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Comparison of Capillary and Schirmer Strip Tear Fluid Sampling Methods Using SWATH-MS Proteomics Approach

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Purpose: The purpose of this study was to examine the protein profile differences between capillary and Schirmer strip tear fluid samples.

Methods: Both capillary and Schirmer strip tear samples were collected from 31 healthy participants at the same visit, and the samples were analyzed with nanoflow liquid chromatography coupled with time-of-flight mass spectrometer (NanoLC-MSTOF), implementing a sequential window acquisition of all theoretical fragment ion spectra mass spectrometry (SWATH-MS). Sample type-specific and combined spectral libraries were used to evaluate the differences between the sample types in protein expression levels and biological functions.

Results: In proportion, more extracellular proteins connected to immune response were quantified from the capillary samples while Schirmer strip samples contained more intracellular proteins. The sample types yielded similar counts of quantified proteins when a combined spectral library including both sample types was implemented. The differential expression analysis between the sample types identified proteins increased in the capillary samples (e.g., immunoglobulins) and Schirmer strip samples (e.g., heat-shock proteins, annexins, and S100 proteins).

Conclusions: Tear proteomics data originating from the same participants vary depending on whether the sample is collected with capillary or Schirmer strip, although there is also overlap between the two sample types when a combined spectral library is implemented in the SWATH-MS analysis. In discovery-based proteomics research of tear fluid, appropriate sampling method should be chosen carefully based on the research focus.

Translational Relevance: Currently, there is no consensus on how the tear fluid sampling methods affect the resulting proteomics data, and hence, identification of the most suitable sampling methods for clinical researchers with varying research interests is important.

Introduction

Tear fluid is an ideal sampling material in ocular surface studies as it can be used to efficiently examine the condition of the underlying tissue while the sampling itself is noninvasive and fast. In modern mass spectrometry, efficient techniques have been developed to reliably identify and quantify large numbers of distinct proteins from tear fluid samples,[1–3] and therefore, it has been implemented in several recent studies examining the health status of the ocular surface.[4–14] Although the results from these studies indicate similar protein expression level and functional changes, there is uncertainty on how much variability exists due to various technical reasons (e.g., different tear fluid sampling methods, use of topical anaesthesia,[15,16] and other sampling and analysis parameters[17]).

The most common tear fluid sampling methods include Schirmer strip, microcapillary tube, absorbent-based methods such as sponges, and eye flush,[18] the first two being the most used. Schirmer strip samples are collected with filter paper, which is placed partially under the lower eyelid, in the lower cul-de-sac, typically for 5 minutes. In addition to tear fluid sampling, Schirmer strip is a standard clinical test tool in ophthalmology clinics as it can be also used to evaluate the tear fluid volume in the eye. Due to this

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familiarity, it is often implemented in clinical studies examining the tear fluid. In capillary sampling, the tip of the glass capillary is placed in the lower tear meniscus, and via capillary action, the tear fluid can be sampled from the conjunctival sac. This method requires more experience; can be challenging, especially with dry eye patients with small volumes of tear fluid; and is considered less pleasant by patients.[19] However, it does minimize the contact and resulting irritation to the ocular surface tissue, unlike Schirmer strips, which are known to collect some additional cellular proteins during the sampling.[20]

In addition to the differences in the physical and practical attributes of capillary and Schirmer strip sampling, the two methods may also result in different proteomics data and analysis results. Capillary and Schirmer strip sampling methods have been previously examined in studies implementing two-dimensional electrophoresis (2-DE), liquid chromatography, enzyme-linked immunosorbent assay (ELISA), Western blotting, mass spectrometry, and protein chip arrays, as well as methods focusing on lipidomics.[4,20–28] However, to our knowledge, our study is the first to implement a wider discovery proteomics approach in the form of sequential window acquisition ion spectra mass spectrometry (SWATH-MS), enabling individual protein profile comparisons between capillary and Schirmer strip samples originating from the same patients. In SWATH-MS, the quantitative MS data are decoded with the help of pregenerated spectral reference libraries,[29] and in this study, the samples were processed using both separate sample type–specific (capillary or Schirmer strip) spectral libraries as well as a combined (capillary and Schirmer strip), comprehensive spectral library. This enabled us to examine the protein profiles using each of the sampling methods alone and, in addition, the relative protein expression-level differences between the two sampling methods. Therefore, the main aim of this study was to provide information to researchers about the potential protein profile differences between the sample types, particularly in SWATH-MS, and thus enabling them to choose the most appropriate sampling and analysis method for their study.

Methods

Study Population

Tear fluid samples were collected from 31 strabismus surgery patients, who had no prior diagnosed ocular surface conditions. Schirmer strip and capillary samples were collected during the same visit, and all tear fluid samples were taken from the same eye prior to surgery, without topical administration of any surgery-related medication. All patients included in this study had a preoperative ophthalmic examination, including biomicroscopy, fluorescein staining, conjunctival redness, and Schirmer's test, to identify any clinical pathologies, which would be an exclusion criterion for this study. No patients younger than 18 years or pregnant were included. This study was conducted in accordance with the tenets of the Declaration of Helsinki and a written informed consent was obtained from the patients. The study was also approved by the Ethics Committee at Tampere University Hospital (ethical permission number R13074).

Tear Fluid Sampling and Sample Preparation

For each patient, tear fluid samples were collected using two different sampling techniques during the same visit. More specifically, tear samples were collected from patients using 2- or 3-µL Microcap tubes (Drummond, Broomall, PA, USA) and Schirmer strips (Tear Touch, Madhu Instruments, New Delhi, India) without anesthesia. The capillary samples were collected from the lower conjunctival sac. The Schirmer strip samples were collected from closed eyes by inserting a strip under patients' lower eyelid and removing it after 5 minutes. All tear samples were stored at –80°C until further proteomic analyses were conducted. The sample preparation steps were carried out as described in our previously published studies.[12,30]

Samples were flushed from capillaries with 0.5% sodium dodecyl sulphate (SDS) in 50 mM ammonium bicarbonate supplemented with protease inhibitor cocktail. The same solution was used to solubilize samples from Schirmer strips. Total protein concentration of the tear samples was measured by the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

