

**Proteomic Analysis of Aqueous Humor from Primary Open Angle Glaucoma Patients on  
Drug Treatment Revealed Altered Complement Activation Cascade**

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## **Abstract**

Primary open angle glaucoma (POAG) is a complex disease and a leading cause of irreversible blindness, and its underlying pathophysiology remains poorly understood. Proteomic characterization of the protein composition of aqueous humor (AH) may identify prognostic candidate proteins involved in pathogenesis and progression of the disease. To delineate the possible mechanisms that lead to POAG, this study adopted state-of-art mass spectrometric technique and analyzed AH of POAG and their respective controls. In total, more than 1000 proteins were identified with false discovery rate of less than 1%. Numerous proteins of complement cascade, immunoglobulin, neuronal and amyloidogenic proteins, which were part of processes like acute-phase and inflammatory response, humoral immune and acute inflammatory response, regulation of complement activation and protein processing were identified. Proteins of complement system underwent significant changes, which correlate to pathogenic events characterizing POAG, including altered complement cascade, astrocyte activation, neural degeneration, and apoptosis. Further, protein modification such as deamidation of complement subcomponent was noted, particularly in POAG. Proteomic analysis of AH allows a better understanding of the mechanism involved in the pathogenesis of POAG.

**Keywords:** Aqueous humor, Glaucoma, POAG, Complement, Proteomics

## Introduction

Glaucoma is a complex neurodegenerative ocular disease with multiple genetic and non-genetic risk factors. It is characterized by chronic neurodegeneration of the optic nerve, death of retinal ganglion cells (RGCs) and their axons. In 2013, peoples with glaucoma (aged 40 to 80 years) worldwide were estimated to be 64.3 million, which may further increase to 76.0 million in 2020 and 111.8 million people by 2040.<sup>1</sup> More than 3% of the global population over 40 years of age has glaucoma. It will cost significant personal healthcare costs and a societal economic burden.<sup>2</sup> Of different types, open-angle glaucoma remains the most common form, accounting for at least two-thirds of all glaucoma cases. Elevated intraocular pressure (IOP) and IOP-generated vascular stress are considered as a hallmark of glaucoma,<sup>3</sup> but how IOP leads to RGC death and optic nerves damage is still unclear. Therapeutics have been developed to reduce IOP, but bringing IOP to normal levels could not always stop progressive loss of RGCs, optic nerve fiber damage and visual impairment. Moreover, glaucomatous changes in the optic nerve in the absence of high IOP, called normal tension glaucoma (NTG) occur. This indicates an IOP-independent mechanism of damage.<sup>4</sup> Therefore, in addition to IOP, there may exist another pathogenic process of primary open angle glaucoma (POAG).

The accumulated literature recapitulate major glaucoma risk factors such as aging,<sup>5</sup> genetic predispositions,<sup>6</sup> epigenetic risk factors,<sup>7</sup> compartmentalized subcellular processes and secondary neurodegenerative events due to oxidative stress,<sup>8</sup> glial activation/dysfunction,<sup>9</sup> and glia-mediated inflammation.<sup>10</sup> Furthermore, mitochondrial dysfunction,<sup>11</sup> oxidative stress,<sup>12</sup> inflammation,<sup>13</sup> involvement of the immune system<sup>8, 14</sup> have been linked to glaucoma indicating a major shift in research focus to find out the molecular mechanisms underlying disease progression and pathology. Recent mouse models of glaucoma suggest the innate immune and complement system<sup>15</sup> may be

vital in the development and progression of the disease. In short, the precise etiology of POAG remains unclear and the factors that initiate the degeneration of the optic nerve, RGCs death, and their axons remain elusive. It is essential to identify the factors that initiate and contribute to the pathogenesis and progression of this disease.

Aqueous humor (AH) is a clear, gelatinous fluid secreted by the non-pigmented ciliary body into posterior chamber and it flows between the lens and iris into the anterior chamber and then exits via the trabecular meshwork (TM). It remains in close proximity to the avascular structures of the eye and help in supplying nutrients and disposal of metabolic wastes. In addition, AH dynamics maintain the IOP of the eye which is required for retaining its optical and refractive properties.<sup>16</sup> It is important to note that the blood-aqueous barrier strictly controls the composition of AH by restricting large molecules. Due to small sampling volume and minimal protein concentration of 120-500 ng/ $\mu$ l,<sup>17</sup> defining AH proteome; and identifying and quantifying AH proteins is challenging. However, proteomics technology is highly sensitive and provides a high-throughput analysis of protein level in biological samples.

Proteomics offers the opportunity to discover novel disease-specific proteins, and hence, this technique has been used to study neurodegenerative diseases,<sup>18-20</sup> as well as eye diseases like acute corneal rejection,<sup>21</sup> myopia,<sup>22</sup> cataracts,<sup>17</sup> POAG<sup>23-25</sup> and many more. Altered protein level within AH may have a significant association with abnormal cellular function, biological process, and cell-matrix communication. Being in a close proximity to glaucoma disease sites, defining comprehensive AH proteome and profiling quantitative proteins abundances will provide an in-depth understanding of POAG pathogenesis. Therefore, the main objective of this study was to adopt the discovery-based proteomic approach and define the quantitative expression of human AH samples of POAG (N= 5) and control (N=5) by using a state-of-the-art high-performance

liquid chromatography coupled tandem mass spectrometry (LC-MS/MS). Based on the quantitative expression profile of AH and their relative regulation in the glaucomatous eye, affected cellular pathways and their correlation with disease pathogenesis have been established and discussed.

## **Materials and Methods**

### **Study participants and collection of aqueous humor**

POAG patients were recruited by the glaucoma department of TTSH between January 2012 and January 2013. Ten subjects including five POAG and five age- and gender-matched cataract patients as a control were recruited for this study. All subjects underwent a thorough ophthalmic evaluation including glaucoma-related measurements such as IOP, best corrected visual acuity, gonioscopic examination (to confirm open iridocorneal angles), fundus photography of the optic nerves, preoperative cup-disc ratio and Humphrey visual field analysis. Patient's clinical characteristics are tabulated in Table S1. Controls subjects did not have any clinical features of POAG, as determined by a glaucoma specialist using standard diagnostic criteria, such as normal optic nerve and visual field. Exclusion criteria were patients with secondary angle-closure glaucoma (e.g. uveitis, trauma, or lens subluxation), pre-existing ocular disease (e.g. retinal vein occlusion, retinal artery occlusion, diabetic retinopathy or age-related macular degeneration) and a history of previous ocular surgery.

This study was in agreement with the Declaration of Helsinki and approved by the Institutional Review Board at the Tan Tock Seng Hospital (TTSH). Informed consent was obtained from each patient. In this case-control prospective study, AH samples were obtained from POAG patients (cases) prior to undergoing combined cataract and trabeculectomy surgery or

trabeculectomy alone or cataract surgery alone and patients with senile cataracts (controls) undergoing cataract surgery. A mean volume of 100-150  $\mu$ l of AH from each eye was collected from anterior chamber limbal paracentesis by inserting a 30-gauge needle attached to 1 ml syringe just before surgery. All samples were stored at -80°C until processing.

