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**Author:** Raus, P.P.M.

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Chapter 7: 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 FT™), 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

