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miR-15a: a potential diagnostic biomarker and a candidate for non-operative therapeutic modality for age-related cataract

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ABSTRACT

Introduction: In order to better understand the role of hsa-miR-15a in the pathogenesis of age-related cataracts, we hypothesised altered expression, and of target anti-apoptotic genes, *BCL-2* and *MCL-1*, in lens epithelial cells amongst age-related cataract patients.

Material and methods: Reverse transcription quantitative polymerase chain reaction (RT-qPCR) quantified the expression of hsa-miR-15a and the target genes *BCL-2* and *MCL-1* in lens epithelial cells of 120 age-related cataract patients (40 patients with cortical cataracts, 40 patients with nuclear cataracts and 40 patients with posterior subcapsular cataracts) and 40 controls. Sixty specimens (15 normal and 45 cataracts) were stained immunohistochemically with *BCL-2* and *MCL-1* markers.

Results: The expression of hsa-miR-15a was significantly increased ($p = 0.003$) in lens epithelial cells of cataract patients compared to the control group. *BCL-2* and *MCL-1* expression levels were significantly decreased in cataract patients ($p < 0.001$). A significant increase in hsa-miR-15a expression in the cortical subtype compared to the posterior subcapsular subtype ($p = 0.003$) and a significant decrease in *BCL-2* and *MCL-1* expressions in the cortical subtype compared to the nuclear and the posterior subcapsular subtype was detected.

Conclusions: The increased expression of hsa-miR-15a in lens epithelial cells of cataract patients may repress the expression of *BCL-2* and *MCL-1*. The expression of hsa-miR-15a and the subsequent apoptosis of lens epithelial cells are part of the pathogenesis of age-related cataracts.

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Introduction

Cataract, one of the prevalent age-related eye diseases, is the prominent cause of blindness worldwide [1]. The group of age-related cataract includes nuclear, cortical and posterior subcapsular cataract [2]. Nuclear cataract is the most common subtype of cataracts, and accounts for approximately 60% of age-related cataract cases, cortical cataract accounts for about 30% and the remaining 10% of age-related cataract cases are of the posterior subcapsular type [3]. Varying levels of apoptosis are present in the epithelium of human cataract lenses [4].

MicroRNAs are small RNAs (20 to 25 nucleotides) which are non-coding but play an important role in the regulation of gene expression through a post-transcriptional modification [5,6]. miRNAs regulate degradation or translation of mRNA in binding to a complementary sequence in the 3′-untranslated regions (UTR) of mRNAs of the target gene [7]. Many previously published studies have revealed that abnormal miRNAs expression is associated with the development of many age-related pathological conditions, including cataract [8,9].

miRNA hsa-miR-15a is involved in the apoptosis of several cell types. It can negatively control the expression of anti-apoptotic genes like *BCL-2* and *MCL-1* which inhibits cell growth and arrests the cell cycle causing apoptosis [10]. In order to better understand the role of this miRNA in the pathogenesis of age-related cataract disease, we hypothesised (a) altered expression of hsa-miR-15a and its target anti-apoptotic genes *BCL-2* and *MCL-1* as apoptotic markers in age-related cataract patients compared to a group of control patients with no cataract disease, and (b) differences in expression in the sub-types of cataract disease.

Patients and methods

This study included 120 lenses from 120 patients with age-related cataracts; 40 with cortical cataracts, 40 with nuclear cataracts, and 40 with posterior subcapsular cataract. Forty-five specimens (15 from each patient group) were fixed in 10% formalin for immunostaining. Exclusion criteria were previous ophthalmic injuries or surgeries, glaucoma, diabetes, intraocular tumours, intraocular inflammation, either infectious or autoimmune, or long-term eye exposure to radiation.

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A control group of 40 age matched normal lens patients indicated for refractive lens exchange were used to compare with the age-related cataract disease group (Table 1). Fifteen specimens were fixed in 10% formalin for immunostaining. The study was approved by Benha University Research Ethics Committee and the 1964 Helsinki Declaration including later amendments to the study design, and written informed consent was obtained from each participant.

All patients had a pre-operative examination of a visual acuity test, intra-ocular pressure, fundus examination and slit lamp to examine the anterior eye segment and determine the cataract subtype. The anterior capsule including anterior sub-capsular epithelial cells was obtained during cataract extraction by phacoemulsification. The control patients' anterior capsules were harvested during a refractive lens exchange to treat high myopia.

