RESEARCH ARTICLE

Proteomic Analysis of Vitreous Humor in Retinal Vein Occlusion

Michael Reich¹, Ivanka Dacheva², Matthias Nobl², Justyna Siwy³, Joost P. Schanstra⁴,⁵, William Mullen⁶, Frank H. J. Koch⁷, Jürgen Kopitz⁸, Florian T. A. Kretz⁹, Gerd U. Auffarth², Michael J. Koss²,⁹*

¹ Eye Center, Albert-Ludwigs-University Freiburg, Freiburg, Germany, ² Department of Ophthalmology, University of Heidelberg, Heidelberg, Germany, ³ Mosaik Diagnostics GmbH, Hannover, Germany, ⁴ Institut National de la Santé et de la Recherche Médicale (INSERM), U1048, Institut of Cardiovascular and Metabolic Disease, Toulouse, France, ⁵ Université Toulouse III Paul-Sabatier, Toulouse, France, ⁶ BHF Glasgow Cardiovascular Research Centre, University of Glasgow, Glasgow, United Kingdom, ⁷ Department of Ophthalmology, University of Frankfurt, Frankfurt am Main, Germany, ⁸ Department of Pathology, University of Heidelberg, Heidelberg, Germany, ⁹ Department of Ophthalmology, University of Southern California, Los Angeles, California, United States of America

* Michael.koss@med.uni-heidelberg.de

Abstract

Purpose
To analyze the protein profile of human vitreous of untreated patients with retinal vein occlusion (RVO).

Methods
Sixty-eight vitreous humor (VH) samples (44 from patients with treatment naïve RVO, 24 controls with idiopathic floaters) were analyzed in this clinical-experimental study using capillary electrophoresis coupled to mass spectrometer and tandem mass spectrometry. To define potential candidate protein markers of RVO, proteomic analysis was performed on RVO patients (n = 30) and compared with controls (n = 16). To determine validity of potential biomarker candidates in RVO, receiver operating characteristic (ROC) was performed by using proteome data of independent RVO (n = 14) and control samples (n = 8).

Results
Ninety-four different proteins (736 tryptic peptides) could be identified. Sixteen proteins were found to be significant when comparing RVO and control samples (P = 1.43E-05 to 4.48E-02). Five proteins (Clusterin, Complement C3, Ig lambda-like polypeptide 5 (IGLL5), Opticin and Vitronectin), remained significant after using correction for multiple testing. These five proteins were also detected significant when comparing subgroups of RVO (central RVO, hemi-central RVO, branch RVO) to controls. Using independent samples ROC-Area under the curve was determined proving the validity of the results: Clusterin 0.884, Complement C3 0.955, IGLL5 1.000, Opticin 0.741, Vitronectin 0.786. In addition, validation through ELISA measurements was performed.

Citation: Reich M, Dacheva I, Nobl M, Siwy J, Schanstra JP, Mullen W, et al. (2016) Proteomic Analysis of Vitreous Humor in Retinal Vein Occlusion. PLoS ONE 11(6): e0158001. doi:10.1371/journal.pone.0158001

Editor: Jon M. Jacobs, Pacific Northwest National Laboratory, UNITED STATES

Received: December 30, 2015

Accepted: June 8, 2016

Published: June 30, 2016

Copyright: © 2016 Reich et al. 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.

Data Availability Statement: All relevant data are within the paper or its Supporting Information files.

Funding: The research group was supported by the Adolf Messer Stiftung, Königstein, Hessen, Germany. Parts of this research were supported by a research grant from Bayer Vital GmbH, Leverkusen, Germany. Michael Reich is a recipient of scholarships from the Studienstiftung des deutschen Volkes and the Dr. Gabriele Lederle-Stiftung. He wants to thank these organizations for their support. Matthias Nobl is a recipient of scholarships from the PRO RETINAStiftung zur Verhütung von Blindheit and the Dr. Gabriele Lederle-Stiftung. The funders had no
Conclusion

The results of the study reveal that the proteomic composition of VH differed significantly between the patients with RVO and the controls. The proteins identified may serve as potential biomarkers for pathogenesis induced by RVO.

Introduction

Retinal vein occlusion (RVO) is the second most common cause of vision loss in older patients due to retinal vascular disease after diabetic retinopathy [1,2]. Despite major achievements in diagnosis and treatment perspectives based on spectral OCT, there is still limited understanding of the pathophysiology of RVO [3–6]. A number of cytokines including vascular endothelial growth factor (VEGF) and interleukin-6 (IL-6) have been shown to be associated to RVO. However these cytokines display high interindividual variations and their concentrations often do not correspond to the associated clinical RVO phenotype [7,8]. Even in non diseased eyes, reproducible vitreous protein profiling is quite complicated as the aging vitreous incorporates different stages of liquefaction and syneresis, which is aggravated after treatment with intravitreal injections [9]. Therefore, many aspects of the molecular mechanism of RVO remain poorly described.

Proteomics is a promising approach allowing the analysis of the total protein content of a sample and in combination with robust statistical analyses can lead to the identification of proteins associated to specific diseases in ophthalmology [10–13].

In the context of RVO the current proteomic data are incomplete. To the best of our knowledge, only one proteome study in RVO exists. Yao et al. 2013 described the proteome of aqueous humor from patients with branch RVO (BRVO; n = 6) compared to controls (n = 6) [14].

Therefore, to potentially improve upon the insight in the pathophysiology of RVO we performed high-resolution proteome analysis of vitreous humor (VH) of a high number of patients with RVO (n = 44).

Materials and Methods

Study design

This was a clinical-experimental study. Samples were collected after the approval from local institutional review board (351/12) of the Goethe University Frankfurt am Main (Germany) in accordance with the European Guidelines for Good Clinical Practice and the Declaration of Helsinki. Written informed consent was obtained from each patient before the start of therapy (linked to the source file of each patient, which was approved by a local ethics committee).

Patient characterization

In our retrospective case series we analyzed a total of 68 undiluted samples from previously untreated (any intravitreal drug application) patients for VH proteome identification and quantitative verification: 44 from RVO patients and 24 randomly chosen controls with idiopathic floaters. All patients consulted our Department of Ophthalmology between April and August 2012. Patients suffering neovascular complications such as rubeosis iridis, vascularisations of iridocorneal chamber, neovascularisations of the papilla or in the retina perimeter were considered ineligible for this study as well as patients with vitreo-macular traction or patients previously treated with intravitreal anti-VEGF, intraocular steroids, coagulation with
photo laser, vitrectomy or previous intraocular operations, such as cataract operation at the included or not included eye in the last six months. Patients with diabetic retinopathy, uveitis, glaucoma or other compromising ocular conditions were also excluded. For all participants personal data, such as gender and patient-age, were collected as well as information regarding type of RVO (central-RVO (CRVO), hemi-central RVO (H-CRVO), BRVO), lens status, averaged and central retinal thickness, posterior attachment of the vitreous body and presence of retinal cysts with spectral domain OCT (SD-OCT; Topcon 200, Tokyo, Japan).

