Age-related aqueous humor (AH) and lens epithelial cell/capsule protein carbonylation and AH protein concentration in cataract patients who have pseudoexfoliative diseases

Garyfallia Papadopoulou, Dimitrios Zisimopoulos, Electra Kalaitzopoulou, Olga E. Makri, Foteini N. Tsapardoni, Constantinos D. Georgakopoulos, Christos D. Georgiou

(The second and third authors contributed equally to this work.)

1Department of Biology, University of Patras, Patras, Greece; 2Department of Ophthalmology, University of Patras, Patras, Greece

Purpose: The aim of this study is to investigate the age-correlation of oxidative stress (OS, assessed by the accumulative OS damage marker protein carbonyls) in aqueous humour (AH; together with protein concentration) and lens epithelial cells plus capsule (LECs/capsule) in patients with cataract (CAT), and also suffering from pseudoexfoliation syndrome (PEX), primary open-angle glaucoma (POAG) and pseudoexfoliation glaucoma (PXG).

Methods: AH samples from 78 male/female patients (21, 20, 19 and 18 with CAT, PEX, PXG, and POAG, respectively), and LECs/capsule samples from 104 male/female patients (34, 32, 18, and 20 with CAT, PEX, PXG and POAG, respectively) were collected during phacoemulsification CAT surgery. Average protein carbonyl concentrations were measured in patients grouped in 5-year age intervals (ranging from 56-60 to 86-90). The non-overlapping age ranges and numbers of the tested subjects did not allow comparative follow up studies for the tested diseases.

Results: There is an age-dependent increase of protein carbonyls in AH (nmol mg⁻¹ protein and ml⁻¹), and in the order CAT<PEX<POAG<PXG. The slow rate of change of protein carbonyls strongly suggests a long-term implication of OS in ocular disease pathogenesis. Additionally, protein concentration in AH accumulates in the order CAT<PEX<POAG<PXG but is not age-related. An age-dependent increase of protein carbonyls (nmol mg⁻¹ protein) is also observed in LECs/capsule, and in the order CAT<PEX<POAG<PXG.

Conclusions: The present study shows for the first time an age-increased OS-induced protein damage (protein carbonyl formation) in the AH and LECs/capsule of CAT patients with PEX, POAG or PXG. The slow rate of change of protein carbonyls strongly suggests a long-term implication of OS in ocular disease pathogenesis. Additionally, protein concentration levels in the AH of CAT patients increase independently of age, and in same as with protein carbonyls increasing order levels for CAT<POAG<PXG in AH and LECs/capsule. This may suggest a protein cross-diffusion taking place between AH and LECs/capsule, most likely originating from PEX deposition and/or necrotic/apoptotic LECs/capsule. Moreover, the findings of the present study establish the use of protein carbonyls (together with a methodology for their more accurate quantification, which overcomes serious unreliability problems of past methods) as an age accumulative marker of OS damage, for future studies that investigate long-term OS involvement in pseudoexfoliative ocular disorders.

Although the pathogenesis of cataract, pseudoexfoliation (PEX), secondary open-angle glaucoma (PXG), and primary open-angle glaucoma (POAG) is not fully understood, there is growing evidence that oxidative stress plays an important role, given that it is implicated in the etiology of many ocular diseases [1-9], such as PEX [10-14], PXG [15], and POAG [16,17]. However, the central question is how reliably oxidative stress has been measured in these studies. The most reliable evaluation of oxidative stress is via the direct quantification of the reactive oxygen species (ROS) components. The most representative are the superoxide and hydroxyl free radicals. However, the in vivo quantification of these free radicals in patients is not applicable due to bioethical restrictions. Alternatively, indirect methods for assessing oxidative stress have been applied and focus mainly on the oxidative damage ROS cause in biomolecules, such as lipids, proteins, and DNA. However, more reliable are those oxidative modifications that are not repairable and thus, accumulated, with protein carbonylation the most representative.

This necessitates the investigation of past experimental approaches in their attempt to correlate oxidative stress and ocular diseases in humans in both the epithelial cells plus the capsule (LECs/capsule) and in the aqueous humor (AH). LECs/capsule exposure to various oxidative stress factors has been associated to several conditions, with CAT being more extensively studied [6,18-22]. Oxidative stress development in LECs/capsule has been assessed by certain biological indicators: activity of certain antioxidant enzymes...
OMST1 (superoxide dismutase, SOD, catalase, CAT) [23,24]; genes encoding antioxidant defense enzymes (aldehyde dehydrogenase 1-ALDH1A1, OMIM 603687; microsomal glutathione S-transferase 1-MGST1, OMIM 138330; superoxide dismutase 2-SOD2, OMIM 147460) [25]; proteins involved in multiple protection paths (e.g., metallothioneins) [26,27]; levels of non-enzymatic antioxidants (reduced and oxidized glutathione, GSH and GSSG, respectively) [12,28]; DNA oxidative damage (8-OH-dG, 8-oxo-Gua, DNA strand breaks, pyrimidine dimers, and telomeres [29-34]); lipid peroxidation (malondialdehyde [MDA]) [12,28]; and protein oxidative modification (i.e., carbonyl groups) [21,28].

Similarly, oxidative stress changes in the AH may also be associated with ocular diseases [6,20,35-37]. Oxidative stress in human aqueous humor has been also assessed with certain biochemical indicators: DNA oxidative damage evaluation (8-OH-dG, 8-oxo-Gua) [17,38]; lipid peroxidation (MDA, 8-iso-PGF_2α, conjugated dienes, and lipofuscin-like fluorescent end products) [10,39-42]; antioxidant enzymes (SOD, CAT, glutathione peroxidase [GPx], and arylesterase, paraoxonase) [14,39,40,42-46]; prooxidant enzymes (xanthine oxidase) [47]; antioxidant enzyme gene expression (for SOD and glutathione transferase [GST]) [48]; non-enzymatic antioxidants (ascorbic acid, vitamin E, selenium, uric acid, GSH, and GSSG) [3,10,11,46,49-51]; superoxide radical (O_2^-) scavenging activity [52]; total antioxidant capacity [14,17,39,41,43,50,53-55]; total oxidant status [14,54]; oxidant/antioxidant balance [44]; ROS levels (e.g., H_2O_2) [44,52]; total inorganic/lipid/protein/nucleic acid hydroperoxides [52]; and protein oxidative modification (carbonyl groups) [3,56,57].

Oxidative carbonylation of proteins is the most reliable indicator of oxidative stress for the following reasons: Proteins that have been carbonylated are accumulated because they are not readily recognized by proteasomes. Although moderately carbonylated proteins are degraded by the proteasomal system [58], the heavily carbonylated ones tend to form high-molecular-weight aggregates that escape proteolytic degradation by the proteasome [59,60]. This is possibly because carbonylated protein aggregates exhibit structural constraints that prevent recognition of the aggregates by the catalytic sites inside the cylinder of the proteasome complex. Such aggregates accumulate in cells as damaged or unfolded proteins [61]. Moreover, these carbonylated protein aggregates can inhibit proteasome activity [60], cause a progressive further increase in protein aggregation and cross-linking in non-dividing (post-mitotic) cells, and may eventually induce apoptosis [61,62]. Carbonylated aggregates can also become cytotoxic and have been associated with numerous diseases, including cataractogenesis [63,64]. Another reason for the limited carbonylated protein withdrawal by proteasomes is that they can become the target of carbonylation (e.g., subunit S6 ATPase) and other oxidative modifications (e.g., glycoxidation and modification with lipid peroxidation products) [65], ending up (e.g., 26S proteasome) in decreased activity [66]. Other advantages of using carbonylated proteins as indicators of oxidative stress is their chemical stability which allows easy detection even after long storage [67] and the existence of many assays for detection [68-75].

