Identifying Quantitative Protein Changes in Iris Biopsies of Glaucoma Patients Using Label Free Proteomics

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ABSTRACT

Purpose: Label free quantification (LFQ) of iris biopsies from patients with primary angle closure glaucoma (PACG) compared to healthy eyes.

Methods: Quadrupole-Orbitrap mass spectrometry quantitative proteomics.

Results: LFQ identified difference in protein concentrations between PACG and control iris samples, providing new insights into pathogenesis of glaucoma.

INTRODUCTION

Glaucoma is a group of eye diseases that research has shown damages the optic nerve (the bundle of nerve fibers that carries information from the eye to the brain) and can lead to vision loss and blindness. The disease first causes a loss of peripheral vision and over time can damage central vision. If undetected and untreated, irreversible damage to the optic nerve occurs. Early treatment of symptoms can slow the progression of the disease.

Figure 1

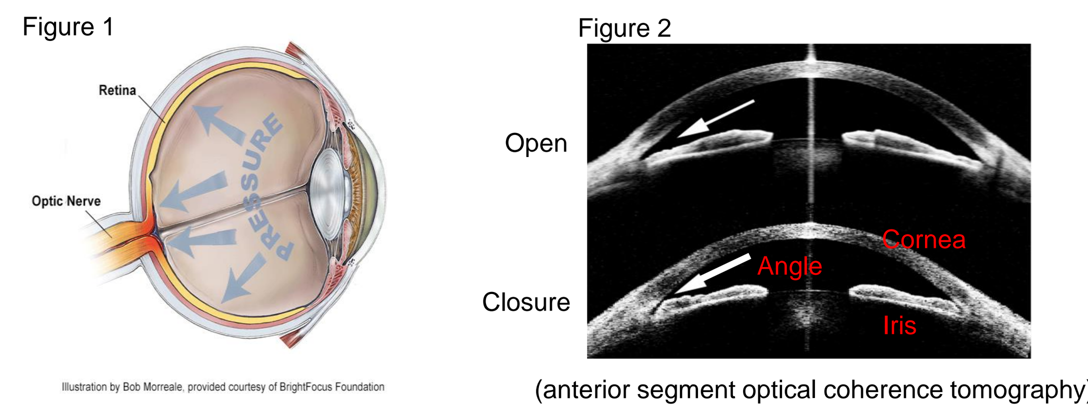


Figure 2

Open

Closure

Cornea

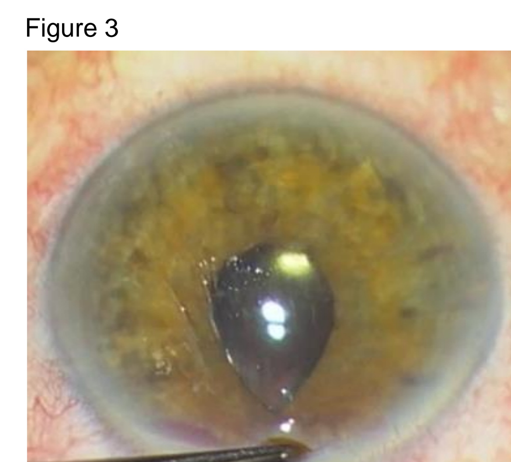
Angle

Iris

(anterior segment optical coherence tomography)

Primary open angle glaucoma (POAG) is the major form of glaucoma. A smaller proportion of patients have primary angle closure glaucoma (PACG), a more visually devastating form of glaucoma. Angle closure eyes are generally smaller in all dimensions. The drainage of aqueous humor from the eye can blocked in passage through the trabecular meshwork (Figure 2, arrow), causing an increase in intraocular pressure (IOP), as shown in Figure 1. There are a large number of medications available to patients to lower eye pressure and alleviate symptoms of glaucoma. In addition, an immediate lowering of the pressure can be obtained with surgical iridectomy (Figure 3). In this procedure, a small section of the iris is removed to allow passage of aqueous humor through the iris; the discarded tissue provides the samples in this work.