Tryptic digestion of vitreous

Sampling of VH, sample preparation and analysis was conducted as described in Koss et al. 2014 [12]. In short: 10 μL of the thawed samples were diluted 1:10 with 0.1% SDS, 20 mM DTT, and 0.1 M Tris–HCl (pH = 7.6). Samples were sonicated at room temperature for 30 minutes. Afterwards, the samples were denaturated at 95°C for 3 min and then were incubated for 30 min at room temperature in the absence of light with 0.05 M Iodoacetamide. Eight M urea, 0.2 M Tris–HCl and 50 mM ammonium bicarbonate buffer solution was added and the samples were applied to NAP-5 columns equilibrated in 50 mM ammonium bicarbonate buffer solution. Trypsin solution was added to the desalted samples. Trypsin digestion was carried out overnight at a temperature of 37°C. Subsequently, the samples were lyophilized. Afterwards the samples were stored at 4°C and were resuspended with 15 μl distilled water shortly before mass spectrometry analysis.

CE-MS analysis

Capillary electrophoresis coupled to mass spectrometer (CE-MS) analysis was carried out as described by Theodorescu et al. 2006 [15]. A P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Brea, CA) being linked online to a micro-TOF MS (Bruker Daltonik, Leipzig, Germany) was used. The sprayer (Agilent Technologies, Santa Clara, CA) interfacing the CE and MS was grounded. 256 nl of the sample was injected hydrodynamically on an untreated silica capillary (New Objective, Woburn, USA, 90 cm x 50 μm). A solution of 20% acetonitrile (Sigma-Aldrich, Taukirchen, Germany) in HPLC-grade water (Roth, Karlsruhe, Germany) supplemented with 0.94% formic acid (Sigma-Aldrich) was used as running buffer. Interface potential was adjusted to -4.5 kV. The capillary temperature was held at 35°C. Mass spectra were recorded for three seconds with signals at an m/z range between 350 and 3000. The detection limit of the TOF-Analyzer was 1 fmol [15].

Data processing of CE-MS analysis

Analysis of raw CE-MS data was carried out via MosaiquesVisu version 2.1.0 (mosaiques diagnostics GmbH, Hannover, Germany) which uses isotope identification and conjugated mass detection for mass deconvolution [16]. Only signals observed in a minimum of 3 consecutive spectra with a signal-to-noise (SNR) ratio of at least 4 were considered. The observed minimal signal intensity with SNR >4 was 1.2. The software automatically eliminates all signals that can be detected only as singly charged species. The software employs a probabilistic clustering algorithm and uses both, isotopic distribution and conjugated masses for charge-state determination of peptides/proteins. The resulting peak list characterizes each polypeptide by its molecular mass, CE-migration time, and ion signal intensity (amplitude) value. Mass spectral ion peaks from the same molecule at different charge states were deconvoluted and summarized into a single mass. To minimize effects of biological and analytical variability between the different lots, a normalization of retention time, signal intensity and mass was performed. In total, 292 signals for mass and CE-time with a frequency ≥35% could be determined that
served as reference signals for normalization of peptide CE-time using local regression. The detected signal intensities for each individual peptide were normalized to the total ion count (total intensity) per individual sample analysis (ppm normalization).

All normalized peptides were deposited, matched, and annotated in a Microsoft SQL database, allowing further analysis and comparison of multiple samples [17]. Peptides were considered identical when deviation of mass was ≤±50 ppm (parts per million) for an 800 Da protein fragment, respectively ≤±75 ppm for a 15 kDa protein fragment. Peptides were considered identical if the CE-migration time window did not exceed 2–5%, continuously increasing between 10 and 60 min.

Peptide sequencing

Nine lyophilized, tryptic-digested randomly selected vitreous samples were dissolved in 15 μL distilled water for MS/MS analysis. Separation was carried out via Dionex Ultimate 3000 RSLS nano flow system (Dionex, Camberly UK) as described by Metzger et al. [18]. After loading 5 μl onto a Dionex 0.1×20 mm 5 μm C18 nano trap column at a flowrate of 5 μl/min in 98% 0.1% formic acid and 2% acetonitrile, sample was eluted onto an Acclaim PepMap C18 nano column 75 μm×15 cm, 2 μm 100 Å at a flow rate of 0.3 μl/min. The trap and nano flow column were maintained at 35°C. The samples were eluted with a gradient of solvent A: 98% 0.1% formic acid, 2% acetonitrile versus solvent B: 80% acetonitrile, 20% 0.1% formic acid starting at 1% B for 5 minutes rising to 20% B after 90 min and finally to 40% B after 120 min. Subsequently, the column was washed and re-equilibrated prior to the next injection. The Proxeon nano spray ESI source (Thermo Fisher Hemel UK) in positive ion mode was used for samples ionisation. Ionization voltage was 2.6 kV, the capillary temperature was 200°C. The samples were analysed using an LTQ Orbitrap Velos (Thermo Finnigan, Bremen, Germany). The mass spectrometer was operated in MS/MS mode scanning from 380 to 2000 amu. The samples were analysed using both CID and HCD to obtain the maximum number of sequence identifications. The top 20 multiply charged ions were selected from each scan for MS/MS analysis using either CID or HCD at 40% collision energy. The resolution of ions in MS1 was 60,000 and 7,500 for mass fragmentation MS2. The searches were performed by the use of Proteome Discoverer (version 1.3, Thermo Fisher Scientific, Bremen, Germany) with the use of the SEQUEST algorithm and against the human, non redundant IPI database (version 3.87, entry count: 91464) for each data file separately. Trypsin was used as the enzyme while screening for proteins. Hydroxylated proline from collagen fragments and oxidation of methionine were accepted as variable modifications and carbamidomethylated cysteine as fixed modification. A maximal mass deviation of 10 ppm for precursor ions and 0.8 Da for product ions was accepted. The allowed false discovery rate was 1% and the number missed cuts one. In addition, only peptides with medium or high confidence, rank one and XCorr factor >0.8 were accepted.

The sequences were matched to the detected CE-MS data according to Zürbig et al. [19]. Charge of the peptides was used in this matching procedure instead of the LC retention time. On the basis of their charge, discrimination between peptides with similar masses can be performed even when having identical or very close masses in the liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis. This is due to the fact that the number of basic and neutral polar amino acids of peptide sequences distinctly correlates with their CE-MS migration time/molecular weight coordinates. This enables linking a unique LC-MS MS peptide to a CE-MS peptide in nearly all cases [19]. This procedure has been successfully used in a number of studies linking specific CE-MS-identified peptides to sequences obtained by LC-MS/MS [12,18,20–22].
CE-MS peptides with sequencing information were combined for each protein. Proteins were accepted when being represented by a minimum of two peptides. Protein abundance was calculated as the average of all normalized CE-MS peptide intensities for the given protein.