Protein oxidative damage is exerted on ROS-labile aromatic and sulfur-containing amino acids in proteins. This is especially relevant to lens proteins as they contain large amounts of tryptophan, tyrosine, phenylalanine, histidine, methionine, and cysteine amino acids, which can be modified by ROS, forming adducts and aggregates and affect enzyme function [76]. These events may be related to an increase in the protein concentration in AH, which has been observed in some studies on cataract [45], PEX [15,77-81], and PXG [78,82-84].

The use of protein carbonylation to assess oxidative stress in ocular diseases is limited, and the methodologies employed quite unreliable. Specifically, protein carbonyl levels were assessed in the lens epithelial cell fraction of smokers or diabetic patients with cataract [21], in non-cataractous human LEC cultures [28], and in the AH of patients with PEX [57] with an unreliable version of the 2,4-dinitrophenylhydrazine (DNPH)-based assay [85,86]. In another study, carbonyl groups were measured semiquantitatively (with enzyme-linked immunosorbent assay [ELISA] using an anti-DNP antibody) in the AH of patients with cataract [56]. These studies did not investigate oxidative stress as a function of a patient’s sex and age.

The present study proposes the very reliable oxidative stress marker protein carbonyls, and the methodology for its quantitative assessment, in order to evaluate more accurately the role of oxidative stress in pseudoexfoliative ocular diseases. The study specifically evaluates the protein carbonyl levels, as a function of age and sex, in the AH and LECs/capsule of CAT patients suffering also from PEX, POAG or PXG, and also correlates the protein carbonyls in AH with protein concentration. Since some of the carbonyls measured in the LEC samples may have come from oxidized proteins in the capsule basement membrane, this is why these samples is more appropriate to be referred as LECs/capsule.
METHODS

Reagents: Bovine serum albumin (BSA), dithiothreitol (DTT), ethyl acetate, sodium deoxycholate (DOC), urea, and 2,4-dinitrophenyldihydrazine (DNPH prod. no. D198501, ≥97%, ≥15% water) were obtained from Sigma-Aldrich (Taufkirchen, Germany); acetic acid was obtained from Carlo Erba, Val de Reuil, France; coomassie brilliant blue G-250 (CBB G-250) was obtained from SERVA (Heidelberg, Germany); ethanol absolute (EtOH), hexane, hydrochloric acid (HCl, concentrated, 37% w/w, 12 M), sodium hydroxide (NaOH), trichloroacetic acid (TCA), Na₂PO₄·12H₂O was obtained from MERCK (Darmstadt, Germany); sodium dodecyl sulfate (SDS) was obtained from Bio-Rad Laboratories (Hercules, CA); Tris-base was obtained from MP Biomedicals (Illkirch Cedex, France). All other chemicals used were reagent grade.

Study design: All human studies were performed following the guidelines of the Declaration of Helsinki and were approved by the Ethics Committee of the University Hospital of the University of Patras. All participating patients provided informed written consent for use of their biologic material in the present study. This experimental material consisted of AH and lens capsule samples that were obtained (between July 2015 and March 2016) from patients with cataract (the control group) and from patients with cataract and PEX, POAG, or PXG. The study adhered to the ARVO statement on human subjects.

All patients underwent phacoemulsification with posterior chamber intraocular lens (IOL) implantation. Before surgery, patients underwent a complete ophthalmologic examination, including applanation tonometry, gonioscopy, slit-lamp examination under complete pharmaceutical mydriasis for the identification of pseudoexfoliative material on the anterior chamber structures, and dilated fundus examination to evaluate any possible posterior segment pathology (e.g., diabetic retinopathy, age-related macular degeneration, or glaucomatous damage of the optic nerve). Exclusion criteria that might have influenced the analysis of the present study were pathological myopia, laser treatment, history of previous intraocular surgery or uveitis, any posterior segment pathology (e.g., related macular degeneration), or any other systemic disease (e.g., diabetes). Patients under treatment with topical or systemic nonsteroidal anti-inflammatory drugs or steroids were also excluded.

Patients were all of Caucasian ancestry. Patients with PXG and POAG were all under treatment with topical antiglaucoma agents; those with PXG had a mean intraocular pressure (IOP) of 19.1 ± 2.50 mmHg and had been treated with a median number of two agents (range one to three). Those with POAG had an IOP of 17.6 ± 3.20 mmHg and were treated with a median number of one agent (range one to three). Healthy patients with cataract had a mean IOP of 15.2 ± 2.70 mmHg. Data from patients (averages from males and females in approximately equal numbers) were derived from 5-year interval age groups: Total AH samples were 78, out of which 21, 20, 19 and 18 came from patients with CAT (year range/number of patients: 61-65/5, 66-70/5, 71-75/5, 76-80/6), PEX (year range/number of patients: 71-75/5, 76-80/5, 81-85/5, 86-90/5), PXG (year range/number of patients: 71-75/6, 76-80/6, 81-85/7) and POAG (year range/number of patients: 61-65/6, 66-70/6, 71-75/6), respectively. Moreover, a total of 104 lens capsule samples was analyzed; 34, 32, 18 and 20 from patients with CAT (year range/number of patients: 56-60/5, 61-65/5, 66-70/6, 71-75/6, 76-80/7, 81-85/5), PEX (year range/number of patients: 71-75/7, 76-80/11, 81-85/8, 86-90/6), PXG (year range/number of patients: 71-75/6, 76-80/6, 81-86/6) and POAG (year range/number of patients: 66-70/6, 76-80/7, 86-90/7), respectively. Minimum number of patients per measured 5-year interval age group was no less than 5. The non-overlapping 5-year interval age and the limited patient numbers did not allow at present comparative follow up studies for the tested diseases.

The AH sample (30 to 70 μl) was drawn by aspiration from the anterior chamber through a corneal side port with a tuberculin syringe (with a 27-gauge needle). Special care was taken to avoid needle contact with the iris, lens, and corneal endothelium. The second sample was a piece (average diameter about 5.5 mm) of the central portion of the anterior lens capsule, which was obtained through capsulorhexis, removed immediately, and washed with a sterile solution to remove any blood and viscoelastic material residues. Both samples were collected in 1.5-ml microcentrifuge tubes and immediately stored at −80 °C. The analysis was performed within a week after initial storage.