Figure 3



Label free quantification provides a way to compare peptide expression levels across a large number of sample conditions. Since the experimental comparisons do not have to be set up prior to data collection, more or unexpected comparisons can be made. Hybrid Orbitrap™ technologies provide a high enough (generally less than 3 ppm) mass accuracy to allow label free quantification using full MS extracted ion chromatogram peak areas without a large degree of interferences.

Processing of the high resolution label free data was done using the Minora algorithm. The algorithm finds all features (a distinct molecule at a particular charge state) and maps them across their elution profile against their PSM (peptide spectral match). Features don't have to match a PSM for quantification; those features were not analyzed in this study. The resulting peak areas were consolidated across the individual samples and statistics can be calculated to determine what changes are significant.

MATERIALS AND METHODS

Sample Preparation

One hundred and three iris samples were obtained from surgical iridectomy of patients with primary angle closure glaucoma and primary open angle glaucoma. Iridectomy samples from normal eye bank eyes were used as controls. The PACG and POAG patient samples were pooled into nine and eight iris samples respectively. The tissue was suspended in lysis buffer, homogenized using a motorized handheld Eppendorf mortar/pestle until tissue was no longer clumping, sonicated, reduced and alkylated, and digested overnight with trypsin/LysC. Protein concentrations were normalized using a Thermo Scientific™ Pierce™ Micro BCA™ kit.

LCMS

Samples were separated on a Thermo Scientific™ EASY-nLC™ 1200 nano-flow UPLC system using a four hour gradient of 0.1% formic acid to 80% acetonitrile/0.1% formic acid on a 25 cm, 2 um particle Thermo Scientific™ EASY-Spray™ C_{18} column with an Thermo Scientific™ Acclaim™ PepMap™ 100 75 µm x 2 cm trap. The column temperature was thirty-five Celsius at a flow rate of 350 nL/min. Each pooled sample was injected twice. Detection of the full ms data on the Thermo Scientific™ Q Exactive™ Plus Hybrid Orbitrap™ mass spectrometer scanning from 400-2000 m/z was acquired at a resolution of 70,000 (m/z 200) with an AGC target of 1e6. The top eight ions passing Peptide Match 'preferred' were selected for fragmentation at a NCE of 28 with an isolation width of 1.3. The target for fragmentation was 5e5 with a Max IT of 55 msec. Dynamic Exclusion was enabled with a time of 120 seconds.

Data Analysis

The raw data files were analyzed using the Minora algorithm in Thermo Scientific™ Proteome Discoverer™ 2.2 Software for relative quantification and protein annotation. Spectra were searched using the SEQUEST™ search engine against the UniProt human database using a precursor tolerance of +/- 10 ppm and fragment ions were searched in a 0.02 Dalton window. Carbamidomethylation of cysteine was a static modification and methiones were searched as a variable modification with oxidation. Proteins were filtered using stringent criteria (two or more peptides with a 1% False Discovery Rate form Percolator) with maximum parsimony. Up regulation and down regulation were filtered for a two fold difference in protein expression with a $P < 0.05$.

