

Oxidative Stress–Related Molecular Biomarker Candidates for Glaucoma

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PURPOSE. Glaucoma-related molecular biomarkers can improve clinical testing to diagnose the disease early, predict its prognosis, and monitor treatment responses. Based on the evidence of increased oxidative stress in glaucomatous tissues, this study analyzed oxidative stress–related biomarker candidates in blood and aqueous humor samples with or without glaucoma.

METHODS. The blood and aqueous humor samples collected from carefully selected groups of 96 patients with glaucoma and 64 healthy subjects without glaucoma were included in the study. The samples were analyzed for protein carbonyls and advanced glycation end products (AGEs) through ELISA-based quantification assays. To allow proper comparisons, the Goldmann-Witmer coefficient that reflects the ratio of aqueous humor to blood values corrected to total protein concentration in individual samples was calculated.

RESULTS. Blood and aqueous humor levels of protein carbonyls and AGEs were found significantly higher in glaucomatous samples compared with age-matched nonglaucomatous controls ($P < 0.001$). The glaucoma-related increase in protein carbonyls and AGEs was more prominent in aqueous humor samples than blood samples (2.6-fold versus 1.9-fold for protein carbonyls, and 3.1-fold versus 1.9-fold for AGEs; $P < 0.001$). Comparison of the Goldmann-Witmer coefficients indicated greater values for protein carbonyls (1.37 ± 0.3 vs. 3.07 ± 0.8) and AGEs (1.2 ± 0.3 vs. 3.2 ± 1.1) in the glaucoma group ($P < 0.001$).

CONCLUSIONS. Findings of this study encourage further validation studies of oxidative stress–related biomarkers in glaucoma. Analysis of protein carbonyls and AGEs in longitudinal studies of larger and heterogeneous patient cohorts should better assess the value of these promising candidates as molecular biomarkers of glaucoma for clinical predictions.

Keywords: glaucoma, biomarker, oxidative stress, protein carbonyl, advanced glycation end products

To improve clinical testing of glaucoma, specific biomarkers are needed for early diagnosis of this blinding disease and prediction of its prognosis. Such diagnostic and prognostic biomarkers have been critically useful for timely treatment of many different diseases. For glaucoma, currently available clinical analysis tools have limitations, there is no gold standard to detect disease progression, and it may take several years to statistically confirm a treatment effect on clinical outcomes. The inadequacy in monitoring the treatment responses has also been a challenge in translation from preclinical studies to human trials of neuroprotective interventions for glaucoma. To overcome such difficulties, clinical research aims to develop new techniques and structure-function models for early and more accurate detection of glaucoma.¹ Patients' symptoms, various objective or functional signs, or imaging parameters may be useful as clinical biomarkers to determine the presence and severity of glaucoma. However, our interest has been focused on searching for “molecular biomarkers” that can assist early diagnosis of glaucoma, help predict disease prognosis, and serve as surrogate endpoints in new drug trials.

The common approach to molecular biomarkers is to initially identify biomarker candidates and then pursue validation studies, the most challenging step, to verify their value for

clinical predictions. With respect to etiological complexity of glaucoma and significant variability among patients, our first-step studies toward biomarker candidates have followed different approaches in parallel. Our initial studies have included direct analysis of undepleted serum proteins by quantitative mass spectrometry. This has been followed by the analysis of proteins co-eluted with IgG and the targeted analysis of selected molecules.^{2–4} Although the IgG-based analysis is grounded on increased production of autoantibodies reacting to ocular antigens in glaucoma, depending on the experimental evidence of T cell-mediated immune responses, an additional approach to molecular biomarkers of glaucoma has recently included the analysis of T lymphocyte subset markers (Yang X, et al. *IOVS* 2017;58:ARVO E-Abstract 2562). Besides providing the lists of candidate molecules, these biomarker studies through proteomics analysis of blood samples also have detected a greater abundance of protein oxidation in the glaucomatous blood than age-matched healthy controls.² Based on this observation, along with the growing evidence of increased oxidative stress in glaucomatous tissues (including glaucomatous human retina and optic nerve^{5–9}), we thought that oxidatively modified proteins or oxidative stress end products may particularly be valuable as glaucoma-related

biomarkers. Our more recent work presented herein has therefore focused on the analysis of oxidative stress-related biomarker candidates. The studied markers included protein carbonyls and advanced glycation end products (AGEs), both exhibiting a significant increase in glaucomatous human eyes⁷ and ocular tissues of animal models.⁵

Blood samples are easily accessible, and biomarker screening in blood samples is a common and suitable choice for clinical applications; however, analysis of more proximal fluids to disease sites, such as aqueous humor for glaucoma, seems more advantageous to gain disease-specific information. We therefore studied protein carbonyls and AGEs in both blood and aqueous humor samples collected from the same patients with glaucoma and nonglaucomatous controls. The oxidative stress-related biomarker candidates that presented higher levels in glaucomatous samples than controls await further validation studies to value them as clinically useful biomarkers of glaucoma.

MATERIALS AND METHODS

Study Groups

This study of the oxidative stress-related biomarker candidates included 96 patients with glaucoma and a control group of 64 healthy volunteers without glaucoma.

All patients in the glaucoma group had primary open-angle glaucoma. The diagnosis of glaucoma was based on elevated intraocular pressure (IOP) (>22 mm Hg), glaucomatous cupping of the optic disc on fundusoscopic assessment, open anterior chamber angles by gonioscopy, and reliable visual field defects (with either a pattern SD <5%, or a glaucoma hemifield test result outside the 99% normal limits) detected by a visual field analyzer (Humphrey Visual Field Analyzer; Carl Zeiss Meditec, San Francisco, CA, USA). There was no clinical evidence for alternative causes of optic neuropathy in any of the patients with glaucoma. Control subjects had aging-related cataract with no clinical evidence of glaucoma, or a family history of glaucoma.

As an attempt to minimize the effects of individual heterogeneity, we carefully selected our study groups. Because glaucoma is more common in the elderly and oxidative stress is an important component of aging-related processes, a donor age of older than 55 years was a preselection criterion for both study groups. Additional inclusion or exclusion criteria were related to other diseases. The eyes with a coexisting retinal or corneal endothelial disease or uveitis were not included in the study groups. Moreover, none of study participants had previous vitrectomy, endothelial keratoplasty, or cataract surgery. Regarding systemic diseases, patients or control subjects with diabetes mellitus, or any cardiovascular or cerebrovascular disease (except for a history of mild systemic hypertension in the past) were also excluded from the study to eliminate potential effects of such diseases or their treatment on oxidative stress-related markers. None of the study participants was smoking or taking any antioxidant supplements at the time of sampling.