Determination of protein carbonyls and protein concentration in LECs/capsule and AH:

Lens capsule sample treatment—Each lens capsule sample was sonicated for 30 sec (using the UP50H sonicator by Hielscher Ultrasonics GmbH, Teltow, Germany, connected with a 2-mm diameter MS2 microtip) in the presence of 92 μl double-distilled water (ddH₂O) and 8 μl 1% DOC stock solution (made in ddH₂O). Then, the sonicated homogenate was mixed with 300 μl ddH₂O, and the mixture was centrifuged at 16,000 ×g for 5 min at 4 °C to remove any insoluble material. The resulting supernatant was incubated for 10 min at 25 °C, followed by the addition of 46 μl ice-cold 100% TCA (final 10% TCA, 0.019% DOC), incubation for 15 min in an
ice-water bath, and centrifugation at 16,000 ×g for 5 min at 4 °C. This DOC-TCA protein precipitation procedure is a modification of a procedure previously reported [87-89], which is able to precipitate minute quantities of proteins (as low as 3 µg) with ≥90% recovery. The resulting protein pellet was dissolved in 50 µl 50 mM NaOH containing 4 M urea and stored at −80 °C for measuring protein carbonyls and protein concentration.

AH sample treatment—The AH samples were also subjected to the DOC-TCA protein precipitation procedure as follows. Each sample was adjusted to final 0.02% DOC (using the 1% DOC stock) and incubated for 10 min at 25 °C. Then, each DOC-treated sample was adjusted to final 10% TCA (using the ice-cold 100% TCA stock in a proportion of, e.g., 115 µl per 1,000 µl), incubated for 15 min in an ice-water bath, and centrifuged at 16,000 ×g for 5 min at 4 °C. The resulting protein pellet was dissolved in 200 µl 50 mM NaOH containing 4 M urea and stored at −80 °C for the quantification of the protein carbonyls and the protein concentration.

Protein concentration—The concentration of the NaOH-urea solubilized total isolated proteins from each AH and lens capsule sample was assayed with a Coomassie Brilliant Blue G-250-based ultrasensitive photometric assay [90]. Given the small volumes of the NaOH-urea solubilized LECs/capsule and AH samples (50 µl out of total 200 µl were used for this assay; the rest was used for protein carbonyl determination), the standard or microplate version of this assay was followed.

Protein carbonyl determination—Oxidative damage to AH and LECs/capsule proteins was assessed by the quantification of their carbonyl groups via their derivatization (hydrazine formation) with DNPH via a new photometric ntrDNPH assay [89]. By following the assay’s 3.1.2. Procedure section, 50 to 150 µl NaOH-urea-solubilized LECs/capsule and AH samples (S) were brought to 200 µl with 50 mM NaOH containing 4 M urea in a microcentrifuge tube. In two more microcentrifuge tubes (one for the reagent blank, RB, and the other for the reagent used for zeroing the spectrophotometer, designated RZ), 200 µl 50 mM NaOH to 4M urea were added; a sample blank was not needed. Then, S, RB, and RZ were treated as in step 2 in the procedure and the following steps [89].

Statistical analysis: Age and ocular disease–related straight line data in Figure 1A,B, Figure 2, and Figure 3 and their corresponding slopes and ± standard error of the mean (SEM) were determined with linear regression analysis, while the slopes’ multiple comparisons tests between diseases were statistically analyzed for differences (a p value of less than 0.05 was considered statistically significant) using one-way ANOVA (ANOVA) via Tukey’s multiple comparisons test [91]. The latter was also used for determining the mean values ± standard error of the mean (SEM) in Figure 3 (see Table 1). Statistical calculations were performed using the linear regression analysis tools in GraphPad Prism (Mac version 7.0a by GraphPad Software, San Diego, CA).

RESULTS
LEC/capsule and AH average carbonyl value for each 5-year interval age group in patients with CAT, PEX, PXG, and POAG was plotted as a curve (fitted straight line) vs the studied 5-year-age intervals. Age-correlated protein carbonyl content was expressed as the resulting straight line curve slope (representing the carbonyl content change versus increasing 5-year age interval), and defined as nmoles carbonyl groups

Figure 1. Age-related association of protein carbonyls in aqueous humor (AH). The association is expressed as a linear function of the change (rate) in the protein carbonyl concentration (nmoles ml⁻¹ and mg⁻¹ in A and B, respectively) versus the patient’s 5-year age intervals. Data are presented as mean ± standard error of the mean (SEM).
Figure 2. Age-related association of protein carbonyls in lens epithelial cells plus the capsule (LECs/capsule). The association is expressed as a linear function of the change (rate) in the protein carbonyl concentration (nmoles mg⁻¹) versus the patient’s 5-year age intervals. Data are presented as mean ± standard error of the mean (SEM).

Figure 3. Age-related association of protein concentration in AH. The association is expressed as the protein concentration (mg ml⁻¹) versus the patient’s 5-year age intervals. Note that the straight lines are nearly horizontal, and the corresponding slopes are near zero, showing no age correlation. Data are presented as mean ± standard error of the mean (SEM).
mg⁻¹ aqueous protein vs age for AH and LECs/capsule, while for AH as nmoles carbonyls ml⁻¹ versus age. In addition, the study investigated the age-correlated association of protein concentration in AH.

**Change in protein carbonyl levels versus age and sex in AH and LECs/capsule:** Protein carbonyl-level data are presented for each age interval per disease (cataract, PEX, POAG, and PXG) in Figure 1 and Figure 2. It was found that there was an age-dependent (and sex independent; data not shown) increase in the protein carbonyl levels per milliliter in the AH (Figure 1A) and per milligram of protein in the AH (Figure 1B) and in the LECs/capsule (Figure 2) of patients with PEX, POAG, and PXG, using those with cataract as control. Comparing the carbonyl-level change rates versus increasing age (i.e., the slopes of the straight lines in Figure

### Table 1. Protein carbonyl level and concentration change versus age in CAT, PEX, POAG and PXG.

| Sample | Ocular Disease | Experimental values |
|--------|----------------|---------------------|
|        |                | Tukey's multiple comparisons test¹ | Adjusted P-value / samples (n1/n2) | Significance degree |
|        |                |                     |                                    |                     |
| A. Rate of protein carbonyl level increase per age versus disease |
|        |                | Slope rate values (± SE) from Figure 1A (in nmoles carbonyls ml⁻¹ per 5-year interval) | CAT vs. PEX | <0.0001 (33/31) | **** |
| AH     | CAT            | 0.803 ± 0.060       | CAT vs. POAG                       | <0.0001 (33/19)    | **** |
|        | PEX            | 1.770 ± 0.110       | CAT vs. PXG                        | <0.0001 (33/17)    | **** |
|        | POAG           | 2.049 ± 0.127       | PEX vs. POAG                       | =0.2021 (31/19)    | ns² |
|        | PXG            | 2.489 ± 0.096       | PEX vs. PXG                        | <0.0001 (31/17)    | **** |
|        |                | from Figure 1B (in nmoles carbonyls mg⁻¹ protein per 5-year interval) | CAT vs. PEX | <0.0001 (20/19) | **** |
| AH     | CAT            | 4.939 ± 0.310       | CAT vs. POAG                       | =0.0328 (20/17)    | *  |
|        | PEX            | 8.768 ± 0.423       | CAT vs. PXG                        | =0.2351 (20/18)    | ns  |
|        | POAG           | 6.403 ± 0.476       | PEX vs. POAG                       | =0.0002 (19/17)    | *** |
|        | PXG            | 5.921 ± 0.234       | PEX vs. PXG                        | <0.0001 (19/18)    | **** |
| LECs/  | CAT            | 1.506 ± 0.116       | CAT vs. PEX                        | =0.1225 (20/19)    | ns  |
| capsule| PEX            | 4.524 ± 0.137       | CAT vs. POAG                       | <0.0001 (33/19)    | **** |
|        | POAG           | 5.048 ± 0.139       | CAT vs. PXG                        | <0.0001 (33/17)    | **** |
|        | PXG            | 8.364 ± 0.322       | POAG vs. PXG                       | =0.1425 (31/19)    | ns  |
|        |                | from values for horizontal straight lines in Figure 3 (in mg protein ml⁻¹) | CAT vs. PEX | <0.0001 (20/17) | **** |
| AH     | CAT            | 0.16 ± 0.0074       | CAT vs. POAG                       | <0.0001 (20/17)    | **** |
|        | PEX            | 0.23 ± 0.0093       | CAT vs. PXG                        | <0.0001 (20/18)    | **** |
|        | POAG           | 0.34 ± 0.0136       | PEX vs. POAG                       | =0.0003 (19/17)    | *** |
|        | PXG            | 0.42 ± 0.0162       | PEX vs. PXG                        | <0.0001 (19/18)    | **** |
|        |                | POAG vs. PXG        | PEX vs. PXG                        | =0.0001 (19/18)    | **** |
|        |                |                     | POAG vs. PXG                       | =0.0303 (17/18)    | *   |

