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

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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<PXG=~POAG<PEX and CAT<PEX=~POAG<PXG respectively. Moreover, 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 are 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

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(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$_{2a}$, 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$_2^{\cdot-}$) 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$_2$O$_2$) [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-dinitrophenylhydrazine (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), $Na_3PO_4 \cdot 12H_2O$ 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 anti-glaucoma 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 (ddH_2O) and 8 μl 1% DOC stock solution (made in ddH_2O). Then, the sonicated homogenate was mixed with 300 μl ddH_2O, 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

LECs/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
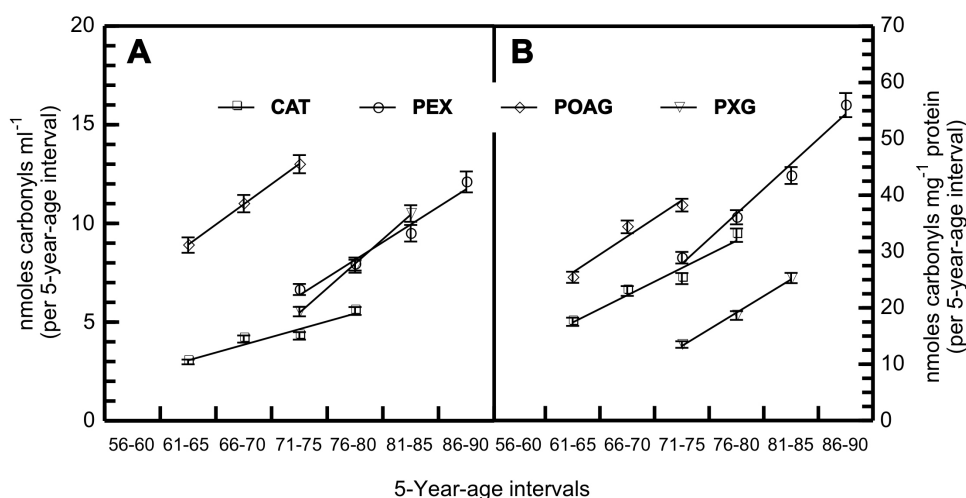

Figure 1. Age-related association of protein carbonyls in aqueous humor (AH). The association is expressed as a linear function of the change (rate) in the protein carbonyl concentration (nmoles ml^{-1} and mg^{-1} in **A** and **B**, respectively) versus the patient's 5-year age intervals. Data are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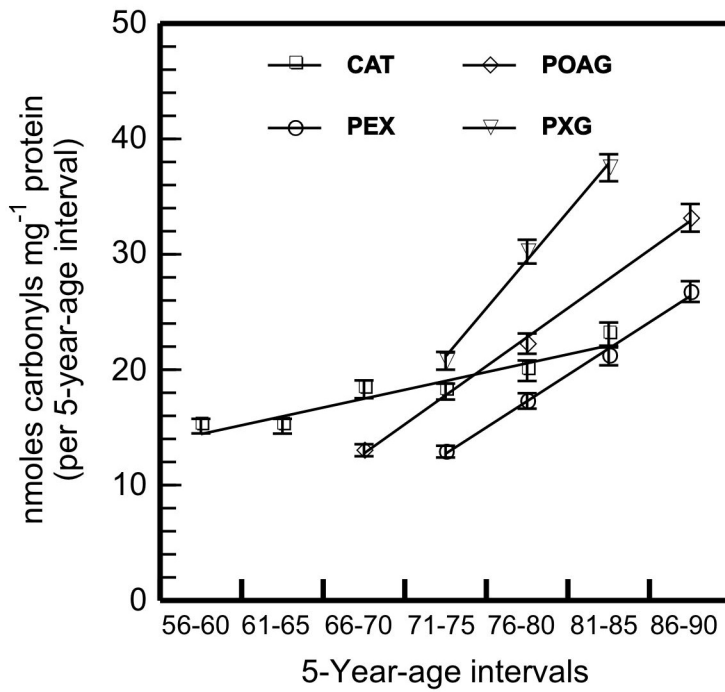
Figure 2. Age-related association of protein carbonyls in lens epithelial cells plus the capsule (LECs/capsule). The association is expressed as a linear function of the change (rate) in the protein carbonyl concentration (nmoles mg^{-1}) versus the patient's 5-year age intervals. Data are presented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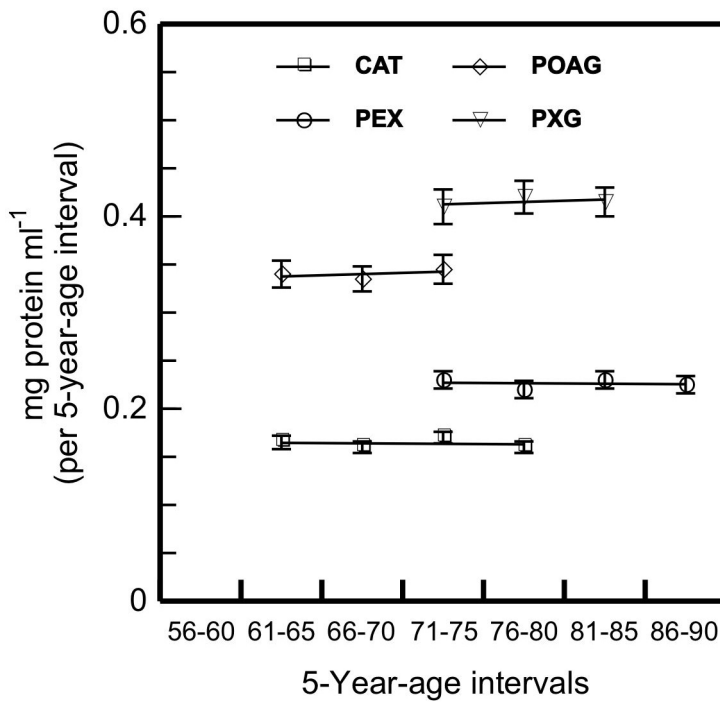