As described below, we collected blood and aqueous humor samples from patients with glaucoma and nonglaucomatous controls at the time of their ocular surgeries (with a 10- to 15-minute interval between the blood and aqueous humor sampling). Sample collection from the glaucoma group was at the time of their cataract surgery by phacoemulsification ($n = 30$), trabeculectomy ($n = 43$), or combined trabeculectomy with cataract surgery ($n = 23$). Glaucoma patients who underwent repeated filtration surgery or drainage device implantation were not included in sampling for this study.

Blood and aqueous humor samples of the control subjects were collected at the time of their cataract surgery by phacoemulsification.

Sample Collection

All samples were collected after a written informed consent was obtained according to the protocols approved by the Columbia University Institutional Review Board, and all procedures were conducted in accordance with the tenets of the Declaration of Helsinki.

The blood samples (~7 mL) were withdrawn by routine venipuncture before each ocular surgery using a butterfly needle and syringe to avoid hemolysis and were collected into EDTA-containing tubes. As a requirement of preoperative preparation, all participants were fasted for at least 8 hours overnight before sample collection. The aqueous humor (100–200 μ L) was aspirated at the beginning of ocular surgery by limbal paracentesis. A small syringe attached to a 30-gauge needle was entered into the peripheral anterior chamber at the corneoscleral limbus. The aqueous humor was aspirated at approximately the middle of the anterior chamber after moving the needle on a plane parallel with the lens by avoiding lens injury or bleeding. The serum was separated from blood samples by centrifugation (2000g for 10 minutes at room temperature). All samples were immediately stored at -80°C for up to 4 weeks until their analysis.

The analyses included the presence and abundance of protein carbonyls and AGEs using targeted quantification by specific ELISAs.

Enzyme-Linked Immunosorbent Assay

Total protein concentration was determined in all samples by a colorimetric assay based on the Bradford protein assay (Bio-Rad, Hercules, CA, USA). Protein carbonyl content was measured using an OxiSelect protein carbonyl ELISA kit (Cell Biolabs, San Diego, CA, USA), as we previously described.^{5,9} Briefly, the samples and standards adsorbed to wells of a 96-well plate at 4°C overnight were reacted with 2,4-dinitrophenylhydrazine (DNPH) for 45 minutes at room temperature in the dark. After blocking and washing steps, wells were incubated with 100 μ L of an anti-DNP antibody (1:1000) for 1 hour at room temperature. Wells were next exposed to 100 μ L horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000) for 1 hour, followed by developing with the substrate solution. After termination of the enzyme reaction, the absorbance was read by a plate reader (iMarkMicroplate Reader; Bio-Rad) at 450 nm. The average absorbance was calculated from sample duplicates. The protein carbonyl content in samples was calculated from the standard curve that was generated with pre-optimized concentration ranges of reduced and oxidized BSA standards. The sensitivity of ELISA was approximately 3 nmol/mg, consistent with previous studies.^{10–12}

The level of AGEs was similarly assessed with a OxiSelect AGE competitive ELISA kit (Cell Biolabs). Briefly, samples (50 μ L) and AGE-BSA standards were added onto 96-wells of the AGE conjugate-coated plates for incubation at room temperature for 10 minutes. This was followed by incubation with 50 μ L anti-AGE antibody (1:1000) at room temperature for 1 hour at room temperature. After washing, 100 μ L HRP-conjugated secondary antibody (1:1000) was added to wells for another 1 hour. After developing with the substrate solution and stopping the enzyme reaction, the absorbance was read using a plate reader (Bio-Rad) at 450 nm. The AGE level in samples was determined from the AGE-BSA standard curve generated

TABLE. Demographics of the Study Groups and Clinical Characteristics of Glaucoma

	Glaucoma	Control	Significance
<i>n</i>	96	64	
Age, y	73.1 ± 8.0	70.20 ± 8.4	<i>P</i> > 0.05
Sex, female (%)	54 (56)	36 (56)	<i>P</i> > 0.05
Race, white (%)	81 (84)	57 (89)	<i>P</i> > 0.05
IOP, mm Hg	16.3 ± 4.3 (28.8 ± 4.9)	14.6 ± 3.1	<i>P</i> > 0.05 (<i>P</i> > 0.01)
Visual field mean deviation, dB	12.6 ± 4.8	N/A	-
Duration of glaucoma, y	9.6 ± 3.4	N/A	-
Number of IOP-lowering medications	1.6 ± 0.7	N/A	-

Data are presented as mean ± SD when applicable. The elevated IOP (>22 mm Hg at glaucoma diagnosis) was under control by treatment or remained elevated despite IOP-lowering treatment (given in parenthesis). Statistical significance was tested by a two-tailed *t*-test (for age and IOP) or χ^2 test (for sex and race). Note that glaucoma-related clinical characteristics, such as visual field defects, disease duration, or IOP-lowering medications, were not applicable (N/A) in the control group and therefore not tested for statistical significance of group differences.

using pre-optimized concentration ranges. The sensitivity of ELISA was approximately 0.1 µg/mL, similar to previously described.^{13,14}

All concentrations that were calculated from a standard curve for each assay were corrected to the protein concentration in individual samples of serum and aqueous humor samples. Moreover, to allow proper comparison of serum and aqueous humor concentrations between study groups, the Goldmann-Witmer coefficient (GWC) was calculated for each sample. This coefficient (commonly used as a diagnostic tool to evaluate local antibody production in ocular inflammatory diseases^{15,16}) is defined as $GWC = X/Y$, where *X* is the aqueous humor (AH) concentration of the tested molecule divided by the total protein concentration in aqueous humor, and *Y* is the serum concentration of the same molecule divided by the total protein concentration in serum:

$$GWC = \frac{AH \text{ concentration}/AH \text{ total protein concentration}}{Serum \text{ concentration}/Serum \text{ total protein concentration}}$$

Statistical Analysis

The main outcome of this study was the level of protein carbonyls and AGEs in blood and aqueous humor samples from the glaucoma group in comparison with nonglaucomatous controls.