ELISA
Clusterin, Complement C3, Ig lambda-like polypeptide 5 (IGLL5), Opticin and Vitronectin concentrations were assayed in sandwich-ELISAs (Cloud-Clone Corporation, Houston, TX, USA) where antibodies specific to the target proteins were precoated to a microtiter plate. After antigen binding to the precoated plates biotin-conjugated antibodies specific for Clusterin, Complement C3, Opticin or Vitronectin were used for detection of the bound antigens. Horseradish peroxidase conjugated to avidine and TMB substrate were applied for quantification. Measurements were conducted spectrophotometrically at 450 nm. Minimum detectable doses were 3 ng/ml for Clusterin, 2 ng/ml for Complement C3, 0,2 ng/ml for IGLL5, 0,1 ng/ml for Opticin, and 0,5 pg/ml for Vitronectin.

Statistical methods
SPSS version 21.0 was used for statistical analysis. A P-value of $\alpha<5.00E-02$ was considered statistically significant.

Descriptive Statistics. For the descriptive data analysis, mean ± standard deviation (SD), median values, and minimal and maximal values were calculated.

Patient Characterization. Clinical and demographic characteristics of control patients and each subgroup of RVO (CRVO, H-CRVO, BRVO) was compared by using the Kruskal Wallis test where appropriate.

Proteomic Analysis. Candidate RVO biomarkers were defined by examination of differences in signal intensity of the proteins between the RVO-patients and the controls. Mean CE-MS based protein signal intensity was used as a measure for relative abundance. Mann-Whitney test was used for analysis. Multiple hypotheses testing correction was performed by using the Benjamini-Hochberg test for false discovery rate [23].

Subgroup Analysis. Patients with RVO were subdivided in following subgroups: CRVO, H-CRVO, BRVO. The closed testing procedure was used for subgroup analysis [24]. For detailed information see Fig 1.

Influence of past time period since suffering RVO on biomarker discovery. To analyze the influence of past time period since suffering RVO we compared protein signal intensity between fresh (patients suffered RVO $<8$ weeks ago) and old RVO-samples (patients suffered RVO $\geq$8 weeks ago) versus controls. The Mann-Whitney test was used for analysis.

Validation of Selected Potential Biomarker Candidates. To determine validity of potential biomarker candidates in RVO, receiver operating characteristic (ROC) was performed by using data of randomly selected, independent control and RVO samples not being used in proteomic analysis and biomarker definitions. Area under the curve (AUC) was determined. Furthermore, the protein abundance in VH of the selected potential biomarkers was measured by ELISA. Mann-Whitney test was used to analyze changes in the protein abundance between RVO and controls in the ELISA.

Results
Patient characterization
A total of 44 RVO samples and 24 controls with idiopathic floaters were analyzed. Patients with RVO were additionally subdivided in the following subgroups: CRVO (n = 20), H-CRVO
(n = 9), BRVO (n = 15). Epidemiologic data and further patient information such as fresh/old RVO, lens status, retinal thickness, attachment of the vitreous body, and presence of retinal cysts are listed in Table 1 (for detailed information see S1 Table). No differences between subgroups regarding patient-age and average or center retinal thickness were observed ($P = 1.60E^{-01}$ to $5.48E^{-01}$).

**Proteomic analysis**

The study layout is depicted in Fig 1. Samples were randomly subdivided for selection and verification of potential biomarker candidates. Two thirds of all samples were used as a discovery set and selection of potential biomarker candidates and subgroup analysis (16 controls, 14 CRVO, 6 H-CRVO, 10 BRVO); one third was used for the validation using ROC analysis (8 controls, 6 CRVO, 3 H-CRVO, 5 BRVO). The two mass spectrometry techniques employed in

---

**Fig 1. Study layout.**

doi:10.1371/journal.pone.0158001.g001
this study are used in a complementary fashion. CE-MS, due to its high reproducibility, is used for candidate peptide selection while LC-MS/MS is used for identification of the biomarker peptides. Using LC-MS/MS analysis, 736 of the tryptic peptides detected with CE-MS could be sequenced. The mass of sequenced peptides ranged between 800.4 and 3619.0 Da. Migration time ranged between 20.1 and 38.0 min. The 736 tryptic peptides corresponded to 94 different proteins in VH (S2 Table, Fig 1). For detailed information regarding all raw data see S3 Table.

Selection of potential biomarker candidates

In the discovery study, the comparison of protein data between RVO (irrespective of subgroup) and control samples resulted in 16 significant proteins ($P = 1.43 \times 10^{-5}$ to $4.48 \times 10^{-2}$; Table 2). After using correction for multiple testing, five proteins remained significant (Table 2, see *; Fig 1): Clusterin, Complement C3, IGLL5, Opticin and Vitronectin.

Subgroup analysis

Signal intensity of the proteins of each subgroup (CRVO, H-CRVO, BRVO) and the controls was compared by using the closed testing procedure (see S1 Fig). Proteins being detected significantly different in any of the analysis are listed in Table 3. In step 3 ten proteins were expressed significantly different in each, CRVO and BRVO samples versus controls, 9 proteins were expressed significantly different in H-CRVO versus controls. Five proteins remained significant in all comparisons of step 1 to step 3: Clusterin, Complement C3, IGLL5, Opticin and Vitronectin. For detailed information regarding subgroup analysis see S4 Table.

Influence of past time period since suffering RVO on biomarker discovery

When analyzing the influence of past time period since suffering RVO Clusterin, Ig lambda-like polypeptide 5 (IGLL5), Opticin and Vitronectin remained significantly different between control samples ($n = 16$) versus fresh ($n = 22$) or old RVO-samples ($n = 8$) (S5 Table). Except for Apolipoprotein A-II ($P = 1.75 \times 10^{-2}$) no difference was detected of the potential biomarkers.

| Table 1. Epidemiology. | CRVO | Hemi-CRVO | BRVO | Control |
|-------------------------|------|-----------|------|---------|
| N                       | 20   | 9         | 15   | 24      |
| Fresh/Old               | 14/6 | 7/2       | 11/4 | -       |
| Female/Male             | 8/12 | 4/5       | 10/5 | 13/11   |
| Age in years (mean ± SD)| 67.6 ± 14.3* | 69.2 ± 14.2* | 68.4 ± 12.2* | 62.7 ± 11.7* |
| Phakic/Pseudophakic      | 16/4 | 8/1       | 13/2 | 12/12   |
| Average Thickness in μm (mean ± SD) | 376.3 ± 82.8* | 351.3 ± 44.2* | 351.5 ± 69.6* | -         |
| Center Thickness in μm (mean ± SD) | 513.8 ± 196.9* | 443.4 ± 142.2* | 462.0 ± 149.9* | -         |
| Posterior Vitreous attached/ detached/unrecognizable | 6/14/0 | 3/5/1 | 0/14/1 | - |
| Cysts yes/no            | 15/5 | 8/1       | 13/2 | -       |

* Kruskal Wallis test was used for analyses; $P$-value of $\alpha < 5.00 \times 10^{-2}$ was considered significant. No significant difference was tested: Age $P = 3.71 \times 10^{-1}$; Average Thickness $P = 1.60 \times 10^{-1}$; Center Thickness $P = 5.48 \times 10^{-1}$

doi:10.1371/journal.pone.0158001.t001
### Table 2. Significant proteins in vitreous humor.