¹Tukey's multiple comparisons test and statistical significance at p<0.05 (more details in the “Statistical analysis” section). ²ns designates not significant.
1 and Figure 2) among the studied ocular diseases (shown and statistically analyzed in Table 1), it can be concluded that the carbonyl rate change values per milligram of protein are higher than those for the control cataract, and in the increasing disease order cataract<PXG=~POAG<PEX and cataract<PEX=~POAG<PXG for AH and LECs plus the capsule, respectively (Table 1). In addition, the carbonyl level change per volume (milliliter) for AH was also higher than that for the control cataract and in the increasing order cataract<PEX=~POAG<PXG (Table 1).

Protein concentration changes in AH versus age and sex: The present study also showed that there was an increase in the protein concentration in the AH of patients with PEX, POAG, and PXG, compared to the control patients (with cataract). This increase appears in the increasing order cataract<PEX=~POAG<PXG (deduced from data in Table 1). This increase was solely dependent on the ocular disease type, and not on the patient’s age (and sex; data not shown), and this is shown by the fact that the slopes of the straight lines in Figure 3 are near zero. Interestingly, the observed increasing order of the protein concentration in AH is in agreement with that of the protein carbonyl level change per milligram of protein and per volume (milliliter) for cataract, POAG, and PXG.

DISCUSSION

The present study investigated for the first time the age (and sex)-related association of ROS in the AH and LECs/capsule of CAT patients that also suffer from PEX, POAG, or PXG. The study used the specific oxidative marker of protein carbonylation, which is a very reliable representative of high oxidative stress mainly due to its intracellular accumulation as not being repairable. It should be noted that it became possible to quantify this marker with high reliability and sensitivity only until recently [89].

Until now, the association of ocular diseases with oxidative stress via protein carbonyls in AH was limited to three studies [3,56,57], which used methodologies that are known to have serious reliability problems [89]. This may be one reason that the findings of these studies are partially correlated to those of the present study. Other reasons are that (a) protein carbonyl levels were measured only in PEX (also using cataract as control), (b) these levels were not correlated with age (or sex), and (c) measurements were performed with either ELISA [56] or a version of the standard DNPH photometric assay [57], both known to be unreliable [89].

Specifically, Yagci et al. showed a 66% increase in the protein carbonyl concentration (per volume) over control cataract in AH of patients with PEX with an age span of 66.9 ± 8.30 years [57]. This result is in agreement with the approximate 60% increase observed in the present study (after the carbonyl values were averaged for the similar patient age span 56 to 70 years). However, the protein carbonyl level increase (0.87 nM) over that of the cataract control derived from the data presented in the Yagci et al. study is about 2,300-fold lower (in absolute number) than that for PEX in the present study (Figure 1A). Nonetheless, this carbonyl value (0.87 nM) is questionable because it is about 1,000-fold lower than the sensitivity limit 0.91 µM of the standard DNPH assay (determined at minimum absorbance 0.02 and for DNPH molar absorptivity 22,000 M⁻¹ cm⁻¹ at 360 nm [85,86]). Similarly, Alamdari et al. showed a 47% increase in the protein carbonyl concentration per milligram of AH protein (over control patients with cataract) in patients with PEX of unspecified, however, age [56]. This is also in agreement with the approximate 48% increase using the average value of the patients tested in the present study (covering an age span of 71 to 90 years; Figure 1B). However, the protein carbonyl increase over control derived from the data from the Alamdari et al. study in absolute number (0.32 nmol mg⁻¹) is about 40-fold lower (Figure 1B). The present study, however, for the first time presented systematic data for the carbonyl rate change values per AH milligram of protein and volume in the increasing disease order cataract<PXG=~POAG<PEX and cataract<PEX=~POAG<PXG, respectively (Table 1).

Similarly, there are no studies available on the association of PEX, POAG and PXG neither with oxidative stress (via protein carbonyls) nor with age (and sex) in patients’ LECs/capsule. Thus, there are no data from other studies to corroborate the findings of the present study that there is an age-related increase of protein carbonyl levels per mg protein and in the increasing order CAT<PEX=~POAG<PXG (Figure 2). Previous studies limited protein carbonyl assessment in smokers or diabetic patients with cataract (an age group of 58 to 75 years [21]) and in non-cataractous human LEC cultures [28], in both cases determined with an unreliable version of the DNPH-based assay [89].

The present study also investigated the association of the protein concentration in AH and found that it is not age-related and increased among the studied ocular diseases in the following order cataract<PEX=~POAG<PXG (Figure 3). Interestingly, this increasing order in AH is in agreement with that of the protein carbonyl level change per volume for POAG and PXG. Most of the studies on AH-associated ocular diseases have determined the protein concentration in one or more of the same diseases [15,77-80,82-84,92-102]. The only study of patients who had the same diseases (male and female, non-age correlated, and
using an age mean value of 72.1 ± 2.7 years) [78] reported a different order (cataract<POAG<PEX<PXG) than that of the present study. However, when grouping in the same disease type and averaging the protein concentration values reported by the previous studies, their resulting mean values showed an increasing protein concentration order (cataract<PEX<POAG<PXG with corresponding values 0.28<0.326<0.431<0.473 mg ml⁻¹), that coincides (in terms of increasing order and absolute values) with those of the present study.

LECs/capsule and AH are of great interest for oxidative stress related studies in ocular diseases since LECs/capsule are strongly metabolizing and in contact with AH, which is also involved in metabolic exchange (i.e., supplies nutrients and oxygen through diffusion, removes metabolic wastes etc.) [103,104]. Moreover, pseudoexfoliation disorders are characterized by abnormal fibrillary material production and its deposition in the anterior segment, such as the iris, lens surface, ciliary body, zonula, trabecular meshwork, and corneal endothelium, which can lead to the development of PXG and POAG.