### **LC-MS/MS analysis and Discovery proteomic data processing**

After protein estimation, 20  $\mu$ g of protein samples were separated by SDS PAGE at 100V and bands were visualized using coomassie brilliant blue. The gel was further processed for in-gel protein digestion after reduction with dithiothreitol (10 mM) and alkylation with iodoacetamide (55 mM) as described previously.<sup>26</sup> The gel pieces were subjected to sequencing grade modified trypsin (Promega, Madison, WI) digestion at 37 °C overnight. The peptides were extracted using 50% acetonitrile/ 5% acetic acid, vacuum centrifuged to dryness. LC-MS/MS analysis of digested peptides was performed using QExactive mass spectrometers (Thermo Fisher, MA) coupled with a Dionex UltiMate 3000 UHPLC system as described previously<sup>18</sup>. Briefly, peptides were analyzed on a Dionex EASY-spray column (PepMap® C18, 3 $\mu$ m, 100 ° A) using an EASY nanospray at an electrospray potential of 1.7 kV. The MS was recorded at 350-1600 m/z at resolution 70,000 m/z. Maximum accumulation time was 100 ms and dynamic exclusion was 30 s. MS/MS spectra resolution was 35,000 at m/z 200; ACG was 1E6 for the full MS and 2E5 for the MS2 scan. For HCD, 10 most intense ions above 1000 count threshold were chosen and 120 ms was set for maximum ion accumulation. The collision energy was 28 and an isolation width of 2 Da for the MS2 scan. Single charged and unassigned ions were excluded from MS/MS.

ProteomeDiscoverer™ (PD, Thermo Scientific, San Jose, USA) ver 1.4 software was used to analyze MS data acquired by QExactive. The MS/MS spectra were deisotoped and deconvoluted using the MS2 spectrum processor. Protein identification was performed by comparing MS/MS

spectra against Uniprot Human database (Released on 7/25/2016, 70,849 sequences, 23,964,784 residues) using Sequest HT and Mascot. ProteomeDiscoverer's workflow included an automatic target-decoy search tactic along with the Percolator to score peptide spectral matches to estimate the false discovery rate (FDR). The Percolator parameters are set to maximum delta Cn = 0.05; false discovery rate (FDR) of <1%, validation based on q-value<sup>27</sup>. Maximum two missed cleavage for trypsin was used. The peptide precursor mass tolerances of 10 ppm and 0.02 Da mass tolerance was used during data search. The fixed modification was carbamidomethylation (+57.021 Da) of cysteine residues, while deamidation (+0.984 Da) of asparagine and glutamine residues and oxidation (+15.995 Da) of methionine residues were variable. The area under the curve (AUC) for each precursor ion peak was calculated using algorithm embedded in PD 1.4 by setting mass precision of 2 ppm. The resultant search output was exported to an Excel file by applying filters like peptide confidence-high, peptide per protein –rank 1 peptide in top score protein etc. for further analysis. The protein list is provided in table S2 while shortlisted protein list for further analysis list is provided in S3. Further raw data was searched using mascot search engine and data is shown in table S4 and S5.

### **Multiple Reaction Monitoring Mass Spectrometry (MRM-MS) analysis and data processing**

For MRM analysis, peptides (two or three) and transitions for selected proteins were extracted from the MS/MS data derived from QExactive. Initially, LC-MRM-MS analysis of the selected proteins was performed in triplicate using pooled POAG and control samples. Then, individual POAG and their respective control LC-MRM-MS analysis was also performed in triplicates on a TSQ Vantage triple quadrupole mass spectrometer coupled to an EASY-nLC™ 1000 nanoflow UHPLC system (Thermo Scientific Inc., Bremen, Germany). The details of peptide sequences, Q1,

Q3 m/z and charge, ion, collision energy, retention time etc. are tabulated in Table S6. Peptides were injected onto an Acclaim® PepMap100 trap column (75µm x 2cm; nanoViper C18, 3µm, 100Å) and resolved on an Acclaim® PepMap RSLC C18 column (75µm x 15cm; nanoViper C18, 2µm, 100Å) (Thermo Scientific, USA), at a flow rate of 300nL/min. A 60 min gradient was established using mobile phase A (0.1% FA in HPLC water) and mobile phase B (0.1% FA in acetonitrile). The TSQ Vantage was set to perform data acquisition in positive ion mode. An electrospray potential of 1.1kV, the capillary temperature of 250° C and collision gas pressure of argon for Q2 was set at 1.2mTorr. The selectivity for both Q1 and Q3 were set to 0.8 Da.

The raw data files generated by TSQ Vantage were processed using Skyline that provides support for selecting peptides and optimization of transitions, and downstream data analysis.<sup>28</sup> Peptides selected for protein quantitation were evaluated based on the intensity, background interferences, and reproducibility through visualization of the extracted ion chromatogram across individuals and technical replicates that were processed automatically in Skyline software. Based on the pooled samples, the retention time for each peptide was established, which was further used to set an isolation window of 8 min for individual POAG and controls sample analysis. The ion chromatograms for each targeted peptides were examined closely with reference to the MS/MS spectral library generated from QExactive raw data to ensure accurate integration of the peaks. The peptide areas were computed by integrating the peak area of all respective transitions that were included for each of the peptides for the respective proteins. The relative abundance of each complement and house-keeping protein was determined by considering mean peptide areas of replicates. To compare quantitative protein abundance between control and glaucoma, the mean peak area (obtained by summation of peptide peak areas) of each complement protein was

normalized against the mean peak area of two house-keeping proteins (serotransferrin and serum albumin) separately.

## **Results and Discussion**

### **Proteomic profiles of AH proteome in Glaucoma**

Identifying proteins in AH remains challenging due to minimal protein concentration. This study adopted the high-throughput proteomic approach and identified 865 protein with false discovery rate of less than 1% (Table S3). Adopting a combination of LC-MS/MS and antibody-based protein arrays, Chowdhury *et al.*,<sup>23</sup> identified 676 proteins in human AH. Using a combination of in-gel digestion, in-solution digestion followed by basic pH-RPLC coupled with mass spectrometry, Murthy *et al.*<sup>29</sup> identified 763 proteins. Compared to published literature that identified 7-819 proteins in AH (reviewed in Table 1), our proteomic data provides improved coverage of AH proteome. The study design used AH of 5 cataract and 5 glaucoma patients, adopted label-free quantitative proteomics approach and further validated protein abundances using LC-MRM-MS/MS. The Volcano plot of the p-values and data reproducibility is depicted in Fig 1. The AH is in direct contact with the critical site of pathogenesis in glaucoma and it may provide vital information on the pathophysiology of this disease. However, a low sample number remains the limitation of this study. Hierarchical cluster analysis based on the protein quantities as estimated by AUC revealed different clusters of the proteins (Fig 2). Proteins clustered under cluster C2 (all glaucoma patients) and C3 (four glaucoma patients) were significantly low abundant in POAG patients when compared to controls. Proteins clustered under cluster C2 were complement component C6, C8, C3, C4-A, C4-B, complement C1q and complement factor H. To study relative abundances of identified proteins, fold change in the protein level was determined by dividing AUC of each protein identified in POAG by the AUC of the same protein in their

respective controls. Based on the frequency distribution (Fig S1), 2-fold change that corresponds to 1 in log<sub>2</sub> scale was set as an up-regulation and 0.5 fold change that corresponds to -1 in log<sub>2</sub> scale as down-regulation. Based on these criteria, 150 proteins were differentially regulated including 43±18 up-regulated and 105±45 as down-regulated.

