalmol. Eye Res. 5: 313-315]

OPEN ACCESS http://scidoc.org/IJOES.php

SciDoc Publishers
Infer, Interpret & Inspire Science

International Journal of Ophthalmology & Eye Science (IJOES)
ISSN 2332-290X

Transconjunctival Lower Lid Blepharoplasty Made Easy by Everting the Eyelid

Research Article

Peter PM Raus*

Miró Center for Ophthalmology and Aesthetics, Geel, Belgium.

Abstract

Purpose: To evaluate the transconjunctival lower lid blepharoplasty using a Desmarres lid retractor to evert the eyelid.
Design: Prospective study.
Methods: Twenty-one cases of inferior blepharoplasty in 11 patients, performed through a transconjunctival approach are reported. The author describes the surgical technique with Radio Frequency surgery, the use of a Desmarres lid retractor to evert the eyelid and the results.
Results: Radio Frequency surgery and the use of a Desmarres lid retractor appear to give excellent results in transconjunctival lower lid blepharoplasty.
Everting instead of retracting the eyelid provides excellent access to the fat pockets in the lower lids with less bleeding thanks to the traction on the eyelid.
The transconjunctival approach has a lower complication rate than the classic transcutaneous approach, with no visible scar and less risk of postoperative scleral show or ectropion.
Conclusion: Radiosurgically assisted transconjunctival blepharoplasty of the lower lid with the use of a Desmarres lid retractor is associated with a good functional and cosmetic outcome.

Keywords: Transconjunctival Approach; Radio Frequency Surgery; Desmarres; Lower Lid Blepharoplasty.

Introduction

A prolapse of orbital fat in the lower eyelid is the most frequent reason for patients to ask for a lower bleph. In the lower lid only in very rare cases a resection of skin is needed and that is why in the lower lid the transconjunctival blepharoplasty is out favorite approach. Also, in transconjunctival blephs there is no visible scar. No sutures have to be placed (or removed) and the *orbicularis* muscle does not need to be incised [1]. For this reason the risk for postoperative ectropion and/or scleral show is minimal. Indeed: not only excessive skin resection can cause ectropion. Even a simple incision in the *orbicularis* muscle-especially in the pretarsal part when making a subciliary incision-or the orbital septum can pull the eyelid down (scleral show) or out (ectropion) as it heals [7]. This complication can be very difficult to correct [2]. Also, an incision in the highly vascularized tarsal conjunctiva can induce a significant bleeding, especially in patients with a short lower eyelid and shallow *cul de sac* where the fat pockets are not easily accessible [3]. Our technique of transconjunctival blephs of the lower eyelid tries to deal with all these aspects.

Materials and Methods

Anesthesia

Even with surgery under general anesthesia, we start with a subcutaneous injection of Xylocaïne 2% with epinephrine. Then the lower eyelid is everted using a Desmarres lid retractor and subsequently Xylocaïne 2% with epinephrine is injected subconjunctivally. The purpose of a local anesthesia both from the outside as transconjunctivally is fourfold:

- Additional anesthesia (in patients under local anesthesia).
- Vasoconstriction by epinephrine.
- Hydrodissection of the conjunctiva and the retractor muscle.
- Wetting the tissues with the injection of anesthetic facilitates the radiosurgical incision.

***Corresponding Author**:
Dr. Peter P.M. Raus,
Miró Center for Ophthalmology and Aesthetics, 130 Stationsstraat , 2440, Geel, Belgium.
Tel: +32 (0)486 22 14 09
E-mail: Peter.raus@gmail.com

Received: April 10, 2017
Accepted: May 03, 2017
Published: May 05, 2017

Citation: Peter PM Raus (2017) Transconjunctival Lower Lid Blepharoplasty Made Easy by Everting the Eyelid. *Int J Ophthalmol Eye Res.* 5(5), 313-315.
doi: http://dx.doi.org/10.19070/2332-290X-1700064

Copyright: Peter PM Raus© 2017. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution and reproduction in any medium, provided the original author and source are credited.

Although theoretically surgery can be performed both under local and general anesthesia, we prefer the latter: the transconjunctival incision appears to be more stressful for severel patients. This can induce the blood pressure to rise with more bleeding. An intervention under general anesthesia therefore is not only safer but also more comfortable for both surgeon and patient. Besides, in dialogue with the anesthesiologist blood pressure can be lowered maximally during general anesthesia.

Surgical Technique

The lower lid is not retracted but everted wit a Desmarres lid retractor. Additional Xylocaïne 2% with epinephrine is injected subconjunctivally and as superficially as possible. Thanks to both the stretching of the lower eyelid with the lid retractor and the effect of the epinephrine an almost bloodless area is created to further diminish the bleeding during the intervention. The stretching of the lid also facilitates the identification of the lower tarsal border. Also, the incision of the lower lid layer by layer becomes much easier. For the radiosurgical incision in the conjunctiva we use a relatively thick electrode with the unit in the cut/coagulate mode which gives 50% cutting and 50% coagulation.

The thicker electrode than the one that is used for skin incisions induces an extra coagulation so that the incision is almost bloodless. If vessels or possible bleeders are seen, they can be grasped with a forceps and coagulated by touching the forceps with the electrode and activating the unit shortly. We design this indirect coagulation.

The Incision

The incision in the conjunctiva is very superficial, parallel to the lower tarsal border but 2mm lower. The incised conjunctiva is lifted with the forceps and then a similar incision is made through the retractor muscles. This technique procures a direct access to the inferior orbital fat without wounding the septum. This is an extra advantage since damaging the orbital septum may lead to eyelid malposition postoperatively. The Desmarres lid retractor is then removed. For maximal dilation of the wound, a wound hook with 3 blunt edges on the caudal side and a forceps in the cranial side are used.

Removal of the Fat

By pushing slightly on the globe, the fat now will prolapse. Sometimes additional membranes need to be cut before the fat pockets pop out. With the unit in the cut/coagulate mode, direct resection of the fat without the use of a clamp is possible. It is important to cut very slowly to have a maximal coagulation effect. Thicker blood vessels, when encountered, can be coagulated before cutting, in the same way as described for the conjunctival incision. Care has to be taken to excise only pure fat. Indeed: the inferior oblique muscle lies between the nasal and central fat pocket and must not be harmed [4, 5]. Fat resection has to be very conservative: the pockets in the lower eyelid are not only caused by excess of fat but primarily by a herniation of the anatomical fat pockets. Besides: the quantity of orbital fat can diminish with age and we have to take care not to cause a 'sunken' eye. They can also be partially repositioned and fixated more nasally or over the inferior orbital rim to correct a tear trough [6-8].

Final Steps

At the end of the intervention we check the wound for possible bleeders. With a forceps the lid is pulled upwards to loosen eventual adhesions. We do not suture the conjunctiva but only put some antibiotic ointment. One gram of Tranexamin is administered intravenously and a cooling compress is put on the eye.

Postoperative checks

An ambulatory check up is done after 6 days. At this moment it is not unusual to notice some conjunctival edema. The skin can be moderately wrinkled. If wrinkles persist, a superficial peeling can be performed after a month. Only in very rare cases a (conservative) excision of skin is needed. This can be combined with a suspension of the *orbicularis* muscle to the *periosteum* of the temporal superior border of the orbit [11, 12]. An external subciliary incision is then made in the lateral third of the lower eyelids extending into the crow's feet followed by a very conservative skin excision. Then a small excision of skin can be performed in the tail of the upper lid crease. A small piece of *orbicularis* is resected in the lower eyelid. A double armed polyglactin 5/0 suture is passed through the caudal part of the incised orbicularis, passed under the skin of the external cantus and recovered in the upper lid to be fixated at the periosteum of the orbital rim in the temporal superior sector. These skin incisions are closed with a few separate polypropylene 6/0 sutures.

Results

Between Augsut 2015 to May 2016, 11 patients underwent esthetic blepharoplasty with a transconjunctival approach. We performed all blepharoplasties personally. Only one patient was male and 10 female, with a mean age of 52 years. All patients underwent a retroseptal approach. In 18 eyelids, there were isolated lower lid bags and without excess skin. In three eyelids with excess skin and orbital septum laxity a transconjunctival approach was preferred to avoid postoperative retraction (scleral show) [10]. In these cases we additionally performed a suspension of the *orbicularis* of the lower eyelid to the periosteum of the superior orbital rim.

Surgery was performed under general anesthesia conditions in all cases. The average operating time for bilateral lower eyelid transconjunctival approach was 40 min. All patients were followed-up for at least 2 months with medical control at day 1, day 15, and day 60 after surgery.

Moderate edema of the lower eyelid in the weeks after surgery was reported in seven patients and fast resolved. A postop limited ecchymosis has been noted in 4 patients.

The main complication of this approach is under correction of the orbital bags, due to an insufficient fat resection. That happened in three patients and concerned the nasal fat pocket in one case and the temporal fat pocket in 2 cases. The patient with the under-corrected nasal fat pocket underwent a second transconjunctival blepharoplasty through the same approach to resect some more fat. In the case of the temporal fat pocket, a transcutaneous reintervention was performed under local anesthesia.

Discussion

Transconjunctival inferior blepharoplasty is our preferred technique for the correction of orbital bags. The transconjunctival approach allows a safe and precise excision of orbital fat. By avoiding a skin incision in the lower eyelid, there is no visible scar. Especially in younger patients with only limited wrinkles and skin folds this is an important aspect. Also the risk of retraction and scleral show is minimal.

Postoperative hemorrhage, although rare too, must be identified early. The risk of a deep or even sight threatening retrobulbar hematoma is reduced with the use of Radio Frequency surgery in the cut-coagulation mode. Also, the traction on the eyelid structures created by the Desmarres lid retractor creates a relatively bloodless surgical field. Thanks to the eversion of the eyelid a precise dissection layer by layer can be achieved avoiding a too deep incision and damaging thicker blood vessels.

Indeed, retraction of the lower eyelid can occur in patients even without skin resection, just by the incision of the skin, septum and *orbicularis oculi* muscle. The transconjunctival approach avoids incision of these tissues, and minimizes thus he retraction risk. In patients with excess skin, wrinkles of the lower eyelid can persist after surgery for up to three months. After 6 weeks these wrinkles can be treated with laser or superficial chemical peeling [9]. If the excess of skin is too pronounced, a minimal skin excision can be performed [10].

Entropion is exceptional after a transconjunctival approach, despite the incision of palpebral retractile tissues. We have seen a transitory postoperative diplopia in a few cases. It seemed to have been induced by the injected anesthetic. It never lasted more than 2h. However, prolonged and even permanent diplopia has been reported after damaging the inferior oblique and even the inferior rectus muscle [3, 4].

Wounding of the eyeball is always possible and should be prevented by the use of corneoscleral contact lenses.

The most common complication is undercorrection with underexcision of the orbital fat, while overcorrection is particularly rare [4]. For proper evaluation of the fat excision, the lipectomy should begin with the medial fat pocket to give easier access to the central and lateral pocket.

Conclusions

For the lower lid blepharoplasty we prefer the transconjunctival approach. It reduces the risk of postoperative eyelid retraction and scleral show. Especially in younger patients with good skin quality and only few wrinkles and skin folds, it is very attractive because there is no visible scar on the eyelid skin. Everting the lower eyelid with a Desmarres lid retractor and the use of Radio Frequency surgery in the cut/coag mode limit intra-and postoperative bleeding. The transconjunctival approach allows the reposition of orbital fat pockets to correct a tear trough and can be used in conjunction with a conservative skin resection if needed.

References

[1]. Korchia D, Braccini F, Paris J, Thomassin JM (2003) Transconjunctival approach in lower eyelid blepharoplasty. Can J Plast Surg. 11(3): 166-170.
[2]. Verity DH, Collin JR (2001) Eyelid reconstruction: the state of the art. Curr Opin Otolaryngol Head Neck Surg. 2(4): 344-348.
[3]. Pack S, Quereshy FA, Altay MA, Baur DA (2016) Transconjunctival Lower Blepharoplasty. Atlas Oral Maxillofac Surg Clin North Am. 24(2): 147-151.
[4]. Mowlavi A, Neumeister MW, Wilhelmi BJ (2002) Lower blepharoplasty using bony anatomical lasndmarks to identify and avoid injury to the inferior oblique muscle. Plast Reconstr Surg. 110(5): 1318-22; discussion 1323-1324.
[5]. Morax S, Touitou V (2006) Complications of blepharoplasty. Orbit. 25(4): 303-318.
[6]. Goldberg RA (2000) Transconjunctival orbital fat repositioning: transposition of orbital fat pedicles into a subperiosteal pocket. Plast Reconstr Surg. 105(2):743-8. discussion 749-751.
[7]. Davison SP, Irio M, Oh C (2015) Transconjunctival lower lid blepharoplasty with and without fat repositioning. Clin Plast Surg. 42(1): 51-56.
[8]. Zoumalan CI, Roostaeian J (2016) Simplifying Blepharoplasty. Plast Reconstr Surg. 137(1): 196e-213e.
[9]. Bosniak S, Cantisano-Zilkha M, Purewal BK, Zdinak LA (2006) Combination therapies in oculofacial rejuvenation. Orbit. 25(4): 319-326.
[10]. Kim EM, Bucky LP (2008) Power of the pinch: pinch lower lid blepharoplasty Ann Plast Surg. 60(5): 532-537.
[11]. Bajaj MS, Pushker N, Balasubramanya R (2004) Lower eyelid dermatochalasis with Massive postural herniation of orbital fat. Orbit. 23(1): 41-44.
[12]. Lee AS, Thomas JR (2005) Lower lid blepharoplasty and canthal surgery. Facial Plast Surg Clin North Am. 13(4): 541-551.

2.4 Punctal Ectropion repair using the Raus-Garito clamp.

[P.P.M. Raus, N. Bral, R. Collin (2017) Orbit doi: 10.1080/01676830.2017.1353112]

ORBIT
https://doi.org/10.1080/01676830.2017.1353112

Taylor & Francis
Taylor & Francis Group

ORIGINAL ARTICLE

Check for updates

Punctal Ectropion repair using the Raus–Garito clamp

Peter P. M. Raus[a], Nathalie Bral[b], and Richard Collin[c]

[a]Miró Centrum voor Oogheelkunde en Esthetiek, Geel, Belgium; [b]Department of Ophthalmology, Vrije Universiteit Brussel, Brussels, Belgium; [c]Department of Oculoplastic Surgery, Moorfields Eye Hospital, London, United Kingdom

ABSTRACT

Purpose: To evaluate the results from the correction of ectropion of the punctum lacrimale in lower eyelids with a new surgical clamp. Design: Prospective study. Methods: Eighty eight eyelids in 55 patients with mild and moderate ectropion were included in the study. An excision of a diamond of tarso-conjunctiva with retractor reattachment and concomitant correction of horizontal lid laxity, if present, was performed. Results: Resolution of tearing was obtained in 77 eyes. In 11 eyes, persistent tearing was reported. Conclusion: Repair of early to intermediate ectropion of the lacrimal punctum using the Raus–Garito clamp is associated with a good functional and cosmetic outcome.

KEYWORDS

ectropion; epiphora; eyelid; instrument; surgery

Introduction

A tearing eye due to an ectropion is very frequently encountered but under-diagnosed in everyday practice. Involutional ectropion and horizontal lid laxity is not always generalized. It can be limited to the medial or lateral canthus or even to the area of the lower punctum. It can also occur in paralytic, cicatricial, and mechanical ectropion.

Lower lid retractor desinsertion or laxity can contribute to a variable extent to eversion of the punctum.[1] We speak of 'ectropion' as soon as the lower punctum is not in apposition to the globe and visible on simple slit lamp examination. When this occurs, blinking no longer allows the physiological tear drainage pump to function, normally leading to tear overflow[2] The resulting epiphora and the repeated need to wipe aggravates the lid laxity because most patients wipe their eyes from the nose laterally in the direction of the ear. The exposed conjunctiva may induce secondary blepharoconjunctivitis and edema which in turn further accentuates the ectropion. This condition can also be of cosmetic concern to the patients.[3] In patients with involutional ectropion, the surgeon can perform a lateral wedge excision or tarsal strip procedure[4], but if the punctum is not apposed to the globe with simple lateral traction of the lower eyelid, none of these techniques will be enough to achieve a good functional result and an additional procedure will be mandatory. Several techniques are described in the literature:

(1) A transconjunctival plication or reinsertion of the retractor muscle to the tarsus in the area below the lacrimal punctum, without shortening of the posterior lamella[4]
(2) A tarso-conjunctival diamond excision below the punctum with tightening of the lower lid retractors by one internal suture passing through the retractors, the conjunctiva at the lower edge of the diamond excision, the pretarsal conjunctiva and the tarsus and making a buried knot.[3]
(3) A tarso-conjunctival diamond excision below the punctum with tightening of the lower lid retractors by a double armed vircyl 5/0 suture passing through the retractors, the tarsus, the pretarsal conjunctiva to reinsert the retractor muscle to the tarsus. The two needles are then externalized to the skin at the lower border of the diamond with a knot on the eyelid.[3,5]

Most surgeons will evert the lower eyelid with a Bowman probe placed in the lower punctum and canaliculus[3–5,7] and a suture placed several millimeters temporal to the punctum through skin, orbicularis muscle, and superficial tarsus. In that case, care has to be taken not to tear the punctum. An advantage of our clamp to evert the lower eyelid is that it creates a bloodless surgical field.

CONTACT Peter P. M. Raus Peter.raus@gmail.com Miró Centrum voor Oogheelkunde en Esthetiek, Stationsstraat 130, Geel, Belgium 2440.
Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/iorb.
© 2017 Taylor & Francis

Ferguson and coworkers have described a technique to combine a wedge excision, just lateral to the punctum and a modified lazy-T procedure. First, a pentagonal wedge excision 3 mm lateral to the lower punctum is made. The retractors are then picked up, pulled medially through a subconjunctival tunnel and tied off on the conjunctiva 3 mm medial and below the inferior punctum.[8,9] Although all these techniques can give excellent results and have their proper indications, the lower lid retractors are not easily accessible. It also is an extremely vascularized area that tends to bleed easily during surgery. That is why a lot of surgeons are disappointed after trying this type of surgery. However, in many cases of ectropion of the lower lid, a 'traditional' lateral wedge exicsion or tarsal strip procedure is not yet necessary to obtain a good apposition of the punctum lacrimale after surgery. Moreover, sometimes a horizontal shortening of the lower eyelid alone is not enough to correct the ectropion and an additional diamond excision of tarso-conjunctiva (DET) appears to be mandatory to bring the punctum in contact with the globe again. For those cases, we developed the Raus clamp (Figure 1). It makes the surgery easier and faster. Moreover, its use *is easy to learn* like in the philosophy of John Dewey: "See one, do one, teach one". Another advantage is that the administration of blood thinning drugs does not have to be interrupted before surgery. We combine the use of the Raus/Garito clamp with high frequency (4MHz) radio wave surgery. We think that our clamp is very versatile and can be helpful for multiple techniques of ectropion surgery. This type of surgery can even be used as intervention on its own in all cases of punctal ectropion without significant horizontal lid laxity. Moreover, it can be used repeatedly on the same eyelid during the same intervention. It can also be used in combination with a lateral wedge excision or tarsal strip procedure in all cases with pronounced horizontal lid laxity except in patients with an extreme medial canthal tendon laxity

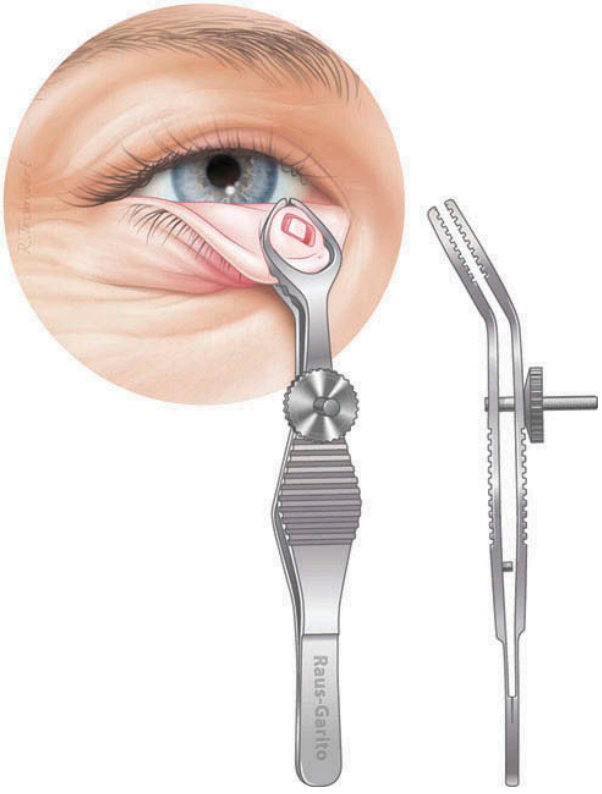


Figure 1. Diamond excision of conjunctiva.

Materials and methods

Patients with involutional ectropion can be divided into different groups:

(1) patients with mild or punctal ectropion and no or very limited horizontal lid laxity
(2) patients with pronounced ectropion with good apposition of the punctum upon lateral traction on the lower eyelid
(3) patients with pronounced ectropion and remaining punctal ectropion upon lateral traction on the ower eyelid

Lid laxity was evaluated with the pinch test whereby if the lid could be pulled more than 4 mm away from the globe we considered it to be lax. Sometimes this test is also called 'Eyelid distraction test'. It was described in 1975 by Hill.[10] Another valuable test to measure lower lid laxity is the snap-back test. We pull the lower lid away and down from globe for a few seconds and check how long it takes before it returns to its original position without the patient blinking.[11] When it takes more than 2–3 seconds for the eyelid to return to its original position a DET surgery can be considered.

Although the horizontal lid length appears not to increase with age[12] even if the lateral canthal tendon is elongated *we* try to avoid a lateral tarsal strip procedure but only perform a full thickness lid shortening laterally. Indeed when the tarsal strip is not fixated properly, the procedure tends to induce a lateral ectropion postoperatively with pooling of the tears in a lateral tear lake. With limited medial lid laxity a tarso-conjunctival diamond excision is performed in the medial part of the lid.[3] When the lower lid retractors appear to function properly (snap-back test of <3 seconds or pinch test not more than 4 mm) a simple excision of tarso-conjunctiva with an internal suture that includes the retractors, tarsus and the conjunctiva can be sufficient to re-establish proper evacuation of tears. If not, an externalization of the suture can additionally invert

the lower eyelid to achieve an apposition of the punctum against the globe. *Indeed, when the suture exits the skin more inferiorly then the internal wound, the eyelid will be inverted when making the knot.*

For the patients of group 1 we only performed a tarsoconjunctival diamond excision with tightening of the lower lid retractors (DET) but without a lateral wedge excision.

The patients of group 2 can be helped by a simple lateral wedge excision or a tarsal strip procedure and or not good candidates for a DET. None such cases were included in this article.

The patients of group 3 underwent a combination of lateral wedge excision and a DET. Written informed consent from all the patients was obtained before surgery. All patients underwent a standard eye examination and an irrigation test was performed to test the patency of the tear ducts. In patients with lacrimal obstruction a Ritleng Monoka silicone tube was inserted during the same intervention but in these cases the surgery was done under general anesthesia and with the cooperation of an ENT surgeon.

Surgical technique of diamond excision with the Raus–Garito clamp

In patients under local anesthesia, first a topical anesthetic is instilled. The skin around the eye is desinfected e.g. with a 10% Povidone-iodine solution and the area is draped. For patients under local anesthesia, a local anesthetic with epinephrine is injected under the skin and deep under the orbicularis muscle. This deep infiltration is preferable to anesthetize the muscle and tarsus and to limit discomfort when the clamp is closed and the eyelid is everted. The lid margin of the lower eyelid in the nasal third is pulled upwards using a forceps with teeth. The complete loop of the clamp is slid over the eyelid with the concave side on the skin side (Figure 1). The arms of the clamp are pinched together before the clamp is closed with the screw. Thanks to the angulated form of the clamp, less traction is needed to evert the eyelid until the clamp rests on the skin. *This means less traction on the medial canthal tendon*. The aim of the teeth in the loops of the clamp is to prevent slipping of the clamp from the eyelid during eversion. Then a superficial diamond-shaped fusiform wedge of conjunctiva is excised inferior to the lower margin of the tarsal plate with the tip op the diamond pointing to the lacrimal punctum (Figure 1). The height of this excision is determined by the amount of punctal ectropion but is limited by the size of the loops of the clamp. The greatest vertical dimension should lie beneath the punctum.

However, when this surgery is combined with the implantation of a silicone plug or a silicone tube, the plug or silicone stent should be inserted first and the clamp will be placed a little more laterally to avoid the plug/stent popping out when the clamp is closed. We have found that the inverting effect of the surgery will be nearly as good when placing the clamp a little more laterally. *So, although we have not seen any problems of canalicular patency after surgery with our clamp, to avoid crushing the canalicular tissue, the surgeon could decide to place the clamp somewhat laterally to the lower punctum*. Also, with the clamp more laterally, everting of the lid is easier. The inverting technique can be used or repeated elsewhere on the same eyelid.

With the excision of a fragment of conjunctiva, the anterior expansion of the lower lid retractors is exposed. For the inverting suture we use a double-armed 5/0 Vicryl suture. Both needles are initially passed through the retractors in the center of the diamond excision Then the two arms have to be passed backhanded through the apex of the diamond, adjacent to the punctum (Figure 2.). In this way the lower lid retractors are advanced to the lower border of the tarsus. In a next step each suture arm is passed though the full thickness of the lid at the inferior apex of the diamond (Figure 3). When the clamp is lifted from a horizontal to a vertical position, it is easy to switch from the conjunctival to the cutaneous side of the eyelid to pick up the needles on the skin side (Figure 4.). With a patient under blood diluting drugs, the surgeon can prefer to make the knot before taking out the clamp. However, the slit in the outer arm of the clamp makes it possible to take the clamp off and adjust the position of the punctum when tying the knot. For a minimal ectropion, limited to the punctal area, one could prefer not to externalize the suture with a knot on the skin but just to place one conjunctival suture. In this case one arm of the *double* armed Vicryl 5/0 suture is passed through the apex of the diamond,

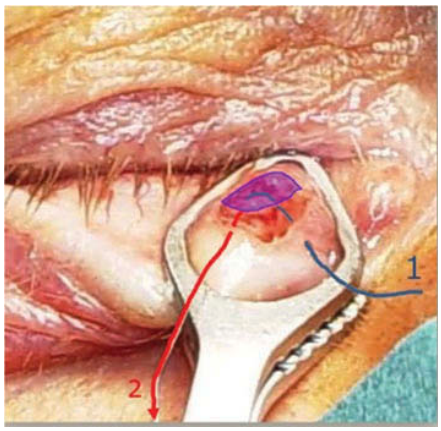


Figure 2. Reinsertion of retractor muscle.

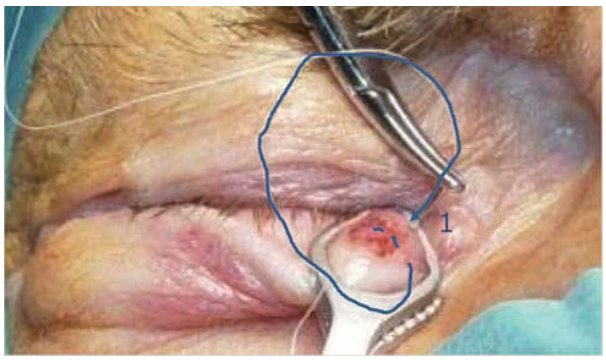


Figure 3. Inversion of the eyelid.

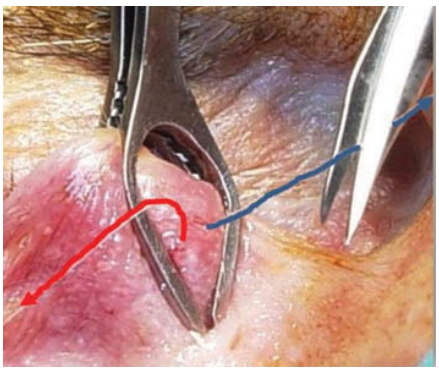


Figure 4. Sutures passed through the eyelid.

immediately below the punctum. The other arm is passed through the conjunctiva below the inferior apical cut. With this second arm the lower lid retractors are picked up and after 'preparing' the knot, the clamp can be removed thanks to the slit in the top of (both arms of) the clamp. The knot can then be tied and buried in the wound. The diamond excision can be combined with a horizontal shortening technique, but it should precede it: if the diamond excision would be performed after the wedge excision, everting the lid to do the diamond excision is very difficult and there is a risk of breaking the sutures of the wedge excision. To prevent a (temporary) pit in the skin, the surgeon can tie the suture over a bolster. In this case the suture can be taken out after 10–14 days.

An antibiotic ointment is applied in the lower fornix and on the skin. Inverting sutures in combination with excision of a diamond of conjunctiva can be used anywhere on upper or lower eyelid to aid other types of ectropion repair.

Results

In this study 88 eyelids in 55 patients with mild and moderate ectropion and different degrees of laxity of the lower eyelids were included. Patients with severe laxity of the medial canthal tendon were excluded. Although paralytic ectropion is not an exclusion criterion for our technique, in this article, no patients with paralytic ectropion were included. All patients underwent a shortening of the internal lamella below the punctum and a retractor muscle advancement. Patients with pronounced ectropion, a pinch test of >4mm, and remaining punctal ectropion upon lateral traction on the lower eyelid also underwent a lateral wedge excision (25 eyes). Thirty-one patients were female, 24 were male. The average age at the time of surgery was 72.56 years (52–85). Most of the patients presented with the chief complaint(s) of tearing (48 patients), eyelid malposition (3 patients), itching (2 patients), nasal BCC (1 patient, 1 eyelid), aesthetic problem of ectropion (1patient). Additional presenting chief complaints included dry eye, red eye (with or without tearing or irritation) and ocular irritation or a combination of symptoms. We recorded all complaints individually and for each eye separately because patients with bilateral disease sometimes present with different symptomatology for each eye.

Concomitant procedures at the time of initial surgery were:

- Placement of punctum plugs ('clou trou') to dilate a small punctum: 11 patients (20 eyes)
- Lateral wedge excision: 15 patients (24 eyes)
- Monocanalicular intubation: 8 patients (11 eyes)
- Upper lid blepharoplasty: 2 patients (4 eyes)
- Excision of Xanthelasmata: 1patient, 1 eye
- Pediculated flap from upper to lower eyelid: 1 patient (2 eyes)
- Shortening of caruncula: 2 patients (2 eyes)
- Ptosis correction: 1 patient (2 eyes)

When we insert a silicone tube or perforated punctal plug, the stent is usually taken out after 6 weeks. That is why we scheduled the last postoperative examination about 8 weeks after surgery. At that time, 16 of the 88 eyes still showed a visible punctum with slit lamp examination with 1 eye presenting a residual ectropion due to a broken suture; so in 16 eyelids the ectropion was not anatomically corrected. However as much as 77 eyes reported complete resolution of tearing. *Of the 16 patients where the punctum was still visible with slit lamp examination after surgery, only 11 had reduced or occasional tearing. In patients where no concomitant silicone intubation was performed, the lower canaliculus might be injured by closing the clamp and pinching the canaliculus. However, in the first 17 eyelids we encountered no problems in passing a Bowman 00 probe a first time at the end of the intervention and a second time after 4 to 6 weeks.*

punctal Ectropion. *J Craniomaxillofac Surg*. 2013;41(7): e111–6. doi:10.1016/j.jcms.2012.11.036.
8. Ferguson AW, Chadha V, Kearns PP. The not-so-lazy-T: a modification of medial ectropion repair. *Surgeon*. 2006;4(2):87–89. doi:10.1016/S1479-666X(06)80036-3.
9. Smith B. The "lazy-T" correction of ectropion of the lower punctum. *Arch Ophthalmol*. 1976;94(7):1149–1150. doi:10.1001/archopht.1976.03910040061011.
10. Hill JC. Analysis of senile changes in the palpebral fissure. *Trans Ophthalmol Soc UK*. 1975;95(1):49–53.
11. Schaefer AJ. Lateral canthal tendon tuck. *Ophthalmology*. 1979;86(10):1879–1882. doi:10.1016/S0161-6420(79)35342-8.
12. Ousterhout DK, Weil RB. The role of the lateral canthal tendon in lower eyelid laxity. *Plast Reconstr Surg*. 1982;69 (4):620–623. doi:10.1097/00006534-198204000-00007.

Chapter 3: Innovative treatments for DED using RF surgery

3.0 Introduction.

Most current treatments of DED as summarized in **Chapter 1** are common knowledge, but two innovative therapies using RF surgery are described in this chapter:

Chapter 3.1 reports on the in-office occlusion of the tear punctum using the CPO electrode. This electrode, developed by Dr. Bouzouaya and Dr. Garito, is designed for use in combination with RF surgery. RF surgery is less invasive for the permanent occlusion of the *punctum lacrimale* and the *canaliculus lacrimalis* (lacrimal canal; see Fig. 1.3) than the usual coagulation with electrosurgery, limited to the *punctum* [see Bouzouaya and Raus, 2012; **Appendix 3.1**].

Chapter 3.2 deals with a technique of minor salivary gland transplantation to the eyelids which we perfectionized.

Several salivary gland surgeries had been published before:

- transposition of the parotid duct to the eyelids [Pierce et al., 1960];
- transplantation of the submandibular salivary gland to the eyelids [Geerling et al., 1998];
- transplantation of minor (labial) salivary glands to the eyelids [Murube, 1998; Raus, 2003];
- transplantation of the sublingual salivary glands [Geerling and Sieg, 2008].

Conclusion: Transplantation of the labial salivary glands appears to be the least invasive of these techniques.

References:

Bouzouaya C, Raus P (2012) The Chedly punctal occluder: A new treatment modality for dry eyes. PRIME 2: 30-37.

Filatov VP, Chevaljev VE (1951) Surgical treatment of parenchymatous ophthalmoxerosis. J. Oftalmol (Odessa) 3: 131–137.

Geerling G, Sieg P, Bastian GO, Laqua H (1998)Transplantation of the autologous submandibular gland for most severe cases of keratoconjunctivitis sicca. Ophthalmology 105: 327-335.

Geerling G, Sieg P (2008) Transplantation of the major salivary glands. Dev. Ophthalmol. 41: 255-268.

Murube J (1998) Labial salivary gland transplantation in severe dry eye. Oculoplast. Orbital Reconstr. Surg. 1: 104-110.

Pierce MK, Goldberg JL, Brooks CJ (1960) A direct approach for transposition of the parotid duct. A description of an intra-oral approach to the inferior conjunctival cul-de-sac. Arch. Ophthalmol. 64: 566-570.

Raus P (2003) Radiosurgery aids in salivary gland transplants for severe dry eye. Ocular Surg. News. 12: 15-16.

3.1 In office occlusion of the tear *punctum* using the CPO electrode (Current surgical DED treatment 1).

[P.P.M. Raus, P.D.E.M. Verhaert (2017) Int. J. Ophthalmol. Eye Res. 5: 283-286.]

SciDoc Publishers
Infer, Interpret & Inspire Science

International Journal of Ophthalmology & Eye Science (IJOES)
ISSN 2332-290X

In Office Occlusion of the Tear *Punctum* using the CPO Electrode

Research Article

Raus PPM[1*], Verhaert PDEM[2]

[1] Miró Center for Ophthalmology and Oculoplastic Surgery, Geel, Belgium.
[2] Pathology Unity, General Hospital Herentals (AZ Herentals), Herentals, Belgium.

Abstract

Dry eye syndrome (DES) is a relatively common condition with signs and symptoms varying from light irritation to functional blindness as a result of corneal opacification. The disorder generally occurs in subjects over 40 years of age. DES management is related to the severity of the symptoms. In most cases treatment focuses on tear substitution.

However, permanent punctal occlusion may be indicated for patients in which these conventional therapies fail. The procedure is simple and can be performed in the office. To cauterize and close the *punctum*, a high-frequency low-temperature surgical device and a specifically designed electrode [Chedly Punctal Occluder (CPO)] are employed. The very same CPO electrode, may be utilized to achieve dilation of the *punctum* and sealing of the the lumen of the lacrimal duct.

In cases where a permanent punctal occlusion is indicated, the CPO, in combination with a high frequency device, permanently seals the *punctum*. In most cases a scar is only visible by close slit lamp examination.

So far no cases of *punctum* reopening have been observed.
Although permanent punctal occlusion in DES is not the first treatment of choice, it sometimes has to be considered in severe DES cases, where other therapies remain unsuccessful.

Keywords: Dry Eyes; Punctal Occlusion; Radio Frequency Surgery; CPO.

Introduction

Dry eye disease (dry eye syndrome, DES) arguably is the most frequently encountered ocular disorder in our daily ophthalmological practice. The prevalence varies between 5.5% [1] and 33.7 % [2].

Yeta correct diagnosis appears to be extremely difficult and no single test is known to achieve this [3].

In 1995 the National Eye Institute of the USA proposed two main categories of 'dry eyes' and this division is still applied [4]:

- 'Aqueous tear deficiency', characterized by insufficient secretion of tears.
- 'Evaporative dry eyes' attributable to abnormally fast evaporation of tears.

Ten years later, a first classification for practical and clinical use was published by Murube et al., [5]. The International Dry Eye Workshop (DEWS) updated the definition and classification of dry eyes in 2007 [6]:

'*Dry eye 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*'

A common symptom in both aqueous tear deficiency and evaporative dry eyes is increased tear osmolarity, and hence osmolarity of tears is considered a valuable parameter for clinical diagnosis and evaluation the degree of dry eyes [7].

***Corresponding Author**:
Peter PM Raus,
Miró Center for Ophthalmology and Oculoplastic Surgery, Geel, Belgium.
Tel: (32) 14 31 26 60
E-mail: peter.raus@gmail.com

Received: January 30, 2017
Accepted: March 11, 2017
Published: March 15, 2017

Citation: Raus PPM, Verhaert PDEM (2017) In office Occlusion of the Tear *Punctum* using the CPO Electrode. *Int J Ophthalmol Eye Res.* 5(3), 283-286.

Copyright: Raus PPM© 2017. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution and reproduction in any medium, provided the original author and source are credited.

As such, assessing the degree of dry eyes is an important and decisive factor in determining the DES patient's therapy. Until a superior cure for dry eye disease will be discovered, therapeutic treatments limited to:

1. Try and raise the quantity of tears.
2. To prevent the loss or evacuation of tears.

Examples to raise the quantity of tears are:

- Substitution therapy with artificial tears or gels,
- Stimulation of secretion of tears or components of tears (e.g., Rebamipide, Diquafosol, cholinergic agonists), or
- Transplantation of labial salivary glands to the eyelids [10].

Examples to limit tear evacuation are:

- Induction of ptosis,
- Lateral or medial tarsorrhaphy
- Botulinum toxin injection around the inferior *punctum*,
- Obstruction of one or more puncta lacrimalia by inserting a silicone plug [8, 9], or
- The use of the 'Chedly punctal Occluder' (CPO), developed by Bouzouaya to coagulate a *punctum* [8].

DES Diagnosis

Many test are reported to evaluate the quality and quantity of tears but not all of them are usable in the office to assess cause and degree of our patients' dry eyes. For research purposes we are analyzing the protein composition of tears in healthy and dry eye patients by mass spectrometry. The underlying idea is that this could yield biomarkers to assist in a correct differential diagnosis for the many different forms of dry eyes [19-21]. In addition, we anticipate that comparing the proteins in tears of dry eye patients with those of healthy subjects will yield invaluable information about DES pathophysiology and consequently suggestions for a better or more personalized therapy [11]. In the office the most frequently used tests are:

- Evaluation of the tear meniscus height: less than 1mm thick is considered as suggestive for DES.
- The tear break-up time (BUT) with fluorescein: has to be higher than 10 sec (the tear BUT is the time measured from the blink to the appearance of tear film defects).
- Schirmer's test without topical anaesthesia indicates basal and reflex tearing (the norm usually used is more than 15mm at 5 minutes).
- Schirmer's test with topical anaesthesia measures basal tearing only (the norm is: greater than 10mm at 5 minutes).

Surgical Anatomy of the Tear Duct

Tears enter the lacrimal drainage system through the upper and lower puncta lacrimalia of the eyelids. They are located on the medial lid margin. The *papillae* appear pale in contrast to the surrounding tissue because they contain more connective tissue and are less vascularized. They are centered on the eyelid margin in line with the mucocutaneous junction. The upper *papillae* are located approximately 6mm from the medial canthal angle and the lower *papillae* are 6.5mm from the medial canthus. The punctal opening measures 0.2-0.3mm in diameter and is surrounded by a ring of dense connective tissue that normally maintains a patent entrance. In youth the aperture is round or oval, whereas with age, this often collapses into a slit, which typically delays normal evacuation of tears. The *puncta* are normally directed some what posterior towards the globe and do not become visible unless the lid is slightly everted. As soon as the *punctum* is visible, we already call it ectropion. From each *punctum*, a *canaliculus* passes perpendicular to the lid margin at a distance of 2mm, where it dilates to form the *ampulla*, an irregular sac 2mm in diameter. From the *ampulla*, the *canaliculi* turn horizontally and run medially, parallel to the lid margin for a distance of approximately 8mm. In 90% of individuals, the two *canaliculi* join at an angle of approximately 25 degrees to form a common *canaliculus* 3-5mm in length. In the remaining 10%, the two join the lacrimal sac independently [12, 13].

The lacrimal sac passes inferiorly into a bony canal of 12mm in length and 3-5mm in width. From the lacrimal sac the lacrimal duct goes down to the nose where it ends below the inferior nasal concha.

Treatment in DES

Since in the majority of cases a causal therapy is not possible yet, the treatment will be limited to a substitution of normal tears or an attempt to limit the consequences of the dry eye.

Generally speaking, the treatment can be divided into 5 groups:

- Substitution of normal tears (e.g. artificial tears, gels,…),
- Stimulation of secretion of tears or tear components (e.g. cholinergic agents, Diquafosol, Rebamipide,…),
- Prevention of evaporation or outflow of tears by, e.g., punctal plugs, coagulation of the tear *puncta*),
- In cases of severe dry eyes, symptomatic treatmentwith anti-inflammatory drugs…),
- Avoidance and/or reduction of additional factors triggering dry eyes (e.g. dry air, computer work, …).

In this paper we focus on the prevention of tear outflow by using punctal plugs versus coagulation of the *puncta*.

Although at first sight the prevention of outflow of tears seems to be an elegant solution to increase the quantity of tears, the technique has is limitations:

- Reduced tear outflow also slows down the elimination of microorganisms and allergens. Especially in younger patients this can cause bacterial or allergic conjunctivitis.
- In older patients the secretion of tears might be so limited that obstruction of the tear outflow has only negligible effect.
- In DES patients without a known cause, the problem might be temporary; in such cases it is safer to opt for a reversible obstruction of the tear duct.

In case of doubt a soluble collagen plug can be implanted in one lower *punctum*. In the hours after implantation the plug will swell and obstruct the tear duct for about one week, the time for the plug to be dissolved. If the plug relieves the symptoms of the pa-

tient to an acceptable level, a silicone plug can be implanted. One should always choose for the smallest diameter of plug to close the *punctum*. If the plug must be taken out afterwards or pops out spontaneously, a thick plug will have dilated the *punctum* and the dilated *punctum* will aggravate the problem by increasing the outflow of tears through a dilated *punctum*. Some of the drawbacks of punctal plugs are migration into the nasolacrimal system, which can cause *canaliculus* and lacrimal sac inflammation and recurrent extrusion [7, 8]. In those cases and with a patient with severe dry eyes permanent occlusion of the tear duct is to be considered. Several techniques for permanent occlusion have been described.

Potentially reversible methods mostly involve mobilization or transfer of tissue with the possibility to reverse all or parts of the made changes. Examples are:

- Transfer of the *punctum* towards the external lid margin [14]
- Punctaltarsoraphy [15]
- Punctal patch with a pedunculated flap of adjacent tarsal *conjunctiva* [14]
- *Punctum* switch graft [16]

Irreversible methods mostly use heat-induced damage or shrinkage of tissue. For this coagulation cautery probes, diathermy and laser can be used to occlude the tear duct by heat-induced damage to the canalicular and pericanalicular tissues. The more lateral heat spread to the pericanalicular tissues the less the chance that the technique used is reversible. On the other side, not all techniques are as effective to occlude the tear duct. The efficacy of permanent closure depends on the technique used and is limited to 7% when only the vertical portion of the *canaliculus* is cauterized [17].

Also an Argon laser can be used to co close the *punctum*. This technique is known to give less postoperative discomfort, but the risk of recanalization appears to be significantly greater [18]. Moreover, the high cost of a laser may be a limiting factor to decide for this kind of treatment. That is why we prefer (and exclusively use) the high-frequency, low-temperature Surgitron® Dual radio frequency (RF) device (Ellman International Inc.) for this type of intervention. The RF device is more cost-effective than the laser therapy and has only limited side-effects in comparison with thermal cautery. With the Surgitron®, which we use for eyelid interventions, we prefer the CPO electrode specifically designed to cauterize and close the *punctum by ChedlyBouzouaya and developed by Jon Garito* (Figure 1). The CPO, made of tungsten, is designed to both dilate the *punctum* and to be inserted in the lumen of also the horizontal portion of the lacrimal duct. The CPO is the cone-shaped Ellman RF electrode TNAEE287, patented and approved by the US Food and Drug Administration (FDA). Because it also gently coagulates the lateral part of the horizontal *canaliculus*, the CPO is very effective in permanently closing the tear duct in DES patients with only one brief in office treatment. The electrode is autoclavable and thus re-usable.

Technique to Seal the *punctum*

The lower eyelid is infiltrated in the nasal side with Xylocaine 2% with epinephrine, first on the external side and then on the conjunctival side. Because the tip of the CPO is cone-shaped, no dilation of the *punctum* is required.

The CPO is mounted on the handpiece of radiosurgical unit that is put in a cut/coagulation mode. Only the non coated, metallic tip of the electrode is inserted in the *canaliculus*. Then the unit is activated for a few seconds while the CPO is slowly withdrawn. If the *punctum* is still visible, the tip of the CPO is applied on the lumen to coagulate and seal it [8]. An antibiotic ointment can be applied once or twice a day for a few days. The patient is seen after one week for a check-up. No patch is necessary. Because the procedure is easy, it can be performed in the office.

Results

During the last 16 months 11 inferior puncta were coagulated with the CPO;

Indications for permanent occlusion were:

- Recurrent loss of silicone plugs: 8 cases,
- Granuloma around punctalplug :1 case,
- Severe dry eye with excellent results after implantation of temporary collagen plug: 2 cases.

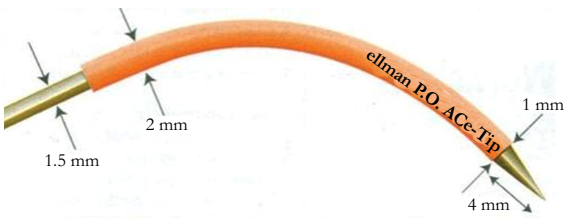
All patients were over 60 years old and had severe dry eyes with Schirmer's test + topical anaesthesia < 5 mm and insufficient response to artificial tears application of 6 or more times daily.

The result was excellent in 8 cases. In two cases, where a perfect occlusion of the *punctum* was achieved, the patient did not report subjective improvement. Schirmer's test was about the same. In one case the *punctum* re-opened a few weeks after coagulation. In that patient the *canaliculus* was cut and the stumps were coagulated. The wound was closed with three sutures of Vicryl 6/0. This led to an acceptable but limited improvement.

Conclusion

Despite the limited indications for permanent occlusion of the *punctum lacrimale*, the CPO appears to be a valuable instrument in our daily practice. When used for the right indications, coagula-

Figure 1. The CPO Electrode.



tion of the *lacrimal punctum* using the CPO, in combination with a high frequency low-temperature radiofrequency device, yields excellent results. The therapy can be performed in the office and is very cost-effective when compared to a laser coagulation.

Acknowledgement

The authors gratefully acknowledge support by Z. de Silva (Melbourne, Australia) and Allergan (Hoeilaart, Belgium).

References

[1]. Mc Carty CA, Bansal AK, Livingston PM, Stanislavsky YL, Taylor HR (1998) The epidemiology of dry eye in Melbourne, Australia. Ophthalmol. 105(6): 1114-1119.

[2]. Lin PY, Tsai SY, Cheng CY, Liu JH, Chou P, et al., (2003) Prevalence of dry eye among an elderly Chinese population in Taiwan: the Shihpai Eye Study. Ophthalmol. 110(6): 1096-1101.

[3]. Savini G, Prabhawasat P, Kojima T, Grueterich M, Espana E, Goto E (2008) The challenge of dry eye diagnosis. Clin Ophthalmol. 2(1): 31–55.

[4]. Lemp MA (1995) Report of the National Eye Institute/Industry workshop on Clinical Trials in Dry Eyes. CLAO J. 21: 221-232.

[5]. Murube J, Németh J, Höh H, Kaynak-Hekimhan P, Horwath-Winter J, et al., (2005) The triple classification of dry eye for practical clinical use. Eur J Ophthalmol. 15(6): 660-667.

[6]. Report of the 2007 International Dry Eye Workshop. Ocul Surf. 5: 65-204.

[7]. Lemp MA, Bron AJ, Baudouin C, Benítez Del Castillo JM, Geffen D, et al., (2011) Tear osmolarity in the diagnosis and management of dry eye disease. Am J Ophthalmol. 151(5): 792-798.

[8]. Bouzouaya C, Raus P (2012) The Chedly punctal occluder: A new treatment modality for dry eyes. PRIME. 2: 30-37.

[9]. Ervin AM, Wojciechowski R, Schein O (2010) Punctal occlusion for dry eye syndrome. Cochrane Database Syst Rev. 9: CD006775.

[10]. Geerling G, Raus P, Murube J (2008) Minor salivary gland transplantation. Dev Ophthalmol. 41: 243-2.

[11]. Raus P, Kumar Raghumaran B, Pinkse M, Verhaert P (2015) Bottom–up protein identifications from microliter quantities of individual human tear samples. Important steps towards clinical relevance. EuPA Open Proteomics. 9: 8-13.

[12]. Paulsen F (2008) Anatomy and physiology of efferent tear ducts. Ophthalmologe. 105(4): 339-345.

[13]. Tucker NA, Tucker SM, Linberg JV(1996) The anatomy of the common *canaliculus*. Arch Ophthalmol. 114(10): 1231-1234.

[14]. Murube-del-Castillo J, Hernandez-King J (1993) Treatment of dry eye by moving the lacrimal *punctum* to dry dock. Ophthalmic Surg. 24(1): 53-58.

[15]. MurubeJ, Murube E (1996) Treatment of dry eye by blocking the lacrimal canaliculi. Surv Ophthalmol. 40(6): 463-480.

[16]. Geerling G, Tost FHW (2008) Surgical Occlusion of the Lacrimal Drainage System. Dev Ophthalmol. 41: 213-229.

[17]. Knapp ME, Frueh BR, Nelson CC, Musch DC (1989) A comparison of two methods of punctalocclusion. Am J Ophthalmol. 108(3): 315-318.

[18]. Benson DR, Hemmady PB, Snyder RW (1992) Efficacy of laser punctual occlusion. Ophthalmol. 99(4): 618-621.

[19]. Zhou L, Beuerman RW, Chan CM, Zhao SZ, Li XR, et al., (2009) Identification of tear fluid biomarkers in dry eye syndrome using iTRAQ quantitative proteomics. J Proteome Res. 8(11): 4889-4905.