The fact that the age-dependent increasing rate of change in the protein carbonyl levels (in AH and LECs/capsule) and the age-independent increase of the protein levels (in AH) coincide in increasing order with the increasing levels of protein carbonyls at a disease order CAT<POAG<PXG (with the levels in PEX being variable; Table 1), the following can be suggested: The causative factors of oxidative stress in CAT lens may produce proteins (as part of the deposited PEX material or possibly originating from necrotic/apoptotic LECs/capsule), with some of them being carbonylated and possibly become dissolved in the AH and also cross-diffused between AH and LECs/capsule.

Concluding, the present study correlates, for the first time, increasing age-associated oxidative stress in the AH and LECs/capsule of patients with CAT, PEX, POAG and PXG. The relatively slow age-related rate of change observed for the levels of protein carbonyls in the AH and LECs/capsule of patients with the tested ocular diseases, together with the accumulative nature of protein carbonylation, strongly suggest a long-term implication of OS in their pathogenesis. Moreover, the findings of the present study establish the use of protein carbonyls (together with a new methodology for their more accurate quantification, which overcomes serious unreliability problems of past methods) as an age accumulative marker of OS damage. Nonetheless, protein carbonyls assess only indirectly the long-term association of OS with the pathogenesis of each of the aforementioned pseudoexfoliative ocular disorders. Moreover, AH and LECs/capsule extremely small sample size (30-60µl and 5mm diameter single-layer cells, respectively) limits the simultaneous complementary testing, ideally in each sample, of other indirect OS markers. Therefore, this association needs to be verified also by direct markers and initiators of OS, such as the superoxide and hydroxyl free radicals. We plan to conduct such studies upon finalizing the development of specific methodologies for the in vivo quantification of these free radicals.

ACKNOWLEDGMENTS

The present study was institutionally supported (by the Biology Department of the University of Patras, Greece).

REFERENCES

1. Zhou L, Li Y, Yue BY. Oxidative stress affects cytoskeletal structure and cell-matrix interactions in cells from an ocular tissue: the trabecular meshwork. J Cell Physiol 1999; 180:182-9. [PMID: 10395288].
2. Izzotti A, Saccà SC, Cartiglia C, De Flora S. Oxidative deoxyribonucleic acid damage in the eyes of glaucoma patients. Am J Med Sci 2003; 114:638-46. [PMID: 12798451].
3. Campochiaro PA, Strauss RW, Lu L, Hafiz G, Wolfson Y, Shah SM, Sophie R, Mir TA, Scholl HP. Is there excess oxidative stress and damage in eyes of patients with retinitis pigmentosa? Antioxid Redox Signal 2015; 23:643-8. [PMID: 25820114].
4. Truscott RJ, Augusteyn RC. Oxidative changes in human lens proteins during senile nuclear cataract formation. Biochim Biophys Acta 1977; 492:43-52. [PMID: 861252].
5. Varma SD, Chand D, Sharma YR, Kuck JF Jr, Richards RD. Oxidative stress on lens and cataract formation: role of light and oxygen. Curr Eye Res 1984; 3:35-57. [PMID: 6360540].
6. Spector A. Oxidative stress-induced cataract: mechanism of action. FASEB J 1995; 9:1173-82. [PMID: 7672510].
7. Spector A. Review: Oxidative stress and disease. J Ocul Pharmacol Ther 2000; 16:193-201. [PMID: 10803430].
8. Ottonello S, Foroni C, Carta A, Petrucco S, Maraini G. Oxidative stress and age-related cataract. Ophthalmologica 2000; 214:78-85. [PMID: 10657746].
9. Beebe DC, Holekamp NM, Shui YB. Oxidative damage and the prevention of age-related cataracts. Ophthalmic Res 2010; 44:155-65. [PMID: 20829639].
10. Koliakos GG, Konstas AG, Schlotzer-Schrehardt U, Hollo G, Katsimbris IE, Georgiadis N, Ritch R. 8-Isoprostaglandin F2a and ascorbic acid concentration in the aqueous humour of patients with exfoliation syndrome. Br J Ophthalmol 2003; 87:353-6. [PMID: 12598453].
11. Gartaganis SP, Georgakopoulos CD, Patsoukis NE, Gotsis SS, Gartaganis VS, Georgiou CD. Glutathione and lipid peroxide changes in pseudoexfoliation syndrome. Curr Eye Res 2005; 30:647-51. [PMID: 16109644].
12. Gartaganis SP, Patsokis NE, Nikolopoulos DK, Georgiou CD. Evidence for oxidative stress in lens epithelial cells in pseudoexfoliation syndrome. Eye (Lond) 2007; 21:1406-11. [PMID: 17001325].

13. Yagci R, Gurel A, Ersoz I, Keskin UC, Hepsen IF, Duman S, Yigitoglu R. Oxidative stress and protein oxidation in pseudoexfoliation syndrome. Curr Eye Res 2006; 31:1029-32. [PMID: 17169841].

14. Dursun F, Vural Ozec A, Aydin H, Topalkara A, Dursun A, Toker MI, Erdogan H, Arici MK. Total oxidative stress, paraoxonase and arylesterase levels at patients with pseudoexfoliation syndrome and pseudoexfoliative glaucoma. Int J Ophthalmol 2015; 8:985-90. [PMID: 26558214].

15. Schlotzer-Schrehardt U. Oxidative stress and pseudoexfoliation glaucoma. Klin Monatsbl Augenheilkd 2010; 227:108-13. [PMID: 20155654].

16. Aslan M, Cort A, Yucel I. Oxidative and nitrative stress markers in glaucoma. Free Radic Biol Med 2008; 45:367-76. [PMID: 18489911].

17. Sorkhabi R, Ghorbaniaghjoo A, Javadzadeh A, Rashtchizadeh N, Moharrery M. Oxidative DNA damage and total antioxidant status in glaucoma patients. Mol Vis 2011; 17:41-6. [PMID: 21245957].

18. Li WC, Kuszak JR, Dunn K, Wang RR, Ma W, Wang GM, Spector A, Leib M, Cotlar AM, Weiss M, Espj J, Howard G, Farris RL, Auran J, Donn A, Hofeldt A, Mackay C, Merriam J, Mittl R, Smith TR. Lens epithelial cell apoptosis appears to be a common cellular basis for non-congenital cataract development in humans and animals. J Cell Biol 1995; 130:169-81. [PMID: 7790371].

19. Li WC, Spector A. Lens epithelial cell apoptosis is an early event in the development of UVB-induced cataract. Free Radic Biol Med 1996; 20:301-11. [PMID: 8720900].

20. Reddan JR, Steiger CA, Dziedzic DC, Gordon SR. Regional differences in the distribution of catalase in the epithelium of the ocular lens. Cell Mol Biol 1996; 42:209-19. [PMID: 8696259].

21. Balog Z, Klepac R, Sikic J, Jukic-Lesina T. Protein carbonylation and glycation in human lenses. Coll Antropol 2001; 25(Suppl)145-8. [PMID: 11817006].

22. Dudek EJ, Shang F, Taylor AH. O2- mediated oxidative stress activates NF-kappa B in lens epithelial cells. Free Radic Biol Med 2001; 31:651-8. [PMID: 11522450].