The statistical analysis was carried out using a commercial statistical software (SigmaPlot, version 12.5; Systat Software, Inc., San Jose, CA, USA). All data are presented as mean ± SD, and bar graphs are accompanied by univariate scatterplots for more complete presentation of data distribution.¹⁷ A two-tailed *t*-test and χ^2 test were used for comparison of demographics and clinical characteristics between glaucoma and control groups. Differences in protein carbonyls and AGEs between glaucoma and control groups were analyzed by the Kruskal-Wallis 1-way ANOVA on ranks. Linear regression analysis was used to determine the relationship between the studied markers and demographic or glaucoma-related variables. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Demographic features of the study groups and clinical characteristics of glaucoma are presented in the Table. The mean (±SD) age was 73.1 ± 8.0 years in the glaucoma group and 70.2 ± 8.4 years in the control group (*P* > 0.05). Similarly, there were no sex or race differences between the study groups (56% female in both groups; *P* > 0.05, and 84% vs. 89% white in glaucoma and control groups, respectively; *P* > 0.05).

As also shown in the Table, in 30 patients out of 96 with glaucoma, the elevated IOP at diagnosis (>22 mm Hg) was under control by IOP-lowering treatment (IOP, 16.3 ± 4.3 mm Hg). In these patients, blood and aqueous humor samples were collected at the time of their cataract surgery by phacoemulsification. However, in 66 patients, IOP remained elevated (IOP, 28.8 ± 4.9 mm Hg) and/or their disease was progressing despite IOP-lowering treatment. These patients underwent trabeculectomy (*n* = 43) or combined trabeculectomy with cataract surgery (*n* = 23) at which study samples were collected. As noted in the “Study Groups” section, more complicated patients requiring repeated filtration surgery or drainage device implantation were not included in sampling for this study. IOP was in normal limits in the control group (14.6 ± 3.1 mm Hg), whose samples were collected at the time of their cataract surgery.

Based on the Glaucoma Staging System scale,^{18,19} the glaucoma in 42 eyes out of 96 was categorized as moderate (mean deviation, −5.01 to −12.00 dB), whereas 54 eyes were at more advanced stage (mean deviation, −12.01 to −20.00 dB). The distribution of moderate or advanced stages of injury was similar between the glaucoma patients whose samples were collected at the time of their cataract surgery (14 patients with moderate injury and 16 patients with advanced injury, 47% vs. 53%; *P* > 0.05), or a trabeculectomy (28 patients with moderate injury and 38 patients with advanced injury; 42% vs. 58%; *P* > 0.05).

Duration of glaucoma was more than 5 years for all patients (9.6 ± 3.4 years), during which they were given an altered combination of multiple medications to lower their IOP (1 to 3 medications at the time of sampling for this study, including beta-adrenergic blockers, alpha 2-adrenergic receptor agonist, prostaglandin analogs, or carbonic anhydrase inhibitors). In addition, 9 patients with glaucoma (in the cataract surgery subgroup) had previously undergone trabeculectomy (2 years or more before sampling for this study), although patients with repeated filtration surgery or other previous ocular surgery were excluded from the study.

The oxidative stress-related biomarker candidates studied in blood serum and aqueous humor, namely protein carbonyls and AGEs, presented significant association to glaucoma. When the data from the group of patients with primary open-angle glaucoma was compared with age-matched nonglaucomatous controls, both serum (584.1 ± 162.5 nmol/mg versus 1085.8 ± 144.8 nmol/mg) and aqueous humor (8.8 ± 2.1 nmol/mg versus 23.0 ± 7.9 nmol/mg) levels of protein carbonyls were found significantly higher in the glaucoma group (*P* < 0.001; Fig. 1). The level of AGEs was also significantly higher in the glaucomatous serum (471.3 ± 106.1 µg/mL versus 917.1 ± 111.6 µg/mL) and aqueous humor (5.7 ± 2.3 µg/mL versus 17.6 ± 4.6 µg/mL) than

