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

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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.

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.

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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

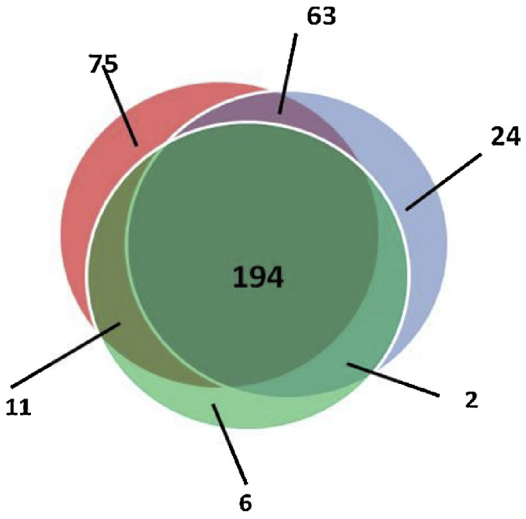

Fig. 1. Numbers of protein groups identified by nanoUHPLC quadrupole-Orbitrap MSMS from sub-microliter tear volumes of healthy human volunteer. Triplicate analysis of a single unpooled individually collected tear sample, yielding an overall protein number of 375, with 194 proteins identified consistently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

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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.

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