[20]. Tong L, Zhou L, Beuerman RW, Zhao SZ, Li XR (2011) Association of tear proteins with Meibomian gland disease and dry eye symptoms. Br J Ophthalmol. 95(6): 848-852.

[21]. Zhou L, Zhao SZ, Koh SK, Chen L, Vaz C, et al., (2012) In-depth analysis of the human tearproteome. J Proteomics. 75(13): 3877-3885.

Geerling G, Brewitt H (eds): Surgery for the Dry Eye.
Dev Ophthalmol. Basel, Karger, 2008, vol 41, pp 243–254

Minor Salivary Gland Transplantation

Gerd Geerling[a], *Peter Raus*[b], *Juan Murube*[c]

[a]Department of Ophthalmology, University of Würzburg, Würzburg, Germany; [b]MIRO Centrum voor Ooglidchirurgie en Esthetiek, Mol, Belgium; [c]Department of Ophthalmology, University of Alcala, Madrid, Spain

Abstract

Introduction: Minor oral salivary glands exist in large numbers in the labial, buccal and palatal mucosa, and account for approximately half of the baseline secretion of saliva. They can be transplanted together with the overlying mucosa as a complex graft to the posterior lamella of the eyelids to increase ocular surface lubrication and reduce discomfort in dry eyes. **Material and Methods:** The surgical methods and the results of this technique in 17 patients are described. All patients had been retractive to medical treatment. The recipient bed over the lower or upper lid retractors and a donor tissue of lower labial mucosa with its submucosal minor salivary glands of approximately 2.5 × 2 cm were prepared by means of a surgical knife or Ellman Surgitron high-frequency/low-temperature radiosurgical device. The graft was cut in two strips of approximately 2.5 × 1 cm size and sutured to the recipient site with interrupted or running sutures. The labial wound was left open for second intention healing. **Results:** All grafts remained viable and vascularised within 1 week. Vascularisation of the graft was associated with an improvement of symptoms and increased ocular surface lubrication. Complications included temporary labial hypaesthesia, partial necrosis of the graft (n = 1), herpes simplex virus keratitis (n = 1) and epiphora (n = 1). Viable glandular tissue was found in specimens taken 18 and 36 months postoperatively. Other reported complications include lid malpositioning such as ptosis and entropium. **Conclusion:** Transplantation of minor salivary glands is a promising new treatment option for severe dry eyes. The procedure is simple with minimal surgical risks. These grafts remain viable in over 90% and seem to be capable of sustaining a basal secretion for up to 36 months. Since experience with this technique is still very limited, prospective controlled studies have to be performed to establish the long-term survival of the glands and to characterise the salivary tear film and its impact on the ocular surface.

Copyright © 2008 S. Karger AG, Basel

General Considerations

With absolute tear deficiency, defined by ourselves as a Schirmer test (without prior application of a topical anaesthetic) of <2 mm, ocular surface

disease may progress despite frequent application of unpreserved tear substitutes and occluded lacrimal puncta [1]. Such patients frequently suffer from permanent and sometimes excruciating symptoms of discomfort, but also experience severe loss of vision. In this situation, mucous membrane grafting (see chapter 16) alone may not provide sufficient lubrication to alleviate symptoms or to successfully enable ocular surface reconstruction with e.g. a corneal graft.

Apart from artificial devices termed 'dacryoreservoirs' (see chapter 19), transplantation of significant amounts of actively fluid secreting tissue is the only option available to provide a sufficient volume of substitute lubrication. Various forms of mucous membrane grafts (oral, nasal, ileac) have been used, but failed to provide sufficient amounts of lubrication. Several studies have shown that oral and nasal mucosa could successfully be used to reconstruct the fornices and to provide mucin for lubrication. However, neither after oral nor nasal mucosal transplantation did lubrication increase to a level sufficient to sustain a healthy corneal graft [2–5].

The composition of saliva and tears is fairly similar in its complexity as well as in several specific parameters (table 1 [6–14] and table 2 [15–35]). It contains large amounts of albumin, immunoglobulins, growth factors, mucins and lipids, all of which are also present in tears. In addition, saliva is also characterised by the presence of enzymes such as amylase. However, due to their substrate specificity the latter have not been found to be deleterious for the ocular surface [10, 36]. It is thus not surprising that whole or compounds of saliva have been used to treat dry eye since biblical times (Mark 8:23) [37].

A number of salivary glands have been reported repeatedly as a source of substitute lubrication for the ocular surface [37–40]. These include minor as well as major salivary glands from the oral cavity and neck. Major salivary glands are the paired (right/left) parotid, sublingual and submandibular glands. Minor oral salivary glands exist in large numbers in the labial, buccal and palatal mucosa. Major and minor salivary glands account for approximately half of the baseline secretion of saliva (i.e. without gustatory stimulus) [40]. Other tissues, such as gastrointestinal mucosa, have also been tried but harvesting the graft as well as donor site morbidity and insufficient success have limited further use.

The idea of transplanting minor salivary glands was first conceived by Juan Murube [40], who in 1998 described the use of various complex grafts of mucosa with attached salivary glands. Of these he found the combination of labial minor salivary glands with their covering mucosal sheath most promising in experimental and clinical use (fig. 1). In a group of 6 patients he observed increased surface lubrication and moderate to significant reduction of dry eye symptoms in 4 cases [40].

Table 1. Flow rate and general type of secretion of unstimulated tears, whole and specific parts of saliva. NDA = No Data Available.

	Tears	Whole saliva	Sublingual gland saliva	Parotid gland saliva	Submandibular gland saliva normal	Submandibular gland saliva transplanted (salivary tears)	Labial gland saliva
Flow rate, μl/min (stimulated)	0.6–1.4 (4.4)	~400 ± 200 (~2,000 ± 1,000)	~20	60–80 (260–330 ± 13)	108–134 (450 ± 0.4)	0.12 (0.74)	~300 0.4–5 μl/cm^2 (2.7–3.0 ± 1.3)
Type of secretion	Seromucinous + lipids	Mixed	Mucoserous	Serous	Seromucinous	Mucoserous	Seromucinous
Surface tension, dyn/cm	62 ± 6	15–26		50–70	70 ± 5	66 ± 1	NDA

Table 2. Composition of unstimulated tears, whole and specific parts of saliva. NDA = No Data Available.

	Tears	Whole saliva	Parotid gland saliva	Submandibular grand saliva normal	Submandibular grand saliva transplanted (salivary tears)	Labial saliva
Osmolality mosm/l	298 ± 10	200–310	NDA	108 ± 4	165 ± 74	NDA
Potassium mosm/l	18.7 ± 5.5	20 ± 6	30 ± 21	20 ± 6	NDA	NDA
pH	7.47	6.5–7.25	NDA	NDA	NDA	NDA
Glucose mmol/l	0.16 ± 0.03	1.3 ± 0.3		NDA	NDA	NDA
Total protein, g/l (stimulated)	6–23.3 ± 3.4	1.8 ± 0.4	~800 (247 ± 15/min)	0.38 ± 0.16 (0.54 ± 0.34)	1.8 ± 2.5 (1.2 ± 0.56)	NDA
Albumin, g/l (stimulated)	4 ± 0.5	0.04 ± 0.02	(0.0025 ± 0.24)	(2.7 ± 0.26)	NDA	0.23 ± 0.13
EGF, ng/) (stimulated)	5,090–8,466 ± 3,740 (2,763)	864 ± 135	NDA	357 ± 60	NDA	NDA
Vitamin A, ng/ml	16	NDA	NDA	NDA	NDA	NDA
Fibronectin, ng/ml	19 ± 24 (open) 4,127 ± 3,222 (closed eyes)	149 ± 46.2	2.5 ± 1.4	NDA	NDA	NDA
sIgA, µg/ml (stimulated)	1,189 ± 904	89 ± 55	(131 ± 7)	127 ± 178 (42 ± 45)	643 ± 1,183 (828 ± 1041)	69 ± 56
Lactoferrin, µg/ml	3,080 ± 930	10 ± 7	(1.81 ± 0.31)	(1.63 ± 0.21)	NDA	8.5 ± 6.1
Lysozyme, µg/ml (stimulated)	1.4 ± 0.2	1.39 ± 1.37	(1.82 ± 0.16)	6.6 ± 2.7 (8 ± 2.9)	87.7 ± 144.1 (39.8 ± 25.6)	NDA
Amylase, kU/l (stimulated)	NDA	494 ± 44	590	45 ± 182 139 ± 212	41 ± 57 (112 ± 69)	NDA

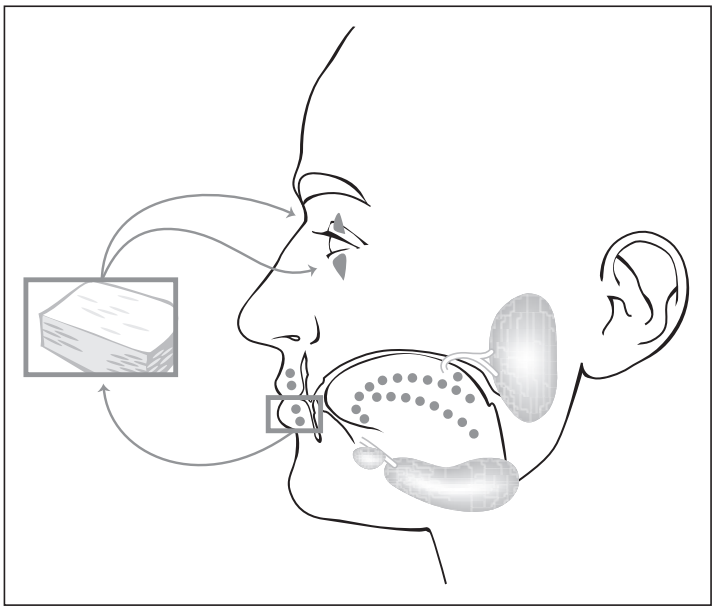


Fig. 1. Diagram showing general distribution of minor salivary glands (grey dots), donor and recipient site of fabial mucosa with minor salivary gland transplant.

The minor salivary glands of the lip are present in such large numbers that they form an almost compact layer of single lobules between the quadratus labii muscle and the oral mucosa. Each lobule measures approximately $2 \times 2 \times 3$ mm and has a short excretory duct exiting onto the surface of the oral mucosa. The secretion of these glands is seromucinous and has a viscosity higher than tears. It contains large amounts of antimicrobial peptides such as IgA and growth factors such as epidermal growth factor [41, 42] as well as a lipid level 4–5 times higher than in other salivary gland secretions.

Surgical Techniques

The complex graft of labial mucosa with attached minor salivary glands is usually taken from the lower lip, since access is easier here than in the upper lip. Also the glandular density per surface area is higher in the lower lip [40, 43]. We have also observed that more salivary glands are found in the lateral compared to the central part of the lip. However, if severe aqueous tear deficiency is bilateral – as is often the case in such disorders as graft-versus-host disease or Stevens-Johnson syndrome – additional graft material has to be harvested and the upper lip or buccal mucosa can be considered.

Preferably surgery is performed under general anaesthesia. First the recipient bed, either in the upper, lower or usually both lids is prepared. The lid is

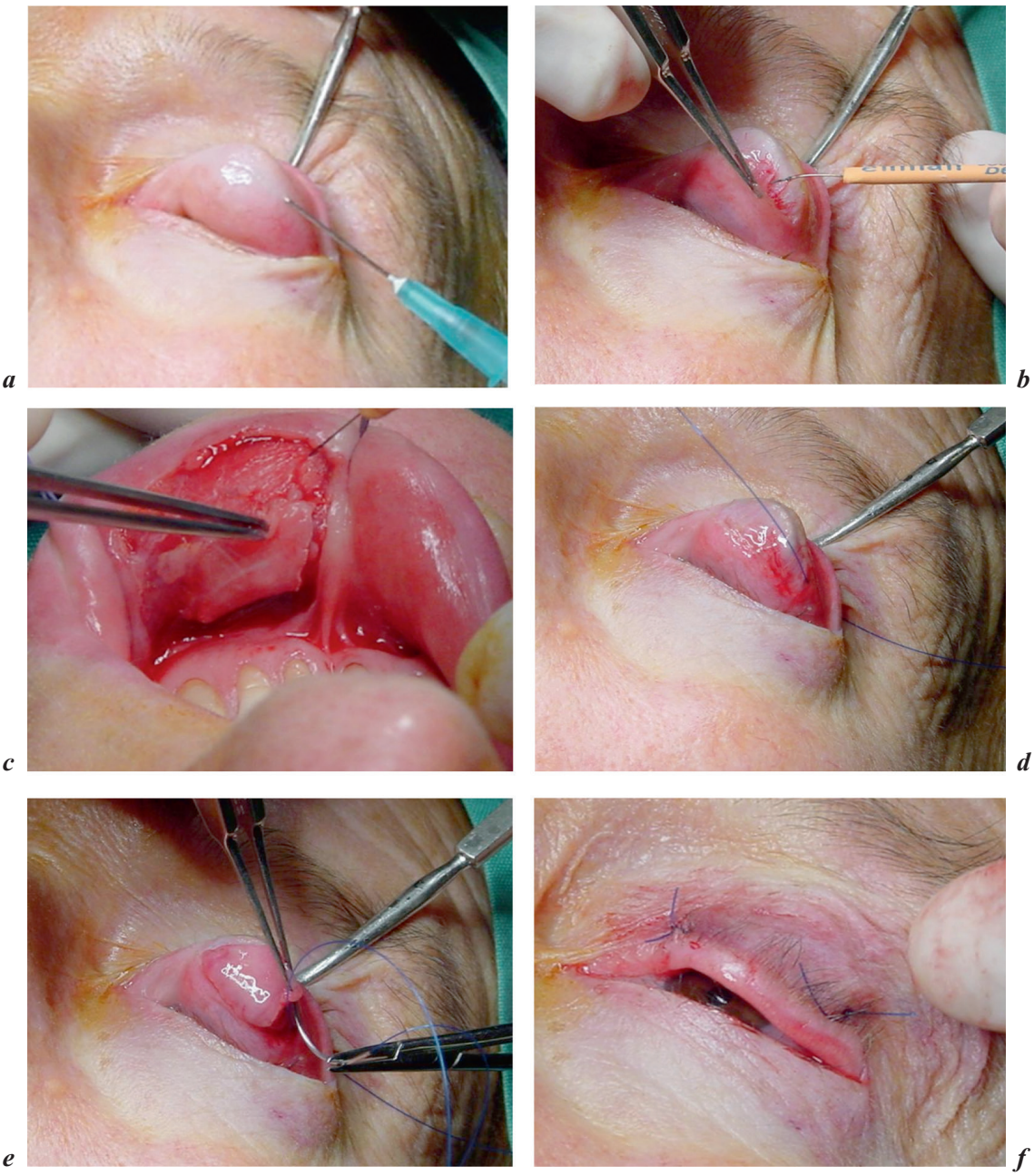


Fig. 2. (***a***) Injection of fluid into the subconjunctival space to ease surgical dissection performed with (***b***) Ellman® radiosurgical device. (***c***) The graft is harvested from the lower lip and fixed with a (***d–f)*** transpalpebral running 4.0 prolene suture in the upper and lower lid.

everted using a Desmarres lid retractor. Isotonic saline or xylocaine 1% solution with epinephrine is injected under the conjunctiva to separate it from the underlying Müller's muscle in the upper lid or the retractor muscle in the lower lid (fig. 2a). The epinephrine causes a local vasoconstriction to (further) limit

bleeding, but on the down side it potentially may reduce oxygen supply to the graft in the early postoperative phase. Next, an incision of approximately 2.5 cm is made along the rim of the tarsal plate using either a surgical blade or a fine wire electrode in the cut/coagulation mode of a radiosurgical unit (fig. 2b). The conjunctiva is dissected posteriorly for approximately 1.5 cm and any major bleeding vessel coagulated without cauterising large areas of the recipient site.

To harvest the donor tissue, the inferior lip is everted either with a large chalazion-style clamp or with two 4-0 silk traction sutures, which are placed at the inner aspect of and parallel to the labial rim. The labial mucosa is exposed with gentle traction. A first horizontal full-thickness incision is made in the mucosa, not closer than 1 cm from the mucocutaneous junction, starting 0.5 cm lateral of the midline of the lip and extending 2–2.5 cm laterally. The first salivary lobules will become visible in the incision, which is then extended at both ends backwards over approximately 1.5–2 cm towards the gingivolabial sulcus. By means of blunt or radiofrequency-assisted dissection, the graft is then lifted from the quadratus labii muscle (fig. 2c). The high-frequency radiosurgery device (4 MHz) has a fine wire electrode, a low power setting to cut, coagulate or remove soft tissues, while minimising collateral tissue damage. For this particular intervention we use the 50:50 cut-coagulate mode, which is helpful in limiting bleeding from the highly vascularised mucosa. Great care is taken to include as many salivary gland lobules as possible, while at the same time avoiding damage to the underlying muscle. The graft is finally severed completely by a fourth incision parallel to the gingivolabial sulcus and placed in a polyvidone solution (1–10%) for several minutes. All obvious bleeders are carefully coagulated. The labial wound is left unsutured. It usually rapidly granulates and is closed by secondary intention healing within 2–4 weeks.

The donor tissue obtained is carefully cleaned and cut into two strips of approximately 2.5 × 1 cm size. All glandular structures are carefully preserved. The upper lid is again double everted with the Desmarres retractor, and the donor piece is sutured to the recipient site. Care is taken to establish good contact between the graft and the underlying lid retractors to support rapid oxygen supply and revascularisation of the mucosa. The procedure is then repeated in the lower lid with the second strip of the donor tissue.

Suturing can be done with 7.0 or 8.0 long-acting absorbable sutures such as polyglactin. To avoid damage to the corneal epithelium, especially from the graft in the upper lid, a bandage contact lens should be applied until complete resorption of the sutures. During the first postoperative month the upper lid is usually fairly edematous and should not be everted to avoid damage to the graft. Alternatively, each graft can be held in place with two horizontal submucosal running prolene 4–0 sutures along their upper and lower horizontal border.

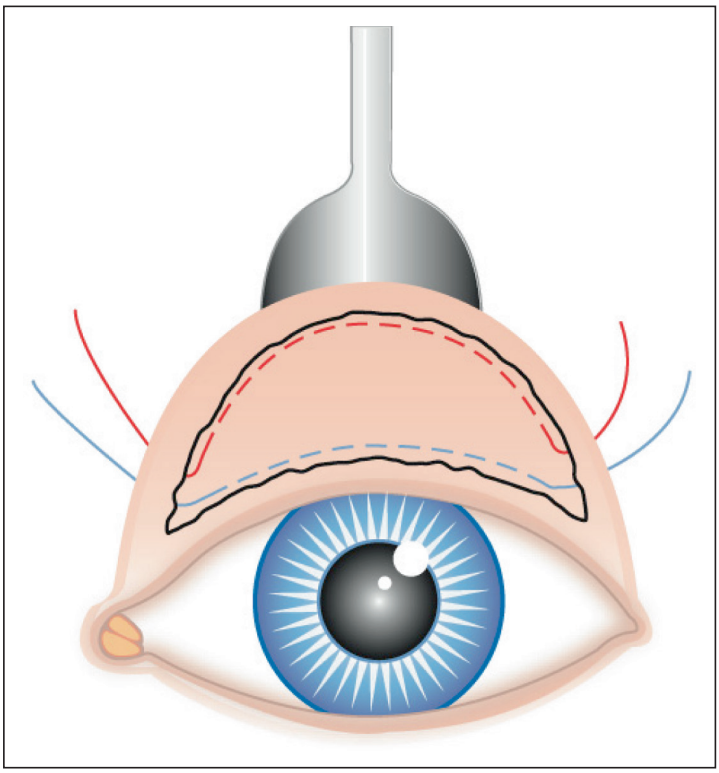


Fig. 3. Position of graft and sutures in the upper lid.

Both sutures are passed transpalpebral out to the skin, where both externalised ends are tied together (figs 2d–f, 3). This technique avoids the use of a bandage contact lens and reduces the amount and duration of the postoperative palpebral oedema.

Antibiotic eyedrops are instilled and a light pressure bandage is applied for 24 h up to 1 week. A temporary tarsorrhaphy suture can be equally placed to reduce palpebral motility and prevent any bleeding that could reduce the chances of the graft taking. Postoperative treatment includes continued application of topical antibiotics and lubricants 4–8 times daily for usually 2 weeks. At the end of this period the prolene sutures can be removed by simply cutting the ends and pulling the sutures out from the skin.

Results

So far we have performed this procedure in 17 patients with severe dry eyes using the Ellman Surgitron® high-frequency/low-temperature radiosurgical device. All patients had suffered from severe to absolute aqueous-deficient dry

eye, e.g. due to alkali burn, congenital alacrimia and other severe disorders. All patients had previously used various types of artificial tears, gels and/or ointments in most of the cases with insufficient results.

Initially the graft was pale and became chemotic, but showed signs of vascularisation within 1 week, which was when patients began to report relief from dry eye symptoms. After 2 weeks, secretion of saliva often became apparent. This was initially fairly viscous, but became more serous by 1 month. Schirmer test and fluorescein break-up increased in all cases, the latter being potentially a result of the lipid component of the labial gland saliva (table 1).

Complications included almost obligatory temporal local hypaesthesia of the lip, which can persist for a few months. Recipient site complications included 1 case of a partial necrosis (<10% of the graft) which had no effect on the final result as well as 1 case of herpes simplex virus keratitis on the fourth day postoperatively which rapidly resolved with topical acyclovir ointment 5 times a day. One patient also complained of 'watery' eyes, which 18 months postoperatively required partial resection of the graft in both lower lids under local anaesthesia. Histology showed normal glandular acinar tissue in both specimens. In 1 patient a part of the graft became externally visible presenting with a papillomatous appearance. For cosmetic reasons this portion was resected after 36 months. Again, histology revealed normal glandular structures with only minimal local inflammation. Other complications observed include a bulkiness of the lids, which in association with lower lid laxity can lead to entropium and a reduced labial volume.

Murube et al. [40] reported about 6 patients with a mean follow-up of 11 months (6–24 months) who received a labial salivary gland transplant for Sjögrens syndrome (n = 3), ocular cicatricial pemphigoid (1), Stevens-Johnson syndrome (1) or seventh nerve palsy (1). One graft became atrophic, but five grafts remained viable and led to increased lubrication and a reduction of symptoms in four. Mean preoperative Schirmer test improved from 5.7 ± 3.8 to 11.7 ± 9.5 mm. Güerrissi and Belmonte [44] reported a patient with a 2-year history of dry eye secondary to Sjögren's syndrome and severe dry eye symptoms retractive to any medical treatment, who showed significant relief after minor salivary gland transplantation. A biopsy taken 3 months after surgery confirmed the presence of viable salivary gland acini and ducts with mucin content [44]. Soares and Franca [45] reported a graft survival rate of 97% in 37 transplants and a maximum follow-up of 4.5 years. Graft viability was associated with improvement of signs and symptoms including improved vision in 92% of the cases. Complications included infection (n = 1) and ptosis (n = 3) [45].

Conclusion

Transplantation of minor salivary glands seems to be a promising new treatment option for severe cases of dry eye. The procedure is simple to perform with only minimal surgical risks. These grafts remain viable in over 90% and seem to be capable of sustaining a basal secretion for up to 36 months. Since experience with this technique is still very limited, prospective controlled studies have to be performed to establish the long-term survival of the glands and to characterise the salivary tear film and its impact on the ocular surface.

References

1 Schroeder C, Hakim SG, Collin JRO, Sieg P, Geerling G: Long-term follow-up after autologous submandibular gland transplantation in scarring keratoconjunctivitis with absolute dry eyes (in German). Ophthalmologe 2003;100:1079–1084.

2 Heiligenhaus A, Shore JW, Rubin PA, Foster CS: Long-term results of mucous membrane grafting in ocular cicatricial pemphigoid. Implications for patient selection and surgical considerations. Ophthalmology 1993;100:1283–1288.

3 Wenkel H, Rummelt V, Naumann GOH: Long-term results after autologous nasal mucosal transplantation in severe mucus deficiency syndromes. Br J Ophthalmol 1999;84:279–284.

4 Kuckelkorn R, Wenzel M, Lamprecht J, Bocking B, Reim M: Autologous nasal mucosa transplantation after severe chemical and thermal eye burns. Klin Monatsbl Augenheilkd 1994;204: 155–161.

5 Kuckelkorn R, Schrage N, Redbrake C, Kottek A, Reim M: Autologous transplantation of nasal mucosa after severe chemical and thermal eye burns. Acta Ophthalmol Scand 1996;74:442–448.

6 Birkhead EL, Carlén ÖT: Minor salivary gland secretion rates and immunolgobulin A in adults and the elderly. Eur J Oral Sci 2006;114:494–499.

7 Borrelli M, Schröder C, Dart JKG, Collin JRO, Sieg P, Cree IA, Matheson MA, Tiffany JM, Proctor G, van Best J, Hyde N, Geerling G: Long-term follow-up after submandibular gland transplantation in severe dry eyes secondary to cicatrising conjunctivitis. PR J Ophthalmol (submitted).

8 Eliasson L, Almstahl A, Lingstroöm P, Wikström M, Carlén A: Minor gland saliva flow rate and proteins in subjects with hyposalivation due to Sjögren's syndrome and radiation therapy. Arch Oral Biol 2005;50:293–299.

9 Ferguson DB: The flow rate and composition of human labial gland saliva. Arch Oral Biol 1999; 44:S11–S14.

10 Geerling G, Honnicke K, Schroder C, Framme C, Sieg P, Lauer I, et al: Quality of salivary tears following autologous submandibular gland transplantation for severe dry eye. Graefes Arch Clin Exp Ophthalmol 1999;237:546–553.

11 Humphrey SP, Williamson RT: A review of saliva: normal composition, flow, and function. J Prosthet Dent 2001;85:162–169.

12 Proctor GB, Hamdan S, Carpenter GH, Wilde P: A statherin and calcium enriched layer at the air interface of human parotid saliva. Biochem J 2005;389:111–116.

13 Sorensen T, Jensen FT: Tear flow in normal human eyes. Determination by means of radiosotope and gamma camera. Acta Ophthalmol (Copenh) 1979;57:564–581.

14 Pijpe J, Kalk WWO, Bootsma H, Spijkervet FKL, Kallenberg CGM, Vissink A: Progression of salivary gland dysfunction in patients with Sjögren's syndrome. Ann Rheum Dis 2007;66: 107–112.

15 Aydin S: A comparison of ghrelin, glucose, α-amylase and protein levels in saliva from diabetics. J Biochem Mol Biol 2007;40:29–35.

16 Dodds MWJ, Johnson DA, Yeh CK: Health benefits of saliva: a review. J Dent 2005;33:223–233.
17 Ferguson DB: The flow rate and composition of human labial gland saliva. Arch Oral Biol 1999;44:S11–S14.
18 Fukuda M, Fullard RJ, Willcox MD, Baleriola-Lucas C, Bestawros F, Sweeney D, Holden BA: Fibronectin in the tear film. Invest Opthahlmol Vis Sci 1996;37:459–467.
19 Fullard RJ, Tucker DL: Changes in human tear protein levels with progressively increasing stimulus. Invest Ophthalmol Vis Sci 1991;32:2290–2301.
20 Jentsch H, Sievert Y, Göcke R: Lactoferrin and other markers from gingival crevicular fluid and saliva before and after periodontal treatment. J Clin Periodontol 2004;31:511–514.
21 Kalk WWI, Vissink A, Stegenga B, Bootsma H, Nieuw Amerongen AV, Kallenberg CGM: Sialometry and sialochemistry: a non-invasive approach for diagnosing Sjögren's syndrome. Ann Rheum Dis 2002;61:137–144.
22 Kanehisa J, Doi S, Yamanak T, Takeuchi H: Salivary fibronectin in man: an immunoblotting, radioimmunoassay and immunohistochemial study. Arch Oral Biol 1991;36:265–272.
23 Lane JD, Krumholz DM, Sack RA, Morris C: Tear glucose dynamics in diabetes mellitus. Curr Eye Res 2006;31:895–901.
24 Lenander-Lumikari M, Laurikainen K, Kuusisto P, Vilja P: Stimulated salivary flow rate and composition in asthmatic and non-asthmatic adults. Arch Oral Biol 1998;43:151–156.
25 Lew H, Yun YS, Lee SY: Electrolytes and electrophoretic studies of tear proteins in tears of patients with nasolacrimal duct obstruction. Ophthalmologica 2005;219:142–146.
26 Lin AL, Johnson DA, Stephan KT, Yeh CK: Alteration in salivary function in early HIV infection. J Dent Res 2003;82:719–724.
27 Ng V, Cho P, Mak S, Lee A: Variability of tear protein levels in normal young adults: between-day variation. Graefes Arch Clin Exp Ophthalmol 2000;238:892–899.
28 Obahashi Y, Ishida R, Kojima T, Goto E, Matsumoto Y, Watanabe K, Ishida N, Nakata K, Tekeuchi T, Tsubota K: Abnormal protein profiles in tears with dry eye syndrome. Am J Ophthalmol 2003;136: 291–299.
29 Pflugfelder SC, Jones D, Ji Z, Afonso A, Monroy D: Altered cytokine balance in the tear fluid and conjunctiva of patients with Sjögren's syndrome keratoconjunctivitis sicca. Curr Eye Res 1999;19:201–211.
30 Seemann R, Hägewald SJ, Sztankay V, Drews J, Bizhang M, Kage A: Levels of parotid and submandibular/sublingual salivary immunoglobulin A in response to experimental gingivitis in humans. Clin Oral Invest 2004;8:233–237.
31 Ship JA, Fischer DJ: Metabolic indicators of hydration status in the prediction of parotid salivary gland function. Arch Oral Biol 1999;44:343–350.
32 Shoji N, Sasano T, Inukai K, Satoh-Kuwada S, Jikubo M, Furuuchi T, Sakamoto M: A simple, yet accurate method for detecting and quantifying secretions from human minor salivary glands using the iodine-starch reaction. Arch Oral Biol 2003;48:761–765.
33 Thesleff I, Viinikka L, Saxen L, lehtonen E, Perheentupa J: The parotid gland is the main source of human salivary epidermal growth factor. Life Sci 1988;43:13–18.
34 Ubels JL, MacRae SM: Vitamin A is present as retinol in the tears of humans and rabbits. Curr Eye Res 1984;3:815–822.
35 Van Setten GB: Epidermal growth factor in human tear fluid: increased release but decreased concentrations during reflex tearing. Curr Eye Res 1990;19:79–83.
36 Geerling G, Daniels JT, Dart JK, Cree IA, Khaw PT: Toxicity of natural tear substitutes in a fully defined culture model of human corneal epithelial cells. Invest Ophthalmol Vis Sci 2001;42:948–956.
37 Filatov VP, Chevaljev VE: Surgical treatment of parenchymatous ophthalmoxerosis. J Ophthalmol (Odessa) 1951;3:131–137.
38 Bennett JE: The management of total xerophthalmia. Arch Ophthalmol 1969;81:667–682.
39 Murube J: Transplantation of salivary gland to the lacrimal basin. Scand J Rheumatol Suppl 1986;61:264–267.
40 Murube J, Manyari A, Chen-Zhuo L, Rivas L, Murbue I: Labial salivary gland transplantation in severe dry eye. Oper Tech Oculoplast Orbit Reconstr Surg 1998;1:104–110.
41 Crawford JM, Taubman MA, Smith DJ: Minor salivary glands as a major source of secretory immunoglobulin A in the human oral cavity. Science 1975;190:1206–1209.

42 Koski H, Konttinen YT, Hietanen J, Tervo T, Malmstrom M: Epidermal growth factor transforming growth factor-α, and epidermal growth factor receptor in labial salivary glands in Sjögren's syndrome. J Rheumatol 1997;24:1930–1935.
43 Gaubenshtok LM, Lent'ev VK: The quantitative topographic characteristics of the minor salivary glands of the lips (in Russian). Stomatologiia (Mosk) 1990;69:28–31.
44 Güerrissi JO, Belmonte J: Surgical treatment of dry eye syndrome: conjunctival graft of the minor salivary gland. J Craniofac Surg 2004;15:6–10.
45 Soares EJ, Franca VP: Transplantation of labial salivary glands for severe dry eye treatment (in Portuguese). Arq Bras Oftalmol 2005;68:481–489.

Prof. Dr. Gerd Geerling
Klinik für Augenheilkunde, Julius-Maximilians-Universität Würzburg
Josef-Schneider-Str. 11, DE-97080 Würzburg (Germany)
Tel. +49 931 201 20 610, Fax +49 931 20 120 490
E-Mail g.geerling@augenklinik.uni-wuerzburg.de

Appendix 3.1 The Chedly Punctal Occluder. A new treatment modality for Dry Eyes.

[C. Bouzouaya, P. Raus (2012) Prime-Journal.com March 2012: 30-37.]



"Severe forms of dry eye syndrome associated with Sjögren's syndrome or skin diseases such as rosasea, can lead to vision-threatening complications, such as keratitis, corneal scarring, and vision disruption."

THE CHEDLY PUNCTAL OCCLUDER: A NEW TREATMENT MODALITY FOR DRY EYES

Chedly Bouzouaya and Peter Raus discuss the treatment options available to treat dry eye syndrome—a common condition treated by ophthalmologists

ABSTRACT

Objective
Dry eye syndrome is a common disorder, generally occurring in those over 40 years of age. The management of this condition is related to the severity of the symptoms. Permanent punctal occlusion is indicated for those patients experiencing severe dry eye syndrome.

Methods
With the use of a high-frequency, low-temperature surgical device and electrode specifically designed to cauterise and close the punctum, a large number of patients were treated and relieved from the symptoms of dry eye syndrome. The Chedly Punctal Occluder (CPO) electrode is designed to both dilate the punctum and be inserted in the lumen of the lacrimal duct to seal it.

Results
When there is an indication for a permanent punctal occlusion, the CPO, with the use of a high frequency device, seals the punctum permanently. There are no cases of reopening of the punctum and no lid notch as seen with the use of electrocautery or lasers.

Conclusions
Permanent punctal occlusion in severe dry eye syndrome using silicone plugs, argon laser, diode laser or cautery have drawbacks, are not cost-effective, and showed lower success rates in comparison with an easy-to-use and cost-effective punctal occluder electrode.





CHEDLY BOUZOUAYA, MD is Oculoplastic and Ophthalmic Surgeon, Tunisia; and **PETER RAUS, MD** is Ophthalmologist and Oculoplastic Surgeon, Belgium.

email: chedly@bouzouaya.com; Peter.raus@skynet.be

KEYWORDS
dry eye syndrome, Chedly Punctal Occluder, radiofrequency, safety, cost-effective

DRY EYE SYNDROME IS ONE OF THE most common problems treated by ophthalmologists. It is estimated that over 10 million Americans suffer from dry eye syndrome (also known as keratoconjunctivitis sicca), and especially those older than 40 years of age. It is estimated that nearly 75% of people over age 65 years will experience dry eye syndrome[1]. Dry eye is a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, and tear film instability with potential damage to the ocular surface[2].

Dry eye is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface, and has been classified into two main categories by the US National Eye Institute[3]:

- Aqueous tear deficiency (ATD)
- Evaporative dry eye.

Tear-deficient dry eye can be further separated into Sjögren's syndrome-related dry eye, an autoimmune disorder affecting the lacrimal and salivary glands, and non-Sjögren's syndrome dry eye, which encompasses the range of other causes of tear deficiency.

Evaporative dry eye is caused by deficiency and/or alterations in lipid secretions as a result of meibomian gland dysfunction (MGD). Although differentiating patients according to the main causative factor is useful for diagnosis and therapy, clinical presentation is often a mixture of both pathogenic pathways[3,4].

Severe forms of dry eye syndrome associated with Sjögren's syndrome or skin diseases such as rosasea, can lead to vision-threatening complications, such as keratitis, corneal scarring, and vision disruption; therefore, early differential diagnosis is important. Most cases of dryness develop gradually over many years, with decreasing tear production and ocular surface damage as the final stages. Non-Sjögren's syndrome dry eye seems to deteriorate more slowly and less profoundly than dry eye associated with primary and secondary Sjögren's syndrome[3]. Owing to its intractable development, therapy of dry eye syndrome is often frustrating for patients and unrewarding for ophthalmologists. This makes the assessment of prognosis and patient counselling difficult. Therefore, it is paramount to find the most efficient therapeutic solutions to assist patients—even surgical solutions such as the salivary gland transplantation[5].

Diagnosing dry eye syndrome

The signs of dry eye can include conjunctival pleating, conjunctival vascular dilation, irregular corneal surface, increased debris in the tear film, and epithelial keratopathy. Rose bengal staining is an indicator of ▷

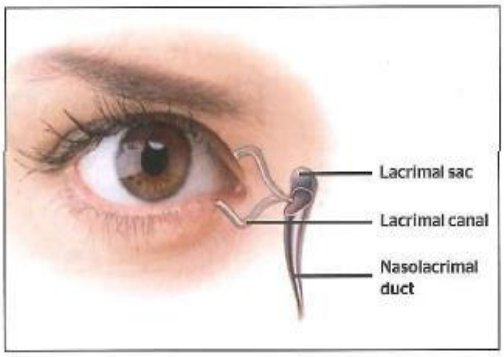


Figure 1 Anatomy of the lacrimal drainage system in frontal view

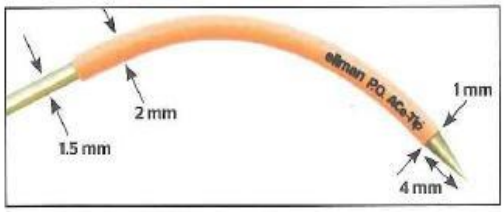


Figure 2 The Chedly Punctal Occluder

▷ early or mild cases of dry eye syndrome. It will stain the epithelial cells that are not protected by mucin tear coating. Other indicators include:

- The tear meniscus is less than 1 mm thick and is losing its convex shape
- The tear break-up time (BUT) with fluorescein stain is less than 10 seconds (the tear BUT is the time measured from a blink to the appearance of tear film defect)
- Schirmer's test shows unanaesthetised measures of basal and reflex tearing (the norm would be more than 15 mm at 5 minutes)
- Anaesthetised measures of basal tearing only (the norm is greater than 10 mm at 5 minutes).

The authors prefer to use anaesthetised methods to assess the patient's condition.

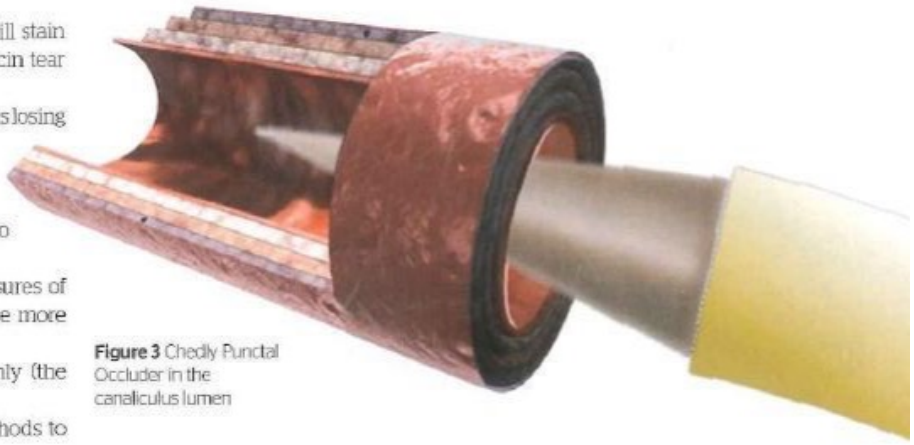


Figure 3 Chedly Punctal Occluder in the canaliculus lumen

Surgical anatomy of the lacrimal system

Tears in the marginal tear strip enter the lacrimal drainage system through the punctal opening of both eyelids (*Figure 1*). The puncta are located on the medial lid margin at the apex of small mounds of soft tissue. The papillae appear pale in contrast to the surrounding tissue as they contain more connective tissue and less vasculature. They are centred on the eyelid margin in line with the mucocutaneous junction. The upper papillae are located approximately 6 mm from the medial canthal angle and the lower papillae are 6.5 mm from the medial canthus. The punctal opening measures 0.2-0.3 mm in diameter and is surrounded by a ring of dense connective tissue that normally maintains a patent entrance. The aperture is round or oval in youth, but often collapses into a slit configuration with age. The puncta are normally directed somewhat posterior towards the globe and do not become visible unless the lid is slightly everted. For each punctum, a canaliculus passes perpendicular to the lid margin at a distance of 2 mm, where it dilates to form the ampulla, an irregular sac 2 mm in diameter. From the ampulla, the canaliculi turn horizontally and run medially, parallel to the lid margin for a distance of approximately 8 mm. In 90% of individuals[7], the two canaliculi join at an angle of approximately 25 degrees to form a common canaliculus 3-5 mm in length[8]. In the remaining 10%, the two canaliculi join the lacrimal sac independently.

The lacrimal sac passes inferiorly into the bony canal 12 mm in length and 3-5 mm in width. Intranasally, the canal forms the lacrimal ridge, which runs in front of the middle turbinate and the medial wall of the ethmoid labyrinth.

> **"Thermal cautery has a higher success rate than laser, but also has severe drawbacks. The energy cannot be adjusted easily in order to provide the right amount."**

Management strategies for severe dry eye syndrome

The management strategy for dry eye syndrome will often depend on the severity of symptoms. The mainstay is the use of tear substitutes, such as artificial tears. Pharmacologic stimulation of tear secretion, such as with topical cyclosporine, has been tried with only minimal success[9,10].

Permanent punctal occlusion is indicated for severe dry eye syndrome and minimal tear secretion. It is possible to achieve punctal occlusion using collagen or silicone plugs[3]. However, one of the drawbacks of punctal plugs is migration into the nasolacrimal system. This migration can cause canaliculus and lacrimal sac ▷

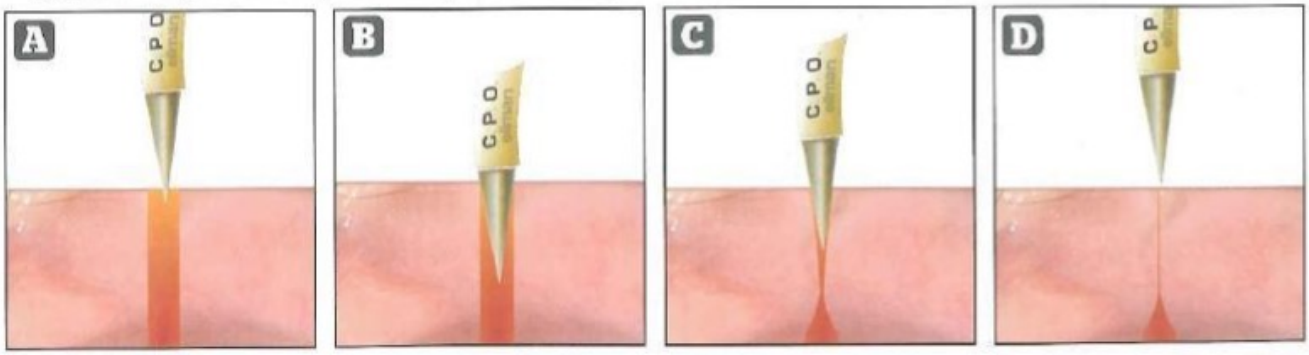


Figure 4 Four-step diagram of insertion and retrieval of CPO



Figure 5 Local anaesthesia on conjunctival side

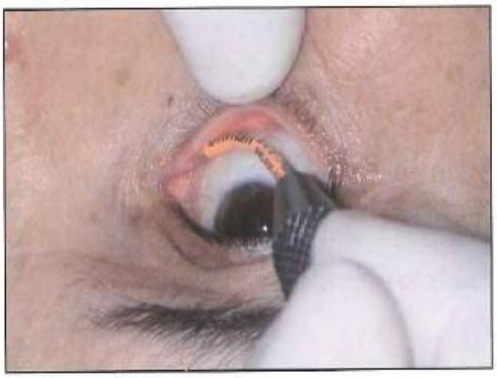


Figure 6 CPO inserted in the lumen of lower canaliculus



Figure 7 CPO in the lumen of the lower canaliculus



Figure 8 RF activated with CPO in the canaliculus lumen

▷ inflammation and infection (canaliculitis and dacryocystitis), caused by bacterial adherence to the silicone plug. They can also cause irritation, epiphora or extrusion[12, 13]. Prior to permanent punctal occlusion, collagen plugs can be used as diagnostic adjuncts; but the collagen may dissolve within days. Permanent punctal occlusion[14, 15, 16, 17] can be performed using the Argon laser, diode laser, electrocautery, or by using a high-frequency, low-temperature device[18].

In a study by Hutnic and Probst[19], comparing the use of Argon laser to electrocautery, the laser-treated patients reported a reduction in symptoms by 14% at 6 months, while symptoms in the electrocautery group were reduced by 64%. Therefore, it can be said that Argon laser punctal closure has a low success rate and is not cost-effective; thermal cautery has a higher reported success rate, but it is difficult to precisely control the depth of cauterisation and the exact temperature, often resulting in tissue burn, lid notch, and deformity.

> "Thermal cautery has a higher success rate than laser, but also has severe drawbacks. The energy cannot be adjusted easily in order to provide the right amount of energy. High energy will cause tissue destruction, a burn and a lid notch."

For the use of a diode laser, the canalicular area needs to be first stained with gentian violet, promoting absorption of the laser energy (532nm wavelength). The cornea needs to be protected with a metal corneal shield. It takes 2-3 weeks to have a complete closure with a slight opening of the punctum at 4-5 months[20]. With the use of the high-frequency radiosurgical device and the Chedly Punctal Occluder (CPO), the punctal closure was immediate, permanent and right into the lumen of the canaliculus (*Figures 2-4*). As a result, there is no tissue burn, no lid notch, and no additional precautions are needed, as is the case with lasers.

For the past 5 years the authors have been using the high-frequency, low-temperature Surgitron Dual radiofrequency (RF) device (Ellman International, Inc.)[21,22,23] with a higher success rate than that of laser[24] or thermal cautery. The RF device is more cost-effective than laser and has no side-effects in comparison with thermal cautery. Although thermocautery is not costly and has a reasonable success rate, the aforementioned drawbacks make the intervention with the RF device, together with the CPO, a much better treatment option. In addition to the low success rate, lasers can cost 20-30-times more than the price of a RF unit.

Thermal cautery has a higher success rate than laser, but also has severe drawbacks. The energy cannot be adjusted easily in order to provide the right amount of energy. High energy will cause tissue destruction, a burn and a lid notch.

In conjunction with the Surgitron, the authors use an electrode specifically designed to cauterise and close the punctum. The CPO, made from an alloy material, is designed to both dilate the punctum and be inserted in the lumen of the horizontal portion of the lacrimal ▷

Key points

- Using the Chedly Punctal Occluder and radiofrequency is a safe, quick and cost-effective treatment modality for dry eye syndrome
- The procedure can be carried out in an office environment
- Studies have shown that the procedure allows for a greater comfort among patients compared with traditional treatment methods



Figure 9 Postoperative result. Sealed lower punctum on the lower right lid

> When there is an indication for permanent punctal occlusion, RF with the use of the CPO was more efficient and more cost-effective than any of the other modalities.

▷ duct. The CPO is the cone-shaped Ellman RF electrode TNAEE287, patented and approved by the US Food and Drug Administration (FDA). With the CPO, a very effective treatment modality has been added to the treatment methods that are available for dry eye syndrome.

Technique

The eyelid is infiltrated with Xylocaine from the conjunctival side (*Figure 5*). The CPO—mounted on the Ellman handpiece—is inserted in the lumen of the canaliculus (*Figures 6* and *7*). The Surgitron is set on 'cut/coag' mode, the unit is activated for a few seconds (*Figure 8*), and then the CPO is pulled out. If the punctum is still visible, the tip of the CPO is applied on the lumen to seal it. At the end of the procedure, an antibiotic ointment is applied and used for a few days. No patches are necessary following treatment. This is a safe, fast, successful and cost-effective technique. One eye is treated at a time, addressing the lower punctum (*Figures 9* and *10*). If dry eye symptoms do not resolve, the upper punctum is occluded in the same manner. With the punctum completely sealed, the patient is relieved from the risk of irritation, inflammation, foreign body sensation, and light sensitivity for example.



Figure 10 Postoperative result. Sealed lower punctum on both sides

Discussion

When there is an indication for permanent punctal occlusion, RF with the use of the CPO was more efficient and more cost-effective than any of the other modalities. The RF unit operates at a high frequency of 4.0 Mhz. The patented microprocessor circuit matches the wattage, waveform, alloy and patient's impedance to the optimum 4.0 Mhz. As the thicker diameter electrode produces slightly higher temperatures in tissue, the CPO has a thick cone-shaped design, eliminating the need to use a punctum dilator.

The CPO is 5mm long and is inserted nearly 4mm into the lumen. The punctum is sealed permanently. The authors have not experienced a reopening of the punctum or a lid notch as sometimes seen with cautery or lasers (*Figures 11* and *12*).

Conclusions

In the experience of the authors, surgical punctal occlusion using the Argon laser has a very low success rate in comparison with the diode laser. Both lasers were not cost-effective and showed a lower success rate compared with the Ellman RF unit.

When there is an indication for permanent punctal occlusion, RF with the CPO has been more efficient and cost-effective than other treatment modalities that

References

1. [No authors listed]. Report of the International Dry Eye WorkShop (2007). Ocul Surf 2007; 5(2): 65-204
2. Lemp MA. Report of the National Eye Institute/Industry Workshop on Clinical Trials in Dry Eyes. CLAO J 1995; 221-32
3. Mathers WD. Ocular evaporation in meibomian gland dysfunction and dry eye. Opthalmology 1993; 100(3): 347-51
4. Sullivan DA, Sullivan BD, Evans JE et al. Androgen deficiency, Meibomian gland dysfunction, and evaporative dry eye. Ann N Y Acad Sci 2002; 966: 211-22
5. Kruize AA, van Bijsterveld OP, Hené RJ et al. Long-term cause of tear gland function in patients with keratoconjunctivitus sicca and Sjögren's syndrome. Br J Ophthalmol 1997; 81(6): 435-8
6. Raus P. Radiosurgery aids in salivary gland transplants for severe dry eye. Ocular Surg News 2003; 12: 15-6
7. Jones LT. An anatomical approach to problems of eyelids and lacrimal apparatus. Arch Ophthalmol 1961; 66: 111-24
8. Werb A. The anatomy of lacrimal system. In: Milder B, Weil BA. eds. The Lacrimal System. Appleton-Century Crofts: Connecticut, 1982
9. Gündüz K, Ozdemir O. Topical cyclosporin treatment of keratoconjunctivitis sicca in secondary Sjögren's syndrome. Acta Ophthalmol (Copenh) 1994; 72(4): 438-42
10. Kunert KS, Tisdale AS, Stern ME, Smith JA, Gipson IK. Effect of cyclosporine ophthalmic emulsion on the number of inflammatory cells and goblet cells in conjunctival biopsies of patients with dry eye. Invest Ophthalmol Vis Sci 1999; 40: S771
11. Willis RM, Folberg R, Krachmer JH, Holland EJ. The treatment of aqueous-deficient dry eye with removable punctal plugs. A clinical impression-cytologic study. Ophthalmology 1987; 94(5): 514-8
12. Tsubota K, Fujihara T, Takeuchi T. Soluble Interleukin-2 Receptors and Serum Autoantibodies in Dry Eye Patients: Correlation with Lacrimal Gland Function. Cornea 1997; 16(3): 339-44
13. Soparkar CN, Patrinely JR, Hunts J, Linberg JV, Kersten RC, Anderson R. The perils of permanent punctal plugs. Am J Ophthamol 1997; 123(1): 120-1
14. [No authors listed] Punctal occlusion for the dry eye. Three-year revision. American Academy of Ophthalmology. Ophthalmology 1997; 104(9): 1521-4
15. Tuberville AW, Frederick WR, Wood TO. Punctal occlusion in tear deficiency syndromes. Ophthalmology 1982; 89(10): 1170-2
16. Dohiman CH. Punctal occlusion in keratoconjunctivitis sicca. Ophthalmology 1978; 85(12): 1277-81
17. Lamberts DW. Punctal occlusion. Int Ophthalmol Clin 1994; 34(1): 145-50
18. Harris DR, Noodelman R. Using a low current radiosurgical unit to obliterate facial telangiectasias. J Dermatol Surg Oncol 1991; 17(4): 382-4.
19. Hutnic CM, Probst LE. Argon laser punctal therapy versus thermal cautery for the treatment of aqueous deficiency dry eye syndrome. Can J Ophthalmol 1998; 33(7): 365-72
20. Rise CD. Diode laser punctal closure an option for dry eye. 2005. OSN Europe/Asia-Pacific Edition
21. Bouzouaya C. Mini-incision lower lid blepharoplasty reduces postoperative complications. Ocular Surgery News 2001; 1: 71
22. Bouzouaya C. Radiosurgery can effectively remove xanthelasma. Ocular Surgery News 2004: 76
23. Bouzouaya C. Radiosurgery an effectively and efficient technique for cosmetic eyelid surgery. Ocular Surgery News 1993; 37
24. Awan KJ. Laser punctoplasty for the treatment of punctal stenosis. Am J Ophthalmol 1985; 100(2): 341-2



Figure 11 One-year postoperative result



Figure 12 Two-year postoperative result

the authors have tried. It is also reusable. Laser punctal occlusion should be discouraged because it is less effective and more expensive than other methods of treatment.

