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Supplementary Information for

Selective Permeability of Mouse Blood-Aqueous Barrier as Determined by ^{15}N-Heavy Isotope Tracing and Mass Spectrometry

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www.pnas.org/cgi/doi/10.1073/pnas.1807982115

Supplementary Materials and Methods

Mass spectrometry

The peptides were dissolved in 20uL of Buffer A (94.785% H_2O, 5% ACN, 0.125% FA) and quantified using Micro BCA Protein Assay. 3 ug were loaded with Easy nLC 1200 onto nanoViper C18 trap column (0.075mm x 2cm). Following a 2 hour gradient with increasing Buffer B (99.875% ACN, 0.125% FA) concentration, peptides eluted from the NanoViper analytical column (0.075mm x 50cm) were elctrosprayed with 2,000V from the stainless steel emitter tip on the Nanospray Flex Ion Source. Each sample was analyzed on the Orbitrap Fusion Tribrid mass spectrometer. MS parameters were as follows: ion transfer tube temp = 300ºC, Easy-IC internal mass calibration, default charge state = 2. Detector type set to Orbitrap, with 60K resolution, wide quad isolation, mass range = normal, scan range = 300-1500 m/z. Max injection time = 50 ms, AGC target = 200,000, microscans = 1, S-lens RF level = 60. Without source fragmentation, datatype = positive and centroid, MIPS was on, included charge states = 2-6 (reject unassigned). Dynamic exclusion enabled with n =1. Precursor selection decision = most intense, top 20, isolation window = 1.6, scan range = auto normal, first mass = 110, collision energy 30%, CID, Deterctor type = ion trap, max injection time = 75 ms, AGC target – 10,000, inject ions for all available parallelizable time.

Spectra analysis, protein quantification

Protein identification, quantification, and analysis were done using Integrated Proteomics Pipeline (IP2). The raw files were extracted to MS1 and MS2 spectra using RawConverter (http://fields.scripps.edu/downloads.php) and ProLuCID searched against the RefSeq mouse dataset on IP2. Basic parameters of 50 ppm precursor mass tolerance and 600 ppm for fragmented ions were used. Searches were filtered with DTAselect containing one peptide per protein, at least one tryptic end and unlimited missed cleavages of a minimum of 6 amino acid, with a false discovery rate (FDR) < 0.001, fixed modification of +57.02146Da on cysteine residues, and all precursor mass within 10 ppm of expected. To estimate peptide FDRs accurately, target/decoy database was used containing the reversed sequences of all the proteins appended to the target database(1). After the matches were filtered, the protein FDRs were below 1% for each sample analysis. With IDCompare, measurements of abundance were generated across all samples, such as peptide count, NSAF and emPAI. Searches were done for combined light and heavy peptides and Census quantified(2).

To calculate the $^{15}N/^{14}N$ peptide ion intensity, the ProLuCID results were used to reconstruct MS1 ion chromatograms in the m/z range that included both the heavy and light peptide (*SI Appendix*, Fig. S1) using the Census software(3). Calculating the elemental compositions and corresponding isotopic distribution for the heavy and light peptides determined the m/z range of the chromatogram. Using the MS1 files the chromatograms were generated that included both the labeled and unlabeled precursor peptides. The intensity ratios were then calculated per peptide using the reconstructed chromatogram. Census also allows for filtration of poor-quality peptide ratio measurements with a correlation coefficient (ranging between 0-1) that represents the quality of the correlation between the labeled and unlabeled chromatograms. In this analysis, the peptide ratios with correlation values greater than 0.5 were used. When more than two peptides were found for the same protein, Census used an outlier algorithm by calculating the SDs for the proteins. By applying the Grubbs test (p value < 0.01) the outliers were removed. With QuantCompare, the final peptide ratios were generated. For each protein, its heavy *vs*. light ratios were represented by the composite of all peptide ratios identified by MS that are assigned to the protein.

Laser- and alkali burn-induced eye injury

Laser illumination and alkali burn was applied to inflict injuries to one of the two eyes of the subject mice (the ^{15}N-serum recipient mice). In brief, mice were anesthetized by an intraperitoneal injection of ketamine (100 mg/kg; Butler Schein Animal Health, Dublin, OH, USA) and xylazine (10 mg/kg; Lloyd, Inc., Iowa, Shenandoah, IA, USA), and a drop of 0.5% proparacaine hydrochloride (Thermo-Fisher) was applied to the corneal surface for local analgesia. Laser illumination (approximately 80 to 100 laser spots) was applied to the corneal limbus to photocoagulate its vascular plexus (details of the method described previously(4)). Two weeks after surgery to permit wound healing, the mice were subjected to infusion of ^{15}N-labeled serum. For alkali burn-induced corneal neovascularization study, mice were systemically and locally anesthetized as in laser illumination model. Filter papers of 2 mm in diameter were soaked with 1 M NaOH and placed on the central cornea for 30 seconds, followed by flushing the eyes twice with 10 mL PBS (more details in (5)). Two weeks after treatment, the mice were sacrificed and aqueous was collected for western blotting and eye tissues were collected for imaging.

Vascular imaging

The method for whole mount imaging of the corneal vascular plexus was published previously(6, 7). Briefly, following the extraction of the aqueous, eyes were immersion fixed in 4% paraformaldehyde for overnight. The hemispheres were then dissected and mounted onto glass slide to be stained with CD31 (R&D Systems) for confocal imaging.

Western blotting

Aqueous (2 μL) and serum (0.5 μL) samples were resolved by 4−12% SDS-PAGE and transferred to PVDF membrane. The membrane was blocked with 5% non-fat milk for 1 hour at room temperature, and then probed with complement factor H/CFH antibody (R&D Systems) or complement factor B/CFB antibody (Novus Biologicals) followed by HRP-conjugated secondary antibodies. The membrane was developed using the Clarity™ ECL substrate (Bio-Rad Laboratories).

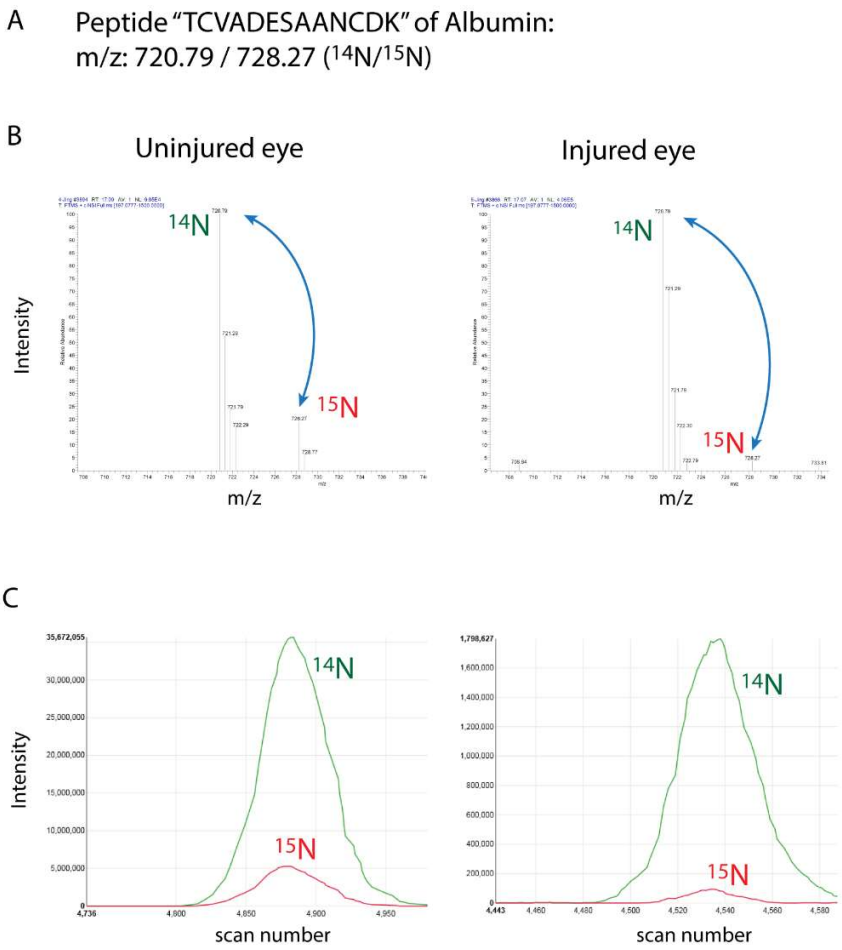


Fig. S1. Relative quantification of ^{15}N-labeled proteins by reconstructed MS1 chromatograms. A. Tryptic Albumin peptide sequence. B. MS1 spectra of the Albumin peptide with heavy ^{15}N and light ^{14}N atoms. C. Relative quantification was based on area-under-curve values of reconstructed chromatograms.