Significant proteins were detected by capillary electrophoresis coupled to mass spectrometer (CE-MS) and identified by tandem mass spectrometry (LC-MS/MS) when comparing protein signal intensity of retinal vein occlusion (RVO)-samples compared to control-samples.

| Protein Identification | Statistical analysis | Protein Identification | Statistical analysis |
|-----------------------|----------------------|-----------------------|----------------------|
| **Protein** | **UniProt** | **RVO (n = 30)** | **Control (n = 16)** | **P-Value** | **Benjamini-Hochberg (adjusted P-Value)** |
| **Pep-tides** | **Cover-age** | **N** | **Mean intensity** | **SD** | **Pep-tides** | **Cover-age** | **N** | **Mean intensity** | **SD** | **P-Value** | **Benjamini-Hochberg (adjusted P-Value)** |
| Ig lambda-like polypeptide 5* | B9A064 | 3 | 20 | 30 | 358.2 | 422.5 | 2 | 13 | 11 | 93.4 | 75.7 | 1.43E-05 | 1.37E-03 |
| Vitronectin* | P04004 | 4 | 10 | 25 | 89.6 | 136.0 | 3 | 8 | 8 | 8.8 | 11.0 | 2.74E-04 | 8.77E-03 |
| Clusterin* | P10909 | 9 | 26 | 30 | 524.1 | 194.2 | 10 | 28 | 15 | 282.3 | 174.6 | 4.97E-04 | 1.19E-02 |
| Complement C3* | P01024 | 43 | 29 | 30 | 582.3 | 134.3 | 41 | 27 | 16 | 390.2 | 194.5 | 1.85E-03 | 3.55E-02 |
| Collagen alpha-2(IX) chain | P13942 | 3 | 6 | 30 | 740.4 | 678.7 | 5 | 9 | 13 | 377.1 | 528.5 | 8.55E-03 | 1.10E-01 |
| Collagen alpha-1(VII) chain | Q02388 | 3 | 2 | 5 | 29.3 | 101.6 | 2 | 1 | 8 | 25.1 | 57.2 | 2.94E-02 | 1.88E-01 |
| Collagen alpha-2(I) chain | Q08123 | 4 | 7 | 18 | 15.1 | 15.8 | 4 | 7 | 6 | 5.1 | 8.1 | 4.32E-02 | 2.53E-01 |
| Collagen alpha-1(III) chain | P02461 | 3 | 4 | 23 | 93.4 | 91.6 | 3 | 4 | 10 | 37.9 | 41.8 | 4.48E-02 | 2.53E-01 |
| Opticin* | Q9UBM4 | 6 | 20 | 23 | 82.8 | 207.2 | 5 | 16 | 16 | 163.7 | 95.1 | 7.07E-05 | 3.39E-03 |
| Neuroblast differentiation associated protein AHNAK | Q06695 | 2 | 1 | 6 | 1.4 | 3.9 | 4 | 1 | 9 | 14.0 | 24.8 | 3.34E-03 | 5.34E-02 |
| Alpha-crystallin B chain | P02511 | 2 | 11 | 3 | 1.9 | 7.4 | 5 | 30 | 7 | 30.1 | 75.5 | 9.20E-03 | 1.10E-01 |
| Complement factor B | P00751 | 2 | 3 | 13 | 18.1 | 60.4 | 4 | 6 | 11 | 44.9 | 61.4 | 1.23E-02 | 1.31E-01 |
| Obscurin | Q5VST9 | 2 | <1 | 21 | 520.3 | 1358.1 | 2 | <1 | 16 | 1767.1 | 3857.7 | 1.51E-02 | 1.31E-01 |
| Apolipoprotein A-II | P02652 | 2 | 29 | 10 | 8.1 | 14.4 | 4 | 41 | 10 | 50.1 | 67.6 | 1.78E-02 | 1.41E-01 |
| Complement C4-A | P0C0L4 | 2 | 2 | 4 | 2.0 | 6.9 | 4 | 3 | 7 | 29.3 | 78.3 | 1.91E-02 | 1.41E-01 |
| Serum albumin | P02768 | 40 | 61 | 30 | 2109.7 | 657.6 | 42 | 65 | 16 | 2910.3 | 1163.1 | 2.11E-02 | 1.45E-01 |

---

**Notes:**

1. Listed in the universal protein resource (UniProt), a central repository of protein data.
2. Number of peptides observed by CE-MS analysis and sequenced by LC-MS/MS for each protein.
3. Percentage (%) of peptide coverage of the protein sequence.
4. Number of samples with a signal intensity >0
5. P-Value was analyzed by using the Mann-Whitney test. A P of α<5.00E-02 was considered statistically significant.
6. Proteins which remained significant after performing multiple hypotheses testing correction, analyzed by using the Benjamini-Hochberg test for false discovery rate. An adjusted P-Value of α<5.00E-02 was considered statistically significant.

[DOI:10.1371/journal.pone.0158001.t002]
Table 3. Significant proteins in vitreous humor of subgroups of retinal vein occlusion-samples compared to control-samples. Significant proteins were detected by capillary electrophoresis coupled to mass spectrometer (CE-MS) and identified by tandem mass spectrometry (LC-MS/MS) when comparing protein signal intensity of subgroups of retinal vein occlusion (RVO)-samples (central RVO (CRVO), branch RVO (BRVO)) compared to control-samples.

| Protein                      | UniProt† | Control (n = 16) | CRVO (n = 14) | H-CRVO (n = 6) | BRVO (n = 10) |
|------------------------------|----------|------------------|---------------|----------------|---------------|
|                              |          | N°               | N°            | N°             | N°            |
|                              |          | Mean             | SD            | Mean           | SD            |
|                              |          | Mean             | SD            | Mean           | SD            |
| Clusterin*                  | P10909 15| 282.3            | 174.6         | 487.5          | 187.6         |
| Collagen alpha-1(V) chain† | P20906 13| 64.7             | 142.9         | 14.3           | 24.0          |
| Collagen alpha-2(XI) chain† | P13642 13| 377.1            | 528.6         | 1097.0         | 856.5         |
| Complement C3*†             | P01024 16| 390.2            | 194.5         | 569.1          | 167.8         |
| Complement C4-A††           | P006L4 7 | 29.3             | 78.3          | 0              | 0             |
| Complement factor B††        | P00751 11| 44.9             | 61.4          | 13.3           | 21.2          |
| Fibrinogen alpha chain†      | P02671 6 | 12.0             | 31.1          | 6.3            | 8.7           |
| Haptoglobin†                 | P00738 10| 30.6             | 51.3          | 14.9           | 23.8          |
| Ig lambda-2 chain C regions†| P00780 12| 452.7            | 305.4         | 484.6          | 494.0         |
| IgGFC-binding protein†       | Q09Y6R7 14| 181.5            | 268.0         | 9              | 170.1         |
| Ig lambda-like polypeptide 5†| B9A064 11| 39.4             | 75.7          | 14          | 215.4         |
| Neuroblast differentiation-associated protein AHNAK†    | Q09666 9 | 14.0             | 24.8          | 3              | 2.1           |
| Obscurin†                   | Q5V879 16| 1767.1           | 3857.7        | 8              | 775.6         |
| Opticin†                    | Q9UBMF4 16| 163.7            | 95.1          | 9              | 111.6         |
| Pigment epithelium-derived factor†   | P36955 15| 161.2            | 134.1         | 14          | 145.8         |
| Serum albumin†               | P02788 16| 2910.3           | 1163.1        | 14           | 1759.2        |
| Vitronectin††                | P04004 8 | 8                 | 8.6           | 10            | 71.9          |