### **Differentially Expressed proteins and functional annotation**

Differentially altered protein analysis indicated a significant impact on the positive regulation of cholesterol esterification as evidenced by the upregulation of apolipoprotein A-IV (Fig 3a). ApoE that mediates the binding, internalization and catabolism of lipoproteins was also significantly upregulated in glaucomatous AH. Recently, Mirzaei *et al.*,<sup>30</sup> found significant up-regulation of apolipoprotein including ApoE, Apo A-IV in glaucoma indicating induction of cholesterol metabolism and protein transport. Interestingly, *APOE* allele is involved in controlling Aβ proteolysis and its clearance and has been allied with glaucoma and Alzheimer's disease.<sup>31</sup> This study found relatively higher abundances of amyloid beta (A4)-like protein 2 in glaucoma. An accumulation of amyloid beta in the retina of glaucomatous and AD patients has been reviewed by Ghiso *et al.*<sup>32</sup> Proteases like cathepsin D which remains active in intracellular protein breakdown and plays a major role in amyloid precursor protein processing was upregulated in POAG (Fig 3a). The proteomic analysis AH of glaucoma patients revealed enrichment of critical mediators of oxygen homeostasis including hemoglobins like HBB, HBA2, HBD. The elevated level of catalase in glaucoma indicates higher oxidative stress in glaucoma patients.

To decode biochemical pathways significantly impacted by glaucoma, down-regulated proteins were analyzed using Gene Ontology (GO), which pointed out that these proteins are involved in the processes like regulation of complement activation; acute-phase and inflammatory response;

regulation of complement activation, humoral immune and acute inflammatory response; protein processing, neutrophil activation involved in immune response etc. (Fig 3b). The cellular localization of the down-regulated proteins revealed that these proteins are the components of membrane attack complex, high-density lipoprotein, platelet alpha granule, extracellular exosome and vesicles, and secretory granule etc. (Fig 3c). The complement cascade is a part of the innate immune system and is composed of over 30 proteins with diverse role including initiators, regulators, and inhibitors. In this study 25 complement proteins including C1q, C1s, C2, C3, C4A, C4B, C5, C6, C8, C9, c-type lectin, galectin, Mannan-binding lectin serine protease 2 (MASP2); complement factor C1 (CFI), H (CFH) and I (CFI) were identified and quantified. The integration of the LC-MS/MS identified proteins into complement activation pathway revealed that this study identified proteins of classical, lectin or alternative pathway (Figure 4a). C1s belongs to the first complement component (C1 complex), which initiates a cascade of host defense events during an immune response, was found to be significantly up-regulated in AH of POAG (Table S7). C1s have been implicated in retinal neurodegeneration in eye diseases.<sup>33</sup> The proteins like galectin-3-binding protein, isoform 4 of clusterin, C9 and CFI were also upregulated in glaucoma when compared with their respective controls.

Atomic-force microscopy examination of lenses with exfoliation syndrome revealed significant elevation of complement components C3a and soluble C5b-9, complement inhibitors clusterin and vitronectin.<sup>34</sup> Similarly, proteomic analysis of AH found elevated levels of complement including C1q, C1r, C2, C6 and C8.<sup>35</sup> On the contrary, the mice (DBA/2J mice) with mutation in complement component C1qa prevents RGC loss and were protective from glaucoma.<sup>36</sup> While determining the role of C1 in early synaptic loss and dendritic atrophy during glaucoma, Williams *et al.*,<sup>37</sup> inhibited C1q functions using either genetic ablation (DBA/2 J mutant for C1qa) or pharmacological

inhibition (C1 esterase inhibitor in the rat ocular hypertension model) and found that inhibition of classical pathway of the complement cascade prevents dendritic and synaptic degeneration in glaucoma. In short, knock-out of C1q or inhibition of C1q function prevents retinal ganglion cell degeneration. In this study, the proteomic analysis of AH of POAG found down-regulation of C1q, C3, C4A, C4B, C5, C8, and C6 (Fig 4b and 4c). In addition to label-free quantitation, LC-MRM-MS/MS also revealed down-regulation of C1r, C1q, C1s, C4A, C5 and SERPING1 protein in pooled POAG samples (Fig 5). However, an influence of drugs (timolol and xalatan) on the regulatory profile of proteins cannot be completely ruled out since glaucoma patients were receiving these drugs for treatment. It's important to note that the timolol and xalatan reduces IOP and lessens the risk of damage to the optic nerve and loss of vision in patients with glaucoma.<sup>38</sup>

<sup>39</sup> Thus, these drugs acts as a neuroprotective compound. Zymosan A is an activator of alternative pathway of complement while heat-aggregated IgG activate classical pathway<sup>40</sup>. While assessing *in vitro* effects of anti-glaucoma medications on the complement system, timolol was found to significantly inhibit complement activation in both zymosan A as well as IgG-induced complement activation.<sup>40</sup> Restated, timolol has the potential to inhibit activation of both pathways. The complement C4A was noted in lower concentrations in POAG and pseudoexfoliation glaucoma (PEXG) when compared with controls ( $p < 0.05$ ), where both POAG and PEXG patients were receiving drug treatments timolol, xalatan or combination of both.<sup>41</sup> While studying the effects of different drugs such as xalatan and timolol on tear proteomics of glaucoma patients by cytokine arrays, Lopilly Park et al<sup>42</sup> found significantly decreased levels of matrix metalloproteinases like TIMP-1 and TIMP-2. Li *et al*,<sup>43</sup> found significantly lower levels of C3 in plasma of PACG in the female, but not in male, suggesting disordered complement system in an older PACG woman. Similar to Li *et al*,<sup>43</sup> this study found a lower level of C3 in AH of POAG as the study constitutes

three female groups whose age were in the range of 79 to 89 years. Although limited data is available in the literature regarding the association of complement C3 levels in woman, support comes from the existence of ocular autoantibodies in the serum<sup>44, 45</sup> and retinas<sup>46</sup> of glaucoma patients. IgG can also activate the complement system,<sup>47</sup> the activation of ocular autoantibodies in glaucoma may contribute to the depleted levels of C3. Further, recent technical advances discovered pathways other than established pathways of complement activation. The serine protease thrombin can also cleave C3 and C5 in a dose- and time-dependent manners leading to the generation of biological active C3a/C5a.<sup>48</sup> The phagocytic cells are able to cleave C5 into biologically active C5a.<sup>48</sup>

The crystallins (CRY) are the predominant structural proteins in the lens and are mainly grouped into three families like alpha, beta and gamma. When proteomic data of glaucoma and their control was searched using PD and mascot search engine, it revealed identification of different members of crystallin family including CRYGB, CRYGD, CRYGC, CRYGS, CRYAB, CRYBB1, CRYBB3, CRYAA, CRYBA4, that were significantly down regulated in glaucoma. Crystallins belongs to the small heat-shock protein superfamily and function as molecular chaperones preventing aggregation of proteins in the lens and also protect neurons by suppressing signals linked to stress mediated apoptosis. The reduced abundances of CRY in glaucoma may make the retinal neurons more vulnerable to cytotoxic injury and cell death.