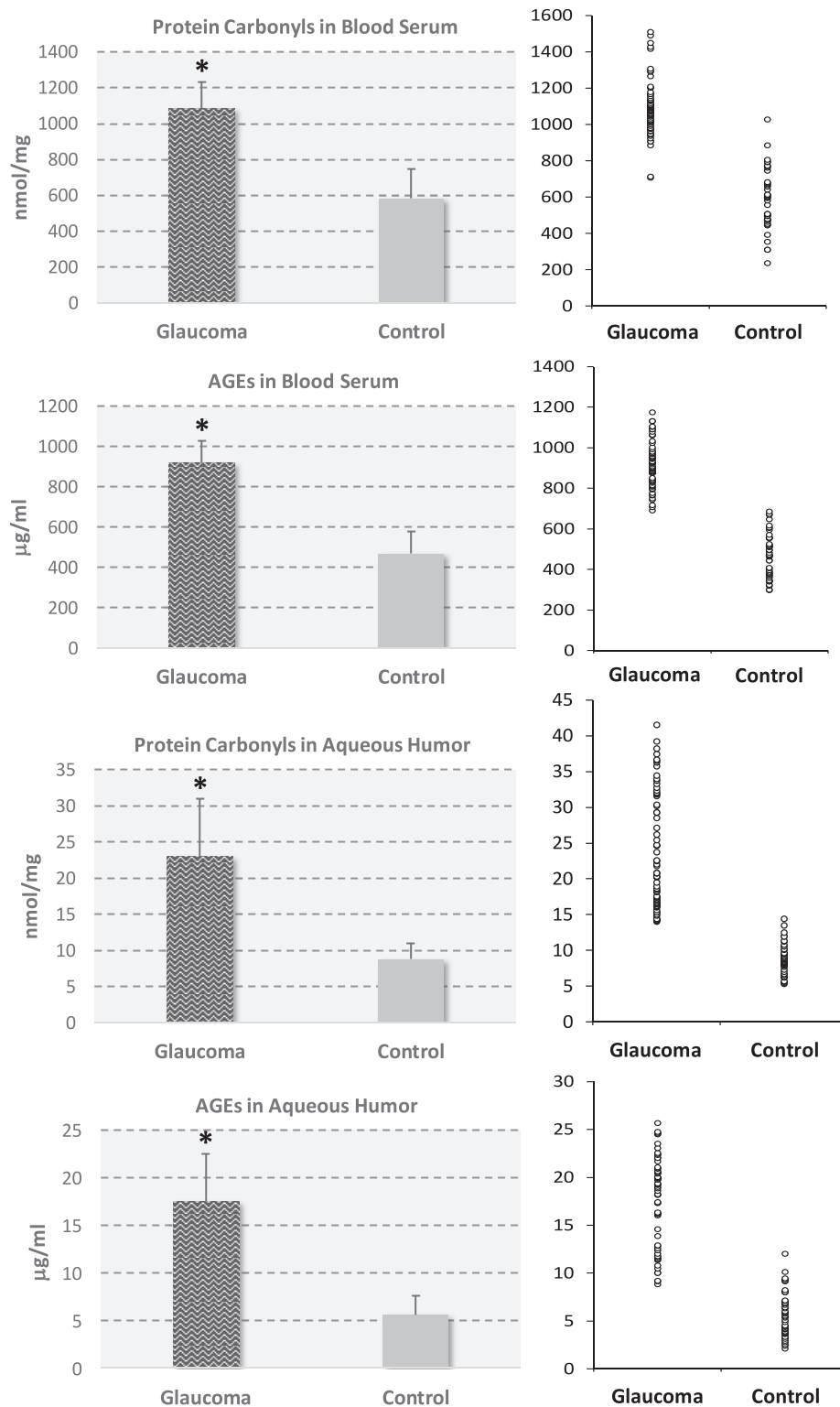


FIGURE 1. Oxidative stress-related biomarker candidates in glaucoma. Protein carbonyls (*top*) and AGEs (*bottom*) were analyzed in samples of the blood serum and aqueous humor collected from patients with glaucoma ($n = 96$) and age-matched nonglaucomatous controls ($n = 64$) by specific ELISAs. Data are presented as mean \pm SD in *bar graphs* and *univariate scatterplots* present data distribution. Compared with controls, glaucomatous samples exhibited significantly higher titers of these oxidative stress-related biomarker candidates (*Statistical significance; the Kruskal-Wallis 1-way ANOVA on ranks, $P < 0.001$).

controls ($P < 0.001$; Fig. 1). As shown in Figure 2, the difference detected in protein carbonyls and AGEs between the glaucoma and control groups was more prominent in aqueous humor samples than blood samples (2.6-fold versus

1.9-fold for protein carbonyls; and 3.1-fold versus 1.9-fold for AGEs). This study was not longitudinal and therefore did not allow the comparison of individual aqueous humor or blood samples over time; however, to determine the validity of this

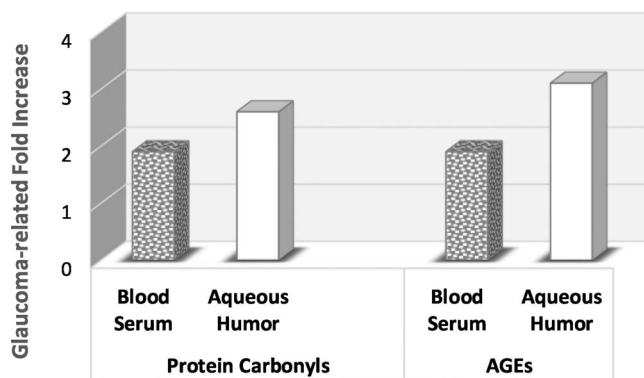


FIGURE 2. Oxidative stress-related biomarker candidates in glaucoma. Based on the data presented in Figure 1, the glaucoma-related increase in ELISA titers of protein carbonyls and AGEs was more prominent in aqueous humor samples than blood serum.

observation, we calculated the GWCs that reflect the ratio of aqueous humor to blood values corrected to total protein concentration in individual samples.

Comparison of the GWCs between the age-matched groups of patients with primary open-angle glaucoma and nonglaucomatous controls indicated greater values for protein carbonyls (1.37 ± 0.3 vs. 3.07 ± 0.8) and AGEs (1.2 ± 0.3 vs. 3.2 ± 1.1) in glaucomatous samples ($P < 0.001$; Fig. 3). A positive correlation was detectable between the GWCs for protein carbonyls and AGEs in the glaucoma group ($R = 0.549$, $P < 0.001$).

As shown in Figure 4, there was no significant relationship between blood and aqueous humor levels of protein carbonyls in the glaucoma group ($R = 0.07$, $P > 0.05$). However, blood and aqueous humor levels of AGEs exhibited a positive correlation among glaucoma patients ($R = 0.28$, $P = 0.02$).

After comparison of protein carbonyl and AGE levels in blood and aqueous humor samples between the glaucoma group and age-matched nonglaucomatous controls, we analyzed if any of the demographic or glaucoma-related parameters could affect these oxidative stress-related biomarker candidates.

When we analyzed the relationship between the donor age and GWCs for protein carbonyls or AGEs, no significant correlation was detected ($R = 0.01$; $P > 0.05$, and $R = 0.09$; $P > 0.05$, respectively). However, note that the study groups included a restricted age group as preselection criterion. Similarly, no significant relationship was detectable between the duration of glaucoma and GWCs for protein carbonyls or AGEs ($R = 0.08$; $P > 0.05$, and $R = 0.04$; $P > 0.05$, respectively).

As detailed above, the glaucoma group included patients with controlled ($n = 30$; 16.3 ± 4.3 mm Hg) or uncontrolled ($n = 66$; 28.8 ± 4.9 mm Hg) IOP with treatment, or patients with moderate ($n = 42$; mean deviation, -5.01 to -12.00 dB) or more advanced stages of injury ($n = 54$; mean deviation, -12.01 to -20.00 dB). No significant relationship was detected between the oxidative stress-related biomarker candidates studied and the level of IOP ($R = 0.05$, $P > 0.05$ for protein carbonyls; $R = 0.06$, $P > 0.05$ for AGEs) or the stage of glaucomatous injury ($R = 0.07$, $P > 0.05$ for protein carbonyls; $R = 0.09$, $P > 0.05$ for AGEs). Although the GWCs for protein carbonyls or AGEs were not significantly different between glaucoma subgroups (2.90 ± 0.7 vs. 3.11 ± 1.0 , $P > 0.05$, and 3.26 ± 0.9 vs. 3.34 ± 1.2 , $P > 0.05$, respectively), the correlation we detected between protein carbonyls and AGEs in the glaucoma group (noted above) was similarly detectable in subgroups with moderate ($R = 0.487$, $P = 0.003$) or more advanced injury ($R = 0.591$, $P = 0.001$).