For protein analysis, acetone-precipitated proteins were dissolved in 2% SDS in 0.05 M triethylammonium bicarbonate buffer (TEAB) and reduced by tris-(2-carboxyethyl)phosphine for 1 hour at +60°C. Reduced samples were transferred into 30-kDa molecular weight cutoff filters (Pall Corporation, Port Washington, NY, USA) and flushed with 8 M urea in 0.05 M Tris-HCl (Thermo Fisher Scientific, Waltham, MA, USA). Cysteine residue blocking was done by iodoacetamide at room temperature in the dark. Alkylation was terminated by centrifugation, and the samples were washed with urea followed by 0.05 M TEAB prior to digestion with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (Sciex, Framingham, MA, USA) for 16 hours

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at +37°C at a trypsin-to-protein ratio of 1:25. Digests were eluted from filters with 0.05 M TEAB followed by 0.5 M NaCl and dried in a speed vacuum concentrator. Samples were reconstituted in 0.1% trifluoroacetic acid, cleaned, and desalted with Pierce C18 tips (Thermo Fisher Scientific) according to the manufacturer's instructions. After cleanup, the samples were vacuum dried and stored at –20°C until reconstituted to loading solution (2% acetonitrile (ACN), 0.1% formic acid (FA)) at equal concentrations. Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Quantitative Mass Spectrometry Analysis

Digested peptides were analyzed by NanoLC-MSTOF instrumentation using an Eksigent 425 NanoLC coupled to a high-speed TripleTOF 5600+ mass spectrometer (AB Sciex, Concord, Canada). A nano cHiPLC column (cHiPLC ChromXP C18-CL, 3-μm particle size, 120 Å, 75 μm i.d. × 15 cm; Eksigent, Concord, Canada) was used for the liquid chromatography separation of peptides. The samples were first loaded onto a trap column (cHiPLC ChromXP C18-CL, 3-μm particle size, 120 Å, 75 μm i.d. × 5 mm). After 10 minutes of loading at 2 μL/min (2% ACN, 0.1% FA), the trap column was switched in line with the analytical column. The peptide mix was introduced into the mass spectrometer via nanospray source with a fused silica emitter (New Objective, Woburn, MA, USA) and analyzed with 120-minute six-step gradients using eluent A (0.1% FA in 1% ACN) and eluent B (0.1% FA in ACN; eluent B from 5% to 7% over 2 minutes, 7% to 24% over 55 minutes, 24% to 40% over 29 minutes, 40% to 60% over 6 minutes, 60% to 90% over 2 minutes and kept at 90% for 15 minutes, 90% to 5% over 0.1 minutes and kept at 5% for 13 minutes) at 300 nL/min. The same amount of protein was loaded to MS analysis with both sample types. The key parameters for the TripleTOF mass spectrometer were as follows: ion spray voltage floating, 2300 V; curtain gas, 30; interface heater temperature, +150°C; ion source gas 1, 13; and declustering potential, 100 V. Additional information for the NanoLC and MSTOF methodology has been published in our previous studies.[12,30]

SWATH Library Creation and Peak Integration

Spectral libraries were created by the data-dependent acquisition (DDA) method to decode the SWATH spectra and obtain relative quantitation data. Spectral libraries for relative protein quantification were created using 34 capillary or 34 Schirmer strip sample identification runs, which originated from this study as well as other, previously published studies.[12–14] The spectral libraries were created using Protein Pilot 4.5 (Sciex, Redwood City, CA, USA) using a false discovery rate of 1%, and all DDA run MS/MS spectra were identified against the UniProtKB/Swiss-Prot human database. In DDA runs, 0.25-second MS survey scans in the mass range of 350 to 1250 m/z were followed by 60 MS/MS scans in the mass range of 100 to 1500 Da (with total cycle time of 3.302 seconds). Switching criteria were set to ions greater than 350 m/z and smaller than 1250 m/z with charge state 2 to 5 and an abundance threshold of more than 120 counts. Former target ions were excluded for 12 seconds, and mass tolerance was set to 50 mDa. DDA rolling collision energy (CE) parameters script was used for automatically controlling CE. SWATH quantification analysis parameters were the same, with the following exceptions: cycle time of 3.332 seconds and MS parameters set to 15-Da windows with a 1-Da overlap between mass range of 350 to 1250 Da followed by 40 MS/MS scans in the same mass range. Protein quantification was performed using PeakView and MarkerView software (Sciex). Retention time calibration was implemented for all samples using up to three proteins identified specifically for each of the processes (capillary specific, Schirmer strip specific, and combined), and 1 to 15 peptides were used for peak area calculations. Protein quantification results are presented as a combination of protein-specific peptide peak intensities from SWATH-MS measurement and referred to here as protein expression.

Data Processing and Statistical Analysis

Data sets originating from capillary tube and Schirmer strip samples, as well as their combined data, were all processed and analyzed using the same methods to obtain comparative results. Log_2 transformation and central tendency normalization were performed on all data sets as necessary. Quality checks were performed by chromatogram inspection for all samples and replicate MS runs. In addition, intraclass correlation (ICC) and permuted P values were calculated for samples including at least two replicate MS runs, in order to evaluate the quality of the runs as well as to identify runs, which had failed during the MS process. Replicate MS runs were combined by calculating the geometric mean for each protein.

Successfully quantified proteins from capillary and Schirmer strip data sets (processed separately with sample type–specific spectral libraries) were examined by comparing gene ontology (GO) terms and their proportions of associated proteins obtained from Panther Classification System.[31] Pearson's chi-squared