23. Gajjar D, Patel D, Alapure B, Praveen MR, Patel A, Johar K, Sr, Vasavada AR. Rapid action of oestradiol against hydrogen peroxide-induced oxidative stress in cataractous lens epithelium: an in vitro study. Eye (Lond) 2009; 23:1456-63. [PMID: 18806765].

24. Ucakhan OO, Karel F, Kanpolat A, Devrim E, Durak I. Superoxide dismutase activity in the lens capsule of patients with pseudoexfoliation syndrome and cataract. J Cataract Refract Surg 2006; 32:618-22. [PMID: 16698483].

25. Strzalka-Mrozik B, Prudlo L, Kimsa MW, Kimsa MC, Kapral M, Nita M. Mazurek U. Quantitative analysis of SOD2, ALDH1A1 and MGST1 messenger ribonucleic acid in anterior lens epithelium of patients with pseudoexfoliation syndrome. Mol Vis 2013; 19:1341-9. [PMID: 23805041].

26. Kantorow M, Yaks T, Horwitz J, Huang Q, Sun J, Piatiqorsky J, Carper D. Differential display detects altered gene expression between cataractous and normal human lenses. Invest Ophthalmol Vis Sci 1998; 39:2344-54. [PMID: 9804143].

27. Oppermann B, Zhang W, Magabo K, Kantorow M. Identification and spatial analysis of metallothioneins expressed by the adult human lens. Invest Ophthalmol Vis Sci 2001; 42:188-93. [PMID: 11133866].

28. Gao S, Qin T, Liu Z, Caceres MA, Ronchi CF, Chen CY, Yeum KJ, Taylor A, Blumberg JB, Liu Y, Shang F. Lutein and zeaxanthin supplementation reduces H2O2-induced oxidative damage in human lens epithelial cells. Mol Vis 2011; 17:3180-90. [PMID: 22194644].

29. Babizhayev MA, Yegorov YE. Telomere attrition in human lens epithelial cells associated with oxidative stress provide a new therapeutic target for the treatment, dissolving and prevention of cataract with N-acetylcarnosine lubricant eye drops. Kinetic, pharmacological and activity-dependent separation of therapeutic targeting: transcorneal penetration and delivery of L-carnosine in the aqueous humor and hormone-like hypothalamic antiaging effects of the instilled ophthalmic drug through a safe eye medication technique. Recent Pat Drug Deliv Formul 2016; 10:82-129. [PMID: 26084629].

30. Xu B, Kang L, Zhang G, Wu J, Zhu R, Yang M, Guan H. The changes of 8-OHdG, hOGG1, APE1 and Pol beta in lenses of patients with age-related cataract. Curr Eye Res 2015; 40:378-85. [PMID: 24911554].

31. Kang L, Zhao W, Zhang G, Wu J, Guan H. Acetylated 8-oxoguanine DNA glycosylase 1 and its relationship with p300 and SIRT1 in lens epithelium cells from age-related cataract. Exp Eye Res 2015; 135:102-8. [PMID: 25660075].

32. Osnes-Ringen O, Azqueta AO, Moe MC, Zetterstrom C, Roger M, Nicolaissen B, Collins AR. DNA damage in lens epithelium of cataract patients in vivo and ex vivo. Acta Ophthalmol 2013; 91:652-6. [PMID: 22994213].

33. Kim J, Kim NH, Sohn E, Kim CS, Kim JS. Methyglyoxal induces cellular damage by increasing argpyrimidine accumulation and oxidative DNA damage in human lens epithelial cells. Biochem Biophys Res Commun 2010; 391:346-51. [PMID: 19915307].

34. Sorte K, Sune P, Bhake A, Shivkumar VB, Gangane N, Basak A. Quantitative assessment of DNA damage directly in lens epithelial cells from senile cataract patients. Mol Vis 2011; 17:1-6. [PMID: 21224996].

35. Gibin FJ, McCready JP, Reddan JR, Dziedzic DC, Reddy VN. Detoxification of H2O2 by cultured rabbit lens epithelial cells: participation of the glutathione redox cycle. Exp Eye Res 1985; 40:827-40. [PMID: 4018167].

36. Ozcan AA, Ozdemir N, Canataroglu A. The aqueous levels of TGF-beta2 in patients with glaucoma. Int Ophthalmol 2004; 25:19-22. [PMID: 15085971].
37. Fleenor DL, Shepard AR, Hellberg PE, Jacobson N, Pang IH, Clark AF. TGFbeta2-induced changes in human trabecular meshwork: implications for intraocular pressure. Invest Ophthalmol Vis Sci 2006; 47:226-34. [PMID: 16384967].

38. Lau LJ, Liu CJ, Wei YH. Increase of 8-hydroxy-2'-deoxyguanosine in aqueous humor of patients with exudative age-related macular degeneration. Invest Ophthalmol Vis Sci 2010; 51:5486-90. [PMID: 20538979].

39. Zanon-Moreno V, Garcia-Medina JJ, Gallego-Pinazo R, Vinuesa-Silva I, Moreno-Nadal MA, Pinazo-Duran MD. Antioxidant status modifications by topical administration of dorzolamide in primary open-angle glaucoma. Eur J Ophthalmol 2009; 19:565-71. [PMID: 19551670].

40. Ghanem AA, Arafa LF, El-Baz A. Oxidative stress markers in patients with primary open-angle glaucoma. Curr Eye Res 2010; 35:295-301. [PMID: 20373896].

41. Nucci C, Di Pierro D, Varesi C, Ciufofoli E, Russo R, Gentile R, Cedrone C, Pinazo Duran MD, Coletta M, Mancino R. Increased malondialdehyde concentration and reduced total antioxidant capacity in aqueous humor and blood samples from patients with glaucoma. Mol Vis 2013; 19:1841-6. [PMID: 23946639].

42. Miric DJ, Kistic BM, Zoric LD, Miric BM, Mirkovic M, Mitic R. Influence of cataract maturity on aqueous humor lipid peroxidation markers and antioxidant enzymes. Eye (Lond) 2014; 28:72-7. [PMID: 24097121].

43. Ferreira SM, Lerner SF, Brunzini R, Evelson PA, Llesuy SF. Oxidative stress markers in aqueous humor of glaucoma patients. Am J Ophthalmol 2004; 137:62-9. [PMID: 14700645].

44. Koliakos GG, Befani CD, Mikropoulos D, Ziakas NG, Konstas AG. Prooxidant-antioxidant balance, peroxide and catalase activity in the aqueous humour and serum of patients with exfoliation syndrome or exfoliative glaucoma. Graefes Arch Clin Exp Ophthalmol 2008; 246:1477-83. [PMID: 18607617].

45. Sawada H, Fukuchi T, Abe H. Oxidative stress markers in aqueous humor of patients with senile cataracts. Curr Eye Res 2009; 34:36-41. [PMID: 19172468].

46. Goyal A, Srivastava A, Sihota R, Kaur J. Evaluation of oxidative stress markers in aqueous humor of primary open angle glaucoma and primary angle closure glaucoma patients. Curr Eye Res 2014; 39:823-9. [PMID: 24912005].