Cataractous epithelial tags were extracted and graded within minutes of cataract surgery. Contaminating fibre cells were gently removed from both normal and cataractous lens epithelia and the resulting tissues were washed to remove potential contaminants as described previously [11,12]. All samples were immediately stored at −80°C after dissection for further molecular assays.

Total RNA including miRNA was extracted from human lens epithelial cells using the RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Two-step RT-PCR for hsa-miR-15a, *BCL-2*, and *MCL-1* was performed using Qiagen miScript preAMP RT-PCR kit (Qiagen GmbH, Hilden, Germany) for the conversion of microRNA to cDNA in a G-storm thermocycler (Gene technologies Ltd., Essex, UK). Then, amplification and quantification of RNAs were done by real-time PCR in ABI7900 (Applied Biosystem, CA, USA) using SuperReal Premix Plus QuantiTect. Kit, SYBR Green (Tianjen, Shanghai) according to the manufacturer's instructions and using the specific primers for each. Real-time cycler conditions were 95°C for 15 min for initial denaturation, followed by 40 cycles of 95°C within 30 secs for denaturation, 55°C for 1 min for annealing and 72°C for 1 min for extension step. The target sequences were hsa-miR-15a, *BCL-2*, and *MCL-1* and the calibrator sample is normal lens. The reference gene (housekeeping gene) was RUN6B. PCR primer for miR-15a: forward 5′-GCGGCTAGCAGCACATAATGG-3′, reverse 5′-GTGCAGGGTCCGAGGT-3′ [13]. Primers for apoptotic genes: *BCL-2* forward: 5′ – GGAGGATTGTGGC CTTCTTT-3′; *BCL-2* reverse: 5′-GGCCGTACAGTTCCACA AAT-3′ [14]. *MCL-1* forward: 5′-TGGTGCCTTTGTGGCTA AA-3′; *MCL-1* reverse: 5′- CCACCTTCTAGGTCCTCTACAT-3′ [15]. Primer sequence of RUN6B: forward 5′- CTC GCT TCG GCAGCACA −3′, reverse 5′-AACGCTTCACGAAT TTGCGT −3′ [16].

The Threshold Cycle (CT) serves as a tool for the calculation of the starting template amount in each sample and gene fold expression changes are calculated using the equation $2^{-\Delta\Delta ct}$. Due to the relative nature of quantification using the $2^{-\Delta\Delta CT}$ method an adjustment is required for each sample. Briefly, cDNA was diluted 10-, 10^2-, 10^3-, 10^4-, 10^5-, and 10^6-fold prior to amplification by real-time PCR and a standard curve was derived in order to obtain optimal amplification conditions [17].

Capsulotomy specimens (n = 60, 45 cataractous specimens and 15 non-cataractous) were processed for conventional light microscopy and immunohistochemistry. Specimens were processed and embedded in Paraffin blocks. Three slides of each block of 4 μm thick sections were cut. The sections were dewaxed at 56°C for 2 h and then one slide processed for staining with haematoxylin-eosin. The other two slides were processed to detect *BCL-2* and *MCL-1* by immunohistochemical method. Envision to FLEX/HRP (Envision Flex Target Retrieval solution High PH, Dako) method was used according to manufacturers' instruction for immunostaining. The two sections from the paraffin block were microwave treated to unmask epitopes. The monoclonal antibody *BCL-2* (Thermo Scientific, USA) conc. in dilution 1:100 and polyclonal *MCL-1* antibody (Thermo Scientific, USA) concentration in dilution 1:50 were used.

The number of immune-positive cells was counted in four representative fields, the total number of cells in one microscopic field was counted and the percentage of positively stained cells was detected (positive cells/total cells × 100%). The average of the four fields was calculated for both *BCL-2* and *MCL-1* and then analysed for scoring. The scoring system was based on a scale of 0 to 4+ as follows: 4+: very high (75% to 100% positive cells); 3+: high (50% to 75% positive cells); 2+: moderate (25% to 50% positive cells); 1+:

Table 1. Comparison between cases and control groups regarding age and sex, fold change and median (IQR) levels of the studied parameters expressed in FRU.