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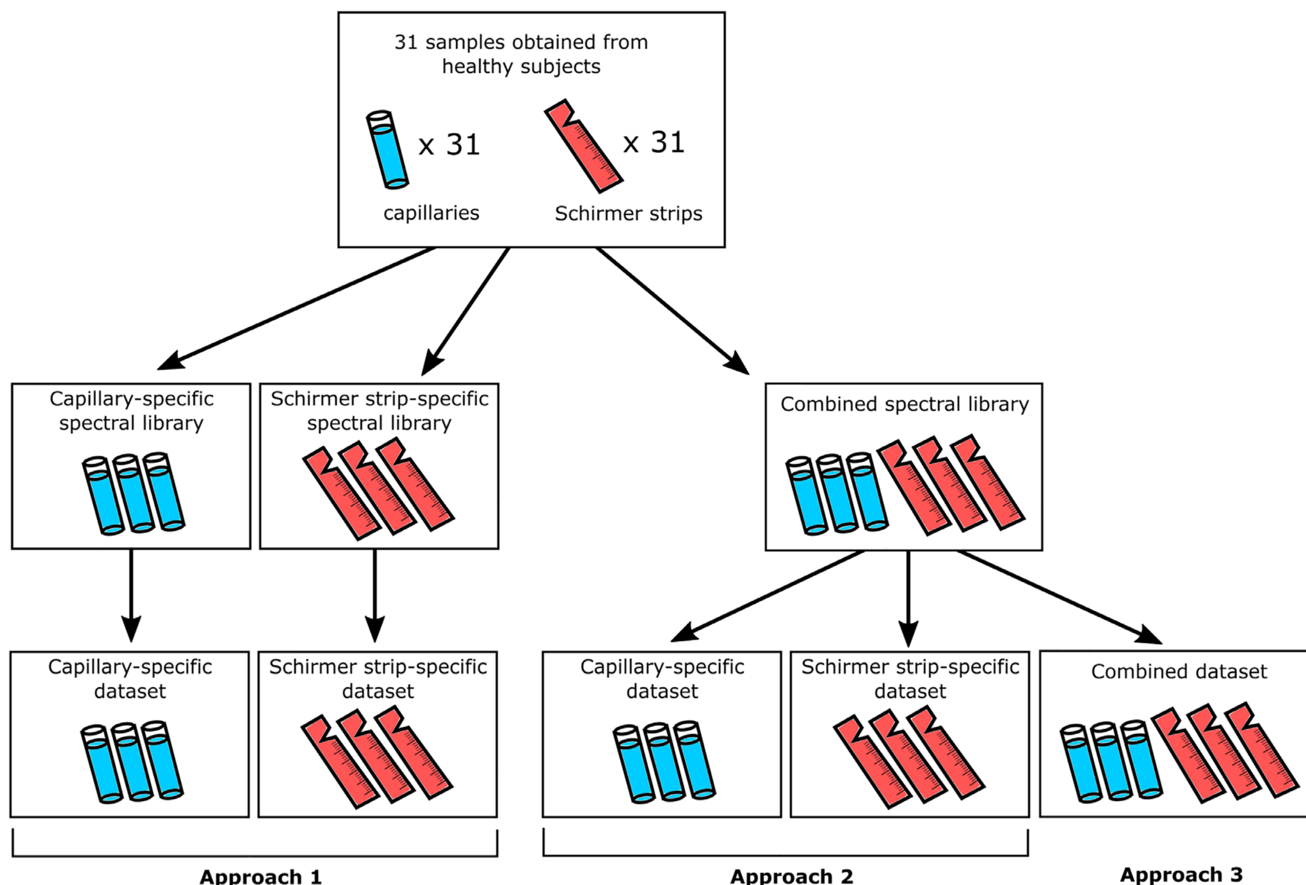

Figure 1. Study outline showing the types of data sets produced and analyzed. In the first approach, separate capillary and Schirmer strip spectral libraries were used to process the capillary and Schirmer strip samples separately, resulting in sample type–specific proteomics data. Next, in the second approach, the capillary and Schirmer strip samples were processed separately, both with a combined spectral library, containing identification runs from both sample types. In the third and final approach, the capillary and Schirmer strip samples were processed together with the combined spectral library, enabling a comparison of the relative expression levels.

test was used to estimate the differences in protein proportions connected to specific GO terms between capillary and Schirmer strip data sets. Similar analyses of the separate capillary and Schirmer strip data sets were processed also with the combined spectral library.

For the combined data set, which was processed using both capillary and Schirmer strip samples and the combined spectral library, differential expression analysis between the two sampling methods was performed using the Wilcoxon signed rank test assuming dependence between the capillary and Schirmer strip samples originating from the same patient (at the same time point). *P* values were adjusted using the Benjamini–Hochberg procedure to control the false discovery rate. *P* value (adjusted) threshold of 0.05 and mean fold change (FC) threshold of 1.5 and 0.667 ($\log_2$ FC = ±0.585) were used to filter the statistically significant results, which were then used to evaluate the functional differences between the sample types as well as the associated protein families and groups with the Panther Classification System and QIAGEN's Ingenuity's pathway analysis (IPA) (QIAGEN, Redwood City, CA, USA). All other statistical analyses for the proteomics data were performed using R software version 3.5.3 (R Core Team, Vienna, Austria).[32]

Results

The total tear protein amount was estimated to be on average 19.7 µg and ranging from 8.6 to 50.1 µg in capillary samples, and in Schirmer strip samples, the mean total protein was 199 µg and ranged from 71.8 to 517.4 µg. The tear proteomics data were processed implementing three different approaches illustrated in Figure 1. Of the 31 patients, 30 Schirmer strips were successfully processed while respective value for capillary samples was 28. In protein expression-level comparisons, only patients with both capillary and Schirmer strip samples were included, resulting in 27 patients for these analyses.

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Sample Type–Specific Data Sets and Libraries

Successfully quantified proteins differed in both number and content between the two data sets originating from different sample types. Capillary-specific spectral library contained 445 proteins, of which 404 were quantified, whereas the Schirmer strip–specific spectral library had 1076 proteins and its analysis produced a data set with 908 quantified proteins. Data quality checks resulted in mean ICC values of 0.99 for both capillary and Schirmer strip data sets. All, except one capillary, sample run pairs had a permuted correlation-associated P value of <0.05. The two data sets had 316 proteins in common, leaving the capillary data with 88 and Schirmer strip data with 592 unique proteins (Fig. 2a). Supplementary Table S1 lists the proteins from the two sample type–specific data sets in more detail.