### **Validation of Selected protein Candidates of the complement system**

Multiple Reaction Monitoring-Tandem Mass Spectrometry (MRM-MS/MS) technique is a highly sensitive and selective method for the targeted quantification of proteins/peptides abundances in complex biological samples. In addition to pooled samples, we also analyzed

individual patient samples and their respective controls to validate abundances of C1r, C1q, C1s, C4A, C5 and SERPING1 using LC-MRM-MS/MS assay. The results of an individual analysis (Fig 6a-6e) also suggested significant down-regulation of the protein of classical pathways of the complement system. The classical pathway of complement is triggered by C1 which is constituted from recognition protein C1q and C1s-C1r-C1r-C1s, a tetramer of two modular proteases, C1r and C1s. LC-MRM-MS/MS showed downregulation of C1q, C1s, and C1r. The classical pathway is antibody-dependent and only IgM and IgG upon binding to a specific pathogen can activate this pathway. In addition to antibody-induced activation, this pathway can also be activated by several other factors including amyloid P, mitochondrial fragments, apoptotic cells,<sup>49</sup> and cardiolipin vesicles.<sup>50</sup> The release of inflammatory cytokines and upregulation of complement cascade has been noted only in the early stages of glaucoma in several disease models.<sup>15</sup> In a mouse glaucoma model, C1q was upregulated and synaptically localized in the retina very early in the disease.<sup>15</sup> The low abundances of C1q, C1s, and C1r in POAG may be due to the severity of disease as up-regulation of the complement cascade was noted only at the early stages of glaucoma in DBA/2J mice.<sup>36</sup> The disease severity was further supported by the detection of tumor necrosis factor (TNF) in POAG. The release of TNF that promote apoptosis, is the indication of late glaucoma.<sup>51</sup>

### **Protein deamidation and its role in the pathogenesis**

We analyzed deamidation profile of all identified and quantified complement components in POAG and controls and found that complement C1s was exclusively deamidated in four of five POAG (Table S8) patients. The spectrums are provided in Annexure I. No deamidated C1s was noted in their respective controls. The deamidation spectrums of POAG patients are provided in the supplementary information. It's important to note that protein modification including

deamidation critically regulates a wide variety of pathological processes by modulating protein activity, charge, hydrophobicity, stability, specificity, transport, etc.; and also promote protein misfolding, aggregation, and accumulation.<sup>20</sup> The classical pathway is triggered by activation of the C1-complex which is composed of C1q, C1r, and C1s. However, C1s was found to be deamidated, particularly in POAG, which may affect the C1-complex formation, influence its activity and downstream complement pathway. Extensive studies have correlated the degree of eye lens crystalline deamidation with its functional worsening.<sup>52</sup> Although, unlike healthy eye lenses in age-matched controls, cataract exhibit significant accumulation of deamidated crystalline,<sup>53</sup> but the site of modification varied. We found C1s deamidated but at two different sites, however, more detailed study to establish the role of site-specific deamidation in the activation of the complement pathway is required.

### **Pathological progress in POAG**

In rat glaucoma model of elevated IOP, astrocyte activation is the earliest event and depletion of neurotrophins results in axonal degeneration and RGCs loss.<sup>54</sup> With the progression of glaucoma, RGC death begins in the periphery and extends to the center of visual field which activates inflammatory response.<sup>15, 55</sup> As revealed by the proteomic data, the level of an anti-inflammatory protein, alpha-1-acid glycoprotein 2, that play a major role in modulating the activity of the immune system was elevated in POAG patients. Glial cells respond quickly to damage or infection and provide protection to neurons.<sup>56</sup> However, our proteomics data discovered higher quantities of vimentin (VIM) and glial fibrillary acidic protein (GFAP) indicating the presence of reactive astrocyte in POAG patients. Reactive astrocytes result in hypertrophy, hyperplasia, as well as increased expression of GFAP and vimentin.<sup>57</sup> Glial cell activation may trigger processes that

cause further injury, as illustrated by glutamate excitotoxicity.<sup>58,59</sup> Growth factors such as neuronal growth regulator 1 (NEGR1) that play a major role in regenerative axon sprouting were also identified in glaucoma patients. Based on these discoveries, it can be interpreted that glial cell activation contributes to the pathogenesis of glaucoma. Early stage glaucoma can be characterized by changes in astrocyte proliferation, upregulation and activation of components of the complement cascade and synapse loss prior to visual impairment;<sup>15,54</sup> mid-stage glaucoma can be diagnosed through increased levels of inflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$  that leads to disruption in blood-brain barrier;<sup>55</sup> while late stage may be indicated by RGCs death via apoptosis and vision impairment. The data presented in this work is indicative of severe glaucoma. Down-regulation of complement cascade may be due to drug treatment which offer neuroprotection by reducing the expression of the proteins involved in complement pathways.

## **Conclusions**

This study adopted the discovery based-proteomics approach and profiled AH proteome from POAG patients and their age- and gender-matched controls to investigate disease mechanism. We identified 1123 proteins including numerous proteins of the complement cascade, immunoglobulin, neuronal and amyloidogenic proteins in aqueous humor. GO analysis indicated that these proteins were part of processes like an inflammatory response, humoral response, complement activation, protein processing etc. Proteins of aqueous humor proteome, particularly, proteins of the complement system, astrocyte activation, neural degeneration, and apoptosis were significantly altered in primary open angle glaucoma indicating these processes contribute to the pathogenesis. Further, this study identified C1s as deamidated exclusively in POAG but the more detailed study is required to establish the role of site-specific protein deamidation in the pathogenesis of POAG.

**Acknowledgments:** This work is in part supported by grants from the Singapore Ministry of Education (MOE2014-T2-2-043 and MOE2016-T2-2-018), the National Medical Research Council of Singapore (NMRC-OF-IRG-0003-2016).

**Conflict of Interest:** None

### **Supporting Information**

The following supporting information is available free of charge at ACS website <http://pubs.acs.org>

Table S1: Demographic and disease characteristics of cases and controls.