		All cataract patients (n = 120)	Control group (n = 40)	*p-value*
Age (years)	Mean ± SD	58.8 ± 2.9	57.9 ± 1.2	0.06
Sex	Male N (%)/Female N (%)	63 (52.5%)/57 (47.5%)	21 (52.5%)/19 (47.5%)	0.9
miRNA-15a (FRU)	Median (IQR)	4.6 (6.9)	2.0 (1.6)	0.003
Bcl-2 mRNA (FRU)	Median (IQR)	559 (282)	7549 (9812)	<0.001
Mcl-1 mRNA (FRU)	Median (IQR)	1.8 (2.8)	21.7 (12.4)	<0.001

FRU = fluorescence units.

low (<25% positive cells) and 0: negative (0 positive cells) [18].

Data were analysed on Statistical package for social science version 24; the results were expressed as the median and interquartile range (IQR), numbers and percent. Normality was verified by the Kolmogorov–Smirnov test. In the statistical comparison of quantitative data between the different groups, the significance of difference was tested using Student's t-test to compare between mean of two groups of numerical (parametric) data. For non-parametric data, Mann–Whitney U-test was used, Kruskal–Wallis was used to compare between more than two groups of non-parametric data. While in the statistical comparison of qualitative data between the different groups, chi-square and Fisher-exact test were used. Significance was accepted at $p < 0.05$.

Results

Patients with cataracts showed a significant increase in hsa-miR-15a expression compared to the non-cataractous control group (Table 1). The comparison between cataract subtypes showed a significant increase in hsa-miR-15a expression in the cortical subtype compared to the posterior subcapsular subtype (Table 2). Patients with cataracts showed a significant decrease in *BCL-2* expression compared to the control group (Table 1). The comparison between cataract subtypes showed a significant decrease in *BCL-2* expression in the cortical subtype compared to the nuclear and the posterior subcapsular subtype (Table 2). Patients with cataracts showed a significant decrease in *MCL-1* expression compared to the control group (Table 1). The comparison between cataract subtypes showed a significant decrease in *MCL-1* expression in the cortical subtype compared to the nuclear and the posterior subcapsular subtype (Table 2). There is an inverse correlation between hsa-miR-15a expression and the expression level of both *BCL-2* genes ($r = -0.82$, $p < 0.001$) and *MCL-1* genes ($r = -0.87$, $p < 0.001$). These results show irrespective of subtype of cataract the expression levels of genes *BCL-2* and *MCL-1* were significantly reduced.

Cytoplasmic staining in the lining epithelium of the lens capsule showed *BCL-2* expression in 7/15 (46.6%) of cortical cataract patients, 9/15 (60%) of nuclear cataract patients and 9/15 (60%) of posterior

Table 2. Comparison between cataract subtypes regarding age, sex and median (IQR) levels of the studied parameters expressed in FRU.

		Cortical cataract (n = 40)	Nuclear cataract (n = 40)	Posterior subcapsular cataract (n = 40)	*p-value*
Age (years)	Mean ± SD	58.3 ± 2.5	58.6 ± 2.8	59.5 ± 3.3	0.2
Sex	Male N (%)	20 (50%)	21 (52.5%)	22 (55%)	0.9
	Female N (%)	20 (50%)	19 (47.5%)	18 (45%)	
miRNA-15a (FRU)	Median (IQR)	5.7 (10.5) †	4.6 (7.2)	5 (5.9)	0.003
BCL-2 mRNA (FRU)	Median (IQR)	442 (284) *†	605 (527)	607 (552)	0.002
MCL-1 mRNA (FRU)	Median (IQR)	1.8 (2.8) *†	2.9 (2)	3.6 (4.9)	0.001

* $p < 0.05$ to nuclear cataract, †$p < 0.05$ to posterior subcapsular cataract. FRU = fluorescence units.