RESULTS

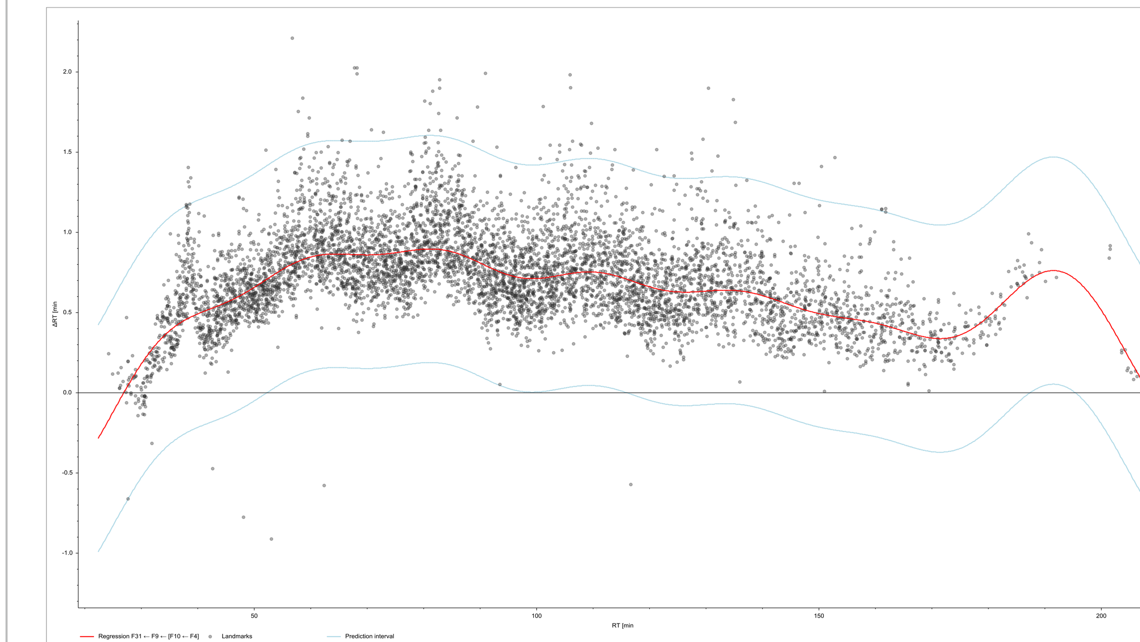
Search Results

599,491 Unique Isotopic Clusters Found ('Averagine' Matched)
4,056,043 MSMS Spectra Acquired in 37 Injections
812,285 PSMs (Peptide Spectral Match, MSMS Match to a Sequence) Matched
40,373 Unique Sequences (Not Including Modifications of Previous Matches)
4,986 Protein Groups (Includes 'One-hit Wonders', "Leads", Would Require Further Validation)
3,855 Proteins Identified with Stringent Criteria

Retention Time Alignment

The Minora Feature Detector node of Proteome Discoverer detects LC/MS peaks in the raw data files and then maps them to identified PSMs. It calculates the theoretical isotope pattern of a PSM, finds the LC/MS peaks mapping to the isotope pattern, and creates a feature from the fitting peaks. It uses the file with the most features as its reference file. It takes each file and matches features from the reference file in descending order of intensity, recording their retention times and retention time differences which are plotted. It plots the data and fits a regression curve into the retention time and differences data points. The node uses this curve to correct the retention times of the file relative to the chosen reference file. An example of the worst error in the chromatographic alignment is shown in Figure 4.