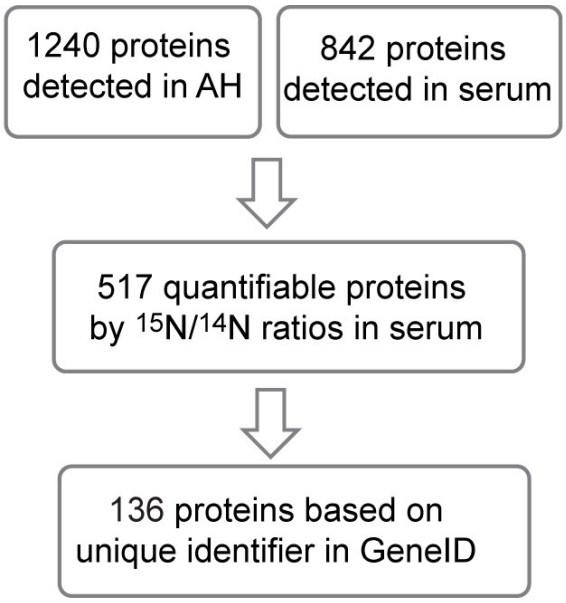
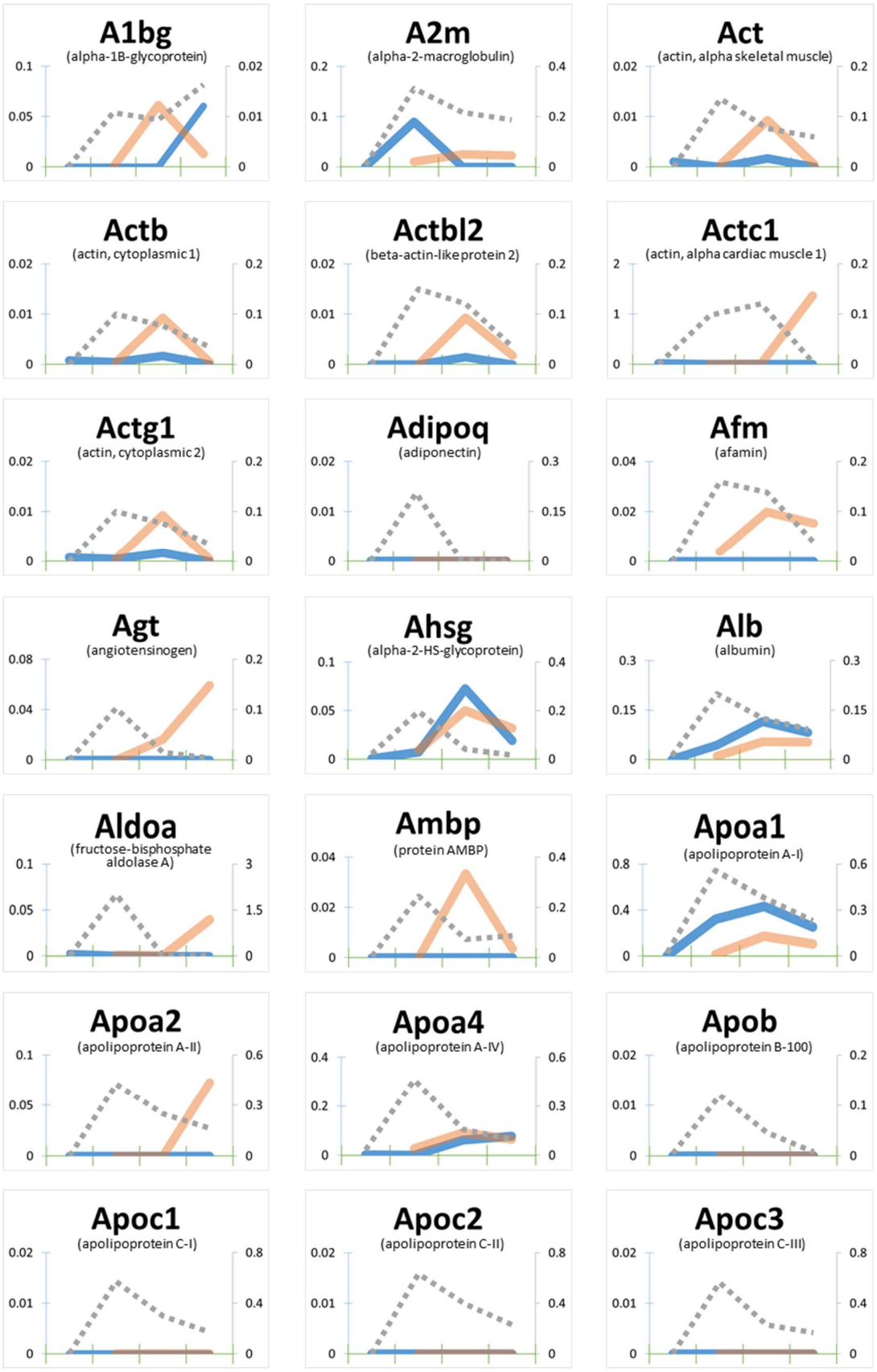
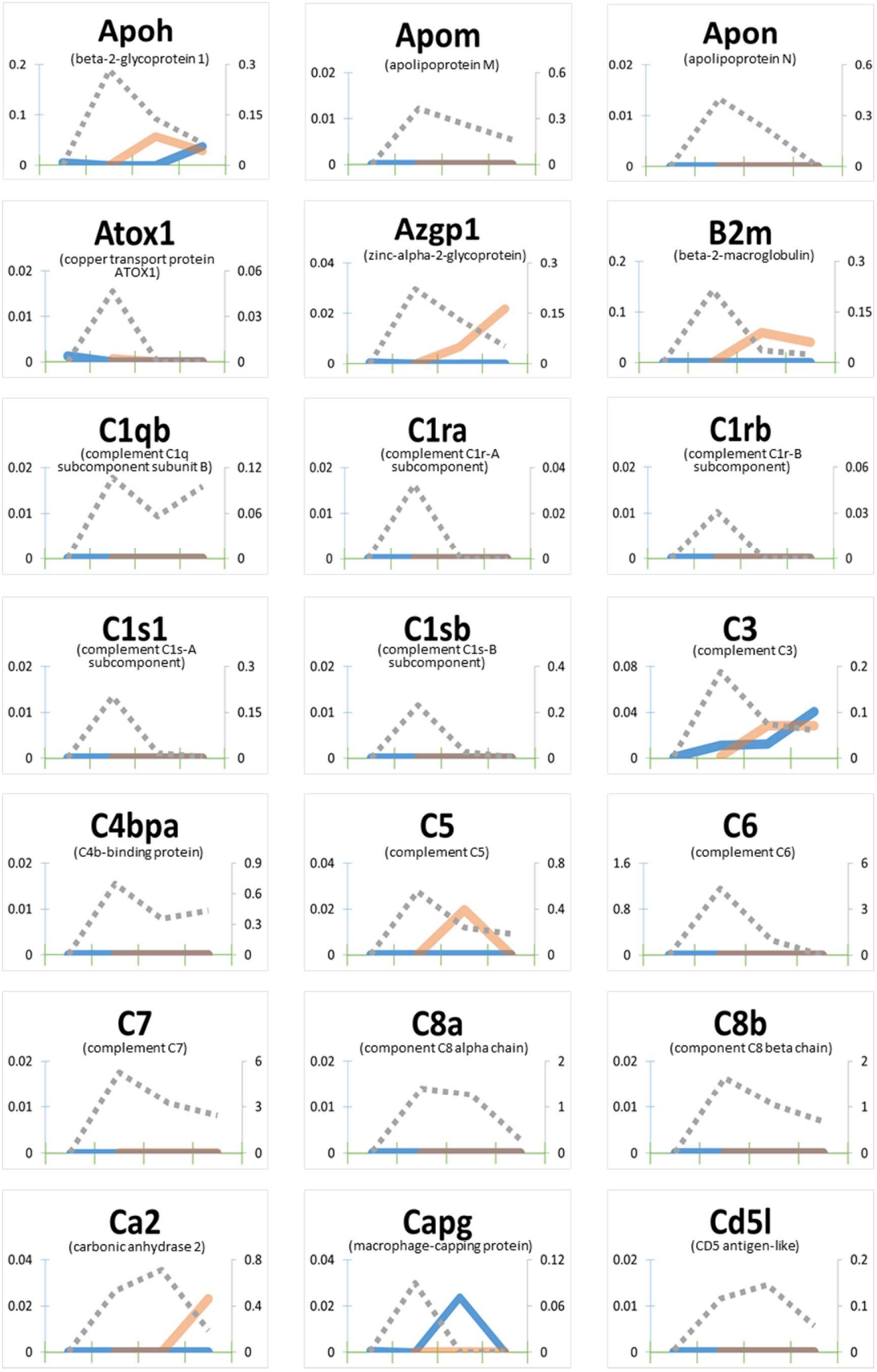