47. Simavli H, Tosun M, Bucak YY, Erdurmus M, Oacak Z, Onder HI, Acar M. Serum and aqueous xanthine oxidase levels, and mRNA expression in anterior lens epithelial cells in pseudoexfoliation. Graefes Arch Clin Exp Ophthalmol 2015; 253:1161-7. [PMID: 25957764].

48. Bagnis A, Izzotti A, Centofanti M, Sacca SC. Aqueous humor oxidative stress proteomic levels in primary open angle glaucoma. Exp Eye Res 2012; 103:55-62. [PMID: 22974818].

49. Karakucuk S, Ertugrul Mirza G, Faruk Ekinçiler O, Saraymen R, Karakucuk I, Ustdal M. Selenium concentrations in serum, lens and aqueous humour of patients with senile cataract. Acta Ophthalmol Scand 1995; 73:329-32. [PMID: 8646578].

50. Aksoy H, Keles S, Kocer I, Akcay F. Diabetic cataract and the total antioxidant status in aqueous humor. Clin Chem Lab Med 2001; 39:143-5. [PMID: 11347148].

51. Yilmaz A, Ayaz L, Tamer L. Selenium and pseudoexfoliation syndrome. Am J Ophthalmol 2011; 151:272-6. [PMID: 2168825].

52. Hayashi R, Hayashi S, Araikai K, Sakai M, Okamoto H, Chikuda M. The gender-differentiated antioxidant effects of a lutein-containing supplement in the aqueous humor of patients with senile cataracts. Exp Eye Res 2014; 129:5-12. [PMID: 25305576].

53. McLauchlan WR, Sanderson J, Quinlan M, Williamson G. Measurement of the total antioxidant activity of human aqueous humor. Clin Chem 1998; 44:888-9. [PMID: 9554070].

54. Beyazylizidiz E, Cankaya AB, Ergun E, Anayol MA, Ozdamar Y, Sezer S, Turhis MH, Yilmazbas P, Ozturk F. Changes of total antioxidant capacity and total oxidant status of aqueous humor in diabetes patients and correlations with diabetic retinopathy. Int J Ophthalmol 2013; 6:531-6. [PMID: 23991392].

55. Martinez-Fernandez de la Camara C, Salom D, Sequejo MD, Hervas D, Marin-Lambies C, Aller E, Jaijo T, Diaz-Llopis M, Millan JM, Rodrigo R. Altered antioxidant-oxidant status in the aqueous humor and peripheral blood of patients with retinitis pigmentosa. PLoS One 2013; 8:e74223-[PMID: 24069283].

56. Alamdari DH, Kostidou E, Paletas K, Sarigianni M, Konstas AG, Karapiperidou A, Koliakos G. High sensitivity enzyme-linked immunosorbent assay (ELISA) method for measuring protein carbonyl in samples with low amounts of protein. Free Radic Biol Med 2005; 39:1362-7. [PMID: 16257645].

57. Yakici R, Ersoz I, Erdurmus M, Gurel A, Duman S. Protein carbonyl levels in the aqueous humour and serum of patients with pseudoexfoliation syndrome. Eye (Lond) 2008; 22:128-31. [PMID: 17293783].

58. Grune T, Merker K, Sandig G, Davies KJ. Selective degradation of oxidatively modified protein substrates by the proteasome. Biochem Biophys Res Commun 2003; 305:709-18. [PMID: 12763051].

59. Bota DA, Davies KJ. Lon protease preferentially degrades oxidized mitochondrial aconitate by an ATP-stimulated mechanism. Nat Cell Biol 2002; 4:674-80. [PMID: 12198491].

60. Dalle-Donne I, Aldini G, Carini M, Colombo R, Rossi R, Milzani A. Protein carbonylation, cellular dysfunction, and disease progression. J Cell Mol Med 2006; 10:389-406. [PMID: 16796807].

61. Grune T, Jung T, Merker K, Davies KJ. Decreased proteolysis caused by protein aggregates, inclusion bodies, plaques, lipofuscin, ceroid, and `aggresomes' during oxidative stress, aging, and disease. Int J Biochem Cell Biol 2004; 36:2519-30. [PMID: 15325589].
62. Powell SR, Wang P, Divald A, Teichberg S, Haridas V, McCloskey TW, Davies KJ, Katzeff H. Aggregates of oxidized proteins (lipofuscin) induce apoptosis through proteasome inhibition and dysregulation of proapoptotic proteins. Free Radic Biol Med 2005; 38:1093-101. [PMID: 15780767].

63. Levine RL. Carbonyl modified proteins in cellular regulation, aging, and disease. Free Radic Biol Med 2002; 32:790-6. [PMID: 11978480].

64. Dalle-Donne I, Giustarini D, Colombo R, Rossi R, Milzani A. Protein carbonylation in human diseases. Trends Mol Med 2003; 9:169-76. [PMID: 12727143].

65. Jung T, Höhn A, Grune T. The proteasome and the degradation of oxidized proteins: Part III—Redox regulation of the proteasomal system. Redox Biol 2014; 2:388-94. [PMID: 24563857].

66. Ishii T, Sakurai T, Usami H, Uchida K. Oxidative modification of proteasome: identification of an oxidation-sensitive subunit in 26 S proteasome. Biochemistry 2005; 44:13893-901. [PMID: 16229478].

67. Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R. Protein carbonyl groups as biomarkers of oxidative stress. Clin Chim Acta 2003; 329:23-38. [PMID: 12589963].

68. Shaeter E. Protein oxidative damage. Methods Enzymol 2000; 319:428-36. [PMID: 10907531].

69. Madian AG, Myracle AD, Diaz-Maldonado N, Rochelle NS, Janle EM, Regnier FE. Differential carbonylation of proteins as a function of in vivo oxidative stress. J Proteome Res 2011; 10:3959-72. [PMID: 21800835].

70. Moller IM, Rogowska-Wrezinska A, Rao RS. Protein carbonylation and metal-catalyzed protein oxidation in a cellular perspective. J Proteomics 2011; 74:2228-42. [PMID: 21601020].

71. Yan L-J, Forster MJ. Chemical probes for analysis of carbonylated proteins: a review. J Chromatogr B Analyt Technol Biomed Life Sci 2011; 879:1308-15. [PMID: 20732835].

72. Baraibar MA, Ladouce R, Friguet B. Proteomic quantification and identification of carbonylated proteins upon oxidative stress and during cellular aging. J Proteomics 2013; 92:63-70. [PMID: 23689083].

73. Cabisco E, Tamarit J, Ros J. Protein carbonylation: proteomics, specificity and relevance to aging. Mass Spectrom Rev 2014; 33:21-48. [PMID: 24114980].

74. Fedorova M, Bollineni RC, Hoffmann R. Protein carbonylation as a major hallmark of oxidative damage: update of analytical strategies. Mass Spectrom Rev 2014; 33:79-97. [PMID: 23832618].

75. Weber D, Davies MJ, Grune T. Determination of protein carbonyls in plasma, cell extracts, tissue homogenates, isolated proteins: focus on sample preparation and derivatization conditions. Redox Biol 2015; 5:367-80. [PMID: 26149212].

76. Cabrera MP, Chihuailaf RH. Antioxidants and the integrity of ocular tissues. Vet Med Int 2011; 2011:437207. [PMID: 21789267].