Table S2: Proteins identified in POAG and controls. The data was searched using Proteome Discoverer ver1.4

Table S3: Proteins identified in POAG and controls. Proteins with more than one unique peptide and at least two peptide spectrum match were considered.

Table S4: Protein identified in glaucoma and their controls by Mascot search engine. Proteins identified with  $\geq 30$  protein score are considered

Table S5: Protein groups identified using Mascot search engine and their fold changes

Table S6: The details of MRM peptide, their sequences and related parameters.

Table S7: LC-MS/MS analysis of complement activation in human primary open angle glaucoma

Table S8: Deamidation profile of complement components and complement factors identified and quantified in LC-MS/MS.

Figure S1: Protein identification in control and glaucoma subjects. Frequency distributions of the proteins fold change in log<sub>2</sub> scale.

Annexure I: The deamidation spectrums and fragmentation profile of complement C1s subcomponent that was detected in POAG patients

### **Supplementary data**

Project Name: Human aqueous humor of Primary open-angle glaucoma LC-MS/MS

Project accession: PXD007624

Project DOI: Not applicable

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Table 1. Proteomic analysis of Aqueous humor samples in various eye diseases and proteins identified

Sample	Method used	Disease	Number of proteins identified	Reference
Aqueous Humor	2D- LC-MS/MS	Primary open-angle glaucoma	7	Duan et al <sup>24</sup>
Aqueous Humor	SELDI-TOF-MS	Primary open-angle glaucoma	250	Grus <sup>60</sup>
Aqueous Humor	iTRAQ LC-MS/MS, (combination of 1D-G-CID, 2D-SG-CID, 2D-SG-HCD)	Cataract	198	Bennett <sup>17</sup>
Aqueous Humor	LC-ESI-MS/MS + antibody-based protein arrays	Cataract	676	Chowdhury <sup>61</sup>
Aqueous Humor	iTRAQ-LC-MS/MS	Cataract development	445	Xiang <sup>62</sup>
Aqueous Humor	LC-MS/MS	Cataract, glaucoma, and pseudoexfoliation syndrome	269	Kliuchnikova <sup>63</sup>
Aqueous Humor	iTRAQ LC-MS/MS	Cataract development	445	Ji et al <sup>64</sup>
Aqueous Humor	LC-MS/MS	Cataract	763	Murthy <sup>29</sup>
Aqueous Humor	2D- MALDI TOF MS and tandem TOF/TOF MS	Macular degeneration	68	Yao et al <sup>65</sup>
Aqueous Humor	LTQ Orbitrap XL mass spectrometer	Proliferative diabetic retinopathy	39	Balaiya <sup>66</sup>
Aqueous Humor	1DE LC-MS/MS	Cataract: Gender difference	147	Perumal et al <sup>67</sup>
Aqueous Humor	iTRAQ based LC/MS/MS	Coats' disease	819	Yang et al <sup>68</sup>
Aqueous Humor	LC-MS/MS	Keratoconus	242	Soria et al <sup>69</sup>
Aqueous Humor	2D- LC-MS/MS	Branch retinal vein occlusion-induced macular edema	49	Yao et al <sup>70</sup>
Aqueous Humor	iTRAQ LC-MS/MS	Idiopathic epiretinal membranes	323	Pollreisz et al <sup>71</sup>
Aqueous Humor	LC-MS/MS	Primary open angle glaucoma	>1000	This study

## Figure legends

**Figure 1.** Statistical analysis and reproducibility, a) Volcano plot of the p-values as a function of protein fold change. Data statistical significance (p-value in a  $-\log_{10}$  scale) is plotted as a function of the protein fold change (in a  $\log_2$  scale), b) data reproducibility expressed as a protein fold change between replicates of G009/CG075, c) data reproducibility expressed as a protein fold change between replicates of G010/CG072, d) data reproducibility expressed as a protein fold change between replicates of G016/CG078, e) data reproducibility expressed as a protein fold change between replicates of G039/CG065, f) data reproducibility expressed as a protein fold change between replicates of G041/CG070.

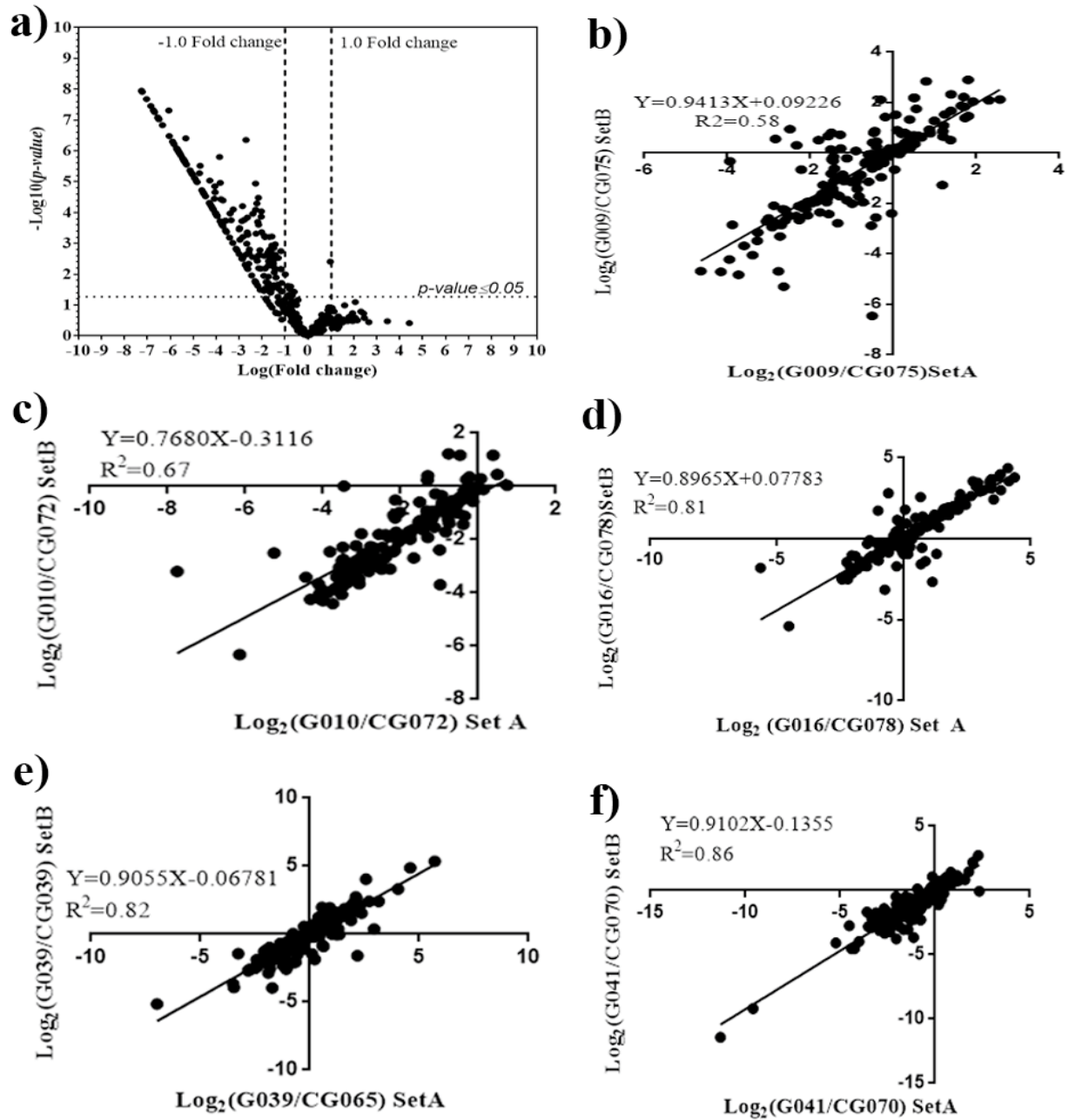
**Figure 2.** Hierarchical clustering of the differentially expressed proteins identified in AH proteome of individual POAG patients and controls. Up-regulated protein expression values are displayed in red, the down-regulation values are in blue, and the intermediate values are in shades of red and blue.