The protein types also differed based on GO terms and, more specifically, the protein proportions associated with them. Cellular component GO term results showed that the protein group unique to capillary samples contained a higher percentage of proteins originating from extracellular region, protein-containing complexes and membrane (Fig. 2b). It should be noted, however, that the absolute numbers of sample type–specific proteins did not always indicate as large differences (e.g., extracellular region had 34 proteins unique to capillary samples and 37 proteins unique to Schirmer strip samples). The protein group unique to Schirmer strip samples contained more cell- and organelle-specific proteins, although no statistically significant differences were present for cell-related proteins' proportions. Biological processes differed most notably between the sample types in immune system process, biological regulation, response to stimulus, and localization, which were associated more commonly with capillary-originating proteins, whereas cellular- and metabolic process–associated proteins had a similar proportion in all protein sets (Fig. 2c). GO terms under the molecular function domain had a differing proportion of proteins associated with catalytic activity, which were higher in Schirmer strip–specific proteins (Fig. 2d).

Sample Type–Specific Data Sets and a Combined Library

Separate capillary and Schirmer strip data sets, generated using the same combined spectral library, had relatively similar amounts of quantified proteins. The combined library itself contained 958 proteins, and of these proteins, the capillary data set had 770 and the Schirmer strip data set 841 successfully quantified proteins. Altogether, 761 proteins were common between the two data sets, leaving the capillary data set with 9 unique proteins and Schirmer strip data set with 80 (Fig. 3). The proteins from the two data sets are listed in Supplementary Table S2. The quality measured with the replicate MS runs indicated fairly similar results as with the data generated with sample type–specific spectral libraries as ICC means were 0.98 for both, and 88.5% and 96.4% of replicate MS run pairs had permuted P values of <0.05 for capillary and Schirmer strip, respectively. The number of unique proteins for capillary and Schirmer strip data sets were so small that further comparison between them was not considered feasible.

Combined Data Set and Library

The combined spectral library, generated using both capillary and Schirmer strip samples, contained 958 proteins, and as both capillary and Schirmer strip samples were processed together, 855 proteins were in the final quantified data. Mean ICC for the run pairs was 0.98, and 97.2% of pairs had a permuted correlation-associated P value of <0.05, indicating good quality. Preliminary analysis showed that the capillary and Schirmer strip samples differed in their protein expression profiles, notably according to the hierarchical clustering dendrogram (Fig. 4a) and principal component analysis (Fig. 4b), which clustered the two sample types very clearly into two groups.

Next, the relative expression levels between capillary and Schirmer strip samples were compared. Out of the 855 proteins, 521 proteins had an adjusted P value of <0.05 when expression levels were compared between capillary and Schirmer strip samples (Fig. 5a). In total, 221 proteins had a higher expression level in the capillary samples, and 300 were elevated in the Schirmer strip samples; once filtered, based on the adjusted P value as well as mean FC (adjusted $P < 0.05$ and mean FC >1.5 or <0.667), the respective values were 191 and 251 (Supplementary Table S3). Supplementary Figures S1 to S3 illustrate total ion chromatograms as well as extracted ion chromatograms and associated mass spectra of two proteins, albumin (ALB) and lactotransferrin (LTF), for the same patient. Biological functions associated with these two protein groups were again evaluated with the Panther Classification System, and it was noted that higher proportions of proteins associated with extracellular region, protein-containing complex and membrane were upregulated in capillary samples, and conversely, proteins associated with cells or organelles were more often upregulated in Schirmer strip samples (Fig. 5b). In biological processes, immune response proteins were upregulated

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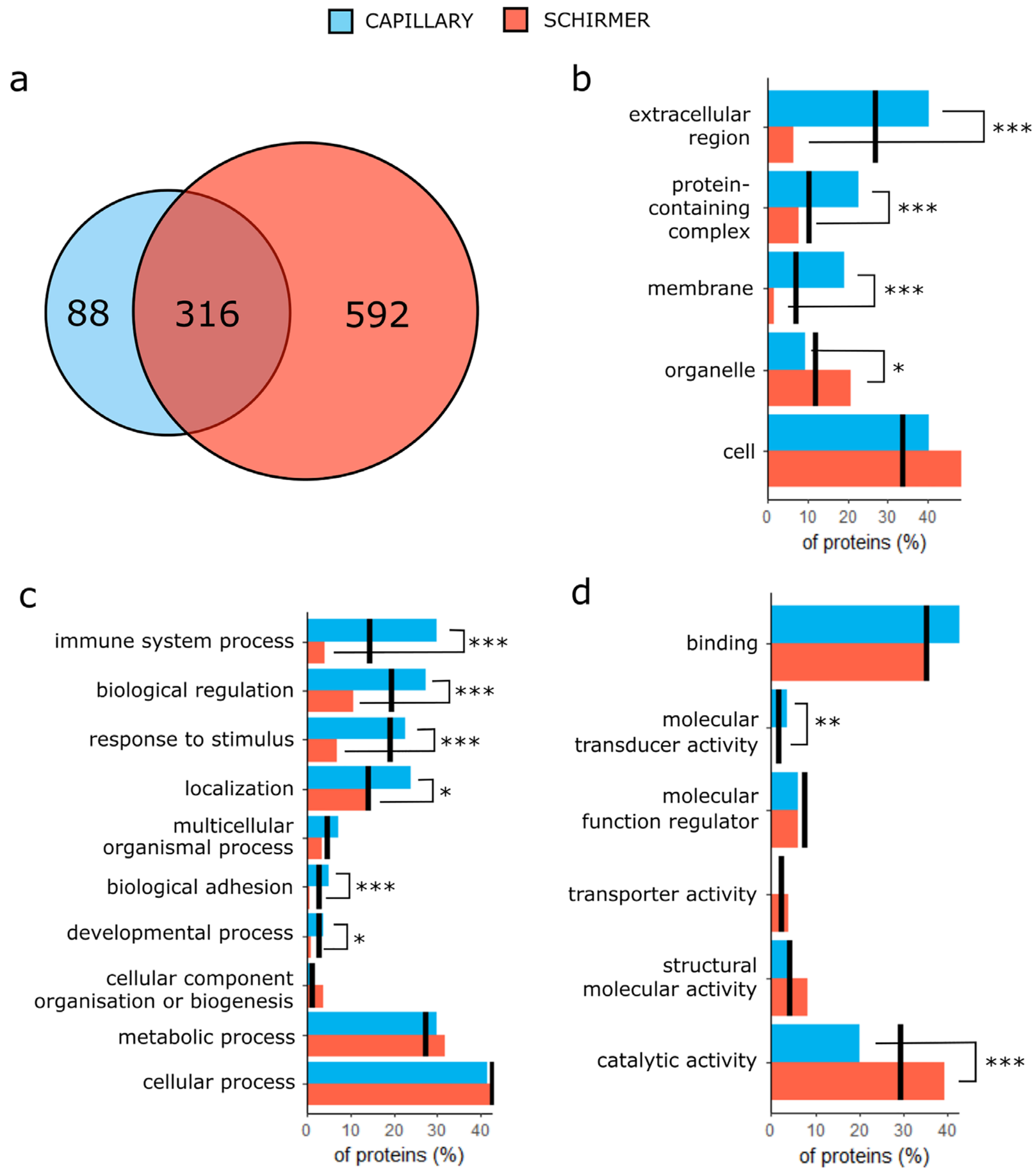