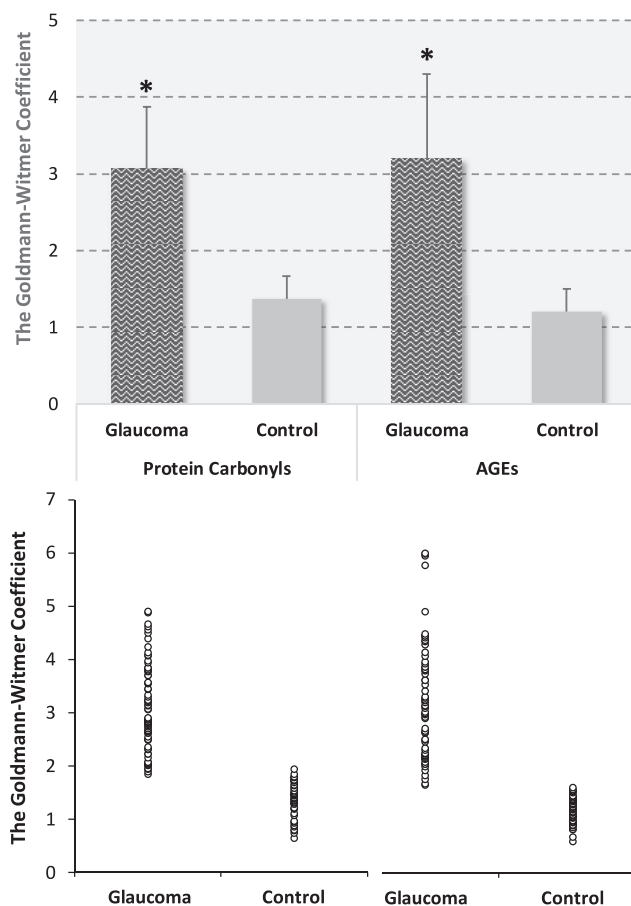


FIGURE 3. Oxidative stress-related biomarker candidates in glaucoma. To allow proper comparison of blood and aqueous humor levels of protein carbonyls and AGEs between study groups, the GWC was calculated for each sample, which is defined as $GWC = X/Y$, where X is the aqueous humor concentration divided by the total protein concentration in aqueous humor, and Y is the blood serum concentration divided by the total protein concentration in blood serum. Data are presented as mean \pm SD in bar graphs and univariate scatterplots present data distribution. Group comparison of the GWCs indicated significantly higher values in the glaucoma group than controls (*Statistical significance; the Kruskal-Wallis 1-way ANOVA on ranks, $P < 0.001$).

However, as shown in Figure 5, the GWCs for protein carbonyls (2.8 ± 0.8 vs. 3.2 ± 0.8 ; $P = 0.015$) and AGEs (2.7 ± 0.7 vs. 3.4 ± 1.2 ; $P = 0.03$) were significantly higher in patients who underwent trabeculectomy with or without cataract surgery (because they had uncontrolled IOP and/or progressing injury) compared with patients who underwent only cataract surgery (because their IOP was controlled by treatment).

DISCUSSION

Our findings in this study indicated significantly higher levels of protein carbonyls and AGEs in samples of the blood serum and aqueous humor collected from patients with glaucoma compared with age-matched nonglaucomatous controls. Interestingly, the glaucoma-related increase in these oxidative stress-related biomarker candidates was more prominent in aqueous humor samples than blood samples. This is verified by calculating the Goldman-Witmer coefficients that display the ratio of aqueous humor to blood values corrected to total

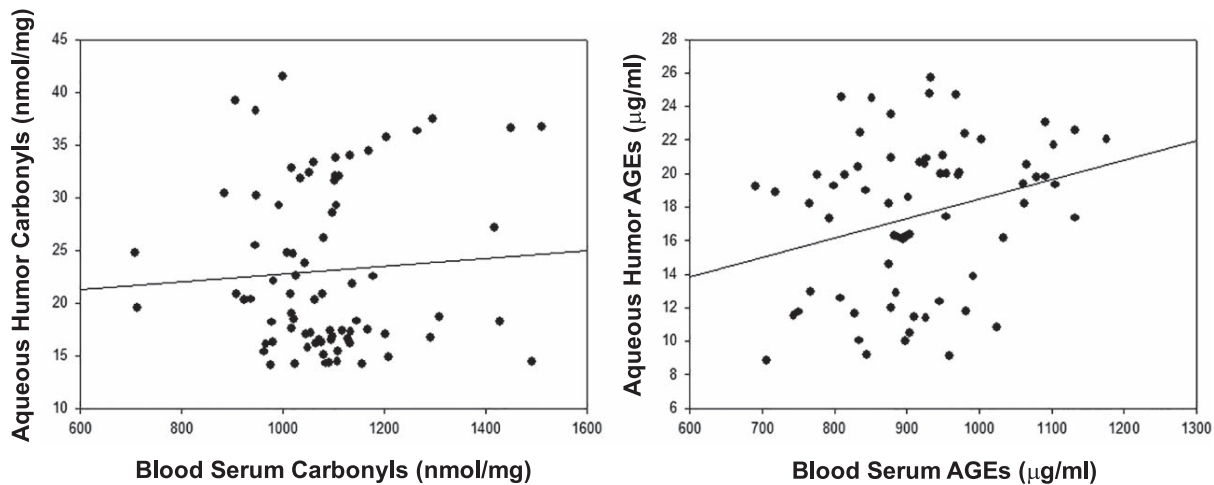


FIGURE 4. Relationship between the blood and aqueous humor levels of oxidative stress-related biomarker candidates in glaucoma. *Scatterplots* show blood serum and aqueous humor levels of protein carbonyls, and AGEs in glaucomatous samples. No significant relationship was detected between the blood and aqueous humor levels of protein carbonyls in the glaucoma group (linear regression analysis, $R = 0.07$, $P > 0.05$). However, blood and aqueous humor levels of AGEs exhibited a positive correlation among glaucoma patients (linear regression analysis, $R = 0.28$, $P = 0.02$).

protein concentration in individual samples. It is also worth noting that the greater values of the Goldman-Witmer coefficients for protein carbonyls and AGEs in the glaucoma group may support intraocular generation of oxidative stress as an outcome of glaucoma. This auxiliary observation bolsters previous research findings, pointing out the pathogenic and therapeutic importance of oxidative stress in glaucoma.^{5-9,20}