**Figure 3.** Protein abundances in POAG and gene ontology analysis of downregulated proteins. A) The abundance of the proteins in POAG. B) Down-regulation of proteins involved in complement activation, protein activation cascade, and immunity. Shown are significantly enriched GO terms (p-Value  $< 0.001$  with Bonferroni correction for multiple comparisons) with enrichment defined as a number of proteins found/number of proteins expected based on the human reference genome. C) Down-regulated proteins of glaucomatous aqueous humor were analyzed for cellular components. Shown are significantly enriched GO terms (p-Value  $< 0.01$  with Bonferroni correction for multiple comparisons).

**Figure 4.** Complement pathway with integrated LC-MS/MS identified and quantified proteins of complement pathways. A) Complement pathway with identified proteins. Proteins in green were identified in our data, B) abundances of complement C3, C4, and C5 in AH of PAOG. C) The abundance of complement C8, C1Q, and C6 in Ah of POAG.

**Figure 5.** MRM of complementary cascade proteins in pooled POAG (n=5) and their respective controls. The significance was determined by using unpaired t-test.

**Figure 6.** MRM of complementary cascade proteins in individual POAG (n=3) and their respective controls. The significance was determined by using unpaired t-test.



**Figure 1**

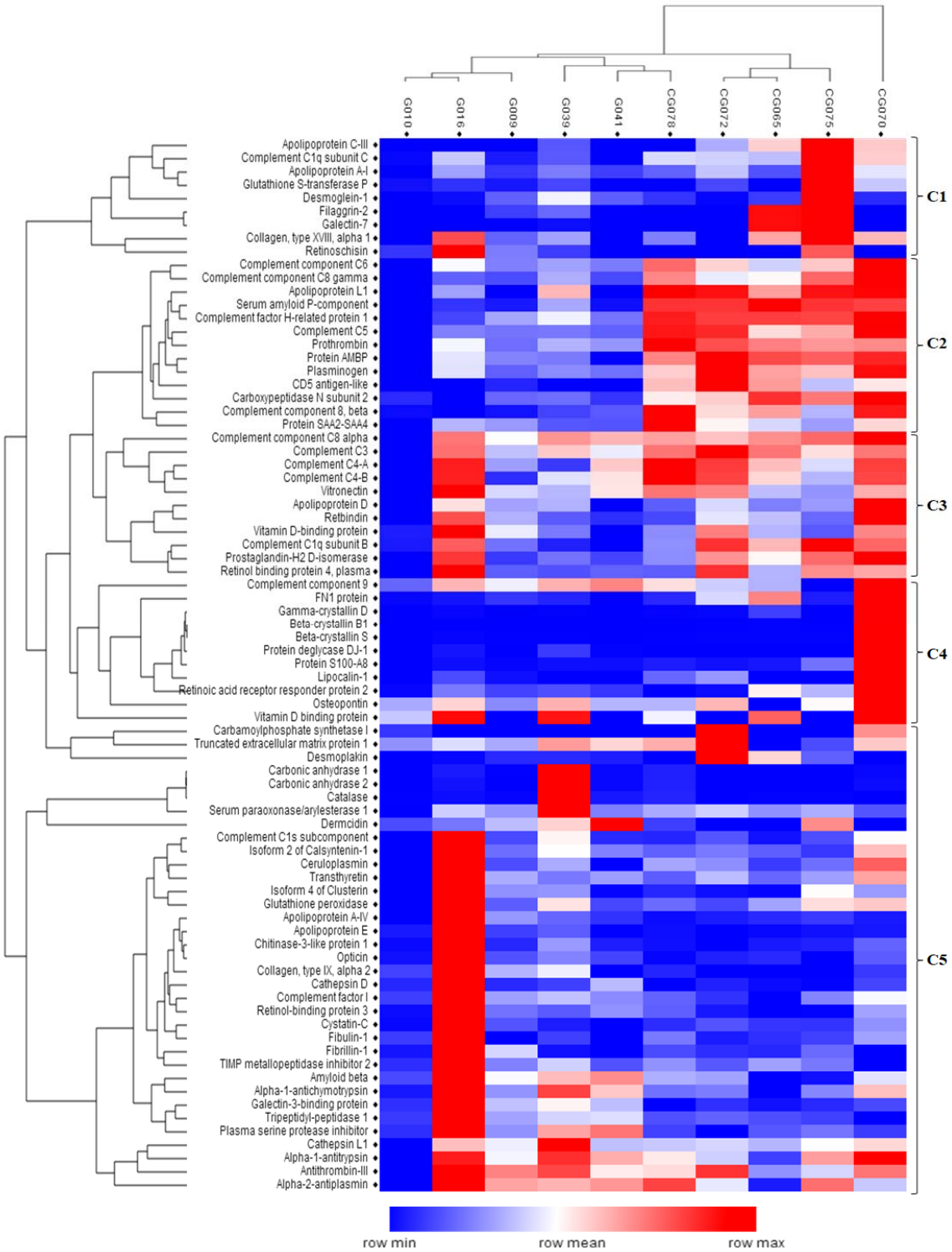
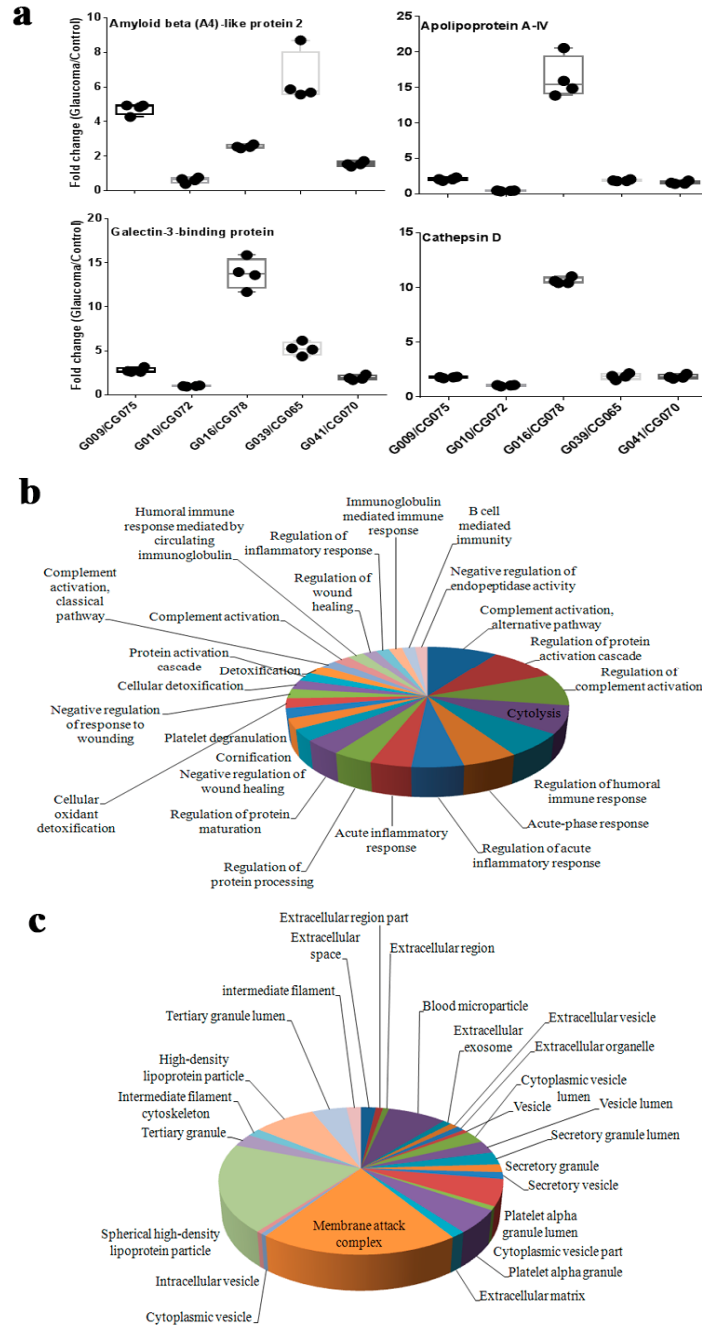
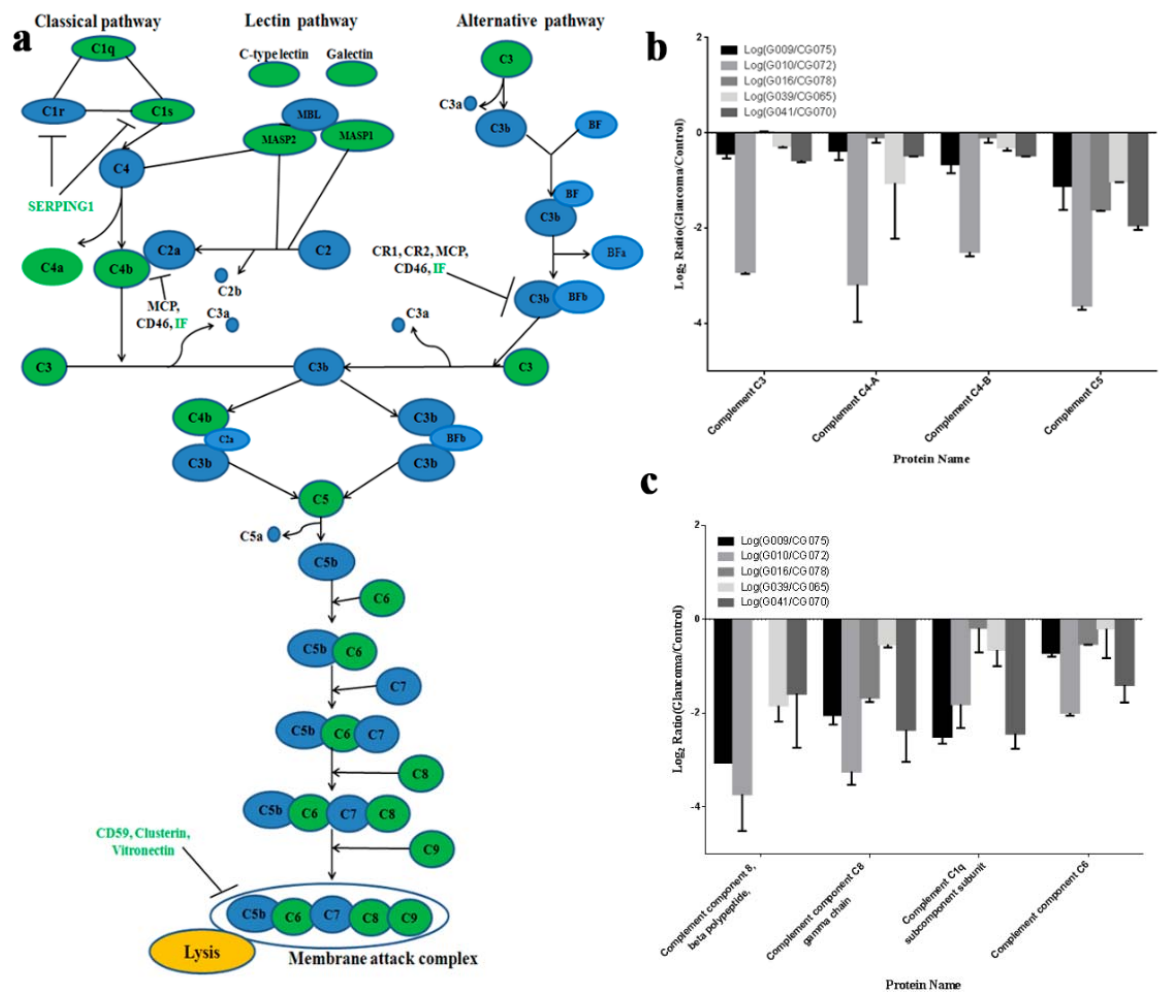