Figure 2. Comparison of the sample type–specific proteomics data originating from capillary (blue) and Schirmer strip (red) tear fluid samples. (a) Venn diagram displays the numbers of unique and common proteins between the two data sets, indicating a much higher proportion of unique proteins in the Schirmer strip data set when sample type–specific spectral libraries are implemented. Bar charts illustrate the percentage of proteins (x-axis) associated with a given gene ontology (GO) term subgroup (y-axis) by category: (b) cellular component, (c) biological process, and (d) molecular function. Pearson's chi-squared test was used to estimate differences between the capillary- and Schirmer strip–specific proteins' distributions. In addition to showing the proportions of proteins originating only from capillary (blue, 88 proteins) or Schirmer strip (red, 592 proteins) samples, the bar charts also include the associated value for proteins, which are common between the two data sets (black vertical lines, 316 proteins). GO terms, which had protein proportions $<2.5\%$ for both Schirmer strip and capillary data sets, were excluded from the charts. $^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$.

more frequently in capillary samples, and cellular process proteins were upregulated in Schirmer strip samples (Fig. 5c). Connections to catalytic activity were again more frequent among proteins upregulated in Schirmer strip samples (Fig. 5d). Table 1 further lists the protein families and groups (with number of proteins ≥ 3), which were found to have a higher protein expression in either capillary or Schirmer strip samples in the comparisons.

The protein groups were also analyzed using IPA tool by using only the protein name lists as the input. The top 20 biological functions (lowest adjusted *P* value) are shown in Table 2, which excludes all disease-specific terms and focuses on more general categories.

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Table 1. Protein Families and Groups with Differing Expression Levels between Capillary and Schirmer Strip Samples

Higher Expression in	Family/Group	No. of Proteins	Proteins
Capillary samples	Immunoglobulin heavy/light/J chain	46	[a]
	Keratin, type I	7	KRT10, KRT12, KRT14, KRT16, KRT17, KRT36, KRT9
	Keratin, type II	6	KRT1, KRT2, KRT6B, KRT78, KRT82, KRT86
	Cysteine protease family C1 related	3	CTSC, CTSS, CTSZ
	Secretoglobin	3	SCGB2A1, SCGB2A2, SCGB1D1
	Complement component	3	C1R, C1S, C2
Schirmer strip samples	Heat-shock protein	7	HSP90AA1, HSP90AB1, HSPA1B, HSPA4, HSPA8, HSPB1, HSPD1
	Annexin	6	ANXA1, ANXA11, ANXA2, ANXA3, ANXA4, ANXA5
	Chaperonin (T-complex proteins)	6	CCT2, CCT3, CCT5, CCT6A, CCT8, TCP1
	S100 calcium-binding protein	6	S100A11, S100A4, S100A6, S100A8, S100A9, S100P
	Proteasome subunit α/β	5	PSMA3, PSMA4, PSMA6, PSMB1, PSMB10
	Thioredoxin peroxidase	4	PRDX1, PRDX2, PRDX5, PRDX6
	Serpin	4	SERPINA1, SERPINA7, SERPINB1, SERPINB5
	Tubulin	4	TUBA1B, TUBA4A, TUBB, TUBB4B
	ARP2/3 complex 16-kD subunit	4	ARPC3, ARPC5, ARPC4, ARPC5L
	Ras-related protein Rab	4	RAB10, RAB1B, RAB27B, RAB5B
	Superoxide dismutase [Cu-Zn]	3	SOD1, SOD2, SOD3
	Actin or actin related	3	ACTG1, ACTR2, ACTR3
	14-3-3 protein	3	SFN, YWHAG, YWHAZ
	Immunoglobulin heavy chain	3	IGHG1, IGHG2, IGHG4
	Aldehyde dehydrogenase related	3	ALDH1A1, ALDH3A1, ALDH9A1
	Fetuin	3	AHSG, FETUB, KNG1

[a] Protein names omitted due to large number of proteins and missing protein abbreviations.

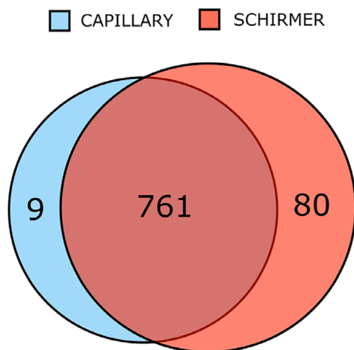

Figure 3. Comparison of the capillary- and Schirmer strip–collected tear fluid samples processed separately with a combined library. Most of the proteins (761) are shared between the two data sets, leaving only a few proteins unique to the sample types.

These results suggested that proteins associated with immune response and complement pathway in particular as well as endocytosis, keratinization, and tissue development had higher expression levels in capillary samples in comparison to Schirmer strips. Cell death and survival as well as free radical scavenging were top terms associated with proteins with a higher Schirmer strip expression. Inflammatory responses, cell migration, and protein synthesis and degradation were present in the top results of both groups.