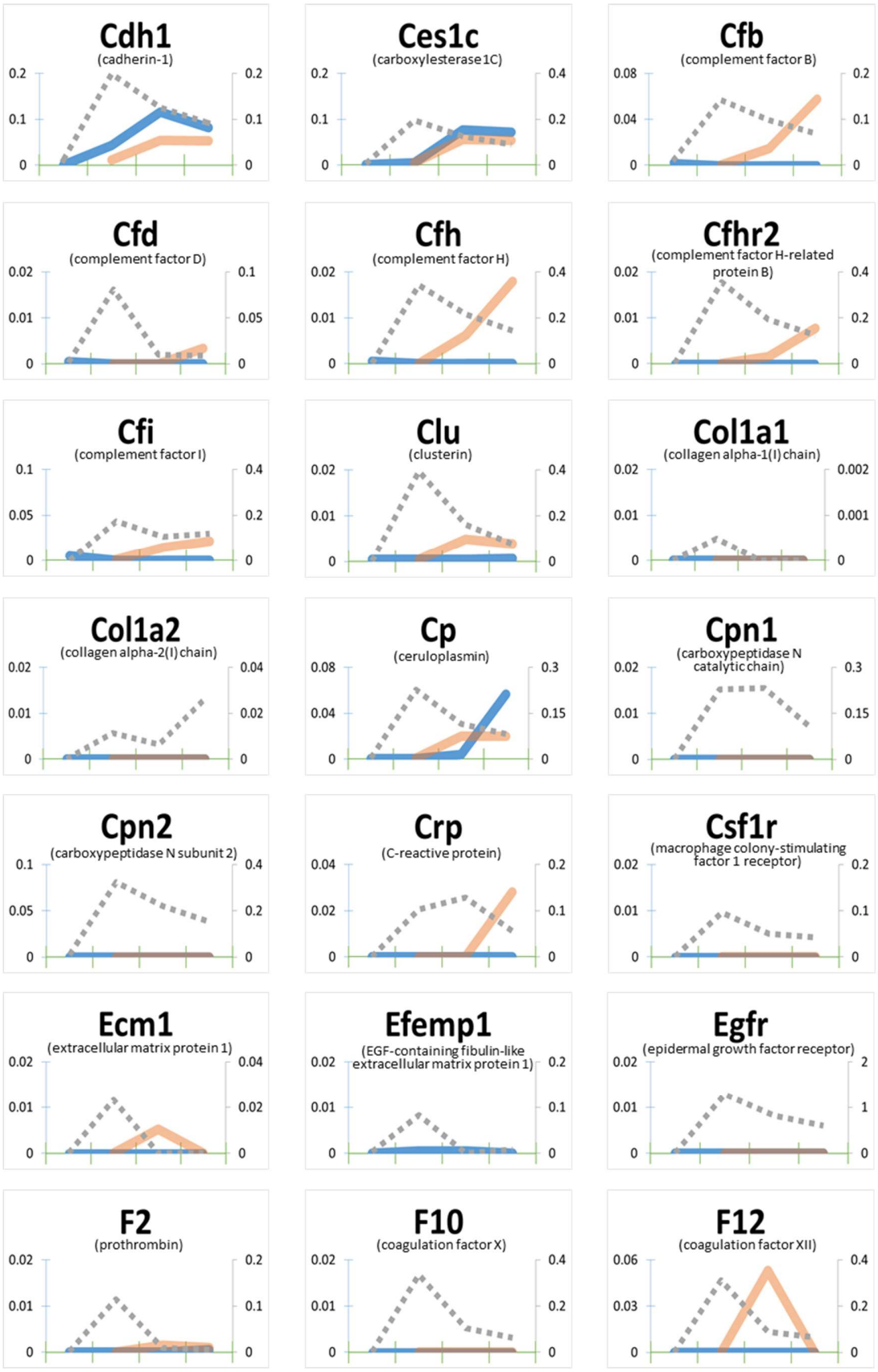
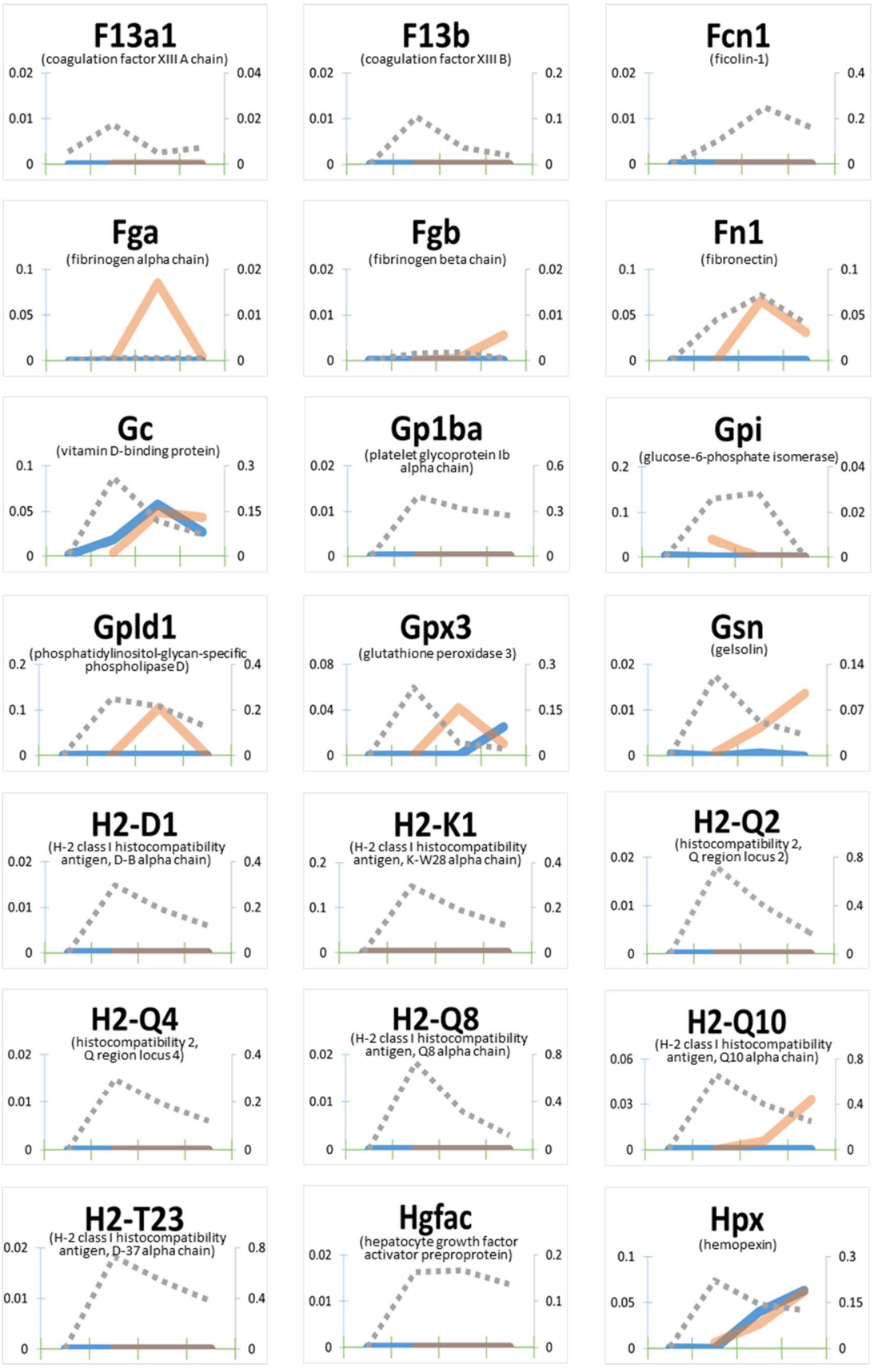