Some systemic or genetic factors (such as aging-related or vascular factors, tendency for autoimmune disease, or a family history of glaucoma) may affect the glaucoma risk in many patients, and related blood analysis may be useful for risk assessment (such as analysis of blood pressure, blood glucose or autoimmune panel, or genetic testing). For glaucoma-related protein/peptide biomarkers, however, analysis of aqueous humor seems to provide more specific information. This is because representation of ocular proteins or glaucoma-related biomarkers within the large and complex pool of blood that exhibits a dynamic spectrum of numerous molecules is highly limited. Yet, analysis of aqueous humor, more proximal to disease sites of glaucoma, may present the advantage to gain more specific information about injurious conditions and secreted molecules. Regrettably, however, aqueous humor is not as easily accessible as blood for routine clinical sampling. It is for the same reason that our glaucoma group included patients with moderate to severe glaucoma only (but did not include patients with ocular hypertension or early-stage glaucoma), because aqueous humor sampling required cataract or glaucoma surgery that is mostly indicated in patients with more advanced disease stages.

Our previous study of the oxidative modification of serum proteins in human blood has specifically determined methionine oxidation,² because methionine residues are important targets for oxidation by various reactive oxygen species^{21,22} and their analysis may reflect the extent of oxidative stress.²³ We searched the acquired mass spectra of serum proteins for a mass shift corresponding to addition of the oxidation moiety to methionine. After normalizing the number of methionine oxidation to the number of mass spectra corresponding to modified protein, we detected a greater abundance of methionine oxidation in glaucomatous samples than age-matched healthy controls.² This observation, along with the evidence of increased oxidative stress in glaucomatous tissues,^{5-9,20} motivated us to study oxidative stress-related

biomarker candidates in glaucomatous versus nonglaucomatous samples. For targeted analysis of oxidative stress-related biomarker candidates, this present study used specific ELISAs that require a small sample volume and allow simultaneous and quick analysis of a large batch of clinical samples with high sensitivity.

The oxidative stress-related biomarker candidates we studied in blood and aqueous humor samples included protein carbonyls. Protein carbonyl formation is an important marker for protein oxidation that results from free radical attack on amino acid side chains.²⁴ The carbonyl content of proteins increases with aging,^{25,26} and the accumulating carbonyls cause protein damage and dysfunction^{26,27} in several aging-related neurodegenerative disorders.^{25,28,29} The protein carbonyl content has also been found significantly elevated in the glaucomatous human donor retina⁷ and ocular hypertensive retinas in animal models.⁵ Based on growing experimental data, increased protein carbonyls in glaucomatous tissues support the likelihood of oxidative stress-related neurodegenerative and neuroinflammatory mechanisms in glaucoma.^{6,8,9,30}

Another oxidative stress-related biomarker candidate studied herein was AGEs. AGEs accumulate in all different compartments of the aged human eye, including the retina and optic nerve head.^{7,31} Although the pathogenic implications of AGEs in ocular diseases have initially been investigated in relation to diabetic retinopathy,³² it has later become clear that the role of AGEs in ocular damage is not restricted to diabetic retinopathy. Due to their synergism with oxidative stress, AGEs have been associated with all major ocular neurodegenerative diseases, including human glaucoma. Augmented accumulation of AGEs in the glaucomatous human donor retina and optic nerve head over age-matched nonglaucomatous controls may indicate an accelerated process of aging-related deterioration that likely accompanies neurodegeneration.⁷ However, it should be noted that AGEs mainly accumulate on long-lived proteins in specific tissues, such as lamina cribrosa in human glaucoma.⁷ The analysis of AGEs in blood or aqueous humor samples may therefore not necessarily reflect their tissue levels, yet circulating AGEs keep their biomarker potential.

Some previous studies of human samples have analyzed antioxidant enzymes in blood^{33,34} or aqueous humor^{33,35-38} and detected a decrease in the overall antioxidant capacity with glaucoma. Previous studies have also reported increased