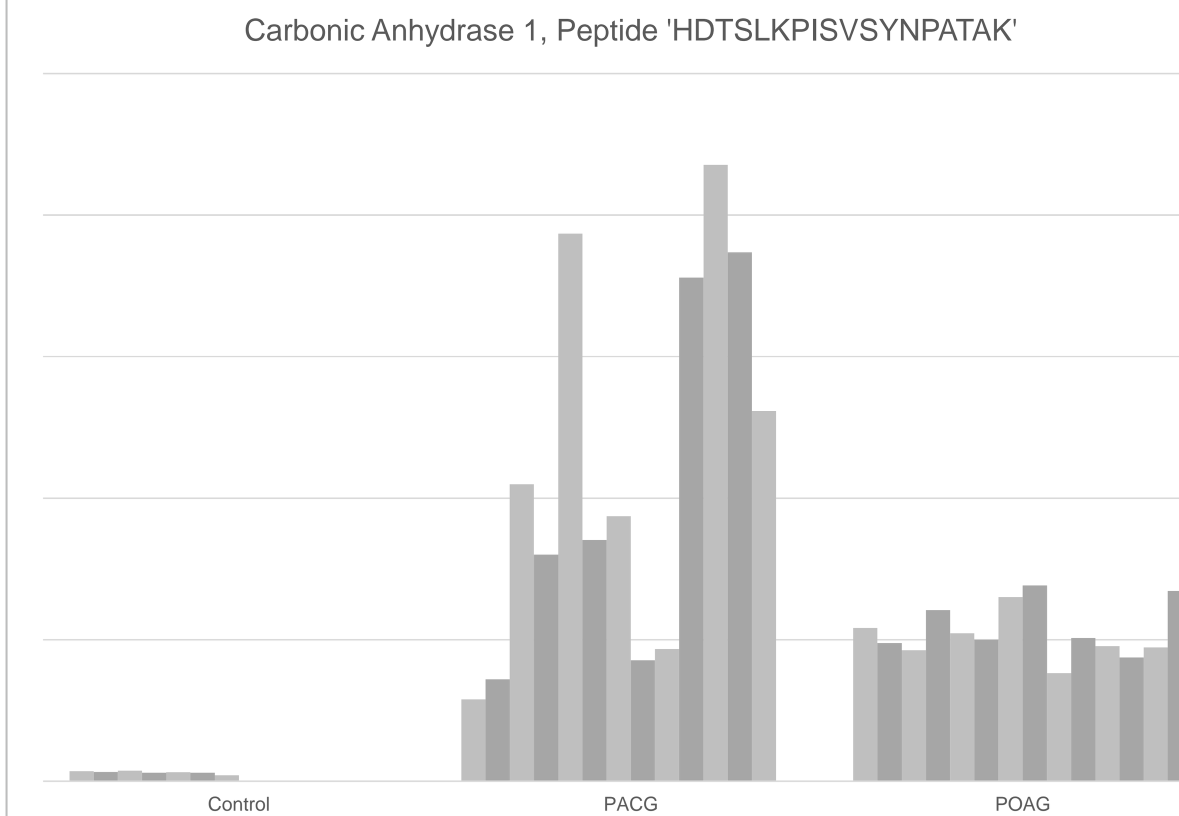
Figure 4



Peak Area Integration and Comparison for a Single Feature

The Minora algorithm then finds the integrated peak area of each isotopic cluster that matches its theoretical 'Averagine' fit. The peak area is calculated across all time aligned files and displayed for statistical analysis. An example from a peptide of carbonic anhydrase 1 is shown in Figure 5. Each bar represents a pool of samples.