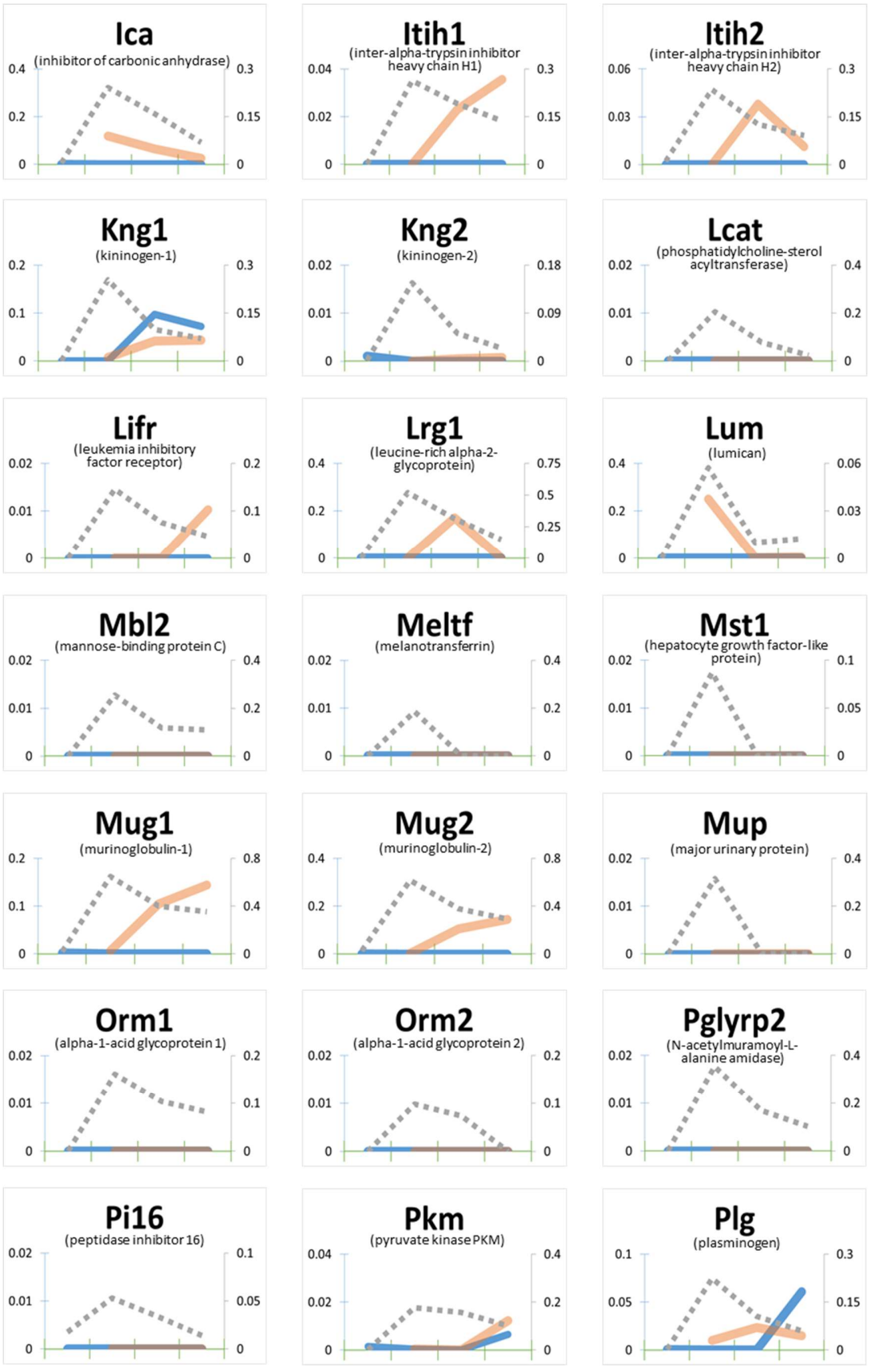
Fig. S2. The total numbers of proteins measured in aqueous and in serum. Middle box: Proteins showing both ^{15}N and ^{14}N signals (serum extracted at the 15 min time point) were analyzed, of which a manually annotated non-redundant set of 136 proteins were followed up (bottom box).