This technique is again easy to use, with no great deal of time needed to learn the technique. It is an office procedure which takes only a few minutes to complete, and with no drawbacks or experience of reopening. All these advantages make the technique suitable to the growing numbers of dry eye patients following the widely used excimer laser refractive surgery.

▶ ***Declaration of interest*** *Dr. Bouzouaya has no direct financial or any other interest in the products mentioned in the article, nor is he a paid consultant for any companies mentioned.*

▶ ***Figures 1-12*** *© Chedly Bouzouaya*

Chapter 4: First contact with Proteomics

4.0 Introduction: The age of Q-TOFs.

The proteomics techniques utilized today (2017-2018), are quite different from those at the time of our first proteomics experiments. We started with GeLC MS/MS to study the protein composition of tears and saliva, by employing so-called shotgun or bottom-up analyses on a quadrupole time-of-flight (QTOF) mass spectrometer.
It is clear that with the more advanced and higher resolution MS based analytical technology of today, an increased depth of analysis is readily achieved. Yet we feel that, in the global context of this thesis, our first proteomics experiments merit a separate chapter.
Indeed, the results of these initial tests, which were designed to support a possible surgical intervention to treat severe DED, comprised the start of the analytical section of this thesis. We would like to emphasize here that, retrospectively speaking, all of the protein identifications realized by the technology of the past, were later confirmed (and thus validated) by MS systems exhibiting higher performance in terms of mass accuracy as well as sensitivity. We particularly refer to (hybrid) orbitrap based MS analyzers, including (linear) ion trap/orbitrap and quadrupole/orbitrap combinations (**Chapters 5 and 6**).

4.1. Comparative GeLC MS/MS bottom-up proteomics analysis of tears and saliva on a quadrupole time-of-flight mass spectrometer. Towards tear protein analysis in the clinical context of dry eye disease.

[Peter Raus[1], Remco Crefcoeur[2], Martijn Pinkse[2], Leen Slaets[3], Peter Verhaert[2,4]. (1) Miró Center for Eyelid Surgery and Esthetics, Geel (Belgium); (2) Laboratory for Analytical Biotechnology and Innovative Peptide Biology, Delft University of Technology, Delft (Netherlands); (3) BioMedical Research Institute, Hasselt University, Hasselt (Belgium); (4) ProteoFormiX, Beerse (Belgium)]

4.1.1 Abstract

We report the initial data obtained in a comparative study of the protein composition of tears and labial saliva of the same individual, employing a workflow historically referred to as GeLC MS/MS, i.e. shotgun tandem MS of polyacrylamide gel electrophoresis separated proteins. A quadrupole TOF mass spectrometer was used for precursor ion detection and product ion analyses. The study yielded the confident identification of 132 lacrimal proteins, whereas in the labial saliva sample 191 proteins were identified. Of the 81 proteins which were found to be common in both secretory fluids, the identification of salivary lacritin was most interesting in the context of an innovative but still controversial treatment of dry eye disease (DED) which involves the auto-transplantation of a labial salivary gland into the dry eye. Validation of salivary lacritin was performed by immunohistochemistry.

4.1.2 Introduction

Although the transplantation of labial salivary glands is a relatively simple technique with very limited surgical risks [Murube, 1998], its value as a treatment for patients suffering from severe forms of DED, has been debated since its initial use. Even today the technique is still being criticized, at least, by several conventional ophthalmologists [Conrady et al., 2016].
To objectively support the excellent results from this remarkably simple unconventional surgical procedure for DED [see also **Chapter 3.2**], additionally confirmed by a score of

patients who underwent this treatment in our clinic [Geerling et al., 2008], we set out to evaluate whether comparative protein analysis of tear and saliva could provide added value.
It is evident that analysis of the tear composition before and after surgery, may yield objective data indicating that transplanted salivary glands remain functional post-operatively. Indeed, salivary gland contribution to post-operative tear protein composition, even after surgery-induced denervation (Chapter 3.2), should be reflected in an increased salivary protein component, confirming transplant survival.
We initiated a study in 2006 to assess the performance of the protein workflows operational at the TUDelft Department of Biotechnology to analyze proteins in human tears and (labial) saliva. The purpose was to compile a list of identifiable proteins with the proteomics analytics available to us at that time.

4.1.3 Material and methods

Human tears and labial saliva of the same (adult male) individual were collected using glass capillaries.
Prior to analysis, the samples were stored at -20°C.
In this first set of proteomics analyses, tear and labial saliva samples were separated by one-dimensional denaturing (sodium dodecyl sulfate) polyacrylamide gel electrophoresis (1D SDS PAGE).

After electrophoresis (10 µl of each sample loaded), the gel lanes containing saliva and tear proteins respectively were individually cut into 12 equal slices for so-called GeLC MS/MS analysis (see Fig. 4.1). Briefly, gel pieces were shrunk and equilibrated in 0.1 M ammonium bicarbonate (pH 8.0) prior to in-gel digestion (IGD) overnight at 37°C, as described by Piersma and coworkers [2013]. Porcine trypsin (Promega) was used as proteolytic enzyme.
The resulting tryptic peptides were analyzed by LC MS/MS on a C_{18} reversed phase HPLC directly in-line with a nano-electrospray quadrupole time-of-flight (TOF) hybrid mass spectrometer (QTof Premier, Waters, Manchester, UK) as described before [Thevelein et al., 2008].
The data (lists of detected tryptic peptide precursor ion masses and their associated MS/MS fragment ions) were searched against the SwissProt database using Mascot™ (Matrix Science, Ltd., London, UK).
Proteins were considered to be confidently identified when represented in the acquired LC MS/MS dataset by, at least, 2 distinct peptides mapping to the respective protein database entry. The following preset mass measurement tolerances were employed: 50 ppm for the precursor ion mass, and 0.2 Da for the m/z of the fragment ions.
One specific protein (lacritin) identified in human saliva was of special interest. We, therefore, decided to go for a conventional validation by immunohistochemistry on an extra labial salivary gland of a DED patient which was not transplanted. The patient gave his informed consent for use of this material in our validation study. The labial salivary gland under investigation was fixed in formalin and histological sections were analyzed using a polyclonal (rabbit) antiserum to recombinant human lacritin (a generous gift by Prof. G. Laurie (University of Virginia, USA)), following a procedure described by Sanghi and coworkers [2001].

4.1.4 Results and Discussion

In the original idea of Murube, the election of labial salivary glands for transplantation was for multiple reasons:
(1) Prior studies, corroborated by more recent publications, confirmed that saliva is not toxic for the ocular surface [Murube et al., 1998].
(2) The composition of the secretion by the labial salivary glands is very similar to natural tears [Geerling et al., 2001].
(3) Compared to other salivary glands, the labial salivary glands appear to best maintain their secretion capacity in Sjögren's syndrome (one of the known types of DED) [Pennec et al., 1990].
(4) The minor salivary glands of the lower lip are easily accessible to surgery.
(5) Murube [personal communication] was convinced that the secretion itself of the transplanted glands represents a suitable substitution for the lack of tears in DED.

Figure 4.1 shows the 1D PAGE of both samples studied.

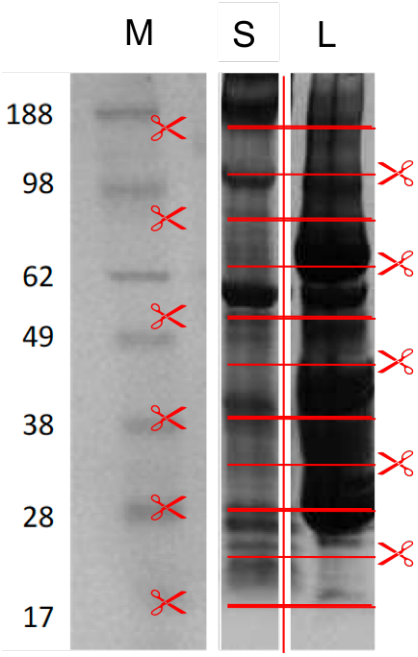


Fig. 4.1 One dimensional PAGE of human saliva (S) and tear (lacrima, L). M lane shows molecular weight markers (in kDa).

In the tear sample studied, 132 different proteins could be confidently identified, whereas in the labial saliva sample the number of positively identified proteins was 191. Between both body fluids 81 protein identities overlapped (see Fig. 4.2).

Table 4.1 provides the list of all, in this study, confidently identified proteins, in saliva (Table 1a), and in tears (Table 4.1b). The overlap between proteins in both fluids is depicted in Figure 2. The table is arbitrarily limited to protein sequences with a tryptic peptide coverage of >20%. The full list of proteins the identifications of which were matching the confidence criteria outlined in the Materials and methods section is provided as Supplemental Data in Section 4.2.

Gene Identifier / Protein Name	Sequence coverage (%)
S100A8 / Protein S100-A8	77.40
CST4 / Cystatin-S precursor	71.60
AMY1A; AMY1C; AMY2A; AMY1B / Alpha-amylase 1 precursor	71.20
PIP / Prolactin-inducible protein precursor	69.90
LOC728320 / similar to lactotransferrin	69.70
LTF / Lactotransferrin precursor	69.60
ALB / Isoform 1 of Serum albumin precursor	67.70
CSTA / Cystatin-A	67.30
S100A9 / Protein S100-A9	66.70
ALB / Uncharacterized protein ALB	66.20
SMR3B / Submaxillary gland androgen-regulated protein 3 homolog B precursor	65.80
ANXA1 / Annexin A1	62.10
CST2 / Cystatin-SA precursor	61.00
CST1 / Cystatin-SN precursor	60.30
HIST2H2AA3; HIST2H2AA4 / Histone H2A type 2-A	57.70
AZGP1 / alpha-2-glycoprotein 1, zinc	57.40
LYZ / Lysozyme C precursor	56.10
IGJ / immunoglobulin J chain	55.30
IGHA1 / IGHA1 protein	53.80
LOC124220 / Protein UNQ773/PRO1567 precursor	52.90
HIST1H4A; HIST1H4K; HIST1H4J; HIST1H4D; HIST1H4E; HIST1H4H; HIST1H4C; HIST4H4; HIST1H4I; HIST1H4F; HIST2H4B; HIST1H4B; HIST2H4A; HIST1H4L / Histone H4	52.40
IGHA2 / IGHA2 protein	50.10
ACTB / Actin, cytoplasmic 1	49.60
KLK1 / Kallikrein-1 precursor	49.60
- / CDNA FLJ41981 fis, clone SMINT2011888, highly similar to Protein Tro alpha1 H, myeloma	49.50
CST5 / Cystatin-D precursor	49.30
LCN1 / Lipocalin-1 precursor	48.90
KRT9 / Keratin, type I cytoskeletal 9	47.70
CSTB / Cystatin-B	45.90
KRT1 / Keratin, type II cytoskeletal 1	44.90
LCN2 / Neutrophil gelatinase-associated lipocalin precursor	44.40
PIGR / Polymeric immunoglobulin receptor precursor	44.20
IGKV2-24 / IGKV2-24 protein	41.00
TF / Serotransferrin precursor	41.00
IGKV1-5 / IGKV1-5 protein	40.80
IGKV1-5 / IGKV1-5 protein	40.00
SCGB2A1 / Mammaglobin-B precursor	40.00
IGHA2 / Uncharacterized protein IGHA2 (Fragment)	39.90
KRT13 / Isoform 1 of Keratin, type I cytoskeletal 13	39.30
FABP5; FABP5L7 / Fatty acid-binding protein, epidermal	38.50
KRT6A / Keratin, type II cytoskeletal 6A	37.80
PFN1 / Profilin-1	37.10
APOA1 / Apolipoprotein A-I precursor	36.30
B2M / Beta-2-microglobulin	36.30
PPIA; PPIAL3; LOC654188 / Peptidyl-prolyl cis-trans isomerase A	35.80
C20orf70 / Short palate, lung and nasal epithelium carcinoma-associated protein 2 precursor	35.70
C6orf58 / Uncharacterized protein C6orf58 precursor	35.20

CTSG / Cathepsin G precursor	34.90
KRT16 / Keratin, type I cytoskeletal 16	34.90
KRT6B / Keratin, type II cytoskeletal 6B	34.80
HP / Haptoglobin precursor	33.80
CFL1 / Cofilin-1	33.70
KRT4 / keratin 4	32.50
ANXA3 / Annexin A3	32.50
IGHM / IGHM protein	32.40
CST3 / Cystatin-C precursor	31.50
SERPINA1 / Isoform 1 of Alpha-1-antitrypsin precursor	30.90
GSTP1 / Glutathione S-transferase P	30.50
- / Putative uncharacterized protein	29.70
SAA2; SAA1 / Serum amyloid A protein precursor	29.50
GAPDH / Glyceraldehyde-3-phosphate dehydrogenase	29.00
IGHG1 / IGHG1 protein	28.70
KRT10 / Keratin, type I cytoskeletal 10	28.00
TTR / Transthyretin precursor	27.90
MPO / Isoform H17 of Myeloperoxidase precursor	27.00
HIST1H2BL / Histone H2B type 1-L	27.00
LPO / Lactoperoxidase precursor	26.70
ANXA2 / annexin A2 isoform 1	25.80
KRT2 / Keratin, type II cytoskeletal 2 epidermal	25.70
- / Ig heavy chain V-III region BRO	25.00
CRISP3 / Cysteine-rich secretory protein 3 precursor	24.90
- / Ig kappa chain V-III region SIE	24.80
MYL6 / Isoform Non-muscle of Myosin light polypeptide 6	24.50
CA6 / Carbonic anhydrase 6 precursor	24.40
IGL@ / IGL@ protein	24.40
- / Putative uncharacterized protein	24.30
MGP / Matrix Gla protein precursor	23.30
ACTA1 / Actin, alpha skeletal muscle	23.10
DBI / Isoform a 1 of Acyl-CoA-binding protein	23.00
- / Rheumatoid factor D5 light chain (Fragment)	22.90
LACRT / Extracellular glycoprotein lacritin precursor	22.50
CALM3; CALM1; CALM2 / Calmodulin	22.10
KRT14 / Keratin, type I cytoskeletal 14	22.00
PRR4; PRH1; PRH2; PRB4 / Proline-rich protein 4 precursor	21.60
TCN1 / Transcobalamin-1 precursor	20.10

Table 4.1 a. Proteins identified in saliva (with a sequence coverage of >20%)

Gene Identifier / Protein Name	Sequence coverage (%)
LTF / Lactotransferrin precursor	80.40
LOC728320 / similar to lactotransferrin	78.70
IGHA1 / IGHA1 protein	53.30
HSPG2 / Basement membrane-specific heparan sulfate proteoglycan core protein precursor	21.90
IGHA1 / IGHA1 protein	50.60
IGHA1 / IGHA1 protein	51.30
IGHA1 / Putative uncharacterized protein DKFZp686J11235 (Fragment)	52.60
ALB / Isoform 1 of Serum albumin precursor	59.40
PIGR / Polymeric immunoglobulin receptor precursor	37.60
KRT1 / Keratin, type II cytoskeletal 1	46.20

IGHA2 / IGHA2 protein	46.80
KRT1 / Keratin, type II cytoskeletal 1	39.30
LYZ / Lysozyme C precursor	56.80
IGKC / IGKC protein	43.00
IGKV1-5 / IGKV1-5 protein	40.80
IGKV1-5 / IGKV1-5 protein	40.00
KRT10 / Keratin, type I cytoskeletal 10	45.00
LCN1 / Lipocalin-1 precursor	56.30
KRT9 / Keratin, type I cytoskeletal 9	48.60
C3 / Complement C3 precursor (Fragment)	21.50
SCGB2A1 / Mammaglobin-B precursor	58.90
IGHM / Putative uncharacterized protein DKFZp686I15196	38.20
IGHG1 / Putative uncharacterized protein DKFZp686P15220	39.40
CP / Ceruloplasmin precursor	21.60
HP / HP protein	38.30
PRR4; PRH1; PRH2; PRB4 / Proline-rich protein 4 precursor	32.10
AZGP1 / alpha-2-glycoprotein 1, zinc	46.00
IGHA2 / Uncharacterized protein IGHA2 (Fragment)	35.80
PIP / Prolactin-inducible protein precursor	69.90
KRT13 / keratin 13 isoform b	39.00
FGG / Isoform Gamma-B of Fibrinogen gamma chain precursor	34.90
FGB / Fibrinogen beta chain precursor	42.40
KRT2 / Keratin, type II cytoskeletal 2 epidermal	22.60
- / Putative uncharacterized protein	33.10
CST4 / Cystatin-S precursor	66.00
KRT14 / Keratin, type I cytoskeletal 14	33.10
KRT5 / Keratin, type II cytoskeletal 5	23.40
TF / Serotransferrin precursor	29.20
LOC124220 / Protein UNQ773/PRO1567 precursor	22.10
KRT6B / Keratin, type II cytoskeletal 6B	23.20
APOA1 / Apolipoprotein A-I precursor	46.80
KRT16 / Keratin, type I cytoskeletal 16	25.60
IGHM / IGHM protein	26.10
IGJ / immunoglobulin J chain	27.70
- / Anti-(ED-B) scFV (Fragment)	23.10
PLA2G2A / Phospholipase A2, membrane associated precursor	30.60
SCGB1D1 / Secretoglobin family 1D member 1 precursor	23.30
IGHM / IGHM protein	24.80
- / Ig kappa chain V-III region SIE	39.40
S100A8 / Protein S100-A8	53.80
GAPDH / Glyceraldehyde-3-phosphate dehydrogenase	27.80
IGHG2 / Putative uncharacterized protein DKFZp686I04196 (Fragment)	25.40
LCN2 / Neutrophil gelatinase-associated lipocalin precursor	31.30
LACRT / Extracellular glycoprotein lacritin precursor	22.50
CST2 / Cystatin-SA precursor	29.80
CST1 / Cystatin-SN precursor	38.30
ACTB / Actin, cytoplasmic 1	21.90
- / Ig heavy chain V-III region BRO	25.00
S100A9 / Protein S100-A9	26.30
CTSD / Cathepsin D precursor	20.40

Table 4.1 b. Proteins identified in tears (with a sequence coverage of >20%)

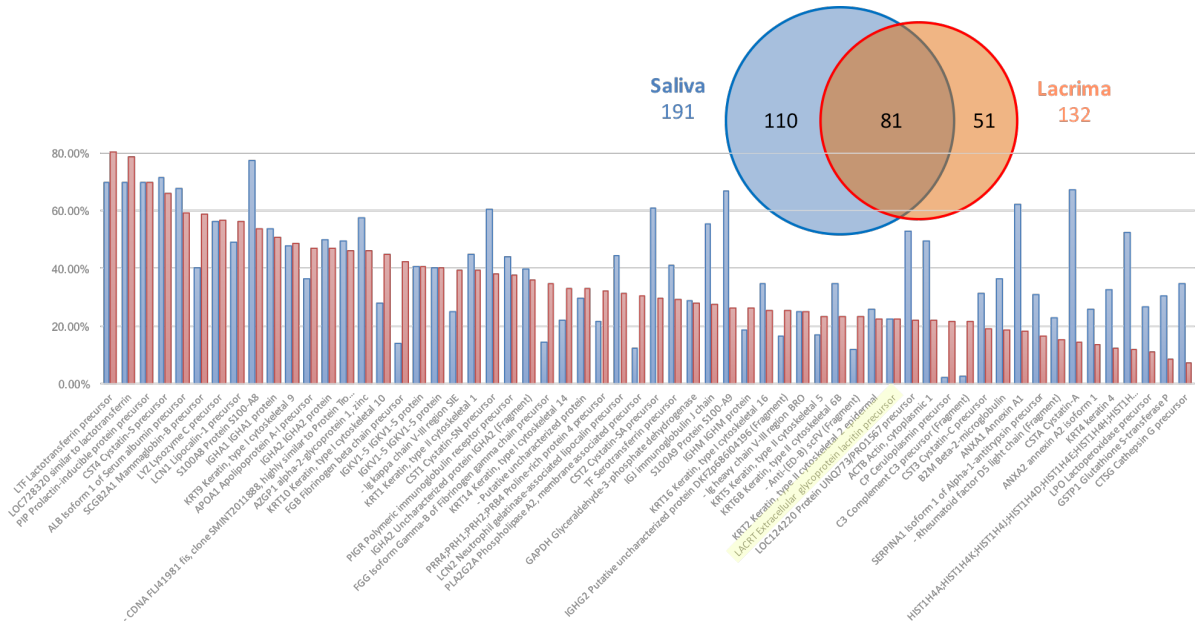


Fig. 4.2. Proteins identified both in saliva and tears (with a sequence coverage of >20% in, at least, one of the samples). Y-axis indicates protein sequence coverage (with lacritin highlighted in pale yellow). Venn diagram shows total numbers of all confidently identified proteins in both samples.

We acknowledge that today (end of 2017) the numbers of confident protein identifications achieved with this workflow may seem rather low, especially when compared to cutting-edge proteomic study reports. However, we believe that the data we acquired over a decade ago (in the course of 2006) are still highly valid. Actually, none of the protein identifications we here report have not been reported in more recent saliva, respectively tear protein studies published in the last years (see references below). It was indeed not unexpected that, with the employment of more sensitive mass spectrometers, more proteins were to be identified, as less and less abundant species would reach the detection limit. Our study was well in line with the benchmark study by Li and coworkers [2015], who did a technological comparison of the state-of-the-art proteomic analyses of the time showing that a mere total of 54 proteins could be confidently identified using less than 5 µl of tear fluid. Still today, our GeLC MS/MS approach is being used for discovery-type differential proteome studies [Perumal et al., 2016].
The salivary protein list nicely matches published lists [Xie et al., 2005], with amylase not unexpectedly being on top of the list of 110 proteins uniquely identified in saliva compared to tears.

Of the 81 proteins which we found to be shared between tears and saliva, our attention was particularly triggered by the appearance in the labial saliva of extracellular glycoprotein lacritin (LACRT_HUMAN, IPI 00020487; NX_Q9GZZ8).
Lacritin is a 12.3 kDa glycoprotein known as one of the abundant proteins secreted in tears (hence its name, derived from the Latin for tear *lacrima*). One of its prominent roles is to positively modulate the secretion of gland cells, in all three tear gland types around the human eye [Samudre et al., 2011]. This includes active stimulation of the main (aqueous) component of the tear by the major and accessory lacrimal glands, the mucus secretion by

epithelial cells and goblet cells of the conjunctiva, as well as the lipid secretion by the Meibomian glands.
The immunopositive results with anti-lacritin obtained in immunohistochemistry of the DED patient minor salivary gland (Fig. 4.3) validate our MS/MS identifications.

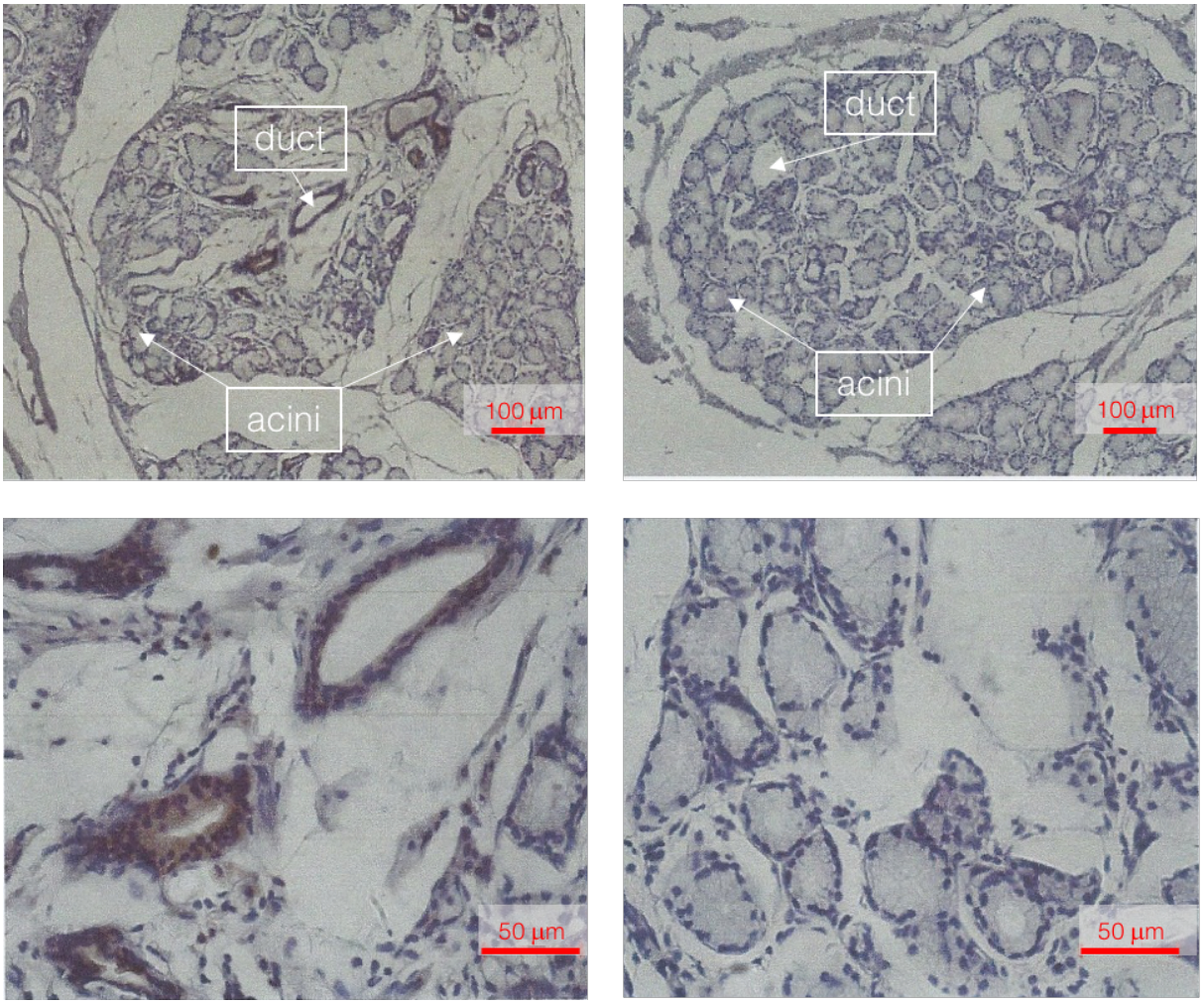


Fig.4.3 Photomicrographs (two different magnifications) of formalin-fixed paraffin embedded histological sections of human minor salivary gland labeled with rabbit anti-human lacritin immunoperoxidase (diaminobenzidine) staining. Cell nuclei were counterstained with hematoxylin. Left panel shows lacritin immunohistochemistry, whereas right panel was treated with non-immune rabbit serum as primary antibody (negative control). Lacritin immunoreactivity is primarily evident in (larger) secretory duct epithelia.

Lacritin immunopositive labeling is particularly present at the level of the epithelium of the large salivary gland ducts. In the negative control (without specific primary antibody), the tissue remains entirely negative. Comparable data have been described by the group of Laurie, who reported immunoreactive lacritin in acini of parotis and submandibular salivary glands [Karnati et al., 2013].
Our data thus suggest that labial salivary gland transplantation into a dry eye effectively represents the translocation of an active lacritin-secreting tissue at the site of the diseased tear gland. The prosecretory properties of lacritin, which is known to stimulate lacrimal gland secretion of both the aquaeous, mucous as well as lipid components of the tear fluid, may well (partly) contribute to the positive effects observed with this treatment. As a matter of fact, studies by the research group of Prof. Laurie [Samudre et al, 2011] suggest for certain

forms of DED in which lacritin seems selectively downregulated (including Sjögren's disease), a replacement therapy in which (topically applied) lacritin is administered into the diseased eye(s) [Vijmasi et al., 2014]. In this context, we want to refer to the ongoing clinical trial of LACRIPEP™, in which a specific bioactive fragment of lacritin is added to artificial tears as DED treatment [Basha, 2017].
Indeed, for such subpopulation of DED patients, the success of minor salivary gland transplantation may be attributed to the secretion of salivary gland lacritin onto the surface of the treated eye.

4.1.5 Conclusion

A relatively primitive bottom-up GeLC MS/MS workflow yields a limited but relevant set of proteins allowing a basic comparison of the protein composition of human saliva (191 identified proteins) and tears (132 identified proteins) of the same individual. The large overlap between both samples confirms earlier literature data that the composition of tears and saliva is very similar.
We predict that future more in-depth proteome (as well as peptidome) analyses will undoubtedly yield highly valuable biochemical information on the natural composition of the healthy (as well as the diseased) tear, which may ultimately lead to even more elegant (and personalized) treatment of patients suffering from DED. These may include innovative peptide and/or protein additives to artificial tears.

4.1.6 Acknowledgements

We are indebted to the Netherlands Proteomics Centre (NPC) for financial support to the Delft NPC Analytical Hotel.

4.1.7 References:

Basha AA (2017) Lacripep for tears of joy. EC Ophthalmol. 8: 125-126.

Conrady CD, Joos ZP, Patel BC (2016) Review: The lacrimal gland and its role in dry eye. J. Ophthalmol. 2016: 7542929; doi: 10.1155/2016/7542929.

Geerling G, Daniels JT, Dart JKG, Cree IA, Khaw PT (2001) Toxicity of natural tear substitutes in a fully defined culture model of human corneal epithelial cells. Invest. Ophthalmol. Vis. Sci. 42: 948-956.

Geerling G, Raus P, Murube J (2008) Minor salivary gland transplantation. Dev. Ophthalmol. 41: 243-254.

Geerling G, Garrett JR, Paterson KL, Sieg P, Collin JRO, Carpenter GH, Hakim SG, Lauer I, Proctor, GB (2008) Innervation and secretory function of transplanted human submandibular salivary glands. Transplantation, 85: 135-140.

Thevelein JM, Bonini BM, Castermans D, Haesendonckx S, Kriel J, Louwet W, Thayumanavan P, Popova Y, Rubio-Texeira M, Schepers W, Vandormael P, Van Zeebroeck G, Verhaert, P, Versele M, Voordeckers K (2008) Novel mechanisms in nutrient activation of the yeast protein kinase A pathway. Acta Microbiol. Immunol. Hung. 55: 75-89.

Xie H, Rhodu NL, Griffin RJ, Carlis JV, Griffin TJ (2005) A catalogue of human saliva proteins identified bt free flow electrophoresis-based peptide separation and tandem mass spectrometry. Mol. Cell. Proteomics 4: 1826-1830.

Karnati R, Laurie DE, Laurie GW (2013) Lacritin and the tear proteome as natural replacement therapy for dry eye. Exp. Eye Res. 117: 39-52.

Li N, Wang N, Zheng J, Liu XM, Lever OW, Erickson PM, Li L (2005) Characterization of human tear proteome using multiple proteomic analysis techniques. J. Proteome Res. 4: 2052-2061.

Murube J (1998) Labial salivary gland transplantation in severe dry eye. Oculoplast. Orbital Reconstr. Surg. 1: 104-110.

Pennec YL, Leroy JP, Jouquan J, Lelong A, Katsikis P, Youinou P (1990) Comparison of labial and sublingual salivary gland biopsies in the diagnosis of Sjögren's syndrome. Ann. Rheum. Dis. 49:37-39.

Perumal N, Funke S, Pfeiffer N, Grus FH (2016) Proteomics analysis of human tears from aqueous-deficient and evaporative dry eye patients. Scientific Reports. 6: 29629 DOI: 10.1038/srep29629.

Piersma SR, Warmoes MO, Wit M, Reus I, Knol JC, Jiménez CR (2013) Whole gel processing procedure for GeLC-MS/MS based proteomics. Proteome Sci. 11: 1-8.

Samudre S, Lattanzio FA, Lossen V, Hosseini A, Sheppard JD, Mc Cown RL, Laurie GW, Williams PB (2011) Lacritin, a novel human tear glycoprotein, promotes sustained basal tearing and is well tolerated. Invest. Ophthalmol. Vis. Sci. 52: 6265–6270.

Sanghi S, Kumar R, Lumsden A, Dickinson D, Klepeis V, Trinkaus-Randall V, Friersor HJ Jr., Laurie GW (2001) cDNA and genomic cloning of lacritin, a novel secretion enhancing factor from the human lacrimal gland. J. Mol. Biol. 310: 127-139.

Stevenson W, Chauhan SK, Dana R (2013) Dry eye disease: an immune-mediated ocular surface disorder. Arch. Ophthalmol. 130: 90-100.

Vijmasi T, Chen FYT, Balasubbu S, Gallup M, McKown RL, Laurie GW (2014) Topical administration of lacritin Is a novel therapy for aqueous-deficient dry eye disease. Invest. Ophthalmol. Vis. Sci. 55: 5401-5409.

4.2. Supplemental data: list of all confidently identified proteins from section 4.1.

4.2.1 Proteins identified in human tear sample.

Origin	prot_hit_	prot_acc	prot_desc	prot_score	prot_mass	prot_matches	prot_cover
Lacrima	1	IPI00848342	Tax_Id=9606 Gene_Symbol=LTF Lactotransferrin precursor	8520	80014	233	80,4
Lacrima	2	IPI00788271	Tax_Id=9606 Gene_Symbol=LOC728320 similar to lactotransferrin	6333	57494	167	78,7
Lacrima	3	IPI00166866	Tax_Id=9606 Gene_Symbol=IGHA1 IGHA1 protein	2931	54483	67	53,3
Lacrima	4	IPI00024284	Tax_Id=9606 Gene_Symbol=HSPG2 Basement membrane-specific heparan sulfate proteoglycan core protein precursor	2788	479248	67	21,9
Lacrima	5	IPI00744561	Tax_Id=9606 Gene_Symbol=IGHA1 IGHA1 protein	2784	54327	64	50,6
Lacrima	6	IPI00430842	Tax_Id=9606 Gene_Symbol=IGHA1 IGHA1 protein	2782	53858	64	51,3
Lacrima	7	IPI00426060	Tax_Id=9606 Gene_Symbol=IGHA1 Putative uncharacterized protein DKFZp686J11235 (Fragment)	2757	55394	63	52,6
Lacrima	8	IPI00745872	Tax_Id=9606 Gene_Symbol=ALB Isoform 1 of Serum albumin precursor	2580	71317	71	59,4
Lacrima	9	IPI00004573	Tax_Id=9606 Gene_Symbol=PIGR Polymeric immunoglobulin receptor precursor	2224	84429	62	37,6
Lacrima	10	IPI00784830	Tax_Id=9606 Gene_Symbol=- CDNA FLJ41981 fis, clone SMINT2011888, highly similar to Protein Tro alpha1 H,myeloma	2189	53810	51	46,2
Lacrima	11	IPI00783993	Tax_Id=9606 Gene_Symbol=IGHA2 IGHA2 protein	2134	52511	49	46,8
Lacrima	12	IPI00220327	Tax_Id=9606 Gene_Symbol=KRT1 Keratin, type II cytoskeletal 1	2019	66149	56	39,3
Lacrima	13	IPI00019038	Tax_Id=9606 Gene_Symbol=LYZ Lysozyme C precursor	1978	16982	54	56,8
Lacrima	14	IPI00430808	Tax_Id=9606 Gene_Symbol=IGKC IGKC protein	1803	25915	43	43
Lacrima	15	IPI00419424	Tax_Id=9606 Gene_Symbol=IGKV1-5 IGKV1-5 protein	1791	26503	43	40,8
Lacrima	16	IPI00430820	Tax_Id=9606 Gene_Symbol=IGKV1-5 IGKV1-5 protein	1683	26034	43	40
Lacrima	17	IPI00009865	Tax_Id=9606 Gene_Symbol=KRT10 Keratin, type I cytoskeletal 10	1581	59703	44	45
Lacrima	18	IPI00009650	Tax_Id=9606 Gene_Symbol=LCN1 Lipocalin-1 precursor	1486	19409	65	56,3
Lacrima	19	IPI00019359	Tax_Id=9606 Gene_Symbol=KRT9 Keratin, type I cytoskeletal 9	1263	62320	29	48,6
Lacrima	20	IPI00783987	Tax_Id=9606 Gene_Symbol=C3 Complement C3 precursor (Fragment)	1128	188569	35	21,5
Lacrima	21	IPI00418512	Tax_Id=9606 Gene_Symbol=DMBT1 Isoform 4 of Deleted in malignant brain tumors 1 protein precursor	1092	170672	31	18,9
Lacrima	22	IPI00026126	Tax_Id=9606 Gene_Symbol=SCGB2A1 Mammaglobin-B precursor	1039	11104	36	58,9
Lacrima	23	IPI00816314	Tax_Id=9606 Gene_Symbol=IGHM Putative uncharacterized protein DKFZp686I15196	1031	51579	26	38,2
Lacrima	24	IPI00645363	Tax_Id=9606 Gene_Symbol=IGHG1 Putative uncharacterized protein DKFZp686P15220	1011	52376	23	39,4
Lacrima	25	IPI00017601	Tax_Id=9606 Gene_Symbol=CP Ceruloplasmin precursor	860	122983	19	21,6
Lacrima	26	IPI00478493	Tax_Id=9606 Gene_Symbol=HP HP protein	813	38940	20	38,3
Lacrima	27	IPI00027019	Tax_Id=9606 Gene_Symbol=PRR4;PRH1;PRH2;PRB4 Proline-rich protein 4 precursor	811	15116	22	32,1
Lacrima	28	IPI00166729	Tax_Id=9606 Gene_Symbol=AZGP1 alpha-2-glycoprotein 1, zinc	803	34465	25	46
Lacrima	29	IPI00829711	Tax_Id=9606 Gene_Symbol=IGHA2 Uncharacterized protein IGHA2 (Fragment)	792	43202	23	35,8
Lacrima	30	IPI00022974	Tax_Id=9606 Gene_Symbol=PIP Prolactin-inducible protein precursor	779	16847	29	69,9
Lacrima	31	IPI00171196	Tax_Id=9606 Gene_Symbol=KRT13 keratin 13 isoform b	661	46181	19	39
Lacrima	32	IPI00021891	Tax_Id=9606 Gene_Symbol=FGG Isoform Gamma-B of Fibrinogen gamma chain precursor	644	52106	18	34,9
Lacrima	33	IPI00298497	Tax_Id=9606 Gene_Symbol=FGB Fibrinogen beta chain precursor	626	56577	18	42,4
Lacrima	34	IPI00021304	Tax_Id=9606 Gene_Symbol=KRT2 Keratin, type II cytoskeletal 2 epidermal	611	66110	20	22,6
Lacrima	35	IPI00785200	Tax_Id=9606 Gene_Symbol=- Putative uncharacterized protein	569	25350	20	33,1
Lacrima	36	IPI00412044	Tax_Id=9606 Gene_Symbol=DMBT1 143 kDa protein	534	147875	13	10,5
Lacrima	37	IPI00032294	Tax_Id=9606 Gene_Symbol=CST4 Cystatin-S precursor	513	16489	16	66
Lacrima	38	IPI00168728	Tax_Id=9606 Gene_Symbol=IGHM FLJ00385 protein (Fragment)	511	57272	11	18,3
Lacrima	39	IPI00384444	Tax_Id=9606 Gene_Symbol=KRT14 Keratin, type I cytoskeletal 14	490	51875	13	33,1
Lacrima	40	IPI00009867	Tax_Id=9606 Gene_Symbol=KRT5 Keratin, type II cytoskeletal 5	473	62568	17	23,4
Lacrima	41	IPI00022463	Tax_Id=9606 Gene_Symbol=TF Serotransferrin precursor	455	79280	13	29,2
Lacrima	42	IPI00021885	Tax_Id=9606 Gene_Symbol=FGA Isoform 1 of Fibrinogen alpha chain precursor	450	95656	12	13,9
Lacrima	43	IPI00060800	Tax_Id=9606 Gene_Symbol=LOC124220 Protein UNQ773/PRO1567 precursor	447	22725	7	22,1
Lacrima	44	IPI00293665	Tax_Id=9606 Gene_Symbol=KRT6B Keratin, type II cytoskeletal 6B	443	60247	17	23,2
Lacrima	45	IPI00550640	Tax_Id=9606 Gene_Symbol=IGHG4 IGHG4 protein	427	52637	8	17,8
Lacrima	46	IPI00021841	Tax_Id=9606 Gene_Symbol=APOA1 Apolipoprotein A-I precursor	413	30759	14	46,8
Lacrima	47	IPI00103397	Tax_Id=9606 Gene_Symbol=MUC5AC Mucin-5AC precursor (Fragment)	386	543092	13	3,1
Lacrima	48	IPI00217963	Tax_Id=9606 Gene_Symbol=KRT16 Keratin, type I cytoskeletal 16	385	51578	10	25,6
Lacrima	49	IPI00549291	Tax_Id=9606 Gene_Symbol=IGHM IGHM protein	348	66998	14	26,1
Lacrima	50	IPI00178926	Tax_Id=9606 Gene_Symbol=IGJ immunoglobulin J chain	333	18543	8	27,7
Lacrima	51	IPI00290078	Tax_Id=9606 Gene_Symbol=KRT4 keratin 4	327	64442	9	12,3
Lacrima	52	IPI00478003	Tax_Id=9606 Gene_Symbol=A2M Alpha-2-macroglobulin precursor	326	164600	12	10,4
Lacrima	53	IPI00418163	Tax_Id=9606 Gene_Symbol=C4B C4B1	316	194170	10	9,9
Lacrima	54	IPI00828062	Tax_Id=9606 Gene_Symbol=- Anti-(ED-B) scFV (Fragment)	312	25357	8	23,1
Lacrima	55	IPI00003469	Tax_Id=9606 Gene_Symbol=- Ig kappa chain V-I region WEA	311	11947	6	16,7
Lacrima	56	IPI00026962	Tax_Id=9606 Gene_Symbol=PLA2G2A Phospholipase A2, membrane associated precursor	309	16870	6	30,6
Lacrima	57	IPI00001468	Tax_Id=9606 Gene_Symbol=SCGB1D1 Secretoglobin family 1D member 1 precursor	309	10234	10	23,3
Lacrima	58	IPI00829768	Tax_Id=9606 Gene_Symbol=IGHM IGHM protein	308	68823	13	24,8
Lacrima	59	IPI00387115	Tax_Id=9606 Gene_Symbol=- Ig kappa chain V-III region SIE	304	11882	7	39,4
Lacrima	60	IPI00007047	Tax_Id=9606 Gene_Symbol=S100A8 Protein S100-A8	303	10885	8	53,8
Lacrima	61	IPI00219018	Tax_Id=9606 Gene_Symbol=GAPDH Glyceraldehyde-3-phosphate dehydrogenase	288	36201	8	27,8
Lacrima	62	IPI00399007	Tax_Id=9606 Gene_Symbol=IGHG2 Putative uncharacterized protein DKFZp686I04196 (Fragment)	285	46716	10	25,4
Lacrima	63	IPI00299547	Tax_Id=9606 Gene_Symbol=LCN2 Neutrophil gelatinase-associated lipocalin precursor	282	22745	8	31,3
Lacrima	64	IPI00020487	Tax_Id=9606 Gene_Symbol=LACRT Extracellular glycoprotein lacritin precursor	259	14237	9	22,5
Lacrima	65	IPI00291262	Tax_Id=9606 Gene_Symbol=CLU Clusterin precursor	255	53031	8	12
Lacrima	66	IPI00013382	Tax_Id=9606 Gene_Symbol=CST2 Cystatin-SA precursor	247	16719	5	29,8
Lacrima	67	IPI00305477	Tax_Id=9606 Gene_Symbol=CST1 Cystatin-SN precursor	238	16579	6	38,3
Lacrima	68	IPI00218918	Tax_Id=9606 Gene_Symbol=ANXA1 Annexin A1	236	38918	5	18,2
Lacrima	69	IPI00553177	Tax_Id=9606 Gene_Symbol=SERPINA1 Isoform 1 of Alpha-1-antitrypsin precursor	231	46878	6	16,7
Lacrima	70	IPI00021439	Tax_Id=9606 Gene_Symbol=ACTB Actin, cytoplasmic 1	230	42052	6	21,9
Lacrima	71	IPI00450768	Tax_Id=9606 Gene_Symbol=KRT17 Keratin, type I cytoskeletal 17	224	48361	5	11,3
Lacrima	72	IPI00382480	Tax_Id=9606 Gene_Symbol=- Ig heavy chain V-III region BRO	205	13332	6	25
Lacrima	73	IPI00749245	Tax_Id=9606 Gene_Symbol=SFRP1 Secreted frizzled-related protein 1 precursor	199	36274	3	15,3
Lacrima	74	IPI00027462	Tax_Id=9606 Gene_Symbol=S100A9 Protein S100-A9	193	13291	4	26,3
Lacrima	75	IPI00032293	Tax_Id=9606 Gene_Symbol=CST3 Cystatin-C precursor	185	16017	3	19,2
Lacrima	76	IPI00479145	Tax_Id=9606 Gene_Symbol=KRT19 Keratin, type I cytoskeletal 19	181	44065	5	12,5
Lacrima	77	IPI00783024	Tax_Id=9606 Gene_Symbol=- Myosin-reactive immunoglobulin heavy chain variable region (Fragment)	169	14247	4	19,8
Lacrima	78	IPI00025023	Tax_Id=9606 Gene_Symbol=LPO Lactoperoxidase precursor	167	81149	6	11
Lacrima	79	IPI00816799	Tax_Id=9606 Gene_Symbol=- Rheumatoid factor D5 light chain (Fragment)	162	12872	4	15,3
Lacrima	80	IPI00418169	Tax_Id=9606 Gene_Symbol=ANXA2 annexin A2 isoform 1	150	40671	4	13,7
Lacrima	81	IPI00023673	Tax_Id=9606 Gene_Symbol=LGALS3BP Galectin-3-binding protein precursor	146	66202	5	12,1
Lacrima	82	IPI00011229	Tax_Id=9606 Gene_Symbol=CTSD Cathepsin D precursor	144	45037	6	20,4
Lacrima	83	IPI00179330	Tax_Id=9606 Gene_Symbol=RPS27A;UBC;UBB ubiquitin and ribosomal protein S27a precursor	138	18296	3	16
Lacrima	84	IPI00022488	Tax_Id=9606 Gene_Symbol=HPX Hemopexin precursor	138	52385	3	12,1
Lacrima	85	IPI00009682	Tax_Id=9606 Gene_Symbol=PROL1 Proline-rich protein 1 precursor	121	22970	3	18,9
Lacrima	86	IPI00025512	Tax_Id=9606 Gene_Symbol=HSPB1 Heat shock protein beta-1	106	22826	2	12,7
Lacrima	87	IPI00217778	Tax_Id=9606 Gene_Symbol=PLTP Isoform 2 of Phospholipid transfer protein precursor	104	49412	3	3,4
Lacrima	88	IPI00026314	Tax_Id=9606 Gene_Symbol=GSN Isoform 1 of Gelsolin precursor	103	86043	2	7
Lacrima	89	IPI00004656	Tax_Id=9606 Gene_Symbol=B2M Beta-2-microglobulin	99	14521	2	18,5
Lacrima	90	IPI00022429	Tax_Id=9606 Gene_Symbol=ORM1 Alpha-1-acid glycoprotein 1 precursor	95	23725	1	7,5