† Listed in the universal protein resource (UniProt), a central repository of protein data.
* Number of samples with a signal intensity >0
§ P-Value was analyzed by using the Mann-Whitney test. A P of α=5.00E-02 was considered statistically significant.
Closed testing procedure was used to verify the results. Kruskal-Wallis Test was used for analysis. A P of α=5.00E-02 was considered statistically significant.
Proteins are marked when being significant in listed steps: Step 1:
† Control vs. CRVO vs. H-CRVO vs. BRVO
Step 2:
†† Control vs. CRVO vs. H-CRVO
††† Control vs. CRVO vs. BRVO
†††† Control vs. H-CRVO vs. BRVO
Step 3: For detailed information of step 3 see §
* Proteins which remained significant after performing multiple hypotheses testing correction, analyzed by using the Benjamini-Hochberg test for false discovery rate.

doi:10.1371/journal.pone.0158001.t003
when comparing signal intensity of the proteins between the fresh versus old RVO-samples ($P = 8.27 \times 10^{-2}$ to $9.68 \times 10^{-1}$; S5 Table).

**Verification of selected potential biomarker candidates**

Fig 2 shows the comparison of signal intensity of Clusterin, Complement C3, IGLL5, Opticin and Vitronecctin in the control group ($n = 16$) compared to the three subgroups in the discovery cohort (14 CRVO, 6 H-CRVO, 10 BRVO) and compared to the total discovery cohort (30 RVO). All these proteins remained significant after using correction for multiple testing when comparing RVO versus controls (Table 2) and were listed significant in each comparison of the subgroup analysis (Table 3). Therefore, these proteins were considered for verification of potential biomarker candidates (Fig 1).

To determine validity of potential biomarker candidates in RVO, we performed receiver operating characteristic (ROC) analysis (Fig 3) using randomly selected, independent samples. For analyses we used data of 8 controls and 14 RVO patients that were not included in analyses of biomarker definitions. AUCs were: Clusterin 0.884 ($P = 3.34 \times 10^{-3}$), Complement C3 0.955 ($P = 5.00 \times 10^{-4}$), IGLL5 1.000 ($P = 1.32 \times 10^{-4}$), Opticin 0.741 ($P = 6.54 \times 10^{-2}$), Vitronecin 0.786 ($P = 2.90 \times 10^{-2}$). Fig 4 shows the comparison of signal intensity of the 5 proteins in the control group ($n = 8$) compared to the three subgroups in the discovery cohort (6 CRVO, 3 H-CRVO, 5 BRVO) and compared to the total discovery cohort (14 RVO).

The vitreal protein concentration of Clusterin, Complement C3, IGLL5, Opticin and Vitronecctin was also measured using ELISA. Twelve RVO and twelve control samples were used. All Clusterin, Complement C3, Opticin and Vitronecctin analyzed proteins were successfully detected in all vitreous samples. Unfortunately, we were not able to detect IGLL5 in any of the vitreous samples. Opticin displayed reduced abundance, whereas Clusterin, Complement C3 and Vitronecctin showed increased abundance in the VH in the RVO group (see Table 4). Thus, the variations in the protein concentration observed by ELISA corresponded to the changes in signal intensity identified by mass spectrometry analysis. All four detected proteins showed a significant difference in the protein abundance between the RVO and the control group (see Table 4). Thus, the ELISA results further affirm our primary findings by mass spectrometry.

**Discussion**

The aim of this study was to analyze the protein profile of undiluted human vitreous of untreated patients with RVO and to identify potential biomarkers that are associated with the pathophysiology of the disease. CE-MS and LC-MS/MS were used for sample analysis in this study. CE-MS is a powerful and very reproducible technology platform with known performance characteristics [25] and therefore is used as one of the most advanced techniques for the discovery of new protein biomarkers of clinical significance [25–28]. Furthermore, CE-MS allows the characterization of highly complex samples in a consistent and reproducible way and has a high reproducibility allowing the comparison of the protein content of samples over time [25,29]. LC-MS/MS was used in this study to provide sequence information (i.e. identify) of the CE-MS detected peptides.

**Potential biomarker candidates**

Comparing the proteome of 30 RVO patients and 16 controls, we could define 16 proteins that were significantly up- or downregulated in RVO. Thrombosis and thrombolysis are involved in RVO as well as inflammatory processes due to hypoxic induced cell death [30]. This could be confirmed in our study as inflammatory response pathway, complement activation (classical pathway) as well as complement and coagulation cascades were identified as
Fig 2. Comparison of signal intensity of potential biomarker proteins. Comparison of signal intensity of proteins remained significant after using correction for multiple testing (Benjamini-Hochberg test) when comparing retinal vein occlusion (RVO) versus controls (see *, Table 2) of samples used for biomarker candidates identification (discovery set). Mann-Whitney test was used for analysis. A P of $\alpha<5.00E-02$ was considered statistically significant. RVO was subdivided in following subgroups: Central-RVO (CRVO), hemi-central RVO (H-CRVO), branch RVO (BRVO).

doi:10.1371/journal.pone.0158001.g002
Fig 3. Biomarker validation—Receiver operating characteristic (ROC) curves of selected candidate proteins. Independent samples of 14 patients suffered a retinal vein occlusion (RVO) and 8 controls were used. The area under the curve (AUC) was shown for RVO at 95% confidence level (95% CI).

| Protein                          | AUC  | SE  | 95% CI         |
|----------------------------------|------|-----|----------------|
| Clusterin                        | 0.884| 0.081| 0.726 to 1.000 |
| Complement C3                    | 0.955| 0.040| 0.877 to 1.000 |
| Ig lambda-like polypeptide 5     | 1.000| 0.000| 1.000 to 1.000 |
| Opticin                          | 0.741| 0.128| 0.489 to 0.993 |
| Vitronectin                      | 0.786| 0.103| 0.583 to 0.988 |

† area under the curve
‡ standard error
§ 95% confidence interval

doi:10.1371/journal.pone.0158001.g003
Fig 4. Comparison of signal intensity of proteins used for biomarker validation. Mann-Whitney test was used for analysis. A P of $\alpha<5.00E-02$ was considered statistically significant. Significant values are written in bold. Retinal vein occlusion (RVO) was subdivided in following subgroups: Central-RVO (CRVO), hemi-central RVO (H-CRVO), branch RVO (BRVO).

doi:10.1371/journal.pone.0158001.g004
primary pathways of the 16 significant up- or downregulated proteins in VH of RVO patients compared to controls.