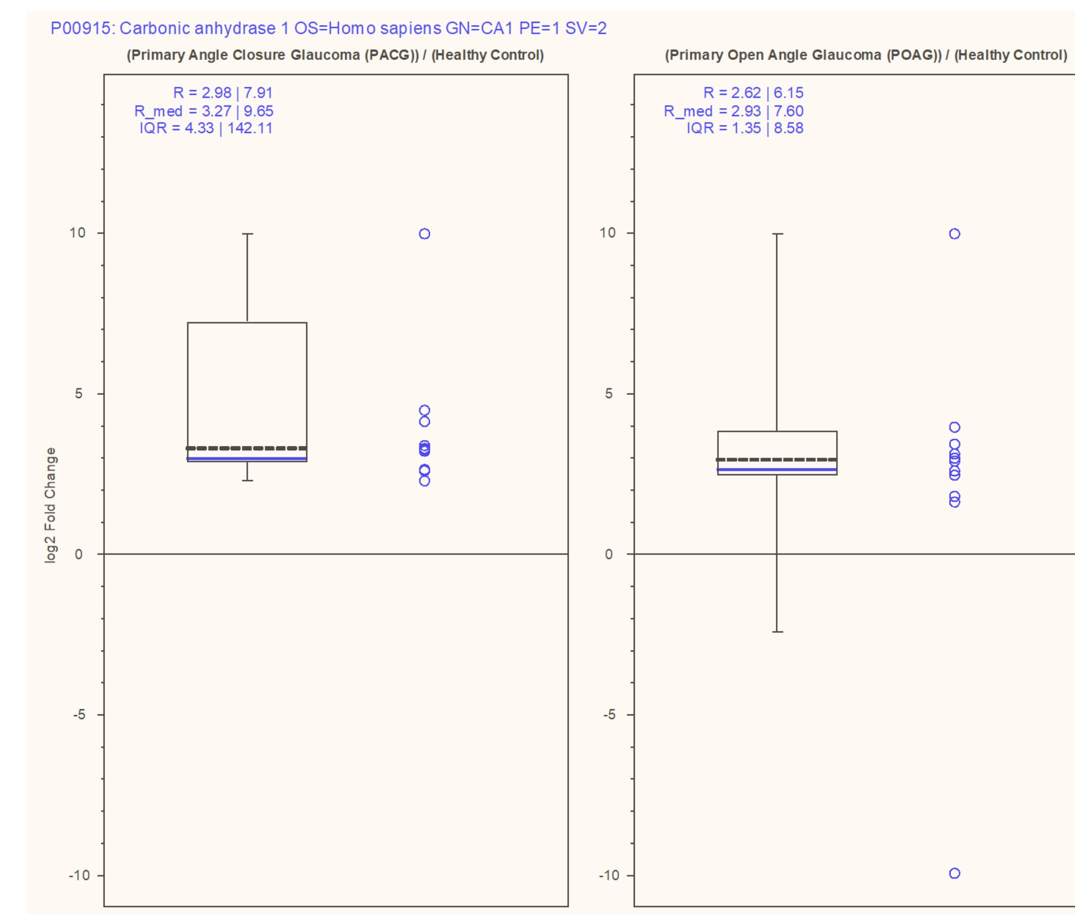
Figure 5



Mapping of Single Peptides to Proteins by Parsimony

Proteome Discoverer sorts peptide spectral matches (PSMs) into the smallest set of proteins possible using rules of strict parsimony. It then creates whisker plots showing the variation in the individual quantifications. An example for the carbonic anhydrase 1 is shown in Figure 6.