Lacrima	91	IPI00000874	Tax_Id=9606 Gene_Symbol=PRDX1 Peroxiredoxin-1	90	22324	2	9,5
Lacrima	92	IPI00296777	Tax_Id=9606 Gene_Symbol=SPARCL1 SPARC-like protein 1 precursor	87	76025	1	3,2
Lacrima	93	IPI00022752	Tax_Id=9606 Gene_Symbol=AQP5 Aquaporin-5	85	42399	1	4
Lacrima	94	IPI00022204	Tax_Id=9606 Gene_Symbol=SERPINB3 Serpin B3	83	44594	3	10,8
Lacrima	95	IPI00006662	Tax_Id=9606 Gene_Symbol=APOD Apolipoprotein D precursor	82	21547	3	19
Lacrima	96	IPI00024138	Tax_Id=9606 Gene_Symbol=- Uncharacterized protein ENSP00000374816	81	14171	2	7
Lacrima	97	IPI00298547	Tax_Id=9606 Gene_Symbol=PARK7 Protein DJ-1	79	20050	1	8,5
Lacrima	98	IPI00387120	Tax_Id=9606 Gene_Symbol=- Ig kappa chain V-IV region Len	78	12746	2	15,8
Lacrima	99	IPI00032325	Tax_Id=9606 Gene_Symbol=CSTA Cystatin-A	77	11000	1	14,3
Lacrima	100	IPI00219757	Tax_Id=9606 Gene_Symbol=GSTP1 Glutathione S-transferase P	75	23569	1	8,6
Lacrima	101	IPI00465028	Tax_Id=9606 Gene_Symbol=TPI1 Isoform 1 of Triosephosphate isomerase	73	31057	1	4,5
Lacrima	102	IPI00219806	Tax_Id=9606 Gene_Symbol=S100A7 Protein S100-A7	72	11564	1	18,8
Lacrima	103	IPI00295741	Tax_Id=9606 Gene_Symbol=CTSB Cathepsin B precursor	72	38766	2	5
Lacrima	104	IPI00029739	Tax_Id=9606 Gene_Symbol=CFH Isoform 1 of Complement factor H precursor	69	143680	2	2,8
Lacrima	105	IPI00018230	Tax_Id=9606 Gene_Symbol=SMR3A Submaxillary gland androgen-regulated protein 3 homolog A precursor	67	14165	1	11,2
Lacrima	106	IPI00297900	Tax_Id=9606 Gene_Symbol=DDX10 Probable ATP-dependent RNA helicase DDX10	60	101168	2	1
Lacrima	107	IPI00382440	Tax_Id=9606 Gene_Symbol=- Ig lambda chain V-IV region Hil	59	11624	1	17,8
Lacrima	108	IPI00171411	Tax_Id=9606 Gene_Symbol=GOLM1 Golgi phosphoprotein 2	59	46416	1	2,9
Lacrima	109	IPI00019533	Tax_Id=9606 Gene_Symbol=CHI3L2 Chitinase-3-like protein 2 precursor	58	43701	2	7,4
Lacrima	110	IPI00009792	Tax_Id=9606 Gene_Symbol=IGHV1OR15-1 Ig heavy chain V-I region V35 precursor	58	13114	1	10,3
Lacrima	111	IPI00008603	Tax_Id=9606 Gene_Symbol=ACTA2 Actin, aortic smooth muscle	57	42381	2	7,2
Lacrima	112	IPI00382436	Tax_Id=9606 Gene_Symbol=- Ig lambda chain V-III region SH	56	11500	1	16,7
Lacrima	113	IPI00152442	Tax_Id=9606 Gene_Symbol=RNASET2 Extra-cellular ribonuclease	54	27049	1	5,8
Lacrima	114	IPI00304273	Tax_Id=9606 Gene_Symbol=APOA4 Apolipoprotein A-IV precursor	52	45371	1	2,3
Lacrima	115	IPI00013885	Tax_Id=9606 Gene_Symbol=CASP14 Caspase-14 precursor	51	27947	1	4,5
Lacrima	116	IPI00028064	Tax_Id=9606 Gene_Symbol=CTSG Cathepsin G precursor	49	29161	1	7,1
Lacrima	117	IPI00003470	Tax_Id=9606 Gene_Symbol=- Ig kappa chain V-I region Wes	48	11715	1	16,7
Lacrima	118	IPI00021997	Tax_Id=9606 Gene_Symbol=CREG1 Protein CREG1 precursor	47	24173	1	9,5
Lacrima	119	IPI00745363	Tax_Id=9606 Gene_Symbol=- Immunglobulin heavy chain variable region (Fragment)	46	14555	1	6,9
Lacrima	120	IPI00000816	Tax_Id=9606 Gene_Symbol=YWHAE 14-3-3 protein epsilon	44	29326	1	3,1
Lacrima	121	IPI00328587	Tax_Id=9606 Gene_Symbol=- Enolase	44	42657	1	1,8
Lacrima	122	IPI00453473	Tax_Id=9606 Gene_Symbol=HIST1H4A;HIST1H4K;HIST1H4J;HIST1H4D;HIST1H4E;HIST1H4H;HIST1H4C;HIST4H4;HIST1H4I;HIST1H	42	11360	1	11,7
Lacrima	123	IPI00398625	Tax_Id=9606 Gene_Symbol=HRNR Hornerin	39	283140	1	1
Lacrima	124	IPI00009123	Tax_Id=9606 Gene_Symbol=NUCB2 Nucleobindin-2 precursor	39	50305	1	2,1
Lacrima	125	IPI00761105	Tax_Id=9606 Gene_Symbol=MGC21874 transcriptional adaptor 2 (ADA2 homolog, yeast)-beta	37	49010	1	1,7
Lacrima	126	IPI00747459	Tax_Id=9606 Gene_Symbol=PDE6C Uncharacterized protein PDE6C	36	99939	1	0,9
Lacrima	127	IPI00027547	Tax_Id=9606 Gene_Symbol=DCD Dermcidin precursor	35	11391	1	10
Lacrima	128	IPI00028344	Tax_Id=9606 Gene_Symbol=BRSK2 Isoform 3 of BR serine/threonine-protein kinase 2	35	75321	1	1,2
Lacrima	129	IPI00008580	Tax_Id=9606 Gene_Symbol=SLPI Antileukoproteinase precursor	34	15228	1	9,1
Lacrima	130	IPI00424460	Tax_Id=9606 Gene_Symbol=ZFYVE16 Isoform 1 of Zinc finger FYVE domain-containing protein 16	32	171138	1	0,8
Lacrima	131	IPI00555812	Tax_Id=9606 Gene_Symbol=GC Vitamin D-binding protein precursor	31	54526	1	1,5
Lacrima	132	IPI00021855	Tax_Id=9606 Gene_Symbol=APOC1 Apolipoprotein C-I precursor	31	9326	1	10,8

4.2.2 Proteins identified in human saliva sample.

Origin	prot_hit_	prot_acc	prot_desc	prot_score	prot_mass	prot_matches	prot_cover
Saliva	1	IPI00300786	Tax_Id=9606 Gene_Symbol=AMY1A;AMY1C;AMY2A;AMY1B Alpha-amylase 1 precursor	6337	58415	143	71,2
Saliva	2	IPI00848342	Tax_Id=9606 Gene_Symbol=LTF Lactotransferrin precursor	5158	80014	140	69,6
Saliva	3	IPI00004573	Tax_Id=9606 Gene_Symbol=PIGR Polymeric immunoglobulin receptor precursor	4627	84429	123	44,2
Saliva	4	IPI00788271	Tax_Id=9606 Gene_Symbol=LOC728320 similar to lactotransferrin	3820	57494	94	69,7
Saliva	5	IPI00022434	Tax_Id=9606 Gene_Symbol=ALB Uncharacterized protein ALB	3632	73881	97	66,2
Saliva	6	IPI00745872	Tax_Id=9606 Gene_Symbol=ALB Isoform 1 of Serum albumin precursor	3596	71317	96	67,7
Saliva	7	IPI00218918	Tax_Id=9606 Gene_Symbol=ANXA1 Annexin A1	2973	38918	76	62,1
Saliva	8	IPI00419424	Tax_Id=9606 Gene_Symbol=IGKV1-5 IGKV1-5 protein	2773	26503	69	40,8
Saliva	9	IPI00744561	Tax_Id=9606 Gene_Symbol=IGHA1 IGHA1 protein	2674	54327	73	53,8
Saliva	10	IPI00440577	Tax_Id=9606 Gene_Symbol=IGKV2-24 IGKV2-24 protein	2583	26514	67	41
Saliva	11	IPI00430820	Tax_Id=9606 Gene_Symbol=IGKV1-5 IGKV1-5 protein	2551	26034	67	40
Saliva	12	IPI00783993	Tax_Id=9606 Gene_Symbol=IGHA2 IGHA2 protein	2433	52511	65	50,1
Saliva	13	IPI00784830	Tax_Id=9606 Gene_Symbol=- CDNA FLJ41981 fis, clone SMINT2011888, highly similar to Protein Tro alpha1 H,myeloma	2416	53810	64	49,5
Saliva	14	IPI00220327	Tax_Id=9606 Gene_Symbol=KRT1 Keratin, type II cytoskeletal 1	2256	66149	69	44,9
Saliva	15	IPI00021439	Tax_Id=9606 Gene_Symbol=ACTB Actin, cytoplasmic 1	2235	42052	63	49,6
Saliva	16	IPI00019359	Tax_Id=9606 Gene_Symbol=KRT9 Keratin, type I cytoskeletal 9	2207	62320	52	47,7
Saliva	17	IPI00060800	Tax_Id=9606 Gene_Symbol=LOC124220 Protein UNQ773/PRO1567 precursor	1915	22725	53	52,9
Saliva	18	IPI00166729	Tax_Id=9606 Gene_Symbol=AZGP1 alpha-2-glycoprotein 1, zinc	1879	34465	48	57,4
Saliva	19	IPI00022974	Tax_Id=9606 Gene_Symbol=PIP Prolactin-inducible protein precursor	1724	16847	60	69,9
Saliva	20	IPI00027462	Tax_Id=9606 Gene_Symbol=S100A9 Protein S100-A9	1569	13291	43	66,7
Saliva	21	IPI00855918	Tax_Id=9606 Gene_Symbol=MUC5B mucin 5, subtype B, tracheobronchial	1319	611987	34	8,1
Saliva	22	IPI00448925	Tax_Id=9606 Gene_Symbol=IGHG1 IGHG1 protein	1192	60862	34	28,7
Saliva	23	IPI00021428	Tax_Id=9606 Gene_Symbol=ACTA1 Actin, alpha skeletal muscle	1190	42366	31	23,1
Saliva	24	IPI00009866	Tax_Id=9606 Gene_Symbol=KRT13 Isoform 1 of Keratin, type I cytoskeletal 13	1187	49898	31	39,3
Saliva	25	IPI00007047	Tax_Id=9606 Gene_Symbol=S100A8 Protein S100-A8	1171	10885	38	77,4
Saliva	26	IPI00019038	Tax_Id=9606 Gene_Symbol=LYZ Lysozyme C precursor	1170	16982	41	56,1
Saliva	27	IPI00472610	Tax_Id=9606 Gene_Symbol=IGHM IGHM protein	1168	53317	34	32,4
Saliva	28	IPI00374315	Tax_Id=9606 Gene_Symbol=C6orf58 Uncharacterized protein C6orf58 precursor	1153	38244	45	35,2
Saliva	29	IPI00007244	Tax_Id=9606 Gene_Symbol=MPO Isoform H17 of Myeloperoxidase precursor	1078	84784	32	27
Saliva	30	IPI00022463	Tax_Id=9606 Gene_Symbol=TF Serotransferrin precursor	1018	79280	30	41
Saliva	31	IPI00453473	Tax_Id=9606 Gene_Symbol=HIST1H4A;HIST1H4K;HIST1H4J;HIST1H4D;HIST1H4E;HIST1H4H;HIST1H4C;HIST4H4;HIST1H4I;	957	11360	25	52,4
Saliva	32	IPI00009865	Tax_Id=9606 Gene_Symbol=KRT10 Keratin, type I cytoskeletal 10	934	59703	28	28
Saliva	33	IPI00295105	Tax_Id=9606 Gene_Symbol=CA6 Carbonic anhydrase 6 precursor	908	35459	22	24,4
Saliva	34	IPI00305477	Tax_Id=9606 Gene_Symbol=CST1 Cystatin-SN precursor	904	16579	26	60,3
Saliva	35	IPI00418512	Tax_Id=9606 Gene_Symbol=DMBT1 Isoform 4 of Deleted in malignant brain tumors 1 protein precursor	897	170672	22	13,8
Saliva	36	IPI00032294	Tax_Id=9606 Gene_Symbol=CST4 Cystatin-S precursor	882	16489	30	71,6
Saliva	37	IPI00021304	Tax_Id=9606 Gene_Symbol=KRT2 Keratin, type II cytoskeletal 2 epidermal	878	66110	31	25,7
Saliva	38	IPI00829711	Tax_Id=9606 Gene_Symbol=IGHA2 Uncharacterized protein IGHA2 (Fragment)	852	43202	31	39,9
Saliva	39	IPI00009650	Tax_Id=9606 Gene_Symbol=LCN1 Lipocalin-1 precursor	830	19409	31	48,9
Saliva	40	IPI00304557	Tax_Id=9606 Gene_Symbol=C20orf70 Short palate, lung and nasal epithelium carcinoma-associated protein 2 precursor	800	27166	23	35,7
Saliva	41	IPI00412044	Tax_Id=9606 Gene_Symbol=DMBT1 143 kDa protein	790	147875	14	9,5
Saliva	42	IPI00785200	Tax_Id=9606 Gene_Symbol=- Putative uncharacterized protein	762	25350	32	29,7
Saliva	43	IPI00028064	Tax_Id=9606 Gene_Symbol=CTSG Cathepsin G precursor	747	29161	20	34,9
Saliva	44	IPI00300725	Tax_Id=9606 Gene_Symbol=KRT6A Keratin, type II cytoskeletal 6A	719	60293	20	37,8
Saliva	45	IPI00290078	Tax_Id=9606 Gene_Symbol=KRT4 keratin 4	714	64442	17	32,5
Saliva	46	IPI00018534	Tax_Id=9606 Gene_Symbol=HIST1H2BL Histone H2B type 1-L	704	13944	20	27
Saliva	47	IPI00293665	Tax_Id=9606 Gene_Symbol=KRT6B Keratin, type II cytoskeletal 6B	672	60247	19	34,8
Saliva	48	IPI00807428	Tax_Id=9606 Gene_Symbol=- Putative uncharacterized protein	653	25016	30	24,3
Saliva	49	IPI00025023	Tax_Id=9606 Gene_Symbol=LPO Lactoperoxidase precursor	648	81149	30	26,7
Saliva	50	IPI00032293	Tax_Id=9606 Gene_Symbol=CST3 Cystatin-C precursor	627	16017	13	31,5
Saliva	51	IPI00013382	Tax_Id=9606 Gene_Symbol=CST2 Cystatin-SA precursor	612	16719	17	61
Saliva	52	IPI00217963	Tax_Id=9606 Gene_Symbol=KRT16 Keratin, type I cytoskeletal 16	602	51578	15	34,9
Saliva	53	IPI00641737	Tax_Id=9606 Gene_Symbol=HP Haptoglobin precursor	600	47378	17	33,8
Saliva	54	IPI00550640	Tax_Id=9606 Gene_Symbol=IGHG4 IGHG4 protein	594	52637	17	17,5
Saliva	55	IPI00304808	Tax_Id=9606 Gene_Symbol=KLK1 Kallikrein-1 precursor	578	29498	15	49,6
Saliva	56	IPI00296654	Tax_Id=9606 Gene_Symbol=BPIL1 Bactericidal/permeability-increasing protein-like 1 precursor	539	49256	17	12,7
Saliva	57	IPI00719373	Tax_Id=9606 Gene_Symbol=IGL@ IGL@ protein	525	25475	20	24,4
Saliva	58	IPI00178926	Tax_Id=9606 Gene_Symbol=IGJ immunoglobulin J chain	494	18543	14	55,3
Saliva	59	IPI00399007	Tax_Id=9606 Gene_Symbol=IGHG2 Putative uncharacterized protein DKFZp686I04196 (Fragment)	476	46716	18	16,5
Saliva	60	IPI00219018	Tax_Id=9606 Gene_Symbol=GAPDH Glyceraldehyde-3-phosphate dehydrogenase	470	36201	14	29
Saliva	61	IPI00027444	Tax_Id=9606 Gene_Symbol=SERPINB1 Leukocyte elastase inhibitor	450	42829	7	17,4
Saliva	62	IPI00013890	Tax_Id=9606 Gene_Symbol=SFN Isoform 1 of 14-3-3 protein sigma	442	27871	8	16,5
Saliva	63	IPI00553177	Tax_Id=9606 Gene_Symbol=SERPINA1 Isoform 1 of Alpha-1-antitrypsin precursor	432	46878	13	30,9
Saliva	64	IPI00024095	Tax_Id=9606 Gene_Symbol=ANXA3 Annexin A3	427	36524	12	32,5
Saliva	65	IPI00021841	Tax_Id=9606 Gene_Symbol=APOA1 Apolipoprotein A-I precursor	415	30759	12	36,3
Saliva	66	IPI00009867	Tax_Id=9606 Gene_Symbol=KRT5 Keratin, type II cytoskeletal 5	405	62568	13	17,1
Saliva	67	IPI00299729	Tax_Id=9606 Gene_Symbol=TCN1 Transcobalamin-1 precursor	372	48689	12	20,1
Saliva	68	IPI00297056	Tax_Id=9606 Gene_Symbol=CRNN Cornulin	359	53730	7	12,1
Saliva	69	IPI00219757	Tax_Id=9606 Gene_Symbol=GSTP1 Glutathione S-transferase P	355	23569	8	30,5
Saliva	70	IPI00382480	Tax_Id=9606 Gene_Symbol=- Ig heavy chain V-III region BRO	344	13332	9	25
Saliva	71	IPI00299547	Tax_Id=9606 Gene_Symbol=LCN2 Neutrophil gelatinase-associated lipocalin precursor	330	22745	12	44,4
Saliva	72	IPI00027019	Tax_Id=9606 Gene_Symbol=PRR4;PRH1;PRH2;PRB4 Proline-rich protein 4 precursor	330	15116	10	21,6
Saliva	73	IPI00002851	Tax_Id=9606 Gene_Symbol=CST5 Cystatin-D precursor	318	16355	8	49,3
Saliva	74	IPI00419585	Tax_Id=9606 Gene_Symbol=PPIA;PPIAL3;LOC654188 Peptidyl-prolyl cis-trans isomerase A	313	18229	8	35,8
Saliva	75	IPI00384444	Tax_Id=9606 Gene_Symbol=KRT14 Keratin, type I cytoskeletal 14	300	51875	9	22
Saliva	76	IPI00007797	Tax_Id=9606 Gene_Symbol=FABP5;FABP5L7 Fatty acid-binding protein, epidermal	296	15497	6	38,5
Saliva	77	IPI00026126	Tax_Id=9606 Gene_Symbol=SCGB2A1 Mammaglobin-B precursor	290	11104	9	40
Saliva	78	IPI00020487	Tax_Id=9606 Gene_Symbol=LACRT Extracellular glycoprotein lacritin precursor	284	14237	9	22,5
Saliva	79	IPI00216457	Tax_Id=9606 Gene_Symbol=HIST2H2AA3;HIST2H2AA4 Histone H2A type 2-A	275	14087	8	57,7
Saliva	80	IPI00829767	Tax_Id=9606 Gene_Symbol=IGHG2 Uncharacterized protein IGHG2 (Fragment)	274	44520	12	16,5
Saliva	81	IPI00003469	Tax_Id=9606 Gene_Symbol=- Ig kappa chain V-I region WEA	258	11947	6	16,7
Saliva	82	IPI00023673	Tax_Id=9606 Gene_Symbol=LGALS3BP Galectin-3-binding protein precursor	256	66202	8	13,2
Saliva	83	IPI00783024	Tax_Id=9606 Gene_Symbol=- Myosin-reactive immunoglobulin heavy chain variable region (Fragment)	251	14247	6	19,8
Saliva	84	IPI00387115	Tax_Id=9606 Gene_Symbol=- Ig kappa chain V-III region SIE	245	11882	10	24,8
Saliva	85	IPI00783987	Tax_Id=9606 Gene_Symbol=C3 Complement C3 precursor (Fragment)	232	188569	5	2,6
Saliva	86	IPI00021828	Tax_Id=9606 Gene_Symbol=CSTB Cystatin-B	230	11190	7	45,9
Saliva	87	IPI00549291	Tax_Id=9606 Gene_Symbol=IGHM IGHM protein	223	66998	7	18,8
Saliva	88	IPI00152154	Tax_Id=9606 Gene_Symbol=MUC7 Mucin-7 precursor	223	39432	10	6,6
Saliva	89	IPI00291262	Tax_Id=9606 Gene_Symbol=CLU Clusterin precursor	219	53031	7	10,2
Saliva	90	IPI00000045	Tax_Id=9606 Gene_Symbol=IL1RN Isoform 1 of Interleukin-1 receptor antagonist protein precursor	217	20384	3	19,8
Saliva	91	IPI00216691	Tax_Id=9606 Gene_Symbol=PFN1 Profilin-1	210	15216	7	37,1
Saliva	92	IPI00021891	Tax_Id=9606 Gene_Symbol=FGG Isoform Gamma-B of Fibrinogen gamma chain precursor	210	52106	7	14,3
Saliva	93	IPI00012011	Tax_Id=9606 Gene_Symbol=CFL1 Cofilin-1	203	18719	7	33,7
Saliva	94	IPI00418169	Tax_Id=9606 Gene_Symbol=ANXA2 annexin A2 isoform 1	202	40671	6	25,8

Saliva	95	IPI00002459	Tax_Id=9606 Gene_Symbol=ANXA6 annexin VI isoform 2	202	75571	2	2,4
Saliva	96	IPI00075248	Tax_Id=9606 Gene_Symbol=CALM3;CALM1;CALM2 Calmodulin	188	16827	2	22,1
Saliva	97	IPI00465439	Tax_Id=9606 Gene_Symbol=ALDOA Fructose-bisphosphate aldolase A	186	39851	4	15,7
Saliva	98	IPI00008359	Tax_Id=9606 Gene_Symbol=KRT76 Keratin, type II cytoskeletal 2 oral	184	66400	5	9,6
Saliva	99	IPI00643920	Tax_Id=9606 Gene_Symbol=TKT Transketolase	174	68519	5	12,8
Saliva	100	IPI00022432	Tax_Id=9606 Gene_Symbol=TTR Transthyretin precursor	170	15991	3	27,9
Saliva	101	IPI00032325	Tax_Id=9606 Gene_Symbol=CSTA Cystatin-A	168	11000	4	67,3
Saliva	102	IPI00004798	Tax_Id=9606 Gene_Symbol=CRISP3 Cysteine-rich secretory protein 3 precursor	164	28524	5	24,9
Saliva	103	IPI00816799	Tax_Id=9606 Gene_Symbol=- Rheumatoid factor D5 light chain (Fragment)	163	12872	5	22,9
Saliva	104	IPI00005721	Tax_Id=9606 Gene_Symbol=LOC728358;DEFA1 Neutrophil defensin 1 precursor	163	10536	5	19,1
Saliva	105	IPI00022488	Tax_Id=9606 Gene_Symbol=HPX Hemopexin precursor	159	52385	4	11,3
Saliva	106	IPI00027769	Tax_Id=9606 Gene_Symbol=ELA2 Leukocyte elastase precursor	154	29127	4	10,1
Saliva	107	IPI00298497	Tax_Id=9606 Gene_Symbol=FGB Fibrinogen beta chain precursor	152	56577	6	13,8
Saliva	108	IPI00554711	Tax_Id=9606 Gene_Symbol=JUP Junction plakoglobin	143	82376	2	5,1
Saliva	109	IPI00478003	Tax_Id=9606 Gene_Symbol=A2M Alpha-2-macroglobulin precursor	143	164600	5	4,9
Saliva	110	IPI00027409	Tax_Id=9606 Gene_Symbol=PRTN3 Myeloblastin precursor	142	28245	5	11,3
Saliva	111	IPI00465248	Tax_Id=9606 Gene_Symbol=ENO1 Isoform alpha-enolase of Alpha-enolase	138	47481	4	12
Saliva	112	IPI00828062	Tax_Id=9606 Gene_Symbol=- Anti-(ED-B) scFV (Fragment)	136	25357	6	11,8
Saliva	113	IPI00217465	Tax_Id=9606 Gene_Symbol=HIST1H1C Histone H1.2	134	21352	3	15,5
Saliva	114	IPI00027547	Tax_Id=9606 Gene_Symbol=DCD Dermcidin precursor	132	11391	2	12,7
Saliva	115	IPI00179330	Tax_Id=9606 Gene_Symbol=RPS27A;UBC;UBB ubiquitin and ribosomal protein S27a precursor	131	18296	4	16
Saliva	116	IPI00555874	Tax_Id=9606 Gene_Symbol=- Actin-like protein (Fragment)	128	11603	2	10,7
Saliva	117	IPI00004656	Tax_Id=9606 Gene_Symbol=B2M Beta-2-microglobulin	127	14521	2	36,3
Saliva	118	IPI00419215	Tax_Id=9606 Gene_Symbol=A2ML1 alpha-2-macroglobulin-like 1	124	162430	5	4,7
Saliva	119	IPI00171611	Tax_Id=9606 Gene_Symbol=HIST2H3C;HIST2H3A Histone H3.2	124	15436	6	16,9
Saliva	120	IPI00296183	Tax_Id=9606 Gene_Symbol=ALDH3A1 Aldehyde dehydrogenase, dimeric NADP-preferring	120	50746	1	4,2
Saliva	121	IPI00217182	Tax_Id=9606 Gene_Symbol=DSP Isoform DPII of Desmoplakin	117	262166	2	1,2
Saliva	122	IPI00291792	Tax_Id=9606 Gene_Symbol=ITGB2 Integrin beta-2 precursor	114	87976	3	5,1
Saliva	123	IPI00219806	Tax_Id=9606 Gene_Symbol=S100A7 Protein S100-A7	112	11564	2	11,9
Saliva	124	IPI00552432	Tax_Id=9606 Gene_Symbol=PRB2 Basic salivary proline-rich protein 2 precursor	111	40775	3	4,8
Saliva	125	IPI00216984	Tax_Id=9606 Gene_Symbol=CALML3 Calmodulin-like protein 3	110	16937	2	19,5
Saliva	126	IPI00418471	Tax_Id=9606 Gene_Symbol=VIM Vimentin	109	53676	4	9,9
Saliva	127	IPI00006995	Tax_Id=9606 Gene_Symbol=P11 Placental protein 11 precursor	107	42892	1	5,1
Saliva	128	IPI00169383	Tax_Id=9606 Gene_Symbol=PGK1 Phosphoglycerate kinase 1	106	44985	2	4,3
Saliva	129	IPI00335168	Tax_Id=9606 Gene_Symbol=MYL6 Isoform Non-muscle of Myosin light polypeptide 6	103	17090	3	24,5
Saliva	130	IPI00010182	Tax_Id=9606 Gene_Symbol=DBI Isoform a 1 of Acyl-CoA-binding protein	102	10038	2	23
Saliva	131	IPI00022246	Tax_Id=9606 Gene_Symbol=AZU1 Azurocidin precursor	99	27325	3	8
Saliva	132	IPI00016593	Tax_Id=9606 Gene_Symbol=CCL28 C-C motif chemokine 28 precursor	93	14670	1	11,8
Saliva	133	IPI00021263	Tax_Id=9606 Gene_Symbol=YWHAZ 14-3-3 protein zeta/delta	92	27899	4	10,6
Saliva	134	IPI00024138	Tax_Id=9606 Gene_Symbol=- Uncharacterized protein ENSP00000374816	90	14171	3	7
Saliva	135	IPI00025512	Tax_Id=9606 Gene_Symbol=HSPB1 Heat shock protein beta-1	89	22826	2	12,7
Saliva	136	IPI00744692	Tax_Id=9606 Gene_Symbol=TALDO1 Transaldolase	88	37688	2	6,5
Saliva	137	IPI00552578	Tax_Id=9606 Gene_Symbol=SAA2;SAA1 Serum amyloid A protein precursor	86	13581	2	29,5
Saliva	138	IPI00827847	Tax_Id=9606 Gene_Symbol=BPI Bactericidal permeability-increasing protein precursor	85	54120	2	4,9
Saliva	139	IPI00291005	Tax_Id=9606 Gene_Symbol=MDH1 Malate dehydrogenase, cytoplasmic	85	36631	1	3,9
Saliva	140	IPI00219525	Tax_Id=9606 Gene_Symbol=PGD 6-phosphogluconate dehydrogenase, decarboxylating	85	53619	2	4,1
Saliva	141	IPI00017526	Tax_Id=9606 Gene_Symbol=S100P Protein S100-P	85	10450	1	13,7
Saliva	142	IPI00292532	Tax_Id=9606 Gene_Symbol=CAMP Antibacterial protein FALL-39 precursor	84	19806	2	12,7
Saliva	143	IPI00008580	Tax_Id=9606 Gene_Symbol=SLPI Antileukoproteinase precursor	83	15228	2	9,1
Saliva	144	IPI00219221	Tax_Id=9606 Gene_Symbol=LOC732032;LGALS7;LOC653499;LOC728910 Galectin-7	83	15123	2	11,8
Saliva	145	IPI00028714	Tax_Id=9606 Gene_Symbol=MGP Matrix Gla protein precursor	83	12516	2	23,3
Saliva	146	IPI00032292	Tax_Id=9606 Gene_Symbol=TIMP1 Metalloproteinase inhibitor 1 precursor	81	23840	4	15,5
Saliva	147	IPI00291410	Tax_Id=9606 Gene_Symbol=C20orf114 Isoform 1 of Long palate, lung and nasal epithelium carcinoma-associated protei	78	52580	2	3,1
Saliva	148	IPI00029699	Tax_Id=9606 Gene_Symbol=RNASE4 Ribonuclease 4 precursor	75	17286	1	8,8
Saliva	149	IPI00024915	Tax_Id=9606 Gene_Symbol=PRDX5 Isoform Mitochondrial of Peroxiredoxin-5, mitochondrial precursor	74	22298	3	15
Saliva	150	IPI00216298	Tax_Id=9606 Gene_Symbol=TXN Thioredoxin	73	12015	1	12,4
Saliva	151	IPI00003865	Tax_Id=9606 Gene_Symbol=HSPA8 Isoform 1 of Heat shock cognate 71 kDa protein	72	71082	1	2,6
Saliva	152	IPI00550991	Tax_Id=9606 Gene_Symbol=SERPINA3 Alpha-1-antichymotrypsin precursor	70	50737	1	4,2
Saliva	153	IPI00011692	Tax_Id=9606 Gene_Symbol=IVL Involucrin	69	68541	1	2,2
Saliva	154	IPI00017601	Tax_Id=9606 Gene_Symbol=CP Ceruloplasmin precursor	69	122983	1	2
Saliva	155	IPI00414896	Tax_Id=9606 Gene_Symbol=RNASET2 Isoform 1 of Ribonuclease T2 precursor	69	30089	2	9,8
Saliva	156	IPI00783625	Tax_Id=9606 Gene_Symbol=SERPINB5 Serpin B5 precursor	68	42568	2	6,9
Saliva	157	IPI00026314	Tax_Id=9606 Gene_Symbol=GSN Isoform 1 of Gelsolin precursor	68	86043	1	2,8
Saliva	158	IPI00013163	Tax_Id=9606 Gene_Symbol=MNDA Myeloid cell nuclear differentiation antigen	67	46092	2	6,6
Saliva	159	IPI00026962	Tax_Id=9606 Gene_Symbol=PLA2G2A Phospholipase A2, membrane associated precursor	66	16870	1	12,5
Saliva	160	IPI00013972	Tax_Id=9606 Gene_Symbol=CEACAM8 Carcinoembryonic antigen-related cell adhesion molecule 8 precursor	66	38415	2	9,7
Saliva	161	IPI00787362	Tax_Id=9606 Gene_Symbol=LOC730839 similar to Hornerin	65	188565	2	2,2
Saliva	162	IPI00010471	Tax_Id=9606 Gene_Symbol=LCP1 Plastin-2	62	70815	2	3,8
Saliva	163	IPI00478198	Tax_Id=9606 Gene_Symbol=PRB1 proline-rich protein BstNI subfamily 1 isoform 1 precursor	60	32594	1	6
Saliva	164	IPI00382440	Tax_Id=9606 Gene_Symbol=- Ig lambda chain V-IV region Hil	59	11624	1	17,8
Saliva	165	IPI00414884	Tax_Id=9606 Gene_Symbol=C4orf40 hypothetical protein LOC401137	56	22820	3	16
Saliva	166	IPI00010402	Tax_Id=9606 Gene_Symbol=SH3BGRL3 Putative uncharacterized protein	56	24108	2	8,4
Saliva	167	IPI00025427	Tax_Id=9606 Gene_Symbol=RNASE3 Eosinophil cationic protein precursor	54	18887	1	10
Saliva	168	IPI00373937	Tax_Id=9606 Gene_Symbol=SBSN Suprabasin	53	60562	1	3,1
Saliva	169	IPI00217966	Tax_Id=9606 Gene_Symbol=LDHA Isoform 1 of L-lactate dehydrogenase A chain	53	36950	2	5,7
Saliva	170	IPI00029739	Tax_Id=9606 Gene_Symbol=CFH Isoform 1 of Complement factor H precursor	51	143680	1	1
Saliva	171	IPI00387024	Tax_Id=9606 Gene_Symbol=- Ig kappa chain V-I region CAR	49	11810	1	16,8
Saliva	172	IPI00003734	Tax_Id=9606 Gene_Symbol=LOC347701 Putative S100 calcium-binding protein H_NH0456N16.1	48	11673	1	15,4
Saliva	173	IPI00010270	Tax_Id=9606 Gene_Symbol=RAC2 Ras-related C3 botulinum toxin substrate 2 precursor	47	21814	1	4,2
Saliva	174	IPI00028946	Tax_Id=9606 Gene_Symbol=RTN3 Isoform 3 of Reticulon-3	47	25764	1	4,7
Saliva	175	IPI00009123	Tax_Id=9606 Gene_Symbol=NUCB2 Nucleobindin-2 precursor	46	50305	1	3,1
Saliva	176	IPI00004524	Tax_Id=9606 Gene_Symbol=GCA Grancalcin	45	24223	1	4,1
Saliva	177	IPI00082931	Tax_Id=9606 Gene_Symbol=SPRR3 Small proline-rich protein 3	43	18598	1	9,5
Saliva	178	IPI00183695	Tax_Id=9606 Gene_Symbol=S100A10 Protein S100-A10	42	11310	1	17,5
Saliva	179	IPI00293276	Tax_Id=9606 Gene_Symbol=MIF Macrophage migration inhibitory factor	42	12639	1	7,8
Saliva	180	IPI00465028	Tax_Id=9606 Gene_Symbol=TPI1 Isoform 1 of Triosephosphate isomerase	41	31057	1	4,5
Saliva	181	IPI00023011	Tax_Id=9606 Gene_Symbol=SMR3B Submaxillary gland androgen-regulated protein 3 homolog B precursor	41	8239	2	65,8
Saliva	182	IPI00329801	Tax_Id=9606 Gene_Symbol=ANXA5 Annexin A5	41	35971	1	3,8
Saliva	183	IPI00300376	Tax_Id=9606 Gene_Symbol=TGM3 Protein-glutamine gamma-glutamyltransferase E precursor	37	76926	1	2,6
Saliva	184	IPI00000041	Tax_Id=9606 Gene_Symbol=RHOB Rho-related GTP-binding protein RhoB precursor	36	22565	1	8,7
Saliva	185	IPI00332947	Tax_Id=9606 Gene_Symbol=- Uncharacterized protein ENSP00000313541 (Fragment)	35	31442	1	2,9
Saliva	186	IPI00745363	Tax_Id=9606 Gene_Symbol=- Immunglobulin heavy chain variable region (Fragment)	34	14555	1	6,9
Saliva	187	IPI00010303	Tax_Id=9606 Gene_Symbol=SERPINB4 Serpin B4	34	44997	1	1,8
Saliva	188	IPI00154664	Tax_Id=9606 Gene_Symbol=PIWIL2 Piwi-like protein 2	32	111089	1	1,2
Saliva	189	IPI00646410	Tax_Id=9606 Gene_Symbol=AOF1 Isoform 2 of Flavin-containing amine oxidase domain-containing protein 1	31	66830	1	1,7
Saliva	190	IPI00022890	Tax_Id=9606 Gene_Symbol=IGLV7-43 Ig lambda chain V region 4A precursor	31	12600	1	7,7
Saliva	191	IPI00219365	Tax_Id=9606 Gene_Symbol=MSN Moesin	31	67892	1	2,9

4.2.3 Proteins identified in human tear, but not in human saliva sample.

origin	prot_hit	prot_acc	prot_desc	ot_sco	ot_ma	ot_match	rot_cove	overlap
Lacrima	4	IPI00024284	Tax_Id=9606 Gene_Symbol=HSPG2 Basement membrane-specific heparan sulfate proteoglycan core protein precursor	2788	5E+05	67	21,9	0
Lacrima	3	IPI00166866	Tax_Id=9606 Gene_Symbol=IGHA1 IGHA1 protein	2931	54483	67	53,3	0
Lacrima	6	IPI00430842	Tax_Id=9606 Gene_Symbol=IGHA1 IGHA1 protein	2782	53858	64	51,3	0
Lacrima	7	IPI00426060	Tax_Id=9606 Gene_Symbol=IGHA1 Putative uncharacterized protein DKFZp686J11235 (Fragment)	2757	55394	63	52,6	0
Lacrima	14	IPI00430808	Tax_Id=9606 Gene_Symbol=IGKC IGKC protein	1803	25915	43	43	0
Lacrima	23	IPI00816314	Tax_Id=9606 Gene_Symbol=IGHM Putative uncharacterized protein DKFZp686I15196	1031	51579	26	38,2	0
Lacrima	24	IPI00645363	Tax_Id=9606 Gene_Symbol=IGHG1 Putative uncharacterized protein DKFZp686P15220	1011	52376	23	39,4	0
Lacrima	26	IPI00478493	Tax_Id=9606 Gene_Symbol=HP HP protein	813	38940	20	38,3	0
Lacrima	31	IPI00171196	Tax_Id=9606 Gene_Symbol=KRT13 keratin 13 isoform b	661	46181	19	39	0
Lacrima	47	IPI00103397	Tax_Id=9606 Gene_Symbol=MUC5AC Mucin-5AC precursor (Fragment)	386	5E+05	13	3,1	0
Lacrima	58	IPI00829768	Tax_Id=9606 Gene_Symbol=IGHM IGHM protein	308	68823	13	24,8	0
Lacrima	42	IPI00021885	Tax_Id=9606 Gene_Symbol=FGA Isoform 1 of Fibrinogen alpha chain precursor	450	95656	12	13,9	0
Lacrima	38	IPI00168728	Tax_Id=9606 Gene_Symbol=IGHM FLJ00385 protein (Fragment)	511	57272	11	18,3	0
Lacrima	57	IPI00001468	Tax_Id=9606 Gene_Symbol=SCGB1D1 Secretoglobin family 1D member 1 precursor	309	10234	10	23,3	0
Lacrima	53	IPI00418163	Tax_Id=9606 Gene_Symbol=C4B C4B1	316	2E+05	10	9,9	0
Lacrima	82	IPI00011229	Tax_Id=9606 Gene_Symbol=CTSD Cathepsin D precursor	144	45037	6	20,4	0
Lacrima	71	IPI00450768	Tax_Id=9606 Gene_Symbol=KRT17 Keratin, type I cytoskeletal 17	224	48361	5	11,3	0
Lacrima	76	IPI00479145	Tax_Id=9606 Gene_Symbol=KRT19 Keratin, type I cytoskeletal 19	181	44065	5	12,5	0
Lacrima	95	IPI00006662	Tax_Id=9606 Gene_Symbol=APOD Apolipoprotein D precursor	82	21547	3	19	0
Lacrima	85	IPI00009682	Tax_Id=9606 Gene_Symbol=PROL1 Proline-rich protein 1 precursor	121	22970	3	18,9	0
Lacrima	94	IPI00022204	Tax_Id=9606 Gene_Symbol=SERPINB3 Serpin B3	83	44594	3	10,8	0
Lacrima	87	IPI00217778	Tax_Id=9606 Gene_Symbol=PLTP Isoform 2 of Phospholipid transfer protein precursor	104	49412	3	3,4	0
Lacrima	73	IPI00749245	Tax_Id=9606 Gene_Symbol=SFRP1 Secreted frizzled-related protein 1 precursor	199	36274	3	15,3	0
Lacrima	91	IPI00000874	Tax_Id=9606 Gene_Symbol=PRDX1 Peroxiredoxin-1	90	22324	2	9,5	0
Lacrima	111	IPI00008603	Tax_Id=9606 Gene_Symbol=ACTA2 Actin, aortic smooth muscle	57	42381	2	7,2	0
Lacrima	109	IPI00019533	Tax_Id=9606 Gene_Symbol=CHI3L2 Chitinase-3-like protein 2 precursor	58	43701	2	7,4	0
Lacrima	103	IPI00295741	Tax_Id=9606 Gene_Symbol=CTSB Cathepsin B precursor	72	38766	2	5	0
Lacrima	106	IPI00297900	Tax_Id=9606 Gene_Symbol=DDX10 Probable ATP-dependent RNA helicase DDX10	60	1E+05	2	1	0
Lacrima	98	IPI00387120	Tax_Id=9606 Gene_Symbol=- Ig kappa chain V-IV region Len	78	12746	2	15,8	0
Lacrima	120	IPI00000816	Tax_Id=9606 Gene_Symbol=YWHAE 14-3-3 protein epsilon	44	29326	1	3,1	0
Lacrima	117	IPI00003470	Tax_Id=9606 Gene_Symbol=- Ig kappa chain V-I region Wes	48	11715	1	16,7	0
Lacrima	110	IPI00009792	Tax_Id=9606 Gene_Symbol=IGHV1OR15-1 Ig heavy chain V-I region V35 precursor	58	13114	1	10,3	0
Lacrima	115	IPI00013885	Tax_Id=9606 Gene_Symbol=CASP14 Caspase-14 precursor	51	27947	1	4,5	0
Lacrima	105	IPI00018230	Tax_Id=9606 Gene_Symbol=SMR3A Submaxillary gland androgen-regulated protein 3 homolog A precursor	67	14165	1	11,2	0
Lacrima	132	IPI00021855	Tax_Id=9606 Gene_Symbol=APOC1 Apolipoprotein C-I precursor	31	9326	1	10,8	0
Lacrima	118	IPI00021997	Tax_Id=9606 Gene_Symbol=CREG1 Protein CREG1 precursor	47	24173	1	9,5	0
Lacrima	90	IPI00022429	Tax_Id=9606 Gene_Symbol=ORM1 Alpha-1-acid glycoprotein 1 precursor	95	23725	1	7,5	0
Lacrima	93	IPI00022752	Tax_Id=9606 Gene_Symbol=AQP5 Aquaporin-5	85	42399	1	4	0
Lacrima	128	IPI00028344	Tax_Id=9606 Gene_Symbol=BRSK2 Isoform 3 of BR serine/threonine-protein kinase 2	35	75321	1	1,2	0
Lacrima	113	IPI00152442	Tax_Id=9606 Gene_Symbol=RNASET2 Extra-cellular ribonuclease	54	27049	1	5,8	0
Lacrima	108	IPI00171411	Tax_Id=9606 Gene_Symbol=GOLM1 Golgi phosphoprotein 2	59	46416	1	2,9	0
Lacrima	92	IPI00296777	Tax_Id=9606 Gene_Symbol=SPARCL1 SPARC-like protein 1 precursor	87	76025	1	3,2	0
Lacrima	97	IPI00298547	Tax_Id=9606 Gene_Symbol=PARK7 Protein DJ-1	79	20050	1	8,5	0
Lacrima	114	IPI00304273	Tax_Id=9606 Gene_Symbol=APOA4 Apolipoprotein A-IV precursor	52	45371	1	2,3	0
Lacrima	121	IPI00328587	Tax_Id=9606 Gene_Symbol=- Enolase	44	42657	1	1,8	0
Lacrima	112	IPI00382436	Tax_Id=9606 Gene_Symbol=- Ig lambda chain V-III region SH	56	11500	1	16,7	0
Lacrima	123	IPI00398625	Tax_Id=9606 Gene_Symbol=HRNR Hornerin	39	3E+05	1	1	0
Lacrima	130	IPI00424460	Tax_Id=9606 Gene_Symbol=ZFYVE16 Isoform 1 of Zinc finger FYVE domain-containing protein 16	32	2E+05	1	0,8	0
Lacrima	131	IPI00555812	Tax_Id=9606 Gene_Symbol=GC Vitamin D-binding protein precursor	31	54526	1	1,5	0
Lacrima	126	IPI00747459	Tax_Id=9606 Gene_Symbol=PDE6C Uncharacterized protein PDE6C	36	99939	1	0,9	0
Lacrima	125	IPI00761105	Tax_Id=9606 Gene_Symbol=MGC21874 transcriptional adaptor 2 (ADA2 homolog, yeast)-beta	37	49010	1	1,7	0

4.2.4 Proteins identified in human saliva, but not in human tear sample.

origin	prot_hit	prot_acc	prot_desc	ot_sco	ot_ma	ot_match	rot_cove	overlap
Saliva	1	IPI00300786	Tax_Id=9606 Gene_Symbol=AMY1A;AMY1C;AMY2A;AMY1B Alpha-amylase 1 precursor	6337	58415	143	71,2	0
Saliva	5	IPI00022434	Tax_Id=9606 Gene_Symbol=ALB Uncharacterized protein ALB	3632	73881	97	66,2	0
Saliva	181	IPI00023011	Tax_Id=9606 Gene_Symbol=SMR3B Submaxillary gland androgen-regulated protein 3 homolog B precursor	41	8239	2	65,8	0
Saliva	79	IPI00216457	Tax_Id=9606 Gene_Symbol=HIST2H2AA3;HIST2H2AA4 Histone H2A type 2-A	275	14087	8	57,7	0
Saliva	55	IPI00304808	Tax_Id=9606 Gene_Symbol=KLK1 Kallikrein-1 precursor	578	29498	15	49,6	0
Saliva	73	IPI00002851	Tax_Id=9606 Gene_Symbol=CST5 Cystatin-D precursor	318	16355	8	49,3	0
Saliva	86	IPI00021828	Tax_Id=9606 Gene_Symbol=CSTB Cystatin-B	230	11190	7	45,9	0
Saliva	10	IPI00440577	Tax_Id=9606 Gene_Symbol=IGKV2-24 IGKV2-24 protein	2583	26514	67	41	0
Saliva	24	IPI00009866	Tax_Id=9606 Gene_Symbol=KRT13 Isoform 1 of Keratin, type I cytoskeletal 13	1187	49898	31	39,3	0
Saliva	76	IPI00007797	Tax_Id=9606 Gene_Symbol=FABP5;FABP5L7 Fatty acid-binding protein, epidermal	296	15497	6	38,5	0
Saliva	44	IPI00300725	Tax_Id=9606 Gene_Symbol=KRT6A Keratin, type II cytoskeletal 6A	719	60293	20	37,8	0
Saliva	91	IPI00216691	Tax_Id=9606 Gene_Symbol=PFN1 Profilin-1	210	15216	7	37,1	0
Saliva	74	IPI00419585	Tax_Id=9606 Gene_Symbol=PPIA;PPIAL3;LOC654188 Peptidyl-prolyl cis-trans isomerase A	313	18229	8	35,8	0
Saliva	40	IPI00304557	Tax_Id=9606 Gene_Symbol=C20orf70 Short palate, lung and nasal epithelium carcinoma-associated protein 2 precursor	800	27166	23	35,7	0
Saliva	28	IPI00374315	Tax_Id=9606 Gene_Symbol=C6orf58 Uncharacterized protein C6orf58 precursor	1153	38244	45	35,2	0
Saliva	53	IPI00641737	Tax_Id=9606 Gene_Symbol=HP Haptoglobin precursor	600	47378	17	33,8	0
Saliva	93	IPI00012011	Tax_Id=9606 Gene_Symbol=CFL1 Cofilin-1	203	18719	7	33,7	0
Saliva	64	IPI00024095	Tax_Id=9606 Gene_Symbol=ANXA3 Annexin A3	427	36524	12	32,5	0
Saliva	27	IPI00472610	Tax_Id=9606 Gene_Symbol=IGHM IGHM protein	1168	53317	34	32,4	0
Saliva	137	IPI00552578	Tax_Id=9606 Gene_Symbol=SAA2;SAA1 Serum amyloid A protein precursor	86	13581	2	29,5	0
Saliva	22	IPI00448925	Tax_Id=9606 Gene_Symbol=IGHG1 IGHG1 protein	1192	60862	34	28,7	0
Saliva	100	IPI00022432	Tax_Id=9606 Gene_Symbol=TTR Transthyretin precursor	170	15991	3	27,9	0
Saliva	29	IPI00007244	Tax_Id=9606 Gene_Symbol=MPO Isoform H17 of Myeloperoxidase precursor	1078	84784	32	27	0
Saliva	46	IPI00018534	Tax_Id=9606 Gene_Symbol=HIST1H2BL Histone H2B type 1-L	704	13944	20	27	0
Saliva	102	IPI00004798	Tax_Id=9606 Gene_Symbol=CRISP3 Cysteine-rich secretory protein 3 precursor	164	28524	5	24,9	0
Saliva	129	IPI00335168	Tax_Id=9606 Gene_Symbol=MYL6 Isoform Non-muscle of Myosin light polypeptide 6	103	17090	3	24,5	0
Saliva	33	IPI00295105	Tax_Id=9606 Gene_Symbol=CA6 Carbonic anhydrase 6 precursor	908	35459	22	24,4	0
Saliva	57	IPI00719373	Tax_Id=9606 Gene_Symbol=IGL@ IGL@ protein	525	25475	20	24,4	0
Saliva	48	IPI00807428	Tax_Id=9606 Gene_Symbol=- Putative uncharacterized protein	653	25016	30	24,3	0
Saliva	145	IPI00028714	Tax_Id=9606 Gene_Symbol=MGP Matrix Gla protein precursor	83	12516	2	23,3	0
Saliva	23	IPI00021428	Tax_Id=9606 Gene_Symbol=ACTA1 Actin, alpha skeletal muscle	1190	42366	31	23,1	0
Saliva	130	IPI00010182	Tax_Id=9606 Gene_Symbol=DBI Isoform a 1 of Acyl-CoA-binding protein	102	10038	2	23	0
Saliva	96	IPI00075248	Tax_Id=9606 Gene_Symbol=CALM3;CALM1;CALM2 Calmodulin	188	16827	2	22,1	0
Saliva	67	IPI00299729	Tax_Id=9606 Gene_Symbol=TCN1 Transcobalamin-1 precursor	372	48689	12	20,1	0
Saliva	90	IPI00000045	Tax_Id=9606 Gene_Symbol=IL1RN Isoform 1 of Interleukin-1 receptor antagonist protein precursor	217	20384	3	19,8	0
Saliva	125	IPI00216984	Tax_Id=9606 Gene_Symbol=CALML3 Calmodulin-like protein 3	110	16937	2	19,5	0
Saliva	104	IPI00005721	Tax_Id=9606 Gene_Symbol=LOC728358;DEFA1 Neutrophil defensin 1 precursor	163	10536	5	19,1	0
Saliva	178	IPI00183695	Tax_Id=9606 Gene_Symbol=S100A10 Protein S100-A10	42	11310	1	17,5	0
Saliva	61	IPI00027444	Tax_Id=9606 Gene_Symbol=SERPINB1 Leukocyte elastase inhibitor	450	42829	7	17,4	0
Saliva	119	IPI00171611	Tax_Id=9606 Gene_Symbol=HIST2H3C;HIST2H3A Histone H3.2	124	15436	6	16,9	0
Saliva	171	IPI00387024	Tax_Id=9606 Gene_Symbol=- Ig kappa chain V-I region CAR	49	11810	1	16,8	0
Saliva	80	IPI00829767	Tax_Id=9606 Gene_Symbol=IGHG2 Uncharacterized protein IGHG2 (Fragment)	274	44520	12	16,5	0
Saliva	62	IPI00013890	Tax_Id=9606 Gene_Symbol=SFN Isoform 1 of 14-3-3 protein sigma	442	27871	8	16,5	0
Saliva	165	IPI00414884	Tax_Id=9606 Gene_Symbol=C4orf40 hypothetical protein LOC401137	56	22820	3	16	0
Saliva	97	IPI00465439	Tax_Id=9606 Gene_Symbol=ALDOA Fructose-bisphosphate aldolase A	186	39851	4	15,7	0
Saliva	146	IPI00032292	Tax_Id=9606 Gene_Symbol=TIMP1 Metalloproteinase inhibitor 1 precursor	81	23840	4	15,5	0
Saliva	113	IPI00217465	Tax_Id=9606 Gene_Symbol=HIST1H1C Histone H1.2	134	21352	3	15,5	0
Saliva	172	IPI00003734	Tax_Id=9606 Gene_Symbol=LOC347701 Putative S100 calcium-binding protein H_NH0456N16.1	48	11673	1	15,4	0
Saliva	149	IPI00024915	Tax_Id=9606 Gene_Symbol=PRDX5 Isoform Mitochondrial of Peroxiredoxin-5, mitochondrial precursor	74	22298	3	15	0
Saliva	141	IPI00017526	Tax_Id=9606 Gene_Symbol=S100P Protein S100-P	85	10450	1	13,7	0
Saliva	99	IPI00643920	Tax_Id=9606 Gene_Symbol=TKT Transketolase	174	68519	5	12,8	0
Saliva	56	IPI00296654	Tax_Id=9606 Gene_Symbol=BPIL1 Bactericidal/permeability-increasing protein-like 1 precursor	539	49256	17	12,7	0
Saliva	142	IPI00292532	Tax_Id=9606 Gene_Symbol=CAMP Antibacterial protein FALL-39 precursor	84	19806	2	12,7	0
Saliva	150	IPI00216298	Tax_Id=9606 Gene_Symbol=TXN Thioredoxin	73	12015	1	12,4	0
Saliva	68	IPI00297056	Tax_Id=9606 Gene_Symbol=CRNN Cornulin	359	53730	7	12,1	0
Saliva	111	IPI00465248	Tax_Id=9606 Gene_Symbol=ENO1 Isoform alpha-enolase of Alpha-enolase	138	47481	4	12	0
Saliva	144	IPI00219221	Tax_Id=9606 Gene_Symbol=LOC732032;LGALS7;LOC653499;LOC728910 Galectin-7	83	15123	2	11,8	0
Saliva	132	IPI00016593	Tax_Id=9606 Gene_Symbol=CCL28 C-C motif chemokine 28 precursor	93	14670	1	11,8	0
Saliva	110	IPI00027409	Tax_Id=9606 Gene_Symbol=PRTN3 Myeloblastin precursor	142	28245	5	11,3	0