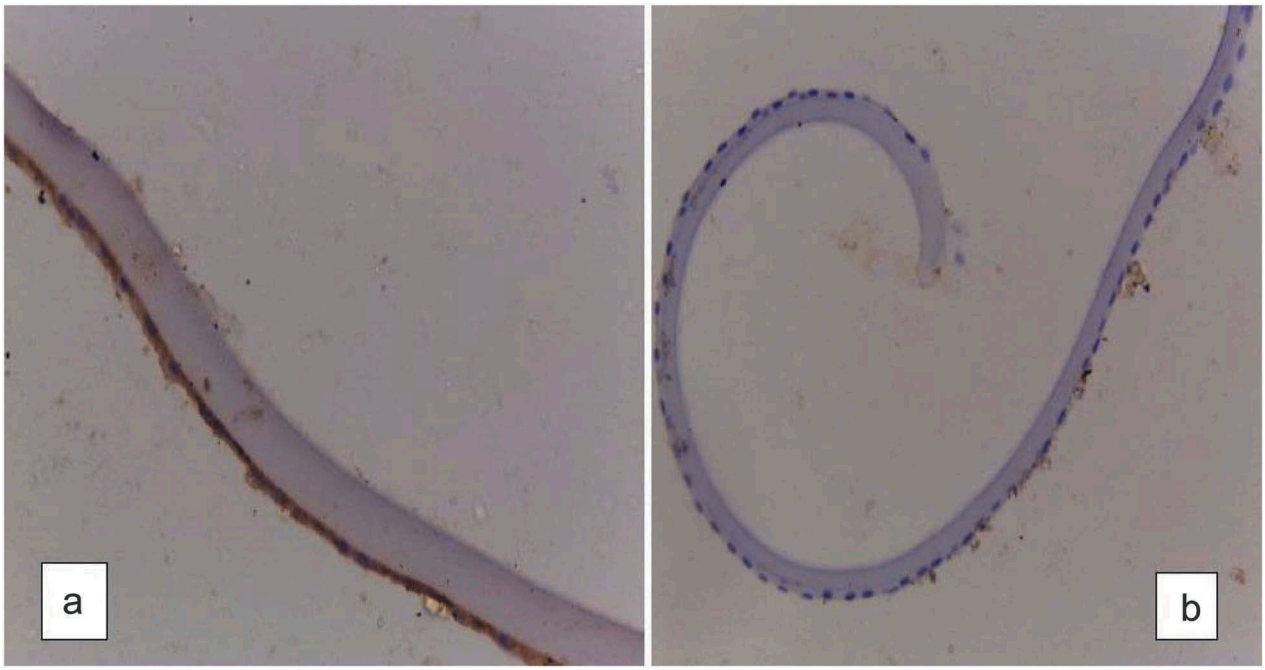

Figure 1. (a) lens capsule of control group showing positive cytoplasmic immunostaining of *BCL-2*, (b) lens capsule of cortical cataract patients showing negative immunostaining of *BCL-2* (IHCX400).

Table 3. Immunohistochemical staining for *BCl-2* and *MCL-1* in the lens capsule of control and cataract groups.

		Cataract patients				
Positive expression	Score	Control No = 15	Cortical No = 15	Nuclear No = 15	Posterior subcapsular No = 15	*p-value*
BCL-2 N (%)	**0**	3 (20%)	8 (53.3%)	6 (40%)	6 (40%)	<0.001
	+1	0 (0%)	6 (40%)	7 (46.7%)	7 (46.7%)	
	+2	2 (13.3%)	1 (6.7%)	0 (0%)	1 (6.7%)	
	+3	8 (53.3%)	0 (0%)	2 (13.3%)	1 (6.7%)	
	+4	2 (13.3%)	0 (0%)	0 (0%)	0 (0%)	
MCL-1 N (%)	**0**	2 (13.3%)	8 (53.3%)	7 (46.7%)	8 (53.3%)	<0.001
	+1	1 (6.7%)	4 (26.7%)	5 (33.3%)	3 (20%)	
	+2	1 (6.7%)	3 (20%)	3 (20%)	4 (26.7%)	
	+3	6 (40%)	0 (0%)	0 (0%)	0 (0%)	
	+4	5 (33.3%)	0 (0%)	0 (0%)	0 (0%)	