Discussion

Results from our study demonstrated that the tear fluid proteomics data originating from the same patients at the same time point vary depending on whether the samples are collected using capillary or Schirmer strip. In addition, it should be noted that at least with the SWATH-MS method, the further

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Table 2. Top 20 Biological Functions (Lowest Adjusted *P* Value) of Proteins with Differing Expression Levels between Capillary and Schirmer Strip Samples

Categories	Diseases or Functions Annotation	Higher Expression in Capillary *P* (Adjusted)	Capillary No. of Proteins	Higher Expression in Schirmer Strip *P* (Adjusted)	Schirmer Strip No. of Proteins
Antimicrobial response	Antibacterial response	3.68E-06	12		
Cell death and survival	Necrosis			2.91E-16	106
	Apoptosis			4.06E-13	99
	Cell survival			2.53E-11	66
	Cell viability			1.98E-10	62
Cell-to-cell signaling and interaction	Adhesion of blood cells	7.16E-06	19		
Cellular function and maintenance	Receptor-mediated endocytosis	6.58E-31	36		
	Endocytosis	1.42E-23	45		
Cellular movement	Migration of cells	6.67E-19	78	8.17E-12	82
	Cell movement	8.31E-18	81	8.41E-20	104
	Leukocyte migration	2.87E-28	64		
Free radical scavenging	Metabolism of reactive oxygen species			2.69E-13	38
	Synthesis of reactive oxygen species			9.09E-12	35
Humoral immune response	Complement activation	1.51E-44	36		
	Classical complement pathway	4.89E-40	30		
Inflammatory response	Degranulation of neutrophils	2.72E-14	29	7.74E-37	56
	Degranulation of cells	1.41E-13	35	2.99E-41	72
	Degranulation			8.23E-42	73
	Degranulation of phagocytes			3.08E-33	58
	Degranulation of blood platelets			1.22E-14	20
	Inflammation of organ			2.95E-14	69
	Inflammation of body cavity			1.01E-09	45
Nucleic acid metabolism	Metabolism of nucleic acid component or derivative			6.68E-10	32
Posttranslational modification/protein degradation/protein synthesis	Metabolism of protein	1.1E-10	46	8.66E-18	68
	Catabolism of protein	7.47E-07	27	5.52E-10	37
	Cross-linkage of peptide	4.64E-06	6		
	Proteolysis	5.31E-06	16		
	Metabolism of cellular protein	6.26E-06	13		
	Cleavage of protein fragment	2.55E-05	14		
	Folding of protein			2.95E-14	19
	Synthesis of protein			1.50E-10	35
Tissue development/cell morphology	Keratinization	3.81E-11	18		
	Keratinization of epidermis	1.1E-09	15		
	Formation of skin	4.36E-06	21		

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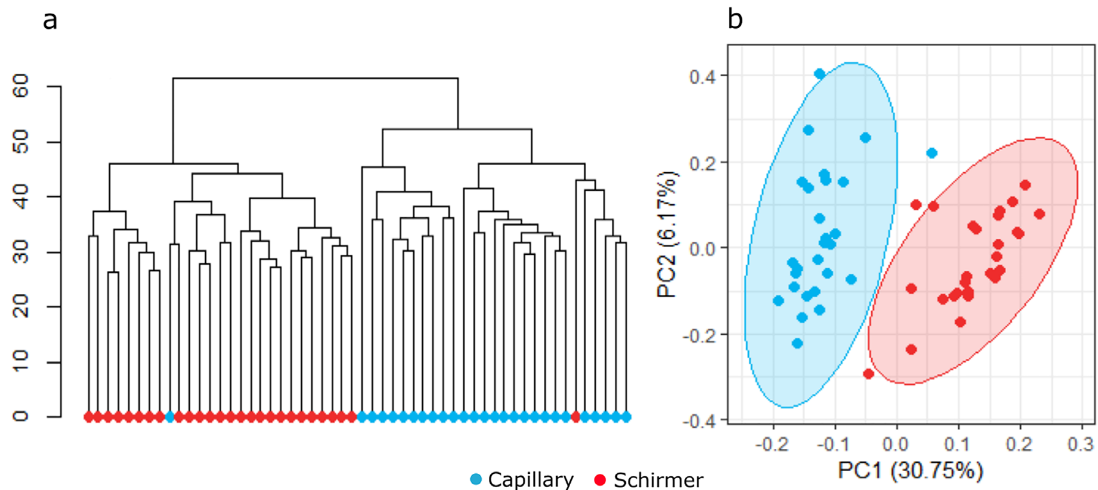

Figure 4. Hierarchical clustering (a) and principal component analysis (PCA) (b) displaying the grouping between the sample types. The capillary (blue) and Schirmer strip (red) tear fluid samples clearly separate to individual clusters based on the hierarchical clustering using the Euclidean distance metric with complete linkage as well as by the first two components of the PCA, which together explain 36.92% of the total variance. The normal confidence ellipses have been generated at the 95% confidence level.

processing steps, such as the choice of spectral library, are also crucial in the analysis. Although the tear fluid sampling methods, capillary and Schirmer strip in particular, have been compared in several earlier studies, to our knowledge, discovery proteomics studies using SWATH-MS on this topic have not been published previously. In fact, the earlier studies on tear fluid sampling methods have expressed differing opinions on how comparable the two sampling methods are in terms of proteomics studies and in general. Some consider that both approaches are equally suitable at least for comparative case-control analyses,[4,19,25] whereas others argue that the Schirmer strip results in increased tear fluid flow and altered tear fluid composition, as well as causes reflex tearing and "contamination" of cellular proteins through contact irritation.[23,33]

We analyzed data, which were produced with both sample type–specific spectral libraries as well as with a combined library consisting of both capillary and Schirmer strip samples' identification runs. In the first approach, which implemented capillary and Schirmer strip samples separately with sample type–specific spectral libraries, the Schirmer strip data set had notably a higher number of successfully quantified proteins in comparison to capillary sample–specific data. There are several possible reasons for the uneven protein counts. The varying sample and thus protein amounts between the sampling methods, which differed notably according to our results, are likely to have an effect. In this case, Schirmer strip samples had ten times higher total protein mean in comparison to capillary samples. Recovery of proteins from glass capillary tube and Schirmer strip filter paper may differ, resulting in varying protein amounts. In addition, although at the end, all tear samples were analyzed using the same protein concentration, sample-processing efficiency may vary between the sample types due to protein amount differences at the beginning. These differences in the early sample processing steps can affect the spectral libraries and quantified proteins at least partially due to the DDA method, which always identifies the most abundant proteins in a given sample, also demonstrated by Jylhä et al.[30] This may be a particular issue with the sample type–specific spectral libraries, which are constructed with a lower amount of identification (ID) runs, thus potentially resulting in a loss of low-abundance proteins. Therefore, when concentration differences between proteins are high, which is likely the case in capillary samples, smaller amounts of cellular proteins, smaller spectral library, and lower quantified protein amount are obtained in comparison to the Schirmer strip. Finally, it should be noted that the Schirmer strip, as mentioned, does come in contact with the ocular surface tissue, possibly irritating the eye in the process and resulting in sample type differences originating from varying tear types (reflex vs. basal tear) or, perhaps more important, sample sources (tissue vs. tear).[23,33–36] Sample source variation has in fact been proposed as one of the main differences between capillary and Schirmer strip samples, and Green-Church et al.[20] have previously shown that although the proteins identified from capillary samples were mainly extracellular, Schirmer strip sample data included also cellular proteins.