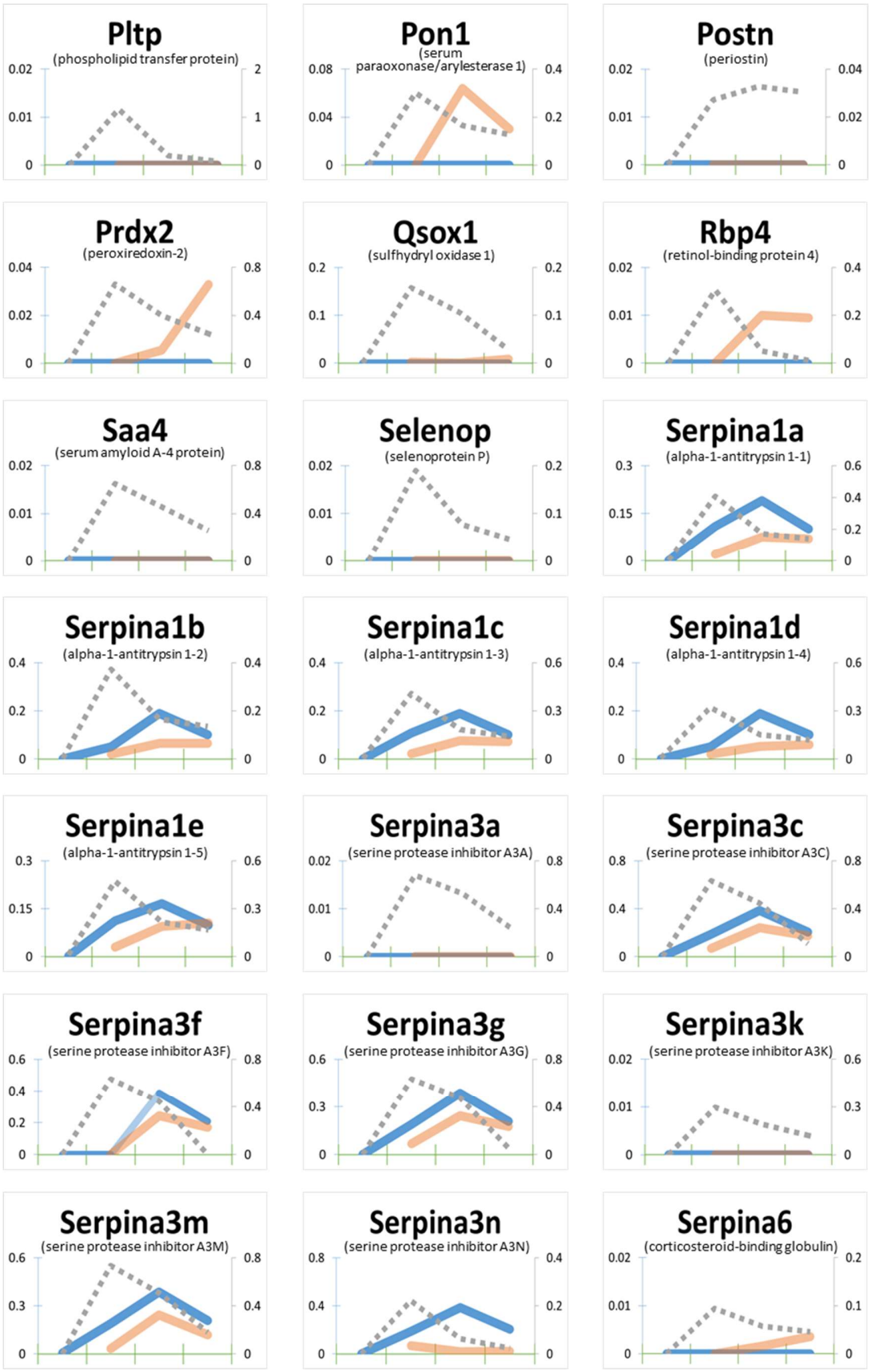

A1bg
(alpha-1B-glycoprotein)
A2m
(alpha-2-macroglobulin)
Act
(actin, alpha skeletal muscle)
Actb
(actin, cytoplasmic 1)
Actbl2
(beta-actin-like protein 2)
Actc1
(actin, alpha cardiac muscle 1)
Actg1
(actin, cytoplasmic 2)
Adipoq
(adiponectin)
Afm
(afamin)
Agt
(angiotensinogen)
Ahsg
(alpha-2-HS-glycoprotein)
Alb
(albumin)
Aldoa
(fructose-bisphosphate aldolase A)
Ambp
(protein AMBP)
Apoa1
(apolipoprotein A-I)
Apoa2
(apolipoprotein A-II)
Apoa4
(apolipoprotein A-IV)
Apob
(apolipoprotein B-100)
Apoc1
(apolipoprotein C-I)
Apoc2
(apolipoprotein C-II)
Apoc3
(apolipoprotein C-III)


Apoh
(beta-2-glycoprotein 1)
0.2
0.1
0
0.3
0.15
0
Apom
(apolipoprotein M)
0.02
0.01
0
0.6
0.3
0
Apon
(apolipoprotein N)
0.02
0.01
0
0.6
0.3
0
Atox1
(copper transport protein ATOX1)
0.02
0.01
0
0.06
0.03
0
Azgp1
(zinc-alpha-2-glycoprotein)
0.04
0.02
0
0.3
0.15
0
B2m
(beta-2-macroglobulin)
0.2
0.1
0
0.3
0.15
0
C1qb
(complement C1q subcomponent subunit B)
0.02
0.01
0
0.12
0.06
0
C1ra
(complement C1r-A subcomponent)
0.02
0.01
0
0.04
0.02
0
C1rb
(complement C1r-B subcomponent)
0.02
0.01
0
0.06
0.03
0
C1s1
(complement C1s-A subcomponent)
0.02
0.01
0
0.3
0.15
0
C1sb
(complement C1s-B subcomponent)
0.02
0.01
0
0.4
0.2
0
C3
(complement C3)
0.08
0.04
0
0.2
0.1
0
C4bpa
(C4b-binding protein)
0.02
0.01
0
0.9
0.6
0.3
0
C5
(complement C5)
0.04
0.02
0
0.8
0.4
0
C6
(complement C6)
1.6
0.8
0
6
3
0
C7
(complement C7)
0.02
0.01
0
6
3
0
C8a
(component C8 alpha chain)
0.02
0.01
0
2
1
0
C8b
(component C8 beta chain)
0.02
0.01
0
2
1
0
Ca2
(carbonic anhydrase 2)
0.04
0.02
0
0.8
0.4
0
Capg
(macrophage-capping protein)
0.04
0.02
0
0.12
0.06
0
Cd5l
(CD5 antigen-like)
0.02
0.01
0
0.2
0.1
0


Cdh1
(cadherin-1)
0.2
0.1
0
0.2
0.1
0
Ces1c
(carboxylesterase 1C)
0.2
0.1
0
0.4
0.2
0
Cfb
(complement factor B)
0.08
0.04
0
0.2
0.1
0
Cfd
(complement factor D)
0.02
0.01
0
0.1
0.05
0
Cfh
(complement factor H)
0.02
0.01
0
0.4
0.2
0
Cfhr2
(complement factor H-related protein B)
0.02
0.01
0
0.4
0.2
0
Cfi
(complement factor I)
0.1
0.05
0
0.4
0.2
0
Clu
(clusterin)
0.02
0.01
0
0.4
0.2
0
Col1a1
(collagen alpha-1(I) chain)
0.02
0.01
0
0.002
0.001
0
Col1a2
(collagen alpha-2(I) chain)
0.02
0.01
0
0.04
0.02
0
Cp
(ceruloplasmin)
0.08
0.04
0
0.3
0.15
0
Cpn1
(carboxypeptidase N catalytic chain)
0.02
0.01
0
0.3
0.15
0
Cpn2
(carboxypeptidase N subunit 2)
0.1
0.05
0
0.4
0.2
0
Crp
(C-reactive protein)
0.04
0.02
0
0.2
0.1
0
Csf1r
(macrophage colony-stimulating factor 1 receptor)
0.02
0.01
0
0.2
0.1
0
Ecm1
(extracellular matrix protein 1)
0.02
0.01
0
0.04
0.02
0
Efemp1
(EGF-containing fibulin-like extracellular matrix protein 1)
0.02
0.01
0
0.2
0.1
0
Egfr
(epidermal growth factor receptor)
0.02
0.01
0
2
1
0
F2
(prothrombin)
0.02
0.01
0
0.2
0.1
0
F10
(coagulation factor X)
0.02
0.01
0
0.4
0.2
0
F12
(coagulation factor XII)
0.06
0.03
0
0.4
0.2
0


F13a1
(coagulation factor XIII A chain)
0.02
0.01
0
0.04
0.02
0
F13b
(coagulation factor XIII B)
0.02
0.01
0
0.2
0.1
0
Fcn1
(ficolin-1)
0.02
0.01
0
0.4
0.2
0
Fga
(fibrinogen alpha chain)
0.1
0.05
0
0.02
0.01
0
Fgb
(fibrinogen beta chain)
0.02
0.01
0
0.02
0.01
0
Fn1
(fibronectin)
0.1
0.05
0
0.1
0.05
0
Gc
(vitamin D-binding protein)
0.1
0.05
0
0.3
0.15
0
Gp1ba
(platelet glycoprotein Ib alpha chain)
0.02
0.01
0
0.6
0.3
0
Gpi
(glucose-6-phosphate isomerase)
0.2
0.1
0
0.04
0.02
0
Gpld1
(phosphatidylinositol-glycan-specific phospholipase D)
0.2
0.1
0
0.4
0.2
0
Gpx3
(glutathione peroxidase 3)
0.08
0.04
0
0.3
0.15
0
Gsn
(gelsolin)
0.02
0.01
0
0.14
0.07
0
H2-D1
(H-2 class I histocompatibility antigen, D-B alpha chain)
0.02
0.01
0
0.4
0.2
0
H2-K1
(H-2 class I histocompatibility antigen, K-W28 alpha chain)
0.2
0.1
0
0.4
0.2
0
H2-Q2
(histocompatibility 2, Q region locus 2)
0.02
0.01
0
0.8
0.4
0
H2-Q4
(histocompatibility 2, Q region locus 4)
0.02
0.01
0
0.4
0.2
0
H2-Q8
(H-2 class I histocompatibility antigen, Q8 alpha chain)
0.02
0.01
0
0.8
0.4
0
H2-Q10
(H-2 class I histocompatibility antigen, Q10 alpha chain)
0.06
0.03
0
0.8
0.4
0
H2-T23
(H-2 class I histocompatibility antigen, D-37 alpha chain)
0.02
0.01
0
0.8
0.4
0
Hgfac
(hepatocyte growth factor activator preproprotein)
0.02
0.01
0
0.2
0.1
0
Hpx
(hemopexin)
0.1
0.05
0
0.3
0.15
0