Saliva	116	IPI00555874	Tax_Id=9606 Gene_Symbol=- Actin-like protein (Fragment)	128	11603	2	10,7	0
Saliva	133	IPI00021263	Tax_Id=9606 Gene_Symbol=YWHAZ 14-3-3 protein zeta/delta	92	27899	4	10,6	0
Saliva	106	IPI00027769	Tax_Id=9606 Gene_Symbol=ELA2 Leukocyte elastase precursor	154	29127	4	10,1	0
Saliva	167	IPI00025427	Tax_Id=9606 Gene_Symbol=RNASE3 Eosinophil cationic protein precursor	54	18887	1	10	0
Saliva	126	IPI00418471	Tax_Id=9606 Gene_Symbol=VIM Vimentin	109	53676	4	9,9	0
Saliva	155	IPI00414896	Tax_Id=9606 Gene_Symbol=RNASET2 Isoform 1 of Ribonuclease T2 precursor	69	30089	2	9,8	0
Saliva	160	IPI00013972	Tax_Id=9606 Gene_Symbol=CEACAM8 Carcinoembryonic antigen-related cell adhesion molecule 8 precursor	66	38415	2	9,7	0
Saliva	98	IPI00008359	Tax_Id=9606 Gene_Symbol=KRT76 Keratin, type II cytoskeletal 2 oral	184	66400	5	9,6	0
Saliva	177	IPI00082931	Tax_Id=9606 Gene_Symbol=SPRR3 Small proline-rich protein 3	43	18598	1	9,5	0
Saliva	148	IPI00029695	Tax_Id=9606 Gene_Symbol=RNASE4 Ribonuclease 4 precursor	75	17286	1	8,8	0
Saliva	184	IPI00000041	Tax_Id=9606 Gene_Symbol=RHOB Rho-related GTP-binding protein RhoB precursor	36	22565	1	8,7	0
Saliva	166	IPI00010402	Tax_Id=9606 Gene_Symbol=SH3BGRL3 Putative uncharacterized protein	56	24108	2	8,4	0
Saliva	21	IPI00855918	Tax_Id=9606 Gene_Symbol=MUC5B mucin 5, subtype B, tracheobronchial	1319	6E+05	34	8,1	0
Saliva	131	IPI00022246	Tax_Id=9606 Gene_Symbol=AZU1 Azurocidin precursor	99	27325	3	8	0
Saliva	179	IPI00293276	Tax_Id=9606 Gene_Symbol=MIF Macrophage migration inhibitory factor	42	12639	1	7,8	0
Saliva	190	IPI00022890	Tax_Id=9606 Gene_Symbol=IGLV7-43 Ig lambda chain V region 4A precursor	31	12600	1	7,7	0
Saliva	156	IPI00783625	Tax_Id=9606 Gene_Symbol=SERPINB5 Serpin B5 precursor	68	42568	2	6,9	0
Saliva	88	IPI00152154	Tax_Id=9606 Gene_Symbol=MUC7 Mucin-7 precursor	223	39432	10	6,6	0
Saliva	158	IPI00013163	Tax_Id=9606 Gene_Symbol=MNDA Myeloid cell nuclear differentiation antigen	67	46092	2	6,6	0
Saliva	136	IPI00744692	Tax_Id=9606 Gene_Symbol=TALDO1 Transaldolase	88	37688	2	6,5	0
Saliva	163	IPI00478198	Tax_Id=9606 Gene_Symbol=PRB1 proline-rich protein BstNI subfamily 1 isoform 1 precursor	60	32594	1	6	0
Saliva	169	IPI00217966	Tax_Id=9606 Gene_Symbol=LDHA Isoform 1 of L-lactate dehydrogenase A chain	53	36950	2	5,7	0
Saliva	122	IPI00291792	Tax_Id=9606 Gene_Symbol=ITGB2 Integrin beta-2 precursor	114	87976	3	5,1	0
Saliva	108	IPI00554711	Tax_Id=9606 Gene_Symbol=JUP Junction plakoglobin	143	82376	2	5,1	0
Saliva	127	IPI00006995	Tax_Id=9606 Gene_Symbol=P11 Placental protein 11 precursor	107	42892	1	5,1	0
Saliva	138	IPI00827847	Tax_Id=9606 Gene_Symbol=BPI Bactericidal permeability-increasing protein precursor	85	54120	2	4,9	0
Saliva	124	IPI00552432	Tax_Id=9606 Gene_Symbol=PRB2 Basic salivary proline-rich protein 2 precursor	111	40775	3	4,8	0
Saliva	118	IPI00419215	Tax_Id=9606 Gene_Symbol=A2ML1 alpha-2-macroglobulin-like 1	124	2E+05	5	4,7	0
Saliva	174	IPI00028946	Tax_Id=9606 Gene_Symbol=RTN3 Isoform 3 of Reticulon-3	47	25764	1	4,7	0
Saliva	128	IPI00169383	Tax_Id=9606 Gene_Symbol=PGK1 Phosphoglycerate kinase 1	106	44985	2	4,3	0
Saliva	173	IPI00010270	Tax_Id=9606 Gene_Symbol=RAC2 Ras-related C3 botulinum toxin substrate 2 precursor	47	21814	1	4,2	0
Saliva	120	IPI00296183	Tax_Id=9606 Gene_Symbol=ALDH3A1 Aldehyde dehydrogenase, dimeric NADP-preferring	120	50746	1	4,2	0
Saliva	152	IPI00550991	Tax_Id=9606 Gene_Symbol=SERPINA3 Alpha-1-antichymotrypsin precursor	70	50737	1	4,2	0
Saliva	140	IPI00219525	Tax_Id=9606 Gene_Symbol=PGD 6-phosphogluconate dehydrogenase, decarboxylating	85	53619	2	4,1	0
Saliva	176	IPI00004524	Tax_Id=9606 Gene_Symbol=GCA Grancalcin	45	24223	1	4,1	0
Saliva	139	IPI00291005	Tax_Id=9606 Gene_Symbol=MDH1 Malate dehydrogenase, cytoplasmic	85	36631	1	3,9	0
Saliva	162	IPI00010471	Tax_Id=9606 Gene_Symbol=LCP1 Plastin-2	62	70815	2	3,8	0
Saliva	182	IPI00329801	Tax_Id=9606 Gene_Symbol=ANXA5 Annexin A5	41	35971	1	3,8	0
Saliva	147	IPI00291410	Tax_Id=9606 Gene_Symbol=C20orf114 Isoform 1 of Long palate, lung and nasal epithelium carcinoma-associated protein 1 precursor	78	52580	2	3,1	0
Saliva	168	IPI00373937	Tax_Id=9606 Gene_Symbol=SBSN Suprabasin	53	60562	1	3,1	0
Saliva	191	IPI00219365	Tax_Id=9606 Gene_Symbol=MSN Moesin	31	67892	1	2,9	0
Saliva	185	IPI00332947	Tax_Id=9606 Gene_Symbol=- Uncharacterized protein ENSP00000313541 (Fragment)	35	31442	1	2,9	0
Saliva	151	IPI00003865	Tax_Id=9606 Gene_Symbol=HSPA8 Isoform 1 of Heat shock cognate 71 kDa protein	72	71082	1	2,6	0
Saliva	183	IPI00300376	Tax_Id=9606 Gene_Symbol=TGM3 Protein-glutamine gamma-glutamyltransferase E precursor	37	76926	1	2,6	0
Saliva	95	IPI00002459	Tax_Id=9606 Gene_Symbol=ANXA6 annexin VI isoform 2	202	75571	2	2,4	0
Saliva	161	IPI00787362	Tax_Id=9606 Gene_Symbol=LOC730839 similar to Hornerin	65	2E+05	2	2,2	0
Saliva	153	IPI00011692	Tax_Id=9606 Gene_Symbol=IVL Involucrin	69	68541	1	2,2	0
Saliva	187	IPI00010303	Tax_Id=9606 Gene_Symbol=SERPINB4 Serpin B4	34	44997	1	1,8	0
Saliva	189	IPI00646410	Tax_Id=9606 Gene_Symbol=AOF1 Isoform 2 of Flavin-containing amine oxidase domain-containing protein 1	31	66830	1	1,7	0
Saliva	121	IPI00217182	Tax_Id=9606 Gene_Symbol=DSP Isoform DPII of Desmoplakin	117	3E+05	2	1,2	0
Saliva	188	IPI00154664	Tax_Id=9606 Gene_Symbol=PIWIL2 Piwi-like protein 2	32	1E+05	1	1,2	0

4.2.5 Proteins identified both in human saliva and in human tear sample.

origin	prot_hit	prot_acc	prot_desc	ot_sco	ot_ma	ot_match	rot_cove	overlap
Saliva	81	IPI00003469	Tax_Id=9606 Gene_Symbol=- Ig kappa chain V-I region WEA	258	11947	6	16,7	1
Lacrima	55	IPI00003469	Tax_Id=9606 Gene_Symbol=- Ig kappa chain V-I region WEA	311	11947	6	16,7	1
Saliva	3	IPI00004573	Tax_Id=9606 Gene_Symbol=PIGR Polymeric immunoglobulin receptor precursor	4627	84429	123	44,2	1
Lacrima	9	IPI00004573	Tax_Id=9606 Gene_Symbol=PIGR Polymeric immunoglobulin receptor precursor	2224	84429	62	37,6	1
Saliva	117	IPI00004656	Tax_Id=9606 Gene_Symbol=B2M Beta-2-microglobulin	127	14521	2	36,3	1
Lacrima	89	IPI00004656	Tax_Id=9606 Gene_Symbol=B2M Beta-2-microglobulin	99	14521	2	18,5	1
Saliva	25	IPI00007047	Tax_Id=9606 Gene_Symbol=S100A8 Protein S100-A8	1171	10885	38	77,4	1
Lacrima	60	IPI00007047	Tax_Id=9606 Gene_Symbol=S100A8 Protein S100-A8	303	10885	8	53,8	1
Saliva	143	IPI00008580	Tax_Id=9606 Gene_Symbol=SLPI Antileukoproteinase precursor	83	15228	2	9,1	1
Lacrima	129	IPI00008580	Tax_Id=9606 Gene_Symbol=SLPI Antileukoproteinase precursor	34	15228	1	9,1	1
Saliva	175	IPI00009123	Tax_Id=9606 Gene_Symbol=NUCB2 Nucleobindin-2 precursor	46	50305	1	3,1	1
Lacrima	124	IPI00009123	Tax_Id=9606 Gene_Symbol=NUCB2 Nucleobindin-2 precursor	39	50305	1	2,1	1
Saliva	39	IPI00009650	Tax_Id=9606 Gene_Symbol=LCN1 Lipocalin-1 precursor	830	19409	31	48,9	1
Lacrima	18	IPI00009650	Tax_Id=9606 Gene_Symbol=LCN1 Lipocalin-1 precursor	1486	19409	65	56,3	1
Saliva	32	IPI00009865	Tax_Id=9606 Gene_Symbol=KRT10 Keratin, type I cytoskeletal 10	934	59703	28	28	1
Lacrima	17	IPI00009865	Tax_Id=9606 Gene_Symbol=KRT10 Keratin, type I cytoskeletal 10	1581	59703	44	45	1
Saliva	66	IPI00009867	Tax_Id=9606 Gene_Symbol=KRT5 Keratin, type II cytoskeletal 5	405	62568	13	17,1	1
Lacrima	40	IPI00009867	Tax_Id=9606 Gene_Symbol=KRT5 Keratin, type II cytoskeletal 5	473	62568	17	23,4	1
Saliva	51	IPI00013382	Tax_Id=9606 Gene_Symbol=CST2 Cystatin-SA precursor	612	16719	17	61	1
Lacrima	66	IPI00013382	Tax_Id=9606 Gene_Symbol=CST2 Cystatin-SA precursor	247	16719	5	29,8	1
Saliva	154	IPI00017601	Tax_Id=9606 Gene_Symbol=CP Ceruloplasmin precursor	69	1E+05	1	2	1
Lacrima	25	IPI00017601	Tax_Id=9606 Gene_Symbol=CP Ceruloplasmin precursor	860	1E+05	19	21,6	1
Saliva	26	IPI00019038	Tax_Id=9606 Gene_Symbol=LYZ Lysozyme C precursor	1170	16982	41	56,1	1
Lacrima	13	IPI00019038	Tax_Id=9606 Gene_Symbol=LYZ Lysozyme C precursor	1978	16982	54	56,8	1
Saliva	16	IPI00019359	Tax_Id=9606 Gene_Symbol=KRT9 Keratin, type I cytoskeletal 9	2207	62320	52	47,7	1
Lacrima	19	IPI00019359	Tax_Id=9606 Gene_Symbol=KRT9 Keratin, type I cytoskeletal 9	1263	62320	29	48,6	1
Saliva	78	IPI00020487	Tax_Id=9606 Gene_Symbol=LACRT Extracellular glycoprotein lacritin precursor	284	14237	9	22,5	1
Lacrima	64	IPI00020487	Tax_Id=9606 Gene_Symbol=LACRT Extracellular glycoprotein lacritin precursor	259	14237	9	22,5	1
Saliva	37	IPI00021304	Tax_Id=9606 Gene_Symbol=KRT2 Keratin, type II cytoskeletal 2 epidermal	878	66110	31	25,7	1
Lacrima	34	IPI00021304	Tax_Id=9606 Gene_Symbol=KRT2 Keratin, type II cytoskeletal 2 epidermal	611	66110	20	22,6	1
Saliva	15	IPI00021439	Tax_Id=9606 Gene_Symbol=ACTB Actin, cytoplasmic 1	2235	42052	63	49,6	1
Lacrima	70	IPI00021439	Tax_Id=9606 Gene_Symbol=ACTB Actin, cytoplasmic 1	230	42052	6	21,9	1
Saliva	65	IPI00021841	Tax_Id=9606 Gene_Symbol=APOA1 Apolipoprotein A-I precursor	415	30759	12	36,3	1
Lacrima	46	IPI00021841	Tax_Id=9606 Gene_Symbol=APOA1 Apolipoprotein A-I precursor	413	30759	14	46,8	1
Saliva	92	IPI00021891	Tax_Id=9606 Gene_Symbol=FGG Isoform Gamma-B of Fibrinogen gamma chain precursor	210	52106	7	14,3	1
Lacrima	32	IPI00021891	Tax_Id=9606 Gene_Symbol=FGG Isoform Gamma-B of Fibrinogen gamma chain precursor	644	52106	18	34,9	1
Saliva	30	IPI00022463	Tax_Id=9606 Gene_Symbol=TF Serotransferrin precursor	1018	79280	30	41	1
Lacrima	41	IPI00022463	Tax_Id=9606 Gene_Symbol=TF Serotransferrin precursor	455	79280	13	29,2	1
Saliva	105	IPI00022488	Tax_Id=9606 Gene_Symbol=HPX Hemopexin precursor	159	52385	4	11,3	1
Lacrima	84	IPI00022488	Tax_Id=9606 Gene_Symbol=HPX Hemopexin precursor	138	52385	3	12,1	1
Saliva	19	IPI00022974	Tax_Id=9606 Gene_Symbol=PIP Prolactin-inducible protein precursor	1724	16847	60	69,9	1
Lacrima	30	IPI00022974	Tax_Id=9606 Gene_Symbol=PIP Prolactin-inducible protein precursor	779	16847	29	69,9	1
Saliva	82	IPI00023673	Tax_Id=9606 Gene_Symbol=LGALS3BP Galectin-3-binding protein precursor	256	66202	8	13,2	1
Lacrima	81	IPI00023673	Tax_Id=9606 Gene_Symbol=LGALS3BP Galectin-3-binding protein precursor	146	66202	5	12,1	1
Saliva	134	IPI00024138	Tax_Id=9606 Gene_Symbol=- Uncharacterized protein ENSP00000374816	90	14171	3	7	1
Lacrima	96	IPI00024138	Tax_Id=9606 Gene_Symbol=- Uncharacterized protein ENSP00000374816	81	14171	2	7	1
Saliva	49	IPI00025023	Tax_Id=9606 Gene_Symbol=LPO Lactoperoxidase precursor	648	81149	30	26,7	1
Lacrima	78	IPI00025023	Tax_Id=9606 Gene_Symbol=LPO Lactoperoxidase precursor	167	81149	6	11	1
Saliva	135	IPI00025512	Tax_Id=9606 Gene_Symbol=HSPB1 Heat shock protein beta-1	89	22826	2	12,7	1
Lacrima	86	IPI00025512	Tax_Id=9606 Gene_Symbol=HSPB1 Heat shock protein beta-1	106	22826	2	12,7	1
Saliva	77	IPI00026126	Tax_Id=9606 Gene_Symbol=SCGB2A1 Mammaglobin-B precursor	290	11104	9	40	1
Lacrima	22	IPI00026126	Tax_Id=9606 Gene_Symbol=SCGB2A1 Mammaglobin-B precursor	1039	11104	36	58,9	1
Saliva	157	IPI00026314	Tax_Id=9606 Gene_Symbol=GSN Isoform 1 of Gelsolin precursor	68	86043	1	2,8	1
Lacrima	88	IPI00026314	Tax_Id=9606 Gene_Symbol=GSN Isoform 1 of Gelsolin precursor	103	86043	2	7	1
Saliva	159	IPI00026962	Tax_Id=9606 Gene_Symbol=PLA2G2A Phospholipase A2, membrane associated precursor	66	16870	1	12,5	1
Lacrima	56	IPI00026962	Tax_Id=9606 Gene_Symbol=PLA2G2A Phospholipase A2, membrane associated precursor	309	16870	6	30,6	1
Saliva	72	IPI00027019	Tax_Id=9606 Gene_Symbol=PRR4;PRH1;PRH2;PRB4 Proline-rich protein 4 precursor	330	15116	10	21,6	1
Lacrima	27	IPI00027019	Tax_Id=9606 Gene_Symbol=PRR4;PRH1;PRH2;PRB4 Proline-rich protein 4 precursor	811	15116	22	32,1	1
Saliva	20	IPI00027462	Tax_Id=9606 Gene_Symbol=S100A9 Protein S100-A9	1569	13291	43	66,7	1
Lacrima	74	IPI00027462	Tax_Id=9606 Gene_Symbol=S100A9 Protein S100-A9	193	13291	4	26,3	1
Saliva	114	IPI00027547	Tax_Id=9606 Gene_Symbol=DCD Dermcidin precursor	132	11391	2	12,7	1
Lacrima	127	IPI00027547	Tax_Id=9606 Gene_Symbol=DCD Dermcidin precursor	35	11391	1	10	1
Saliva	43	IPI00028064	Tax_Id=9606 Gene_Symbol=CTSG Cathepsin G precursor	747	29161	20	34,9	1
Lacrima	116	IPI00028064	Tax_Id=9606 Gene_Symbol=CTSG Cathepsin G precursor	49	29161	1	7,1	1
Saliva	170	IPI00029739	Tax_Id=9606 Gene_Symbol=CFH Isoform 1 of Complement factor H precursor	51	1E+05	1	1	1
Lacrima	104	IPI00029739	Tax_Id=9606 Gene_Symbol=CFH Isoform 1 of Complement factor H precursor	69	1E+05	2	2,8	1
Saliva	50	IPI00032293	Tax_Id=9606 Gene_Symbol=CST3 Cystatin-C precursor	627	16017	13	31,5	1
Lacrima	75	IPI00032293	Tax_Id=9606 Gene_Symbol=CST3 Cystatin-C precursor	185	16017	3	19,2	1
Saliva	36	IPI00032294	Tax_Id=9606 Gene_Symbol=CST4 Cystatin-S precursor	882	16489	30	71,6	1
Lacrima	37	IPI00032294	Tax_Id=9606 Gene_Symbol=CST4 Cystatin-S precursor	513	16489	16	66	1
Saliva	101	IPI00032325	Tax_Id=9606 Gene_Symbol=CSTA Cystatin-A	168	11000	4	67,3	1
Lacrima	99	IPI00032325	Tax_Id=9606 Gene_Symbol=CSTA Cystatin-A	77	11000	1	14,3	1
Saliva	17	IPI00060800	Tax_Id=9606 Gene_Symbol=LOC124220 Protein UNQ773/PRO1567 precursor	1915	22725	53	52,9	1
Lacrima	43	IPI00060800	Tax_Id=9606 Gene_Symbol=LOC124220 Protein UNQ773/PRO1567 precursor	447	22725	7	22,1	1
Saliva	18	IPI00166729	Tax_Id=9606 Gene_Symbol=AZGP1 alpha-2-glycoprotein 1, zinc	1879	34465	48	57,4	1
Lacrima	28	IPI00166729	Tax_Id=9606 Gene_Symbol=AZGP1 alpha-2-glycoprotein 1, zinc	803	34465	25	46	1
Saliva	58	IPI00178926	Tax_Id=9606 Gene_Symbol=IGJ immunoglobulin J chain	494	18543	14	55,3	1
Lacrima	50	IPI00178926	Tax_Id=9606 Gene_Symbol=IGJ immunoglobulin J chain	333	18543	8	27,7	1
Saliva	115	IPI00179330	Tax_Id=9606 Gene_Symbol=RPS27A;UBC;UBB ubiquitin and ribosomal protein S27a precursor	131	18296	4	16	1
Lacrima	83	IPI00179330	Tax_Id=9606 Gene_Symbol=RPS27A;UBC;UBB ubiquitin and ribosomal protein S27a precursor	138	18296	3	16	1

Saliva	52	IPI00217963	Tax_Id=9606 Gene_Symbol=KRT16 Keratin, type I cytoskeletal 16	602	51578	15	34,9	1
Lacrima	48	IPI00217963	Tax_Id=9606 Gene_Symbol=KRT16 Keratin, type I cytoskeletal 16	385	51578	10	25,6	1
Saliva	7	IPI00218918	Tax_Id=9606 Gene_Symbol=ANXA1 Annexin A1	2973	38918	76	62,1	1
Lacrima	68	IPI00218918	Tax_Id=9606 Gene_Symbol=ANXA1 Annexin A1	236	38918	5	18,2	1
Saliva	60	IPI00219018	Tax_Id=9606 Gene_Symbol=GAPDH Glyceraldehyde-3-phosphate dehydrogenase	470	36201	14	29	1
Lacrima	61	IPI00219018	Tax_Id=9606 Gene_Symbol=GAPDH Glyceraldehyde-3-phosphate dehydrogenase	288	36201	8	27,8	1
Saliva	69	IPI00219757	Tax_Id=9606 Gene_Symbol=GSTP1 Glutathione S-transferase P	355	23569	8	30,5	1
Lacrima	100	IPI00219757	Tax_Id=9606 Gene_Symbol=GSTP1 Glutathione S-transferase P	75	23569	1	8,6	1
Saliva	123	IPI00219806	Tax_Id=9606 Gene_Symbol=S100A7 Protein S100-A7	112	11564	2	11,9	1
Lacrima	102	IPI00219806	Tax_Id=9606 Gene_Symbol=S100A7 Protein S100-A7	72	11564	1	18,8	1
Saliva	14	IPI00220327	Tax_Id=9606 Gene_Symbol=KRT1 Keratin, type II cytoskeletal 1	2256	66149	69	44,9	1
Lacrima	12	IPI00220327	Tax_Id=9606 Gene_Symbol=KRT1 Keratin, type II cytoskeletal 1	2019	66149	56	39,3	1
Saliva	45	IPI00290078	Tax_Id=9606 Gene_Symbol=KRT4 keratin 4	714	64442	17	32,5	1
Lacrima	51	IPI00290078	Tax_Id=9606 Gene_Symbol=KRT4 keratin 4	327	64442	9	12,3	1
Saliva	89	IPI00291262	Tax_Id=9606 Gene_Symbol=CLU Clusterin precursor	219	53031	7	10,2	1
Lacrima	65	IPI00291262	Tax_Id=9606 Gene_Symbol=CLU Clusterin precursor	255	53031	8	12	1
Saliva	47	IPI00293665	Tax_Id=9606 Gene_Symbol=KRT6B Keratin, type II cytoskeletal 6B	672	60247	19	34,8	1
Lacrima	44	IPI00293665	Tax_Id=9606 Gene_Symbol=KRT6B Keratin, type II cytoskeletal 6B	443	60247	17	23,2	1
Saliva	107	IPI00298497	Tax_Id=9606 Gene_Symbol=FGB Fibrinogen beta chain precursor	152	56577	6	13,8	1
Lacrima	33	IPI00298497	Tax_Id=9606 Gene_Symbol=FGB Fibrinogen beta chain precursor	626	56577	18	42,4	1
Saliva	71	IPI00299547	Tax_Id=9606 Gene_Symbol=LCN2 Neutrophil gelatinase-associated lipocalin precursor	330	22745	12	44,4	1
Lacrima	63	IPI00299547	Tax_Id=9606 Gene_Symbol=LCN2 Neutrophil gelatinase-associated lipocalin precursor	282	22745	8	31,3	1
Saliva	34	IPI00305477	Tax_Id=9606 Gene_Symbol=CST1 Cystatin-SN precursor	904	16579	26	60,3	1
Lacrima	67	IPI00305477	Tax_Id=9606 Gene_Symbol=CST1 Cystatin-SN precursor	238	16579	6	38,3	1
Saliva	164	IPI0038244(	Tax_Id=9606 Gene_Symbol=- Ig lambda chain V-IV region Hil	59	11624	1	17,8	1
Lacrima	107	IPI0038244(	Tax_Id=9606 Gene_Symbol=- Ig lambda chain V-IV region Hil	59	11624	1	17,8	1
Saliva	70	IPI0038248(	Tax_Id=9606 Gene_Symbol=- Ig heavy chain V-III region BRO	344	13332	9	25	1
Lacrima	72	IPI0038248(	Tax_Id=9606 Gene_Symbol=- Ig heavy chain V-III region BRO	205	13332	6	25	1
Saliva	75	IPI00384444	Tax_Id=9606 Gene_Symbol=KRT14 Keratin, type I cytoskeletal 14	300	51875	9	22	1
Lacrima	39	IPI00384444	Tax_Id=9606 Gene_Symbol=KRT14 Keratin, type I cytoskeletal 14	490	51875	13	33,1	1
Saliva	84	IPI00387115	Tax_Id=9606 Gene_Symbol=- Ig kappa chain V-III region SIE	245	11882	10	24,8	1
Lacrima	59	IPI00387115	Tax_Id=9606 Gene_Symbol=- Ig kappa chain V-III region SIE	304	11882	7	39,4	1
Saliva	59	IPI00399007	Tax_Id=9606 Gene_Symbol=IGHG2 Putative uncharacterized protein DKFZp686I04196 (Fragment)	476	46716	18	16,5	1
Lacrima	62	IPI00399007	Tax_Id=9606 Gene_Symbol=IGHG2 Putative uncharacterized protein DKFZp686I04196 (Fragment)	285	46716	10	25,4	1
Saliva	41	IPI00412044	Tax_Id=9606 Gene_Symbol=DMBT1 143 kDa protein	790	1E+05	14	9,5	1
Lacrima	36	IPI00412044	Tax_Id=9606 Gene_Symbol=DMBT1 143 kDa protein	534	1E+05	13	10,5	1
Saliva	94	IPI00418169	Tax_Id=9606 Gene_Symbol=ANXA2 annexin A2 isoform 1	202	40671	6	25,8	1
Lacrima	80	IPI00418169	Tax_Id=9606 Gene_Symbol=ANXA2 annexin A2 isoform 1	150	40671	4	13,7	1
Saliva	35	IPI00418512	Tax_Id=9606 Gene_Symbol=DMBT1 Isoform 4 of Deleted in malignant brain tumors 1 protein precursor	897	2E+05	22	13,8	1
Lacrima	21	IPI00418512	Tax_Id=9606 Gene_Symbol=DMBT1 Isoform 4 of Deleted in malignant brain tumors 1 protein precursor	1092	2E+05	31	18,9	1
Saliva	8	IPI00419424	Tax_Id=9606 Gene_Symbol=IGKV1-5 IGKV1-5 protein	2773	26503	69	40,8	1
Lacrima	15	IPI00419424	Tax_Id=9606 Gene_Symbol=IGKV1-5 IGKV1-5 protein	1791	26503	43	40,8	1
Saliva	11	IPI0043082(	Tax_Id=9606 Gene_Symbol=IGKV1-5 IGKV1-5 protein	2551	26034	67	40	1
Lacrima	16	IPI0043082(	Tax_Id=9606 Gene_Symbol=IGKV1-5 IGKV1-5 protein	1683	26034	43	40	1
Saliva	31	IPI00453473	Tax_Id=9606 Gene_Symbol=HIST1H4A;HIST1H4K;HIST1H4J;HIST1H4D;HIST1H4E;HIST1H4H;HIST1H4C;HIST4H4;HIST1H4I;HIST1H4F;HIST	957	11360	25	52,4	1
Lacrima	122	IPI00453473	Tax_Id=9606 Gene_Symbol=HIST1H4A;HIST1H4K;HIST1H4J;HIST1H4D;HIST1H4E;HIST1H4H;HIST1H4C;HIST4H4;HIST1H4I;HIST1H4F;HIST	42	11360	1	11,7	1
Saliva	180	IPI00465028	Tax_Id=9606 Gene_Symbol=TPI1 Isoform 1 of Triosephosphate isomerase	41	31057	1	4,5	1
Lacrima	101	IPI00465028	Tax_Id=9606 Gene_Symbol=TPI1 Isoform 1 of Triosephosphate isomerase	73	31057	1	4,5	1
Saliva	109	IPI00478003	Tax_Id=9606 Gene_Symbol=A2M Alpha-2-macroglobulin precursor	143	2E+05	5	4,9	1
Lacrima	52	IPI00478003	Tax_Id=9606 Gene_Symbol=A2M Alpha-2-macroglobulin precursor	326	2E+05	12	10,4	1
Saliva	87	IPI00549291	Tax_Id=9606 Gene_Symbol=IGHM IGHM protein	223	66998	7	18,8	1
Lacrima	49	IPI00549291	Tax_Id=9606 Gene_Symbol=IGHM IGHM protein	348	66998	14	26,1	1
Saliva	54	IPI0055064(	Tax_Id=9606 Gene_Symbol=IGHG4 IGHG4 protein	594	52637	17	17,5	1
Lacrima	45	IPI0055064(	Tax_Id=9606 Gene_Symbol=IGHG4 IGHG4 protein	427	52637	8	17,8	1
Saliva	63	IPI00553177	Tax_Id=9606 Gene_Symbol=SERPINA1 Isoform 1 of Alpha-1-antitrypsin precursor	432	46878	13	30,9	1
Lacrima	69	IPI00553177	Tax_Id=9606 Gene_Symbol=SERPINA1 Isoform 1 of Alpha-1-antitrypsin precursor	231	46878	6	16,7	1
Saliva	9	IPI00744561	Tax_Id=9606 Gene_Symbol=IGHA1 IGHA1 protein	2674	54327	73	53,8	1
Lacrima	5	IPI00744561	Tax_Id=9606 Gene_Symbol=IGHA1 IGHA1 protein	2784	54327	64	50,6	1
Saliva	186	IPI00745363	Tax_Id=9606 Gene_Symbol=- Immunglobulin heavy chain variable region (Fragment)	34	14555	1	6,9	1
Lacrima	119	IPI00745363	Tax_Id=9606 Gene_Symbol=- Immunglobulin heavy chain variable region (Fragment)	46	14555	1	6,9	1
Saliva	6	IPI00745872	Tax_Id=9606 Gene_Symbol=ALB Isoform 1 of Serum albumin precursor	3596	71317	96	67,7	1
Lacrima	8	IPI00745872	Tax_Id=9606 Gene_Symbol=ALB Isoform 1 of Serum albumin precursor	2580	71317	71	59,4	1
Saliva	83	IPI00783024	Tax_Id=9606 Gene_Symbol=- Myosin-reactive immunoglobulin heavy chain variable region (Fragment)	251	14247	6	19,8	1
Lacrima	77	IPI00783024	Tax_Id=9606 Gene_Symbol=- Myosin-reactive immunoglobulin heavy chain variable region (Fragment)	169	14247	4	19,8	1
Saliva	85	IPI00783987	Tax_Id=9606 Gene_Symbol=C3 Complement C3 precursor (Fragment)	232	2E+05	5	2,6	1
Lacrima	20	IPI00783987	Tax_Id=9606 Gene_Symbol=C3 Complement C3 precursor (Fragment)	1128	2E+05	35	21,5	1
Saliva	12	IPI00783993	Tax_Id=9606 Gene_Symbol=IGHA2 IGHA2 protein	2433	52511	65	50,1	1
Lacrima	11	IPI00783993	Tax_Id=9606 Gene_Symbol=IGHA2 IGHA2 protein	2134	52511	49	46,8	1
Saliva	13	IPI0078483(	Tax_Id=9606 Gene_Symbol=- CDNA FLJ41981 fis, clone SMINT2011888, highly similar to Protein Tro alpha1 H,myeloma	2416	53810	64	49,5	1
Lacrima	10	IPI0078483(	Tax_Id=9606 Gene_Symbol=- CDNA FLJ41981 fis, clone SMINT2011888, highly similar to Protein Tro alpha1 H,myeloma	2189	53810	51	46,2	1
Saliva	42	IPI0078520(	Tax_Id=9606 Gene_Symbol=- Putative uncharacterized protein	762	25350	32	29,7	1
Lacrima	35	IPI0078520(	Tax_Id=9606 Gene_Symbol=- Putative uncharacterized protein	569	25350	20	33,1	1
Saliva	4	IPI00788271	Tax_Id=9606 Gene_Symbol=LOC728320 similar to lactotransferrin	3820	57494	94	69,7	1
Lacrima	2	IPI00788271	Tax_Id=9606 Gene_Symbol=LOC728320 similar to lactotransferrin	6333	57494	167	78,7	1
Saliva	103	IPI0081679(	Tax_Id=9606 Gene_Symbol=- Rheumatoid factor D5 light chain (Fragment)	163	12872	5	22,9	1
Lacrima	79	IPI0081679(	Tax_Id=9606 Gene_Symbol=- Rheumatoid factor D5 light chain (Fragment)	162	12872	4	15,3	1
Saliva	112	IPI00828062	Tax_Id=9606 Gene_Symbol=- Anti-(ED-B) scFV (Fragment)	136	25357	6	11,8	1
Lacrima	54	IPI00828062	Tax_Id=9606 Gene_Symbol=- Anti-(ED-B) scFV (Fragment)	312	25357	8	23,1	1
Saliva	38	IPI00829711	Tax_Id=9606 Gene_Symbol=IGHA2 Uncharacterized protein IGHA2 (Fragment)	852	43202	31	39,9	1
Lacrima	29	IPI00829711	Tax_Id=9606 Gene_Symbol=IGHA2 Uncharacterized protein IGHA2 (Fragment)	792	43202	23	35,8	1
Saliva	2	IPI00848342	Tax_Id=9606 Gene_Symbol=LTF Lactotransferrin precursor	5158	80014	140	69,6	1
Lacrima	1	IPI00848342	Tax_Id=9606 Gene_Symbol=LTF Lactotransferrin precursor	8520	80014	233	80,4	1

Chapter 5: Further exploration of Proteomics for clinical tear analysis.

5.0 Introduction: Entering the Orbitrap age.

Three years after our initiation to proteomics (Chapter 4) we got access to a newer type of mass spectrometer, which we were keen to evaluate for its performance in clinical tear analysis. Meanwhile, we had been working on improvements of the tear collection procedure, making it more amenable to the ophthalmological daily practice. We selected to sample donor or patient tears no longer with glass capillaries but with Schirmer strips, which are very well accepted for clinical monitoring of DED.
As mentioned in chapter 1, Schirmer strips allow prelevation of tears with minimal irritation (even without the prior instillation of anaesthetics).

Our experiments demonstrate that only 20 µl collected on a Schirmer strip are sufficient for multiple analyses of single patient tears, thus without the need to pool samples.
This is extremely useful when one intends to evaluate intra-individual changes in protein composition under different conditions, such as there are:
- technical duplicates/triplicates of the same sample;
- changes with time (diurnal, age, …);
- effects of clinical treatments;
- correlations with patient-specific DED severity measurements (Chapter 1).

It is interesting to remark here that the bottom-up analysis data reported in Chapter 5.1 seem to further support the positive effects of our transplantation of labial salivary glands. When considering the panel of 4 biomarker proteins the absence of which can be correlated with dry eye disease as described by the well-known Singapore Eye Research Institute (Beuerman group) [Zhou et al., 2009]. Whereas none of these biomarkers were detectable in the untreated diseased state, they partly seem to get restored after the transplantation.

In addition, we want to remark here that using these modern MS systems, all our previously obtained tear protein identifications obtained by QTOF GeLC MSMS could be confirmed.

Reference

Zhou L, Zhao SZ, Koh SK, Chen L, Vaz C, Tanavde V, Li XR, Beuerman RW (2012) In-depth analysis of the human tear proteome. J. Proteomics 75: 3877-3885.

5.1 Bottom-up protein identifications from microliter quantities of individual human tear samples. Important steps towards clinical relevance.

[P. Raus, B. Raghuraman Kumar, M. Pinkse, P. Verhaert (2015) EuPA Open Proteomics 9: 8-13.]

EuPA Open Proteomics 9 (2015) 8–13

Contents lists available at ScienceDirect

EuPA Open Proteomics

journal homepage: www.elsevier.com/locate/euprot

Bottom–up protein identifications from microliter quantities of individual human tear samples. Important steps towards clinical relevance.

Peter Raus[a,b], Bharath Raghuraman Kumar[a], Martijn Pinkse[a], Peter Verhaert[a,c,*]

[a] Department of Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands
[b] Miró, Centre for Ocular Surgery and Esthetics, Stationsstraat 128B, 2240 Geel, Belgium
[c] Department of Biology & Faculty of Biomedical, Pharmaceutical and Veterinary Sciences, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium

ARTICLE INFO

Article history:
Received 17 March 2015
Received in revised form 17 June 2015
Accepted 19 June 2015
Available online 29 June 2015

Keywords:
Human tears
Schirmer strip sampling
Quadrupole Orbitrap
Bottom–up protein identification
Individual sample analysis
Clinical applicability

ABSTRACT

A relatively simple combination of Schirmer strip sampling with straightforward sensitive nanoLC quadrupole-Orbitrap tandem mass spectrometry after a minimum of sample processing steps allows for replicate proteomic analysis of single human tears, i.e., without the requirement for sample pooling. This opens the way to clinical applications of the analytical workflow, e.g., to monitor disease progression or treatment efficacy within individual patients. Proof of concept is provided by triplicate analyses of a singular sampling of tears of a dry eye patient, before and one and two months after minor salivary gland transplantation. To facilitate comparison with the outcome of previously reported analytical protocols, we also include the data from a typical healthy young adult tear sample as obtained by our streamlined method.

With 375 confidently identified proteins in the healthy adult tear, the obtained results are comprehensive and in large agreement with previously published observations on pooled samples of multiple patients. We conclude that, to a limited extent, bottom–up tear protein identifications from individual patients may have clinical relevance.

© 2015 The Authors. Published by Elsevier GmbH. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

Since the recent launch, within the Human Proteome Organization (HUPO), of the Human Eye Proteome Project (HEPP) [10,8], tears are among the body fluids which have gained increasing interest as a source of diagnostic markers not only for ophthalmological diseases, but also for systemic and neurological disorders. Whereas accounts of proteins identified from human tears all resulted from the analyses of pooled samples (see advanced LC MS/MS reports by de Souza et al. [3]; Zhou et al. [15]; Srinivasan et al. [12]; Salvisberg et al. [11]; and the references quoted therein to other earlier (and generally less performant) mass spectrometry based proteomics approaches), we focus on what can be achieved by studying individual tear samples with the latest LC MS/MS.

In order to be compliant with the envisioned clinical application, we compiled an efficient analytical workflow with minimal sample preparation steps.

We opted for Schirmer strips (instead of capillaries) as most convenient clinician friendly tear sampling tools. On these filter paper strips >20 μl volumes of tear can be easily collected. This minimally invasive form of body fluid collection is highly accepted in the primary healthcare setting and has great potential for use in health screening [9]. As such it is already common use in current ophthalmological practice, e.g., for testing the severity of dry eye disease.

Employing straightforward nanoLC tandem MS by a recently introduced high resolution quadrupole-Orbitrap hybrid system, we demonstrate that it is realistically feasible to perform multiple replicate proteomic analyses (in terms of bottom–up protein identifications) on these microliter sample quantities.

As such the overall sensitivity of this optimized analytical protocol permits intra-individual (unpooled) monitoring of e.g., disease progression or treatment, with several hundreds of relevant data points (protein identifications and relative quantitations) collected for each clinical sample.

* Corresponding author at: Department of Biotechnology, Analytical Biotechnology & Innovative Peptide Biology Group, Delft, The Netherlands.
E-mail address: p.d.e.m.verhaert@tudelft.nl (P. Verhaert).

http://dx.doi.org/10.1016/j.euprot.2015.06.005
1876-3820/© 2015 The Authors. Published by Elsevier GmbH. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

As proof-of-concept we monitored tears of a severe case of keratoconjunctivitis sicca before and after surgical treatment. Keratoconjunctivitis sicca, or dry eye syndrome is a very complex multifactorial disease [7], which, as the name indicates, in virtually all cases results in reduced tear volume production, which is reported to be associated with a decreased general lacrimal protein secretion. Very severe cases are uniquely treated by autotransplantation of a minor salivary gland into the eye, a technique originally introduced by Prof. J. Murube and perfectionized over the past ten years [5]. 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.

2. Materials and methods

2.1. Reagents and chemicals

Ammonium bicarbonate, *tris*(2-carboxyethyl)phosphine (TCEP), iodoacetamide, formic acid, dimethyl sulphoxide, as well as trypsin were from Sigma–Aldrich™.

2.2. Tear collection and sample preparation

Human lacrimal fluids were sampled using Schirmer tear test strips (Haag-Streit, UK), principally as published earlier by Zhou et al. [15] and Srinivasan et al. [12]. For this the paper strip was tenderly placed inside the lower eyelid, after which the subject was instructed to gently close the eye. The moistened strip was removed after a maximum of 5 min. The sampling procedure did not include any anesthetizing eye drops. Both during sampling as well as further strip handling, gloves were worn.

Two different individuals provided the tear samples used in this study (Table 1). Tear samples from an individual diagnosed with severe dry eye syndrome (aqueous deficiency subtype) were collected (with the patient's consent) at 3 different time points during disease treatment, i.e., before treatment, and 1, and 2 months after surgery (minor salivary gland transplantation). Consistent with the data in the literature [12] the aqueous deficient dry eye typically scored <5 mm of Schirmer strip wetting. For comparative purposes one additional tear sampling of a healthy young adult male volunteer was included in this method evaluation study. Healthy adult tears have been consistently analyzed by the relevant proteomics methods described in the literature [3,15,12,11]. The healthy tear easily moistened >15 mm during sampling.

After sampling, strips were stored in labeled protease-free Eppendorf vials at −20 °C until further analysis. For analysis 2 mm of the wetted part of the filter paper area which had not been in direct contact with the eye ball and conjunctiva (in order to minimize sample contamination with epithelial proteins) was carefully cut from each strip. During sample processing, care was taken to keep the analysis volume to an absolute minimum, to remain maximally compatible with the limited nanoLC injection volume. After transfer to another protease-free microcentrifuge tube the 2 mm ribbon was carefully cut into minute equally sized pieces using clean scissors. The resulting shreds were submerged in 47 μl of 25 mM ammonium bicarbonate (pH 8.0) for 90 min. Reduction of disulfide bonds was achieved by mixing 1 μl of TCEP (×50 stock solution; 10 mM final) with the sample for 30 min. Subsequent alkylation was allowed to occur for 45 min after addition of 1 μl iodoacetamide (×50 stock solution; 20 mM final). Finally overnight protein digestion (RT) was initiated by adding trypsin (sequencing grade; 1 μl of 200 ng/μl stock). Afterwards 5 μl of a mixture of 5% DMSO and 5% formic acid were added to assist resuspension of tryptic peptides.

Table 1
Tear sample donor list (same color row indicates that sample originated from the same individual).

Sample code	R/L	Sex	Age	Clinical origin
Y	R	M	26	Healthy volunteer
59	R	M	69	Dry eye patient (untreated eye)
60	L	M	69	Dry eye patient (treated eye, 1 month after surgery)
61	L	M	69	Dry eye patient (treated eye, 2 months after surgery)

Table 2
Gradient elution profile (A: Milli Q; B: 80% acetonitrile, 0.1% formic acid).

Time (min)	Flow (nl/min)	% A	% B
0	350	98	2
40	350	70	30
57	350	35	65
59	350	0	100
60	350	0	100

2.3. Sample analysis

Of each sample 5% of the total reaction volume (2.5 μl) were analyzed by Easy-nLC 1000™ ultra performance liquid chromatography on a 200 mm long in-house packed C18 nano HPLC column (50 μm ID). A 60 min elution gradient (solvent B: 80% acetonitrile, 0.1% formic acid; solvent A: Milli Q; 350 nl/min) was applied as detailed in Table 2.

Tandem MS analysis was carried out on a Q Exactive Plus™ quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptide fragmentation was by high energy collision induced dissociation (HCD), with the MS^2 settings are summarized in Table 3.

2.4. Data analysis

Spectral files generated (Xcalibur™, RAW format) were analyzed using Proteome Discoverer™ software version 1.4. Multiply charged peptide spectra were deconvoluted to singly charged spectra and deisotoped. The spectral files were then searched against the Uniprot *Homo sapiens* reference proteome (UP000005640; release Oct, 2014) using the Sequest™ HT algorithm (Thermo Fisher Scientific; parameters see Table 4).

Table 3
Full scan MS and data dependent MS/MS settings of the Q Exactive Plus™ system. Abbreviations: AGC, automatic gain control; dd, data dependent; IT, injection time; NCE, normalized collision energy.

Properties of full scan/dd-MS^2	
Resolution full MS	70,000
AGC target full MS	3e6
Maximum IT	250 ms
Scan range	300-1400 *m/z*
Loop count	10
dd resolution	15,000
dd target	1e5
dd-MS^2 max IT	150 ms
Isolation window	2.5 *m/z*
Fixed first mass	100.0 m/z
NCE	28
dd underfill ratio	0.5%
Charge exclusion	Unassigned, 1, >8
Peptide match	Preferred
Dynamic exclusion	30 s

Table 4
SEQUEST HT parameters.

Database	Uniprot *Homo sapiens* proteins (Oct. 2014 release)
Enzyme	Trypsin (full)
Missed cleavages	2
Precursor mass tolerance	5 ppm
Fragment mass tolerance	0.02 Da
Dynamic N-terminal modification	Gln-> pyro-Glu
Dynamic C-terminal modification	Amidation
Other dynamic modification	Oxidation of H,M,W
Static modification	Carbamidomethyl C

Peptide spectral matches (PSMs) were validated using Percolator™ with a false discovery rate (FDR) <1% and a minimum cross correlation score (Xcorr) of 2 set as obligatory criteria for confident protein identification.

To reveal (semi) quantitative trends in tear protein abundances we employed the basic spectral counting option (summing PSMs per protein identity) available through the Proteome Discoverer™ software suite (Thermo Fisher Scientific, Bremen, Germany).

3. Results and discussion

Since only 5% of the prepared sample, corresponding to less than 0.5 μl of original tear fluid (equivalent to approximately 4 μg of total protein), was injected for each one hour LC/MSMS run, a triplicate analysis could easily be carried out for all individual samplings.

We here report the obtained number of proteins identified, as well as the (semi) quantitation values of some selected (differential) proteins primarily to illustrate the analysis depth obtainable on single (unpooled) samples. We refer to the Supplemental information for the results of all individual analyses.

We also want to mention that MALDI TOF MS data have been previously reported on individual human tears (e.g., Gonzaléz et al. [6]). These studies are typically limited to mere profiling, lacking direct protein identifications.

3.1. Healthy lacrimal fluid

3.1.1. Protein groups in healthy tears

The combined triplicate analysis yields a total of 375 identified protein groups, based on a total of 8020 peptide spectral matches (2506 unique peptides). In spite of the substantial technical variance of the overall analytical workflow (as illustrated in Fig. 1), out of these, 194 protein groups were consistently identified in all 3 separate measurements.

3.1.2. Proteins identified

The protein identifications agree well with those reported in earlier tear proteome investigations [3,12,15]. Remarkably our number of confidently detected proteins in individual tears (375) does not significantly differ from the number (386) identified from the combined pooled Schirmer strip samples in the LS MS/MS study (linear ion trap-orbitrap hybrid; [12]). In comparison the study of de Souza et al. [3] detected 491 proteins from pooled sets of capillary collected tears, after combining different sample preparation methods, including in gel and in solution digestion. The study by Zhou et al. reported the largest number of human tear proteins so far (1543), by combining the results of the analysis of the equivalent of 400 μg of pooled tears by multidimensional HPLC (SCX followed by nanoRP) on a latest generation quadrupole-TOF hybrid. More valuable than mere protein numbers, are the protein identities and (relative) quantities, and with regard to this, our results are highly comparable with the literature studies

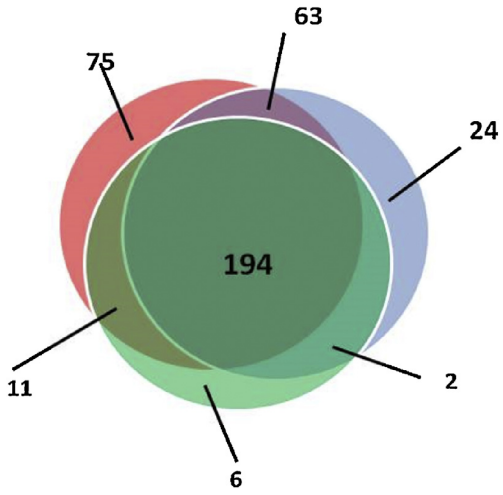


Fig. 1. Numbers of protein groups identified by nanoUHPLC quadrupole-Orbitrap MSMS from sub-microliter tear volumes of healthy human volunteer. Triplicate analysis of a single unpooled individually collected tear sample, yielding an overall protein number of 375, with 194 proteins identified consistently in all separate measurements.

referenced above. We consistently identified the most abundant tear proteins with the largest number of peptides. As an illustration proteins identified with >100 peptide spectral matches (PSMs) are listed in Table 5. The full lists of protein identifications in all triplicate samples are included as Supplemental data.