Figure 2



**Figure 3**



**Figure 4**

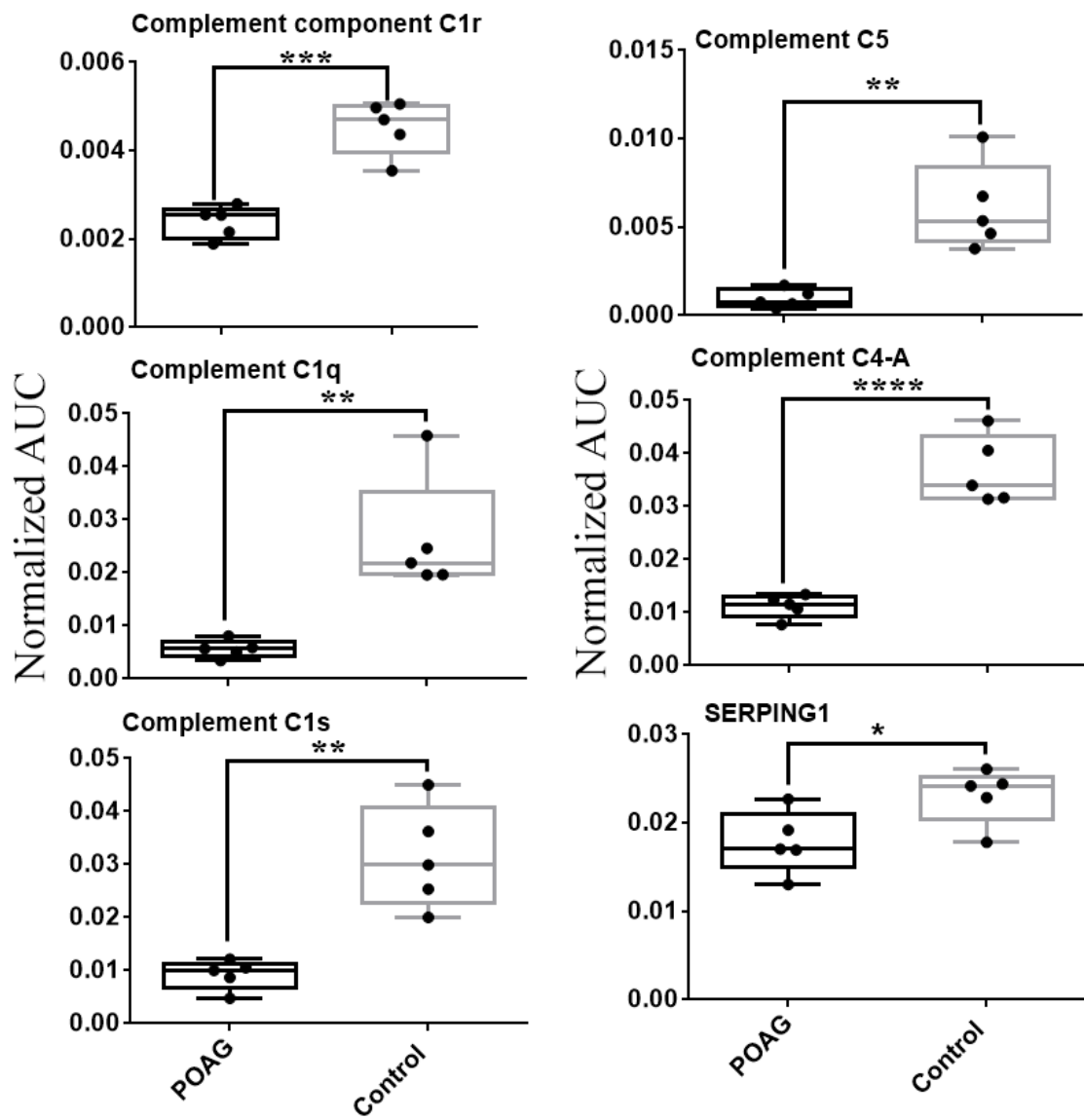
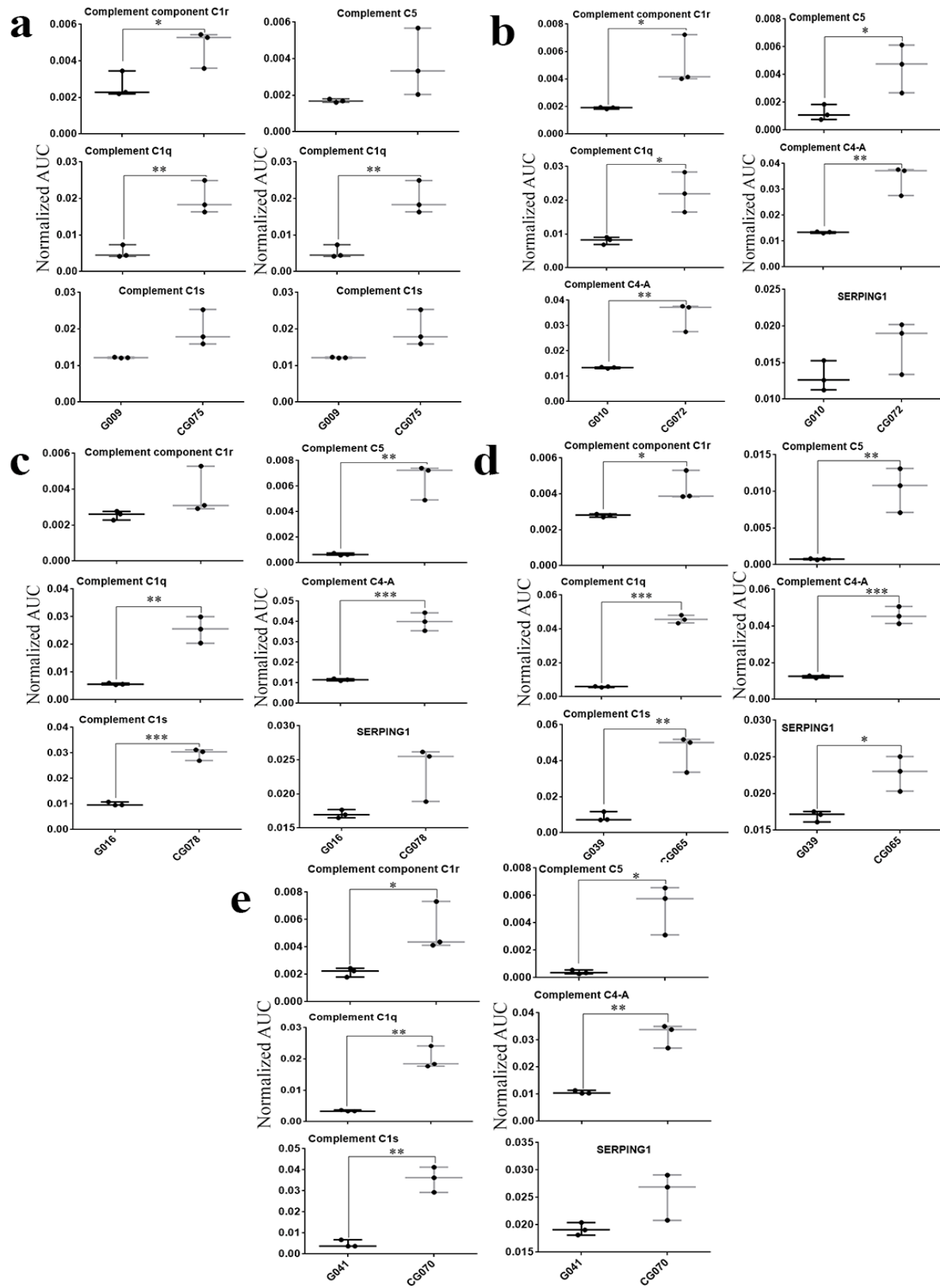


Figure 5



**Figure 6**

**“For TOC only”**

**Graphical abstract**