Ica
(inhibitor of carbonic anhydrase)
0.4
0.2
0
0.3
0.15
0
Itih1
(inter-alpha-trypsin inhibitor heavy chain H1)
0.04
0.02
0
0.3
0.15
0
Itih2
(inter-alpha-trypsin inhibitor heavy chain H2)
0.06
0.03
0
0.3
0.15
0
Kng1
(kininogen-1)
0.2
0.1
0
0.3
0.15
0
Kng2
(kininogen-2)
0.02
0.01
0
0.18
0.09
0
Lcat
(phosphatidylcholine-sterol acyltransferase)
0.02
0.01
0
0.4
0.2
0
Lifr
(leukemia inhibitory factor receptor)
0.02
0.01
0
0.2
0.1
0
Lrg1
(leucine-rich alpha-2-glycoprotein)
0.4
0.2
0
0.75
0.5
0.25
0
Lum
(lumican)
0.4
0.2
0
0.06
0.03
0
Mbl2
(mannose-binding protein C)
0.02
0.01
0
0.4
0.2
0
Meltf
(melanotransferrin)
0.02
0.01
0
0.4
0.2
0
Mst1
(hepatocyte growth factor-like protein)
0.02
0.01
0
0.1
0.05
0
Mug1
(murinoglobulin-1)
0.2
0.1
0
0.8
0.4
0
Mug2
(murinoglobulin-2)
0.4
0.2
0
0.8
0.4
0
Mup
(major urinary protein)
0.02
0.01
0
0.4
0.2
0
Orm1
(alpha-1-acid glycoprotein 1)
0.02
0.01
0
0.2
0.1
0
Orm2
(alpha-1-acid glycoprotein 2)
0.02
0.01
0
0.2
0.1
0
Pglyrp2
(N-acetylmuramoyl-L-alanine amidase)
0.02
0.01
0
0.4
0.2
0
Pi16
(peptidase inhibitor 16)
0.02
0.01
0
0.1
0.05
0
Pkm
(pyruvate kinase PKM)
0.04
0.02
0
0.4
0.2
0
Plg
(plasminogen)
0.1
0.05
0
0.3
0.15
0


Pltp
(phospholipid transfer protein)
0.02
0.01
0
2
1
0
Pon1
(serum paraoxonase/arylesterase 1)
0.08
0.04
0
0.4
0.2
0
Postn
(periostin)
0.02
0.01
0
0.04
0.02
0
Prdx2
(peroxiredoxin-2)
0.04
0.02
0
0.8
0.4
0
Qsox1
(sulfhydryl oxidase 1)
0.2
0.1
0
0.2
0.1
0
Rbp4
(retinol-binding protein 4)
0.02
0.01
0
0.4
0.2
0
Saa4
(serum amyloid A-4 protein)
0.02
0.01
0
0.8
0.4
0
Selenop
(selenoprotein P)
0.02
0.01
0
0.2
0.1
0
Serpina1a
(alpha-1-antitrypsin 1-1)
0.3
0.15
0
0.6
0.4
0.2
0
Serpina1b
(alpha-1-antitrypsin 1-2)
0.4
0.2
0
0.4
0.2
0
Serpina1c
(alpha-1-antitrypsin 1-3)
0.4
0.2
0
0.6
0.3
0
Serpina1d
(alpha-1-antitrypsin 1-4)
0.4
0.2
0
0.6
0.3
0
Serpina1e
(alpha-1-antitrypsin 1-5)
0.3
0.15
0
0.6
0.3
0
Serpina3a
(serine protease inhibitor A3A)
0.02
0.01
0
0.8
0.4
0
Serpina3c
(serine protease inhibitor A3C)
0.8
0.4
0
0.8
0.4
0
Serpina3f
(serine protease inhibitor A3F)
0.6
0.4
0.2
0
0.8
0.4
0
Serpina3g
(serine protease inhibitor A3G)
0.6
0.3
0
0.8
0.4
0
Serpina3k
(serine protease inhibitor A3K)
0.02
0.01
0
0.6
0.3
0
Serpina3m
(serine protease inhibitor A3M)
0.6
0.3
0
0.8
0.4
0
Serpina3n
(serine protease inhibitor A3N)
0.8
0.4
0
0.4
0.2
0
Serpina6
(corticosteroid-binding globulin)
0.02
0.01
0
0.2
0.1
0

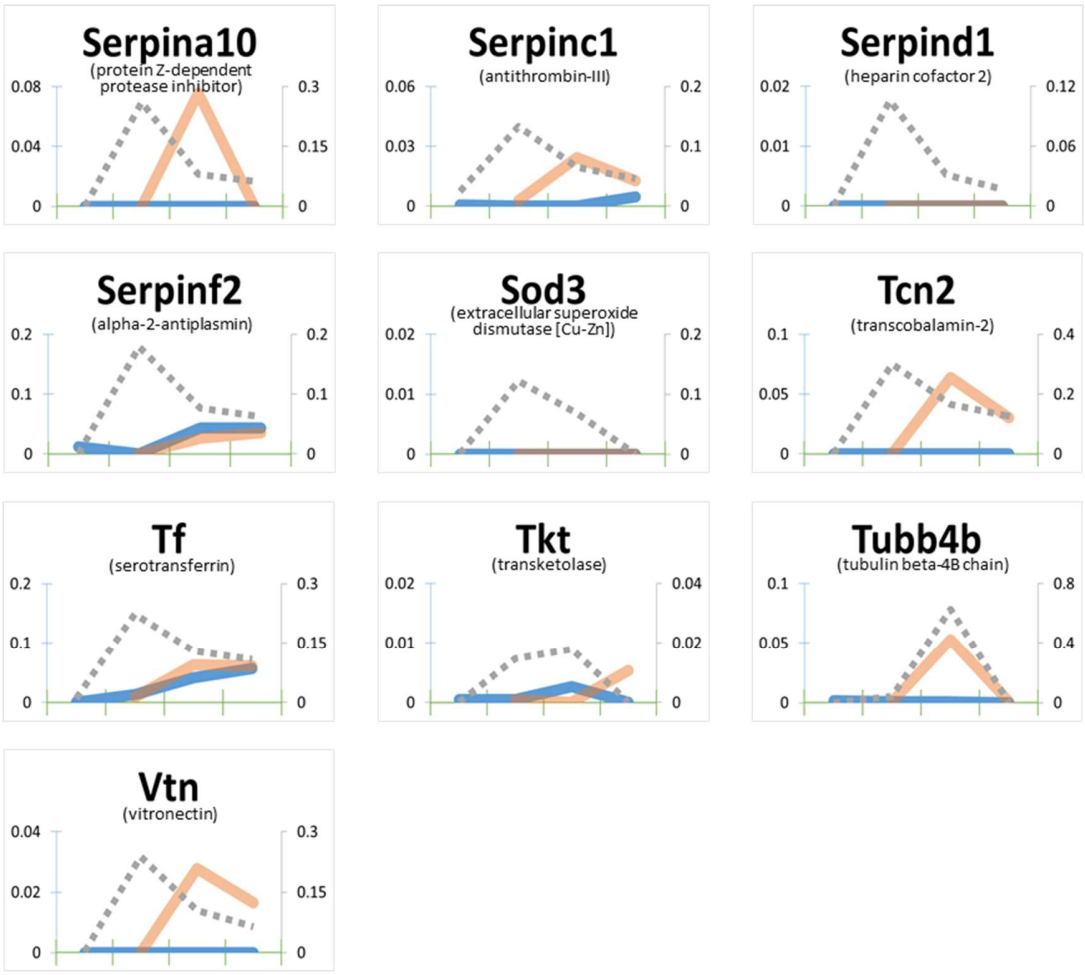


Fig. S3. Aqueous $^{15}N/^{14}N$ ratios of 136 serum proteins. As in Figure 4A, the x-axis shows time points of 0, 15min, 2hr and 5hr; the y-axis shows $^{15}N/^{14}N$ ratios (left scale for aqueous; right scale for serum). Gray dotted line: ratio in serum; blue line: ratio in normal eye; orange line: ratio in injured eye.