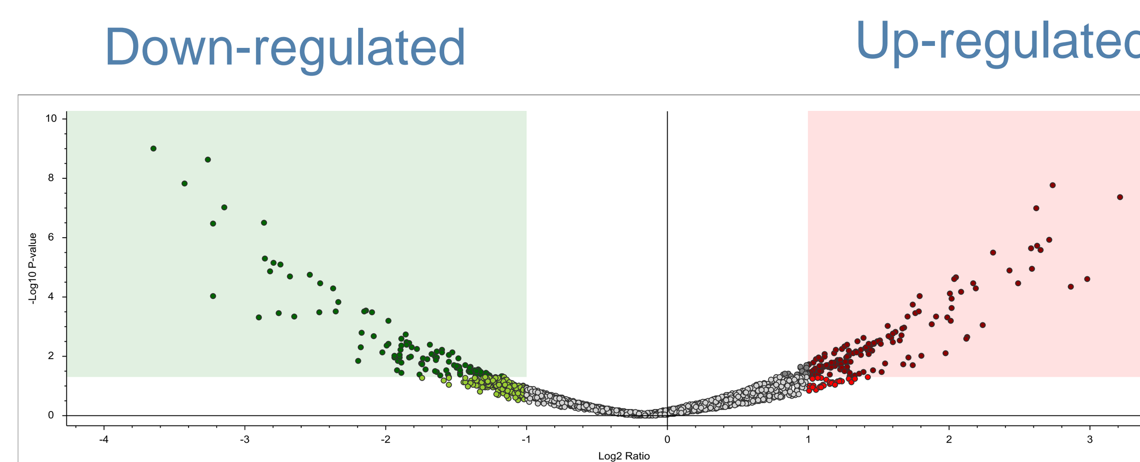
Figure 6



Statistical Analysis of Results

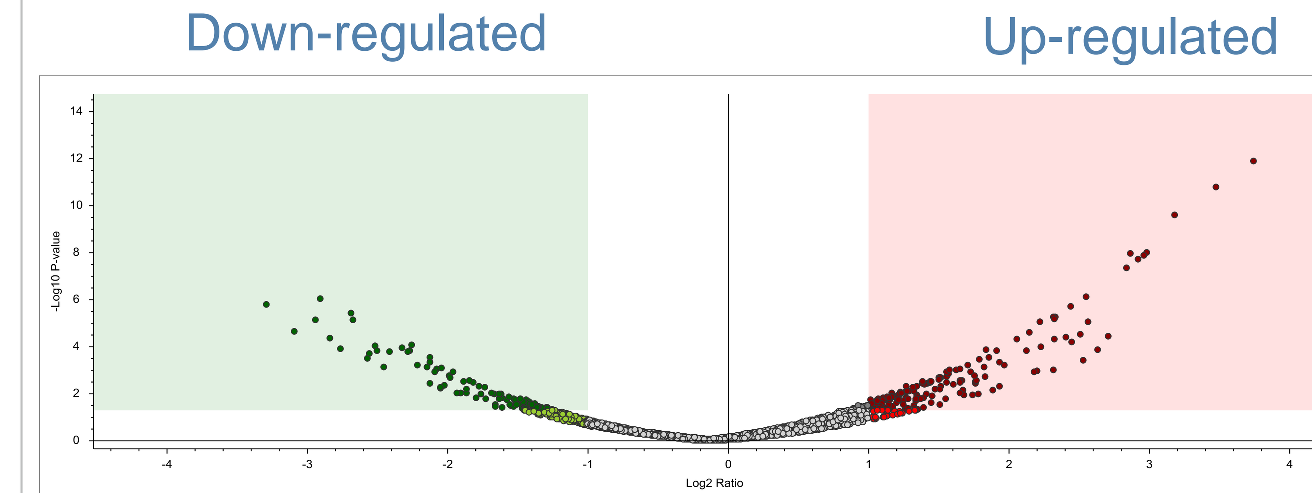
Proteome Discoverer performs a statistical analysis of all proteins across the groups. A P value is calculated as a measure of significance (All proteins were filtered at $P < 0.05$) and highlighted in green or pink. POAG showed up regulation of one-hundred three proteins and down regulation of seventy-four proteins in iris compared with controls. PACG showed up regulation of ninety-six proteins and down-regulation of fifty-nine proteins compared to controls. The top up-regulated protein, carbonic anhydrase, was of particular importance in substantiating the study, since it is a major target for glaucoma treatment with topical carbonic anhydrase inhibitors such as dorzolamide and brinzolamide. There were at least eleven proteins showing significant up-regulation in the PACG irides compared to controls that are linked to extracellular matrix, such as FGB, SCUBE1, and LAMA2. Some of the significant proteins with altered levels have been identified as possible biomarkers using genome-wide association studies (GWAS), thus providing some corroboration between GWAS and proteomics. The proteins involved in extracellular matrix may explain the clinical differences in how PACG irides respond differently to healthy eyes during dilation and have increased susceptibility to angle closure glaucoma attacks.

Figure 7



Primary open angle glaucoma vs Healthy

Figure 8



Primary angle closure glaucoma vs healthy

CONCLUSIONS

- LFQ found over one hundred significant protein expression changes in both PACG and POAG patient samples.
- The largest change, carbonic anhydrase 1 is a known target for topical carbonic anhydrase inhibitors, substantiating the study.
- At least eleven proteins showing significant up-regulation in PACG are linked to extracellular matrix, which may explain the clinical differences with healthy eyes in how they respond to dilation and angle closure attacks.

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