We believe, that five of the 16 significant proteins, Clusterin, Complement C3, IGLL5, Opticin and Vitronectin, are of special interest as they remained significant after multiple testing, analysis of fresh versus old RVO, and subgroup analysis (CRVO, H-CRVO, BRVO versus controls).

Clusterin is a glycoprotein with a nearly ubiquitous tissue distribution and an apparent involvement in a number of biological processes, including inter alia lipid transport, membrane recycling, cell adhesion, programmed cell death, and complement cascade [31,32]. Interestingly, increased blood levels of Clusterin are associated with atrophy of the entorhinal cortex in Alzheimer’s disease for which this protein seems to be a marker of disease severity [33]. Therefore, upregulated Clusterin levels may indicate biochemical signs of a neurodegenerative disease. Our findings indicate for the first time that Clusterin in VH is associated with retinal disease like RVO.

Complement C3 plays a central role in the activation of complement system. Among other things, it is involved in the adaptive immune response to select the appropriate antigens for a humoral response, promotes phagocytosis and supports local inflammatory responses against pathogens [34]. Complement C3 might thus be involved in the well known phagocytic and inflammatory process subsequent to the onset of RVO.

Similar to Complement C3, IGLL5 demonstrated upregulation in RVO. IGLL5 could also be involved in immune processes being associated with cell death due to RVO although IGLL5 seems not to be expressed in pre-B-cells (http://uniprot.org). In general little is known about IGLL5 in the literature and further research is needed to explain the association of high IGLL5 levels in VH of patients with RVO.

Opticin is a protein produced by the non-pigmented ciliary epithelium. It is present in significant quantities in the vitreous of the eye and also localizes at the cornea, the iris, the ciliary body, the optic nerve, the choroid and the retina. It can noncovalently bind collagen fibrils and regulate fibril morphology, spacing, and organization [35]. Furthermore, Opticin was found to be located, besides various ocular tissues, especially in basal and cortical vitreous and adjacent basement membranes, like the internal limiting membrane, suggesting a possible role in vitreoretinal adhesion [36]. Therefore, decreased Opticin levels may be correlated with macula edema due to RVO.

Vitronectin is an abundant glycoprotein which promotes cell adhesion and spreading, inhibits the membrane damaging effect of the terminal cytolytic complement pathway, and binds to

### Table 4. Biomarker validation—Enzyme Linked Immunosorbsent Assay (ELISA).

Vitreal levels of our five potential biomarkers in retinal vein occlusion (RVO, n = 12) and control samples (n = 12) measured by ELISA.

| Protein                          | UniProt† | RVO (n = 12) | Control (n = 12) | P-Value‡ RVO vs. Control |
|----------------------------------|----------|--------------|-----------------|-------------------------|
| Clusterin (ng/ml)                | P10909   | 12 16.7 2.4 12.7 19.9 12 6.6 2.0 3.2 9.9 | 3.12E-05 |
| Complement C3 (ng/ml)            | P01024   | 12 3659.6 336.0 3188.0 4111.0 12 3241.3 464.3 2268.4 3657.8 | 3.77E-02 |
| Immunoglobulin lambda-like polypeptide | B9A064  | 0 - - - - 0 - - - - - | - |
| Opticin (pg/ml)                  | Q9UBM    | 12 524.3 83.6 412.0 632.0 12 579.7 90.2 450 734 | 4.94E-02 |
| Vitronectin (ng/ml)              | P04004   | 12 74.3 21.7 51.8 117.0 12 26.2 17.7 8.1 55.7 | 1.36E-04 |

† Listed in the universal protein resource (UniProt), a central repository of protein data.
‡ Number of samples in which the proteins were detected with ELISA
§ P-Value was analyzed by using the Mann-Whitney test. A P of α<5.00E-02 was considered statistically significant

doi:10.1371/journal.pone.0158001.t004
several serpins. By its localization in the extracellular matrix and its binding to plasminogen activation inhibitor-1, vitronectin can potentially regulate the proteolytic degradation of this matrix. In addition, vitronectin binds to complement, to heparin and to thrombin-antithrombin III complexes, showing its participation in the immune response and in the regulation of clot formation [37]. Therefore, upregulation of Vitronectin in RVO potentially plays a role in the disease.

Advantages and limitations of the study

To our knowledge, this is the first proteomics study using a high number (n = 30 and 16 idiopathic vitreous floaters as controls) of undiluted VH of RVO patients and validation of the 5 most significant proteins in an independent data set of an additional 8 controls and 14 RVO patients. We could only confirm 3 of the proteins 36 different proteins of the Yao et al. study [14] (Alpha-crystallin B chain, Beta-crystallin B2, Serum albumin) mostly due to this difference in power and the fact that they used 2-dimensional electrophoresis coupled with MS providing a different coverage of the proteome. Furthermore, we compared our results with other high throughput studies on VH. We have identified 85 proteins by other studies of vitreous humor proteome [12,38–40]. In addition, we have identified 9 novel VH proteins (see S2 Table) not described in previous studies, although our study fails to detect more than 94 proteins compared to the studies of Aretz et al. and Murthy et al. which detected in total 1111, respectively 1205 proteins in VH.

Nevertheless, there are some limitations of our study. First of all, there might be some bias in biomarker definitions by using patients with idiopathic vitreous floaters as controls. The existence of idiopathic vitreous floaters might have an influence on protein profile in vitreous body and therefore might have an influence of biomarker definitions in this study. Furthermore, highly abundant VH proteins such as albumin and immunoglobulin were not depleted in this study, possibly preventing the detection of less abundant proteins [41]. This seems to be the reason why only 94 proteins were identified although one would expect a higher number of proteins in human VH and why the number of detected peptides and coverage is low for some proteins (see S2 Table and Table 2), limiting statistical analysis of these proteins. In addition, depletion of highly abundant proteins does not necessarily lead to increased detection of low abundance proteins. On the contrary it has been shown that depletion can lead to co-depletion of many other proteins as shown for the analysis of the plasma proteome [42]. Another limitation of our study is the high variability of the mean intensity in some identified proteins resulting in a high SD (see for example Tables 2 and 3). The high variability may be induced by accepting identification of proteins when being represented by only two peptides. In future studies variability of the mean intensity of the proteins could be reduced by accepting identification of proteins when being represented in more than two peptides or by higher group sizes, leading to the next limitation of our study, the limited subgroup sizes. Although performing proteomic analysis of in total 68 participants (Proteomic analysis: 30 RVO, 16 controls; ROC analysis: 14 RVO, 8 controls) bigger group sizes would be desirable to further improve the power. Furthermore, with bigger group sizes one would expect significant results in all analyses of verification of potential biomarker candidates.