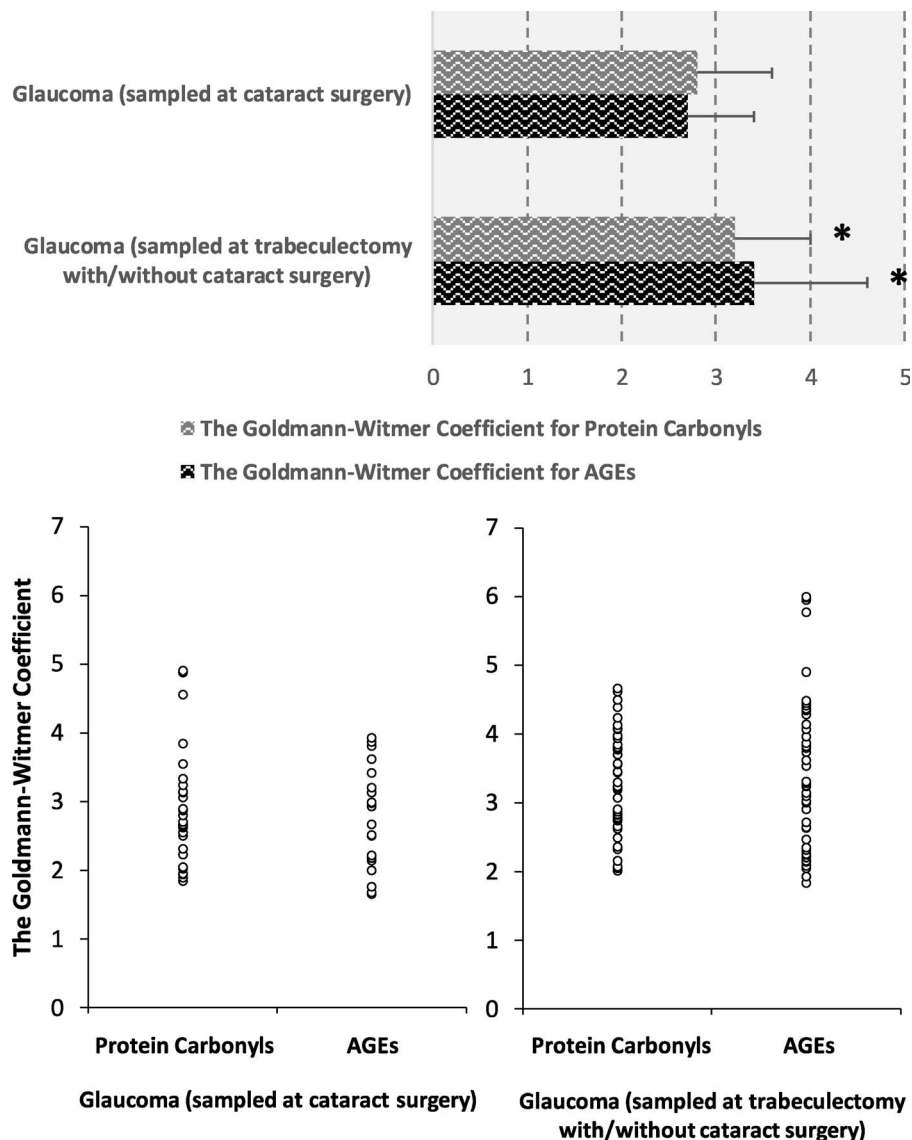


FIGURE 5. Oxidative stress-related biomarker candidates in glaucoma. The GWCs for protein carbonyls and AGEs were significantly higher in samples collected from glaucoma patients who underwent trabeculectomy (alone or combined with cataract surgery, $n = 66$) compared with glaucoma patients who underwent only cataract surgery ($n = 30$). Data are presented as mean \pm SD in *bar graphs*, and *univariate scatterplots* present data distribution. *Statistical significance; the Kruskal-Wallis 1-way ANOVA on ranks, $P = 0.015$ and $P = 0.03$ for protein carbonyls and AGEs, respectively.

protein carbonyls in blood samples^{39,40} and increased malondialdehyde, a lipid peroxidation end product, in blood^{39,41} or aqueous humor^{36,37} samples of patients with glaucoma. More recently, a systematic review of the published work and meta-analysis of 22 eligible studies have demonstrated an overall increase in oxidative stress markers in glaucoma.⁴² However, most of these studies have reported the measurements relative to sample volume without normalization to total protein concentration in blood or aqueous humor samples. This makes it difficult to obtain a meaningful comparison between multiple samples from glaucoma and control groups. Our study applied a normalization strategy and calculated the Goldman-Witmer coefficients to determine the relative amounts of protein carbonyls and AGEs in blood and aqueous humor samples with or without glaucoma.

Our approach for relative assessment of biomarker candidates in blood and aqueous humor samples should enable

gaining the more specific information. However, there are many challenges of biomarker studies, which mainly include interindividual variability, unavoidably. To minimize the effects of individual heterogeneity, we carefully selected our study groups. The glaucoma and control groups were matched and distribution-balanced for demographic variables, including age, sex, and race. Our exclusion criteria should also minimize any vulnerability related to individual differences in systemic or ocular diseases and their treatment. Because protein carbonyls and AGEs have been found elevated in other patient groups, including those with diabetes mellitus,^{43,44} such patients, as well as patients with other ocular diseases, were excluded from our study groups. In addition, our study participants did not have previous vitrectomy, endothelial keratoplasty, or cataract surgery, which have been suggested to increase oxidative stress in the anterior chamber.^{45,46} For consideration of any potential effect of IOP-lowering treatments (that typically included an altered combination of multiple eye

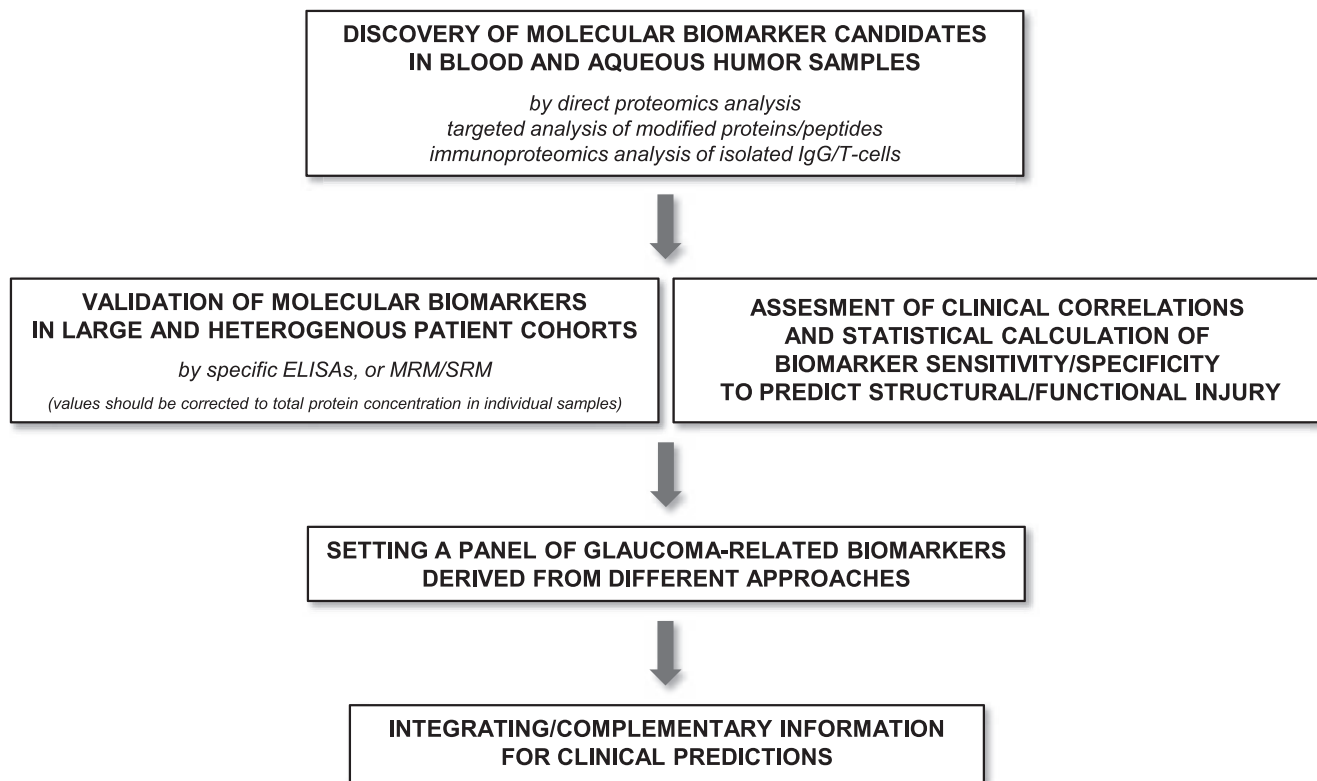


FIGURE 6. Working scheme toward protein/peptide biomarkers of glaucoma. The clinical validation phase after discovery of biomarker candidates aims to eliminate false positivity and calculate the biomarker sensitivity and specificity through the targeted analysis of candidate molecules in large and heterogeneous populations. This challenging process requires the collaborative efforts of basic scientists, physicians, and funding organizations. Due to etiologic complexity of glaucoma and significant variability among patients, instead of a single molecule, a panel of biomarkers including those derived from different approaches, such as analysis of blood samples for targeted molecules, analysis of oxidative stress-related biomarkers, analysis of isolated IgGs and T cells (perhaps also including the genetic/epigenetic markers), should provide integrating and complementary information for clinical predictions.