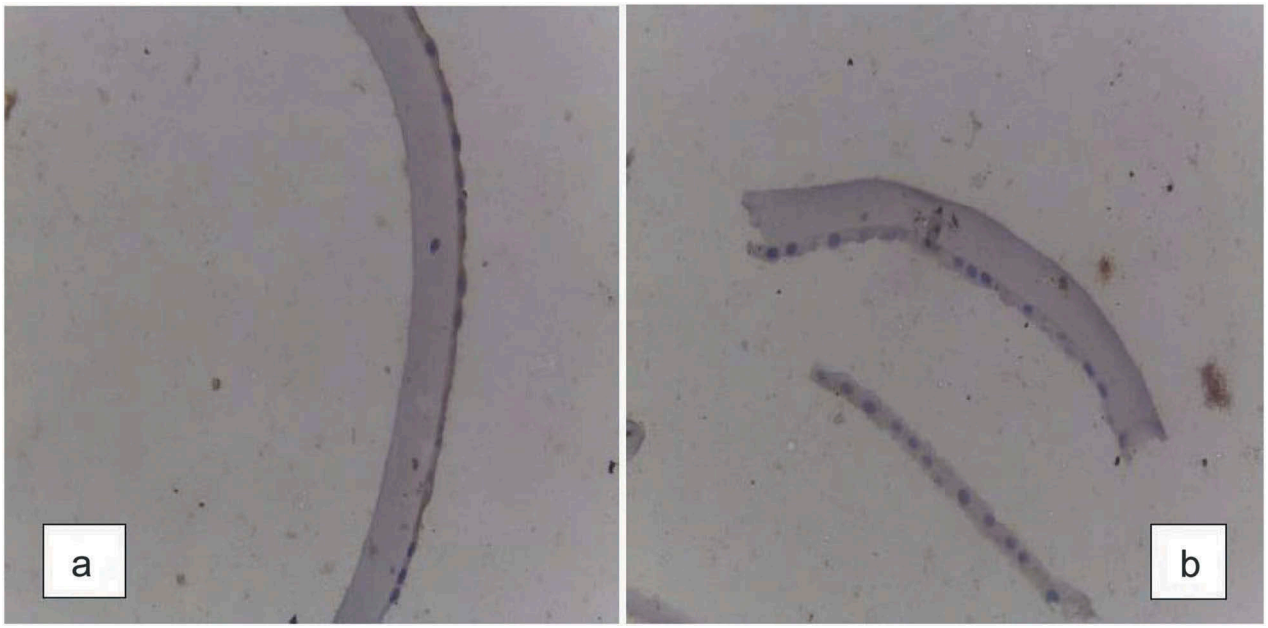

Figure 2. (a) lens capsule of control group showing positive cytoplasmic immunostaining of *MCL-1*, (b) lens capsule of cortical cataract patients showing negative immunostaining of *MCL-1* (IHCX400).

subcapsular cataract patients compared to 12/15 (80%) in the control group (see Figure 1 and Table 3). Cytoplasmic staining in the lining epithelium of the lens capsule showed *MCL-1* expression in 7/15 (46.6%) of cortical cataract patients, 8/15 (53.3%) of nuclear cataract patients and 7/15 (46.6%) of posterior subcapsular cataract patients compared to 13/15 (86.6%) of the control group (see Figure 2 and Table 3).

Discussion

Studying the molecular base of lens epithelial cell apoptosis may shed new light in understanding the mechanism of cataract development and progression. This, in turn, could provide new insights into a potential non-operative therapeutic modality for cataracts.

Abnormal miRNAs expression is associated with the development of many age-related pathological conditions [19–22] including cataract formation [8,9]. Information gained about miRNA has provided new opportunities in studying lens epithelial cell apoptosis, which appears to be a common cellular basis for non-congenital cataract development [23]. B cell lymphoma 2 (*BCL-2*) is a central player in the genetic program of eukaryotic cells favouring survival by inhibiting cell death [24] and known to have protective effects against varied age-related diseases [25,26]. *MCL-1* is a pro-survival member of the *BCL-2* family that also plays a role in antagonising apoptosis [15].

The dysregulation of *BCL-2* protein family expression and function has been implicated in all malignancies and a number of other pathologies [27]. Cimmino et al. showed that miR-15a and miR-16–1 expression were inversely correlated with *BCL-2* expression in Chronic Lymphocytic Leukaemia and they both negatively regulated *BCL-2* at a post-transcriptional repression level. They also demonstrated that *BCL-2* repression by these miRNAs promoted apoptosis in a leukemic cell line model. Therefore, miR-15 and miR-16 have been considered as natural antisense *BCL-2* interactors that could have a valuable therapeutic significance in *BCL-2* over-expressing tumours [28].