Conclusions

In this retrospective, clinical-experimental study we applied a reproducible proteomics detection method in a high number of undiluted vitreous humor of patients with RVO. Our findings were thoroughly statistically evaluated and yielded five potential biomarker candidates associated with the pathophysiology of RVO: Clusterin, Complement C3, IGLL5, Opticin and
Vitronectin. Future studies are needed to validate our substantial findings, which might be helpful regarding future diagnostic or therapeutic approaches in RVO. These studies should deplete highly abundant VH proteins such as albumin and immunoglobulin to determine more precisely potential biomarker candidates associated with the pathophysiology of RVO.

Supporting Information
S1 Fig. Closed testing procedure. (TIF)
S1 Table. Epidemiology—detailed patient information. (DOCX)
S2 Table. 94 proteins being detected in vitreous humor. (DOCX)
S3 Table. Raw data. (XLS)
S4 Table. Subgroup analysis—detailed information. (DOCX)
S5 Table. Influence of past time period since suffering retinal vein occlusion on biomarker discovery. (DOCX)

Acknowledgments

Funding/support
The research group was supported by the Adolf Messer Stiftung, Königstein, Hessen, Germany. A research grant from Bayer Vital GmbH, Leverkusen, Germany, was obtained to cover parts of the experimental costs which did not influence study design, data collection and analysis, decision to publish, or preparation of the manuscript. Michael Reich is a recipient of scholarships from the Studienstiftung des deutschen Volkes and the Dr. Gabriele Lederle-Stiftung. He wants to thank these organizations for their support. Matthias Nobl is a recipient of scholarships from the PRO RETINA-Stiftung zur Verhütung von Blindheit and the Dr. Gabriele Lederle-Stiftung. The funding organizations had no role in the design or conduct of the research.

Financial disclosures
The research group was supported by the Adolf Messer Stiftung, Königstein, Hessen, Germany. Parts of this research were supported by research grant from Bayer Vital GmbH, Leverkusen, Germany. Michael Reich is a recipient of scholarships from the Studienstiftung des deutschen Volkes and the Dr. Gabriele Lederle-Stiftung. He wants to thank these organizations for their support. Matthias Nobl is a recipient of scholarships from the PRO RETINA-Stiftung zur Verhütung von Blindheit and the Dr. Gabriele Lederle-Stiftung. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Mosaiques diagnostics GmbH provided support in the form of salaries for authors [JS], but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific roles of these authors are articulated in the 'author contributions' section.
Integrity and accuracy of the data

Michael J. Koss had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Each author meets the four criteria set by the ICMJE required to claim authorship.

Author Contributions

Conceived and designed the experiments: MR JS MJK. Performed the experiments: MR ID MN JS JK MJK. Analyzed the data: MR ID MN MJK. Contributed reagents/materials/analysis tools: MR ID MN JS JPS WM JK FHJK FTAK GUA MJK. Wrote the paper: MR ID MN JS JPS WM JK FHJK FTAK GUA MJK. Data interpretation: MR. Preparation of manuscript: MR MJK.