Figure 3. Age-related association of protein concentration in AH. The association is expressed as the protein concentration (mg ml^{-1}) versus the patient's 5-year age intervals. Note that the straight lines are nearly horizontal, and the corresponding slopes are near zero, showing no age correlation. Data are presented as mean ± standard error of the mean (SEM).

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 test[1]	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)	****
			CAT vs. POAG	<0.0001 (33/19)	****
AH	CAT	0.803 ± 0.060	CAT vs. PXG	<0.0001 (33/17)	****
	PEX	1.770 ± 0.110	PEX vs. POAG	=0.2021 (31/19)	ns[2]
	POAG	2.049 ± 0.127	PEX vs. PXG	<0.0001 (31/17)	****
	PXG	2.489 ± 0.096	POAG vs. PXG	=0.0146 (19/17)	*
		from Figure 1B (in nmoles carbonyls mg^{-1} protein per 5-year interval)	CAT vs. PEX	<0.0001 (20/19)	****
			CAT vs. POAG	=0.0328 (20/17)	*
AH	CAT	4.939 ± 0.310	CAT vs. PXG	=0.2351 (20/18)	ns
	PEX	8.768 ± 0.423	PEX vs. POAG	=0.0002 (19/17)	***
	POAG	6.403 ± 0.476	PEX vs. PXG	<0.0001 (19/18)	****
	PXG	5.921 ± 0.234	POAG vs. PXG	=0.8056 (17/18)	ns
		from Figure 2 (in nmoles carbonyls mg^{-1} protein per 5-year interval)	CAT vs. PEX	<0.0001 (33/31)	****
			CAT vs. POAG	<0.0001 (33/19)	****
LECs/	CAT	1.506 ± 0.116	CAT vs. PXG	<0.0001 (33/17)	****
capsule	PEX	4.524 ± 0.137	PEX vs. POAG	=0.1425 (31/19)	ns
	POAG	5.048 ± 0.139	PEX vs. PXG	<0.0001 (31/17)	****
	PXG	8.364 ± 0.322	POAG vs. PXG	<0.0001 (19/17)	****
B. Protein concentration versus disease (non-age dependent)					
		Mean concentration (± SE)			
		from values for horizontal straight lines in Figure 3 (in mg protein ml^{-1})	CAT vs. PEX	=0.1225 (20/19)	ns
			CAT vs. POAG	<0.0001 (20/17)	****
AH	CAT	0.16 ± 0.0074	CAT vs. PXG	<0.0001 (20/18)	****
	PEX	0.23 ± 0.0093	PEX vs. POAG	=0.0003 (19/17)	***
	POAG	0.34 ± 0.0136	PEX vs. PXG	<0.0001 (19/18)	****
	PXG	0.42 ± 0.0162	POAG vs. PXG	=0.0303 (17/18)	*

[1]Tukey's multiple comparisons test and statistical significance at p<0.05 (more details in the "Statistical analysis" section).[2]ns designates not significant.

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

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

$\pm$ 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 ± 7.2 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.

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