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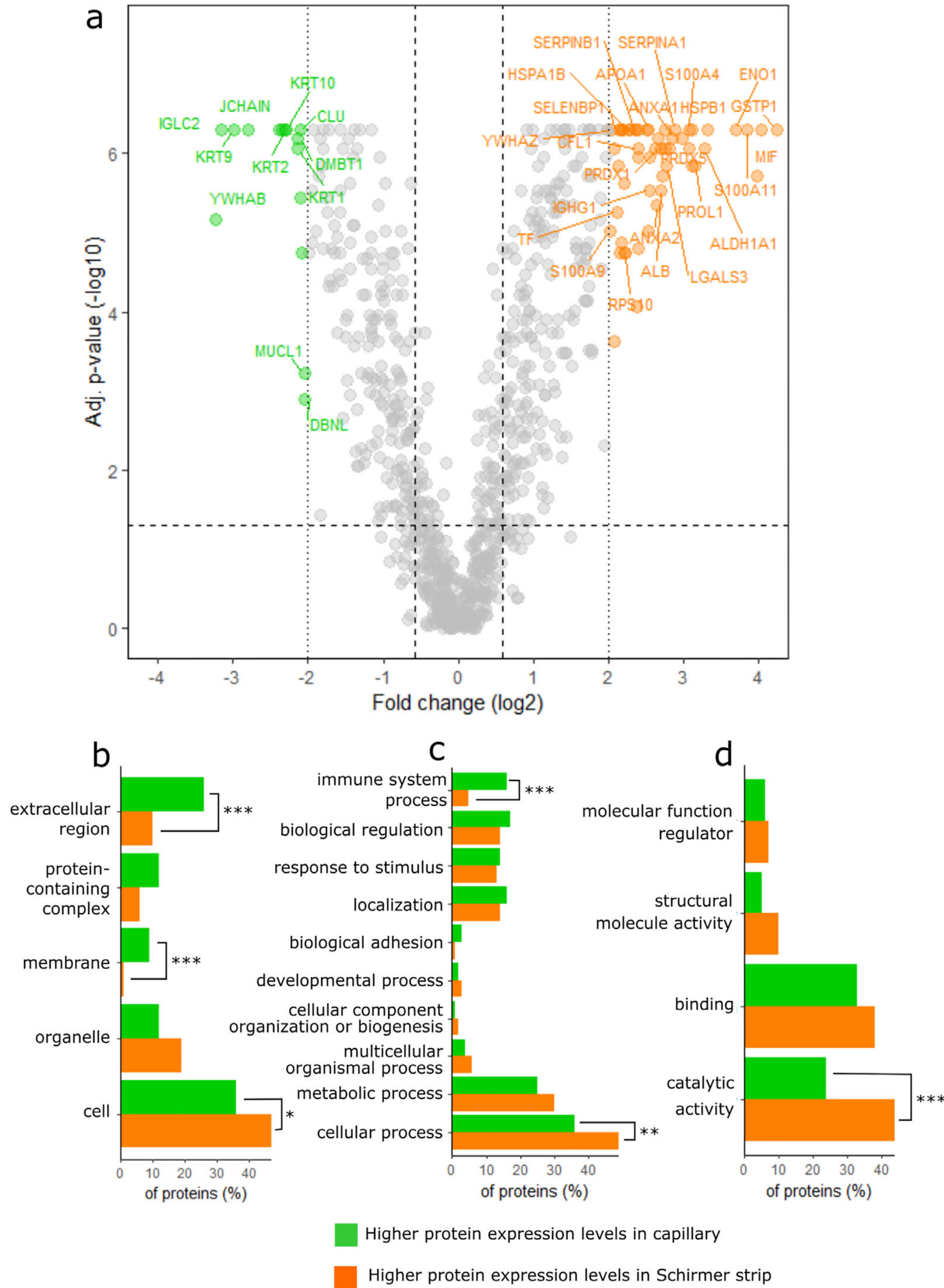

Figure 5. Volcano plot of differential expression analysis results comparing the capillary and Schirmer strip samples (a) and associated gene ontology (GO) (b–d). In the volcano plot (a), the y-axis represents the adjusted *P* value (displayed in $-\log_{10}$ scale) and x-axis the associated fold change (FC, $\log_2$ scale). Chosen proteins with an adjusted $P < 0.05$ (horizontal dashed line) and FC >2 or <0.25 (vertical dotted lines), indicating proteins with higher expression levels in capillary (green) or Schirmer strip samples (orange), are labeled. The vertical dashed lines indicate the 1.5 FC threshold, which was used to filter data together with the adjusted *P* values. Bar charts (b–d) display the percentage of proteins (x-axis) associated with a given GO term subgroup (y-axis) by category. Pearson's chi-squared test was used to estimate differences between distributions. GO categories are (b) cellular component, (c) biological process, and (d) molecular function. GO terms, which had protein proportions <2.5% for both Schirmer strip and capillary data sets, were excluded from the plots. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$.