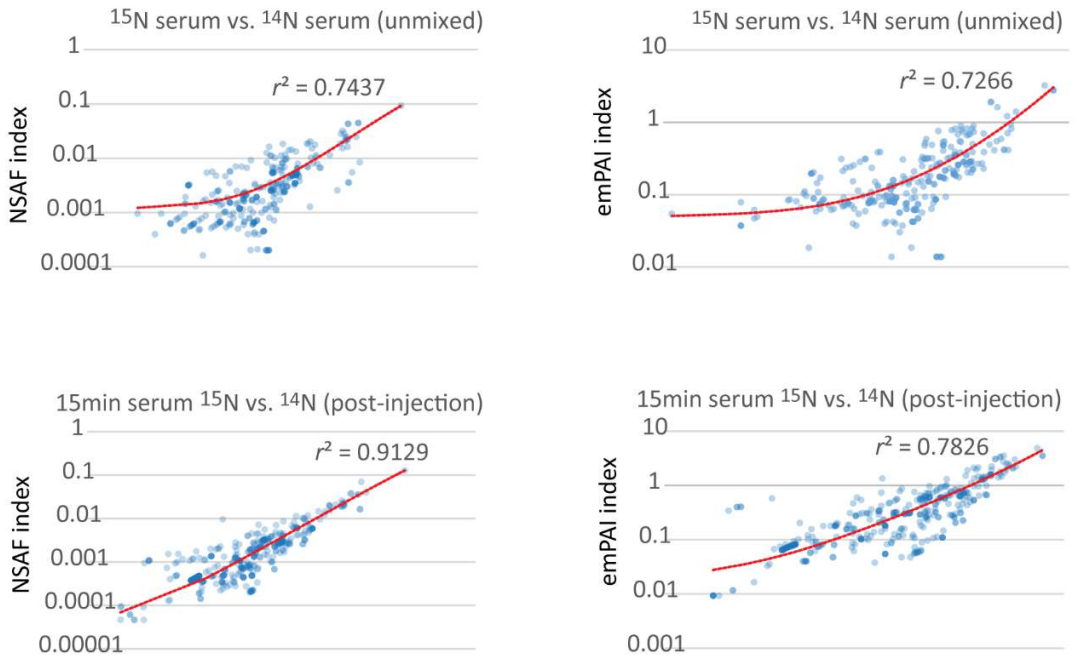


Fig. S4. Comparison of MS parameters for protein quantification. The x-axis direction is for $^{15}N/^{14}N$ ratios calculated based on under the curve area from reconstructed chromatogram. The y-axes values are either NSAF or emPAI as indicated. The r^2 values represent correlation coefficient between x- vs. y- axis.

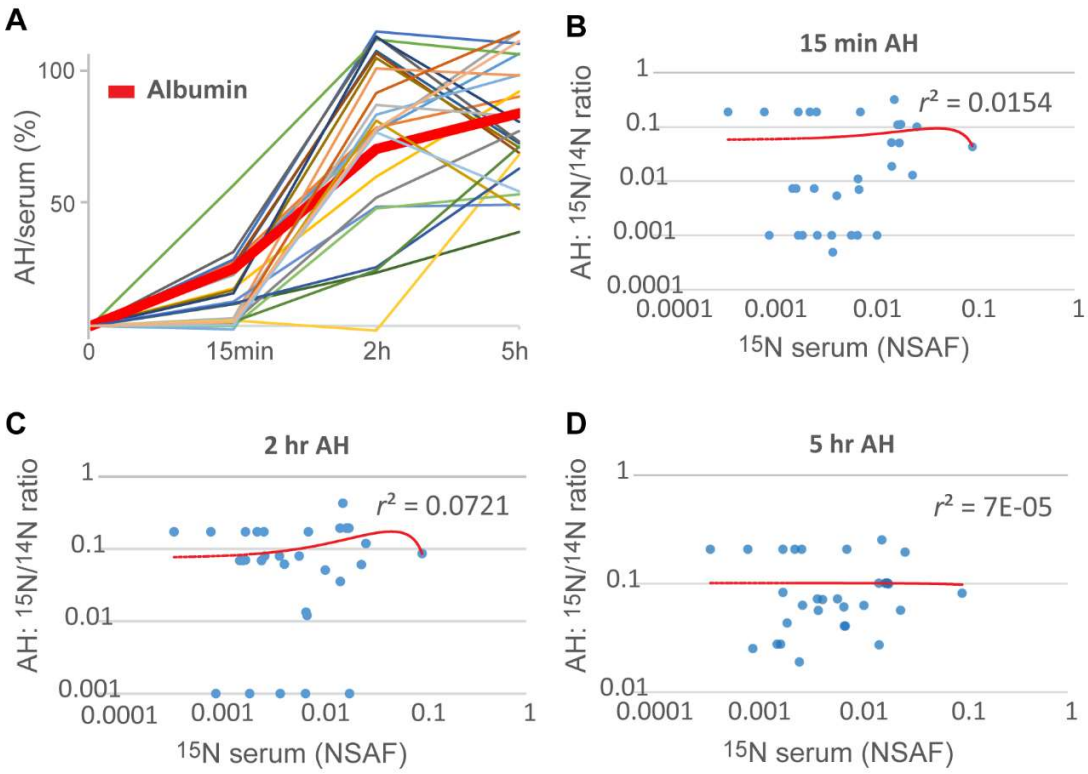


Fig. S5. Time-dependent accumulation of ^{15}N-labeled proteins in aqueous. A. Using ^{15}N/^{14}N ratios in serum as the benchmark (arbitrarily adjusted to as 100% on y-axis) for each time point, the aqueous ^{15}N/^{14}N ratios relative to the corresponding serum benchmarks were calculated. As a representative example, Albumin (the highlighted red line) in the aqueous reached 70-80% of its serum levels 1 hour after injection of ^{15}N-labeled serum. B-D. No overall correlation between serum abundance (x-axis: quantified using label-free NSAF indices of ^{15}N-labeled proteins in serum.) and the aqueous ^{15}N/^{14}N ratio (y-axis)–correlation coefficient r^2 >0.95 is considered as positive correlation.