3.2. Diseased lacrimal fluids

3.2.1. Protein groups in patient tears

The untreated condition yielded 126 protein groups (2817 PSMs; 788 unique peptides), the one and two month treated samples yielded respectively 161 (4261 PSMs; 1162 unique peptides) and 135 protein groups (3605 PSMs; 894 unique peptides). Considering that these tear analyses were performed on equal volumes of material (equal size wetted Schirmer strip surface areas) as for the healthy samples, these data suggest markedly less (minus 50%) identifiable proteins in the diseased tear. Whether or not this lower number of tear protein identifications is due to an effect of the age difference between the donor of the healthy tear sample and the patient (the amount of tear proteins is known to decrease with age [14]), to a direct effect of the disease, or to potential disease-specific PTMs which were not considered in our typical database search (Table 4), is not assessed by this study. It shall be clear that for such inter-individual comparisons a large cohort of properly matched (sex, age, race, . . .) donors need to be investigated.

Rather than comparing different individuals, it is much more appropriate to compare protein compositions within the very same subject, as we did for the dry eye patient under investigation, the tears of whom were analysed before and at two time points after surgical treatment. Thanks to the fact that following the described workflow, a satisfactory analysis depth can be achieved from unpooled, individual donor samples (see above, healthy young adult male), such intra-individual comparisons now become feasible and yield meaningful results. Although the label-free method for relative protein quantitations has its clear limitations in terms of accuracy, it is clear that this simple cost-effective and sample saving approach does have its merit (see Ref. [4], especially when the purpose is to uncover relative protein abundance trends rather than to reveal accurate up- or downregulation factors. In this respect we find it interesting to note that proteins which, by label-free quantitative evaluation, appear originally

Table 5
Most abundant proteins (identified with >100 peptide spectral matches, PSMs) in individual tear sample collected from healthy young adult male volunteer.

Accession	Description	Sum (coverage)	Sum (# proteins)	Sum (# unique peptides)	Sum (# peptides)	Sum (# PSMs)
P61626	Lysozyme C OS = Homo sapiens GN = LYZ PE = 1 SV = 1—[LYSC_HUMAN]	84.46%	1	26	26	433
Q9GZZ8	Extracellular glycoprotein lacritin OS = Homo sapiens GN = LACRT PE = 1 SV = 1—[LACRT_HUMAN]	43.48%	1	14	14	384
E7ER44	Lactoferrin (Kaliocin-1) OS = Homo sapiens GN = LTF PE = 2 SV = 1—[E7ER44_HUMAN]	74.29%	2	64	64	284
P31025	Lipocalin-1 OS = Homo sapiens GN = LCN1 PE = 1 SV = 1—[LCN1_HUMAN]	78.41%	1	22	22	234
P60709	Actin, cytoplasmic 1 OS = Homo sapiens GN = ACTB PE = 1 SV = 1—[ACTB_HUMAN]	64.53%	2	11	27	217
Q5T8M8	Actin, alpha skeletal muscle OS = Homo sapiens GN = ACTA1 PE = 2 SV = 1—[Q5T8M8_HUMAN]	45.64%	4	5	22	189
P14618	Pyruvate kinase PKM OS = Homo sapiens GN = PKM PE = 1 SV = 4—[KPYM_HUMAN]	64.03%	2	34	34	150
P06733	Alpha-enolase OS = Homo sapiens GN = ENO1 PE = 1 SV = 2—[ENOA_HUMAN]	65.67%	1	37	37	136
P14555	Phospholipase A2, membrane associated OS = Homo sapiens GN = PLA2G2A PE = 1 SV = 2—[PA2GA_HUMAN]	71.53%	1	23	23	125
P04083	Annexin A1 OS = Homo sapiens GN = ANXA1 PE = 1 SV = 2—[ANXA1_HUMAN]	80.64%	1	32	32	122
Q09666	Neuroblast differentiation-associated protein AHNAK OS = Homo sapiens GN = AHNAK PE = 1 SV = 2—[AHNK_HUMAN]	29.86%	1	52	53	110
Q99935	Proline-rich protein 1 OS = Homo sapiens GN = PROL1 PE = 1 SV = 2—[PROL1_HUMAN]	33.06%	1	8	8	104

downregulated in the untreated diseased state, (re) appear in the tear with treatment. Unraveling the exact medical/physiological background of what these observations entail is not the scope of this paper, but interestingly, several additional agreements with earlier published semiquantative data are remarkable.

For example, Zhou et al. [14] described a biomarker panel of proteins the level of which can be correlated with dry eye disease. Although it should be clear that, when considering the number of representative peptides reflecting the protein presence, an inter-individual comparison of the present limited data set (we here show only a single non-age matched healthy sample) cannot be conclusive, it is interesting to note that all 4 biomarker proteins which Zhou reports as downregulated in dry eye disease (i.e., lysozyme, lactotransferrin, lipocalin and prolactin-inducible

Table 6
Lysosyme C (P61626, LYSC_HUMAN), lactotransferrin (E7ER44, LTF_HUMAN); lipocalin (P31025, LCN1_HUMAN); prolactin-inducible protein (P12273, PIP_HUMAN) spectral countings. Note that same color row indicates that sample originated from same individual: comparisons between pink rows thus represent intra-individual comparisons, whereas any comparison with grayish-green column is an intra-individual (not age-matched) comparison.

Sample	UniProt accession E7ER44	Sum (coverage)	Sum (# proteins)	Sum (# unique peptides)	Sum (# peptides)
Healthy	Lactotransferrin (Kaliocin-1) OS = Homo sapiens GN = LTF PE = 2 SV = 1—[E7ER44_HUMAN]	74.29%	2	64	64
Diseased untreated	Lactotransferrin (Kaliocin-1) OS = Homo sapiens GN = LTF PE = 2 SV = 1—[E7ER44_HUMAN]	65.68%	2	63	63

Sample	UniProt accession P31025	Sum (coverage)	Sum (# proteins)	Sum (# unique peptides)	Sum (# peptides)	Sum (# PSMs)
Healthy	Lipocalin-1 OS = Homo sapiens GN = LCN1 PE = 1 SV = 1—[LCN1_HUMAN]	78.41%	1	22	22	234
Diseased untreated	Lipocalin-1 OS = Homo sapiens GN = LCN1 PE = 1 SV = 1—[LCN1_HUMAN]	60.23%	1	19	19	89
One month treated	Lipocalin-1 OS = Homo sapiens GN = LCN1 PE = 1 SV = 1—[LCN1_HUMAN]	78.41%	1	22	22	130
Two months treated	Lipocalin-1 OS = Homo sapiens GN = LCN1 PE = 1 SV = 1—[LCN1_HUMAN]	60.23%	1	19	19	131

Sample	UniProt accession P12273	Sum (coverage)	Sum (# proteins)	Sum (# unique peptides)	Sum (# peptides)	Sum (# PSMs)
Healthy	Prolactin-inducible protein OS = Homo sapiens GN = PIP PE = 1 SV = 1—[PIP_HUMAN]	19.86%	1	2	2	7
Diseased untreated	Prolactin-inducible protein OS = Homo sapiens GN = PIP PE = 1 SV = 1—[PIP_HUMAN]	ND	ND	ND	ND	ND
One month treated	Prolactin-inducible protein OS = Homo sapiens GN = PIP PE = 1 SV = 1—[PIP_HUMAN]	19.86%	1	3	3	9
Two month treated	Prolactin-inducible protein OS = Homo sapiens GN = PIP PE = 1 SV = 1—[PIP_HUMAN]	7.53%	1	2	2	4

Sample	UniProt accession P61626	Sum (coverage)	Sum (# proteins)	Sum (# unique peptides)	Sum (# peptides)	Sum (# PSMs)
Healthy	Lysozyme C OS = Homo sapiens GN = LYZ PE = 1 SV = 1—[LYSC_HUMAN]	84.46%	1	26	26	433
Diseased	Lysozyme C OS = Homo sapiens GN = LYZ PE = 1 SV = 1—[LYSC_HUMAN]	81.08%	1	18	18	142
One month treated	Lysozyme C OS = Homo sapiens GN = LYZ PE = 1 SV = 1—[LYSC_HUMAN]	84.46%	1	28	28	442
Two months treated	Lysozyme C OS = Homo sapiens GN = LYZ PE = 1 SV = 1—[LYSC_HUMAN]	84.46%	1	28	28	487

Table 7
Protein S100-A9 (P06702, S10A9_HUMAN) spectral countings.

Sample	UniProt Accession P06702	Sum (coverage)	Sum (# proteins)	Sum (# unique peptides)	Sum (# peptides)	Sum (# PSMs)
Healthy	Protein S100-A9 OS = Homo sapiens GN = S100A9 PE = 1 SV = 1—[S10A9_HUMAN]	66.67%	1	6	6	26
Diseased	Protein S100-A9 OS = Homo sapiens GN = S100A9 PE = 1 SV = 1—[S10A9_HUMAN]	74.56%	1	8	8	32
One month treated	Protein S100-A9 OS = Homo sapiens GN = S100A9 PE = 1 SV = 1—[S10A9_HUMAN]	42.98%	1	4	4	25
Two months treated	Protein S100-A9 OS = Homo sapiens GN = S100A9 PE = 1 SV = 1—[S10A9_HUMAN]	42.98%	1	4	4	27

Table 8
Proline-rich proteins 1 and 4 (Q99935, PROL1_HUMAN; Q16378, PROL4_HUMAN) spectral countings.

Sample	UniProt accession	Sum (coverage)	Sum (# proteins)	Sum (# unique peptides)	Sum (# peptides)	Sum (# PSMs)
Healthy	Q99935, Proline-rich protein 1 OS = Homo sapiens GN = PROL1 PE = 1 SV = 2—[PROL1_HUMAN]	33.06%	1	8	8	104
Diseased	Q99935, Proline-rich protein 1 OS = Homo sapiens GN = PROL1 PE = 1 SV = 2—[PROL1_HUMAN]	–	–	–	–	–
One month treated	Q99935, Proline-rich protein 1 OS = Homo sapiens GN = PROL1 PE = 1 SV = 2—[PROL1_HUMAN]	33.06%	1	5	5	29
Two months treated	Q99935, Proline-rich protein 1 OS = Homo sapiens GN = PROL1 PE = 1 SV = 2—[PROL1_HUMAN]	31.85%	1	5	5	39
Healthy	Q16378, Proline-rich protein 4 OS = Homo sapiens GN = PRR4 PE = 1 SV = 3—[PROL4_HUMAN]	28.36%	1	5	5	36
Diseased	Q16378, Proline-rich protein 4 OS = Homo sapiens GN = PRR4 PE = 1 SV = 3—[PROL4_HUMAN]	–	–	–	–	–
One month treated	Q16378, Proline-rich protein 4 OS = Homo sapiens GN = PRR4 PE = 1 SV = 3—[PROL4_HUMAN]	20.90%	1	2	2	9
Two months treated	Q16378, Proline-rich protein 4 OS = Homo sapiens GN = PRR4 PE = 1 SV = 3—[PROL4_HUMAN]	23.88%	1	3	3	30

protein) also appear downregulated in the untreated diseased eye compared to the healthy eye (of a considerably younger donor). Moreover. the more appropriate intra-individual comparison seems to indicate that after treatment the amounts of these proteins all show a tendency of getting restored (Table 6).

Of the 8 proteins which Zhou reported as upregulated, protein S100 A9 exhibited the corresponding trend, when comparing the number of peptides between healthy, diseased and treated eyes (Table 7).

In view of the nature of the treatment (minor salivary gland transplantation) it is noteworthy that in the untreated diseased eye, none of the typical proline rich proteins (PROL1 or PRR4) described in tears [15] were detected. Both proteins, which are also known as abundant saliva proteins [1], (re) appear in the treated eye (Table 8).

In the same context we would like to mention the trend observed for lacritin. This is known to be a potent secretagogue for the various tear glands [13], and we previously demonstrated that minor salivary glands secrete substantial amounts of lacritin [2]. This gave rise to the hypothesis that part of the success of the transplantation surgery could be due to the beneficial effect of increased lacritin concentration in the treated eye. The present data are in line with this, as they show a consistent trend when focusing on the peptide spectral matches for lacritin (Table 9). Lacritin is detected with more representative peptides in the healthy versus the dry eye tear, whereas after minor salivary gland transplantation the number of lacritin peptide spectral matches steadily increases from 1 to 2 months post surgery.

Another observation (data not detailed) is that various immunoglobulin chains appear upregulated in the untreated diseased eye (consistent with inflammation), compared to both the healthy and the treated condition.

4. Concluding remarks

It is evident that the high sensitivity and precision as well as the robustness of the analytics described above allows one to observe relevant differences in the protein composition of tears without the need for sample pooling, thus for each sampling individually. Nonetheless, it shall be clear that larger numbers of samples in

Table 9
Lacritin (Q9GZZ8, LACRT_HUMAN) spectral countings.

Sample	UniProt accession Q9GZZ8	Sum (coverage)	Sum (# proteins)	Sum (# unique peptides)	Sum (# peptides)	Sum (# PSMs)
Healthy	Extracellular glycoprotein lacritin OS = Homo sapiens GN = LACRT PE = 1 SV = 1—[LACRT_HUMAN]	43.48%	1	14	14	384
Diseased	Extracellular glycoprotein lacritin OS = Homo sapiens GN = LACRT PE = 1 SV = 1— [LACRT_HUMAN]	34.78%	1	13	13	71
One month treated	Extracellular glycoprotein lacritin OS = Homo sapiens GN = LACRT PE = 1 SV = 1—[LACRT_HUMAN]	43.48%	1	13	13	114
Two months treated	Extracellular glycoprotein lacritin OS = Homo sapiens GN = LACRT PE = 1 SV = 1— [LACRT_HUMAN]	43.48%	1	17	17	239

combination with extensive standardized (reference) libraries of identities (as well of quantities) of human tear proteins are required to enable clinicians to deduce medically or pharmaceutically pertinent information from nanoUPLC MSMS data acquired according to the above described protocol. As such this paper is not a conventional biomarker discovery study, but purely a proof-of-concept technical note that demonstrates and evaluates the analytical feasibility for identifying medically relevant proteins from individual patient samplings of clinically obtained tear fluid. We moreover show that the workflow allows a differential protein analysis in tear fluid from a single individual during clinical treatment.

Having shown this, we actually have strong reservations whether a bottom–up proteomics approach, as in the typical scientific literature cited above, i.e., semi-quantitative protein identifications per se, is appropriate to provide the physician with the type of data (biomarker (candidate) s) desired. In many cases not the mere presence or absence of a gene product, but rather the (relative) quantities of specifically modified proteoforms and/or protein fragments/peptides will hold the clinically relevant information, and these are generally not distinguishable in bottom–up analyses.

We are, therefore, currently optimizing a complementary (alternative) top–down strategy, which is likewise compatible with the Schirmer strip sampling method, and which focuses on the qualitative as well as quantitative analysis of the many endogenous tear peptides and protein fragments/isoforms which can be detected by nanoUPLC quadrupole-Orbitrap tandem MS. Instead of relying on protein identifications by database searching, the latter approach much more depends on de novo sequencing.

Acknowledgements

The authors thankfully appreciate the stimulating discussions and support by Dr. Dagmar Hoeben and Mr. Lieven De Baere (Allergan N.V., Hoeilaart, Belgium). Also the continued analytical care and assistance by Dr. Markus Kellmann, Dr. Tabiwang Arrey, and Dr. Thomas Moehring (Thermo Fisher, Bremen, Germany) are gratefully acknowledged. Finally we greatly value the encouragement and sponsorship by Mr. Zachary de Silva (http://australian-dryeye.webs.com).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.euprot.2015.06.005.

Reference

[1] F.M.L. Amado, R.P. Ferreira, R. Vitorino, One decade of salivary proteomics: current approaches and outstanding challenges, Clin. Biochem. 46 (2013) 506–517.
[2] R. Crefcoeur, P. Raus, M. Krishnan, M. Pinkse, L. Slaets, G. Laurie, N. Hellings, P. Verhaert, Quantitative comparative proteomics of human tear fluid and labial saliva provides physiological/biochemical evidence supporting salivary gland transplantation for dry eye treatment, Conference Program and Abstract Book, Taormina, Sicily (Italy), Sep. 18–21, in: D.A. Sullivan (Ed.), 7th International Conference on the Tear Film & Ocular Surface: Basic Science and Clinical Relevance, 20132013, pp. 104–105.
[3] G.A. de Souza, L.M.F. de Godoy, M. Mann, Identification of 491 proteins in the tear fluid proteome reveals a large number of proteases and protease inhibitors, Genome Biol. 7 (2006) R72.
[4] A.M. Falick, W.S. Lane, K.S. Lilley, M.J. MacCoss, B.S. Phinney, N.E. Sherman, S.T. Weintraub, H.E. Witkowska, N.A. Yates, ABRF-PRG07: advanced quantitative proteomics study, J. Biomol. Technol. 22 (2011) 21–26.
[5] G. Geerling, J. Murube, P. Raus, Minor salivary gland transplantation, in: G. Geerling, H. Brewitt (Eds), Surgery for the dry eye, Karger, Zürich, Dev. Ophthalmol. (2008) 41: 243-254.
[6] N. González, I. Iloro, J.A. Durán, F. Elortza, T. Suárez, Evaluation of inter-day and inter-individual variability of tear peptide/protein profiles by MALDI-TOF MS analyses, Mol. Vis. 18 (2012) 1572–1582.
[7] J. Murube, J. Németh, H. Höh, P. Kaynak-Hekimhan, J. Horwath-Winter, A. Agarwal, C. Baudouin, J.M. Benítez del Castillo, S. Cervenka, L. ChenZhuo, A. Ducasse, J. Durán, F. Holly, R. Javate, J. Nepp, F. Paulsen, A. Rahimi, P. Raus, O. Shalaby, P. Sieg, H. Soriano, D. Spinelli, S.H. Ugurbas, G. Van Setten, The triple classification of dry eye for practical clinical use, Eur. J. Ophthalmol. 15 (2005) 660–667.
[8] G.S. Omenn, The human eye proteome project, Proteomics 13 (2013) 2375–2376.
[9] J.H.M. Quah, L. Tong, S. Barbier, Patient acceptability of tear collection in the primary healthcare setting, Optom. Vis. Sci. 91 (2014) 452–458, doi:http://dx.doi.org/10.1097/OPX.0000000000000188.
[10] R.D. Semba, J.J. Enghild, V. Venkatraman, T.F. Dyrlund, J.E. Van Eyk, The human eye proteome project: perspectives on an emerging proteome, Proteomics 13 (2013) 2500–2511.
[11] C. Salvisberg, N. Tajouri, A. Hainard, P.R. Burkhard, P.H. Lalive, N. Turck, Exploring the human tear fluid: discovery of new biomarkers in multiple sclerosis, Prot. Clin. Appl. 8 (2014) 185–194, doi:http://dx.doi.org/10.1002/prca.201300053.
[12] S. Srinivasan, M. Thangavelu, L. Zhang, K.B. Green, K.K. Nichols, iTRAQ quantitative proteomics in the analysis of tears in dry eye patients, Invest. Ophthalmol. Vis. Sci. 53 (2012) 5052–5059, doi:http://dx.doi.org/10.1167/iovs.11-9022.
[13] T. Vijmasi, F.Y. Chen, S. Balasubbu, M. Gallup, R.L. McKown, G.W. Laurie, N.A. McNamara, Topical administration of lacritin is a novel therapy for aqueous-deficient dry eye disease, Invest. Ophthalmol. Vis. Sci. 55 (2014) 5401–5409.
[14] L. Zhou, R.W. Beuerman, C.M. Chan, S.Z. Zhao, X.R. Li, H. Yang, L. Tong, S. Liu, M. E. Stern, D. Tan, Identification of tear fluid biomarkers in dry eye syndrome using iTRAQ quantitative proteomics, J. Proteome Res. 8 (2009) 4889–4905.
[15] L. Zhou, S.Z. Zhao, S.K. Koh, L. Chen, C. Vaz, V. Tanavde, X.R. Li, R.W. Beuerman, In-depth analysis of the human tear proteome, J. Proteomics 75 (2012) 3877–3885.

Chapter 6: Modern high-performance mass spectrometry approaches for future diagnostics in the ophthalmology clinic, and beyond.

6.0 Introduction

We have recently shown that modern proteomics employing high resolution MS systems such as orbitrap based instruments enables in depth bottom-up profiling of the protein content of the human tear from single subjects (individual donors or patients; see **Chapter 5**), we became convinced that the actual use of tear protein information for clinical purposes can become reality in the future.

With this vision in mind, we set out a series of exploratory experiments to assess the very latest MS based analytical technologies for their potential clinical value in the ophthalmological practice of the future. Three of these experiments, which are all described in **this Chapter**, were selected as separate key presentations at the 2017 Congress of the American Society for Mass Spectrometry (June 2017, Indianapolis, IN, USA). This further supports the frontline nature of our experiments, contributing to novel and innovating developments in clinically oriented diagnostics.

First, we evaluated the power and performance of a recent quadrupole-orbitrap combination (QExactive Plus™) for so-called 'top-down' proteomics analysis of the human tear. A fair size comparative experiment was designed to assess both bottom-up and top-down analyses of the same tear samples collected from 6 individuals. Samples taken from right and left eye of each donor served as intra-individual biological duplicates (**Chapter 6.1**). The results suggest that top-down proteomics employing high energy collision dissociation (HCD) allows for the identification of a significant, albeit limited, number of proteins compared to the bottom-up approach. Whereas bottom-up (shotgun) proteomics identifies between 300 and 400 proteins in a human tear, as shown in chapter 5, top-down analysis of the same sample set allows for the identification of roughly a third of these proteins. The most striking finding, however, was that, between the 6 donors, the 90 proteins that were confidently 'top-down' identified were observed in no less than 770 different so-called proteoforms.

Based on the results of this initial study, we intensified our collaboration with Thermo Fisher Scientific (in particular the team of Dr. Lopez-Ferrer, San Jose, CA, USA) and evaluated the performance of today's top-of-the-bill hybrid orbitrap system, i.e. the ultraviolet photon dissociation (UVPD) enabled Fusion™ Lumos™ Tribrid™ interfaced with a Vanquish™ UHPLC (**Chapter 6.2**). Comparative evaluation of various precursor ion fragmentation strategies, including CID, HCD, EThCD and UVPD indicated that the extra energy for protein precursor ion fragmentation provided by the UV laser has considerable benefit for more efficient top-down fragmentation, and subsequent extended identification of supplementary proteoforms.

To assess the analysis speed which may realistically be achieved when clinically (top-down) profiling of human tear proteins, we tested the very same samples which were extensively analysed by the Fusion Lumos Tribrid (in **Chapter 6.2**) on a QExactive HF™ fitted with a capillary electrophoresis sample introduction device (ZipChip™ by 908 Devices). In this way, we demonstrated that high resolution proteoform MS profiling can be achieved on a benchtop instrument (the small footprint of which allows it to be positioned in the cabinet of the ophthalmologist), in, literally, less than 5 minutes (**Chapter 6.3**).

Having previously shown that the use of Schirmer strips is a highly efficient and most elegant way for clinical tear collection, we finally wanted to establish whether or not it would make

sense for such harvested human tear samples to include a heat-stabilization step for prolonged storage prior to MS proteoform analysis (Chapter 6.4). In a collaboration with the group of Dr. Neil Kelleher at Northwestern University (Chicago, IL, USA), we performed top-down analyses of a sample treated in a Stabilizor™ (Denator) immediately following Schirmer strip collection versus the same sample non-stabilized. Our results indicate that heat denaturation indeed helps to preserve specific post-translational modifications on the proteoforms during prolonged storage prior to MS analysis.

As such these pilot experiments set the stage for what we envisage as future reality in the ophthalmological analysis lab, by demonstrating what can already be achieved today, and the promise/consequences these possibilities have for future personalized diagnostics and patient treatment and care.

6.1 "Proteoformics" of the human tear: Modern orbitrap based mass spectrometry detection of an unexpected multitude of proteoforms in (human) secretory fluids.

[P.D. Verhaert, P.P. Raus, M.W. Pinkse, P. Thomas, N. L. Kelleher ASMS2017_1]

6.1.1 Abstract

We have performed a first-ever comparative analysis by hybrid orbitrap-based bottom-up and top-down proteomics on the very same human tear samples collected clinically from a variety of donors. Results are beyond expectation.
Bottom-up analysis yielded the expected results with between 300 and 400 different proteins identified in tear samples of various donors. Top-down analysis yielded "only" 90 proteins identified with a 1% FDR, i.e. approx. one third to one quarter of the bottom-up identified gene products. However, the latter analysis clearly indicated that these 90 proteins may be represented by no less than 770 different proteoforms.

6.1.2 Introduction

Less than 5 years ago [Smith et al., 2013] Neil Kelleher together with the Consortium for Top-Down Proteomics (CTDP; www.topdownproteomics.org) introduced the term 'proteoforms', to designate the specific molecular, post-translationally modified, forms of a protein product arising from a specific gene.
Today, the mass spectrometry and proteomics communities progressively become aware that top-down proteomics, which characterizes the proteoform composition rather than just establishing protein identities detectable in a biological sample, is more than 'yet another' complementary sub-proteomics discipline [Gregorich and Ge, 2014]. Biologists and clinicians alike are employing the latest proteomics approaches, and it has become increasingly clear that many, if not all, proteins which constitute the living system under investigation occur in a multitude of so-called proteoforms. Whereas formerly such proteoforms were often interpreted as being truncated breakdown products of the fully processed and translated gene transcripts, current insights reveal that not seldom the actual modified proteoforms determine whether a specific protein is biologically active or not. Using human tear fluid as an example we suggest that 'Proteoformics' can be justified as a valid one-word replacement term for top-down proteomics.

6.1.3 Materials and Methods

The experimental strategy is summarized in Fig. 1.

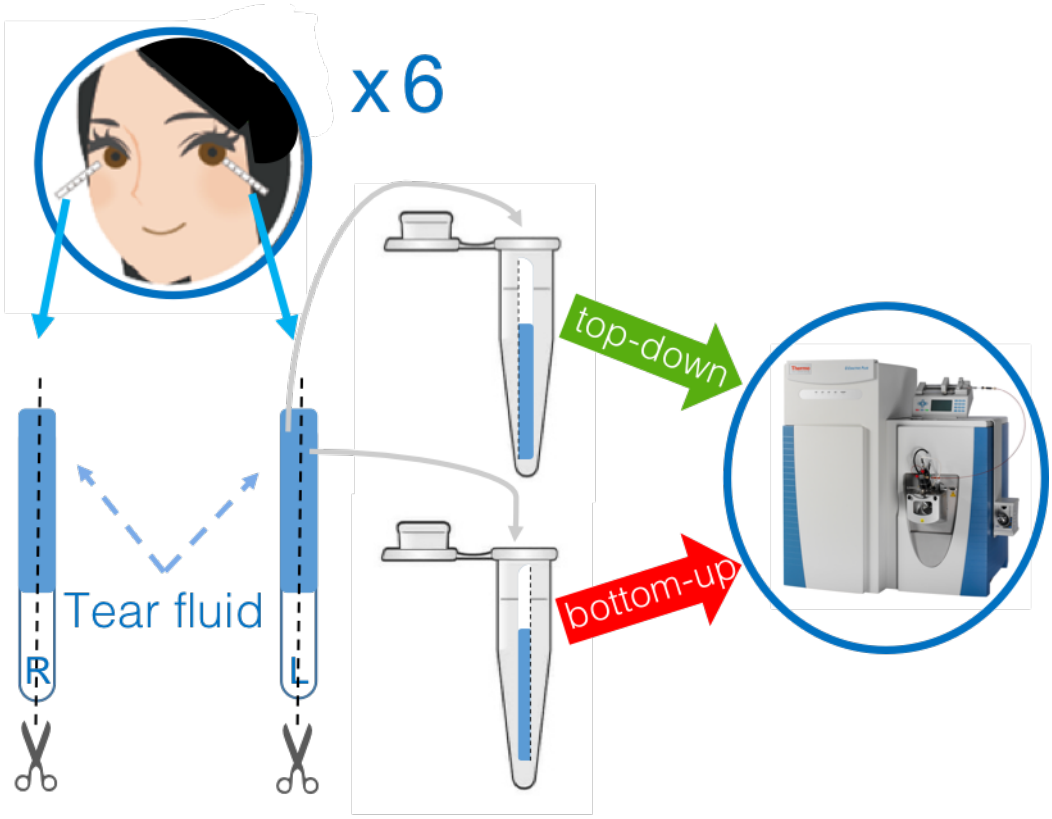


Fig. 6.1 Design of comparative proteomics experiment.

In summary, 6 genetically unrelated donors of different age and sex were asked to participate in a clinical study by donating a few tens of microliters of tear collected on Schirmer strips. The donors included 2 adult females and 4 adult males between 20 and 55 years old. Each sample was analyzed both top-down, without any sample pre-treatment, as well as bottom-up. As such 6x2 different biological samples (both left and right eye tears were handled as separate samples), were analyzed in duplicate by RP capillary LC (C4 for top-down; C18 for bottom-up) on-line to a quadrupole-orbitrap analyzer (QExactive Plus™ Thermo Fisher Scientific, Bremen, Germany).
Bottom-up analysis was basically following the protocol described before for single donor tear proteome characterization [Raus et al., 2015, **Chapter 5**].
Top-down data were searched using the TDPortal, and visualized using TDViewer (both available at http://nrtdp.northwestern.edu/resource-software/).

6.1.4 Results

Bottom-up analysis did not reveal specifically noteworthy novelties compared to our previously published analysis [Raus et al., 2015]. The top-down data, on the other hand, were remarkable in that they revealed brand new, unexpected insights.
Whereas only 90 of the more than 350 proteins identified by the bottom-up strategy were identified in the top-down approach, the information uncovered with regard to the multitude of proteoforms detected (in total no less than 770) was overwhelming.
Proteoform identification and characterization was achieved through searches of the MS and MS/MS data against the human UniProt database. Three different search modes were employed ('Tight Absolute Mass', 'BioMarker', and 'Find Unexpected Modifications') available

in TDPortal 1.3 [Savaryn et al., 2016]. The *Tight Absolute Mass search* employs a MS tolerance of 2.2 Da, and a MS/MS tolerance of 10 ppm. The *BioMarker search* has both MS and MS/MS tolerances set at 10 ppm. The *Find Unexpected Modifications search* used a MS tolerance of 200 Da, and a MS/MS tolerance of 10 ppm, which allows the detection of an unknown modification in a protein. Table 1 lists the number of identified proteoforms divided over the different donors.

The data are so rich that it is virtually impossible to show, let alone discuss all in detail. Instead, we prefer to discuss the results generally based on 3 figures (Figs. 6.2-6.4) featuring representative summarizing views on the data which are made possible through the Top-Down Viewer software, recently developed by the team of Prof. Kelleher at Northwestern University (Chicago) [http://topdownviewer.northwestern.edu/].

Sample code	L/R	# of proteoforms detected
F_{35} MV	L	173
	R	N/A
F_{40} KR	L	374
	R	340
M_{23} WH	L	368
	R	321
M_{32} MP	L	453
	R	364
M_{53} PV	L	257
	R	401
M_{55} PR	L	86
	R	N/A

Table 6.1 Number of proteoforms broken down per donor sample. Sample codes include gender (F, female; M, male) and age (subscript, in years); L/R = left/right eye tear sample; N/A = not analyzed.

Figure 6.2 shows the top 26 of the 90 top-down identified proteins, with a schematic representation (right panel) of the 21 different proteoforms associated with thymosin beta 4 (highlighted in left column), the protein which was identified with the highest overall confidence (highest top-down sequence coverage; best Q-value, a statistical metric obtained from a procedure to determine the global false discovery rate). Another example, lipocalin, one of the abundant tear proteins, was detected with a comparable number (20) of distinct proteoforms.

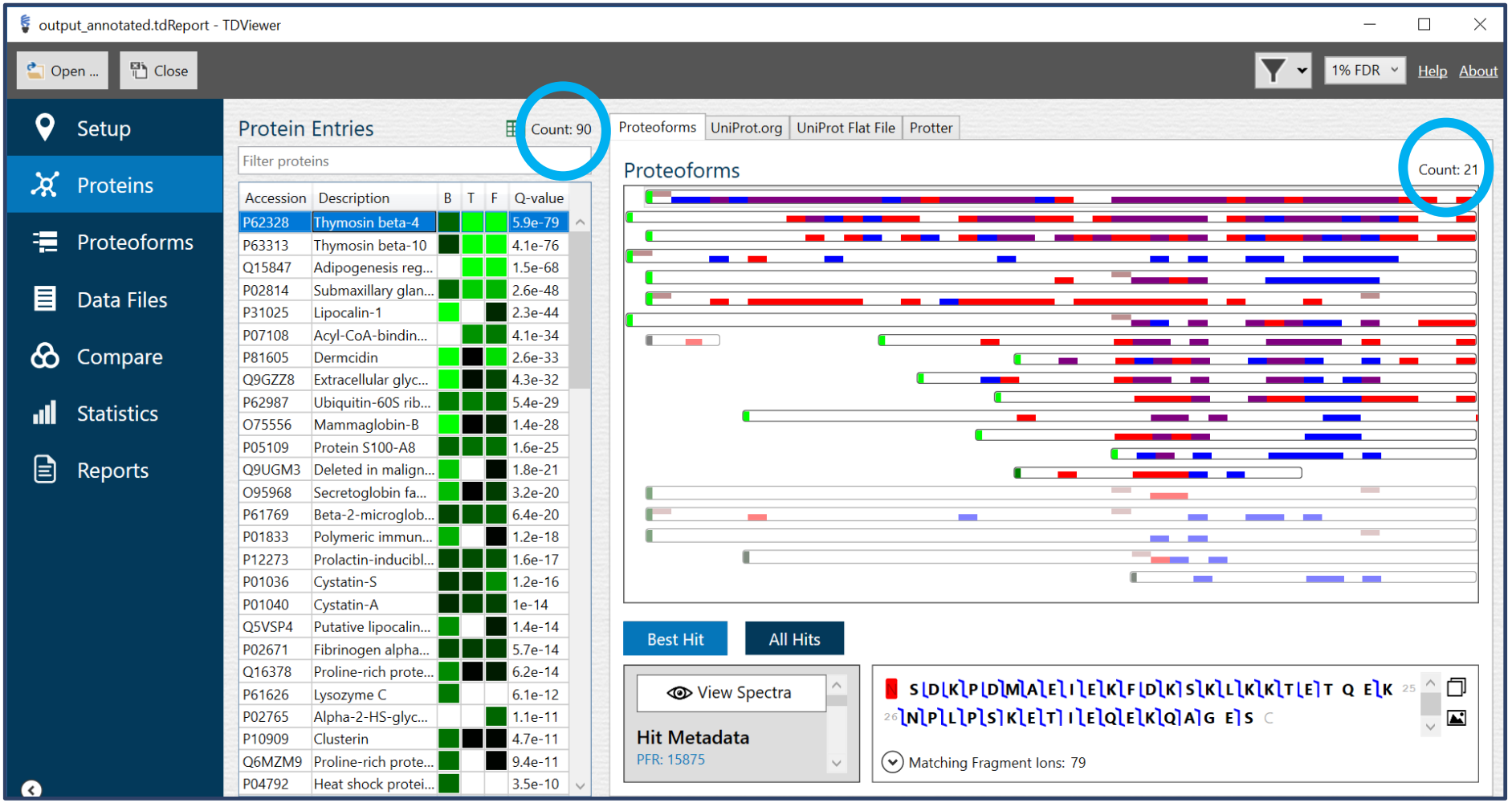


Fig. 6.2 Overall top-down data summary (TDViewer screenshot). Left column, Protein Entries displaying upper 26 of a total of 90 identified proteins, with matching database entry (UniProt accession code), and indication of confidence level (Q-value); B, T and F represent Biomarker, Tight absolute mass, and Find Unexpected Modifications search mode respectively; Right Panel, Proteoforms associated with protein entry highlighted in left column, displayed schematically as color coded bars, representing proteoform sequence matching database entry (accession code in left column), with experimentally observed protein fragment ions. Red and blue colors indicate b- and y-type ions respectively. Lower right panel shows actual amino acid sequence of 'best hit', i.e. proteoform with most complete sequence coverage (showing on top).

Only one protein detected in all samples of all different donors, was observed in a single proteoform (Fig. 6.3). This protein, i.e. adipogenesis regulating factor (UniProt accession Q15847), apparently is a rather unique example of a protein detected as its canonical sequence.

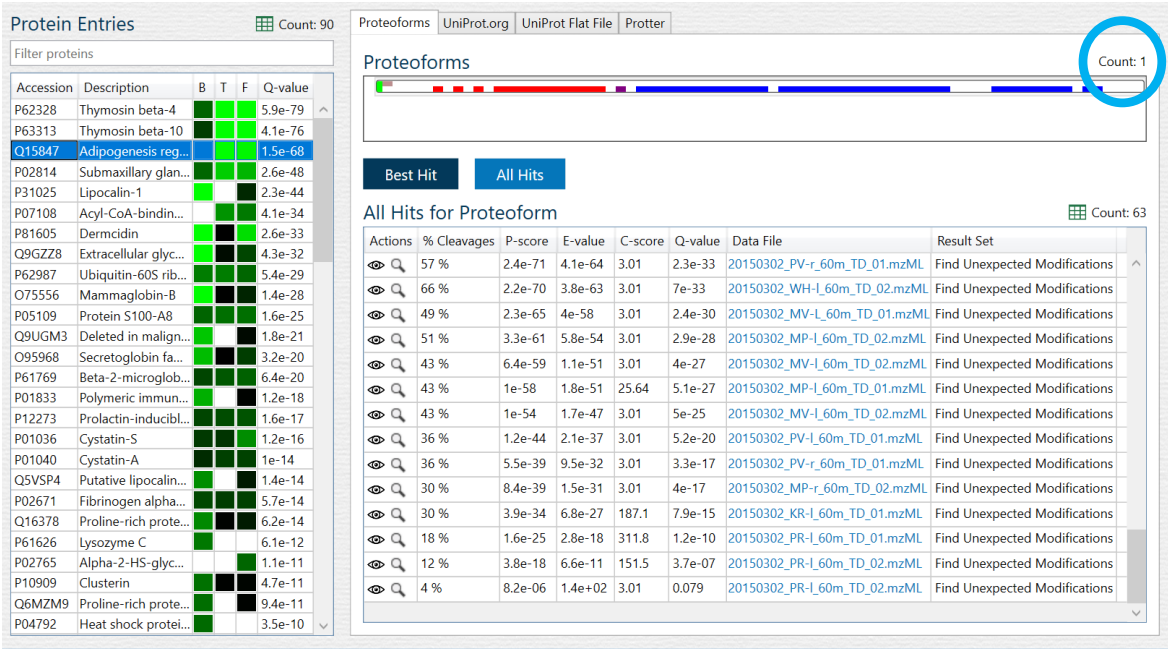


Fig. 6.3 TDViewer output showing protein identified in human tear (i.e. adipogenesis regulating factor), represented in all 6 donors by one and the very same proteoform.

Lacritin, an intensely studied tear glycoprotein because of its biological activity promoting the tear secretion by the 3 different exocrine gland types in the eye [Samudre et al., 2011; see also **Chapter 4**], was represented in the study by 176 proteoforms, distributed over the different individual 'donors' (Fig. 6.4).

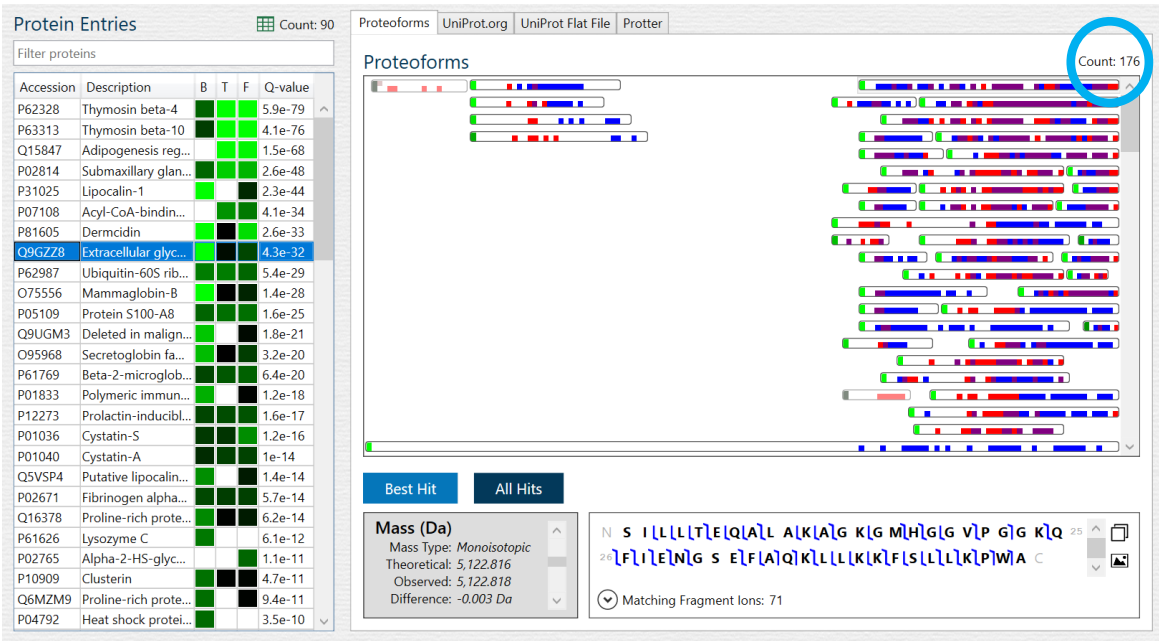


Fig. 6.4 TDViewer output showing summary of human tear lacritin (Q9GZZ8) data identified by top-down proteomics.

6.1.5 Discussion / Conclusion

With the goal of a proteomics experiment being the identification of all the proteins present in a sample, the result of a bottom-up proteomics experiment typically comprises a list containing a preferentially high number of “identified proteins”. Considering the workflow followed in a typical shotgun LC MS/MS experiment, the so-called identified proteins in such a bottom-up list represent actually not more than the identification (ID) of the respective expressed genes.

The identification of a proteoform may hold substantially more information about the detected biomolecule than the mere ID of the gene the translation of which was responsible for its synthesis. For adipogenesis regulating factor (Fig. 3) the information of our top-down analyses does not yield much extra information than a typical bottom-up result. Indeed, in all samples the same (canonical) protein sequence is observed. All other top-down identifications yield extra information compared to their bottom-up counterparts. In terms of molecular information on the identified protein (forms) in each different sample, the top-down strategy is superior to the bottom-up approach, as multiple molecular forms are detected as products from the same genes.

A considerable proportion of proteoforms were found to be common to all donors, others were found to be unique for a specific donor. When present both in the left and right eye tear sample, this observation may be the result of SNPs distributed over the genome.

Considering the sequence coverage of the top-down analysis, some striking observations were made. Some proteoforms have nearly full MSMS sequence coverage, others lack coverage in well-defined areas of the protein, not seldom rich in Cysteines, most probably involved in disulfide bridge formation. Of certain Cys rich (and other) proteins only the so-called signal peptide was detected. Hence, although there is still room for further improvement and development, a wealth of novel biological information may become uncovered by this top-down approach, which we here suggest to designate "Proteoformics".

6.1.6 References

Gregorich ZR, Ge Y (2014) Top-down proteomics in health and disease: Challenges and opportunities. Proteomics 14: 1195-1210.

Raus P, Raghuraman Kumar B, Pinkse M, Verhaert P (2015) Bottom–up protein identifications from microliter quantities of individual human tear samples. Important steps towards clinical relevance. EuPA Open Proteomics 9: 8-13.

Samudre S, Lattanzio FA, Lossen V, Hosseini A, Sheppard JD, Mc Cown RL, Laurie GW, Williams PB (2011) Lacritin, a novel human tear glycoprotein, promotes sustained basal tearing and is well tolerated. Invest. Ophthalmol. Vis. Sci. 52: 6265–6270.

Savaryn JP, Toby TK, Catherman AD, Fellers RT, LeDuc RD, Thomas PM, Friedewald JJ, Salomon DR, Abecassis MM, Kelleher NL (2016) Comparative top down proteomics of peripheral blood mononuclear cells from kidney transplant recipients with normal kidney biopsies or acute rejection. Proteomics 16: 2048-2058.

Smith LM, Kelleher NL, Linial M, Goodlett D, Langridge-Smith P, Goo AY, Safford G, Bonilla L, Kruppa G, Zubarev R, Rontree J, Chamot-Rooke J, Garavelli J, Heck A, Loo J, Penque D, Hornshaw M, Hendrickson C, Pasa-Tolic L, Borchers C, Chan D, Young N, Agar J, Masselon C, Gross M, McLafferty F, Tsybin Y, Ge Y, Sanders I, Langridge J, Whitelegge J, Marshall A (2013) Proteoform: a single term describing protein complexity. Nat. Meth. 10:186-187.

6.2 Rapid characterization of the proteoform landscape in human tear samples using top down mass spectrometry with multiple MS/MS strategies.

[D. Lopez-Ferrer, R. Huguet, D. Horn, P. Raus, V. Zabrouskov, A.F.R. Huhmer, P. Verhaert ASMS2017_2]

6.2.1 Abstract

Combination of nanoLC with a modern quadrupole-orbitrap hybrid (Q Exactive Plus™), allows for the top-down identification of several hundreds of proteoforms originating from 90 protein genes (Chapter 6.2). Here we report our preliminary data using the top-of-the-bill liquid chromatography - hybrid orbitrap mass spectrometer combination with the ultimate performance achievable today, i.e., the Orbitrap Fusion™ Lumos™ Tribrid™, equipped with a Vanquish capillary HPLC unit. The goal of the experiment was twofold: [1] to benchmark this newest high-performance orbitrap based system for top-down (tear) proteome analysis, and [2] to evaluate the ultraviolet photon dissociation (UVPD) option for additional proteoform fragmentation, and concurrent additional proteoform identification. The results indicate a superior performance of the system over any other previously employed (tear) proteoform analysis mass spectrometry method. The UVPD set-up significantly enhanced the protein/proteoform fragmentation, and, consequently, identification performance, particularly in comparison to conventional fragmentation mechanisms, including CID, HCD, ECD/ETD, or ETHCD.
The proposed research workflow for top-down proteomics to map the proteoform diversity in clinical tear fluid samples includes the use of Schirmer strips for sample collection, sample handling, and protein extraction in conjunction with various top-down fragmentation techniques, including UVPD, on a chromatographic time scale.

6.2.2 Introduction

Tear fluid maintains the health of the front of the eye and provide clear vision [Suchi et al., 2015]]. Dry eye disease is defined as the low-volume or low-quality production of tears to sufficiently lubricate and nurture the eye, and affects up to 20% of the population above age 60 [McDonnell et al., 2017]. Currently, the presence of DED is determined either by subject self-reported history, or by measuring tear flow using Schirmer strips. Here, we explore the use of top-down proteomics as a viable biomarker detection strategy for various eye related conditions / diseases.

6.2.3 Materials and Methods

Tear samples were purchased from Lee Biosolutions (Maryland Heights, MO, USA), Schirmer Tear Test strips from Clement Clarke International (Harlow, UK). Specific volumes of tear fluid were directly pipetted on Schirmer strips. No further sample treatment was performed to the samples.

Sample Preparation

LC-MS

Tear proteins extracted from the Schirmer strips in aqueous solution (5% acetonitrile in 0.1% TFA; LC solvent A) were directly analyzed by LC-MS using an Orbitrap Fusion™ Tribrid™ mass spectrometer (Thermo Scientific™, San Jose, CA, USA) modified with 'UVPD' (Fig. 6.5),

ultraviolet photodissociation capacity, a novel way of high-energy precursor ion fragmentation [Cleland et al., 2017].
Sample introduction was achieved by a Thermo Scientific™ Vanquish™ UHPLC pump system via autosampler into the Thermo Scientific™ MAbPac™ RP 2.1x100 mm column hyphenated to a Orbitrap Fusion™ Tribrid™. MS^1 and MS^2 scans were acquired at 120k resolving power.

Data Analysis
Raw files were analyzed using Proteome Discoverer™ 2.2 software (Thermo Fisher Scientific) using the ProsightPD™ and Protein Center™ nodes.

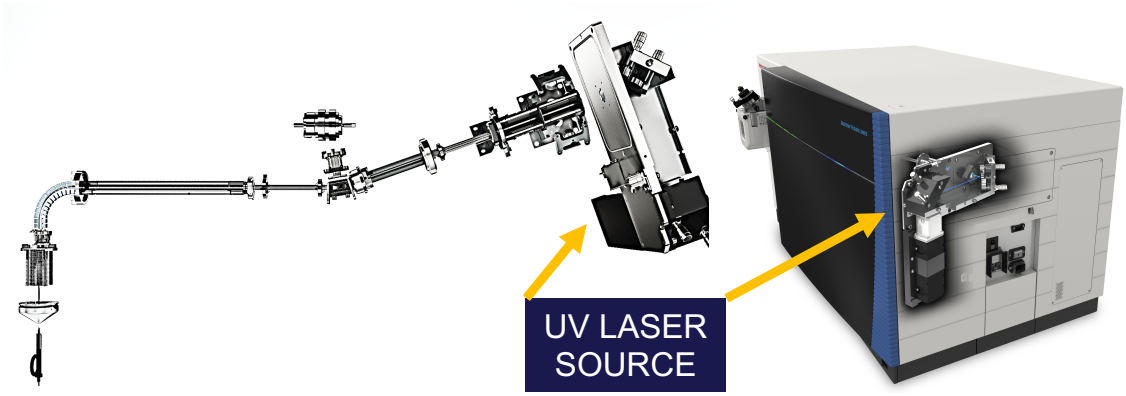


Fig. 6.5 Orbitrap Fusion Lumos Tribrid (schematic) with UV laser source allowing UVPD capabilities. Scheme shows different hardware parts including UVPD source

MS/MS acquisitions were performed using classical collision induced dissociation (CID), electron transfer dissociation (ETD), higher energy collision induced dissociation (HCD), electron transfer/higher energy collision dissociation (EThcD), and ultraviolet photon dissociation (UVPD) fragmentations, detecting precursor and fragment ions with a resolution of 120,000 at m/z 200. Data analysis was performed using Thermo Scientific™ Deconvolution™ 4.0 software and ProsightPD™ nodes in the Thermo Scientific™ Proteome Discoverer™ 2.2 software (Fig. 6.6).