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As we evaluated the GO terms associated with capillary- and Schirmer strip–specific proteins obtained from our data, the hypothesis about the sample source variations between the two sampling methods gained support, although many GO terms were still found in similar proportions from both sample types. The results suggested that a higher proportion of quantified capillary-specific proteins were extracellular proteins associated with the immune system process, whereas Schirmer strip–specific proteins were more often proteins associated with metabolic and catalytic activity originating from cells or organelles. The absolute numbers of proteins associated with these GO terms did suggest that some of the large proportional differences were affected by the different protein counts in the data sets. However, our differential expression analysis results with the combined data set containing both capillary and Schirmer strip samples similarly suggested that the cell- and organelle-associated proteins had increased expression levels in Schirmer strip samples, whereas proteins with increased expression in capillary samples were more often extracellular and membrane proteins associated with complement pathway and tissue development. These results again support previous studies with similar findings[20]—that is, that capillary samples contain proportionally more tear fluid, whereas the Schirmer strip, due to its contact with the conjunctiva, has a higher abundance of tissue material mixed together with the tear fluid. It should be noted that the different segments of the Schirmer strip (e.g., parts directly touching the conjunctiva and parts outside the lid margin) are also likely to differ in protein profiles.[33]

Despite these differences between the sample types, many of the well-known tear fluid biomarkers can be identified from both capillary- and Schirmer strip–specific data sets. For example, proteins from S100 and 14-3-3 protein families, as well as cystatins, LTF, complement C3, lacritin, ALB, lysozyme, and α-enolase 1, which are all proteins known to play roles in protection and inflammation as well as pathophysiologic conditions of the ocular surface, could be found from both capillary- and Schirmer strip–specific data sets.[4–11] Therefore, in theory, proteomics analyses evaluating the ocular surface health and conditions could be performed implementing either of the two sampling methods, although the relative protein expression-level differences between, for example, disease and control participants may still produce varying results.

Another approach implemented in our study consisted of separate capillary and Schirmer strip data sets processed using a combined spectral library. This enabled us to see whether using the same library produces similar results regardless of the sample type. The number of proteins quantified this way produced similar protein counts between the capillary and Schirmer strip data sets (770 and 841 proteins, respectively), and most of the proteins were quantified in both data sets (761 proteins). This indicates the crucial role that the SWATH spectral library has in the processing of the raw data and that with a large and diverse enough spectral library, both sampling methods can be used to quantify similar amounts and types of proteins. However, a combined spectral library may still result in the loss of some sample type–specific proteins with lower abundance, and therefore the most suitable sampling method and SWATH spectral library should be carefully selected depending on the focus and requirements of the study.

When comparing the relative expression levels between capillary and Schirmer strip samples, several proteins had significantly differing expression levels. As mentioned, the differences were most notably associated with the sample source differences (i.e., Schirmer strip samples had higher expression levels in cell- and organelle-originating proteins, whereas capillary samples yielded higher expression levels among extracellular proteins). Previously, Stuchell et al.[37] discovered that the concentrations of plasma proteins ALB, IgG, and transferrin (TF) were significantly higher in Schirmer strip sampling, and Choy et al.[23] found increased levels of antioxidants. Our results were in line with the results by Stuchell et al. as we also identified an increase of ALB and TF in the Schirmer strip samples. These results could indicate higher mechanical irritation and plasma protein leakage in Schirmer strip sampling, as suggested by Stuchell et al., despite our careful attempts to optimize and standardize our sampling methods. However, as also noted by Posa et al.,[19] all tear fluid sampling methods can cause a response in the ocular surface physiology despite careful collection. A study by Zhou et al.[2] also discovered that of 1543 proteins identified in Schirmer strip–collected tear fluid samples, 540 were also found from a high-confidence plasma proteome reference set, indicating that plasma proteins could also be considered an essential part of the tear fluid samples.

Many other tear protein markers were also noted to be upregulated in Schirmer strip samples, such as heat-shock proteins, annexins, S100 proteins, serpins, and aldehyde dehydrogenase–related proteins. Proteins from all of these protein families have been previously identified to be increased in various ocular surface conditions.[5,10,38–41] Protein groups upregulated in capillary samples included immunoglobulins, keratin proteins, and secretoglobin proteins, and at least secretoglobins have been identified as decreased among

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patients with dry eye.[6,8,9,38] More research is needed to establish whether these differences are due to ocular surface irritation caused by Schirmer strip sampling or whether they are caused at least partially by the sample source variations. Studies focusing on absolute protein expression levels should also note these likely differences when establishing, for example, thresholds. Changed expression levels between, for example, treatment groups and the associated differences between the sample types cannot be evaluated based on our study. Collection and comparison of both capillary and Schirmer strip samples from a sample population with diagnosed ocular surface disease would hence enable further evaluation of the sample type differences.

The overall quality between the samples was quite similar, but it should be noted that three of the capillary samples had to be removed due to MS analyzing failure, whereas only one Schirmer strip sample was excluded. The ICC and the proportion of significant ($<$0.05) permuted *P* values between the replicate MS runs were relatively high in all data sets, but there was a slight drop with the capillary data set produced with the combined spectral library. This could mean that when both Schirmer strip and capillary samples are used in the library creation, it is likely that some cell and organelle proteins are included in the library, which are not readily as abundant in tear fluid samples collected with capillaries and could affect the data quality. In addition, with the combined libraries, some potentially informative proteins unique to capillary samples may be lost from the data in the process. For the aforementioned reasons, the choice of spectral library should be carefully considered when processing Schirmer strip or especially capillary samples using SWATH-MS.

In this study, we did not collect open-eye Schirmer strips, and instead patients were asked to close their eyes during the sampling. Because capillary samples are necessarily open-eye samples, some differences observed between the two sampling methods can also be due to this variation as it has been suggested that the tear composition does change depending on the eye closure.[34–36] Further studies could include open- and closed-eye Schirmer strip samples (with and without anesthesia) and capillary samples with and without stimulation to establish in more detail the effects of these variables.

In conclusion, the sampling method used to collect the tear fluid samples does affect the protein profiles obtained with SWATH-MS, although there is an overlap between the two data set types. In particular, if the research was specifically focused on the extracellular proteins connected to immune response in the ocular surface, capillary samples would be sufficient for a proteomics analysis. However, if intracellular proteins are equally of interest and the aim is to identify a large quantity of proteins from the ocular surface, the Schirmer strip would be more advisable. Larger quantities of proteins can still be achieved with capillary samples, if a combined spectral library is implemented in the SWATH-MS process. Expression-level differences between the two sample types are also notable and this should be acknowledged especially in studies focusing on absolute tear protein expression levels, and researchers should be wary of the possible irritation effects caused by Schirmer strip sampling. Further studies are needed on establishing whether both sample types are able to identify similar protein changes in varying ocular surface conditions.

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