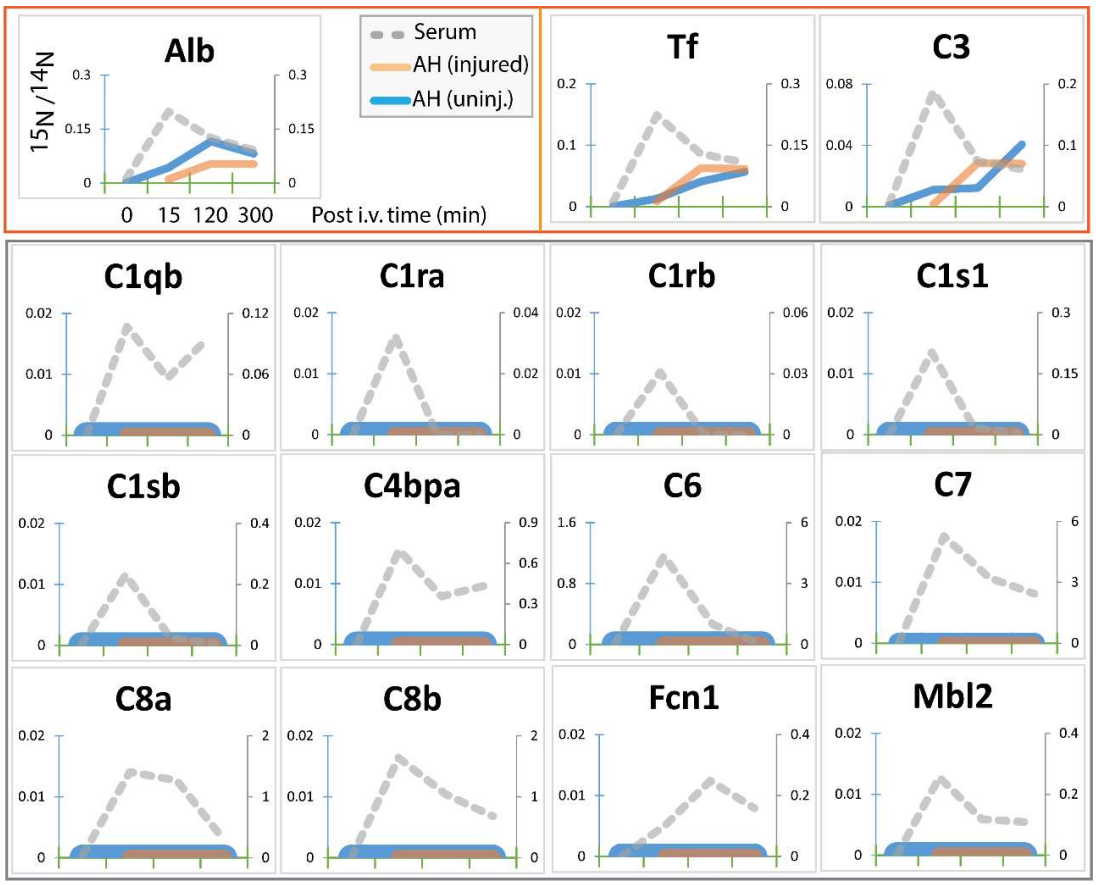


Fig. S6. Serum and aqueous $^{15}N/^{14}N$ ratios of complement proteins. This figure is related to Figure 4A showing a separate set of proteins, mostly complement proteins that did not enter normal or injured eyes. From the top: The orange color boxes contain proteins that entered aqueous of both injured and uninjured eyes (legends shown with the example of Albumin, Transferrin/Tf and C3; left scale for aqueous $^{15}N/^{14}N$ ratios and the right scale for serum. The black color box contains proteins that did not cross the barrier regardless of injury status, despite that the labeled fractions (measured as $^{15}N/^{14}N$) could be detected in serum.

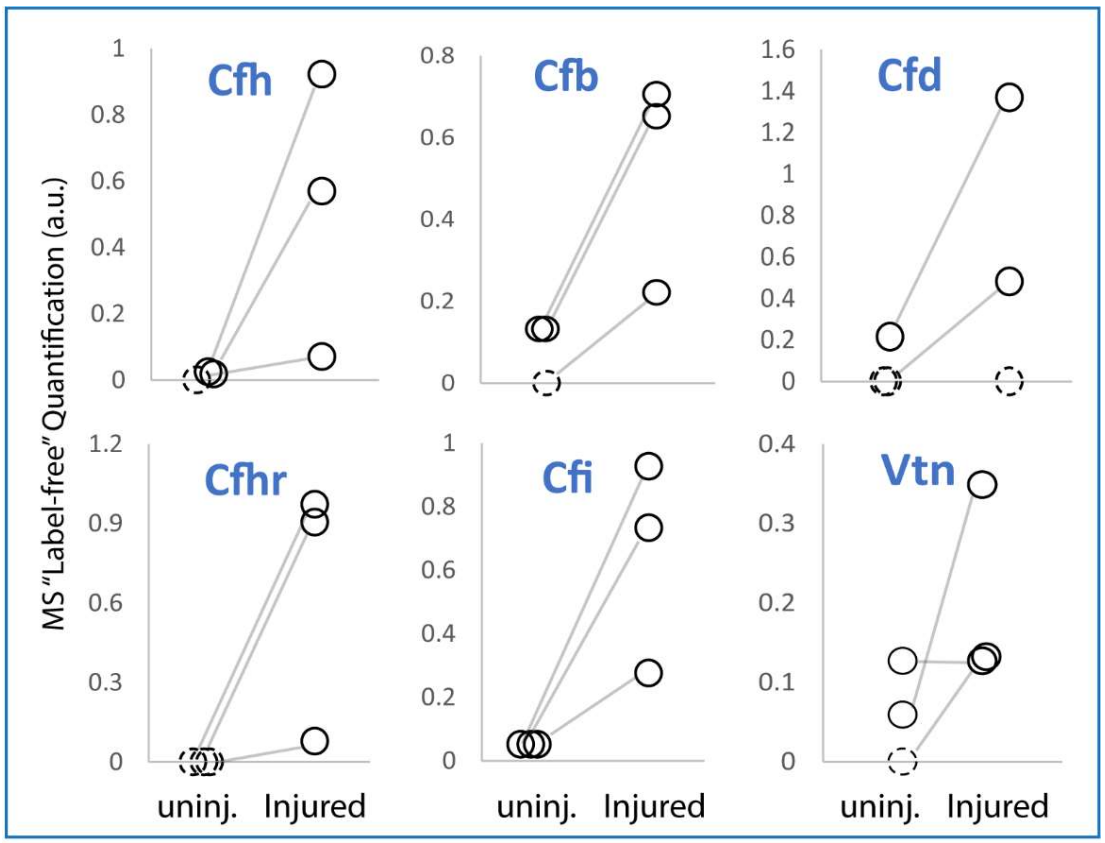


Fig. S7. Steady state levels of CRegs between uninjured and injured eyes as measured by label-free MS quantification (y-axis. Solid circle: data obtained; broken circle: protein was not detected and was arbitrarily assigned a zero value)**.**

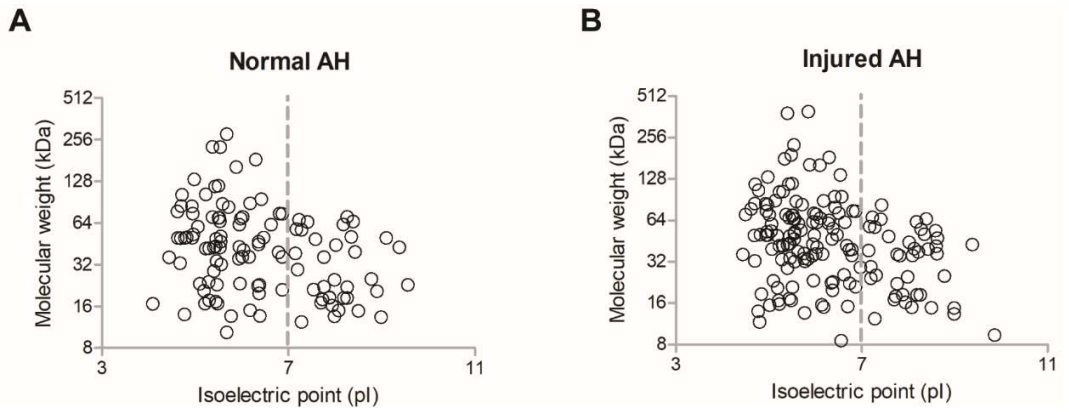


Fig. S8. Molecular weight and isoelectric point distribution of BAB permeable proteins in uninjured (A) and injured (B) eyes.

Table S1. The most abundant aqueous proteins measured by MS. Quantification of individual proteins was based on label-free NSAF indices.

Abundant AH Proteins	Function
Albumin	Carrier protein
Transferrin	Carrier protein
Aphrodisin	Aphrodisiac pheromone
Odorant-binding protein IB	Response to stimulus
Alpha-2-HS-glycoprotein	Involve in differentiation
Cystatin-C	Proteinases inhibitor
Hemoglobin subunit alpha	Oxygen transport
Vitamine D-binding protein	Carrier protein
Prostaglandin-H2 D-isomerase	Prostaglandin D2 synthase
Apolipoprotein	Transporter
Crystallin	Lens and cornea protein
Glutathione S-transferase	Oxidation/reduction
Retinoschisin	Maintain retina structure
Clusterin	Extracellular chaperon
Serine protease inhibitor A3K	Proteinases inhibitor

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