References

1. Klein R, Moss SE, Meuer SM, Klein BE (2008) The 15-year cumulative incidence of retinal vein occlusion: the Beaver Dam Eye Study. Arch Ophthalmol 126: 513–518. doi:10.1001/archopht.126.4.513 PMID: 18413521
2. Cugati S, Wang J, Rochtchina E, Mitchell P (2006) Ten-year incidence of retinal vein occlusion in an older population: the Blue Mountains Eye Study. Arch Ophthalmol 124: 726–732. PMID: 16682596
3. Rehak J, Rehak M (2008) Branch retinal vein occlusion: pathogenesis, visual prognosis, and treatment modalities. Curr Eye Res 33: 111–131. doi:10.1016/j.cejr.2007.09.008 PMID: 20103055
4. Margolis R, Singh RP, Kaiser PK (2006) Branch retinal vein occlusion: clinical findings, natural history, and management. Compr Ophthalmol Update 7: 265–276. PMID: 17244442
5. Tsujikawa A, Sakamoto A, Ota M, Kotera Y, Oh H, Miyamoto K, et al. (2010) Serous retinal detachment associated with retinal vein occlusion. Am J Ophthalmol 149: 291–301 e295. doi:10.1016/j.ajo.2009.09.007 PMID: 20103055
6. Yamaike N, Tsujikawa A, Ota M, Sakamoto A, Kotera Y, Kita M, et al. (2008) Three-dimensional imaging of cystoid macular edema in retinal vein occlusion. Ophthalmology 115: 355–362 e352. PMID: 17675242
7. Koss MJ, Pfister M, Rothweiler F, Michaelis M, Cinatl J, Schubert R, et al. (2012) Comparison of cytokine levels from undiluted vitreous of untreated patients with retinal vein occlusion. Acta Ophthalmol 90: e98–e103. doi:10.1111/j.1755-3768.2011.02292.x PMID: 22066978
8. Pfister M, Rothweiler F, Michaelis M, Cinatl Jr., Schubert R, Koch FH, et al. (2013) Correlation of inflammatory and proangiogenic cytokines from undiluted vitreous samples with spectral domain OCT scans, in untreated branch retinal vein occlusion. Clin Ophthalmol 7: 1061–1067. doi:10.2147/OPHT.S42786 PMID: 23766628
9. Stefansson E (2009) Physiology of vitreous surgery. Graefes Arch Clin Exp Ophthalmol 247: 147–163. doi:10.1007/s00417-008-0980-7 PMID: 19034481
10. Garcia-Ramirez M, Canals F, Hernandez C, Colome N, Ferrer C, Carrasco E, et al. (2007) Proteomic analysis of human vitreous fluid by fluorescence-based difference gel electrophoresis (DIGE): a new strategy for identifying potential candidates in the pathogenesis of proliferative diabetic retinopathy. Diabetologia 50: 1294–1303. PMID: 17380318
11. Gorg A, Weiss W, Dunn MJ (2004) Current two-dimensional electrophoresis technology for proteomics. Proteomics 4: 3665–3685. PMID: 15543535
12. Koss MJ, Hoffmann J, Nguyen N, Pfister M, Mishak H, Mullen W, et al. (2014) Proteomics of vitreous humor of patients with exudative age-related macular degeneration. PLoS One 9: e96895. doi: 10.1371/journal.pone.0096895 PMID: 24828375
13. Cryan LM, O’Brien C (2008) Proteomics as a research tool in clinical and experimental ophthalmology. Proteomics Clin Appl 2: 762–775. doi: 10.1002/prca.200780094 PMID: 21136873
14. Yao J, Chen Z, Yang Q, Liu X, Chen X, Zhuang M, et al. (2013) Proteomic analysis of aqueous humor from patients with branch retinal vein occlusion-induced macular edema. Int J Mol Med 32: 1421–1434. doi: 10.3892/ijmm.2013.1509 PMID: 24068204
15. Theodorescu D, Wittke S, Ross MM, Walden M, Conaway M, Just I, et al. (2006) Discovery and validation of new protein biomarkers for urothelial cancer: a prospective analysis. Lancet Oncol 7: 230–240. PMID: 16510332
16. Neuhoff N, Kaiser T, Wittke S, Krebs R, Pitt A, Burchard A, et al. (2004) Mass spectrometry for the detection of differentially expressed proteins: a comparison of surface-enhanced laser desorption/ionization and capillary electrophoresis/mass spectrometry. Rapid Commun Mass Spectrom 18: 149–156. PMID: 14745763
17. Siwy J, Mullen W, Golovko I, Franke J, Zurbig P (2011) Human urinary peptide database for multiple disease biomarker discovery. Proteomics Clin Appl 5: 367–374. doi: 10.1002/prca.201000155 PMID: 21591268
18. Metzger J, Negm AA, Plentz RR, Weismuller TJ, Wedemeyer J, Karlsen TH, et al. (2013) Urine proteomic analysis differentiates cholangiocarcinoma from primary sclerosing cholangitis and other benign biliary disorders. Gut 62: 122–130. doi: 10.1136/gutjnl-2012-302047 PMID: 22580416
19. Zurbig P, Renfrow MB, Schiffer E, Novak J, Walden M, Wittke S, et al. (2006) Biomarker discovery by CE-MS enables sequence analysis via MS/MS with platform-independent separation. Electrophoresis 27: 2111–2125. PMID: 16645980
20. Mansor R, Mullen W, Albalat A, Zerefos P, Mischak H, Barrett DC, et al. (2013) A peptidomic approach to biomarker discovery for bovine mastitis. J Proteomics 85: 89–98. doi: 10.1016/j.jprot.2013.04.027 PMID: 23639846
21. Neuhaus J, Schiffer E, von Wicke P, Bauer HW, Leung H, Siwy J, et al. (2013) Seminal plasma as a source of prostate cancer peptide biomarker candidates for detection of indolent and advanced disease. PLoS One 8: e67514. doi: 10.1371/journal.pone.0067514 PMID: 23826311
22. Weissinger EM, Metzger J, Dobbelstein C, Wolff D, Schleuning M, Kuzmina Z, et al. (2014) Proteomic peptide profiling for preemptive diagnosis of acute graft-versus-host disease after allogeneic stem cell transplantation. Leukemia 28: 842–852. doi: 10.1038/leu.2013.210 PMID: 23842427
23. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society, Series B: Methodological 57: 289–300.
24. Marcus R, Peritz E, Gabriel KR (1976) On closed testing procedures with special reference to ordered analysis of variance. Biometrika 63: 655–660.
25. Mischak H, Vlahou A, Ioannidis JP (2013) Technical aspects and inter-laboratory variability in native peptide profiling: the CE-MS experience. Clin Biochem 46: 432–443. doi: 10.1016/j.clinbiochem.2012.09.025 PMID: 23041249
26. Stalmach A, Husi H, Mosbahi K, Albalat A, Mullen W, Mischak H (2015) Methods in capillary electrophoresis coupled to mass spectrometry for the identification of clinical proteomic/peptidomic biomarkers in biofluids. Methods Mol Biol 1243: 187–205. doi: 10.1007/978-1-4939-1872-0_11 PMID: 25384747
27. Mischak H, Julian BA, Novak J (2007) High-resolution proteome/peptidome analysis of peptides and low-molecular-weight proteins in urine. Proteomics Clin Appl 1: 792. PMID: 20107618
28. Mischak H, Schanstra JP (2011) CE-MS in biomarker discovery, validation, and clinical application. Proteomics Clin Appl 5: 9–23. doi: 10.1002/prca.201000058 PMID: 21280234
29. Latosinska A, Frantzi M, Vlahou A, Mischak H (2013) Clinical applications of capillary electrophoresis coupled to mass spectrometry in biomarker discovery: Focus on Bladder Cancer. Proteomics Clin Appl.
30. Browning DJ (2012) Retinal Vein Occlusions: Evidence-Based Management. New York: NY: Springer.
31. Jones SE, Jomary C (2002) Clusterin. Int J Biochem Cell Biol 34: 427–431. PMID: 11906815
32. Park S, Mathis KW, Lee IK (2014) The physiological roles of apolipoprotein J/clusterin in metabolic and cardiovascular diseases. Rev Endocr Metab Disord 15: 45–53. doi: 10.1007/s11154-013-9275-3 PMID: 24097125
33. Thambisetty M, Simmons A, Velayudhan L, Hye A, Campbell J, Zhang Y, et al. (2010) Association of plasma clusterin concentration with severity, pathology, and progression in Alzheimer disease. Arch Gen Psychiatry 67: 739–748. doi: 10.1001/archgenpsychiatry.2010.78 PMID: 20603455
34. Sahu A, Lambris JD (2001) Structure and biology of complement protein C3, a connecting link between innate and acquired immunity. Immunol Rev 180: 35–48. PMID: 11414361
35. Reardon AJ, Le Goff M, Briggs MD, McLeod D, Sheehan JK, Thornton DJ, et al. (2000) Identification in vitreous and molecular cloning of opticin, a novel member of the family of leucine-rich repeat proteins of the extracellular matrix. J Biol Chem 275: 2123–2129. PMID: 10636917
36. Ramesh S, Bonshek RE, Bishop PN (2004) Immunolocalisation of opticin in the human eye. Br J Ophthalmol 88: 697–702. PMID: 15090426
37. Schwartz I, Seger D, Shaltiel S (1999) Vitronectin. Int J Biochem Cell Biol 31: 539–544. PMID: 10399314
38. Murthy KR, Goel R, Subbannayya Y, Jacob HK, Murthy PR, Manda SS, et al. (2014) Proteomic analysis of human vitreous humor. Clin Proteomics 11: 29. doi: 10.1186/1559-0275-11-29 PMID: 25097467
39. Gao BB, Chen X, Timothy N, Aiello LP, Feener EP (2008) Characterization of the vitreous proteome in diabetes without diabetic retinopathy and diabetes with proliferative diabetic retinopathy. J Proteome Res 7: 2516–2525. doi:10.1021/pr800112g PMID: 18433156

40. Aretz S, Krohne TU, Kammerer K, Warnken U, Hotz-Wagenblatt A, Bergmann M, et al. (2013) In-depth mass spectrometric mapping of the human vitreous proteome. Proteome Sci 11: 22. doi:10.1186/1477-5956-11-22 PMID: 23688336

41. Angi M, Kalirai H, Coupland SE, Damato BE, Semeraro F, Romano MR (2012) Proteomic analyses of the vitreous humour. Mediators Inflamm 2012: 148039. doi:10.1155/2012/148039 PMID: 22973072

42. Shen Y, Kim J, Strittmatter EF, Jacobs JM, Camp DG 2nd, Fang R, et al. (2005) Characterization of the human blood plasma proteome. Proteomics 5: 4034–4045. PMID: 16152657