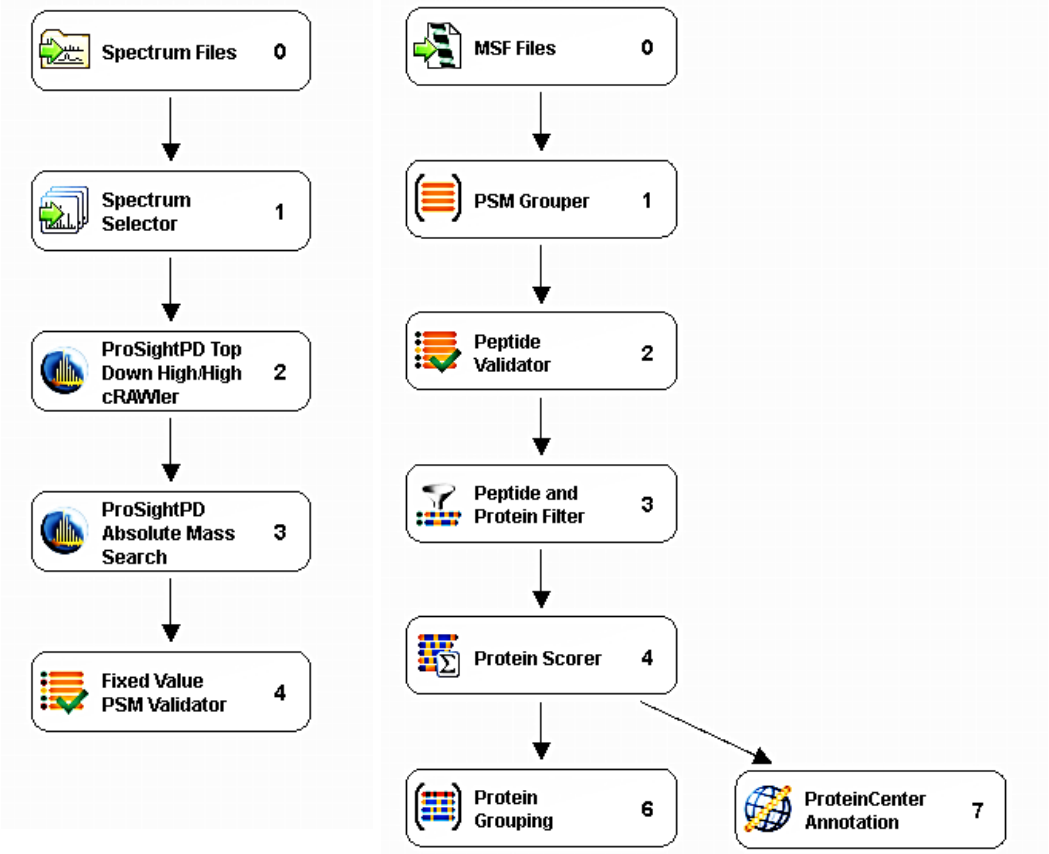


Fig. 6.6 Processing and Consensus workflows for top-down proteomics analysis in Proteome Discoverer™ 2.2 software using ProsightPD™ node.

6.2.4 Results

In the sample, which consisted of tear fluid from a single donor, the UVPD protein dissociation strategy allowed for the highest number (>500) of protein spectral matches, and, correspondingly for the highest number of proteoforms to be identified (50 different ones), i.e. mapped to the respective human gene. The overall relatively low number of proteoforms identified in this study, when compared to our previous study with a system only applying HCD as fragmentation technique (Chapter 6.1), prompted us to also extend this study to a set of tears from not one but a number of different donors. This extensive study is currently ongoing, and promises to give a more complete view on the number and types of human lacrimal proteoforms identifiable with the current superior analytical approach. Therefore, rather than to go into detail about the different proteoforms identified in this Orbitrap Fusion Lumos Tribrid pilot study, we limit ourselves to show here a summary of our results, using a number of figures, which illustrate the trends which are discernible when overlooking the different proteoform fragmentation strategies and comparing the various analytical strategies.

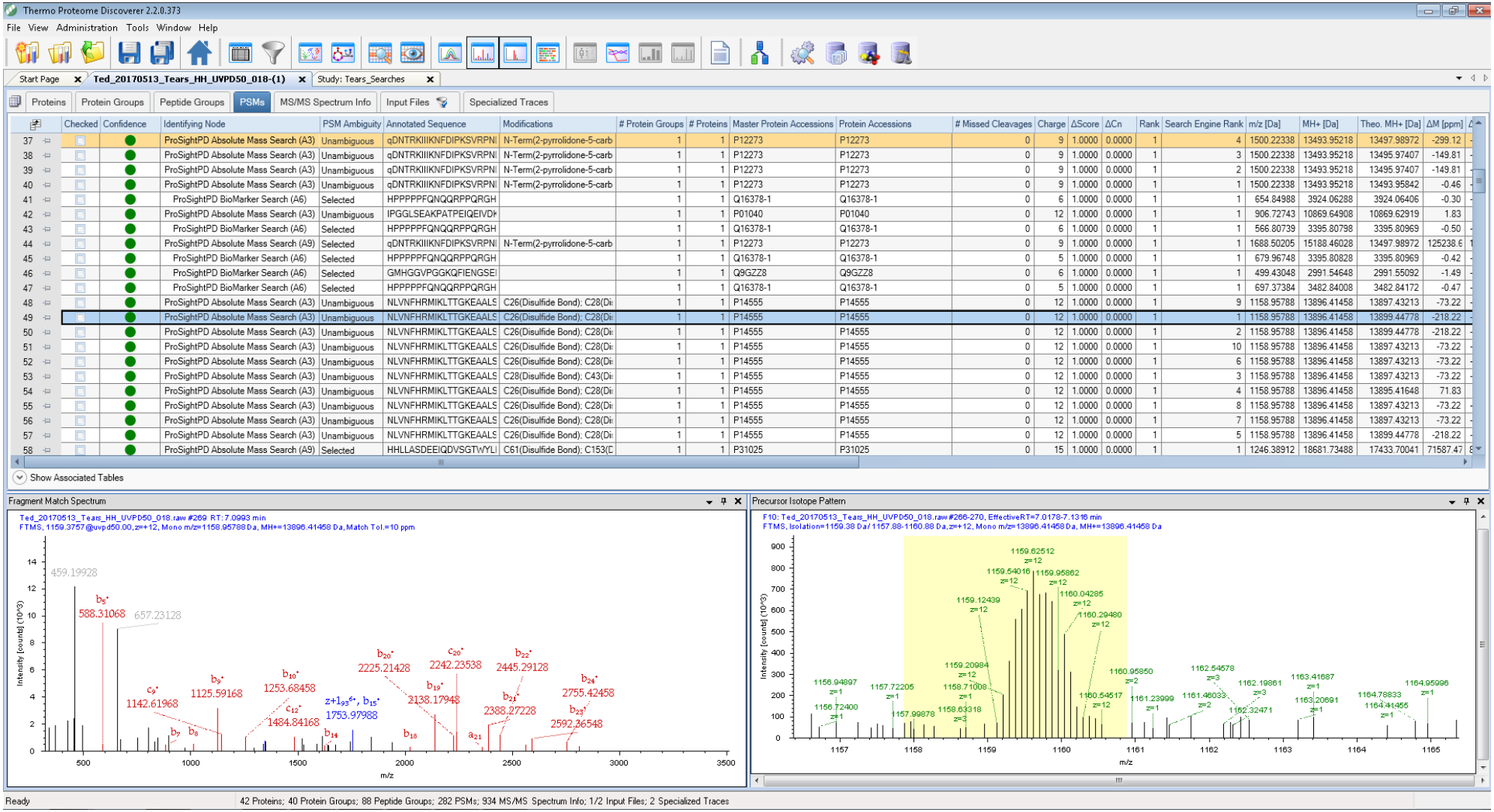


Fig. 6.7 Exemplar analysis output of Proteome Discoverer 2.2 software (screenshot), showing identified proteoform list (partial), MS^2 fragmentation spectrum for highlighted protein (*i.c.* proteoform of antimicrobial protein phospholipase A2 [UniProt Accession P14555]), and full MS^1 spectrum highlighting isolation window of precursor ion mass.

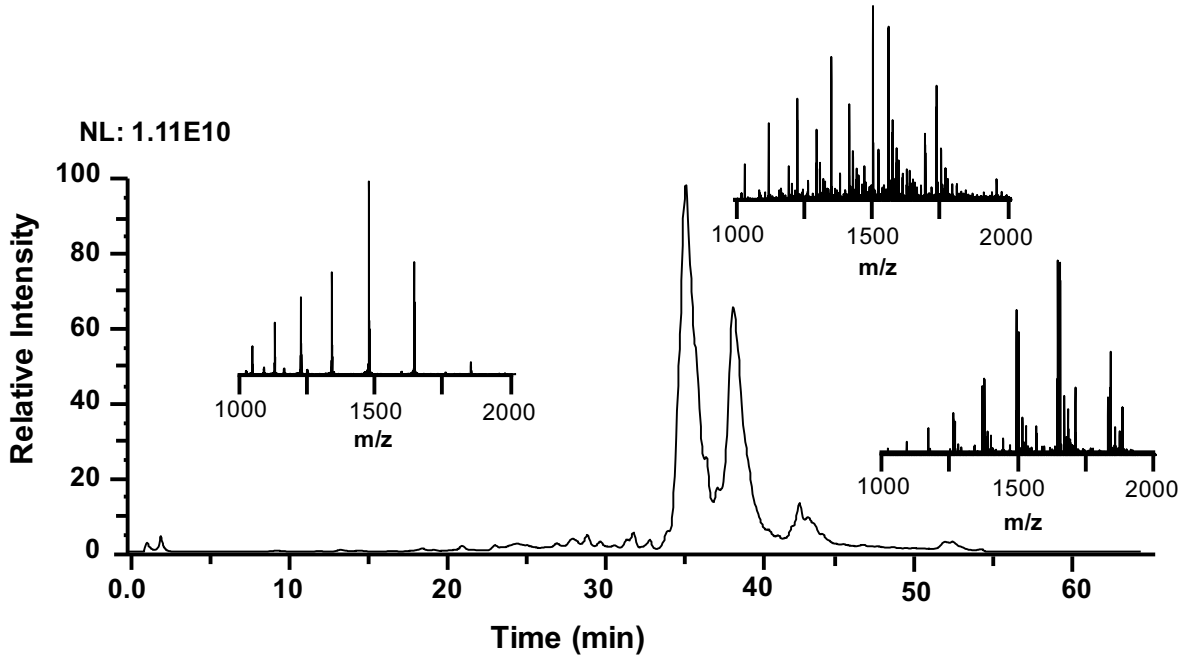


Fig. 6.8 Representative chromatogram from a 60 min reverse phase UHPLC gradient of tear fluid without any extra sample processing (i.e., no reduction or alkylation, no other sample component enrichment). Inserts show different full MS scans across the chromatogram.

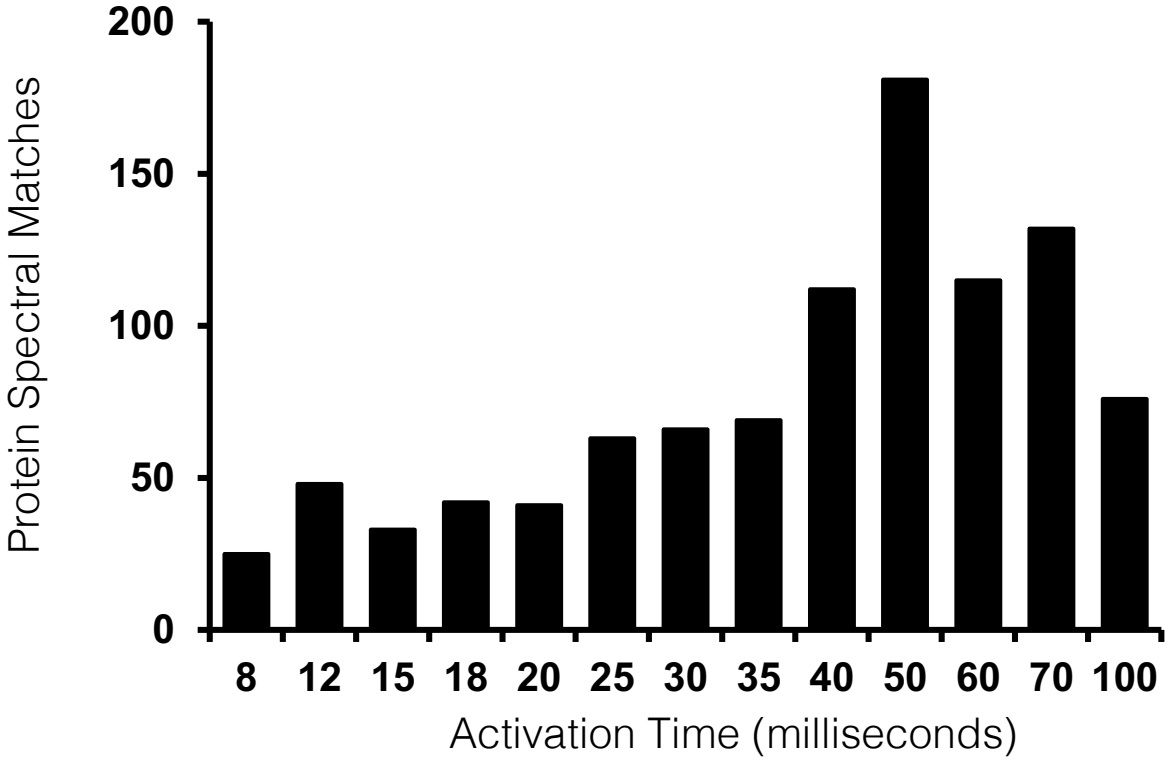


Fig. 6.9 Histogram showing number of protein spectral matches in function of activation time during UVPD irradiation.

Several proteoforms which do not yield any fragmentation via CID, HCD or ETHCD, did exhibit extensive fragmentation in UVPD. An example is shown in Fig. 6.10. Tear fluid prolactin-induced protein (PIP; UniProt accession number P12273) is an endoprotease playing a role in host defense by binding to microorganisms. PIP has a very rigid secondary structure, stabilized

by two disulfide bridges, which is probably important for its molecular function. This makes the proteoform very difficult to fragment in the gas phase. Interestingly, UVPD allows for a full characterization of the respective proteoform, allowing not only identification of the disulfide bridges which add to the proteoform stability, but also providing evidence for the conversion of the aminoterminal glutamine residue to 2-pyrrolidone-5-carboxylic acid (pyroglutamic acid), as noted in NextProt (https://www.nextprot.org/entry/NX_P12273/proteomics).

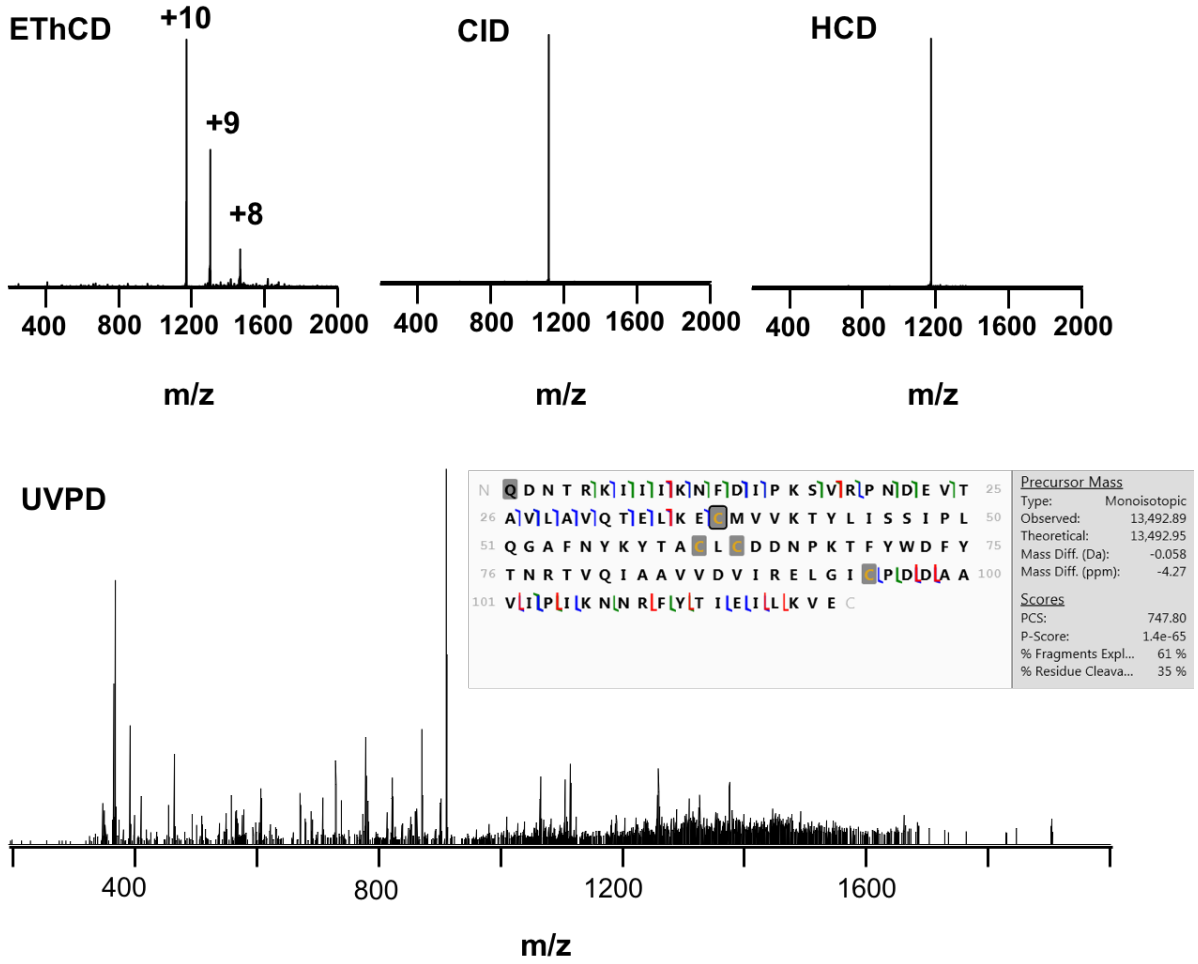


Fig. 6.10 Comparison of 4 fragmentation spectra on a chain of tear fluid protein (a prolactin-induced protein (PIP) proteoform) applying different dissociation techniques.

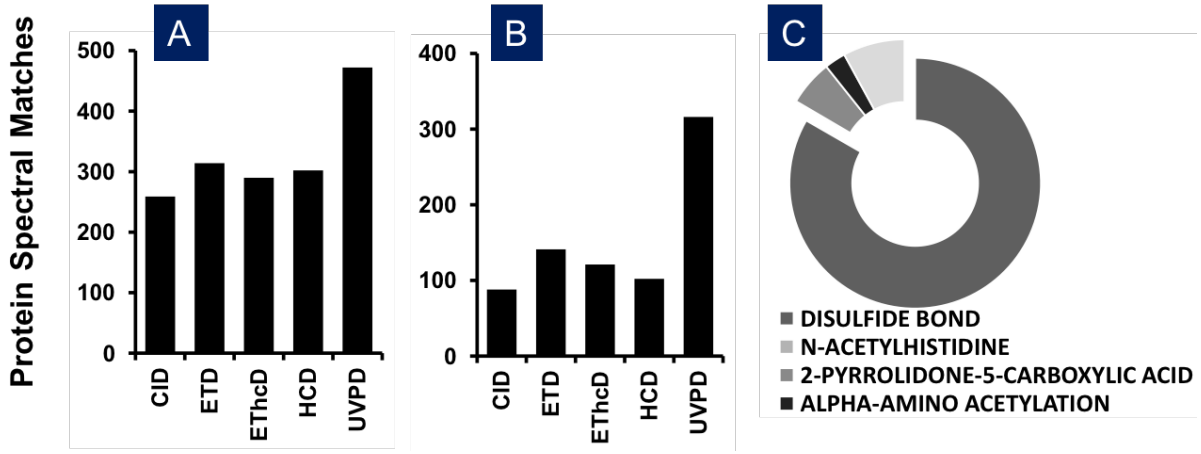


Fig. 6.11 (A) Histogram showing total number of protein spectral matches in function of dissociation technique. (B) Histogram showing number of protein spectral matches containing post-translational modifications on function of dissociation technique. (C) Pie chart showing the distribution of major modifications found in human tear proteoform landscape.

6.2.5 Discussion

Compared to the number of proteoforms confidently identified by employing the Q Exactive Plus™ instrument (**Chapter 6.1**), i.e. 770, the number of 50 in this Orbitrap Fusion™ Lumos™ Tribrid™ study appears relatively low.
However, it should be noted that the tear sample studied here originated from a single individual, whereas the top-down proteomics dataset acquired in **Chapter 6.1** comprised 6 donors. This means that the current dataset does not include any inter-individual protein sequence differences.
The proteins identified here match those identified in **Chapters 4, 5 & 6.1**. To aid visualization of the overall results, data can be represented in so-called protein networks. Fig. 6.12 shows a summary of the identified proteins clustered as such. This bioinformatics exercise yields 4 major clusters. Most significant are the cystatin cluster (around lacritin) containing proteins with endopeptidase inhibitor activity, and the Mut cluster containing proteins with endopeptidase and DNA binding functions.
Another bioinformatics way to overview the data is through a so-called GO ('gene ontology') representation [http://www.geneontology.org/], as shown in Fig. 6.13. This analysis indicates that the top-down analysis does not exhibit an obvious bias towards a specific protein class.

We anticipate that with a technological setup as performant and sensitive as the UHPLC/orbitrap MSMS system employed in this experiment, it will not be long before an extensive human tear fluid top-down proteomics data repository will be filled with accurate masses of identified lacrimal proteoforms. To make a thorough start with this, a large(r) scale study on human tear proteoforms employing the current high-performance system has been initiated in San Jose and the first data will be presented at the 2018 edition of the Annual Conference of the American Society for Mass Spectrometry.
We foresee that such proteoform database will be of great value from the moment that mass spectrometry-based tear proteoform profiling will become common practice in the cabinet of the ophthalmologist. Yet, it is clear that the installation of extensive/expensive equipment with a considerable footprint will be beyond reach of the average physician. This is why we decided

to run a trial with the very same human tear sample as analysed in the present study, on a novel high-performance benchtop orbitrap instrument, with an innovative sample separation device. The preliminary data from this pilot study have likewise been presented at the 2017 Conference of the American Society for Mass Spectrometry, and are described in Chapter 6.3.

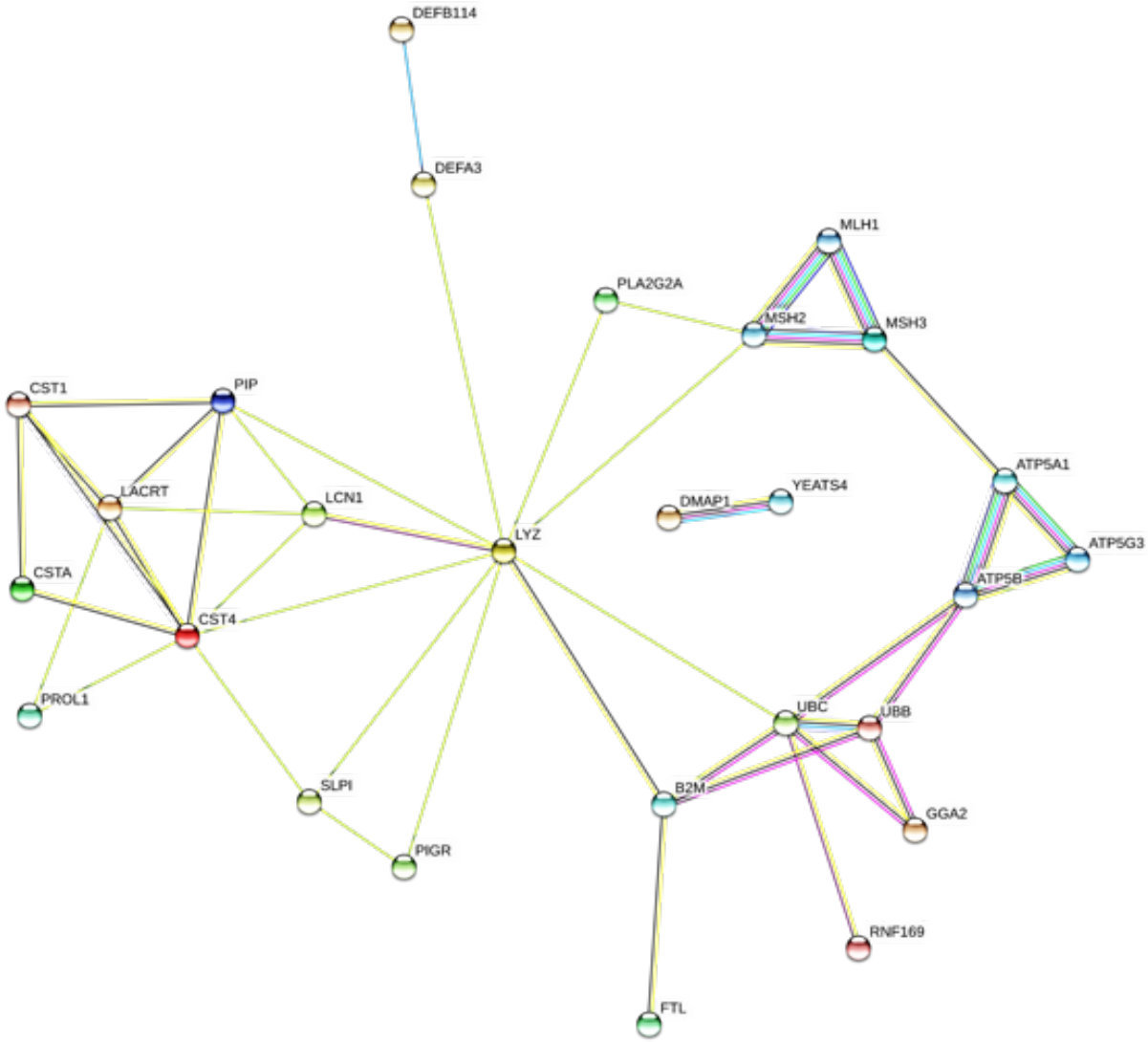


Fig. 6.12 Protein network built from identified proteoforms, highlighting 4 major clusters, including cystatin/lacritin cluster (left side) and Mut cluster (upper right.

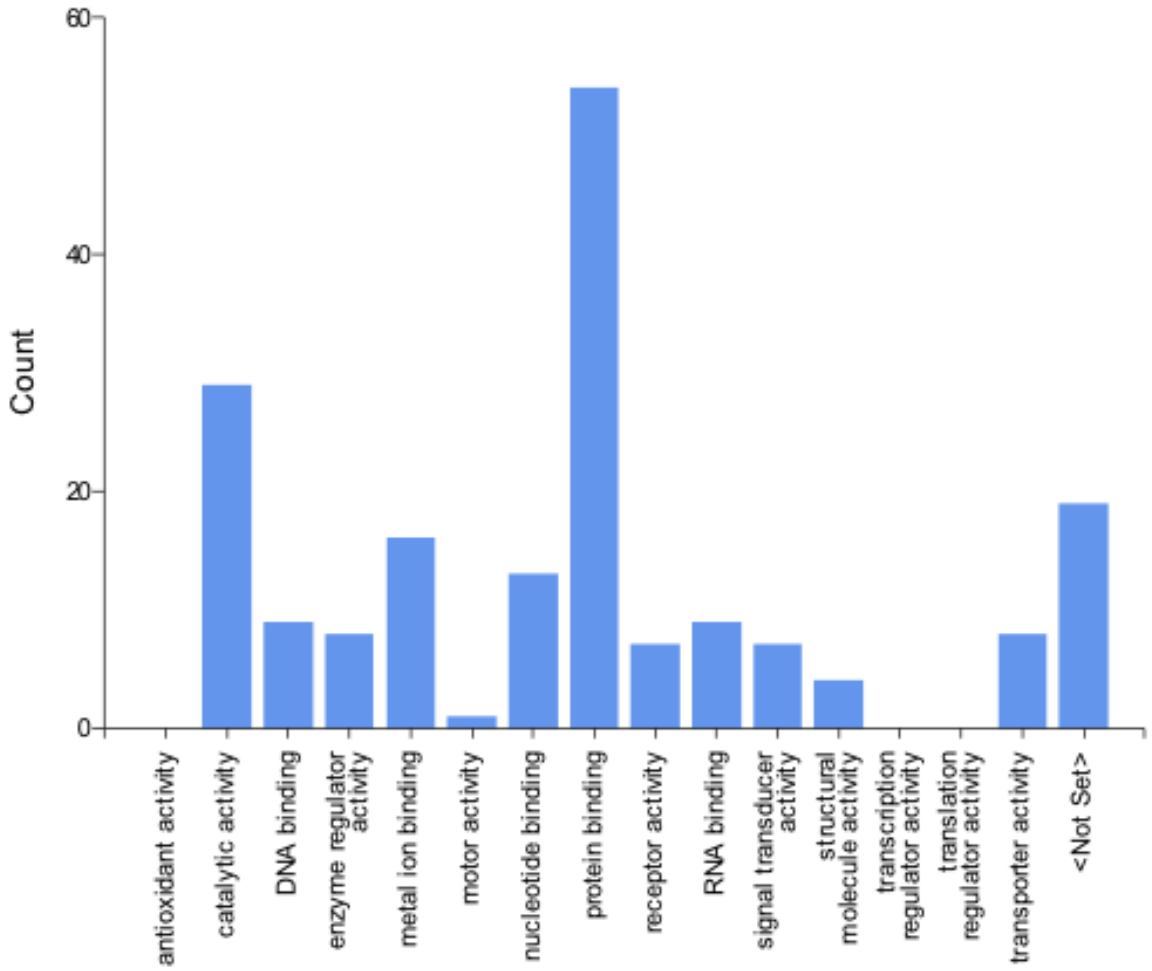


Fig. 6.13 Histogram showing number of significant 'gene ontology' terms for (top-down) identified proteins as classified by molecular function.

6.2.6 Conclusions

UVPD coupled to high resolution mass spectrometry and sophisticated bioinformatics tools is shown to represent a relatively easy and versatile combination for (top-down) proteoform profiling of human tears. We show that UVPD provides significantly more protein identifications than any other dissociation technique. The combination of UVPD dissociation, high resolution and accurate mass Orbitrap™ MS, and Proteome Discoverer™ data processing, allow for extremely sensitive analysis using minute amounts of sample.

Our results illustrate that robust profiling and monitoring of many tens of proteoforms in tear fluid in barely 60 min is possible, opening up this technology for single donor tear analysis in ophthalmological clinical research applications.

6.2.7 References

Cleland TP, DeHart CJ, Fellers RT, VanNispen AJ, Greer JB, LeDuc RD, Parker WR, Thomas PM, Kelleher NL, Brodbelt JS (2017) High-throughput analysis of intact human proteins using UVPD and HCD on an orbitrap mass spectrometer. J. Proteome Res. 16: 2072-2079.

McDonnell PJ, Pflugfelder SC, Stern ME, Hardten DR, Conway T, Villanueva L, Hollander DA (2017) Study design and baseline findings from the progression of ocular findings (PROOF) natural history study of dry eye. BMC Ophthalmol. 17:265.

Suchi S, Harsha J (2015) Prevalence and associated factors of dry eye: Our experience in patients above 40 years of age at a Tertiary Care Center. Oman J. Ophthalmol. 8: 151-156.

6.3 Clinically relevant profiling of proteoforms in human tear fluid using chip-based capillary electrophoresis coupled to mass spectrometry.

[D. Lopez-Ferrer, R. Huguet, A. Krupke, C.-H. Chen, A. Paulus, P.P.M. Raus, V. Zabrouskov, A.F.R. Huhmer, P.D.E.M. Verhaert ASMS2017_3]

6.3.1 Abstract

With the aim to demonstrate the potential of capillary electrophoresis hyphenated to high-performant orbitrap mass spectrometry for monitoring lacrimal proteoforms, we designed an experiment to combine a microfluidic chip (ZipChip™) capable of highly efficient, and in very short time, electrokinetically driven separations [Redman et al., 2016] with a Q Exactive HF™ quadrupole-orbitrap hybrid MS/MS instrument.
The same tear sample, which had been extensively top-down characterized in Chapter 6.2, was applied to Schirmer strips, and eluted in aqueous solution and directly analyzed by capillary electrophoresis (CE) coupled to mass spectrometry in without further sample processing.
We demonstrate that highly reproducible electropherograms can be generated using very small protein samples. These CE-separated tear proteoform MS profiles can be generated in less than 5 min, and, employing the database of 50 proteoforms identified previously, all these proteoforms in the tear sample profile can be readily identified.

6.3.2 Introduction

Currently, Dry Eye Disease (DED) is diagnosed either by subject self-reported history, or by measuring tear quantity using Schirmer strips. Here, we evaluate the use of an attractive instrument combination for top-down proteomics as clinically viable biomarker detection strategy for DED, and, by extension, various eye-related and other diseases. We propose the combination of Schirmer strips for sample collection, protein extraction, and CE-MS/MS as an accurate and sensitive way to identify proteoform biomarkers in tear fluid.

6.3.3 Materials and Methods

Sample Preparation

Protein standards (Pierce™ Intact Protein Standard Mix; see Table 6.2) to evaluate protein separations in the microfluidic CE device (Fig. 6.14) were purchased from Pierce. Tears were purchased from Lee Biosolutions (Maryland Heights, MO, USA), Schirmer Tear Test strips from Clement Clarke International (Harlow, UK).

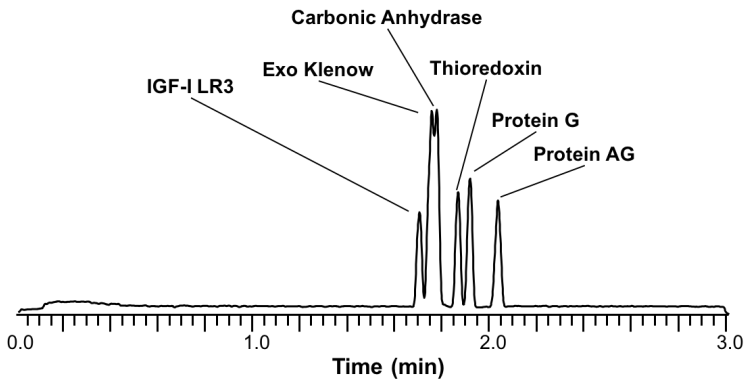


Fig. 6.14 Electropherogram of the Pierce™ Intact Protein Standard Mix in a 3 min analysis.

Test Method(s)

Samples were introduced via autosampler into a CE microchip (ZipChip™, 908 Devices, Boston, MA, USA) attached to the ESI-MS. MS was performed on a Thermo Scientific™ Q Exactive HF™ hybrid quadrupole-orbitrap MS.

A schematic of the instrumentation is shown in Fig. 6.15.

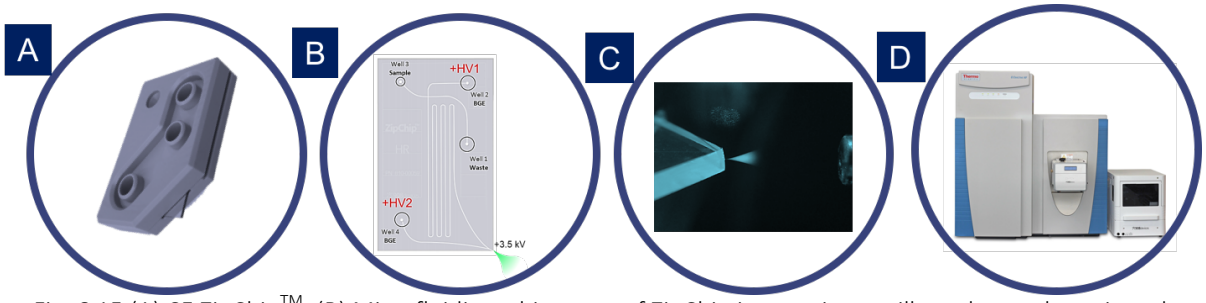


Fig. 6.15 (A) CE ZipChip™; (B) Microfluidic architecture of ZipChip integrating capillary electrophoresis and electrospray mass spectrometry; (C) Picture of electrospray plume illuminated with green laser; (D) benchtop QExactive HF with ZipChip CE source and CE autosampler for automated injections.

Six proteins were selected that (1) evenly covered a MW range of 12kD – 66kD; (2) presented mostly clean, modification and adduct-free ESI spectra; and (3) the ESI charge state distributions of which covered a wide m/z range between 500-2000 (Fig. 6.15).

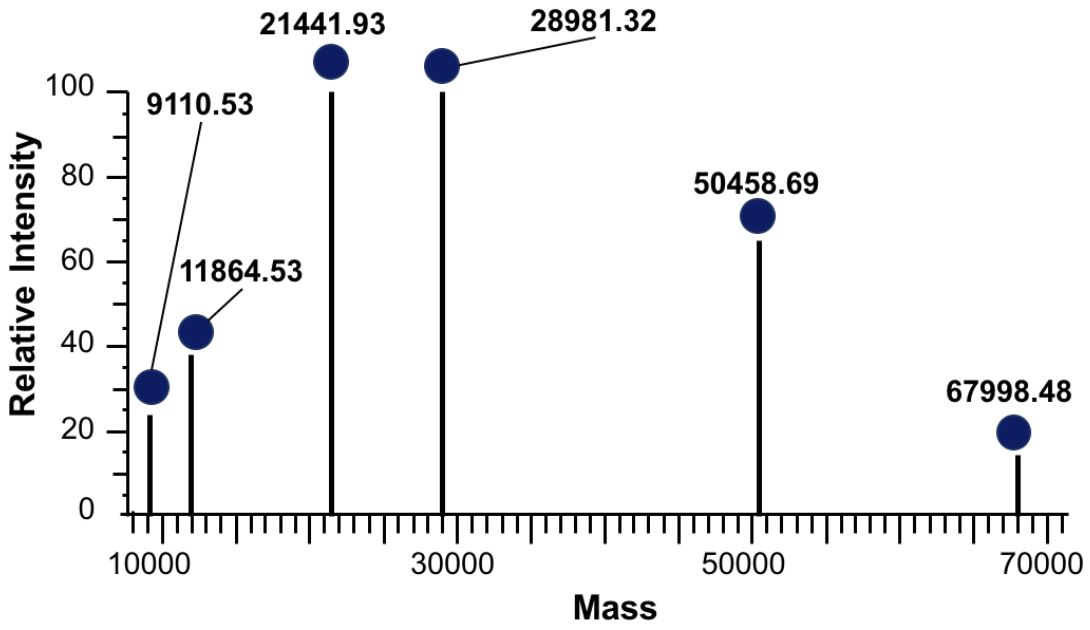


Fig. 6.16 Biopharma Finder™ software deconvoluted masses for of each of the six proteins using sliding window deconvolution feature.

Data Analysis

Raw MS files were deconvoluted using BioPharma Finder™ Mass Informatics Platform (Thermo Fisher Scientific, San Jose, CA, USA) and deconvoluted masses were matched to a database populated with tear proteoforms that were identified previously using top down proteomics applying high resolution high mass measurement accuracy LC-MSMS-based approaches (Chapter 6.2).

6.3.4 Preliminary Results

Ten replicate injections were done subsequently to assess the robustness of the analytical setup (Table 6.2). Five representative runs are shown in Fig. 6.17.

Protein ID	Accession number	Time	CV	Intensity	CV
Human IGF-1	P05019	1.68	1%	1.83E9	25%
E. coli Exo Klenow	P00582	1.73	0%	2.5E7	16%
Bovine carbonic anhydrase II	P00921	1.78	0%	2.4E9	18%
Human Thioredoxin	Q99757	1.85	0%	3.4E9	10%
Streptococcus Protein G	Q54181	1.90	0%	3.3E9	24%
Streptococcus Protein AG	P02976	2.02	0%	1E9	39%

Table 6.2 Standard proteins and their CE migration time and raw MS intensity variation over 10 different analyses.

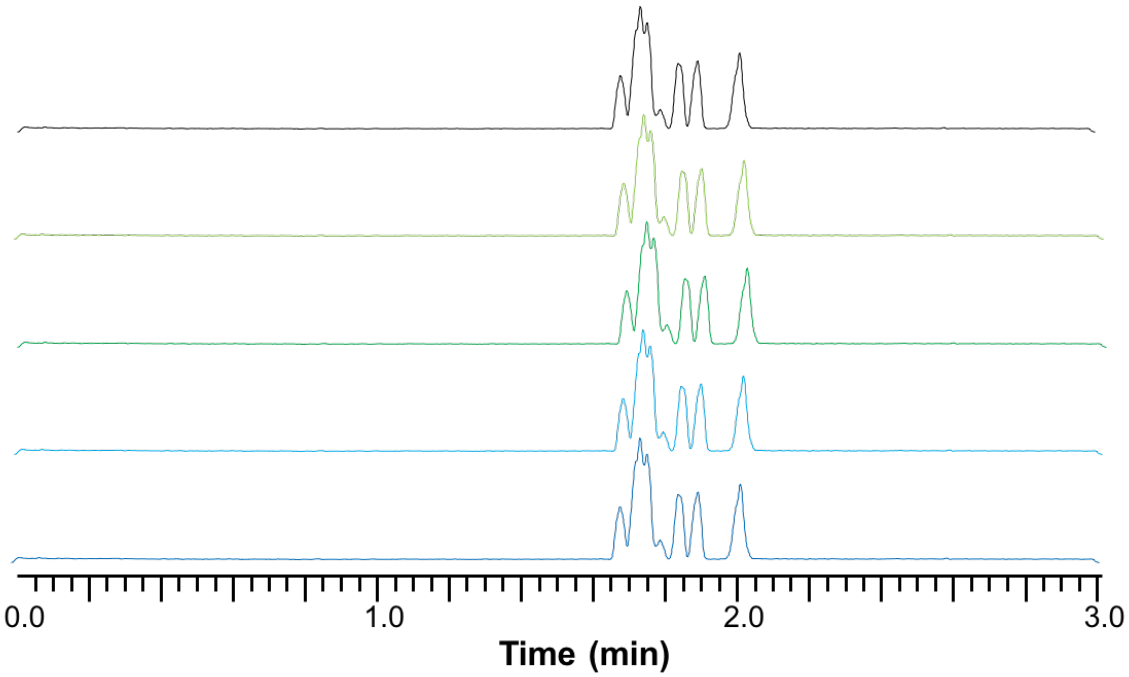


Fig. 6.17 Five representative electropherograms from 10 replicate injections of the Pierce™ Intact Protein Standard Mix

The tear sample analyses yielded highly reproducible CE-MS profiles (Fig. 6.18). It is clear that, in contrast to the standard peak analysis which showed 6 separate peaks on 6 (carefully selected) proteins, each electropherogram peak of the true biological sample contains multiple mass spectral peaks. As a matter of fact, previous analysis of the exact same sample indicated that the sample contains, at least, 50 different proteoforms. This is illustrated by the inserts in Fig. 6.18, which represent mass spectra across selected electropherogram peaks.

Matching the (high accuracy) mass measurements of the CE separated proteoforms with the database, which was populated with the proteoforms extensively analyzed from this sample before, enabled for a confident identification of a substantial number of proteoform peaks. Not surprisingly these contain some of the 'usual suspects', i.e. some of the typical and relevant lacrimal proteins. These are listed in Table 6.3.

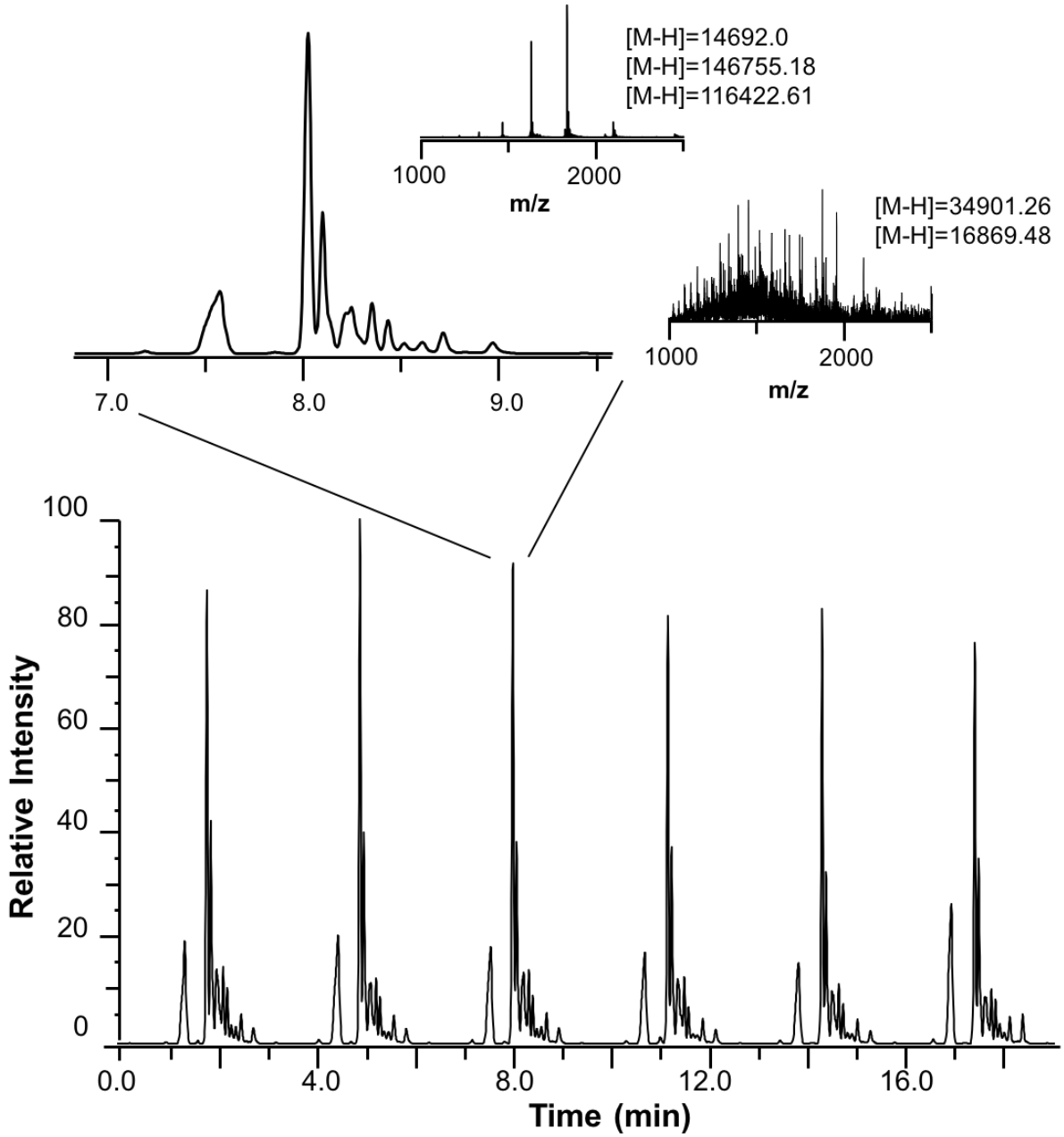


Fig. 6.18 Six consecutive replicate injections from 5 µL of human tears as starting material, illustrating high reproducibility of analysis with real biological samples.

UNIPROT	NAME	FUNCTION [Literature source]
O75368	SH3 domain binding glutamic acid-rich protein like	Related to glutaredoxins and expressed in retina [Bowes Rickman et al., 2006]
P01037	Cystatin SN	Cysteine proteinase inhibitor [Barka et al., 1991]
P05109	S100 calcium binding protein A8	Proinflammatory protein with potential antimicrobial activity [Zhou et al., 2009]
P06702	S100 calcium binding protein A9	Proinflammatory proteins with potential antimicrobial activity [Zhou et al., 2009]
P12273	Prolactin-induced protein	Potent antiangiogenic, proapoptotic effects [Priyadarsini et al., 2014]
P31025	Lipocalin 1-like 1; lipocalin 1 (tear prealbumin)	Primary lipid binding protein in tears, overproduced in response to multiple stimuli including infection and stress [Dartt, 2011]
Q9GZZ8	Lacritin	Secretion modulation by lacrimal acinar cells [Samudrfe et al., 2011]
Q9Y5E8	Protocadherin beta 15	Crucial role in development of retina [Alagramam et al., 2001]
P01009-1	alpha1-antitrypsin (SERPINA1)	Controls several types of chemical reactions by blocking (inhibiting) activity of certain enzymes [Sen et al., 1988]
Q02505-1	Mucin-3a	Maintaining homeostasis of wet ocular surface [Hodges and Dartt, 2013]

Table 6.3 List of relevant proteins in eye health monitored as identified proteoform MS peaks by our ZipChip-MS platform

6.3.5 Discussion / Conclusion

Whereas the potential of (chip-based) capillary electrophoresis has been recognized for several years [Carrilho and Garcia, 2014], its practical implementation is only recently becoming commercially available (Redman et al., 2016). Coupled to performant orbitrap mass spectrometry, CE using microfluidic devices represents a very easy and versatile technology for top-down tear analysis, such as for rapid screening of potential disease biomarkers. The combination of the ZipChip^{TM} and Q Exactive HF^{TM} instrumentation allows for extremely sensitive assays using very little amount of sample. The ZipChip MS technology allows for the robust monitoring of more than 30 proteoforms in tears in merely 5 min. and using minimal sample material.

Our analyses, moreover, demonstrate that CE-MS is a highy reproducible and sensitive technology for intact protein (proteoform) identification and assaying.

6.3.6 References

Alagramam KN, Yuan H, Kühn MH, Murcia CL, Wayne S, Srisailpathy CRS, Lowry RB, Knaus R, Van Laer L, Bernier FP, Schwartz S, Lee C, Morton CC, Mullins RF, Ramesh A, Van Camp G, Hagemen GS, Woychik RP, Smith RJH (2001) Mutations in the novel proptocadherin PCDH15 cause Usher syndrome type 1F. Human Mol. Genetics 10: 1709-1718.

Barka T, Asbell PA, van der Noen H, Prasad A (1991) Cystatins in human tear fluid. Curr. Eye Res. 10: 25-34

Bowes Rickman C, Ebright JN, Zavodni ZJ, Yu L, Wang T, Daiger SP, Wistow G, Boon K, Hauser MA (2006) Defining the human macula transcriptome and candidate retinal disease genes using EyeSAGE. Invest. Ophthalmol. Vis. Sci. 47: 2305-2316.

Carrilho E, Garcia CD (2014) Instrumentation for capillary electrophoresis and microchip electrophoresis. Electrophoresis 35: 2067.

Dartt DA (2011) Tear lipocalin: structure and function. Ocul Surf. 9: 126-138.
Hodges RR, Dartt DA (2013) Tear Film Mucins: Front Line Defenders of the Ocular Surface; Comparison with airway and gastrointestinal tract mucins. Exp. Eye Res. 117: 62–78.
Priyadarsini S, Hjortdal J, Sarker-Nag A, Sejersen H, Asara JM, Karamichos D (2014) Gross cystic disease fluid protein-15/prolactin-Inducible protein as a biomarker for keratoconus disease. PLoS ONE 9: e113310.
Redman EA, Mellors JS, Starkey JA, Ramsey M (2016) Characterization of intact antibody drug conjugate variants using microfluidic capillary electrophoresis – mass spectrometry. Anal. Chem. 88: 2220-2226.
Samudre S, Lattanzio FA, Lossen V, Hosseini A, Sheppard JD, Mc Cown RL, Laurie GW, Williams PB (2011) Lacritin, a novel human tear glycoprotein, promotes sustained basal tearing and is well tolerated. Invest. Ophthalmol. Vis. Sci. 52: 6265–6270.
Sen D K, Sarin G S, Mathur M D (1988) Alpha-1 antitrypsin and serum albumin levels in tear fluid of healthy subjects and in persons with conjunctival diseases. Indian J. Ophthalmol. 36: 22-26.
Zhou L, Beuerman RW, Ang LP, Chan CM, Li SF, Chew FT, Tan DT (2009) Elevation of human alpha-defensins and S100 calcium-binding proteins A8 and A9 in tear fluid of patients with pterygium. Invest Ophthalmol Vis Sci. 50:2077-2086.

6.4 Evaluation of heat stabilization in human tear sample preparation for top-down proteomics.

[P. Raus, H. Park, P. Thomas, N. Kelleher, P. Verhaert]

6.4.1 Abstract

We report a pilot experiment to assess the value of heat stabilization as sample preparation step to store clinical tear samples prior to proteoform profiling. Top-down proteomics data obtained in a single series of analytical measurements on tear fluid samples, simultaneously collected on Schirmer strips from a single human volunteer donor (2 left eye, and 2 right eye samples) were compared. Half of the samples were rapidly heated; the other half of the samples were left to air dry. All subsequent sample storage and processing steps were kept strictly identical. Top-down proteomics analysis of all samples was performed half a year after tear sampling.
The relatively limited data set suggests that post-translational modifications like acetylations remain detectable after 6 months storage of heat stabilized samples, whereas several such proteoforms disappear from the non-stabilized versions of the same sample.