MCL-1 is a member of the *BCL-2* protein family and the *MCL-1* transcript is also a target of miR-15a and miR-16–1. Calin et al. showed that *MCL-1* expression is inhibited by these miRNAs [29]. *BCL-2* and *MCL-1* regulation by miR-15a has been widely investigated on malignancies and a number of pathological conditions other than age-related cataract. So, in this study, we aimed to demonstrate the expression levels of hsa-miR-15a in lens epithelial cells and investigate the associations of this miRNA with the expression of its target anti-apoptotic genes *BCL-2* and *MCL-1* and subsequently with lens epithelial cells apoptosis among age-related cataract patients.

We found that the expression of hsa-miR-15a was significantly increased in lens epithelial cells of cataract patients compared to normal lens epithelial cells. These results are consistent with a trial by Yuanbin et al. who revealed that hsa-miR-15a-5p, hsa-miR-15a-3p, and hsa-miR-16–1-5p were expressed at lower levels in normal lens epithelial cells compared to patients with age-related cataract of all subtypes (cortical cataracts, nuclear cataracts and posterior subcapsular cataracts) [15]. As regards *BCL-2* and *MCL-1*, their mRNA expression levels were significantly decreased in lens epithelial cells in age-related cataract patients compared to control patients. These findings were further supported by the results of immunohistochemistry (IHC) staining, which revealed that *BCL-2* and *MCL-1* proteins were highly expressed in lens epithelial cells of the non-cataractous control group compared to patients with all subtypes of cataract. This corroborates the study of Liu et al. who demonstrated reduced protein levels of *BCL-2* and *MCL-1* in the miR-15a-3p mimic transfected HLE-B3 human lens epithelial cell line [30]. Regarding the comparison between cataract subtypes, there was a significant increase in hsa-miR-15a expression in the cortical subtype compared to the posterior subcapsular subtype and a significant decrease in *BCL-2* and *MCL-1* expressions in the cortical subtype compared to the nuclear and the posterior subcapsular subtype. We think these differences between cataract subtypes could be related to differences in the disease severity as reported by Chien et al. who demonstrated a positive correlation between high miR-34a levels and high lense opacity severity in nuclear, cortical or posterior subcapsular cataracts [31]. Further studies are needed to evaluate the correlation between the severity of lens opacity and hsa-miR-15a, *BCL-2* and *MCL-1* expression levels in the lens epithelium of age-related cataracts.

This study demonstrated an inverse correlation between hsa-miR-15a expression and the expression of *BCL-2* and *MCL-1* as their expression levels were significantly decreased in cataract patients, irrespective of subtype. This agrees with findings previously shown by Cimmino et al. [28], Li et al. [15] and Willimott and Wagner [32] who reported that decreased expression of miR-15a and miR-16–1 were associated with increased *BCL-2* expression and resistance to apoptosis.

In conclusion, this study identifies the impact of hsa-miR-15a and its target genes, *BCL-2* and *MCL-1*, on age-related cataract patients. These findings may help understand the molecular mechanisms implicated in the pathogenesis of cataract development and progression that could provide new insights into a potential non-operative therapeutic modality. miRNA is a good candidate for therapeutic applications through antisense inhibition or replacement that could significantly affect disease progression.

This work represents an advance in biomedical science because it provides further understanding of the molecular mechanisms involved in the pathogenesis of cataract development and progression.

Summary table

What is known about this subject:

- Apoptosis is associated with human cataractogenesis.
- Both *BCL-2* and *MCL-1* play a role in the genetic program of eukaryotic cells antagonising apoptosis.
- hsa-miR-15a is in the apoptosis of several cell types and may target *BCL-2* and *MCL-1* at the posttranscriptional level.

What this paper adds:

- *BCL-2* and *MCL-1* expressions are significantly decreased in lens epithelial cells in age-related cataract patients compared to control patients.
- hsa-miR-15a expression was significantly increased in lens epithelial cells of patients with all subtypes of cataract.

Disclosure statement

No potential conflict of interest was reported by the authors.

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