77. Hardenborg J, Botling-Taube A, Hanrieder J, Andersson M, Alm A, Bergquist J. Protein content in aqueous humor from patients with pseudoexfoliation (PEX) investigated by capillary LC MALDI-TOF/TOF MS. Proteomics Clin Appl 2009; 3:299-306. [PMID: 26238748].

78. Maatta M, Tervahartiala T, Harju M, Airaksinen J, Autio-Haromain H, Sorsa T. Matrix metalloproteinases and their tissue inhibitors in aqueous humor of patients with primary open-angle glaucoma, exfoliation syndrome, and exfoliation glaucoma. J Glaucoma 2005; 14:64-9. [PMID: 15650607].

79. Kuchle M, Ho TS, Nguyen NX, Hannappel E, Naumann GO. Protein quantification and electrophoresis in aqueous humor of pseudoexfoliation eyes. Invest Ophthalmol Vis Sci 1994; 35:748-52. [PMID: 8113026].

80. Kuchle M, Nguyen NX, Hannappel E, Beck W, Ho ST, Naumann GO. Tyndallometry with the laser flare cell meter and biochemical protein determination in the aqueous humor of eyes with pseudoexfoliation syndrome. Ophthalmologe 1994; 91:578-84. [PMID: 7812086].

81. Kuchle M, Nguyen NX, Hannappel E, Naumann GO. The blood-aqueous barrier in eyes with pseudoexfoliation syndrome. Ophthalmic Res 1995; 27:suppl 1136-42. [PMID: 8577452].

82. Howell KG, Vrabel AM, Chowdhury UR, Stamer WD, Fautsch MP. Myocilin levels in primary open-angle glaucoma and pseudoexfoliation glaucoma human aqueous humor. J Glaucoma 2010; 19:569-75. [PMID: 20179615].

83. Ghanem AA, Arafa LF, El-Baz A. Connective tissue growth factor and tissue inhibitor of matrix metalloproteinase-2 in patients with exfoliative glaucoma. Curr Eye Res 2011; 36:540-5. [PMID: 21591863].

84. Picht G, Welge-Luessen U, Grehn F, Lutjen-Drecoll E. Transforming growth factor beta 2 levels in the aqueous humor in different types of glaucoma and the relation to filtering bleb development. Graefes Arch Clin Exp Ophthalmol 2001; 239:199-207. [PMID: 11405069].

85. Levine RL, Garland D, Oliver NC, Amici A, Climent I, Lenz G-A, Ahn W-B, Shaltiel S, Stadtman RE. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol 1990; 186:464-79. [PMID: 21800835].

86. Levine RL, Williams JA, Stadtman ER, Shaeter E. Carboxyl assays for determination of oxidatively modified proteins. Methods Enzymol 1994; 233:346-57. [PMID: 8015469].

87. Bensadoun A, Weinstein D. Assay of proteins in the presence of interfering materials. Anal Biochem 1976; 70:241-50. [PMID: 1259145].

88. Arnold U, Ulbrich-Hofmann R. Quantitative protein precipitation from guanidine hydrochloride-containing solutions by sodium deoxycholate/trichloroacetic acid. Anal Biochem 1999; 271:197-9. [PMID: 10419639].

89. Georgiou CD, Zisimopoulos D, Argyropoulou V, Kalaitzopoulou E, Salachas G, Grune T. Protein and cell wall polysaccharide carbonyl determination by a neutral pH
90. Georgiou CD, Grintzas K, Zervoudakis G, Papapostolou I. Mechanism of Coomassie brilliant blue G-250 binding to proteins: a hydrophobic assay for nanogram quantities of proteins. Anal Bioanal Chem 2008; 391:391-403. [PMID: 18327568].

91. Zar JH. Comparing simple linear regression equations. Biostatistical Analysis. 5th ed: Prentice Hall; 2010. p. 363–78.

92. Rohde E, Tomlinson AJ, Johnson DH, Naylor S. Comparison of protein mixtures in aqueous humor by membrane preconcentration - capillary electrophoresis - mass spectrometry. Electrophoresis 1998; 19:2361-70. [PMID: 9788321].

93. Janciauskiene S, Brandl L, Wallmark A, Westin U, Krakau T. Secreted leukocyte protease inhibitor is present in aqueous humours from cataracts and other eye pathologies. Exp Eye Res 2006; 82:505-11. [PMID: 16202405].

94. Berlau J, Lorenz P, Beck R, Makovitzky J, Schlötzer-Schrehardt U, Thiesen H-J, Guthoff R. Analysis of aqueous humour proteins of eyes with and without pseudoexfoliation syndrome. Graefes Arch Exp Ophthalmol 2001; 239:743-6. [PMID: 11760034].

95. Chowdhury UR, Madden BJ, Charlesworth MC, Fautsch MP. Proteome analysis of human aqueous humor. Invest Ophthalmol Vis Sci 2010; 51:4921-31. [PMID: 20463327].

96. Duan X, Lu Q, Xue P, Zhang H, Dong Z, Yang F, Wang N. Proteomic analysis of aqueous humor from patients with myopia. Mol Vis 2008; 14:370-7. [PMID: 18334949].

97. Duan X, Xue P, Wang N, Dong Z, Lu Q, Yang F. Proteomic analysis of aqueous humor from patients with primary open angle glaucoma. Mol Vis 2010; 16:2839-46. [PMID: 21203405].

98. Escoffier P, Paris L, Bodaghi B, Danis M, Mazier D, Marinach-Patrice C. Pooling aqueous humor samples: bias in 2D-LC-MS/MS strategy? J Proteome Res 2010; 9:789-97. [PMID: 19929026].

99. Funding M, Vorum H, Honore B, Nexø E, Ehlers N. Proteomic analysis of aqueous humour from patients with acute corneal rejection. Acta Ophthalmol Scand 2005; 83:31-9. [PMID: 15715554].

100. Hadjistilianou T, Gigioni S, Micheli L, Vannoni D, Brogi E, Cevenini G, Cortelazzo A, De Francesco S, Menicacci F, Leoncini R. Analysis of aqueous humour proteins in patients with retinoblastoma. Clin Experiment Ophthalmol 2012; 40:e8-15. [PMID: 22003840].

101. Prata TS, Navajas EV, Melo LAJ, Martins JR, Nader HB, Belfort RJ. Aqueous humor protein concentration in patients with primary open-angle glaucoma under clinical treatment. Arq Bras Oftalmol 2007; 70:217-20. [PMID: 17589689].

102. Tripathi RC, Borisuth NS, Tripathi BJ, Gotsis SS. Quantitative and qualitative analyses of transferrin in aqueous humor from patients with primary and secondary glaucomas. Invest Ophthalmol Vis Sci 1992; 33:2866-73. [PMID: 1526736].

103. To CH, Kong CW, Chan CY, Shahidullah M, Do CW. The mechanism of aqueous humour formation. Clin Exp Optom 2002; 85:335-49. [PMID: 12452764].

104. Shahidullah M, Al-Malki WH, Delamere NA. Mechanism of aqueous humor secretion, its regulation and relevance to glaucoma. In: Rumelt S, editor. Glaucoma - Basic and Clinical Concepts: InTech; 2011. p. 3–32.