6.4.2 Introduction

With this thesis, we make the argument that modern analytics of body fluids (with tears as concrete example), can be extremely powerful, when focusing on the multiplicity of protein species, proteoforms, present. In this context, we enthusiastically advocate the future potential of top-down proteomics in medical diagnostics in general, and in ophthalmology in particular.

We previously established that, using state-of-the-art MS, proteoform profiles of clinically sampled tear fluid can be generated in a robust and reproducible way, and in relatively short timeframes of several minutes, with a significant fraction of the proteoforms identifiable (Chapter 6.3). As such the proposed analytical workflow meets a number of crucial requirements, essential for a method which has the ambition to be useful in the future ophthalmological practice.

Obviously and importantly, the applicability and potential of any analytical, diagnostic procedure is intrinsically dependent on the extent to which the (analytes in the) samples to be investigated can be preserved between the collection moment (patient sampling) and the actual analysis.

In the context of the eye clinic, this is a highly relevant point. Indeed, we are not in a situation (yet), where each (or any) ophthalmology clinic is equipped with the necessary high-end analytics. This means that samples will have to be transported to the analytical lab, and stored until the moment of the actual analysis. It is evident that analyte degradation should be maximally avoided. This is particularly challenging for peptides (and other proteoforms) which are extremely prone to proteolytic/catabolic breakdown (Fridjonsdottir et al., 2018).

Tears are in principle secreted into the environment, and are known to naturally contain inhibitors to proteases and other protein modifying enzymes which could damage the function of specific secretory proteins. At the same time, tears also contain a variety of peptidases and protein modifying enzymes themselves. Hence, the risk of sample modification after prelevation is real. We have setup a pilot test to assess whether, for samples that need to be

stored for prolonged times, heat stabilization could be a valuable sample processing step as a prelude towards a future validated clinical tear sampling standard operating procedure. Our experiment involved the creative adaptation of a commercial Stabilizor™ system, originally developed to thermally stabilize tissue samples [Svensson et al., 2009; Colgrave et al., 2011], towards liquid samples collected on Schirmer strips.

6.4.3 Materials and Methods

Sample Preparation

Tear samples were collected on Schirmer strips (see also **Chapters 5-6.1-6.3**) simultaneously from both the left and right eye of the same individual. Of these, one left and one right eye sample were heat-stabilized in a Stabilizor™ T1 (Denator, Uppsala, Sweden), which was programmed to heat the sample directly to 95C for 5 minutes. For this experiment, the Schirmer strip was folded to be entirely inserted into the Maintainor® tissue cartridges. The equivalent second sample was left unprocessed to air dry on the Schirmer strip. All samples were subsequently stored in individually sealed separate plastic bags in one and the same envelope, until the actual proteoform analysis. As such storage conditions were kept strictly equal for all samples. When in the lab, the envelope containing the samples was stored refrigerated at 4C. Transport between labs (from Belgium to US) was at ambient temperature with no specific precautions with respect to cooling. Six months after collection, all 4 samples were processed simultaneously for analysis as described (**Chapter 6.1**). Briefly, this involved elution from the Schirmer strip and analysis using a top-down proteomics protocol employing off-line GELFREE 8100 size exclusion cartridges essentially as described by Fornelli et al. [2017], followed by on-line RP capillary LC MSMS on an Orbitrap Elite™ (Thermo Fisher Scientific, San Jose, CA USA) ion trap orbitrap hybrid mass spectrometer (analyses performed in triplicate).
We specifically note here that the MS system employed for this study, was different from any of the instruments employed in the previous Chapters. This was due to practical reasons of instrumentation availability. Therefore, we emphasize here that the purpose of the experiment was solely to compare the analytical data of heat stabilized versus non-stabilized samples. For this, the very same clinically collected sample, in stabilized and not-stabilized versions were scrutinized in a single analytical series on one and the same instrument, where all 4 samples were analysed in triplicate. The limited reach of the experiment does not allow or justify any conclusion with respect to comparisons with data obtained on different mass spectrometers.

Top-Down Proteomic Data Analysis

Raw data files were processed using TDPortal 1.3, a Galaxy [Afgan et al., 2016]-based software allowing estimation of accurate false discovery rates (FDRs) for protein and proteoform identification in top down proteomics. The cRAWler™ algorithm (Thermo Fisher Scientific) was first used to assign precursor masses and the corresponding fragment masses. Mass-to-charge data for each precursor/fragmentation scan pair were deconvoluted to monoisotopic neutral mass values using the Xtract™ algorithm (Thermo Fisher Scientific).
For proteoform identification and characterization, the deconvoluted MS and MS/MS queries were searched against the (well annotated) human UniProt database using a three-tiered search mode tree ('Tight Absolute Mass', 'BioMarker', and 'Find Unexpected Modifications' search modes) in TDPortal 1.3 [Savaryn et al., 2016; Fornelli et al., 2017; Cleland et al., 2017; Toby et al., 2017]. The so-called *Tight Absolute Mass search* was the first search programmed with a MS tolerance of 2.2 Da, and a MS/MS tolerance of 10 ppm. The second search was the

BioMarker search which has both MS and MS/MS tolerances set at 10 ppm. The last search was a *Find Unexpected Modifications search* with a MS tolerance of 200 Da, and a MS/MS tolerance of 10 ppm, using *Delta M mode*, allowing the detection of proteins with one unknown modification. All search results were filtered using a 1% global FDR threshold at both the protein and the proteoform levels. Search results were visualized using TDViewer software (http://topdownviewer.northwestern.edu).

6.4.4 Results and Discussion

The experiment allowed for 46 different proteoforms to be confidently identified, mapping to 17 different proteins. Table 6.4 shows a summary of the proteoform identifications. It includes only the identified proteoforms which were detected in, at least, 2 of the 3 technological replicate analyses *and* in both biological duplicates (left and right tear sample). All data per individual sample measurement are provided through Appendix 6.5.7.

Thus, a relatively limited number of proteoforms were identified in the samples stored on Schirmer strips for over 6 months. This number appears limited in view of our work described in Chapters 6.2. and 6.3.

Several reasons for this can immediately be hypothesized, and to test these, different experiments would be required. As indicated above, the goal of this experiment was not to compare the performance of different orbitrap based analyses (the configuration of the current Elite™ is quite different from the QEPlus™ and Lumos Tribrid™ as used in Chapters 6.2 and 6.3). Neither would it be valid to employ the data from this experiment to compare 6 months old tear samples with freshly collected ones as analysed before (Chapter 6.2, 6.3). Besides the difference in storage time (and conditions), the samples from this experiment came from a different donor, which may contribute to the incomplete overlap between the proteoform-based protein identifications (Tables 6. and 6.4).

The only valid comparison to be made is the one we focus on here, i.e. the different proteoforms detectable in the heat stabilized versus non-stabilized samples, which were all stored in identical conditions for half a year prior to analysis.

Within this relevant comparison, some interesting trends can be observed.

Post-translational modifications, including aminoterminal pyroglutamic acid (2-pyrrolidone-5-carboxylic acid) and acetylated residues can still be detected in samples kept ‘dry’ for several months and at varying ambient temperatures. Interestingly pyroglutamic acid modifications are detectable in the non-stabilized proteoform of prolactin-inducible protein (P12273) after 6 months storage, but not in the stabilized samples.

When considering the proteoforms which represent thymosin beta-4 and polymeric immunoglobulin receptor gene products (Figs. 6.19 and 6.20), it is remarkable that certain site-specific acetylations are exclusively detected in the stabilized samples. These are significant differences, and no coincidence, as for all these proteoforms the profile was consistent for the right and left eye sample (biological replicate), with detection in all 3 technical replicate analyses.

The relatively scarce differences between stabilized and non-stabilized samples suggest that an important proportion of the proteoforms detectable in tear fluid is already relatively stable, even without specific stabilization treatment. In this context, it may be relevant to remark that, unlike blood or lymph, the tear fluid is a ‘body fluid’ which is secreted into the environemnt, and the proteins in this secretome have been optimized to be functional in this setting through *evolution*.

UNIPROT Accession	Stabilized		Non-stabilized	
	PROTEIN NAME	PROTEOFORM #	PROTEIN NAME	PROTEOFORM #
P62328	Thymosin beta-4	8	Thymosin beta-4	3
P31025	Lipocalin-1	4	Lipocalin-1	7
Q9UGM3	Deleted in malignant brain tumors 1 protein	1	Deleted in malignant brain tumors 1 protein	1
P01036	Cystatin-S	2	Cystatin-S	1
P02768			Serum albumin	3
P06702	Protein S100-A9	1	Protein S100-A9	1
P01833	Polymeric immunoglobulin receptor	10	Polymeric immunoglobulin receptor	2
P61769	Beta-2-microglobulin	1	Beta-2-microglobulin	2
P10909			Clusterin	1
Q16378	Proline-rich protein 4	3	Proline-rich protein 4	3
P05109			Protein S100-A8	1
P63313	Thymosin beta-10	2		
P01037			Cystatin-SN	1
P12273			Prolactin-inducible protein	1

Table 6.4 Comparison of top-down identified proteins and proteoform numbers between stabilized and non-stabilized samples. Only proteoforms identified in two or more technical replicates of both left and right tear sample are included. As such identifications of proteoforms of acid phosphatase type 7 [Q6ZNF0], protein S100-A7 [P31151], and transforming growth factor-beta-induced protein ig-h3 [Q15582] are not included in this table.

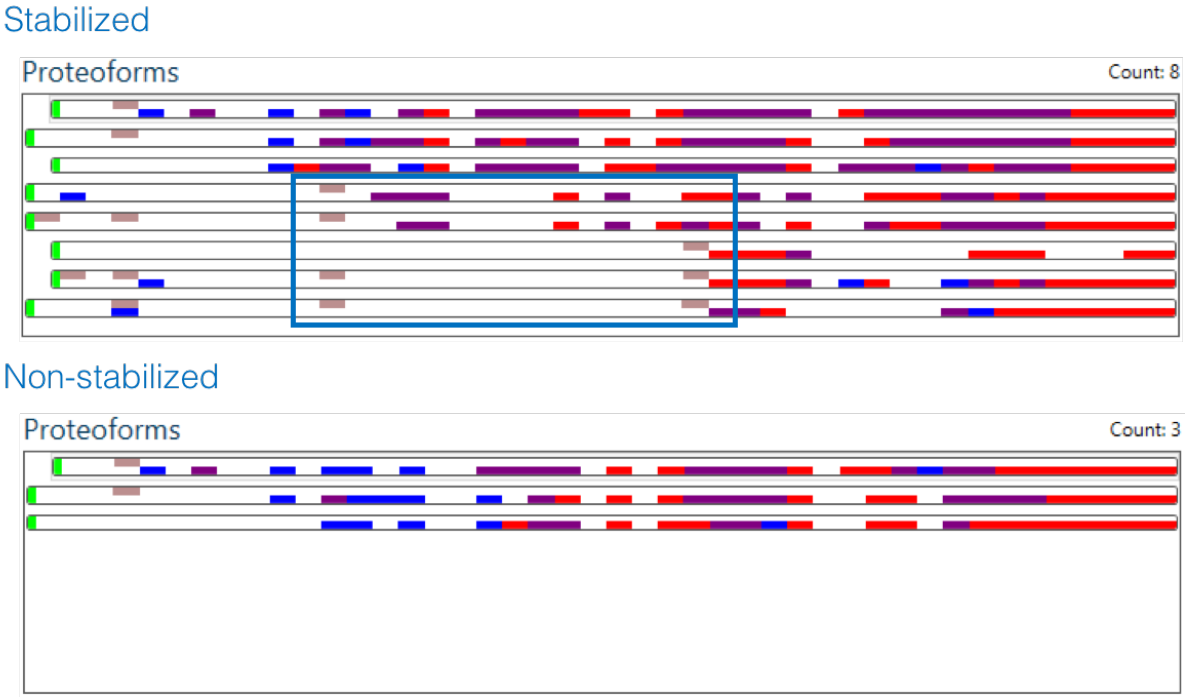


Fig. 6.19 Proteoforms detected of thymosin beta-4. Stabilized sample yields 8 different proteoforms, whereas non-stabilized sample yields only 3. Remarkably all extra proteoforms picked up in stabilized sample contain acetylated residues (color coded in greyish pink (upper half of sequence bar)).

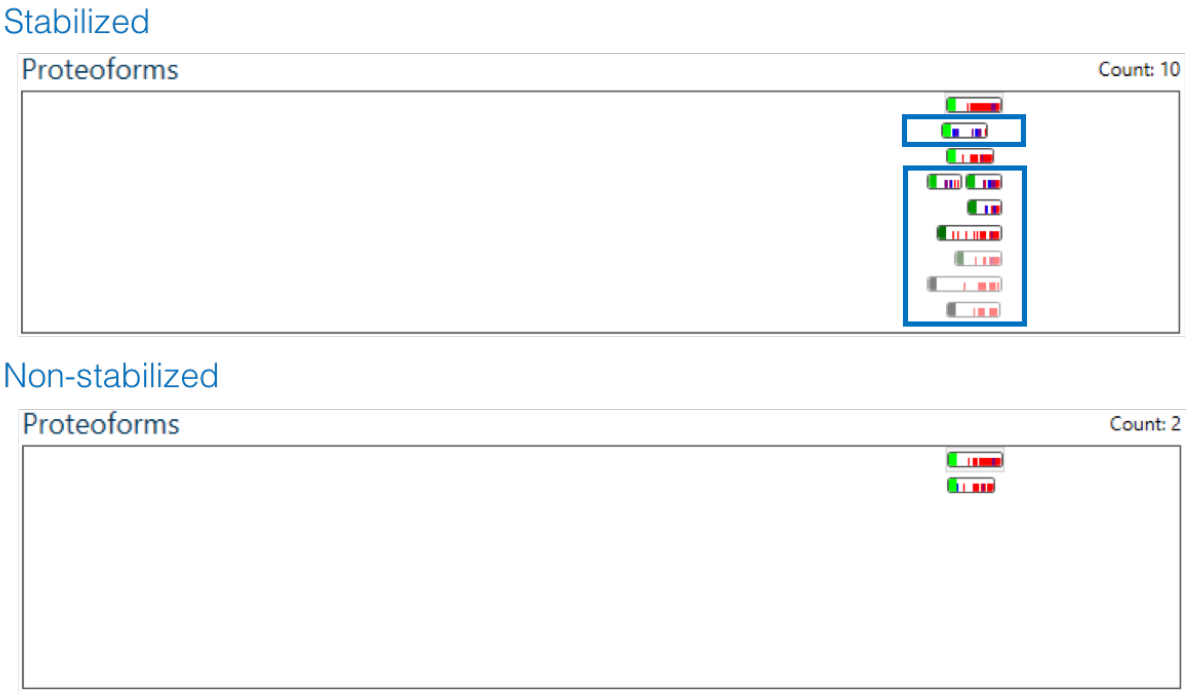


Fig. 6.19 Proteoforms detected of polymeric immunoglobiulin receptor. Stabilized sample yields 10 different proteoforms, whereas non-stabilized sample yields only 2. Remarkably 7 out of 8 of extra proteoforms picked up in stabilized sample contain acetylated residues (color coded in grey).

6.4.5 Discussion / Conclusion

Our data indicate that several tear proteoforms are remarkably stable, as they are detectable in untreated as well as heat-stabilized samples. Our results moreover suggest that heat stabilization may be a valuable step to ensure the maintaining of certain post-translational modifications like acetylations in proteoforms of clinically collected tear samples.

6.4.6 References

Afgan E, Baker D, Van den Beek M, Blankenberg D, Bouvier D, Čech M, Chilton J, Clements D, Coraor N, Eberhard C, Grüning B, Guerler A, Hillman-Jackson J, Von Kuster G, Rasche E, Soranzo N, Turaga N, Taylor J, Nekrutenko A, Goecks J (2016) The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. Nucleic Acids Res. 44: W3-W10, doi: 10.1093/nar/gkw343.

Cleland TP, DeHart CJ, Fellers RT, VanNispen AJ, Greer JB, LeDuc RD, Parker WR, Thomas PM, Kelleher NL, Brodbelt JS (2017) High-throughput analysis of intact human proteins using UVPD and HCD on an orbitrap mass spectrometer. J. Proteome Res. 16: 2072-2079.

Colgrave ML, Xi L, Lehnert SA, Flatscher-Bader T, Wadensten H, Nilsson A, Andren PE, Wijffels G (2011) Neuropeptide profiling of the bovine hypothalamus: thermal stabilization is an effective tool in inhibiting post-mortem degradation. Proteomics 11: 1264-1276.

Fornelli L, Durbin KR, Fellers RT, Early BP, Greer JB, LeDuc RD, Compton PD, Kelleher NL (2017) Advancing Top-down Analysis of the Human Proteome Using a Benchtop Quadrupole-Orbitrap Mass Spectrometer. J Proteome Res. 16: 609-618.

Fridjonsdottir E, Nilsson A, Wadensten H, Andrén PE (2018) Brain tissue sample stabilization and extraction strategies for neuropeptidomics. In "Peptidomics: Methods and Strategies" (Schrader M, Fricker L, eds.), Meth. Mol. Biol. 1719, doi: https://doi.org/10.1007/978-1-4939-7537-2_2.

Savaryn JP, Toby TK, Catherman AD, Fellers RT, LeDuc RD, Thomas PM, Friedewald JJ, Salomon DR, Abecassis MM, Kelleher NL (2016) Comparative top down proteomics of peripheral blood mononuclear cells from kidney transplant recipients with normal kidney biopsies or acute rejection. Proteomics 16: 2048-2058.

Svensson M, Boren M, Sköld K, Fälth M, Sjögren B, Andersson M, Svenningsson P, Andren PE (2009) Heat stabilization of the tissue proteome: a new technology for improved proteomics. J. Proteome Res. 8: 974-981.

Toby TK, Abecassis M, Kim K, Thomas PM, Fellers RT, LeDuc RD, Kelleher NL, Demetris J, Levitsky J (2017) Proteoforms in Peripheral Blood Mononuclear Cells as Novel Rejection Biomarkers in Liver Transplant Recipients. Am. J. Transplant. 17: 2458-2467.

6.4.7 Appendix

List of all proteoform identification hits (from triplicate analytical measurements) detected in the total experiment described, from the three modes of database searching in TDPortal 1.3. To aid data interpretation, all heat-stabilized samples are highlighted in yellow.

All hits from the entire runs_annotated.xlsx
File downloadable from
URL: https://wordpress.com/page/proteoformix.wordpress.com/207

Chapter7: Conclusion & Perspectives.
Analytical innovation in the ophthalmological practice - from novel diagnostics to new treatment and beyond.

Introduction

In the previous Chapters of this thesis, we have described (co-)development and implementation of novel mass spectrometry (MS) based protein analytics that promise to bring innovation in the ophthalmological practice. The technology and insight described will further enhance our knowledge and understanding of human tear composition in healthy donors, as well as in DED patients. As such, this will bring into scope the possibility of efficient, personalized treatment for these patients which currently is not standing practice. By adopting this novel technological approach, ophthalmologists will gain access to the invaluable amount of information contained in the patients' tears, which, until to date is, literally, left to flow away.

The examples and results outlined in this thesis underscore the promise that future diagnostics and treatment in the medical practice will be relying on modern molecular and analytical technology. Physiology and functioning of living cells is dependent on the expression of genes into proteins. The expression of genetic information into physiological output ultimately determines functioning of cells and organs, as well as metabolism and behavior. Modern (top-down) proteomics technology will help bridging the gap in our understanding of the transition between human genetic predisposition and cellular behavior.

Through the research and results presented in this thesis, we hope to 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. What is more, 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.

Future Perspectives

In this final Chapter we take the liberty to elaborate on a few provocative statements into the future.

1. Proteoform analyses of clinically collected tears will be invaluable to elucidate the various different molecular mechanisms causing DED.
2. Proteoform profiling of clinically collected tears will lead to innovative diagnostic and/or prognostic biomarkers for DED subtypes.
3. Novel therapeutics, amongst which specific proteoforms that may be identical to or derived from originally identified proteins in human tears, will find their way to the DED patient.
4. The wealth of information locked up in tear proteoform profiles promises to become useful in the monitoring of other diseases, with no obvious link to ophthalmology.

In order for these statements to become reality, a healthy and efficient interaction between treating physicians and researchers in the clinic, on the one side, and proteoform analysts on the other, has to be maintained.
With DED as example case, the importance of the collaboration between clinicians and analytical biotechnologists, is underlined. We believe that it is essential that ophthalmologists and physicians in general become aware of (and get familiar with) the advent and possibilities as well as with the limitations of mass spectrometry (MS) based analytics. At the same time, other areas in medicine where mass spectrometry and other modern analytics can provide an added value, should team up with the (top-down) analytical (proteomics) scientist. With this thesis we sincerely hope to encourage this dialogue.

Hurdles and bottlenecks

A combination of several factors has until now, caused a significant bottleneck in the introduction of MS-based applications into the clinical practice. One of those is the complexity of the typical sample pretreatment required for MS analysis, which often involves time-consuming and labor-intensive sample manipulations including extraction, purification and chromatographic separation. This has long been considered out of reach for common practice in the physician's office. In addition, the expertise necessary for MS operation and data interpretation has been an inhibiting factor. The efforts by analytical researchers to help simplify sample preparations, and by clinicians to become acquainted with, and engage in, this novel analytical domain, together with a determined attitude by the industry to produce, not only better performing, but particularly more user-friendly MS analytical systems, will ultimately lead to the more widespread use of the technology in the medical field.

Innovative developments

Of course, only some of the many developments in analytical technology will ultimately find an application in the framework of DED research. An interesting illustration of the rapid progress with which the combination of novel technologies may lead to innovative instruments, is the so-called **'iKnife'** [Balog *et al*., 2013]. Although we do not immediately envisage an iKnife application in the context of DED, we certainly want to mention it here. This recently developed clinical device directly links electrosurgery, very comparable to radiofrequency surgery as described in the first part of this thesis (Chapters 2-3), to MS, which is employed in Chapters 4-6. The device couples 'rapid evaporative ionization mass spectrometry' (REIMS) technology to an electrosurgery probe and, as such, enables the analysis of the surgery by-products in the 'evaporating' tissue as it is being incised or resected. Rather than disposing of this (irritant and potentially toxic) 'surgical smoke' through vacuum tubing into charcoal or other filters, the iKnife analyzes, in real-time, specific sets of metabolites. We envisage that in the future also small proteoforms, such as neuropeptides, will become analyzable through such an 'advanced biomarker discovery' system!

Throughout this thesis a number of technological advances were introduced, which, in our opinion, are leading the way forward to significant progress in ophthalmology, and by extension, to medicine in general. Three different sample preparation developments were described.

(i) Whereas different tear sampling protocols were reported in the literature, our proteomic analyses described in Chapters 4, 5, 6.1-6.4 demonstrate that Schirmer strips, a clinically well-accepted tool, rather than glass capillaries (or other less comfortable tissue wicks), represent the most elegant way to collect the often extremely small volumes of tears of human subjects, especially DED patients, prior to protein analysis. In this context, it is appropriate to mention the recent development of paper spray [see review by Lin *et al.*, 2014]. This ambient ionization principle directly sprays ions from a triangular piece of paper. We do foresee a future paper spray source directly accommodating a Schirmer strip, which would simplify and speed-up the MS analysis of tear proteins even more.

(ii) Schirmer strip sample collection can readily be combined with optional tear proteoform stabilization by immediate (*in officio*) controlled heat-treatment (Chapter 6.4). The instrument required for this step has a small footprint, which makes it easily fit in any ophthalmologist's cabinet.
We realize that, especially in the early days of technology adoption, not every private practice or small hospital will have direct access to the required MS analytical technology. Therefore, we believe that heat-stabilization sample treatment may come in quite useful, as it will allow the collection of samples in the doctor's office and sending them off to specialized labs without losing critical information prior to the final MS analysis.

(iii) Finally, and importantly, simplification of the proteomics sample preparation to a top-down approach, leaving out protein trypsin digestion (Chapters 5, 6.2-6.4) may prove to be the most important innovation we adopted in our study of the human tear proteome.

All of those advances were only possible thanks to concurrent improvements in the instrumentation hardware (from Q-TOFs to a variety of hybrid orbitrap systems with higher sensitivity and enhanced precursor ion fragmentation capabilities) as well as in the data analysis and interpretation software (from Mascot™ to ProSight™ and TopDown Viewer™), which enable the identification of scarce proteoforms, together with their post-translational modifications (PTMs). Especially the notion that the novel approach allows for the direct analysis of entire native tear proteoforms including their PTMs and, as such, their correlation with specific clinical observations, promises to be important, e.g. to help clarify what actually initiates the DED condition, before secondary inflammation due to the dry eye starts. This way the actual cause of the disease, rather than secondary phenomena may become treatable, before turning into vision threatening complications.

The discovery of the existence of multiple proteoforms in tear samples of some proteins with importance in DED (among which lacritin), is likely to be pertinent. Specific (sets of) proteoforms may indeed be biomarkers for certain subgroups or stages of DED, and to target them and track their changes in different forms or stages of DED may, therefore, be highly relevant. Moreover, the administration with artificial tear fluid of a disease-related missing proteoform, may well represent a novel therapeutic strategy, as illustrated by the ongoing clinical trial of LacriPep [https://clinicaltrials.gov/ct2/show/NCT03226444].

Top-down proteomics – a promising future?

We advocate that, rather than traditional bottom-up proteomics, the complementary top-down approach should be implemented in the daily ophthalmological practice. We realize

that a number of aspects in the proposed (top-down) proteomics workflow are still prone to optimization, before it may become available for routine clinical use. At the same time, the availability of a human tear proteoform repository will have to be established.

Whereas work by the Beuerman group, validated by our own (bottom-up) experiments (Chapters 4, 5, 6.1) has piloted a useful database of proteins identified in the human tear [Zhou *et al*., 2012], it is clear from our work that, as a matter of fact, such database is a mere reflection of the possibly expressed human genes rather than a list of the actual resulting gene products. We propose to start building a database/repository of all proteoforms which can be identified in tears of healthy donors as well as of patients.

To populate the human tear proteoform repository, extremely well performant LC-MS/MS combinations, such as the Orbitrap Fusion Lumos™ will be of great help. At the same time, such ultimate instrument set up is (currently) way beyond the capacity of the average ophthalmologist, both price-, labour-, and footprint-wise. This is why we are very enthusiastic about the progress made with a much smaller, benchtop instrument (like the QExactive HF™), which we anticipate will have a much better chance to make it to the future doctor's lab, particularly in combination with a reliable and detailed tear proteoform database. When interfaced with an extremely fast and user-friendly capillary electrophoresis inlet (such as the ZipChip™ system (Chapter 6.3), this system's performance appears to come within realistic proportions. Theoretically, with the chip-based capillary electrophoresis coupled to MS, the generation of a patient-specific proteoform profile can be sped up significantly to be completed within minutes rather than hours, meaning that the process of getting relevant information to the clinician promises to be no longer the limiting factor.

Outlook

We have firm confidence in tear protein MS to help gather novel information on various pathologies, the cause of which today still remain elusive. Examples include various eye-related illnesses, like glaucoma, Fuchs' heterochromic uveitis [Mohamed et al*.,* 2005], Posner-Schlossman Syndrome [Green, 2007], macular degeneration, keratoconus, and others. For some of these pathologies, the first tear proteomic data have in the meantime been published [Lema et al., 2010; Pieragostino et al., 2013]. In addition, low-abundant secretory proteins and (neuro)peptides are becoming directly accessible for exploration in tears (rather than indirectly via targeted immunoassays [Lambiase *et al.*, 2011]). Hence, we expect that certain metabolic diseases, obesity, hypertension, but also neurodegenerative disorders like multiple sclerosis, Alzheimer's disease (AD), Parkinson's Disease (PD), and others, may be reflected in specific protein biomarker patterns in tears. The first indications for this have already been reported for diabetes [Kim et al., 2012; Li et al., 2014], and even breast cancer [Bohm et al., 2012].

Differential occurrence of tear proteoforms may promise to provide new insights into a potential metabolic and/or neurological deficit of the patient's condition. Still, virtually all literature data available today in this respect rely on classical bottom-up proteomics studies, and, hence, fail to specify the actual respective biomarker proteoforms involved [see Hagan *et al.*, 2016; Azkargorta *et al*., 2017]. We here refer to a limited selection of recently published and ongoing studies which would very much benefit from a complementary top-

down approach. This includes the ongoing clinical trial for differential protein measurement in tears of PD patients versus healthy subjects [https://clinicaltrials.gov/ct2/show/NCT03037463] and the related work by Börger *et al.* [2015] on PD biomarker discovery; a tear fluid biomarker study for AD [Lim *et al*., 2016]; a study on multiple sclerosis and other CNS infecting inflammatory disorders [Salvisberg *et al*., 2014]; and a tear analysis study aiming to diagnose vitamin deficiencies in neonates [Khaksari *et al*., 2016].

Proteoform analysis through top-down proteomics approaches can be applied, *mutatits mutandis*, to other body fluids like the aqueous humor and vitreous fluid of the eye, saliva, serum, plasma, cerebrospinal fluid, etc. Yet, we judge that the easy accessibility and relative cleanliness of the tear fluid, makes it one of the least complicated, and hence preferred, sources for biomarkers for disease diagnosis, progression, prognosis and therapeutic response. Nevertheless, even when specific (panels of) proteoform biomarkers will finally have been annotated in correlation with a specific DED or other disease state, the amount of work which will need to be carried out before such discovery will have been translated into a validated and clinically qualified test that is approved by the health authorities, should not be underestimated.

Experiencing on a day-to-day basis how DED can dominate a person's life (as illustrated by the patient's testimony recorded in Chapter 1), we sincerely hope that this thesis will positively contribute to the ultimate goal of establishing a cure rather than a treatment for the many DED patients worldwide, thereby offering them a 'healthier' future.

Geel, April 2018

References

Azkargorta M, Soria J, Acera A, Iloro I, Elortza F (2017) Human tear proteomics and peptidomics in ophthalmology: Toward the translation of proteomic biomarkers into clinical practice. J. Proteom. 150: 359-367.

Balog J, Sasi-Szabó L, Kinross J, Lewis MR, Muirhead LJ, Veselkov K, Mirnezami R, Dezső B, Damjanovich L, Darzi A, Nicholson JK, Takáts Z (2013) Intraoperative tissue identification using rapid evaporative ionization mass spectrometry. Sci. Transl. Med. 17: 194ra93

Böhm D, Keller K, Pieter J, Böhm N, Wolters D, Siggelkow W, Lebrecht A, Schmidt M, Kölbl H, Pfeiffer N, Grus FH. (2012) Comparison of tear protein levels in breast cancer patients and healthy controls using a *de novo* proteomic approach. Oncol. Rep. 28: 429-438.

Börger M, Funke S, Bähr M, Grus F, Lingor P (2015) Biomarker sources for Parkinson's disease: time to shed tears? Basal Ganglia 5: 63-69.

Green RJ (2007) Posner-Schlossman syndrome (glaucomatocyclitic crisis): Clin. Exp. Optom. 90: 53-56.

Hagan S, Martin E, Enríquez-de-Salamanca A (2016) Tear fluid biomarkers in ocular and systemic disease: potential use for predictive, preventive and personalised medicine. EPMA J. 7: 15. doi: 10.1186/s13167-016-0065-3.

Khaksari M, Mazzoleni LR, Ruan C, Kennedy RT, Minerick AR (2016) Determination of water-soluble and fat-soluble vitamins in tears and blood serum of infants and parents by liquid chromatography/mass spectrometry. Exp. Eye Res. 155: 54-63.

Kim HJ, Kim PK, Yoo HS, Kim CW (2012) Comparison of tear proteins between healthy and early diabetic retinopathy patients. Clin. Biochem. 45: 60-67.

Lambiase A, Micera A, Sacchetti M, Cortes M, Mantelli F, Bonini S (2011) Alterations of tear neuromediators in dry eye disease. Arch. Ophthalmol. 129: 981-986.

Lema I, Brea D, Rodríguez-González R, Díez-Feijoo E, Sobrino T. (2010) Proteomic analysis of the tear film in patients with keratoconus. Mol. Vis. 16: 2055-2061.

Li B, Sheng M, Xie L, Liu F, Yan G, Wang W, Lin A, Zhao F, Chen Y (2014) Tear proteomic analysis of patients with type 2 diabetes and dry eye syndrome by two-dimensional nano-liquid chromatography coupled with tandem mass spectrometry. Invest. Ophthalmol. Vis. Sci. 55: 177-186.

Lim JKH, Li Q-X, He Z, Vingrys AJ, Wong VHY, Currier N, Mullen J, Bui BV, Nguyen CTO (2016). The eye as a biomarker for Alzheimer's disease. Front. Neurosci. 10: 536 doi: 10.3389/fnins.2016.00536.

Lin CH, Liao WC, Chen HK, Kuo TY (2014) Paper spray-MS for bioanalysis. Bioanalysis. 6: 199-208. doi: 10.4155/bio.13.310.

Pieragostino D, Agnifili L, Fasanella V, D'Aguanno S, Mastropasqua R, Di Ilio C, Sacchetta P, Urbani A, Del Boccio P. (2013) Shotgun proteomics reveals specific modulated protein patterns in tears of patients with primary open angle glaucoma naïve to therapy. Mol. Biosyst. 9: 1108-1116.

Mohamed Q, Zamir E (2005) Update on Fuchs' uveitis syndrome. Curr. Opin. Ophthalmol. 16: 356-363.

Salvisberg C, Tajouri N, Hainard A, Burkhard PR, Lalive PH, Turck N (2014) Exploring the human tear fluid: discovery of new biomarkers in multiple sclerosis. Proteom. Clin. Appl. 8: 185-194.

Zhou L, Zhao SZ, Koh SK, Chen L, Vaz C, Tanavde V, Li XR, Beuerman RW (2012) In-depth analysis of the human tear proteome. J. Proteom. 75: 3877-3885.

Summary

"Innovative strategies to clinically characterize the human tear proteome; from fundamental exploration to clinical ophthalmological relevance"

Ophthalmologists are seriously concerned about the chronic and frequently progressive course in many patients of a problematic and possibly vision-threatening chronical condition known as Dry Eye Disease (DED) [DEWS, 2017, Chapter 2]. This multifactorial ophthalmologic disorder is known to affect a large number of people.
Prevalence of DED is reported to vary from 7% in the United States to 33% in Taiwan and Japan. A grade 1 or mild DED condition may even be observed in 100% of patients over 60 years of age [Shah et al., 2015].
DED may have many causes, and, as such, occurs in various forms and degrees of severity [Murube et al., 2005 & Chapter 2]. Currently, in a majority of cases no clear diagnosis can be made. As a consequence, many patients are not effectively treated and their condition does not improve or even worsens.

In recent years, several lines of clinical as well as molecular and cell biological research have yielded relatively limited novel insights into cause and progression of DED [Olligris et al., 2014]. Clinical research has largely focused on the classification of various disease grades. Extensive pathophysiological studies including the cellular and molecular alterations in DED pathogenesis are rare [Wei et al., 2014].
One of the issues, which we feel to be hampering progress in the field of DED, is the apparent difficulty for clinicians and cell and molecular biology research scientists to find, let alone understand, each other.

This thesis wants to contribute to the noble goal of bridging the gap between clinical practice in the field of DED and present day (as well as future) bio-analytical research science. In particular, we elected to focus on the analytical discipline of proteomics, which complements other "omics" approaches, including genomics, transcriptomics and metabolomics.
As such we attempt to help establish an integrated approach of clinical and bio-analytical protein research, to further our understanding of DED causes and progression.

Chapter 1 introduces the field of DED, by describing some fundamental knowledge of the disease, its diagnosis and standard treatments. We focus on the *status praesens* concerning clinical knowledge of DED, including several recent developments in diagnostics and treatment. Besides the assessment of a current practicing ophthalmologist, also the vision of an analytical molecular biologist as well as the view of a contemporary patient is given.

Chapter 2 includes a review on radiofrequency surgery and elaborates on various aspects of our earlier personal eye surgical practice, which were instrumental (sometimes literally, as in Chapter 2.4) in our later development of innovative DED treatments.

Chapter 3 continues by describing the various innovative treatments which we introduced in our personal practice treating DED patients. This includes the so-called 'Chedly punctal Occluder' [Chapter 3.1], and the, in our eyes, most important example in this respect, i.e. the

first transplantation in Belgium (one of the first in Europe) of labial salivary glands to the conjunctival side of the eyelids [Chapter 3.2].

Initial mass-spectrometric analyses and (proteomics) comparison of tears and saliva from healthy individuals revealed the different protein compositions of tears and saliva, confirming a high degree of similarity between both 'secretomes' [Chapter 4], as previously suggested by Murube et al. [1998].
As such Chapter 4 of this thesis constitutes the first effective pillar of our bridge between clinical and molecular (protein) biological understanding of DED.

Throughout our thesis work we tried to keep track of the numerous analytical and technological developments in the field of proteomics, and we got more and more convinced of the huge potential modern mass spectrometry (MS) approaches in the analysis of tears have for continued research leading to improved diagnostics for DED and to novel insights into DED pathogenesis.
As such, Chapter 5 reports on our first use of an orbitrap equipped MS system. This work was the first published record that proteomics analysis of human tears can be successfully and reproducibly performed on material from a single (DED) patient.

Chapter 6 summarizes a set of current ongoing research, in which we set off to explore the potential which the recently made available top-down proteomics tools (both hardware as well as software) are bringing to the field. The preliminary results which we have obtained so far are overwhelming. These include a number of novel developments and improvements of top-down mass spectrometric analysis of the tear proteome for future use in the clinical practice. It describes innovative approaches for the full identification of tear proteins with all the post-translational modifications present on them, i.e. the actual tear 'proteoforms'.

As such we are confident to conclude (Chapter 7) that (top-down) MS will live up to its promise of being a very valuable analytical tool to further our understanding of DED pathophysiology, enabling the discovery of innovative diagnostics as well as therapeutics for DED. At the end of this thesis, we give our personal view on future research directions and novel clinical developments (including MS applications) in DED.
We prophesize that the top-down proteomics study of tear proteins ('tear proteoforms') will represent an innovative approach to help evaluate the general health and disease state of, not only, DED patients, but of patients suffering from a wide variety of metabolic, age-related, and other diseases which characterize our modern society.

References:

DEWS (2007) The definition and classification of dry eye disease. Report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop. Ocul. Surf. 5: 75-92.

Murube J (1998) Labial salivary gland transplantation in severe dry eye. Oculoplast. Orbital Reconstr. Surg. 1: 104-110.

Murube J, Németh J, Höh H, Kaynak-Hekimhan P, Horwath-Winter J, Agarwal A, Baudouin C, Benítez del Castillo JM, Cervenka S, ChenZhuo L, Ducasse A, Durán J, Holly F, Javate R, Nepp J, Paulsen F, Rahimi A, Raus P, Shalaby O, Sieg P, Soriano H, Spinelli D, Ugurbas

SH, Van Setten G (2005) The triple classification of dry eye for practical clinical use. Eur. J. Ophthalmol. 15: 660-667.

Olligris B, Alkozi HA, Pintor J (2014) Recent developments on dry eye disease treatment compounds. Saudi J. Ophthalmol. 28: 19-30.

Shah S, Jani H (2015) Prevalence and associated factors of dry eye: Our experience in patients above 40 years of age at a Tertiary Care Center. Oman J. Ophthalmol. 8: 151-156.

Wei Y, Asbell PA (2014) The core mechanism of Dry Eye Disease (DED) is inflammation. Eye Contact Lens 40: 248-256.

Samenvatting

"Innovatieve strategieën voor de karakterisatie van het proteoom van de humane traan; van fundamenteel onderzoek tot klinische oftalmologische relevantie"

Oogartsen zijn bijzonder begaan met het chronisch en veelal progressief verloop van het klinisch beeld bij de vele patiënten met droge ogen. Deze mogelijks zichts-bedreigende aandoening wordt meestal omschreven met de Engelse term Dry Eye Disease (DED [DEWS, 2017, Hoofdstuk 2].
De frequentie van DED zou variëren van 7% in de VS tot 33% in Taiwan en Japan. Eén studie maakt zelfs gewag van een graad 1 of mildere vorm van DED bij 100% van de patiënten ouder dan 60 jaar [Shah et al., 2015].
DED heeft multipele mogelijke oorzaken en kan zich presenteren in verschillende ernstgraden [Murube et al., 2005 & Hoofdstuk 2]. Tot op heden kan in de meerderheid van de gevallen geen eenduidige diagnose worden gesteld. Bijgevolg worden vele patiënten niet adequaat behandeld waardoor hun toestand niet verbetert of zelfs achteruit gaat.

Klinisch onderzoek evenmin als research op moleculair en celbiologisch vlak hebben de laatste jaren nieuwe inzichten kunnen verschaffen in de oorzaak en het ziekteproces van DED [Olligris et al., 2014].
Klinisch onderzoek was vooral gefocust op de ernst van de verschillende vormen van droge ogen en uitgebreide fysio-pathologische studies met inbegrip van de cellulaire en moleculaire veranderingen bij DED zijn bijzonder schaars [Wei et al., 2014].
Een verbeterde communicatie tussen clinici en biomoleculaire analytici zou de voortgang in het onderzoek naar droge ogen ten goede kunnen komen. Hiertoe willen we met dit proefschrift een bijdrage leveren.

We een willen de kloof helpen overbruggen tussen de oogheelkundige praktijk en de *status praesens* (alsook de perspectieven) in de analytische biotechnologie. In het bijzonder hebben we ons toegespitst op de analytische mogelijkheden van proteomics, en dit als aanvulling van de andere beschikbare "omics" data, zoals uit genomics, transcriptomics en metabolomics.
Op die manier hopen we te komen tot een integrale aanpak van klinisch en bio-analytisch proteïnen-onderzoek om meer inzicht te verwerven in de problematiek van DED.

Hoofdstuk 1 maakt ons wegwijs In de wereld van droge ogen: de voornaamste klachten, hoe de diagnose wordt gesteld en wat de klassieke actuele behandelingen zijn. We focussen zowel op de huidige kennis ter zake als op enkele recente ontwikkelingen in diagnose en behandeling. Droge ogen worden bekeken vanuit het standpunt van de patiënt, de oogarts en de analytische moleculaire bioloog.

Hoofdstuk 2 gaat over de chirurgie met hoogfrequente radiogolven (RF) en illustreert hoe ikzelf deze techniek aanwend voor ooglidchirurgie in het algemeen. Het belang van deze oculoplastische technieken was dat ze de basis vormden voor een aantal chirurgische behandelingen voor DED die aan bod komen in Hoofdstuk 3.

Hoofdstuk 3 spitst zich toe op 2 innovatieve toepassingen van RF in de heelkundige

behandeling van DED, m. n. de permanente occlusie van het traanpunt met de 'Chedly punctal occluder' [Hoofdstuk 3.1] maar ook, en vooral, de eerste transplantatie in België van lipspeekselklieren naar het ooglid [Hoofdstuk 3.2].

Een eerste massaspectrometrische analyse en vergelijkende studie van proteïnen in tranen en speeksel van gezonde personen bevestigde de belangrijke overeenkomsten in proteïnen-samenstelling tussen beide vloeistoffen, zoals eerder gesuggereerd door Murube en medewerkers [Murube et al., 1998].
Zodoende is Hoofdstuk 4 de eerste pijler van de brug tussen kliniek en moleculaire benadering van DED.

Gedurende mijn doctoraal onderzoek hebben we de vele analytische en technologische ontwikkelingen gevolgd op het vlak van proteomics en meer en meer geraakten we overtuigd van het uitgebreide potentieel van moderne massaspectrometrie (MS) naar de toekomst toe in de analyse van tranen. Dit kan leiden tot een betere (differentiële) diagnose van DED en een beter begrip van het ziekteproces.
Zo brengt Hoofdstuk 5 onze eerste resultaten, gebruik makend van een MS-opstelling met daarin een orbitrap. Het was de eerste publicatie waarbij de mogelijkheid en reproduceerbaarheid van de tranen-analyse van een individuele patiënt kon worden aangetoond.

Hoofdstuk 6 vat een aantal lopende onderzoeken samen die de mogelijke toepassingen illustreren van enkele recente nieuwe technologische ontwikkelingen, zowel wat betreft hardware als software, m.n. in top-down proteomics. De eerste resultaten hiervan zijn overweldigend. Zo boren we nieuwe mogelijkheden in top-down proteomics aan die toekomstige toepassingen in de dagelijkse klinische praktijk mogelijk moeten maken. E.e.a. moet ons toelaten in een minimum van tijd de eiwitten in de tranen van onze patiënten met al hun post-translationele modificaties, de zogenaamde proteoformen, te profileren van patiënten op onze raadpleging.

In Hoofdstuk 7 menen wij dan ook te mogen concluderen dat (top-down) MS zijn belofte kan waarmaken een bijzonder analytisch instrument te zijn om meer inzicht te krijgen in het ziekteproces van DED.
Dit kan leiden tot nieuwe middelen voor diagnose en behandeling van DED.
Op het einde van dit proefschrift reiken wij een aantal richtlijnen aan voor verder onderzoek in DED, met inbegrip van MS-toepassingen.
En we maken ons sterk dat de top-down proteomics-studie van traan-eiwitten en hun proteoformen ook mogelijkheden inhoudt voor de evaluatie van andere, niet louter oog-gerelateerde/oftalmologische, gezondheidsproblemen.
Tranen kunnen aldus een toegankelijke en rijke bron zijn voor biomarkers voor een pleïade aan pathologieën.

Referenties:

DEWS (2007) The definition and classification of dry eye disease. Report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop. Ocul. Surf. 5: 75-92.

Murube J (1998) Labial salivary gland transplantation in severe dry eye. Oculoplast. Orbital

Reconstr. Surg. 1: 104-110.
Murube J, Németh J, Höh H, Kaynak-Hekimhan P, Horwath-Winter J, Agarwal A, Baudouin C, Benítez del Castillo JM, Cervenka S, ChenZhuo L, Ducasse A, Durán J, Holly F, Javate R, Nepp J, Paulsen F, Rahimi A, Raus P, Shalaby O, Sieg P, Soriano H, Spinelli D, Ugurbas SH, Van Setten G (2005) The triple classification of dry eye for practical clinical use. Eur. J. Ophthalmol. 15: 660-667.
Olligris B, Alkozi HA, Pintor J (2014) Recent developments on dry eye disease treatment compounds. Saudi J. Ophthalmol. 28: 19-30.
Shah S, Jani H (2015) Prevalence and associated factors of dry eye: Our experience in patients above 40 years of age at a Tertiary Care Center. Oman J. Ophthalmol. 8: 151-156.
Wei Y, Asbell PA (2014) The core mechanism of Dry Eye Disease (DED) is inflammation. Eye Contact Lens 40: 248-256.

Dankwoord

Bij het indienen van mijn doctoraal proefschrift hoort een dankwoord voor alle familieleden, vrienden en collega's zonder wie dit werk nooit tot stand zou zijn gekomen.
In de eerste plaats mijn echtgenote Manuela die me deed geloven in mijn ideeën en me aanmoedigde ze uit te werken in een doctoraal proefschrift.
Zij was de drijvende kracht om me ook in moeilijke periodes te doen blijven geloven in het project.
In de praktijk was het mijn promotor Prof. Peter Verhaert die me de mogelijkheden leerde kennen van de massaspectrometrie om mijn transplantatie van speekselklieren naar de oogleden een stevige wetenschappelijke basis te geven.
Hij bracht mij met engelengeduld de basisbegrippen bij van massaspectrometrie.
Tijdens mijn aanvullende zomercursus in Dubrovnik begreep ik pas echt dat ik de eer had met een wereld-autoriteit te mogen samenwerken.
Ook gewezen assistent professor Dr. Martijn Pinkse en enkele hulpvaardige studenten wil ik hierbij graag bij naam noemen voor hun zeer gewaardeerde praktische hulp bij de LC MSMS traananalyses: Remco Crefcoeur en Bharath Kumar Raguraman.
Bijzondere dank ook aan mijn promotor Prof. Han de Winde voor de gastvrijheid mij aangeboden als buiten-promovendus aan de Universiteit Leiden.
Niet alleen met hem mogen werken aan deze hoog aangeschreven Universiteit was een eer, ook het vriendschappelijke karakter van de contacten tijdens mijn PhD periode heeft een bijzondere indruk op mij gemaakt.
A special thank you to Dr. Paul Thomas and Dr. Neil Kelleher, for their expert assistance in the analysis of high content top-down proteomics data (Chapters 6.1 and 6.4), as well as to Dr. Caroline De Hart and Mr. Haemin Park, all at Northwestern University in Evanston (Chicago, Illinois), for their help with the analyses described in Chapter 6.4.
The unique analyses with the very latest exceptional instrumentation which we have been able to perform during this PhD study, have only been possible thanks to the visionary support by Dr. Daniel Lopez-Ferrer and his team of experts at ThermoFisher Scientific in San Jose (California), who realized the possibilities of the novel analytical instruments in the context of ophthalmology.

Ik ben ook veel dank verschuldigd aan de mensen die helemaal niet in mijn project geloofden. Koppig als ik ben waren zij het die me extra stimuleerden om niet op te geven en te bewijzen dat we op het juiste spoor zitten met de transplantatie van speekselklieren en de doorgedreven analyse van traanvocht.

Zeker mag ik niet vergeten al de mensen in mijn naaste omgeving te bedanken voor de tolerantie als ik in de laatste 4 jaar niet altijd even vrolijk rondliep.
Vooral mijn jongste zoon Linus heeft zijn papa veel moeten missen in deze periode maar ook de broers Kasper, Bregt, Ruben en Maurits hebben hun (plus)papa in soms lastige periodes moeten ondergaan.

Hetzelfde geldt voor de medewerkers op mijn poli: Ria, Nannie, Kristel, Martine en Vicky van wie heel wat flexibiliteit werd gevraagd om naast 'de thesis' een meer dan voltijds draaiende raadpleging te organiseren.

Last but not least, a special thank you to my paranymph from New York, Dr. Jon Garito, with whom I enjoy, for already more than 25 years, an extraordinary collaboration in the domain of radio frequency surgery. Thanks for introducing me to the main radio surgeons on the planet. This formed the foundation for a number of my techniques in eyelid surgery, including the autotransplantation of labial salivary glands, which started this whole exciting journey.

Curriculum Vitae

Peter Paul Magdalena Raus (°1957, Mol, Belgium) started his medical studies at Hasselt University in 1975. He obtained his medical degree at the university of Leuven in 1982.
He received his ophthalmology training (1982-1986) at the Universities of Leuven (Belgium), El Mansourah (Egypt) and at the Barraquer Institute in Barcelona (Spain).
After being introduced to radio frequency surgery by Dr. Stephen Bosniak (New York, USA), he became a performant 'Radiosurgeon' and lectured in 26 countries on that subject.
As an eyelid surgeon, he performed the first transplantation in Belgium of labial salivary glands to the eyelids of a patient with very dry eyes. This type of surgery lead to the study that resulted in this manuscript.
Peter Raus is the owner of the Miró Institute for ophthalmolgy, cardiology and eyelid surgery in Geel (Belgium).
He developed the Raus-Garito clamp for eyelid surgery and is TFOS (Tear Film and Ocular Surface Society) ambassador for Belgium.

"*A tear is made of 1% water and 99% feelings*" [Anonymous, not an analytical chemist]