drops in all patients with glaucoma), however, further studies specifically designed by ethical guidelines are needed. Nevertheless, it is noteworthy that IOP-lowering medications, including beta blockers,^{47,48} alpha 2-adrenergic receptor agonist,^{49,50} prostaglandin analogs,^{51,52} or carbonic anhydrase inhibitors,^{53,54} would be expected to lower oxidative stress, not to increase, as we detected in the glaucoma group. With regard to the role for aqueous outflow in removal of the cytotoxic products generated through pathogenic processes, any effect of treatments reducing the aqueous inflow versus treatments increasing the aqueous outflow⁵⁵ also would be interesting to further study. Thus, many aspects stimulate additional studies to elucidate the influence of different variables on oxidative stress-related biomarkers, so that the usefulness of these promising biomarker candidates for glaucoma can be better valued.

An interesting observation that also awaits further validation in longitudinal studies was higher values of protein carbonyls and AGEs in glaucoma patients who underwent trabeculectomy (alone or combined) in comparison with glaucoma patients who underwent cataract surgery alone. This observation is interesting, because the distribution of moderate or advanced stages of injury was similar between these two subgroups of patients with glaucoma, and no significant relationship was detectable between the oxidative stress-related biomarker candidates studied and the level of IOP or the stage of glaucomatous injury (which is influenced by individual differences in etiological components, cellular stress duration, or susceptibility to injury). Although this

study was cross-sectional, because decision for trabeculectomy might have been due to uncontrolled IOP and/or progressing injury, the higher values of oxidative stress-related biomarker candidates in glaucoma patients who underwent trabeculectomy may be considered correlative to their progressive disease state. This observation seems consistent with previous studies that supported the oxidative stress-related mechanisms of neurodegeneration and neuroinflammation in human glaucoma and animal models.^{6-9,10,56} Besides glaucoma, the protein expression pattern in the ocular hypertensive human retina was suggestive of increased oxidative stress as a molecular risk factor for glaucoma development.⁵⁷ Moreover, the baseline age has been found as a major risk factor for glaucoma development in ocular hypertensive individuals,⁵⁸ which also may be related to oxidative stress-related pathogenic processes, because aging augments oxidative stress and oxidative stress is the key component of aging-related deterioration. These collectively warrant that the oxidative stress-related biomarker candidates studied herein, protein carbonyls and AGEs, may well serve as “glaucoma susceptibility markers” for clinical testing. Regarding their utility as surrogate endpoints for assessing the success of a new therapeutic intervention, oxidative stress-related biomarkers should well reflect a direct effect of antioxidant treatments or treatments targeting the mitochondrial dysfunction in glaucoma. In this regard, it would be of particular interest to analyze these oxidative stress-related biomarker candidates before and after antioxidant supplementation in glaucoma patients. It is similarly appealing to

further determine as to whether the lack of correlation between blood and aqueous humor levels of protein carbonyls (different than AGEs that exhibited a positive correlation between blood and aqueous humor levels) may suggest a higher specificity as a glaucoma-related biomarker. Particularly respecting the relation of protein carbonyls or AGEs to other diseases, such as diabetes mellitus,^{43,44} continued longitudinal analysis in larger and heterogeneous cohorts of patients, with or without glaucoma, should help proper calculation of biomarker sensitivity and specificity for clinical prediction of glaucoma and its progression.

Thus, many features arouse enthusiasm for oxidative stress-related molecules as susceptibility markers or surrogate endpoints in glaucoma, but their clinical utility remains to be further evaluated. Although our findings suggest the usefulness of oxidative stress-related biomarkers for assessing the glaucoma risk or monitoring treatment efficacy, because our study groups did not include ocular hypertensive patients, potential usefulness of these biomarker candidates for initial diagnosis of glaucoma needs further studies.

It should be clarified that molecular biomarkers may not necessarily be relevant to specific pathology, and some biomarkers may be an indirect or even remote sign of a molecular interaction. However, as discussed above, oxidative stress-related biomarker candidates present epidemiologic (aging, accompanied by oxidative stress, is a major risk factor for glaucoma^{58,59}), pathophysiologic (oxidative stress, evident in glaucomatous tissues, may result in neurodegenerative and neuroinflammatory outcomes^{6,56}), and therapeutic (anti-oxidant treatment may provide neuroprotection and immunomodulation in glaucoma⁹) relevance to glaucoma. There is an additional aspect to clarify that is independent from the value of oxidative stress-related molecules for clinical predictions, which is the focus of biomarker studies, specifically focused experimental studies should further evaluate whether they are a consequence or cause (or perhaps both) of the disease.

In conclusion, this study presenting a glaucoma-related increase in blood and aqueous humor levels of protein carbonyls and AGEs delivers promising information for future clinical applications. Presented findings encourage longitudinal studies of larger patient groups to analyze clinical correlations with structural and functional outcomes of glaucoma and assess the diagnostic and prognostic value of protein carbonyls and AGEs as glaucoma biomarkers. Figure 6 schematizes the workflow toward glaucoma-related molecular biomarkers. It is important to emphasize that due to etiologic complexity of glaucoma and significant variability among patients, instead of a single molecule, a panel of biomarkers including those derived from different approaches, such as analysis of blood samples for targeted molecules, analysis of oxidative stress-related biomarkers, analysis of isolated IgGs and T cells (perhaps also including the genetic/epigenetic markers), should provide integrating and complementary information for clinical predictions. We hope that once validated, molecular biomarkers can offer a clinical tool to facilitate glaucoma diagnosis, predict disease prognosis, and monitor treatment responses in glaucoma patients.

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